

**A study of p120-catenin and its tyrosine phosphorylation in cancer
cell adhesion and invasion.**

By

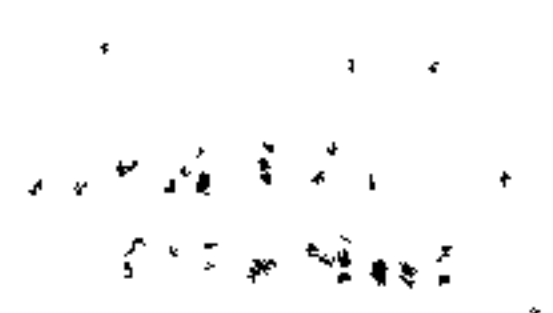
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April 2007

**This thesis is submitted to the University of Glasgow in part fulfilment of the requirements
for the degree of Doctor of Philosophy.**

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ABSTRACT

The acquisition of an invasive phenotype by neoplastic cells is often associated with increased activity of tyrosine kinases, such as Src or epidermal growth factor receptor (EGFR). Additionally, cell-cell adhesion, mediated via cadherin-based adherens junctions (AJs), profoundly influences whether and how invasion might occur. p120-catenin, which undergoes prominent phosphorylation on tyrosine in response to growth factor signalling or activation of Src, has emerged as a key player in promoting E-cadherin stability and AJ integrity. In this work we address the role of p120, and specifically its tyrosine phosphorylation, in determining the adhesive and invasive phenotype of neoplastic cells.

As an initial step we generated polyclonal site-specific phosphorylation state-specific antibodies to recently identified Src phosphorylation sites in p120. Antibodies to two of eight sites, phospho-tyrosine-228 and phospho-tyrosine-296 were successfully produced and validated and, together with newly available commercial monoclonal p120 phospho-specific antibodies, were used to characterise p120 phosphorylation events occurring in response to activation of Src or EGFR.

Over-expression of constitutively active Src in a colon cancer cell line (KM12C) caused phosphorylation of p120 at tyrosine residues 228, 280, 291 and 296 and this was dependent on Src kinase activity and the presence of an intact Src SH2 domain. Simultaneous phosphorylation of the same sites occurred in a squamous cell carcinoma cell line (A431) stimulated with EGF, most probably via activation of Src kinase. In both models tyrosine phosphorylation of p120 correlated with disruption of AJs. However, studies utilising phosphorylation-defective p120 mutants suggested that tyrosine phosphorylation of p120 was not required for Src-induced inhibition of AJ formation in KM12C cells but, along with other events occurring at the p120 N-terminal region, was required for growth factor induced scattering of A431 cells.

RNAi-mediated knockdown of p120 in A431 cells impaired EGF-induced invasion into 3-dimensional matrix gels or in organotypic culture, while re-expression of RNAi-resistant p120, or phosphorylation defective p120, restored the collective mode of invasion employed by A431 cells in vitro. The requirement for p120 was not due to its effects on stabilizing E-cadherin, since RNAi-mediated E-cadherin knockdown did not cause a similar reduction in A431 invasion. Thus, we describe a novel requirement for p120 in mediating the collective invasion of squamous cancer cells via mechanisms which are independent of p120's ability to be phosphorylated or to stabilise E-cadherin.

ACKNOWLEDGEMENTS

I would to thank several people who have made this work possible:

First and foremost my supervisors, Margaret Frame and Val Brunton, for their ceaseless enthusiasm, support and guidance. Every member of R01 helped me in some way at some time but, in particular, Val Fincham, Alan Serrels, Bryan Serrels and Gordon McLean. Lynn McGarry for her expert assistance in the performance and analysis of Matrigel invasion assays. Erik Sahai for undertaking the organotypic invasion assays and allowing the use of unpublished data. Gaby Ashton and Owen Sansom who also freely shared unpublished data. All the staff of the Beatson Institute support services without whose assistance little of this work could have been performed.

I am indebted to Jeff Evans who, via his enthusiasm for experimental cancer medicine, helped guide me towards a career in Medical Oncology and who introduced me to Margaret in the first place.

Finally, this work was funded by a generous fellowship from Cancer Research UK.

DECLARATION

I declare that all the work in this thesis was performed personally unless otherwise acknowledged.

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ABBREVIATIONS

AJ	Adherens junction
APC	Adenomatous polyposis coli
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
C	Carboxy
CBD	Catenin binding domain
cDNA	Complementary DNA
CFP	Cyan fluorescent protein
CHO	Chinese hamster ovary
CSF	Colony stimulating factor
Dia	Diaphanous
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulfoxide
DA	Dominant active
DN	Dominant negative
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Extracellular cadherin
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGF(R)	Epidermal growth factor (receptor)
EMT	Epithelial-mesenchymal transition
ERK	Extracellular regulated kinase
F	Phenylalanine
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FGF(R)	Fibroblast growth factor (receptor)
GAP	GTPase-activating protein
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GSK-3 β	Glycogen synthase kinase-3 β
GTP	Guanosine 5'-triphosphate
HEPES	N-[hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HER	Human epidermal growth factor receptor
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
IGF(R)	Insulin-like growth factor (receptor)
IP	Immunoprecipitation
IPTG	Isopropylthiogalactoside
JMD	Juxtamembrane domain
KGM	Keratinocyte growth medium
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MDCK	Madin-Darby canine kidney
MEM	Minimum essential medium

MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
N	Amino
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PBMC	Peripheral blood mononuclear cell
PE	PBS / EDTA
PH	Pleckstrin homology domain
PI 3-K	Phosphatidylinositol 3-kinase
PIP-3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
PMSF	Phenylmethylsulphonyl fluoride
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RBD	Rhotekin binding domain
RNA	Ribodeoxynucleic acid
RNAi	RNA interference
ROCK	Rho associated kinase
SDS	Sodium dodecyl sulphate
SFK	Src family kinase
SH	Src Homology
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline
TCF	T cell factor
TEMED	Tetramethylethylenediamine
TGFβ	Transforming growth factor β
TNFα	Tumour necrosis factor α
TRIS	Tris-[hydroxymethyl]aminomethane
TRITC	Tetramethyl rhodamine isocyanate
VEGF(R)	Vascular endothelial growth factor (receptor)
WASP	Wiskott Aldrich syndrome protein
WT	Wild type
Y	Tyrosine

1 Introduction: p120-catenin, the adherens junction and invasion.

Invasion, the ability of neoplastic cells to breach tissue barriers, is a hallmark of malignancy (1). Whether by infiltration and compromise of adjacent tissues, or as an initial step in the process of metastasis, invasion is directly responsible for much of the morbidity and mortality of cancer. Over the past two decades an understanding of the molecular processes fundamental to invasion and metastasis has begun to emerge. It is clear that invasion requires co-ordinated changes in cell-cell and cell-matrix adhesion, remodelling of the extracellular matrix, reorganisation of the actin cytoskeleton and increased motility. Furthermore, current evidence suggests cancer cells are able to utilise differing strategies of invasion which are characterised by the relative contribution of each of these factors. Although complex these processes are ultimately coordinated and are regulated, to a large extent, by tyrosine kinases and their numerous substrates. In this piece of work we have attempted to identify the contribution of one such tyrosine kinase substrate, the adherens junction (AJ) protein p120-catenin (hereafter referred to as p120), which would seem well placed to co-ordinately regulate altered cell-cell adhesion, motility and invasion. Work to understand the biology of invasion and metastasis is timely, not least because of the recognition that this particular hallmark of malignancy may be susceptible to molecularly targeted therapies. To use such agents appropriately will require translation from bench to bedside and back again and will demand a sound understanding of the fundamental biology of invasion.

1.1 Structure and function of the adherens junction.

Epithelial cell-cell adhesion is determined by three types of specialised structure located at the basolateral membrane: AJs, tight junctions and desmosomes (Figure 1-1). Of these the AJ is the key determinant of cell-cell adhesion, epithelial differentiation and tissue morphogenesis (2) and, at least in vitro, formation of AJs is a prerequisite for the formation of tight junctions and desmosomes (3). However, far from simply providing inert physical adhesion it is increasingly clear that AJs are dynamically regulated sites of bi-directional signalling which regulate and are regulated by tyrosine kinases and the actin cytoskeleton (2, 4). The core components of the AJ are cadherins and their cytoplasmic binding partners known as catenins (Figure 1-2). However, it should be borne in mind that diverse molecules are physically and functionally associated with the AJ including tyrosine and lipid kinases, phosphatases, integrins, nectins and actin regulatory proteins.

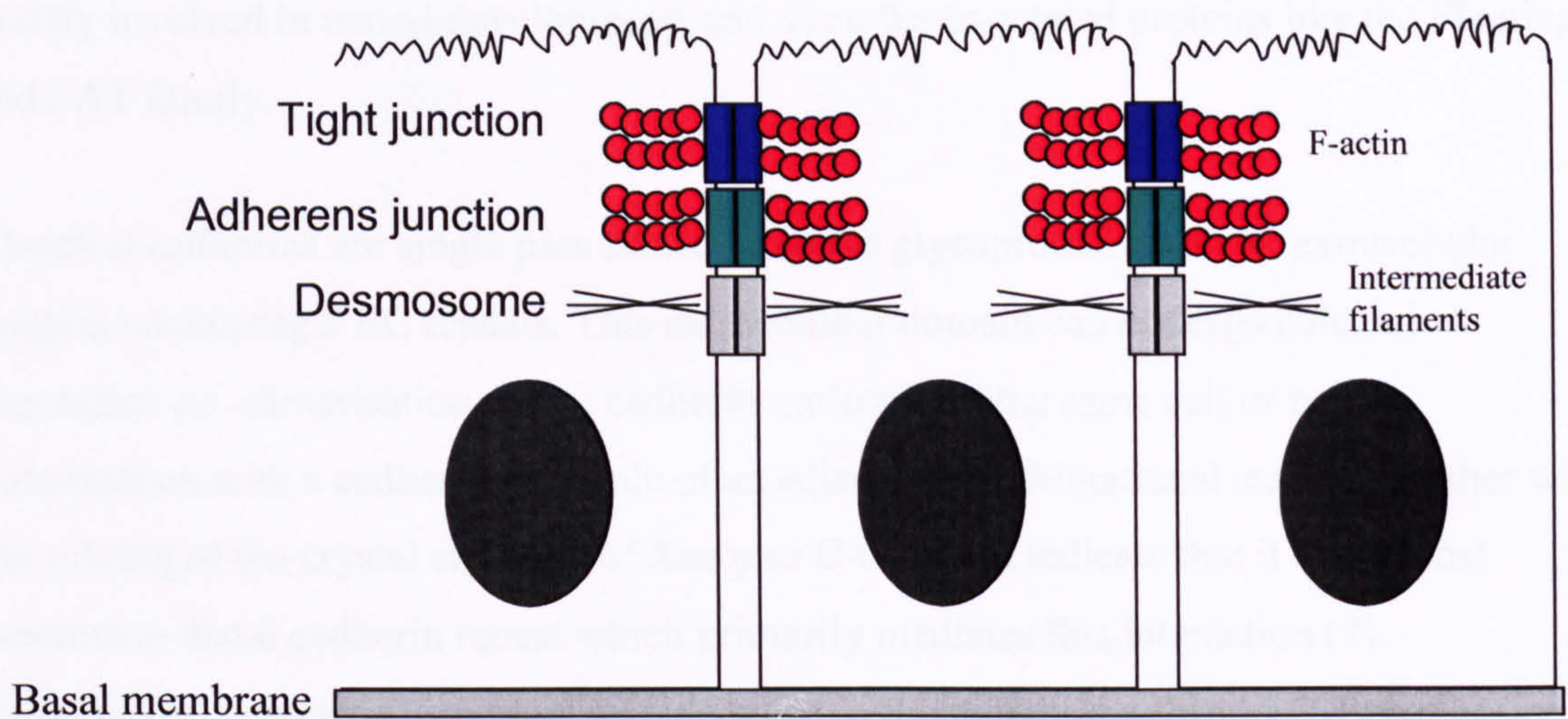


Figure 1-1 Adhesive structures in epithelia.

The tight junction (blue) is composed of transmembrane proteins such as claudin and occludin. It forms a virtually impenetrable barrier preventing the passage of most dissolved molecules from one side of the epithelial sheet to the other. The AJ (green) is formed by classical cadherins and catenins. Both AJs and tight junctions are linked to the actin cytoskeleton (red circles). The desmosome (grey) is composed of desmosomal cadherins and plakophilins and links sites of cell-cell adhesion to intermediate filaments.

1.1.1 Cadherins

Cadherins form a diverse superfamily of transmembrane glycoproteins characterised by the presence of one or more extracellular cadherin (EC) repeats. These 110 amino acid long conserved sequences contain specific calcium-binding motifs and the binding of calcium ions is essential for the proper folding, rigidity and hence function of the cadherin repeat (5). Cadherins can be divided into 4 sub-families (6): 1) classical cadherins, including E-, N-, and P-cadherin which participate in AJs and to which this discussion is limited; 2) desmosomal cadherins which participate in desmosomes; 3) protocadherins which are mainly involved in neural development; and 4) cadherin-related proteins like the Flamingo and FAT family.

Classical cadherins are single pass transmembrane glycoproteins with an extracellular domain containing 5 EC repeats. This extracellular domain can undergo calcium-dependant *cis* -dimerisation with a cadherin molecule of the same cell or *trans*-dimerisation with a cadherin molecule of an adjacent cell. Mutational studies together with the solving of the crystal structure of *Xenopus* C-cadherin indicate that it is the most membrane-distal cadherin repeat which primarily mediates this interaction (7). Specifically, a conserved tryptophan side chain in the membrane-distal EC repeat of one molecule inserts into a hydrophobic pocket in a separate region of the membrane-distal repeat of the second cadherin molecule.

The cadherin cytoplasmic domain contains two distinct and non-overlapping binding domains via which it interacts with members of the Armadillo family of proteins known as catenins. The highly-conserved membrane-proximal *juxtamembrane domain* (JMD) interacts with p120-catenin via residues 758-773 (8-10) and other regulatory proteins such as Hakai (11) and presenilin (12) whilst residues 815-839 of the membrane distal *catenin-binding domain* (CBD) interact with β -catenin (13) (numbering refers to E-cadherin).

E-cadherin, the prototypic classical cadherin, is ubiquitously expressed by epithelial tissues while other classical cadherins such as N-, P- and VE cadherin have a more restricted

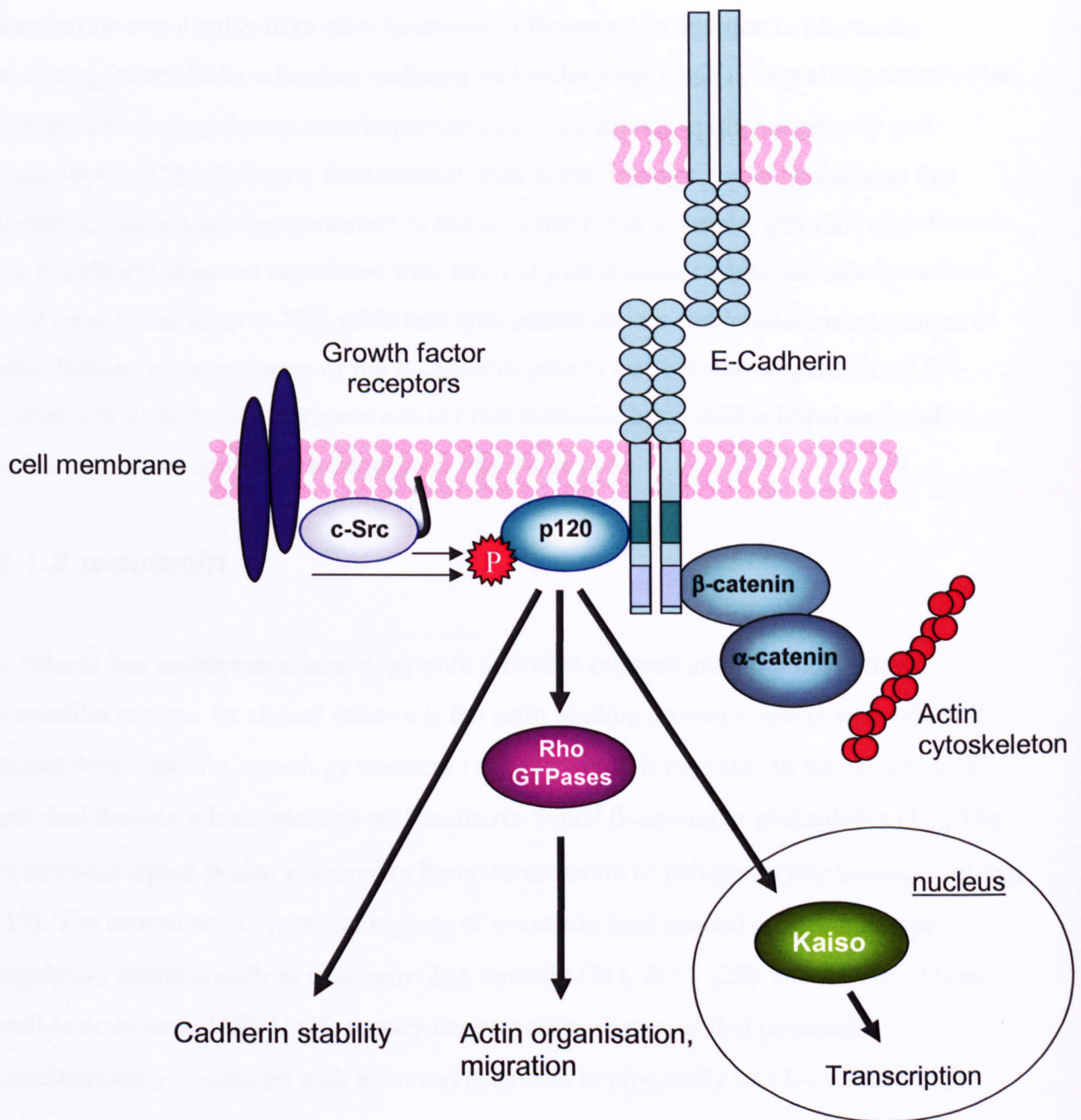


Figure 1-2 p120 and the adherens junction (AJ)

Cadherins are single pass transmembrane proteins which form calcium-dependent homophilic interactions with cadherin molecules of an adjacent cell via the first extracellular cadherin repeat. β -catenin interacts with the cadherin catenin-binding domain. α -catenin is recruited to the AJ via β -catenin and regulates interaction of the AJ with the actin cytoskeleton. p120 binds the cadherin juxtamembrane domain and this physical interaction is essential for the stable retention of cadherin at the plasma membrane. p120 can also directly modulate the activity of the Rho-family GTPases RhoA, Rac and Cdc42. In addition, p120 may enter the nucleus where it binds to and inhibits the transcriptional repressor activity of Kaiso. p120 is phosphorylated on tyrosine in response to the activation of receptor and non-receptor tyrosine kinases but the consequences of this for p120 function are enigmatic.

distribution and display important functional differences. In addition to physically mediating intercellular adhesion, cadherin molecules form a hub of signalling activity (14), mediate cell sorting during morphogenesis (2), and maintain epithelial polarity and homeostasis (15). With such fundamental roles in cell biology it is not surprising that cadherin function is often perturbed in human cancer. For example, germline mutations in the E-cadherin gene are associated with familial gastric cancer whilst somatic mutations have been found in up to 50% of diffuse type gastric cancer and lobular breast cancer. In other tumour types mutation of the E-cadherin gene is rare but downregulation of E-cadherin is a common occurrence and is often mediated by promoter hypermethylation or by the activity of specific transcriptional repressors (16).

1.1.2 α -catenin

α -catenin has no sequence homology with the other catenins and does not contain Armadillo repeats. Its closest relative is the actin binding protein vinculin with which it shares three vinculin homology domains (17). α -catenin is recruited to the AJ via its N-terminal domain which interacts with cadherin-bound β -catenin or plakoglobin (18). The N-terminal region is also involved in homodimerisation of unbound cytoplasmic α -catenin (19). The central and C-terminal regions of α -catenin bind several actin-binding or regulatory proteins such as α -actinin (20), vinculin (21), ZO-1 (22) and formin (23) as well as actin itself (24). Until recently the prevailing view was that α -catenin simultaneously associated with actin and β -catenin to physically link E-cadherin to the actin cytoskeleton. However, recent work demonstrating that the α -catenin- β -catenin and α -catenin-actin interactions are mutually exclusive has challenged this dogma (25). Current data suggests that the true role of α -catenin is to act as a molecular switch controlling the pattern of local actin polymerisation which is essential for AJ formation (26, 27). Loss of function mutations in the α -catenin gene are associated with impaired cell-cell adhesion and have been found in tumour cell lines derived from lung, prostate, ovarian and colon cancers (28).

1.1.3 β -catenin

β -catenin contains a central Armadillo domain flanked by an amino-terminal domain of approximately 130 amino acids and a carboxyl-terminal domain of 110 amino acids. The Armadillo domain consists of 12 Armadillo repeats, each of approximately 42 residues, which are packed together to form a long positively charged central groove via which β -

catenin interacts with cadherins, APC or TCF transcription factors in a mutually exclusive manner (29). The amino-terminus mediates the interaction with α -catenin and also contains several serine and threonine residues whose phosphorylation is important in regulating β -catenin stability. The carboxyl terminus has transcriptional activator properties (30).

Taken together current evidence indicates dual roles for β -catenin in cell adhesion and transcription (31). Firstly, β -catenin serves as an adaptor protein simultaneously binding E-cadherin and α -catenin, and hence is necessary for the recruitment of α -catenin to the AJ (32). Secondly, a tightly regulated nuclear pool of β -catenin can act as a transcriptional coactivator in the well described Wnt / β -catenin / TCF pathway (Figure 1-3) (33). In the absence of Wnt signalling, cytoplasmic β -catenin is bound by the APC / axin / glycogen synthase kinase-3 β (GSK3- β) complex and undergoes N-terminal serine / threonine phosphorylation. This targets β -catenin for ubiquitination and destruction (34-38). Wnt, an extracellular matrix-associated growth factor signals via its receptor, Frizzled, to inhibit GSK3- β causing stabilisation of cytoplasmic β -catenin. This pool of cytoplasmic β -catenin can then translocate to the nucleus where it cooperates with TCF transcription factors to induce expression of target genes including myc (39), cyclin D1 (40, 41) and MMP7 (42, 43). Importantly, the interactions of β -catenin with cadherin, APC and TCFs are mutually exclusive. Thus, by sequestering β -catenin in AJs, E-cadherin may act as an inhibitor of this pathway (44-46). Indeed, the ability to coordinate cell-cell adhesion with diverse cellular pathways via mutually exclusive competitive molecular interactions is a recurring theme in catenin biology.

The Wnt signalling pathway, with nuclear β -catenin as its main effector, has a critical role in morphogenesis and organogenesis via the regulation of migration, proliferation and differentiation (31). Deregulated activity of the Wnt pathway is a common event in oncogenesis (47). Although upregulation of Wnt-1 expression is an important event in mouse mammary tumour virus-induced carcinogenesis (48), increased production of Wnts does not appear to be a common event in human cancer. Rather, mutations in APC or β -catenin are the dominant mechanism by which the Wnt / β -catenin / TCF pathway is upregulated. For example, a loss of function mutation in APC can be detected in approximately 80% of spontaneous colorectal cancers (49). Furthermore, germline mutations of APC cause the cancer syndrome familial adenomatous polyposis coli (49). In-keeping with a critical role of the Wnt / β -catenin / TCF pathway in colorectal carcinogenesis, those tumours which retain functional APC often contain mutated β -

catenin which is resistant to GSK3- β -dependent phosphorylation and, consequently, degradation (50). Stabilising mutations in the β -catenin gene and, less commonly loss of function mutations in APC, have also been reported with varying incidence in many other human tumour types including breast, endometrial, gastric, hepatocellular, ovarian and pancreatic cancer (28).

1.1.4 Plakoglobin

Plakoglobin, also called γ -catenin, can substitute for β -catenin but preferentially binds to desmosomal cadherins (51). Plakoglobin shares overall 70% amino acid identity with β -catenin and as much as 80% within the Armadillo domain (29). Plakoglobin binds E-cadherin, α -catenin, APC, and TCF transcription factors, and is involved in cell adhesion as well as Wnt signalling. However, differences exist in both the N-terminal regulatory events and in the pattern of TCF target gene expression (29). The potential role of plakoglobin in cancer has been less extensively studied.

1.1.5 p120-catenin

p120 was initially identified as a Src substrate whose tyrosine phosphorylation correlated with transformation (52) and was subsequently recognised to be a catenin (8, 53, 54) and the prototypic member of a new subfamily of Armadillo proteins (55). Whilst p120 is ubiquitously expressed other family members, namely ARVCF (Armadillo Repeat gene deleted in Velo-Cardio-Facial syndrome), δ -catenin, p0071 and the plakophilins tend to have a tissue specific distribution and have been less extensively studied (55). Although over-expression of p120 can rescue depletion of ARVCF and vice versa (56) there is currently no strong evidence for functional redundancy in vivo.

In common with β -catenin, p120 exists in membrane, cytoplasmic and nuclear fractions and cadherins are both necessary and sufficient for recruitment of p120 to the cell membrane (9, 10). It is increasingly clear that p120 is a fundamental regulator of epithelial biology with functions which place it at a critical intersection in the regulation of cell-cell adhesion and, potentially, invasion. For clarity these regulatory roles will be considered in turn but, by virtue of crosstalk occurring at multiple levels, any such distinction is somewhat arbitrary.

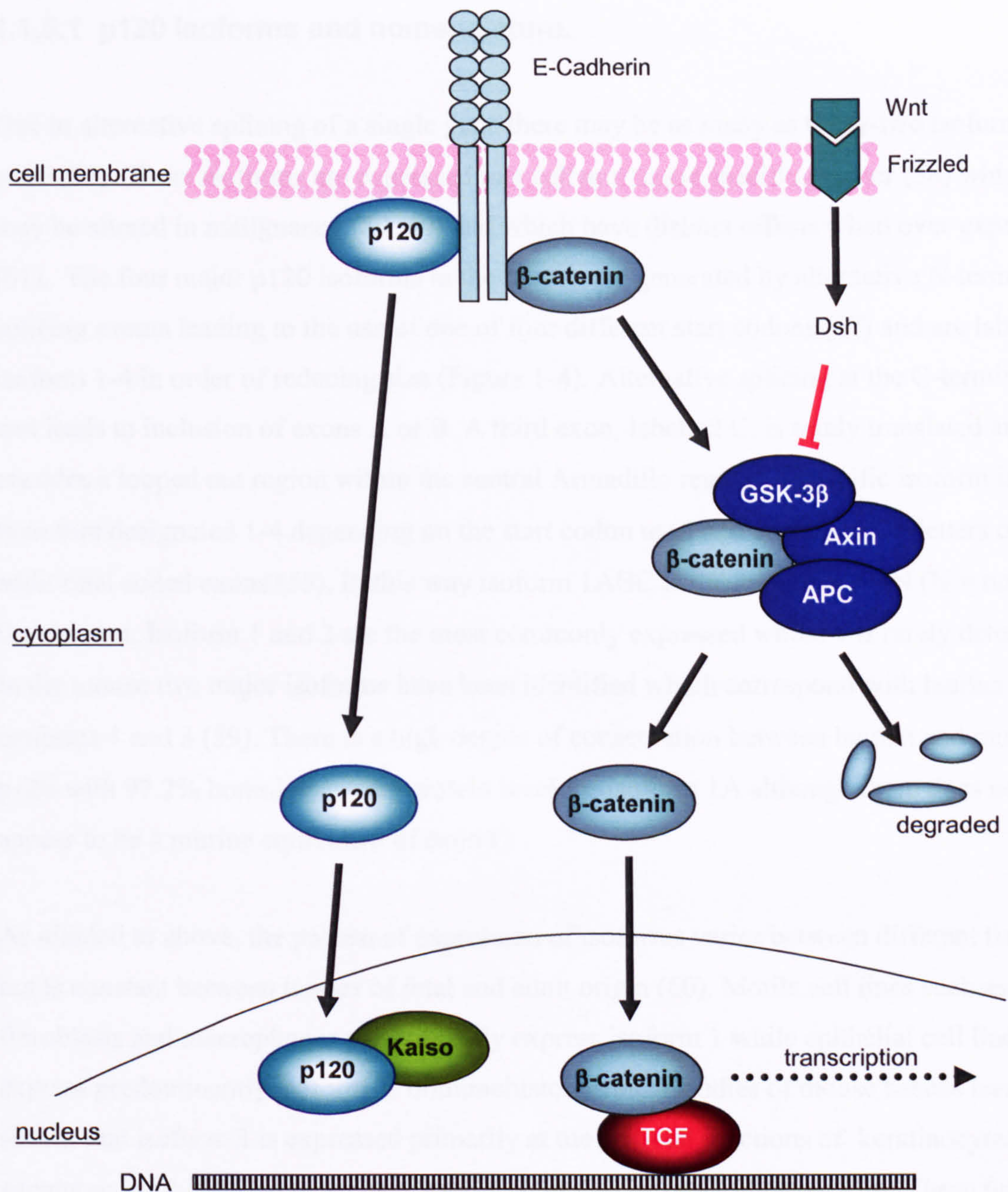


Figure 1-3 Catenins regulate gene expression.

In the absence of Wnt signalling cytoplasmic β-catenin is bound by the APC / axin / GSK-3β complex and phosphorylated by GSK-3β. Phosphorylation targets β-catenin for degradation by the ubiquitin-proteasome pathway. Wnt signalling via its receptor Frizzled activates Dishevelled (Dsh) which inhibits GSK-3β thus causing accumulation of β-catenin. Loss of function mutations in APC or stabilising mutations in β-catenin have a similar effect. Cytoplasmic β-catenin can then translocate to the nucleus where it interacts with the TCF family of transcription factors to promote transcription of target genes. Unlike β-catenin, p120 has no specific degradative pathway and its level in the cytoplasm is determined primarily by cadherin levels. In the nucleus p120 competitively inhibits the interaction of the transcriptional repressor Kaiso with DNA hence increasing transcription.

1.1.5.1 p120 isoforms and nomenclature.

Due to alternative splicing of a single gene there may be as many as thirty-two isoforms of p120 (57). These isoforms are expressed in a cell and tissue specific manner (58) which may be altered in malignancy (59, 60) and which have distinct effects when over-expressed (61). The four major p120 isoforms in the human are generated by alternative N-terminal splicing events leading to the use of one of four different start codons (57) and are labelled isoform 1-4 in order of reducing size (Figure 1-4). Alternative splicing at the C-terminal end leads to inclusion of exons A or B. A third exon, labelled C, is rarely translated and encodes a looped out region within the central Armadillo region. A specific isoform is therefore designated 1-4 depending on the start codon used and attributed the letters of any additional coded exons (55). In this way isoform 1ABC is the longest and 4N (N = none) is the shortest. Isoform 1 and 3 are the most commonly expressed whilst 4 is rarely detected. In the mouse two major isoforms have been identified which correspond with human isoforms 1 and 3 (59). There is a high degree of conservation between human and murine p120 with 97.2% homology at the protein level for isoform 1A although there does not appear to be a murine equivalent of exon C.

As alluded to above, the pattern of expression of isoforms varies between different tissues but is constant between tissues of fetal and adult origin (60). Motile cell lines such as fibroblasts and macrophages preferentially express isoform 1 while epithelial cell lines express predominantly isoform 3. Immunohistochemical studies of mouse tissues have shown that isoform 3 is expressed primarily at the cell-cell junctions of keratinocytes, squamous epithelium of oesophagus and also in villi of small intestine (58). These tissues stained weakly or not at all for isoform 1 which was present at cell-cell junctions of vascular endothelial cells, serosal epithelium, retina and intercalated discs of cardiomyocytes.

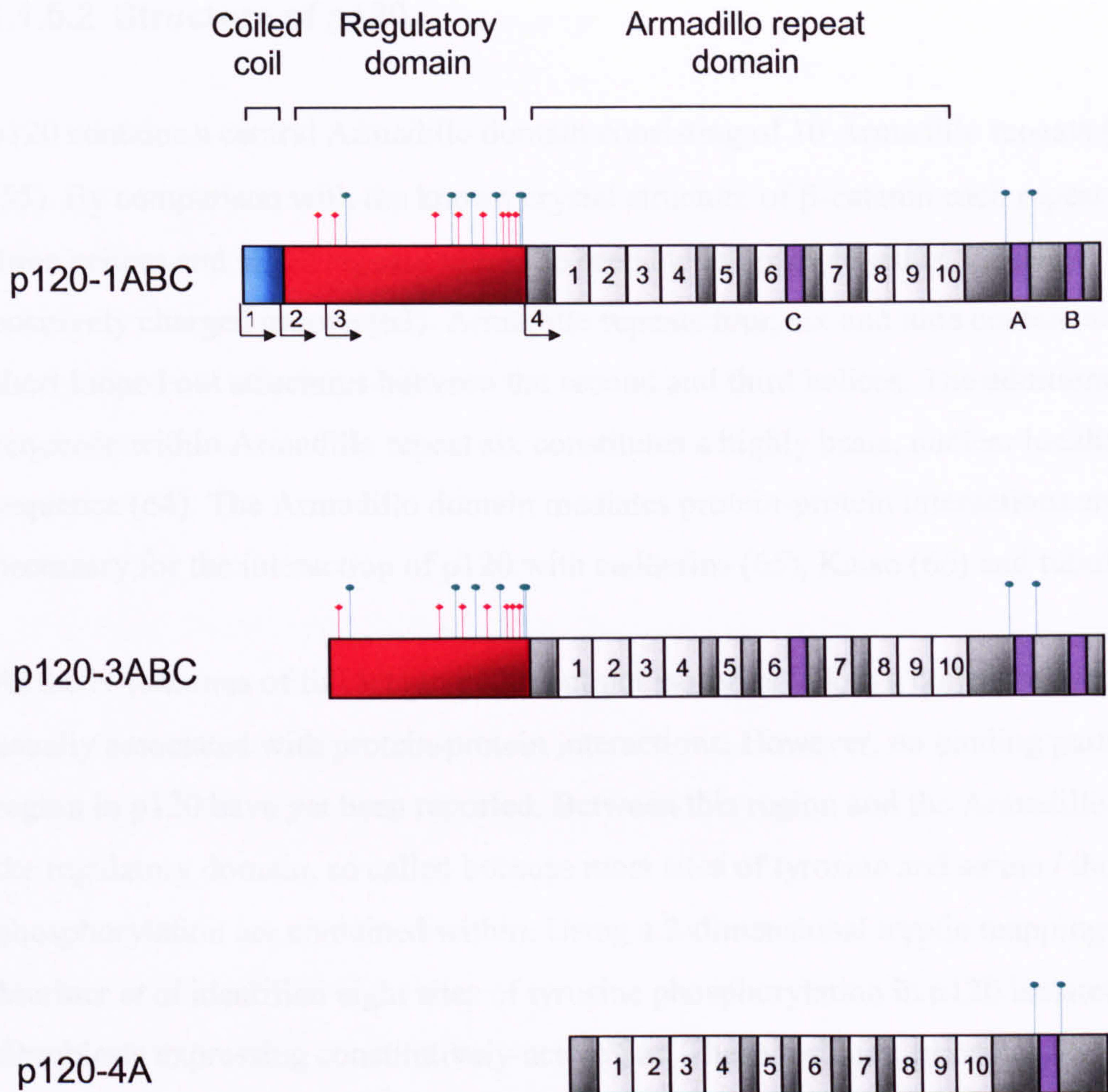


Figure 1-4 p120 structure and isoforms.

Four major p120 isoforms exist due to alternative N-terminal splicing events resulting in the use of different start codons. Furthermore, alternative splicing also determines the inclusion of exons A, B or C (purple boxes). The longest isoform, p120-1ABC, is illustrated along with two shorter isoforms for comparison. Arrows indicate the start sites for each major isoform. p120 isoform 1, but not isoforms 2-4, contains an N terminal coiled-coil domain (blue box). The regulatory domain (red box) contains all identified sites of tyrosine phosphorylation (96, 112, 228, 257, 280, 291, 296, 302; red diamonds) and six of the eight identified sites of serine / threonine phosphorylation (122, 252, 268, 288, 310, 312; but not 873 and 910; green circles). Tyrosine-96 is present in isoforms 1 and 2 but not 3. Isoform 4 lacks the entire regulatory domain. All isoforms contain a central Armadillo domain which consists of 10 Armadillo repeats. Exons A and B encode additional sequences in the carboxy terminal region whilst exon C encodes a short sequence within the Armadillo domain (adapted from (62)).

1.1.5.2 Structure of p120.

p120 contains a central Armadillo domain consisting of 10 Armadillo repeats (Figure 1-4) (55). By comparison with the known crystal structure of β -catenin each repeat consists of three helices and all 10 repeats would be predicted to pack together forming a long positively charged groove (63). Armadillo repeats four, six and nine contain additional short looped out structures between the second and third helices. The additional looped sequence within Armadillo repeat six constitutes a highly basic, nuclear localisation sequence (64). The Armadillo domain mediates protein-protein interactions and is necessary for the interaction of p120 with cadherins (65), Kaiso (66) and tubulin (67).

At the N-terminus of full length p120, but not isoforms 2-4, is a coiled-coil region, a motif usually associated with protein-protein interactions. However, no binding partners for this region in p120 have yet been reported. Between this region and the Armadillo domain lies the regulatory domain, so called because most sites of tyrosine and serine / threonine phosphorylation are contained within. Using a 2-dimensional tryptic mapping approach Mariner *et al* identified eight sites of tyrosine phosphorylation in p120 isolated from fibroblasts expressing constitutively-active Src. These residues, tyrosines 96, 112, 228, 257, 280, 291, 296 and 302 are all contained within the regulatory region (68). Of these only tyrosine-291 is not conserved in human, mouse and chicken. Subsequently, serine residues 122, 252, 268, 288, 312, and 873 and threonine residues 310 and 910 were also identified as sites of phosphorylation (69). Again, with the exception of C-terminal serine-873 and threonine-910, all of these sites are contained within the regulatory region.

Exon A encodes a twenty-one amino acid sequence in the carboxyl terminal region adjacent to serine-873. When present this sequence reveals a protein kinase C phosphorylation consensus sequence for serine-873 (69). Exon B encodes a highly negatively charged stretch of twenty-nine amino acids at the carboxyl terminus which contains a bona fide nuclear export signal (70). Exon C codes for six amino acids which, when expressed, may disrupt the nuclear localisation signal within Armadillo repeat six.

1.1.5.3 p120 regulates the surface retention of cadherin.

To date the main function identified for p120 at the AJ is to stabilise membrane-bound cadherin via the regulation of cadherin trafficking.

1.1.5.3.1 Cadherin Trafficking is a continuous process.

AJs are not static but rather must be amenable to rapid remodelling during, for example, embryogenesis or wound healing. Even in a confluent epithelial monolayer there is a continual endocytosis and recycling of cadherin molecules, albeit to a lesser extent than in subconfluent cells (71). A variety of proteins have been implicated in the exocytic and endocytic trafficking of cadherins although the mechanisms of cadherin trafficking are not yet fully elucidated (72). Exocytosis of cadherin requires sorting of cadherin and delivery in a polarised manner to the basolateral membrane. This requires a conserved dileucine motif in the cadherin JMD which is recognised by a specific adaptor complex at the trans golgi network which directs cadherin molecules into clathrin coated vesicles for polarised exocytosis (73). Several routes of cadherin endocytosis have been identified including clathrin-dependent (71, 74-76) and independent mechanisms (77) and caveolae (78, 79). Thus, multiple pathways of cadherin endocytosis and exocytosis may exist, the relative contribution of which may be determined by cellular context or specific regulatory events.

1.1.5.3.2 p120 regulates cadherin trafficking.

Recent evidence has identified p120 as a key determinant of cadherin trafficking and suggests that regulating the membrane retention of cadherin is the core function of p120 at the AJ (80). The first clue linking p120 to cadherin stability was the finding that the SW48 colon cancer cell line lacked p120 due to mutations in both alleles, had reduced levels of E-cadherin, and displayed impaired intercellular adhesion (81). Reconstitution of SW48 cells with exogenous p120 caused a 10-fold increase in E-cadherin levels and a rescue of epithelial morphology. Levels of E-cadherin RNA transcripts were unaltered but the half-life of E-cadherin was more than doubled indicating a post-transcriptional effect of p120. Deletional studies of the p120 Armadillo repeats showed this effect to be dependent on direct interaction of p120 with E-cadherin (81). Subsequently Xiao and Davis independently utilised siRNA to demonstrate a dose-dependent reduction in E-, P-, N- and VE-cadherin levels in response to p120 silencing in a variety of cell lines (82, 83). In the absence of p120 there was no difference in the rate of synthesis of cadherin molecules or in the rate of their delivery to the plasma membrane. However, on arrival at the plasma membrane cadherin molecules were rapidly endocytosed and degraded by a proteosomal / lysosomal pathway (82, 83). The level of p120 and the levels of cadherins were strictly related indicating that p120 functions as a “rheostat” to control the steady state level of cadherins (80). Furthermore, β -catenin and α -catenin are degraded in the absence of E-cadherin and thus the p120-cadherin interaction determines the stability of the entire complex. In this respect p120 has been called the “master-regulator” of the AJ (62).

This appreciation of p120's role has also provided a prism through which to re-evaluate earlier literature on cadherin biology. For example, the observation that expression of dominant negative cadherins (which have intact cytoplasmic but non-functional extracellular domains) causes downregulation of endogenous cadherin (84-86) is now explained by competition of exogenous and endogenous cadherin molecules for a limiting pool of p120 (82, 87). Importantly, work utilising DN-cadherins has also provided further evidence for the key role of p120, rather than β -catenin, as master regulator of the AJ (82). Firstly, forced expression of p120 but not β -catenin prevents downregulation of endogenous cadherin by DN-cadherins. Secondly, DN-cadherins require an intact JMD but not CBD if they are to cause downregulation of endogenous cadherin.

1.1.5.3.3 Mechanisms by which p120 may regulate cadherin trafficking.

Several non-exclusive models with varying levels of evidence have been proposed by which p120 could inhibit the internalisation and destruction of cadherin (80, 88).

1. p120 acts as a “cap” to inhibit endocytosis.

Fusion of the VE-cadherin cytoplasmic domain to the interleukin 2 receptor (IL-2R) extracellular domain dramatically enhanced IL-2R endocytosis suggesting that the cadherin cytoplasmic region encodes a signal for constitutive internalisation (75). Internalisation of the chimeric protein occurred via a clathrin mediated pathway and was inhibited by the over-expression of p120 and enhanced by a mutation in the cadherin JMD which prevented p120 binding. This inhibition of endocytosis was specific for VE-cadherin as overexpression of p120 did not interfere with the internalisation of other proteins known to undergo clathrin mediated endocytosis such as the transferrin receptor. p120 could not be detected with internalised cadherin. Taken together this suggested a model where p120 might function to “cap” the cytoplasmic domain, preventing the access of adaptor proteins which would recruit VE-cadherin to clathrin mediated endocytosis (75). It is not known whether the cytoplasmic domains of other cadherins contain similar signals for constitutive endocytosis. However, the JMD of E-cadherin interacts with Hakai, an E3 ubiquitin ligase, in a mutually exclusive manner with p120 (11). On binding E-cadherin Hakai induces ubiquitination and endocytosis of the cadherin complex. The effect of Hakai is specific for E-cadherin and whether similar molecules regulate other cadherins is unknown. As will be discussed below, both the interaction of p120 with VE-cadherin and Hakai with E-cadherin are regulated by tyrosine phosphorylation (11, 89).

2. p120 stabilises cadherin via the promotion of stable adhesions.

The constitutive turnover of cadherin is diminished as cells become confluent suggesting that the engagement of cadherin molecules in AJs might directly inhibit their endocytosis (71). Several lines of evidence implicate p120 in the conversion of initial cell-cell contacts into mature AJs. For example, cadherin molecules with a p120 uncoupling mutation in the JMD can mediate some cell-cell adhesion but cells typically display defects in cadherin clustering (90), cadherin mediated signalling (91), local organisation of the actin cytoskeleton and formation of stable cell-cell junctions (10). Thus by promoting the formation of stable AJs p120 may reduce the pool of free cadherin available for endocytosis.

3. p120 promotes recycling from the endocytic pathway to the plasma membrane.

Experimental evidence so far has not been of sufficient temporal or spatial resolution to definitively demonstrate whether p120 acts to inhibit internalisation or whether p120 is involved in trafficking already endocytosed cadherin away from destructive pathways back to the plasma membrane. This possibility is suggested by the finding that p120 promotes the trafficking of newly synthesised N-cadherin to intercellular junctions (92). Specifically, N-cadherin associates with p120 (via the p120 Armadillo domain) in the perinuclear golgi region immediately after it is synthesised (93). The N-terminal domain of p120 then interacts with kinesin heavy chain thus facilitating transport of the N-cadherin-catenin complex along the microtubule network to the cell membrane (67, 92). However, whether p120 may similarly regulate the trafficking of endocytosed cadherin is not known.

1.1.5.3.4 p120 similarly stabilises cadherins in vivo.

Recently the in vitro role of p120 as master regulator of the AJ has been confirmed in vivo. The depletion of p120 with morpholino oligonucleotides (stable oligonucleotides complementary to the translation start site which effectively block translation of the target gene) in *Xenopus* embryos caused a loss of AJ proteins which was associated with gross defects in early embryogenesis (56). Similarly, knockout of p120 was lethal at an early stage of murine embryogenesis (62). However, the creation of transgenic mice with targeted deletion of p120 in the skin or salivary gland was possible and resulted in loss of E-cadherin, α - and β -catenin in these tissues (94, 95). In the salivary gland this was

associated with severe defects in epithelial polarisation and glandular morphogenesis. Depletion of p120 in the skin also caused loss of AJ components although, surprisingly, no overt defect in morphology. Ultrastructural analysis showed desmosomes to be intact and functional studies identified no defect in barrier function. However, in-keeping with the phenotype of p120 deficient cell lines, primary keratinocytes derived from p120 null skins were unable to form effective junctions when cultured in vitro. The requirement for AJs to exist for the formation of tight junctions and desmosomes in vitro but not in vivo is intriguing and may reflect the fundamentally different environmental contexts. Specifically, keratinocytes in vivo are packed together and adopt a cuboid morphology whereas cultured cells in vitro are less densely packed and are flat rather than columnar so that the surface area for adhesive interaction is reduced. In vitro there may also be cell-substratum signalling or actin dynamics which do not favour AJ formation.

Despite this evidence confirming p120 as a master-regulator of the AJ in vertebrates, the depletion of p120 did not alter levels of AJ proteins in *Drosophila* or *C. elegans*.

Furthermore, p120 was not required for their development even though the genomes of these organisms lack potentially redundant p120 family members (96, 97). However, in both organisms loss of p120 potentiated the effect of mutations in cadherin or other catenins suggesting a positive but non-critical role for p120 at the AJ. The critical requirement for p120 in vertebrate, but not invertebrate, development is likely to reflect the increased complexity and regulation of the AJ in evolutionary higher organisms.

1.1.5.4 p120 regulates Rho GTPases.

Multiple lines of evidence link p120 to the Rho family of small GTPases which includes RhoA, Rac and Cdc42. Rho GTPases control a wide variety of signal transduction pathways impacting on cell polarity, microtubule function, membrane trafficking and survival (98). However, it is their key regulatory role in control of the actin cytoskeleton, adhesion and motility which is most relevant in understanding the potential role of Rho GTPases in neoplastic invasion (99, 100).

1.1.5.4.1 The activity of Rho GTPases is tightly regulated.

Rho family GTPases cycle between an inactive GDP-bound state and an active GTP-bound form (Figure 1-5). These processes are controlled by specific regulatory proteins. Rho GTPase activating proteins (GAPs) catalyse the hydrolysis of GTP to GDP and therefore cause inactivation. Conversely, Rho guanine nucleotide exchange factors (GNEFs) promote exchange of GDP for GTP and hence increase activity. Rho GTPases are often

bound to a third class of regulatory protein, guanine dissociation inhibitors (GDIs), which stabilise the Rho-GDP interaction maintaining the inactive state. Binding of the Rho-GDI complex to an activated receptor promotes release of the Rho GTPase which is then free to be activated by local GNEFs. The activity of Rho GTPases is thus tightly controlled both spatially and temporally within the cell to ensure specific and localised effects (98, 101).

1.1.5.4.2 Rho GTPases regulate the actin cytoskeleton.

The activity of each Rho GTPase is associated with a specific type of remodelling of the actin cytoskeleton (102). Increased activity of RhoA results in the formation of contractile actin-myosin filaments known as stress fibres and promotes the formation of integrin-containing cell-matrix focal contacts. Rac activity promotes the formation of a meshwork of actin filaments at the cell periphery which form lamellipodia. Cdc42 activity causes the formation of peripheral actin rich protrusions known as filopodia. Integrin dependent cell-matrix adhesion, cadherin-based cell-cell adhesion and cellular motility are all processes dependent on the integrity and appropriate remodelling of the actin cytoskeleton and Rho GTPases are therefore in a pivotal position to regulate these activities. For this reason aberrant activity of Rho GTPases might be expected in invasive carcinomas and indeed increased levels and activity of Rho GTPases have been reported in breast, colon and lung cancer (103). In vitro studies have demonstrated a direct role for Rho and Rac proteins in invasion (103). However, the influence of Rho GTPases on adhesion and invasion is complex and may vary depending on the particular cell context or be influenced by specific integrin-derived signals (104). One such example is the activation of Rac by its GNEF, Tiam1, which promotes adhesion in Ras- or Src-transformed MDCK cells (105, 106) but has the opposite effect in a breast cancer cell line (107).

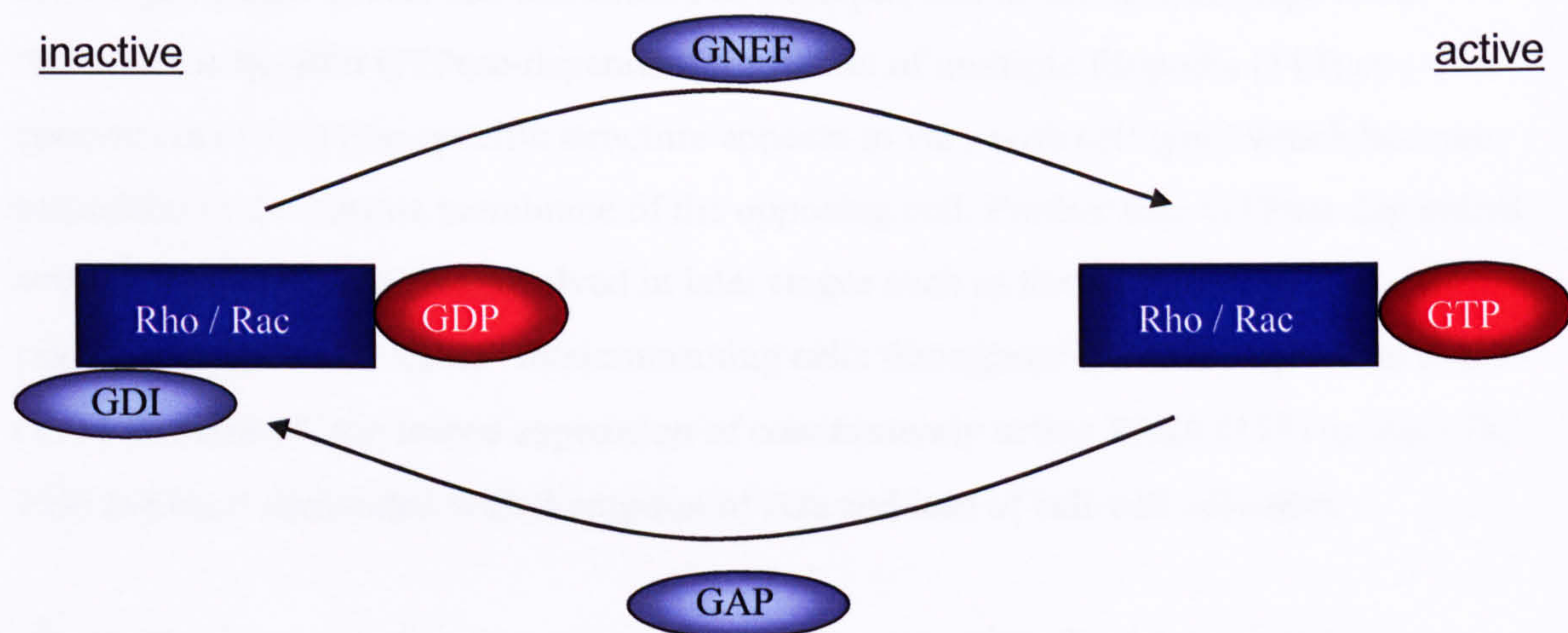


Figure 1-5 Regulation of Rho GTPases

Rho GTPases exist in either an inactive GDP-bound or active GTP-bound state. Guanine nucleotide exchange factors (GNEFs) promote the exchange of GDP for GTP thus favouring activation whilst GTPase-activating proteins (GAPs) catalyse the hydrolysis of GTP to GDP causing inactivation. GDP-dissociation inhibitors (GDIs) associate with GDP bound Rho proteins keeping them in the inactive state. p120 can activate Rac and Cdc42 via the recruitment of Vav2, a GNEF. p120 also directly interacts with and inhibits RhoA in a GDI-like manner.

1.1.5.4.3 Rho GTPases regulate the AJ.

The activity of Rho GTPases has been associated both positively and negatively with the AJ. Several studies have demonstrated a requirement for Rho GTPases in AJ formation (108-111). Indeed, Rho GTPase driven actin polymerisation directly determines the physical strength of intercellular adhesion (112). This activity of Rho GTPases is required at multiple stages of cell-cell adhesion. For example, one of the earliest steps in AJ formation is the Rho GTPase-dependent formation of multiple filopodia (113) or lamellipodia (114) (the specific structure appears to vary with cell type) which become embedded in the plasma membrane of the opposing cell. Further Rho GTPase dependent actin reorganisation is also involved in later stages such as the formation of a circumferential actin “cable” interconnecting cells throughout the entire epithelial sheet (113). In contrast, the forced expression of constitutively active RhoA (115) or Rac (78, 116) has been associated with disruption of AJs and loss of cell-cell adhesion.

The molecular mechanisms by which Rho-GTPases regulate the AJ, positively or negatively, are poorly defined although actin regulation appears to be a critical event (103, 117, 118). The apparently contradictory data showing both positive and negative effects on adhesion presumably reflects a need for tight spatio-temporal regulation if productive adhesions are to be formed. One possible molecular explanation was provided by Sahai who identified two opposing pathways by which Rho signalling may regulate AJs (115). Firstly, Dia-dependent actin polymerisation stabilised AJs. Secondly, ROCK-mediated acto-myosin contractility disrupted AJs. Which pathway dominated depended on the level of Rho activity and on the expression of particular Rho members. Low levels of RhoA activity favoured signalling through Dia whereas higher levels preferentially signalled to ROCK. In contrast, even low levels of activity of RhoC, a close relative of RhoA linked to metastasis (119), favoured signalling to ROCK. (115).

A potential mechanism by which Rac and Cdc42 may promote AJ formation is provided by IQGAP1 which localises to cell-cell junctions and negatively regulates adhesion by binding β -catenin and displacing α -catenin from the AJ (120). Active Rac and Cdc42 bind and sequester IQGAP1 thus preventing its interaction with β -catenin and positively regulating adhesion (121). Rho GTPases may also regulate the endocytic trafficking of AJ proteins (122) and expression of dominant negative (DN)-Rac or Ccd42 mutants in MDCK cells effectively blocked trafficking of E-cadherin from the trans-Golgi network to the basolateral membrane (123).

1.1.5.4.4 Rho-family GTPases are regulated by cytoplasmic p120.

The first suggestion that p120 may act in concert with Rho family GTPases to regulate the actin cytoskeleton came from the distinct phenotype observed in NIH3T3 fibroblasts forced to over-express p120 (124). This caused cells to adopt a so-called dendritic morphology characterised by long branching processes suggestive of active actin remodelling. Subsequently three groups independently demonstrated that over-expression of p120 in fibroblasts resulted in loss of actin stress fibres and focal adhesions, acquisition of a dendritic phenotype and increased cell motility (125-127). Critically, this phenotype could be suppressed by co-transfection of cells with DN-Rac or DN-Cdc42 and was exacerbated by expression of DN-RhoA. Affinity precipitation assays confirmed that Rac and Cdc42 activity was increased whereas RhoA activity was diminished. Finally, sequestration of p120 from the cytoplasm to the membrane by over-expression of E-cadherin abrogated any effects on GTPase activity. Although these experiments utilised supra-physiological amounts of p120 they provide proof-of-principle that p120 regulates Rho GTPases and that this effect is mediated by free cytoplasmic rather than cadherin-bound p120.

Similar effects of cytoplasmic p120 have subsequently been identified in epithelial cells. For example, RNAi-mediated depletion of p120 in the SKBR-3 breast cancer cell line (which lacks E-cadherin and in which p120 is cytoplasmic) caused an increase in RhoA activity but no change in Rac activity (128); re-localisation of p120 from the membrane to the cytoplasm increased Rac and Cdc42 activity in a pancreatic cancer cell line (129); and TGF β -treated LIM1863 colorectal cancer cells have increased cytoplasmic p120 which is associated with inhibition of RhoA activity (130). Despite the evidence that over-expression of p120 appears to inhibit RhoA and activate Rac in most cell types there are exceptions. For example, in keratinocytes the over-expression of p120 activated RhoA in a Ras / PI 3-K dependent manner but had no effect on Rac or Cdc42 (131). In MCF-10A breast epithelial cells RNAi-mediated depletion of p120 increased Rac activity whilst over-expression of p120 or the introduction of a p120 uncoupling mutation in the E-cadherin JMD, both of which caused cytoplasmic accumulation of p120, inhibited Rac activity (132).

A potential molecular explanation for Rac and Cdc42 activation by p120 was elucidated by Noren *et al* who observed the co-immunoprecipitation and hence potential recruitment of Vav2, a GNEF for Rac1 and Cdc42, by p120 (125). Co-expression of a dominant negative Vav2 mutant with p120 was sufficient to abrogate p120's ability to induce a dendritic phenotype in fibroblasts. Taken together this strongly suggested that activation occurred

via the p120 / Vav2 complex (125). The mechanism by which cytoplasmic p120 might inhibit Rac activation in MCF-10A cells has not been addressed (132).

p120 appears to inhibit RhoA by direct GDI activity although p120 shares no sequence homology with known Rho GDIs. However, using purified proteins, p120 demonstrated *in vitro* RhoGDI activity (127). Similarly, in *Drosophila* p120 was found to interact directly with Rho1 the *Drosophila* homolog of RhoA. Consistent with a GDI role, p120 interacted preferentially with GDP rather than GTP-bound Rho1 (133). The inhibition of RhoA activity by cytoplasmic p120 in LIM1863 cells was associated with formation of a p120-RhoA complex, again consistent with p120 having direct GDI activity.

1.1.5.5 p120 and RhoGTPases cooperate in AJ formation and cadherin-activated signalling.

In addition to the studies linking cytoplasmic p120 to the regulation of Rho GTPases there is also data suggesting a more spatially restricted and perhaps more physiological role for membrane localised p120 in mediating the activation of RhoGTPases following cadherin engagement. This might reflect a situation where cytoplasmic p120 binds RhoA causing direct inhibition via its RhoGDI activity. On recruitment to cadherin complexes at the basolateral membrane p120 could then release RhoA creating a pool available for activation by local GNEFs which may then contribute to AJ function (62). A paradigm for this type of spatio-temporal control is the binding of Rho-GDI complexes to CD44 which is coupled to release and local activation of Rho (101). Alternatively, as discussed below, cadherin-bound p120 may be an integral part of the cadherin-activated signal transduction machinery as evidenced by the inability of cadherins with a p120 uncoupling mutation to activate Rac when ligated (91, 134).

1.1.5.5.1 Cadherin-activated signalling.

Rac, PI 3-K, SFKs and EGFR are recruited to and activated at nascent AJs (135-138). These events can be blocked by an E-cadherin neutralising antibody (DECMA-1). However, to clarify whether such events were cadherin-*dependent* (that is cadherin engagement simply functioned to bring cell membranes into apposition facilitating juxtacrine signalling events) or whether they were truly cadherin-*activated*, several groups developed model systems where cadherin expressing cells are plated at low density and in the absence of serum on to an artificial surface expressing recombinant cadherin extracellular domains (14, 139-142). This reductionist approach has provided proof that cadherin ligation in the absence of other juxtacrine interactions is sufficient to initiate

intracellular signalling and that this is important for coordinated changes in the actin cytoskeleton required for the formation of stable cell-cell adhesions. The two molecules most implicated are the small GTPase, Rac and the lipid kinase, PI-3 kinase.

E-cadherin expressing cells plated on a substrate coated with E-cadherin extracellular domain underwent rapid cell spreading with the development of prominent lamellipodial protrusions (143). Both Rac and PI 3-K were activated and were co-localised with AJ components at the leading edge of lamellipodia. Rac activation consisted of an early PI 3-K independent phase followed by a PI 3-K dependent amplification phase. The conversion of initial contacts to broad zones of adhesion required the activity of both Rac1 and PI 3-K. PI 3-K inhibition could be overcome by a DA-Rac mutant consistent with PI 3-K acting as an upstream activator of Rac (143). Critically, a minimal p120-uncoupling mutation in the E-cadherin JMD prevented recruitment or activation of Rac and the formation of stable adhesions although activation of PI 3-K was not altered (91). This was highly suggestive of a role for p120 in cadherin-mediated Rac activation. Subsequent work by Gavard *et al* has further implicated p120 as an essential player in this process. N-cadherin expressing myogenic C2 or S180 cells plated on a recombinant N-cadherin substrate typically underwent two experimentally distinguishable responses (134). Firstly, the induction of lamellipodial protrusions associated with new actin assembly which was PI 3-K and Rac-dependent. Secondly, the PI 3-K-independent / Rac-dependent reorganisation of N-cadherin, catenins and actin filaments into higher order “cadherin adhesions” located at the tips of lamellipodia. The formation of “cadherin adhesions” was inhibited by activated RhoA. Silencing of p120 by RNAi prevented both lamellipodium extension and cadherin adhesion formation from occurring. Lamellipodia extension but not maturation of contacts into “cadherin adhesions” could be rescued by expression of DA-Rac. Additionally, high level over-expression of p120 had an inhibitory effect, apparently by inhibition of Rac and this could be prevented by sequestration of p120 at the membrane by a cadherin JMD construct. This would suggest that membrane-localised p120 is essential for the activation of Rac by cadherin engagement and that an excess of cytoplasmic p120 may inhibit this activation, perhaps by sequestering GEFs.

1.1.5.5.2 Regulation of Rho GTPases by p120 in vivo.

Several lines of evidence suggest that the effects of p120 on Rho GTPases identified in vitro may be relevant in vivo. Over-expression of p120 in *Drosophila* embryos enhances the Rho1 null phenotype consistent with it being a negative regulator of Rho1 activity (133). In *Xenopus* the carefully titrated expression of DN-RhoA, DA-Rac or C-cadherin was sufficient to rescue the defect seen in gastrulation resulting from p120 depletion (56).

This would be consistent with a role for endogenous p120 in inhibiting RhoA, activating Rac and stabilising cadherin. Whether DN-RhoA and DA-Rac acted by enhancing the adhesive or signalling function of residual cadherin or by regulating the trafficking of cadherin was not addressed. Finally, levels of active RhoA were increased in the epidermis of mice with conditional deletion of p120 in the skin (95). Taken together, these in vivo findings are in keeping with the in vitro data suggesting that, in many cell types, p120 inhibits RhoA and activates Rac.

1.1.5.6 p120 regulates gene transcription via Kaiso.

Utilising a yeast-two hybrid assay with p120 as bait, Daniel *et al.* identified a novel transcriptional repressor which they named Kaiso (66). Kaiso was an addition to the BTB/POZ (Broad complex, Tramtrak, Bric a brac, Pox virus and Zinc finger) family, containing an amino-terminal protein-protein interaction POZ domain and a carboxy-terminal DNA-binding zinc finger domain. Other members of this family include BCL-6 and PLZF which have been causally implicated in non-Hodgkin lymphoma and acute promyelocytic leukaemia respectively (144). Kaiso is a bi-modal DNA binding protein able to recognise both methyl-CpG sequences and the non-methylated sequence CTGCNA (145, 146). The interaction of p120 with Kaiso is mediated by p120 ARM repeats 1-7 and, interestingly, the carboxy-terminal DNA-binding region of Kaiso rather than the N-terminal protein-protein interaction domain. Consequently the interaction of p120 with Kaiso inhibits Kaiso-DNA binding in vitro either via direct steric hindrance or conformational change (146). Similarly, in vivo studies demonstrated that both Kaiso-DNA interaction and Kaiso-mediated transcriptional repression are inhibited by over-expressed p120 (147-149). Inhibition of Kaiso-mediated transcriptional repression requires an intact p120 nuclear localisation sequence suggesting that p120 inhibits Kaiso in the nucleus rather than by sequestering Kaiso in the cytoplasm (64, 147, 149). This action of p120 is reminiscent of, but mechanistically different to, that of β -catenin. Specifically, in the absence of β -catenin TCFs repress transcription either by acting in an endogenous dominant negative fashion or by recruiting transcriptional repressors such as Groucho. Binding of β -catenin does not displace TCFs from DNA but rather functions to contribute a transactivation domain and to recruit other components of a larger promoter complex (47). In contrast, Kaiso appears to repress transcription via recruitment of the histone deacetylase-containing N-CoR complex and the role of p120 is to dissociate this entire complex from DNA, hence facilitating transcription (148, 150).

Several Kaiso target genes with potential roles in development and / or cancer have now been identified including MMP7 (149), rapsyn (151), MTA2 (150), Wnt11 (147), Siamois, c-Fos, Cyclin D1 and c-Myc (148). Crucially, some of these are also targets of the canonical Wnt pathway and therefore p120-Kaiso and β -catenin-TCF may regulate target genes in parallel (Figure 1-3) (152). A further level of interaction is provided by the ability of Kaiso to interact directly with TCFs thus providing a second mechanism by which it can recruit N-CoR to Wnt target genes (148). It is unclear what upstream pathways might regulate the p120-Kaiso interaction. Unlike β -catenin, a dedicated destructive pathway has not been identified for p120 and there is no evidence that p120 acts downstream of Wnt receptor activation (144). Therefore potential levels of regulation could include: 1) altered cadherin-p120 binding thus leading to an increased pool of cytoplasmic p120; 2) altered nucleo-cytoplasmic shuttling of p120; or 3) a p120 isoform switch which can, in itself, influence nuclear localisation. An exciting but untested hypothesis is that parallel regulation of canonical Wnt target genes by TCFs and Kaiso may allow for differential regulation by cadherins, acting via β -catenin and p120, versus Wnt signalling acting via β -catenin alone (152).

Recent evidence suggests biologically significant roles for Kaiso both in development and cancer. Depletion of Kaiso caused severe defects in gastrulation of *Xenopus* embryos (147, 153). In contrast deletion of the mouse Kaiso gene did not result in any obvious phenotype perhaps due to redundancy with the protein Kaiso-like 1 which is absent in amphibians. Alternatively, as zygotic transcription is detected at the two cell stage in mice but not until the 5000 cell mid-blastula stage in frogs, a requirement for Kaiso in ensuring delayed activation of genes may have no murine counterpart (51).

When the Kaiso-null mouse was crossed with the *Apc*^{Min/+} mouse, a model for human adenomatous polyposis (154), a significant reduction in intestinal tumorigenesis was observed (51). In addition Kaiso was found to be upregulated in invasive tumours developing in *Muc2*^{-/-} mice and was present, although not consistently upregulated, in human colorectal tumours (51). Tumorigenesis in the mouse intestine is dependent on DNA methylation (155) and it seems likely that Kaiso could promote intestinal tumorigenesis via the repression of methylated tumour suppressor genes. The elucidation of p120's role in this process and how it might be regulated will be fascinating.

1.1.5.7 p120 is altered in human cancer.

An alteration in the protein level or localisation of p120 is a common observation in human cancers. However, the incidence of other potential tumour related changes such as altered p120 isoform expression or elevated tyrosine phosphorylation are less well defined.

1.1.5.7.1 p120 is commonly reduced or mis-localised in cancer.

In normal epithelial tissues p120 co-localises with E-cadherin at cell-cell junctions and is not detectable in the cytoplasm. To date there are almost 20 reports in the pathology literature describing altered levels or localisation of p120 in colorectal, oesophageal, gastric, pancreatic, bladder, prostate, endometrial, cutaneous squamous cell, melanoma, non-small cell lung and breast tumours (summarised in (156)). The most useful studies attempt to correlate p120 staining with that of other AJ components although a significant number have studied p120 in isolation. Also differences in the categorisation of immunohistochemical staining patterns by different authors hinders cross comparison of studies. The largest series is that of Bellocq who, utilising a tissue array containing 557 colorectal tumours, reported complete loss of p120 in 5.4% of cases, re-localisation of p120 from the membrane to the cytoplasm in 52.9% and normal membranous distribution in 41.7% (130). All tumours with absent p120 demonstrated absent or cytoplasmic E-cadherin. In tumours with cytoplasmic localisation of p120, E-cadherin was absent in 36%, cytoplasmic in 62% and membrane-localised in only 2%. Smaller colorectal studies report a higher incidence of complete loss of p120 expression in 18-23% of cases (157-159).

In a study of 193 non-small cell lung cancer specimens Bremnes reported absent or low membranous p120 staining in 61% of cases, intermediate staining in 33% and high in 6% . Again, p120 and E-cadherin expression were significantly correlated. A second study confirmed cytoplasmic redistribution of p120 in 114 of 143 (79.7%) of non-small cell lung cancers (160).

In invasive ductal breast cancers complete loss of p120 occurs in 2.1-10% of cases with reduced membranous staining in 40-57% and normal membranous staining in 30.8-50% (161, 162). Re-localisation of p120 to the cytoplasm appears to be a relatively uncommon event, occurring in approximately 10% of cases. In contrast, lobular breast cancers in which E-cadherin was undetectable in 78.3% and cytoplasmic in 8.7% demonstrated re-localisation of p120 to the cytoplasm in 88% of cases (162). Similar results were obtained in lobular carcinoma in situ and even atypical lobular hyperplasia underlining this as an early event in the neoplastic process (163). A high incidence of cytoplasmic redistribution

(90%) also occurred in diffuse-type gastric cancers, another tumour type characterised by loss of E-cadherin expression (164).

In most studies the reduction or loss of p120 correlated with the grade and stage of the tumour (157, 158, 165-169). Unsurprisingly then, several studies also report altered p120 to correlate with poor prognosis (130, 158, 165, 166, 168). However, in only one study did multivariate analysis suggest aberrant p120 expression to be an independent prognostic factor (168).

Viewed in their entirety these descriptive studies are consistent with two key observations arising from the mechanistic p120 studies described above. Namely, 1) in the absence of p120, E-cadherin cannot be stably retained at the AJ; and 2) E-cadherin is necessary and sufficient to recruit p120 to the membrane and in its absence p120, which is relatively stable, can accumulate in the cytoplasm. The potential consequences of these observations for cancer will be considered more fully below.

Finally, two important questions arise from these studies. Firstly, the mechanism by which p120 is lost in a minority of tumours, whether mutation, promoter methylation, transcriptional repression or post-translational modification is not known. Secondly, despite the evidence that approximately 5-20% of colon, breast, pancreatic and bladder cancers have undetectable p120, to date only one tumour derived cell line has been identified in which p120 is absent (81). Possible explanations are that the downregulation of p120 is a reversible event and is dependent on the tumour microenvironment. Alternatively, p120 null cells may, for as yet unknown reasons, be selected against during the establishment of cell lines in vitro.

1.1.5.7.2 Alternative splicing leads to altered p120 isoform expression in cancer.

Malignant cell lines often express an altered pattern of p120 isoforms in comparison to their tissue of origin (60). One such example is the absence of isoform 4A in cell lines derived from melanoma and cutaneous squamous cell carcinomas despite its presence in normal keratinocytes and melanocytes (61). Similarly, exon B which is present in p120 from healthy pancreatic, prostate and colonic epithelia was absent in tumour tissues from the corresponding organs (61). In comparison to normal thyroid epithelium, four anaplastic thyroid carcinoma cell lines had an upregulation of p120 isoform 1 (170). Similarly, cell lines derived from a colorectal primary tumour and metastases from the same patient demonstrated upregulation of isoform 1 in the metastatic line (171). Interestingly expression of constitutively-active Src in MDCK cells is sufficient to cause an

upregulation of isoform 1 and concomitant downregulation of shorter isoforms (59). Furthermore, Snail, Slug or E47 induced epithelial-mesenchymal transition (EMT) (discussed in 1.3.1.2) in MDCK cells was associated with a switch from p120 isoform 3 to isoform 1 (162, 172). A similar isoform switch occurred during c-Fos induced EMT in mouse mammary epithelial cells (173) although in other models of EMT such as TGF β and TNF α induced EMT in LIM1863 colon cancer cells no change in p120 isoform occurred (130).

Unfortunately, despite the existence of antibodies which specifically detect p120 isoform 1 (174) no large scale immunohistochemical study to identify the prevalence of p120 isoform switching in human cancer specimens has been performed.

1.1.5.7.3 Tyrosine Phosphorylation of p120.

A constitutive increase in p120 tyrosine phosphorylation has been detected in a variety of tumour derived cell lines (175, 176). However, whether and with what frequency elevated tyrosine phosphorylation of p120 may occur in cancers has barely been addressed.

Nishimura *et al* demonstrated increased tyrosine phosphorylation of 100-130 kDa proteins in cell lysates from lung cancer tissue in comparison to adjacent normal mucosa and showed that a significant component of this could be attributed to p120 (177). However, large scale immunohistochemical studies have been impossible to date due to the lack of p120 phosphorylation state specific antibodies.

1.2 Tyrosine phosphorylation regulates the AJ.

The AJ is an active hub of tyrosine kinase signalling. Specifically, Src family kinases (178, 179) and EGFR (137) are activated on cadherin engagement and participate in “outside-in” signalling pathways. Conversely, phosphorylation of AJ proteins by SFKs, EGFR or other receptor tyrosine kinases (RTKs) can mediate “inside-out” signalling by influencing the adhesive state or stability of the AJ (180).

The major tyrosine kinase substrates within the AJ are p120, β -catenin, and plakoglobin, but not α -catenin (180). For example, p120 is heavily phosphorylated in v-Src transformed cells (52) and in response to EGF, PDGF, CSF-1 and VEGF (181-184). Similarly β -catenin and / or plakoglobin are phosphorylated in v-Src transformed cells (185, 186) and in response to EGF (187-190), HGF (187), TGF- α (191) and IGF-1 (192). In most models

cadherins are only weakly phosphorylated (185-187, 193). Nevertheless, cadherin phosphorylation has recently emerged as a significant regulatory event.

In this section we consider some of the tyrosine kinases linked to phosphorylation of AJ proteins and consider the functional consequences of tyrosine phosphorylation for the AJ. Kinases may also regulate the AJ via mechanisms other than direct phosphorylation of AJ proteins such as via the regulation of acto-myosin contractility. In addition aberrant kinase activity can drive changes in gene transcription leading to the phenomenon of EMT and this is considered separately in section 1.3.1.2.

1.2.1 *Src family kinases (SFKs)*

1.2.1.1 Structure function relationships of SFKs.

There are nine members of the Src family of kinases: Src, Yes, Fyn, Lyn, Lck, Hck, Blk, Fgr and Yrk. Most are expressed primarily in haematopoietic cells whilst Src, Yes and Fyn have a ubiquitous distribution with particularly high levels in platelets, neurons and osteoclasts and are also enriched at epithelial cell-cell junctions (138, 194). SFKs are basic components of the cell signalling machinery and are involved in diverse signalling pathways promoting growth, survival, motility and invasion which originate from receptor tyrosine kinases, integrins, cadherins, cytokine receptors and G-protein coupled receptors.

c-Src (hereafter referred to as Src) consists of an N-terminal unique region containing a myristylation site which is essential for membrane localisation, an SH3 domain, an SH2 domain, a kinase domain and a C-terminal regulatory sequence (195). The SH3 domain mediates protein-protein interaction by binding proline rich sequences whilst the SH2 domain binds regions containing phosphorylated tyrosines. Tyrosine-416 (by convention numerical positioning refers to chicken Src, the corresponding tyrosine residue in human Src is at position 418) which lies within the activation loop of the kinase domain undergoes autophosphorylation and is often used as a surrogate marker of Src kinase activity.

Tyrosine-527 (tyrosine-530 in human Src) lies in the C-terminal regulatory region and its phosphorylation is associated with inactivation of Src (196).

The activity of Src is regulated by several mechanisms which have in common the ability to influence the stability of inhibitory intra-molecular interactions (195). Phosphorylated tyrosine-527 forms an intra-molecular interaction with the SH2 domain. A further intra-molecular interaction occurs between the SH3 domain and a short stretch of amino acids

between the SH2 domain and kinase domain. In this conformation not only is the kinase domain inaccessible but access of potential binding partners to the protein-protein interacting domains is sterically hindered. v-Src, the oncogene isolated from Rous Sarcoma virus, lacks the C-terminal regulatory region and so is constitutively active (197). Similarly, targeted mutation of tyrosine-527 to phenylalanine (Src527F) is sufficient to render the proto-oncogene c-Src constitutively active. Consequently the activity of Src can be regulated in several ways (198). Firstly, the SH2 and SH3 domains may bind ligands with greater affinity than their intra-molecular binding sites thus forcing Src to adopt an open conformation. Alternatively dephosphorylation of Src tyrosine-527 may occur via decreased activity of C-terminal Src kinase (Csk) or increased activity of protein tyrosine phosphatases such as PTP- α and SHP-1.

1.2.1.2 Src expression and activity is increased in cancer.

Src expression or activity is upregulated in many tumours (199). For example, the activity of Src is elevated in colorectal cancer specimens when compared to adjacent normal mucosa and the degree of activity increases in parallel with advancing stage of disease (200-202). Elevated Src kinase activity has also been reported in pathological specimens from human breast cancer (203, 204), gastric cancer (205), pancreatic cancer (206) and oesophageal cancer (207). However, the molecular mechanisms by which Src activity may be elevated in tumours are poorly understood. Although an activating mutation in a subset of colorectal cancers has been reported by one group (208) other studies have failed to replicate this finding (209-211) suggesting that mutational activation of Src is an infrequent event. Increased expression of Src frequently occurs but Src activity is usually increased by a relatively greater extent than protein levels indicating additional regulation at the post-translational level. In many cancers the activity of RTKs such as EGFR or HER2 are increased, commonly by activating mutation or gene amplification (212). Src is recruited and activated via displacement of tyrosine-527 as the Src SH2 domain binds to phosphotyrosine residues in the receptor with higher affinity (213). Therefore elevated Src activity may be secondary to activation of a more upstream receptor to which it is coupled (214, 215). Alternatively cytoplasmic proteins such as focal adhesion kinase (FAK) are often over-expressed in tumours and could similarly activate Src by competition with Src tyrosine-527 for the Src SH2 domain (216). Other potential mechanisms include reduced phosphorylation of tyrosine-527 either by decreased expression of Csk which has been reported in some hepatocellular cancers (217) or increased expression of phosphatases such as PTP-1B as has been reported in breast cancer cell lines (218).

1.2.2 Receptor tyrosine kinases.

As alluded to above, signalling from various RTKs is associated with phosphorylation of AJ proteins. However, with regard to potential roles in modulating cell-cell adhesion, members of the EGFR family and c-Met have been the most intensively studied. EGFR directly associates with the AJ via β -catenin (188) and is the prototypic member of a family of RTKs consisting of EGF receptor/HER1, neu/ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. All consist of an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic domain which, with the exception of HER3, has intrinsic tyrosine kinase activity. Binding of ligand to the extracellular domain of EGFR causes homo- or hetero-dimerisation and intermolecular phosphorylation of tyrosine residues within the cytoplasmic tail including positions 992, 1068, 1086, 1148 and 1173. Phosphotyrosine binding adaptor or signalling proteins such as Src, PI 3-K, Shc and Grb2 are then recruited and propagate downstream signals influencing growth, survival and invasion (219). EGFR activity is aberrantly increased in many cancers by mechanisms including gene amplification (220, 221), increased ligand stimulation via autocrine loops (222) and activating mutations within the kinase domain (223, 224). Like EGFR, HER2 also associates with the AJ via β -catenin (225). HER2 is over-expressed in a subset of breast and ovarian cancers due to gene amplification events (226). Activating mutations in the HER2 kinase domain have also been reported but this appears to be less frequent than for EGFR (227). The potential role of HER3 and HER4 in cancer is less well defined.

c-Met, the receptor for hepatocyte growth factor consists of an α and β chain dimer held together by disulphide bonds. Ligation elicits intramolecular phosphorylation of two critical tyrosines residues (tyrosine-1349 and tyrosine-1356) located at the C-terminal of the β chain, forming a docking site for multiple SH2-containing signal transducers, including Src, PI 3-K, and the adaptor proteins Grb2, Shc and Gab1 (228). Signalling via c-Met in several models appears to trigger a biological program favouring invasive growth (228). Aberrant c-Met signalling in human cancer may occur via gene amplification (229, 230), activating mutation (231), or an autocrine signalling loop (232).

1.2.3 EGFR and SFKs cooperate in signal transduction.

Src is activated on stimulation of the EGFR (233) and this is thought to be primarily due to recruitment of Src via its SH2 domain to phosphorylated tyrosine residues in the cytoplasmic tail of the receptor. Over-expression of Src potentiates EGF-stimulated DNA

synthesis and proliferation (234). Conversely, inhibition of Src activity by dominant negative Src mutants or pharmacological inhibition can block EGF-dependent activation of the downstream STAT3 pathway and inhibit proliferation (235). Interestingly, most of the actin remodelling that occurs downstream of EGFR appears to involve Src substrates such as FAK, p130Cas and cortactin (236). As well as being an EGFR effector, Src may contribute to activation of the EGFR itself. Src phosphorylates EGFR at several positions including tyrosine-845 although the role of this is controversial with mutation of tyrosine-845 to phenylalanine either inhibiting EGF-dependent proliferation (237, 238) or having no effect (239) in different models. In addition to directly modulating EGFR activity by phosphorylation Src may also regulate the EGFR via receptor endocytosis or ubiquitination (240). Src also associates with HER2 (241) and c-Met (242) via its SH2 domain in a tyrosine phosphorylation dependent manner and similarly participates in their downstream signalling.

1.2.4 Protein tyrosine phosphatases.

At any time the degree of tyrosine phosphorylation of AJ proteins is determined not just by kinase activity but also by the relative activity of protein tyrosine phosphatases (PTPs) (243). Several receptor and non-receptor PTPs interact with and dephosphorylate components of the AJ (244-249). Of these DEP-1 (249), rPTP μ (247) and SHP-1 (248) are recruited specifically by p120. Phosphatase activity is essential for AJ formation as demonstrated by the ability of vanadate, a phosphatase inhibitor, to cause disruption of AJs in association with increased phosphorylation of AJ proteins. Consistent with this is the upregulation of phosphatase activity in a confluent monolayer as compared to more sparsely plated cells. For example, the expression of DEP-1 is increased more than 10-fold in various cell lines as they approach confluence (250).

1.2.5 Regulated tyrosine phosphorylation occurs in nascent AJs.

A transient increase in phosphotyrosine content occurs at nascent AJs (138, 251) whilst the phosphotyrosine content of mature junctions in a confluent monolayer is diminished (251). Activation of both SFKs (178, 252) and EGFR (137) can be detected at nascent junctions. Cadherin engagement alone is sufficient to activate SFKs as it occurs in cells plated on cadherin ectodomain-coated cover slips which effectively excludes any contribution due to juxtacrine activation of other cell surface receptors (252). Phosphorylated proteins in nascent junctions include cadherins, β -catenin and p120 (138, 176, 251). The physiological

importance of AJ phosphorylation and the identity of potential kinases was demonstrated by the finding that keratinocytes in mice with a disruption of the Src and Fyn genes have reduced phosphorylation of p120 and β -catenin and are defective in AJ formation (138).

A molecular explanation of the requirement for SFK activity in AJ formation has recently been proposed. Initial work showed that pharmacological SFK inhibition or the dominant negative expression of a kinase-defective Src mutant prevented the recruitment and activation of PI 3-K at nascent cadherin contacts and impaired the development of initial cadherin contacts into broad zones of adhesion (179). The effects of Src inhibition could be reversed by expression of constitutively active PI-3 kinase. Thus Src appeared to influence AJ formation via the recruitment of PI 3-K (179). Subsequently Fukayama *et al* confirmed that inhibition of Src activity prevented the cadherin-dependent activation of Rac and the conversion of initial contacts to broad areas of adhesion. In this model Src phosphorylated Vav2 (a Rac GNEF), although this phosphorylation was not sufficient for Vav2 activation. Src also phosphorylated the Crk adaptor protein which recruited C3G, an exchange factor for Rap1 (253), resulting in the local activation of Rap1 at nascent sites of cadherin engagement. Rap1 activation caused activation of PI 3-K and both PI 3-K signalling and tyrosine phosphorylation of Vav2 were required for full activation of Vav2 and subsequent activation of Rac (252). Thus the regulated activity of SFKs is required for the activation of Rac at nascent cadherin contacts via a Src-Rap1-PI 3-K-Vav2 pathway.

1.2.6 Deregulated tyrosine phosphorylation disrupts the AJ.

In contrast to the role of SFKs in AJ formation many studies have reported a negative effect of kinase activity on AJ function. In general these studies utilise constitutively active kinases or cells which over-express growth factor receptors. Deregulated tyrosine phosphorylation of AJs by SFKs (185, 186, 189, 193, 254-257) or RTKs (187, 189, 190, 258, 259) causes disruption of cell-cell adhesion, often accompanied by increased invasiveness. These effects are reversible with pharmacological inhibition of kinase activity (185, 186, 189, 193, 254-256). Similarly, the ectopic expression of protein tyrosine phosphatases favours cell-cell adhesion (260, 261) whilst the expression of dominant negative phosphatase-deficient mutants (262) or pharmacological inhibition of phosphatase activity (254) causes increased phosphorylation and disruption of AJs.

Conflicting evidence for the role of tyrosine phosphorylation at the AJ can be reconciled if tightly regulated phosphorylation has a physiological role in AJ formation and turnover whereas non-physiological sustained or elevated phosphorylation has a deleterious effect.

For example, although endogenous Fyn was required for AJ assembly in keratinocytes, expression of constitutively active Src impaired AJ formation (138). Such sustained kinase signalling is a common pathological event in oncogenesis.

Multiple signalling pathways with pleotropic effects are activated in Src-transformed cells (198) or in response to growth factor signalling (228). It has therefore been a challenge to causally link phosphorylation of an individual substrate with regulatory changes at the AJ. However, several plausible models can be proposed by which aberrant tyrosine phosphorylation might disrupt the AJ (180, 263). The simplest model proposes that phosphorylation, via changes in charge or conformation may interfere with cadherin – catenin binding to cause direct physical disruption of the AJ. Alternatively tyrosine phosphorylation could trigger the recruitment of other signalling proteins to the AJ as, for example, occurs at focal adhesions. In addition tyrosine phosphorylation might “target” proteins for increased turnover or degradation. Finally, tyrosine kinases may exert their effects on the AJ by mechanisms which are spatially distinct from the AJ and which may be independent of phosphorylation of the AJ itself. Examples of this would include unfavourable alterations in acto-myosin contractility or alterations in gene transcription. These potential mechanisms are not mutually exclusive and indeed may overlap at multiple levels.

1.2.6.1 Direct effects of cadherin phosphorylation.

In most models cadherins are only weakly phosphorylated (185-187, 193). However several lines of evidence now support an important role for the tyrosine phosphorylation of cadherins. Interestingly, distinct mechanisms have been identified for different cadherins. Whether this indicates genuine differences or simply reflects the experimental models used is not yet clear.

Phosphorylation of VE-cadherin at tyrosine-658 and tyrosine-731 is sufficient to cause dissociation of p120 and β -catenin respectively (89). The JMD of VE-cadherin contains a sequence motif for constitutive endolysosomal processing which is “unmasked” on detachment of p120 (75). Thus the ability of p120 to retain VE-cadherin at the membrane is determined by the phosphorylation state of VE-cadherin tyrosine-658.

In E-cadherin the phosphorylation of tyrosine residues 755 and 756 promotes interaction with the E3 ubiquitin ligase, Hakai, which targets cadherin for endocytosis with a resulting decrease in cell-cell adhesion (11). These residues are within the p120-binding cadherin

JMD so, reminiscent of the situation for VE-cadherin, it has been suggested that p120 may act to limit access of Hakai and hence stabilise E-cadherin.

Phosphorylation of tyrosine-860 in N-cadherin causes dissociation of β -catenin and was important for the transendothelial migration of melanoma cells, an EMT-like process which is driven by the accumulation of nuclear β -catenin (264). This residue is within the core β -catenin interacting region and is conserved in all classical cadherins (29) although its equivalent residue in VE-cadherin, tyrosine-733, did not influence β -catenin binding (89). Taken together these experiments demonstrate an important regulatory role for cadherin phosphorylation.

1.2.6.2 Direct effects of β -catenin phosphorylation.

Tyrosine phosphorylation of β -catenin causes dissociation of the cadherin / β -catenin / α -catenin complex, loss of cell-cell adhesion, and increased β -catenin-dependent transcription (262, 265-268). This phenotype is specifically mediated by the phosphorylation state of tyrosine-142 in the first Armadillo repeat and tyrosine-654 in the last Armadillo repeat (266, 268). Phosphorylation of tyrosine-654 prevents binding of β -catenin to E-cadherin but does not inhibit binding of β -catenin to TCFs and so transcriptional activation is promoted (267). Phosphorylation of β -catenin at tyrosine-142 inhibits its interaction with α -catenin (268). In addition, the phosphorylation of tyrosine-142 is also important for the transcriptional activity of β -catenin by promoting binding of β -catenin to BCL9-2 (269). BCL9-2 has transcriptional cofactor and nuclear localisation activity and is essential for the nuclear localisation and transcriptional activity of β -catenin (269). Consequently, tyrosine phosphorylation of β -catenin drives a switch from the adhesive function of cadherin-bound β -catenin to the transcriptional function of the β -catenin / TCF / BCL9 complex (270).

1.2.6.3 Direct effects of p120 phosphorylation.

Ironically, given its discovery as a prominent kinase substrate, the effects of tyrosine phosphorylation of p120 are largely unknown. Despite repeated assertions in the literature that tyrosine phosphorylation is likely to regulate the activity of p120 at the AJ (55, 271) definitive evidence is lacking. Early work reported increased cell-cell adhesion in cell lines, including HT29 and Colo205, when cadherin with a p120-uncoupling JMD mutation or p120 with a deleted N-terminal region were expressed (9, 272). Treatment with

staurosporine, a relatively non-specific kinase inhibitor also promoted adhesion. This was interpreted as demonstrating a negative modulatory role for p120 and specifically its N-terminal region, perhaps due to phosphorylation mediated by a constitutively active staurosporine-sensitive kinase. However this work did not distinguish the contribution of tyrosine phosphorylation, serine/threonine phosphorylation, or indeed other events requiring the p120 regulatory region. Subsequently Ozawa *et al* demonstrated that the reduction in E-cadherin dependent cell-cell adhesion in L cells (a cadherin-deficient fibroblast cell line) occurring when E-cadherin and active Src were co-expressed compared to L-cells expressing E-cadherin alone required an intact cadherin JMD (273). Moreover, expression of p120 with a tyrosine to phenylalanine substitution at position 217 was sufficient to abrogate the effects of active Src, although this mutation did not diminish the tyrosine phosphorylation of p120. In the light of our current knowledge of the p120 tyrosine phospho-acceptor sites (which do not include tyrosine-217) it appears that this substitution altered p120 conformation or function in an as yet undefined, but phosphorylation-independent, manner.

The recent identification of p120 tyrosine phospho-acceptor residues has facilitated more targeted investigation of p120 phosphorylation. For example, over-expression of p120 rendered phosphorylation-defective by mutation of all 8 tyrosine phospho-acceptor residues to phenylalanine did not inhibit the Src induced transformation of fibroblasts (68) and did not impair the ability of A431 cells to form AJs (176). However, a possible adaptor role for phosphorylated p120 is suggested by the finding that the phosphatase SHP-1 could be recruited by wild type but not phosphorylation defective p120 (68). Interestingly, the total number of tyrosine residues which were phosphorylated rather than the phosphorylation state of any specific residue determined the strength of binding.

It has been suggested that one consequence of the tyrosine phosphorylation of p120 may be to reduce the affinity of its interaction with cadherin, perhaps exposing signals for internalisation on the JMD. However, most evidence suggests that tyrosine phosphorylation of p120 either increases its affinity for E-cadherin (138, 265, 266, 268) or has no effect (8, 176, 273-275). Piedra has suggested that increased binding of p120 to cadherin may impair AJ stability if p120 functions as an adaptor to recruit the tyrosine kinase Fer, which may be constitutively associated with p120, to the AJ (276). In this model phosphorylation of p120 promotes its association with E-cadherin and hence recruits Fer which subsequently negatively regulates the AJ by phosphorylation of β -catenin at tyrosine-142 (268). Directly contradicting this model is work by Xu *et al* which demonstrated that Fer, recruited to the AJ via p120, phosphorylated the tyrosine

phosphatase PTP1B at tyrosine-152 enabling it to bind the cytoplasmic domain of cadherin (277). PTP1B then promoted AJ stability by maintaining tyrosine-654 of β -catenin in a dephosphorylated state. This inconsistency may reflect the different experimental systems with Xu *et al* using chick retinal cells and fibroblasts which predominantly expressed N-cadherin whilst Piedra used E-cadherin expressing epithelial cells. Thus whether tyrosine phosphorylation of p120 is involved in regulation of the AJ remains enigmatic.

1.2.6.4 Kinase-driven acto-myosin contractility may disrupt the AJ.

Several lines of evidence now suggest that increased RhoA activity may influence tensile forces within the cell in such a way as to be either unfavourable to AJ formation or, in some cases, to literally pull established AJs apart. In this context Sahai has shown that constitutively active RhoA disrupts AJs via its effector kinase ROCK in a myosin light chain kinase (MLCK) and myosin ATPase dependent manner consistent with a mechanism requiring contractile force (115). Src appears able to induce similar effects as expression of constitutively active Src in a colorectal cancer cell line disrupted AJ formation and this was associated with localised activation of ERK, MLCK and ROCK at integrin-based cell-matrix adhesions and accumulation of phospho-myosin at these sites (278). Blockade of ERK, ROCK or MLCK activity inhibited accumulation of phosphomyosin and was sufficient to allow AJs to form. A strikingly similar mechanism has been elicited for cell scattering mediated by RTKs (279). To assay cadherin-mediated adhesion without interference from other adhesion receptors De Rooij *et al* plated MDCK cells on coverslips coated with a cadherin extracellular domain fusion protein. Similar to the N-cadherin adhesions reported by Gavard *et al* (134) E-cadherin plaques formed at the termini of F-actin bundles and contained vinculin, α -, β - and p120-catenin. Surprisingly, stimulation with HGF increased rather than inhibited cadherin-mediated homotypic adhesion to the substrate. Furthermore, live cell imaging of small clusters of cells plated on extracellular matrix and treated with HGF showed that GFP-labelled E-cadherin remained localised at cell-cell contacts until cells suddenly pulled apart with no alteration in cadherin localisation preceding the moment of detachment. Subsequently, as migrating cells contacted one another new junctions formed with rapid accumulation of GFP-cadherin, again suggesting that HGF did not impair the intrinsic ability of E-cadherin to form homotypic adhesions. HGF treated cells formed thick bundles of actin perpendicular to areas of cell-cell contact which terminated in focal adhesions rather than in relation to the AJ itself and which accumulated phospho-myosin light chain. Taken together this suggested that HGF-induced scattering was mediated mainly via increased myosin-dependent tension literally pulling cells apart rather than by direct impairment of cadherin

homotypic adhesion. In keeping with these findings was the observation that AJs were rapidly disrupted in a TGF β -treated mouse mammary epithelial cell line in a RhoA and ROCK dependent manner (280).

Unfavourable acto-myosin contractility has also been proposed to explain the adhesive defect seen in primary enterocytes derived from the Apc^{Min/+} mouse. When cultured in vitro they display impaired cadherin-dependent cell-cell adhesion resulting from elevated EGFR and Src activity. This was associated with increased MAPK and RhoA activity. Pharmacological inhibition of EGFR or of ROCK suppressed the expression of actomyosin contractility markers and simultaneously restored E-cadherin adhesion (281).

Taken together these findings strongly suggest that SFKs or RTKs can cooperate with RhoA to promote acto-myosin contractility which physically impairs AJ stability.

1.3 Tyrosine kinases and the AJ regulate the invasive phenotype.

The ability of neoplastic cells to penetrate into and survive in neighbouring tissues defines cancer. Local invasion may cause destruction or compromise of surrounding tissues and is a prerequisite for malignant cells to enter the circulation and hence metastasise. Invasion requires coordinated changes in several aspects of the cells phenotype. Namely, alterations in cell-cell and cell-matrix adhesion, remodelling of the actin cytoskeleton, increased motility and the production of substances capable of degrading extracellular matrix. As described below it is the particular combination of these factors which determines the mode of invasion utilised. The AJ and tyrosine kinases, both RTKs and cytoplasmic kinases such as Src, play critical roles in the regulation of invasion.

1.3.1 The invasive phenotype is modulated by the AJ.

1.3.1.1 E-cadherin is frequently downregulated in cancer.

Complete loss of E-cadherin is unusual in human cancer. However reduced levels or mislocalisation of E-cadherin occurs in a high proportion of cancers and this may occur by several mechanisms (28, 282).

1. Germline mutation of the E-cadherin gene (CDH1)

CDH1 lies on chromosome 16q22.1. Germline mutations have been causally linked to hereditary diffuse-type gastric cancer (283), a tumour type characterised by scattered growth with loss of cell-cell cohesion. These kindreds also have an increased incidence of colorectal, lobular breast and prostate cancer. Mutations are dispersed throughout CDH1 and are usually frameshift, nonsense or splice site resulting in truncated proteins. A smaller proportion are missense mutations and these are clustered in the second and third EC repeats. In cancers the wild type allele is usually inactivated by promoter hypermethylation (284). Thus CDH1 follows Knudson's two-hit model for inactivation of a tumour suppressor gene (285).

2. Somatic mutation of CDH1

Although rare in most tumour types somatic mutation of CDH1 occurs in approximately half of all diffuse-type gastric and lobular breast cancers (286-288). A lower frequency of mutations has also been identified in endometrial and ovarian cancer (289). In contrast to hereditary diffuse-type gastric cancer the second hit in sporadic gastric and breast cancers is usually via loss of heterozygosity of the wild type allele (287, 288).

3. Epigenetic silencing / transcriptional repression

Hypermethylation of the CDH1 promoter region has been reported in a variety of tumour types including breast (290), ovarian (291), oesophageal (292), colorectal (293), non-small cell lung (294), hepatocellular (295), bladder (296), gastric (297) and oral squamous cell carcinoma (298). Treatment of various E-cadherin deficient cancer cell lines with the demethylating agent 5-azacytidine is sufficient to restore expression (299-302) suggesting that the promoter methylation is a cause rather than effect of gene downregulation (303). Silencing of E-cadherin can also occur due to the binding of specific transcriptional repressors such as Snail (304, 305), SIP1 (306) and Twist (307) to E box motifs in the E-cadherin promoter. In general this occurs in the context of EMT and is discussed below.

4. Post-translational modification

Cadherin levels or activity are also regulated at the post-translational level. Increased degradation may occur via increased endocytosis and targeting to the degradative compartment. As discussed above, Src kinase activity may drive this process via the action of the E3 ubiquitin ligase Hakai (11), or via ARF6-GTPase dependent endocytosis (308). Alternatively proteolytic cleavage of cadherin may occur via the action of presenilin-1 (309), calpain (310), ADAM10 (311) or MMPs (312, 313). The potential role of direct tyrosine phosphorylation of AJ proteins in impairing AJ function has been described above. Finally, as already alluded to, the correlation between p120 loss and downregulation of E-cadherin in tumour specimens provides circumstantial evidence that loss of p120 as a primary event may account for some cases of E-cadherin downregulation.

1.3.1.2 Epithelial-mesenchymal transition is characterised by downregulation of E-cadherin.

EMT describes a phenotypic change occurring naturally during development and which, it has been suggested, may be adopted by tumour cells as a means of increasing their invasiveness. Whilst mechanisms such as post-translational modification of E-cadherin may contribute to the EMT phenotype, full EMT represents a coordinated program of genetic changes coordinated by transcription factors such as Snail (304, 305), Slug (314), SIP1 (306) and Twist (307) and by the nuclear translocation of β -catenin (315). The hallmarks of EMT are loss of apico-basal polarity and cell-cell adhesive architecture, often associated with a decrease or switch in cadherin expression from E-cadherin to mesenchymal cadherins such as N-cadherin. Typically there is a change in intermediate filaments from cytokeratins to vimentin (316).

1.3.1.2.1 EMT occurs in human cancers.

EMT was first identified as a developmental process occurring, for example, at gastrulation when the primitive ectoderm invaginates at the primitive streak and undergoes transition to migratory mesenchymal cells which form the mesodermal layer (316). Although EMT type transitions have been extensively modelled in vitro both the exact definition of EMT and whether it plays any role in human cancer has been the subject of controversy (317, 318). This is in part, due to the difficulty in relating mechanistic research on model systems to

the inherently descriptive studies of human cancer specimens. It has been argued that as metastases usually recapitulate the cell-cell adhesion architecture of the primary tumour EMT must be an uncommon process (317). However, recent detailed immunohistochemical studies of common human tumours have identified coordinated patterns of expression of proteins such as cadherins, vimentin, Snail and Twist which bear a strong resemblance to the phenotypic changes of EMT induced in model systems (307, 319, 320). In colorectal cancer Brabletz *et al* have identified a transient EMT driven by nuclear β -catenin which occurs only at the invasive front of the tumour whilst the remainder of the tumour and metastases do not show evidence of mesenchymal change (315). Thus EMT in cancer, as in development, may be a transient and reversible process. The transient nature of EMT may explain the infrequency with which EMT is recorded by diagnostic pathologists. In addition carcinoma cells which have undergone EMT may easily be mistaken for stromal cells (321).

1.3.1.2.2 Kinase activity drives EMT.

Multiple lines of evidence suggest that the sustained aberrant activation of kinases can initiate EMT. In most models increased RTK signalling cooperates with signalling via TGF β , a receptor serine/threonine kinase, to cause EMT (322, 323). The effects of TGF β are mediated via Smad transcription factors and via Smad-independent activation of PI3-K and MAPK (324). Diverse mechanisms contribute to the cooperation between TGF β and RTKs including convergence at the level of transcriptional regulation, joint regulation of Smad-independent signalling effectors and via autocrine production of TGF β and other ligands (322). RTKs implicated include c-Met (228), EGFR (325-327), HER2 (328, 329), IGFR (330) and FGFR (331, 332). Downstream events include activation of Src, Ras and PI3-K signalling along with increased activity of the Raf / MAPK pathway (316, 323, 333-335). In addition increased activity of the β -catenin / TCF transcriptional pathway which may occur via Wnt signalling, secondary to downregulation of E-cadherin, or via inhibition of GSK-3 β by PI 3-K also favours EMT (31).

1.3.1.3 E-cadherin inhibits invasion.

Multiple lines of evidence indicate that E-cadherin acts to suppress invasion although the mechanisms by which it exerts this effect are poorly understood. For example, many cadherin deficient cell lines are invasive and expression of exogenous E-cadherin caused reversion to a well differentiated and non-invasive phenotype (336, 337). Conversely, treatment with E-cadherin blocking antibodies (336, 337), expression of DN-E-cadherin (338), or depletion of E-cadherin by anti-sense (337) or RNAi techniques (339) increased

invasiveness. Although these experiments confirmed a critical role for E-cadherin in the suppression of invasion in cultured tumour cells they could not demonstrate whether loss of E-cadherin occurring in vivo was required for tumour progression or whether it was a consequence of de-differentiation. This question was elegantly addressed by Christofori, utilising a transgenic mouse model of pancreatic β cell carcinogenesis (340). Expression of the SV40 large-T antigen under control of the rat insulin promoter (RIP1TAG2) caused development of pancreatic β cell tumours (341). Progression of tumours from the adenoma to invasive carcinoma stage was invariably associated with loss of E-cadherin (340). The role of E-cadherin loss was then directly tested by crossing RIP1TAG2 mice with transgenic mice expressing either wild type E-cadherin or DN-E-cadherin, also under the rat insulin promoter (340). Maintained expression of wild type E-cadherin arrested tumour progression at the adenoma stage whereas DN-cadherin induced early invasion. This confirmed a causal role for E-cadherin loss in the development of the invasive phenotype.

Further support for an invasion suppressor role for E-cadherin comes from studies characterising the effect of germline CDH1 mutations on E-cadherin function. Wild type E-cadherin when expressed in CHO epithelial cells (which lack endogenous cadherin), inhibits their ability to invade a 3 dimensional extracellular matrix. In contrast expression of a variety of E-cadherin mutants identified in hereditary diffuse-type gastric cancer was associated with impaired cell-cell adhesion and an inability to inhibit in vitro invasiveness (342-344).

Despite these findings it should be reiterated that complete loss of E-cadherin is uncommon in most human cancers. Although some detailed studies do show a localised loss of E-cadherin at the invasive front of tumours, for example, in oesophageal squamous cell carcinoma (345), the invasion of coherent groups of cells which retain E-cadherin at sites of cell-cell contact is also a frequent finding in human cancer specimens. Furthermore, although E-cadherin expression often correlates with low grade and low invasiveness this is not always the case. For example, inflammatory breast cancer which is extremely invasive is characterised by the retention of E-cadherin containing cell-cell contacts both in infiltrative tumour and in tumour emboli detected in the lymphovascular space (346, 347). Thus the invasion-suppressor activity of E-cadherin is not absolute but must reflect the specific cellular context.

Finally it is perhaps misleading to regard E-cadherin solely as an “invasion-suppressor” when the evidence also suggests direct tumour-suppressor activity. Firstly, germline mutations in CDH1 are sufficient to cause cancer in a high proportion of carriers. The loss

of the wild type allele is required following Knudson's two hit hypothesis for a tumour suppressor gene. Secondly, loss of E-cadherin expression occurs at high frequency in lobular breast carcinoma in situ and thus precedes invasiveness (348).

1.3.1.4 Mechanisms by which E-cadherin may inhibit invasion.

Despite the overwhelming evidence that E-cadherin can inhibit invasion the molecular mechanisms by which this effect is exerted remain elusive. Seemingly contradictory data exist although perhaps this is not surprising if invasion is viewed simply as the phenotypic end result of disparate and mechanistically distinct pathways. Therefore, whilst no overarching mechanism can be proposed several potential mechanisms have been described.

1.3.1.4.1 Inhibition via direct cell adhesion.

The idea that E-cadherin solely acts as a physical "glue" to prevent cells invading is probably simplistic (349). Indeed the adhesive and anti-invasive roles of E-cadherin have been experimentally separated by Gumbiner (350). In MDA-MB-231 breast cancer and TSU-Pr1 prostate cancer cell lines (both of which lack E-cadherin and are invasive) the adhesive activity of E-cadherin was neither necessary nor sufficient to mediate suppression of invasion. Specifically, chimeric constructs containing the IL2 receptor extracellular domain fused to the E-cadherin cytoplasmic domain were able to inhibit invasion to the same extent as wild type E-cadherin without conferring any cell-cell adhesiveness.

However, these results would appear to be inconsistent with reports that antibodies which bind E-cadherin and prevent adhesion can induce invasiveness. This probably reflects genuine differences in cell lines as it has been reported in MDCK (351) and some carcinoma cells (336) but not in others (350, 352). It therefore seems possible that inhibition of cell-cell adhesion may be sufficient to cause invasiveness in some, but not all, cellular contexts.

1.3.1.4.2 Inhibition via sequestration of β -catenin.

In Gumbiner's work only E-cadherin constructs with an intact β -catenin binding domain could inhibit invasion (350). Furthermore, Gumbiner showed that RNAi-mediated depletion of β -catenin was also sufficient to inhibit invasion. Taken together this suggested a model where E-cadherin suppressed invasion by sequestering β -catenin at the AJ.

However, other experimental data directly challenge any model where membrane delocalisation of β -catenin is invoked as a mechanism by which loss of E-cadherin promotes invasiveness. For example, in the RIP1TAG2 transgenic mouse model the DN-cadherin which was expressed effectively sequestered β -catenin at the cell membrane and yet promoted invasiveness (340).

Interestingly, in Gumbiner's work the p120 binding site was not required to inhibit invasion. However, in the light of current knowledge of p120's function this result in no way excludes an important anti-invasive role for p120. Single cell clones expressing wild type or JMD mutant cadherin were selected to have equal protein levels of E-cadherin. Presumably the selected JMD mutant clones had very high expression levels to maintain an identical amount of cadherin at the membrane despite its rapid turnover secondary to loss of interaction with p120. In consequence, this approach would be inherently unable to identify an anti-invasive role for p120 if that role were solely mediated via stabilisation of anti-invasive E-cadherin.

Both Wnt / β -catenin / TCF pathway dependent and independent signalling have been proposed as mechanisms by which β -catenin might promote invasion:

1.3.1.4.2.1 Modulation of Wnt Signalling.

It has been proposed that E-cadherin, by sequestering β -catenin, may reduce its transcriptional activity and thereby inhibit invasion. This hypothesis poses two questions: firstly does β -catenin-TCF transcription influence invasiveness and secondly can E-cadherin negatively regulate this pathway by sequestering β -catenin. In answer to the first question, β -catenin-TCF target genes such as MMP7 (42), c-Met (353), Nr-CAM (354), osteopontin (354), laminin (355) and urokinase plasminogen activator (356) have been linked to invasion. Furthermore, β -catenin-TCF signalling is involved in developmental EMTs and circumstantial evidence suggests a similar role in malignant invasion (356).

As alluded to above, activation of the Wnt- β -catenin-TCF pathway in human cancer usually occurs secondary to mutations in APC or β -catenin. Whether E-cadherin, via sequestration of β -catenin, actually participates in this pathway in human cancer is controversial. Proof-of-principle that cadherins can antagonise β -catenin transcriptional activity exists in *Drosophila* (357) and *Xenopus* (44, 358). An identical pattern of competitive inhibition of β -catenin transcriptional activity via cadherin binding has been demonstrated in human malignant and non-malignant cells in vitro (45, 338, 359, 360).

Whilst phenotypic changes such as a reduction in proliferation were reported in these studies no assessment of invasion or motility was made. In some models of EMT downregulation of E-cadherin was associated with nuclear accumulation of β -catenin (173) (79) and increased invasiveness (79). However, in such models multiple pathways would have been expected to be induced in addition to downregulation of E-cadherin and the increased invasiveness was not proven to be dependent on nuclear β -catenin.

Refuting the participation of E-cadherin in the Wnt / β -catenin / TCF pathway is the observation that none of 10 breast cancer cell lines with mutated or absent E-cadherin displayed activation of β -catenin-TCF transcription (361). Similarly gastric and pancreatic cancer cell lines with loss of E-cadherin did not display upregulated transcriptional activity of TCF targets (362). Rather constitutive transcriptional activity, when it occurred, was always associated with APC or β -catenin mutations. Expression of E-cadherin in cadherin-negative MDA-MB-231 breast cancer and TSU-Pr1 prostate cancer cell lines was not associated with any reduction in β -catenin-TCF transcriptional activity (350). Furthermore the expression of DA- or DN-TCFs did not influence invasion in this system. Interestingly, the expression in CHO cells of E-cadherin mutants isolated from hereditary diffuse-type gastric cancer kindreds promoted invasion in comparison to wild type E-cadherin whilst actually reducing β -catenin-TCF transcription to the same degree as the wild type cadherin (342).

On balance it seems probable that, at least in some cellular contexts, E-cadherin can participate in the Wnt- β -catenin-TCF pathway and that upregulation of this pathway via loss of E-cadherin may contribute to the invasive phenotype. However, this does not appear to be a universal mechanism.

1.3.1.4.2.2 Wnt-independent signalling by β -catenin.

Gumbiner's work suggested a key role for cytoplasmic β -catenin that was not related to alterations in transcription of TCF target changes (350). The molecular mechanisms underlying this are undefined although a variety of cytoplasmic β -catenin binding partners including NF- κ B (363) have potential roles in invasion. Alternatively, β -catenin may mediate the interaction of EGF (225) or HER2 (225) with the AJ and Gumbiner's data does not exclude a model where β -catenin was required to mediate this interaction. Indeed, disruption of the AJ-HER2 complex by expression of an N-terminally truncated β -catenin which binds HER2 but not cadherin inhibited the recruitment of HER2 to the AJ and suppressed invasion (191).

1.3.1.5 Cadherin switching promotes invasion.

Recent studies have demonstrated increased expression of non-epithelial cadherins such as N-cadherin or P-cadherin in gastric (320), prostate (364) and breast (365) tumour specimens and in various cancer derived cell lines (366-368). Expression of N-cadherin induced an invasive phenotype in squamous carcinoma cells (366) and increased motility, invasion and metastasis of breast cancer cells (369, 370). This is reminiscent of the well characterised developmental EMT when epiblast cells switch from E to N-cadherin to ingress the primitive streak (2), although upregulation of N-cadherin is not a universal event in other developmental EMTs (16). Interestingly a cadherin hierarchy seems to exist with N-cadherin trumping E-cadherin as expression of even small amounts of N-cadherin promoted motility (371) and increased invasion in E-cadherin expressing cell lines (369, 370) whereas increased expression of E-cadherin could not inhibit the motile and invasive properties of an N-cadherin expressing breast cancer cell line (369). Increased expression of cadherin-11, a related cadherin, is also associated with increased invasiveness in breast cancer cell lines (367, 369).

The role of P-cadherin in invasion is less clear with data supporting both a pro- and anti-invasive role. In a breast cancer cell line an increase in P-cadherin was associated with a gain of invasiveness (372). In contrast, other studies have reported an anti-invasive role for P-cadherin when expressed in lung or melanoma cell lines (354, 373, 374).

The molecular mechanisms by which cadherin switching may favour invasion are incompletely understood. However, in general terms these may include: 1) downregulation of endogenous anti-invasive E-cadherin via competition for p120 by newly expressed mesenchymal cadherins; or 2) dominant pro-invasive mechanisms of mesenchymal cadherins which occur despite maintained E-cadherin expression. Several such pro-invasive mechanisms may exist:

1. N-cadherin may mediate more dynamic intercellular adhesions allowing single cells to dissociate from the tumour and invade (375).
2. N-cadherin expressed by neoplastic cells may participate in homophilic interactions with N-cadherin expressed on stromal cells including myofibroblasts and endothelial cells to promote invasion (376). One such example is the intravasation of melanoma cells which required the formation of N-cadherin dependent contacts with endothelial cells (264, 377). A similar phenomenon may occur in breast cancer

cell lines in which expression of Twist causes an EMT with an E- to N-cadherin switch. The orthotopic injection of breast cancer cells transfected with Twist into mouse mammary fat pad was shown to specifically enhance intravasation whilst having no effect on survival, growth, or extravasation (307).

3. N-cadherin may potentiate pro-invasive signalling through the FGF receptor. N-cadherin associates with the FGF receptor and inhibits its ligand-induced internalisation resulting in sustained MAPK-ERK activation which leads to increased MMP9 gene transcription and increased invasiveness (378). This may represent the co-opting by malignant cells of a pathway whose physiological role is in neurite outgrowth (379).
4. Cadherin switching may be associated with alterations in Rho GTPase activity and it has been suggested this may reflect altered affinity of mesenchymal cadherins for p120. For example, the expression of R-cadherin in tumour cells was associated with downregulation of endogenous cadherins, decreased association of p120 with R-cadherin compared to E-cadherin, and increased motility and sustained activation of Rac (380). Similarly, over-expression of P-cadherin in a pancreatic cancer cell line was associated with cytoplasmic accumulation of p120, activation of Rac and Cdc42, and increased motility (129).

The processes which regulate the coordinated expression of cadherins and, in particular, the often inverse relationship between E-cadherin and mesenchymal cadherins are poorly understood. The co-ordinated transcriptional reprogramming of EMT provides one explanation. The reciprocal regulation of cadherins may also occur via competition by different cadherins for a limiting amount of p120 (82, 87). An additional level of regulation might be provided by the pattern of p120 isoform expression. This is suggested by the observation that p120 isoform 1 preferentially associates with N-cadherin whereas isoform 3 preferentially associates with E-cadherin (381). Thus a switch in expression from p120 isoform 3 to 1 could conceivably contribute to an increase in N-cadherin and simultaneous downregulation of E-cadherin, although this has not been experimentally investigated.

1.3.2 Deregulated kinase activity promotes invasion.

1.3.2.1.1 Src promotes invasion and metastasis.

Increased activity of SFKs may have multiple effects in cancer including proliferation, survival and angiogenesis (198). However, several lines of evidence support there being a

critical role for Src in invasion and metastasis. Specifically, the over-expression of Src in a rat colon epithelial cell line (382) and in human breast or colon tumour derived cell lines (256) increased in vitro invasiveness. Similarly, expression of Src in MDCK cells increased invasiveness (186) and was sufficient to render them metastatic to lung when injected orthotopically in the murine kidney (383). Conversely, inhibition of Src activity by over-expression of Csk (a negative regulator of Src activity) in a mouse colon adenocarcinoma cell line did not inhibit the formation of primary tumours in mice but prevented the development of lung metastases in vivo and inhibited in vitro invasion (384). In-keeping with this, the expression of Csk inhibited the in vitro invasiveness of two human colorectal cancer cell lines (385) whilst the expression of Csk or of a kinase-deficient dominant-negative Src mutant in a rat carcinoma cell line specifically prevented metastasis without preventing the formation of primary tumours (386). Furthermore, depletion of Src by RNAi was sufficient to reduce the incidence of metastases in a pancreatic cancer orthotopic model (387).

Recently several relatively specific ATP-based small molecule inhibitors of Src kinase activity have been developed (388). Treatment of an MCF-7 derived cell line with AZD0530 inhibited in vitro motility and invasion (389). Treatment of pancreatic, head and neck squamous cell carcinoma and non-small cell lung cancer derived cell lines with dasatinib was associated with inhibition of migration and invasion of all cell lines (390, 391) and with a reduction in metastases in vivo (387). Invasion of NSCLC cell lines was inhibited by M475271 (392). CGP77675 and CGP76030 inhibited in vitro invasion of prostate cancer cell lines (393). The in vitro invasion and in vivo metastases of a breast cancer cell line was inhibited by PP2 (394). Similarly the development of lymph node or liver metastases in an orthotopic pancreatic cancer model was prevented by administration of AZM475271 (395). Importantly, the pharmacological inhibition of invasion in SW620 and HT29 colorectal cancer cell lines was mimicked by RNAi-mediated depletion of Src protein (335).

1.3.2.1.2 The EGFR family promote invasion.

Like Src, EGFR family members can regulate many of the hallmarks of malignancy (212). However, several lines of evidence suggest that the aberrant activity of EGFR can specifically induce invasiveness. For example, exposure to EGF increased invasiveness of oral (396) and cutaneous (397) squamous cell carcinoma, renal (398), pancreatic (399), breast (256, 400) and oesophageal cancer cell lines (189). Interestingly, several studies suggest that HER2 may be required to heterodimerise with EGFR for efficient induction of invasion (401-403).

Gefitinib, a small molecule inhibitor of EGFR kinase activity, blocked in vitro invasiveness and establishment of bone metastases in an in vivo nude mouse model (404) and inhibited invasion of prostate (405), pancreatic (406), head and neck squamous cell and breast carcinoma derived cell lines (407). As alluded to above Src and EGFR signalling are intimately linked. In this context cooperation of Src and EGFR in mediating invasiveness has been reported (214, 256) and pharmacological inhibition of Src activity can prevent EGF-dependent invasion (408).

1.3.3 Malignant cells use diverse invasion strategies.

EMT provides one mechanism by which cancer cells may adopt an invasive phenotype which is characterised by detachment of cells from their neighbours and the subsequent invasion of single cells. However, in many cases invasion occurs without EMT and malignant cells may remain in contact with their neighbours and invade as coherent sheets or clusters. Importantly, invasive cells often demonstrate plasticity in response to environmental cues or in response to pharmacological inhibition. In vitro studies combined with in vivo imaging of labelled tumour cells have defined at least three broad categories of malignant migration and invasion (Figure 1-6) (409, 410).

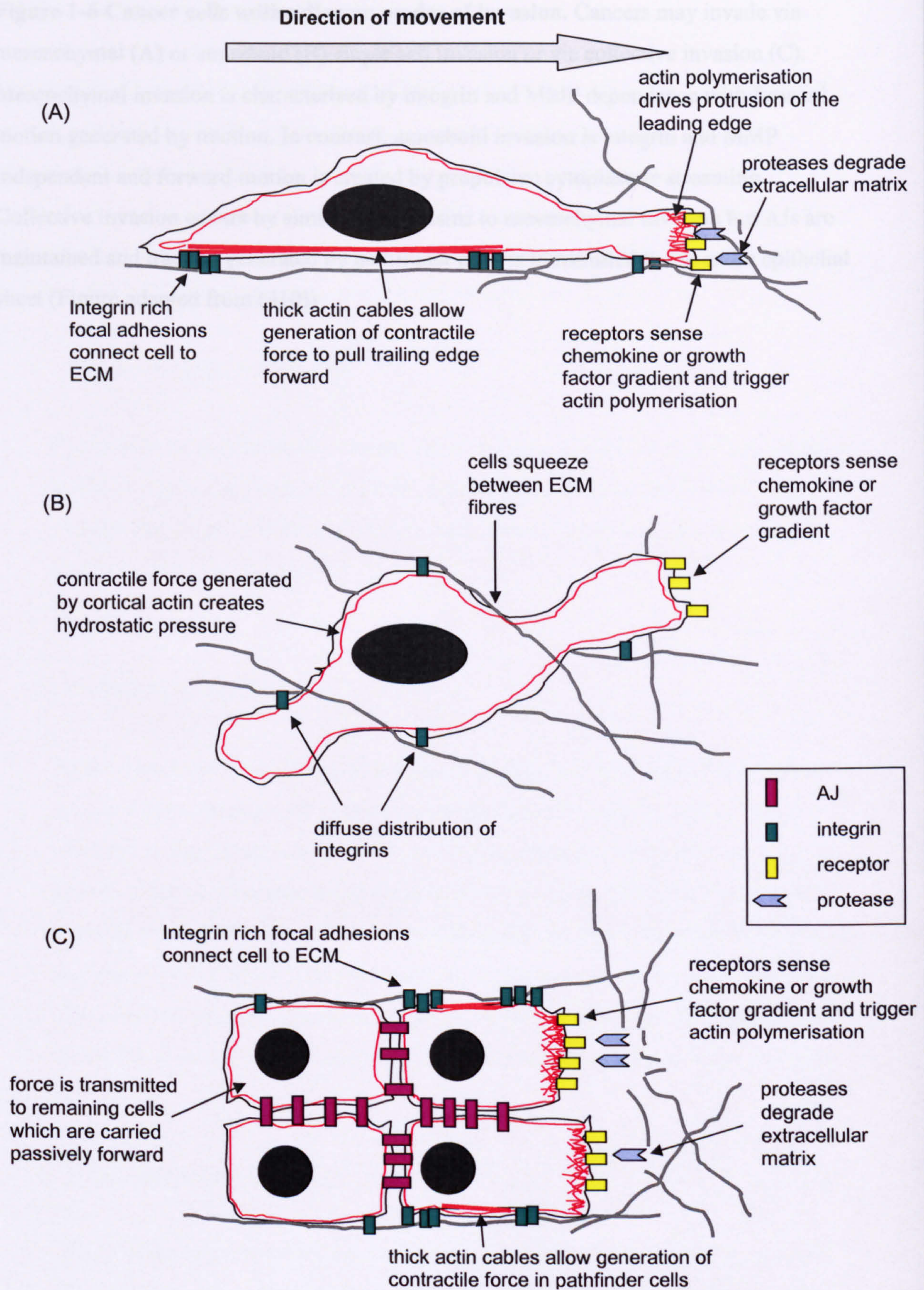


Figure 1-6 Cancer cells utilise diverse modes of invasion.

Figure 1-6 Cancer cells utilise diverse modes of invasion. Cancers may invade via mesenchymal (A) or amoeboid (B) single cell invasion or via collective invasion (C). Mesenchymal invasion is characterised by integrin and MMP dependence with forward motion generated by traction. In contrast, amoeboid invasion is integrin and MMP independent and forward motion is created by propulsive cytoplasmic streaming. Collective invasion occurs by similar mechanisms to mesenchymal invasion but AJs are maintained and traction generated by pathfinder cells is transmitted to the entire epithelial sheet (Figure adapted from (410))

1.3.3.1 Mesenchymal Invasion.

Mesenchymal invasion has been the most intensively studied, largely because it can be partially recapitulated in 2-dimensions and hence has been more readily accessible.

Mesenchymal invasion requires cells from an epithelial cancer to have undergone an EMT. and is characterised by loss of the AJ and cell-cell cohesion with migration of individual elongated and polarised cells moving at relatively slow speeds. This pattern of migration has been divided into a continuous cycle of five phases (409, 410):

1. Protrusion of the leading edge.

Chemokine or growth factor receptor signalling activates PI 3-K and PI 4,5-K whose products recruit the Arp2/3-WASP actin-nucleating complex and GNEFs which activate Rho-family GTPases. This results in forward propulsion of a pseudopod driven by actin polymerisation.

2. Formation of focal contacts.

As the pseudopod forms $\beta 1$ and $\beta 3$ integrins, along with other adhesion receptors such as CD44, are engaged by their extracellular matrix ligands and become clustered in the cell membrane. Various adaptor, signalling and actin regulatory proteins such as α -actinin, talin, vinculin, FAK, paxillin, PI 3-K, protein kinase C and Rho-family GTPases are recruited. This guides the local polymerisation of actin and cross-linking of actin into filaments. Cytoplasmic bundles of actin, termed stress fibres, extend back from the focal contact along the longitudinal axis. Stress fibre formation is predominantly controlled by Rho and its downstream effector ROCK.

3. Focalized proteolysis.

Whilst actin polymerisation is occurring proteases are recruited to focal contacts. Soluble proteases such as separase, MMP1 and MMP2 bind directly to integrins and, together with membrane bound proteases such as MT-MMP1 (411), cause localised degradation of the extracellular matrix creating a defect which is occupied by the advancing cell body (412).

4. Acto-myosin contraction.

Myosin II binds actin filaments and generates contraction thus generating force to pull the cell body and trailing edge forward. Myosin II is activated by phosphorylation of the myosin light chain by myosin light-chain kinase and deactivated by myosin light-chain phosphatase (MLCPtase). This is regulated by Rho via its effector ROCK which inhibits MLCptase (413).

5. Detachment of the trailing edge.

Focal contacts at the trailing edge are disassembled by a variety of mechanisms including the activity of actin severing proteins such as cofilin (414) and cleavage of focal contact components by the protease calpain (415).

Mesenchymal invasion is thus characterised by its dependence on cell-matrix interactions, the generation of traction forces via actin stress fibres and extensive extracellular matrix remodelling. A requirement for Src kinase activity has been demonstrated perhaps acting via its effector calpain to promote the turnover of focal adhesions (416).

1.3.3.2 Amoeboid Invasion

A second mode of invasion utilised by single cells is amoeboid migration. This differs in several important respects from mesenchymal migration. It has been most extensively characterised in the single cell amoeba *Dictyostelium*, but has also been observed in leukocytes and tumour cells, both in vitro and in vivo (409, 410). Amoeboid movement is characterised by weak diffuse cell matrix interaction and cannot be inhibited by integrin blocking antibodies. Mature focal contacts and stress fibres are lacking. The initial step in amoeboid migration occurs via the activation of chemokine or growth factor receptors which triggers formation of a pseudopod. A wave of cortical actin polymerisation then occurs around the cell cortex and the cell undergoes contraction to generate propulsive force with streaming forward of the cytoplasm. Rather than degrading matrix, cells squeeze through gaps between fibres and achieve far higher speeds than in mesenchymal invasion (417). Mesenchymal cells treated with protease inhibitors, integrin blocking antibodies or inhibitors of Src kinase activity (416, 418, 419) have been shown to switch to an amoeboid invasive phenotype. This may have relevance for therapeutic anti-invasion strategies.

1.3.3.3 Collective Invasion

On histopathological examination many human tumours clearly retain cell-cell junctions and invade either as clusters of cells which have detached from the primary tumour or as strands or sheets remaining in contact with the primary tumour (410, 420). It is likely that this process recapitulates developmental processes such as migration of cell sheets in the blastoderm or ectoderm following closure of the neural tube. This form of invasion shares certain traits with mesenchymal invasion. A subset of cells at the invasive front, termed pathfinder cells, generate pseudopods which adhere to extracellular matrix via clustered $\beta 1$ and $\beta 3$ integrins. Focal contacts form and traction is generated by actomyosin contractility whilst localised extracellular matrix degradation occurs in a similar manner to mesenchymal invasion (421, 422). AJs are maintained and actin organised at a supracellular level so that traction force is transmitted across the entire cohort (422). Cells in the centre and trailing edge of the cohort are largely non-motile, maintain identical positions relative to each other and are pulled along passively (423). Perhaps the greatest advantage of this mode of invasion is the maintenance of a favourable microenvironment for tumour cells via high local concentrations of growth and survival signals. For example, E-cadherin mediated intercellular adhesion is able to promote anchorage independent growth and prevent apoptosis of carcinoma cells in vitro (424) perhaps via ligand independent activation of EGFR which causes upregulation of the anti-apoptotic protein Bcl-2 (425). The in vivo relevance of this is demonstrated by the observation that clusters of 6-7 tumour cells injected into the tail vein of mice generate significantly more metastatic foci than injection of an identical number of single tumour cells (426, 427). Such circulating tumour cell clusters are relatively common in the peripheral blood of patients with common solid tumours such as colorectal (428) or prostate cancer (429).

The mechanisms regulating collective invasion are poorly understood. Recent work suggests a pivotal role for the mucin-like transmembrane glycoprotein, podoplanin, in inducing collective invasion (430). Expression of podoplanin in the previously discussed RIP1TAG2 transgenic mouse model of pancreatic carcinogenesis (341) caused a significant increase in the number of invasive tumours forming. Crucially, these tumours retained AJs and invaded in a collective manner. This was in complete contrast to tumours forming in the absence of podoplanin which undergo EMT with loss of E-cadherin and in which loss of E-cadherin is a rate-limiting step for the development of invasive tumours (340, 430). Thus, expression of a single protein, podoplanin, was sufficient to override the requirement for EMT and to promote collective invasion.

Immuno-histochemical analysis of 189 human squamous cell carcinoma specimens (oesophageal, cutaneous, cervical and lung) demonstrated that in 80% of cases invasion adopted a collective morphology (430). Whilst E-cadherin was retained at cell-cell junctions in all layers podoplanin was found exclusively in the outermost cell layer of the invasive front. Thus the expression of podoplanin by an individual cell is affected by the tumour microenvironment, perhaps by growth factors produced by stromal cells. EGF, for example, increased expression of podoplanin in cultured cells in vitro. As would be predicted the collective invasion induced by podoplanin was sensitive to matrix metalloproteinase inhibition. The mechanism by which podoplanin promotes invasion awaits definition but may be related to alterations in Rho family GTPase activity (430).

Integrins also appear to play an important role in collective invasion, not just in attachment of the leading edge to the matrix but also to promote cell-cell cohesion. For example, when primary melanoma explants were treated with a $\beta 1$ -integrin blocking antibody collective invasion was inhibited and individual cells dispersed by amoeboid migration (422). Indeed several lines of evidence suggest that integrins are important in maintaining cell-cell adhesion in collective migration (431, 432). Potential mechanisms include integrin signalling pathways which modulate Rho GTPase activity (431) or incorporation of $\alpha 3\beta 1$ integrin into the AJ where it interacts with the tetraspanin CD151 and recruits PTP μ to stabilise AJs. Alternatively $\alpha 5\beta 1$ integrin may remodel fibronectin into an extracellular scaffold which functionally links individual cells to each other (432).

1.3.4 Potential roles for p120 in invasion.

Based on current knowledge of p120's central role in cadherin and epithelial biology two major scenarios by which p120 might influence invasion have been proposed (156).

1.3.4.1.1 p120 as an invasion suppressor.

As described above there is overwhelming evidence that, in many situations, E-cadherin acts to inhibit invasion. Consequently, p120 via its core function in maintaining E-cadherin levels may logically be expected to similarly inhibit invasion. Indeed, the finding that E-cadherin is almost always absent or redistributed to the cytoplasm in tumours lacking p120 provides circumstantial evidence that loss of p120 may be an important mechanism for the downregulation of E-cadherin in human tumours.

No invasive behaviour was detected in the skin or salivary gland of mice with targeted knockout of p120 in these tissues (94, 95) although the effect of knockdown of p120 in

combination with manipulations of known tumour suppressor or oncogenes has not been directly addressed. However, as alluded to above, an intriguing hypothesis is that, by effectively sequestering p120, the transgenic expression of DN-cadherin may phenocopy p120 knockout. In this regard it is tempting to speculate that the ability of DN-E-cadherin to promote invasion in the RIP1TAG2 mouse model may predict the effects of knocking out p120 in this model (271).

1.3.4.1.2 p120 as an invasion promoter.

A second situation may occur, as described for example in lobular breast and diffuse-type gastric cancers where E-cadherin is downregulated by mutation or transcriptional repression. In these tumours, as in vitro (10), p120 appears relatively stable and a redistribution of p120 to the cytoplasm occurs. In vitro, cytoplasmic re-localisation of p120 is often associated with alterations in Rho GTPase activity and an increase in 2-dimensional planar migration (433). Whilst it is tempting to speculate that this may translate into increased invasion, no test of this hypothesis has yet been reported.

A second mechanism by which re-localisation of p120 from the membrane to the cytoplasm or nucleus could influence cellular phenotype is via its interaction with Kaiso. Loss of E-cadherin causes increased entry of p120 into the nucleus (434) whilst loss of the nuclear export signal encoded by p120 exon B, as reported in colon and prostate cancer (61), may also favour nuclear accumulation. An increase in nuclear p120 would be predicted to release Kaiso-mediated transcriptional repression of its target genes. Although these include target genes with known roles in invasion, recent in vivo data has shown that Kaiso, rather than inhibiting oncogenesis, was required for intestinal tumorigenesis in the *Apc^{Min/+}* mouse (51). Whether Kaiso has similar or divergent roles in other tissues and how this might be regulated by p120 remains unexplored.

Finally, the potentially pro-invasive role of the switch from p120 isoform 3 to 1 which has been detected in some tumour cell lines and which may occur during EMT is intriguing. p120 isoform 1 is found naturally in more motile cell types and it associates preferentially with the pro-invasive N-cadherin. Whether a p120 isoform switch may causally contribute to the invasive phenotype is unknown.

1.4 Aims.

p120 as “master-regulator” of the AJ, prominent tyrosine kinase substrate, and regulator of Rho GTPase activity would appear well placed to integrate and disseminate signals leading to altered cell-cell adhesion and invasion. However, the biological consequences of p120 phosphorylation and the role, if any, of p120 in invasion are unknown. We therefore set out to:-

1. Develop new tools to facilitate the study of p120 tyrosine phosphorylation.
2. Identify the role of p120 tyrosine phosphorylation in Src or EGFR induced disruption of the AJ.
3. Investigate role of p120 and its tyrosine phosphorylation in growth factor dependent invasion.

2 Materials

Materials used in the experiments described in this thesis are listed below.

2.1 Plasmids and oligonucleotides

Supplier: Dr J Collard, The Netherlands Cancer Institute, The Netherlands

pGEX2TK-GST-PAK-CD

pGEX2TK-GST-C21

Supplier: Prof A. Reynolds, Vanderbilt University Medical School, Nashville, USA

pLZRS-IRES-murine (m) p120-3A / HA-GFP

pLZRS-IRES-(m) p120-3A / 7F / HA-GFP

pLZRS-IRES- (m) p120-4A / HA-GFP

Supplier: Dr J. van Hengel, Flanders Interuniversity Institute for Biotechnology, Belgium

pEFBOS-human p120-1A

Supplier: BD Biosciences, Oxford, UK

pECFP

Supplier: Dharmacon, Colorado, USA

siCONTROL Non-targeting siRNA duplexes

Supplier: Invitrogen, Paisley, UK

pCR-Blunt II-TOPO

Supplier: Oligoengine, Seattle, USA

pRetroSuper

2.2 Cell lines

Supplier: American Type Culture Collection, LGC Promochem, Middlesex, UK

A431 cutaneous squamous cell carcinoma cell line

Supplier: Dr V. Brunton and Dr E. Avizientye, Beatson Institute

KM12C cell lines expressing Src constructs (listed in Table 5)

Supplier: Prof I. Fidler, M.D. Anderson Center, Texas, USA

KM12C colon cancer cell line

Supplier: Dr G Nolan, Stanford University, California, USA

Phoenix Ampho packaging cell line

2.3 Antibody generation

Supplier: Affiniti Research Products Ltd., Exeter, UK

Free phosphorylated peptides

Free non-phosphorylated peptides

KLH-conjugated phosphorylated peptides

Agarose-bound non-phosphorylated peptides

Supplier: Amersham International, Little Chalfont, UK

HiTrap™ Protein G Column

Supplier: Bio-Rad Laboratories, California, USA

Coomassie Brilliant Blue R-250 Staining Solution

Supplier: Harlan UK Ltd, Oxfordshire, UK

Rabbits, New Zealand White

Supplier: Sigma Chemical Co, Poole, UK

Freund's Adjuvant, complete
Freund's Adjuvant, incomplete

2.4 Cell culture reagents

Supplier: Amaxa GmbH, Cologne, Germany

Amaxa Nucleofector System

Supplier: Beatson Institute Central Services

Sterile PBS

Sterile PBS/1mM EDTA

Supplier: Clonetics Corporation, distributed by TCS Biologicals, Botolph Claydon, UK

Keratinocyte Growth Medium

Calcium chloride solution

Bovine pituitary extract

Human EGF

Hydrocortisone

Insulin

Amphotericin-B

Gentamicin

Supplier: Gibco Europe Life Technologies Ltd, Paisley, UK

Dulbecco's Modified Eagle Medium

Eagles' Minimal Essential Medium

Hanks' buffered saline solution

L-glutamine

Non-essential amino acids

Trypsin solution

Supplier: Invitrogen

Oligofectamine Transfection Reagent

Supplier: Roche Diagnostics Ltd, Sussex, UK

DOTAP Liposomal Transfection Reagent

Supplier : Sigma Chemical Co.

Foetal bovine serum (FBS)

HEPES-buffered saline (HBS)

MEM vitamin solution

Puromycin

Polybrene

Sodium pyruvate

2.5 Cell culture plasticware

Supplier: BD Biosciences, Oxford, UK

Falcon tissue culture dishes (60mm, 90mm and 120mm)

Supplier: TCS biologicals

Eight well chamber slides

Nunc tissue culture flasks

Nunc cryotubes

2.6 Growth factors and drugs

Supplier: Ariad pharmaceuticals, Massachusetts, USA

AP23464

Supplier: CN Biosciences Ltd, Nottingham, UK

AG1478

Supplier: R&D Systems, Abingdon, UK

Recombinant human EGF

2.7 Primary antibodies

Supplier: Abcam Ltd, Cambridge, UK

Rabbit anti- α -catenin

Rabbit anti-p120-catenin

Supplier: BD Biosciences

Mouse anti- β -catenin

Mouse anti- γ -catenin

Mouse anti-Cdc42

Mouse anti-E-cadherin

Mouse anti-EGFR

Mouse anti-phospho-EGFR

Mouse anti-FAK

Mouse anti-N-cadherin

Mouse anti-P-cadherin

Mouse anti-p120

Mouse anti-p120-phospho-Y96

Mouse anti-p120-phospho-Y228

Mouse anti-p120-phospho-Y280

Mouse anti-p120-phospho-Y291

Mouse anti-paxillin

Mouse anti-phosphotyrosine (clone PY20)

Mouse anti-p130^{cas}

Mouse anti-Rac1

Supplier: B. Serrels, Beatson Institute

Anti-Src kinase domain antibody (435)

Supplier: New England Biolabs (UK) Ltd, Hertfordshire, UK

Mouse anti-phospho-Ser/Thr 44/42 MAP Kinase

Mouse anti-Myc Tag (clone 9B11)

Mouse anti-STAT3

Rabbit anti-STAT3-phospho-Y705

Rabbit anti-Src-phospho-Y416

Supplier: Roche Diagnostics Ltd

Mouse anti-HA tag

Supplier: Santa Cruz Biotechnology, Santa Cruz, USA

Mouse anti-RhoA

Supplier: Sigma Chemical Co.

Mouse anti-Actin

Rabbit anti-MAP kinase

2.8 Immunofluorescence

Supplier: Fisher Scientific UK Ltd, Loughborough, UK

Formaldehyde

Supplier: Jackson ImmunoResearch, Luton, UK

FITC labelled sheep anti-mouse IgG

FITC labelled goat anti-rabbit IgG

TRITC labelled sheep anti-mouse IgG

TRITC labelled goat anti-rabbit IgG

Supplier: Sigma Chemical Co.

Phalloidin-FITC

Supplier: Vector Laboratories Ltd, Peterborough, UK

Vectashield mounting medium

2.9 Protein immunoprecipitation, electrophoresis and Western blotting

Supplier: Amersham International

Sheep anti-mouse/horseradish peroxidase conjugate

Donkey anti-rabbit/horseradish peroxidase conjugate

ECL reagent

High molecular weight rainbow markers

Supplier: Chemicon International, Harrow, UK

Re-blot kit

Supplier: CN Biosciences, Nottingham, UK

Anti-v-Src conjugated agarose

Recombinant LAR protein tyrosine phosphatase

Supplier: PERBIO, Glasgow, UK

Micro BCA protein assay kit

Supplier: Premier Foods, St Albans, UK

Marvel milk powder

Supplier: Schleicher and Schuell, London, UK

Nitrocellulose membrane

Supplier: Severn Biotech Ltd, Kidderminster, UK

Design-a-gel 30% acrylamide

Supplier: Sigma Chemical Co.

Ammonium persulphate (APS)

Anti-mouse IgG agarose

Aprotinin

Bovine serum albumin (BSA)

IgG conjugated agarose beads

Phenylmethylsulphonyl fluoride

N,N,N',N'-Tetramethylethylenediamine (TEMED)

Supplier: Whatman, Maidstone, UK

3MM filter paper

2.10 Manipulation of DNA

Supplier: Bioline Ltd., London, UK

Hyperladder I Quantitative Markers

Supplier: Gibco Life Technologies

DH5 α competent cells

Phosphatase and buffer

Restriction endonucleases and buffers

Supplier: Invitrogen, Paisley, UK

Zero Blunt Topo PCR cloning kit

Supplier: Melford Laboratories Ltd, Suffolk, UK

Electrophoresis grade agarose

Supplier: Promega, Madison, USA

Wizard® PureFection Plasmid DNA Purification System

Supplier: Qiagen, Crawley, UK

QIAquick gel extraction kit

QIAprep miniprep kit

Supplier: Roche Diagnostics Ltd

Rapid DNA ligation kit

Supplier: Sigma Chemical Co.

Ampicillin

Kanamycin

Supplier: Stratagene, La Jolla, USA

PFU Turbo DNA polymerase

Quickchange Site Directed Mutagenesis Kit

XL10-Gold Ultracompetent cells

2.11 Invasion assays

Supplier: BD Biosciences, Oxford, UK

BD Matrigel Basement Membrane Matrigel

Supplier: Corning Life Sciences, Acton, MA, USA

Transwell (8.0 μ m Pore)

Supplier: Gibco Europe Life Technologies Ltd

HEPES-buffered Dulbecco's Modified Eagle Medium

Supplier: Invitrogen

Calcein AM

2.12 Proliferation assay

Supplier : Promega UK Ltd, Southampton, UK

CellTiter 96® Non-Radioactive Cell Proliferation Assay kit

2.13 Rho GTPase activity assay

Supplier: Amersham International

Glutathione Sepharose 4B

2.14 Chemotactic migration assay

Supplier: CN Biosciences Ltd

Innocyte™ Cell Migration Assay kit, 96 well

Supplier: Corning Life Sciences, Koolhovenlaan, Netherlands

Clear bottom black 96 well plate

2.15 Stock solutions and buffers

2.15.1 Cell culture solutions

2.15.1.1 A431 / Phoenix Ampho growth medium

DMEM (1x) supplemented with:

Foetal bovine serum	10%
L-glutamine	2 mM

2.15.1.2 KM12C growth medium

Eagles' minimal essential medium supplemented with:

Foetal bovine serum	10%
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MEM vitamin solution	2x (final concentration)
Non-essential amino acids	1x
L-glutamine	2 mM
Sodium pyruvate	1 mM

2.15.1.3 Low calcium growth medium

KGM supplemented with:

Bovine pituitary extract	30 µg/ml
Human EGF	10 ng/ml
Hydrocortisone	0.5 µg/ml
Insulin	5 µg/ml
Amphotericin-B	50 ng/ml
Gentamicin	50 µg/ml
Calcium	0.03 mM

2.15.1.4 Trypsinization solution

PBS supplemented with:

Trypsin	0.25% (w/v)
EDTA	1 mM

2.15.2 *Immunoprecipitation, electrophoresis and Western blotting solutions.*

2.15.2.1 RIPA lysis buffer

Tris/HCl, pH 7.4	50 mM
NaCl	150 mM
Triton X-100	1%
Sodium deoxycholate	0.5% (w/v)
Sodium dodecyl sulphate (SDS)	0.1% (w/v)
EDTA	2 mM
Sodium orthovanadate	100 µM
PMSF	2 mM

Aprotinin	10 µg/ml
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2.15.2.2 Co-immunoprecipitation lysis buffer

Tris-HCl, pH 7.6	50 mM
NaCl	200 mM
MgCl ₂	5 mM
NP40	0.1%
DTT	1 mM
NaF	25 mM
PMSF	1 mM
Aprotinin	10 µg/ml
Sodium orthovanadate	100 µM

2.15.2.3 LAR phosphatase buffer

Imidazole, pH 7.0	25 mM
NaCl	50 mM
EDTA	2.5 mM
DTT	5 mM
BSA	100 µg/ml

2.15.2.4 Resolving gel buffer

Tris-HCl, pH 8.9	1.5 M
SDS	0.4% (w/v)

2.15.2.5 Acrylamide gel – 10%

Resolving gel buffer	12 ml
Acrylamide (30% w/v)	10.7 ml
H ₂ O	20 ml
APS (10% w/v)	400 µl
TEMED	30 µl

2.15.2.6 Acrylamide gel – 7%

Resolving gel buffer	12 ml
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Acrylamide (30% w/v)	11.2 ml
H ₂ O	24.8 ml
APS (10% w/v)	400 µl
TEMED	30 µl

2.15.2.7 Stacking Gel Buffer

Tris-HCl, pH 6.7	0.5 M
SDS	0.4 % (w/v)

2.15.2.8 Stacking gel

Stacking gel buffer	4.5 ml
Acrylamide (30% w/v)	2.4 ml
H ₂ O	11.1 ml
APS (10% w/v)	200 µl
TEMED	15 µl

2.15.2.9 Laemmli Sample buffer – 4x

Tris-HCl, pH 6.8	150 mM
SDS	6% (w/v)
Glycerol	30%
2-mercaptoethanol	15%
Bromophenol blue to colour	

2.15.2.10 Tank buffer - 5x

Tris-HCl	0.26 M
Glycine	0.26 M
SDS	0.5% (w/v)

2.15.2.11 Transfer Buffer – 10x

Tris-HCl	0.48 M
Glycine	0.39 M
SDS	0.37% (w/v)

Diluted to 1x with H₂O containing 20% methanol

2.15.2.12 Tris buffered saline (TBS) 10x

Tris-HCl, pH 7.6	20 mM
NaCl	150 mM

2.15.2.13 Wash Buffer

TBS	
Tween 20	0.2%

2.15.3 *Molecular biology solutions*

2.15.3.1 TAE Buffer

Tris-acetate	40 mM
Sodium acetate	5 mM
EDTA	1 mM

pH to 7.8 with glacial acetic acid

3 Methods

3.1 Production of sequence-specific phosphorylation-state specific antibodies.

Peptides corresponding to the amino acid sequences encompassing human p120 tyrosine residues 96, 112, 228, 257, 280, 291, 296 and 302 were synthesised with the addition of an amino-terminal cysteine residue to facilitate coupling to the carrier protein keyhole limpet haemocyanin (KLH) (Table 1). Murine p120 is fully homologous over these sequences. Peptides were estimated to be greater than 90% pure by high performance liquid chromatography, and composition was confirmed by amino acid analysis (synthesis and analysis of peptides was carried out by Affiniti Research Products Ltd). KLH-conjugated peptide was emulsified in Freund's adjuvant, either complete for the primary immunization or incomplete for subsequent immunizations. Two rabbits were then immunised with 200 µg of peptide by subcutaneous inoculation on four occasions over a three to four month period and sacrificed 14 days after the final immunisation. All animal handling including immunisation was performed by trained staff of the Beatson Institute animal facility and was in accordance with a Home Office license for this project (Home Office license no: PPL603576).

After collection, blood was allowed to clot for 60 minutes at room temperature. The clot was separated from the sides of the glass collection vessel using a Pasteur pipette and the vessel incubated at 4°C overnight to allow the clot to contract. Serum was then aspirated and centrifuged at 10 000g for 10 minutes to remove any remaining particulate material. To isolate the IgG fraction 1 ml of serum was diluted in 14 ml of binding buffer (20 mM sodium phosphate, pH 7.0), any insoluble material removed with a 0.45 µm filter and ran through a Protein G Sepharose column. After washing with 10 column volumes of binding buffer the IgG was eluted with 0.1 M glycine-HCl, pH 2.7 into 1 ml aliquots pre-filled with 60 µl 1M Tris-HCl, pH 9.0. The IgG-containing fraction was dialysed against phosphate-buffered saline (PBS) at 4°C for 16 hours. Non-phospho-specific antibodies were then removed by absorption against a Sepharose column containing the immunizing peptide in its non-phosphorylated form and the remaining fraction was again absorbed against a protein G column, eluted and dialysed against PBS. Purified IgG was aliquoted and stored at -70°C. Approximate quantification was obtained by comparison of the density of bands produced by purified IgG and BSA standards on electrophoresis and subsequent Coomassie Brilliant Blue staining of the gel.

Tyrosine	Sequence	Residues
96	QDHSLL p YSTIPRM	89-102
112	QIVET p YTEEDPEG	107-119
228	CGGSDN p YGSLSRV	222-234
257	CSRQDV p YGPQPQVR	251-264
280	HRFHPEP p YGLEDDQR	273-287
291	DDQRSMG p YDDL DY	284-296
296	GYDDL p YGMMSD	290-301
302	GMMSD p YGTARRTG	297-309

Table 1 Peptide sequences used for immunisation.
In each case the phosphorylated tyrosine residue is highlighted.

3.2 Generation of plasmids

Full length human p120 isoform 1A with a C-terminal myc tag was generated by PCR using pEFBOS-p120-1A as a template and the 5' primer TTACCCTGCCCT-GCGGCGGCTCCGC and the 3' primer CGGTCGACTTACAGATCCTCTTCTGAGATGAGTTTTTGTTC AATCTTCTGCATC AAGGG which includes a myc tag and stop codon. The PCR product was cloned directly into pCR-Blunt II-TOPO and sequenced to ensure no mutations had occurred during amplification. The insert was then subcloned into the retroviral vector pLZRS-IRES-GFP using *EcoRI* sites to generate pLZRS-p120-1A / myc-IRES-GFP.

For the generation of pRetroSuper constructs oligonucleotides were synthesised which contained a 19 nucleotide human p120-specific RNAi sequence (GCCAGAGGTGGTTCGGATA) or scrambled control sequence (GATAAGGTGCTGCGTGGAC) in sense and antisense orientations separated by a 9 nucleotide spacer sequence. Oligonucleotides were inserted into pRetroSuper using the *Hind III* and *Bgl II* sites.

To digest plasmids 1.0 µg of DNA was incubated for 90 minutes at 37°C with 5-10 units of the appropriate restriction endonuclease and buffer in a final volume of 20 µl. Where vector was being linearised 1 µl of shrimp alkaline phosphatase and 2 µl 10X buffer were then added and the reaction incubated for a further 30 minutes at 37°C. Fragments were separated by electrophoresis on a 0.8 - 1.5% agarose gel run in 1X TAE at 90V for 30-90 minutes and were visualised by UV transillumination. Gels were prepared by dissolving electrophoresis grade agarose in 1X TAE buffer by microwaving. After cooling to 50°C 5 µg/ml ethidium bromide was added and the molten gel poured into an appropriate gel former. The band of interest was excised with a scalpel and extracted using the QIAquick Gel Extraction kit according to the manufacturer's instructions and resuspended in 20µl TE (10 mM Tris-HCl pH7.4, 1 mM EDTA pH 8.0). Purified DNA was quantified by agarose gel electrophoresis and comparison with a quantitative DNA ladder. Subsequent DNA ligation was performed with the Rapid DNA Ligation kit using 20-100 ng of linearised vector and a molar ratio of vector to insert of 1:3.

3.3 Site-directed mutagenesis

Site-directed mutagenesis of tyrosine residues 96, 112, 228, 257, 280, 291, 296 and 302, either singly or in combination, was performed in myc-tagged p120 in pCR-Blunt II-TOPO prior to subcloning into pLZRS-IRES-GFP and the sequence verified by sequencing. Mutagenesis was performed using the QuickChange Site-directed mutagenesis kit and the following primers incorporating the desired point mutation (synthesised by TAGN Ltd):-

CCAGGATCACAGTCACCTTCTATTTAGCACCATCCCCAGG	(Y96F)
GGCAGATTGTGGAGACCTTTACGGAGGAGGATCCTGAGGG	(Y112F)
CCAGGTGGCAGTGATAACTTTGGCAGTCTGTCCCCGGG	(Y228F)
CCTAGTAGACAGGATGTGTTTGGGCCCCCAACCCCAGG	(Y257F)
GCTTTCATCCAGAGCCTTTTGGGCTAGAGGATGACCAGCG	(Y280F)
CTAGAGGATGACCAGCGTAGTATGGGCTTTGATGACCTGG	(Y291F)
TGATGACCTGGATTTTGGTATGATGTCTGATTATGGCACTGCC	(Y296F)
TGGTATGATGTCTGATTTTGGCACTGCCCCGTCGGACTGGG	(Y302F)

100 ng of primer was combined with 100 ng of ds-DNA template, 1 µl of dNTP mix, 1 µl QuickChange multi enzyme blend and buffer in a final volume of 25 µl. 30 thermal cycles were performed using an extension time of 14 minutes. The reaction mixture was then incubated with *Dpn I* at 37°C for 1 hour. 1.5 µl of the *Dpn I* treated DNA was subsequently used to transform XL10-Gold competent cells.

3.4 Preparation of DNA

E.coli (DH5α or XL10-Gold) competent cells, stored at -70°C, were thawed on ice and 20 µl aliquoted into pre-chilled Eppendorf tubes. Various amounts of DNA, ranging from 0.1 to 1 µg, were added to the competent cells and the solution was gently mixed with a pipette tip. The mixture was incubated on ice for 30 minutes, after which the cells were heat-shocked for 30 seconds in a 42°C water bath. After an additional 2 minutes on ice 250 µl of pre-heated L-broth was added and cells were incubated for one hour at 37°C in a shaking incubator (at 225 rpm). The mixture was then spread on agar plates containing 100 µg/ml of ampicillin or 50 µg/ml of kanamycin (depending on the resistance cassette in the plasmid) and incubated overnight at 37°C. Single colonies were picked and grown overnight in L-broth containing 100 µg/ml of ampicillin or 50 µg/ml of kanamycin. DNA

extraction was carried out by Wizard PureFection plasmid DNA Purification System according to the manufacturer's instructions.

3.5 Retroviral infection

pLZRS-IRES-GFP and pRetroSuper based constructs were introduced into KM12C-Src527F or A431 cells by retroviral infection. 5-7.5 μ g of DNA was incubated with 30 μ l of DOTAP Liposomal Transfection Reagent in a final volume of 150 μ l HBS for 15 minutes at room temperature. The transfection mixture was then added to Phoenix Ampho packaging cells which had been plated at 5×10^5 cells per 60 mm tissue culture dish 6 hours earlier. The transfection medium was removed 16 hours later and replaced with fresh DMEM and 10% FBS. After 24 hours the viral supernatant was collected, filtered through a 0.45 μ m membrane and added to sparsely plated cells in the presence of 4 μ g/ml polybrene. Fresh medium was added to the Phoenix Ampho cells and a second infection carried out 24 hours later. Cells with stable retroviral integration were selected on the basis of GFP expression (KM12C-Src527F / pLZRS-IRES-p120-GFP constructs) or with 2 μ g/ml puromycin (A431-pRetroSuper).

3.6 Nucleofection

Nucleofection of A431-p120⁻ cells with pLZRS-murine (m)p120-3A-IRES-GFP, pLZRS-mp120-3A/7F-IRES-GFP and pLZRS-mp120-4A-IRES-GFP was performed with the Amaxa Nucleofector System in accordance with the manufacturer's instructions. Briefly, 2×10^6 cells were suspended in 100 μ l Nucleofector solution V in a cuvette and 5 μ g DNA added. The chemical composition of Solution V was proprietary information and not available from Amaxa. Nucleofection was performed using program P-20 and cells immediately transferred to a culture dish at 37°C. Pooled populations with uniform levels of expression were selected according to GFP expression using a BD FACSVantage SE flow cytometer.

3.7 Routine cell culture

A431 and Phoenix Ampho cell lines were routinely grown in DMEM supplemented with 10% FBS and L-glutamine. KM12C-derived cell lines were cultured in MEM supplemented with 10% FBS, L-glutamine, MEM vitamin solution, non-essential amino acids and sodium pyruvate. All cell lines were maintained in a humidified 37°C / 5% CO₂

incubator. To sub-culture adherent cells the medium was removed by aspiration and the monolayer rinsed with PBS followed by 10% trypsin/PE solution. Upon detachment the cells were resuspended in complete medium, counted, and then transferred into tissue culture flasks, plates or chamber slides.

3.8 Growth factors and drugs

Recombinant human EGF was dissolved in 10 mM acetic acid / 0.1% BSA to a stock concentration of 100 µg/ml and diluted in serum-free medium to a final concentration of 10 or 100 ng/ml immediately prior to use. AP23464 was dissolved in DMSO to a stock concentration of 10 mM. Immediately prior to use it was diluted in medium to achieve a final concentration of 1 µM. AG1478 was dissolved in DMSO to a stock concentration of 10 mM and was further diluted in medium to a final concentration of 300 nM immediately prior to use.

3.9 Immunofluorescence

Cells plated on 8-well glass (A431) or plastic (KM12C) chamber slides were washed in PBS then fixed in 3.7% formaldehyde for 10 minutes. They were then washed twice in PBS and permeabilised with PBS/0.5% Triton X-100/1% BSA for 15 minutes. After blocking with PBS/10% FCS for 1 hour, cells were incubated with primary antibodies (Table 2) in blocking buffer overnight at 4°C. Detection of p120-phospho-Y228 mAb was by reaction with anti-mouse biotin (diluted 1 in 750 in blocking buffer) for 60 minutes followed by Alexa-488 Streptavidin (diluted 1 in 200 in blocking buffer) for 40 minutes. For all other primary antibodies detection was with FITC- or TRITC-conjugated secondary antibodies (diluted 1 in 100 in blocking buffer) for 45 minutes. Cells were visualised using a Leica DM IRBE confocal microscope (Leica UK Ltd., Milton Keynes, UK).

Antibody	Dilution
Anti-E-cadherin	1:100
Anti-HA tag	1:200
Anti-myc tag	1:2000
Anti-p120	1:1000
Anti-p120-phospho-Y228 (monoclonal)	1:200

Table 2 Primary antibodies used for immunofluorescence

3.10 Preparation of protein extracts

Dishes were transferred directly from the incubator onto ice. Cells were then washed twice with PBS and then lysed in ice-cold RIPA buffer or co-IP buffer for 10 minutes. Cells were scraped off the tissue culture plastic using a disposable cell scraper and the lysate transferred to a microcentrifuge tube. The lysate was then clarified by centrifugation at 14,000g for 15 minutes at 4⁰C. Protein concentration was determined using the Micro BCA Protein Assay Kit and light absorbance then measured with a DU 650 spectrophotometer at a wavelength of 562 nm.

3.11 Western blot analysis

Laemmli sample buffer was added to lysates which were then boiled for 5 minutes. Protein separation was achieved by running the samples and molecular weight markers on an SDS-PAGE gel consisting of a short stacking gel and a longer resolving gel. The resolving gel contained 7% or 10% acrylamide depending on the size of the proteins being separated. Gels were typically run at 200V for 1 hour and 15 minutes. Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane, while being buffered by 3MM filter paper saturated in transfer buffer, using semi-dry blotting apparatus at 20V for 45 minutes. After the proteins had been transferred onto nitrocellulose the membrane was blocked for one hour at room temperature in 5% low fat milk in TBS / 0.2% Tween 20. The primary antibody in 3% BSA / TBS / 0.2% Tween 20 was then added for 1 hour at room temperature or overnight at 4⁰C (Table 3). The blots were then washed four times in TBS / 0.2% Tween 20 (10 minutes each) and incubated with horseradish peroxidase conjugated secondary antibodies at 1 in 10 000 in 5% low fat milk / TBS / 0.2% Tween 20 for 45 minutes. The blots were again washed four times in TBS / 0.2% Tween 20 (15 minutes each) and ECL reagent added for 1 minute. The proteins were then visualized using a Kodak X-OMAT 480 RA film processor. When necessary, blots were stripped using Re-Blot Plus Strong Antibody Stripping Solution and reprobed with additional antibodies.

Antibody	Dilution
Anti-actin	1:500
Anti- α -catenin	1:400
Anti- β -catenin	1:500
Anti- γ -catenin	1:2000
Anti-E-cadherin	1:2000
Anti-N-cadherin	1:2500
Anti-P-cadherin	1:250
Anti-CDC42	1:1000
Anti-EGFR	1:5000
Anti-phospho-EGFR	1:1000
Anti-FAK	1:1000
Anti-HA tag	1:500
Anti-MAPK	1:10 000
Anti-phospho-MAPK	1:1000
Anti-myc tag (9B11)	1:1000
Anti-p120	1:1000
Anti-p120-pY96	1:1000
Anti-p120-pY228 (monoclonal)	1:5000
Anti-p120-pY228 (polyclonal)	1:250
Anti-p120-pY280	1:250
Anti-p120-pY291	1:500
Anti-p120-pY296	1:5000
Anti-p130 ^{cas}	1:1000
Anti-paxillin	1:10 000
Anti-phosphotyrosine (PY20)	1:1000
Anti-Rac1	1:1000
Anti-RhoA	1:500
Anti-Src Kinase Domain	1:5000
Anti-Src-pY416	1:1000
Anti-STAT3	1:1000
Anti-phospho-STAT3	1:250

Table 3 Primary antibodies used for Western blotting

3.12 Immunoprecipitation

250-1000 µg of cleared lysate was incubated with the appropriate primary antibody at 4°C overnight (Table 4). Anti-mouse IgG-agarose or protein-G-agarose (for rabbit primary antibodies) was then added to immunoprecipitates for 1 hour at 4°C. Immunoprecipitates were washed three times, resuspended in Laemmli sample buffer, and boiled for 5 minutes. Where indicated immunoprecipitates were first washed in LAR reaction buffer then incubated with 2 µl / 10 units LAR phosphatase at 30°C for 30 minutes prior to addition of Laemmli sample buffer. Denatured proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes for immunoblotting.

Antibody	Dilution
Anti-E-cadherin	1:125
Anti-HA tag	1:125
Anti-myc tag	1:500
Anti-p120	1:250

Table 4 Primary antibodies used for immunoprecipitation.

3.13 Rho-family GTPase activity assay

E. Coli were transformed with the pGEX2TK vector encoding either the Rac- and Cdc42-binding region from human PAK1B (GST-PAK-CD) (104) or the Rho binding domain, from the Rho effector protein Rhotekin (GST-C21) (436) fused to GST. Cells were grown to OD₆₀₀ 0.3 and then induced to express the recombinant protein by the addition of 0.1 mM IPTG for 2 hours. Cells were then harvested, resuspended in bacterial lysis buffer (50 mM Tris-HCl pH 7.5, 1mM EDTA, 100 mM NaCl, 0.1% triton X-100, 5% glycerol, 1 mM dithiothreitol, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM benzamidine, and 1 mM PMSF) and sonicated for 2-3 minutes. Lysates were centrifuged at 4°C for 20 minutes at 10 000g and the supernatant incubated with glutathione-coupled Sepharose beads for 30 minutes at 4°C with gentle agitation. Beads were then washed three times with lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 1% NP-40, 10% glycerol, 100 mM NaCl, 1 mM benzamidine, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 1 mM DTT, 1 mM PMSF) and retained at 4°C until used. Cells were serum starved for 16 hours then stimulated with 100 ng/ml EGF or vehicle (10 µM acetic acid / 0.0001% BSA) for 5

minutes (Rac / Cdc42) or 15 minutes (RhoA), washed in ice-cold PBS (containing 1 mM MgCl₂ and 0.5 mM CaCl₂), incubated for 5 minutes on ice in lysis buffer, and then centrifuged for 5 min at 14 000 g at 4°C. Aliquots were taken from the supernatant to compare protein amounts. The remaining supernatant was incubated at 4°C with either GST-PAK beads for 30 minutes or GST-RBD beads for 60 minutes. Beads were then washed three times in lysis buffer, eluted in Laemmli sample buffer, and analyzed for bound Rho family molecules by Western blotting using anti-RhoA, anti-Rac1, or anti-CDC42 antibodies as described in section 3.11. Films were scanned and the density of bands quantified using Total Labs software.

3.14 Cell proliferation assay

Proliferation was determined using the CellTiter 96 Non-Radioactive Cell Proliferation Assay kit. Briefly, 1×10^3 cells / well were plated per well on multiple 96-well plates in a volume of 100 μ l. At 24 hour intervals 15 μ l of dye solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added. After a 4 hour incubation at 37°C 100 μ l of solubilization solution (an organic solvent, exact composition proprietary information of Promega) was added and the absorbance at 570 nm determined using a FlexStation II 96 well plate reader (Molecular Devices, CA, USA). Mean and S.D. values were calculated from quadruplicate wells.

3.15 Cell motility assay

1×10^4 cells / well were plated on a 6-well plate and allowed to adhere for 8 hours. Images were then captured every 15 minutes using an Axiovert 200 M Zeiss microscope with a x20 objective and AQM Advance software (Kinetic Imaging, Nottingham, UK). The motility of individual cells was analysed using Tracking Analysis software (Kinetic Imaging). In all experiments the data was positively skewed and therefore data is reported as median and interquartile range and the non-parametric Mann-Whitney U test used for statistical comparison.

3.16 Wound healing assay

1×10^6 cells / well were plated on 6 well plates. 24 hours later a wound was made with a single pass of a sterile pipette tip and images of cells migrating into the wound recorded by time-lapse video-microscopy at 15 minute intervals until complete wound closure. For

quantification, the distance travelled by the leading edge just prior to wound closure was measured and divided by time to calculate speed of migration. 10 individual points in two different wounds were measured and results expressed as mean \pm S.D.

Alternatively cells were plated on glass chamber slides and grown to confluence prior to wounding with a sterile pipette tip. After 6 hours cells were fixed with 3.7% formaldehyde and immunofluorescence performed.

3.17 Chemotactic migration assay

Directional migration of cells in response to EGF was measured using the InnoCyte Cell Migration Assay Kit according to the manufacturer's instructions. Briefly, 5×10^4 cells were resuspended in serum-free DMEM and plated on the upper surface of an uncoated transwell with 8 μ m pore size. The lower surface of the transwell was placed in serum free DMEM with or without EGF (1-100 ng/ml). After incubation for 24 hours at 37°C in a humidified incubator the transwell was removed and placed in a fresh chamber containing Cell Detachment buffer and Calcein-AM solution. Following a 45 minute incubation at 37°C the transwell was gently tilted back and forth to facilitate dislodgement of any cells still adherent to the lower surface of the transwell and then removed. The lower chamber was then incubated for a further 45 minutes at 37°C before determining the fluorescence with excitation 485 nm and emission 520 nm. Mean and S.D. values were calculated from triplicate wells.

3.18 Matrigel invasion assay

60 μ l of growth factor-depleted Matrigel was added to the upper chamber of a Transwell 8 μ m pore size filter and allowed to set at 37°C for 90 minutes. Cells were trypsinised, washed and resuspended in DMEM at 1×10^5 cells/ml. 100 μ l of cell suspension was then placed on an inverted Transwell and the cells allowed to adhere for 4 hours. The Transwell was then carefully washed twice with serum-free DMEM before being placed in a well containing serum free DMEM with the cells adherent to the lower surface. Serum free DMEM with or without EGF (10 ng/ml) was then added to the centre of the Transwell and the plate left at 37°C in a humidified incubator for 72 hours. To stain cells Calcein AM (diluted in HEPES-buffered serum-free DMEM to a final concentration of 4 μ M) was added to both upper and lower chambers and the plate returned to the 37°C incubator for 60 minutes. Horizontal z-sections through the Matrigel were then taken at 15 μ m intervals

using a Leica DM IRBE confocal microscope. The number of positive pixels in each image was determined using “Image J” software (NIH) using a value of 150 for background subtraction. The sum of the values obtained for individual z-sections (excluding the first section which corresponds to the upper surface of the filter) was then expressed as a percentage of the control cell value. For each experiment samples were run in duplicate and at least 4 z-series were taken per sample.

3.19 Organotypic invasion assay

pECFP was introduced into A431-Scr or A431-p120⁻ cells using the Amaxa Nucleofector System and pooled populations with uniform expression selected by FACS. These were plated on top of collagen/Matrigel (4 mg/ml collagen/2.5 mg/ml Matrigel) gels containing 10% FBS. Where indicated 5×10^5 tumour derived fibroblasts were added to the gel. After 24 hours the gel was transferred onto a nylon filter and placed on a mesh support. This was partially submerged in media containing 10% FBS, while the rest of the gel including the A431 cells was in contact with air. After 5 days the gels were fixed with 4% paraformaldehyde / 0.25% glutaraldehyde / PBS, permeabilised with 0.2% Triton X-100/PBS and stained with TRITC-phalloidin. A multi-photon laser scanning microscope was used to take 5 optical xz sections of each gel and the area of non-invading cells and the total area over which cells had spread was measured. The invasion index = $1 - \text{area of non-invading cells} / \text{total area over which cells had disseminated}$. Where indicated E-cadherin specific siRNA sequences (#1 GGAGAGCGGUGGUCAAAGAUU, #2 ACCAGAACCUCGAACUAUAUU) or non-targeting control sequences were introduced into the cells using Oligofectamine according to the manufacturers instructions. All organotypic invasion assays were performed by Dr S Hooper in the laboratory of Dr E Sahai, London Research Institute.

4 Results: Generation and validation of novel p120 phospho-specific antibodies.

4.1 Aim

p120-catenin is phosphorylated on tyrosine residues following activation of RTKs including EGF, PDGF, CSF-1 and VEGF (181, 182, 184) and cytoplasmic tyrosine kinases such as Src (52). Using a 2-dimensional tryptic mapping approach eight tyrosine residues (at positions 96, 112, 257, 280, 291, 296 and 302) which are phosphorylated in Src transformed fibroblasts have been identified (68). However the regulation and consequences of phosphorylation events occurring at each of these individual sites is largely unknown (176).

Sequence-specific phosphorylation-state specific antibodies (hereafter referred to as phospho-specific antibodies) have proved useful tools in enabling the feasible dissection of signalling pathways and, specifically, in defining events occurring at individual residues rather than global phosphorylation of a protein. In addition, for the in-situ study of protein phosphorylation in tissues or cells phospho-specific antibodies are essential. At the outset of this work no p120 phospho-specific antibodies existed. We therefore aimed to produce novel phospho-specific antibodies to facilitate the study of p120 site-specific phosphorylation events occurring in response to receptor and non-RTK activation in cancer cell lines and tissues.

4.2 Results

4.2.1 p120 is phosphorylated in KM12C cells expressing constitutively active c-Src.

To validate novel phospho-specific antibodies a source of phosphorylated p120 was required. Tyrosine phosphorylation of p120 has been best defined in cell lines expressing the active form of the cytoplasmic tyrosine kinase Src (52, 68). The generation of a KM12C colon carcinoma cell line with stable expression of a constitutively active Src527F mutant has previously been reported (257). The level and activity of Src (as measured by auto-phosphorylation of tyrosine-416) was dramatically increased in KM12C-Src527F cells as compared to the parental cell line (Figure 4-1 A). Using an antibody that recognises all tyrosine phosphorylation events, p120 immunoprecipitated from Src527F expressing cells was seen to be heavily phosphorylated on tyrosine residues as compared to p120 in the parental cells (Figure 4-1 B, upper panel). Equivalent levels of total p120 were present in the immunoprecipitates (Figure 4-1 B, lower panels). The presence of two

distinct bands reflects the presence of two dominant p120 isoforms in this cell line due to alternative splicing events which generate p120 isoform 3 with or without inclusion of the additional exons A, B or C.

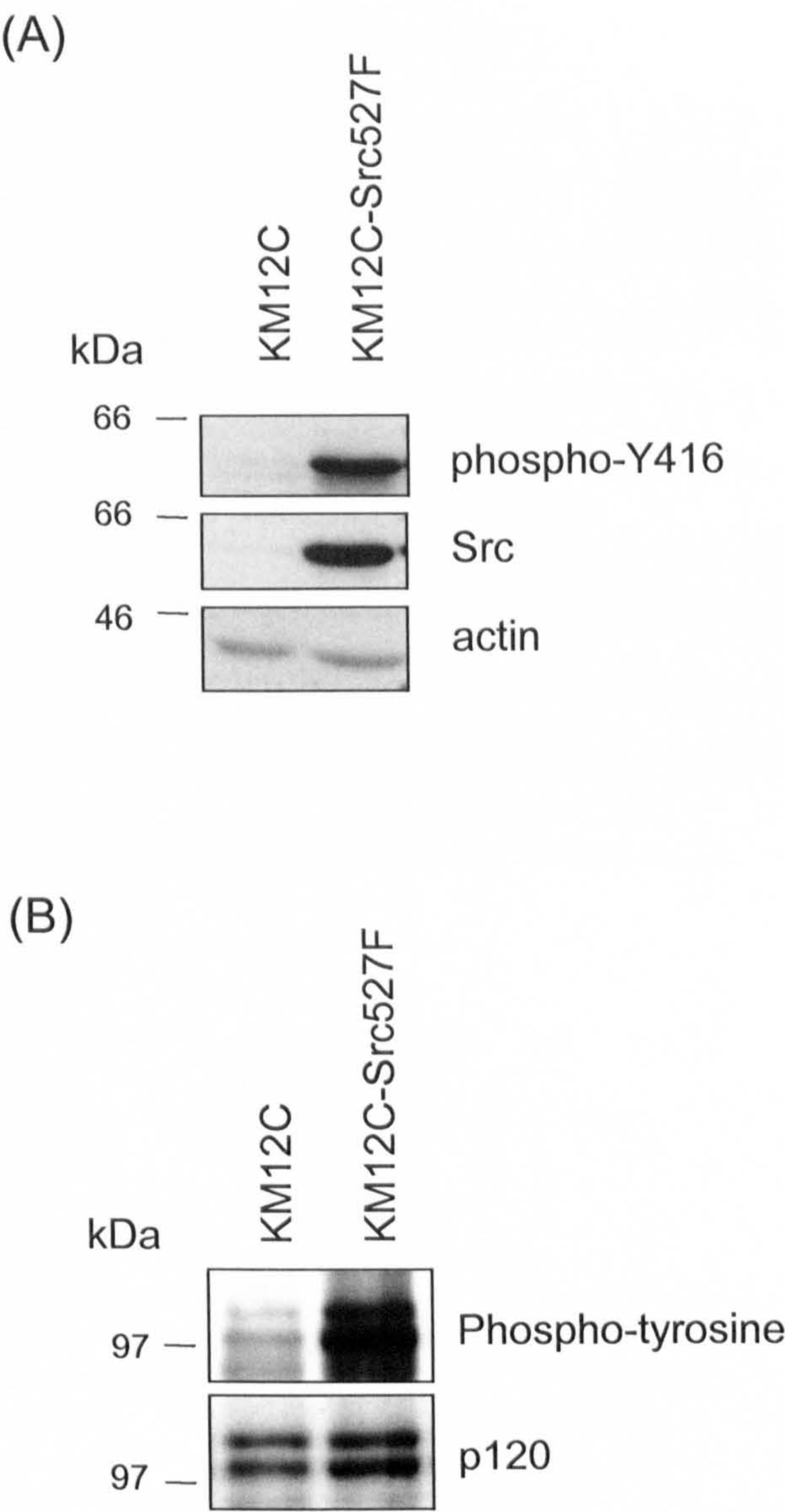


Figure 4-1 Expression of active Src in KM12C cells causes tyrosine phosphorylation of p120.

A) Lysates from KM12C cells or KM12C cells expressing constitutively active Src (KM12C-Src527F) were prepared and analyzed by Western blotting with Src phospho-Y416 antibody. After removal of the phospho-specific antibody the blot was reprobed with Src antibody. Actin is a loading control.

(B) p120 was immunoprecipitated from KM12C or KM12C-Src527F cells and analysed by Western blotting with a pan-phosphotyrosine antibody. The blot was stripped and reprobed with total p120 antibody.

4.2.2 Production of antibodies.

Serum was obtained from rabbits immunised with short peptides encompassing p120 tyrosine residues 96, 112, 257, 280, 291, 296 and 302. In each case the specific tyrosine residue had been phosphorylated at the time of synthesis and two rabbits were immunised with each peptide. We attempted to remove antibodies able to recognise non-phosphorylated peptide by immuno-affinity absorption and the remaining purified IgG fraction was then tested for its ability to immunoprecipitate p120 from KM12C-Src527F lysate (Figure 4-2 A). In addition, as KM12C cells express primarily isoform 3 which lacks tyrosine 96, exogenous p120 isoform 1A was co-expressed (see section 1.2.5) and used to test sera from rabbits immunised with peptide 89-102 (Figure 4-2 B). For each peptide at least one rabbit mounted a specific anti-p120 antibody response. The remainder of this chapter describes the validation performed to obtain robust evidence for the specificity of these antibodies.

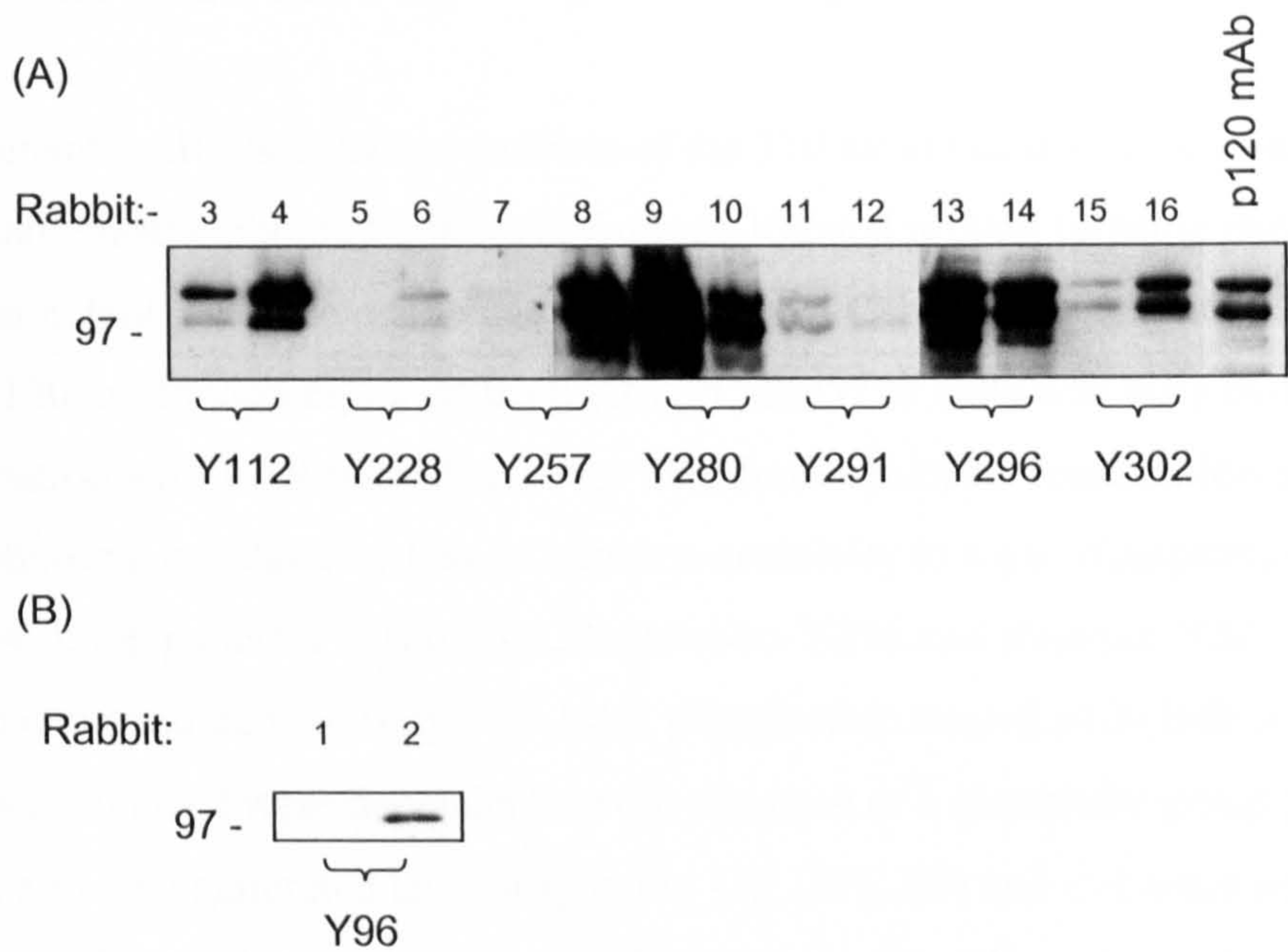


Figure 4-2 Generation of phospho-specific antibodies.

(A) p120 was immunoprecipitated from KM12C-Src527F lysate using total p120 mAb or antisera from duplicate rabbits immunised with the p120 phospho-peptides shown. The membrane was then probed with total p120 antibody. (B) Exogenously expressed myc-tagged p120-1A was immunoprecipitated from KM12C-Src527F cells with anti-myc antibody and the membrane probed with anti-phospho-Y96 antisera.

4.2.3 p120 is effectively de-phosphorylated by LAR phosphatase.

Recombinant LAR phosphatase consists of the 350 amino acid soluble catalytic domain of the human transmembrane Leukocyte Antigen Related protein tyrosine phosphatase and is known to potently release phosphate groups from phospho-tyrosine residues in proteins (437). p120 immunoprecipitated from KM12C-Src527F cells was fully de-phosphorylated by incubation with LAR phosphatase for 30 minutes prior to denaturation and gel electrophoresis as judged by loss of immuno-reactivity to a pan-phosphotyrosine antibody (Figure 4-3, top panel). Phospho-Y228, phospho-Y296 and phospho-Y302 antibodies failed to demonstrate reactivity with LAR phosphatase treated p120 indicating that the epitopes recognised were dependent on the presence of a phosphate group (Figure 4-3). In contrast anti-sera generated against tyrosine 112, 257, 280 and 291 were able to effectively immunoprecipitate dephosphorylated p120 indicating that they recognised epitopes independent of p120 phosphorylation status and thus were not phospho-specific (Figure 4-3, bottom panel shows anti-phospho-Y280 as an example.)

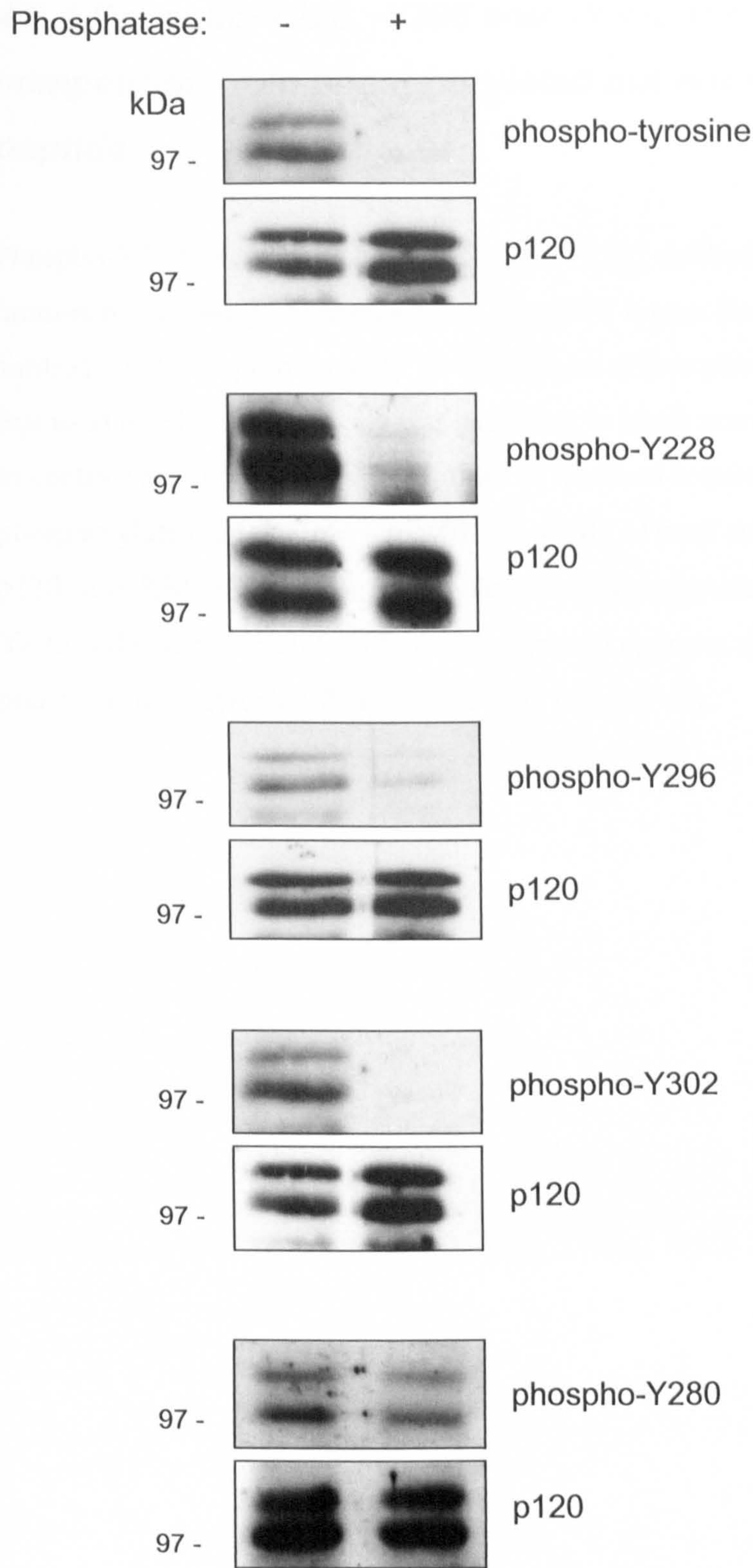


Figure 4-3 Phospho-Y228, -Y296 and -Y302 antibodies only detect phosphorylated p120.

p120 was immunoprecipitated from KM12C-Src527F cell lysate and incubated with LAR phosphatase for 30 minutes prior to denaturation and electrophoresis. The membrane was probed with anti-phosphotyrosine, phospho-Y228, phospho-Y296, phospho-Y302 or phospho-Y280 antibodies. After removal of the phospho-specific antibody the blots were reprobed with anti-p120 antibody.

4.2.4 Phospho-Y228, -Y296 and –Y302 antibodies are sensitive to competition from phosphorylated but not non-phosphorylated peptide.

Phospho-Y228, phospho-Y296 and phospho-Y302 antibodies were able to immunoprecipitate p120 from KM12C-Src527F lysate. However, pre-incubation of the antibody with an approximately 10-fold excess of free phosphorylated peptide identical to that used to raise that antibody was sufficient to block immunoprecipitation (Figure 4-4). In contrast pre-incubation with peptides of identical sequence which were not phosphorylated did not interfere with the ability of each antibody to immunoprecipitate p120 from KM12C-Src527F lysate. This strongly suggested that phospho-Y228, phospho-Y296 and phospho-Y302 antibodies recognised epitopes specifically containing phosphorylated tyrosine-228, -296 or -302 respectively.

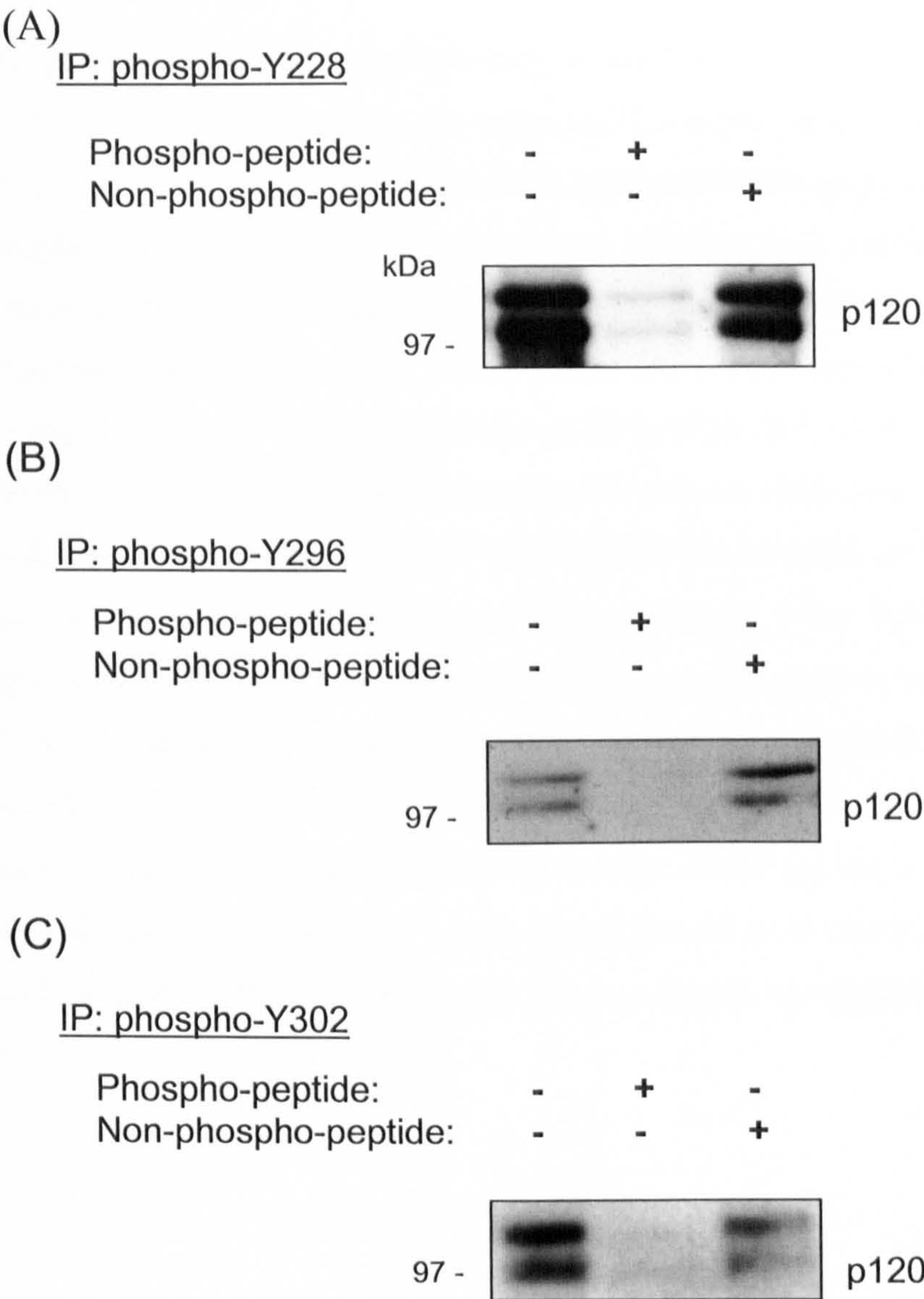


Figure 4-4 Immunoprecipitation of p120 by phospho-antibodies is specifically inhibited by pre-incubation with phospho-peptide.

Phospho-Y228 (A), phospho-Y296 (B), or phospho-Y302 antibodies were used to immunoprecipitate p120 from KM12C-Src527F lysate (left lane). Alternatively, the antibodies were first incubated for one hour with an excess of phosphorylated (middle lane) or non-phosphorylated peptide (right lane) of identical sequence to the immunising peptide prior to immunoprecipitation. The membrane was probed with anti-p120 antibody.

4.2.5 Generation of phosphorylation defective p120 constructs.

Phenylalanine is an aromatic amino acid which differs from tyrosine only in that it lacks the reactive hydroxyl group in the *ortho* position on the benzene ring. Substitution of tyrosine with phenylalanine therefore has minimal effects on protein structure but prevents phosphorylation occurring (438). Therefore, substitution of a specific tyrosine residue with phenylalanine is a commonly used technique to prevent phosphorylation at a specific candidate residue. Myc-tagged p120 isoform 1A constructs were generated in the bicistronic retroviral expression vector pLZRS-IRES-GFP. p120 cDNA was sited upstream, and GFP cDNA downstream, of an internal ribosomal entry site (IRES). As a result translation of both p120 and GFP occurs from the same mRNA transcript and the levels of the two products are directly proportional (81). Site directed mutagenesis of all eight putative sites of tyrosine phosphorylation to phenylalanine was performed (p120-1A/8F). In addition each tyrosine residue was mutated to phenylalanine in isolation (p120-1A/96F, p120-1A/228F, p120-1A/296F, p120-1A/302F). Stable expression of all constructs in KM12C-Src527F cells was achieved and single cell clones derived on the basis of GFP expression. Next we confirmed that all p120 constructs were correctly localised to the cell membrane as this is a prerequisite for significant phosphorylation of p120 to occur (Figure 4-5) (273).

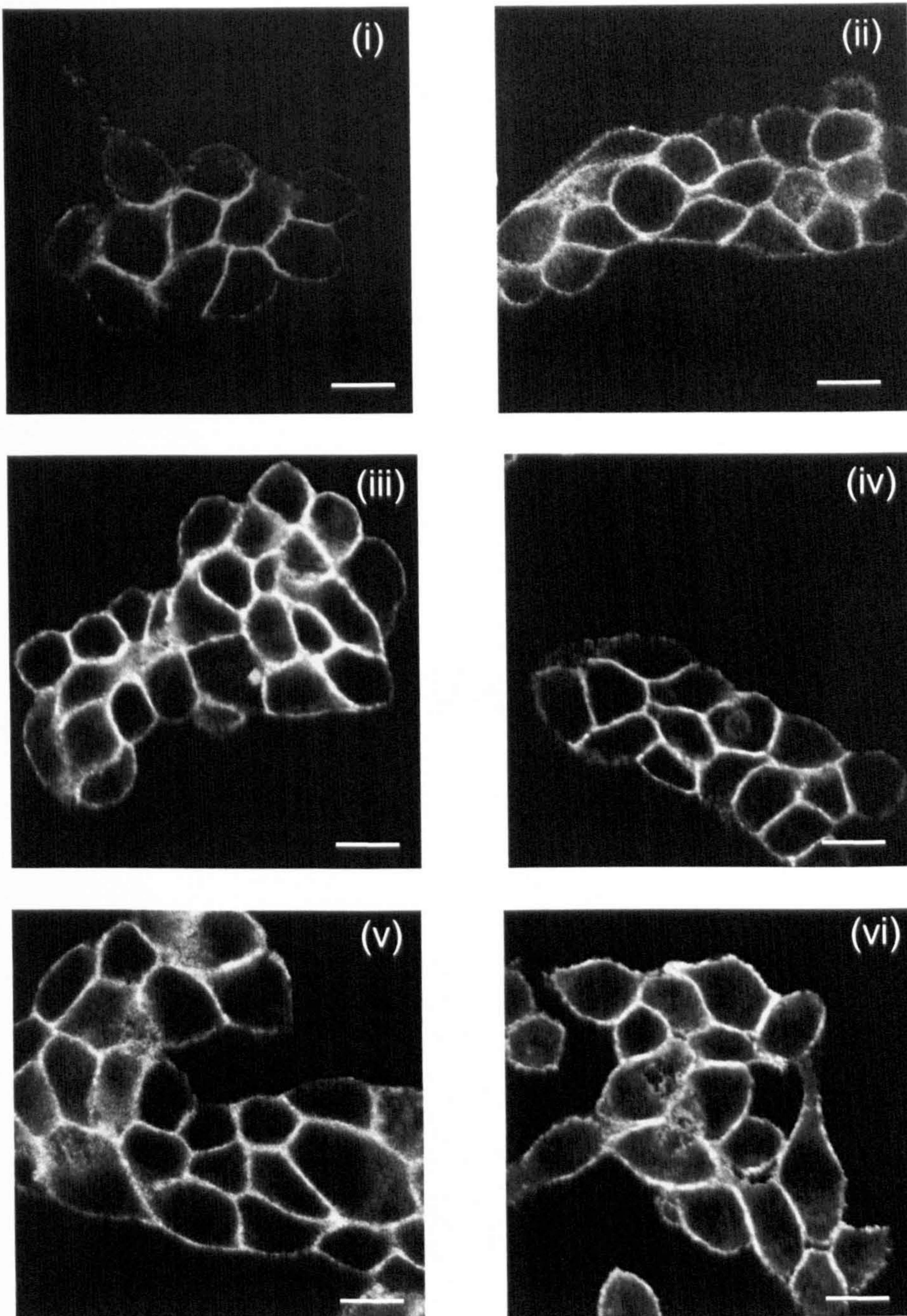


Figure 4-5 Myc-tagged p120 localises to cell-cell contacts.

Immunofluorescence using anti-myc antibody was performed on KM12C-Src527F cells with stable expression of: (i) myc-tagged wt-p120-1A, (ii) p120-1A / 8F, (iii) p120-1A / 96F, (iv) p120-1A / 228F, (v) p120-1A / 296F, (vi) p120-1A / 302F. Bar 20μm.

4.2.6 Phospho-Y228 and phospho-Y296 antibodies specifically recognise phosphorylated Y228 and Y296 respectively.

Both phospho-Y228 (Figure 4-6 A) and phospho-Y296 (Figure 4-6 B) antibodies were able to recognise myc-tagged wild type p120-1A. Neither antibody was able to recognise p120-1A in which all eight sites of tyrosine phosphorylation had been mutated to phenylalanine. Crucially, neither phospho-Y228 nor phospho-Y296 antibody recognised p120 in which only tyrosine-228 or tyrosine-296, respectively, had been mutated to phenylalanine. Thus both antibodies were truly specific for p120 only when phosphorylated on the specific residue intended and there was no cross-reactivity for other phosphorylated p120 tyrosine residues.

4.2.7 Phospho-Y96 and phospho-Y302 antibodies were not site-specific.

Antibodies generated against phospho-Y96 (Figure 4-6 C) and phospho-Y302 (Figure 4-6 D) were unable to recognise p120-1A in which all eight sites of tyrosine phosphorylation had been mutated to phenylalanine. However, mutation to phenylalanine of the individual tyrosine residue to which the antibody had been raised did not prevent immunoreactivity. Indeed no differential ability to recognise wild type versus mutant p120 was elicited despite the use of increasing dilutions of antibody (data not shown). Taken together with the results of the phosphatase and peptide competition experiments this suggested that these antibodies recognised phosphorylated tyrosine residues present in p120 in addition to the specific target residue to which they had been raised. For this reason these antibodies were not used further.

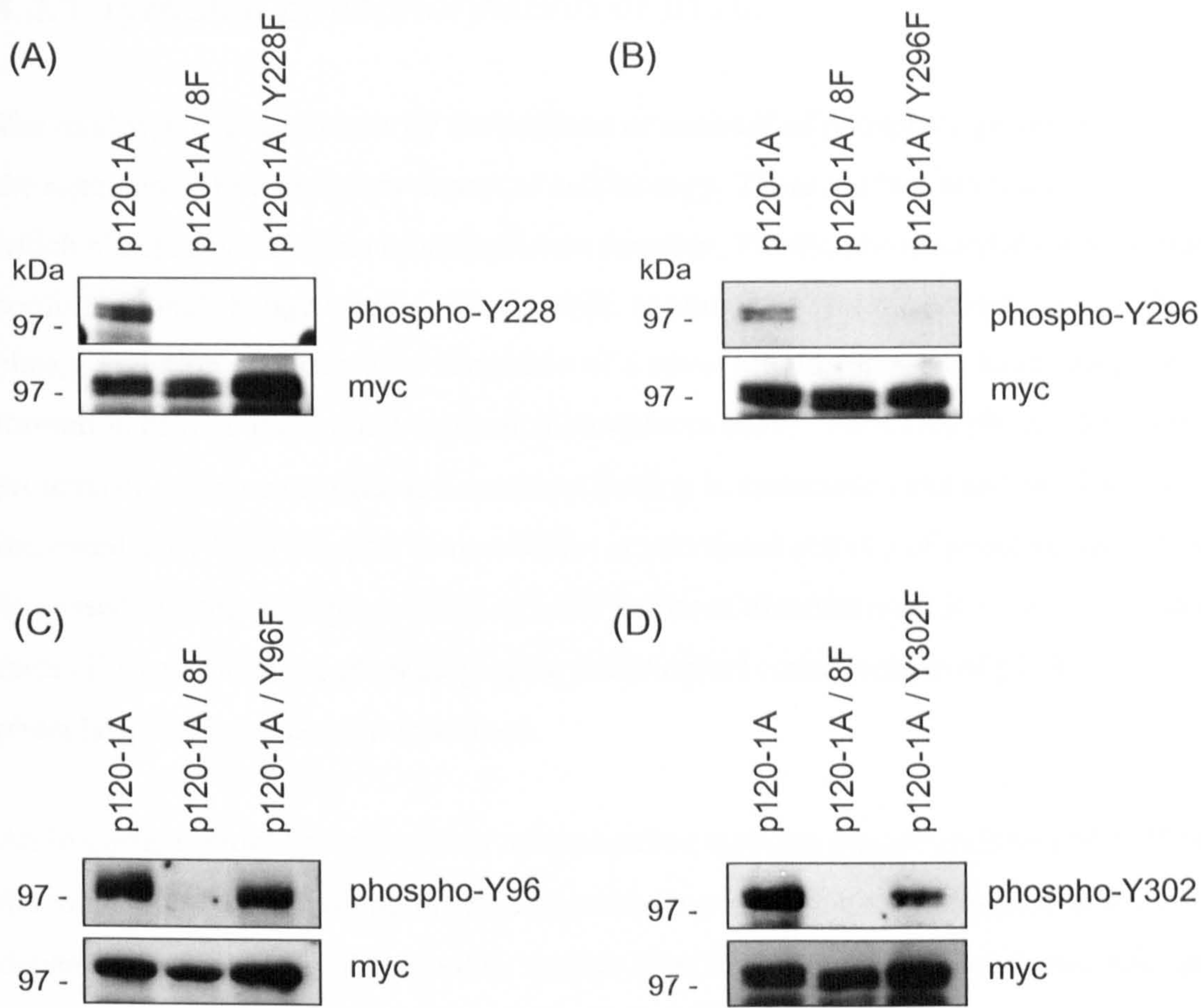


Figure 4-6 Tyrosine to phenylalanine mutants are not recognised by phospho-Y228 and phospho-Y296 antibodies.

Lysates prepared from KM12C cell lines co-expressing constitutively active Src (KM12C-Src527F) with myc-tagged wild type p120-1A (p120-1A) or p120-1A in which either all eight tyrosine phospho-acceptor residues (p120-1A/8F) or only the residue to which each antibody had been raised (p120-1A/96F, p120-1A/228F, p120-1A/296F and p120-1A/302F) were mutated to phenylalanine. Immunoprecipitations were performed with anti-myc antibody. Membranes were analysed by immuno-blotting with phospho-Y228 (A), phospho-Y296 (B), phospho-Y96 (C) or phospho-Y302 (D) antibodies. After removal of the phospho-specific antibody the blots were reprobed with anti-myc antibody.

4.3 Discussion

4.3.1 Tyrosine phosphorylation of p120.

The modification of proteins by the addition or removal of phosphate groups is involved in the regulation of nearly every aspect of cell biology. There are two main mechanisms by which phosphorylation can modify protein function. Firstly, phosphorylation may induce a conformational change leading, for example, to altered enzymatic activity. Secondly, phosphorylation may cause the formation of a novel binding site thus facilitating the formation of functional multi-molecular complexes (439). Increased phosphorylation of proteins on tyrosine residues is a common finding in cancerous cells and results from increased activity of receptor or non-RTKs or decreased activity of phosphatases (440). Increased tyrosine phosphorylation of p120 has been demonstrated in various tumour cell lines (176) although the physiological or pathological consequences of p120 phosphorylation are largely undefined.

An important initial contribution in understanding tyrosine phosphorylation of p120 was the mapping of eight individual tyrosine residues which are phosphorylated in a Src-dependent manner (68). Prior to this, studies of p120 tyrosine phosphorylation have relied on the use of pan-phosphotyrosine antibodies and studied global phosphorylation of the protein. However, many examples exist of differential regulation of individual tyrosine residues within a single protein and therefore attempts to correlate phenotype with changes in overall phosphorylation, rather than phosphorylation at individual sites, risk being uninformative. Indeed, a paradigm for this already exists for p120 in the regulation of serine / threonine phosphorylation. Activation of protein kinase C (PKC) in epithelial or endothelial cells was associated with dephosphorylation of p120 on serine / threonine residues using a general phospho-serine / threonine antibody (441, 442). However, two-dimensional tryptic mapping techniques showed that PKC dependent de-phosphorylation of several p120 residues was, unexpectedly, accompanied by a potent increase in phosphorylation at serine-873 (69). Furthermore, a variety of kinases and phosphatases may impact on p120 phosphorylation and the extent to which individual tyrosine residues are independently regulated in response to varying stimuli or cellular context is unknown.

4.3.2 Generation of phospho-specific antibodies.

The development of phospho-specific antibodies since the early 1990's has been a major technological advance in the study of protein phosphorylation (443). Earlier techniques employing ^{32}P -radiolabelling are laborious, difficult to control, can use large amounts of radio-isotope and are not feasible methods to study individual events in proteins containing multiple phospho-acceptor residues. Drawbacks also exist with the use of general anti-phosphotyrosine antibodies. Firstly, to study phosphorylation of a particular protein the antibody must be used in conjunction with a protein-specific antibody, usually by incorporating a specific immuno-precipitation step and thus the study of phosphorylation in cells or tissues in-situ is not possible. Secondly, phosphorylation occurring at different residues within the molecule cannot be distinguished. Hence phospho-specific antibodies have several theoretical and practical advantages. Firstly, they allow the easy assessment by Western blotting of protein phosphorylation which has occurred in a cell.

Phosphorylation events occurring at different residues within the same protein are readily distinguished. The sub-cellular localisation of phosphorylated proteins can be identified by immunofluorescence. Finally, the presence and localisation of phosphorylated proteins in tissues can be determined by immuno-histochemical analysis.

To generate phospho-specific antibodies to p120 we utilised short peptides encompassing each of the eight tyrosine residues recently identified as sites of tyrosine phosphorylation. Peptides of 12-15 residues are long enough to form an epitope including the phosphorylated tyrosine but minimise the chances of providing other, phosphorylation-independent epitopes (444). Peptides of this length are poorly immunogenic and require to be linked to a carrier protein for efficient elicitation of an antibody response. For this reason our immunising peptides were conjugated to the carrier protein, keyhole limpet haemocyanin via the sulphydryl group of a cysteine residue which was added to the N-terminus.

Both monoclonal and polyclonal phospho-specific antibodies can be successfully generated (444). Due to the relative simplicity of production we opted to produce polyclonal rather than monoclonal antibodies in the first instance. We conducted immunisations in rabbits which are easy to maintain and handle and in which polyclonal antibody production has been well characterised (445). An outbred strain was used in which the range of class II major histocompatibility molecules is greater and hence the likelihood of successfully generating an immune response is enhanced.

Despite the use of a short phosphorylated peptide for immunisations the sera obtained could theoretically still contain antibodies which recognised non-phosphorylated p120. For example, the immunising peptide may contain additional epitopes not encompassing the phosphorylated tyrosine residue. Alternatively, dephosphorylation of the peptide may occur *in vivo* following its subcutaneous injection with the creation of epitopes containing the non-phosphorylated tyrosine residue.

4.3.3 Validation of phospho-specific antibodies.

To identify potential phospho-specific antibodies we assayed the ability of sera to recognise p120 in which the phosphorylation state was experimentally manipulated. As a source of phosphorylated p120 we used both endogenous and exogenous myc-tagged p120 from a colon cancer cell line expressing constitutively active Src. Although this approach tests the ability of sera to recognise the epitope in the context of the entire protein a possible weakness is the risk of rejecting a potentially useful antibody should the residue it recognises not undergo phosphorylation in this specific cellular context. However, all of the sites of interest are known to be good Src substrates both *in vitro* and *in vivo* (68) and there was no *a priori* reason to expect them not to be phosphorylated in KM12C-Src527F cells. Furthermore, this concern would primarily apply where antibodies were rejected as unsuccessful because they failed to demonstrate any reactivity with lysate from KM12C-Src527F cells. In fact, as discussed below unsuccessful antibodies were generally rejected due to their ability to recognise a specific tyrosine residue equally well when non-phosphorylated as phosphorylated or due to their ability to recognise phosphorylated tyrosine residues in a non-specific manner.

Several techniques were used to identify sera which reacted with p120 only when phosphorylated on a specific tyrosine residue:

1. As an initial screen p120 was fully dephosphorylated using recombinant LAR phosphatase. Antibodies which recognised p120 equally well before and after phosphatase treatment (those generated to phospho-Y112, phospho-Y257 and phospho-Y280 and phospho-Y291) were considered not to be phospho-specific.
2. The ability of sera to immunoprecipitate phosphorylated p120 from KM12C-Src527F lysate after pre-incubation with an excess of immunising antigen was then assessed. Phosphorylated but not non-phosphorylated peptide competitively inhibited immunoprecipitation of p120 by phospho-Y228, phospho-Y296 and

phospho-Y302 antibodies. This suggested that these antibodies recognised the immunising peptide only when phosphorylated and did not recognise epitopes present on non-phosphorylated peptide.

3. Antibodies were tested for their ability to recognise p120 in which the target tyrosine residue either individually, or in combination with all eight phospho-acceptor residues, had been mutated to phenylalanine. For phospho-Y228 and phospho-Y296 antibodies an isolated tyrosine to phenylalanine substitution was sufficient to prevent immuno-reactivity. In contrast phospho-Y96 and phospho-Y302 antibodies failed to recognise p120 in which all eight sites were mutated, yet maintained immuno-reactivity with p120 in which the specific tyrosine residue was mutated in isolation. These antibodies therefore demonstrated cross-reactivity with other phosphorylated tyrosine residues in p120 and were not used further.

Thus antibodies were essentially rejected for one of two reasons. Firstly, because the antibody displayed immunoreactivity with dephosphorylated p120 indicating that epitopes not dependent on the phosphorylation of the target tyrosine residue were recognised. The presence of such sera demonstrates that affinity absorption against column-bound non-phosphorylated peptide was not fully effective. In future, an additional affinity purification step could be performed in which the fraction adsorbed to column-bound phosphorylated peptide is retained. This may reduce background due to non-specific antibodies present in serum. However, as antibodies may still recognise any non-phosphorylation-dependent epitopes present on bound phosphorylated peptide it is not clear whether this would offer significant advantages.

Secondly, as was the case for phospho-Y96 and phospho-Y302, the antibody appeared to have pan-phosphotyrosine activity as evidenced by the ability to recognise p120 in which the target residue was mutated to phenylalanine but not p120 in which either all 8 residues were mutated or which had been enzymatically dephosphorylated. Interestingly a similar phenomenon was identified even for phospho-Y228 and phospho-Y296 antibodies when used at inappropriately high concentrations. However, in contrast to the phospho-Y96 and phospho-Y302 antibodies it was possible to identify dilutions of phospho-Y228 and phospho-Y296 antibodies at which this activity was effectively titrated out whilst maintaining strong reactivity for wild type phospho-p120.

When Western blotting cell lysates multiple non-specific bands were detected by both phospho-228 and phospho-296 antibodies. This may reflect cross reactivity with other

phosphorylated proteins due to homology between a specific p120 phosphorylation site and the consensus sequence for that tyrosine kinase. Alternatively, this may indicate non-specific interactions with other antibodies present in rabbit serum at the time of sacrifice. For this reason phospho-Y228 and phospho-Y296 antibodies were primarily used in subsequent work for immunoprecipitation of p120 or for immunoblotting of immunoprecipitated p120 in preference to immunoblotting of whole cell lysate.

Attempts to generate monoclonal phospho-specific antibodies were undertaken on our behalf by the Beatson Institute Antibody Production Service but were unsuccessful. However, during the time course of this work monoclonal phospho-specific antibodies to p120 tyrosine residues 96, 228, 280 and 291 became commercially available.

For the remainder of this thesis the following p120 phospho-specific antibodies were used: monoclonal phospho-Y96; monoclonal phospho-Y228; monoclonal phospho-Y280; monoclonal phospho-Y291; rabbit polyclonal phospho-Y296.

4.4 Summary

Immunoglobulin was purified from rabbits immunised with chemically phosphorylated peptides encompassing each of the eight previously identified sites of p120 tyrosine phosphorylation. Several tests of validity were then employed to define antibodies which were truly phospho-specific. Firstly, phospho-specific antibodies were shown to recognise phosphorylated but not enzymatically de-phosphorylated p120. Secondly, phosphorylated but not non-phosphorylated immunising peptide competitively inhibited the ability of phospho-specific antibodies to immunoprecipitate p120. Finally, p120 in which specific tyrosine residues had been mutated to phenylalanine could not be recognised by phospho-specific antibodies. Using these criteria two polyclonal antibodies which specifically recognised p120 only when phosphorylated on tyrosine residues 228 or 296 were successfully produced. Concurrent with this work, p120 phospho-specific monoclonal antibodies to phospho-tyrosine-96, -228, -280 and -291 became commercially available. Together these facilitated a study of site-specific phosphorylation events in p120 as described in chapter five.

5 Results: Regulation of site-specific tyrosine phosphorylation events in p120.

5.1 Aim

p120-catenin is phosphorylated on tyrosine residues in response to activation of both RTKs and the cytoplasmic tyrosine kinase, Src. Although eight potential sites of tyrosine phosphorylation have recently been mapped (68), the regulation of phosphorylation events at these individual sites is largely unknown (176). We therefore used the novel phospho-specific antibodies described in chapter four to define the p120 site-specific phosphorylation events occurring in response to Src activation in KM12C cells and to EGFR activation in the A431 cell line.

5.2 Results

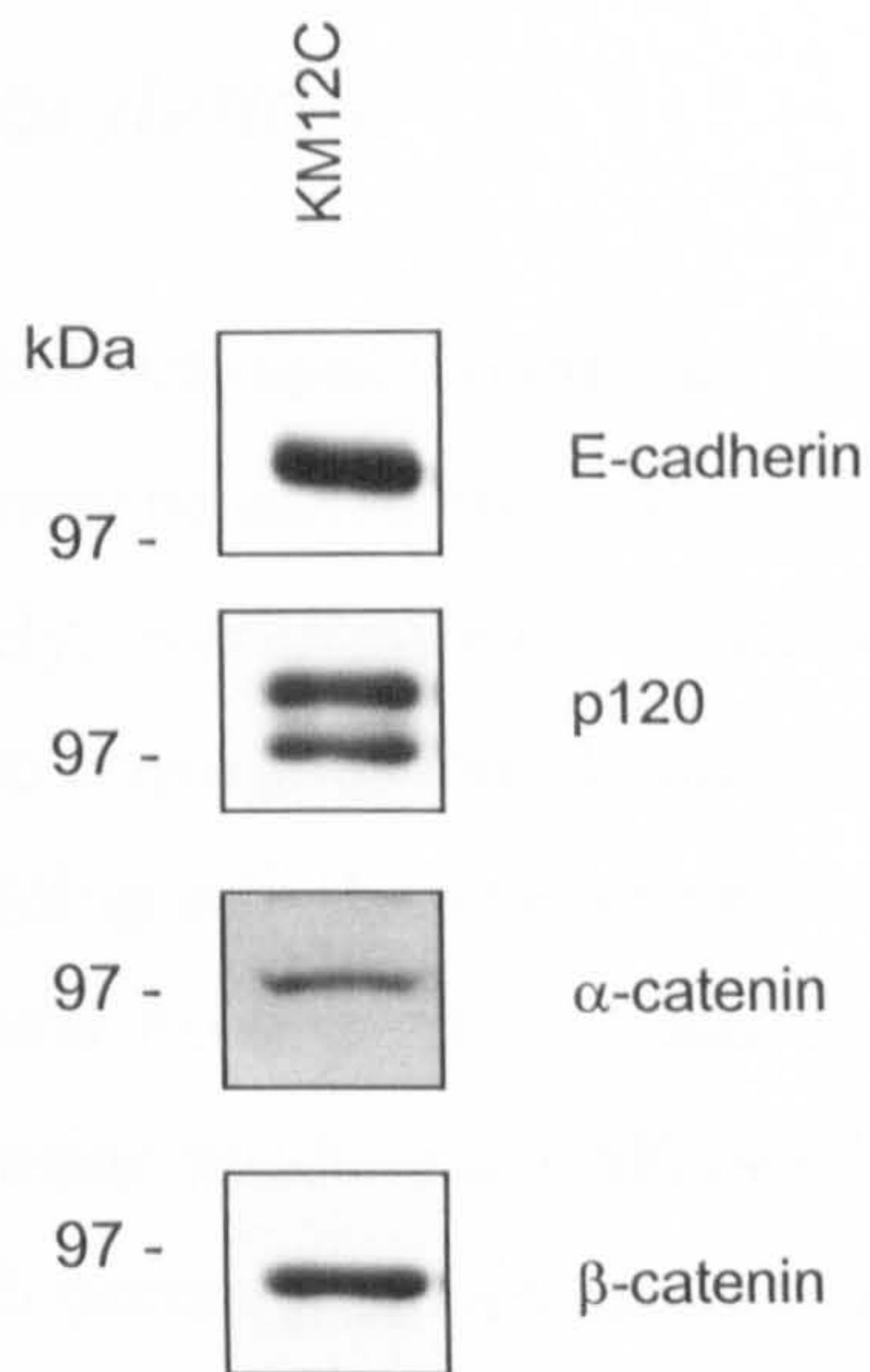
5.2.1 The KM12C cell line provides a model system in which to study Src-dependent phosphorylation of p120.

KM12C cells are derived from a human colorectal carcinoma (446) and have low endogenous Src family kinase activity (257). KM12C cells retain expression of core components of the AJ (Figure 5-1 A) and form functional AJs in culture (Figure 5-1 B). Previous work by this group has demonstrated disruption of the AJ, an increase in cell-matrix adhesion and expression of mesenchymal markers in response to increasing Src levels and activity in KM12C cells (257, 447). In-keeping with p120's known status as a Src substrate, p120 immunoprecipitated from KM12C-Src527F lysate demonstrated a dramatic increase in phosphorylation on tyrosine as compared to p120 in cells transfected with empty vector alone (Figure 4-1 B). Thus KM12C cells provide an appropriate model in which to investigate the requirements for site-specific tyrosine phosphorylation of p120 by Src in a carcinoma cell line.

5.2.2 p120 is phosphorylated at multiple sites in Src527F-expressing KM12C cells.

Using phospho-specific antibodies the expression of Src527F in KM12C cells was shown to increase phosphorylation of p120 at tyrosine residues 228, 280, 291 and 296 in comparison to cells expressing empty vector (Figure 5-4). As tyrosine-96 occurs only in p120 isoform 1 which is expressed at very low levels in KM12C cells phosphorylation at this site was not assessed.

(A)



(B)

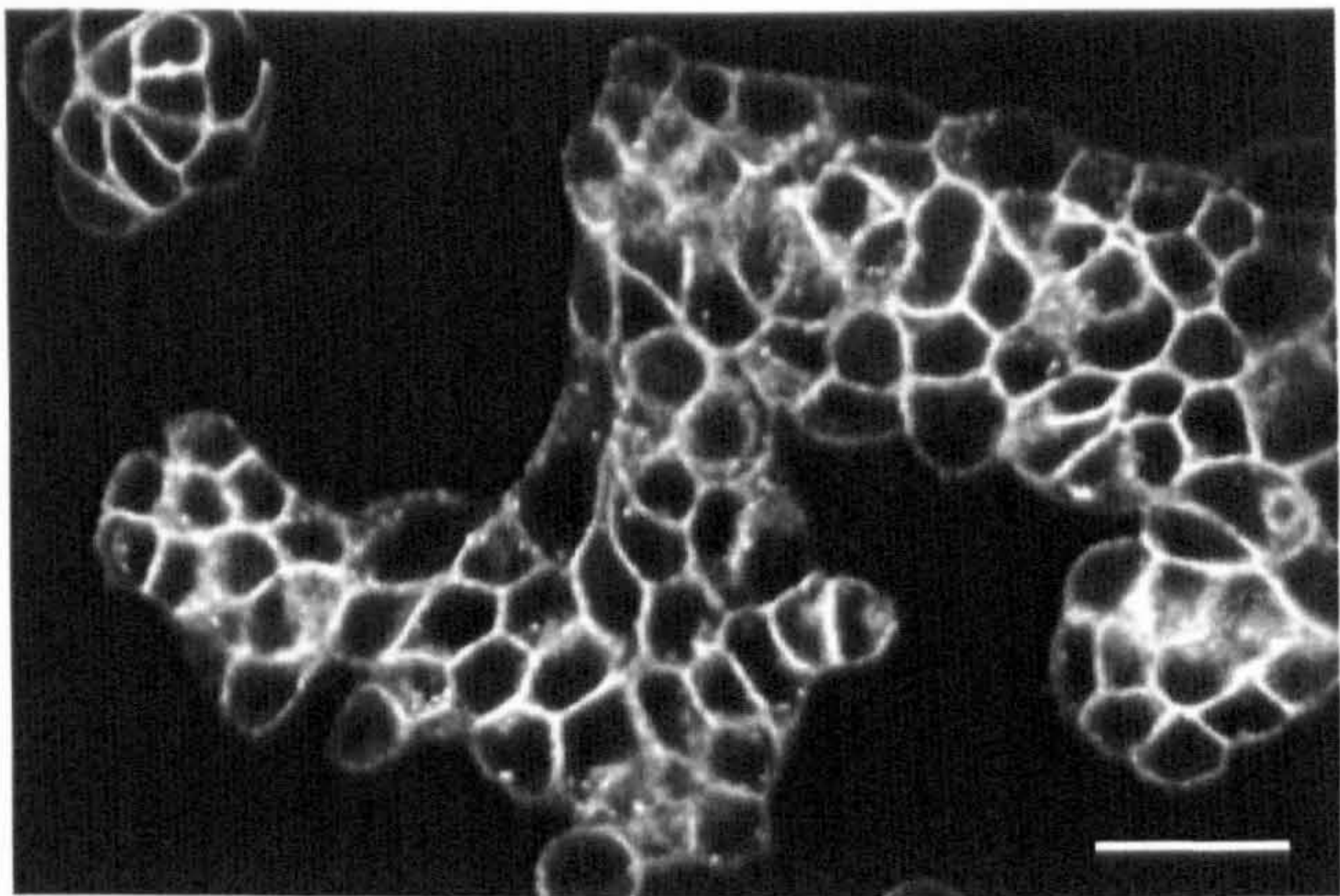


Figure 5-1 KM12C cells form adherens junctions

(A) KM12C lysate was prepared and protein levels analysed by Western blotting using the antibodies indicated.

(B) KM12C cells were fixed and immunofluorescence performed using E-cadherin antibody. Bar 50 μ m.

5.2.3 Requirement of Src kinase activity for p120 phosphorylation.

The phosphorylation of a particular tyrosine residue which occurs when Src is over-expressed may be due, directly or indirectly, to the kinase activity of Src itself.

Alternatively, over-expression of the non-catalytic SH2 and SH3 domains may be sufficient to cause phosphorylation independently of Src's kinase activity, presumably due to a scaffolding activity which enables the recruitment of additional kinase molecules. For example, over-expression of kinase-deficient Src causes increased phosphorylation of several tyrosine residues in FAK (448). It is not known whether the phosphorylation of p120 which occurs when Src is over-expressed in epithelial cells results from Src kinase activity or from a scaffolding function of the non-catalytic domains. Therefore, to define the specific role of Src's kinase activity in p120 phosphorylation in KM12C cells we utilised both kinase-deficient Src constructs (Figure 5-3 and Table 5) and a novel and selective small molecule inhibitor of Src kinase activity.

5.2.3.1 Pharmacological inhibition with AP23464 inhibits the phosphorylation of p120.

AP23464 is a potent and selective ATP-based inhibitor of Src-family kinases, Abl and KIT (448-450). Using auto-phosphorylation at tyrosine-416 as a marker of Src's kinase activity, AP23464 was seen to fully inhibit Src at a concentration of 1 μ M with no effect on protein levels (Figure 5-2 A). This correlated with complete inhibition of p120 phosphorylation at tyrosine residues 228, 280 and 296 as well as inhibition of global phosphorylation of p120 as detected by a pan-phosphotyrosine antibody (Figure 5-2 B). Interestingly treatment of cells with AP23464 for as little as two minutes prior to lysis was sufficient to fully inhibit tyrosine phosphorylation of p120 (Figure 5-2 C).

5.2.3.2 Kinase-deficient Src promotes moderate phosphorylation of p120.

Expression of either Src-251 (Figure 5-4 A) or Src-527F/K295M (Figure 5-4 B) was sufficient to increase tyrosine phosphorylation of p120, albeit to a significantly lesser degree than that achieved by the expression of kinase-active Src. Levels of total p120 were unaffected (Figure 5-4 A,B). All of the p120 tyrosine residues studied (Y228, Y280, Y291, Y296) were phosphorylated synchronously with no evidence of any differential regulation occurring at any site. Significantly, the tyrosine phosphorylation of other Src substrates

such as paxillin (451) and p130^{Cas} (452) was reduced in the presence of kinase-deficient Src (Figure 5-5).

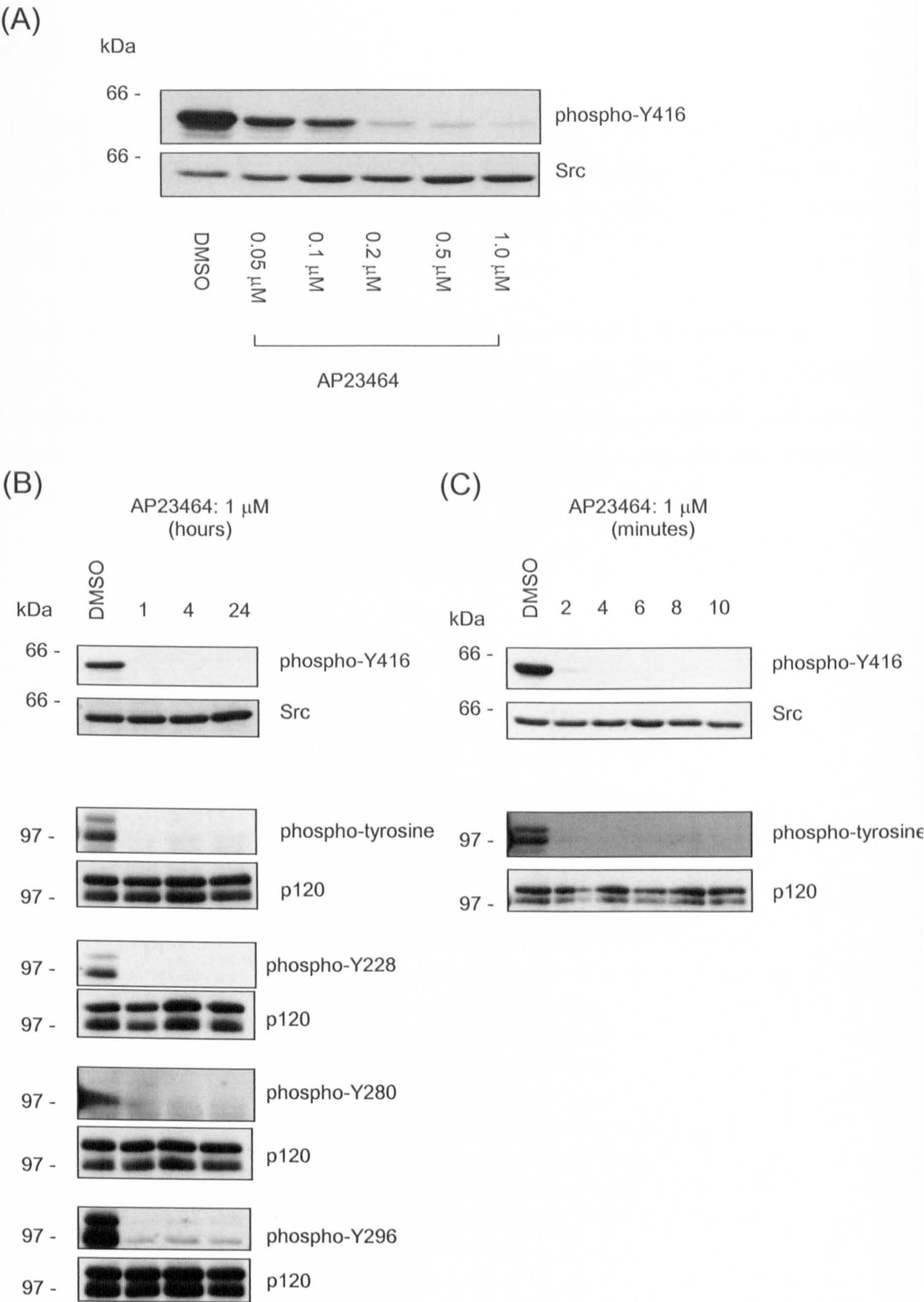


Figure 5-2 Src-family kinase activity is required for phosphorylation of p120 in KM12C-Src527F cells.

Figure 5-2 Src family kinase activity is required for phosphorylation of p120 in KM12C-Src527F cells.

(A) KM12C-Src527F cells were treated with vehicle (DMSO) or increasing concentrations of AP23464 for 60 minutes after which lysates were prepared and analyzed by Western blotting with Src phospho-Y416 antibody. After removal of the phospho-specific antibody the blot was reprobed with Src antibody.

(B) and (C) KM12C-Src527F cells were incubated with 1 μ M AP23464 for the time intervals shown prior to lysis. p120 was then immunoprecipitated and analysed by Western blotting with site specific phospho-p120 antibodies or anti-phosphotyrosine antibody. In each case after removal of the phospho-specific antibody the blot was reprobed with p120 antibody.

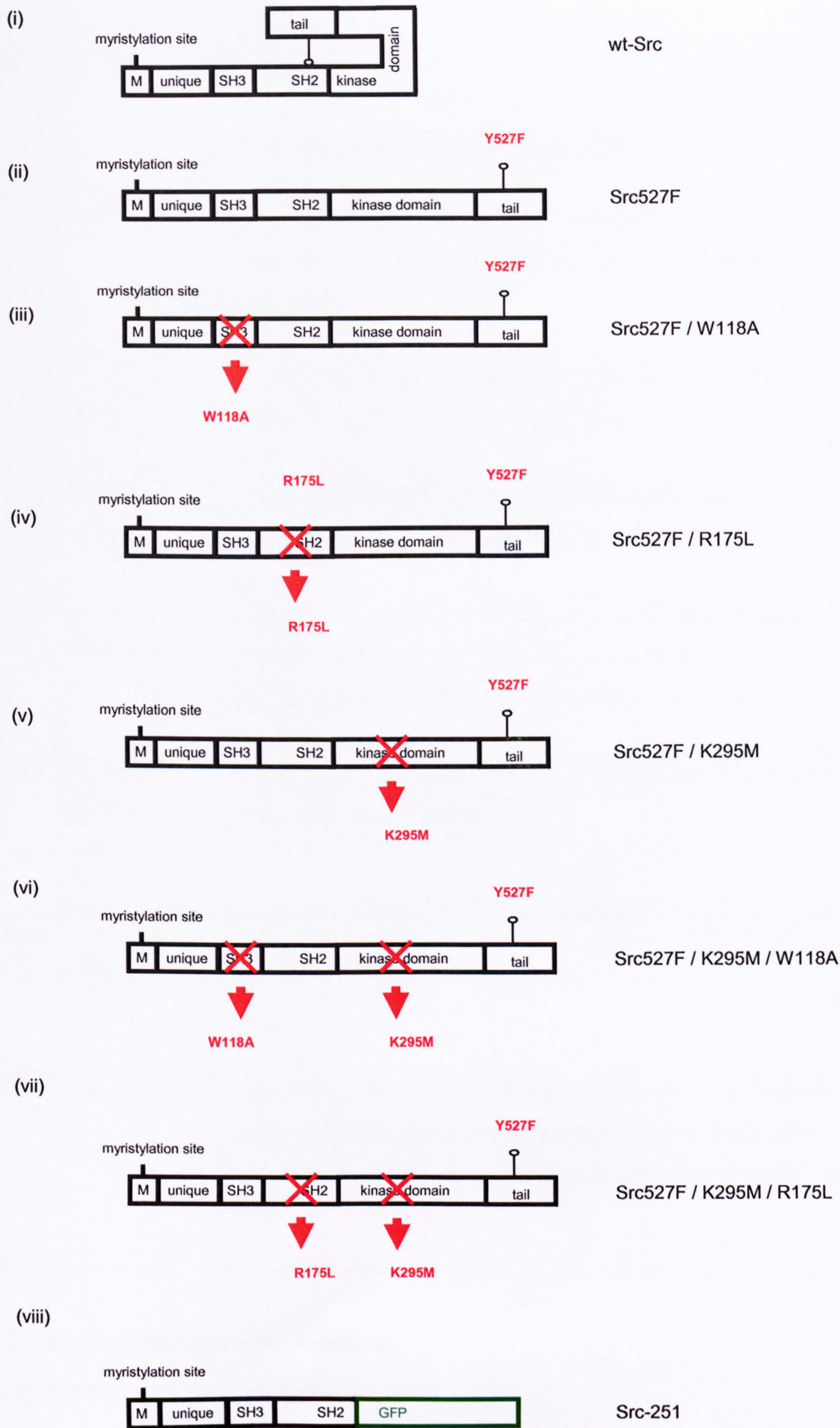


Figure 5-3 Schematic diagram of Src constructs.

	Name	Comments
(i)	wt-Src	<i>Wild type c-Src. In the absence of an activating event intramolecular interactions render the kinase domain, SH2 and SH3 domain conformationally unavailable.</i>
(ii)	Src527F	<i>Substitution of Y527 to F results in constitutive activity of the kinase domain and availability of SH2 and SH3 domains for protein-protein interaction</i>
(iii)	Src527F / W118A	<i>As (ii) except substitution of W118 to A prevents SH3 domain interaction with its binding partners (453).</i>
(iv)	Src527F / R175L	<i>As (ii) except substitution of R175 to L prevents SH2 domain interaction with its binding partners (454).</i>
(v)	Src527F / K295M	<i>Substitution of K295 to M abrogates kinase function therefore kinase deficient (454). SH2 and SH3 domains available for protein-protein interaction due to Y527 to F substitution.</i>
(vi)	Src527F / K295M / W118A	<i>As (v) except substitution of W118 to A prevents SH3 domain interaction with its binding partners.</i>
(vii)	Src527F / K295M / R175L	<i>As (v) except substitution of R175 to L prevents SH2 domain interaction with its binding partners.</i>
(viii)	Src-251	<i>Entire kinase domain deleted and replaced with GFP therefore kinase deficient. SH2 and SH3 domains available for protein-protein interaction due to deletion of intramolecular binding sites (455).</i>

Table 5 Characteristics of Src constructs

All constructs were derived from chicken Src.

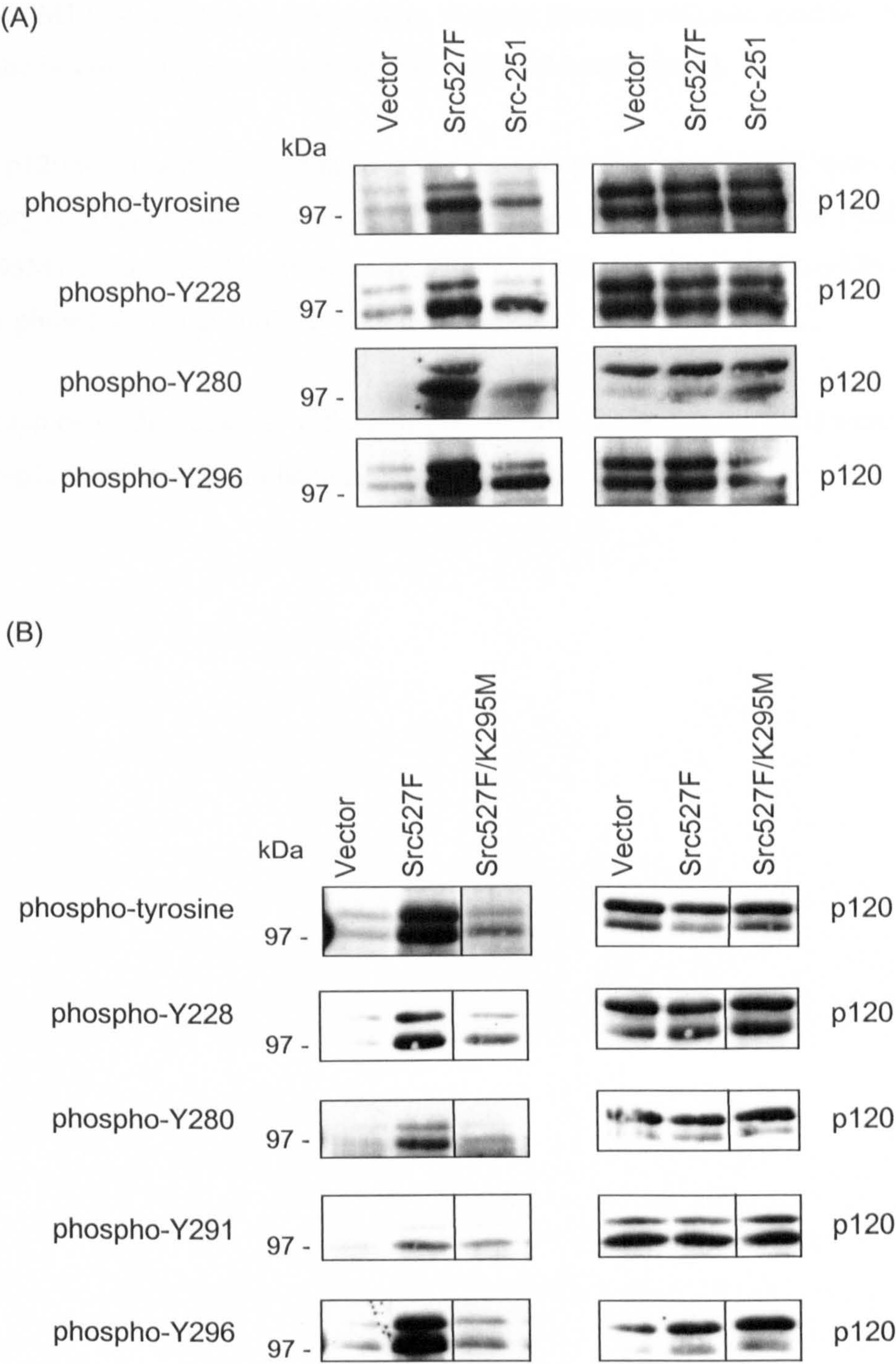


Figure 5-4 Expression of kinase-deficient Src promotes p120 phosphorylation.

Figure 5-4 Expression of kinase-deficient Src promotes p120 phosphorylation.

(A) p120 was immunoprecipitated from lysate prepared from KM12C cells transfected with empty vector (vector), constitutively active Src (KM12C-Src527F) or kinase-deficient Src (KM12C-Src251) and analysed by Western blotting with site specific phospho-p120 antibodies or anti-phosphotyrosine antibody (left hand panels).

(B) p120 was immunoprecipitated from lysate prepared from KM12C cells expressing empty vector, constitutively active Src or a second kinase-deficient Src (KM12C-Src527F / K295M) and analysed by Western blotting with site specific phospho-p120 antibodies or anti-phosphotyrosine antibody (left hand panels).

In each case after removal of the phospho-specific antibodies the blots were reprobed with anti-p120 antibody (right hand panels).

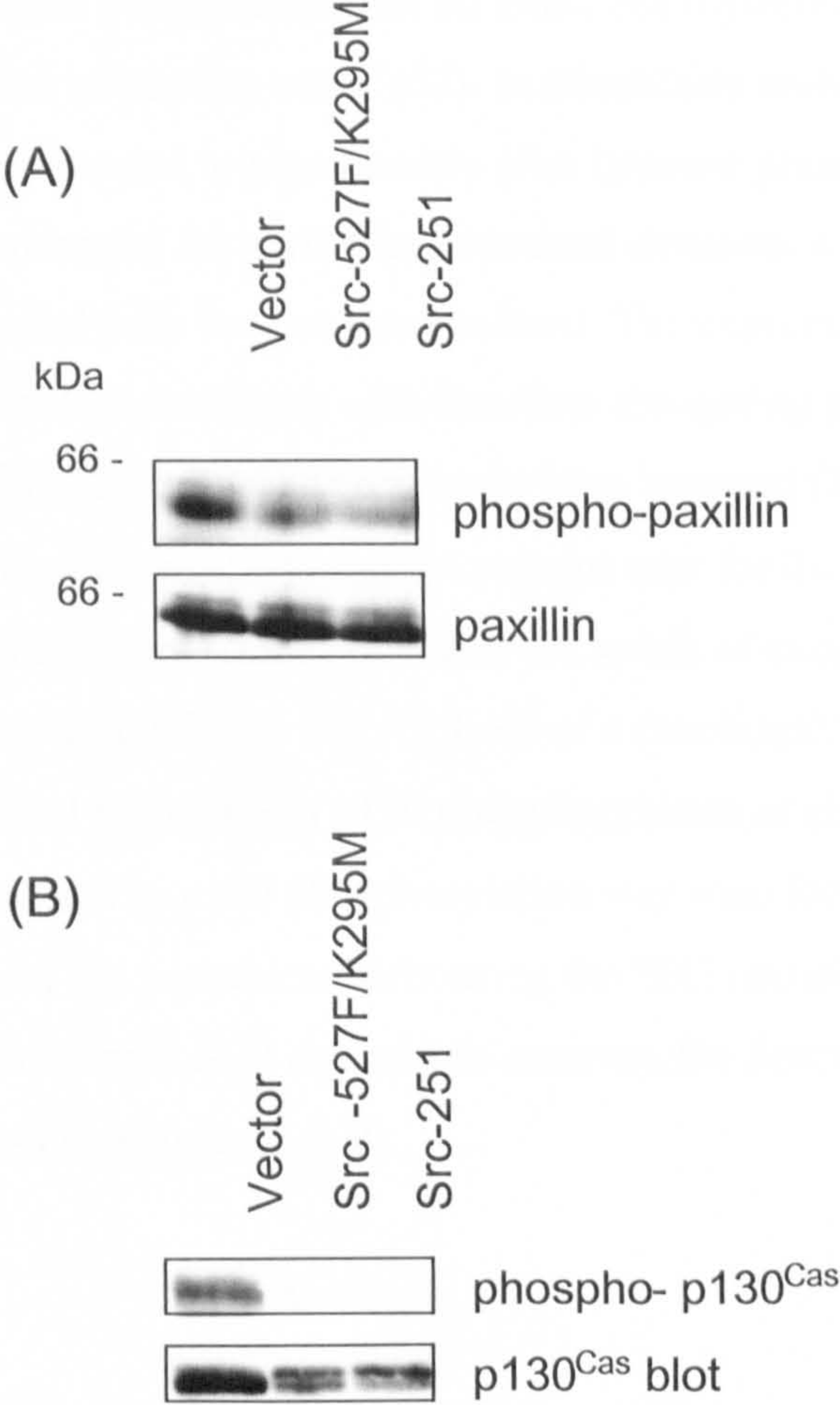


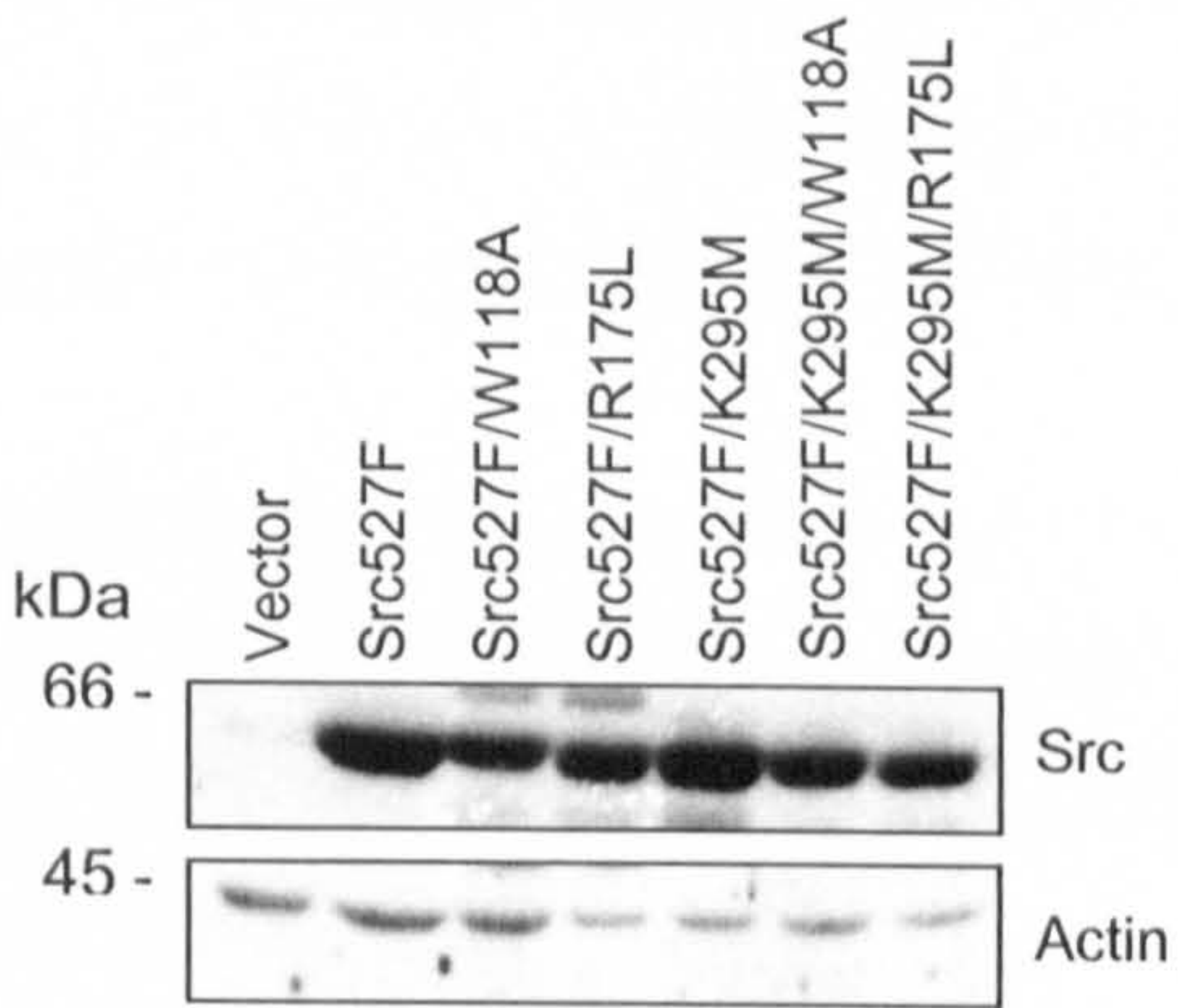
Figure 5-5 Kinase-deficient Src inhibits phosphorylation of Src substrates

Paxillin (A) or p130^{Cas} (B) were immunoprecipitated from lysate prepared from KM12C cells containing empty vector or two different kinase deficient Src constructs. Blots were probed with anti-phosphotyrosine antibody then stripped and reprobed with anti-paxillin (A) or anti-p130^{Cas} antibody (B).

5.2.4 The Src SH2 but not SH3 domain is required for phosphorylation of p120.

Previous work has identified a requirement for an intact Src myristylation site for Src-dependent phosphorylation of p120 to occur (52). In fibroblasts mutation of the Src SH2 and SH3 domain was not reported to significantly alter tyrosine phosphorylation of p120 (182). However, the requirement for particular structural elements within Src for p120 phosphorylation in epithelial cells has not been defined. The expression of constitutively active and kinase-deficient Src constructs with function-abrogating point mutations in the SH2 or SH3 domain in KM12C cells has previously been reported (278, 448). These constructs provided a means by which to test the requirement for the Src non-catalytic domains in p120 phosphorylation. Clones in which the levels of exogenous Src expression were broadly similar were used (Figure 5-6 A). Loss of a functional SH2 domain was associated with a significant reduction in p120 phosphorylation at all tyrosine sites studied (Figure 5-6 B). This reduction in p120 phosphorylation was seen for both constitutively active and kinase-deficient Src constructs harbouring the SH2 mutation as compared to the same constructs with a functional SH2 domain. In contrast, the function of the Src SH3 domain appeared dispensable (Figure 5-6 B).

(A)



(B)

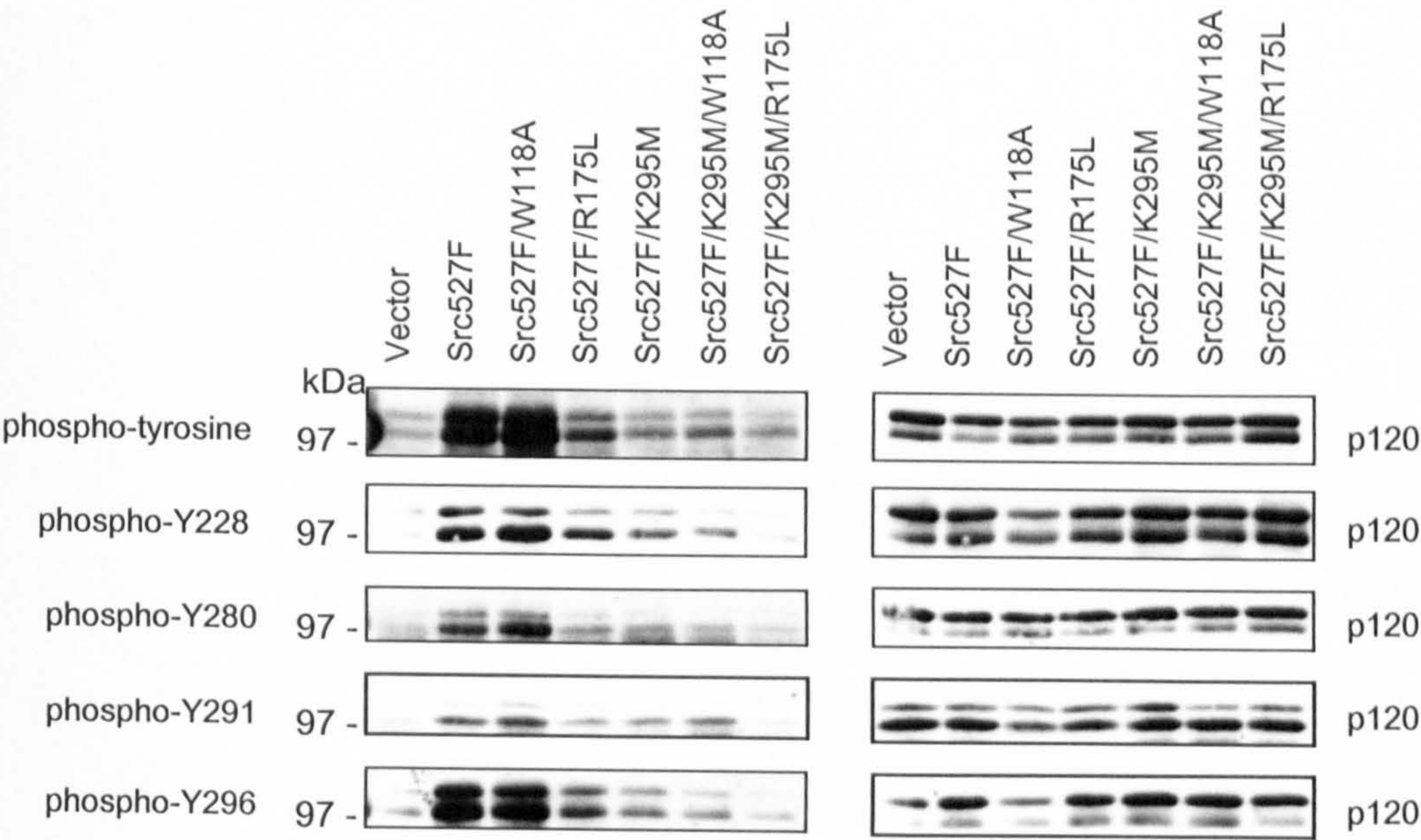


Figure 5-6 The Src SH2 domain is necessary for Src-dependent phosphorylation of p120.

Figure 5-6 The Src SH2 domain is necessary for Src-dependent phosphorylation of p120.

(A) Lysates were prepared from KM12C cells transfected with empty vector (vector) or constitutively active Src (Src527F), constitutively active Src with a loss of function mutation in the SH3 (Src527F / W118A) or SH2 domain (Src527F / R175L), kinase-deficient Src (Src527F / K295M) or kinase-deficient Src with a loss of function mutation in the SH3 (Src527F / K295M / W118A) or SH2 domain (Src527F / K295M / R175L) and analysed by Western blotting using anti-Src antibody and anti-actin antibodies.

(B) p120 was immunoprecipitated from the same cell lines and analysed by Western blotting with site specific phospho-p120 antibodies or anti-phosphotyrosine antibody (left hand panels). In each case after removal of the phospho-specific antibodies the blots were reprobed with anti-p120 antibody (right hand panels).

5.2.5 Phosphorylation of p120 by EGF

Phosphorylation of p120 in response to growth factor stimulation has been reported in a variety of cell lines. However, treatment of KM12C cells with EGF did not cause a significant increase in total or site specific tyrosine phosphorylation of p120 (Figure 5-7 A). This was despite the readily detectable phosphorylation of EGFR itself and of its downstream effector, MAPK (Figure 5-7 B).

Therefore, to better define site specific phosphorylation events occurring due to EGF signalling we utilised the A431 cell line in which EGF-dependent phosphorylation of p120 has previously been reported (176). A431 cells are derived from an epidermal squamous cell carcinoma and express high levels of EGFR due to a gene amplification event (456). In common with the KM12C cell line, A431 cells maintain all major components of the AJ and grow as epithelial colonies (described in chapter 6; Figure 6-10, 6-11). Similarly, they predominantly express p120 isoform 3 (457) which does not contain tyrosine-96.

Phosphorylation of p120 at tyrosine-228 in EGF-stimulated A431 cells has recently been identified (176). However, whether other sites are similarly regulated is unknown. Following EGF stimulation of A431 cells, EGFR phosphorylation (Figure 5-8 A) and simultaneous phosphorylation of p120 at tyrosine residues 228, 280, 291 and 296 (Figure 5-8 B) occurred. Spatially, phosphorylation of p120 occurred mainly at AJs (Figure 5-8 C, dotted arrow) but also occurred at the leading edge of lamellipodia (Figure 5-8 C, filled arrow).

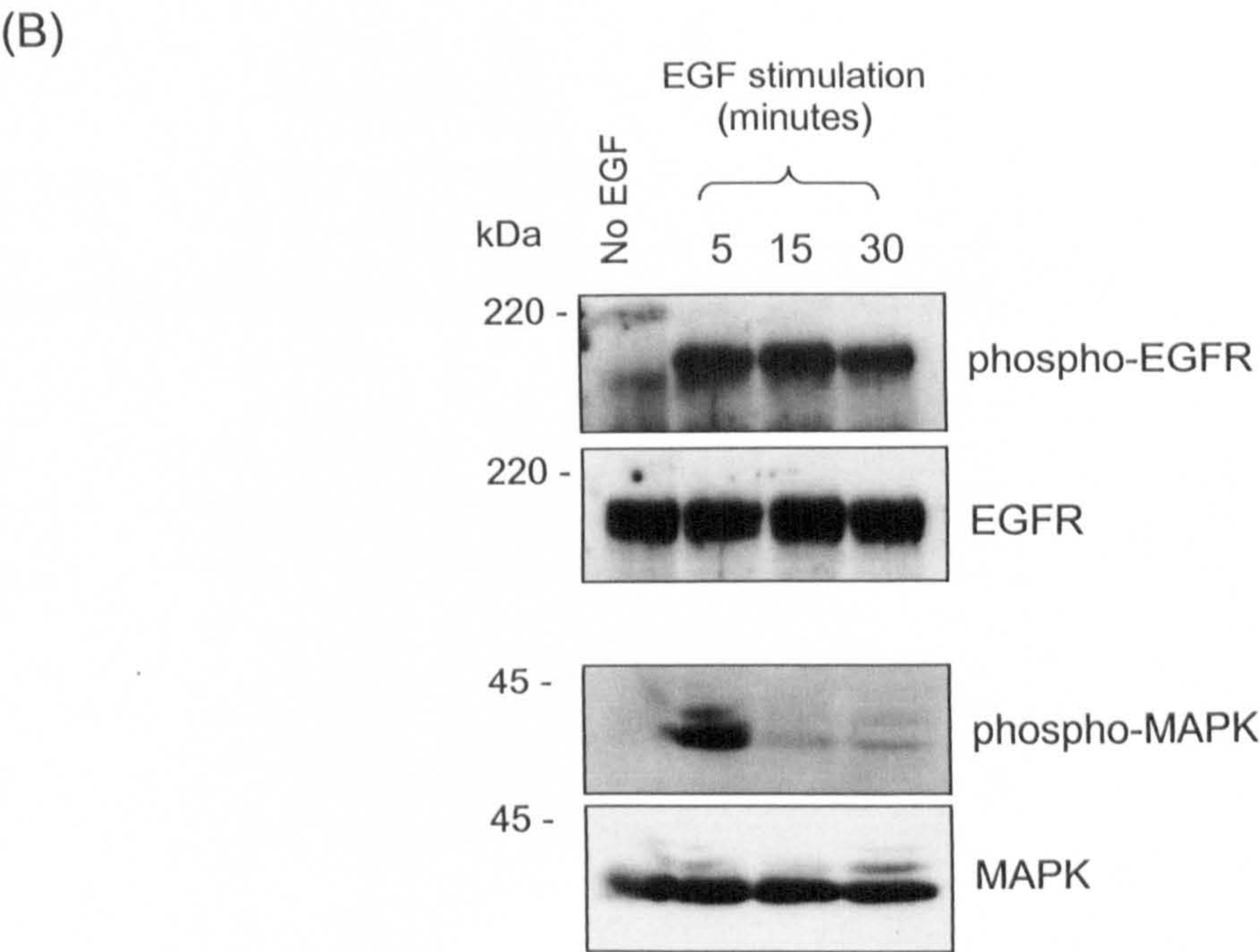
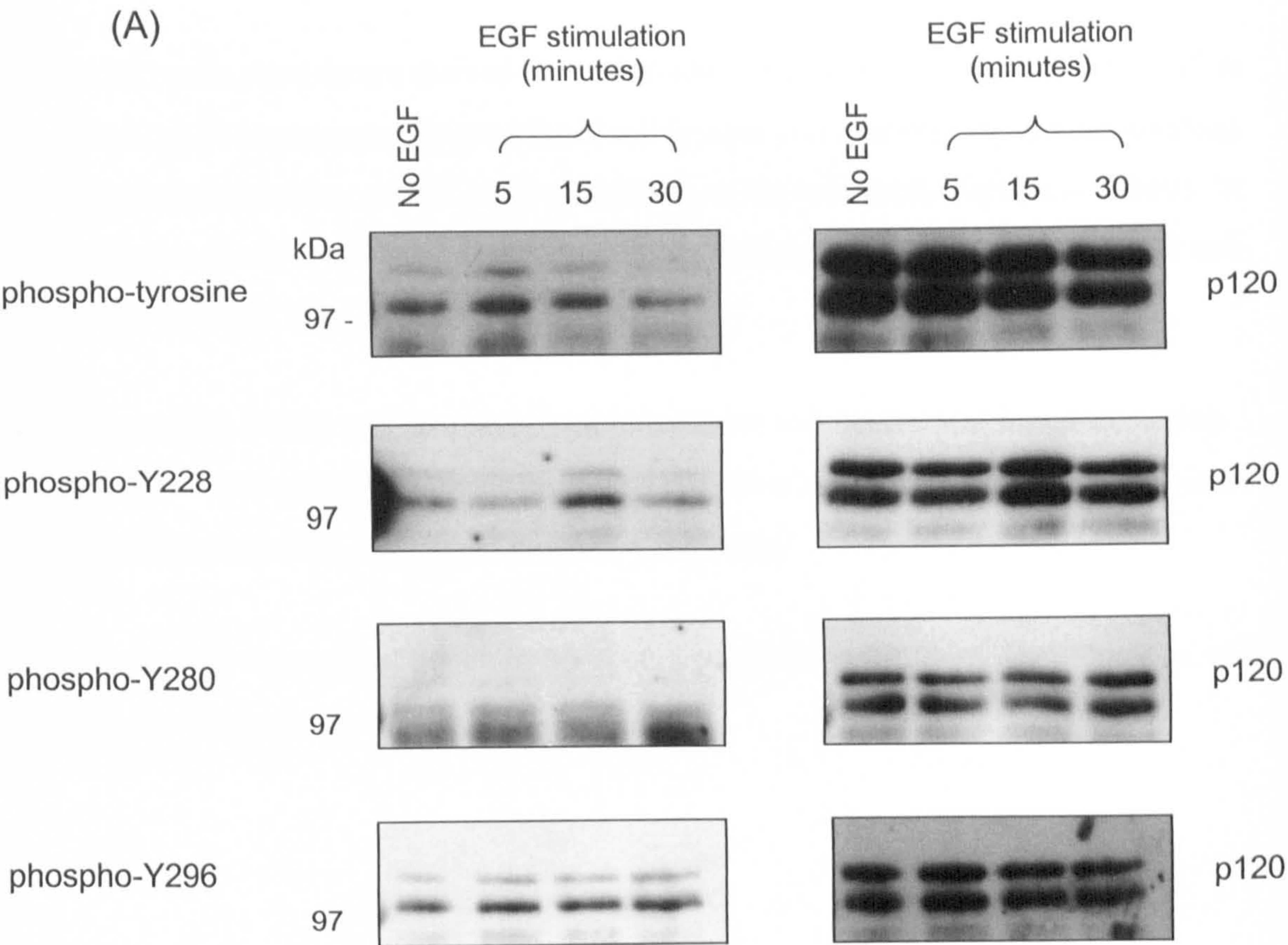


Figure 5-7 p120 is not phosphorylated in EGF stimulated KM12C cells.

Figure 5-7 p120 is not phosphorylated in EGF stimulated KM12C cells.

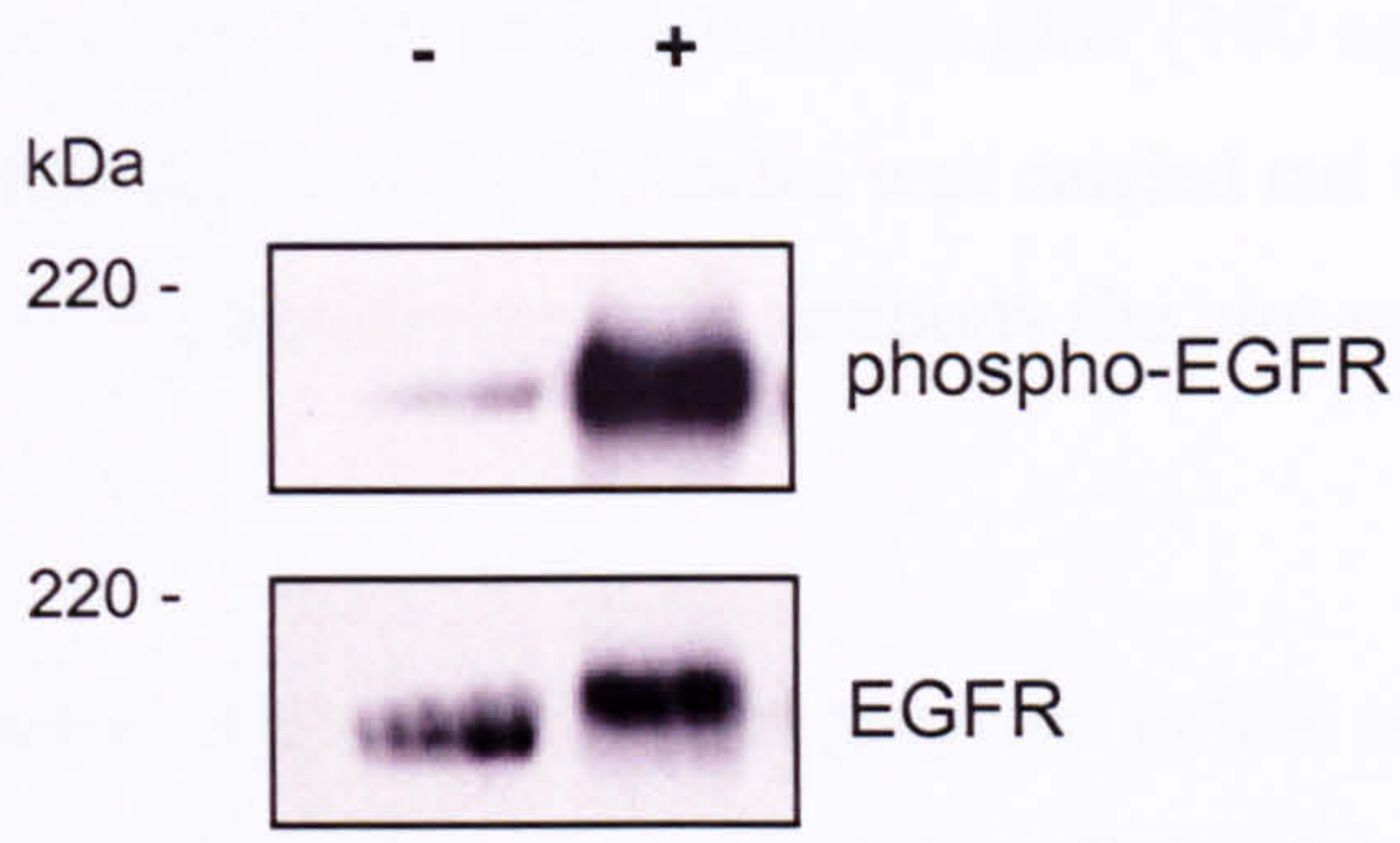
(A) KM12C cells were serum starved then stimulated with EGF (100 ng/ml) for 5, 15 or 30 minutes. p120 immunoprecipitated from cell lysates was analysed by immunoblotting with anti-phosphotyrosine or anti-p120 phospho-specific antibodies (left hand panels). In each case after removal of the phospho-specific antibody the blot was reprobed with anti-p120 antibody (right hand panels).

(B, upper panels) EGFR was immunoprecipitated from cell lysates and immunoblotting performed with anti-phosphotyrosine antibody. (B, lower panels) Cell lysate was analysed by Western blotting with anti-phospho-MAPK antibody.

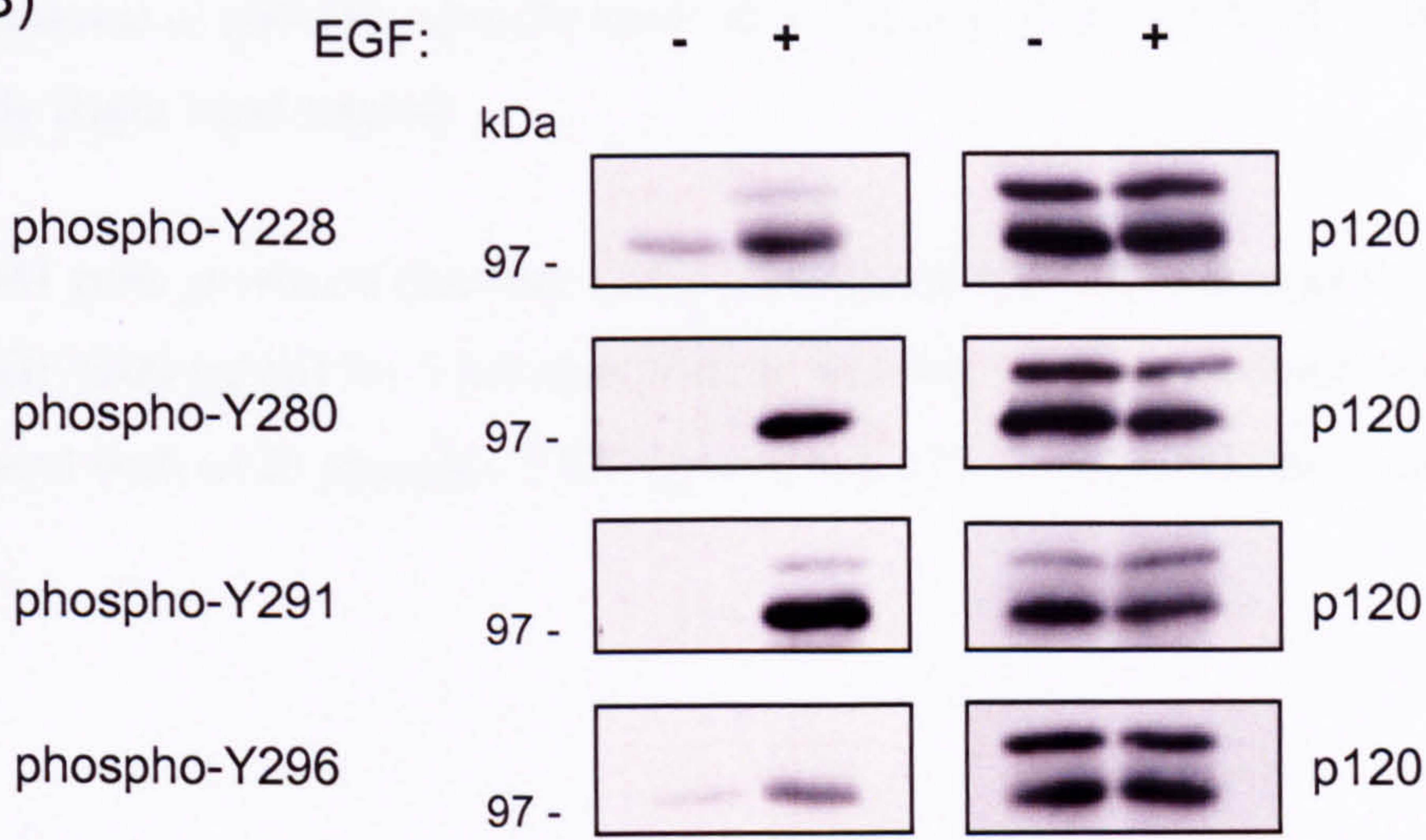
In each case after removal of the phospho-specific antibodies the blots were reprobed with the corresponding non-phospho-specific antibody.

(A)

EGF (100ng/ml)



(B)



(C)

phospho-Y228

p120

merge

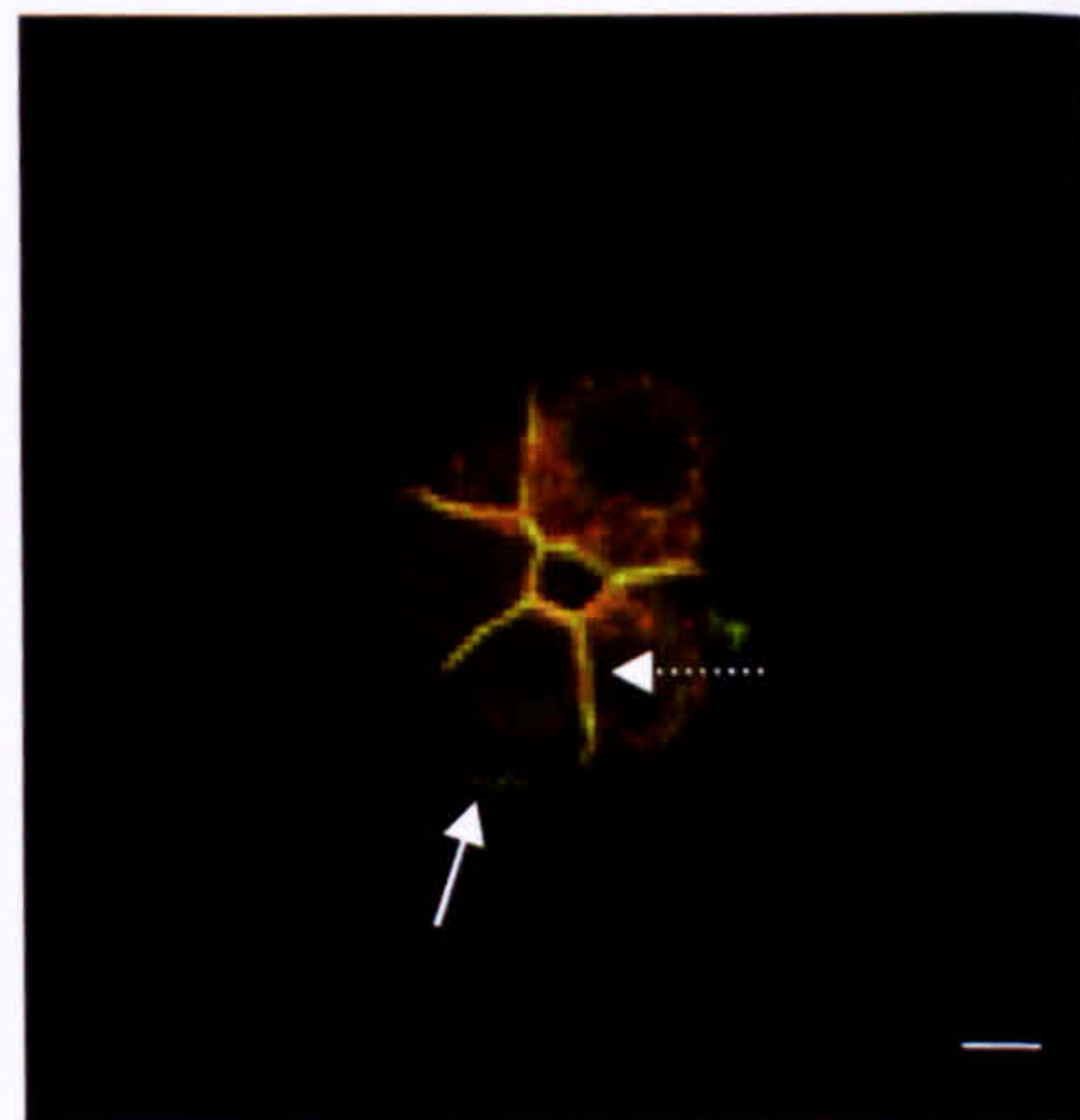
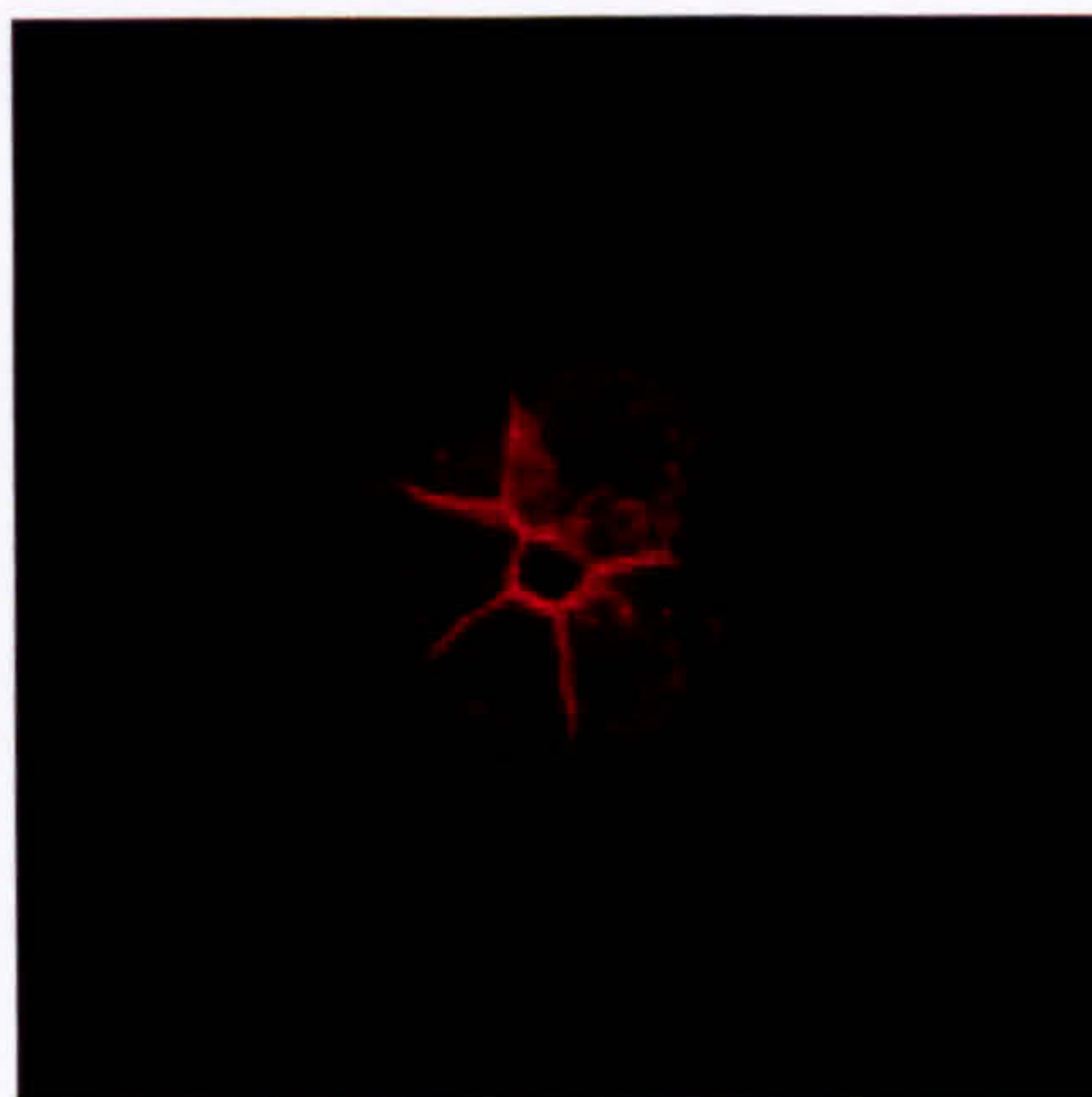
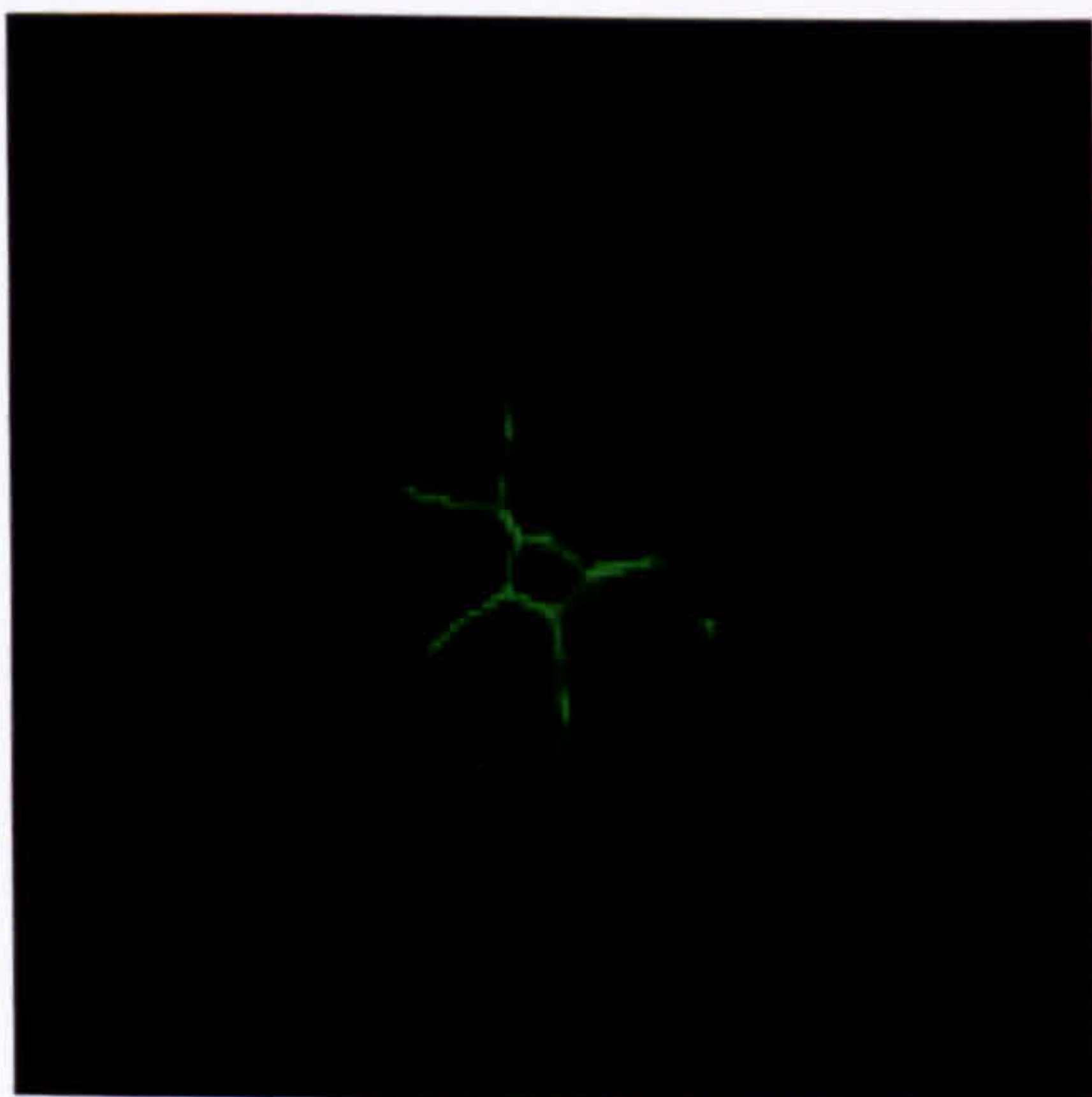


Figure 5-8 EGF stimulation results in phosphorylation of p120 at multiple sites.

Figure 5-8 EGF stimulation results in phosphorylation of p120 at multiple sites. (A)

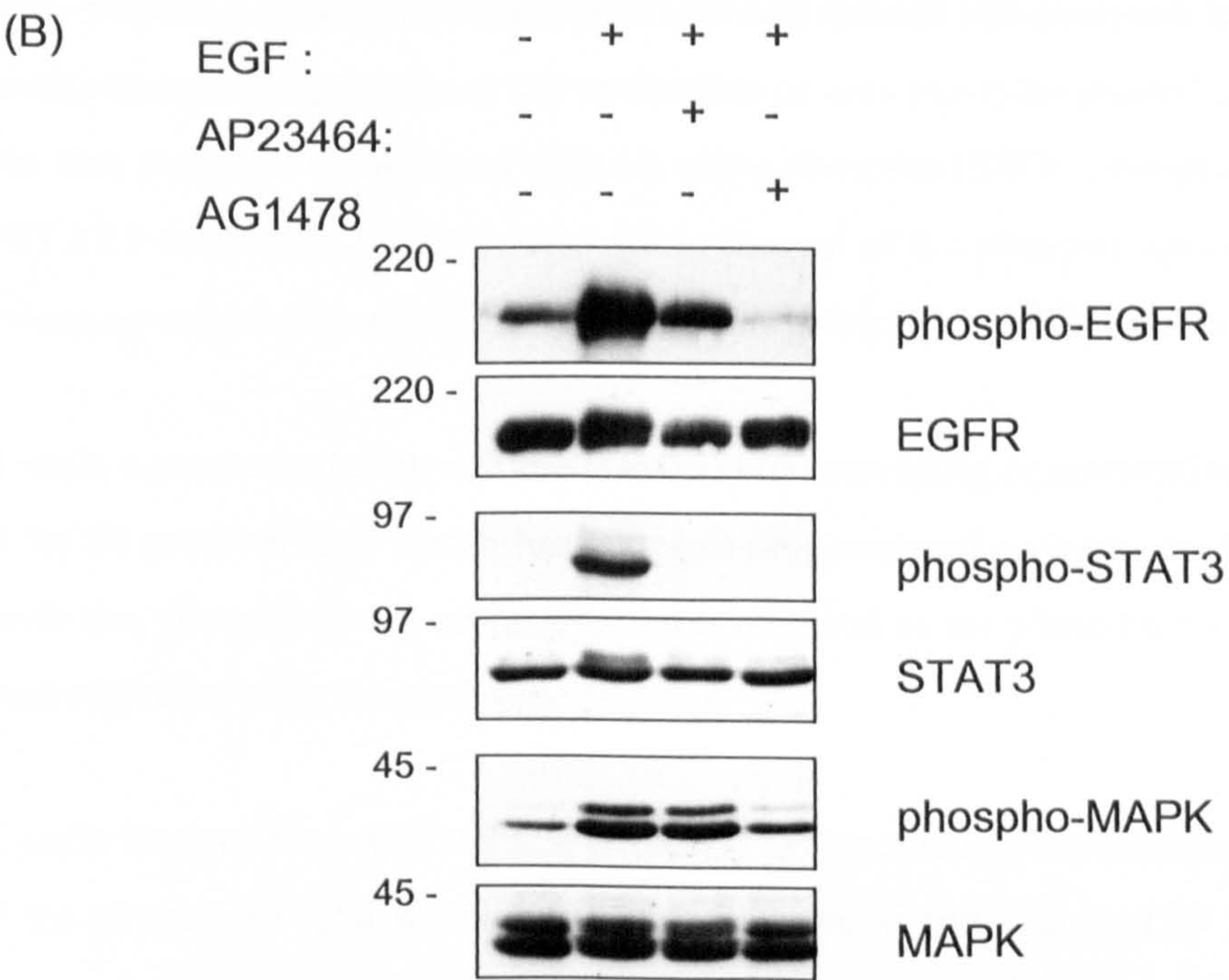
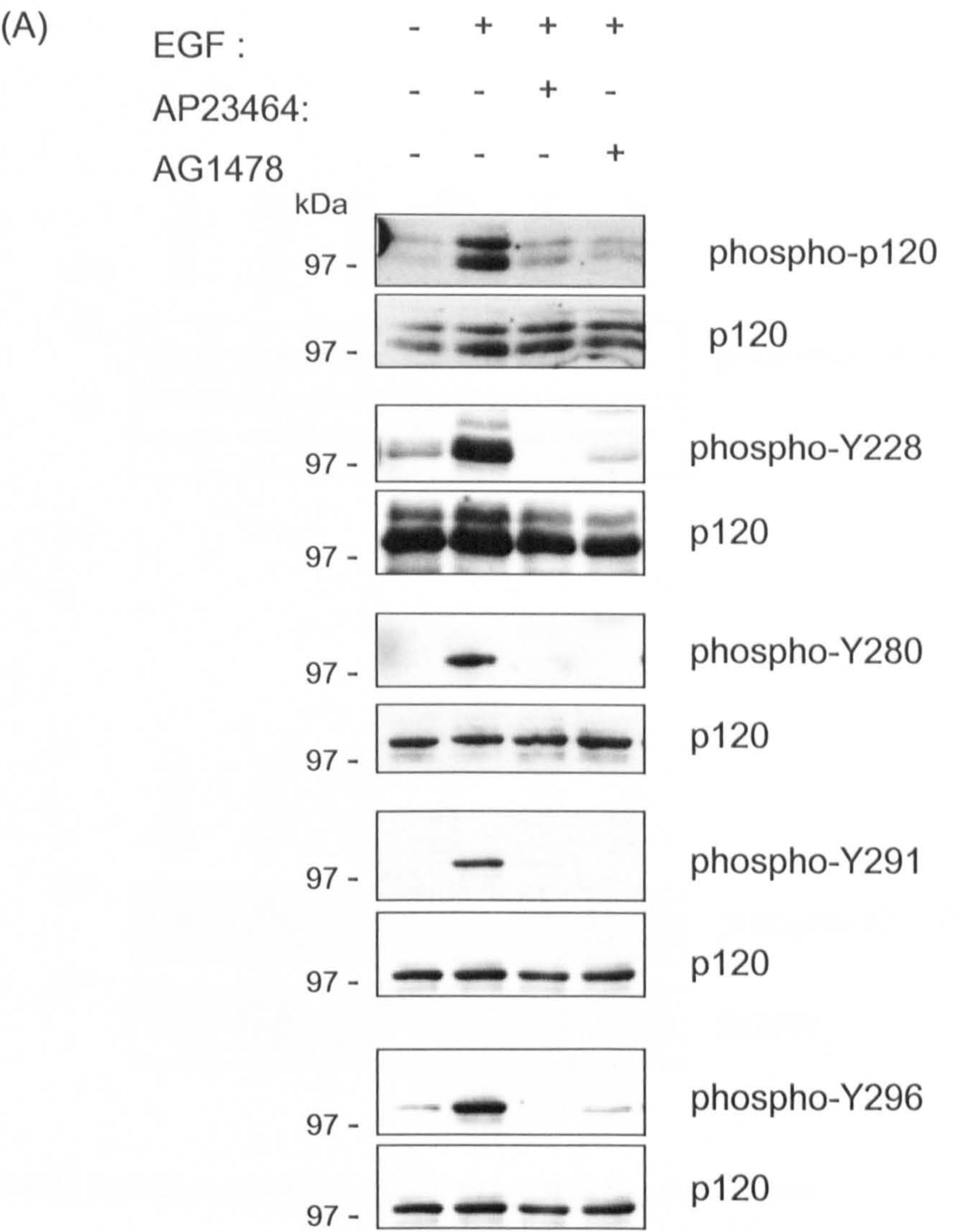
A431 cells were serum starved overnight then stimulated with EGF (100 ng/ml) for 5 minutes prior to lysis. Western blot analysis of cell lysates was carried out with phospho-EGFR antibody. After removal of the phospho-specific antibody the blot was reprobed with EGFR antibody.

(B) A431 cells were serum-starved overnight and lysates prepared before or 5 minutes after stimulation with EGF (100 ng/ml). p120 was immunoprecipitated from cell lysates and immuno-blotting performed with p120 phospho-specific antibodies (left hand panels). After removal of phospho-specific antibodies the blots were reprobed with anti-p120 antibody (right hand panels).

(C) A431 cells grown on chamber slides were serum starved overnight then stimulated with EGF (100 ng/ml) for 5 minutes prior to fixation. Co-immunofluorescence was performed with p120 phospho-Y228 (green) and p120 (red) antibodies. Bar, 10 μ m.

5.2.6 EGF induced phosphorylation of p120 requires an AP23464-sensitive kinase.

As all 4 sites appeared to be phosphorylated in tandem, we used pharmacological inhibitors to investigate whether any differential regulation could be unmasked. As expected, treatment of A431 cells with AG1478, a specific inhibitor of EGFR kinase activity, prevented the EGF-dependent phosphorylation of p120 on all 4 sites (Figure 5-9 A). This corresponded with the ability of AG1478 to prevent phosphorylation of the EGFR following stimulation with EGF as well as inhibiting the activation of the well-defined EGF-dependent signalling pathways to MAPK and STAT3 (Figure 5-9 B). The EGF-dependent phosphorylation of p120 was also prevented in cells treated with AP23464, a selective inhibitor of Src family tyrosine kinases (Figure 5-9 A). The basal phosphorylation of p120 on residues 228 and 296 was also abrogated in AP23464 treated cells (Figure 5-9 A). Using phosphorylation of the auto-phosphorylation site of Src (tyrosine-416) as a measure of Src activity, we showed that Src activity was inhibited in A431 cells treated with AP23464 at the concentration (1 μ M) used in these experiments (Figure 5-9 C). AP23464 did not inhibit EGFR phosphorylation at the concentration used (Figure 5-9 D). Interestingly, pre-treatment with AP23464 was sufficient to block EGFR signalling to both p120 and to STAT3, a known Src substrate, while phosphorylation of MAPK was not reduced (Figure 5-9 B). Thus p120 is phosphorylated on multiple residues in an EGF-and AP23464-sensitive kinase-dependent manner.



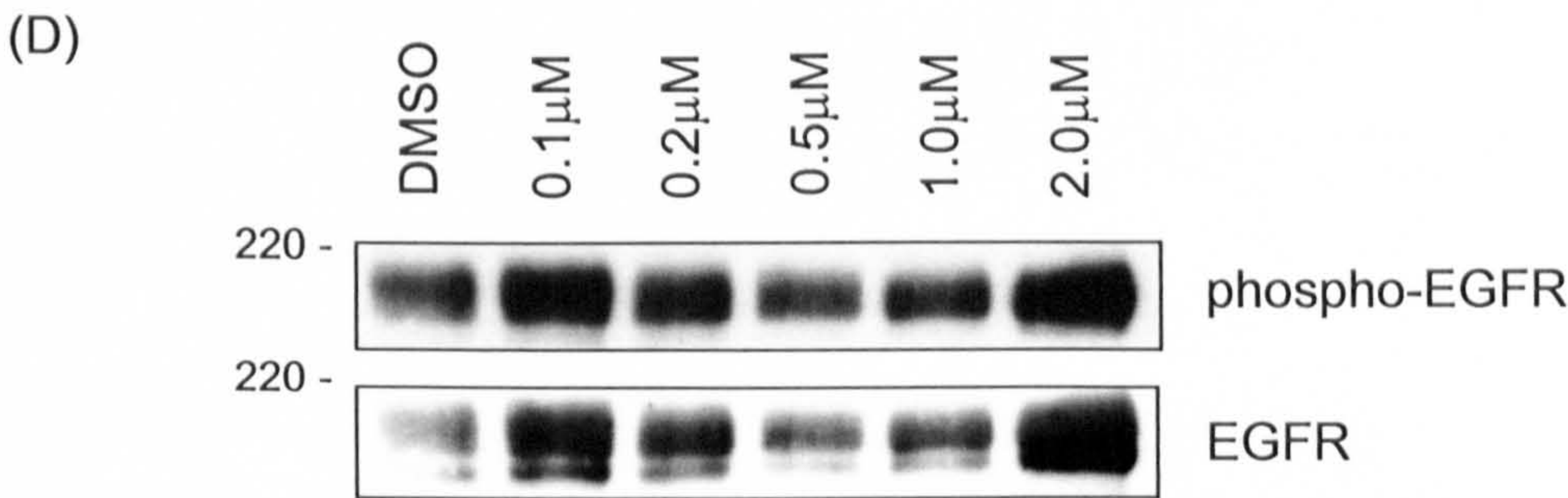
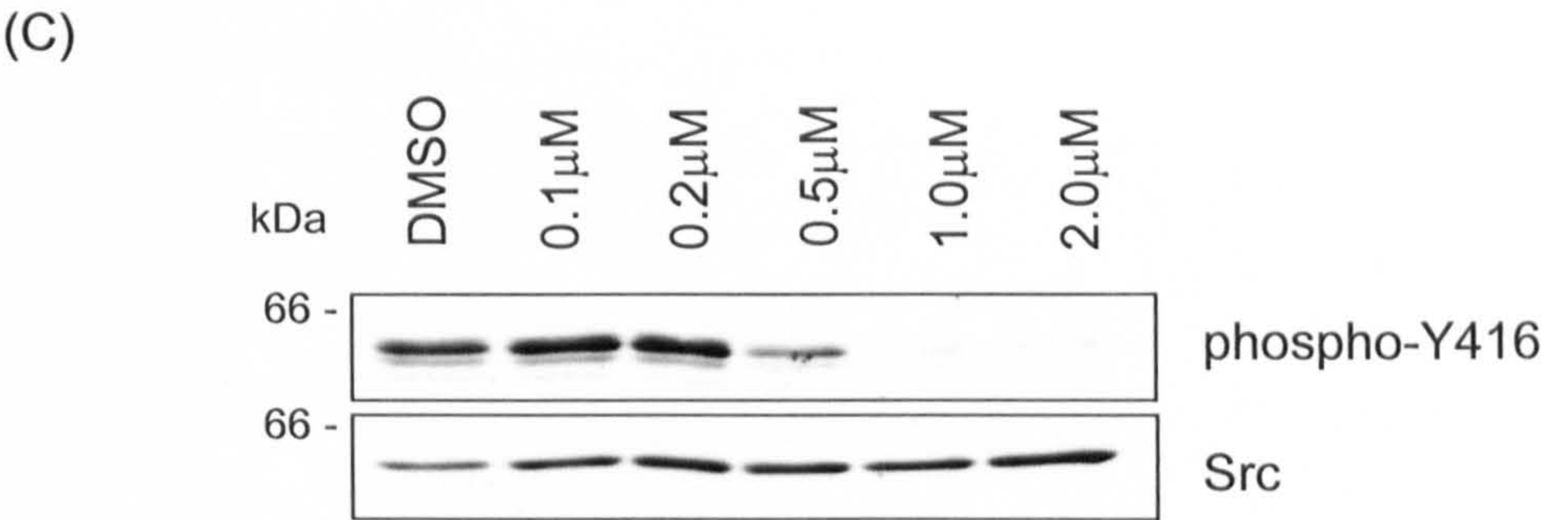


Figure 5-9 EGF induced phosphorylation of p120 is Src dependent.

(A, B) A431 cells were serum starved overnight, incubated with AP23464 (1 μM) or AG1478 (300 nM) for 1 hour, then lysed before, or 5 minutes after, stimulation with EGF (100 ng/ml). p120 was immunoprecipitated from cell lysates and analysed by Western blotting with site specific phospho-p120 antibodies or anti-phosphotyrosine antibody. Lysate was also analysed by Western blotting using phospho-EGFR, phospho-MAPK and phospho-STAT3 antibodies. In each case after removal of the phospho-specific antibodies the blots were reprobed with the corresponding non-phospho-specific antibodies.

(C) A431 cells were treated with vehicle (DMSO) or increasing concentrations of AP23464 for 60 minutes after which lysates were prepared and analyzed by Western blotting with Src phospho-Y416 antibody. After removal of the phospho-specific antibody the blot was reprobed with Src antibody.

(D) A431 cells were treated with vehicle (DMSO) or increasing concentrations of AP23464 for 60 minutes after which lysates were prepared and analyzed by Western

blotting with phospho-EGFR antibody. After removal of the phospho-specific antibody the blot was reprobed with EGFR antibody.

5.3 Discussion

p120 is an excellent Src substrate both in vitro and in vivo. However, whether the phosphorylation of individual tyrosine residues may be differentially regulated is unknown. Furthermore, the relative contributions of the Src catalytic domain and of the non-catalytic SH2 and SH3 domains have not been defined in epithelial cells. Finally, whether Src kinases are required to mediate RTK signalling to p120 is unclear. We therefore used the novel p120 phospho-specific antibodies described in chapter four to systematically study p120 phosphorylation in epithelial cancer cell lines in response to both Src and EGFR signalling.

5.3.1 *Src-dependent phosphorylation of p120.*

p120 was initially identified as a prominent phospho-protein in v-Src transformed chicken embryo fibroblasts (52). The phosphorylation of p120 correlated with, but was not necessary for, transformation (68). Src in which glycine at position 2 is mutated to alanine is unable to undergo myristylation, fails to localise to the plasma membrane and is unable to phosphorylate p120 (52). In keeping with this is the observation that p120 is not phosphorylated in cadherin-deficient cell lines (176, 273). Thus, Src-dependent phosphorylation of p120 requires both molecules to co-localise at the cell membrane, Src via myristylation and p120 via its interaction with the cadherin JMD.

The rapidity with which p120 underwent dephosphorylation in AP23464 treated KM12C-Src527F cells was striking. Complete loss of tyrosine phosphorylation occurred after as little as two minutes of drug treatment suggesting that the phosphorylation state of p120 may be highly dynamic. When over-expressing constitutively active Src in fibroblasts Mariner *et al* found that a five minute treatment with the phosphatase inhibitor pervanadate was required for the optimal detection of phosphorylation events suggesting that p120 tyrosine residues were subject to rapid dephosphorylation (68). A possible mediator is the cytoplasmic phosphatase, SHP-1, which interacts with phosphorylated p120 via its SH2 domain and mediates its dephosphorylation (248). SHP-1 is itself phosphorylated and activated by Src and so its activity would be expected to be high in Src527F expressing KM12C cells (273).

5.3.1.1 Tyrosine phosphorylation of p120 is predominantly mediated via Src kinase activity.

Although purified Src can phosphorylate p120 *in vitro* (68) it has not been shown, *in vivo*, whether Src-dependent phosphorylation of p120 is entirely mediated via Src's kinase activity or whether the recruitment of additional kinases to a signalling complex via the Src SH2 and / or SH3 domains may be involved. This is exemplified by another Src substrate, FAK, which undergoes phosphorylation at tyrosine residues 407, 576, 577 and 861 when kinase-deficient Src is over-expressed (448). Phosphorylation of these sites is not inhibited by AP23464 indicating that their phosphorylation is independent of Src's kinase activity. In contrast phosphorylation of FAK at tyrosine-925 specifically requires Src kinase activity. Understanding the relative contributions of Src kinase and non-kinase domains to signalling pathways is now particularly timely with the entry of Src inhibitors such as dasatinib into phase I clinical trials (458). In cancers both the absolute amount, as well as the activity, of Src may be increased (198) and drugs which competitively inhibit ATP binding to inhibit Src kinase activity would not necessarily be expected to block pathways aberrantly activated in response to over-expressed Src non-catalytic domains.

To investigate the structural requirements for p120 phosphorylation in a colorectal cancer cell line we utilised both a novel small molecule Src inhibitor as well as a variety of over-expressed Src constructs. As expected, the over-expression of constitutively active c-Src in KM12C cells resulted in a dramatic increase in tyrosine phosphorylation of p120. This was evident at all tyrosine residues studied. Interestingly, the over-expression of Src constructs rendered kinase-deficient, either by introduction of a function-abrogating point mutation in the kinase domain or by deletion of the entire kinase domain, was sufficient to promote moderate tyrosine phosphorylation of p120. Again, this increase in phosphorylation was seen at all tyrosine residues studied albeit to a lesser extent than that achieved by constitutively active Src. Importantly, the phosphorylation events caused by kinase-deficient Src appeared to be selective rather than a non-specific effect involving all Src substrates as phosphorylation of the Src substrates paxillin and p130^{cas} was clearly reduced in the presence of kinase-deficient Src. Furthermore, as discussed above, our group has previously reported differential phosphorylation of FAK tyrosine residues in KM12C cells in the presence of kinase-deficient Src as discussed above (448).

We next used AP23464 to pharmacologically inhibit Src kinase activity in KM12C cells expressing constitutively active Src. This resulted in rapid and complete dephosphorylation of p120 at all residues studied. No residual phosphorylation of p120 remained indicating

that in the presence of pharmacological inhibition of SFK kinase activity the over-expression of Src SH2 and SH3 domains is not sufficient to cause p120 phosphorylation. Taken together this data suggests that phosphorylation of p120 is primarily directly dependent on Src family kinase activity with a minor component attributable to over-expression of the non-catalytic domains. As this component was also inhibited by AP23464 it appears to represent recruitment or activation of an endogenous SFK or other AP23464-sensitive kinase. The only cytoplasmic kinase other than SFKs which has been shown to phosphorylate p120 in vitro and in vivo is Fer (276, 459) although the sensitivity of this kinase to AP23464 has not been reported. Another potential cytoplasmic kinase might be Abl which interacts with and phosphorylates the p120 family member δ -catenin in neuronal cell lines (460) and which is also potently inhibited by AP23464 (450). However, SFKs activate Abl (461) and in other systems the expression of kinase-deficient Src has inhibited rather than activated Abl (462, 463). Therefore we propose that the moderate increase in phosphorylation of p120 mediated by the over-expression of Src non-catalytic domains occurs via the recruitment and activation of either an endogenous SFK or unknown AP23464-sensitive kinase which can then phosphorylate p120.

5.3.1.2 An intact SH2 domain is required for Src-dependent phosphorylation of p120.

To define the role of the Src SH2 and SH3 domains in p120 phosphorylation we employed Src constructs with function-abrogating point mutations in the SH2 and SH3 domain. These mutations have been shown to prevent binding to a variety of SH2 and SH3 targets respectively (453, 454). Loss of SH3 function did not interfere with Src's ability to phosphorylate p120. However, for both constitutively active and kinase-deficient Src proteins loss of function of the SH2 domain was associated with a significant reduction in p120 phosphorylation.

In contrast to our results, an early study reported no reduction in p120 phosphorylation in response to mutation of the Src SH2 or SH3 domain (182). There are at least two plausible explanations for the discrepancy between this result and our work. Firstly, the earlier work was performed in chick embryo fibroblasts which represent a very different cellular context to KM12C colon cancer cells. In particular, the structure of the actin cytoskeleton and of cell-cell junctions as well as the localisation and activation of endogenous kinases may be fundamentally different in these two cell types. Secondly, the constructs used in the earlier work contained short deletions of residues 92-95 (c-Src *d*192, SH3 domain) or 155-157 (c-Src *d*155, SH2 domain), neither of which overlap the W118A or R175L point

mutations we used. These constructs cannot, therefore be regarded as synonymous and it is possible that the spectrum of proteins to which binding is perturbed does not fully overlap. Expression of the c-Src *dl92* and *dl155* constructs in KM12C cells could help to answer this question. However, it is clear that the possession of a functional SH2 domain is important for Src to cause phosphorylation of p120 in this epithelial cancer cell line.

5.3.2 EGFR-dependent phosphorylation of p120.

Stimulation of KM12C cells with EGF did not result in significant changes in p120 tyrosine phosphorylation despite evidence of receptor activation as determined by EGFR phosphorylation and robust activation of other downstream signalling pathways. A more tractable model for studying the regulation of EGFR-dependent phosphorylation of p120 was therefore provided by the A431 epidermal squamous cell carcinoma cell line.

Stimulation of A431 cells with EGF resulted in the simultaneous phosphorylation of p120 at all tyrosine residues studied. As expected this was completely abrogated by inhibition of EGFR kinase activity with AG1478. Treatment with AP23464 was also sufficient to fully block EGF-dependent phosphorylation of p120, thus implying a signalling pathway from EGFR to p120 involving an AP23464-sensitive kinase. This data contradicts work recently published by Mariner *et al* who reported EGFR signalling to p120 tyrosine-228 (176).

They reported no reduction in phosphorylation of p120 tyrosine-228 in EGF-stimulated A431 cells which had been pre-treated with an alternative Src inhibitor, SU6656 (464). Several possible explanations exist for this discrepancy. Firstly, inappropriate concentrations of inhibitor may have been used by one or other group. Mariner chose a concentration of SU6656 based on its ability to inhibit Src kinase activity in an in vitro kinase assay. The strength of this approach is that it provides a direct measure of biochemical inhibition. However, the relationship between extracellular and intracellular concentration of inhibitor is not known and therefore it cannot be assumed that a dose based on inhibition in a cell free system will be equally potent against intracellular Src. In contrast we defined the concentration of AP23464 to be used on the basis of inhibition of Src auto-phosphorylation at tyrosine-416 which has been widely used and validated as a surrogate marker of Src activity (465). However, it is conceivable that a small molecule Src kinase inhibitor which occupies the ATP binding cleft may block kinase activity whilst allowing Y416 to remain in a phosphorylated state. If this were the case we could potentially have used an excessively large dose of inhibitor, in which case “off-target” inhibition of additional kinases might occur. A second explanation would be that both inhibitors were used at appropriate concentrations but that Src family kinases are not

involved in EGFR signalling to p120. Rather an unidentified kinase targeted by AP23464 but not SU6656 would be the mediator of p120 phosphorylation. Abl is activated downstream of EGF signalling (461) and AP23464 is approximately equipotent in its inhibition of Abl and Src. In contrast SU6656 is 6-fold more potent for Src than Abl (466). However, it is not known if p120 is a substrate of Abl. Our demonstration that EGFR signalling to STAT3, a known Src substrate, was inhibited whilst signalling to MAPK was unaltered strongly suggests that the concentration of AP23464 used demonstrated selectivity and did not globally inhibit EGFR signalling. It also suggests that an AP23464-sensitive kinase operates downstream of EGFR rather than being required to co-operate in EGFR activation. Further work is required to definitively identify the kinase recruited by EGFR activation to phosphorylate p120. If this kinase is indeed Src then the over-expression of kinase-deficient Src protein might be expected to inhibit this in a dominant-negative fashion. However, as we have demonstrated the overexpression of Src non-catalytic domains may, in itself, promote a degree of p120 phosphorylation. Alternatively, RNAi-mediated depletion of Src would be possible but might be confounded by redundancy with other SFKs within this cell type. A head to head comparison of various Src inhibitors with known IC_{50} s for a variety of kinases may be useful to suggest potential non-SFK candidates.

5.4 Summary

Tyrosine phosphorylation of p120 by Src in epithelial cells is dependent on Src's kinase activity and SH2 domain. However, two different kinase-deficient Src constructs were able to induce moderate p120 phosphorylation suggesting that over-expressed Src non-catalytic domains may recruit endogenous Src-family kinases or other AP23464-sensitive kinase to a signalling complex. EGFR-dependent phosphorylation of p120 appears to be indirect via a pathway sensitive to inhibition by AP23464. Whether this reflects inhibition of Src or an as yet unidentified AP23464-sensitive kinase warrants further investigation.

6 Results: An investigation of the effects of p120 phosphorylation on cell-cell adhesion.

6.1 Aim

Despite p120's initial identification 16 years ago as a major phospho-protein in Src transformed cells the biological sequelae of its phosphorylation on tyrosine have proved enigmatic. In cancer cell lines increased activity of Src or EGFR, which often correlates with disruption of cell-cell adhesion and increased invasiveness, also causes prominent phosphorylation of p120. However, whilst p120 can profoundly influence the AJ and the motile phenotype of a cell it is not known whether the tyrosine phosphorylation state of p120 has any role in this regulation. We therefore used phosphorylation-defective p120 constructs to specifically define the role of p120 tyrosine phosphorylation in cell-cell adhesion in KM12C and A431 cell lines.

6.2 Results

6.2.1 p120 is phosphorylated at tyrosine-228 in nascent AJs.

Tyrosine phosphorylation appears to have a dual function at the AJ with tightly regulated phosphorylation of AJ proteins occurring as a physiological event during the formation of AJs, whilst deregulated kinase activity is often associated with disruption of AJs. The extracellular domain of E-cadherin must bind calcium to stabilise its conformation and facilitate adhesive interaction with the cadherin molecule of an adjacent cell (2). A common experimental manipulation involves the reduction of calcium in tissue culture media to 0.03 mM which results in rapid loss of cell-cell adhesion and redistribution of E-cadherin to a diffuse pattern around the membrane. On subsequent re-addition of calcium to achieve a concentration of 1-2 mM AJs reform. This type of "calcium switch" was utilised to study the phosphorylation of p120 by endogenous kinases in KM12C cells during AJ formation.

Following a switch from low to high calcium, tyrosine phosphorylation of p120 was most apparent at residue 228 (Figure 6-1). The temporal kinetics of phosphorylation were identical to those of morphological AJ formation as determined by the recruitment of p120 to linear areas of cell-cell contact (Figure 6-2). Phosphorylation was not detectable at residues 280 and 291. Basal phosphorylation of tyrosine 296 was identified but was not altered during the time course of AJ formation. These phosphorylation events required long exposures for detection and hence were of a significantly lower order of magnitude than that seen constitutively in Src527F expressing cells. Spatially, the phosphorylated

p120 was localised at nascent junctions as determined by immunofluorescence with phospho-Y228 antibody (Figure 6-2A). In low calcium medium p120 was localised primarily at the membrane with a diffuse distribution. In contrast E-cadherin was localised both at the membrane and internalised in apparent vesicular structures in the cytoplasm (Figure 6-2B). This observation is consistent with previous reports that dissociation of p120 from cadherin is an early event during cadherin internalisation (75).

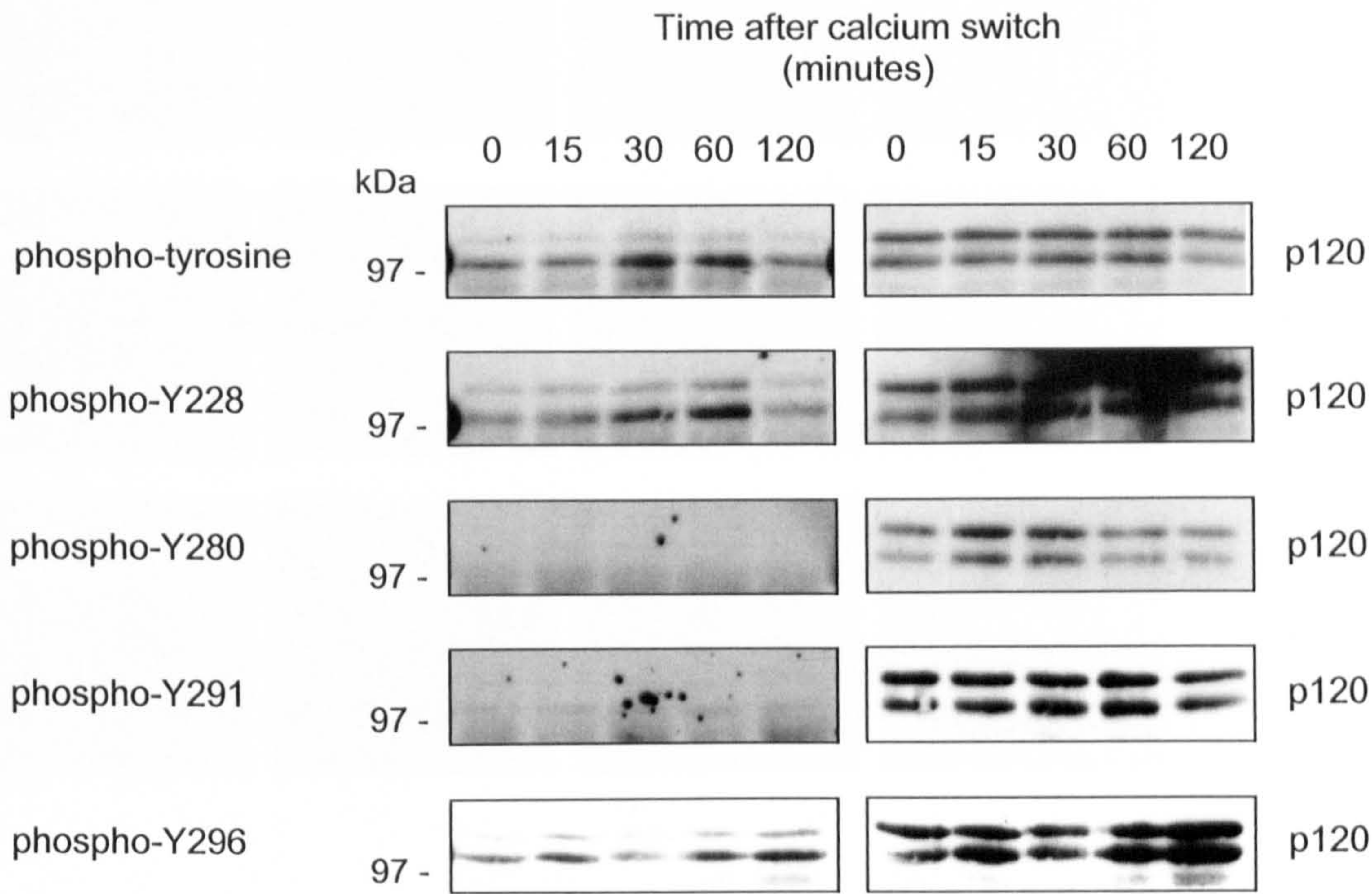


Figure 6-1 p120 is phosphorylated during AJ formation.

KM12C cells were maintained in low calcium-containing medium (KGM supplemented with 0.03 mM CaCl₂) for 24 hours then cell lysate prepared before or 15, 30, 60 or 120 minutes after being switched to high calcium-containing medium (KGM supplemented with 1.5 mM CaCl₂). p120 was immunoprecipitated and immuno-blotting performed with a pan-phosphotyrosine antibody (left upper panel) or with the site-specific phospho-p120 antibodies shown (left remaining panels). After removal of the phospho-specific antibodies each blot was reprobed with anti-p120 antibody (right panels).

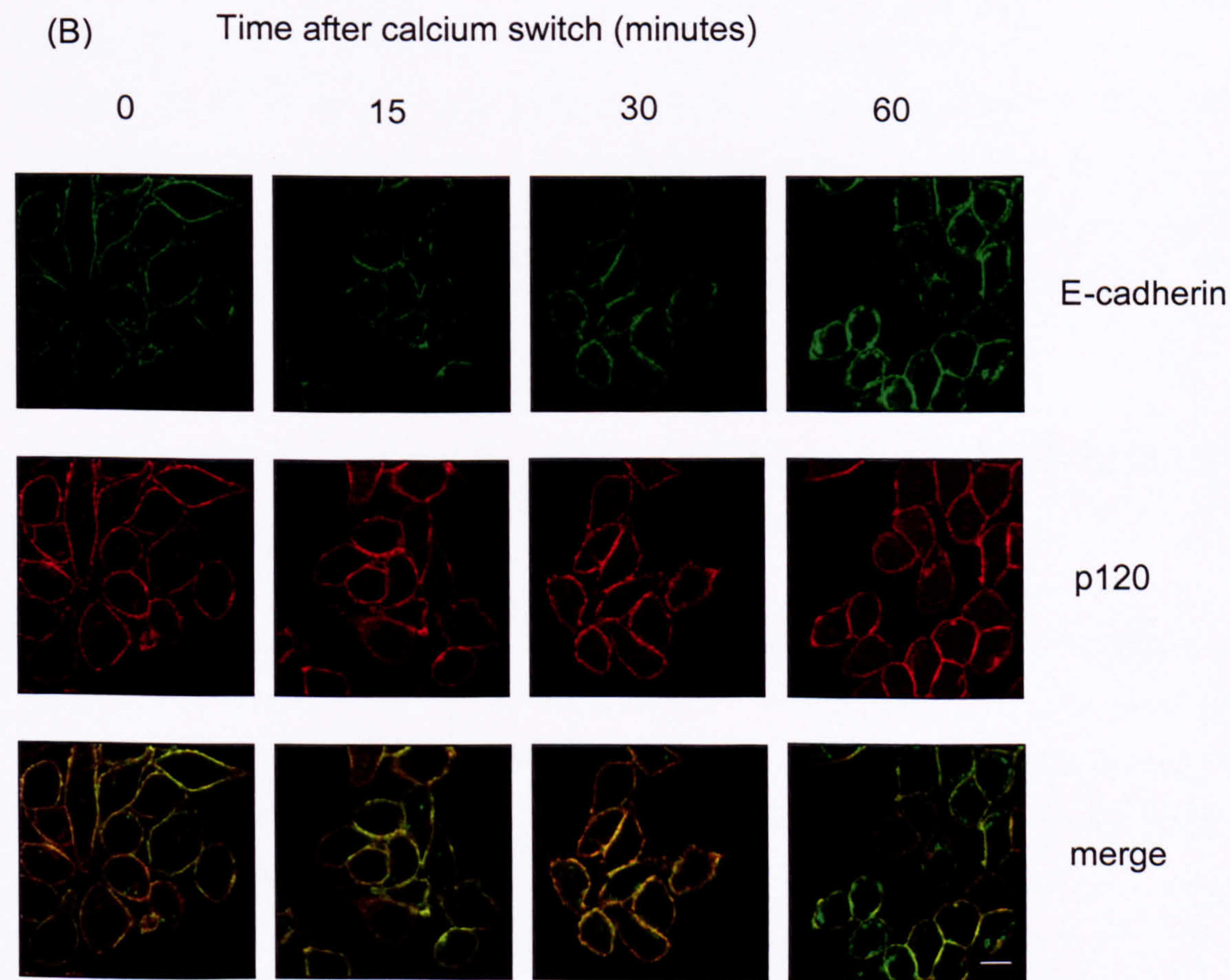
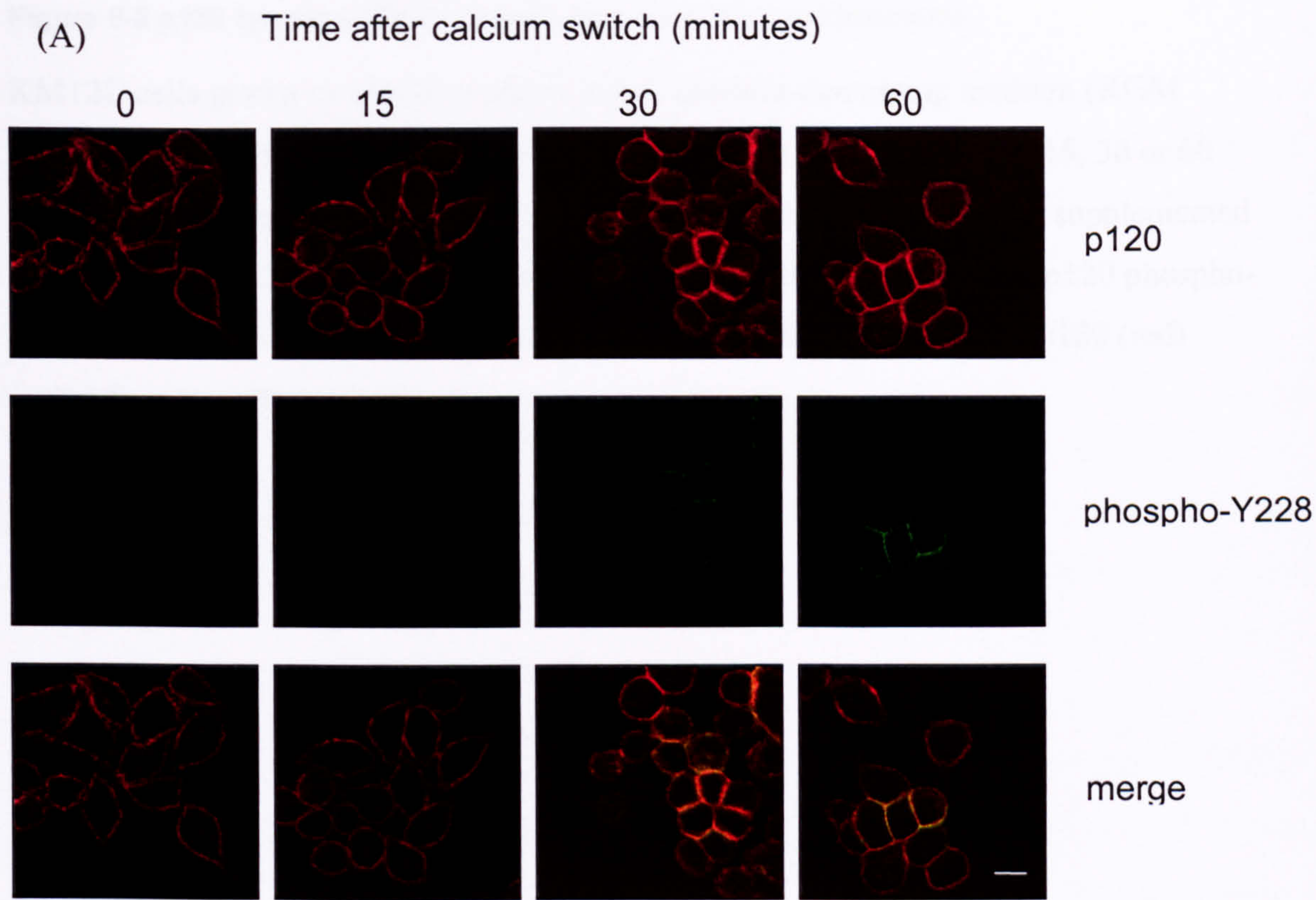


Figure 6-2 p120 tyrosine-228 is phosphorylated at nascent junctions.

Figure 6-2 p120 tyrosine-228 is phosphorylated at nascent junctions.

KM12C cells grown on chamber slides in low calcium-containing medium (KGM supplemented with 0.03 mM CaCl_2) for 24 hours were fixed prior to, or 15, 30 or 60 minutes after being switched to high calcium-containing medium (KGM supplemented with 1.5 mM CaCl_2). Co-immunofluorescence was performed with (A): p120 phospho-Y228 (red) and p120 (green) antibodies; or (B): E-cadherin (green) and p120 (red) antibodies. Bar, 20 μm .

6.2.2 p120 is a major Src-dependent target at the AJ.

Our group has previously identified an “adhesion-switch” phenotype in KM12C colorectal carcinoma cells when constitutively active Src (Src527F) is expressed (257, 278, 447). Specifically, Src527F blocks the proper assembly of AJs after cells are switched from media containing low levels of calcium to media containing high levels of calcium. In parallel there is an increased prominence of cell-matrix adhesions and increased expression of mesenchymal markers such as vimentin. As described in chapter five, the expression of Src527F in KM12C colorectal cancer cells is associated with a marked increase in the phosphorylation of several p120 tyrosine residues. Tyrosine phosphorylation of E-cadherin and β -catenin in cell lines expressing active Src has previously been reported (180). To identify whether these other components of the AJ were similarly phosphorylated in KM12C cells expressing Src527F (and the other Src constructs described in chapter five) E-cadherin and β -catenin were immunoprecipitated and analysed by immunoblotting with an anti-phosphotyrosine antibody. Expression of Src527F was associated with increased phosphorylation of E-cadherin (Figure 6-3). Interestingly, and in contrast to p120, the introduction of an SH2 function abrogating mutation did not reduce the ability of Src527F to cause phosphorylation of E-cadherin. As is the case for p120, the expression of kinase-defective Src caused a small but consistent increase in phosphorylation of E-cadherin. However, for all Src constructs the relative increase in E-cadherin phosphorylation above that in parental KM12C cells was far less than that for p120 (Fig. 6-3). No alteration in β -catenin phosphorylation was detected. In all cases total levels of the proteins were not changed. Thus p120 appeared to be the most prominently phosphorylated major component of the AJ in KM12C cells expressing Src527F.

6.2.3 Expression of active Src in KM12C cells perturbs formation of AJs.

KM12C cells grown in low calcium containing medium readily reformed junctions on re-addition of calcium (76.7% at 4 hours) as judged by localisation of E-cadherin to linear areas of cell-cell contact (Figure 6-4 A). The expression of Src527F potently inhibited the reformation of AJs (12.6% at 4 hours) (Figure 6-4 A) with most E-cadherin remaining diffusely localised around the cell membrane and internalised in the cytoplasm (Figure 6-4 B).

Treatment of KM12C-Src527F cells with concentrations of AP23464 which inhibit SFK kinase activity abrogated the negative impact of expression of Src527F on calcium-induced AJ formation in a dose dependent manner (Figure 6-4 A, B). The concentrations of AP23464 were similar to those in which tyrosine phosphorylation of p120 was inhibited (Figure 5-5). Thus increased Src kinase activity in KM12C cells caused deregulation of the AJ and this correlated with phosphorylation of p120. Conversely, pharmacological inhibition of Src kinase activity effectively blocked Src induced inhibition of AJ formation and this similarly correlated with prevention of p120 hyper-phosphorylation.

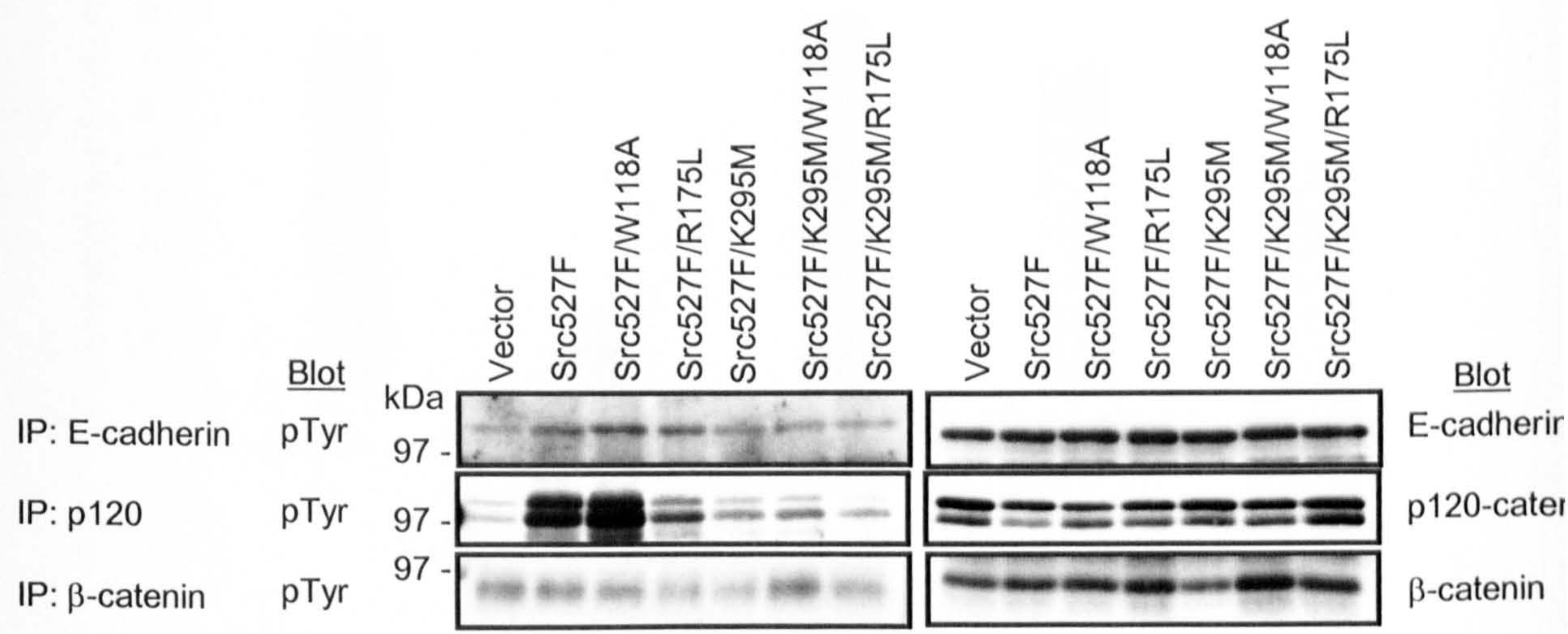


Figure 6-3 p120 is a major phosphorylated protein at the AJ.

p120, E-cadherin and β-catenin were immunoprecipitated from KM12C cells expressing empty vector (vector) or constitutively active Src (Src527F), constitutively active Src with a loss of function mutation in the SH3 (Src527F / W118A) or SH2 domain (Src527F / R175L), kinase-deficient Src (Src527F / K295M) or kinase-deficient Src with a loss of function mutation in the SH3 (Src527F / K295M / W118A) or SH2 domain (Src527F / K295M / R175L). Western blotting with anti-phosphotyrosine antibody was performed (left hand panels). After removal of the phosphotyrosine antibody the blots were reprobed with E-cadherin, p120, or β-catenin antibody (right hand panels).

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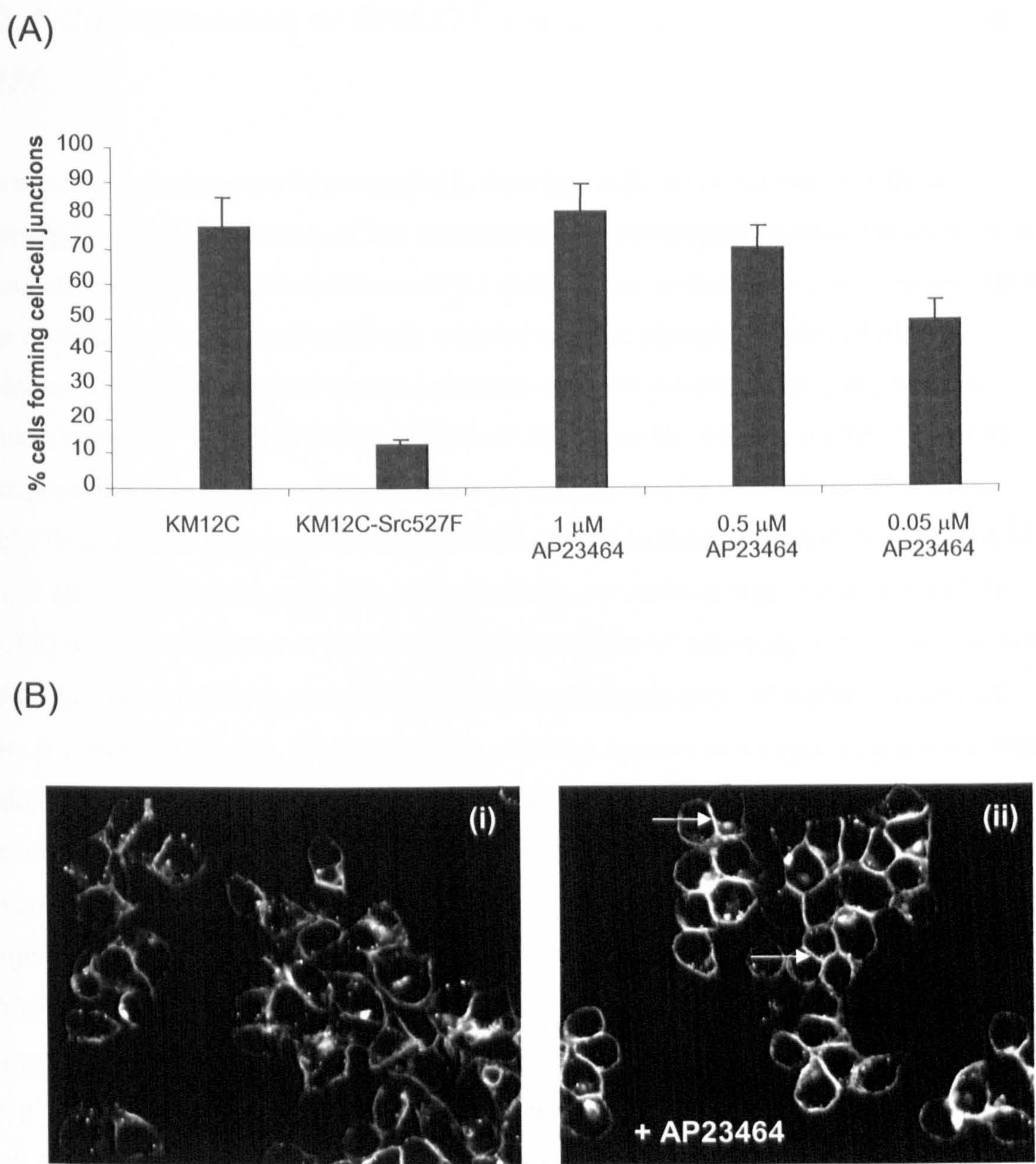


Figure 6-4 Src induces deregulation of the AJ which is reversed by AP23464.

(A) KM12C cells or KM12C cells expressing constitutively active Src (Src527F) were plated for 24 hours in high calcium-containing medium (MEM), further maintained for 24 hours in low calcium-containing medium (KGM supplemented with 0.03 mM CaCl₂) then switched to high calcium-containing medium (KGM supplemented with 1.5 mM CaCl₂) with or without increasing concentrations of AP23464. After 4 hours cells were fixed and stained with E-cadherin antibody. For quantification, 100 cells were assessed for their ability to form AJs as assessed by contacts with adjacent cells delineated by E-cadherin staining. Results represent the mean +/- S.D. of three independent experiments. (B) Representative confocal images demonstrating prominent accumulation of E-cadherin at cell-cell contacts (arrows) in KM12C-Src527F cells switched from low to high calcium in the absence (i) or presence (ii) of 0.5 μ M AP23464.

6.2.4 Co-expression of Src527F and phosphorylation-defective p120.

Src has many downstream targets and it is therefore difficult to attribute a cellular phenotype to phosphorylation of any one substrate. To determine whether the correlation seen between p120 phosphorylation and AJ deregulation indicated a causal relationship it was necessary to interfere specifically with the tyrosine phosphorylation of p120 in isolation. The replacement of tyrosine phospho-acceptor residues with phenylalanine renders a protein “phosphorylation-defective” and hence the relative contribution of its phosphorylation to the overall Src-induced phenotype may be ascertained. Therefore, Src527F and either wild type HA-tagged murine p120 isoform 1A or murine p120-1A in which all eight sites of tyrosine phosphorylation were mutated to phenylalanine (p120-1A/8F) were co-expressed in KM12C cells with the aim of achieving a dominant negative effect. In common with most epithelial cells, the predominant p120 isoform in KM12C cells is isoform 3 (58-60). Thus exogenous p120-1A appears as a slightly slower migrating additional band (Figure 6-5 A, arrow). Expression of p120-1A or p120-1A/8F did not alter the levels of other core components of the AJ (Figure 6-5 A). Both wild-type and p120-8F constructs localised correctly to the cell membrane, specifically at sites of cell-cell contact (Figure 6-5 B). An anti-HA antibody was used to immunoprecipitate only exogenous p120 which was then analysed by immuno-blotting with an anti-phosphotyrosine antibody. As expected, significant phosphorylation on tyrosine was detected for wild type p120-1A but not p120-1A/8F (Figure 6-5 C). Immunoprecipitation with an antibody which recognises both endogenous and exogenous p120 demonstrated that expression of p120-1A/8F was associated with a significant reduction in phosphorylation of endogenous p120 suggesting a dominant negative effect was at least partially achieved (Figure 6-5 D).

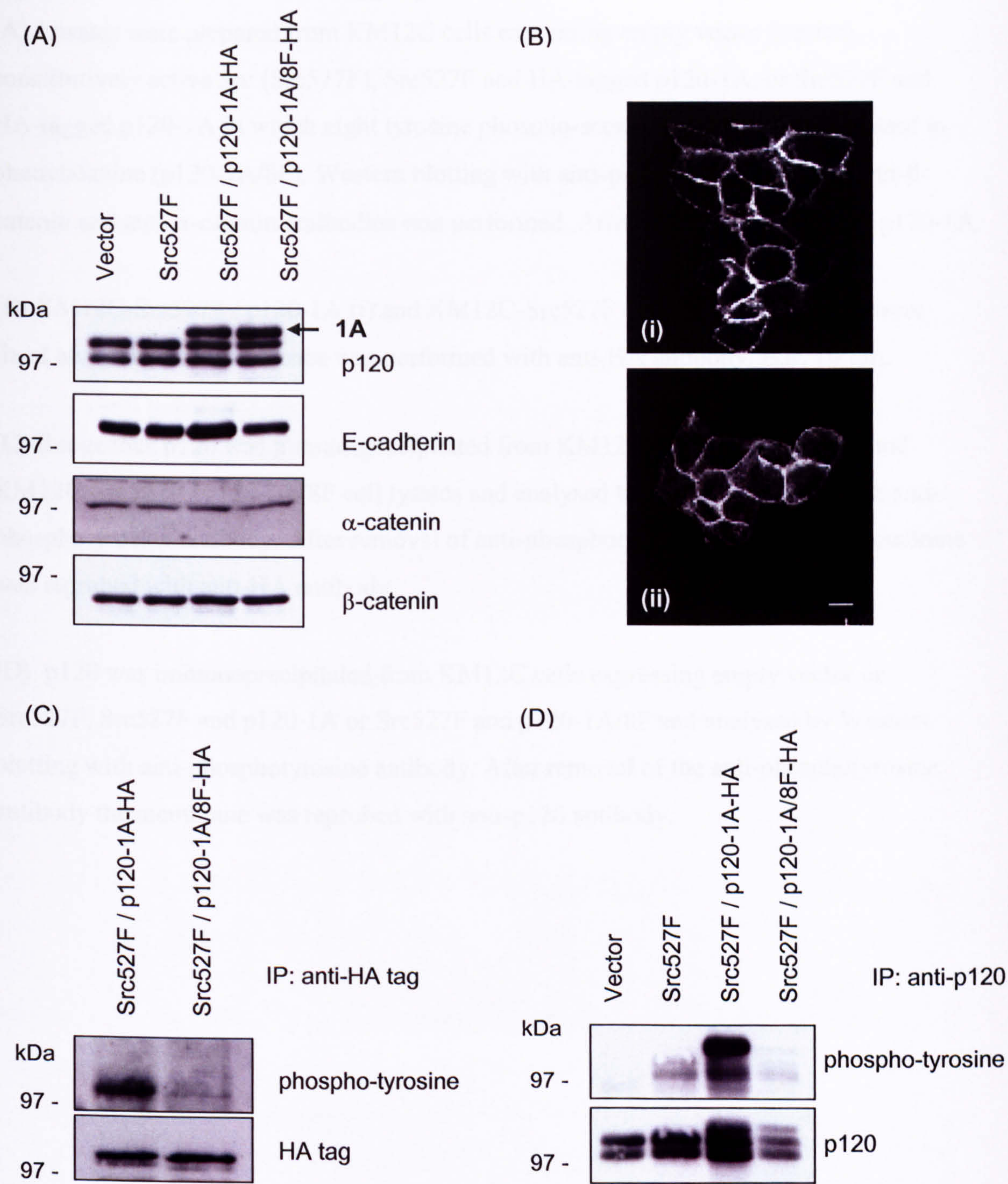


Figure 6-5 Expression of HA-tagged p120 constructs in KM12C-Src527F cells.

Figure 6-5 Expression of HA-tagged p120 constructs in KM12C-Src527F cells.

(A) Lysates were prepared from KM12C cells expressing empty vector (vector), constitutively active Src (Src527F), Src527F and HA-tagged p120-1A, or Src527F and HA-tagged p120-1A in which eight tyrosine phospho-acceptor residues were mutated to phenylalanine (p120-1A/8F). Western blotting with anti-p120, anti-E-cadherin, anti- β -catenin and anti- α -catenin antibodies was performed. Arrow indicates exogenous p120-1A.

(B) KM12C-Src527F / p120-1A (i) and KM12C-Src527F / p120-1A/8F (ii) cells were fixed and immunofluorescence was performed with anti-HA antibody. Bar, 10 μ m.

(C) Exogenous p120 was immunoprecipitated from KM12C-Src527F / p120-1A and KM12C-Src527F / p120-1A/8F cell lysates and analysed by Western blotting with anti-phosphotyrosine antibody. After removal of anti-phosphotyrosine antibody the membrane was reprobed with anti-HA antibody.

(D) p120 was immunoprecipitated from KM12C cells expressing empty vector or Src527F, Src527F and p120-1A or Src527F and p120-1A/8F and analysed by Western blotting with anti-phosphotyrosine antibody. After removal of the anti-phosphotyrosine antibody the membrane was reprobed with anti-p120 antibody.

6.2.5 Phosphorylation-defective p120 does not reverse Src-induced inhibition of AJ formation.

We predicted that if increased tyrosine phosphorylation of p120 was an important mechanism of Src-induced inhibition of AJ formation then expression of p120-1A/8F, which we had shown to act in a dominant-negative manner to prevent phosphorylation of p120, should abrogate the Src-induced phenotype. Therefore, the ability of p120-1A/8F to reverse the Src-induced inhibition of cell-cell contact formation in response to a calcium switch was determined (Figure 6-6). KM12C cells containing empty vector readily reformed cell-cell contacts (82% +/- 8.6% of cells at 4 hours). Expression of Src527F potently reduced the percentage of cells forming cell-cell contacts (10.3% +/- 1.7%) but co-expression of p120-1A/8F did not abrogate the effect of Src527F (5% +/- 2.5%). Co-expression of wild type p120-1A, included as a control, also had no discernable effect (2.7% +/- 1.9%).

6.2.6 Phosphorylation-defective p120 does not inhibit disassembly of AJs.

As the expression of phosphorylation-defective p120 did not alter Src's ability to inhibit cell contact formation we asked whether phosphorylation-defective p120 might interfere with the initial disassembly of the AJ occurring when KM12C cells are switched from high to low calcium-containing medium. After four hours in low calcium-containing medium 34.7% +/- 15.3% of KM12C cells containing empty vector retained cell-cell contacts. In cells expressing Src527F approximately 10.2% +/- 12.3% of cells retained cell-cell contacts and the co-expression of p120-1A/8F (12.0% +/- 13.1%) or wild type p120-1A (12.5% +/- 6.75%) did not influence this process (Figure 6-7).

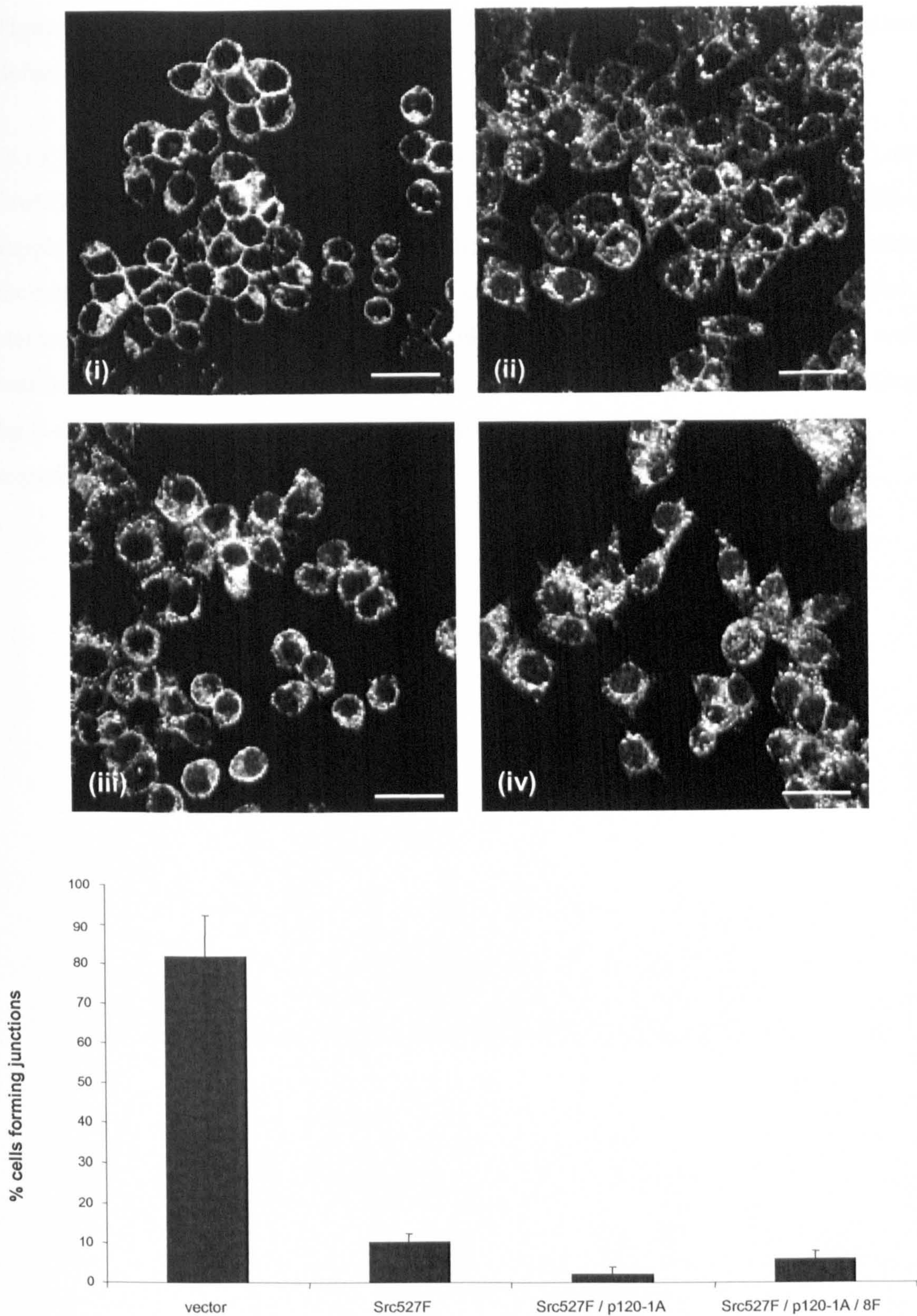


Figure 6-6 Phosphorylation-defective p120 does not reverse the Src-induced adhesion defect.

Figure 6-6 Phosphorylation-defective p120 does not reverse the Src-induced adhesion defect.

(A) KM12C cells containing empty vector (i), Src527F (ii), Src527F / p120-1A (iii), or Src527F / p120-1A/8F (iv) were maintained in low calcium-containing medium (KGM supplemented with 0.03 mM CaCl_2) for 24 hours then switched to high calcium-containing medium (KGM supplemented with 1.5 mM CaCl_2). After 4 hours cells were fixed and stained with anti-E-cadherin antibody. Bar 50 μm . (B) For quantification 100 cells were assessed for their ability to form AJs as assessed by contacts with adjacent cells delineated by E-cadherin staining. Results represent the mean \pm S.D. of three independent experiments.

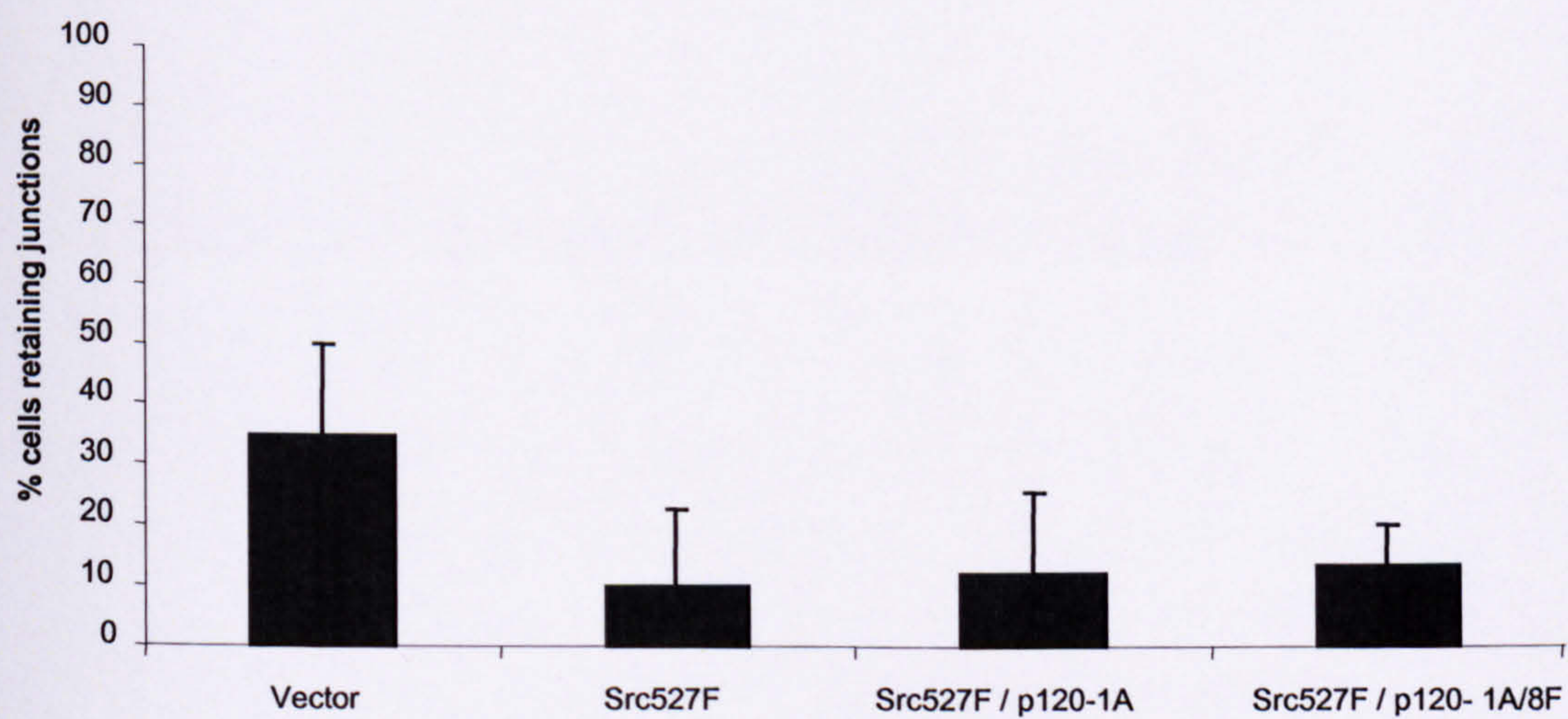
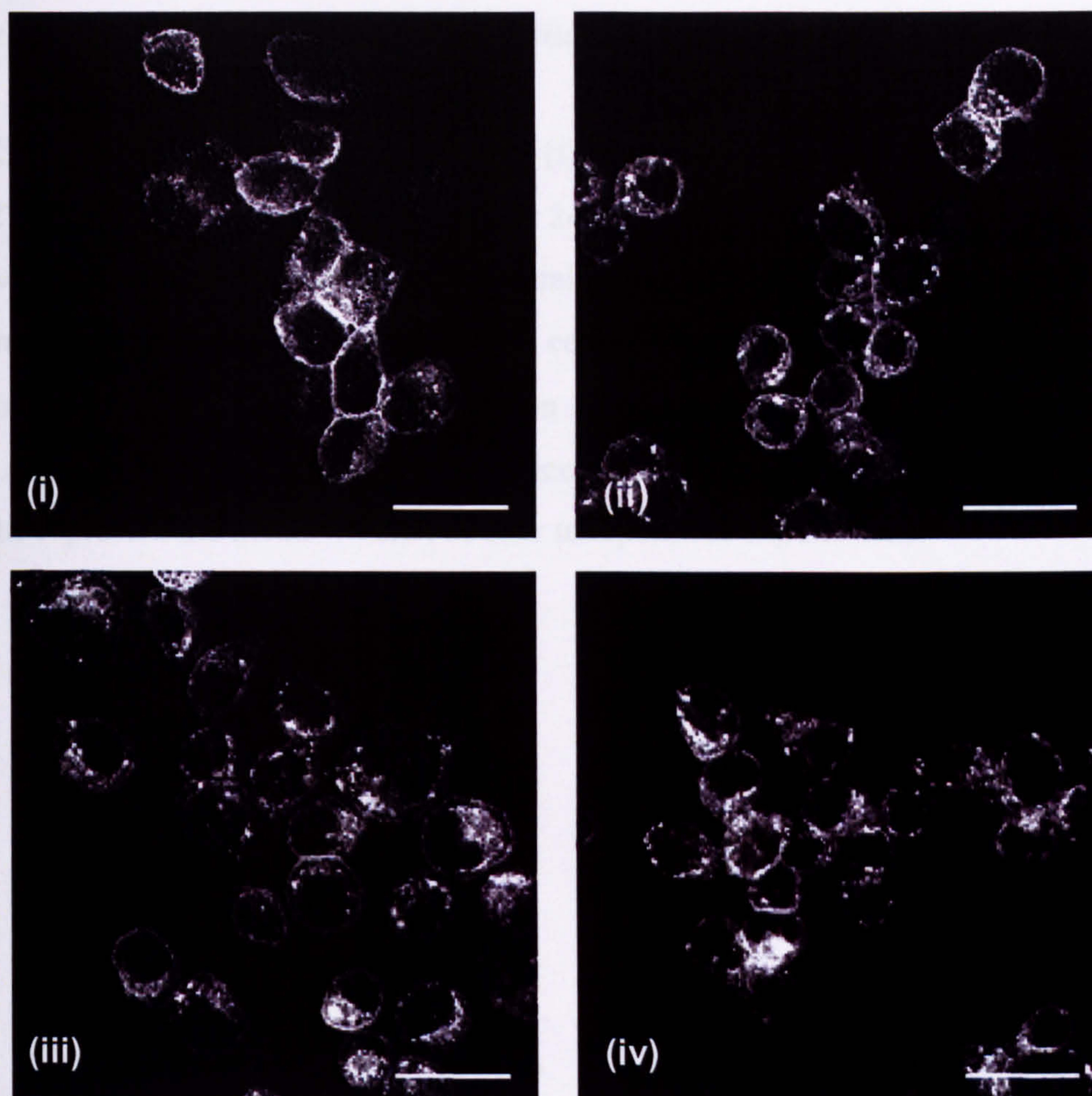


Figure 6-7 p120-8F does not interfere with disassembly of AJs.

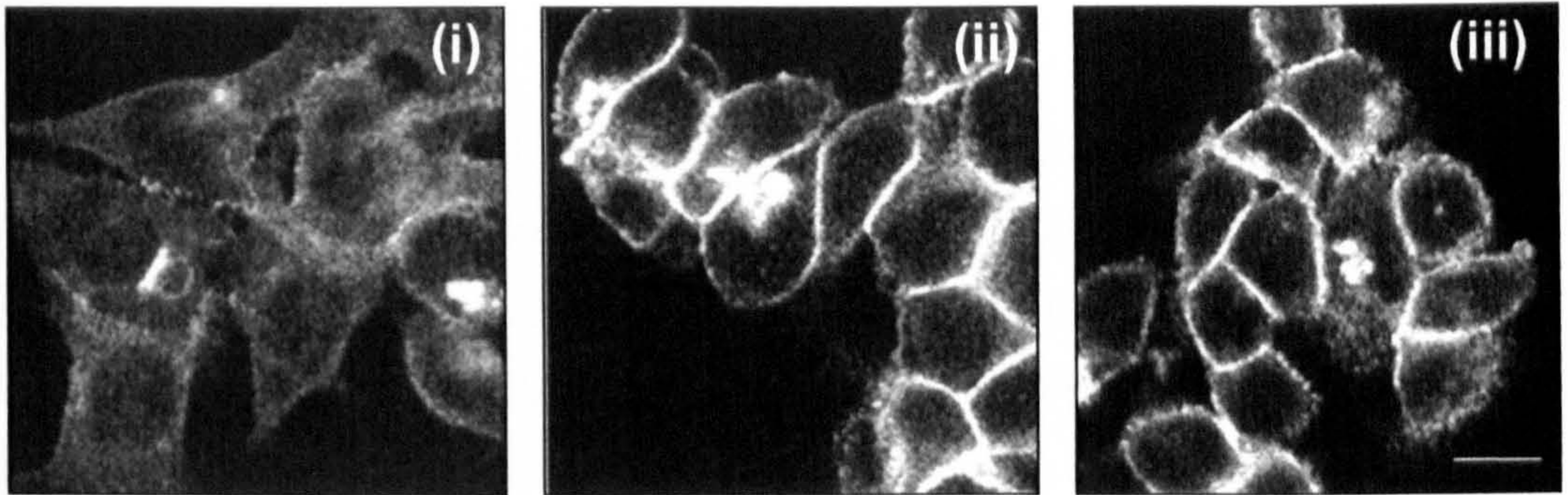
Figure 6-7 p120-8F does not interfere with disassembly of AJs.

(A) KM12C cells containing empty vector (i), Src527F (ii), Src527F / p120-1A (iii), or Src527F / p120-1A/8F (iv) were plated for 24 hours in high calcium-containing medium (MEM), then switched to low calcium-containing medium (KGM supplemented with 0.03mM CaCl₂) for 4 hours. After fixation cells were stained with anti-E-cadherin antibody. Bar 40 μ m. (B) For quantification 100 cells were assessed for their ability to form AJs as assessed by contacts with adjacent cells delineated by E-cadherin staining. Results represent the mean \pm S.D. of four independent experiments.

6.2.7 Functional Src non-catalytic domains are required for disruption of the AJ.

Although the phosphorylation state of p120 correlated with Src's ability to inhibit AJ formation in KM12C-Src527F cells the preceding experiments suggested there was not a causal link. Interestingly, the introduction of a function abrogating point mutation in the SH2 (Src527F / R175L) or SH3 (Src527F / W118A) domain rendered Src unable to inhibit cell-cell contact formation in response to a calcium switch (Figure 6-8, data courtesy of Dr E Avizienyte (278)). However, both of these Src constructs caused an increase in tyrosine phosphorylation of p120 which, in the case of Src527F / W118A, was as great as Src527F (Figure 5-6).

(A)



(B)

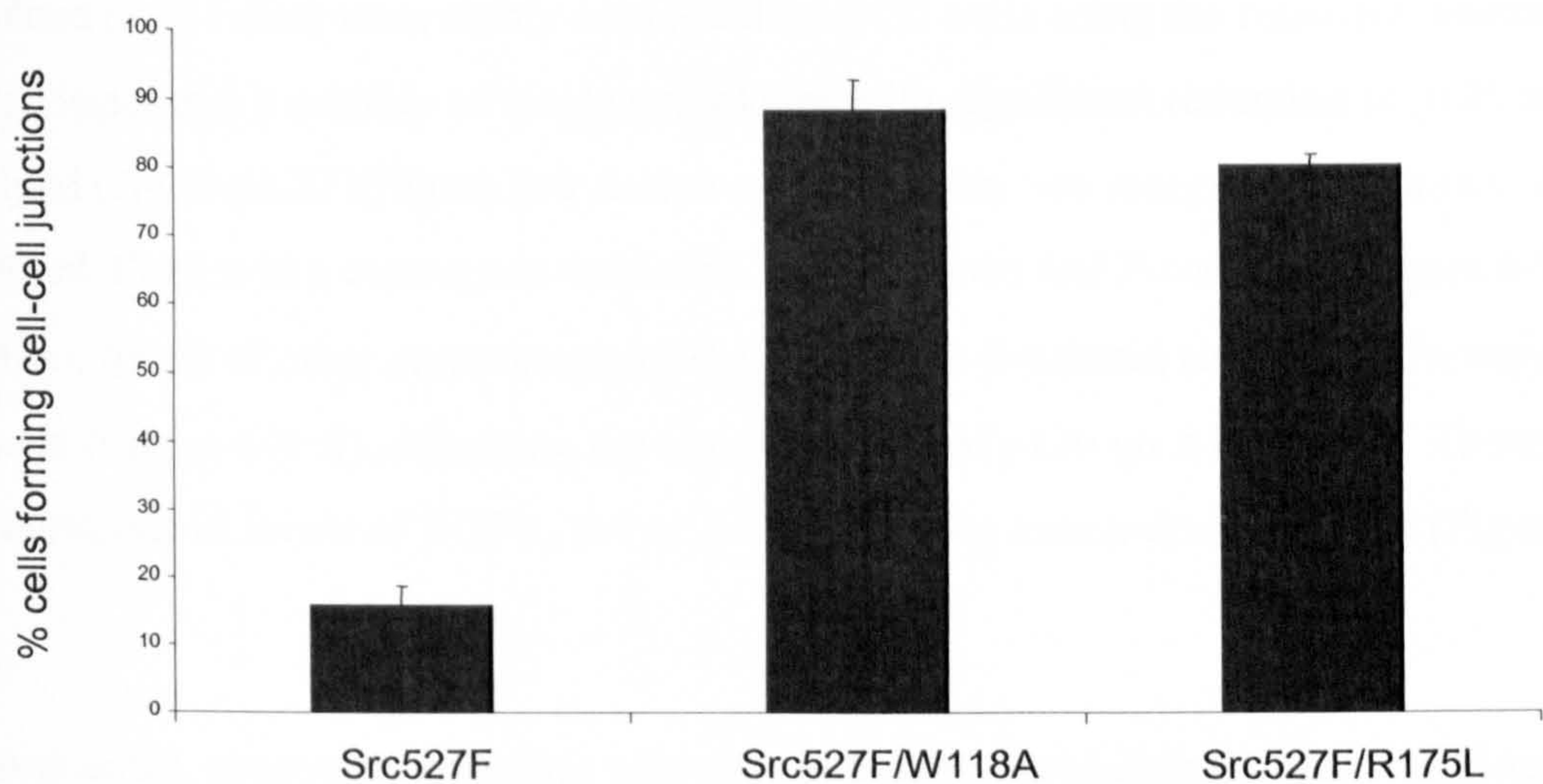


Figure 6-8 Loss of function of the Src SH2 or SH3 domain blocks inhibition of AJ formation.

(A) KM12C cells containing Src527F (i), Src527F / W118A (ii), or Src527F / R175L (iii) were maintained in low calcium-containing medium (KGM supplemented with 0.03 mM CaCl_2) for 24 hours then switched to high calcium-containing medium (KGM supplemented with 1.5 mM CaCl_2). After 4 hours cells were fixed and stained with anti-E-cadherin antibody. Bar, 20 μm . (B) For quantification 100 cells were assessed for their ability to form AJs as assessed by contacts with adjacent cells delineated by E-cadherin staining. Results represent the mean \pm S.D. of three independent experiments.

6.2.8 RNAi-mediated depletion and reconstitution of p120 in A431 cells.

To further investigate the role of p120 phosphorylation in cell-cell adhesion, we utilised a powerful RNAi-based substitution system recently described by Mariner *et al* (176). The magnitude of RNAi effect was cell line specific and, although a potent reduction in p120 levels was achieved in A431 cells, significant knockdown of p120 could not be achieved in KM12C cell lines (data not shown). Importantly, this system avoids the potential confounding factor of sub-cellular mis-localisation of over-expressed p120 due to saturation of cadherin binding sites and hence facilitates direct comparison of the function of different p120 constructs expressed at physiological levels. Human-specific p120 RNAi sequences (which do not alter the stability of murine p120) or a scrambled control sequence (A431-Scr) were stably expressed in A431 cells using the retroviral vector pRetroSuper and a number of single cell clones with significant reduction in p120 levels obtained (A431-p120⁻) (Figure 6-9 A shows results from two independent clones). As expected, there was a concurrent reduction in E-cadherin and P-cadherin (Figure 6-9 A). In addition, levels of other components of the AJ such as β -catenin and α -catenin were also reduced (Figure 6-9 A), reflecting the known effects of p120 on AJ stability. There were no alterations in levels of EGFR, Src or FAK following knock-down of p120 (Figure 6-9 B).

Subsequently, constructs encoding wild type and phosphorylation defective murine p120 isoform 3A were reintroduced into a single cell clone in which a high level of knockdown of endogenous p120 had been achieved (clone 7) using . Unlike isoform 1, p120 isoform 3 does not contain tyrosine-96 but does contain tyrosines 112 – 302. Therefore the corresponding phosphorylation defective mutant was labelled “7F” in contrast to the isoform 1A “8F” mutant described in chapter four. We also introduced isoform 4A which lacks the entire N-terminal regulatory domain containing all identified sites of tyrosine and most sites of serine/threonine phosphorylation (Figure 6-9 C) (69). This isoform undergoes no detectable tyrosine phosphorylation in A431 cells (data not shown). Due to the presence of an intra-ribosomal entry site in the pLZRS-IRES-GFP vector the expression of p120 and GFP was directly proportional. This facilitated the selection of pooled populations with stable expression of p120 at levels similar to parental A431 cells by FACS according to GFP expression. Re-introduction of isoform 3A, 3A/7F or 4A restored levels of cadherins and catenins to those seen in the control cells (Fig. 6-9 A).

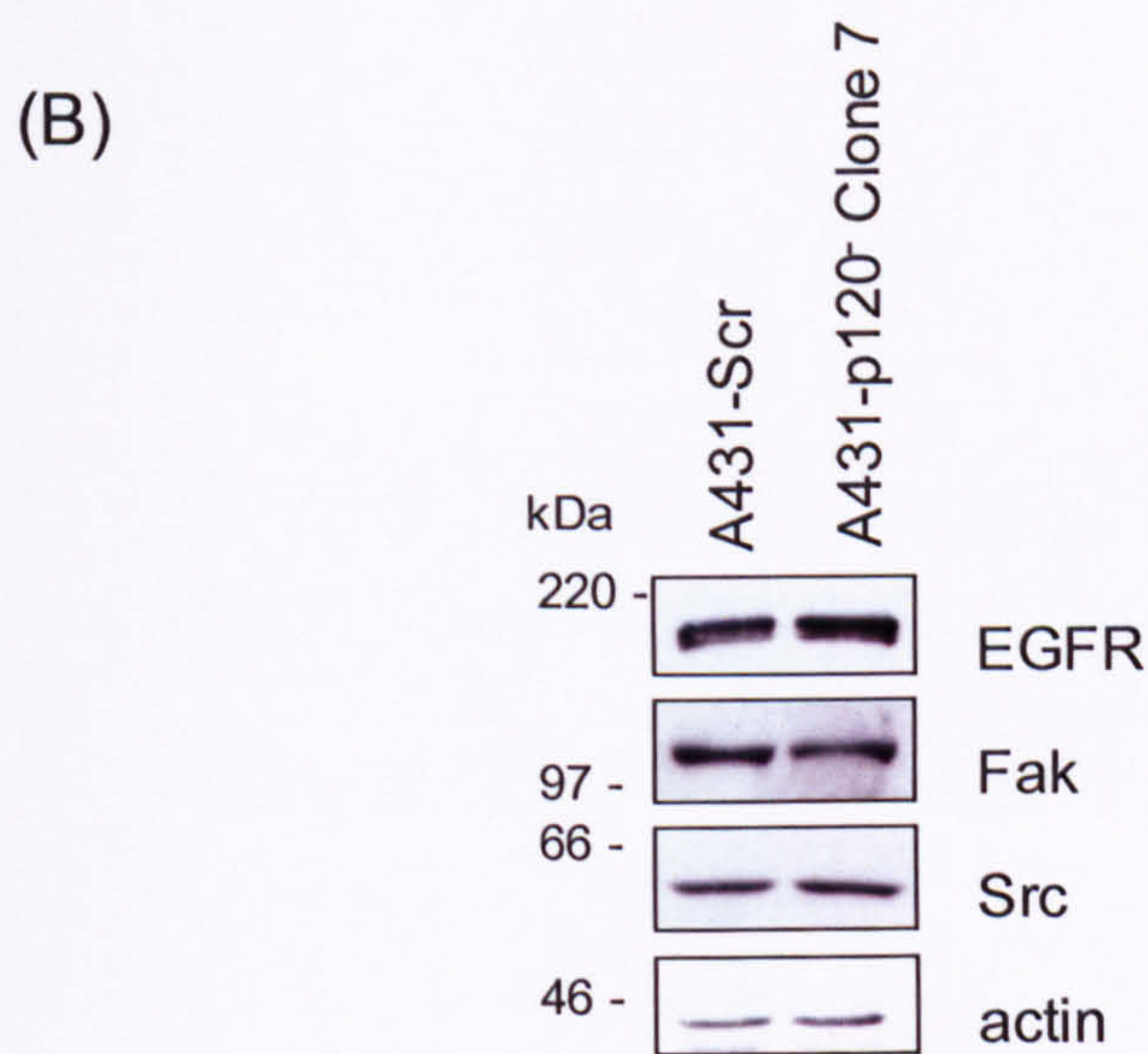
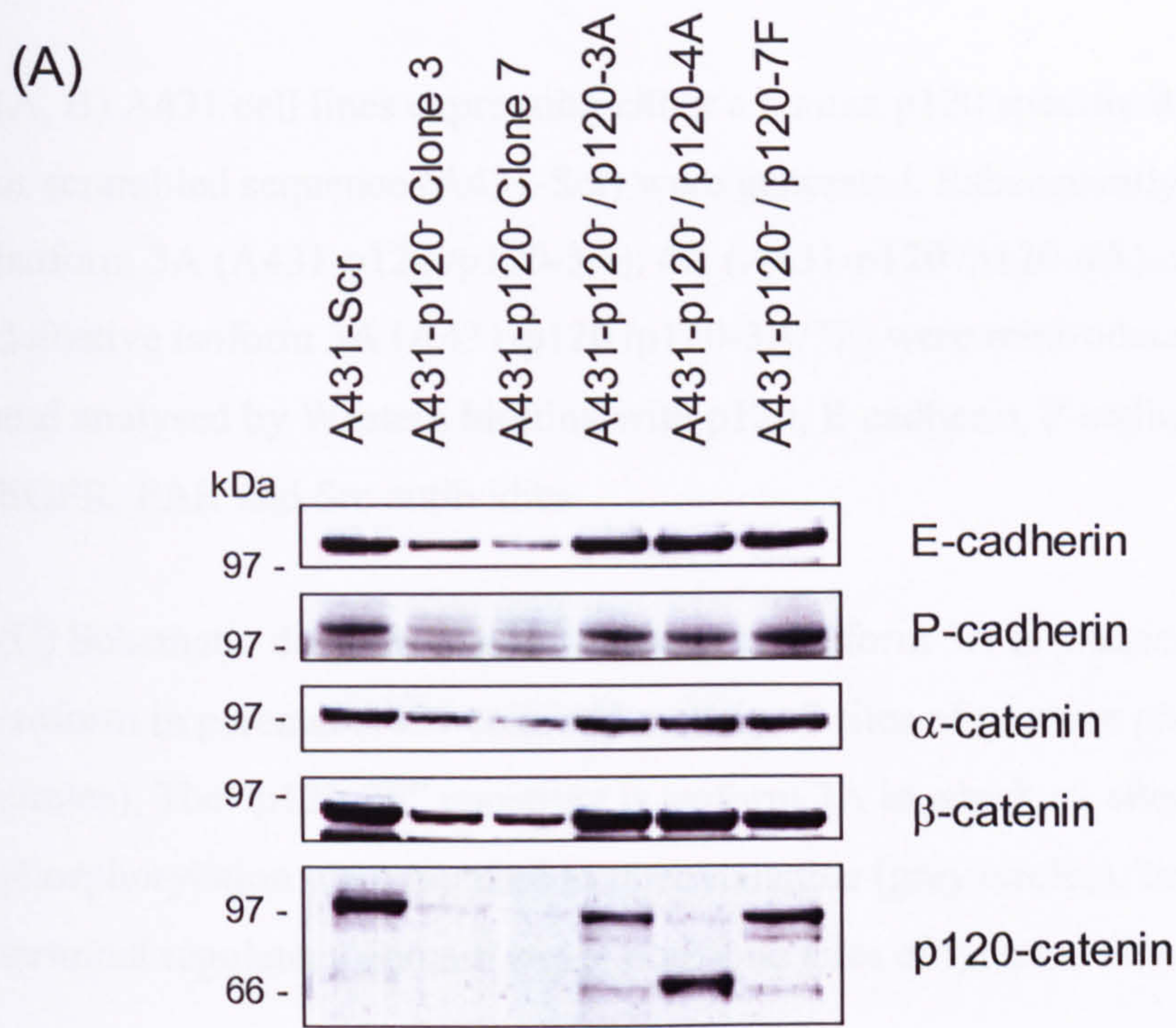


Figure 6-9 RNAi-mediated depletion and reconstitution of p120.

Figure 6-9 RNAi-mediated depletion and reconstitution of p120.

(A, B) A431 cell lines expressing either a human p120 specific RNAi hairpin (A431-p120[~]) or scrambled sequence (A431-Scr) were generated. Subsequently wild type murine p120 isoform 3A (A431-p120[~]/p120-3A), 4A (A431-p120[~]/p120-4A) or phosphorylation defective isoform 3A (A431-p120[~]/p120-3A/7F) were reintroduced. Lysates were prepared and analysed by Western blotting with p120, E-cadherin, P-cadherin, β -catenin, α -catenin, EGFR, FAK and Src antibodies.

(C) Schematic diagram of p120 constructs. Isoform 3A is identical to the predominant isoform in parental A431 cells and contains 7 sites of tyrosine phosphorylation (red circles). The “p120-7F” construct is isoform 3A in which all sites of tyrosine phosphorylation were mutated to phenylalanine (grey circles). Isoform 4A lacks the N-terminal regulatory domain and contains no sites of tyrosine phosphorylation.

6.2.9 Depletion of p120 causes loss of morphological cell-cell cohesion.

As depletion of p120 resulted in a significant reduction in cadherins and other AJ proteins we predicted that morphological cell-cell cohesion would be impaired due to an inability to form AJs. Indeed, whilst parental A431 cells form epithelial colonies when cultured on tissue culture plastic, cells in which p120 was depleted grew in isolation and were unable to form colonies (Figure 6-10). Even when grown at high density morphological cell-cell cohesion did not occur as judged by actin staining (Figure 6-11). Unlike parental cells which adopted a typical cobblestone morphology the p120 depleted cells showed marked variation in shape with no evidence of intercellular coordination of actin organisation. This phenotype was reversed by reconstitution with p120 isoform 3A, 3A/7F or 4A (Figure 6-10). Importantly the ability of A431 cells reconstituted with p120-3A/7F or p120-4A to form cell-cell junctions appeared unimpaired suggesting that the phosphorylation of p120 at tyrosine-228 occurring in nascent junctions (section 6.2.1) was not a necessary event in AJ formation.

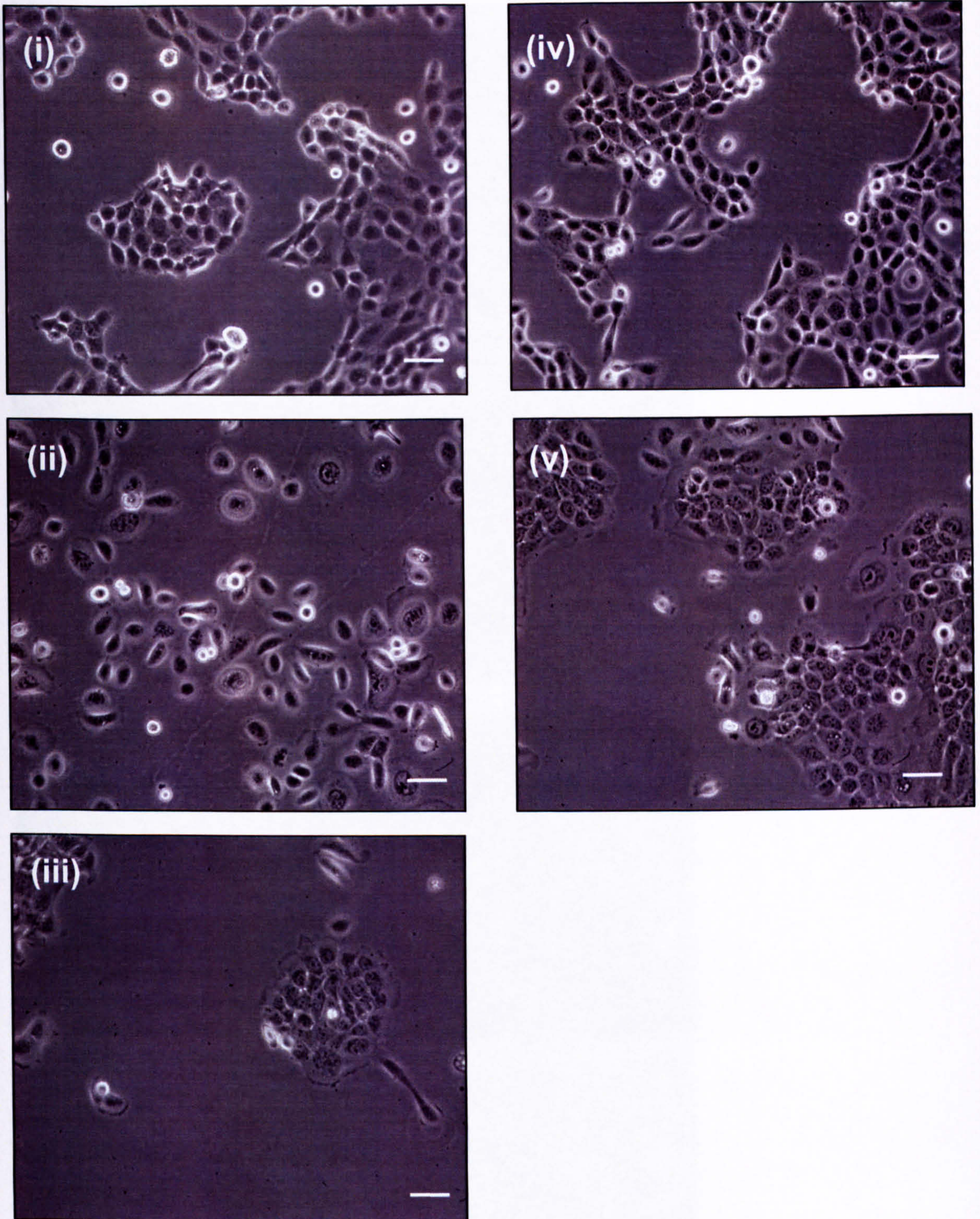


Figure 6-10 Depletion of p120 specifically prevents morphological cell-cell cohesion.

(A) Phase contrast micrographs of A431 cells expressing a scrambled control RNAi sequence (i) or a human p120-specific RNAi sequence (ii - v). p120-depleted cells were reconstituted with murine p120-3A (iii) or murine p120-4A (iv) or murine p120-3A/ 7F (v) (Magnification x10; Bar 50 μ m).

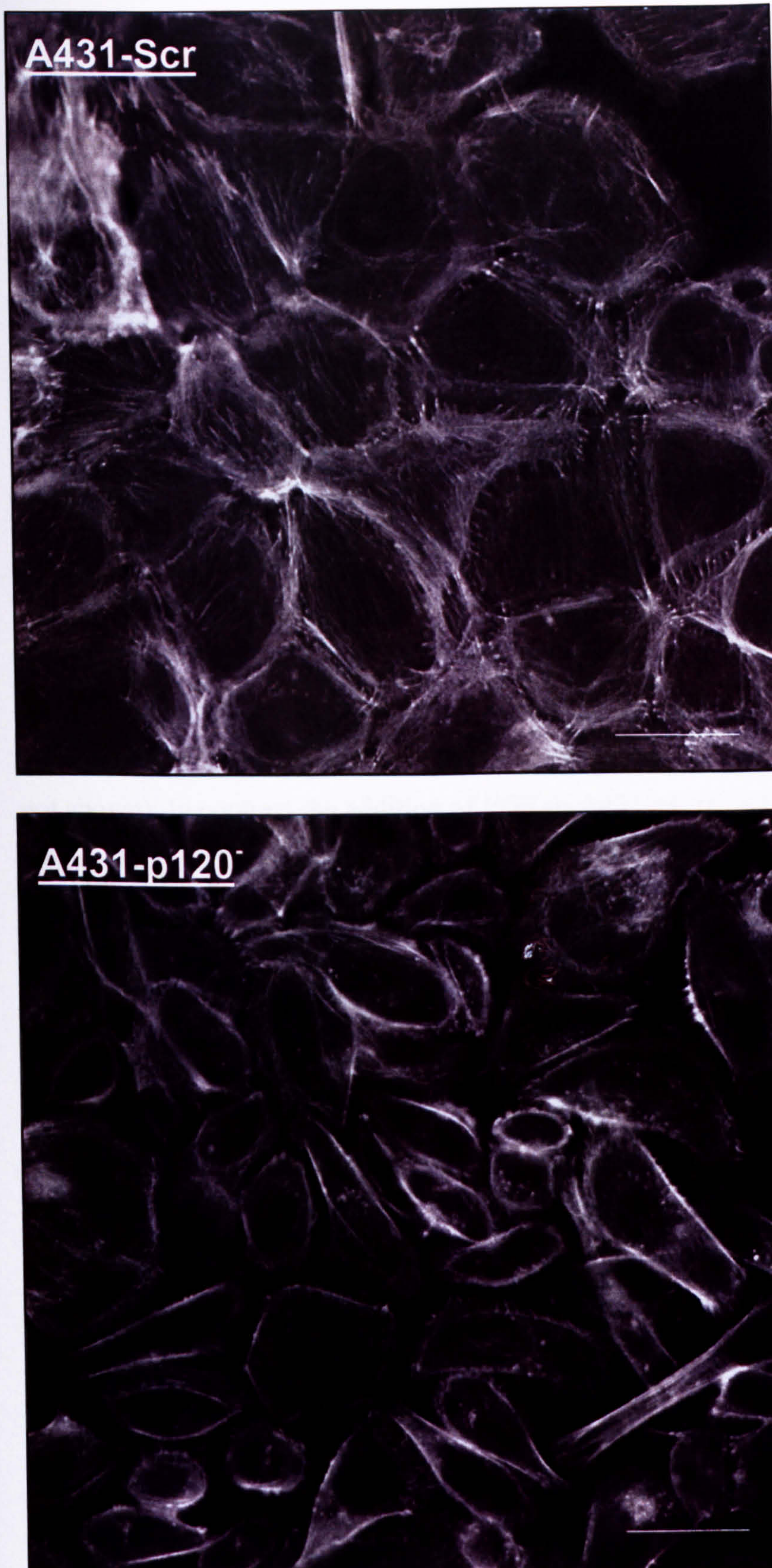


Figure 6-11 Depletion of p120 causes loss of cell-cell adhesion.

A431 cells expressing a scrambled control RNAi sequence (A431-Scr) or a human p120-specific RNAi sequence (A431-p120⁻) were grown to confluence then fixed and actin stained with phalloidin-TRITC. Bar, 20 μ m.

6.2.10 *The p120 regulatory domain is required for growth factor induced scattering.*

Unlike KM12C-Src527F cells, virtually all A431 cells formed cell-cell contacts after switch from low to high calcium containing media. This was not influenced by the expression of p120-4A or p120-3A/7F (data not shown). However, small colonies of A431 cells are known to undergo scattering in response to EGF (397). This process involves the disassembly of AJs followed by migration of individual cells away from the colony in a random fashion. As we had demonstrated phosphorylation of p120 at multiple tyrosine residues in EGF-stimulated A431 cells we wished to ask whether the phosphorylation state of p120 was important for EGF-induced scattering. The ability of EGF to induce scattering of colonies of A431-p120⁻ cells reconstituted with wild type p120-3A, p120-3A/7F or wild type p120-4A was assessed by time-lapse microscopy (Figure 6-12). The addition of EGF to A431-p120⁻ cells reconstituted with wild type p120-3A resulted in complete dissolution of virtually all colonies. This phenotype was identical to that of parental A431 cells (data not shown). In contrast the addition of EGF to A431-p120⁻ / p120-4A colonies caused some contraction and rounding of cells which is characteristic of the A431 cytoskeletal response to EGF but did not disrupt the morphological cohesion of the colony. A subtle but consistent difference was observed for EGF treated A431-p120⁻ / p120-3A/7F colonies which adopted an intermediate phenotype with a less compact appearance and with some scattering but in which complete disruption of morphological cohesion was rare.

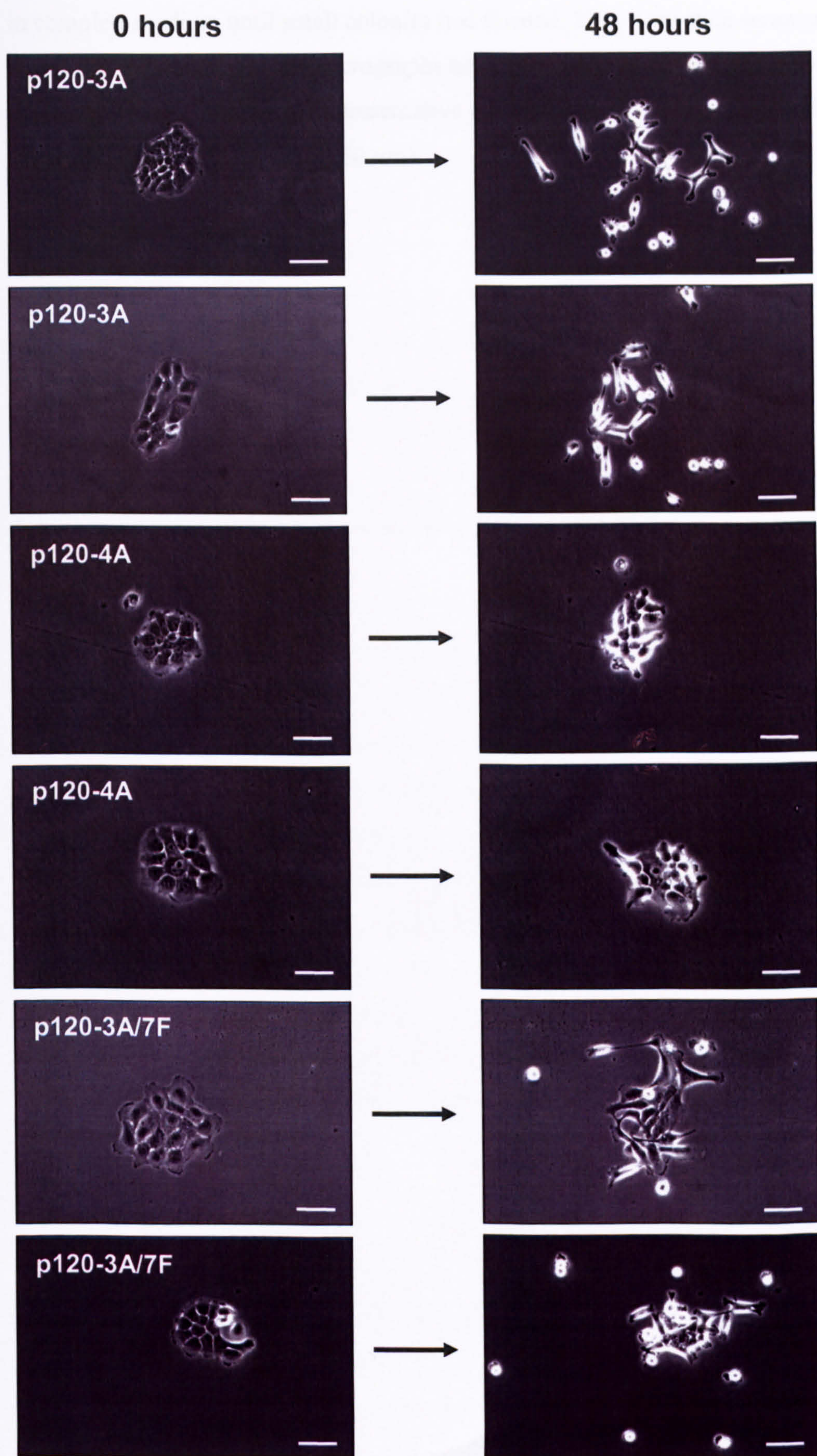


Figure 6-12 p120 isoform influences EGF induced cell scattering.

Figure 6-12 p120 isoform influences EGF induced cell scattering. A431-p120⁻ cells reconstituted with p120-3A, p120-3A/7F, or p120-4A were sparsely plated and maintained in complete medium until small colonies had formed. They were then serum starved overnight and phase contrast micrographs taken immediately before and 48 hours after the addition of EGF (10 ng/ml). Representative examples of two colonies per cell line are shown (x20 magnification; Bar 50 μ m).

6.3 Discussion

The consequences arising from phosphorylation of p120 on tyrosine are largely unknown. This is perhaps surprising given the progress made in understanding the role of tyrosine phosphorylation in other contemporaneously discovered Src substrates such as FAK (467) and cortactin (468) (reviewed in (236)). Alterations in cell-cell adhesion and motility often occur in association with increased Src or RTK activity and are properties which p120, a prominent substrate of such kinases, is in a pivotal position to influence. We set out to investigate the significance of the p120 phosphorylation events we had identified in KM12C and A431 cell lines, with particular reference to intercellular adhesion. Increased activity of Src or EGFR also correlates strongly with the acquisition of a more motile or invasive phenotype and this is addressed separately in chapter seven.

6.3.1 *Phosphorylation of p120 at nascent AJs.*

Src family kinases are enriched at AJs (194) and their regulated activity is necessary for AJ formation (138), perhaps via the activation of PI 3-K and Rac (179, 252). It is not then surprising that p120, as a prime SFK substrate, should undergo phosphorylation during AJ formation. Indeed, the tyrosine phosphorylation of p120 has previously been reported to be increased during AJ formation (138, 176) and, conversely, to be reduced in mature junctions (251). We extend these previous reports to demonstrate that phosphorylation of p120 at nascent AJs appears to occur at tyrosine-228 without significant phosphorylation at tyrosine residues 280, 291 or 296. Whether tyrosine-228 represents the sole site of phosphorylation or whether residues such as 112, 257 and 302 may similarly undergo phosphorylation remains undetermined. Interestingly, phosphorylation of p120 at tyrosine-228 does not appear to be a necessary event for AJ formation in A431 cells as no gross defect was seen in the ability of cells reconstituted with p120-4A to form cell-cell adhesions. This is in-keeping with similar observations published by the Reynolds group (176).

In contrast to the other work described in this chapter which utilised cells over-expressing exogenous Src or a cell line which over-expresses EGFR to high levels, the demonstration of p120 phosphorylation at nascent AJs provided proof-of-principle that p120, and specifically tyrosine-228, is a bona fide substrate of endogenous kinases in epithelial cells.

6.3.2 Phosphorylation of p120 by oncogenic kinases.

In contrast to the spatio-temporally regulated phosphorylation of p120 described above, the stable expression of active Src in the KM12C cell line was associated with constitutive hyper-phosphorylation of p120. Indeed p120 was more prominently phosphorylated than either E-cadherin or β -catenin, the two other major kinase substrates at the AJ. Thus, p120 was a prominent phospho-protein in KM12C-Src527F cells in which cell-cell junction formation is impaired. Furthermore, inhibition of Src kinase activity with the selective inhibitor AP23464 prevented p120 phosphorylation and this correlated with reversal of Src-dependent deregulation of the AJ. This prompted us to ask whether tyrosine phosphorylation of p120 was a necessary event for Src-dependent deregulation of AJs in KM12C cells. The hypothesis that tyrosine phosphorylation of p120 provides a means of exerting dynamic modulation over its key role in the regulation of cadherin trafficking is attractive but remains lacking in direct evidence. For example, Aono *et al* investigated the adhesive defect seen in the Colo 205 cell line which expresses normal levels of E-cadherin and catenins but which is unable to form AJs (272). Treatment with the kinase inhibitor staurosporine or the expression of a p120 construct lacking the regulatory domain (deletion of residues 1-324) was sufficient to promote cell-cell adhesion suggesting that constitutive signalling events impacting on the p120 N-terminal region were the cause of impaired adhesion. However, this approach did not distinguish tyrosine phosphorylation from other events occurring at the p120 N-terminus. Indeed, the major phosphorylated p120 residue in Colo 205 cells was, in fact, serine rather than tyrosine. Secondly, Ozawa *et al* demonstrated that the ability of Src to disrupt AJs in E-cadherin transfected L cells (a cadherin-deficient mouse fibroblast line) was abrogated by the co-expression of p120 lacking most of the N-terminal region (deletion of residues 29-233) (273). Again, this provided circumstantial evidence for p120 tyrosine phosphorylation but, importantly, did not exclude a role for other regulatory events involving the p120 regulatory domain or coiled-coil domain. Both of these studies predated the identification of the sites of p120 tyrosine phosphorylation, a development which now makes it possible to definitively test the specific role of p120 tyrosine phosphorylation via the use of p120 phosphorylation-defective mutants. Consequently we have used both a phosphorylation-defective p120 construct (p120-3A/7F) and p120-4A which lacks the entire N-terminal region containing all sites of tyrosine phosphorylation and most sites of serine/threonine phosphorylation.

We over-expressed phosphorylation-defective p120 in KM12C cells with the aim of achieving a dominant negative effect by competing out endogenous phosphorylation-

competent p120. Although this widely used approach is often instructive it is important to acknowledge its limitations. Firstly, there is no guarantee that a dominant negative effect can be achieved, particularly if only small amounts of phosphorylated protein are sufficient to exert a phenotype. Secondly, even if a dominant negative effect is achieved an excess of over-expressed protein may itself alter the cells phenotype by mechanisms entirely unrelated to phosphorylation. This is a particular concern for p120 where a series of mutually exclusive regulatory interactions with various binding partners occur which may be perturbed with dramatic effect in the presence of an excess of over-expressed p120. Specifically, over-expressed p120 may saturate cadherin binding sites then accumulate in the cytoplasm where it would be free to regulate Rho GTPase and Kaiso activity. Consequently, wild type p120 was also over-expressed as an essential control to differentiate effects caused by altered p120 phosphorylation status from effects simply caused by excess p120. Recently a more elegant and powerful methodology which is likely to supersede the traditional dominant negative approach has been described (176). In brief, this approach exploits non-homology between human and murine cDNA to generate a human-specific RNAi hairpin. Having achieved good knockdown of endogenous human protein, the cell line is then reconstituted with murine cDNA which is unaffected by the human specific RNAi hairpin. An alternative approach to evade RNAi-mediated destruction would be to reconstitute a cell line with cDNA of the same species but in which silent nucleotide substitutions had been introduced. Regardless, the reconstituted cell is forced to use the exogenous protein which can be expressed at levels identical to those of the parental cell. This facilitates a direct and more physiological comparison of protein function. This system was first described by Mariner *et al* in the context of their work on p120 and we have used it in A431 cells without modification other than to include a scrambled control sequence as an additional control (176).

Using a dominant negative approach we could not identify any requirement for p120 phosphorylation in mediating Src-dependent deregulation of cell-cell contacts in KM12C cells. This data is open to at least two interpretations. Firstly, we should acknowledge that the degree of dominant negative effect achieved may simply have been insufficient. Although we demonstrate a clear reduction in phosphorylation of endogenous p120 it is possible that the effect of p120 phosphorylation was in fact so potent that phosphorylation of even a minority of molecules was sufficient to impair AJ formation. In this regard it is unfortunate that the RNAi-mediated substitution system applied effectively to A431 cells was unsuccessful in KM12C-derived cell lines.

A second interpretation would regard phosphorylation of p120, at least for this specific cell-cell adhesive defect, as an epiphenomenon. Supporting this is the finding that mutation of the SH3 domain abrogated the ability of Src527F to deregulate the AJ without any impairment of its ability to phosphorylate p120. In other words, tyrosine phosphorylation of p120 was not sufficient for Src induced disruption of the AJ.

In contrast, tyrosine phosphorylation of p120 was involved in the scattering of EGF-stimulated A431 cells. Substitution of 7 prominent sites of tyrosine phosphorylation with phenylalanine partially abrogated EGF induced scattering. However, a greater inhibitory effect was seen in the presence of p120 isoform 4A. This suggested that additional events in the p120 N-terminal region, perhaps growth factor induced serine / threonine phosphorylation were required for the maximal effect of EGF. This data is in-keeping with that of Cozzolino *et al* who demonstrated an inhibitory effect of over-expressed p120-4A in the HGF induced scattering of MDCK cells (131). Finally, the observation that the motility of A431 cells was unaltered by the substitution of wild type with phosphorylation defective p120 (see section 7.2.3) suggested that the defect in scattering seen in A431 cells expressing phosphorylation defective p120 was due to increased stability of adhesions rather than reduced motility.

There are several mechanisms not requiring p120 phosphorylation by which Src may disrupt AJs in KM12C cells. Recent work has demonstrated that the ability of Src to inhibit junction formation in KM12C cells is at least partially explained by events originating not at the AJ but at cell-matrix adhesions (278). Specifically, elevated Src activity in KM12C cells was associated with localised activation of ERK, MLCK and ROCK at integrin-based cell-matrix adhesions and accumulation of phospho-myosin at these sites (278). Blockade of ERK, ROCK or MLCK activity inhibited accumulation of phospho-myosin and was sufficient to allow AJs to form. Thus, a major mechanism of Src's inhibitory effect on junction formation in KM12C cells may be via the generation of contractile forces which are unfavourable to junction formation.

As was the case in the present work, p120 is generally the most heavily phosphorylated protein at the AJ. Despite this, recent work suggests that phosphorylation of cadherins or β -catenin may be mechanistically more important. For example, phosphorylation of VE-cadherin at tyrosine-658 causes dissociation of p120 (89) and this appears to "unmask" a sequence motif for constitutive endolysosomal processing (75). Thus, the critical role of p120 in maintaining endothelial AJ stability is influenced by tyrosine phosphorylation, but of VE-cadherin rather than p120 itself.

The recent identification of Hakai, a novel E3 ubiquitin ligase, provides a further mechanism for tyrosine kinase mediated AJ disruption (11). Hakai specifically binds the juxtamembrane domain of phosphorylated E-cadherin, targeting it for ubiquitination and endocytosis with a resulting decrease in cell-cell adhesion. These residues are within the p120-binding cadherin JMD so, reminiscent of the situation for VE-cadherin, it has been suggested that p120 may act to limit access of Hakai and hence stabilise E-cadherin.

A potential confounding factor in the work undertaken in KM12C cells was the use of p120 isoform 1A. Although this isoform is expressed at low levels in KM12C cells the predominant isoform, in common with most epithelial cell lines is isoform 3. Isoform 1 is preferentially found in more motile cells types (59), associates preferentially with N-cadherin (381), and is upregulated in snail-driven EMT (172). However, the consequence of this p120 isoform switch, if any, for the AJ is unknown. In p120 deficient SW48 colon carcinoma cells both isoform 1 and 3 were equally able to stabilise cadherin and reconstitute the AJ (81). In addition expression of p120 isoform 1 in Caco-2 colorectal cancer cells which predominantly express lower molecular weight isoforms did not cause any alteration in morphology of cell-cell contacts or any defect in epithelial barrier function (469). Therefore we think it unlikely that we would have obtained different results had we used isoform 3 rather than isoform 1.

Finally, the requirement for p120 tyrosine phosphorylation in EGFR induced scattering of A431 cells but not in Src driven inhibition of AJ formation in KM12C cells likely reflects important differences in the biology of these two processes. Specifically these two models utilised different kinases (Src and EGFR), tested their effects on established (A431) versus nascent (KM12C) AJs and compared sustained steady state kinase activity (stable expression of Src527F) with the acute onset of kinase activity (EGF stimulation of serum starved A431 cells). It is interesting to speculate that kinase driven elevation in actomyosin contractility may be in itself sufficient to prevent the formation of new junctions as occurred in KM12C cells. Conversely in the presence of established AJs, such as in colonies of A431 cells, additional phosphorylation events involving p120 may be necessary for AJ disruption.

6.4 Summary

p120 underwent transient and low level phosphorylation of tyrosine-228 during AJ formation. This does not appear to be a necessary event for AJ formation and its significance is undetermined. In contrast, p120 was hyper-phosphorylated in KM12C cells

expressing Src527F or upon stimulation of A431 cells with EGF. The tyrosine phosphorylation of p120, along with other functions attributable to the N-terminal region were required to mediate EGF-dependent scattering of A431 cells. However, the deregulation of AJ formation in Src-expressing KM12C cells appeared to be due to mechanisms other than p120 phosphorylation.

7 Results: Role of p120 in the motility and invasion of Squamous Cell Carcinoma cells.

7.1 Aim

The ability of tumour cells to invade is a key step in the metastatic process and loss of E-cadherin-mediated cell-cell junctions has often been associated with increased invasive capacity and poor patient survival. p120 has emerged as a key player in regulating cadherin stability, actin re-modelling and cell motility and is a prominent substrate of oncogenic kinases whose activity often correlates with invasion and metastasis. It has been postulated that p120 may be able to either positively or negatively regulate invasion depending on the cellular context. However, the potential role of p120 in invasion has not yet been experimentally tested.

The A431 epidermal squamous cell carcinoma line over-expresses EGFR and invades a 3-dimensional extracellular matrix in response to an EGF gradient, providing a tractable reductionist system in which to investigate cancer cell invasion (397). In chapter five we demonstrated the phosphorylation of p120 at tyrosine residues 228, 280, 291 and 296 in A431 cells exposed to EGF. We therefore set out to define the role, if any, of p120 and its tyrosine phosphorylation in A431 cell motility and invasion.

7.2 Results

7.2.1 Src kinase activity is required for motility.

Inhibition of SFK activity is associated with the inhibition of cellular motility in 2-dimensional culture as well as with the inhibition of invasion, a process occurring in 3-dimensions (335, 384, 388-394). Here we describe the effect of the specific Src kinase inhibitor AP23464 (448) on A431 cell motility whilst the effect of AP23464 on invasion is described in section 7.2.7. Concentrations of AP23464 which blocked SFK activity and inhibited phosphorylation of p120 (described in section 5.2.6) reduced the median speed of A431 cells sparsely plated on tissue culture plastic from 6.33 $\mu\text{m/hr}$ (interquartile range: 4.1 - 11.4 $\mu\text{m/hr}$) to 0.9 $\mu\text{m/hr}$ (interquartile range: 0.4 – 1.6 $\mu\text{m/hr}$) As the data was not normally distributed we utilised the non-parametric Mann-Whitney U test to demonstrate this to be a statistically significant reduction ($p < 0.0001$) (Figure 7-1).

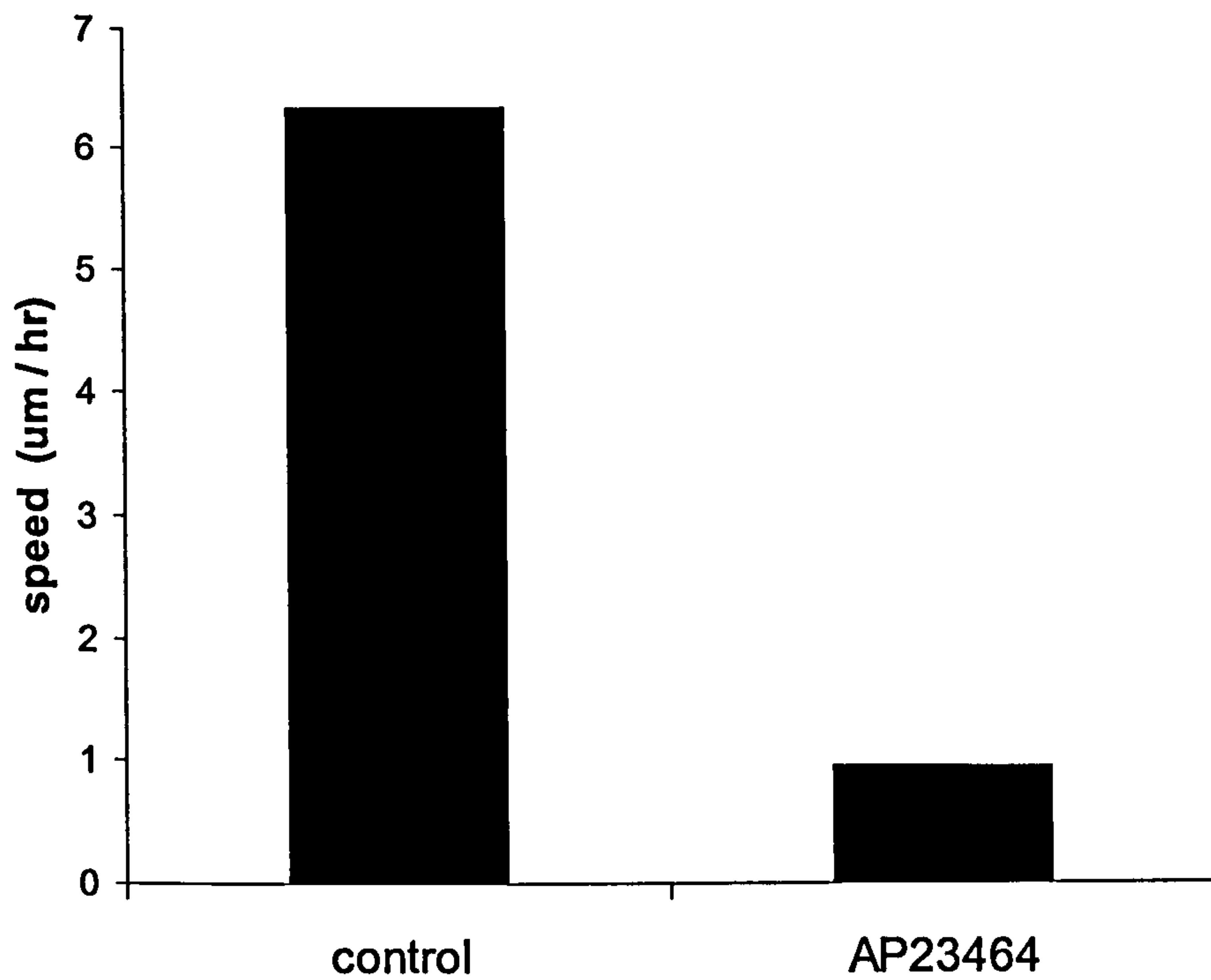


Figure 7-1 AP23464 inhibits motility and invasion of A431 cells.

The migration of sparsely plated A431 cells with or without the addition of 1 μ M AP23464 to the culture media was analysed by time-lapse video microscopy. For quantification of migration 50 individual cells were tracked for 16 hours. Migration is represented as median speed.

7.2.2 Depletion of p120 causes increased motility in 2-dimensions.

p120 has been reported to promote or inhibit the 2D motility of various epithelial cell lines in an isoform and cell context-specific manner (128, 130, 131). The depletion of all p120 isoforms in A431 cells caused an increase in 2-dimensional motility as measured by time lapse video-microscopy of sparsely cultured cells (Figure 7-2 A). The median speed was increased 3.5 fold from 8.0 $\mu\text{m} / \text{hr}$ (interquartile range: 3.5 - 14.4 $\mu\text{m} / \text{hr}$) in A431-Scr cells to 27.3 $\mu\text{m} / \text{hr}$ (interquartile range: 14.1 – 51.6 $\mu\text{m} / \text{hr}$) in A431-p120⁻ cells. The increased motility was confirmed in two clones (clone 3 and 7). This increase was statistically significant ($p < 0.0001$, Mann-Whitney U test).

Next we determined the motility of cells plated on Matrigel-coated tissue culture plastic. The speed (Figure 7-2 B) of both A431-p120⁻ cells and of control cells was increased in comparison to identical cells plated on uncoated tissue culture plastic. The increased motility of p120 depleted cells (77.8 $\mu\text{m} / \text{hr}$, interquartile range: 62.4 – 95.2 $\mu\text{m} / \text{hr}$) compared to control cells (61.2 $\mu\text{m} / \text{hr}$, interquartile range: 52.3 – 76.3 $\mu\text{m} / \text{hr}$) was less apparent than for cells plated on plastic but remained significant ($p < 0.001$, Mann-Whitney U test). The increased motility in p120 depleted cells was reversed by reconstitution with wild type p120-3A indicating that the phenotype observed was due specifically to loss of p120 and not a clonal or “off-target” effect. For qualitative purposes the tracks of 10 individual cells are shown (Figure 7-2 C)

7.2.3 The ability of p120 to be phosphorylated does not influence motility.

As concentrations of AP23464 which inhibited phosphorylation of p120 were sufficient to block motility we wished to determine whether this correlation might indicate any causal relationship. To answer this question we measured the motility of individual A431-p120⁻ cells reconstituted with wild type or phosphorylation-defective p120-3A or with p120-4A. There was no significant difference in median motility whether p120-3A (59.7 $\mu\text{m} / \text{hr}$, interquartile range: 44.5 – 72.6 $\mu\text{m} / \text{hr}$), p120-3A / 7F (60.6 $\mu\text{m} / \text{hr}$, interquartile range: 48.0 – 72.5 $\mu\text{m} / \text{hr}$), or p120-4A (64.7 $\mu\text{m} / \text{hr}$, interquartile range: 51.1 – 77.3 $\mu\text{m} / \text{hr}$) were expressed (Figure 7-3). Thus we concluded that the ability of p120 to undergo tyrosine phosphorylation did not influence the motility of A431 cells.

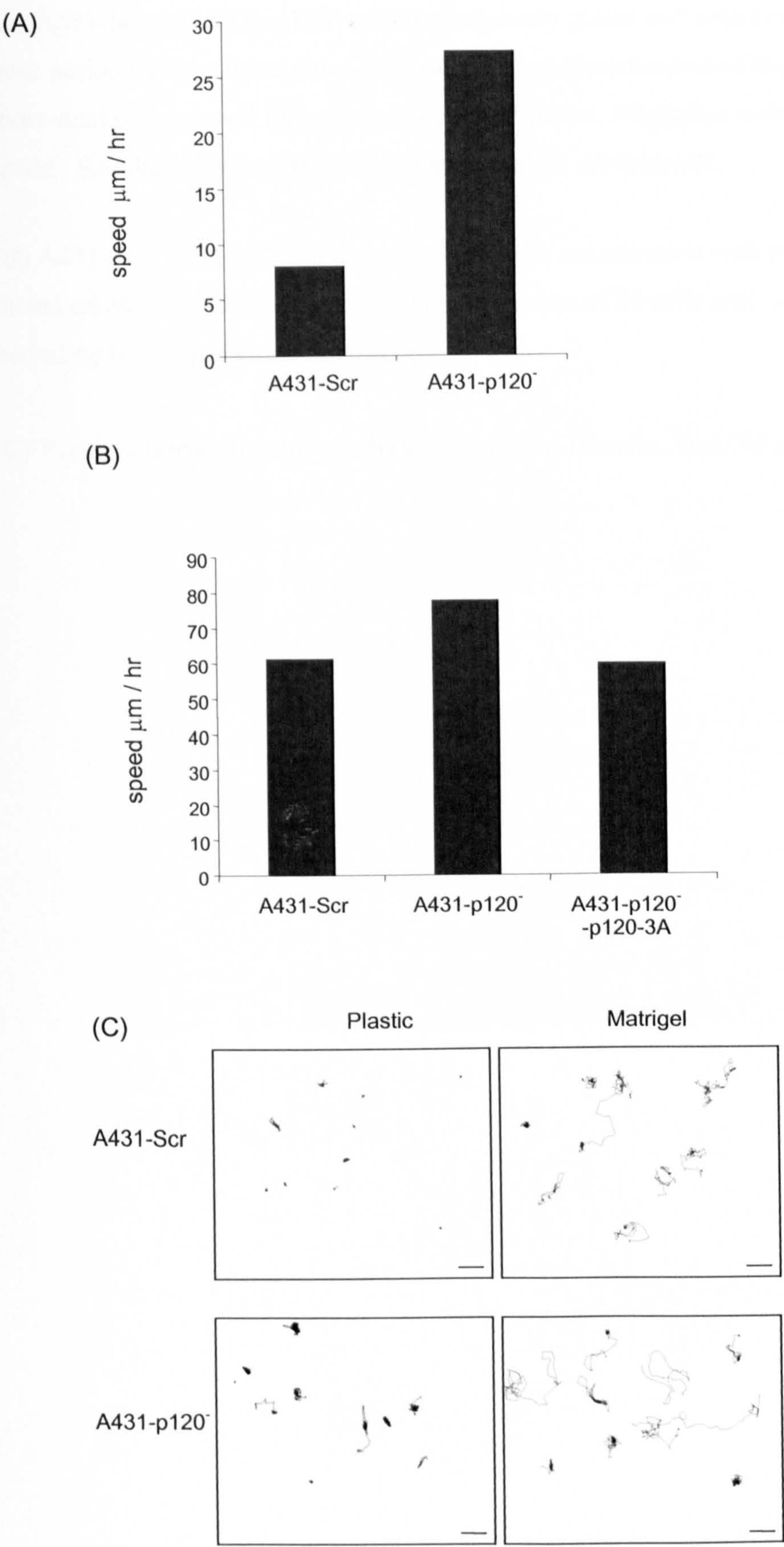


Figure 7-2 Depletion of p120 increases motility of A431 cells.

Figure 7-2 Depletion of p120 increases motility of A431 cells.

(A) A431-Scr and A431-p120⁻ cells were sparsely plated and migration analysed over a 5 hour period by time-lapse video microscopy. For quantification of migration 100 individual cells per cell line were tracked for 5 hours. Migration is represented as median speed. Results are representative of 3 independent experiments.

(B) A431-Scr, A431-p120⁻ and A431-p120⁻ cells reconstituted with p120-3A were sparsely plated on Matrigel-coated plastic and the migration of 50 cells analysed over a 5 hour period by time-lapse video microscopy

(C) Representative migratory paths are shown for 10 cells. Bar 100 μ m.

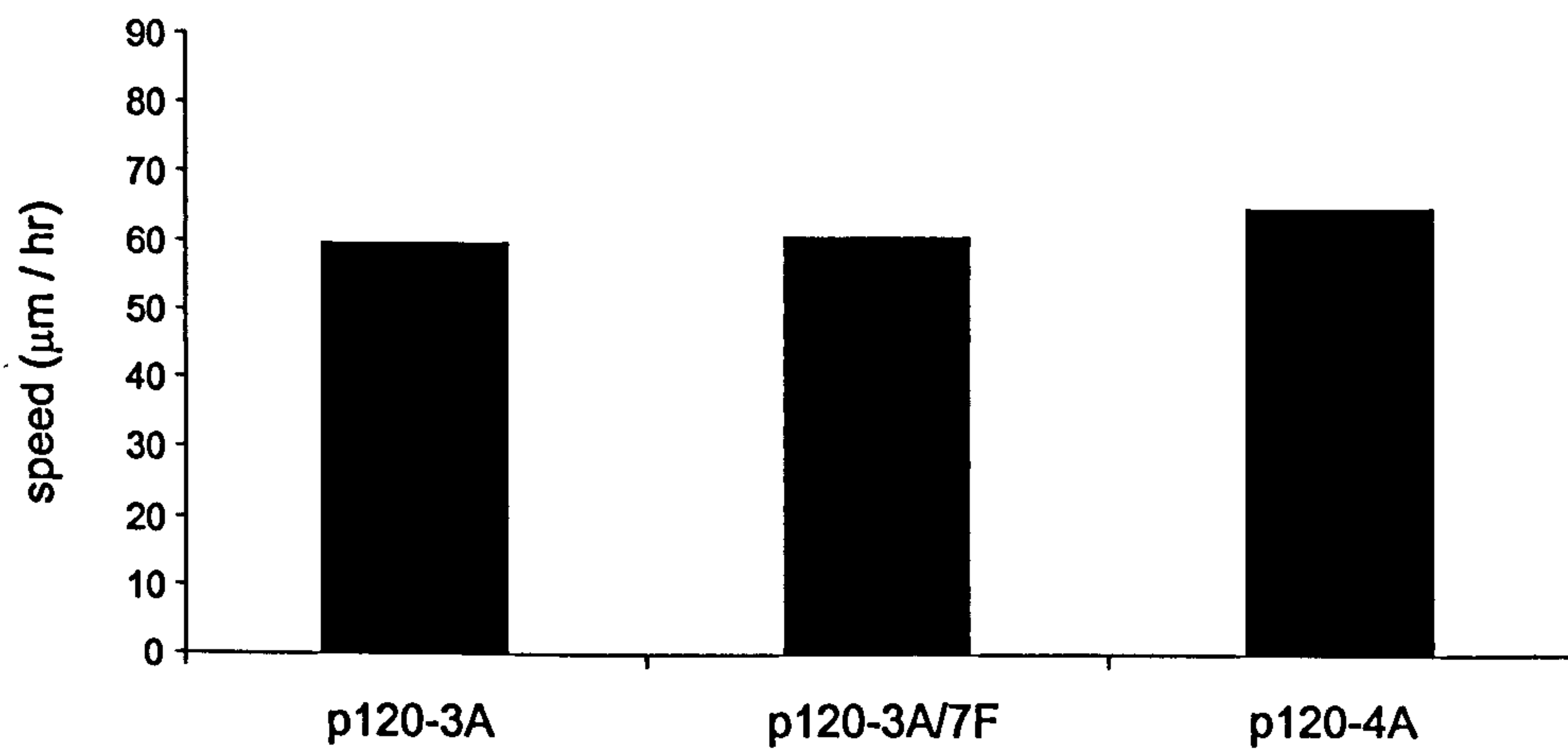


Figure 7-3 The ability of p120 to undergo tyrosine phosphorylation does not influence motility.

A431-p120⁻ cells reconstituted with p120 isoform 3A, 3A/7F or 4A were sparsely plated on Matrigel-coated plastic and migration analysed over a 5 hour period by time-lapse video microscopy. Results represent the median speed of migration of 50 individual cells per cell line and are representative of two independent experiments.

7.2.4 Wound closure by p120-depleted cells is disorganised.

A431 cells migrated into a “wound” created in a confluent monolayer by scratching with a pipette tip. Migration occurred in a collective manner as a confluent sheet of cells with a uniform vector of movement. This was associated with the formation of lamellipodia at the leading edge and with the retention of cell-cell morphological cohesion (Figure 7-4 A, left panel and movie 1). In contrast single A431-p120⁻ cells could clearly be seen to migrate independently into the denuded area. Unlike parental cells, A431-p120⁻ cells appeared to fill the defect by the random movement of single cells. (Figure 7-4A, right panel and movie 2). Staining for actin confirmed that A431-Scr cells retained cell-cell junctions whilst A431-p120⁻ cells adopted an elongated morphology with no apparent cell-cell contacts. However, despite the different mode of migration into the wound the actual speed with which wound closure occurred was not significantly different between the A431-Scr (20.57 +/- 2.26 μm / hour) and A431-p120⁻ cells (18.14 +/- 2.98 μm / hour)(Figure 7-4 B,C).

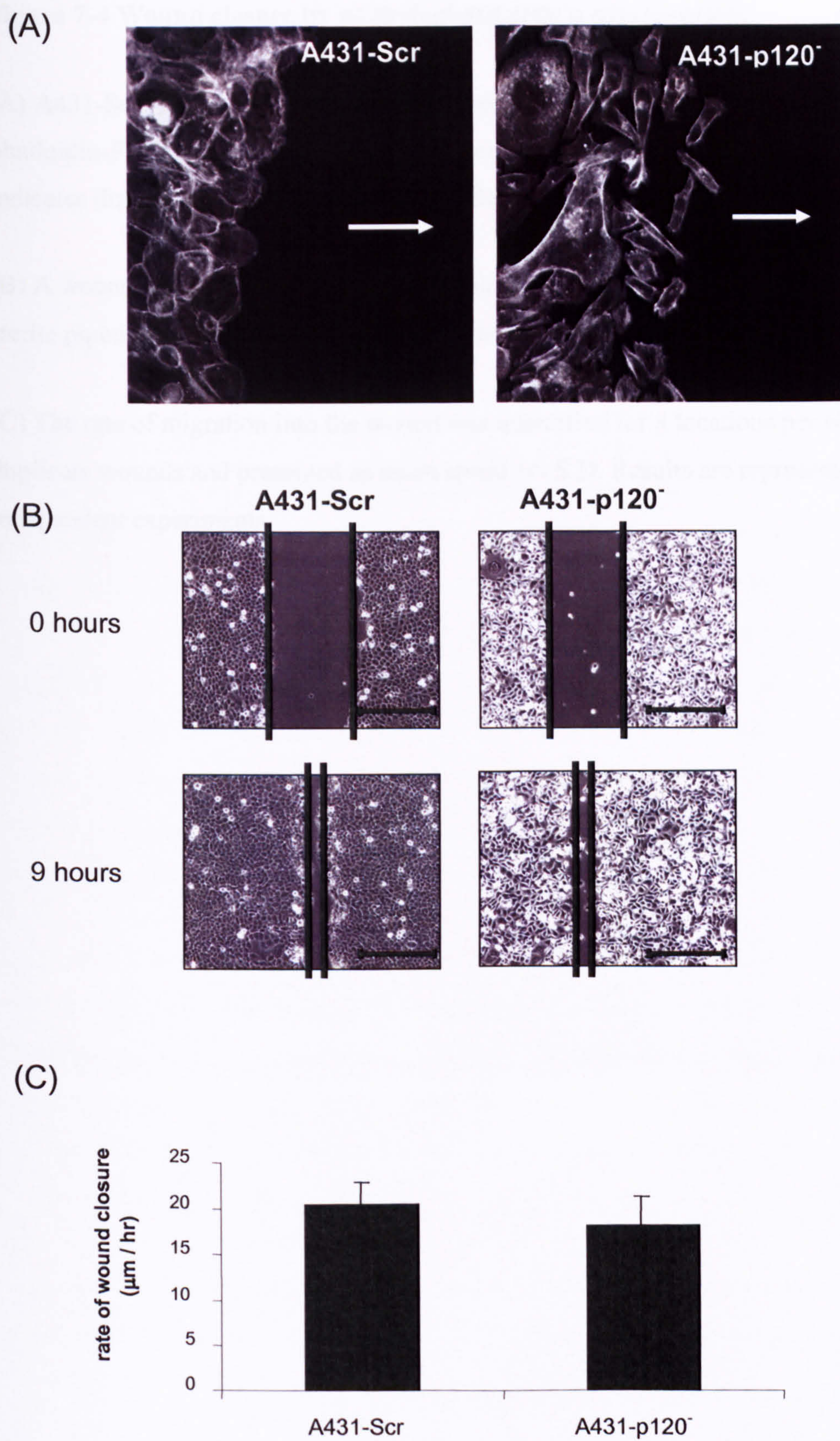


Figure 7-4 Wound closure by p120-depleted cells is disorganised.

Figure 7-4 Wound closure by p120-depleted cells is disorganised.

(A) A431-Scr (left panel) or A431-p120⁻ (right panel) cells were fixed and stained with phalloidin-FITC 6 hours after a wound was made in a confluent monolayer. Arrow indicates direction of migration (x40 magnification).

(B) A wound was made in a confluent monolayer of A431-Scr or A431-p120⁻ cells using a sterile pipette tip and images recorded at 0 and 9 hours. Bar, 400 μm .

(C) The rate of migration into the wound was quantified for 5 locations per wound on duplicate wounds and presented as mean speed \pm S.D. Results are representative of three independent experiments.

7.2.5 Depletion of p120 does not influence proliferation.

The depletion of p120 was previously reported to cause a significant reduction in growth in a pancreatic cancer cell line (470). However, no difference in proliferation was seen between A431-Scr and A431-p120⁻ cells over 6 days (Figure 7-5).

7.2.6 Loss of p120 and the AJ does not impair major EGFR signalling pathways.

Depletion of p120 was not associated with any alteration in the protein levels of EGFR or of other kinases involved in motility and invasion including Src and FAK (Figure 6-9). However as bidirectional cross talk exists between the AJ and the EGFR (471) we investigated the effects of depleting p120, and hence the AJ, on signalling to MAPK and STAT3 which represent two of the major downstream pathways of EGFR activation. As well as providing a readout of EGFR activity both the MAPK (472) and STAT3 (473) pathways are themselves implicated in motility and invasion. Importantly, the EGF-dependent phosphorylation of MAPK and STAT3 was seen in both p120 knock-down and control cells (Figure 7-6). This suggested that EGFR signalling to at least two of its major downstream effector pathways was not perturbed by depletion of p120.

We then turned to Rho-family GTPases which have previously been shown to be activated in EGF stimulated A431 cells (397), are implicated in motility and invasion (474), and which are known to be regulated by p120 (475). The activation of RhoA, Rac and Cdc42 in response to EGF stimulation was not reduced in A431-p120⁻ cells when compared to control cells (Figure 7-7). A reduction in basal levels of active RhoA and Cdc42, but not Rac was noted in p120-depleted cells.

Taken together these results suggested that depletion of p120 did not introduce defects in EGFR signalling to Rho GTPases or to the MAPK or STAT3 pathways, at least in a 2-dimensional context.

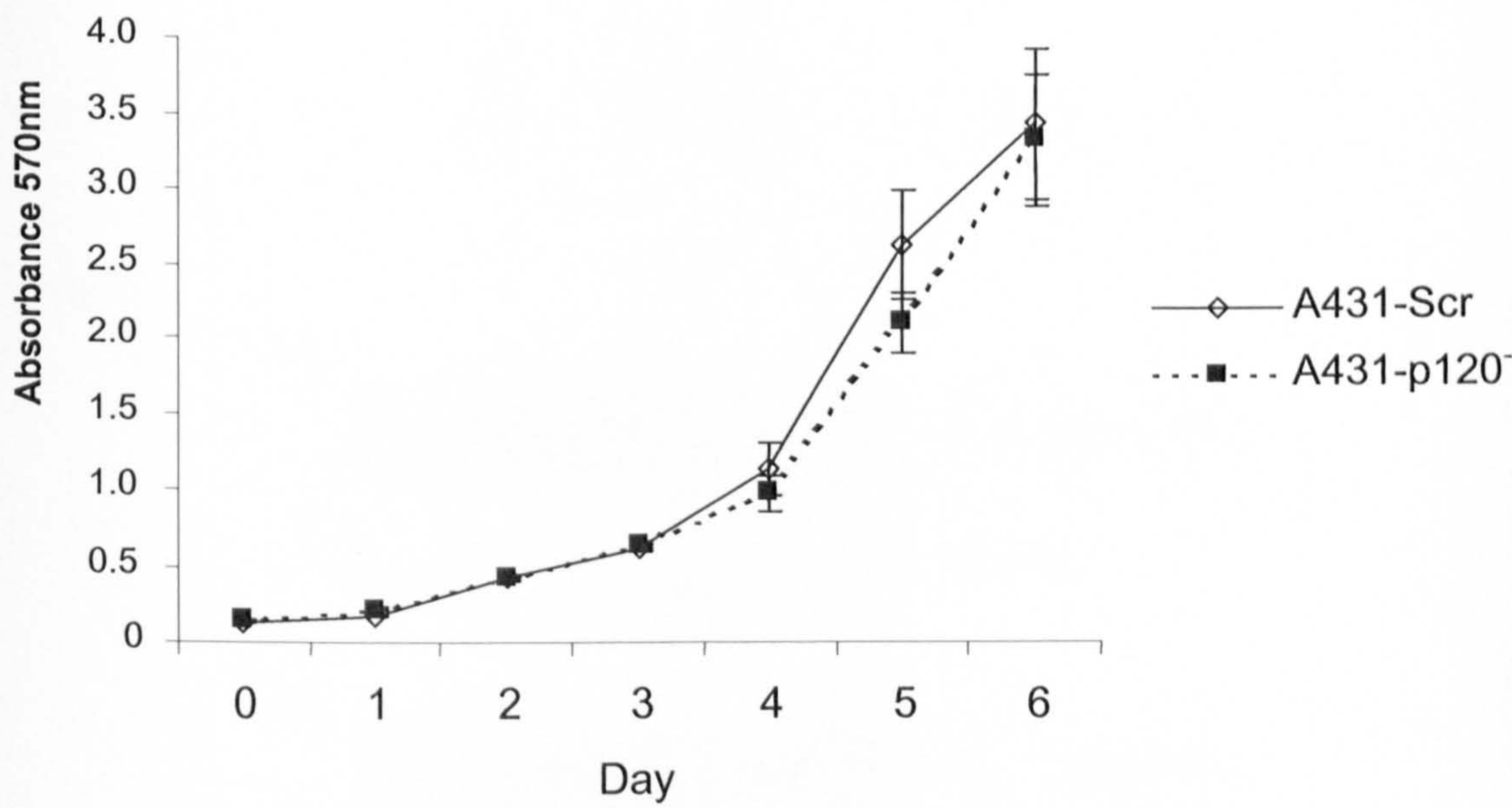


Figure 7-5 Depletion of p120 does not influence cellular proliferation.

A431-Scr or A431-p120⁻ cells were plated at a density of 1×10^3 cells / well in 96 well plates. At 24 hour intervals the number of viable cells was determined using a colorimetric MTT assay where absorbance at 570 nm is directly proportional to cell number. Results are plotted as mean absorbance +/- S.D. for quadruplicate wells.

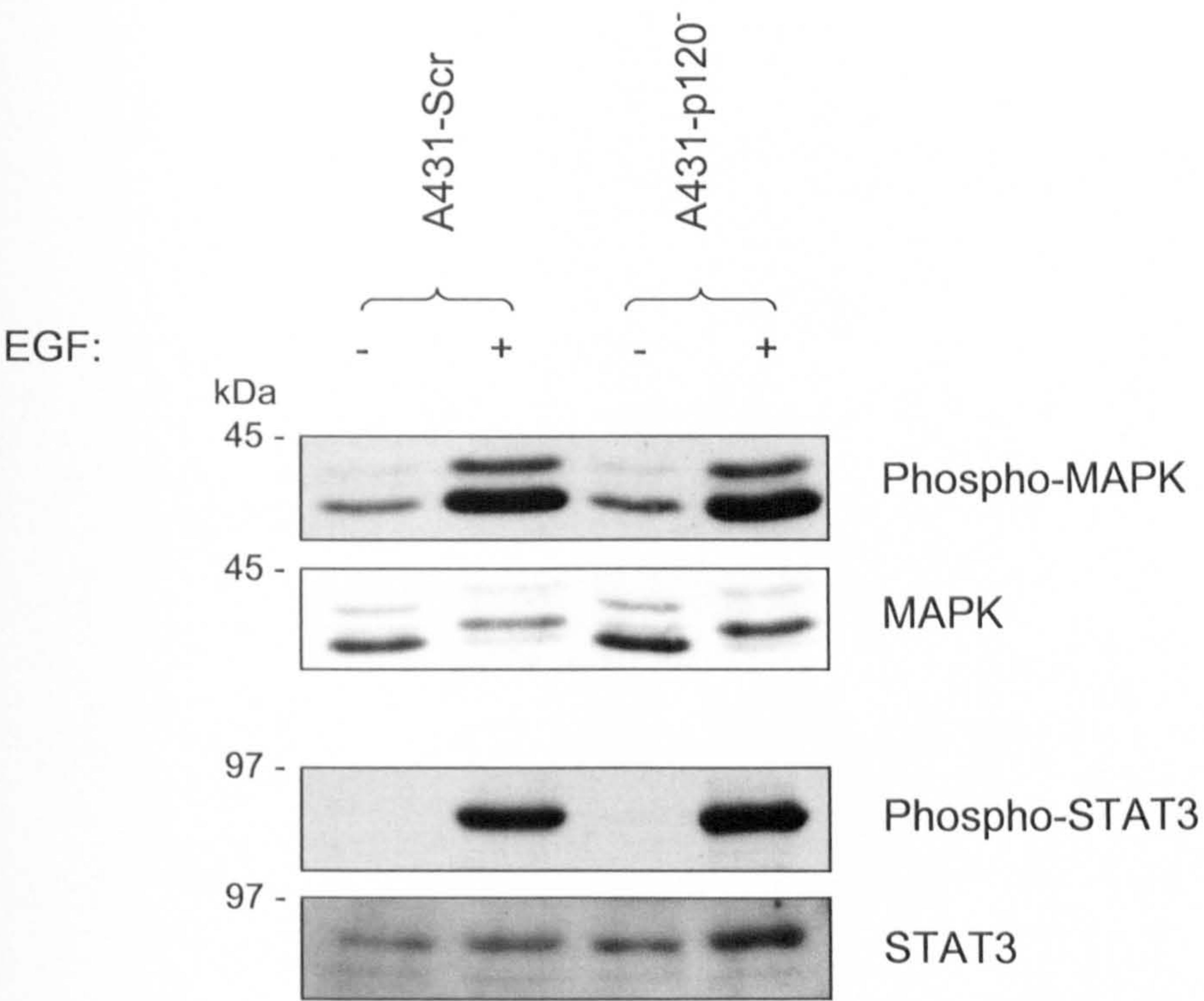


Figure 7-6 p120 is not required for major EGFR signalling pathways.

A431-Scr and A431-p120⁻ cells were serum starved overnight then stimulated with EGF (100 ng/ml) for 5 minutes prior to lysis. Western blot analysis was carried out with phospho-MAPK and phospho-STAT3 antibodies. In each case after removal of the phospho-specific antibodies the blots were reprobed with the corresponding non-phospho-specific antibodies.

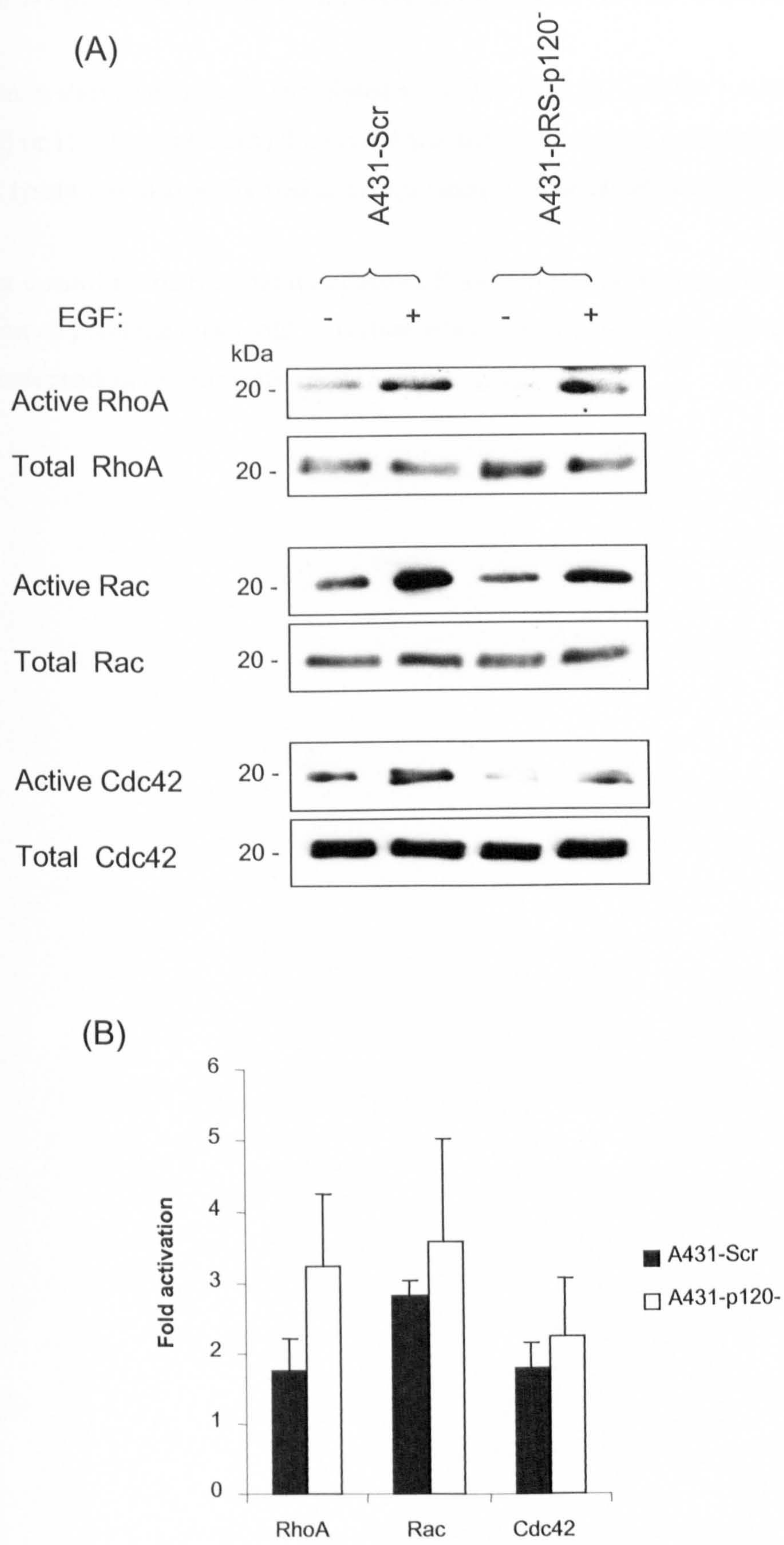


Figure 7-7 p120 is not required for EGF induced Rho GTPase activation.

Figure 7-7 p120 is not required for EGF induced Rho GTPase activation.

(A) Serum starved cells were stimulated with EGF (100 ng / ml) for 5 minutes (Rac1 / Cdc42) or 15 minutes (RhoA) then lysed and pull-down assays performed. Immunoblotting of cell lysate was also performed to ensure equivalent levels of protein were present.

(B) For quantification the amount of active Rho-family GTPase was determined by band densitometry and the mean fold activation relative to serum starved cells displayed for three independent experiments +/- S.D.

7.2.7 AP23464 inhibits invasion into Matrigel.

We next utilised a quantitative in vitro invasion assay to assess the ability of A431 cells to invade into Matrigel in response to EGF. Matrigel is a commercially available preparation consisting primarily of collagen IV and laminins purified from the extracellular matrix produced in vivo by a mouse sarcoma cell line (476). The Matrigel invasion assay is hypothesised to recapitulate three critical steps of invasion: adhesion, proteolytic dissolution of the matrix and migration (477). Invasion only occurred in the presence of an EGF gradient and was maximal for 10 ng/ml (EGF concentrations from 1-100 ng/ml were tested, data not shown). The maximum depth of invasion into Matrigel was typically 60-75 μm . Cells which invaded into the Matrigel appeared to do so collectively, forming multicellular clusters rather than invading as isolated cells. The addition of AP23464 to culture media was sufficient to reduce invasion by 93% in comparison to untreated cells (Fig 7-8 A, B). Thus Src was a necessary effector of EGF-induced invasiveness of A431 cells.

7.2.8 p120 is required for invasion into Matrigel.

As we had demonstrated that depletion of p120 in A431 cells resulted in loss of cell-cell contacts and increased 2-dimensional motility we hypothesised that A431-p120⁻ cells would have a greater capacity for invasion than parental cells. Surprisingly, knock-down of p120 protein resulted in a marked reduction in invasion into Matrigel over 72 hours in comparison to the A431-Scr cells (Figure 7-9 A, B). Quantification showed a 76% reduction in invasion in A431-p120⁻ clone 3 and 71% reduction in A431-p120⁻ clone 7 cells (Figure 7-9). Importantly, the ability of reconstituted p120-3A to reverse the invasion defect confirmed that this phenotype was specifically due to p120 depletion.

7.2.9 The requirement for p120 in EGF-stimulated invasion is phosphorylation-independent.

In chapter five we demonstrated the phosphorylation of p120 at tyrosine residues 228, 280, 291 and 296 in EGF-stimulated A431 cells. As invasion only occurred in the presence of EGF and could be inhibited by concentrations of AP23464 which also blocked the EGF induced phosphorylation of p120, we hypothesised that the requirement of p120 for invasion would specifically depend on its ability to be phosphorylated. However, reconstitution with p120 isoform 4A, which lacks all sites of tyrosine phosphorylation, restored the invasion defect in the A431-p120⁻ cells to a level seen in the control cells

(Figure 7-9 A). Thus, tyrosine phosphorylation of p120 (or other events occurring in the regulatory domain such as serine/threonine phosphorylation) were not required for invasion in response to EGF.

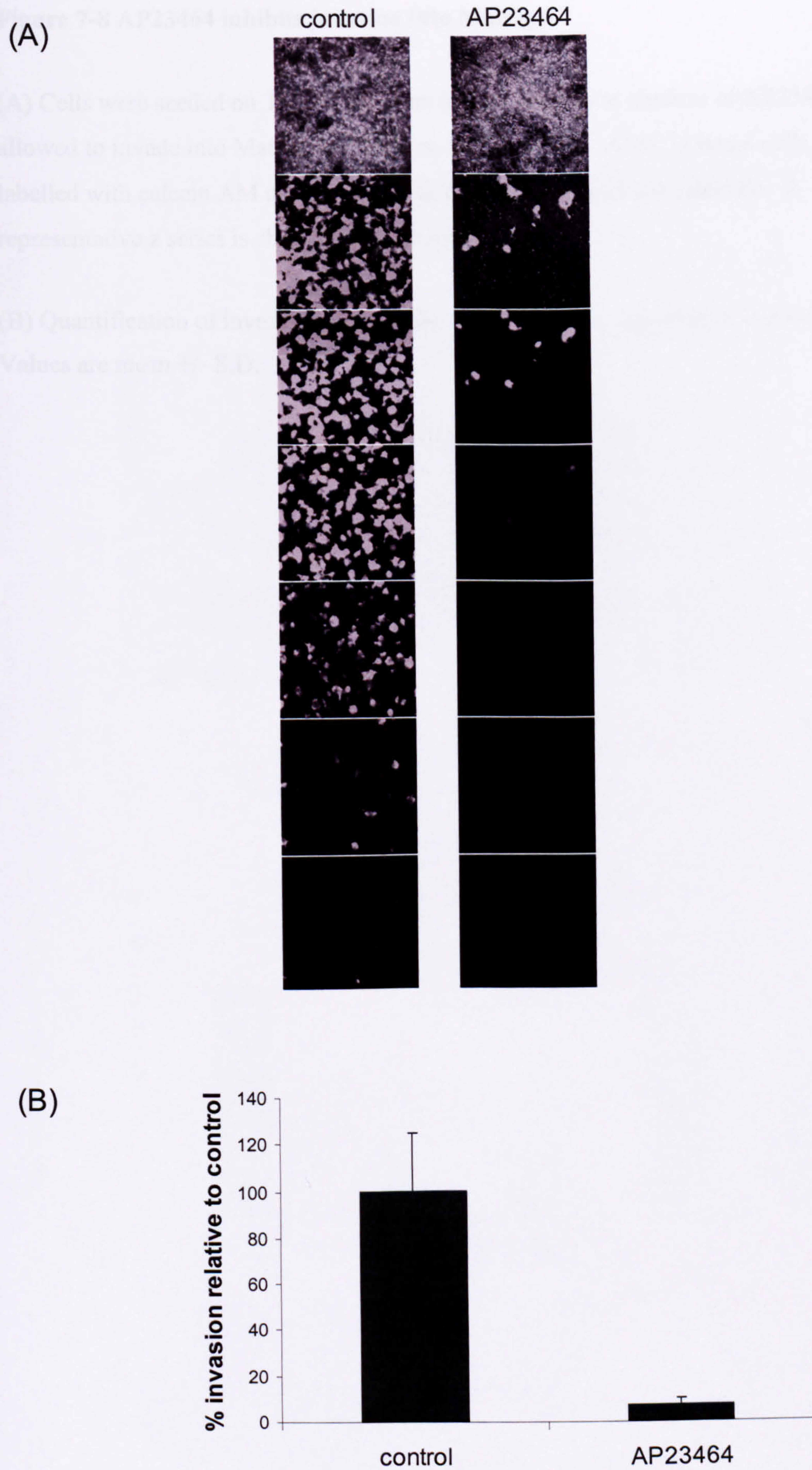


Figure 7-8 AP23464 inhibits invasion into Matrigel

Figure 7-8 AP23464 inhibits invasion into Matrigel

(A) Cells were seeded on Transwell filters in the presence or absence of AP23464 and allowed to invade into Matrigel towards an EGF gradient. After 72 hours cells were labelled with calcein AM and visualised in the Matrigel at 15 μm intervals. A representative z series is shown for qualitative purposes.

(B) Quantification of invasion is shown for a representative experiment in a series of two. Values are mean \pm S.D.

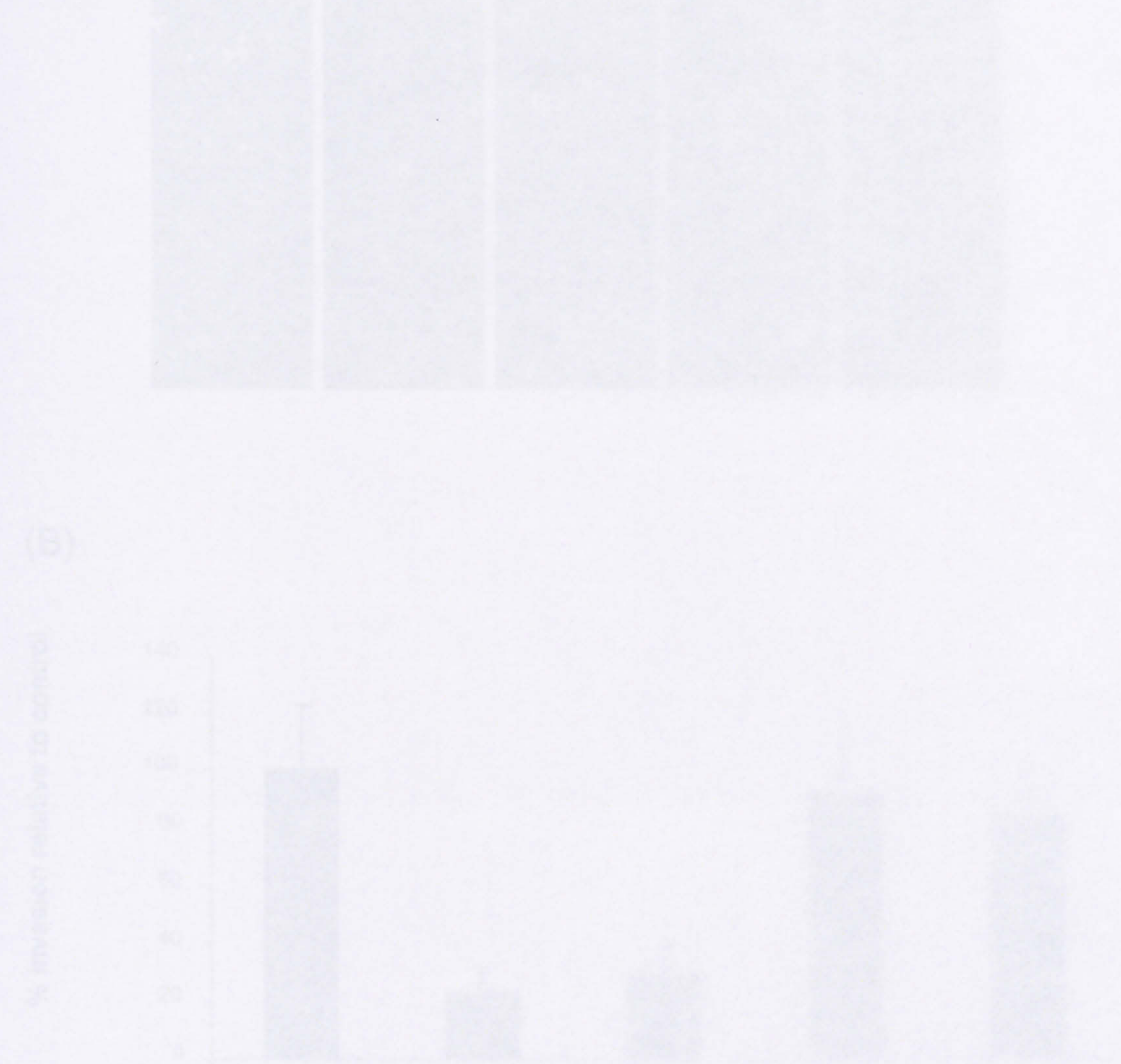


Figure 7-8 p120 is required for invasion in a transwell assay. Cells were seeded on Transwell filters in the presence or absence of AP23464 and allowed to invade into Matrigel towards an EGF gradient. After 72 hours cells were labelled with calcein AM and visualised in the Matrigel at 15 μm intervals. A representative z series is shown for qualitative purposes.

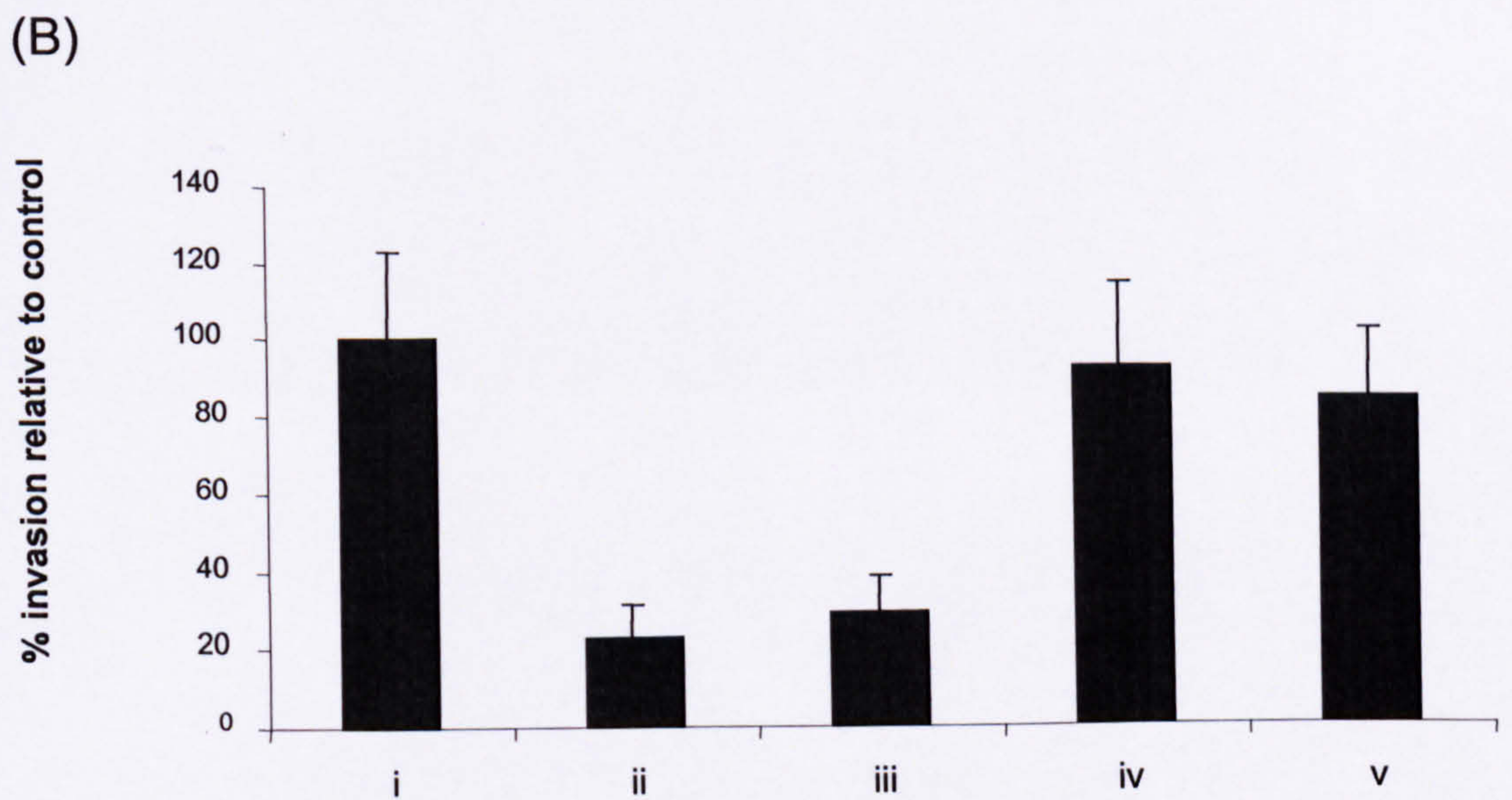
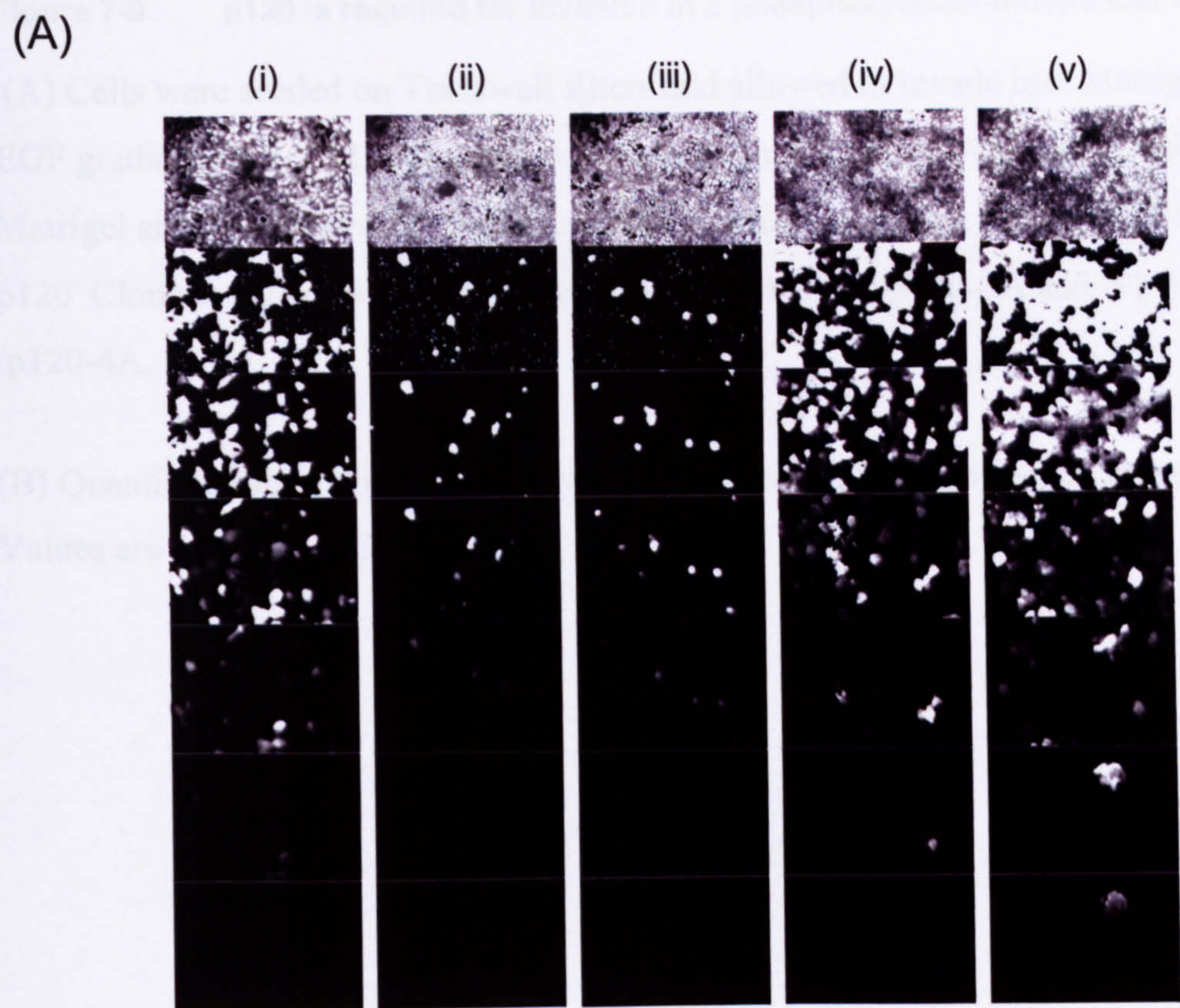


Figure 7-9 p120 is required for invasion in a phosphorylation-independent manner.

Figure 7-9 p120 is required for invasion in a phosphorylation-independent manner.

(A) Cells were seeded on Transwell filters and allowed to invade into Matrigel towards an EGF gradient. After 72 hours cells were labelled with calcein AM and visualised in the Matrigel at 15 μ m intervals. Representative z-series are shown for (i) A431-Scr, (ii) A431-p120⁻ Clone 3, (iii) A431-p120⁻ Clone 7, (iv) A431-p120⁻/p120-3A and (v) A431-p120⁻/p120-4A.

(B) Quantification of invasion is shown for a representative experiment in a series of four. Values are mean \pm S.D.

7.2.10 *p120 is required for invasion in an organotypic assay.*

The reduced ability of A431-p120⁻ cells to undergo invasion was further characterised using an organotypic invasion assay. This 3D environment more closely resembles the in vivo situation and measures invasion of cells exposed to air into a collagen I / Matrigel matrix containing tumour derived fibroblasts (478, 479). Depletion of p120 caused a 71% reduction in invasion in comparison to control cells expressing a scrambled p120 sequence (Figure 7-10). The fibroblast-derived factors which may stimulate invasion in the organotypic assay are poorly defined but may include EGF (480), KGF (481), GM-CSF (481), IGF (482) and TGF- β (480). These fibroblast-derived growth factors were necessary to stimulate invasiveness of A431 cells as there was almost no invasion of control or p120 knock-down cells in the absence of fibroblasts (Figure 7-10).

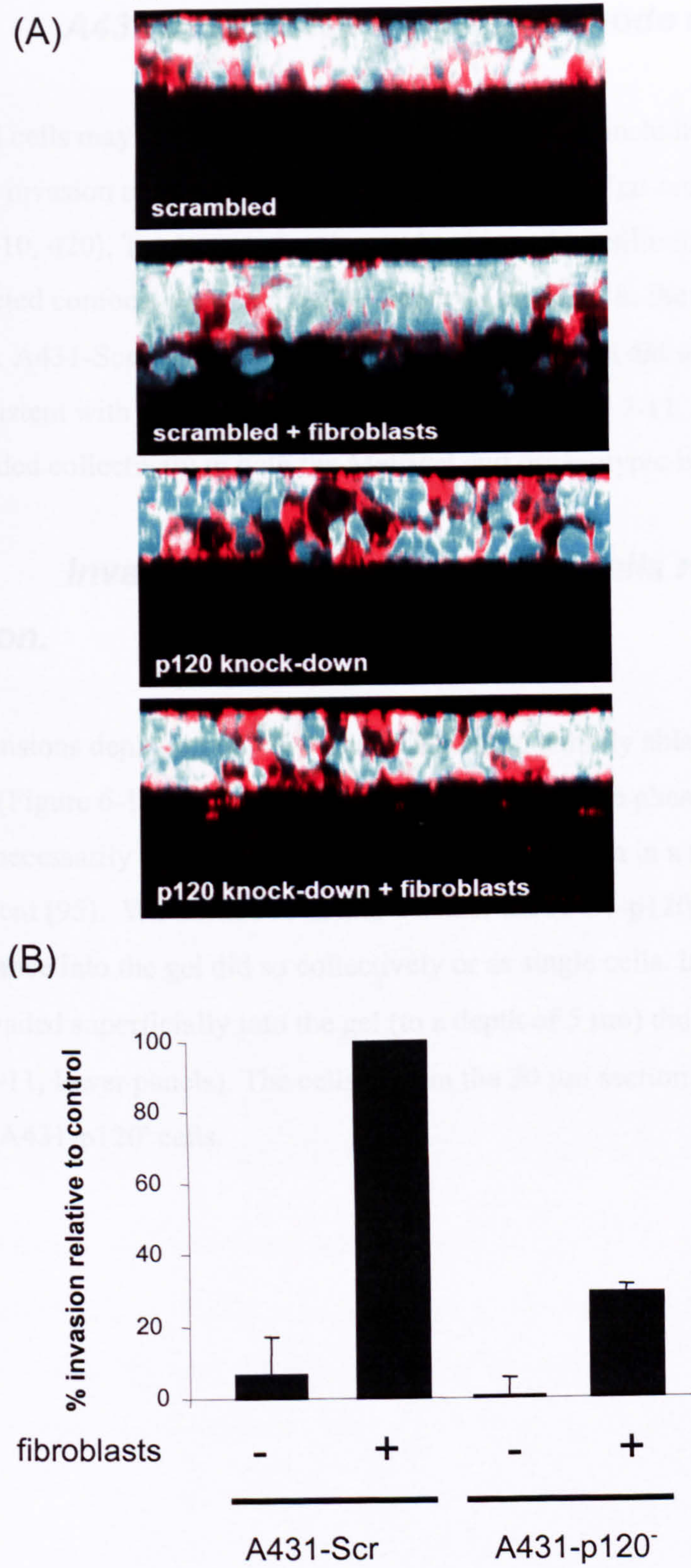


Figure 7-10 p120 is required for organotypic invasion.

CFP-labelled cells (blue) were seeded on collagen / Matrigel gels in the presence or absence of fibroblasts and after 5 days the gels were fixed and labelled with phalloidin-TRITC (red). Quantification of invasion relative to A431-Scr control cells from three separate experiments is shown where values are mean +/- S.D.

7.2.11 *A431 cells utilise a collective mode of invasion.*

Malignant cells may adopt diverse strategies for invasion including mesenchymal or amoeboid invasion of single cells or collective invasion of co-ordinated columns or sheets of cells (410, 420). To characterise the mode of invasion utilised by A431 cells reconstructed confocal images of A431-Scr cells invading in the organotypic assay were generated. A431-Scr cells invading 5 or 30 μm into the gel did so as cohesive groups of cells consistent with a collective mode of invasion (Figure 7-11, upper panels). Thus A431 cells invaded collectively in both the Matrigel and organotypic invasion assays.

7.2.12 *Invading p120-depleted A431 cells retain cell-cell cohesion.*

In 2-dimensions depletion of p120 was sufficient to entirely ablate morphological cell-cell cohesion (Figure 6-10). However the intercellular adhesive phenotype in two dimensions does not necessarily predict the nature of cell-cell adhesion in a three dimensional environment (95). We wished to define whether the A431-p120⁻ cells which invaded a short distance into the gel did so collectively or as single cells. In fact, A431-p120⁻ cells which invaded superficially into the gel (to a depth of 5 μm) did so as collective strands (Figure 7-11, lower panels). The cells seen in the 30 μm section are fibroblasts rather than invading A431-p120⁻ cells.

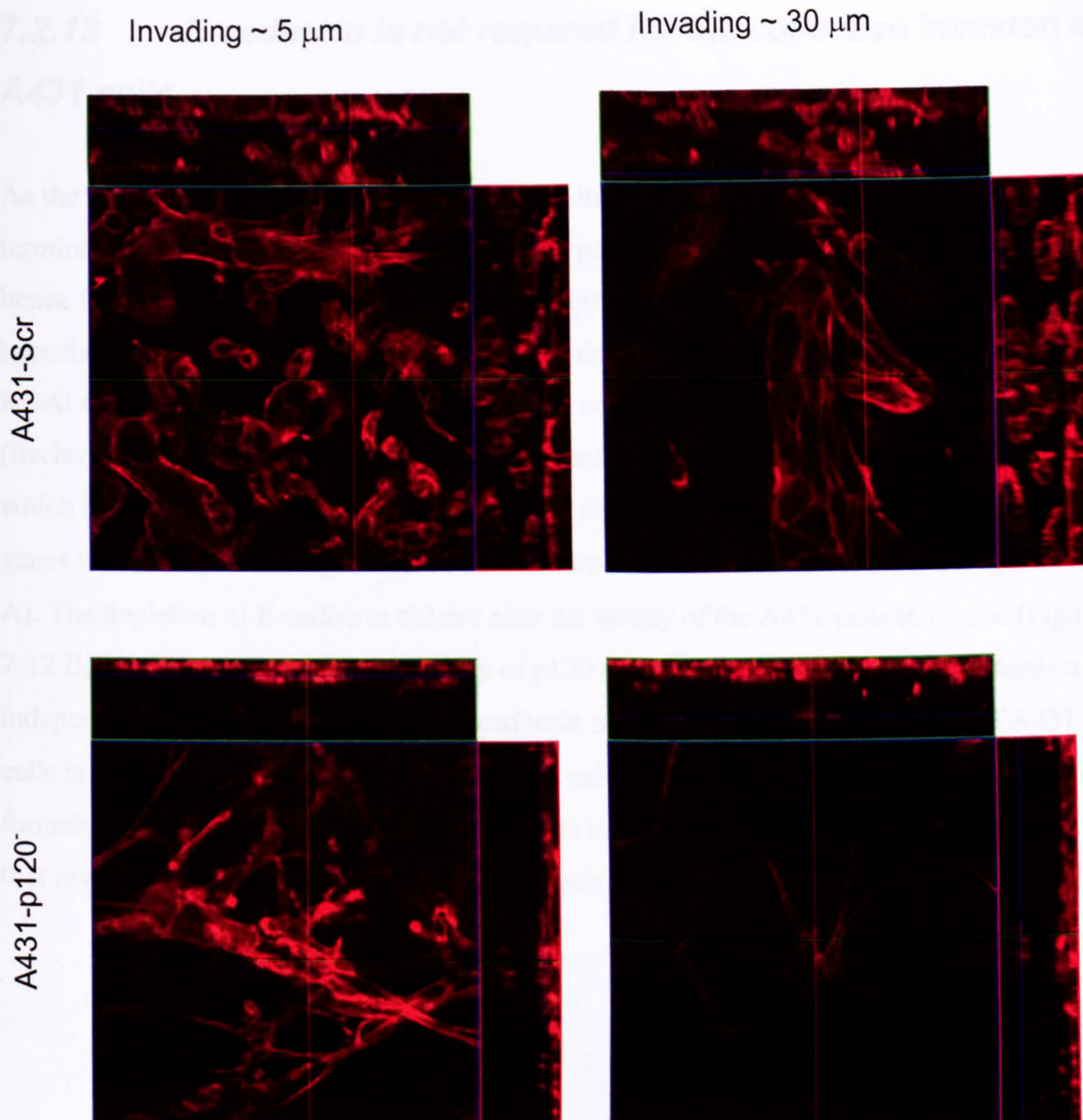


Figure 7-11 A431 cells invade in a collective manner.

A431-Scr or A431-p120⁻ cells were seeded on collagen / Matrigel gels in the presence of fibroblasts and after 5 days the gels were fixed and labelled with phalloidin-TRITC. Confocal images of cells invading approximately 5 μm (left panels) and 30 μm (right panels) are shown.

7.2.13 *E-cadherin is not required for the collective invasion of A431 cells.*

As the requirement for p120 in mediating EGF-induced invasion did not map to its N-terminal regulatory domain it was possible that p120 was acting to stabilise E-cadherin and hence facilitate the intercellular adhesion implicit in collective cell invasion. To test this hypothesis E-cadherin was transiently knocked down in A431-Scr cells using two separate RNAi duplexes (Figure 7- 12). As a control for non-specific effects two duplexes (irrelevant 1 and 2) containing at least 4 mismatches with all known human genes and which have been shown on microarray analysis to have minimal targeting of known human genes were also used. A high level of knockdown of E-cadherin was achieved (Figure 7-12 A). The depletion of E-cadherin did not alter the ability of the A431 cells to invade (Figure 7-12 B,C). This suggests that the ability of p120 to regulate invasion is via a mechanism independent of its ability to stabilise E-cadherin and that the collective invasion of A431 cells is not dependent on E-cadherin mediated cell-cell junctions. This is supported by the formation of collective strands of invasive cells in the small number of A431-p120⁻ cells that invade a small distance into the collagen gels (Figure 7-11).

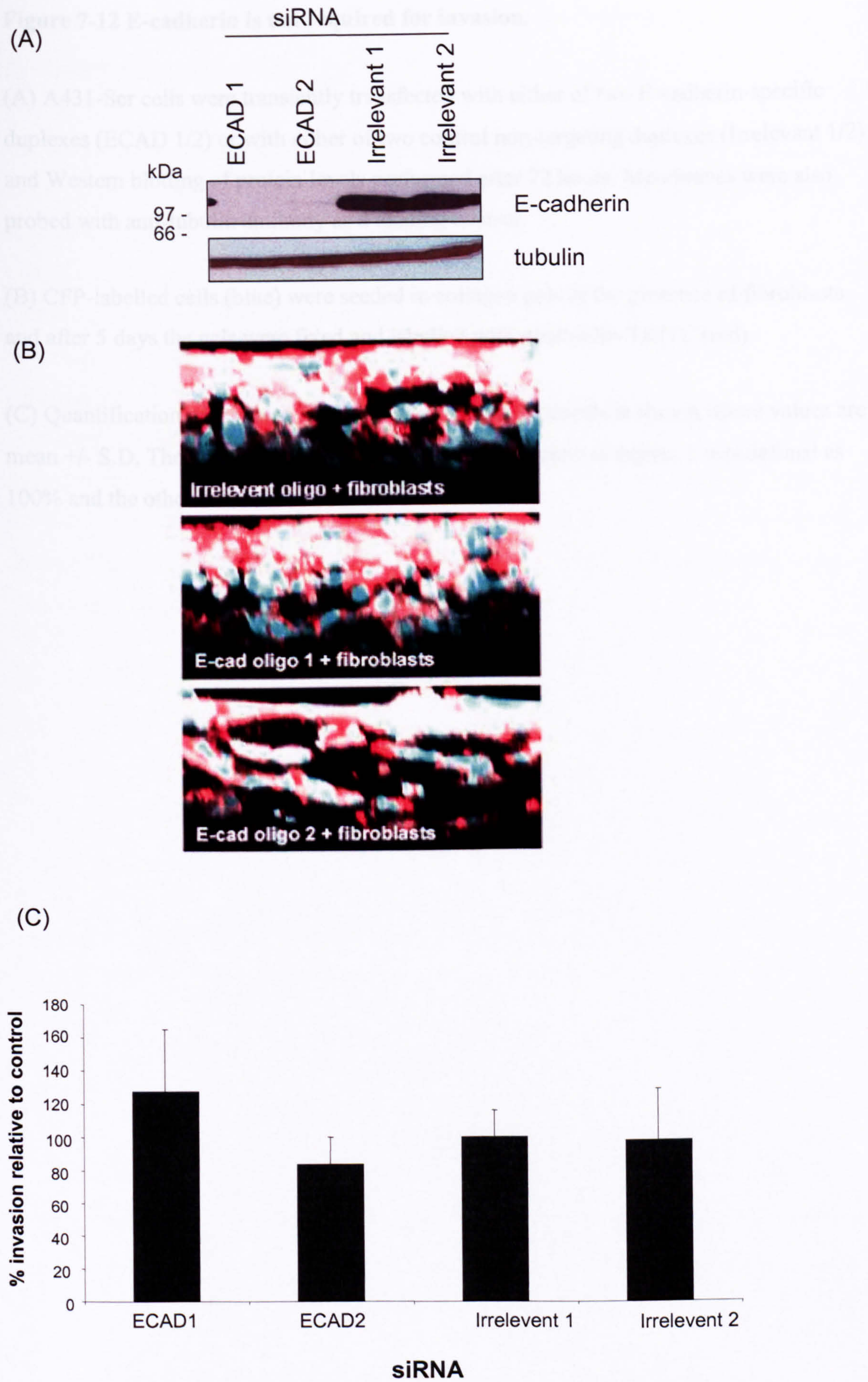


Figure 7-12 E-cadherin is not required for invasion.

Figure 7-12 E-cadherin is not required for invasion.

(A) A431-Scr cells were transiently transfected with either of two E-cadherin-specific duplexes (ECAD 1/2) or with either of two control non-targeting duplexes (Irrelevant 1/2) and Western blotting of protein levels performed after 72 hours. Membranes were also probed with anti-tubulin antibody as a loading control.

(B) CFP-labelled cells (blue) were seeded in collagen gels in the presence of fibroblasts and after 5 days the gels were fixed and labelled with phalloidin-TRITC (red).

(C) Quantification of invasion from three separate experiments is shown where values are mean \pm S.D. The invasion by cells transfected with irrelevant duplex 1 was defined as 100% and the other values normalised to this.

7.2.14 *Reduced invasion is not due to a defect in chemotactic migration.*

The invasive defect seen in p120-depleted A431 cells could represent either a general failure to recognise or respond to a chemotactic EGF gradient or could signify a loss of functions more specifically related to invasion. To discriminate between these two possibilities the migration of A431-Scr and A431-p120⁻ cells across a Transwell filter which had not been coated with extracellular matrix was determined (Figure 7-13). In the presence of endogenous or reconstituted p120 a basal level of migration occurred which, surprisingly, was not increased in the presence of EGF. In contrast, cells in which p120 had been depleted showed significantly increased migration in response to EGF ($p = 0.037$, Two-sample T-Test) and this was maximal for 10 ng/ml EGF, a concentration identical to that used in the Matrigel invasion assays (concentrations of 1-100 ng/ml tested, data not shown). Thus, the depletion of p120 was associated with enhanced rather than diminished chemotactic migration of A431 cells in response to EGF.

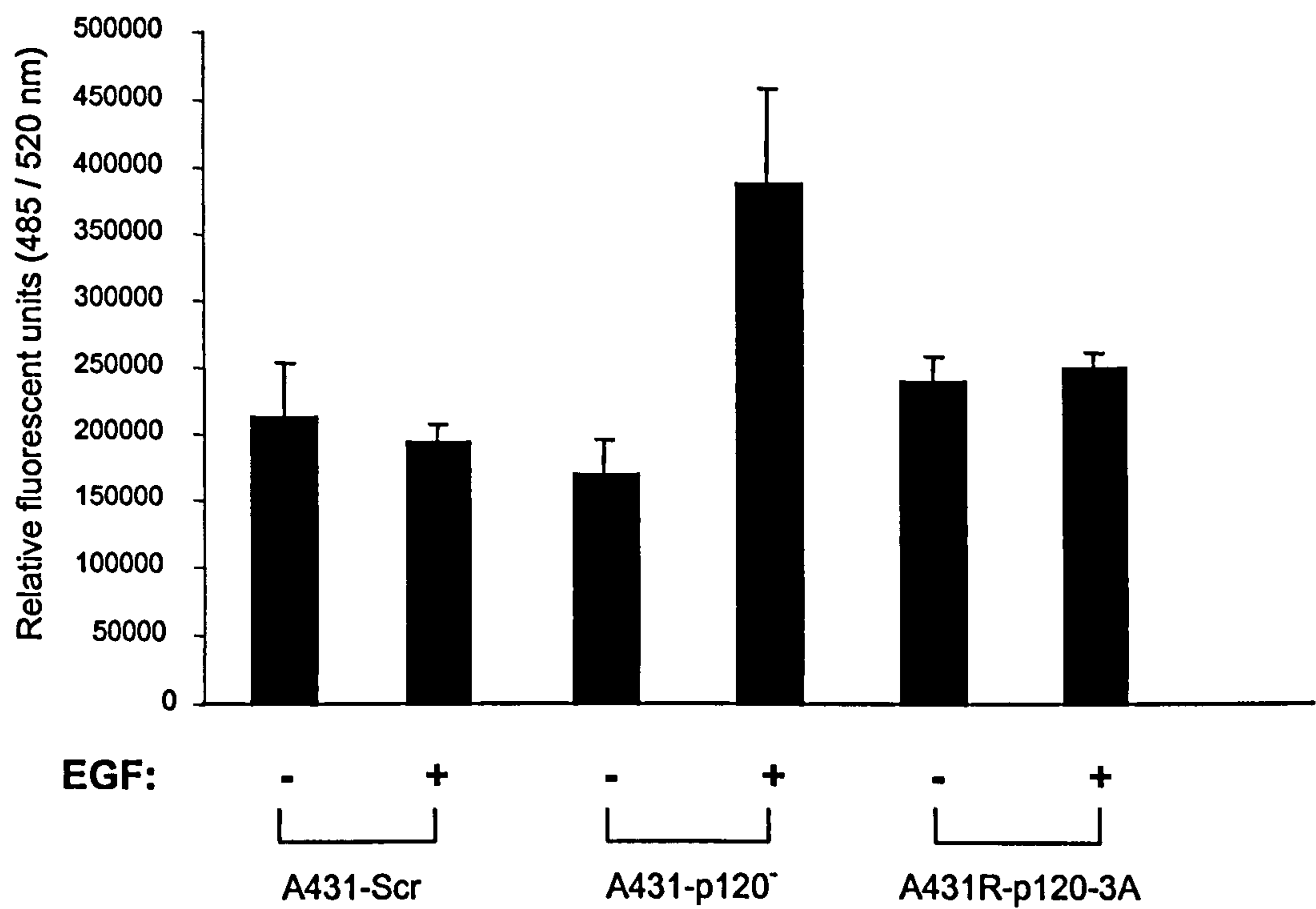


Figure 7-13 p120 is not required for chemotactic migration to an EGF gradient.

Cells were seeded on uncoated Transwell filters and allowed to migrate across the filter with or without an EGF gradient. After 24 hours cells which had crossed to the lower surface of the filter were detached and labelled with a fluorescent dye. Results are plotted as mean absorbance +/- S.D. for triplicate filters.

7.3 Discussion

As a prominent substrate of pro-invasive kinases with the ability to regulate the AJ, Rho GTPases and transcription, p120 appears well placed to integrate and disseminate signals leading to invasion. However, the potential role of p120 in invasion has not been experimentally tested. We utilised the RNAi-based substitution system described in chapter six to recapitulate two distinct scenarios where p120 function may be deregulated in invasive human cancers *in vivo*: 1) Complete loss of p120 which is a relatively infrequent but consistent finding across multiple immunohistochemical studies; and 2) deregulated tyrosine phosphorylation of p120 which is seen in several neoplastic cell lines and which may occur in tumours with upregulated kinase activity. The first question was addressed by RNAi-mediated depletion of p120. The second, by determining whether tyrosine phosphorylation of p120 correlated with invasiveness and then specifically interfering with p120 phosphorylation by replacing wild type p120 with the phosphorylation deficient truncated isoform, p120-4A.

7.3.1 *p120 and 2-dimensional motility.*

The depletion of p120 in A431 cells was associated with a significant increase in two dimensional motility. This contrasts with previous reports in which depletion of p120 by RNAi in a colorectal (130) and lobular breast cancer (128) cell line was associated with increased RhoA activity and decreased motility. However, in both of these cell lines p120 was constitutively cytoplasmic. The depletion of p120 from a cadherin deficient cell line in which p120 is constitutively cytoplasmic and apparently causing tonic inhibition of RhoA may differ from depleting p120 in a cell line such as A431 where it is predominantly membranous. In this regard the effects of depleting p120 on motility may be highly dependent on the specific cellular context. Interestingly, expression of a dominant negative cadherin construct (a substitution of tryptophan to alanine at position 2 leads to an inability of the mutant protein to form *trans* dimers) was previously reported to cause increased motility of A431 cells (483). It has been suggested that, by sequestering p120, dominant negative cadherins may partially phenocopy the effects of p120 depletion (271). Viewed in this light these findings would be consistent with our current data.

7.3.1.1 Tyrosine phosphorylation of p120 is not necessary for cell motility.

We found that the lower rate of motility in p120 expressing cells, compared to cells in which p120 had been depleted, was independent of the ability of the reconstituted p120 to be phosphorylated. This finding is in-keeping with previous reports that expression of p120 isoform 4 in primary keratinocytes did not influence motility as compared to control cells (131). In contrast the motility of HGF stimulated MDCK cells was reported to be inhibited by the expression of p120-4A and increased by expression of p120-1A (131). It is possible that this represents differing requirements for p120 phosphorylation intrinsic to various epithelial cell lines. Alternatively, this may represent distinct pathways activated in response to EGF, HGF, or to the growth factors present in foetal bovine serum (FBS). We found that motility in A431 cells was significantly reduced in serum free medium (median speed 1-2 μm / hour) as compared to complete medium (6-8 μm / hour). However, on addition of even low concentrations of EGF a very high percentage of sparsely plated A431 cells rapidly detached making a direct analysis of EGF-stimulated motility impossible. However, the motility in all cell lines was increased to the same extent in serum containing versus serum free medium suggesting there was no difference in the ability of A431 cells to respond to the growth factors present in FBS regardless of the p120 isoform expressed.

The finding that Src kinase activity was required for motility was in-keeping with the known pro-migratory function of this kinase (484). Previous work has identified the importance of tyrosine phosphorylation of specific Src substrates such as FAK and cortactin in cell migration (236). However, the Src substrate p120 appears to differ in this regard as its ability to undergo phosphorylation did not influence the motile abilities of the cell.

7.3.1.2 A431 cells favour collective migration in 2-dimensional culture.

Striking differences were seen in the mode of wound-closure adopted by control and p120 depleted A431 cells. Unlike control cells which moved as a co-ordinated and coherent sheet, p120 depleted cells migrated into the denuded area independently without evidence of multicellular organisation. Interestingly, despite the 3 fold increase in random motility seen in p120 depleted cells there was no difference in the time taken to achieve wound closure as compared to control cells. This apparent discrepancy is partially explained by the persistent vector of motion displayed by the coherent epithelial sheet. In contrast p120-

depleted cells filled the wound less efficiently as individual cells migrated in various directions rather than uniformly into the defect. Furthermore, the speed of the collective sheet (20.57 μm / hour) was greater than the speed achieved by sparsely plated individual A431 cells (8.0 μm / hour). This suggests that collective motility is a more efficient means of migration for the A431 cell line.

7.3.2 *p120 and invasion.*

7.3.2.1 Increased 2-dimensional planer migration is not predictive of increased invasiveness.

Intuitively, one might expect the depletion of p120 (which caused increased motility in two dimensions and downregulation of the invasion suppressor E-cadherin) to increase invasiveness. On the contrary, p120 depletion was associated with a dramatic reduction in invasiveness both in Matrigel and in collagen gels in an organotypic assay. The finding that two-dimensional planer motility is not predictive of invasion has been seen in other model systems. For example, a study characterising the genotype-phenotype relationship of various E-cadherin germline mutations identified in hereditary diffuse-type gastric cancer demonstrated that 2-dimensional motility was neither necessary nor sufficient for invasion (344). Clearly the ability to invade a 3-dimensional matrix may demand phenotypic changes of a cell in addition to motility such as expression of matrix degrading enzymes or altered expression of cell-matrix adhesion molecules. Furthermore, a cell which is poorly motile in 2-dimensions may become highly motile in a 3-dimensional microenvironment. Indeed molecular signalling pathways leading to invasion or motility have been experimentally dissected. For example, blockade of MAPK signalling in N-cadherin expressing MCF-7 breast cancer cells resulted in the inhibition of FGF-2 induced invasion and MMP-9 production without inhibiting motility (378). Thus caution should be exercised when attempting to infer invasiveness from 2-dimensional studies.

7.3.2.2 p120 is required for the collective invasion of A431 cells.

Visualisation of invading cells within a collagen gel clearly revealed that A431 cells invaded collectively as groups of cells. This type of invasion is common in tumours in vivo and is characterised by the retention of cell-cell contacts, dependence on proteolysis and on integrin-based cell matrix attachments. The cell and molecular biology of collective invasion is poorly defined and we have not yet elicited the mechanism by which p120 is required. However, several plausible explanations may be proposed:

1. Loss of p120 (or other AJ proteins) interferes with EGF signalling.

Various reports have linked the AJ or its component proteins to either positive or negative regulation of growth factor receptor signalling. For example E-cadherin and VE-cadherin dependent cell-cell adhesion has been reported to inhibit EGFR (471, 485) or VEGFR (486) signalling respectively. Conversely a transient increase in EGFR activity has been reported at nascent AJs (137) and the AJ-dependent but EGF independent activation of EGFR appears to be important in the prevention of anoikis in clusters of squamous cell carcinoma cells (425). N-cadherin has been shown to cooperate directly with FGFR in inducing invasiveness and p120 has been implicated in the growth factor dependent activation of RhoA. It was possible then that loss of p120 and the AJ may in some way impair EGF signalling and thus the ability of A431 cells to respond to an EGF gradient. Although, we demonstrated no defect in the activation of major pathways downstream of EGFR signalling such as the STAT3 and MAPK pathways in 2-dimensional culture this does not exclude such a defect occurring within the 3-dimensional environment of an invasion assay.

We demonstrated that A431-p120⁻ cells were able to migrate through an uncoated Transwell towards an EGF gradient; in other words the defect seen was specifically of invasion rather than of chemotactic migration. Interestingly, chemotactic migration of p120 expressing cells was not seen in this system. Clearly, they have the ability to respond chemotactically as evidenced by invasion through a Transwell into Matrigel in response to EGF. It is likely that the intact AJ inhibits migration of cells from a confluent monolayer through the Transwell and that this inhibition is lost in the p120 depleted cells. The addition of Matrigel to the Transwell must provide additional signals which cooperate with EGF to facilitate collective migration and invasion of p120 expressing cells.

2. Loss of p120 alters Rho GTPase activity

As discussed earlier p120 regulates Rho GTPases, signalling molecules whose involvement in motility and invasion are well documented. Previous work has implicated p120 in growth factor dependent activation of RhoA (131). Our failure to detect any difference in the ability of RhoA, Rac or Cdc42 to be activated following EGF stimulation in A431-p120⁻ cells as compared to control cells does not fully eliminate modulation of Rho-GTPase activity as being of relevance. Firstly, it is not clear how the activation of Rho GTPases following 5-15 minutes of stimulation with

EGF in a tissue culture dish compares to the chronic stimulation achieved over several days as in an invasion assay. In this regard the reduction in constitutive activity of RhoA and Cdc42 in serum starved A431-p120⁻ cells in comparison to control cells may be of relevance. This observation at least raises the possibility that a differential activation of Rho GTPases might occur when cells are exposed to lower levels of EGF over a more prolonged time period. Secondly, the regulation of Rho GTPases differs in two and three dimensions. For example, fibroblasts maintained in a 3-dimensional fibronectin matrix have a lower level of Rac activity than when plated in 2 dimensions on fibronectin coated tissue culture plastic (487). Thus, the ability of cells to respond to EGF in two dimensional tissue culture may not necessarily replicate events occurring in a three dimensional extracellular matrix. Furthermore, we measured the global changes in GTPase activity following stimulation of all cells in culture. For collective invasion, polarised activation of Rho GTPases may be required such as in the pathfinder cells at the leading edge and a subtle defect in the spatial regulation of Rho GTPase activity cannot be excluded. Therefore we conclude that there is no gross defect in the ability of EGF to activate Rho GTPases but cannot confidently reject alterations in Rho GTPase signalling as a mechanism by which depletion of p120 inhibits the collective invasion of A431 cells.

3. Loss of p120 alters transcription.

p120 directly links events at the cell surface to gene transcription via the transcriptional repressor Kaiso (144). The physical interaction of p120 with Kaiso negatively regulates the ability of Kaiso to inhibit transcription. Theoretically the depletion of p120 from a cell may therefore cause increased Kaiso-mediated repression of target genes some of which, such as MMP7, may be important for collective invasion. Conversely, in the absence of the AJ β -catenin may also accumulate in the nucleus causing increased transcription of TCF target genes involved in invasion, some of which, such as MMP7, are co-ordinately regulated by Kaiso. However, in A431 cells the APC pathway of β -catenin degradation appears to be intact as, rather than accumulating in the cytoplasm or nucleus, β -catenin levels are reduced in parallel with other AJ components. Furthermore the levels of MMP7 transcripts were unaltered in A431-p120⁻ cells compared to control cells (A Serrels, unpublished data). This does not exclude altered transcription as the cause of impaired invasion and future work should test this further by the use of TCF and Kaiso reporter constructs.

Members of the AP-1 family of transcription factors are required for the EGF-induced invasion of A431 cells (397). However, expression of a dominant negative cadherin construct in A431 cells increased rather than decreased AP-1 activity although whether loss of E-cadherin function via depletion of p120 would have an identical or effect on AP-1 activity is unknown (483). Future work should therefore also clarify the effects of p120 depletion on AP-1 family transcriptional activity.

4. p120 is required to maintain cadherins other than E-cadherin.

E-cadherin was not required for the collective invasion of A431 cells as RNAi mediated depletion of E-cadherin neither increased nor diminished invasiveness. However, A431 cells were shown to also express P-cadherin, which has been reported to have pro-invasive properties in other systems (372). If the collective invasion of A431 cells required pro-invasive cadherins such as P-cadherin then a requirement for p120, without which all cadherins are degraded, would be expected. This hypothesis should be testable by RNAi-mediated depletion of P-cadherin. Should knockdown of P-cadherin inhibit invasion then this would strongly suggest that this is mechanism of p120 action. Conversely, the forced over-expression of P-cadherin in p120-deficient cells might reverse the invasive defect. However, as alluded to above the forced expression of cadherins in the absence of p120 may not replicate the physiological state due to rapid turnover of AJs and impaired cadherin-activated signalling pathways in which p120 is involved. This type of experiment would also be confounded if A431 cells were to express other less well defined cadherins which may also exhibit pro-invasive activity such as cadherin-11 (488).

7.4 Summary

Using an RNAi based substitution system p120 knock-down resulted in loss of AJs and increased motility of A431 cells. This correlated with inhibition of EGF-dependent invasion which was restored by reconstitution of wild type p120. A truncated isoform of p120, which cannot be phosphorylated, was equally able to restore invasive capacity indicating that p120 phosphorylation is not required for EGF-dependent invasion of A431 cells. Visualisation of invading cells within a collagen gel revealed that A431 cells invaded collectively as groups of cells. Knock-down of E-cadherin protein levels by RNAi

demonstrated that this was not dependent on E-cadherin mediated cell-cell adhesion. Thus we identify a requirement for p120 to regulate collective tumour cell invasion which is independent of its known effects on E-cadherin stability and, surprisingly, is independent of its ability to undergo tyrosine phosphorylation.

8 Concluding remarks.

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The data presented and discussed in this thesis raise several points for further consideration:

8.1 Is the tyrosine phosphorylation of p120 increased in cancer?

As a substrate of oncogenic kinases the phosphorylation of p120 might be expected to be increased in human cancers. Whether, and with what frequency, this may occur has not yet been defined. A single study has suggested that increased phosphorylation of p120 may be a common occurrence in lung cancer (177). However, a biochemical approach as was utilised in this study is time consuming and constrained by the availability of fresh-frozen tissue. The development of p120 phospho-specific antibodies, as described in this thesis, should now facilitate the direct immunohistochemical study of p120 tyrosine phosphorylation in human tumour specimens. However, we would argue that the study of protein tyrosine phosphorylation in archival tumour specimens should be performed with caution. Tyrosine phosphorylation is a highly dynamic process and, as a consequence, artefactual alterations in protein phosphorylation may occur (443). Indeed, the snapshot provided by immunohistochemical analysis may be more revealing of factors surrounding tissue collection than of the true phosphorylation status of the protein in vivo. For example, dephosphorylation of a protein may occur due to a predominance of phosphatase activity (which is ATP-independent) over ATP-dependent kinase activity in the face of diminishing cellular energy stores in devascularised tissue. Indeed, a reduction in protein tyrosine phosphorylation correlates with increasing time to fixation. Conversely, transient increases in tyrosine phosphorylation may also occur. This presumably reflects profound and rapid cellular responses to devascularisation. An example of such changes is the rapid increase in expression of acute phase response genes shown to occur in prostate tissue derived from radical prostatectomy (in which the median time from ligation of arterial supply to fixation was 28 minutes) as compared to that obtained by a more rapid biopsy procedure (489). The size of specimen is also of relevance with a common finding being maintained phosphorylation in tissue from the rim but not the centre of larger specimens due to the time taken for penetration of the formalin fixative (443). For these reasons the use of existing tumour arrays, in which the provenance of samples is largely unknown, to address the frequency of protein tyrosine phosphorylation risks being uninformative or even misleading. Ideally biopsy derived material collected according to a validated protocol should be preferred for this type of study. For this reason we have not yet performed a survey of p120 tyrosine phosphorylation in human tumours.

However, recent work utilising the APC^{Min/+} mouse model of colorectal carcinogenesis has provided proof-of-principle that p120 hyper-phosphorylation may occur during in vivo oncogenesis (O Sansom and G Ashton, unpublished observations). A marked increase in tyrosine phosphorylation of p120 at tyrosine-228 was detected by immunohistochemical examination in 10 out of 10 colonic adenomas in comparison to adjacent normal colonic mucosa (Figure 8-1). Levels of p120 protein were unchanged (data not shown). Critically, this shows p120 phosphorylation to occur in a realistic model of oncogenesis which is likely to be predictive of events in human colorectal neoplasia. The nature of the kinases involved is suggested by recent work identifying activation of both EGFR and Src in this model (490). That increased phosphorylation of p120 should occur at such an early point in oncogenesis is intriguing. By definition adenomas are non-invasive. Thus, in the APC^{Min/+} mouse model increased tyrosine phosphorylation of p120 is not sufficient to confer invasive growth.

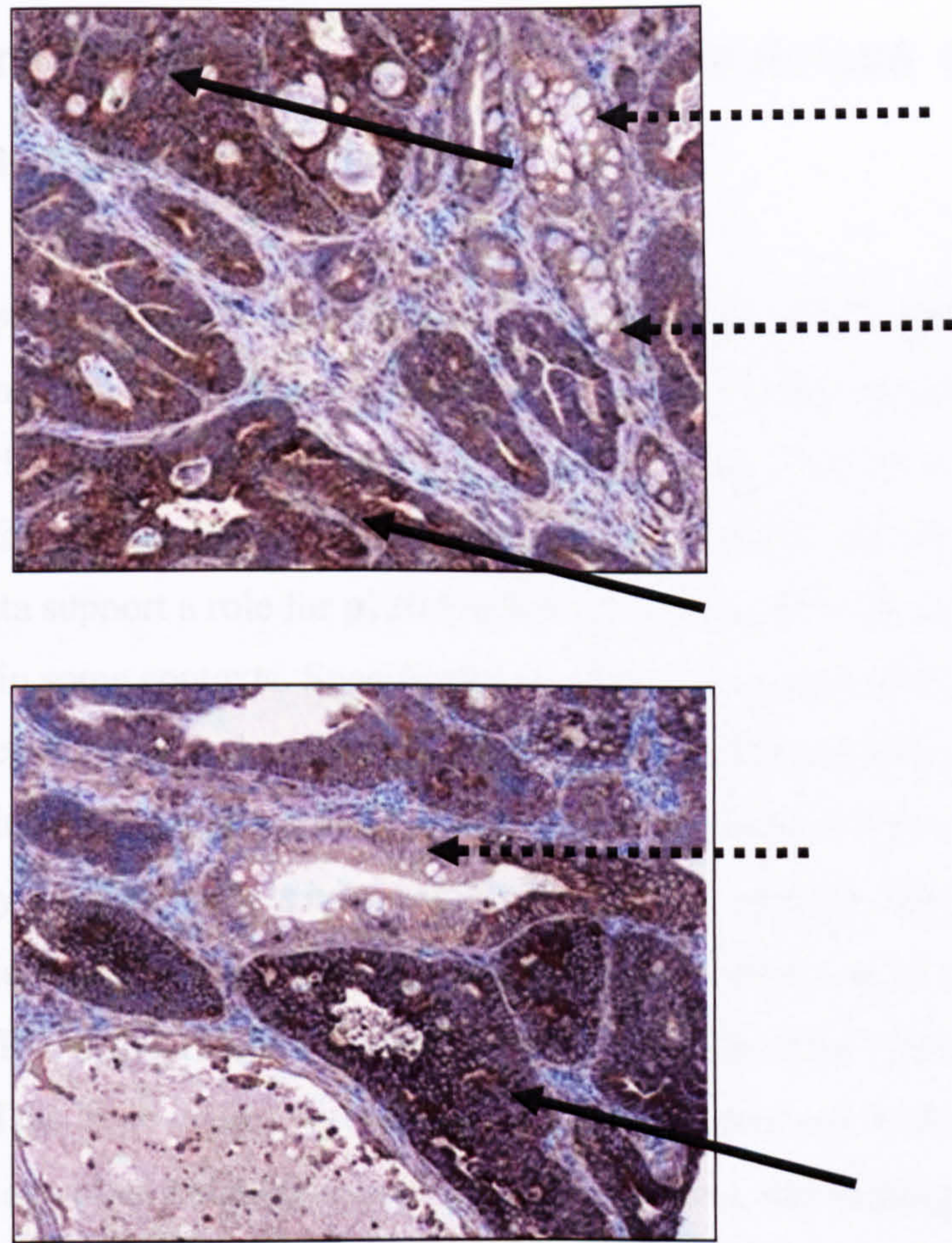


Figure 8-1 p120 is hyper-phosphorylated in colonic adenomas.

Colonic adenomas arising in the APC^{Min/+} mouse were fixed and stained with p120-phospho-Y228 antibody. Of ten adenomas examined all exhibited increased phospho-Y228 staining. Examples of two adenomas demonstrating strong staining with phospho-Y228 antibody are shown (filled arrows). For comparison, an area of morphologically normal mucosa in which only a low level of phospho-Y228 is detectable is shown (upper panel, dotted arrow). In eight of ten adenomas examined a uniform pattern of increased staining was seen, with a patchy increase seen in the remaining two cases. An example of patchy staining is shown in the lower panel where an area of adenoma not displaying elevated phosphorylation of p120 is present (lower panel, dotted arrow).

(unpublished data, courtesy of G Ashton and O Sansom, Beatson Institute)

8.2 What are the physiological consequences of p120 tyrosine phosphorylation?

The tyrosine phosphorylation of AJ proteins such as cadherins and β -catenin appears to provide a rapid and reversible means of regulating the AJ. Whether phosphorylation of p120 may similarly influence AJ function has been less clear. Indeed evidence for a critical modulatory role for tyrosine phosphorylation has been lacking for any of p120's accepted functions. Our data support a role for p120 tyrosine phosphorylation in modulating AJ function, at least in some contexts. Specifically, we have shown that p120 phosphorylation is required for the scattering induced by EGFR signalling and that interfering with this phosphorylation inhibits scattering, most probably via stabilisation of the AJ rather than by inhibiting motility. Further work is now required to identify whether this property maps to the phosphorylation state of one or more specific tyrosine residues or, as is the case for the interaction of SHP-1 with p120, is a property dependent on the total number of residues phosphorylated. This issue is directly testable using p120 constructs with individual and combined tyrosine to phenylalanine substitutions. In addition, our finding that p120 isoform 4 inhibited scattering more fully than the p120-3A/7F construct suggests that other events occurring in the regulatory domain are important. One possibility is phosphorylation on serine which has been shown to occur in response to EGFR activation (181). The role of serine / threonine phosphorylation would similarly be amenable to study by targeted substitution of the appropriate residues.

It is interesting to speculate in what way the role of p120 phosphorylation in two-dimensional scattering may reflect the function of p120 phosphorylation in 3-dimensional culture or, ultimately, in vivo. The effects of growth factor treatment in 2 and 3-dimensions may be markedly different. For example, small colonies of MDCK cells cultured in 2-dimensions rapidly dissociate in response to HGF. In contrast, the same cells cultured in a 3-dimensional collagen matrix form small cysts which, on exposure to HGF, develop branching tubules in which cell-cell adhesion is maintained (491). Furthermore, over-expression of EGFR in oesophageal squamous cells was associated with increased association of p120 with E-cadherin and increased cell-cell adhesion in 3-dimensional organotypic culture (492). Therefore, whilst a role for p120 phosphorylation in the dynamic modulation or remodelling of cell adhesions appears plausible, it is clear that effects observed in 2-dimensional culture are not necessarily directly representative of events occurring in vivo.

Studies incorporating greater spatio-temporal resolution are necessary to further define the consequences of p120 phosphorylation at the AJ. In particular this may address the relevance of p120 phosphorylation at tyrosine-228 which occurs in nascent AJs but which does not appear to be required for AJ formation. Specifically, the use of GFP-labelled p120, E-Cadherin and actin may allow the detection of more subtle defects in the kinetics of AJ formation or turnover.

As was acknowledged in the title of a recent review article, the consequences of p120 phosphorylation remain an unresolved issue (493). However, as our knowledge of p120 biology increases we may be able to better design experiments to test the involvement of tyrosine phosphorylation in various aspects of p120 function.

8.3 Might a requirement for p120 define collective invasion?

Despite the recognition that many common solid tumours invade collectively, the molecular mechanisms underlying this type of invasion are poorly understood. The extent to which cadherins may be involved in collective invasion, either by facilitating cell-cell adhesion or by the generation of specific pro-invasive signals, is not known. Although the AJ is required for cell-cell adhesion in tissue culture this does not appear to be an absolute requirement in 3-dimensional culture (86) or in vivo (95) and so it cannot be assumed that collective invasion is necessarily cadherin (and by implication p120)-dependent.

Our demonstration that p120 was required for the invasion of A431 cells was at first surprising. Indeed, as depletion of p120 caused downregulation of E-cadherin, a frequent invasion suppressor, and increased 2-dimensional motility we had predicted that invasion would be increased and, most likely, associated with a collective to mesenchymal switch. However, A431 cells did not appear able to exhibit plasticity in their mode of invasion in response to depletion of p120. Interestingly, the phosphorylation of p120 was neither required for collective invasion of A431 cells into collagen or Matrigel, nor was sufficient to cause invasion in adenomas in the APC^{Min/+} mouse.

Previous work in A431 cells has shown that EMT induced by the forced expression of Snail caused loss of E-cadherin and increased invasiveness, although whether these cells invaded collectively or displayed mesenchymal single cell invasion was not determined (494). Thus it appears that it is the underlying mechanism by which E-cadherin is lost,

rather than simply its presence or absence, which determines invasiveness. Three distinct scenarios can be considered:

1. Loss of E-cadherin in isolation.

In our hands loss of E-cadherin due to RNAi-mediated depletion had no significant effect, either positive or negative, on invasiveness. It should be noted, however, that effects of transient and long term knockdown of E-cadherin may differ. For example expression of a DN-cadherin in A431 cells caused a switch in intermediate filaments from cytokeratin to vimentin consistent with EMT but this occurred over a period of weeks rather than days (483). Thus it is possible that stable rather than transient knockdown of E-cadherin may have yielded different results.

2. Loss of E-cadherin secondary to depletion of p120.

The depletion of p120 significantly reduced invasiveness. As depletion of E-cadherin had no effect this indicates that the contribution of p120 to invasiveness was unrelated to its role in stabilising E-cadherin. In this regard observations made in biopsies of oral squamous cell carcinomas are intriguing. Some collectively invading oral squamous cell carcinomas appear to lose expression of E-cadherin yet retain membranous expression of p120 (Figure 8-2). As cadherins are necessary and sufficient for the recruitment of p120 to the membrane this implies that in the absence of E-cadherin the expression of other cadherins is maintained. This could suggest a model where p120 is required for the stability and function of pro-invasive N- or P-cadherins. However, the potential role of various cadherins in collective invasion awaits definition.

3. Loss of E-cadherin due to transcriptional downregulation in the context of EMT.

Snail induced EMT was reported to increase invasiveness in A431 cells (494). Although this was associated with downregulation of E-cadherin other transcriptional events such as increased MMP2 production may underlie the increase seen in invasion. An important question to be addressed is whether Snail induced EMT is able to override the requirement for p120 for A431 cell invasion.

Although the invasion of A431 cells reconstituted with p120 isoform 3 or 4 was equal it would be informative to test the effect of reconstitution with p120 isoform 1. Over-expression of this isoform has been associated with increased 2-dimensional motility (131), although as discussed previously this does not necessarily have predictive value for invasion. In addition, however, p120 isoform 1 is upregulated in some metastatic cell lines (171), associates preferentially with pro-invasive N-cadherin (381) and is also upregulated in EMT (172). These observations raise the possibility that a switch from p120 isoform 3 to 1 might increase invasiveness. Again, this hypothesis could be readily tested using the methods described in this thesis.

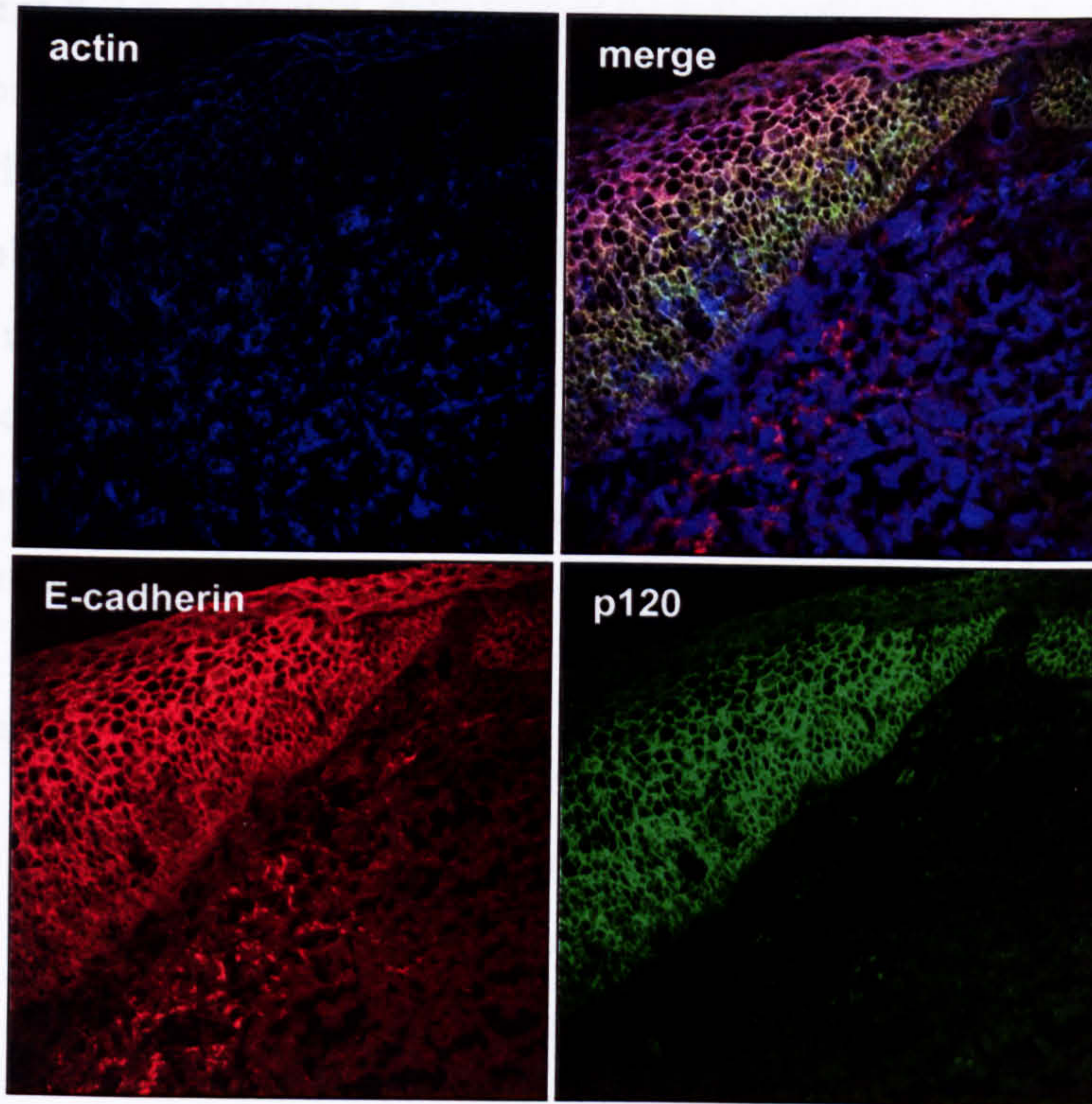
It is also interesting to speculate that the requirement for p120 in the collective invasion of A431 cells may in some way recapitulate the requirement for p120 in morphogenetic movements in vertebrate embryogenesis. In *Xenopus* the effects of loss of p120 could be corrected by carefully titrated expression of DA-Rac and cadherin (56). Whether such manipulations could rescue invasiveness in this system could be readily tested.

A matter of priority is to determine whether the requirement identified for p120 in the collective invasion of A431 cells exists in other cell lines which are known to utilise a collective mode of invasion. Should this be the case then it would be appropriate to test the role of p120 in invasion in vivo. However, several difficulties may hinder this approach. Invasion, by definition, influences and is influenced by tissue micro-architecture. Therefore, invasion should be studied in tumours arising within their tissue of origin; a xenograft approach is likely to be of limited relevance. For this reason a genetic model of carcinogenesis or, alternatively, a chemical carcinogenesis model should be used. Unfortunately targeted deletion of p120 may have profound effects on the development of that tissue and indeed, as in the case of the GI tract, may be lethal. This might be circumvented, at least in part, by developing an inducible targeted knockout of p120. For example, a floxed-p120 transgenic mouse could be crossed with a transgenic mouse expressing ER-Cre recombinase under the control of a keratin promoter. p120 could then be deleted from the skin in a temporally and spatially controlled manner by the direct application of tamoxifen. In combination with a well defined two stage chemical carcinogenesis model (495) the results of knocking out p120 at various stages of carcinogenesis could be determined.

In conclusion, further study to unravel the mechanisms by which p120 is involved in collective invasion is likely to increase our understanding of the complex biology of this

process. Ultimately, this understanding may have an impact on our ability to therapeutically target this particular hallmark of malignancy.

(A)



(B)

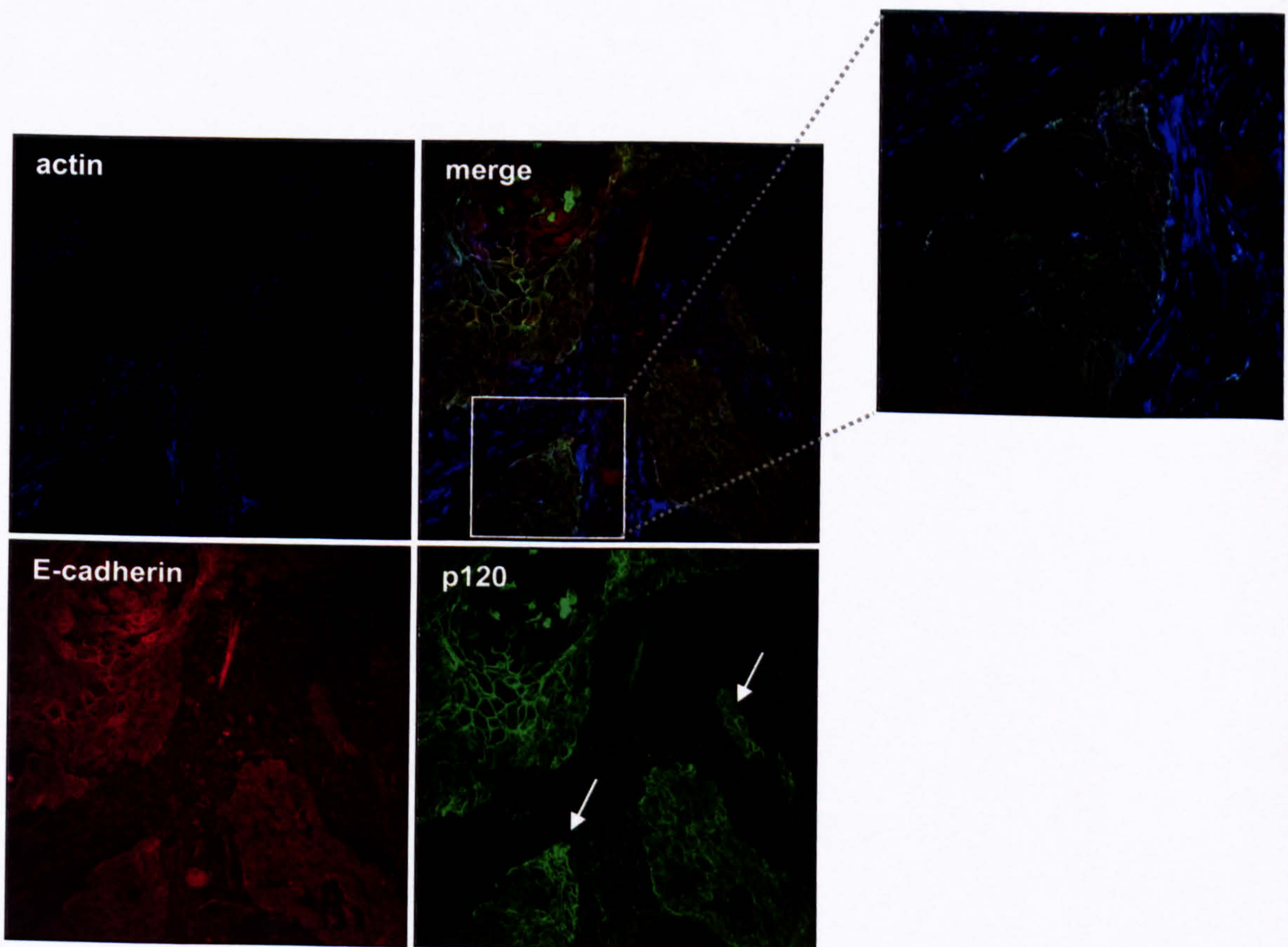


Figure 8-2 E-cadherin deficient squamous cell carcinomas display membranous p120.

Figure 8-2 E-cadherin-deficient squamous cell carcinomas retain membranous p120. Biopsies of non-cancerous oral mucosa (A) or invasive squamous cell carcinoma (B) were fixed and stained with E-cadherin (red) or p120 (green) antibodies or with Alexa633 coupled phalloidin (blue) to visualise actin. Single images are shown with merged images (top right hand panels). Membranous co-localisation of E-cadherin and p120 occurs in non-cancerous mucosa. Note areas of collectively invading tumour in which E-cadherin is downregulated but membranous localisation of p120 is retained (arrows). (C) Higher magnification view of boxed area demonstrating membranous p120.

(unpublished data, courtesy of Dr E Sahai, London Research Institute)

SUPPLEMENTARY MATERIAL

Compact disc containing:-

Movie 1: Wound closure by A431-Scr cells.

A wound was made in a confluent monolayer of A431-Scr cells using a sterile pipette tip and images recorded at 15 minute intervals over a 10 hour period by time-lapse videomicroscopy (x10 magnification). Images were combined using AQM advance software to create the movies shown.

Movie 2: Wound closure by A431-p120⁻ cells.

A wound was made in a confluent monolayer of A431-p120⁻ cells using a sterile pipette tip and images recorded at 15 minute intervals over a 10 hour period by time-lapse videomicroscopy (x10 magnification). Images were combined using AQM advance software to create the movies shown.

REFERENCES

1. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100(1):57-70.
2. Takeichi M. Morphogenetic roles of classic cadherins. *Curr Opin Cell Biol* 1995;7(5):619-27.
3. Gumbiner B, Stevenson B, Grimaldi A. The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J Cell Biol* 1988;107(4):1575-87.
4. Wheelock MJ, Johnson KR. Cadherins as modulators of cellular phenotype. *Annu Rev Cell Dev Biol* 2003;19:207-35.
5. Koch AW, Bozic D, Pertz O, Engel J. Homophilic adhesion by cadherins. *Curr Opin Struct Biol* 1999;9(2):275-81.
6. Angst BD, Marcozzi C, Magee AI. The cadherin superfamily: diversity in form and function. *J Cell Sci* 2001;114(Pt 4):629-41.
7. Boggon TJ, Murray J, Chappuis-Flament S, Wong E, Gumbiner BM, Shapiro L. C-cadherin ectodomain structure and implications for cell adhesion mechanisms. *Science* 2002;296(5571):1308-13.
8. Reynolds AB, Daniel J, McCrea PD, Wheelock MJ, Wu J, Zhang Z. Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes. *Mol Cell Biol* 1994;14(12):8333-42.
9. Ohkubo T, Ozawa M. p120(ctn) binds to the membrane-proximal region of the E-cadherin cytoplasmic domain and is involved in modulation of adhesion activity. *J Biol Chem* 1999;274(30):21409-15.
10. Thoreson MA, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, Hummingbird DK, Reynolds AB. Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. *J Cell Biol* 2000;148(1):189-202.
11. Fujita Y, Krause G, Scheffner M, Zechner D, Leddy HE, Behrens J, Sommer T, Birchmeier W. Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat Cell Biol* 2002;4(3):222-31.
12. Baki L, Marambaud P, Efthimiopoulos S, Georgakopoulos A, Wen P, Cui W, Shioi J, Koo E, Ozawa M, Friedrich VL, Jr., Robakis NK. Presenilin-1 binds cytoplasmic epithelial cadherin, inhibits cadherin/p120 association, and regulates stability and function of the cadherin/catenin adhesion complex. *Proc Natl Acad Sci U S A* 2001;98(5):2381-6.
13. Stappert J, Kemler R. A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhes Commun* 1994;2(4):319-27.
14. Wheelock MJ, Johnson KR. Cadherin-mediated cellular signaling. *Curr Opin Cell Biol* 2003;15(5):509-14.
15. Vidal M, Larson DE, Cagan RL. Csk-deficient boundary cells are eliminated from normal *Drosophila* epithelia by exclusion, migration, and apoptosis. *Dev Cell* 2006;10(1):33-44.
16. Peinado H, Portillo F, Cano A. Transcriptional regulation of cadherins during development and carcinogenesis. *Int J Dev Biol* 2004;48(5-6):365-75.
17. Kobiela A, Fuchs E. Alpha-catenin: at the junction of intercellular adhesion and actin dynamics. *Nat Rev Mol Cell Biol* 2004;5(8):614-25.
18. Obama H, Ozawa M. Identification of the domain of alpha-catenin involved in its association with beta-catenin and plakoglobin (gamma-catenin). *J Biol Chem* 1997;272(17):11017-20.
19. Koslov ER, Maupin P, Pradhan D, Morrow JS, Rimm DL. Alpha-catenin can form asymmetric homodimeric complexes and/or heterodimeric complexes with beta-catenin. *J Biol Chem* 1997;272(43):27301-6.

20. Nieset JE, Redfield AR, Jin F, Knudsen KA, Johnson KR, Wheelock MJ. Characterization of the interactions of alpha-catenin with alpha-actinin and beta-catenin/plakoglobin. *J Cell Sci* 1997;110 (Pt 8):1013-22.
21. Watabe-Uchida M, Uchida N, Imamura Y, Nagafuchi A, Fujimoto K, Uemura T, Vermeulen S, van Roy F, Adamson ED, Takeichi M. alpha-Catenin-vinculin interaction functions to organize the apical junctional complex in epithelial cells. *J Cell Biol* 1998;142(3):847-57.
22. Imamura Y, Itoh M, Maeno Y, Tsukita S, Nagafuchi A. Functional domains of alpha-catenin required for the strong state of cadherin-based cell adhesion. *J Cell Biol* 1999;144(6):1311-22.
23. Kobiela A, Pasolli HA, Fuchs E. Mammalian formin-1 participates in adherens junctions and polymerization of linear actin cables. *Nat Cell Biol* 2004;6(1):21-30.
24. Rimm DL, Koslov ER, Kebriaei P, Cianci CD, Morrow JS. Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc Natl Acad Sci U S A* 1995;92(19):8813-7.
25. Gates J, Peifer M. Can 1000 reviews be wrong? Actin, alpha-Catenin, and adherens junctions. *Cell* 2005;123(5):769-72.
26. Drees F, Pokutta S, Yamada S, Nelson WJ, Weis WI. Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* 2005;123(5):903-15.
27. Yamada S, Pokutta S, Drees F, Weis WI, Nelson WJ. Deconstructing the cadherin-catenin-actin complex. *Cell* 2005;123(5):889-901.
28. Hajra KM, Fearon ER. Cadherin and catenin alterations in human cancer. *Genes Chromosomes & Cancer* 2002;34(3):255-268.
29. Huber AH, Weis WI. The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin. *Cell* 2001;105(3):391-402.
30. Willert K, Nusse R. Beta-catenin: a key mediator of Wnt signaling. *Curr Opin Genet Dev* 1998;8(1):95-102.
31. Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 2004;303(5663):1483-7.
32. Gottardi CJ, Gumbiner BM. Adhesion signaling: how beta-catenin interacts with its partners. *Curr Biol* 2001;11(19):R792-4.
33. Polakis P. Wnt signaling and cancer. *Genes Dev* 2000;14(15):1837-51.
34. Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. beta-catenin is a target for the ubiquitin-proteasome pathway. *Embo J* 1997;16(13):3797-804.
35. Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D, Birchmeier W. Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* 1998;280(5363):596-9.
36. Kishida S, Yamamoto H, Ikeda S, Kishida M, Sakamoto I, Koyama S, Kikuchi A. Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. *J Biol Chem* 1998;273(18):10823-6.
37. Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X, He X. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* 2002;108(6):837-47.
38. Schwarz-Romond T, Asbrand C, Bakkers J, Kuhl M, Schaeffer HJ, Huelsken J, Behrens J, Hammerschmidt M, Birchmeier W. The ankyrin repeat protein Diversin recruits Casein kinase Iepsilon to the beta-catenin degradation complex and acts in both canonical Wnt and Wnt/JNK signaling. *Genes Dev* 2002;16(16):2073-84.

39. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. *Science* 1998;281(5382):1509-12.
40. Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A* 1999;96(10):5522-7.
41. Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 1999;398(6726):422-6.
42. Brabletz T, Jung A, Dag S, Hlubek F, Kirchner T. beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am J Pathol* 1999;155(4):1033-8.
43. Crawford HC, Fingleton BM, Rudolph-Owen LA, Goss KJ, Rubinfeld B, Polakis P, Matrisian LM. The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. *Oncogene* 1999;18(18):2883-91.
44. Heasman J, Crawford A, Goldstone K, Garner-Hamrick P, Gumbiner B, McCrea P, Kintner C, Noro CY, Wylie C. Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. *Cell* 1994;79(5):791-803.
45. Gottardi CJ, Wong E, Gumbiner BM. E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner. *J Cell Biol* 2001;153(5):1049-60.
46. Kuphal F, Behrens J. E-cadherin modulates Wnt-dependent transcription in colorectal cancer cells but does not alter Wnt-independent gene expression in fibroblasts. *Exp Cell Res* 2006;312(4):457-67.
47. Behrens J, Lustig B. The Wnt connection to tumorigenesis. *Int J Dev Biol* 2004;48(5-6):477-87.
48. Callahan R, Smith GH. MMTV-induced mammary tumorigenesis: gene discovery, progression to malignancy and cellular pathways. *Oncogene* 2000;19(8):992-1001.
49. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996;87(2):159-70.
50. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 1997;275(5307):1787-90.
51. Prokhortchouk A, Sansom O, Selfridge J, Caballero IM, Salozhin S, Aithozhina D, Cerchietti L, Meng FG, Augenlicht LH, Mariadason JM, Hendrich B, Melnick A, et al. Kaiso-deficient mice show resistance to intestinal cancer. *Mol Cell Biol* 2006;26(1):199-208.
52. Reynolds AB, Roesel DJ, Kanner SB, Parsons JT. Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cells expressing oncogenic variants of the avian cellular src gene. *Mol Cell Biol* 1989;9(2):629-38.
53. Reynolds AB, Herbert L, Cleveland JL, Berg ST, Gaut JR. p120, a novel substrate of protein tyrosine kinase receptors and of p60v-src, is related to cadherin-binding factors beta-catenin, plakoglobin and armadillo. *Oncogene* 1992;7(12):2439-45.
54. Aghib DF, McCrea PD. The E-cadherin complex contains the src substrate p120. *Exp Cell Res* 1995;218(1):359-69.
55. Anastasiadis PZ, Reynolds AB. The p120 catenin family: complex roles in adhesion, signaling and cancer. *J Cell Sci* 2000;113(Pt 8):1319-34.
56. Fang X, Ji H, Kim SW, Park JJ, Vaught TG, Anastasiadis PZ, Ciesiolka M, McCrea PD. Vertebrate development requires ARVCF and p120 catenins and their interplay with RhoA and Rac. *J Cell Biol* 2004;165(1):87-98.
57. Keirsebilck A, Bonne S, Staes K, van Hengel J, Nollet F, Reynolds A, van Roy F. Molecular cloning of the human p120ctn catenin gene (CTNND1): expression of multiple alternatively spliced isoforms. *Genomics* 1998;50(2):129-46.

58. Montonen O, Aho M, Uitto J, Aho S. Tissue distribution and cell type-specific expression of p120ctn isoforms. *J Histochem Cytochem* 2001;49(12):1487-96.
59. Mo YY, Reynolds AB. Identification of murine p120 isoforms and heterogeneous expression of p120cas isoforms in human tumor cell lines. *Cancer Res* 1996;56(11):2633-40.
60. Aho S, Rothenberger K, Uitto J. Human p120ctn catenin: tissue-specific expression of isoforms and molecular interactions with BP180/type XVII collagen. *Journal of Cellular Biochemistry* 1999;73(3):390-9.
61. Aho S, Levansuo L, Montonen O, Kari C, Rodeck U, Uitto J. Specific sequences in p120ctn determine subcellular distribution of its multiple isoforms involved in cellular adhesion of normal and malignant epithelial cells. *Journal of Cell Science* 2002;115(7):1391-1402.
62. Reynolds AB, Rocznia-Ferguson A. Emerging roles for p120-catenin in cell adhesion and cancer. *Oncogene* 2004;23(48):7947-56.
63. Huber AH, Nelson WJ, Weis WI. Three-dimensional structure of the armadillo repeat region of beta-catenin. *Cell* 1997;90(5):871-82.
64. Kelly KF, Spring CM, Otchere AA, Daniel JM. NLS-dependent nuclear localization of p120ctn is necessary to relieve Kaiso-mediated transcriptional repression. *J Cell Sci* 2004;117(Pt 13):2675-86.
65. Daniel JM, Reynolds AB. The tyrosine kinase substrate p120cas binds directly to E-cadherin but not to the adenomatous polyposis coli protein or alpha-catenin. *Mol Cell Biol* 1995;15(9):4819-24.
66. Daniel JM, Reynolds AB. The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. *Mol Cell Biol* 1999;19(5):3614-23.
67. Yanagisawa M, Kaverina IN, Wang A, Fujita Y, Reynolds AB, Anastasiadis PZ. A novel interaction between kinesin and p120 modulates p120 localization and function. *J Biol Chem* 2004;279(10):9512-21.
68. Mariner DJ, Anastasiadis P, Keilhack H, Bohmer FD, Wang J, Reynolds AB. Identification of Src phosphorylation sites in the catenin p120(ctn). *Journal of Biological Chemistry* 2001;276(30):28006-28013.
69. Xia X, Mariner DJ, Reynolds AB. Adhesion-associated and PKC-modulated changes in serine/threonine phosphorylation of p120-catenin. *Biochemistry* 2003;42(30):9195-204.
70. van Hengel J, Vanhoenacker P, Staes K, van Roy F. Nuclear localization of the p120(ctn) Armadillo-like catenin is counteracted by a nuclear export signal and by E-cadherin expression. *Proc Natl Acad Sci U S A* 1999;96(14):7980-5.
71. Le TL, Yap AS, Stow JL. Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. *J Cell Biol* 1999;146(1):219-32.
72. Bryant DM, Stow JL. The ins and outs of E-cadherin trafficking. *Trends Cell Biol* 2004;14(8):427-34.
73. Miranda KC, Khromykh T, Christy P, Le TL, Gottardi CJ, Yap AS, Stow JL, Teasdale RD. A dileucine motif targets E-cadherin to the basolateral cell surface in Madin-Darby canine kidney and LLC-PK1 epithelial cells. *J Biol Chem* 2001;276(25):22565-72.
74. Ivanov AI, Nusrat A, Parkos CA. Endocytosis of Epithelial Apical Junctional Proteins by a Clathrin-Mediated Pathway into a Unique Storage Compartment. *Mol Biol Cell* 2003.
75. Xiao K, Garner J, Buckley KM, Vincent PA, Chiasson CM, Dejana E, Faundez V, Kowalczyk AP. p120-Catenin regulates clathrin-dependent endocytosis of VE-cadherin. *Mol Biol Cell* 2005;16(11):5141-51.
76. Janda E, Nevolo M, Lehmann K, Downward J, Beug H, Grieco M. Raf plus TGFbeta-dependent EMT is initiated by endocytosis and lysosomal degradation of E-cadherin. *Oncogene* 2006.
77. Paterson AD, Parton RG, Ferguson C, Stow JL, Yap AS. Characterization of E-cadherin endocytosis in isolated MCF-7 and chinese hamster ovary cells: the initial fate of unbound E-cadherin. *J Biol Chem* 2003;278(23):21050-7.

78. Akhtar N, Hotchin NA. RAC1 regulates adherens junctions through endocytosis of E-cadherin. *Mol Biol Cell* 2001;12(4):847-62.
79. Lu Z, Ghosh S, Wang Z, Hunter T. Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. *Cancer Cell* 2003;4(6):499-515.
80. Kowalczyk AP, Reynolds AB. Protecting your tail: regulation of cadherin degradation by p120-catenin. *Curr Opin Cell Biol* 2004;16(5):522-7.
81. Ireton RC, Davis MA, Van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L, Gilbert B, Van Roy F, et al. A novel role for p120 catenin in E-cadherin function. *J Cell Biol* 2002;159(3):465-76.
82. Xiao K, Allison DF, Buckley KM, Kottke MD, Vincent PA, Faundez V, Kowalczyk AP. Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. *J Cell Biol* 2003;163(3):535-45.
83. Davis MA, Ireton RC, Reynolds AB. A core function for p120-catenin in cadherin turnover. *J Cell Biol* 2003;163(3):525-34.
84. Kintner C. Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. *Cell* 1992;69(2):225-36.
85. Nieman MT, Kim JB, Johnson KR, Wheelock MJ. Mechanism of extracellular domain-deleted dominant negative cadherins. *J Cell Sci* 1999;112 (Pt 10):1621-32.
86. Troxell ML, Loftus DJ, Nelson WJ, Marrs JA. Mutant cadherin affects epithelial morphogenesis and invasion, but not transformation. *J Cell Sci* 2001;114(Pt 6):1237-46.
87. Maeda M, Johnson E, Mandal SH, Lawson KR, Keim SA, Svoboda RA, Caplan S, Wahl JK, Wheelock MJ, Johnson KR. Expression of inappropriate cadherins by epithelial tumor cells promotes endocytosis and degradation of E-cadherin via competition for p120(ctn). *Oncogene* 2006;25(33):4595-604.
88. Peifer M, Yap AS. Traffic control: p120-catenin acts as a gatekeeper to control the fate of classical cadherins in mammalian cells. *J Cell Biol* 2003;163(3):437-40.
89. Potter MD, Barbero S, Cheresch DA. Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenin and maintains the cellular mesenchymal state. *J Biol Chem* 2005;280(36):31906-12.
90. Yap AS, Niessen CM, Gumbiner BM. The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. *J Cell Biol* 1998;141(3):779-89.
91. Goodwin M, Kovacs EM, Thoreson MA, Reynolds AB, Yap AS. Minimal Mutation of the Cytoplasmic Tail Inhibits the Ability of E- cadherin to Activate Rac but Not Phosphatidylinositol 3-Kinase: Direct evidence of a role for cadherin-activated rac signaling in adhesion and contact formation. *J Biol Chem* 2003;278(23):20533-9.
92. Chen X, Kojima S, Borisy GG, Green KJ. p120 catenin associates with kinesin and facilitates the transport of cadherin-catenin complexes to intercellular junctions. *J Cell Biol* 2003;163(3):547-57.
93. Wahl JK, 3rd, Kim YJ, Cullen JM, Johnson KR, Wheelock MJ. N-cadherin-Catenin Complexes Form Prior to Cleavage of the Proregion and Transport to the Plasma Membrane. *J Biol Chem* 2003;278(19):17269-76.
94. Davis MA, Reynolds AB. Blocked Acinar Development, E-Cadherin Reduction, and Intraepithelial Neoplasia upon Ablation of p120-Catenin in the Mouse Salivary Gland. *Dev Cell* 2006;10(1):21-31.
95. Perez-Moreno M, Davis MA, Wong E, Pasolli HA, Reynolds AB, Fuchs E. p120-catenin mediates inflammatory responses in the skin. *Cell* 2006;124(3):631-44.
96. Myster SH, Cavallo R, Anderson CT, Fox DT, Peifer M. Drosophila p120catenin plays a supporting role in cell adhesion but is not an essential adherens junction component. *J Cell Biol* 2003;160(3):433-49.

97. Pettitt J, Cox EA, Broadbent ID, Flett A, Hardin J. The *Caenorhabditis elegans* p120 catenin homologue, JAC-1, modulates cadherin-catenin function during epidermal morphogenesis. *J Cell Biol* 2003;162(1):15-22.
98. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature* 2002;420(6916):629-35.
99. Sahai E, Marshall CJ. RHO-GTPases and cancer. *Nat Rev Cancer* 2002;2(2):133-42.
100. Frame MC, Brunton VG. Advances in Rho-dependent actin regulation and oncogenic transformation. *Current Opinion in Genetics & Development* 2002;12(1):36-43.
101. Sasaki T, Takai Y. The Rho small G protein family-Rho GDI system as a temporal and spatial determinant for cytoskeletal control. *Biochem Biophys Res Commun* 1998;245(3):641-5.
102. Hall A. Rho GTPases and the actin cytoskeleton. *Science* 1998;279(5350):509-14.
103. Lozano E, Betson M, Braga VM. Tumor progression: Small GTPases and loss of cell-cell adhesion. *Bioessays* 2003;25(5):452-63.
104. Sander EE, van Delft S, ten Klooster JP, Reid T, van der Kammen RA, Michiels F, Collard JG. Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J Cell Biol* 1998;143(5):1385-98.
105. Hordijk PL, ten Klooster JP, van der Kammen RA, Michiels F, Oomen LC, Collard JG. Inhibition of invasion of epithelial cells by Tiam1-Rac signaling. *Science* 1997;278(5342):1464-6.
106. Palovuori R, Sormunen R, Eskelinen S. SRC-induced disintegration of adherens junctions of madin-darby canine kidney cells is dependent on endocytosis of cadherin and antagonized by Tiam-1. *Lab Invest* 2003;83(12):1901-15.
107. Adam L, Vadlamudi RK, McCrea P, Kumar R. Tiam1 overexpression potentiates heregulin-induced lymphoid enhancer factor-1/beta -catenin nuclear signaling in breast cancer cells by modulating the intercellular stability. *J Biol Chem* 2001;276(30):28443-50.
108. Braga VM, Machesky LM, Hall A, Hotchin NA. The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J Cell Biol* 1997;137(6):1421-31.
109. Kuroda S, Fukata M, Fujii K, Nakamura T, Izawa I, Kaibuchi K. Regulation of cell-cell adhesion of MDCK cells by Cdc42 and Rac1 small GTPases. *Biochem Biophys Res Commun* 1997;240(2):430-5.
110. Braga VM, Del Maschio A, Machesky L, Dejana E. Regulation of cadherin function by Rho and Rac: modulation by junction maturation and cellular context. *Mol Biol Cell* 1999;10(1):9-22.
111. Takaishi K, Sasaki T, Kotani H, Nishioka H, Takai Y. Regulation of cell-cell adhesion by rac and rho small G proteins in MDCK cells. *J Cell Biol* 1997;139(4):1047-59.
112. Chu YS, Thomas WA, Eder O, Pincet F, Perez E, Thiery JP, Dufour S. Force measurements in E-cadherin-mediated cell doublets reveal rapid adhesion strengthened by actin cytoskeleton remodeling through Rac and Cdc42. *J Cell Biol* 2004;167(6):1183-94.
113. Vasioukhin V, Bauer C, Yin M, Fuchs E. Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* 2000;100(2):209-19.
114. Ehrlich JS, Hansen MD, Nelson WJ. Spatio-temporal regulation of Rac1 localization and lamellipodia dynamics during epithelial cell-cell adhesion. *Dev Cell* 2002;3(2):259-70.
115. Sahai E, Marshall CJ. ROCK and Dia have opposing effects on adherens junctions downstream of Rho. *Nat Cell Biol* 2002;4(6):408-15.
116. Braga VM, Betson M, Li X, Lamarche-Vane N. Activation of the small GTPase Rac is sufficient to disrupt cadherin- dependent cell-cell adhesion in normal human keratinocytes. *Mol Biol Cell* 2000;11(11):3703-21.

117. Vasioukhin V, Fuchs E. Actin dynamics and cell-cell adhesion in epithelia. *Curr Opin Cell Biol* 2001;13(1):76-84.
118. Fukata M, Kaibuchi K. Rho-family GTPases in cadherin-mediated cell-cell adhesion. *Nature Reviews Molecular Cell Biology* 2001;2(12):887-897.
119. Clark EA, Golub TR, Lander ES, Hynes RO. Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* 2000;406(6795):532-5.
120. Kuroda S, Fukata M, Nakagawa M, Fujii K, Nakamura T, Ookubo T, Izawa I, Nagase T, Nomura N, Tani H, Shoji I, Matsuura Y, et al. Role of IQGAP1, a target of the small GTPases Cdc42 and Rac1, in regulation of E-cadherin-mediated cell-cell adhesion. *Science* 1998;281(5378):832-5.
121. Fukata M, Kuroda S, Nakagawa M, Kawajiri A, Itoh N, Shoji I, Matsuura Y, Yonehara S, Fujisawa H, Kikuchi A, Kaibuchi K. Cdc42 and Rac1 regulate the interaction of IQGAP1 with beta-catenin. *J Biol Chem* 1999;274(37):26044-50.
122. Ellis S, Mellor H. Regulation of endocytic traffic by rho family GTPases. *Trends Cell Biol* 2000;10(3):85-8.
123. Wang B, Wylie FG, Teasdale RD, Stow JL. Polarized trafficking of E-cadherin is regulated by Rac1 and Cdc42 in Madin-Darby canine kidney cells. *Am J Physiol Cell Physiol* 2005;288(6):C1411-9.
124. Reynolds AB, Daniel JM, Mo YY, Wu J, Zhang Z. The novel catenin p120cas binds classical cadherins and induces an unusual morphological phenotype in NIH3T3 fibroblasts. *Exp Cell Res* 1996;225(2):328-37.
125. Noren NK, Liu BP, Burrridge K, Kreft B. p120 catenin regulates the actin cytoskeleton via Rho family GTPases. *J Cell Biol* 2000;150(3):567-80.
126. Grosheva I, Shtutman M, Elbaum M, Bershadsky AD. p120 catenin affects cell motility via modulation of activity of Rho-family GTPases: a link between cell-cell contact formation and regulation of cell locomotion. *J Cell Sci* 2001;114(Pt 4):695-707.
127. Anastasiadis PZ, Moon SY, Thoreson MA, Mariner DJ, Crawford HC, Zheng Y, Reynolds AB. Inhibition of RhoA by p120 catenin. *Nat Cell Biol* 2000;2(9):637-44.
128. Shibata T, Kokubu A, Sekine S, Kanai Y, Hirohashi S. Cytoplasmic p120ctn regulates the invasive phenotypes of E-cadherin-deficient breast cancer. *Am J Pathol* 2004;164(6):2269-78.
129. Taniuchi K, Nakagawa H, Hosokawa M, Nakamura T, Eguchi H, Ohigashi H, Ishikawa O, Katagiri T, Nakamura Y. Overexpressed P-cadherin/CDH3 promotes motility of pancreatic cancer cells by interacting with p120ctn and activating rho-family GTPases. *Cancer Res* 2005;65(8):3092-9.
130. Bellocin DI, Bates RC, Muzikansky A, Rimm DL, Mercurio AM. Altered localization of p120 catenin during epithelial to mesenchymal transition of colon carcinoma is prognostic for aggressive disease. *Cancer Res* 2005;65(23):10938-45.
131. Cozzolino MS, V. Spinardi, L. Campioni, N. Fiorentini, C. Salvati, E. Alema, S. Salvatore, A.M. p120 catenin is required for growth factor-dependent cell motility and scattering in epithelial cells. *Mol Biol Cell* 2003;14(5):1964-1977.
132. Liu WF, Nelson CM, Pirone DM, Chen CS. E-cadherin engagement stimulates proliferation via Rac1. *J Cell Biol* 2006;173(3):431-41.
133. Magie CR, Pinto-Santini D, Parkhurst SM. Rho1 interacts with p120ctn and alpha-catenin, and regulates cadherin-based adherens junction components in *Drosophila*. *Development* 2002;129(16):3771-82.
134. Gavard J, Lambert M, Grosheva I, Marthiens V, Irinopoulou T, Riou JF, Bershadsky A, Mege RM. Lamellipodium extension and cadherin adhesion: two cell responses to cadherin activation relying on distinct signalling pathways. *J Cell Sci* 2004;117(Pt 2):257-70.
135. Pece S, Chiariello M, Murga C, Gutkind JS. Activation of the protein kinase Akt/PKB by the formation of E-cadherin-mediated cell-cell junctions. Evidence for the association of phosphatidylinositol 3-kinase with the E-cadherin adhesion complex. *J Biol Chem* 1999;274(27):19347-51.

136. Nakagawa M, Fukata M, Yamaga M, Itoh N, Kaibuchi K. Recruitment and activation of Rac1 by the formation of E-cadherin-mediated cell-cell adhesion sites. *J Cell Sci* 2001;114(Pt 10):1829-38.
137. Pece S, Gutkind JS. Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. *J Biol Chem* 2000;275(52):41227-33.
138. Calautti E, Cabodi S, Stein PL, Hatzfeld M, Kedersha N, Paolo Dotto G. Tyrosine phosphorylation and src family kinases control keratinocyte cell-cell adhesion. *J Cell Biol* 1998;141(6):1449-65.
139. Briher WM, Yap AS, Gumbiner BM. Lateral dimerization is required for the homophilic binding activity of C-cadherin. *J Cell Biol* 1996;135(2):487-96.
140. Lambert M, Padilla F, Mege RM. Immobilized dimers of N-cadherin-Fc chimera mimic cadherin-mediated cell contact formation: contribution of both outside-in and inside-out signals. *J Cell Sci* 2000;113 (Pt 12):2207-19.
141. Noren NK, Niessen CM, Gumbiner BM, Burridge K. Cadherin engagement regulates Rho family GTPases. *J Biol Chem* 2001;276(36):33305-8.
142. Yap AS, Kovacs EM. Direct cadherin-activated cell signaling: a view from the plasma membrane. *J Cell Biol* 2003;160(1):11-6.
143. Kovacs EM, Ali RG, McCormack AJ, Yap AS. E-cadherin homophilic ligation directly signals through Rac and phosphatidylinositol 3-kinase to regulate adhesive contacts. *J Biol Chem* 2002;277(8):6708-18.
144. van Roy FM, McCrea PD. A role for Kaiso-p120ctn complexes in cancer? *Nat Rev Cancer* 2005;5(12):956-64.
145. Prokhortchouk A, Hendrich B, Jorgensen H, Ruzov A, Wilm M, Georgiev G, Bird A, Prokhortchouk E. The p120 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor. *Genes Dev* 2001;15(13):1613-8.
146. Daniel JM, Spring CM, Crawford HC, Reynolds AB, Baig A. The p120(ctn)-binding partner Kaiso is a bi-modal DNA-binding protein that recognizes both a sequence-specific consensus and methylated CpG dinucleotides. *Nucleic Acids Res* 2002;30(13):2911-9.
147. Kim SW, Park JI, Spring CM, Sater AK, Ji H, Otchere AA, Daniel JM, McCrea PD. Non-canonical Wnt signals are modulated by the Kaiso transcriptional repressor and p120-catenin. *Nat Cell Biol* 2004;6(12):1212-20.
148. Park JI, Kim SW, Lyons JP, Ji H, Nguyen TT, Cho K, Barton MC, Deroo T, Vleminckx K, Moon RT, McCrea PD. Kaiso/p120-catenin and TCF/beta-catenin complexes coordinately regulate canonical Wnt gene targets. *Dev Cell* 2005;8(6):843-54.
149. Spring CM, Kelly KF, O'Kelly I, Graham M, Crawford HC, Daniel JM. The catenin p120ctn inhibits Kaiso-mediated transcriptional repression of the beta-catenin/TCF target gene matrilysin. *Exp Cell Res* 2005;305(2):253-65.
150. Yoon HG, Chan DW, Reynolds AB, Qin J, Wong J. N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding protein Kaiso. *Mol Cell* 2003;12(3):723-34.
151. Rodova M, Kelly KF, VanSaun M, Daniel JM, Werle MJ. Regulation of the rapsyn promoter by kaiso and delta-catenin. *Mol Cell Biol* 2004;24(16):7188-96.
152. Gumbiner BM. Coordinate gene regulation by two different catenins. *Dev Cell* 2005;8(6):795-6.
153. Ruzov A, Dunican DS, Prokhortchouk A, Pennings S, Stancheva I, Prokhortchouk E, Meehan RR. Kaiso is a genome-wide repressor of transcription that is essential for amphibian development. *Development* 2004;131(24):6185-94.
154. Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA, Dove WF. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* 1992;256(5057):668-70.

155. Eads CA, Nickel AE, Laird PW. Complete genetic suppression of polyp formation and reduction of CpG-island hypermethylation in Apc(Min/+) Dnmt1-hypomorphic Mice. *Cancer Res* 2002;62(5):1296-9.
156. Thoreson MA, Reynolds AB. Altered expression of the catenin p120 in human cancer: implications for tumor progression. *Differentiation* 2002;70(9-10):583-9.
157. Skoudy A, Gomez S, Fabre M, Garcia de Herreros A. p120-catenin expression in human colorectal cancer. *Int J Cancer* 1996;68(1):14-20.
158. Gold JS, Reynolds AB, Rimm DL. Loss of p120ctn in human colorectal cancer predicts metastasis and poor survival. *Cancer Lett* 1998;132(1-2):193-201.
159. Karayiannakis AJ, Syrigos KN, Alexiou D, Kalahanis N, Rosenberg T, Bastounis E, Pignatelli M. Expression patterns of the novel catenin p120cas in gastrointestinal cancers. *Anticancer Research* 1999;19(5C):4401-4405.
160. Wang EH, Liu Y, Xu HT, Dai SD, Liu N, Xie CY, Yuan XM. Abnormal expression and clinicopathologic significance of p120-catenin in lung cancer. *Histol Histopathol* 2006;21(8):841-7.
161. Dillon DA, D'Aquila T, Reynolds AB, Fearon ER, Rimm DL. The expression of p120ctn protein in breast cancer is independent of alpha- and beta-catenin and E-cadherin. *Am J Pathol* 1998;152(1):75-82.
162. Sarrio D, Perez-Mies B, Hardisson D, Moreno-Bueno G, Suarez A, Cano A, Martin-Perez J, Gamallo C, Palacios J. Cytoplasmic localization of p120ctn and E-cadherin loss characterize lobular breast carcinoma from preinvasive to metastatic lesions. *Oncogene* 2004;23(19):3272-83.
163. Mastracci TL, Tjan S, Bane AL, O'Malley FP, Andrulis IL. E-cadherin alterations in atypical lobular hyperplasia and lobular carcinoma in situ of the breast. *Mod Pathol* 2005;18(6):741-51.
164. Jawhari AU, Noda M, Pignatelli M, Farthing M. Up-regulated cytoplasmic expression, with reduced membranous distribution, of the src substrate p120(ctn) in gastric carcinoma. *J Pathol* 1999;189(2):180-5.
165. Shimazui T, Schalken JA, Girolodi LA, Jansen CFJ, Akaza H, Koiso K, Debruyne FMJ, Bringuier PP. Prognostic value of cadherin-associated molecules (alpha-, beta-, and gamma-catenins and p120(cas)) in bladder tumors. *Cancer Research* 1996;56(18):4154-4158.
166. Syrigos KN, Karayiannakis A, Syrigou EI, Harrington K, Pignatelli M. Abnormal expression of p120 correlates with poor survival in patients with bladder cancer. *European Journal of Cancer* 1998;34(13):2037-2040.
167. Nakopoulou L, Zervas A, Gakiopoulou-Givalou H, Constantinides C, Doumanis G, Davaris P, Dimopoulos C. Prognostic value of E-cadherin, beta-catenin, P120(ctn) in patients with transitional cell bladder cancer. *Anticancer Research* 2000;20(6B):4571-4578.
168. Wijnhoven BPL, Vis AN, Tilanus HW, Pignatelli M, Dinjens WNM. Prognostic value of p120(ctn) in Barrett's adenocarcinomas. *Journal of Pathology* 2000;190:3A-3A.
169. Kallakury BVS, Sheehan CE, Winn-Deen E, Oliver J, Fisher HAG, Kaufman RP, Ross JS. Decreased expression of catenins (alpha and beta), p120 CTN, and E-cadherin cell adhesion proteins and E-cadherin gene promoter methylation in prostatic adenocarcinomas. *Cancer* 2001;92(11):2786-2795.
170. Husmark J, Heldin NE, Nilsson M. N-cadherin-mediated adhesion and aberrant catenin expression in anaplastic thyroid-carcinoma cell lines. *International Journal of Cancer* 1999;83(5):692-699.
171. Vecsey-Semjen B, Becker KF, Sinski A, Blennow E, Victor I, Zatloukal K, Beug H, Wagner E, Huber LA. Novel colon cancer cell lines leading to better understanding of the diversity of respective primary cancers. *Oncogene* 2002;21(30):4646-62.
172. Ohkubo T, Ozawa M. The transcription factor Snail downregulates the tight junction components independently of E-cadherin downregulation. *J Cell Sci* 2004;117(Pt 9):1675-85.
173. Eger A, Stockinger A, Schaffhauser B, Beug H, Foisner R. Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of beta-catenin and upregulation of beta-catenin/lymphoid enhancer binding factor-1 transcriptional activity. *J Cell Biol* 2000;148(1):173-88.

174. Wu J, Mariner DJ, Thoreson MA, Reynolds AB. Production and characterization of monoclonal antibodies to the catenin p120^{ctn}. *Hybridoma* 1998;17(2):175-83.
175. Skoudy A, Llosas MDM, deHerreros AG. Intestinal HT-29 cells with dysfunction of E-cadherin show increased pp60^{src} activity and tyrosine phosphorylation of p120-catenin. *Biochemical Journal* 1996;317:279-284.
176. Mariner DJ, Davis MA, Reynolds AB. EGFR signaling to p120-catenin through phosphorylation at Y228. *J Cell Sci* 2004;117(Pt 8):1339-50.
177. Nishimura M, Machida K, Imaizumi M, Abe T, Umeda T, Takeshima E, Watanabe T, Ohnishi Y, Takagi K, Hamaguchi M. Tyrosine phosphorylation of 100-130 kDa proteins in lung cancer correlates with poor prognosis. *British Journal of Cancer* 1996;74(5):780-787.
178. Calautti E, Grossi M, Mammucari C, Aoyama Y, Pirro M, Ono Y, Li J, Dotto GP. Fyn tyrosine kinase is a downstream mediator of Rho/PRK2 function in keratinocyte cell-cell adhesion. *J Cell Biol* 2002;156(1):137-48.
179. Pang JH, Kraemer A, Stehbens SJ, Frame MC, Yap AS. Recruitment of phosphoinositide 3-kinase defines a positive contribution of tyrosine kinase signaling to E-cadherin function. *J Biol Chem* 2005;280(4):3043-50.
180. Daniel JM, Reynolds AB. Tyrosine phosphorylation and cadherin/catenin function. *Bioessays* 1997;19(10):883-91.
181. Downing JR, Reynolds AB. PDGF, CSF-1, and EGF induce tyrosine phosphorylation of p120, a pp60^{src} transformation-associated substrate. *Oncogene* 1991;6(4):607-13.
182. Kanner SB, Reynolds AB, Parsons JT. Tyrosine phosphorylation of a 120-kilodalton pp60^{src} substrate upon epidermal growth factor and platelet-derived growth factor receptor stimulation and in polyomavirus middle-T-antigen-transformed cells. *Mol Cell Biol* 1991;11(2):713-20.
183. McManus MJ, Connolly DC, Maihle NJ. Tissue- and transformation-specific phosphotyrosyl proteins in v-erbB-transformed cells. *J Virol* 1995;69(6):3631-8.
184. Esser S, Lampugnani MG, Corada M, Dejana E, Risau W. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. *J Cell Sci* 1998;111 (Pt 13):1853-65.
185. Hamaguchi M, Matsuyoshi N, Ohnishi Y, Gotoh B, Takeichi M, Nagai Y. p60^{v-src} causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *Embo J* 1993;12(1):307-14.
186. Behrens J, Vakaet L, Friis R, Winterhager E, Van Roy F, Mareel MM, Birchmeier W. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J Cell Biol* 1993;120(3):757-66.
187. Shibamoto S, Hayakawa M, Takeuchi K, Hori T, Oku N, Miyazawa K, Kitamura N, Takeichi M, Ito F. Tyrosine phosphorylation of beta-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adhes Commun* 1994;1(4):295-305.
188. Hoschuetzky H, Aberle H, Kemler R. Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. *J Cell Biol* 1994;127(5):1375-80.
189. Shiozaki H, Kadowaki T, Doki Y, Inoue M, Tamura S, Oka H, Iwazawa T, Matsui S, Shimaya K, Takeichi M, et al. Effect of epidermal growth factor on cadherin-mediated adhesion in a human oesophageal cancer cell line. *Br J Cancer* 1995;71(2):250-8.
190. Fujii K, Furukawa F, Matsuyoshi N. Ligand activation of overexpressed epidermal growth factor receptor results in colony dissociation and disturbed E-cadherin function in HSC-1 human cutaneous squamous carcinoma cells. *Exp Cell Res* 1996;223(1):50-62.
191. Shibata T, Ochiai A, Kanai Y, Akimoto S, Gotoh M, Yasui N, Machinami R, Hirohashi S. Dominant negative inhibition of the association between beta-catenin and c-erbB-2 by N-terminally deleted beta-catenin suppresses the invasion and metastasis of cancer cells. *Oncogene* 1996;13(5):883-9.

192. Andre F, Rigot V, Thimonier J, Montixi C, Parat F, Pommier G, Marvaldi J, Luis J. Integrins and E-cadherin cooperate with IGF-I to induce migration of epithelial colonic cells. *Int J Cancer* 1999;83(4):497-505.
193. Matsuyoshi N, Hamaguchi M, Taniguchi S, Nagafuchi A, Tsukita S, Takeichi M. Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J Cell Biol* 1992;118(3):703-14.
194. Tsukita S, Oishi K, Akiyama T, Yamanashi Y, Yamamoto T. Specific proto-oncogenic tyrosine kinases of src family are enriched in cell-to-cell adherens junctions where the level of tyrosine phosphorylation is elevated. *J Cell Biol* 1991;113(4):867-79.
195. Roskoski R, Jr. Src protein-tyrosine kinase structure and regulation. *Biochem Biophys Res Commun* 2004;324(4):1155-64.
196. Roskoski R, Jr. Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun* 2005;331(1):1-14.
197. Martin GS. The hunting of the Src. *Nat Rev Mol Cell Biol* 2001;2(6):467-75.
198. Frame MC. Src in cancer: Deregulation and consequences for cell behaviour. *Biochimica et Biophysica Acta Reviews on Cancer* 2002;21(2):114-130.
199. Irby RB, Yeatman TJ. Role of Src expression and activation in human cancer. *Oncogene* 2000;19(49):5636-42.
200. Bolen JB, Veillette A, Schwartz AM, DeSeau V, Rosen N. Activation of pp60c-src protein kinase activity in human colon carcinoma. *Proc Natl Acad Sci U S A* 1987;84(8):2251-5.
201. Cartwright CA, Meisler AI, Eckhart W. Activation of the pp60c-src protein kinase is an early event in colonic carcinogenesis. *Proc Natl Acad Sci U S A* 1990;87(2):558-62.
202. Talamonti MS, Roh MS, Curley SA, Gallick GE. Increase in activity and level of pp60c-src in progressive stages of human colorectal cancer. *J Clin Invest* 1993;91(1):53-60.
203. Ottenhoff-Kalff AE, Rijksen G, van Beurden EA, Hennipman A, Michels AA, Staal GE. Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-src oncogene product. *Cancer Res* 1992;52(17):4773-8.
204. Verbeek BS, Vroom TM, Adriaansen-Slot SS, Ottenhoff-Kalff AE, Geertzema JG, Hennipman A, Rijksen G. c-Src protein expression is increased in human breast cancer. An immunohistochemical and biochemical analysis. *J Pathol* 1996;180(4):383-8.
205. Takekura N, Yasui W, Yoshida K, Tsujino T, Nakayama H, Kameda T, Yokozaki H, Nishimura Y, Ito H, Tahara E. pp60c-src protein kinase activity in human gastric carcinomas. *Int J Cancer* 1990;45(5):847-51.
206. Lutz MP, Esser IB, Flossmann-Kast BB, Vogelmann R, Luhrs H, Friess H, Buchler MW, Adler G. Overexpression and activation of the tyrosine kinase Src in human pancreatic carcinoma. *Biochem Biophys Res Commun* 1998;243(2):503-8.
207. Kumble S, Omary MB, Cartwright CA, Triadafilopoulos G. Src activation in malignant and premalignant epithelia of Barrett's esophagus. *Gastroenterology* 1997;112(2):348-56.
208. Irby RB, Mao W, Coppola D, Kang J, Loubeau JM, Trudeau W, Karl R, Fujita DJ, Jove R, Yeatman TJ. Activating SRC mutation in a subset of advanced human colon cancers. *Nat Genet* 1999;21(2):187-90.
209. Daigo Y, Furukawa Y, Kawasoe T, Ishiguro H, Fujita M, Sugai S, Nakamori S, Liefers GJ, Tollenaar RA, van de Velde CJ, Nakamura Y. Absence of genetic alteration at codon 531 of the human c-src gene in 479 advanced colorectal cancers from Japanese and Caucasian patients. *Cancer Res* 1999;59(17):4222-4.
210. Wang NM, Yeh KT, Tsai CH, Chen SJ, Chang JG. No evidence of correlation between mutation at codon 531 of src and the risk of colon cancer in Chinese. *Cancer Lett* 2000;150(2):201-4.

211. Laghi L, Bianchi P, Orbetegli O, Gennari L, Roncalli M, Malesci A. Lack of mutation at codon 531 of SRC in advanced colorectal cancers from Italian patients. *Br J Cancer* 2001;84(2):196-8.
212. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005;5(5):341-54.
213. Abram CL, Courtneidge SA. Src family tyrosine kinases and growth factor signaling. *Exp Cell Res* 2000;254(1):1-13.
214. Brunton VG, Ozanne BW, Paraskeva C, Frame MC. A role for epidermal growth factor receptor, c-Src and focal adhesion kinase in an in vitro model for the progression of colon cancer. *Oncogene* 1997;14(3):283-293.
215. Mao W, Irby R, Coppola D, Fu L, Wloch M, Turner J, Yu H, Garcia R, Jove R, Yeatman TJ. Activation of c-Src by receptor tyrosine kinases in human colon cancer cells with high metastatic potential. *Oncogene* 1997;15(25):3083-90.
216. Jones RJ, Brunton VG, Frame MC. Adhesion-linked kinases in cancer; emphasis on Src, focal adhesion kinase and PI 3-kinase. *European Journal of Cancer* 2000;36(13):1595-1606.
217. Masaki T, Okada M, Tokuda M, Shiratori Y, Hatase O, Shirai M, Nishioka M, Omata M. Reduced C-terminal Src kinase (Csk) activities in hepatocellular carcinoma. *Hepatology* 1999;29(2):379-84.
218. Bjorge JD, Pang A, Fujita DJ. Identification of protein-tyrosine phosphatase 1B as the major tyrosine phosphatase activity capable of dephosphorylating and activating c-Src in several human breast cancer cell lines. *J Biol Chem* 2000;275(52):41439-46.
219. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp Cell Res* 2003;284(1):31-53.
220. Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL, Burkhard C, Schuler D, Probst-Hensch NM, Maiorka PC, Baeza N, Pisani P, et al. Genetic pathways to glioblastoma: a population-based study. *Cancer Res* 2004;64(19):6892-9.
221. Sunpaweravong P, Sunpaweravong S, Puttawibul P, Mitarnun W, Zeng C, Baron AE, Franklin W, Said S, Varella-Garcia M. Epidermal growth factor receptor and cyclin D1 are independently amplified and overexpressed in esophageal squamous cell carcinoma. *J Cancer Res Clin Oncol* 2005;131(2):111-9.
222. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995;19(3):183-232.
223. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350(21):2129-39.
224. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304(5676):1497-500.
225. Ochiai A, Akimoto S, Kanai Y, Shibata T, Oyama T, Hirohashi S. c-erbB-2 gene product associates with catenins in human cancer cells. *Biochem Biophys Res Commun* 1994;205(1):73-8.
226. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989;244(4905):707-12.
227. Willmore-Payne C, Holden JA, Layfield LJ. Detection of epidermal growth factor receptor and human epidermal growth factor receptor 2 activating mutations in lung adenocarcinoma by high-resolution melting amplicon analysis: correlation with gene copy number, protein expression, and hormone receptor expression. *Hum Pathol* 2006;37(6):755-63.
228. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* 2003;4(12):915-25.

229. Nakajima M, Sawada H, Yamada Y, Watanabe A, Tatsumi M, Yamashita J, Matsuda M, Sakaguchi T, Hirao T, Nakano H. The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. *Cancer* 1999;85(9):1894-902.
230. Di Renzo MF, Olivero M, Giacomini A, Porte H, Chastre E, Mirossay L, Nordlinger B, Bretti S, Bottardi S, Giordano S, et al. Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. *Clin Cancer Res* 1995;1(2):147-54.
231. Di Renzo MF, Olivero M, Martone T, Maffe A, Maggiora P, Stefani AD, Valente G, Giordano S, Cortesina G, Comoglio PM. Somatic mutations of the MET oncogene are selected during metastatic spread of human HNSC carcinomas. *Oncogene* 2000;19(12):1547-55.
232. Ferracini R, Di Renzo MF, Scotlandi K, Baldini N, Olivero M, Lollini P, Cremona O, Campanacci M, Comoglio PM. The Met/HGF receptor is over-expressed in human osteosarcomas and is activated by either a paracrine or an autocrine circuit. *Oncogene* 1995;10(4):739-49.
233. Osherov N, Levitzki A. Epidermal-growth-factor-dependent activation of the src-family kinases. *Eur J Biochem* 1994;225(3):1047-53.
234. Maa MC, Leu TH, McCarley DJ, Schatzman RC, Parsons SJ. Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src: implications for the etiology of multiple human cancers. *Proc Natl Acad Sci U S A* 1995;92(15):6981-5.
235. Karni R, Jove R, Levitzki A. Inhibition of pp60c-Src reduces Bcl-XL expression and reverses the transformed phenotype of cells overexpressing EGF and HER-2 receptors. *Oncogene* 1999;18(33):4654-62.
236. Brunton VG, MacPherson IR, Frame MC. Cell adhesion receptors, tyrosine kinases and actin modulators: a complex three-way circuitry. *Biochim Biophys Acta* 2004;1692(2-3):121-44.
237. Biscardi JS, Maa MC, Tice DA, Cox ME, Leu TH, Parsons SJ. c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. *J Biol Chem* 1999;274(12):8335-43.
238. Tice DA, Biscardi JS, Nickles AL, Parsons SJ. Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. *Proc Natl Acad Sci U S A* 1999;96(4):1415-20.
239. Gotoh N, Tojo A, Hino M, Yazaki Y, Shibuya M. A highly conserved tyrosine residue at codon 845 within the kinase domain is not required for the transforming activity of human epidermal growth factor receptor. *Biochem Biophys Res Commun* 1992;186(2):768-74.
240. Bromann PA, Korkaya H, Courtneidge SA. The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* 2004;23(48):7957-68.
241. Muthuswamy SK, Siegel PM, Dankort DL, Webster MA, Muller WJ. Mammary tumors expressing the neu proto-oncogene possess elevated c-Src tyrosine kinase activity. *Mol Cell Biol* 1994;14(1):735-43.
242. Ponzetto C, Bardelli A, Zhen Z, Maina F, dalla Zonca P, Giordano S, Graziani A, Panayotou G, Comoglio PM. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell* 1994;77(2):261-71.
243. Sallee JL, Wittchen ES, Burridge K. Regulation of Cell Adhesion by Protein-tyrosine Phosphatases: II. CELL-CELL ADHESION. *J Biol Chem* 2006;281(24):16189-92.
244. Brady-Kalnay SM, Rimm DL, Tonks NK. Receptor protein tyrosine phosphatase PTPmu associates with cadherins and catenins in vivo. *J Cell Biol* 1995;130(4):977-86.
245. Fuchs M, Muller T, Lerch MM, Ullrich A. Association of human protein-tyrosine phosphatase kappa with members of the armadillo family. *J Biol Chem* 1996;271(28):16712-9.
246. Balsamo J, Leung T, Ernst H, Zanin MK, Hoffman S, Lilien J. Regulated binding of PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of beta-catenin. *J Cell Biol* 1996;134(3):801-13.
247. Zondag GC, Reynolds AB, Moolenaar WH. Receptor protein-tyrosine phosphatase RPTPmu binds to and dephosphorylates the catenin p120(ctn). *J Biol Chem* 2000;275(15):11264-9.

248. Keilhack H, Hellman U, van Hengel J, van Roy F, Godovac-Zimmermann J, Bohmer FD. The protein-tyrosine phosphatase SHP-1 binds to and dephosphorylates p120 catenin. *J Biol Chem* 2000;275(34):26376-84.
249. Holsinger LJ, Ward K, Duffield B, Zachwieja J, Jallal B. The transmembrane receptor protein tyrosine phosphatase DEP1 interacts with p120(ctn). *Oncogene* 2002;21(46):7067-76.
250. Ostman A, Yang Q, Tonks NK. Expression of DEP-1, a receptor-like protein-tyrosine-phosphatase, is enhanced with increasing cell density. *Proc Natl Acad Sci U S A* 1994;91(21):9680-4.
251. Lampugnani MG, Corada M, Andriopoulou P, Esser S, Risau W, Dejana E. Cell confluence regulates tyrosine phosphorylation of adherens junction components in endothelial cells. *J Cell Sci* 1997;110 (Pt 17):2065-77.
252. Fukuyama T, Ogita H, Kawakatsu T, Inagaki M, Takai Y. Activation of Rac by cadherin through the c-Src-Rap1-phosphatidylinositol 3-kinase-Vav2 pathway. *Oncogene* 2006;25(1):8-19.
253. Hogan C, Serpente N, Cogram P, Hosking CR, Bialucha CU, Feller SM, Braga VM, Birchmeier W, Fujita Y. Rap1 regulates the formation of E-cadherin-based cell-cell contacts. *Mol Cell Biol* 2004;24(15):6690-700.
254. Volberg T, Zick Y, Dror R, Sabanay I, Gilon C, Levitzki A, Geiger B. The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *Embo J* 1992;11(5):1733-42.
255. Irby RB, Yeatman TJ. Increased Src activity disrupts cadherin/catenin-mediated homotypic adhesion in human colon cancer and transformed rodent cells. *Cancer Res* 2002;62(9):2669-74.
256. Lotz M, Wang HH, Cance W, Matthews J, Pories S. Epidermal growth factor stimulation can substitute for c-Src overexpression in promoting breast carcinoma invasion. *J Surg Res* 2003;109(2):123-9.
257. Avizienyte E, Wyke AW, Jones RJ, McLean GW, Westhoff MA, Brunton VG, Frame MC. Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling. *Nature Cell Biology* 2002;4(8):632-638.
258. Hazan RB, Norton L. The epidermal growth factor receptor modulates the interaction of E-cadherin with the actin cytoskeleton. *J Biol Chem* 1998;273(15):9078-84.
259. Hiscox S, Jiang WG. Association of the HGF/SF receptor, c-met, with the cell-surface adhesion molecule, E-cadherin, and catenins in human tumor cells. *Biochem Biophys Res Commun* 1999;261(2):406-11.
260. Muller T, Choidas A, Reichmann E, Ullrich A. Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. *J Biol Chem* 1999;274(15):10173-83.
261. Taddei ML, Chiarugi P, Cirri P, Buricchi F, Fiaschi T, Giannoni E, Talini D, Cozzi G, Formigli L, Raugei G, Ramponi G. Beta-catenin interacts with low-molecular-weight protein tyrosine phosphatase leading to cadherin-mediated cell-cell adhesion increase. *Cancer Res* 2002;62(22):6489-99.
262. Balsamo J, Arregui C, Leung T, Lilien J. The nonreceptor protein tyrosine phosphatase PTP1B binds to the cytoplasmic domain of N-cadherin and regulates the cadherin-actin linkage. *J Cell Biol* 1998;143(2):523-32.
263. Gumbiner BM. Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol* 2005;6(8):622-34.
264. Qi J, Wang J, Romanyuk O, Siu CH. Involvement of Src family kinases in N-cadherin phosphorylation and beta-catenin dissociation during transendothelial migration of melanoma cells. *Mol Biol Cell* 2006;17(3):1261-72.
265. Kinch MS, Clark GJ, Der CJ, Burrridge K. Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. *J Cell Biol* 1995;130(2):461-71.
266. Roura S, Miravet S, Piedra J, Garcia de Herreros A, Dunach M. Regulation of E-cadherin/catenin association by tyrosine phosphorylation. *Journal of Biological Chemistry* 1999;274(51):36734-36740.

267. Piedra J, Martinez D, Castano J, Miravet S, Dunach M, de Herreros AG. Regulation of beta-catenin structure and activity by tyrosine phosphorylation. *J Biol Chem* 2001;276(23):20436-43.
268. Piedra J, Miravet S, Castano J, Palmer HG, Heisterkamp N, Garcia de Herreros A, Dunach M. p120 Catenin-associated Fer and Fyn tyrosine kinases regulate beta- catenin Tyr-142 phosphorylation and beta-catenin-alpha-catenin Interaction. *Mol Cell Biol* 2003;23(7):2287-97.
269. Brembeck FH, Schwarz-Romond T, Bakkers J, Wilhelm S, Hammerschmidt M, Birchmeier W. Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions. *Genes Dev* 2004;18(18):2225-30.
270. Brembeck FH, Rosario M, Birchmeier W. Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. *Curr Opin Genet Dev* 2006;16(1):51-9.
271. Reynolds AB, Carnahan RH. Regulation of cadherin stability and turnover by p120ctn: implications in disease and cancer. *Semin Cell Dev Biol* 2004;15(6):657-63.
272. Aono S, Nakagawa S, Reynolds AB, Takeichi M. p120(ctn) acts as an inhibitory regulator of cadherin function in colon carcinoma cells. *J Cell Biol* 1999;145(3):551-62.
273. Ozawa M, Ohkubo T. Tyrosine phosphorylation of p120(ctn) in v-Src transfected L cells depends on its association with E-cadherin and reduces adhesion activity. *Journal of Cell Science* 2001;114(Pt 3):503-12.
274. Shibamoto S, Hayakawa M, Takeuchi K, Hori T, Miyazawa K, Kitamura N, Johnson KR, Wheelock MJ, Matsuyoshi N, Takeichi M, et al. Association of p120, a tyrosine kinase substrate, with E-cadherin/catenin complexes. *J Cell Biol* 1995;128(5):949-57.
275. Papkoff J. Regulation of complexed and free catenin pools by distinct mechanisms. Differential effects of Wnt-1 and v-Src. *Journal of Biological Chemistry* 1997;272(7):4536-43.
276. Kim L, Wong TW. The cytoplasmic tyrosine kinase FER is associated with the catenin-like substrate pp120 and is activated by growth factors. *Mol Cell Biol* 1995;15(8):4553-61.
277. Xu G, Craig AW, Greer P, Miller M, Anastasiadis PZ, Lilien J, Balsamo J. Continuous association of cadherin with beta-catenin requires the non-receptor tyrosine-kinase Fer. *J Cell Sci* 2004;117(Pt 15):3207-19.
278. Avizienyte E, Fincham VJ, Brunton VG, Frame MC. Src SH3/2 domain-mediated peripheral accumulation of Src and phospho-myosin is linked to deregulation of E-cadherin and the epithelial-mesenchymal transition. *Mol Biol Cell* 2004;15(6):2794-803.
279. de Rooij J, Kerstens A, Danuser G, Schwartz MA, Waterman-Storer CM. Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *J Cell Biol* 2005;171(1):153-64.
280. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* 2001;12(1):27-36.
281. Carothers AM, Javid SH, Moran AE, Hunt DH, Redston M, Bertagnolli MM. Deficient E-cadherin adhesion in C57BL/6J-Min/+ mice is associated with increased tyrosine kinase activity and RhoA-dependent actomyosin contractility. *Exp Cell Res* 2006;312(4):387-400.
282. Van Aken E, De Wever O, Correia da Rocha AS, Mareel M. Defective E-cadherin/catenin complexes in human cancer. *Virchows Arch* 2001;439(6):725-51.
283. Oliveira C, Seruca R, Caldas C. Genetic screening for hereditary diffuse gastric cancer. *Expert Rev Mol Diagn* 2003;3(2):201-15.
284. Grady WM, Willis J, Guilford PJ, Dunbier AK, Toro TT, Lynch H, Wiesner G, Ferguson K, Eng C, Park JG, Kim SJ, Markowitz S. Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer. *Nat Genet* 2000;26(1):16-7.
285. Knudson AG, Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 1971;68(4):820-3.

286. Becker KF, Atkinson MJ, Reich U, Becker I, Nekarda H, Siewert JR, Hofler H. E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. *Cancer Res* 1994;54(14):3845-52.
287. Tamura G, Sakata K, Nishizuka S, Maesawa C, Suzuki Y, Iwaya T, Terashima M, Saito K, Satodate R. Inactivation of the E-cadherin gene in primary gastric carcinomas and gastric carcinoma cell lines. *Jpn J Cancer Res* 1996;87(11):1153-9.
288. Berx G, Cleton-Jansen AM, Strumane K, de Leeuw WJ, Nollet F, van Roy F, Cornelisse C. E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. *Oncogene* 1996;13(9):1919-25.
289. Risinger JI, Berchuck A, Kohler MF, Boyd J. Mutations of the E-cadherin gene in human gynecologic cancers. *Nat Genet* 1994;7(1):98-102.
290. Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, Isaacs WB, Pitha PM, Davidson NE, Baylin SB. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res* 1995;55(22):5195-9.
291. Rathi A, Virmani AK, Schorge JO, Elias KJ, Maruyama R, Minna JD, Mok SC, Girard L, Fishman DA, Gazdar AF. Methylation profiles of sporadic ovarian tumors and nonmalignant ovaries from high-risk women. *Clin Cancer Res* 2002;8(11):3324-31.
292. Corn PG, Heath EI, Heitmiller R, Fogt F, Forastiere AA, Herman JG, Wu TT. Frequent hypermethylation of the 5' CpG island of E-cadherin in esophageal adenocarcinoma. *Clin Cancer Res* 2001;7(9):2765-9.
293. Wheeler JM, Kim HC, Efsthathiou JA, Ilyas M, Mortensen NJ, Bodmer WF. Hypermethylation of the promoter region of the E-cadherin gene (CDH1) in sporadic and ulcerative colitis associated colorectal cancer. *Gut* 2001;48(3):367-71.
294. Yanagawa N, Tamura G, Oizumi H, Takahashi N, Shimazaki Y, Motoyama T. Promoter hypermethylation of tumor suppressor and tumor-related genes in non-small cell lung cancers. *Cancer Sci* 2003;94(7):589-92.
295. Kanai Y, Ushijima S, Hui AM, Ochiai A, Tsuda H, Sakamoto M, Hirohashi S. The E-cadherin gene is silenced by CpG methylation in human hepatocellular carcinomas. *Int J Cancer* 1997;71(3):355-9.
296. Bornman DM, Mathew S, Alsrue J, Herman JG, Gabrielson E. Methylation of the E-cadherin gene in bladder neoplasia and in normal urothelial epithelium from elderly individuals. *Am J Pathol* 2001;159(3):831-5.
297. Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H, Hinoda Y, Imai K. Distinct methylation pattern and microsatellite instability in sporadic gastric cancer. *Int J Cancer* 1999;83(3):309-13.
298. Saito Y, Takazawa H, Uzawa K, Tanzawa H, Sato K. Reduced expression of E-cadherin in oral squamous cell carcinoma: relationship with DNA methylation of 5' CpG island. *Int J Oncol* 1998;12(2):293-8.
299. Yoshiura K, Kanai Y, Ochiai A, Shimoyama Y, Sugimura T, Hirohashi S. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc Natl Acad Sci U S A* 1995;92(16):7416-9.
300. Si HX, Tsao SW, Lam KY, Srivastava G, Liu Y, Wong YC, Shen ZY, Cheung AL. E-cadherin expression is commonly downregulated by CpG island hypermethylation in esophageal carcinoma cells. *Cancer Lett* 2001;173(1):71-8.
301. Debruyne PR, Vermeulen SJ, Berx G, Pocard M, Correia da Rocha AS, Li X, Cirnes L, Poupon MF, van Roy FM, Mareel MM. Functional and molecular characterization of the epithelioid to round transition in human colorectal cancer LoVo cells. *Oncogene* 2003;22(46):7199-208.
302. Tsutsumida A, Hamada J, Tada M, Aoyama T, Furuuchi K, Kawai Y, Yamamoto Y, Sugihara T, Moriuchi T. Epigenetic silencing of E- and P-cadherin gene expression in human melanoma cell lines. *Int J Oncol* 2004;25(5):1415-21.

303. Fearon ER. BRCA1 and E-cadherin promoter hypermethylation and gene inactivation in cancer-association or mechanism? *J Natl Cancer Inst* 2000;92(7):515-7.
304. Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* 2000;2(2):84-9.
305. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2000;2(2):76-83.
306. Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, van Roy F. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell* 2001;7(6):1267-78.
307. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 2004;117(7):927-39.
308. Palacios F, Price L, Schweitzer J, Collard JG, D'Souza-Schorey C. An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration. *Embo J* 2001;20(17):4973-86.
309. Marambaud P, Shioi J, Serban G, Georgakopoulos A, Sarner S, Nagy V, Baki L, Wen P, Efthimiopoulos S, Shao Z, Wisniewski T, Robakis NK. A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *Embo J* 2002;21(8):1948-56.
310. Rios-Doria J, Day KC, Kuefer R, Rashid MG, Chinnaiyan AM, Rubin MA, Day ML. The role of calpain in the proteolytic cleavage of E-cadherin in prostate and mammary epithelial cells. *J Biol Chem* 2003;278(2):1372-9.
311. Maretzky T, Reiss K, Ludwig A, Buchholz J, Scholz F, Proksch E, de Strooper B, Hartmann D, Saftig P. ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. *Proc Natl Acad Sci U S A* 2005;102(26):9182-7.
312. Noe V, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, Bruyneel E, Matrisian LM, Mareel M. Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J Cell Sci* 2001;114(Pt 1):111-118.
313. Davies G, Jiang WG, Mason MD. Matrilysin mediates extracellular cleavage of E-cadherin from prostate cancer cells: a key mechanism in hepatocyte growth factor/scatter factor-induced cell-cell dissociation and in vitro invasion. *Clin Cancer Res* 2001;7(10):3289-97.
314. Hajra KM, Chen DY, Fearon ER. The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res* 2002;62(6):1613-8.
315. Brabletz T, Hlubek F, Spaderna S, Schmalhofer O, Hiendlmeyer E, Jung A, Kirchner T. Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. *Cells Tissues Organs* 2005;179(1-2):56-65.
316. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002;2(6):442-54.
317. Tarin D, Thompson EW, Newgreen DF. The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* 2005;65(14):5996-6000; discussion 6000-1.
318. Thompson EW, Newgreen DF, Tarin D. Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition? *Cancer Res* 2005;65(14):5991-5; discussion 5995.
319. Nakajima S, Doi R, Toyoda E, Tsuji S, Wada M, Koizumi M, Tulachan SS, Ito D, Kami K, Mori T, Kawaguchi Y, Fujimoto K, et al. N-cadherin expression and epithelial-mesenchymal transition in pancreatic carcinoma. *Clin Cancer Res* 2004;10(12 Pt 1):4125-33.

320. Rosivatz E, Becker I, Specht K, Fricke E, Lubert B, Busch R, Hofler H, Becker KF. Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer. *Am J Pathol* 2002;161(5):1881-91.
321. Petersen OW, Nielsen HL, Gudjonsson T, Villadsen R, Rank F, Niebuhr E, Bissell MJ, Ronnov-Jessen L. Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. *Am J Pathol* 2003;162(2):391-402.
322. Grunert S, Jechlinger M, Beug H. Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. *Nat Rev Mol Cell Biol* 2003;4(8):657-65.
323. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005;17(5):548-58.
324. Nawshad A, Lagamba D, Polad A, Hay ED. Transforming growth factor-beta signaling during epithelial-mesenchymal transformation: implications for embryogenesis and tumor metastasis. *Cells Tissues Organs* 2005;179(1-2):11-23.
325. Ackland ML, Newgreen DF, Fridman M, Waltham MC, Arvanitis A, Minichiello J, Price JT, Thompson EW. Epidermal growth factor-induced epithelial-mesenchymal transition in human breast carcinoma cells. *Lab Invest* 2003;83(3):435-48.
326. Docherty NG, O'Sullivan OE, Healy DA, Murphy M, O'Neill A J, Fitzpatrick JM, Watson RW. TGF-beta1-induced EMT can occur independently of its proapoptotic effects and is aided by EGF receptor activation. *Am J Physiol Renal Physiol* 2006;290(5):F1202-12.
327. Davies M, Robinson M, Smith E, Huntley S, Prime S, Paterson I. Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-beta1 involves MAPK, Smad and AP-1 signalling pathways. *J Cell Biochem* 2005;95(5):918-31.
328. Khoury H, Dankort DL, Sadekova S, Naujokas MA, Muller WJ, Park M. Distinct tyrosine autophosphorylation sites mediate induction of epithelial mesenchymal like transition by an activated ErbB-2/Neu receptor. *Oncogene* 2001;20(7):788-99.
329. Jenndahl LE, Isakson P, Baeckstrom D. c-erbB2-induced epithelial-mesenchymal transition in mammary epithelial cells is suppressed by cell-cell contact and initiated prior to E-cadherin downregulation. *Int J Oncol* 2005;27(2):439-48.
330. Morali OG, Delmas V, Moore R, Jeanney C, Thiery JP, Larue L. IGF-II induces rapid beta-catenin relocation to the nucleus during epithelium to mesenchyme transition. *Oncogene* 2001;20(36):4942-50.
331. Valles AM, Boyer B, Badet J, Tucker GC, Barritault D, Thiery JP. Acidic fibroblast growth factor is a modulator of epithelial plasticity in a rat bladder carcinoma cell line. *Proc Natl Acad Sci U S A* 1990;87(3):1124-8.
332. Billottet C, Elkhatib N, Thiery JP, Jouanneau J. Targets of fibroblast growth factor 1 (FGF-1) and FGF-2 signaling involved in the invasive and tumorigenic behavior of carcinoma cells. *Mol Biol Cell* 2004;15(10):4725-34.
333. Avizienyte E, Frame MC. Src and FAK signalling controls adhesion fate and the epithelial-to-mesenchymal transition. *Curr Opin Cell Biol* 2005;17(5):542-7.
334. Malek RL, Irby RB, Guo QM, Lee K, Wong S, He M, Tsai J, Frank B, Liu ET, Quackenbush J, Jove R, Yeatman TJ, et al. Identification of Src transformation fingerprint in human colon cancer. *Oncogene* 2002;21(47):7256-65.
335. Irby RB, Malek RL, Bloom G, Tsai J, Letwin N, Frank BC, Verratti K, Yeatman TJ, Lee NH. Iterative microarray and RNA interference-based interrogation of the SRC-induced invasive phenotype. *Cancer Res* 2005;65(5):1814-21.
336. Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, Lochner D, Birchmeier W. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* 1991;113(1):173-85.

337. Vleminckx K, Vakaet L, Jr., Mareel M, Fiers W, van Roy F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 1991;66(1):107-19.
338. Margulis A, Zhang W, Alt-Holland A, Crawford HC, Fusenig NE, Garlick JA. E-cadherin suppression accelerates squamous cell carcinoma progression in three-dimensional, human tissue constructs. *Cancer Res* 2005;65(5):1783-91.
339. Zheng ZH, Sun XJ, Zhou HT, Shang C, Ji H, Sun KL. Analysis of metastasis suppressing function of E-cadherin in gastric cancer cells by RNAi. *World J Gastroenterol* 2005;11(13):2000-3.
340. Perl AK, Wilgenbus P, Dahl U, Semb H, Christofori G. A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* 1998;392(6672):190-3.
341. Hanahan D. Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* 1985;315(6015):115-22.
342. Suriano G, Mulholland D, de Wever O, Ferreira P, Mateus AR, Bruyneel E, Nelson CC, Mareel MM, Yokota J, Huntsman D, Seruca R. The intracellular E-cadherin germline mutation V832 M lacks the ability to mediate cell-cell adhesion and to suppress invasion. *Oncogene* 2003;22(36):5716-9.
343. Suriano G, Oliveira C, Ferreira P, Machado JC, Bordin MC, De Wever O, Bruyneel EA, Moguilevsky N, Grehan N, Porter TR, Richards FM, Hruban RH, et al. Identification of CDH1 germline missense mutations associated with functional inactivation of the E-cadherin protein in young gastric cancer probands. *Hum Mol Genet* 2003;12(5):575-82.
344. Suriano G, Oliveira MJ, Huntsman D, Mateus AR, Ferreira P, Casares F, Oliveira C, Carneiro F, Machado JC, Mareel M, Seruca R. E-cadherin germline missense mutations and cell phenotype: evidence for the independence of cell invasion on the motile capabilities of the cells. *Hum Mol Genet* 2003;12(22):3007-16.
345. Chibana Y, Fujii S, Ichikawa K, Fujita M, Ono Y, Tomita S, Imura J, Kawamata H, Terano A, Fujimori T. Tumor cell dissociation score highly correlates with lymph node metastasis in superficial esophageal carcinoma. *J Gastroenterol Hepatol* 2005;20(9):1371-8.
346. Colpaert CG, Vermeulen PB, Benoy I, Soubry A, van Roy F, van Beest P, Goovaerts G, Dirix LY, van Dam P, Fox SB, Harris AL, van Marck EA. Inflammatory breast cancer shows angiogenesis with high endothelial proliferation rate and strong E-cadherin expression. *Br J Cancer* 2003;88(5):718-25.
347. Van den Eynden GG, Van der Auwera I, Van Laere S, Colpaert CG, van Dam P, Merajver S, Kleer CG, Harris AL, Van Marck EA, Dirix LY, Vermeulen PB. Validation of a tissue microarray to study differential protein expression in inflammatory and non-inflammatory breast cancer. *Breast Cancer Res Treat* 2004;85(1):13-22.
348. Rasbridge SA, Gillett CE, Sampson SA, Walsh FS, Millis RR. Epithelial (E-) and placental (P-) cadherin cell adhesion molecule expression in breast carcinoma. *J Pathol* 1993;169(2):245-50.
349. Cavallaro U, Christofori G. Cell adhesion in tumor invasion and metastasis: loss of the glue is not enough. *Biochim Biophys Acta* 2001;1552(1):39-45.
350. Wong AS, Gumbiner BM. Adhesion-independent mechanism for suppression of tumor cell invasion by E-cadherin. *J Cell Biol* 2003;161(6):1191-203.
351. Behrens J, Mareel MM, Van Roy FM, Birchmeier W. Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J Cell Biol* 1989;108(6):2435-47.
352. Sommers CL, Thompson EW, Torri JA, Kemler R, Gelmann EP, Byers SW. Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities. *Cell Growth Differ* 1991;2(8):365-72.
353. Boon EM, van der Neut R, van de Wetering M, Clevers H, Pals ST. Wnt signaling regulates expression of the receptor tyrosine kinase met in colorectal cancer. *Cancer Res* 2002;62(18):5126-8.
354. Muller T, Bain G, Wang X, Papkoff J. Regulation of epithelial cell migration and tumor formation by beta-catenin signaling. *Exp Cell Res* 2002;280(1):119-33.

355. Hlubek F, Jung A, Kotzor N, Kirchner T, Brabletz T. Expression of the invasion factor laminin gamma2 in colorectal carcinomas is regulated by beta-catenin. *Cancer Res* 2001;61(22):8089-93.
356. Hiendlmeyer E, Regus S, Wassermann S, Hlubek F, Haynl A, Dimmler A, Koch C, Knoll C, van Beest M, Reuning U, Brabletz T, Kirchner T, et al. Beta-catenin up-regulates the expression of the urokinase plasminogen activator in human colorectal tumors. *Cancer Res* 2004;64(4):1209-14.
357. Sanson B, White P, Vincent JP. Uncoupling cadherin-based adhesion from wingless signalling in *Drosophila*. *Nature* 1996;383(6601):627-30.
358. Fagotto F, Funayama N, Gluck U, Gumbiner BM. Binding to cadherins antagonizes the signaling activity of beta-catenin during axis formation in *Xenopus*. *J Cell Biol* 1996;132(6):1105-14.
359. Orsulic S, Huber O, Aberle H, Arnold S, Kemler R. E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. *J Cell Sci* 1999;112 (Pt 8):1237-45.
360. Stockinger A, Eger A, Wolf J, Beug H, Foisner R. E-cadherin regulates cell growth by modulating proliferation-dependent beta-catenin transcriptional activity. *J Cell Biol* 2001;154(6):1185-96.
361. van de Wetering M, Barker N, Harkes IC, van der Heyden M, Dijk NJ, Hollestelle A, Klijn JG, Clevers H, Schutte M. Mutant E-cadherin breast cancer cells do not display constitutive Wnt signaling. *Cancer Res* 2001;61(1):278-84.
362. Caca K, Kolligs FT, Ji X, Hayes M, Qian J, Yahanda A, Rimm DL, Costa J, Fearon ER. Beta- and gamma-catenin mutations, but not E-cadherin inactivation, underlie T-cell factor/lymphoid enhancer factor transcriptional deregulation in gastric and pancreatic cancer. *Cell Growth Differ* 1999;10(6):369-76.
363. Deng J, Miller SA, Wang HY, Xia W, Wen Y, Zhou BP, Li Y, Lin SY, Hung MC. beta-catenin interacts with and inhibits NF-kappa B in human colon and breast cancer. *Cancer Cell* 2002;2(4):323-34.
364. Tomita K, van Bokhoven A, van Leenders GJ, Ruijter ET, Jansen CF, Bussemakers MJ, Schalken JA. Cadherin switching in human prostate cancer progression. *Cancer Res* 2000;60(13):3650-4.
365. Paredes J, Milanezi F, Reis-Filho JS, Leitao D, Athanazio D, Schmitt F. Aberrant P-cadherin expression: is it associated with estrogen-independent growth in breast cancer? *Pathol Res Pract* 2002;198(12):795-801.
366. Islam S, Carey TE, Wolf GT, Wheelock MJ, Johnson KR. Expression of N-cadherin by human squamous carcinoma cells induces a scattered fibroblastic phenotype with disrupted cell-cell adhesion. *J Cell Biol* 1996;135(6 Pt 1):1643-54.
367. Pishvaian MJ, Feltes CM, Thompson P, Bussemakers MJ, Schalken JA, Byers SW. Cadherin-11 is expressed in invasive breast cancer cell lines. *Cancer Res* 1999;59(4):947-52.
368. Bussemakers MJ, Van Bokhoven A, Tomita K, Jansen CF, Schalken JA. Complex cadherin expression in human prostate cancer cells. *Int J Cancer* 2000;85(3):446-50.
369. Nieman MT, Prudoff RS, Johnson KR, Wheelock MJ. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *J Cell Biol* 1999;147(3):631-44.
370. Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. *J Cell Biol* 2000;148(4):779-90.
371. Youn YH, Hong J, Burke JM. Endogenous N-cadherin in a subpopulation of MDCK cells: distribution and catenin complex composition. *Exp Cell Res* 2005;303(2):275-86.
372. Paredes J, Stove C, Stove V, Milanezi F, Van Marck V, Derycke L, Mareel M, Bracke M, Schmitt F. P-cadherin is up-regulated by the antiestrogen ICI 182,780 and promotes invasion of human breast cancer cells. *Cancer Res* 2004;64(22):8309-17.
373. Foty RA, Steinberg MS. Measurement of tumor cell cohesion and suppression of invasion by E- or P-cadherin. *Cancer Res* 1997;57(22):5033-6.

374. Van Marck V, Stove C, Van Den Bossche K, Stove V, Paredes J, Vander Haeghen Y, Bracke M. P-cadherin promotes cell-cell adhesion and counteracts invasion in human melanoma. *Cancer Res* 2005;65(19):8774-83.
375. Cavallaro U, Schaffhauser B, Christofori G. Cadherins and the tumour progression: is it all in a switch? *Cancer Lett* 2002;176(2):123-8.
376. De Wever O, Mareel M. Role of tissue stroma in cancer cell invasion. *J Pathol* 2003;200(4):429-47.
377. Hazan RB, Kang L, Whooley BP, Borgen PI. N-cadherin promotes adhesion between invasive breast cancer cells and the stroma. *Cell Adhes Commun* 1997;4(6):399-411.
378. Suyama K, Shapiro I, Guttman M, Hazan RB. A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor. *Cancer Cell* 2002;2(4):301-14.
379. Williams EJ, Furness J, Walsh FS, Doherty P. Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. *Neuron* 1994;13(3):583-94.
380. Johnson E, Theisen CS, Johnson KR, Wheelock MJ. R-cadherin influences cell motility via Rho family GTPases. *J Biol Chem* 2004;279(30):31041-9.
381. Seidel B, Braeg S, Adler G, Wedlich D, Menke A. E- and N-cadherin differ with respect to their associated p120ctn isoforms and their ability to suppress invasive growth in pancreatic cancer cells. *Oncogene* 2004;23(32):5532-42.
382. Pories SE, Hess DT, Swenson K, Lotz M, Moussa R, Steele G, Jr., Shibata D, Rieger-Christ KM, Summerhayes C. Overexpression of pp60c-src elicits invasive behavior in rat colon epithelial cells. *Gastroenterology* 1998;114(6):1287-95.
383. Kadono Y, Okada Y, Namiki M, Seiki M, Sato H. Transformation of epithelial Madin-Darby canine kidney cells with p60(v-src) induces expression of membrane-type 1 matrix metalloproteinase and invasiveness. *Cancer Res* 1998;58(10):2240-4.
384. Nakagawa T, Tanaka S, Suzuki H, Takayanagi H, Miyazaki T, Nakamura K, Tsuruo T. Overexpression of the csk gene suppresses tumor metastasis in vivo. *Int J Cancer* 2000;88(3):384-91.
385. Rengifo-Cam W, Konishi A, Morishita N, Matsuoka H, Yamori T, Nada S, Okada M. Csk defines the ability of integrin-mediated cell adhesion and migration in human colon cancer cells: implication for a potential role in cancer metastasis. *Oncogene* 2004;23(1):289-97.
386. Boyer B, Bourgeois Y, Poupon MF. Src kinase contributes to the metastatic spread of carcinoma cells. *Oncogene* 2002;21(15):2347-56.
387. Trevino JG, Summy JM, Lesslie DP, Parikh NU, Hong DS, Lee FY, Donato NJ, Abbruzzese JL, Baker CH, Gallick GE. Inhibition of SRC expression and activity inhibits tumor progression and metastasis of human pancreatic adenocarcinoma cells in an orthotopic nude mouse model. *Am J Pathol* 2006;168(3):962-72.
388. Trevino JG, Summy JM, Gallick GE. SRC inhibitors as potential therapeutic agents for human cancers. *Mini Rev Med Chem* 2006;6(6):681-7.
389. Hiscox S, Morgan L, Green TP, Barrow D, Gee J, Nicholson RI. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. *Breast Cancer Res Treat* 2005;1-12.
390. Johnson FM, Saigal B, Talpaz M, Donato NJ. Dasatinib (BMS-354825) tyrosine kinase inhibitor suppresses invasion and induces cell cycle arrest and apoptosis of head and neck squamous cell carcinoma and non-small cell lung cancer cells. *Clin Cancer Res* 2005;11(19 Pt 1):6924-32.
391. Nam S, Kim D, Cheng JQ, Zhang S, Lee JH, Buettner R, Mirosevich J, Lee FY, Jove R. Action of the Src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells. *Cancer Res* 2005;65(20):9185-9.
392. Zheng R, Yano S, Matsumori Y, Nakataki E, Muguruma H, Yoshizumi M, Sone S. SRC tyrosine kinase inhibitor, m475271, suppresses subcutaneous growth and production of lung metastasis via inhibition

of proliferation, invasion, and vascularization of human lung adenocarcinoma cells. *Clin Exp Metastasis* 2005;22(3):195-204.

393. Recchia I, Rucci N, Festuccia C, Bologna M, MacKay AR, Migliaccio S, Longo M, Susa M, Fabbro D, Teti A. Pyrrolopyrimidine c-Src inhibitors reduce growth, adhesion, motility and invasion of prostate cancer cells in vitro. *Eur J Cancer* 2003;39(13):1927-35.

394. Tan M, Li P, Klos KS, Lu J, Lan KH, Nagata Y, Fang D, Jing T, Yu D. ErbB2 promotes Src synthesis and stability: novel mechanisms of Src activation that confer breast cancer metastasis. *Cancer Res* 2005;65(5):1858-67.

395. Yezhelyev MV, Koehl G, Guba M, Brabletz T, Jauch KW, Ryan A, Barge A, Green T, Fennell M, Bruns CJ. Inhibition of SRC tyrosine kinase as treatment for human pancreatic cancer growing orthotopically in nude mice. *Clin Cancer Res* 2004;10(23):8028-36.

396. Shibata T, Kawano T, Nagayasu H, Okumura K, Arisue M, Hamada J, Takeichi N, Hosokawa M. Enhancing effects of epidermal growth factor on human squamous cell carcinoma motility and matrix degradation but not growth. *Tumour Biol* 1996;17(3):168-75.

397. Malliri A, Symons M, Hennigan RF, Hurlstone AF, Lamb RF, Wheeler T, Ozanne BW. The transcription factor AP-1 is required for EGF-induced activation of rho-like GTPases, cytoskeletal rearrangements, motility, and in vitro invasion of A431 cells. *J Cell Biol* 1998;143(4):1087-99.

398. Price JT, Wilson HM, Haites NE. Epidermal growth factor (EGF) increases the in vitro invasion, motility and adhesion interactions of the primary renal carcinoma cell line, A704. *Eur J Cancer* 1996;32A(11):1977-82.

399. Zhang H, Ma G, Dong M, Zhao M, Shen X, Ma Z, Guo K. Epidermal growth factor promotes invasiveness of pancreatic cancer cells through NF-kappaB-mediated proteinase productions. *Pancreas* 2006;32(1):101-9.

400. Price DJ, Avraham S, Feuerstein J, Fu Y, Avraham HK. The invasive phenotype in HMT-3522 cells requires increased EGF receptor signaling through both PI 3-kinase and ERK 1,2 pathways. *Cell Commun Adhes* 2002;9(2):87-102.

401. Spencer KS, Graus-Porta D, Leng J, Hynes NE, Klemke RL. ErbB2 is necessary for induction of carcinoma cell invasion by ErbB family receptor tyrosine kinases. *J Cell Biol* 2000;148(2):385-97.

402. Dittmar T, Husemann A, Schewe Y, Nofer JR, Niggemann B, Zanker KS, Brandt BH. Induction of cancer cell migration by epidermal growth factor is initiated by specific phosphorylation of tyrosine 1248 of c-erbB-2 receptor via EGFR. *Faseb J* 2002;16(13):1823-5.

403. Zhan L, Xiang B, Muthuswamy SK. Controlled activation of ErbB1/ErbB2 heterodimers promote invasion of three-dimensional organized epithelia in an ErbB1-dependent manner: implications for progression of ErbB2-overexpressing tumors. *Cancer Res* 2006;66(10):5201-8.

404. Angelucci A, Gravina GL, Rucci N, Millimaggi D, Festuccia C, Muzi P, Teti A, Vicentini C, Bologna M. Suppression of EGF-R signaling reduces the incidence of prostate cancer metastasis in nude mice. *Endocr Relat Cancer* 2006;13(1):197-210.

405. Bonaccorsi L, Marchiani S, Muratori M, Forti G, Baldi E. Gefitinib ('IRESSA', ZD1839) inhibits EGF-induced invasion in prostate cancer cells by suppressing PI3 K/AKT activation. *J Cancer Res Clin Oncol* 2004;130(10):604-14.

406. Li J, Kleeff J, Giese N, Buchler MW, Korc M, Friess H. Gefitinib ('Iressa', ZD1839), a selective epidermal growth factor receptor tyrosine kinase inhibitor, inhibits pancreatic cancer cell growth, invasion, and colony formation. *Int J Oncol* 2004;25(1):203-10.

407. Yang Z, Bagheri-Yarmand R, Wang RA, Adam L, Papadimitrakopoulou VV, Clayman GL, El-Naggar A, Lotan R, Barnes CJ, Hong WK, Kumar R. The epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 (Iressa) suppresses c-Src and Pak1 pathways and invasiveness of human cancer cells. *Clin Cancer Res* 2004;10(2):658-67.

408. Zhang Q, Thomas SM, Xi S, Smithgall TE, Siegfried JM, Kamens J, Gooding WE, Grandis JR. SRC family kinases mediate epidermal growth factor receptor ligand cleavage, proliferation, and invasion of head and neck cancer cells. *Cancer Res* 2004;64(17):6166-73.
409. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* 2003;3(5):362-74.
410. Sahai E. Mechanisms of cancer cell invasion. *Curr Opin Genet Dev* 2005;15(1):87-96.
411. Sabeh F, Ota I, Holmbeck K, Birkedal-Hansen H, Soloway P, Balbin M, Lopez-Otin C, Shapiro S, Inada M, Krane S, Allen E, Chung D, et al. Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. *J Cell Biol* 2004;167(4):769-81.
412. Nabeshima K, Inoue T, Shimao Y, Sameshima T. Matrix metalloproteinases in tumor invasion: role for cell migration. *Pathol Int* 2002;52(4):255-64.
413. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR. Cell migration: integrating signals from front to back. *Science* 2003;302(5651):1704-9.
414. Wear MA, Schafer DA, Cooper JA. Actin dynamics: assembly and disassembly of actin networks. *Curr Biol* 2000;10(24):R891-5.
415. Potter DA, Tirnauer JS, Janssen R, Croall DE, Hughes CN, Fiacco KA, Mier JW, Maki M, Herman IM. Calpain regulates actin remodeling during cell spreading. *J Cell Biol* 1998;141(3):647-62.
416. Carragher NO, Walker SM, Scott Carragher LA, Harris F, Sawyer TK, Brunton VG, Ozanne BW, Frame MC. Calpain 2 and Src dependence distinguishes mesenchymal and amoeboid modes of tumour cell invasion: a link to integrin function. *Oncogene* 2006.
417. Friedl P, Zanker KS, Bocker EB. Cell migration strategies in 3-D extracellular matrix: differences in morphology, cell matrix interactions, and integrin function. *Microsc Res Tech* 1998;43(5):369-78.
418. Wolf K, Mazo I, Leung H, Engelke K, von Andrian UH, Deryugina EI, Strongin AY, Bocker EB, Friedl P. Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J Cell Biol* 2003;160(2):267-77.
419. Sahai E, Marshall CJ. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat Cell Biol* 2003;5(8):711-9.
420. Friedl P, Hegerfeldt Y, Tusch M. Collective cell migration in morphogenesis and cancer. *Int J Dev Biol* 2004;48(5-6):441-9.
421. Nabeshima K, Inoue T, Shimao Y, Okada Y, Itoh Y, Seiki M, Koono M. Front-cell-specific expression of membrane-type 1 matrix metalloproteinase and gelatinase A during cohort migration of colon carcinoma cells induced by hepatocyte growth factor/scatter factor. *Cancer Res* 2000;60(13):3364-9.
422. Hegerfeldt Y, Tusch M, Bocker EB, Friedl P. Collective cell movement in primary melanoma explants: plasticity of cell-cell interaction, beta1-integrin function, and migration strategies. *Cancer Res* 2002;62(7):2125-30.
423. Friedl P, Noble PB, Walton PA, Laird DW, Chauvin PJ, Tabah RJ, Black M, Zanker KS. Migration of coordinated cell clusters in mesenchymal and epithelial cancer explants in vitro. *Cancer Res* 1995;55(20):4557-60.
424. Kantak SS, Kramer RH. E-cadherin regulates anchorage-independent growth and survival in oral squamous cell carcinoma cells. *J Biol Chem* 1998;273(27):16953-61.
425. Shen X, Kramer RH. Adhesion-mediated squamous cell carcinoma survival through ligand-independent activation of epidermal growth factor receptor. *Am J Pathol* 2004;165(4):1315-29.
426. Liotta LA, Saidel MG, Kleinerman J. The significance of hematogenous tumor cell clumps in the metastatic process. *Cancer Res* 1976;36(3):889-94.

427. Fidler IJ. The relationship of embolic homogeneity, number, size and viability to the incidence of experimental metastasis. *Eur J Cancer* 1973;9(3):223-7.
428. Molnar B, Ladanyi A, Tanko L, Sreter L, Tulassay Z. Circulating tumor cell clusters in the peripheral blood of colorectal cancer patients. *Clin Cancer Res* 2001;7(12):4080-5.
429. Brandt B, Junker R, Griwatz C, Heidl S, Brinkmann O, Semjonow A, Assmann G, Zanker KS. Isolation of prostate-derived single cells and cell clusters from human peripheral blood. *Cancer Res* 1996;56(20):4556-61.
430. Wicki A, Lehembre F, Wick N, Hantusch B, Kerjaschki D, Christofori G. Tumor invasion in the absence of epithelial-mesenchymal transition: podoplanin-mediated remodeling of the actin cytoskeleton. *Cancer Cell* 2006;9(4):261-72.
431. Yano H, Mazaki Y, Kurokawa K, Hanks SK, Matsuda M, Sabe H. Roles played by a subset of integrin signaling molecules in cadherin-based cell-cell adhesion. *J Cell Biol* 2004;166(2):283-95.
432. Robinson EE, Zazzali KM, Corbett SA, Foty RA. Alpha5beta1 integrin mediates strong tissue cohesion. *J Cell Sci* 2003;116(Pt 2):377-86.
433. Braga V. The crossroads between cell-cell adhesion and motility. *Nat Cell Biol* 2000;2(10):E182-4.
434. Roczniak-Ferguson A, Reynolds AB. Regulation of p120-catenin nucleocytoplasmic shuttling activity. *J Cell Sci* 2003;116(Pt 20):4201-12.
435. Arder H, Sandilands E, Machesky LM, Timpson P, Frame MC, Brunton VG. Src-dependent phosphorylation of Scar1 promotes its association with the Arp2/3 complex. *Cell Motil Cytoskeleton* 2006;63(1):6-13.
436. Sander EE, ten Klooster JP, van Delft S, van der Kammen RA, Collard JG. Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior. *J Cell Biol* 1999;147(5):1009-22.
437. Cho HJ, Ramer SE, Itoh M, Winkler DG, Kitas E, Bannwarth W, Burn P, Saito H, Walsh CT. Purification and characterization of a soluble catalytic fragment of the human transmembrane leukocyte antigen related (LAR) protein tyrosine phosphatase from an Escherichia coli expression system. *Biochemistry* 1991;30(25):6210-6.
438. Betts MJ, Russell RB. Amino acid properties and consequences of substitutions. In: Barnes MR, Gray IC, editors. *Bioinformatics for Geneticists*: Wiley; 2003. p. 289-314.
439. Pawson T. Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. *Cell* 2004;116(2):191-203.
440. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. *Nature* 2001;411(6835):355-65.
441. Ratcliffe MJ, Rubin LL, Staddon JM. Dephosphorylation of the cadherin-associated p100/p120 proteins in response to activation of protein kinase C in epithelial cells. *J Biol Chem* 1997;272(50):31894-901.
442. Ratcliffe MJ, Smales C, Staddon JM. Dephosphorylation of the catenins p120 and p100 in endothelial cells in response to inflammatory stimuli. *Biochemical Journal* 1999;338:471-478.
443. Mandell JW. Phosphorylation state-specific antibodies: applications in investigative and diagnostic pathology. *Am J Pathol* 2003;163(5):1687-98.
444. Czernik AJ, Girault JA, Nairn AC, Chen J, Snyder G, Kebabian J, Greengard P. Production of phosphorylation state-specific antibodies. *Methods Enzymol* 1991;201:264-83.
445. Harlow E, Lane D. *Antibodies: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory; 1988.

446. Morikawa K, Walker SM, Jessup JM, Fidler IJ. In vivo selection of highly metastatic cells from surgical specimens of different primary human colon carcinomas implanted into nude mice. *Cancer Res* 1988;48(7):1943-8.
447. Jones RJ, Avizienyte E, Wyke AW, Owens DW, Brunton VG, Frame MC. Elevated c-Src is linked to altered cell-matrix adhesion rather than proliferation in KM12C human colorectal cancer cells. *Br J Cancer* 2002;87(10):1128-35.
448. Brunton VG, Avizienyte E, Fincham VJ, Serrels B, Metcalf CA, 3rd, Sawyer TK, Frame MC. Identification of Src-specific phosphorylation site on focal adhesion kinase: dissection of the role of Src SH2 and catalytic functions and their consequences for tumor cell behavior. *Cancer Res* 2005;65(4):1335-42.
449. Corbin AS, Demehri S, Griswold IJ, Wang Y, Metcalf CA, 3rd, Sundaramoorthi R, Shakespeare WC, Snodgrass J, Wardwell S, Dalgarno D, Iulucci J, Sawyer TK, et al. In vitro and in vivo activity of ATP-based kinase inhibitors AP23464 and AP23848 against activation-loop mutants of Kit. *Blood* 2005;106(1):227-34.
450. O'Hare T, Pollock R, Stoffregen EP, Keats JA, Abdullah OM, Moseson EM, Rivera VM, Tang H, Metcalf CA, 3rd, Bohacek RS, Wang Y, Sundaramoorthi R, et al. Inhibition of wild-type and mutant Bcr-Abl by AP23464, a potent ATP-based oncogenic protein kinase inhibitor: implications for CML. *Blood* 2004;104(8):2532-9.
451. Richardson A, Malik RK, Hildebrand JD, Parsons JT. Inhibition of cell spreading by expression of the C-terminal domain of focal adhesion kinase (FAK) is rescued by coexpression of Src or catalytically inactive FAK: a role for paxillin tyrosine phosphorylation. *Mol Cell Biol* 1997;17(12):6906-14.
452. Ruest PJ, Shin NY, Polte TR, Zhang X, Hanks SK. Mechanisms of CAS substrate domain tyrosine phosphorylation by FAK and Src. *Mol Cell Biol* 2001;21(22):7641-52.
453. Erpel T, Superti-Furga G, Courtneidge SA. Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions. *Embo J* 1995;14(5):963-75.
454. Kaplan KB, Bibbins KB, Swedlow JR, Arnaud M, Morgan DO, Varmus HE. Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. *Embo J* 1994;13(20):4745-56.
455. Felsenfeld DP, Schwartzberg PL, Venegas A, Tse R, Sheetz MP. Selective regulation of integrin--cytoskeleton interactions by the tyrosine kinase Src. *Nat Cell Biol* 1999;1(4):200-6.
456. Lin CR, Chen WS, Kruiger W, Stolarsky LS, Weber W, Evans RM, Verma IM, Gill GN, Rosenfeld MG. Expression cloning of human EGF receptor complementary DNA: gene amplification and three related messenger RNA products in A431 cells. *Science* 1984;224(4651):843-8.
457. Ishizaki Y, Omori Y, Momiyama M, Nishikawa Y, Tokairin T, Manabe M, Enomoto K. Reduced expression and aberrant localization of p120catenin in human squamous cell carcinoma of the skin. *J Dermatol Sci* 2004;34(2):99-108.
458. Evans TRJ, Morgan JA, van den Abbeele AD, Macpherson IRJ, George S, Crawford D, Mastrullo JM, Cheng S, Fletcher JA, Demetri GD. Phase I dose-escalation study of the Src and multi-kinase inhibitor BMS-354825 in patients with GIST and other solid tumours. *Journal of Clinical Oncology* 2005;23(16S):3034.
459. Rosato R, Veltmaat JM, Groffen J, Heisterkamp N. Involvement of the tyrosine kinase fer in cell adhesion. *Mol Cell Biol* 1998;18(10):5762-70.
460. Lu Q, Mukhopadhyay NK, Griffin JD, Paredes M, Medina M, Kosik KS. Brain armadillo protein delta-catenin interacts with Abl tyrosine kinase and modulates cellular morphogenesis in response to growth factors. *J Neurosci Res* 2002;67(5):618-24.
461. Plattner R, Kadlec L, DeMali KA, Kazlauskas A, Pendergast AM. c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF. *Genes Dev* 1999;13(18):2400-11.

462. Shishido T, Akagi T, Ouchi T, Georgescu MM, Langdon WY, Hanafusa H. The kinase-deficient Src acts as a suppressor of the Abl kinase for Cbl phosphorylation. *Proc Natl Acad Sci U S A* 2000;97(12):6439-44.
463. Furstoss O, Dorey K, Simon V, Barila D, Superti-Furga G, Roche S. c-Abl is an effector of Src for growth factor-induced c-myc expression and DNA synthesis. *Embo J* 2002;21(4):514-24.
464. Frank C, Burkhardt C, Imhof D, Ringel J, Zschornig O, Wieligmann K, Zacharias M, Bohmer FD. Effective dephosphorylation of Src substrates by SHP-1. *J Biol Chem* 2004;279(12):11375-83.
465. Sandilands E, Cans C, Fincham VJ, Brunton VG, Mellor H, Prendergast GC, Norman JC, Superti-Furga G, Frame MC. RhoB and actin polymerization coordinate Src activation with endosome-mediated delivery to the membrane. *Dev Cell* 2004;7(6):855-69.
466. Blake RA, Broome MA, Liu X, Wu J, Gishizky M, Sun L, Courtneidge SA. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol* 2000;20(23):9018-27.
467. Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT. pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci U S A* 1992;89(11):5192-6.
468. Wu H, Reynolds AB, Kanner SB, Vines RR, Parsons JT. Identification and characterization of a novel cytoskeleton-associated pp60src substrate. *Mol Cell Biol* 1991;11(10):5113-24.
469. Roura S, Dominguez D. Inducible expression of p120Cas1B isoform corroborates the role for p120-catenin as a positive regulator of E-cadherin function in intestinal cancer cells. *Biochem Biophys Res Commun* 2004;320(2):435-41.
470. Mayerle J, Friess H, Buchler MW, Schnekenburger J, Weiss FU, Zimmer KP, Domschke W, Lerch MM. Up-regulation, nuclear import, and tumor growth stimulation of the adhesion protein p120 in pancreatic cancer. *Gastroenterology* 2003;124(4):949-60.
471. Qian X, Karpova T, Sheppard AM, McNally J, Lowy DR. E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases. *Embo J* 2004;23(8):1739-48.
472. Reddy KB, Nabha SM, Atanaskova N. Role of MAP kinase in tumor progression and invasion. *Cancer Metastasis Rev* 2003;22(4):395-403.
473. Haura EB, Turkson J, Jove R. Mechanisms of disease: Insights into the emerging role of signal transducers and activators of transcription in cancer. *Nat Clin Pract Oncol* 2005;2(6):315-24.
474. Schmitz AA, Govek EE, Bottner B, Van Aelst L. Rho GTPases: signaling, migration, and invasion. *Exp Cell Res* 2000;261(1):1-12.
475. Anastasiadis PZ, Reynolds AB. Regulation of Rho GTPases by p120-catenin. *Curr Opin Cell Biol* 2001;13(5):604-10.
476. Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry* 1982;21(24):6188-93.
477. Albini A. Tumor and endothelial cell invasion of basement membranes. The matrigel chemoinvasion assay as a tool for dissecting molecular mechanisms. *Pathol Oncol Res* 1998;4(3):230-41.
478. Schmeichel KL, Bissell MJ. Modeling tissue-specific signaling and organ function in three dimensions. *J Cell Sci* 2003;116(Pt 12):2377-88.
479. Hooper S, Marshall JF, Sahai E. Tumor cell migration in three dimensions. *Methods Enzymol* 2006;406:625-43.
480. Konig A, Bruckner-Tuderman L. Transforming growth factor-beta promotes deposition of collagen VII in a modified organotypic skin model. *Lab Invest* 1994;70(2):203-9.

481. Werner S, Smola H. Paracrine regulation of keratinocyte proliferation and differentiation. *Trends Cell Biol* 2001;11(4):143-6.
482. Barreca A, De Luca M, Del Monte P, Bondanza S, Damonte G, Cariola G, Di Marco E, Giordano G, Cancedda R, Minuto F. In vitro paracrine regulation of human keratinocyte growth by fibroblast-derived insulin-like growth factors. *J Cell Physiol* 1992;151(2):262-8.
483. Andersen H, Mejlvang J, Mahmood S, Gromova I, Gromov P, Lukanidin E, Kriaievska M, Mellon JK, Tulchinsky E. Immediate and delayed effects of E-cadherin inhibition on gene regulation and cell motility in human epidermoid carcinoma cells. *Mol Cell Biol* 2005;25(20):9138-50.
484. Frame MC, Fincham VJ, Carragher NO, Wyke JA. v-Src's hold over actin and cell adhesions. *Nature Reviews Molecular Cell Biology* 2002;3(4):233-245.
485. Takahashi K, Suzuki K. Density-dependent inhibition of growth involves prevention of EGF receptor activation by E-cadherin-mediated cell-cell adhesion. *Exp Cell Res* 1996;226(1):214-22.
486. Grazia Lampugnani M, Zanetti A, Corada M, Takahashi T, Balconi G, Breviario F, Orsenigo F, Cattelino A, Kemler R, Daniel TO, Dejana E. Contact inhibition of VEGF-induced proliferation requires vascular endothelial cadherin, beta-catenin, and the phosphatase DEP-1/CD148. *J Cell Biol* 2003;161(4):793-804.
487. Pankov R, Endo Y, Even-Ram S, Araki M, Clark K, Cukierman E, Matsumoto K, Yamada KM. A Rac switch regulates random versus directionally persistent cell migration. *J Cell Biol* 2005;170(5):793-802.
488. Feltes CM, Kudo A, Blaschuk O, Byers SW. An alternatively spliced cadherin-11 enhances human breast cancer cell invasion. *Cancer Res* 2002;62(22):6688-97.
489. Lin DW, Coleman IM, Hawley S, Dumpit R, Gifford D, Kezele P, Hung H, Knudsen BS, Kristal AR, Nelson PS. Influence of surgical manipulation on prostate gene expression: implications for molecular correlates of treatment effects and disease prognosis. *J Clin Oncol* 2006;24(23):3763-70.
490. Moran AE, Hunt DH, Javid SH, Redston M, Carothers AM, Bertagnolli MM. Apc deficiency is associated with increased Egfr activity in the intestinal enterocytes and adenomas of C57BL/6J-Min/+ mice. *J Biol Chem* 2004;279(41):43261-72.
491. Pollack AL, Runyan RB, Mostov KE. Morphogenetic mechanisms of epithelial tubulogenesis: MDCK cell polarity is transiently rearranged without loss of cell-cell contact during scatter factor/hepatocyte growth factor-induced tubulogenesis. *Dev Biol* 1998;204(1):64-79.
492. Andl CD, Mizushima T, Nakagawa H, Oyama K, Harada H, Chruma K, Herlyn M, Rustgi AK. Epidermal growth factor receptor mediates increased cell proliferation, migration, and aggregation in esophageal keratinocytes in vitro and in vivo. *J Biol Chem* 2003;278(3):1824-30.
493. Alema S, Salvatore AM. p120 catenin and phosphorylation: Mechanisms and traits of an unresolved issue. *Biochim Biophys Acta* 2006;doi:10.1016/j.bbamcr.2006.06.001.
494. Yokoyama K, Kamata N, Fujimoto R, Tsutsumi S, Tomonari M, Taki M, Hosokawa H, Nagayama M. Increased invasion and matrix metalloproteinase-2 expression by Snail-induced mesenchymal transition in squamous cell carcinomas. *Int J Oncol* 2003;22(4):891-8.
495. McLean GW, Komiyama NH, Serrels B, Asano H, Reynolds L, Conti F, Hodivala-Dilke K, Metzger D, Chambon P, Grant SG, Frame MC. Specific deletion of focal adhesion kinase suppresses tumor formation and blocks malignant progression. *Genes Dev* 2004;18(24):2998-3003.

PUBLISHED MATERIAL

Macpherson IR, Hooper S, Serrels A, McGarry L, Ozanne BW, Harrington K, Frame MC, Sahai E, Brunton VG. p120-catenin is required for the collective invasion of squamous cell carcinoma cells via a phosphorylation-independent mechanism. *Oncogene* 2007

ORIGINAL ARTICLE

p120-catenin is required for the collective invasion of squamous cell carcinoma cells via a phosphorylation-independent mechanismIR Macpherson¹, S Hooper², A Serrels¹, L McGarry¹, BW Ozanne¹, K Harrington³, MC Frame¹, E Sahai² and VG Brunton¹¹The Beatson Institute for Cancer Research, Glasgow, UK; ²Cancer Research UK London Research Institute, London, UK and ³Institute of Cancer Research, London, UK

Loss of E-cadherin-mediated cell–cell junctions has been correlated with cancer cell invasion and poor patient survival. p120-catenin has emerged as a key player in promoting E-cadherin stability and adherens junction integrity and has been proposed as a potential invasion suppressor by preventing release of cells from the constraints imposed by cadherin-mediated cell–cell adhesion. However, it has been proposed that tyrosine phosphorylation of p120 may contribute to cadherin-dependent junction disassembly during invasion. Here, we use small interfering RNA (siRNA) in A431 cells to show that knockdown of p120 promotes two-dimensional migration of cells. In contrast, p120 knockdown impairs epidermal growth factor-induced A431 invasion into three-dimensional matrix gels or in organotypic culture, whereas re-expression of siRNA-resistant p120, or a p120 isoform that cannot be phosphorylated on tyrosine, restores the collective mode of invasion employed by A431 cells *in vitro*. Thus, p120 promotes A431 cell invasion in a phosphorylation-independent manner. We show that the collective invasion of A431 cells depends on the presence of cadherin-mediated (P- and E-cadherin) cell–cell contacts, which are lost in cells where p120 expression is knocked down. Furthermore, membranous p120 is maintained in invasive squamous cell carcinomas in tumours suggesting that p120 may be important for the collective invasion of tumours cells *in vivo*.

Oncogene advance online publication, 5 March 2007; doi:10.1038/sj.onc.1210334

Keywords: p120; E-cadherin; invasion; phosphorylation

Introduction

Invasion, the ability of neoplastic cells to breach tissue barriers, is a hallmark of malignancy and an attractive

process to target therapeutic strategies (Hanahan and Weinberg, 2000). Several lines of evidence implicate E-cadherin, a cell adhesion molecule, as a powerful suppressor of invasion. For example, invasive human cancers often display reduced expression of E-cadherin (Hajra and Fearon, 2002). E-cadherin suppresses metastatic potential when expressed in cadherin-deficient invasive cell lines (Vleminckx *et al.*, 1991) and expression of a dominant-negative E-cadherin is sufficient to cause early invasion and metastasis in a mouse transgenic pancreatic cancer model (Perl *et al.*, 1998). Conversely, intracellular contacts may be maintained resulting in the invasion of coherent clusters or sheets of cells, a phenotype known as collective invasion (Friedl *et al.*, 2004). This allows the migration and invasion of differentiated tumours that have retained cell–cell junctions. It also allows the passive movement of non-migratory cells within these clusters, thus increasing their invasive and metastatic potential.

The extracellular domain of E-cadherin forms homodimers with cadherin molecules of an adjacent cell, whereas the cytoplasmic domain interacts with binding partners called catenins to form a functional unit known as the adherens junction (AJ) (Wheelock and Johnson, 2003). Rather than simply acting as a physical ‘glue’ it is now clear that multiple signalling pathways originate from the AJ and that a bidirectional cross talk exists with growth factor receptors (Wheelock and Johnson, 2003; Brunton *et al.*, 2004). The AJ intimately links events at the cell surface to gene transcription via the β -catenin/T-cell factor (TCF) (Peifer and Polakis, 2000) and the p120-catenin/Kaiso pathways (Daniel and Reynolds, 1999; Kelly *et al.*, 2004), whereas deregulation of the AJ in cancer has been frequently linked to transcriptional repression of E-cadherin (Strathdee, 2002). Alternatively, post-translational mechanisms and in particular the tyrosine phosphorylation of cadherins or catenins by kinases such as the epidermal growth factor (EGF) receptor, or Src can occur and this correlates strongly with invasion in model systems (Mareel and Leroy, 2003).

p120-catenin (hereafter p120) has emerged as a critical regulator of the AJ, both *in vitro* (Ireton *et al.*, 2002; Davis *et al.*, 2003; Xiao *et al.*, 2003) and *in vivo* (Davis and Reynolds, 2006; Perez-Moreno *et al.*, 2006). Specifically, in the absence of a physical interaction with p120,

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Received 4 August 2006; revised 15 December 2006; accepted 4 January 2007

classical cadherins (e.g. E-, VE-, N- and P-cadherin) are rapidly endocytosed and targeted for degradation. Reduced expression of p120 is frequently observed in human cancers and so this may represent one mechanism by which tumours downregulate E-cadherin (Thoreson and Reynolds, 2002). Hence, it has been hypothesized that p120 may act as an invasion suppressor via its ability to stabilize E-cadherin. Alternatively, when the primary event is the loss of E-cadherin expression, cytoplasmic accumulation of p120, which is relatively stable, may occur. Again, this appears to be a common histopathological finding in human cancer specimens (Thoreson and Reynolds, 2002) and has been linked to a pro-invasive role for p120, which was mediated via the regulation of Rho-family GTPase signalling (Yanagisawa and Anastasiadis, 2006).

A431 cells, derived from a vulval squamous cell carcinoma (SCC), invade a three-dimensional matrix in response to an EGF gradient and this provides a tractable reductionist system to investigate cancer cell invasion (Malliri *et al.*, 1998). We therefore set out to test the role of p120 in invasion in cells that retain E-cadherin expression, by reducing the absolute amount of p120 and also by interfering with its phosphorylation. p120 was first identified as an Src substrate and is heavily phosphorylated in Src-transformed cells and also in response to receptor tyrosine kinase activation. However, the role of tyrosine phosphorylation of p120 remains uncertain. Depending on the cellular context, the p120 N-terminal regulatory domain, which contains all known sites of tyrosine phosphorylation, can negatively modulate cell-cell adhesion (Aono *et al.*, 1999; Ozawa and Ohkubo, 2001). Furthermore, a p120 isoform lacking the N-terminal region can act in a dominant-negative manner to inhibit growth factor-dependent motility (Cuzzolino *et al.*, 2003).

We demonstrate here that loss of AJ caused by depletion of p120 protein inhibits, rather than promotes, invasion of A431 cells. Thus, in this system, p120 does not have an invasion-suppressor role. Surprisingly, despite being phosphorylated at several tyrosine residues in response to EGF, the requirement for p120 in EGF-induced invasion is not dependent on its ability to be phosphorylated. We show that A431 cells invade in a collective manner, which is dependent on the presence of cadherin-mediated cell-cell contacts, which in turn are dependent on the presence of p120.

Results

Activation of EGFR causes phosphorylation of p120 at multiple tyrosine residues

We initially set out to address whether phosphorylation of p120 was important for tumour cell invasion. A431 SCC cells overexpress EGF receptor and undergo specific changes in response to EGF stimulation including activation of Rho-family GTPases, actin reorganization and invasion of an extracellular matrix (Malliri *et al.*, 1998; Kurokawa *et al.*, 2004). p120 is a prominent

phospho-protein in EGF-stimulated epithelial cells (Lim *et al.*, 2003; Thelemann *et al.*, 2005) and is an attractive candidate molecule to mediate, or co-ordinate, some of these responses. Phosphorylation of p120 at tyrosine-228 in EGF-stimulated A431 cells has recently been reported (Mariner *et al.*, 2004). However, whether other sites are similarly regulated is unknown. Site-specific phospho-antibodies are also available to tyrosine-280 and tyrosine-291, and here we describe the generation of a new polyclonal antibody that specifically recognizes p120 when phosphorylated on tyrosine-296. Before using this antibody to analyse the EGF-dependent phosphorylation events in A431 cells, we wished to verify its specificity. To do this we utilized KM12C colon carcinoma cells stably transfected with a c-Src construct, which is constitutively active because of mutation of the negative regulatory tyrosine-527 to phenylalanine (Src527F) (Avizienyte *et al.*, 2002). Using an antibody that recognizes all tyrosine phosphorylation events, p120 immunoprecipitated from Src527F-expressing cells was seen to be heavily phosphorylated on tyrosine residues when compared with p120 in the parental cells (Figure 1a, upper panel). A similar result was seen when the immunoprecipitates were blotted with the phospho-Y296-specific antibody. Equivalent levels of total p120 were present in the immunoprecipitates (Figure 1a, lower panel). The presence of two distinct bands reflects the two dominant p120 isoforms in this cell line. Phospho specificity of the phospho-Y296 antibody was validated in several ways. First, the ability of the antibody to recognize p120 immunoprecipitated from KM12C-Src527F cells was abrogated by dephosphorylation of the immunoprecipitate with leukocyte antigen-related (LAR) phosphatase (Figure 1b). Second, the ability of phospho-Y296 to immunoprecipitate p120 from KM12C-Src527F lysates was blocked by preincubation with an excess of phosphorylated but not non-phosphorylated immunizing peptide (Figure 1c). Finally, myc-tagged wild-type p120 isoform1A (p120-1A) or p120-1A in which tyrosine-296 was mutated to phenylalanine (p120-1A/296F) were expressed in KM12C-Src527F cells. Immunoprecipitation of only the exogenously expressed myc-tagged proteins showed that wild-type p120-1A but not p120-1A/296F was recognized by the phospho-Y296 antibody (Figure 1d).

Using the panel of phospho-specific antibodies described above we identified simultaneous phosphorylation of p120 at tyrosine residues 228, 280, 291 and 296 following EGF stimulation (Figure 2a). As all four sites appeared to be phosphorylated in tandem, we used pharmacological inhibitors to investigate whether any differential regulation could be unmasked. Treatment of A431 cells with AG1478, a specific inhibitor of EGF-receptor tyrosine kinase activity, prevented the EGF-dependent phosphorylation of p120 on all four sites (Figure 2b). This corresponded with the ability of AG1478 to prevent the phosphorylation of the EGF receptor following stimulation with EGF as well as inhibiting the activation of the well-defined EGF-dependent signalling pathways to MAPK and STAT3 (Figure 2c). AP23464 is a selective inhibitor of Src

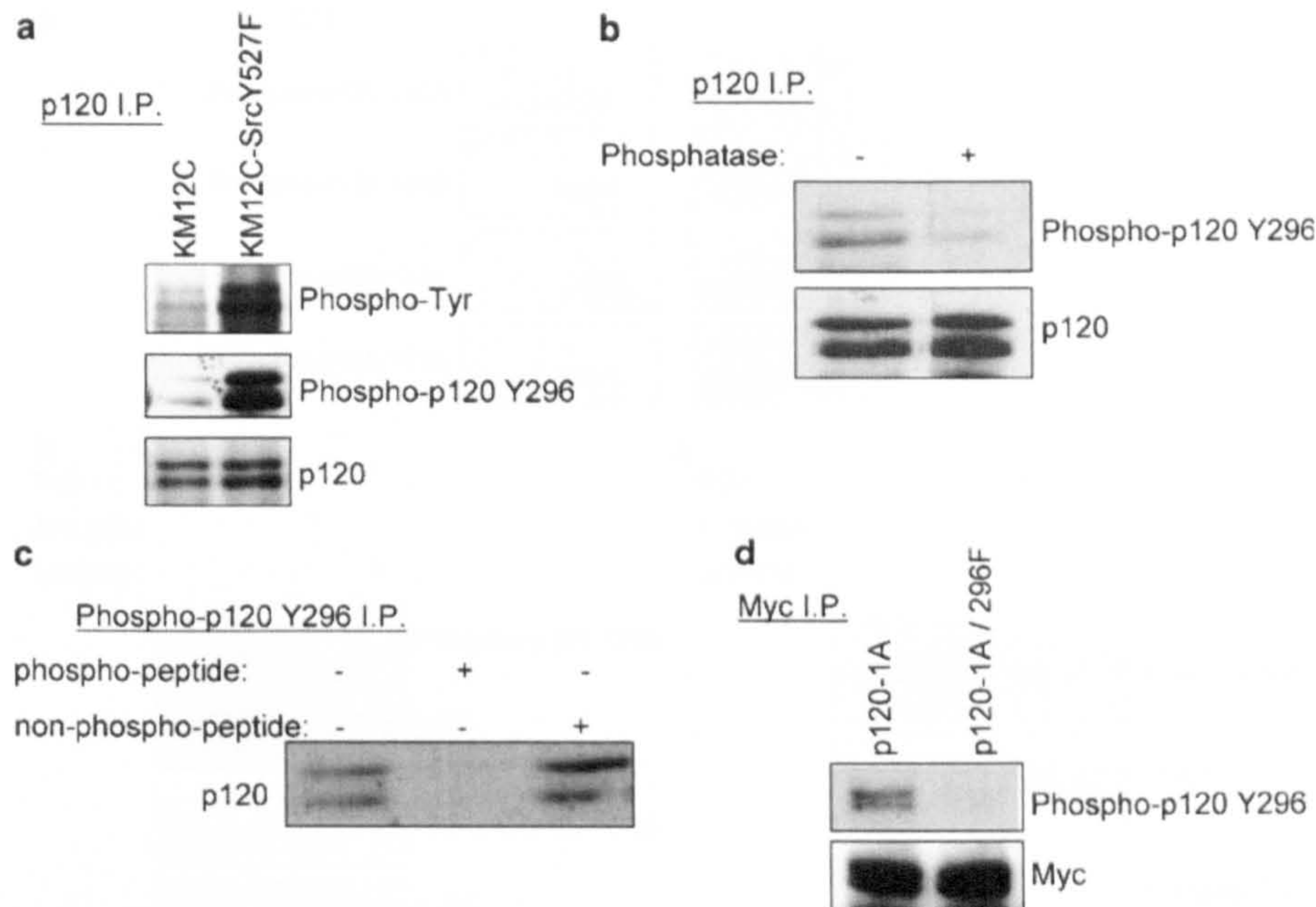


Figure 1 Characterization of a novel p120 Y296 phospho-specific antibody. (a) p120 was immunoprecipitated from KM12C cells or KM12C cells expressing constitutively active Src (KM12C-Src527F) and analysed by Western blotting with anti-phosphotyrosine, anti-phospho-p120 Y296 and anti-p120 antibodies. (b) p120 was immunoprecipitated from KM12C-Src527F cells and incubated with LAR phosphatase for 30 min before denaturation and electrophoresis. The membrane was probed with anti-phospho-p120 Y296 and anti-p120 antibodies. (c) Anti-phospho-p120 Y296 antibody, which had been pre-incubated with an excess of phosphorylated or non-phosphorylated immunising peptide, was used to immunoprecipitate p120 from KM12C-Src527F cell lysates. The membrane was probed with anti-p120 antibody. (d) Myc-tagged p120 proteins were immunoprecipitated from KM12C-Src527F cells and analysed by Western blotting with anti-phospho-p120 Y296 and anti-Myc antibodies.

family tyrosine kinases (Brunton *et al.*, 2005) and the EGF-dependent phosphorylation of p120 was also prevented in AP23464-treated cells (Figure 2b). Phosphorylation of p120 on residues 228 and 296 was reduced to levels below that of basal phosphorylation in unstimulated cells (Figure 2b). Using phosphorylation of the autophosphorylation site of Src (tyrosine-416) as a measure of Src activity, we showed that Src activity was inhibited in cells treated with AP23464 at the concentration (1 μ M) used in these experiments (Figure 2d). Interestingly, pre-treatment with AP23464 was sufficient to block signalling to both p120 and STAT3, a known Src substrate, whereas phosphorylation of MAPK was not reduced (Figure 2c). Thus p120 is phosphorylated on multiple residues in an EGF- and Src-dependent manner, but this pathway does not mediate signalling from the EGF receptor to the MAPK cascade.

RNAi-mediated knockdown and reconstitution of p120

To investigate the role of p120 in EGF-dependent events, particularly invasion, we utilized a powerful RNAi-based substitution system first described by Mariner *et al.* (2004). Importantly, this system avoids the potential confounding factor of subcellular mis-localization of overexpressed p120 due to saturation of cadherin-binding sites, and hence facilitates direct comparison of the function of different p120 constructs expressed at physiological levels. Human-specific p120 RNAi

sequences, which do not alter the stability of murine p120, or a scrambled control sequence (A431-Scr) were stably expressed in A431 cells and a number of single-cell clones with significant reduction in p120 levels were obtained (A431-p120⁻) (Figure 3a, upper panel shows results from two independent clones). As expected, there was a concurrent reduction in E-, P-cadherin and other components of the AJ such as β - and α -catenin (Figure 3a), which corresponds to the known effects of p120 on AJ stability. As a control we showed no alterations in levels of Src following knockdown of p120 (Figure 3a, lower panel).

Owing to alternative splicing events and the use of alternative start codons, four major isoforms of p120 exist (Figure 3b, start codons marked 1–4) (Keirsebilck *et al.*, 1998). A431 cells in common with most epithelial cells, predominantly express p120 isoform 3 (Ishizaki *et al.*, 2004). Therefore constructs encoding murine p120 isoform 3A were reintroduced into cells in which p120 had been knocked down. Pooled populations with stable expression of p120 at levels similar to parental A431 cells were selected by fluorescence-activated cell sorting (FACS) sorting. We also introduced isoform 4A which lacks the entire N-terminal signalling domain containing all identified sites of tyrosine and most sites of serine/threonine phosphorylation (Figure 3b) (Xia *et al.*, 2003). This isoform undergoes no detectable tyrosine phosphorylation in A431 cells (data not shown). Re-introduction of either isoform 3A or isoform 4A

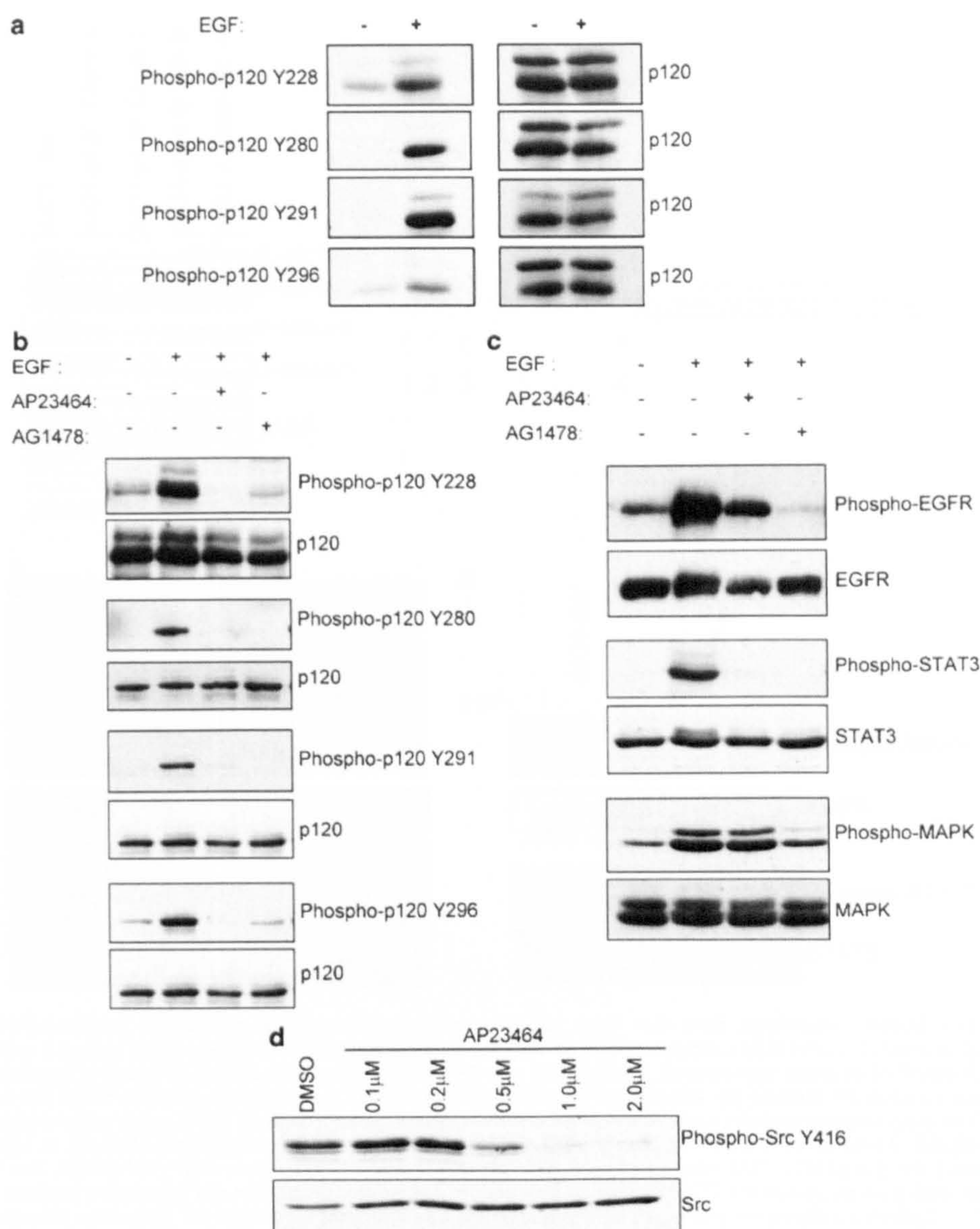


Figure 2 EGF-dependent phosphorylation of p120 at multiple tyrosine residues. (a) A431 cells were serum-starved overnight and then stimulated with EGF (100 ng/ml) for 5 min before lysis. p120 was immunoprecipitated and analysed by Western-blot with site-specific phospho-p120 antibodies. In each case, after removal of the phospho-specific antibodies, the blots were reprobed with total p120 antibody. (b and c) A431 cells were serum-starved overnight, incubated with AP23464 (1 μ M) or AG1478 (300 nM) for 1 h, then lysed 5 min after stimulation with EGF (100 ng/ml). p120 was immunoprecipitated and analysed by Western blotting with site-specific phospho-p120 antibodies or anti-phosphotyrosine antibody (b). Lysate was also analysed by Western blotting using anti-phospho-EGFR, anti-phospho-MAPK and anti-phospho-STAT3 antibodies (c). In each case, after removal of the phospho-specific antibodies, the blots were reprobed with the corresponding non-phospho-specific antibodies. (d) A431 cells were treated with vehicle (DMSO) or increasing concentrations of AP23464 for 60 min after which lysates were prepared and analysed by Western blotting with anti-phospho-SrcY416 antibody. After removal of the phospho-specific antibody, the blot was reprobed with Src antibody.

restored E-cadherin levels to those seen in the control cells (Figure 3a). In parallel, the loss of cell cohesion resulting from knockdown of p120 was reversed by reintroduction of either p120 isoform (Figure 3c).

As cross talk exists between the AJ and the EGF receptor (Qian *et al.*, 2004), we investigated the effects of depleting p120 and hence the AJ on major EGF signalling pathways. Importantly, in A431-p120⁻ cells, there was no defect in EGF-signalling as the

EGF-dependent phosphorylation of MAPK and STAT3 was seen in both p120 knockdown and control cells (Figure 3d).

p120 regulates motility

p120 has been reported to promote or inhibit the motility of various epithelial cell lines in a isoform and cell context-specific manner (Cozzolino *et al.*, 2003; Shibata *et al.*, 2004; Bellovin *et al.*, 2005). In A431 cells,

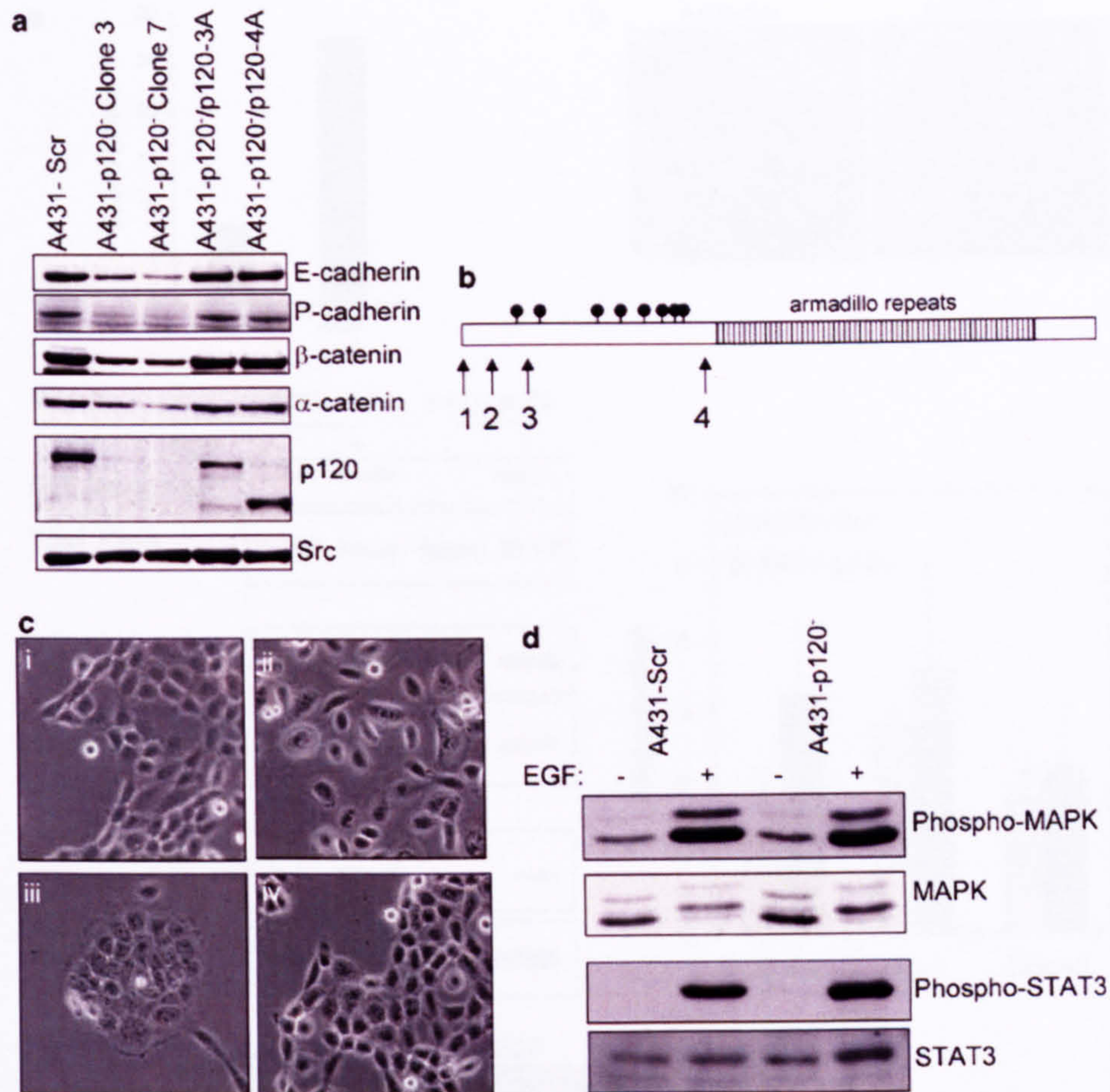


Figure 3 RNAi-mediated knockdown and reconstitution of p120. (a) A431 cells were retrovirally infected with pRetroSuper containing either a human p120-specific RNAi hairpin (A431-p120⁻) or scrambled sequence (A431-Scr). Subsequently, murine p120 isoform 3A (A431-p120⁻/p120-3A) or 4A (A431-p120⁻/p120-4A) were reintroduced. Lysates were analysed by Western blotting using anti-p120, anti-E-, anti-P-cadherin, anti-β-catenin, anti-α-catenin and anti-Src antibodies. (b) Isoform 4A lacks all identified sites of tyrosine phosphorylation (represented by filled circles), whereas isoform 3A contains seven tyrosine phosphorylation sites. (c) Phase-contrast images of (i) A431-Scr, (ii) A431-p120⁻, (iii) A431-p120⁻/p120-3A and (iv) A431-p120⁻/p120-4A. Magnification × 10. (d) A431-Scr and A431-p120⁻ cells were serum-starved overnight, then stimulated with EGF (100 ng/ml) for 5 min before lysis. Western-blot analysis was carried out with anti-phospho-MAPK and anti-phospho-STAT3 antibodies. In each case, after removal of the phospho-specific antibodies, the blots were re probed with the corresponding non-phospho-specific antibodies.

the RNAi-mediated depletion of p120 was associated with an increase in two-dimensional motility of sparsely cultured cells as measured by time-lapse video-microscopy. The median speed was increased 3.5-fold from 8.0 μm/h (interquartile range: 3.5–14.4 μm/h) in A431-Scr cells to 27.3 μm/h (interquartile range: 14.1–51.6 μm/h) in A431-p120⁻ cells (Figure 4a). As the data were not normally distributed, we utilized the non-parametric Mann–Whitney *U*-test to demonstrate this to be a statistically significant increase ($P < 0.0001$).

Using a wound assay to monitor migration of cells from a confluent monolayer into a denuded area, we were also able to see qualitative differences in the mode of migration (Figure 4b and movies in Supplementary material). The A431-Scr cells moved as a sheet of cells keeping contact with adjacent cells in the monolayer (Movie 1, Supplementary Material). In contrast, the movement of A431-p120⁻ was more disorganized with

individual cells moving into the denuded area (Movie 2, Supplementary Material). Cells were fixed and stained with phalloidin to visualize the actin cytoskeleton as they migrated into the wound (Figure 4b). A431-Scr cells maintained tight cell–cell contacts, whereas A431-p120⁻ cells at the edge of the wound had broken away from the neighbouring cells and had become more elongated. Although the individual A431-p120⁻ cells moved more rapidly than the A431-Scr cells, the lack of directionality meant that the speed of wound closure was unaltered between the A431-Scr and A431-p120⁻ cells (A431-Scr 20.57 ± 2.26 and A431-p120⁻ 18.14 ± 2.98 μm/h).

As regulation of Rho family GTPase activity by cytoplasmic p120 has been associated with a more motile phenotype, we measured GTP loading of RhoA, Rac1 and Cdc42 in A431-Scr and A431-p120⁻ cells (Figure 4c). There was no difference in the basal levels of GTP-loaded RhoA, Rac1 and Cdc42 in the A431-Scr

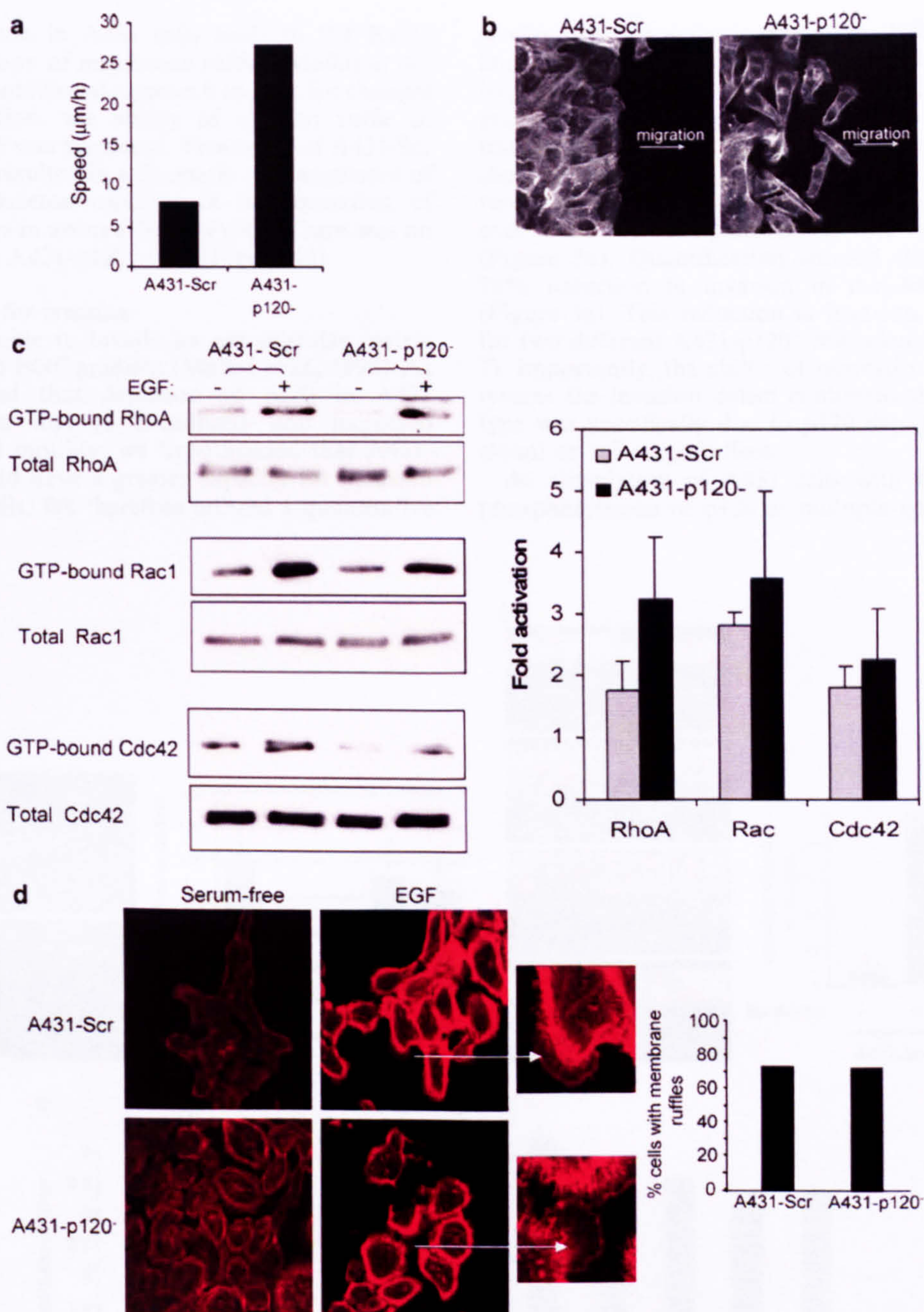


Figure 4 Depletion of p120 regulates motility. (a) A431-Scr and A431-p120⁻ cells were sparsely plated and migration analysed over a 5-h period by time-lapse video microscopy. For quantification of migration, 100 individual cells per cell line were tracked for 5 h. Migration is represented as median speed. Results are representative of three separate experiments. (b) A wound was made in confluent monolayers of A431-Scr and A431-p120⁻ cells and after 6 h the cells were fixed and stained with TRITC-phalloidin. Magnification $\times 40$. (c) A431-Scr and A431-p120⁻ cell lines were serum-starved overnight and then stimulated with EGF (100 ng/ml) for 5 (Rac1/Cdc42) or 15 min (RhoA) before lysis. Total protein extracts were used directly or first incubated with GST-RBD or GST-PAK to selectively pull down active GTP-bound protein. Quantification of band density was performed for three independent experiments and the fold elevation of activity in EGF-stimulated versus serum-starved cells was displayed as mean \pm s.d. (d) A431-Scr and A431-p120⁻ cells were serum-starved overnight and then stimulated with 100 ng/ml EGF. Cells were then fixed and stained with TRITC-phalloidin. The number of cells with membrane ruffles was later counted from at least 100 cells. Values are mean \pm s.d. from three independent experiments. Magnification $\times 63$.

and A431-p120⁻ cells. Furthermore, the increase in GTP-bound RhoA, Rac1 and Cdc42 following EGF treatment was unaltered in cells lacking p120. The

quantitation of the fold activation following EGF treatment is shown from densitometry measurements of three independent experiments (Figure 4c). EGF

receptor activation in A431 cells leads to the Rac1-mediated formation of membrane ruffles (Malliri *et al.*, 1998) and as an additional approach to look for changes in Rac1 activation, the ability of cells to ruffle in response to EGF was measured. Treatment of A431-Scr cells with EGF resulted in a dramatic rearrangement of the actin cytoskeleton resulting in the formation of membrane ruffles in around 70% of cells. There was no difference in the A431-p120⁻ cells (Figure 4d).

p120 is required for invasion

A431 cells are able to invade an extracellular matrix in response to an EGF gradient (Malliri *et al.*, 1998). As we demonstrated that depletion of p120 in A431 cells resulted in loss of E-cadherin and increased two-dimensional motility, we hypothesized that A431-p120⁻ cells would have a greater capacity for invasion than parental cells. We therefore utilized a quantitative

in vitro invasion assay to assess the ability of A431 cell lines to invade into Matrigel in response to EGF. Invasion only occurred in the presence of an EGF gradient and was maximal for 10 ng/ml (EGF concentrations from 1 to 100 ng/ml were tested; data not shown). Surprisingly, knockdown of p120 protein resulted in a marked reduction in invasion into Matrigel over 72 h in comparison to the A431-Scr cells (Figure 5a). Quantification showed that there was a 76% reduction in invasion in the A431-p120⁻ cells (Figure 5a). This reduction in invasion was confirmed for two different A431-p120⁻ cells clones (clones 3 and 7). Importantly, the ability of reconstituted p120-3A to reverse the invasion defect confirmed that this phenotype was specifically due to p120 depletion and not a clonal or 'off-target' effect.

As stimulation of A431 cells with EGF results in phosphorylation of p120 at multiple tyrosine residues,

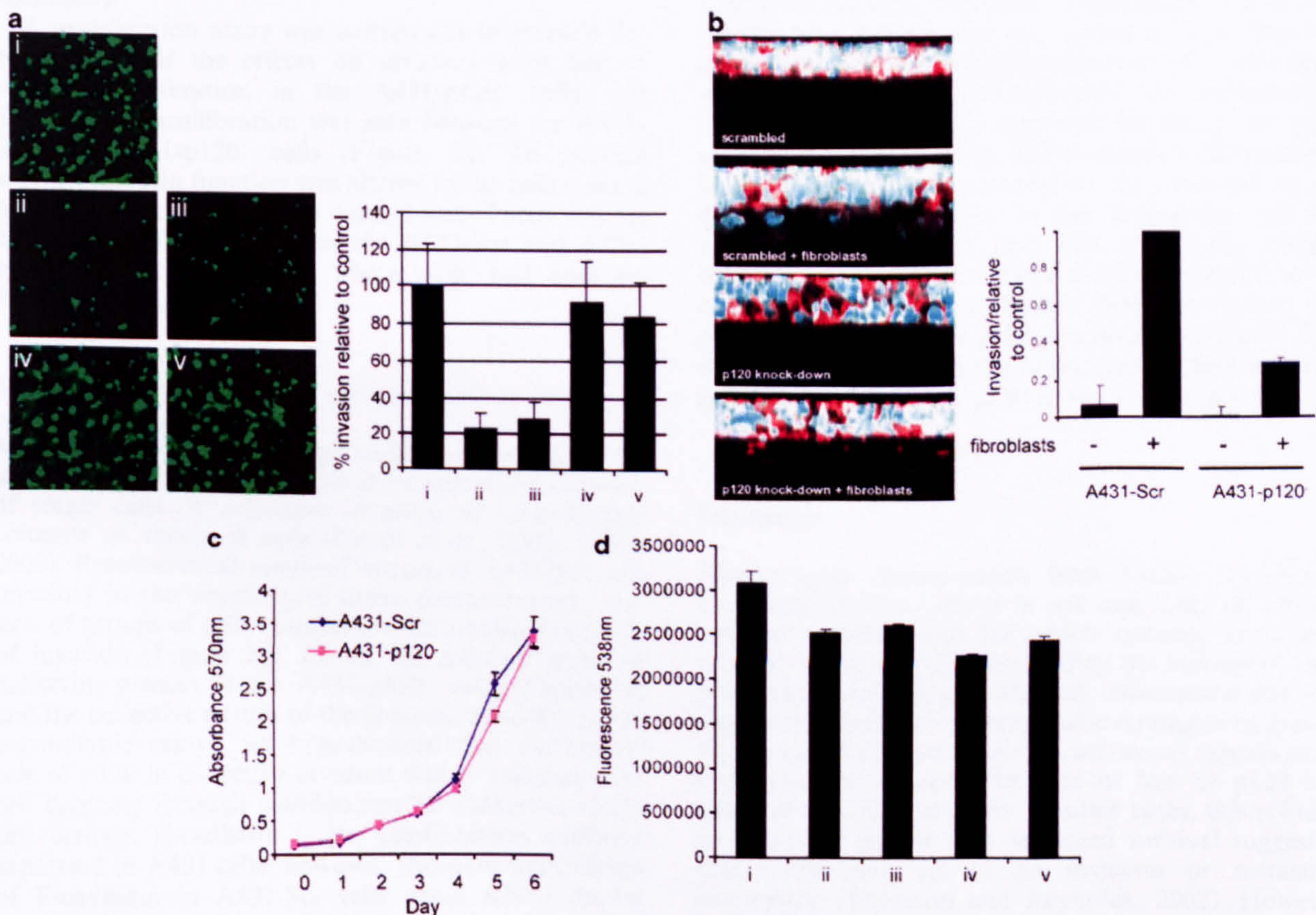


Figure 5 p120 is required for invasion. (a) Cells were seeded on Transwell filters and allowed to invade into Matrigel towards an EGF gradient. After 72 h, cells were labelled with calcein AM and visualized in the Matrigel at 15 μ m intervals. Composite images of invading cells are shown for (i) A431-Scr, (ii) A431-p120⁻ Clone 3, (iii) A431-p120⁻ Clone 7, (iv) A431-p120⁻/p120-3A and (v) A431-p120⁻/p120-4A cells. Quantitation of invasion is shown for a representative experiment in a series of four. Values are mean \pm s.d. (b) CFP-labelled cells (blue) were seeded in collagen/Matrigel gels in the presence or absence of fibroblasts, and after 5 days the gels were fixed and labelled with TRITC-phalloidin (red). Quantitation of invasion is shown where values are mean \pm s.d. collated from three experiments. (c) A431-Scr or A431-p120⁻ cells were plated at a density of 1×10^3 cells/well in 96-well plates. At 24 h intervals, the number of viable cells was determined using a colorimetric MTT assay where absorbance at 570 nm is directly proportional to cell number. Results are plotted as mean absorbance \pm s.d. for quadruplicate wells. (d) Adherent (i) A431-Scr, (ii) A431-p120⁻ Clone 3, (iii) A431-p120⁻ Clone 7, (iv) A431-p120⁻/p120-3A and (v) A431-p120⁻/p120-4A cells were quantified after adhesion to Matrigel for 30 min. Values are mean \pm s.d. of quadruplicate wells taken from a representative experiment.

we hypothesized that this requirement of p120 for invasion would specifically depend on its ability to be phosphorylated. However, reconstitution with p120 isoform 4A, which lacks all sites of tyrosine phosphorylation, restored the invasion defect in the A431-p120⁻ cells to a level seen in the control cells (Figure 5a). Thus, tyrosine phosphorylation of p120 (or other events occurring in the regulatory domain such as serine/threonine phosphorylation (Xia *et al.*, 2003)) was not required for invasion.

The reduced ability of A431-p120⁻ cells to undergo invasion was further characterized using an organotypic invasion assay (Figure 5b). This 3D environment more closely resembles the *in vivo* situation and measures the invasion of cells into collagen/Matrigel gels co-cultured in the presence of tumour-derived fibroblasts. There was a marked reduction in invasion of the p120 knockdown cells when compared with cells expressing scrambled p120 sequences (Figure 5b). There was no invasion of control or p120 knockdown cells in the absence of fibroblasts.

A proliferation assay was carried out to exclude the possibility that the effects on invasion were due to reduced proliferation in the A431-p120⁻ cells. No difference in proliferation was seen between the A431-Scr and A431-p120⁻ cells (Figure 5c). To address whether integrin function was altered in the cells lacking p120, adhesion assays were carried out. There was no difference in the adhesion of the A431-Scr and A431-p120⁻ cells or the cells in which p120 had been re-introduced (Figure 5d).

Invasion is dependent on the ability of cells to form cell-cell contacts

Malignant cells may adopt different strategies for invasion including mesenchymal or amoeboid invasion of single cells or collective invasion of co-ordinated columns or sheets of cells (Friedl *et al.*, 2004; Sahai, 2005). Reconstructed confocal images of A431-Scr cells invading in the organotypic assay demonstrated invasion of groups of cells consistent with a collective mode of invasion (Figure 6a). Given the reduced levels of cadherins present in the A431-p120⁻ cells (Figure 3a) and the collective nature of the invasion observed in the organotypic assays, we hypothesized that the critical role of p120 in collective invasion was to stabilize cell-cell contacts through stabilization of cadherins at the cell surface. E-cadherin is the predominant cadherin expressed in A431 cells; however, transient knockdown of E-cadherin in A431-Scr cells using RNAi duplex oligonucleotides (Figure 6b) did not alter the ability of the A431 cells to invade (Figure 6c). A431 cells also express P-cadherin, but we were unable to detect N-cadherin in any of the conditions tested (results not shown). Knockdown of P-cadherin alone had no effect on the invasion of A431 cells; however, combination of P- and E-cadherin knockdown significantly reduced the ability of A431 cells to invade in the organotypic assay (Figure 6c). Further analysis demonstrated that P-cadherin levels were upregulated when E-cadherin was

knocked down (Figure 6d), suggesting that there is interplay between both cadherin types to maintain cell-cell contacts required for invasion.

To address this we looked at cell-cell contacts in the cells. Loss of either E- or P-cadherin alone did not alter the morphology of the A431 cells and cell-cell contacts were still visible where p120 was present (Figure 7a). However, combined loss of E- and P-cadherin resulted in a loss of cell-cell contacts and p120 was localized diffusely in the cytoplasm. This was similar to the morphology of the A431-p120⁻ cells, which had also lost cell-cell contacts (Figure 7a, right-hand panels) associated with downregulation of both E- and P-cadherin (Figure 3a). Taken together, these data suggest that the pro-invasive role of p120 is at cell-cell contacts where it functions to maintain cadherin-mediated cell junctions that are required for the collective invasion of the A431 cells. Consistent with this we observe that p120 is localized to cell-cell contacts in collectively invading A431 cells (Figure 7b).

SCC of the head and neck often invade in collective clusters and strands and our results indicate that this type of invasion would be dependent on p120. In a small study we found that p120 expression was maintained in 7 out of 7 human SCCs examined including two high-grade tumours (Figure 8). Furthermore, p120 remained localized at cell-cell contacts as we observed in the organotypic assay, even in the high-grade tumours where small groups of cells had collectively invaded the surrounding stromal tissue (Figure 8e and f, arrow heads). These data demonstrate that our finding that p120 is required for the collective invasion of A431 SCC cells is consistent with the expression pattern and sub-cellular distribution of p120 in head and neck SCC.

Discussion

Most cancer deaths result from locally invasive or metastatic disease, which is not amenable to curative surgical resection and for which systemic anti-cancer therapy is required. Understanding the biology of these processes, therefore, provides key information that may aid the development of therapeutic strategies to prevent the spread of disease. There are numerous reports in the literature that describe decrease or loss of p120 in a range of epithelial tumours. In some cases, this is linked to late-stage disease and decreased survival suggesting that p120 may act as an invasion or metastasis suppressor (Thoreson and Reynolds, 2002). However, we have identified a novel role for p120 in promoting collective invasion of epithelial tumour cells as knockdown of p120 by small interfering RNA (siRNA) inhibits invasion of A431 SCC cells into both Matrigel and collagen gels.

The collective movement of tumour cells describes the migration and invasion of groups of tumour cells which have maintained cell-cell junctions. It allows the migration of and invasion of differentiated tumours that have retained cell-cell junctions and also allows the

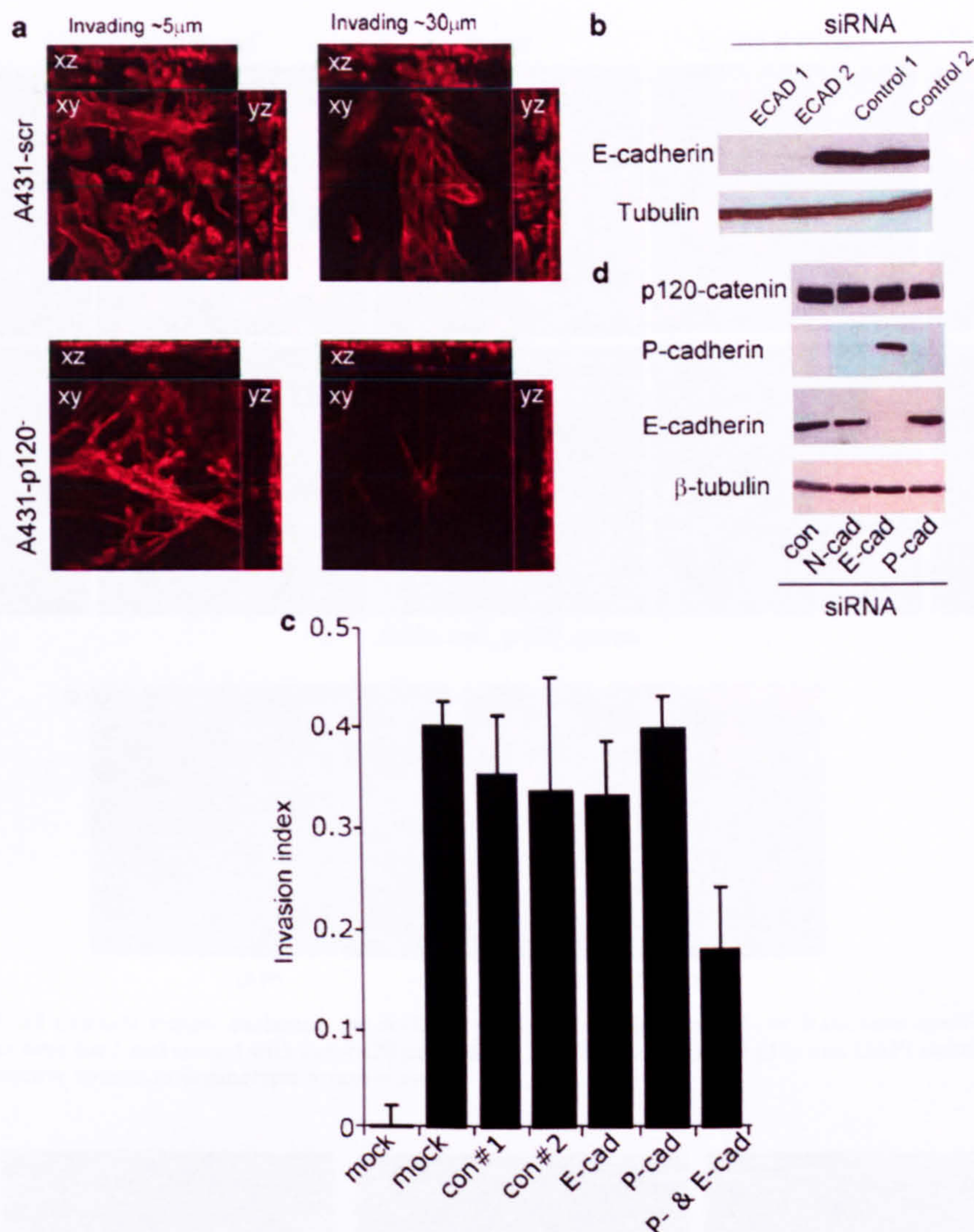


Figure 6 Cell-cell contacts are required for invasion. (a) Phalloidin staining of A431-Scr and A431-p120⁻ cells invading as collective strands in organotypic invasion assay. (b) A431-Scr cells were treated with two independent E-cadherin-specific or two control (irrelevant) RNAi oligonucleotides and lysates prepared. Western-blot analysis was carried out with anti-E-cadherin antibody and anti-tubulin antibody used as a loading control. (c) A431-Scr cells treated with either E- or P-cadherin-specific or control RNAi oligonucleotides were seeded in collagen/Matrigel gels in the presence of fibroblasts and the invasion index calculated after 5 days. Values are mean \pm s.d. from a representative experiment in a series of three. (d) A431-Scr cells were treated with either E- or P-cadherin-specific or control RNAi oligonucleotides and lysates prepared. Western-blot analysis was carried out with p120, P- and E-cadherin antibodies, and anti-tubulin antibody was used as a loading control.

passive movement of non-migratory cells within these clusters. The mechanisms involved in this type of invasion are not fully understood. It shares a number of properties with single-cell migration such as integrin and matrix metalloproteinase requirements, but the extent to which cadherins are involved in collective invasion is not well defined (Friedl *et al.*, 2004; Sahai, 2005).

Here we show that retention of cadherin-mediated cell-cell contacts is required for the collective invasion of A431 cells. Reduction in both P- and E-cadherin expression is required for loss of cell-cell contacts and

an inhibition of invasion of cells, which is consistent with redundancy of function between these two epithelial cadherins (Jensen *et al.*, 1997). *In vitro* studies using either a p120-deficient colon epithelial cell line or siRNA-mediated knockdown of p120 demonstrated that p120 plays a key role in the stability of the AJ by regulating the internalization and subsequent degradation of E-cadherin (Ireton *et al.*, 2002; Davis *et al.*, 2003; Xiao *et al.*, 2003), and the loss of both E- and P-cadherin in the A431-p120⁻ cells supports a pro-invasive role for p120 in collective invasion by maintaining integrity of cadherin-mediated cell-cell contacts.

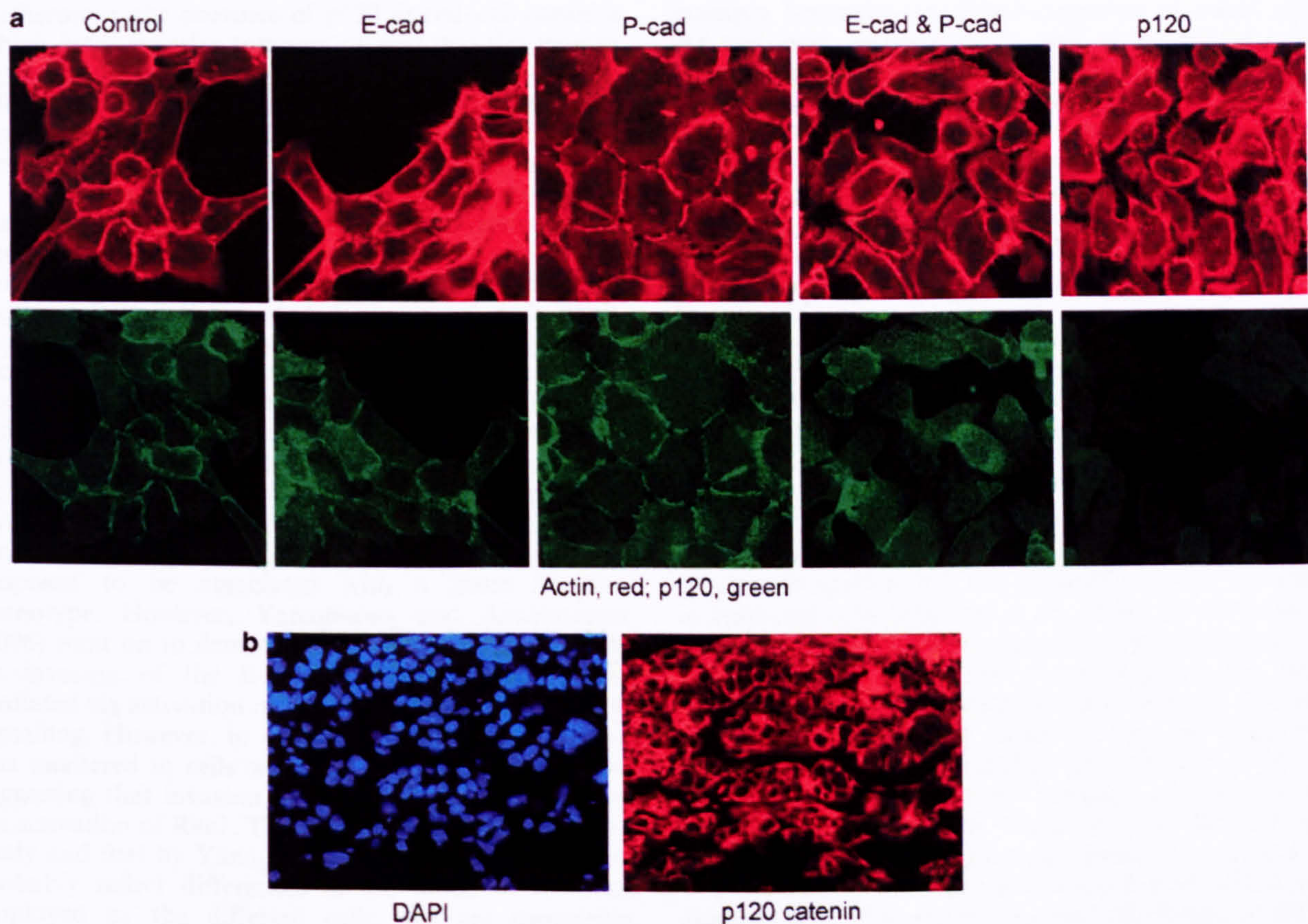


Figure 7 Cell-cell contacts require cadherins. (a) A431-Scr cells treated with either E- or P-cadherin-specific or control RNAi oligonucleotides were fixed and stained with anti-p120 antibody or TRITC-phalloidin. (b) p120 and DAPI staining of A431-Scr cells invading as collective strands in organotypic invasion assay.

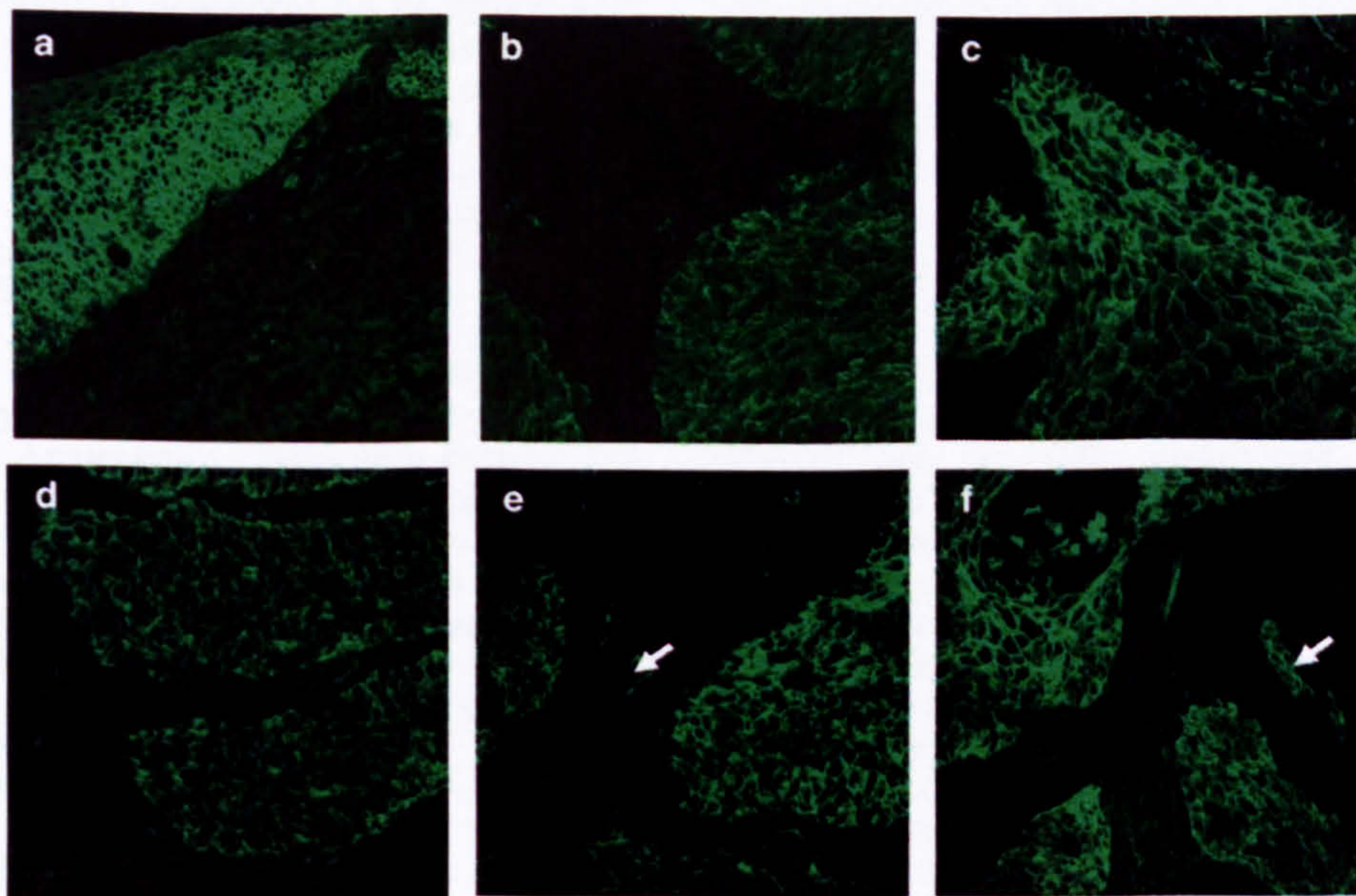


Figure 8 p120 localization in head and neck SCC. Tumour biopsies were processed as described and stained with an anti-p120 antibody. (a) Non-cancerous mucosa (bottom right) adjacent to well-differentiated tumour, (b) non-keratinizing tumour, (c) well-differentiated non-keratinizing tumour, (d) non-keratinizing tumour, (e) high-grade tumour with occasional keratin nests, (f) high-grade tumour. Panels a and f are $500 \times 500 \mu\text{m}$ and panels b–e are $250 \times 250 \mu\text{m}$.

Furthermore, the presence of p120 at cell–cell contacts in high-grade invasive tumours suggest that this may be important mechanism of invasion *in vivo*.

Recently, a requirement for p120 in the invasion of E-cadherin deficient cell lines, which do not have functional cell–cell junctions, has been demonstrated by Yanagisawa and Anastasiadis (2006). In this situation, p120 appears to act by stabilizing expression of the mesenchymal cadherins, N-cadherin and cadherin 11, which have been shown to have pro-invasive functions in other cell types (Islam *et al.*, 1996; Hazan *et al.*, 2000). Regulation of Rho family GTPase activity by cytoplasmic p120 has been associated with a branching morphology and more motile phenotype (Anastasiadis and Reynolds, 2000; Noren *et al.*, 2000; Grosheva *et al.*, 2001; Cozzolino *et al.*, 2003) and has been proposed as the mechanism by which p120 may act to promote invasion. In these reports, no direct measurements of invasion were made but a more motile phenotype was proposed to be associated with a more invasive phenotype. However, Yanagisawa and Anastasiadis (2006) went on to demonstrate that the effects of p120 on invasion of the E-cadherin deficient cells were mediated via activation of Rac1 and inhibition of RhoA signalling. However, in our experiments, Rac1 activity was unaltered in cells where p120 was knocked down suggesting that invasion of A431 cells is not regulated via activation of Rac1. The differences between our own study and that by Yanagisawa and Anastasiadis (2006) probably reflect differences in the mode of invasion employed by the different cells. We are measuring collective invasion of cells that retain cell–cell contacts, whereas the other study was looking at invasion of individual cells that are known to involve different signalling pathways. Furthermore, the ability of p120 to regulate Rac1 activity is dependent on its cytoplasmic localization and we have shown that p120 is predominantly localized at cell–cell junctions in collectively invading cells. It is possible that p120 has an additional role in A431 invasion that involves regulating the levels of phosphorylated myosin light chain as we saw a small reduction in levels of MYPT1 and MLC phosphorylation in A431-p120⁻ cells (results not shown). However, this is unlikely to be mediated through RhoA as we were unable to detect any significant changes in RhoA-GTP levels in our system. Our own work and that of Yanagisawa and Anastasiadis (2006) both define a key role for p120 in tumour cell invasion through stabilization of cadherins, although the downstream signalling pathways are different. Our demonstration that it is important for the collective invasion of tumour cells suggests that p120 may play a pivotal role in tumour cell invasion which show considerable plasticity in terms of their mode of invasion.

Tyrosine phosphorylation of p120 by oncogenic Src and growth factor receptors has been well documented and a number of phosphorylation sites have been mapped to the N-terminal portion of the protein (Mariner *et al.*, 2001, 2004). Use of phosphorylation defective mutants has suggested a requirement for p120 phosphorylation in promoting cell motility. For

example, heregulin-stimulated migration of breast cancer cell lines requires p120 and is associated with tyrosine phosphorylation of p120 (Shibata *et al.*, 2004), whereas HGF-dependent cell scattering and migration of MDCK cells is prevented by expression of a p120 mutant lacking the N-terminal domain phosphorylation sites (Cozzolino *et al.*, 2003). In this case, activation of RhoA by HGF was also abrogated. The same phosphorylation defective mutant was also able to partially suppress cell scattering in v-Src-transformed cells (Ozawa and Ohkubo, 2001), whereas increased motility in keratinocytes upon over-expression of p120 was dependent on EGF (Cozzolino *et al.*, 2003), suggesting a role for phosphorylation. Taken together, these observations indicate that p120 may act in concert with growth factor receptors to regulate cell motility via modulation of phosphorylation and/or Rho GTPase signalling. In contrast, it appears that although phosphorylation of p120 occurs upon junction formation, it is not required for their assembly in epithelial cells (Mariner *et al.*, 2004). In the present study, we show that although p120 is phosphorylated in EGF-stimulated cells, this is not required for their invasion. Thus, the processes involved in cell–cell adhesion, motility and invasion can be separated mechanistically and the ability of p120 to act as an invasion promoter is more complex and cannot be accounted for solely by alterations in RhoGTPase activity or a requirement for phosphorylation events.

The transcription factor Kaiso was first identified in a complex with p120 and is a member of a family of BTB/POZ proteins whose target genes include a number that are involved in tumorigenesis (van Roy and McCrea, 2005). Kaiso acts as a transcriptional repressor and p120 binding to Kaiso relieves this repression. It has therefore been proposed that the aberrant expression of p120 in tumour cells may result in deregulated Kaiso activity. Interestingly, known Kaiso targets include the metalloproteinase, MMP7 (Spring *et al.*, 2005), whereas Kaiso can also repress Wnt gene targets (Park *et al.*, 2005), many of which are known to be involved in tumour cell invasion. This provides an attractive mechanism whereby p120 may regulate tumour cell invasion and this requires further investigation.

Collective migration of tumours has been documented by histologists for many years, although the mechanisms involved are not fully understood. It allows the migration and invasion of differentiated tumours that have retained cell–cell junctions. It also allows the passive movement of non-migratory cells within these clusters, thus increasing their invasive and metastatic potential. The movement of tumour cell clusters in contrast to individual tumour cells may also provide increased survival and migratory signals through autocrine production of such factors. Hence, the collective movement and invasion of tumour cells presents several advantages to the progression of an aggressive invasive tumour. Understanding the signalling pathways that regulate collective cell movement and invasion, such as the role of p120, may present exciting new opportunities to target this process.

Materials and methods

cDNA constructs

Full-length human p120 isoform 1A with a C-terminal myc tag was generated by polymerase chain reaction (PCR) using pEFBOS-p120-1A as a template (van Hengel *et al.*, 1999) (a gift from J van Hengel, Flanders Interuniversity Institute for Biotechnology, Belgium) and the 5' primer TTACCCTGCCC TGC GGCGGCTCCGC and the 3' primer CCGTCGACTTA CAGATCCTCTTCTGAGATGAGTTTTTGTTCATCTTC TGCATC AAGGG. The PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen, Paisley, UK) and sequenced to ensure that no mutations had occurred during amplification. The insert was then subcloned into the bicistronic retroviral vector pLZRS-IRES-GFP (a gift from AB Reynolds, Vanderbilt University Medical School, Nashville, TN) using the *EcoRI* sites to generate pLZRS-p120-1A-IRES-GFP (Ireton *et al.*, 2002). Alternatively, site-directed mutagenesis of tyrosine-296 to phenylalanine was performed using the QuickChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) before subcloning to generate pLZRS-p120-1A/296F-IRES-GFP and the sequence verified. pRetroSuper constructs containing either a human-specific p120 RNAi sequence (GCCAGAGGTGGTTCGGATA) or a scrambled control sequence (GATAAGGTGCTGCGTGGAC) were generated as described previously (Davis *et al.*, 2003).

Cell lines

Generation of KM12C cells (a gift from IJ Fidler, University of Texas, MD, USA; Anderson Center, Houston, TX, USA) expressing constitutively active Src (SrcY527F) was as described previously (Avizienyte *et al.*, 2002). A431 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine and 10% fetal bovine serum (FBS). Retroviral infection of KM12C-SrcY527F or A431 cells was performed as described previously (Avizienyte *et al.*, 2002), and cells with stable retroviral integration were selected on the basis of GFP expression (KM12C-SrcY527F-pLZRS-p120 constructs) or with 2 µg/ml puromycin (Sigma Chemical Co., Poole, UK) (A431-pRetroSuper constructs). pLZRS-IRES-GFP encoding cDNA for murine p120 isoform 3A or 4A (gifts from AB Reynolds) were introduced to single-cell A431 clones with knockdown of p120 using the Amaxa Nucleofector System (Amaxa GmbH, Cologne, Germany) and pooled populations selected according to GFP expression by FACS.

Growth factors and drugs

Human recombinant EGF (R&D Systems, Abingdon, UK) was used at concentrations of 10 or 100 ng/ml. For all experiments, cells were first serum-starved overnight and then stimulated with the appropriate concentration of EGF in serum-free medium. AP23464 (ARIAD Pharmaceuticals, Cambridge, MA, USA) was dissolved in dimethylsulphoxide (DMSO) to a stock concentration of 10 mM. Immediately before use it was diluted in medium to achieve a final concentration of 1 µM. AG1478 (CN Biosciences Ltd, Nottingham, UK) was dissolved in DMSO to a stock concentration of 10 mM and was further diluted in medium to a final concentration of 300 nM immediately before use.

Immunoprecipitation and immunoblotting

Cells were washed and then lysed in ice-cold radioimmuno-precipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 100 µM sodium orthovanadate, 2 mM phenyl-methylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 500 µM

NaF). About 250–1000 µg of cleared lysate was then immunoprecipitated with 1 µg anti-p120 (BD Biosciences, Oxford, UK), 4 µl rabbit anti-p120-pY296 or 4 µl anti-myc clone 9B11 (New England Biolabs, Hertfordshire, UK). Where indicated, immunoprecipitates were first washed in reaction buffer (25 mM Imidazole, pH 7.0, 50 mM NaCl, 2.5 mM Na-EDTA, 5 mM DTT, 100 µg/ml bovine serum albumin (BSA)) and then incubated with 2 µl LAR phosphatase (CN Biosciences Ltd) at 30°C for 30 min before the addition of Laemmli sample buffer. Denatured proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Membranes were blocked and probed with 1:1000 anti-p120, 1:5000 anti-p120-pY228, 1:250 anti-p120-pY280, 1:500 anti-p120-pY291, 1:5000 anti-EGFR, 1:1000 anti-phospho-EGFR, 1:1000 PY20 (anti-phosphotyrosine), 1:2000 anti-E-cadherin, 1:500 anti-β-catenin (all from BD Biosciences, Oxford, UK), 1:10 000 anti-MAPK, 1:500 anti-actin, anti-β-tubulin (all from Sigma Chemical Co., Poole, UK), 1:1000 anti-phospho-MAPK (Thr202/Tyr204), 1:1000 STAT3 (Tyr705), 1:250 anti-phospho-STAT3, 1:1000 9B11 (anti-myc), 1:1000 Src-pY416. (all from New England Biolabs, Hertfordshire, UK), anti-P-cadherin (Merckbiosciences, Nottingham, UK) or 1:5000 anti-Src kinase domain (Arderm *et al.*, 2006). Bound antibody was detected by incubation with horseradish peroxidase conjugated secondary antibodies and visualized by enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). When necessary, blots were stripped using the Re-Blot Plus Strong Antibody Stripping Solution (Chemicon International, Harrow, UK) and reprobed with additional antibodies.

Antibody generation

A polyclonal rabbit antibody against p120 phospho-tyrosine-296 was generated using the peptide 290-(C)GYDDLDPYGMMSD-301. The N-terminal cysteine residue (bracketed) was added to facilitate coupling to the carrier protein keyhole limpet haemocyanin. Peptides were estimated to be >90% pure by high-performance liquid chromatography, and composition was confirmed by amino acid analysis (synthesis and analysis of peptides was carried out by Affiniti Research Products Ltd, Exeter, UK). About 200 µg of conjugated peptide was emulsified in Freund's adjuvant (Sigma Chemical Co., Poole, UK), either complete for the primary immunization or incomplete for subsequent immunizations, as described previously (McLean *et al.*, 1991). Serum was checked for positive reactivity and IgG was purified by affinity absorption to a protein G column (Amersham Biosciences, Buckinghamshire, UK). The elute was dialysed against PBS at 4°C for 16 h. Non-phospho-specific antibodies were removed by absorption against a Sepharose column containing the immunizing peptide in its unphosphorylated form and the remaining fraction was again absorbed against a protein G column, eluted and dialysed against PBS. The antibody was used at 1:125 dilution for immunoprecipitation and 1:5000 for immunoblotting.

Cell migration assays

To monitor movement of individual cells in sparse cultures, cells were plated at 1×10^4 per well of a six-well plate and allowed to adhere for 8 h. Images were then captured every 15 min for 5 h using an Axiovert 200 M Zeiss microscope with a $\times 20$ objective and AQM Advance software (Kinetic Imaging, Nottingham, UK). About 100 individual cells per cell line were analysed using Tracking Analysis software (Kinetic Imaging). For wound-healing assays, cells were plated at a density of 1×10^6 cells per well of a six-well plate. At 24 h later, a wound

was made using a sterile pipette tip and images of cells migrating into the wound recorded by timelapse video-microscopy at 15 min intervals until complete wound closure. Alternatively cells were plated on glass chamber slides and grown to confluence before wounding with a sterile pipette tip. After 6 h, cells were fixed with 3.7% formaldehyde in PBS, permeabilized in PBS containing 0.5% Triton X-100 and 1% BSA, blocked with 10% FBS in PBS, and incubated with FITC-phalloidin for 45 min. Cells were visualized using a Leica DM IRBE confocal microscope with $\times 40$ objective.

Immunofluorescence

Confocal imaging was carried out as described previously (Avizienyte *et al.*, 2002) using anti-p120 antibody (BD Bioscience) and TRITC-phalloidin. For quantification of membrane ruffling, at least 100 cells were counted for each treatment and the results were presented from three independent experiments. Experiments in which cells were transfected with siRNA duplexes were processed 48–72 h after transfection.

Matrigel invasion assays

Inverse invasion assays were carried out as described previously (Scott *et al.*, 2004) using complete Matrigel (BD Biosciences, Oxford, UK). Cells were seeded at 10^4 per Transwell (Corning Life Sciences, Sigma Chemical Co., Poole, Dorset, UK), and 10 ng/ml EGF was added to the DMEM above the Matrigel. After 72 h, cells were stained with Calcein AM (Molecular Probes, Invitrogen, Paisley, UK). Horizontal z-sections through the Matrigel were taken at 15 μ m intervals using a Leica DM IRBE confocal microscope. The number of positive pixels in each image was determined using 'Image J' software (NIH), using a value of 150 for background subtraction. The sum of the values obtained for individual sections (excluding the first section which corresponds to the upper surface of the filter) was then expressed as a percentage of the control cell value. For each experiment, samples were run in duplicate and at least four z-series were taken per sample. Projected images used for display purposes were also created using 'Image J'.

Organotypic invasion assays

pECFP (Clontech, BD Biosciences, Oxford, UK) was introduced into A431-Scr or A431-p120⁻ cells using the Amaxa Nucleofector System (Amaxa GmbH, Cologne, Germany) and pooled populations with uniform expression selected by FACS. These were plated on top of collagen/Matrigel (4 mg/ml collagen/2.5 mg/ml Matrigel) gels containing 10% FBS. Where indicated, 5×10^5 tumour-derived fibroblasts were added to the gel. After 24 h, the gel was transferred onto a nylon filter and placed on a mesh support. This was partially submerged in media containing 10% FBS, whereas the rest of the gel including the A431 cells was in contact with air. After 5 days, the gels were fixed with 4% paraformaldehyde per 0.25% glutaraldehyde per PBS, permeabilized with 0.2% Triton X-100/PBS and stained with TRITC-phalloidin. A multiphoton laser-scanning microscope was used to take five optical xz-sections of each gel and the area of non-invading cells and the total area over which cells had spread were measured. The invasion index = 1 area of non-invading cells per total area

covered by invading and non-invading cells. A score of 0 corresponds to no invasion, whereas the closer the score is to 1, the greater the invasion. Where indicated, E-cadherin-specific siRNA sequences (#1 GGAGAGCGGUGGUCAAAGAUU, #2 ACCAGAACCUCGAACUAUAUU) and P-cadherin-specific sequences (pool of equimolar amounts of #1 GACAACGUCUUCUACUAUGUU, #2 CAUAUGACGUGCACCUUUCUU, #3 CAGCUCAAGUCUAAUAAAGUU) or control sequences (#1 UAGCGACUAAACACAUCAA, #2 UAAGGCUAUGAAGAGAUAC) were introduced into the cells using Oligofectamine (Invitrogen, Paisley, UK) according to the manufacturers instructions.

Cell proliferation assays

Cells were plated at 1×10^3 per well of a 96-well plate. At 24 h intervals, the number of viable cells was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay (Plumb *et al.*, 1989). Mean and s.e.m. values were calculated from quadruplicate wells.

Immunohistochemistry

Specimens were mounted in moulds with tissue-tek OCT compound (Sakura; 4583), and frozen on liquid nitrogen. About 7 or 8 μ m sections were cut and mounted onto slides (Surgipath Superfrost 'Plus' white; 08143G). The slides were fixed in 4% paraformaldehyde/PBS for 10 min followed by permeabilization in 0.2% Triton-X100/PBS for 10 min. Samples were blocked in 2% BSA for 30 min and then incubated overnight at 4°C with antibodies pre-labelled with Zenon Mouse IgG labelling kit (Invitrogen, Alexa488-Z25002) diluted 1:100 in PBS/1% BSA/0.1% Tween, before washing in PBS/1% BSA/0.1% Tween. Specimens were re-fixed in 4% paraformaldehyde/PBS for 10 min followed by washing in PBS/1% BSA/0.1% Tween. Specimens were stained with anti-p120 (BD Biosciences), then counterstained with Alexa633 phalloidin (Invitrogen). Images were captured using a Zeiss LSM510 confocal microscope.

Rho GTP pull-down assays

Bacterially produced GST-PAK-CRIB domain or GST-Rhotekin-binding domain (RBD) fusion protein was bound to glutathione-coupled Sepharose beads by incubation for 30 min at 4°C with gentle agitation. Beads were then washed three times with lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 1% NP-40, 10% glycerol, 100 mM NaCl, 1 mM benzamidine, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 μ g/ml aprotinin, 1 mM DTT and 1 mM PMSF) and retained at 4°C until used. Cells were serum starved for 16 h and then stimulated with 100 ng/ml EGF or vehicle for 5 (Rac/Cdc42) or 15 min (RhoA), washed in ice-cold PBS (containing 1 mM MgCl₂ and 0.5 mM CaCl₂), incubated for 5 min on ice in lysis buffer, and then centrifuged for 5 min at 14 000 g at 4°C. Aliquots were taken from the supernatant to compare protein amounts. The remaining supernatant was incubated at 4°C with either GST-PAK beads for 30 min or GST-RBD beads for 60 min. Beads were then washed three times in lysis buffer, eluted in Laemmli sample buffer, and then analysed for bound Rho family molecules by Western blotting using anti-RhoA, anti-Rac1 or anti-Cdc42 antibodies. Films were scanned and the density of bands quantified using Total Labs software.

References

Anastasiadis PZ, Reynolds AB. (2000). The p120 catenin family: complex roles in adhesion, signaling and cancer. *J Cell Sci* 113(Part 8): 1319–1334.

Aono S, Nakagawa S, Reynolds AB, Takeichi M. (1999). p120(ctn) acts as an inhibitory regulator of cadherin function in colon carcinoma cells. *J Cell Biol* 145: 551–562.

- Ardern H, Sandilands E, Machesky LM, Timpson P, Frame MC, Brunton VG. (2006). Src-dependent phosphorylation of Scar1 promotes its association with the Arp2/3 complex. *Cell Motil Cytoskeleton* 63: 6–13.
- Avizienyte E, Wyke AW, Jones RJ, McLean GW, Westhoff MA, Brunton VG et al. (2002). Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling. *Nat Cell Biol* 4: 632–638.
- Bellovin DI, Bates RC, Muzikansky A, Rimm DL, Mercurio AM. (2005). Altered localization of p120 catenin during epithelial to mesenchymal transition of colon carcinoma is prognostic for aggressive disease. *Cancer Res* 65: 10938–10945.
- Brunton VG, Avizienyte E, Fincham VJ, Serrels B, Metcalf III CA, Sawyer TK et al. (2005). Identification of Src-specific phosphorylation site on focal adhesion kinase: dissection of the role of Src SH2 and catalytic functions and their consequences for tumor cell behavior. *Cancer Res* 65: 1335–1342.
- Brunton VG, MacPherson IR, Frame MC. (2004). Cell adhesion receptors, tyrosine kinases and actin modulators: a complex three-way circuitry. *Biochim Biophys Acta* 1692: 121–144.
- Cozzolino M, Stagni V, Spinardi L, Campioni N, Fiorentini C, Salvati E et al. (2003). p120 Catenin is required for growth factor-dependent cell motility and scattering in epithelial cells. *Mol Biol Cell* 14: 1964–1977.
- Daniel JM, Reynolds AB. (1999). The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. *Mol Cell Biol* 19: 3614–3623.
- Davis MA, Ireton RC, Reynolds AB. (2003). A core function for p120-catenin in cadherin turnover. *J Cell Biol* 163: 525–534.
- Davis MA, Reynolds AB. (2006). Blocked acinar development, E-cadherin reduction, and intraepithelial neoplasia upon ablation of p120-catenin in the mouse salivary gland. *Dev Cell* 10: 21–31.
- Friedl P, Hegerfeldt Y, Tusch M. (2004). Collective cell migration in morphogenesis and cancer. *Int J Dev Biol* 48: 441–449.
- Grosheva I, Shtutman M, Elbaum M, Bershadsky AD. (2001). p120 catenin affects cell motility via modulation of activity of Rho-family GTPases: a link between cell–cell contact formation and regulation of cell locomotion. *J Cell Sci* 114: 695–707.
- Hajra KM, Fearon ER. (2002). Cadherin and catenin alterations in human cancer. *Genes Chromosomes Cancer* 34: 255–268.
- Hanahan D, Weinberg RA. (2000). The hallmarks of cancer. *Cell* 100: 57–70.
- Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA. (2000). Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. *J Cell Biol* 148: 779–790.
- Ireton RC, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA et al. (2002). A novel role for p120 catenin in E-cadherin function. *J Cell Biol* 159: 465–476.
- Ishizaki Y, Omori Y, Momiyama M, Nishikawa Y, Tokairin T, Manabe M et al. (2004). Reduced expression and aberrant localization of p120catenin in human squamous cell carcinoma of the skin. *J Dermatol Sci* 34: 99–108.
- Islam S, Carey TE, Wolf GT, Wheelock MJ, Johnson KR. (1996). Expression of N-cadherin by human squamous carcinoma cells induces a scattered fibroblastic phenotype with disrupted cell–cell adhesion. *J Cell Biol* 135: 1643–1654.
- Jensen PJ, Telegan B, Lavker RM, Wheelock MJ. (1997). E-cadherin and P-cadherin have partially redundant roles in human epidermal stratification. *Cell Tissue Res* 288: 307–316.
- Keirsebilck A, Bonne S, Staes K, van Hengel J, Nollet F, Reynolds A et al. (1998). Molecular cloning of the human p120ctn catenin gene (CTNND1): expression of multiple alternatively spliced isoforms. *Genomics* 50: 129–146.
- Kelly KF, Spring CM, Otchere AA, Daniel JM. (2004). NLS-dependent nuclear localization of p120ctn is necessary to relieve Kaiso-mediated transcriptional repression. *J Cell Sci* 117: 2675–2686.
- Kurokawa K, Itoh RE, Yoshizaki H, Nakamura YO, Matsuda M. (2004). Coactivation of Rac1 and Cdc42 at lamellipodia and membrane ruffles induced by epidermal growth factor. *Mol Biol Cell* 15: 1003–1010.
- Lim YP, Diong LS, Qi R, Druker BJ, Epstein RJ. (2003). Phosphoproteomic fingerprinting of epidermal growth factor signaling and anticancer drug action in human tumor cells. *Mol Cancer Ther* 2: 1369–1377.
- Malliri A, Symons M, Hennigan RF, Hurlstone AF, Lamb RF, Wheeler T et al. (1998). The transcription factor AP-1 is required for EGF-induced activation of rho-like GTPases, cytoskeletal rearrangements, motility, and *in vitro* invasion of A431 cells. *J Cell Biol* 143: 1087–1099.
- Mareel M, Leroy A. (2003). Clinical, cellular, and molecular aspects of cancer invasion. *Physiol Rev* 83: 337–376.
- Mariner DJ, Anastasiadis P, Keilhack H, Bohmer FD, Wang J, Reynolds AB. (2001). Identification of Src phosphorylation sites in the catenin p120ctn. *J Biol Chem* 276: 28006–28013.
- Mariner DJ, Davis MA, Reynolds AB. (2004). EGFR signaling to p120-catenin through phosphorylation at Y228. *J Cell Sci* 117: 1339–1350.
- McLean GW, Owsianka AM, Subak-Sharpe JH, Marsden HS. (1991). Generation of anti-peptide and anti-protein sera. Effect of peptide presentation on immunogenicity. *J Immunol Methods* 137: 149–157.
- Noren NK, Liu BP, Burrridge K, Kreft B. (2000). p120 catenin regulates the actin cytoskeleton via Rho family GTPases. *J Cell Biol* 150: 567–580.
- Ozawa M, Ohkubo T. (2001). Tyrosine phosphorylation of p120(ctn) in v-Src transfected L cells depends on its association with E-cadherin and reduces adhesion activity. *J Cell Sci* 114: 503–512.
- Park JI, Kim SW, Lyons JP, Ji H, Nguyen TT, Cho K et al. (2005). Kaiso/p120-catenin and TCF/beta-catenin complexes coordinately regulate canonical Wnt gene targets. *Dev Cell* 8: 843–854.
- Peifer M, Polakis P. (2000). Wnt signaling in oncogenesis and embryogenesis – a look outside the nucleus. *Science* 287: 1606–1609.
- Perez-Moreno M, Davis MA, Wong E, Pasolli HA, Reynolds AB, Fuchs E. (2006). p120-catenin mediates inflammatory responses in the skin. *Cell* 124: 631–644.
- Perl AK, Wilgenbus P, Dahl U, Semb H, Christofori G. (1998). A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* 392: 190–193.
- Plumb JA, Milroy R, Kaye SB. (1989). Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Res* 49: 4435–4440.
- Qian X, Karpova T, Sheppard AM, McNally J, Lowy DR. (2004). E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases. *EMBO J* 23: 1739–1748.
- Sahai E. (2005). Mechanisms of cancer cell invasion. *Curr Opin Genet Dev* 15: 87–96.
- Scott LA, Vass JK, Parkinson EK, Gillespie DA, Winnie JN, Ozanne BW. (2004). Invasion of normal human fibroblasts

- induced by v-Fos is independent of proliferation, immortalization, and the tumor suppressors p16INK4a and p53. *Mol Cell Biol* 24: 1540–1559.
- Shibata T, Kokubu A, Sekine S, Kanai Y, Hirohashi S. (2004). Cytoplasmic p120ctn regulates the invasive phenotypes of E-cadherin-deficient breast cancer. *Am J Pathol* 164: 2269–2278.
- Spring CM, Kelly KF, O'Kelly I, Graham M, Crawford HC, Daniel JM. (2005). The catenin p120ctn inhibits Kaiso-mediated transcriptional repression of the beta-catenin/TCF target gene matrilysin. *Exp Cell Res* 305: 253–265.
- Strathdee G. (2002). Epigenetic versus genetic alterations in the inactivation of E-cadherin. *Semin Cancer Biol* 12: 373–379.
- Thelemann A, Petti F, Griffin G, Iwata K, Hunt T, Settinar T *et al.* (2005). Phosphotyrosine signaling networks in epidermal growth factor receptor overexpressing squamous carcinoma cells. *Mol Cell Proteomics* 4: 356–376.
- Thoreson MA, Reynolds AB. (2002). Altered expression of the catenin p120 in human cancer: implications for tumor progression. *Differentiation* 70: 583–589.
- van Hengel J, Vanhoenacker P, Staes K, van Roy F. (1999). Nuclear localization of the p120(ctn) Armadillo-like catenin is counteracted by a nuclear export signal and by E-cadherin expression. *Proc Natl Acad Sci USA* 96: 7980–7985.
- van Roy FM, McCrea PD. (2005). A role for Kaiso-p120ctn complexes in cancer? *Nat Rev Cancer* 5: 956–964.
- Vleminckx K, Vakaet Jr L, Mareel M, Fiers W, van Roy F. (1991). Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 66: 107–119.
- Wheelock MJ, Johnson KR. (2003). Cadherin-mediated cellular signaling. *Curr Opin Cell Biol* 15: 509–514.
- Xia X, Mariner DJ, Reynolds AB. (2003). Adhesion-associated and PKC-modulated changes in serine/threonine phosphorylation of p120-catenin. *Biochemistry* 42: 9195–9204.
- Xiao K, Allison DF, Buckley KM, Kottke MD, Vincent PA, Faundez V *et al.* (2003). Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. *J Cell Biol* 163: 535–545.
- Yanagisawa M, Anastasiadis PZ. (2006). p120 catenin is essential for mesenchymal cadherin-mediated regulation of cell motility and invasiveness. *J Cell Biol* 174: 1087–1096.

Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>).