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# **Regulation of E2F**

## **Through the DP Component**

Presented by

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to

The University of Glasgow

For the degree of

a state of the state of the state of the state of the

Doctor of Philosophy

Division of Biochemistry and Molecular Biology

Institute of Biomedical and Life Sciences

University of Glasgow

Scotlaud

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## Abstract

Transcription factor E2F plays an important role in orchestrating early cell cycle progression through its ability to co-ordinate and integrate the cell cycle with the transcriptional apparatus. Physiological E2F arises when members of two distinct families interact as E2F-DP heterodimers, in which the E2F component mediates transcriptional activation and physical interaction with pocket proteins, such as the tumour suppressor protein pRB. However, very little information is available regarding the mechanisms, which control the levels of functional E2F. In this study I have defined the DP component as an integrator of the E2F transcription factor with the nuclear transport pathway and the ubiquitin-proteasome mediated degradation pathway. Specifically, a bipartite nuclear localisation signal in DP is defined and shown to be necessary for nuclear accumulation of a non-NLS-containing heterodimeric partner. Pocket protein association also offers an alternative in trans NLS for translocation of the heterodimer into the nucleus. Surprisingly, the basic region of the NLS is also necessary for efficient nuclear accumulation of NLS-containing E2F heterodimeric partners. The basic region is also instrumental in the association of DP-3 with the epsilon isoform of the 14-3-3 family of signalling molecules. Using a mutant DP that fails to bind 14-3-3 has uncovered a role for 14-3-3 in the E2F pathway. An association targets the E2F heterodimer for ubiquitin-proteasome-mediated degradation to reduce excessive levels of E2F and allow cells to progress into G2 phase of the cell cycle. This study defines new pathways of growth control that are integrated with the E2F pathway through the DP subunit.

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# Abbreviations

APS	Ammonium persulphate
BCIP	5-Bromo-4-chloro-3-inodolyl phosphate
β-gal	β-galactosidase
bp	Base pair
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine mono-phosphate
САК	Cyclin Activating Kinase
CBP	CREB-binding protein
CDI	Cyclin-CDK Inhibitor
CDK-	Cyclin Dependent Kinase -
cDNA	Complementary deoxyribonucleic acid
СК	Casein kinase
cpm	Counts Per Minute
Сус	Cyclin
DBD	DNA binding domain
DHFR	Dihydofolate reductase
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNA pol $\alpha$	DNA polymerase α
DNA-PK	Double-stranded DNA-activated protein kinase

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dNTP	Deoxy-nucleoside triphosphate
DP-	DRTF1 Polypeptide
DTT	Dithiothreitol
E.coli	Eschericha coli
EDTA	Ethylene diamine tetra-acetic acid
FACS	Fluorescence activated cell scanning
FCS	Foetal calf serum
F9EC	F9 embryonal carcinoma
FITC	Fluorescein isothiocyanate
GAD	Gal4 activation domain
GST	Glutathione-S-transferase
HA	Hemagglutinin protein (derived from influenza virus)
HBS	HEPES-buffered saline
HDAC1	Histone deacetylase 1
HEPES	N- [2-Hydroxethyl] piperazine-N'- [2-ethanesulfonic acid]
HPV	Human papilloma virus
в	Immunoblot
IL-3	Interleukin-3
Ъ	Immunoprecipitation
IPTG	Isopropyl-B-D-thiogalactopyranoside
R	Irradiation
IVT	In vitro translated
LOII	Loss of heterozygosity
Luc	Luciferase
MDM 2	Murine double minute 2

NLS	Nuclear localization signal
NP-40	Nonidet P-40
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween-20
PCR	Polymerase chain reaction
РКС	Protein kinase C
PMSF	Phenylmethysulfonyl fluoride
Pol II	RNA polymerase II
pRB	Retinoblastoma gene product
SDS	Sodium dodecyl sulphate
TAg	Simian virus 40 large T antigen
TAD	Transactivation domain
TAF	TBP-associated factor
ТВР	TATA binding protein
TFs	Transcription factors
ТК	Thymidine kinase
TRITC	Tetramethylrhodamine isothiocyanate
Tris	Tris(hydroxymethyl)methylamine
Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet light
V <b>P</b> 16	Virion protein 16 trans activation domain
v/v	Volume per volume
wt	Wild type
w/v	Weight per volume

- (+/+) Wild type
- (+/-) Heterozygous mutant
- (-/-) Homozygous mutant

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## Declaration

All the work presented within this thesis was performed entirely by myself and in no way forms part of any other thesis. The work was carried out while I was a graduate student at the Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Science, University of Glasgow, UK. I was under the supervision of Professor Nicholas B. La Thangue.

Amanda Cruickshank

August 2000

## Chapter 1. Introduction

### 1-1. The E2F pathway

Understanding normal cell cycle control is essential when searching for novel anti-tumour therapeutics. The deregulation of proliferation of a malignant cell is caused by small alterations in the machinery that directs normal cell growth and division. This machinery has the ability to continuously monitor the status of the intra-cellular and extra-cellular environment in order to maintain the integrity of the genome during each cell division cycle. The transition from G1 to S phase is a particularly important period when critical choices, resulting in cell growth or quiescence, are made. Progression into S phase is tightly coupled to the transcriptional control of genes involved in growth and DNA replication (Dyson, 1998) (Figure 1.1). The E2F family of transcription factors principally carries out this temporal control of gene expression. The pathway responsible for regulating E2F is frequently found to be aberrant in human tumour cells, thus, comprehension of the mechanisms involved in regulating the E2F pathway will provide an insight into the workings of the molecular machinery that controls cell proliferation and consequently present suitable targets for anti-cancer therapies.

DNA synthesis	Cell proliferation	Apoptosis
DNA polymerase 0. thymidine kinase dihydrofolate reductase orc 1 cdc6 mcm RanBP1 PCNA topo.1	cyclin D cyclin E cyclin A cdc2 cdk2 p107 pRB c-myc N-Myc B-Myb E2F-1 E2F-2 E2F-3 E2F-3 E2F-4 E2F-5 DP-1 cdc25C cdc25A	p19ARF

## Figure 1.1

## E2F regulated genes

(A) Table showing genes involved in DNA synthesis, cell proliferation and apoptosis that are regulated by E2F through E2F DNA binding sites within their promoter regions.

#### 1-1.1. The E2F family of proteins

The E2F family comprises of two distinctly related subfamilies, E2F and DP. One subunit of E2F and one subunit of DP combine to form a transcriptionally active E2F heterodimer (La Thangue, 1994; Lam and La Thangue, 1994; Wu et al., 1996). To date, six E2F genes and three DP genes have been identified (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992; Ivey-Hoyle et al., 1993; Lees et al., 1993; Beijersbergen et al., 1994; Ginsberg et al., 1994; Sardet et al., 1995; Hijmans et al., 1995; Morkel et al., 1997; Cartwright et al., 1998; Trimarchi et al., 1998; Girling et al., 1993; Ormondrovd et al., 1995; Wu et al., 1995; Zhang and Chellapan, 1995; Rogers et al., 1996) (Figure 1.2). E2F-1, -2, -3, -4 and -5 share 20%-50% identity and have a similar organisation of functional domains but E2F-6 lacks a transactivation domain (Morkel et al., 1997). The DPs are highly homologous (~70%), and each can form a functional heterodimer with any E2F family member. E2F and DP proteins contain highly conserved DNA-binding and dimerisation domains (Helin, 1998) (Figure 1.2). The crystal structure of an E2F-4/DP-3/DNA complex shows that the DNA-binding domains of the E2F and DP proteins both have a fold related to the winged-helix DNAbinding motif and that heterodimerisation aids DNA binding (Zheng et al., 1999).

Expression of the E2F family can be observed in a wide range of tissues and cell lines. The levels of mRNA expression are highly variable and to some extent, tissue restricted (Wu *et al.*, 1995). Synthesis of E2F-1 mRNA is induced in mid-G1 and reaches its highest levels in S phase (Slansky *et al.*, 1993). In fibroblasts, E2F-4 and E2F-5 transcripts are abundant in G0, with E2F-4 expressed considerably higher than



#### Figure 1.2

#### The structures of the E2F and DP family members.

(A) Summary of the structures of the E2F family members E2Fs-1,-2,-3,-4,-5 and -6, indicating the locations of the conserved domains.

(B) Summary of the structures of the DP family members DP-1,-2 and -3 indicating the position of the conserved region with greatest similarity to E2F family members.

E2F-5 in these quiescent cells (Sardet *et al.*, 1995). Following neuronal differentiation, E2F-4 is highly up regulated, while E2F-1, -3 and 5 are down regulated. E2F-5 is up regulated in certain differentiated tissues (Dagnino *et al.*, 1997). DP-1 is a frequent component of E2F, occurring in most forms of the DNA binding activity in many different cell types. The induction of DP-1 mRNA in mid-G1 is almost identical to the induction of E2F-4, although DP-1 mRNA levels do not decrease during S phase (Sardet *et al.*, 1995). DP-2 and DP-3 are less frequent components of E2F and their expression is tissue-restrictive (Girling *et al.*, 1994; Rogers *et al.*, 1996).

#### **1-1.2.** The pRB/E2F complex

The best-studied upstream regulators of E2F are the retinoblastoma family of proteins, or 'pocket proteins', which include pRB, p107, and p130 (Bartek *et al.*, 1996; Sidle *et al.*, 1996). A short, highly conserved domain near the carboxyl-terminus of the E2F proteins mediates binding to pRB family proteins (Helin *et al.*, 1993) (Figure1.2). This binding domain is embedded in the *trans*-activation domain of the E2F subunit, suggesting a model for inhibition of E2F-dependent *trans*-activation without affecting DNA binding ability (Flemington *et al.*, 1993; Zamanian and La Thangue, 1993; Helin *et al.*, 1993). Consistent with the lack of a *trans*-activation domain, E2F-6 is unable to bind to pocket proteins (Trimarchi *et al.*, 1998). The ability of pRB and related proteins to repress E2F activity correlates with their growth suppression function, and intrinsic capacity to repress transcription (Bremner *et al.*, 1995; Zhang *et al.*, 1999), suggesting that E2F repression may be an essential element of pRB-mediated growth suppression (Helin *et al.*, 1993). Different members of the pRB family appear to regulate specific E2F family members, as pRB primarily binds to E2F-1, -2, and -3, although it can also

bind to E2F-4 (Ikeda *et al.*, 1996; Moberg *et al.*, 1996). In contrast, p107 and p130 display higher affinities toward E2F-4 and -5 (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Sardet *et al.*, 1995; Vairo *et al.*, 1995). As a general rule, pRB/E2F complexes can be found in quiescent or differentiated cells, but are most evident as cells progress from G1 into S phase. p107/E2F complexes are most prevalent in S phase cells but can also be found in G1. In mid- to late-G1 the complex also contains cyclin E/cdk2, which is exchanged for cyclin A/cdk2 in S phase. p130/E2F complexes replace p107/E2F complexes when cells exit the cell cycle and differentiate (Smith *et al.*, 1997). Based on these findings it has been suggested that different E2F members, together with their primary pRB protein regulators, control different sets of genes at different stages of the cell cycle.

### 1-1.3. Regulation of the pRB/E2F association

Regulation of the physical interaction between pRB and E2F is integrated with cell cycle progression. In G1 pRB exists in an active, hypophosphorylated state and is complexed with E2F. At the G1/S transition, cyclin D/Cdk4 then cyclin E/Cdk2 or cyclin A/Cdk2 complexes sequentially phosphorylate pRB, thereby reducing its affinity for E2F (Mittnacht 1998; Harbour *et al.*, 1999). Release of free E2F allows activation of transcription of genes required for S phase entry and progression (Figure 1.3).



## Figure 1.3 The E2F pathway

(A) Model showing different E2F complexes that occur as cells progress through the cell cycle: repression complexes recruit RB-family proteins to the DNA, inhibited complexes arise when the binding of RB to E2F physically blocks the activity of the E2F transactivation domain, activating complexes occur when RB-family proteins are phosphorylated by cdks and subsequently loss their affinity for E2F, inactive complexes lose their affinity for DNA upon phosphorylation of the DP component by Cdk2.

The E2F/pRB complex is also subject to regulation by cdk inhibitors (CKIs), which ensure arrest or withdrawal from the cell cycle in response to anti-mitogenic or genotoxic stimuli, or as part of programmes of terminal differentiation (Harper and Elledge, 1996). The INK4 family of CKIs are particularly important in the regulation of pRB. This family includes p15, p16, p18 and p19 that inhibit complexes of cyclin D1, 2, and 3 bound to Cdk4 and Cdk6 to maintain pRB in an active state, thereby, holding cells in G1 (Sherr and Roberts, 1995). The importance of this function of p16 is underscored by the fact that p16-induced cell cycle arrest requires functional pRB (Lukas et al., 1995). The Kip family of CKIs includes p21, p27, and p57, and can inhibit a wider range of Cdks including Cdk2 (Harper and Elledge, 1996). The importance of CKIs in the E2F pathway has been shown by knock-out mice: cells that have lost p16 become immortalised (Serrano et al., 1996) and those that have lost p21 fail to respond to DNA damage (Leder et al., 1995). Mice lacking p27 have an increase in cell numbers in many tissues due to an increase in proliferation, and develop pituitary tumours and retinal abnormalities, reminiscent of Rb-l heterozygotes (Fero *et al.*, 1995).

pRB/E2F complexes represent only a minor fraction of the total E2F complexes present in cell extracts as E2F is not completely deregulated in the absence of pRB. In an attempt to separate the functions of different pocket protein members, primary mouse cells lacking a specific family member were studied. The *Rb-/-* embryos die E13-15 with cells failing to attain a fully differentiated state. Additionally, ectopic cell cycle entry and increased levels of apoptosis are apparent in some tissues (Clarke *et al.*, 1992; Jacks *et al.*, 1992). *Rb-/-* mouse embryo fibroblasts (MEFs) proceed into S phase early but most E2F target genes are induced in time for S phase (Herrera *et al.*, 1996). No differences were observed in *p107-/-* or *p130-/-* cells unless both genes were targeted. The *p107-/-*; *p130-/-* double knock-out mice die *in utero* due to cartilage and bone malformation (Cobrinik *et al.*, 1996). However, extensive functional overlap has been shown to occur between p107 and pRB as pRB-/-; p107-/- double knock-out embryos die 2 days earlier than pRB-/-embryos whilst p107-deficient mice do no show embryonic lethality (Lee *et al.*, 1996). A certain degree of redundancy must occur between p107 and p130 but not between pRB and p107/p130 (Hurford *et al.*, 1997). Until it is possible to confidently identify E2F complexes at endogenous promoters, it will remain unknown if the compensatory effects observed in knock-out mice are a true reflection of functional redundancy that occurs between pRB family members in normal cells.

#### **1-1.4.** E2F-site dependent transcription

It is not yet clear whether different E2F complexes differ in their DNA sequence specificity and in their preference for promoters of different E2F-responsive genes *in vivo*. Most current models rely on the assumption that results seen in over-expression assays in tissue culture cells reflect the properties of the endogenous proteins. Using *in vitro* assays and over-expression systems, all E2F heterodimers will bind to doublestranded DNA encoding the general consensus sequence TTTc/gGCGCg/c (Lees *et al.*, 1993; Buck *et al.*, 1995; Zhang and Chellappan, 1995). A model connects changes in gene expression to the binding of specific E2F complexes: activating complexes, in which the *trans*-activation domain drives transcription; inhibited complexes, in which the activation domain is masked by pRB family proteins; and repression complexes, in which pRB family proteins, and consequently histone deacetylases are recruited to the DNA by E2F, leading to local remodelling of the chromatin structure to make it less accessible to transcription factors (Luo et al., 1998; Ferreira et al., 1998; Harbour et al., 1999) (Figure 1.3). An alternative model to explain pRB-mediated active transcriptional repression proposes that pRB interacts directly with TFIIA and TFIID of the basal transcription initiation machinery. It is possible that both repression mechanisms occur since some promoters tested were insensitive to HDAC1-mediated inhibition (Luo et al., 1998) and it has been shown that pRB is able to repress transcription in a reconstituted in vitro transcription system lacking histones (Dynlacht et al., 1994). E2F binding sites appear to confer specificity for an activating or a repressing complex since mutation of the E2F binding site in the *dhfr* promoter reduces DHFR expression (Means et al., 1992), whereas mutation of the E2F binding site in the promoter of B-myb increases expression of that gene up to wild type levels (Lam and Watson, 1993). Many of the studies of transcriptional activation by E2F use simple promoter constructs containing single E2F sites, however, E2F sites are generally found in considerably more complex promoter regions. The p107 promoter, for example, contains two E2F sites that cooperatively regulate p107 expression (Zhu et al., 1995). In the Ran BP1 promoter, two E2F sites actually exert opposite functions whereby E2F-1 preferentially binds to one site with Sp1 to up-regulate gene expression, and the other site holds E2F-4/pocket protein complexes that repress the activity of the promoter. In another example, an ATF site flanks the two E2F sites in the adenovirus E2a promoter (Loeken et al., 1989) and E2F-1, -2 and -3 have been shown to bind to Sp1 (Karlseder et al., 1996), therefore, E2F and/or pRB may influence flanking transcription factors. Also, it is noteworthy that over 50 other proteins are known to interact with pRB and thus potentially be recruited to the E2F/DNA complex to regulate the basal transcription machinery.

### 1-1.5. Inactivating E2F

Inactivation of E2F by pRB only occurs while pRB is in a hypophosphorylated state in G1, therefore, other mechanisms are needed to switch off the activity of E2F later in the cell cycle. If constitutively active E2F is expressed, then cells will arrest in S phase suggesting that down-regulation of E2F is required for cells to exit S phase (Krek *et al.*, 1995). To this end, E2F-1, E2F-2 and E2F-3 contain a conserved motif that mediates stable association with cyclin A/cdk2 or cyclin E/cdk2 (Krek *et al.*, 1994) (Figure 1.2). Kinases bound through this domain to an E2F/DP heterodimer phosphorylate both the E2F and the DP subunits, and phosphorylation by cyclin A/cdk2 will inhibit DNA-binding activity (Krek *et al.*, 1994, 1995) (Figure 1.3). This down-regulation will not occur until late S phase when cyclin A becomes activated. Preventing cyclin A from binding to and mediating phosphorylation of E2F/DP is sufficient to prevent cells from passing through S phase (Krek *et al.*, 1995).

Ubiquitin-proteasome mediated degradation of E2F may also contribute to the down-regulation of E2F in S phase. Ubiquitination requires sequences within the *trans*-activation/pRB binding domain of E2F and therefore, binding to pocket proteins increases the stability of E2F by blocking access to ubiquitin conjugating enzymes (Martelli and Livingston, 1999). Recently the degradation of E2F-1 was shown to be linked to a specific interaction between E2F-1 and the F-box --containing protein Skp2, which is the cell-cycle-regulated component of the ubiquitin-protein ligase SCF<sup>sKP2</sup> that recognizes substrates for this ligase (Marti *et al* 1999).

#### 1-1.6. Physiological functions of E2F

The importance of E2F in cell cycle regulation has been illustrated in several ways. For instance, over-expression of E2F is sufficient to induce S phase in cells held in quiescence by serum starvation (Johnson et al., 1993; Asano et al., 1996; Lukas et al., 1996; Wang et al., 1998) and dominant negative mutants suggest that E2F activity is required for S phase entry (Wu et al., 1996). Over-expression of E2F-1 can over-ride many different types of cell cycle arrest including that induced by p16, p21, p27, yirradiation, TGFB or dominant negative cdk2 (DeGregori et al., 1995; Schwarz et al., 1995; Lukas et al., 1996; Mann and Jones, 1996). Over-expression of E2F in transgenic mice promotes cell proliferation and blocks some elements of differentiation (Guy et al., 1996). The elevation of E2F activity dissociates the coordination of cell growth and proliferation during *Drosophila* wing development (Neufeld et al., 1998). This ability to drive cells into S phase requires its DP-binding, DNA-binding and *trans*-activation domains, suggesting that the induction of expression of E2F target genes promotes S phase (Johnson et al., 1993; Qin et al., 1994). Consistent with the idea that E2F promotes cell proliferation, E2F-1, E2F-4, E2F-5, DP-1 and DP-2 have all been reported to score in transformation assays, either alone or in combination with an activated ras gene (Beijersbergen et al., 1994; Ginsberg et al., 1994; Johnson et al., 1994; Singh et al., 1994; Jooss et al., 1995; Xu et al., 1995; Polanowska 2000).

As well as promoting cell proliferation, elevated levels of E2F-1 in normal cells can also lead to apoptosis (Shan and Lee 1994). Support for the idea of E2F mediated apoptosis without relying on over-expression, came from E2F-I knock-out mice (Field *et al.*, 1996; Yamasaki *et al.*, 1996). Although these mice developed and reproduced

normally, they exhibited defects in T-cell development. Surprisingly, proliferative failure was not observed, and in fact tumour formation was more prevalent, indicating that E2F-1 functions to positively regulate apoptosis and suppress cell proliferation.

Several studies have shown that E2F-1 can induce apoptosis in either a p53dependent (see below) or p53-independent manner. E2F-1 induction of apoptosis in p53-null cells does not require the *trans*-activation domain of E2F-1 (Hsieh *et al.*, 1997; Philips *et al.*, 1997; Lee *et al.*, 1998). Under these conditions, E2F-1 induces apoptosis by a death receptor-dependent mechanism that sensitises cells to apoptosis in response to tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) by down-regulating expression of TRAF2, thereby inhibiting normal activation of anti-apoptotic signals such as NF- $\kappa$ B and JNK/SAPK (Phillips *et al.*, 1999).

Depending on its level of expression or the cellular environment, E2F-1 can function as an oncogene or a tumour suppressor (Pierce *et al.*, 1999). Oncogenicity is demonstrated by transformation assays (Johnson *et al.*, 1994), the over-expression of E2F-1 in transgenic mice promoting tumourigenesis (Pierce *et al.*, 1998) and the absence of E2F-1 reducing tumour phenotypes resulting from the inactivation of pRB (Yamasaki *et al.*, 1998). On the other hand, germline deletion leads to an increase in the occurrence of tumours (Yamasaki *et al.*, 1996), thus defining E2F-1 as a tumour suppressor.

Recently, it has been shown that differential regulation of E2F-1 and E2F-3 occurs. E2F-1 DNA-binding activity accumulates as cells are stimulated to enter the cell cycle, but does not accumulate in subsequent cell cycles. On the other hand, E2F-3

levels continue to cycle. The fact that immunodepletion of E2F-3 causes cycling cells to arrest suggests that E2F-1 activity is only required for re-entry into the cell cycle from a quiescent state and that E2F-3 regulates expression of S phase genes in proliferating cells (Leone *et al.*, 1998; Wang *et al.*, 1998).

A link between the E2F pathway and the G2/M transition has recently been shown, where E2F dependent expression of cyclin A is needed in S phase to disrupt the ubiquitin-ligase properties of the anaphase promoting complex (APC), thus allowing the accumulation of cyclin B1, a key activator of the cdc2 kinase required to initiate cell division (Lukas, 1999).

### 1-1.7. Integration of E2F with the p53 pathway

In mammalian cells, the p53 tumour suppressor is the archetypal DNA damage checkpoint regulator. Following genomic DNA damage, E2F DNA binding activity increases due to stabilisation of the protein (Blattner *et al.*, 1999), and consequently increases expression of proteins involved in the DNA damage response, such as ribonucleotide reductase (RNR) (Filatov *et al.*, 1996) and p14  $^{ARF}$  (Bates *et al.*, 1998). ARF is a peptide that stabilises p53, increasing transcription of p53-responsive target genes. This leads to up-regulation of p21, GADD45, cyclin G, bax and MDM2, culminating in either cell cycle arrest, to allow time for repair, or apoptosis (El-Diery *et al.*, 1993; Harper *et al.*, 1993).

#### 1-1.8. Regulation of E2F by protein stabilisation

Recent studies have shown that E2F-1 is subject to ubiquitin-proteasome mediated degradation, and the carboxyl terminus has been identified as regulating protein stability (Hateboer *et al.*, 1996). Ectopic overproduction of hypophosphorylated pRB can bind and stabilise E2F-1, protecting it from ubiquitination in a cell cycle dependent manner. In response to gamma irradiation, E2F-1 is stabilised in S phase, parallel to the accumulation of hypophosphorylated pRB (Hofferer *et al.*, 1999).

Like p53, E2F-1 is also subject to degradation when complexed with MDM2. MDM2 carries out its anti-apoptotic role by utilizing its E3 ligase-like activity to stimulate ubiquitin-dependent degradation of p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). MDM2-mediated lowering of the levels of E2F protein consequently switches the position of E2F from a pathway leading to apoptosis to one stimulating growth. This level of regulation of E2F by MDM2 is mediated via the DP partner (Loughran and La Thangue, 2000).

### **1-1.9.** Deregulation of E2F in cancer

Considering the central role of E2F in cell cycle control, manipulation of any of the pathways imparting upon E2F will be sufficient for a tumour cell to deregulate E2F and provide a proliferative advantage (Figure 1.4).

The pRB gene RBI is mutated in approximately 30% of all human cancers. All naturally occurring mutations occur within a region termed the "pocket domain". Although this region is also conserved in p107 and p130, no mutations have been found in the p107 gene, and only a few mutations of the p130 gene, in human cancers (Vairo et al., 1995; Helin et al., 1997). Certain tumours have evolved a mechanism that allows them to escape from the growth-restraining regulation by p16: a missense mutant cdk4 found in human melanomas is unable to associate with p16 rendering it constitutively active (Wolfel et al., 1995). Indeed, the p16 gene itself has also been found mutated. Deletion and loss of function mutations and promoter silencing by DNA methylation are strategies used by turnour cells to thwart negative control of E2F (Merlo et al., 1995). Another critical regulator of pRB, cyclin D, is expressed at high levels in certain tumour cells. This is accomplished through gene amplification, chromosomal rearrangement (Hall and Peters 1996) or mutations that prevent cyclin D degradation (Welcker et al., 1996). These cells possess an abnormally active E2F due to the erroneous inactivation of pRB. However, the most widespread aberration detected in tumour cells is mutation of p53, or inactivation through interaction with viral proteins (Levine 1997). The majority of tumour cells have either a mutation in the p53 gene or alterations in the genes encoding p53 regulatory molecules such as MDM2, ATM and ARF to block initiation of a checkpoint cell cycle arrest or apoptosis.



#### Figure 1.4

#### Major oncogenic aberrations targeting the E2F pathway

(A) Diagram showing some of the mechanisms used by tumour cells to activate the E2F pathway and drive cells into S phase.

DNA tumour viruses, simian virus 40 (SV40), adenovirus, and human papilloma virus (HPV) are all able to induce quiescent cells to enter and progress through the cell cycle (Moran 1993). An infection by these viruses leads very quickly to the expression of their viral oncoproteins T antigen, E1a and E7. All these proteins contain a consensus sequence termed the LxCxE motif that directs the association of the oncoproteins with pRB (Figge *et al.*, 1988). Sequestration of pRB by a viral oncoprotein subverts the E2F pathway by releasing active E2F (Bandara and La Thangue, 1991; Li *et al.*, 1993; Zamanian and La Thangue 1992; 1993). Additionally, the human papillomavirus HPV-16 or HPV-18 E6 protein and cellular E6-AP protein form a complex and function as a ubiquitin ligase for p53 (Scheffner *et al.*, 1993), abolishing the normal p53-dependent stress response.
# **1-2.** Nucleocytoplasmic transport

Eukaryotic cells have a need for continuous exchange of macromolecules between the nucleus and the cytoplasm. As nuclear proteins are synthesized in the cytoplasm, specific transport systems have evolved to allow proteins to be imported from the cytoplasm to the nucleus. Not only do all nuclear proteins need to be imported from the cytoplasm, but tRNA, rRNA and mRNA, which are synthesized in the nucleus, need to be exported to the cytoplasm where they function in translation. A double membrane surrounding the nucleus, tightly guards entry into and exit from the nucleus during interphase. Specific transport signals contribute crucially to the fidelity of nuclear transport to ensure that the one million macromolecules per minute actively transported between the nucleus and the cytoplasm of a growing mammalian cell, are imported or exported correctly (Ohno *et al.*, 1998). Several experimental approaches, including *in vitro* import and export assays in higher eukaryotes, *in vivo* transport studies in frog oocytes and in yeast have been instrumental in the discovery of components and mechanisms of nucleocytoplasmic transport.

# 1-2.1. The nuclear envelope

The nucleus is surrounded by the nuclear envelope, an impermeable double membrane that is continuous with the rough endoplasmic reticulum. To provide access across this barrier, the nuclear envelope is penetrated by nuclear pore complexes (NPCs) (Feldherr *et al.*, 1984). These elaborate protein structures have a mass of ~125 MDa in higher eukaryotes (Reichelt *et al.*, 1990) and are estimated to consist of ~100

different proteins termed nucleoporins (Doye and Hurt, 1997). In principle, the NPC provides a passive diffusion channel for ions, metabolites and macromolecules smaller than ~40-60 kDa. However, the NPC can undergo considerable changes in conformation to expand the diameter of the channel from ~10nm to ~25nm to provide an aqueous channel for active transport of macromolecules greater than 40-60kDa (Feldherr *et al.*, 1984). NPCs can accommodate active transport of particles as large as several million Daltons in molecular weight (Dworetzky *et al.*, 1988).

### **1-2.2.** Nuclear import transporters

Proteins and small ribonucleoproteins (RNPs), that need to traverse the nuclear membrane in order to perform their normal cellular functions, are most often recognised by soluble nuclear transport receptors. These receptors mediate the translocation of cargos through the NPC. Cargos identify themselves to the transport machinery by inherent signals (Nuclear Localisation Signal, NLS) that can be protein- or RNA-based or a composite of both. Transport signals show great variation in complexity, from short peptide motifs like the classical mono-partite NLS up to large protein domains, and even multi-component signals such as those used in U snRNA transport (Figure 1.5).

# A

# Nuclear import signals

Cargo	Signal	
classical signal-monopartite classical signal-bipartite U snRNA histones ribosomal proteins-M9 signal SR proteins	PKKKRKV KRKKKK m3G cap extended basic-rich domain 40aa glycine and aromatic rich SR domains	
Nuclear export signals		*****
leucine rich	LALKLAGLDI	-
ribosomal proteins-M9 signal SR proteins Nuclear export signals leucine rich M9	40aa glycine and aromatic rich SR domains LALKLAGLDI 40aa glycine and aromatic rich	

# B

Import receptors	All A Allon
type	signal recognised
Importin $\beta$ / Importin $\alpha$ Snurportin / importin $\beta$ RIP $\alpha$ / importin $\beta$ Importin 7 / importin $\beta$ Importin $\beta$ TRN1 TRN-SR	monopartite/bipartite NLS U snRNA m3G cap replication protein A histones cyclin NLS, Rev NLS ribosomal proteins SR proteins
Export receptors	
CRM1/cxportin1 Exportin-t CAS	leucine-rich NES tRNA importin α NES

# Figure 1.5

# Import and export signals and receptors

(A) Table showing sequences that target a protein for identification by molecules of the nucleocytoplasmic transport system.

(B) Table showing nucleocytoplasmic transporters and the types of signals they recognize.

These NLSs are recognised by one or more of the nuclear import receptor family (Figure 1.5). Members of this family have been given various names including, karyopherins (Radu et al., 1995), p97 (Chi et al., 1995), PTACS (Imamoto et al., 1995), importins (Gorlich et al., 1995) and transportins (Pollard et al., 1996). Different types of NLS are known to utilise distinct receptors (Nadler et al., 1997). The transportin receptor molecule mediates nuclear translocation of glycine rich M9-NLS-bearing cargos (Siomi and Dreyfuss, 1995; Michael et al., 1995). Three classes of cargo bind indirectly to import  $\beta$  via an adaptor protein: import of basic NLS-containing cargos is mediated by importin  $\alpha$  (Dingwall and Laskey, 1998), U snRNAs bind the Snurportin adaptor (Palacios et al., 1997) and Replication protein A binds the RIPa adaptor (Jullien et al., 1999). These receptors are generally large (90-130kDa) acidic proteins sharing 15-25% sequence identity. They all have an N-terminal RanGTP-binding domain, an N-terminal cargo-binding domain, and the capacity to bind components of the NPC. The genome of *S. cerevisiae* encodes nine receptors and one importin  $\alpha$ adaptor (Corbett and Silver, 1997; Pemberton et al., 1998; Wozniak et al., 1998). In higher eukaryotes, similar numbers of import receptors have been identified but at least 6 importin  $\alpha$  adaptor family members exist (Mattaj and Englmeier, 1998), including one member, hSRP1y, which has been shown to be tissue specific (Nachury et al., 1998).

# 1-2.3. Translocation across the nuclear membrane

Upon arrival at the cytoplasmic face of the nuclear pore complex, the cargoreceptor complex docks through association with the FG-rich domains of nucleoporins (Ohno *et al.*, 1998). Although at present it is far from clear how the complex travels the 200nm length of the NPC, it has been suggested that repeated association and dissociation of the import cargo complex with nucleoporins may drive vectorial translocation across the NPC (Nakielny *et al.*, 1999).

Early studies identified nuclear import as a RanGTP hydrolysis-dependent translocation mechanism (Melchior et al., 1993; Moore and Blobel, 1993) (Figure 1.6). RanGAP1 and RanBP1 carry out activation of Ran's GTPase activity, converting RanGTP to RanGDP in the cytoplasm, RanGDP will form a stable association with cargo-import receptor complexes in the cytoplasm (Hopper et al., 1990; Matunis et al., 1996; Mahajan et al., 1997). Ran's major guanine nucleotide exchange factor is chromatin-bound RCC1, which generates RanGTP inside the nucleus. Release of the cargo from the NPC in the nucleus is triggered when RanGTP binds to the nuclear import receptor (Ohtsubo et al., 1989; Bischoff and Ponstingl, 1991). The importance of the asymmetric distribution of RCC1 and RanGAP1 has been underscored by studies showing that inverting the gradient of Ran GTP across the nuclear membrane in vitro allows the direction of traffic to be reversed (Nachury and Weis, 1999). Although the vectorial nature of nuclear transport is dependent upon the RanGTP/GDP gradient (Gorlich et al., 1996; Koepp et al., 1996), the only step at which GTP hydrolysis is required for the nuclear transport cycle is at the end of a nuclear export event. Thus, it remains uncertain how the translocation event is powered (Koepp and Silver, 1996; Melchior and Gerace, 1998).



### Figure 1.6

#### Nucleocytoplasmic transport and the Ran cycle

Model for import and export of receptor-cargo complexes through the nuclear pore. In the cytoplasm, cargo associates with the receptor via its NLS and is translocated to the nucleus. RanGTP binding to an import receptor through the conserved ran binding domain dissociates the cargo-receptor complex in the nucleus. In the nucleus, cargo will bind to the ranGTP-export receptor complex via its NES. Once this trimeric complex has translocated through the nuclear pore, RanGAP1 and RanBP1 trigger GTP hydrolysis, thereby causing the release of the cargo from the export receptor.

# 1-2.4. Nuclear export

Studying the recycling of importin  $\alpha$  has provided novel insights into nuclear export mechanisms. After depositing their cargos in the nucleus, the import receptor shuttles back through the NPC, as a RanGTP-receptor complex. Export receptors bind cargo with much higher affinity in the presence of RanGTP (i.e., in conditions found in the nucleus) (Fornerod *et al.*, 1997; Kutay *et al.*, 1997; Arts *et al.*, 1998). The exportin CAS (originally identified as 'cellular apoptosis susceptibility protein' or Csc1p in yeast) was identified as a RanGTP-dependent export factor for importin  $\alpha$  (Kutay *et al.*, 1997). When this trimeric complex encounters RanGAP and RanBP1 on the cytoplasmic side of the NPC, it disassembles, releasing the export cargo and the receptor in a form competent for binding NLS-bearing cargo (Rexach and Blobel, 1995; Gorlich *et al.*, 1996; Izurralde and Adam, 1998; Hieda *et al.*, 1999). Following the exporting event, NF12 protein binds to RanGDP in the cytoplasm and delivers Ran back into the nucleus to maintain the RanGTP gradient (Ribbeck *et al.*, 1998; Smith *et al.*, 1998) (Figure 1.6).

The majority of research in the nuclear export field has focused on the CRM1 (exportin 1) pathway since the discovery of an antibiotic, leptomycin B, which has been shown to inhibit all CRM1 dependent export from the nucleus (Wolff *et al.*, 1997; Fornerod *et al* 1997). CRM1 is a receptor that functions in the export of a group of proteins that carries a specific leucine-rich type of nuclear export signal (NES) by targeting them to nucleoporins within the NPC (Fornerod *et al.*, 1997; Moroianu, 1998). The drug is now being used to identify other proteins that exit the nucleus via this pathway.

### 1-2.5. Export of RNPs

The vast majority of cellular RNA molecules are synthesized in the nucleus therefore transportation to the cytoplasm is required before they can function in translation. All four major classes of RNA (mRNA, rRNA, U snRNA, and tRNA) are exported as large RNPs (Nakielny et al., 1997). RNAs have complex NLSs, which become functional only when the RNP is fully processed. This ensures that nonfunctional RNPs do not get into the cytoplasm. Each class of RNA uses preferred export factors as each type of RNA will block only its own export when present in excess (Jarmolowski et al., 1994; Pokrywka and Goldfarb, 1995). Several export factors have now been discovered (Figure 1.5). Independently, Arts et al (1998) and Kutay et al (1998) identified exportin-t, a specific RanGTP-bound factor responsible for directly exporting tRNA from the nucleus. Other RNPs require an adaptor protein: the binding of CBC (cap binding complex) adaptor to the 5' m7G cap of U snRNA is required for its nuclear export by CRM1 (Izaurralde et al., 1995; Fornerod et al., 1997) and the heterogenous nuclear RNP (hnRNP) A1 protein, which commonly complexes with precursor and mature mRNAs, carries a NES within the M9 sequence to bridge mRNA to the CRM1 export pathway (Michael et al., 1995). Studying how retroviruses, such as human immunodeficiency virus (HIV), export their intron-containing RNA genomes and mRNA from the nucleus has uncovered many of the components of the cellular export machinery. Rev is a 13kDa viral NES-bearing RNA-binding protein that was originally identified as being involved in exporting HIV RNA out of the nucleus in a leptomycin B-sensitive pathway. Analysis of leptomycin B action lead to the discovery of the CRM1 export factor (Fischer et al., 1994; Wen et al., 1995).

Evidence exists to support the theory that import receptors and export receptor complexes make distinct contacts with nucleoporins, as some nucleoporin mutations specifically inhibit import or export (Corbett and Silver, 1997). It is unclear at present whether cargos move through an individual NPC in both directions at the same time, or if each NPC operates as a one-way system with only one entrance. It is also unknown what keeps the cargo moving in the right direction through the pore.

### **1-2.6.** Regulating transport

A number of cellular processes are regulated at the level of nucleocytoplasmic transport. Controlling access to the nucleus is an efficient way to regulate the activity of proteins involved in transcription (Morehouse *et al.*, 1999) or DNA replication (Moore *et al.*, 1999). The physiological state of the cell dictates the rate of transport and the maximum aperture of the NPC (Feldherr *et al.*, 1998). For example, passing from quiescence to proliferation to transformation increases both of these parameters. This may be a result of regulation of expression of transport receptors or changes in the Ran system. SV40 large T antigen has also been shown to cause an increase in the aperture of the NPC (Feldherr *et al.*, 1994).

Highly specific regulatory mechanisms exist that affect the localisation of individual proteins in response to stimuli during the cell cycle (Ohno and Mattaj, 1999; Pines, 1999), in signalling pathways and during development (Hood and Silver, 1999) (Figure 1.7). Post-translational modification plays a major role in this regulation by altering the affinity of the cargo for transport receptors. For example, the yeast transcription factor Pho4p must be phosphorylated to be recognised by its export

receptor and dephosphorylated to be recognised by its import receptor. Methylation of arginine residues has also been found to have an effect on the export of some yeast hnRNP proteins (Shen *et al.*, 1998).

Alternatively, an associated factor may determine the cellular localisation of a protein. An accessory protein may hold a potential target in the cytoplasm by masking a NLS/NES domain from the import/export machinery. Stimulation of translocation then involves dissociation of the complex and functional activation of the NLS/NES, generally by modification of either the signal-bearing protein or the accessory protein. For example, phosphorylation of the cytoplasmic retention factor Ikb disrupts its interaction with the transcription factor NFKB, allowing the NLS of NFKB to be recognised by import receptors (Mattaj and Englemeier, 1998). On the other hand, an accessory protein may provide a signal, such as a NLS or a NES, to direct the protein into a specific compartment of the cell. For example, the cyclin component of Cdk2/cyclin E and Cdc2/cyclin B acts as a nuclear targeting subunit for its partner kinase. Cyclin E binds to the importin  $\alpha$  adaptor subunit while cyclin B1 binds directly to import  $\beta$ , the cdk catalytic subunits are dispensable for these interactions (Moore et al., 1999). As well as supplying a NLS, cyclin B1 also provides a NES to an associated kinase (Hagting et al., 1998; Yang et al., 1998). Another example of an accessory protein providing a NES signal can be seen in regulation of p53.



## Figure 1.7

#### **Regulated nuclear import/export**

(A) *Post-translational modification*: Phophorylation and methylation play an important role in signalling proteins as targets for import to or export from the nucleus. *Retention*: Accessory factors may mask an NLS/NES to protect a protein from identification by the nucleocytoplasmic transport machinery.

*Piggyback transport*: proteins may access the nucleocytoplasmic transport machinery through association with an NLS/NES-possessing accessory protein.

In non-stressed cells, p53 associates with MDM2 and moves from the nucleus to the cytoplasm to be degraded by ubiquitin-mediated proteolysis (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). In this situation MDM2 provides a NES for CRM1-dependent p53 export (Roth *et al.*, 1998). In response to DNA damage, DNA-PK (Shieh *et al.*, 1997) and JNK (Fuchs *et al.*, 1998) phosphorylate p53, decreasing its interaction with MDM2. This results in an increased accumulation of p53 in the nucleus where it carries out its roles in apoptotic death or cell cycle arrest. The tumour suppressor protein ARF has also been shown to inhibit the nuclear export of p53/MDM2 (Zhang *et al.*, 1999)

# 1-3. 14-3-3 proteins

14-3-3 proteins were named after their migration pattern on two-dimensional DEAE-cellulose chromatography and starch electrophoresis during a search for small acidic proteins in the brain (Moore *et al.*, 1967). It is now known that 14-3-3 proteins are expressed in a broad range of tissues and cell types (Morrison, 1994) and increasing evidence has shown that 14-3-3 proteins possess diverse biochemical activities related to signal transduction, cell cycle regulation and apoptosis.

### **1-3.1.** The 14-3-3 family of proteins

The 14-3-3 family of proteins comprise of ninc highly homologous members in mammals  $(\alpha, \beta, \gamma, \delta, \varepsilon, \eta, \sigma, \tau, \text{ and } \zeta)$  derived from seven distinct genes ( $\alpha$  and  $\delta$  are phosphorylated forms of  $\beta$  and  $\zeta$  respectively), and two to five members in plants, yeast, and fungi. 14-3-3 molecules are 30-33 kDa proteins that can form homo- and heterodimeric cup-shaped structures with two peptide-binding grooves arranged in an anti-parallel fashion, 30 Angstroms apart (Liu *et al.*, 1995; Xiao *et al.*, 1995; Petosa *et al.*, 1998). (Figure 1.8). Sequence alignment reveals five highly conserved sequence blocks. Blocks 2-5 correlate well with the alpha helices 3, 5, 7, and 9, which form the proposed internal binding domain in the 3-dimensional-structure model of the functioning dimer (Wang and Shakes, 1996) (Figure 1.8). Supporting the current view that 14-3-3 proteins act as adaptor or bridging molecules that modulate interactions between components of signal transduction pathways, 14-3-3 $\zeta$  has been shown to be



# Figure 1.8

### The 14-3-3 dimer

Schematic representation of the 14-3-3 dimer showing predicted position of helices from interacting proteins in red. The central space is lined with highly conserved residues from helices 3, 5, 7 and 9 (shown in yellow and blue) which provide an invariant binding space for an amphipathic alpha helix, the specificity of which being dictated by the less conserved surface loop regions.

capable of binding two different peptide motifs simultaneously, thereby inducing homodimer or heterodimer formation in its target proteins (Petosa *et al.*, 1998). 14-3-3 proteins have been shown to bind to discrete phosphoserine-containing motifs R -(S/Ar) - [+] - pS - [LEAM] - P, present in many signalling molecules (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). Other motifs have been suggested to be important for binding of target proteins to 14-3-3 proteins, including a serine-rich patch on one side of an  $\alpha$ helix (Du *et al.*, 1996) or an amphipathic helix (Xiao *et al.*, 1995).

Although it has been reported that certain signalling proteins such as Cde25A and B (Conklin *et al.*, 1995), Wee-1 (Honda *et al.*, 1997) and Raf (Freed *et al.*, 1994) interact with particular 14-3-3 isotypes, the significance and regulatory role, if any, of these isotype-specific interactions is unclear. In contrast to these results, 14-3-3 isotypes select almost identical phosphopeptide motifs from peptide libraries suggesting that perceived isotype binding-specificity arises not from inherent differences in ligand-binding ability, but from sub-cellular localisation, transcriptional regulation, post-translational modification or cell-specific abundance of particular isoforms (Yaffe *et al.*, 1997; Rittenger *et al.*, 1999). For example, the  $\tau$  isotype is restricted to T-cells (Bonnefoy-Berard *et al.*, 1995) and the  $\delta$  isotype is uniquely expressed in epithelial cells (Leffers *et al.*, 1993).

Interestingly, the 14-3-3 epsilon gene is found on chromosome 17p13.3, which is within a region frequently deleted in several types of cancer, therefore pushing 14-3-3 forward as a reasonable candidate tumour suppressor gene. Although many different biological activities have been attributed to 14-3-3 molecules (Figure 1.9), three pathways have been extensively researched lately (Figure1.10).

INTERACTING	14-3-3	CONSEQUENCE OF	INTERACTION
PROTEIN		INTERACTION	REGULATION
A20	η	14-3-3 bridges interaction with c-	Association mediated by
	ζ	Raf to inhibit TNF-induced	phosphoserine within
	β	apoptosis	RSKSDP
Alpha(2)-	ζ		-
adrenergic	•		
receptor			
Apoptosis signal	jζ	Suppresses apoptotic pathway by	Phosphoserine mediated
-regulated kinase		preventing ASK phosphorylation	binding through
1 (ASK1)		of death promoting targets	RSISLP
BAD		Prevents cell death by	Association in response
		sequestering BAD in the cytosol	to survival factor IL-3
		therefore preventing growth	mediated
		neutralising effects of BAD	phosphorylation of
		binding to BCL-X	RHSSYP and RSRSAP
c-fes	τ		
calmodulin	ε	_	Calcium-dependent
			association
СЫ		_	Interaction mediated by
			phosphorylation within
			RHSLPPS and
			REGSTES in serine rich
<u>a) 260</u>		Die 1. august de marchese hau	regions
Cae25C		Blocks entry into m phase by	Association mediated by
		maintaining cytoplasmic retention	phosphorylation of
			KSISMI
Centrosomes and	3	14-3-3 locates to centrosome in	-
spindle apparatus	Υ	response to seruin-stimulation	SSEC.
Chkl		-	DNA damage induces
			ATM/ATK to
			phosphorylate Chk1 to
Onella dense dense			create site for 14-3-3
listrana litra	ļη	-	-
KITASE-IIKE	τ		
PUTAIKE-I	ξ	· · · · · · · · · · · · · · · · · · ·	
Docking protein	ζ	Associate in response to integrin	Association mediated by
p130(CAS)		ligand binding resulting in cell	phosphoserine
		attachment to the extra-cellular	
		matrix.	

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Figure 1.9 14-3-3 interacting proteins

ExoenzymeS		Mediates ADP-ribosylation of GTP-binding proteins	Association phosphorylation- independent
Forkhead transcription factor FKHRL1	ζ	Suppresses expression of death genes by causing cytoplasmic accumulation of the transcription factor	Binding site created by Akt phosphorylation within RSCTWP and RAVSMD
Glycoprotein Ib	ζ	Mediates platelet adhesion signal transduction pathways	Interaction mediated through serine rich region
Insulin growth factor-1 receptor IGF-1R		-	Interation through serine rich motif SVPLDPSASSSSLP
Insulin receptor substrate-1 (IRS-1)		_	Interaction mediated through phosphorylated Sites RSKSQS, HSRSIP And KSVSAP
Keratin 18		<u> </u>	Interaction mediated through phosphorylated Sites RPVSSAAS
Kinase suppressor of Ras (KSR)			Association mediated by two phosphoserine containing motifs RSKSHE and RTESVP and through the cysteine rich domain
43kDa lipid 5'- phosphatase		Stimulates phosphatase activity	Association through non-phosphorylated motif ELVLRSESEEKVV
MEF-2		Regulates cardiac transcription	
Middle T antigen		Promotes tumours in certain tissues	Association mediated through phosphorylated site RSHSYP
Mitochondrial targeting sequences (MTS)		Mediates mitochondrial localisation of MTS containing proteins	Association mediated through non- phosphorylated MTS
Mitogen-activated protein kinase / extracellular signal regulated kinase kinase kinases (MEKKs)	ε ζ	14-3-3 acts as scaffold for protein-protein interactions	

Figure 1.9 14-3-3 interacting proteins (cont)

Non-structural proteins (NS2) of minute virus	٤	-	-
p53	σ	G2 arrest. Increases affinity of p53 for sequence-specific DNA. Feedback loop is set up whereby p53 induces expression of 14-3-3	Association mediated by dephosphorylation of pS376 in response to DNA damage
Protein kinase C (PKC)		Association inhibits kinase activity	Association mediated by phosphorylated site RLSNVS and RTSSAELS
R18 synthetic peptide		_	Non-phosphorylated WLDLE motif
Raf-1 kinase	η ε ζ	Recruits Raf to the plasma membrane to form ternary complex with Ras leading to activation of Raf-1 kinase activity	Association mediated by phosphoserine in RSTSTP and RSASEP and also through cysteine rich region
Ras	ε	Activation of Ras1 signalling cascade	-
TERT	θ	Enhances nuclear localisation of the telemorase by inhibiting association with CRM1	Phosphorylation independent association
Tyr/trp hydroxylase		Increases activity of enzyme by 40% in neurotransmitter pathways	Association mediated by phosphoserine created by cAMP-dependent protein kinase within RHASSP
Weel	β	Causes an increase in Wee1 kinase activity due to increasing the half-life of Wee1 protein	Association mediated through consensus RSVSLT

Figure 1.9 14-3-3 interacting proteins (cont)


#### Figure 1.10

#### 14-3-3 regulated signalling pathways

(A) 14-3-3 retains cdc25 in the cytoplasm. During interphase, or DNA damage, cdc25 can be phosphorylated by several kinases to create a 14-3-3 binding site. 14-3-3 bound cdc25 is found in the cytoplasm where it cannot act on cdc2, thus preventing mitosis. Phosphatases disrupt the cdc25-14-3-3 interation when mitosis is to be initiated, allowing the cdc25 phosphatase to enter the nucleus and activate cdc2. 14-3-3 $\sigma$  may sequester the cdc2/cyclinB1 complex in the cytoplasm in response to DNA damage.

(B) 14-3-3 sequesters Bad from mitochondrial Bcl-XL/Bcl-2. Bad binds Bcl-XL/ Bcl-2 in the mitochondria, favouring apoptosis. Survival signals stimulate Akt/PKB leading to phosphorylation of Bad. Phosphorylated Bad is found in the cytosol and is unable to induce cell death. Akt/PKB also phosphorylates and inactivates other substrates in the pro-apoptotic pathway.

# 1-3.2. 14-3-3 in cell cycle regulation

During interphase, the activity of the mitotic inducer Cdc25C is suppressed as a result of binding to 14-3-3 proteins (Peng *et al.*, 1997; Kumagai *et al.*, 1998) (Figure 1.10-A). This association is ultimately reversed at mitosis, unless the cell has damaged or unreplicated DNA. Release of the Cdc25C dual-specificity phosphatase activity at the G2/M boundary initiates activation of cyclin B/Cdc2 by removing inhibitory phosphates at Thr-14 and Tyr-15 (Dunphy and Kumagai 1991; Gautier *et al.*, 1991). The cyclin B/Cdc2 complex, also known as MPF (maturation or mitosis phase promoting factor) acts by phosphorylating a myriad of mitotic substrates that orchestrate the committed segregation of replicated chromosomes to daughter cells. MPF remains active until Wee1 and Myt1 kinases replace the inhibitory phosphates on Cdc2 at the metaphase-anaphase transition (Morgan, 1995).

The association of human Cdc25C with 14-3-3 has been shown to be promoted by phosphorylation of Cdc25C on Ser-216, which resides in a consensus 14-3-3 binding site, by Chk1, Cds1 and c-TAK1, although other kinases may also be involved (Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997; Kumagai *et al.*, 1998, Matsuoka *et al.*, 1998).

The binding of 14-3-3 has little or no effect on the *in vitro* phosphatase activity of Cdc25C, suggesting that 14-3-3 proteins do not directly affect catalysis (Peng *et al.*, 1997; Kumagai *et al.*, 1998; Blasina *et al.*, 1999). This lead to the hypothesis that 14-3-3 acts to physically preclude the ability of Cdc25 to interact physically with the Cdc2/cyclin B complex. During interphase, Cdc25 is cytoplasmic, however, the active mitotic form of the phosphatase is nuclear. Abrogation of the 14-3-3 binding region on Cdc25 results in nuclear accumulation of Cdc25, demonstrating that 14-3-3 is responsible for the cytoplasmic localisation of Cdc25 during interphase. Several groups have results to explain this observation.

Studies of *Xenopus* Cdc25 have shown that the binding site for 14-3-3 is immediately adjacent to a putative bipartite nuclear localisation signal sequence, suggesting a mechanism whereby 14-3-3 proteins could influence the intracellular localisation of this regulatory phosphatase (Yang *et al.*, 1999). In accordance with this, Dunphy and Kumagai have shown that the binding of 14-3-3 sterically inhibits the association of Cdc25 with importin  $\alpha$ , implying a function for 14-3-3 in reversibly hindering the nuclear import of Cdc25. In this scenario, dissociation of 14-3-3 would result in nuclear translocation of Cdc25, and if conditions were appropriate for mitosis, Cdc25 would be activated by phosphorylation, by the polo kinase Plx1, in the nuclear export signal of Cdc25 to prevent export of the phosphatase. Should conditions not be favourable for mitosis, then Cdc25 would remain phosphorylated only within the 14-3-3 binding region and promptly be exported from the nucleus to rebind 14-3-3 in the cytoplasm.

In addition, studies in yeast by Lopez-Girona *et al.* (1999) have suggested that 14-3-3, termed Rad24 in yeast, is actively exported from the nucleus via an inherent nuclear export signal. Mutation of this signal resulted in nuclear accumulation of Rad24 and also Cdc25, implying that Rad24 acts as a phosphorylation-dependent attachable NES for Cdc25. In agreement with these results, Rittenger *et al* (1999) have identified, by X-ray crystallography, a putative nuclear export signal located within the ligand-binding domain of 14-3-3. In the absence of bound ligand, 14-3-3 would be exported from the nucleus via Crm1, the nuclear export receptor, however, in the presence of ligand, 14-3-3 would be prevented from interacting with Crm1, leaving the compartmental fate of the complex dependent upon the availability of the NES from the other 14-3-3 subunit or from additional NES/NLS sequences within the bound ligand.

### **1-3.3. 14-3-3** in the DNA damage checkpoint

If DNA is damaged, a delay in the cell cycle facilitates repair. Cells respond to damage by stopping the cell cycle at either G1, before DNA replication, S phase in response to replication failures, or before chromosome segregation in G2 (Hartwell and Weinert, 1989). 14-3-3 proteins link the checkpoint pathway to the cell cycle machinery to aid this arrest.

In epithelial cells, expression of 14-3-3 $\sigma$  is induced in a p53-dependent manner in response to DNA damage and, when over-produced, 14-3-3 $\sigma$  blocks the cell cycle at G2 (Hermeking *et al.*, 1997). This is achieved by the promotion of cytoplasmic accumulation of Cdc2/cyclin B by 14-3-3 $\sigma$  (Chan *et al.*, 1999). In addition, Waterman *et al* (1998) have shown that a physical interaction exists between p53 and 14-3-3 $\sigma$ . Dephosphorylation of p53 occurs in response to DNA-damage, thus creating a consensus binding site for 14-3-3 proteins and leading to most (if not all) p53 becoming complexed with 14-3-3 $\sigma$ . Subsequently, this association increases the affinity of p53 for sequence-specific DNA, possibly by inducing the formation of p53 tetramers. Moreover, DNA damage induced arrest of the cell cycle can also be achieved in a p53-independent manner. Chk1/Cds1 kinases, subsequent to activation by phosphorylation by the *ATM* gene product or other as yet unknown kinases, phosphorylate nuclear Cdc25C, creating a phosphoserine-binding site for 14-3-3 proteins. This checkpoint operates to maintain Cdc25 in a 14-3-3- bound form rather than to induce 14-3-3 binding (Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997). This impedes nuclear accumulation of Cdc25C therefore preventing it from activating Cdc2 (Chan *et al.*, 1999). Chen *et al* (1999) claim that the phosphorylated form of Chk1, generated in response to DNA damage, also forms a direct complex with 14-3-3, presumably to direct Chk1 to a specific substrate or to a particular location within the cell.

Chan *et al* (1999) have shown that initiation of the checkpoint response does not require p53, but depends on the association of Cdc25C with 14-3-3 proteins. Maintenance of the checkpoint does however depend on p53 dependent expression of 14-3-3 $\sigma$  that subsequently causes the exclusion of cdc2 and cyclin B from the nucleus. The p53/14-3-3 association is not required to initiate the G2 arrest, but is critical for cells to sustain and perhaps recover from the delay.

These data present mechanisms for G2 arrest but it is conceivable that 14-3-3 also plays a role in G1 and S phase checkpoints through other checkpoint kinases and Cdks. Arrest in S phase, when DNA replication is blocked, may in some cell types also require Cdc2 phosphorylation (Peng *et al.*, 1997), but in some cases S phase arrest involves additional mechanisms unrelated to Cdk2 phosphorylation (Kumagai and Dunphy 1995; Lee *et al.*, 1996; Amon *et al.*, 1993). Arrest in G1, after DNA damage,

is partly regulated by p21, an inhibitor of Cdk activity, and inhibitory phosphorylation of Cdk4 (Terada *et al.*, 1995). Future work is expected to find a role for 14-3-3 proteins in these checkpoints too.

# **1-3.4. 14-3-3** in the apoptotic response

Apoptosis is a naturally occurring cell death process that is critical for the development of multicellular organisms. The life or death decision made by cells is determined by sensitive balancing of the activities of survival and death promoting factors (Jacobson *et al.*, 1997). Execution of apoptosis is ensured by a series of transcription-independent events that include the activation of cascades of caspases that results in cell disintegration followed by engulfment by surrounding cells (Steller *et al.*, 1995). Transcription-dependent events such as up regulation of p53, Bax, the Fas ligand and TNF $\alpha$  are also part of the apoptotic response pathway (Jacobson *et al.*, 1997). 14–3-3 proteins are involved in the implementation of the cell death/survival choice.

The apoptosis-inducing molecule BAD contains two 14-3-3 binding sites. BAD is a member of the Bcl-2 family of proteins, which is comprised of positive and negative regulators of cell death. The ratio of antagonists (BCL-2, BCL-XL, MCL-1, and A1) to agonists (BAX, BAK, BCL-Xs, and BAD) dictates whether a cell will respond to a proximal apoptotic signal (Oltvai and Korsmeyer, 1994). BAD exerts its death-promoting effects by heterodimerising with, and neutralising the activity of, BCL-2 or BCL-XL survival factors (Yang *et al.*, 1995). In response to the survival factor IL-3, BAD becomes phosphorylated and forms an association with 14-3-3 in the

cytoplasm. Sequestration of BAD protects it from phosphatases and prevents it from heterodimerising with the membrane associated BCL-XL, and thus, protects the cell from undergoing apoptosis (Zha *et al.*, 1996).

Interestingly, one of the kinases responsible for phosphorylating BAD to create a 14-3-3 binding site, Akt (protein kinase B), also induces binding of the forkhead transcription factor FKHRL1 to 14-3-3 (Brunet *et al.*, 1999). The Akt serine/threonine kinase is a general mediator of cell survival, catalysing the phosphorylation, and therefore inactivation, of pro-apoptotic BAD and Caspase 9 (Datta *et al.*, 1997; Cardonc *et al.*, 1998). In the presence of survival factors, Akt phosphorylates FKHRL1, leading to association with 14-3-3 proteins and retention of FKHRL1 in the cytoplasm (Brunet *et al.*, 1999). Cytoplasmic retention and hence inactivation of FKHRL1 function, suppresses the transcription of death genes such as the Fas ligand gene, and thereby promotes cell survival.

A downstream component of Fas-induced apoptosis, apoptosis signal-regulated kinase 1(ASK1), is also subject to regulation by 14-3-3 proteins (Chang *et al.*, 1998). The death-promoting activity of ASK1 is antagonised by its binding to 14-3-3 proteins. Although it is unknown how this is achieved, 14-3-3 does not affect the kinase activity of ASK1, therefore it is assumed that 14-3-3 acts to prevent interactions between ASK1 and its death effectors (Zheng *et al.*, 1999).

These reports all indicate an apoptotic-suppressing role for 14-3-3, where several different components of the apoptotic response pathway are targeted in response to cell survival signals. This places 14-3-3 in a pivotal role in sustaining cellular growth.

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# **1-4.** Ubiquitin-mediated proteolysis in cell cycle control

Regulation of cellular processes, including cell cycle progression, differentiation, and signal transduction, is mediated largely by modulation of steady state levels of key regulatory proteins (Koepp et al., 1999). This is achieved by regulating the level of protein synthesis and the level of degradation. The ubiquitinproteasome pathway is responsible for the majority of specific cellular protein degradation. Degradation of a protein via the ubiquitin system involves two distinct and successive steps. Initially, the target proteins are conjugated to the polypeptide ubiquitin; in the second step, the ubiquitin-conjugated proteins are accepted by the 26S proteasome, a large, multi-catalytic protease, and degraded (Kornitzer and Ciechanover, 2000). Targets of the system comprise of not only soluble cytoplasmic and nuclear proteins, but also membrane-anchored proteins and lumenal endoplasmic reticulum (ER) proteins (Hicke, 1997). The discovery that tumor suppressor proteins such as p53 (Scheffner et al., 1993; Ciechanover et al., 1991, 1994), transcriptional regulators and proto-oncogenes such as c-jun, c-fos (Stancovski et al., 1995; Treier et al., 1994; Tsurumi et al., 1995;), c-myc (Gross-Mesilaty et al., 1998; Salghetti et al., 2000), and β-catenin (Aberle et al., 1997; Jiang and Struhl, 1998; Kitagawa et al., 1999; Orford et al., 1997; Winston et al., 1999), and cell cycle modulators such as the cyclin-dependent kinase (CDK) inhibitor p27 Kip1 (Carrano et al., 1999; Pagano et al., 1995;) are targeted by the system has raised the possibility that aberrations in the process may result in human pathologies. Indeed, the accelerated degradation of p53 induced by the human papillomavirus oncogene product E6 in conjunction with E6-AP has been implicated in the pathogenesis of human uterine cervical carcinoma (Scheffner et al.,

1990, 1993). The levels of another tumor suppressor protein, the CDK inhibitor p27 Kip1, are decreased in many types of malignancies, and this decrease was shown in some cases to be linked to increased degradation of the protein (Loda *et al.*, 1997). The versatility of the ubiquitin-proteasome system derives from both its diversity and its specificity. Because cells possess a diverse collection of ubiquitinating enzymes, ubiquitin-mediated proteolysis targets a wide range of proteins for destruction. The main determinant of degradation by the proteasome is likely to be the level of ubiquitination of the substrate. Regulation at the level of ubiquitination could be achieved via several mechanisms: the activity of specific E2/E3 complexes toward certain substrates, or classes of substrates, could be modulated or the substrate could be modified to alter its affinity for the appropriate E3 complex.

# **1-4.1.** The ubiquitination reaction

Ubiquitination involves the covalent conjugation of ubiquitin, a highly conserved, 76 amino acid residue protein, to a target protein via an isopeptide bond between the carboxyl-terminus of ubiquitin and lysine &-amino groups of the target proteins (Breitschopf *et al.*, 1998). The enzymatic machinery responsible for ubiquitin conjugation consists of at least two distinct catalytic entities, the ubiquitin activating enzyme (E1) and the ubiquitin-carrier protein (or ubiquitin conjugating enzyme), E2 (Hershko *et al.*, 1983). Ubiquitin is initially conjugated in an ATP-requiring reaction to E1 via a thioester bond between its C-terminal carboxyl group and a cysteine side chain in the active site of the enzyme. A *trans*-esterification reaction transfers the activated ubiquitin to a cysteine residue in the active site of the E2, which then transfers the ubiquitin to an amino group of the target protein. A third component, called E3, or

ubiquitin-protein ligase, is responsible for substrate recognition; it serves to bring the E2 enzyme to the vicinity of the substrate and catalyze the transfer of ubiquitin from the thioester intermediate on E2 or E3 to an amide linkage with the substrate. A fourth component of the ubiquitin system, whose activity may be restricted to a specific and limited subset of E3/substrate combinations, has recently been proposed (Koegl *et al.*, 1999). E4 is a protein required for ubiquitin chain elongation: it binds to short ubiquitin chains, and in the presence of E1, E2, and E3, allows the formation of longer chains, which are recognized by the proteasome. In successive reactions, transfer of additional ubiquitin moieties to the  $\varepsilon$ -amino group of Lys48 of the previous conjugated molecule produces a polyubiquitin chain that targets the substrate for degradation by the proteasome (Chau *et al.*, 1989).

# **1-4.2.** The E3 family

A member of the E3 family of proteins recognizes polyubiquitination signals, which may be primary, secondary or post-translationally modified sequences. Because E3s dictate the specificity of ubiquitination reactions, it is likely that protein degradation is substantially controlled via the regulation of E3 activity or the E3-substrate interaction. This family can be subdivided into at least four subtypes with regard to structure and/or class of degradation signal being recognised.

(i) E3 $\alpha$  (UBR1 in yeast) recognizes the destabilizing residues of N-end rule substrates (Bartel *et al.*, 1990; Hershko *et al.*, 1986; Kwon *et al.*, 1998). E3 $\alpha$  has binding sites for either basic (type I) or bulky hydrophobic (type II) N-terminal residues of their substrates. However, E3 $\alpha$  has additional binding sites for other degradation signals as it can also recognize denatured and N-acetylated proteins. A protein related to E3 $\alpha$  has been identified, E3 $\beta$ , which targets proteins with small uncharged N-terminal residues for degradation (Heller and Hershko, 1990).

(ii) HECT (homologous to E6-AP carboxyl terminus) domain proteins carry a 350 amino acid residue domain homologous to the C-terminal domain of the prototypical member of the family, E6-AP (Huibregtse *et al.*, 1995). This domain contains a conserved cysteine residue for attachment of a ubiquitin molecule before it is transferred to the substrate (Scheffner *et al.*, 1995). E6-AP was identified as a protein required for the rapid degradation of the tumor suppressor p53 in the presence of papilloma virus protein E6 (Huibregtse *et al.*, 1993). E6-AP is also the protein mutated in a human genetic disease, Angelman's syndrome. Other members of this family include yeast RSP5, which conjugates RNA polymerase II (Huibregtse *et al.*, 1997).

(iii) The anaphase promoting complex (APC) (Irniger *et al.*, 1995; King et al., 1995) or cyclosome (Sudakin *et al.*, 1995) is a multi-protein complex of at least 10 subunits, which has a ubiquitin-ligase activity specific for degradation of mitotic cyclins, anaphase inhibitors and spindle-associated proteins. Ancillary factors that are not part of the core complex confer further substrate specificity and regulation to the complex. Phosphorylation also appears to play a role in regulation of this complex (Shteinberg *et al.*, 1999). APC substrates contain a degradation signal called the mitotic 'destruction box', with the following consensus sequence: **R**-A/T-A-**L**-G-X-I/V-G/T-N (conserved residues are in bold) (Yamano *et al.*, 1996).

(iv) SCF complexes (Skp1, Cdc53 or Cullin, and F-box protein) are involved in the degradation of certain cell cycle regulators, such as the E2F-1, SIC1 CDK inhibitor, and certain G1 cyclins. They associate with a specific E2, Cdc34/Ubc3 (Feldman et al., 1997; Skowyra et al., 1997), and possibly also with the UbcH5 family of E2s (Yaron et al., 1998; Gonen et al., 1999). Recently, a fourth component of the SCF complex was identified, Rbx1 (Kamura et al., 1999; Skowyra et al., 1999), or Roc1 (Ohta et al., 1999; Tan et al., 1999). Although both yeast and mammals contain additional cullin homologs, only CULI seems to associate with Skp1 (Lisztwan et al., 1998). Recently it was reported that CUL-3 targets cyclin E for degradation, but other components of this ubiquitination complex are unknown (Singer et al., 1999). In contrast, the F-box protein is a variable component of the complex. Given that SCF complexes, with different F-box components, exhibit different substrate specificities, the F-box proteins are thought to carry the substrate recognition site of the complex (Bai et al., 1996). CUL1 recruits the E2 enzyme and Skp1 helps to link the F-box/substrate with the E2 enzyme. With the exception of  $SCF^{\beta TrCP}$  (the superscript denotes the identity of the F box subunit), no specific consensus degradation signal has been recognized so far among the substrates of specific SCF complexes. However, a requirement for phosphorylation of the substrate before recognition by the SCF complex appears to be common to all SCF-substrate interactions.

# 1-4.3. The proteasome

The proteasome is a large, ~2.1MDa, 26S multi-catalytic protease that recognizes, binds, unfolds and degrades polyubiquitinated proteins into short peptides, 4 to 25 residues long (Kisselev *et al.*, 1999). The 26S proteasome complex is composed

of the 20S core catalytic complex flanked on both sides by the two regulatory 19S complexes (Baumeister *et al.*, 1998). Crystal structure of the yeast 20S particle reveals a barrel-shaped structure with catalytic sites located in the  $\beta$ -rings in the interior of the barrel, shielded from the cytoplasm (Groll *et al.*, 1997). Electron microscopy shows that each extremity of the barrel is capped by a 19S particle. The exact number of 19S complex subunits is unknown as some may only associate transiently with the 20S proteasome. One function of the 19S complex is presumably to recognize ubiquitinated proteins and other potential substrates of the proteasome. S5a (Rpn10/Mcb1 in yeast) has been identified as a ubiquitin-binding subunit of the 19S particle (Deveraux *et al.*, 1994). The yeast 20S particle seems to be occluded at both ends (Groll *et al.*, 1997), thus a second function of the 19S complex may be to change the conformation of the 20S particle to create a channel through which substrates can be inserted into the proteolytic interior chamber. Many of the 19S subunits are ATPases (Voges *et al.*, 1999).

### **1-4.4.** Regulation of ubiquitination activity.

Recent data regarding degradation of mitotic substrates by the APC shows that regulated degradation of specific classes of substrates can be achieved by modulating the activity of the ubiquitination machinery. This is achieved through the cell cycle dependent association of ancillary factors with the APC. Protein degradation by the APC is required for progression through anaphase (Irniger *et al.*, 1995; Tugendreich *et al.*, 1995) and the association of different ancillary proteins to the core APC complex prompts target specificity. Cut2 (in *S.pombe*) and Pds1 (in *S. cerevisiae*) (Funabiki *et al.*, 1996; Cohen-Fix *et al.*, 1996), APC substrates that act as inhibitors of the metaphase to anaphase transition, are degraded by the APC at the beginning of anaphase. Binding of the ancillary protein Cdc20 to the APC results in degradation of Pds1, and binding of phosphorylated Hct1/Cdh1 during telophase is required for degradation of cyclin B, as well as other late mitotic substrates such as Ase1 (Schwab *et al.*, 1997; Visintin *et al.*, 1997). In cases of spindle assembly failure, inactivation of Cdc20 through binding to Mad proteins will result in mitotic arrest (Fang *et al.*, 1998; Hwang *et al.*, 1998). Throughout most of the cell cycle, CDK-phosphorylation of Hct1/Cdh1 renders it incapable of binding to the APC, therby preventing degradation of cyclin B (Jaspersen *et al.*, 1999; Zachariae *et al.*, 1998; Lukas *et al* 1999).

# **1-4.5.** Regulation by modification of the substrate.

Post-translational modification of a substrate can regulate its ubiquitination by altering its binding affinity for an E3 enzyme. An increasing number of substrates of the ubiquitin pathway are known to require phosphorylation prior to ubiquitination, for example, the yeast G1 cyclins Cln2 (Lanker *et al.*, 1996) and Cln3 (Yaglom *et al.*, 1995), the yeast cyclin-dependent kinase (CDK) inhibitors Sic1 (Verma *et al.*, 1997) and Far1 (Henchoz *et al.*, 1997), the mammalian G1 cyclins D (Diehł *et al.*, 1997) and E (Clurman *et al.*, 1996), the mammalian CDK inhibitor p27 Kip1 (Carrano *et al.*, 1999; Sheaff *et al.*, 1997; Tsvetkov *et al.*, 1999), and the mammalian transcriptional regulators IkB $\alpha$  (Brown *et al.*, 1995; Yaron *et al.*, 1997) and  $\beta$ -catenin (Rubinfeld *et al.*, 1997). Interestingly, the E3 enzymes that associate with substrates regulated by phosphorylation are all members of the SCF family of ligases. Two of the best-characterised cases are those of IkB $\alpha$  and Sic1 degradation.

The mammalian transcription factor NF-KB is activated by a variety of extracellular stimuli such as tumor necrosis factor, cytokines, bacterial and viral products, and ionizing irradiation (Ghosh et al., 1998; Mercurio and Manning, 1999). Activation is achieved when IkBa, which binds to NFkB and sequesters it in the cytoplasm, is degraded, thus allowing NF $\kappa$ B to translocate to the nucleus. Phosphorylation of NF- $\kappa$ B-bound I $\kappa$ B $\alpha$  on its N-terminal regulatory domain, at two specific serine residues, serines 32 and 36, triggers the polyubiquitination of nearby lysines. The subsequent proteasonal degradation of  $I\kappa B\alpha$  follows association with a specific SCF complex, SCF<sup> $\beta$ TCP</sup> (Fuchs *et al.*, 1999; Kroll *et al.*, 1999; Spencer *et al.*, 1999; Winston *et al.*, 1999; Yaron *et al.*, 1998). Another substrate of SCF<sup> $\beta$ TiCP</sup>, the transcription factor  $\beta$ -catenin, is phosphorylated by a distinct kinase, glycogen synthase kinase (GSK3 $\alpha$ ), on two serines embedded in a sequence similar to the Ser 32 -Ser 36 region of IkBa (Fuchs et al., 1999; Hart et al., 1999; Latres et al., 1999). A consensus recognition motif has been identified for  $\beta$ TrCP binding: D S<sup>P</sup> G L X S<sup>P</sup> (single-letter amino acid code; S<sup>P</sup> stands for phosphoserine). However, no consensus recognition motif has been defined yet for other F-box proteins.

Sic1 is a yeast cyclin B/CDK complex inhibitor whose rapid degradation at the end of G1 enables the initiation of DNA replication (Schwob *et al.*, 1994). Cell cycle regulated phosphorylation of Sic1 by G1 cyclin/CDK complexes is critical for the recognition of Sic1 by the SCF <sup>CDC4</sup> ubiquitination complex (Feldman *et al.*, 1997).

In addition to phosphorylation of the substrate, ubiquitination of an SCF substrate could conceivably be modulated by the presence or absence of the specific F-

box protein. The mammalian CDK inhibitor p27, and the transcription factor E2F-1, are ubiquitinated by a specific SCF complex, SCF <sup>SKP2</sup> (Carrano *et al.*, 1999; Marti *et al.*, 1999; Tsvetkov *et al.*, 1999). Skp2 activity is essential for S-phase entry, and Skp2 expression is itself cell cycle regulated, with a peak of expression during S phase (Zhang et al., 1995). The association of F box proteins with Skp1 can also be regulated (Li and Johnston, 1997).

Inhibition of ubiquitination by phosphorylation has also been described. Ubiquitin-mediated degradation of the proto-oncogene *c-mos* is inhibited by phosphorylation on Ser3 (Nishizawa *et al.*, 1992; Nishizawa *et al.*, 1993). Interestingly, activation of *c-mos* leads to phosphorylation and stabilization of *c-fos*, another substrate of the ubiquitin pathway (Okazaki and Sagata, 1995). Another example is that of the anti-apoptotic protein Bel-2: dephosphorylation of Bel-2 following apoptotic stimuli renders it susceptible to degradation by the ubiquitin pathway (Dimmeler et al., 1999).

### **1-4.6.** Regulation by binding of viral factors.

Certain viruses are capable of manipulating the cellular ubiquitination system to target cellular substrates that may obstruct propagation of the virus. In some instances, the viral protein functions as a bridging protein between the E3 ligase and the substrate. The prototype of such an interaction is the HECT protein, E6-AP (*E6*-Associated *P*rotein). It was isolated as a protein required for the ubiquitination of p53 in the presence of the human papillomavirus (HPV) oncoprotein E6. E6 binds both p53 and E6-AP, and formation of this complex results in the ubiquitination and degradation of p53 (Huibregtse *et al.*, 1991). Removal of the tumour suppressor by the oncoprotein

appears to be a major mechanism utilised by the virus to transform cells. The T-cell CD4 receptor is another example of an endogenous substrate that is targeted for degradation by a viral protein. The Vpu protein of the human immunodeficiency virus type 1 (HTV-1) binds to the CD4 receptor of the T cells infected by the virus, leading to ubiquitination and degradation of CD4 by the SCF<sup>βTCP</sup> complex (Margottin *et al.*, 1998).

# **1-4.7.** Regulation by masking of a degradation signal.

Incorporation of a protein into a complex may protect it from degradation. Cyclin E is protected from ubiquitination when assembled with Cdk2 (Clurman et al., 1996) and E2F-1 is protected from ubiquitination when assembled with pRB (Hoffmann et al., 1996). The  $\alpha 2$  transcription factor in budding yeast is also regulated in this way. When two haploid yeast cells of opposite mating types mate, the resulting diploid expresses both a1 and  $\alpha 2$ , which form a heterodimer with a distinct DNA binding specificity. In haploid cells, the a1 and  $\alpha 2$  proteins are rapidly degraded by the ubiquitin system. It was shown that residues that are important for the interaction with al overlap with the Degl degradation signal (Johnson et al., 1998). Thus, binding of al interferes with the recognition of  $\alpha 2$  by the ubiquitin system because of masking of the degradation signal. Another example was recently shown where the Drosophila homeobox protein *Homothorax* is stabilized in cells expressing its binding partner Extradenticle (Abu-Shaar and Mann, 1998). On the other hand, binding to DNA can stabilize a DNA-binding protein. The myogenic transcription factor MyoD can be efficiently ubiquitinated in vitro; however, binding of MyoD to its specific cognate DNA sequence protects it from ubiquitin-mediated degradation (Abu Hatoum et al.,
1998). Furthermore, Salghetti *et al.*, (2000) propose that an overlap of acidic activation domains and destruction elements in certain transcription factors, presents a mechanism for destruction of excess activators, thereby preventing squelching of the basal transcription machinery.

#### 1-4.8. Deubiquitination.

Deubiquitinating enzymes (DUBs) can either accelerate or inhibit proteolysis. This process is essential for the release of newly synthesized ubiquitin, which is often translated as a linear polyubiquitin molecule, or fused to a ribosomal protein. Following proteolysis of a conjugated substrate, recycling of the ubiquitin molecules by deubiquitinating enzymes is also essential to restore the cellular ubiquitin pool. In addition, deubiquitination has the potential of inhibiting ubiquitin-mediated proteolysis by releasing ubiquitin chains from substrates before they reach the proteasome. This may serve as a proofread facility to prevent degradation of substrates that were falsely ubiquitinated.

The ubiquitin recycling enzymes are generally thiol proteases that recognize the C-terminal domain of ubiquitin (Wilkinson, 1997). Ubiquitin C-terminal hydrolases (UCHs) are ~25kDa enzymes that are involved in the processing of pro-ubiquitin products. Ubiquitin-specific proteases (UBPs) are ~100kDa enzymes that catalyze the release of ubiquitin from polyubiquitin chains or from conjugated cellular proteins. These enzyme families have been shown to target specific substrates. UBP3 has a role in inhibiting gene silencing of transcribed genes following positioning near heterochromatic regions (Moazed and Johnson, 1996). DUB-1 and DUB-2 are induced

by cytokines, and high-level expression of DUB-1 leads to cell cycle arrest (Zhu *et al.*, 1996, 1997).

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#### 1-5. Objectives

Progress in elucidating the molecular composition of E2F has defined a heterodimeric transcription factor composed of an E2F and a DP family member. To date, six E2F proteins and at least three DP proteins have been identified, and each E2F protein can interact combinatorially with a DP protein to generate an array of sequence specific heterodimers (Dyson 1998). Although it is known that the E2F family members perform a physiological role in dictating the nature of the pocket protein that physically interacts with the heterodimer, little is known about the functional differences between heterodimers comprising different DP partners.

With a view towards understanding the physiological role of the DP subunit of the E2F heterodimer, this project firstly focused on a putative nuclear localisation signal present only in certain members of the DP family. An alternatively spliced bipartite nuclear localisation signal was uncovered and a role for this splicing event in E2F function was analysed.

Of particular interest was the C-terminal portion (basic region) of the nuclear localisation signal, which was, unlike the N-terminal portion, present in all the DP family members. The next stage of the project aimed to define a role for this region by assuming it was associated with an interaction domain within the DP protein. It was subsequently found to mediate an interaction between the E2F transcription factor and the epsilon isoform of the 14-3-3 family of signalling molecules.

Finally, the project aimed to propose a functional role for 14-3-3 in the E2F pathway. With the aid of a mutant DP, which failed to bind to 14-3-3, functional analysis revealed a role for 14-3-3 in mediating cell cycle progression by promoting the degradation of E2F in the G2 phase of the cell cycle. This suggested a link between the E2F transcription pathway and a growth regulating pathway, possibly through a G2-phase checkpoint monitor.

In considering the role of the DP protein, it is noteworthy that DP-1 appears to be the predominant DP family member in physiological E2F (Bandara *et al.*, 1993, 1994; Girling *et al.*, 1993). It is possible that DP-3 proteins are rare components of E2F or alternatively, regulate E2F site-dependent transcription in physiological conditions where DP-1 does not play a major role. Another possibility is that DP-3 proteins are down-regulated in tumour cells, a theory which is in keeping with the results of this study. Understanding the nature of the different DP proteins will ultimately discriminate between the various roles of the E2F family members and consequently, yield important insights into cell cycle control.

#### Chapter 2. Materials and methods

#### 2-1.1. Plasmids

The following plasmids have been previously described; pCMV-p107 (Beijersbergen et al., 1994), pCMV-HA-E2F-5 (Allen et al., 1997), pCMVHA-E2F-4 (Beijersbergen et al., 1994), pCMV-βgal (Zamanian et al., 1992), pGDP-3β, pGDP-3δ (de la Luna et al., 1996), pGDP-1 (Bandara et al., 1993), pCE-luc (Botz et al 1996), pG5E1b-luc (Lee et al., 1998), pVP16-TAD (Lee et al., 1998), pG4DP-3δ (de la Luna et al., 1999), pG4DP-1 (de la Luna et al., 1999), pG4-DBD (de la Luna et al., 1999), pVP16-LEU (Shikama et al., 1999), pHIS-LEX-DBD (Buck et al., 1995), pBSK-DP-3 (Ormondroyd et al., 1995), pT7E1A (Allen et al., 1997), pSG5 (Green et al., 1988)

The following plasmids were gratefully received as gifts: pRcCMVHA-E2F-1 encoding E2F-1 (Krek *et al.*, 1994) from Dr W. Krek, pCMV-14-3-3ε-myc, encoding 14-3-3 ε, and pGEX-14-3-3ε, encoding GST tagged 14-3-3ε, from Dr A. Aitken.

pGDP-3δΔb was constructed by adopting the PCR method to delete 18bp from pGDP-38 (bases 174-191 inclusive). The first PCR was carried out using the 5' primer (5' to 3') TCCCCGGGGGATGATTATAAGCACA and the 3' primer (5' to 3') CCCATTTTTATCACTTTCTGAGAA (encompassing the deletion). The second PCR was carried out using the 5' primer (5' to 3') TTCTCAGAAAGTGATAAAAATGGG (encompassing the deletion) and the 3' (5' primer to 3') GAAGTTCTTGTAGCTGGGCTC. The final PCR used the products of PCR 1 and PCR 2 with the 5' primer of PCR 1 and the 3' primer of PCR 2. PCR amplification was carried out using Taq polymerase (Promega) for PCR 1 and PCR 2, as this enzyme did not digest overhangs of the primer after the first round of annealing. PCR 3 was performed using Pfu polymerase (Boehringer Mannheim), as this enzyme has higher fidelity in amplification. PCR was carried out using the manufacturer's incubation buffer for 31 cycles as follows: 5 minutes denaturation at 94°C, followed by 31 cycles of denaturation at 94°C for 30 seconds; annealing at 45°C for 30 seconds; extension at 72°C for 30 seconds (last cycle 5 min). The PCR product was digested with SmaI and BclI and sub-cloned into pBSKDP-38 that had been digested with SmaI and BclI to remove wild-type DP-3 sequence initially. For expression in mammalian cells, the DP-3 sequence was sub-cloned into pG4mpolyII (Stratagene) in place of the GAL4 DNA binding domain sequence.

pG4DP-3 $\beta\Delta b$  was constructed as pG4DP-3 $\delta\Delta b$  using the following primers: PCR 1- 5' primer (5' to 3') TCCCCGGGGGATGATTATAAGCACA and the 3' primer (5' to 3') CCCATTTTTATCACTGGGAACCC (encompassing the deletion). PCR 2- 5' primer (5' to 3') GGGTTCCCAGTGATAAAAATGGG (encompassing the deletion) and the 3' primer (5' to 3') GAAGTTCTTGTAGCTGGGCTC.

For the mammalian two-hybrid assay,  $pVP16TAD14-3-3\epsilon$  was constructed by amplifying  $pCMV-14-3-3\epsilon$ -myc by PCR using the 5' primer (5' to 3') GGCATTACGGCAATTCATGGATGATCGGGGG and the 3' primer (5' to 3') CGCATGCCATGGATCCCAAGTCCTCTTCAG that created digestion sites for EcoRI and BamIII. PCR amplification was carried out using Pfu polymerase (Boehringer Mannheim) and the manufacturer's incubation buffer for 31 cycles as follows: 5minutes denaturation at 94°C, followed by 31 cycles of denaturation at 94°C for 30 seconds; annealing at 70°C for 30 seconds; extension at 72°C for 30 seconds (last cycle 5 min). The PCR product was digested with EcoRI and BamHI and sub-cloned into the vector pCMVHA-VP16TAD which had been digested with BamHI and EcoRI to excise the HA tag.

To construct pHL-DP3δM2E and pHL-DP3δM2EΔb for the yeast two-hybrid assay, pG4-DP3δ (bases 1-388) or pG4-DP3δΔb• (bases 1-370) was amplified by PCR using the 5' primer (5' to 3') TCCCCGGGGGATGATTATAAGCACA and the 3' primer (5' to 3') CATCATAAACTCTTCCGTCTA. PCR amplification was carried out using Pfu polymerase (Boehringer Mannheim) and the manufacturer's incubation buffer for 31 cycles as follows: 5 minutes denaturation at 94°C, followed by 31 cycles of denaturation at 94°C for 30 seconds; annealing at 50°C for 30 seconds; extension at 72°C for 30 seconds (last cycle 5 min). The PCR product was digested with Smal-PvulI and sub-cloned into the vector pHIS-LEXA linearised by SmaI.

The predicted DNA sequence of all constructs was verified by DNA sequencing both strands manually (Sequenase, Amersham) or with an ABI dye terminator cycle sequencing-ready reaction kit (Perkin Elmer) and an automated DNA sequence analyser.

#### **2-1.2.** Luciferase and β-galactosidase reporter assays

For luciferase assay, cells were washed twice with PBS before lysing in 300µl reporter lysis buffer (25mM Tris-H3PO4 pH7.8, 2mM 1,2-diaminocyclohexane tetra

acetic acid, 2mM DTT, 10% glycerol and 1% Triton X-100) for 30 minutes. The lysed cells were then transferred to a micro centrifuge tube and spun to remove cell debris. To assay for luciferase activity 100µl cell extract was added to 300µl luciferase assay reagent (1:4, Promega) and luciferase activity measured using a luminometer (Berthold Lumat).

To measure  $\beta$ -galactosidase activity, 100µl cell extract was incubated with 2 x  $\beta$ -gal substrate buffer (200mM sodium phosphate buffer pH 7.3, 2mM MgCl<sub>2</sub>, 100mM  $\beta$ -mercaptoethanol and 1.33mg/ml O-nitrophenyl- $\beta$ -D-galactopyranoside). The reaction mixtures were incubated at 37°C until faintly yellow in colour and the enzyme activity was quantified by measuring the optical density of the mixture at a light wavelength of 420nm.

#### 2-1.3. Immunofluorescence

Transfected cells were fixed in 4% paraformaldehyde at room temperature for 15min, washed in PBS, then permeabilised in PBS containing 0.2% Triton X-100 for 10min. Fixed cells were then washed three times with PBS-1% FCS and incubated in primary antibody diluted in PBS-5% FCS for 30min. After washing again in PBS-1% FCS, cells were incubated in secondary antibody; goat anti-mouse or anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) (Southern Biotechnology Associates Inc.) diluted in PBS-10% FCS for 30min at room temperature. Finally, coverslips were washed three times in

PBS, mounted on slides using citifluor (Citifluor Ltd), and viewed using a fluorescence microscope (Olympus).

#### 2-1.4. Flow cytometry

For flow cytometry analysis, 10µg of an expression vector for the cell-surface protein CD20 was co-transfected into cells (grown in 10% FCS) together with 4µg of each expression vector of E2F and DP. Cells were washed and fresh full media added after 16h then harvested 24h later by washing in PBS and thereafter treating with cell dissociation medium (Sigma) for 30min at 37°C. Cells were washed in DMEM by centrifugation at 1000rpm for 3min and resuspended in 200µl DMEM containing 20µl anti-CD20 antibody leu16 (Becton Dickinson) coupled to fluorescein isothiocyanate (FITC). Cells were incubated on ice for 20min then washed twice with PBS. Finally, cells were fixed by adding 50% PBS in ethanol drop wise and storing at 4°C overnight. Cells were collected by centrifugation and resuspended in 400µl propidium iodide (20µg/ml) in PBS and 200µl RNase (125U/ml) in PBS and incubated on ice for 30min. Flow cytometry was performed on a Becton Dickinson fluorescence activated cell sorter. Using CellQuest software, the cell cycle profile of the transfected population of cells (at least 10<sup>4</sup> cells per sample) was determined by analysing the intensity of propidium iodide staining in cell populations that were positive for FITC staining. The data presented show a representative example from multiple assays.

#### 2-1.5. Transfection

For transfection, U2OS, COS-7 and CV-1 cells were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 10% foetal calf serum (FCS) and antibiotics, 10mg/ml streptomycin and 100U/ml penicillin (Gibco BRL), at 37°C in a 5% CO<sub>2</sub> / H<sub>2</sub>O-saturated atmosphere. Cells were plated out 24hr before transfection at ~2 x 10<sup>5</sup> cells per 35mm dish (on coverslips for immunofluorescence), ~5 x 10<sup>5</sup> cells per 60mm dish (for luciferase and  $\beta$ -galactosidase assays) or ~1.5 x 10<sup>6</sup> cells per 100mm dish (for immunoprecipitation, stability and flow cytometric analyses). Three hours before the transfection, the medium from the cells was removed and replaced with fresh growth medium.

Transfection of U2OS cells was carried out using the calcium phosphate-DNA precipitate method: A 250mM CaCl2 solution (100µl per 35mm dish, 250µl per 60mm dish and 750µl per 100mm) containing either 6µg (p35), 15µg (p60) or 56µg (p100) plasmid DNA.

For immunostaining assays (p35),  $2\mu g$  of each plasmid DNA was used. Transactivation assays (p60) used 0.5 $\mu g$  reporter, 0.5 $\mu g$  pCMV $\beta$ -gal, 0.5 $\mu g$  DP and E2F and 2 $\mu g$  14-3-3 plasmid. Immunoblotting, immunoprecipitation and gel shift assays (p100) used 10 $\mu g$  each of DP and E2F and 4 $\mu g$  pCMV $\beta$ -gal with empty vector maintaining the final DNA amount constant. DNA/CaCl<sub>2</sub> solution was added drop wise to an equal volume of 2xHBS solution (50mM HEPES pH7.1, 280mM NaCl, and 1.5mM Na2HPO4) and left 20 min to precipitate before adding to media on cells.

4μl Lipofectin Reagent (Gibco) was used to transfect COS-7 and CV-1 cells, <sup>1</sup> according to manufacturer's instructions, with 6μg DNA per 35mm dish.

Whenever required, pcDNA3 (Invitrogen) or pSG5 was used to maintain a constant amount of DNA in each sample. All transfections included pCMV- $\beta$ gal (Invitrogen) as an internal control for transfection efficiency. Cells were washed 3x with PBS 14-17 hr post transfection. For stability analysis, 100mm dishes were trypsinised at this point into 4 x 60mm dishes to maintain equal transfection efficiency levels between samples. U2OS cells were harvested 40-46hrs post-transfection and COS-7 and CV-1 cells were harvested 64-70hrs post- transfection.

#### 2-1.6. Immunoprecipitation

Cells were washed twice in PBS, harvested by scraping into cold Lysis Buffer (20mM HEPES pH7.5, 400mM NaCl, 1mM EDTA, 0.1% NP-40, 10mM NaF, 2.5mM DTT, 0.5mM PMSF, 1.0mM sodium orthovanedate and aprotinin (2.0µg/ml)) and incubated on ice for 30min. The cell extract was centifuged for 15 min at 12,000g then diluted in Lysis Buffer without NaCl to give a final salt concentration of 150mM. The extract was pre-cleared by incubating with protein-G agarose for 1h at 4°C in a vibrax machine. The supernatant was harvested and incubated with 50µl protein-A agarose

(50% v/v) (Boehringer Mannheim) for 2h at 4°C. The protein-A agarose had been previously incubated with primary antibody in 100µl incubation buffer (20mM HEPES pH7.5, 150mM NaCl, 1mM EDTA, 0.1% NP-40, for 1h at room temperature. The agarose beads were collected by centrifugation for 30sec at 5,000g, the supernatant removed and the pellet washed three times in incubation buffer. Bound proteins were released into 2xSDS loading buffer (250mM Tris-HCl, pH6.8, 20% glycerol (v/v), 4% SDS (w/v), 0.1% bromophenol blue (w/v), 200mM DTT and 5% β-mercaptoethanol). The denatured sample was electrophoresed in a 10% SDS-polyacrylamide gel and Western blotted.

#### 2-1.7. Western Blotting

To measure the levels of protein expression in cells, extracts were immobilized on nitrocellulose membranes (Inverclyde Biochemicals Ltd), blocked in PBS-10% milk for 20min then incubated with primary antibody. As secondary antibody in immunoblotting, the blots were incubated with either alkaline phosphatase-conjugated goat anti-rabbit or -mouse IgG (1:5,000, Promega), or horseradish peroxidaseconjugated goat anti-rabbit or -mouse IgG (1:4,000) for ECL (Amersham) as recommended by the manufacturer.

#### 2-1.8. GST recombinant protein purification

Transformed BL21 bacteria were grown to mid-logarithmic stage in LB-broth before protein expression was induced by the addition of 1mM (final concentration) isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) and incubated for 4hr at 30°C. Bacterial pellets were resuspended in 10ml PBS/1% Triton X-100 and 0.5mM PMSF on ice, and then sonicated briefly (3 x 10sec) on ice. Bacterial debris was pelletted by centrifugation and re-centrifugation at 10,000rpm for 15min (at 4°C) each. 300µl of glutathione-agarose beads (33% v/v) in suspension (0.05% NaN3 in PBS) was added to the supernatant and mixed with constant rotation at 4°C for 30min. The suspension was washed twice with 50ml PBS by spinning in a bench-top centrifuge at 5,000rpm for 5min (at 4°C). The washed beads were stored in PBS-azide at 4°C for up to one week. Protein expression was measured by coomassie after SDS-PAGE.

#### 2-1.9. *In vitro* binding assay

For the *in vitro* binding reactions, approximately 10µg of GST or GST-fusion protein bound to glutathione-agarose beads was added to extract from transfected COS-7 cells (as described above). After incubation for 2hr at 4°C, the beads were collected and washed four times in incubation buffer (see 2-1.6.). Proteins were released into SDS-sample buffer, electrophoresed, and detected by immunoblotting.

#### 2-1.10. Yeast two-hybrid Assay

#### <u>Yeast strain</u>

Saccharomyces cerevisiae CTY10.5d with integrated plasmid pSIII8-34. This plasmid carries 4 binding sites for LexA upstream of the transcription start site of a lacZ gene. Genotype is MATa ade2 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3::lexA op-lacZ

#### cDNA library

10.5 d.p.c CD-1 mouse embryo library generaterd by random-primed cDNA synthesis and size selected to have insert sizes in the range 350-700 nucleotides cloned into vector pVP16, which carries the LEU2 gene, to create a VP16-library fusion protein.

#### <u>Bait</u>

DP-3 $\delta$  residues 1-110 cloned into vector pLexA that carries the HIS3 gene to create a LexA-bait fusion protein.

#### <u>Assay</u>

Yeast was transformed simultaneously with bait and library plasmid using the lithium acetate method (based on method of 1to *et al.*, 1983). Approximately  $2x10^5$  transformants were seeded on 16 x 140mm dishes containing synthetic medium lacking leucine and histidine. Colonies were assayed for  $\beta$ -galactosidase activity by filter assay. Blue colonies were isolated and cured of bait plasmid by growing to saturation in selective liquid media in the presence of histidine. After replica plating on selective minimal agar, plasmid DNA from Trp+His' colonies that failed to give a blue colour when assayed for  $\beta$ -galactosidase were recovered into Eschericia coli XL1-blue strain. PCR using vectors from the library backbone vector verified that library plasmid was successfully isolated. A plasmid conferring a Trp+ phenotype that gave a blue colour with an ABI dye terminator cycle sequencing-ready reaction kit (Perkin Elmer) and an automated DNA sequence analyser. Sequences were assembled into contigs with the

Seqman programme from the DNAStar package (DNASTAR, Inc.). Database searches and sequence comparisons were done using the BLAST (Altschul *et al.*, 1990), gapped BLAST and PSI-BLAST (Altschul *et al.*, 1997) programs provided by the National Center for Biotechnology Information (NCBI).

#### 2-1.11. Gel retardation assay

The binding sequence for E2F derived from the adenovirus (ad5) E2a promoter from -71 to -50. 5'-GATCTAGTTTTCGCGCTTAAATTTGA-3' and 3'-ATCAAAAGCGCGAATTTAAACTCTAG-5', was radiolabelled with  $32P-\alpha$ -GTP (Amersham). Approximately 4µg of U2OS or COS-7 cell extract (prepared as above) was used for each gel shift reaction and made up to a final volume of 20µl with reaction buffer (4x; 200mM Tris-HCl pH7.9, 24mM MgCl2, 0.8mM EDTA, 4mM DTT and 60% glycerol (v/v)), 2µg of sonicated-salmon sperm DNA (Sigma) and 200ng of mutant promoter oligo; 5'-GATCTAGTTTTCGATATTAAATTTGA-3' and 3'-ATCAAAAGCTATAAATTTAAACTCTAG-5' to remove the non-specific DNA binding activity. The mixture was incubated for 10min at room temperature before adding 6ng of  $^{32}P-\alpha$ -GTP-labelled oligonucleotide probe for a further 10min incubation at room temperature. Reaction mixtures were loaded on to a non-denaturing 4% polyacrylamide Tris-borate EDTA (TAE) gel at 4°C and ran at 250V for 2h.

#### 2-1.12. Protein stability assay

Transfection was carried out as above. For half-life measurements, cells were incubated with10mg/ml cyclohexamide (Promega) freshly prepared in ethanol. At time points thereafter, dishes were washed twice with PBS before harvesting in SDS sample buffer.

To inhibit ubiquitin-proteasome mediated degradation, two hours before harvesting, cells were incubated with 50mM *N*-acetyl-L-leucinyl-L-leucinyl-N-norleucinal (LLnL) (Promega) in DMSO or as a control, DMSO only.

#### 2-1.13. In vitro protein expression

In vitro transcription and translation was carried out using a TNT T7 coupled reticulocyte lysate system (Promega) as recommended by manufacturer, in the presence of 35S methionine. To check expression 1µl was used in SDS-PAGE and autoradiographed. 10µl was used for each immunoprecipitation reaction.

#### 2-1.14. Single-site *in vitro* mutagenesis

DP-3 $\delta$  target for mutagenesis was produced as anti-sense single stranded DNA by cloning into the M13K07 vector. *In vitro* mutagenesis was carried out using the Sculptor kit (Amersham) using the following oligonucleotides to introduce single site mutations into DP-3 $\delta$  DNA:

#### DP-3 $\delta\!S_{_{75}}\Lambda$ (5' to 3') GAGACATTTTTGCAATGAAGGTG

#### $\text{DP-}3\delta S_{61}\text{A}$ (5' to 3') GAAAGTAAACGAGCCAAAAAAGGAG

The predicted DNA sequence of both constructs was verified by DNA sequencing both strands manually (Sequenase, Amersham) or with an ABI dye terminator cycle sequencing-ready reaction kit (Perkin Elmer) and an automated DNA sequence analyser.

#### 2-1.15. Antibodies

#### Primary

DP-3	rabbit polyclonal serum raised against sequence common to all DP-3s
DP-1	rabbit polyclonal antiserun 098 raised against a C-terminal peptide
ΗΛ	mouse monoclonal HA11 (BabCO)
p107	mouse monoclonal SD9 (SantaCruz)
T7	mouse monoclonal (BabCO)
E2F-1	rabbit polyclonal KH95 (Santa Cruz)
14-3-3	rabbit polyclonal cross reactive with all 14-3-3 isoforms (Santa Cruz)
myc	mouse monoclonal 9E10 (Santa Cruz)

#### Secondary

Anti-Ig-TRITC	rhodamine conjugated (Southern Biotechnology Associates, Inc)
Anti-Ig-FITC	fluorescein conjugated (Southern Biotechnology Associates, Inc
Anti-Ig-hrp	horseradish peroxidase conjugated (Amersham)
Anti-Ig-AP	Alkaline phosphatase conjugated (Promega)
Anti-CD20-FITC	fluorescein conjugated (Becton Dickinson)

# Chapter 3. Nuclear accumulation of E2F is regulated by heterodimerisation

#### 3-1. Introduction

The recent isolation of a novel member of the DP family, DP-3 (Ormondroyd *et al.*, 1995), provided a valuable tool to advance the understanding of the functional regulation of E2F. DP-3 RNA undergoes extensive processing events to generate distinct DP-3 proteins, four of which have been isolated (de la Luna *et al.*, 1996). A sequence of 16 amino acid residues, known as the E region, is one such region subject to alternative splicing (Ormondroyd *et al.*, 1995) and discovery of this uncovered a significant splicing event that occurs throughout the DP family (Figure 3.1-A). Given that the sizes of the E2F and DP families are ever expanding, it is becoming more likely that heterodimers may not be functionally redundant.

This chapter aimed to define the functional significance of the E region by comparing properties of the E+ and E- members of the DP family and by studying the influence these proteins had on a heterodimeric E2F transcription factor. It had previously been shown that the DP proteins that possess the E region required a higher salt concentration to extract the protein from transfected COS-7 cells than was required for extraction of E- DP proteins (de la Luna *et al.*, 1996). As low salt conditions release mostly cytoplasmic proteins, higher salt conditions being required to release nuclear proteins with the cytoplasmic proteins, these results suggested that the different



ii

**DP-3**β

B



DP-1



**DP-3**δ

#### Figure 3.1

iii

Members of the DP family have distinct sub-cellular localisation

(A) Diagrammatic representation of the DP family illustrating the conserved domain mediating DNA binding/dimerisation (in blue) and the E region (in red).

(B) The intracellular distribution of exogenously expressed DP-1 (i), DP-3 $\beta$  (ii) and DP-3 $\delta$  (iii) protein was assessed by immunofluorescence using rabbit anti-peptide serum 7.2 (DP-3) or 098 (DP-1).

biochemical extraction properties reflected distinct intracellular distributions of the DP-3 proteins. This was investigated using indirect immunofluorescence of transfected cells to show the cellular localisation of DP and E2F, and did indeed reveal that the choice of heterodimeric partner was significant in controlling E2F activity by regulating intracellular location. Pocket proteins and viral oncoproteins, which have preferential specificity for particular E2Fs (Sardet *et al.*, 1997), also had an impact on the cellular localisation of E2F.

#### **3-2.** Results

#### 3-2.1. The E-region contributes to the nuclear accumulation of DP

The intracellular distribution of the DP-3 proteins was determined by indirect immunfluorescence of transiently transfected COS-7 cclls using either an anti-DP-3 anti-serum, called 7.2, or an anti-DP-1 antiserum, called 098, both of which only recognise exogenous DP protein (primary antibodies failed to detect any DP protein in non-transfected cells). Results showed that the DP family can be sub-divided into two categories: those containing an E region, DP-3 $\alpha$  and DP-3 $\delta$  (Figure 3.1-A) showed nuclear accumulation (Figure 3.1-Biii), whereas those lacking the E region, DP-1 and DP-3 $\beta$  (Figure 3.1-A) were distributed throughout the cytoplasm (Figure 3.1-Bi and ii). Control experiments where performed to ensure specificity of secondary antibodies (results not shown). These data establish that differences in protein sequence, i.e. the presence or absence of the E region, influenced the ability of the DP to efficiently accumulate in the nucleus.

#### **3-2.2. E2F** proteins differ in their cellular distribution

To address the location of E2F protein, haemagglutinin (HA)-tagged E2F-1, -4, and -5 were expressed, by transient transfection, in COS-7 cells and their location determined by indirect immunofluorescence. In these conditions, E2F-1 was found exclusively in the nucleus (Figure 3.2-Bi). In contrast, the majority of E2F-4 and E2F-5 transfected cells exhibited staining throughout the cytoplasm with a small number of cells showing nuclear staining also (Figure 3.2-Bii and iii). E2F-1 possesses an intrinsic nuclear localization signal (NLS) in the N-terminus of the protein (Krek *et al.*, 1994), a region that is absent from E2F-4 and E2F-5 (Figure 3.2-A). These results support the hypothesis that the E2F family can be divided into two functional subgroups, as suggested from their primary sequence and functional properties (Buck *et al.*, 1995; Krek *et al.*, 1994; Lukas *et al.*, 1996; Mann and Jones, 1996).

#### 3-2.3. E2Fs devoid of an NLS rely on heterodimerisation for translocation.

Although DP-1 was observed to be a cytoplasmic protein, it may not be actively retained in the cytoplasm as attachment of a foreign NLS caused nuclear accumulation (de la Luna *et al.*, 1996). Considering this, a possible mechanism for nuclear accumulation of a cytoplasmic DP could be through heterodimerisation with an NLS containing E2F partner. To test this idea, both HA-tagged E2F-1 and a cytoplasmic DP (DP-1 in figure 3.3-i and -ii or DP-3 $\beta$  in figure 3.3-iii and -iv) were co-expressed and their intracellular distribution determined by double immunostaining with anti-HA monoclonal antibody for E2F-1 and a polyclonal antibody for DP (Neither the rhodamine-conjugated anti-mouse immunoglobulin or fluorescein-conjugated anti-





HA-E2F-1





#### Figure 3.2

Members of the E2F family have distinct sub-cellular localisation

HA-E2F-4

(A) Digrammatic representation of the E2F family, illustrating the conserved domains mediating cyclin A binding (in red), DNA binding/dimerisation (in blue) and trans-activation/pocket protein binding (in green).

(B) The intracellular distribution of exogenously expressed HA-tagged E2F-1 (i), E2F-4 (ii) and E2F-5 (iii) protein was assessed by immunofluorescence using an anti-HA monoclonal antibody 12CA5.

rabbit immunoglobulin cross-reacted with the anti-DP polyclonal or anti-HA monoclonal antibody (data not shown)). There was a striking difference in the distribution of DP-1 and DP-3B upon co-expression of E2E-1. Cells expressing the E2F-1 protein contained nuclear DP-1 (Figure 3.3-ii), in contrast to its cytoplasmic location in the absence of E2F-1 (Figure 3.1-i). Similar results were observed in the nuclear recruitment of DP-3 $\beta$  by E2F-1 (Figure 3.3-iv compared to its location in the absence of E2F-1 shown in figure 3.1-ii). These data strongly suggest that upon forming a DP/E2F-1 heterodimer, E2F-1 has a dominant influence on recruiting DP to a nuclear location. As shown in figure 3.2-Bii and iii, E2F can exist in a cytoplasmic form too, so it was reasoned that heterodimerisation with an NLS-containing DP may influence its cellular location. Indeed, when co-expressed in COS-7 cells, double immunostaining with anti-HA monoclonal antibody for E2F-4 (Figure 3.4-i) or E2F-5 (Figure 3.4-iii) and a polyclonal antibody for DP-38 (Figure 3.4-ii and iv) showed that the non-NLS-containing E2F now accumulated efficiently in the nucleus. Note that in figure 3.4-i, one cell, indicated by an arrow, showed cytoplasmic E2F-4 due to the absence of a co-expressed DP-3 $\delta$  in this cell. These data indicated that the cytoplasmic location of E2F-4 and E2F-5 was likely to be passive since it could be overcome upon interaction with an NLS containing DP partner, defining a dominant role for the DP partner in the context of this heterodimer in regulating the nuclear accumulation of E2F.

#### **3-2.4.** Sub-cellular localization of E2F is affected by co-expression of p107

E2F species are known to complex with pocket proteins (Bagchi et al., 1991; Bandara and La Thangue, 1991; Chellappan et al., 1991; Chittenden et al., 1991; Cao et



#### Figure 3.3

Nuclear E2F can mediate nuclear translocation of a cytoplasmic DP

The intracellular distribution of exogenously expressed DP and HA-tagged E2F partner, in cells expressing both proteins, was detected by immunofluorescence using a rabbit polyclonal anti-DP-1 antibody (ii), anti-DP-3 antibody (iv), and mouse monoclonal anti-HA antibody (i) and (iii). Cells were co-transfected with HA-E2F-1 and either DP-1 (i) and (ii) or DP-3 $\beta$  (iii) and (iv). Note that DP-1 expressing cells that are not co-transfected with HA-E2F-1(ii, the cell on the right) are not located to the nucleus.



#### Figure 3.4

Nuclear DP can mediate nuclear translocation of cytoplasmic E2F

The intracellular distribution of exogenously expressed DP and HA-tagged E2F partner, in cells expressing both proteins, was detected by immunofluorescence using a rabbit polyclonal anti-DP-3 antibody (ii and iv) and mouse monoclonal anti-HA antibody (i and iii). Cells were co-transfected with DP-3\delta and either HA-E2F-4 (i and ii) or HA-E2F-5 (iii and iv). An Arrow indicates cell exogenously expressing HA-E2F-4 alone.

al., 1992; Devoto *et al.*, 1992; Shirodkar *et al.*, 1992; Cobrinik *et al.*, 1993). The cytoplasmic E2Fs -4 and -5 specifically interact with p107 (Sardet *et al.*, 1997), which is a nuclear protein (Figure 3.5-i). To study whether association of p107 with a cytoplasmic heterodimer could result in a nuclear E2F complex, U2OS cells were co-transfected with either HA-E2F-4 or -5 together with DP-3 $\beta$  or DP-1, and the pocket protein p107. Consistent with the absence of NLS activity in each protein, the heterodimer expressed without p107 was cytoplasmic, as determined by double immunostaining with anti-IIA monoclonal antibody for E2F-4 or -5 and a polyclonal antibody for DP-3 $\beta$  or DP-1 (Figure 3.5-ii, iii, vi and vii) Upon addition of p107, both components of the heterodimer were observed to be strongly nuclear (Figure 3.5-iv, v, viii and ix). The nuclear accumulation was dependent upon the expression of E2F since p107 could not mediate nuclear translocation of DP-3 $\beta$  in cells not expressing an E2F (data not shown). These data suggest that the NLS of p107, supplied to the heterodimer *in trans*, is essential for E2F nuclear translocation under the conditions used.

## 3-2.5. E2F, transported by p107 into the nucleus, remains nuclear even after dissociation from p107

It is known that the E2F/p107 complex is a target of viral oncoproteins, such as Adenovirus E1a and SV40 large T antigen, resulting in release of E2F enabling cell cycle progression to ensue (Nevins, 1992). It was therefore of interest to determine the effect of viral oncoproteins on p107-mediated nuclear translocation of E2F. The coexpression of p107 with E2F-4/DP-3 $\beta$  resulted in nuclear accumulation of DP-3 (Figure 3.6-i and ii). Expression of E1a, at levels known to be sufficient to disrupt the



#### Figure 3.5

Pocket proteins can mediate the nuclear accumulation of cytoplasmic E2F heterodimers

The intracellular distribution of exogenously expressed p107 (i), DP-3 $\beta$  and HA-E2F-4 (ii and iii), DP-3 $\beta$  and HA-E2F-4 co-expressed with p107 (iv and v), DP-1 and HA-E2F-5 (vi and vii) or DP-1 and HA-E2F-5 co-expressed with p107 (viii and ix) was detected by immunofluorescence using a rabbit polyclonal anti-DP-1 antibody (vii and ix) or anti-DP-3 (iii and v) or mouse monoclonal anti-HA antibody (i, iv, vi and viii) or anti p107 antibody (i).



#### Figure 3.6

Pocket protein-mediated nuclear translocation of E2F is unaffected by adenovirus E1a

The intracellular distribution of exogenously co-expressed DP-3 $\beta$ , HA-E2F-4 and p107 (i and ii) or DP-3 $\beta$ , HA-E2F-4, p107 co-expressed with E1a (iii and iv) was detected by immunofluorescence using a rabbit polyclonal anti-DP-3 antibody (ii and iv) and mouse monoclonal anti-p107 antibody (i and iii). Distribution of T7 tagged- E1a was assessed with a cytoplasmic heterodimer (v and vi) and with a heterodimer expressed with p107 (vii and viii) using a monoclonal anti-T7 antibody and rabbit polyclonal anti-DP-3 antibody. E1a alone is also shown (xi).

interaction between p107 and E2F, did not affect this nuclear accumulation (Figure 3.6iii and iv). E1a itself is a nuclear protein (Figure 3.6-xi) but cannot transport E2F-4/DP- $3\beta$  to the nucleus (Figure 3.6-v and vi). When expressed with the heterodimer and p107, E1a co-localised to the nucleus with DP-3 $\beta$  (Figure 3.6-vii and viii). Thus, the release of E2F from pocket protein sequestration does not alter the sub-cellular distribution of the E2F heterodimer, suggesting that the pocket protein release occurs once the E2F/pocket protein complex has entered the nucleus. There is also a suggestion here that the E2F heterodimer can be retained in the nucleus after the link with the transporter, p107, has been disrupted.

#### **3-3.** Conclusion

It is known that physiological E2F arises when a member of each of two families of proteins, E2F and DP, interact as E2F/DP heterodimers capable of binding specific sequences of DNA and that transcriptional activity is regulated through physical association with pocket proteins. This chapter showed that these known features all regulate another aspect of E2F activity; that is, in controlling the levels of functional nuclear E2F. For nuclear activity, either the DP or the E2F partner must provide an NLS. In the occasion where both partners are devoid of such a signal, association with a pocket protein will transport the heterodimer into the nucleus. In this case, the heterodimer will be expected to exist in a transcriptionally repressed state. However, should the pocket protein complex be disrupted, by CDKs or viral oncoproteins for example, an active nuclear E2F may be released.

### Chapter 4. The basic region of DP is necessary for efficient nuclear accumulation of E2F heterodimers

#### 4-1. Introduction

The observation that the E region is necessary for nuclear accumulation of DP suggests that it may function as, or contribute to, a nuclear localization signal. The process of alternative splicing of a nuclear localization signal was an exciting and unexpected mechanism uncovered for controlling E2F activity. It was of great interest therefore, to study the DNA sequence of this area in all the DPs (Figure 4.1).

Active transport of proteins into the nucleus is dependent upon short amino acid sequences composed of a high proportion of positively charged residues. An NLS may be a single-cluster, exemplified by SV40 T antigen, or bi-partite, as in nucleoplasmin (Dingwall and Laskey, 1991). Single-cluster sites are defined as comprising at least four arginine and lysine residues, bi-partite motifs consist of two basic amino acids, a spacer region of any ten amino acids, then a basic cluster in which three out of the next five residues must be basic. Figure 4.1 shows that, by sequence alignment, both types of NLS consensus can be identified around the E region.

As sequence analysis revealed two putative NLSs, the aim of this chapter was to determine whether the E region contained a single-cluster SV40 Tag-like NLS or



#### Figure 4.1

#### DPs contain a bipartite nuclear localisation signal

Diagrammatic representation of the DP family of proteins illustrating the conservation of the nuclear localisation signal which is composed of the E region and the basic region. The NLSs of nucleoplasmin and SV40 T antigen are also shown for comparison. Similar and identical residues are indicated. The basic region deleted mutants, DP3- $\beta\Delta b$  and DP3- $\delta\Delta b$  are also shown.

contributed to a bipartite NLS. An NLS is defined as a sequence necessary for nuclear targeting of the protein and it must be sufficient to direct a non-nuclear protein to the nucleus. The previous chapter demonstrated that the E region complies with the first statement. Although the putative NLS sequence within the E region bears resemblance to the SV40 T antigen NLS, it is not sufficient by itself to target a protein to the nucleus (de la Luna *et al.*, 1996). However, the addition of a further eight residues C-terminal to the E region was sufficient to transfer a nuclear phenotype onto a non-nuclear protein. This implies that the E region does not contain an autonomous NLS but may function co-operatively with an additional sequence at the C terminal boundary of the E region. Insertion of the E region by alternative splicing may create a bi-partite NLS. To investigate this, the N-terminal basic cluster of the putative bipartite NLS was deleted and the phenotype of the resulting mutant DP characterised.

#### 4-2. **Results**

#### 4-2.1. The basic region is necessary for nuclear accumulation of E2F

To prove that the basic region forms part of the NLS in DP, DP-3 $\delta$  was mutated using PCR to excise DNA bases encoding six amino acids from the basic region. This protein was named DP-3 $\delta\Delta b$  (Figure 4.1). When expressed in COS-7 cells, wild type DP-3 $\delta$  was located in the nucleus, but the basic region-deleted protein failed to accumulate in the nucleus.



#### Figure 4.2

#### The basic region is necessary for nuclear accumulation of E2F

(A) The intracellular distribution of exogenously expressed DP-3 $\delta$  (i) or DP-3 $\delta\Delta b$  (ii), detected by immunofluorescence using a polyclonal anti-DP-3 peptide antibody.

(B) HA-E2F-4 co-expressed with DP-3 $\delta$  (i and ii) or DP-3 $\delta\Delta b$  (iii and iv) detected by immunofluorescence using a polyclonal anti-DP-3 peptide antibody (ii and iv) and a monoclonal anti-HA antibody (i and iii).

(C) HA-E2F-5 co-expressed with DP-3 $\delta$  (i and ii) or DP-3 $\delta\Delta b$  (iii and iv) detected by immunofluorescence using a polyclonal anti-DP-3 peptide antibody (ii and iv) and a monoclonal anti-HA antibody (i and iii)

The DP-38 $\Delta$ b protein was more abundant, particularly around the nuclear membrane, giving a "cauliflower-like" staining pattern (Figure 4.2-A, compare i with ii). As expected, DP-38 transported non-NLS-containing E2F-4 and E2F-5 into the nucleus (Figure 4.2-Bi and ii and 4.2-Ci and ii). Both the E region and the basic region were required for this function since DP-38 $\Delta$ b was unable to transport E2F-4 and -5 into the nucleus (Figure 4.2-B and 4.2-C, compare i and ii with iii and iv). The staining results do show however, that E2F-4 and -5 co-localised with DP-38 $\Delta$ b in the cytoplasm as the particularly distinctive cauliflower-like pattern was transferred to the E2F protein.

# 4-2.2. Heterodimerisation fails to efficiently transport DP-3δ∆b into the nucleus.

Co-expression of wild type DP-3 $\delta$  with E2F-1 in COS-7 cells resulted in nuclear co-localisation (Figure 4.3-Ai and ii). If DP-3 $\delta$ Δb was co-expressed with E2F-1, one would expect E2F-1 to supply the NLS, and both proteins to translocate to the nucleus, as is the case for E2F-1/DP-3 $\beta$  heterodimers. However, only 50% of cells expressing both proteins exogenously, showed nuclear co-localisation. The remaining 50% double-stained cells show nuclear accumulation of E2F-1, and DP-3 $\delta$ Δb remaining in the cytoplasm (Figure 4.3-Aiii and iv). As the COS-7 cell line is transformed with SV40 large T antigen, these transfections were also carried out in the parental cell line CV-1. The results here implicated T antigen in the observations made in COS-7 cells since CV-1 (and also U2OS) cells showed co-localisation of E2F-1 and DP-3 $\delta$ Δb in the nucleus of all double-stained cells (Figure 4.3-Av and vi). This implies that under specific circumstances, the basic region is either involved in nuclear retention of the DP



#### Figure 4.3

### Co-expression of nuclear E2F is not sufficient to mediate nuclear translocation of basic deletion mutant DP

(A) The intracellular distribution of exogenous HA tagged E2F-1 co-expressed with DP-3 $\delta$  (i and ii), or DP-3 $\delta\Delta b$  (iii, iv, v and vi), detected by immunofluorescence using a polyclonal anti-DP-3 peptide antibody (ii, iv and vi) and a monoclonal anti-HA antibody (i, iii, and v) in either COS-7 cells (i, ii, iii and iv) or CV-1 cells (v and vi)

(B) HA-E2F-4 and DP-3 $\delta\Delta b$  (i and ii), co-expressed with p107 (iii and iv) detected by immunofluorescence using a polyclonal anti-DP-3 peptide antibody (ii and iv) and a monoclonal anti-HA antibody (i and iii).

component, or nuclear export inhibition of DP after transport via E2F-1, or in regulating heterodimerisation in the cytoplasm, a phenotype that is only apparent in the presence of T antigen. As shown previously in figure 4.2-B, DP-3 $\delta$  can provide nuclear translocation to E2F-4 but DP-3 $\delta\Delta$ b was unable to do this. Co-expression of the pocket protein p107 did however, transport the heterodimer to the nucleus in all cells double stained for E2F and DP (Figure 4.3-B, compare i and ii with iii and iv); a role it performs with all non-NLS containing heterodimers. Removal of the basic region did not affect the pocket protein-mediated pathway into the nucleus.

#### 4-2.3. Cytoplasmic DPs require an intact basic region for nuclear transport

Although cytoplasmic DPs do not contain the E region, the basic region is conserved throughout the DP family. To assess the role of the basic region in cytoplasmic DPs, DP-3 $\beta$  was mutated by PCR to excise DNA bases encoding six amino acids from the basic region. This protein was named DP-3 $\beta\Delta b$  (Figure 4.1). When co-expressed in COS-7 cells, wild-type DP-3 $\beta$  was cytoplasmic (Figure 4.4-Ai), however immunostaining of the basic region-depleted-DP-3 $\beta$  protein showed the protein to be absolutely excluded from the nucleus (Figure 4.4-Aii). Around 80% of DP-3 $\beta$ -expressing cells show the protein to be predominantly cytoplasmic with a low level of expression in the nucleus, presumably transported by endogenous E2F/pocket proteins, however, DP-3 $\beta\Delta b$  stained cells show no nuclear staining whatsoever. Reminiscent of DP-3 $\delta\Delta b$  stained cells, some of the DP-3 $\beta\Delta b$ -expressing cells showed a cauliflower-like staining pattern around the nuclear membrane, the DP-3 $\beta\Delta b$  protein expression appeared more abundant than the wild type protein and more cells appeared to express


#### Figure 4.4

### The basic region of cytoplasmic DPs is still necessary for efficient nuclear accumulation of the heterodimer

(A) The intracellular distribution of exogenous DP-3 $\beta$  (i) and DP-3 $\beta\Delta b$  (ii) detected by immunofluorescence using a polyclonal anti-DP-3 peptide antibody.

(B) Sub-cellular distribution of wt and mutant DP-3 $\beta$  co-expressed with an E2F partner. HA-E2F-1 (i, ii, iii and iv) co-expressed with wt DP-3 $\beta$  (i and ii) or mutant DP-3 $\beta$  (iii and iv), and HA-E2F-5 co-expressed with DP-3 $\beta\Delta b$  (v and vi) were detected by immunofluorescence using a polyclonal anti-DP-3 peptide antibody (ii, iv and vi) or a monoclonal anti-HA antibody (i, iii and v). Cells indicated by an arrow show co-localisation of E2F and DP.

the mutant protein. As shown previously, HA-E2F-1 can transport DP-3 $\beta$  into the nucleus, however, when co-expressed with DP-3 $\beta$ Ab, only 50% of co-transfected cells showed co-localisation in the nucleus (Figure 4.4-Biii and iv cells identified by arrows). The remaining 50% exhibited nuclear E2F-1 but DP-3 $\beta$ Ab remained in the cytoplasm (Figure 4.4-B compare i and ii with iii and iv). It appears that the basic region in DP is necessary for efficient nuclear accumulation, and hence functional activity, of the E2F transcription factor, even if the DP does not possess the E region part of the NLS. As expected, DP-3 $\beta$ Ab co-localises with HA-E2F-5 in the cytoplasm (Figure 4.4-B v and vi).

### 4-2.4. The DP basic region is not involved in heterodimerisation or DNA binding

It is clear that the basic region has an important role to play in regulating E2F activity so it was essential to establish whether the mutant affects E2F heterodimerisation, which would subsequently affect Rb-binding, DNA binding, and transcriptional properties (Bandara *et al.*, 1993; 1994; Helin *et al.*, 1993; Krek *et al.*, 1993). To test heterodimerisation capabilities of the basic deletion mutant DP-3, DP- $3\delta$ , DP- $3\delta\Delta b$ , E2F-1 and E2F-5 proteins were in vitro transcribed and translated and immunoprecipitation experiments performed (Figure 4.5-A, lanes 1-8 show the relative expression of E2F and/or DP in the lysates used in the immunoprecipitations in lanes 9-16). Results showed that both DP- $3\delta$  and DP- $3\delta\Delta b$  were immunoprecipitated equally well with the anti-DP-3 antibody (Figure 4.5-A, lanes 9 and 10).





#### Figure 4.5

The basic region of DP-3 $\delta$  is not involved in the heterodimerisation of DP with E2F-1 or E2F-5

(A) Immunoprecipitation of HA-tagged E2F-5 and DP-3 from 35S-labelled *in vitro* translated rabbit reticulocyte lysates using a polyclonal anti-DP-3 peptide antibody, and proteins detected by autoradiography. The postitions of the labelled proteins are shown. Note the absence of E2F bands in the immunopreciptates not containing DP protein (lanes 11 and 14) showing no cross-reactivity occurs between the anti-DP-3 antibody and the HA-tagged E2Fs.

(B) Immunoprecipitation of HA-tagged E2F-5 and DP-3 from transfected cell extracts using a polyclonal anti-DP-3 peptide antibody, and proteins detected by immunoblotting with the anti-HA antibody 12CA5. The position of the co-immunoprecipitated E2F-5 bands are shown. Asterisks indicate the positions of the two hyper-phosphorylated E2F species.

E2F-1 and E2F-5 were both recovered efficiently in anti-DP-3 immunoprecipitates from lysates containing DP-3 $\delta$  or DP-3 $\delta\Delta$ b suggesting that stable complexes of each DP-3 formed with E2F-1 (Figure 4.5-A, lanes 15 and 16), and E2F-5 (Figure 4.5-A, lanes 12 and 13) even when the basic region was deleted. E2F-1 and -5 were not present in anti-DP-3 immunoprecipitates when DP-3 was omitted from the reaction (Figure 4.5-A lanes 11 and 14). Thus, DP-3 $\delta\Delta$ b can form stable complexes with E2F-1 and -5 *in vitro*.

Next, the association was tested *in vivo*. Figure 4.5-B shows that after transient transfection of HA-tagged E2F-5 and either DP-3 $\delta$  or DP-3 $\delta$ Δb into U2OS cells, HA-E2F-5 was detected in anti-DP-3 immunoprecipitates from both extracts (Figure 4.5-B, lanes 3 and 4). Incidentally, co-expression of DP-3 $\delta$  with HA-E2F-5 resulted in stabilization of the two slowest migrating bands corresponding to hyper-phosophorylated species of E2F-5 (Morris *et al.*, 2000) as detected by western blot (Figure 4.5-B, compare lanes 1 and 2, bands indicated with a asterisks). However, co-expression with DP-3 $\delta$ Δb gave a different display of bands with the two top bands less abundant and the appearance of a stronger lower hypo-phosphorylated band. The significance of this is unclear but implies that DP has an influence on the phosphorylation of E2F-5.

Both DP and E2F proteins contain sequence-specific DNA binding domains, located in similar positions of each protein (Girling *et al.*, 1993). Heterodimerisation results in co-operative recognition of, and activation of transcription through, the E2F binding site. Since the basic region was located close to the DNA binding domain of



### Removal of the basic region from DP-3 $\delta$ does not alter the DNA binding activity of E2F

Whole cell extracts of U2OS cells transfected with the indicated expression plasmids were used in gel shift DNA binding assays with a 32P labelled oligonucleotide containing an E2F binding site (see section 2-1.11.). (A) Shows binding of E2F-1 complexes to DNA. DNA binding reactions in lanes 4, 6, 8 and 10 were subjected to supershift by rabbit polyclonal anti-E2F-1 antibody KH95. \* indicates the postion of the shifted complex. NS indicates non-specific binding to the probe. Note that lane 5 was incubated with more cold probe competitor.

(B) Shows binding of E2F-5 complexes to DNA.

DP it was imperative to assess the ability of DP-3 $\delta\Delta$ b to cooperate with E2F in DNA binding. This was determined using gel retardation assays with transfected COS-7 cell extracts. An antibody to E2F-1 was used to super-shift DNA binding complexes containing E2F-1. Figure 4.6-A shows formation of DNA bound heterodimers of E2F-1 with DP-3 $\beta$  (lanes 5 and 6), DP-3 $\delta$  (lanes 7 and 8) and DP-3 $\delta\Delta$ b (lanes 9 and 10). All three DPs increased the DNA binding ability of E2F-1 synergistically (compare lanes 3 and 4 with 5,6,7,8,9, and 10). The observed levels of DNA bound complex correlated with the relative expression levels of each protein. Formation of a DNA binding complex of DP-3 $\delta\Delta$ b with E2F-5 was also assessed. Figure 4.6-B shows that DP-3 $\delta\Delta$  did. Observed levels of complex correlated with relative expression levels of DP-3 $\delta$  is not involved in the association with E2F and does not influence the heterodimers ability to bind to specific DNA sites under the conditions used.

#### 4-3. Conclusion

Although the E region is necessary for nuclear accumulation of DP, it is not sufficient to target a protein to the nucleus. A basic region, C- terminal to the E region, which is conserved in all DPs, was also required. Evidence supporting the theory that these two elements form a bipartite NLS comes from the fact that removal of the basic region from DP-3 $\delta$  altered the protein from a nuclear to a cytoplasmic phenotype. DP-3 $\delta\Delta b$  exhibited the properties expected of a cytoplasmic non-NLS containing protein since it was unable to transport NLS-containing E2Fs to the nucleus. However, unlike other cytoplasmic DPs, hetrodimerisation with an NLS-containing E2F was not sufficient to induce nuclear accumulation of DP-3 $\delta\Delta b$  in SV40 T antigen transformed cells. DP-3 $\delta\Delta b$  was able to co-localise to the nucleus with a non-NLS containing E2F that was bound to pocket protein, implying that downstream E2F functions are regulated differently after pocket protein-mediated nuclear transport compared with free E2F nuclear transport.

# Chapter 5. DP mediates the association of E2F with 14-3-3

#### 5-1. Introduction

With a view towards understanding the role of the DP subunit in the E2F heterodimers it was reasoned that the basic region may be involved in regulating the interaction of the DP subunit with another protein. To test this hypothesis, a yeast two-hybrid system was used to conduct an *in vivo* search for a gene encoding a protein that interacts with DP-3\delta but not with DP-3\delta\Delta b and which may contribute to the properties of this region described in the previous chapters. The results have uncovered a new interacting protein for E2F that integrates the E2F pathway with other growth control pathways.

#### 5-2. Results

#### 5-2.1. Yeast two-hybrid screening

A yeast two-hybrid strategy, described in Material and Methods 2-1.10, was employed to identify DP interacting proteins where the efficiency of interaction was dependent on whether the basic region was present. Figure 5.1-A illustrates the screening system used. A yeast colony phenotype becomes LacZ+, i.e. blue in colour







#### Figure 5.1

#### The yeast two-hybrid interaction assay

(A) Strategy for isolating interactants with DP3 $\delta$  but not DP-3 $\delta\Delta b$ . Interaction between bait and library protein turns yeast colony blue in the presence of  $\beta$ -galactosidase substrate.

(B) Diagrammatic representation of bait and prey used in assay.

upon exposure to an X-Gal substrate, when the LexA DNA binding domain (DBD) and the VP16 activation domain (AD) are brought into close proximity in the promoter region of the *lacZ* gene. This only occurs if there is an interaction between the bait and a library protein. The "baits" were either a fragment of DP-3δ or DP-3δΔb (amino acids 1-110 or equivalent region in DP-3δΔb) fused to the LexA DNA binding domain (Figure 5.1-B). Yeast cells were analysed for LexA-dependent transcription activity following co-transformation of the bait plasmid with a 14.5 dpc mouse embryo cDNA library fused to the VP16 activation domain (Figure 5.1-B).

The sequence of one resulting positive clone revealed the epsilon isoform of 14-3-3. Figure 5.2-B shows the region of 14-3-3 $\varepsilon$ , isolated from yeast, after DNA sequencing and matching to known proteins in the GenBank nucleotide database. Results of the  $\beta$ -gal assay shown in figure 5.2-Ai correspond to the transformations listed in Figure 5.2-Aii. Only filter 3 (Figure 5.2-A) had blue colonies signifying that DP-3 $\delta$  interacted with 14-3-3 $\varepsilon$  whereas DP-3 $\delta\Delta$ b failed to interact with 14-3-3 $\varepsilon$ . The interaction between LexA-DBD-DP3 $\delta$  and VP-16-AD-14-3-3 was specific as binding was not apparent between 14-3-3 and the LexA-DBD or between DP-3 $\delta$ , or DP3 $\delta\Delta$ b and the VP-16-AD.

#### 5-2.2. 14-3-3 association with E2F is regulated through DP

Since 14-3-3 was isolated by screening in a yeast two-hybrid assay for proteins that required the basic region for an interaction with DP-3 $\delta$ , a biochemical approach was next taken to assess if DP-3 $\delta$  could physically interact with 14-3-3 $\epsilon$ . First of all the



ii [	Bait	Prey	Signal
ł	1. Lex DBD	VP16 TAD	white
	2. Lex DBD-DP-38	VP16 TAD	white
	3. Lex DBD-DP-38	VP16 TAD 14-3-3ε	blue
	4. Lex DBD	VP16 TAD 14-3-3ε	white
	5. Lex DBD-DP-3δΔb	VP16 TAD	white
	6. Lex DBD-DP-3δ∆b	VP16 TAD 14-3-3ε	white

B



#### Figure 5.2

#### DP-3 $\delta$ binds to 14-3-3 $\epsilon$ in yeast

(A)(i) Filters of yeast cells transformed as in table (ii) showing VP16 dependent transcriptional activity of an integrated  $\beta$ -galactosidase reporter as a blue colony. (ii) Summary of results from the interaction studies performed in yeast using the indicated bait and prey.

(B) Region of 14-3-3ε identified in yeast two hybrid assay results.

A

interaction was confirmed using GST pull-down experiments with transiently transfected COS-7 cell extracts and bacterially expressed GST-14-3-3 $\varepsilon$  or GST alone (Figure 5.3-Ai), and binding efficiency measured thereafter. In these experiments, HA-E2F-5 was retained by GST-14-3-3 $\varepsilon$  when in the presence of DP-3 $\delta$ . However, HA-E2F-5 was not retained when co-expressed with DP-3 $\delta\Delta$ b. Although the amount of E2F-5 pulled down by GST-14-3-3 $\varepsilon$  was low when in the presence of DP-3 $\delta$ , DP-3 $\delta\Delta$ b heterodimers always scored negative in this assay. GST alone was unable to bind to E2F-5. Immunoblotting for DP-3 showed that GST-14-3-3 $\varepsilon$  pulled down DP-3 $\delta$  (Figure 5.3-B lane 3). Although a low amount of DP-3 $\delta\Delta$ b protein was detected in the GST-14-3-3 precipitate (Figure 5.3-B lane 4), it should be noted that the input lanes show that higher levels of DP-3 $\delta\Delta$ b protein were present in the assay (Figure 15-B, lane 2 compared to lane 1).

Next, the association was tested in mammalian cells using the two-hybrid approach. Gal4-dependent transcription activation was followed after transfecting U2OS cells with VP16-AD-tagged 14-3-3 $\epsilon$  and Gal4-DBD-tagged DP-3 $\delta$  or DP-1. Significant stimulation of activity was apparent when VP16-14-3-3 $\epsilon$  was co-expressed with G4-DP-3 $\delta$  and not with G4-DP-1 (Figure 5.4, compare lanes 2 and 3 with 4 and 5). These data suggest that the interaction between the E2F transcription factor and 14-3-3 is determined by the choice of DP partner.



#### Figure 5.3

14-3-3 $\epsilon$  binds to the E2F-5/DP-3 $\delta$  heterodimer but not the E2F-5/DP-3 $\delta\Delta b$  heterodimer *in vitro*.

(A)(i) Coomassie stained bacterially expressed GST protein (lane1) and GST-14-3-3 $\epsilon$  protein (lane 2). Transfected whole cell extracts were incubated with 1µg GST-14-3-3 $\epsilon$  immobilised on glutathione agarose beads and precipitiates subjected to SDS-PAGE and immunoblotted with anti-HA antibody (A)(ii) or anti-DP-3 peptide antibody (B).



#### Figure 5.4

#### 14-3-3 associates with DP-3 $\delta$ but not efficiently with DP-1

(A) Mammalian two hybrid interaction assay between DP fused to a GAL4 DNA binding domain and 14-3-3 fused to the *trans*-activation domain of VP16. Results are presented as activity of a G5E1b5 luciferase reporter construct containing GAL4 DNA binding sites. Each bait was co-expressed with VP16AD alone (lanes 2 and 4) or with VP16-14-3-3 (lanes 3 and 5). All plates were cotransfected with pCMVβgal as internal control. Values are given as the ratio of luciferase to βgal activities.

#### 5-2.3. The basic region is involved in association of 14-3-3 with E2F

To show that the basic region of DP is influential in the association of E2F with 14-3-3 in mammalian cells, immunoprecipitation experiments were carried out on U2OS cells transfected with HA-E2F-5 and either DP-3 $\delta$  or DP-3 $\delta\Delta b$ . As expected, western blotting analysis of the inputs showed greater protein levels of DP-3 $\delta\Delta b/$  E2F-5 heterodimer than the DP-38/E2F-5 heterodimer but equal protein levels of endogenous 14-3-3 protein (figure 5.5-A, compare lanes 1 and 2, top blot shows DP-3 and 14-3-3 protein levels, bottom blot shows E2F-5 levels). The 14-3-3 antibody is directed towards a common motif found in all isoforms of 14-3-3. Immunoprecipitation of HA-E2F-5 with the anti-HA monoclonal antibody resulted in co-precipitation of both wild type and mutant DP-3; as detected by a rabbit polyclonal antibody to DP-3, equivalent to their input levels, implying equal binding (Figure 5.5-A). Nevertheless, coimmunoprecipitation of endogenous 14-3-3, as detected by a rabbit polyclonal antibody to 14-3-3, was greater from the extract expressing wild type DP-3δ compared to the extract expressing mutant DP-3 $\delta$  (Figure 5.5-A, compare lanes 3 and 4). Again, although a low amount of 14-3-3 protein was precipitated from the extract expressing the mutant DP-3d (Figure 5.5-A lane 3), it should be noted that the input lanes show that far higher levels of DP-3 $\delta\Delta$ b protein were present in the assay (Figure 5.5-A, lane 3) compared to lane 4). Western blot shows a preference for the upper isoform of 14-3-3 in the immunoprecipitate.

Reciprocal immunoprecipitation, utilizing the rabbit polyclonal anti-14-3-3 antibody resulted in co-precipitation of HA-E2F-5 when co-expressed with wild type



#### Figure 5.5

The basic region of DP-3 $\delta$  is necessary for association of heterodimer with 14-3-3

(A) Immunoprecipitation of endogenous 14-3-3 with exogenous DP-3 from extracts of cells transfected with HA-E2F-5 and DP-3. Extracts were immunoprecipitated using an anti-HA monoclonal antibody and proteins detected with a polyclonal anti-DP-3 antibody and a polyclonal anti-14-3-3 antibody raised against a peptide common to all 14-3-3 isoforms. Inputs (lanes 1 and 2) show amounts of exogenous HA-E2F-5 and DP-3 proteins expressed. Positions of DP-3 and 14-3-3 proteins are indicated. Note that only the upper band of 14-3-3 is precipitated (lane 4).

(B) Immunoprecipitation of exogenous HA-E2F-5 from cells transfected with HA-E2F-5, DP-3 and myc-tagged 14-3-3 $\epsilon$ . Extracts were immunoprecipitated using an anti-14-3-3 polyclonal antibody and proteins detected with a monoclonal anti-HA antibody. Inputs (lanes 1 and 2) indicate amounts of HA- E2F-5 and endogenous and exogenous 14-3-3 proteins expressed.

(C) Immunoprecipitation of exogenous myc-tagged 14-3-3ε from cells transfected with HA-E2F-5, DP-3 and myc-tagged 14-3-3e. Extracts were immunoprecipitated using an anti-DP-3 polyclonal antibody and proteins detected with a monoclonal anti-myc antibody (9E10).

DP-38 but not when co-expressed with the mutant DP-38 $\Delta$ b (Figure 5.5-B, compares lanes 3 and 4). The extracts used in this particular experiment contained a similar level of E2F-5 protein, as detected by an anti-HA antibody, however, it should be noted that the pattern of bands differs between the two samples. As observed previously in figure 4.5-B, the top two hyper-phosphorylated species of E2F-5 observed in the wild type heterodimer are less evident in the extract expressing DP-38 $\Delta$ b/HA-E2F-5 (Figure 5.5-B, top blot, compare lanes 1 and 2). Equal levels of endogenous and exogenous 14-3-3 were expressed between the two samples (Figure 5.5-B, bottom blot, compare lanes 1 and 2). Similar results were obtained in an anti-myc immunoblot of proteins immunoprecipitated with an anti-DP-3 antibody from cells transfected with HA-E2F-5, myc-tagged 14-3-3 $\epsilon$  and either DP-38 or DP-38 $\Delta$ b (Figure 5.5-C).

#### 5-2.4. 14-3-3 co-localises with DP-3 in the nucleus

14-3-3 has been shown to function as an attachable nuclear export signal that enhances the nuclear export of Cdc25 (Lopez-Girona *et al.*, 1999) and a forkhead transcription factor in response to phosphorylation by AKT (Brunet *et al.*, 1999), therefore, the cellular location of DP-3 $\delta$  was assessed in COS-7 cells after transient transfection with myc-tagged 14-3-3 $\epsilon$ . Results showed that 14-3-3 $\epsilon$  is a cytoplasmic protein (Figure 5.6-A). After the addition of DP-3 $\delta$ , an increase in nuclear staining of 14-3-3 was observed, (Figure 5.6-Bi) correlating with the co-expression of nuclear DP-3 $\delta$  within the same cell (Figure 5.6-Bi). This illustrated that 14-3-3 does not retain DP-3 in the cytoplasm, as may be assumed from previously documented examples, but that expression of DP-3 $\delta$  can drive 14-3-3 proteins into the nucleus.



#### Figure 5.6

#### 14-3-3 is a cytoplasmic protein which accumulates in the nucleus with E2F-5/DP-38

(A) The intracellular distribution of exogenously expressed myc-tagged 14-3-3ε detected by immunofluorescence using a monoclonal anti-myc antibody.

(B) Myc-tagged 14-3-3 co-expressed with DP-38 detected by immunofluorescence using a polyclonal anti-DP-3 peptide antibody (ii) and amonoclonal antimyc antibody (i) in cells expressing both proteins.

#### 5-3. Conclusion

14-3-3 molecules are highly evolutionarily conserved and expressed in a wide range of tissues and cell-types (Aitken, 1996). They are thought to play key roles in signal transduction pathways as scaffolding or adapter proteins that bring together signalling molecules. E2F associated with 14-3-3 via the DP-component in various different assays. This interaction required an intact basic region. Although little is known about the biological function of 14-3-3 proteins, the crystal structure of two isoforms has been solved (Liu *et al.*, 1995; Xiao *et al.*, 1995). These structures suggest that 14-3-3 proteins exist as dimers, with each monomer containing nine helices that surround a groove (Figure 1.8). One interpretation of the crystal structure suggests that the groove could accommodate an amphipathic helix (Liu et al., 1995). Elucidation of the crystal structure of DP-3 will answer whether the basic region is involved in the formation of an amphipathic helix. Many proteins bind to 14-3-3 subsequent to phosphorylation either within the binding region or flanking it (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). The basic region of DP-38 is within a domain rich in serine residues so its possible that phosphorylation here may regulate binding to 14-3-3.

# Chapter 6. 14-3-3 regulates the cell cycle through E2F

#### 6-1. Introduction

The previous chapter identified 14-3-3 as a novel interacting protein for E2F. 14-3-3 molecules are known to provide a platform to promote communication between proteins involved in a myriad of signalling pathways. However, the exact functions of 14-3-3 molecules are poorly understood and no inherent biological function has been attributed to them.

The aim of this chapter was to identify a role for 14-3-3 in the regulation of E2F activity. This was achieved by characterising the phenotype of a mutant DP that does not bind to 14-3-3. The results propose a role for 14-3-3 in promoting cell-cycle progression through the E2F pathway.

#### 6-2. Results

#### 6-2.1. 14-3-3 influences E2F transcriptional activity

Firstly, the effects of 14-3-3 $\epsilon$  on E2F site-dependent transcription by DP-3/E2F-1 and DP-3/E2F-5 were assessed. 14-3-3 $\epsilon$  was co-expressed with E2F-1 or E2F-5 and either DP-3 $\delta$  or DP-3 $\delta\Delta$ b and the influence on transcriptional activity of the cyclin E promoter, a cellular promoter that is known to be E2F responsive (Botz *et al.*, 1996; Geng *et al.*, 1996) was determined. DP-3 $\beta$ , DP-3 $\delta$  and DP-3 $\delta\Delta$ b clearly co-operated with E2F-5 in activation of the cyclin E promoter (Figure 6.1-A, lanes 2, 4 and 6). Repeatedly, DP-3 $\delta\Delta$ b/ E2F-5 gave a 50% reduction in transcriptional activation compared to both the NLS-containing DP-3 $\delta$  and the non-NLS-containing DP-3 $\beta$ . Coexpression of 14-3-3 $\epsilon$  with E2F-5 and either DP-3 $\beta$  or DP-3 $\delta$  caused a considerable increase in the level of E2F-site dependent transcription (Figure 6.1-A, lanes 3 and 5). However, 14-3-3 $\epsilon$  was unable to significantly increase the activity of the DP-3 $\delta\Delta$ b/E2F-5 heterodimer, supporting the idea that the basic region is required for 14-3-3 to interact with, and exert a functional consequence upon the E2F transcription factor.

Contrasting results were obtained for the E2F-1 heterodimer. Titrating 14-3-3 $\epsilon$  on top of a DP-3 $\delta$ /E2F-1 heterodimer remarkably reduced transcriptional activity of the E2F to almost background levels. However, 14-3-3 $\epsilon$  actually increased the activity of a DP-3 $\delta$ /E2F-1 heterodimer.

In summary, 14-3-3 $\epsilon$  appears to stimulate E2F site-dependent transcriptional activity of an E2F-5 heterodimer, conceivably by providing a growth signal that also results in reducing the activity of an E2F-1 heterodimer. The basic region influences these effects.





#### Figure 6.1

A

The DP component influences 14-3-3 effects on E2F transcriptional activity

The indicated expression vectors were transfected into U2OS cells and the activity of a luciferase reporter, which carries E2F binding sites from the cyclin E promoter, assessed. (A) shows effects on E2F-5 *trans*-activation and (B) shows effects on E2F-1 *trans*-activation.

#### 6-2.2. Transcriptional activity of E2F relates inversely to protein levels

As shown in previous figures (Figure 5.3-B, Janes 1 and 2, and Figure 5.5-A, lanes 1 and 2), DP-3 $\delta\Delta b$  is more abundant than DP-3 $\delta$  in transfected cell extracts, as is E2F-5 when co-expressed with DP-3 $\delta\Delta b$ . However, transcriptional activation of the cyclin E-luciferase reporter construct in cells transfected with the DP-3δΔb/E2F-5 heterodimer was much lower than in cells transfected with the DP-3 $\delta$ /E2F-5 heterodimer (Figure 6.2-A). Increasing levels of wt DP-3 $\delta$  eventually caused a decrease in activation of the reporter, suggesting that this 'squelching' effect may be observed with a lower amount of mutant DP-3. This implied that lower levels of mutant DP-3 might cause an equivalent level of activation seen at greater levels of the wt DP-3 $\delta$ . However, this was not the case: cells transfected with lower amounts of the mutant failed to activate the reporter (results not shown). Figure 6.2-B shows levels of B2F-5 protein after transient transfection in U2OS cells and the corresponding effect on an E2F-site-dependent reporter. E2F-5 alone had little effect on the reporter and low levels of the protein were detected in a western blot (Figure 6.2-B lane 1). Upon addition of DP-38, E2F appears stabilised as heterodimerisation and DNA binding occur and an obvious synergistic increase in transcriptional activity was observed (Figure 6.2-B lane 2). In the presence of DP-3 $\delta\Delta$ b, E2F-5 levels were increasingly stabilised but the transcriptional activity had decreased (Figure 6.2-B lane 3). In accordance with figure 6.2-A, increasing the levels of E2F components resulted in a reduction in transcriptional When 14-3-3 was co-transfected, transcriptional activation increased, activation. however, E2F-5 protein levels decreased (Figure 6.2-C, compare lanes 2 and 3). This suggests that the basic region-dependent association of 14-3-3 with the DP component



#### Figure 6.2

Removing the basic region from DP reduces transactivation of an E2F site dependent reporter although the heterodimer is more abundant

(A)Titration of DP-3 $\delta$  (red line) or DP-3 $\delta\Delta b$  (green line) co-transfected with E2F-5 transactivating a luciferase reporter which carries an E2F binding site from the cyclin E promoter.

(B) and (C) Reporter assays carried out in U2OS cells transfected as indicated. Extracts were also immunoblotted with anti-HA and anti-myc antibodies to show expression of exogenous HA-E2F-5 and 14-3-3myc. of E2F, causes an increase in E2F dependent transcriptional activity by inducing degradation of the heterodimer.

#### 6-2.3. The basic region influences the half-life of DP

When transfected cell extracts corrected for transfection efficiency, were subjected to immunoblotting, DP-3 $\beta\Delta b$  and DP-3 $\delta\Delta b$  protein levels were more abundant than their respective wild-type counterparts (Figure 6.3-A, compare lane 1 with 2 and 3 with 4). It was reasoned that this might be due to an increase in stability of the mutant protein. The degradation of the mutant DP was compared with the wild type DP by western blot analysis of transfected cell extracts after treatment with the translation inhibitor cyclohexamide. While the wild type DP-3 $\delta$  and DP-3 $\beta$  disappeared with a half-life of around 30-60 min (Figures 6.3-B and -D respectively), the mutant DPs remained stable throughout the time-course (Figure 6.3-B and -D). The determination of half-life under these conditions is only a rough estimate since treating cells with cyclohexamide not only blocks the *de novo* synthesis of DP but also the synthesis of proteins required for proteolysis. Nonetheless, the differences in stability, attributed to the basic region were striking.

#### 6-2.4. The basic region of DP influences half-life of E2F

Since the basic region regulates degradation of DP, the role of the basic region within the context of a heterodimer was assessed. When equally transfected cell extracts were subjected to immunoblotting with an anti-HA antibody, E2F-5 levels were



Figure 6.3

The basic region of DP influences protein stability by regulating half-life

(A) Extracts of cells, transfected as indicated, were loaded equally for transfection efficiency and subjected to immunoblotting by an anti-DP-3 peptide antibody to detect exogenous protein.

(B) and (D) Immunoblots of transfected cell extracts. One p100 was transfected with indicated expression plasmids then split into 5 (B) or 4 (D) p60s to maintain equal transfection efficiency between samples. Cells were treated with cyclohexamide for times indicated before harvesting. Note that protein levels between different transfections in (B) and (D) are not comparable.

(C) Relative abundance of DP-3 protein in (B).

(E) Relative abundance of DP-3 protein in (D).

higher when expressed with DP-3 $\beta\Delta b$  and DP-3 $\delta\Delta b$  than when expressed with their wild-type counterparts (Figure 6.4-A, compare lane 2 with 3 and 4 with 5). It appears that the stable basic deletion mutants possess the ability to increase the stability of an associated E2F partner. The degradation of E2F-5, in the presence of the wild type or mutant DP, was assayed by immunoblotting transfected cell extracts after treatment with cyclohexamide. Figure 6.4-C shows quantification of blots 1-4 in figure 6.4-B. Transfection 1 (DP-3 $\delta$  and E2F-5) showed E2F-5 had a half-life of around 100 min while transfection 2 (DP-3 $\delta\Delta b$  and E2F-5) had a very stable E2F-5, even after 2 hours. In the presence of exogenous 14-3-3, the half-life of E2F-5 became shortened to around 60 min when co-expressed with the wild type DP-3 $\delta$  (transfection 3), but remained stable when co-expressed with the mutant DP-3 $\delta$  (transfection 4). These data indicate that DP regulates the degradation of E2F-5 and that 14-3-3 enhances this degradation. Removing the basic region resulted in a more stabilised heterodimer, which no longer responded to 14-3-3.

#### 6-2.5. DP is degraded by the ubiquitin-proteasome mediated pathway

To determine whether DP-3 $\delta$  is degraded by the ubiquitin-proteasome pathway, the abundance of exogenous DP-3 $\delta$  protein in cells that had been exposed to a proteasome-specific inhibitor, LLnL (Rock *et al.*, 1994) was compared with the level of exogenous DP-3 $\delta$  protein in cells that had not been exposed to the inhibitor. COS-7 cells were treated for 2 hours with 50mM LLnL or with the solvent DMSO. As shown in figure 6.5-A, LLnL treatment led to a marked increase in DP-3 $\delta$  abundance (compare lanes 2 and 3) but had little effect on DP-3 $\delta$  $\Delta$ b abundance (compare lanes 5 and 6).





The basic region of DP influences E2F protein stability by regulating half-life

(A) Extracts of cells, transfected as indicated, were loaded equally for transfection efficiency and subjected to immunoblotting by an anti-HA antibody to detect exogenous E2F-5 protein.

(B) Immunoblots of transfected cell extracts. One p100 was transfected with the indicated expression plasmids then split into 3 p60s to maintain equal transfection efficiency between samples. Cells were treated with cyclohexamide for times indicated before harvesting. Positions of HA-E2F-5 and 14-3-3-myc are shown. Note that protein levels between different transfections in (B) are not comparable.

(C) Graph showing rate of degradation of E2F transfections in (B).

Figure 6.5-B investigates the contribution DP-3 $\delta$  makes to 14-3-3-mediated degradation of the heterodimer. In the presence of an E2F partner, DP-3 $\delta$  protein was stabilised (compare figure 6.5-A, lane 1 with figure 6.5-B, lane 4), while DP-3 $\delta\Delta\delta$ b protein levels remained similar to those observed when DP-3 $\delta\Delta\delta$ b was expressed alone (compare figure 6.5-A, lane 4 with figure 6.5-B, lane 10). When 14-3-3 was also expressed (Figure 6.5-B), the relative abundance of DP-3 $\delta$  decreased, whereas DP-3 $\delta\Delta\delta$ b protein levels remained high. This decrease in DP-3 $\delta$  protein abundance, in the presence of 14-3-3 can be attributed to degradation via the ubiquitin-proteasome-mediated pathway since the addition of LLnL restored protein levels (Figure 6.5-B compare lanes 2 and 3). 14-3-3 had little effect on the DP-3 $\delta\Delta$ b heterodimer abundance and so LLnL effects were insignificant.

#### 6-2.6. The basic region is required for cell cycle progression by E2F.

Since it is known that over-expression of E2F promotes cell cycle progression (Beijersbergen *et al.*, 1994; Shan and Lee, 1994) it was important to ascertain the effects of the stabilised E2F heterodimer on cell cycle progression. To investigate this, wild type or mutant DP-3 $\delta$  was transfected alone, or with E2F-5, into the human osteosarcoma cell line U2OS, together with the cell surface marker CD20. Transfected cells were identified with an anti-CD20 antibody, and their DNA content was assayed by flow cytometry analysis to determine cell cycle distribution. The introduction of DP-3 $\delta$  into cells reduced the fraction of G1 cells with concomitant increases in S-phase and G2/M-phase populations (Figure 6.6), suggesting that DP-3 $\delta$  stimulates cell cycle progression. These effects were similar to those reported in previous studies (Wu *et al.*,



#### Figure 6.5

14-3-3 destabilises E2F via the ubiquitin-proteasome mediated degradation pathway

One p100 was transfected with expression plasmids indicated then split into three p60s to maintain equal transfection efficiency between samples. Cells were subjected to either LLnL, DMSO (solvent for LLnL) or untreated for 2 hours before harvesting. All immunoblots are probed with anti-DP-3 peptide antibody.



#### Figure 6.6

The basic region of DP is necessary for efficient progression into S-phase U2OS cells, transfected with the indicated plasmids, were analysed by flow cytometery to determine the cell cycle profiles of CD20 - positive cells. The data are presented as the percentage change of cells in each phase of the cell cycle relative to

CD20 expressing cells transfected with empty expression plasmid.

1996). This effect was also achieved by co-expressing E2F-5 with DP-3 $\delta$ , although alone, E2F-5 had little effect on the cell cycle (data not shown). Expression of DP-3 $\delta\Delta b$  caused a marked difference in cell cycle profile. Although the fraction of cells in S-phase increased slightly, neither a reduction in the G1 fraction or an increase in the G2/M population was observed. This implied that cells were initially stimulated to enter S-phase, but a subsequent increase in G2/M phase population did not follow, therefore as DP-3 $\delta\Delta b$  protein levels rose, cells became stuck in S phase. Cells with high levels of DP are impaired in their ability to exit S-phase. Therefore, degradation of the E2F transcription factor, mediated by 14-3-3 through the basic region of DP3, is necessary before cells with high levels of E2F can progress through the cell cycle.

#### 6-3. Conclusion

The basic region, having been defined in the previous chapter, as influencing the association between 14-3-3 and the E2F transcription factor, was shown to be required for 14-3-3 mediated effects on E2F transcriptional activity, protein turnover, and cell-cycle progression. Removing the basic region from DP resulted in a more stable E2F heterodimer that had lower transcriptional activity, and was less competent in pushing cells through the cell cycle. This may suggest a role for 14-3-3, in a growth promoting pathway, mediating the degradation of E2F via the ubiquitin-proteasome-mediated pathway in S-phase allowing cells to progress into G2/M-phase.

## Chapter 7. The search for the phosphoserine 14-3-3 binding region within DP

#### 7-1. Introduction

This thesis has reported the importance of the basic region in the regulation of the association between 14-3-3 and DP. This region has also been shown to be required for 14-3-3-dependent functional regulation of the E2F transcription factor. It was therefore important to determine the nature of the 14-3-3 binding interaction. Recent findings have suggested that 14-3-3 interactions involve binding to discrete phosphoserine-containing motifs (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). This implies that 14-3-3 could function in a manner analogous to SH2 domains; 14-3-3 binding to a specific phosphoserine motif could result in the assembly of important signalling complexes.

The aim of this chapter was to identify a phosphoserine-containing motif within DP that could mediate an interaction with 14-3-3. To this end, single-site mutagenesis was used to mutate putative phospho-serines to alanines (see materials and methods 2-1.14). Assuming that the basic region was involved in direct association with 14-3-3, serines 61 and 75 appeared plausible phosphorylation targets, which may mediate association with 14-3-3. Ser61 is located within the basic region, and Ser75 lies immediately C terminal to the basic region (Figure 7.1).



#### Figure 7.1

#### Potential 14-3-3 phosphoserine binding motifs in DP-38

Diagrammatic representation of single-site serine to alanine mutations in DP-36; DP-36S61A and DP-36S75A.

Diagram also illustrates the conservation of the mutated serine residues between all the DP family and their positions relative to the E region and the basic region.

#### 7-2. Results

#### 7-2.1. Serines 61 and 75, in DP-38, are not required for heterodimerisation with E2F

Before assessing their 14-3-3-binding status, the DP-3 $\delta$  mutants, DP-3 $\delta$ S<sub>61</sub>A and DP-3 $\delta$ S<sub>75</sub>A, were assayed for their E2F heterodimerisation capabilities. Gel retardation assays, with transfected COS-7 cells extracts, showed that the mutant DP-3 $\delta$ S<sub>75</sub>A was able to bind DNA cooperatively with either E2F-1 or E2F-5 as efficiently as its wild-type counterpart did (Figure 7.2-A compare lane 5 with 3 for E2F-5 complex, and lane 8 with 6 for E2F-1 complex). This demonstrated that Ser75 within DP-3 $\delta$  was dispensable for both heterodimerisation with an E2F partner and for sequence specific DNA binding.

Figure 7.2-B shows that Ser61 within DP-3 $\delta$  is also dispensable for heterodimerisation with an E2F partner. DP-3 $\delta$ S61A, DP-3 $\delta$ , DP-3 $\delta$ \Deltab, E2F-1 and E2F-5 DNAs were in vitro transcribed and translated and immunoprecipitation experiments performed (Figure 7.2-B, lanes 1-6 show the relative expression of E2F and/or DP in the lysates used in the immunoprecipitations in lanes 7-12).





#### Figure 7.2

A

#### Mutation of serine 61 or serine 75 in DP-38 does not alter heterodimerisation of E2F

(A) Whole cell extracts of U2OS cells transfected with the indicated expression plasmids were used in gel shift DNA binding assays with a 32P-labelled oligonucleotide containing an E2F binding site (see section 2-1.11.).

(B) Immunoprecipitation of HA-tagged E2F-5 and DP-3 from 35S-labelled *in vitro* translated rabbit reticulocyte lysates using a polyclonal anti-DP-3 peptide antibody, and proteins detected by autoradiography. The postitions of the labelled proteins are shown.
Results showed that E2F-1 and E2F-5 were both recovered efficiently in anti-DP-3 immunoprecipitates from lysates containing DP-3 $\delta$ , DP-3 $\delta\Delta$ b or DP-3 $\deltaS_{61}A$  suggesting that stable complexes of each DP-3 formed with E2F-1 (Figure 7.2-B, lanes 10, 11 and 12), and E2F-5 (Figure 7.2-B, lanes 6, 7 and 8). Thus, DP-3 $\deltaS_{61}A$  can form stable complexes with E2F-1 and -5 *in vitro*.

# 7-2.2. Ser61 and Ser75 of DP-3δ are not involved in cellular localisation of the E2F transcription factor

Mutating either Ser61 or Ser75 in DP-38 to an alanine residue did not affect the cellular localisation of the DP protein. Like the wild-type protein, both mutants gave a nuclear phenotype when transfected into COS-7 cells with expression detected by indirect immunofluorescence (Figure 7.3-A). When co-expressed with HA-E2F-1, all the DP-3s co-localised with E2F-1 in the nucleus (Figure 7.3-B). Both mutants were also able to transport a cytoplasmic E2F, E2F-5, into the nucleus (Figure 7.3-C). These results demonstrate that mutation of either Ser61 or Ser75 in DP-38 does not affect the cellular transport, of these proteins, or of a heterodimer containing either one of these mutants.



#### Figure 7.3

Serine61 and serine75 of DP-38 are not involved in nucleocytoplasmic targeting of the E2F transcription factor

(A) The intracellular distribution of exogenously expressed DP-3δ, DP-3δS61A or DP-3δS75A was detected by immunofluorescence using a rabbit polyclonal anti-DP-3 antibody.

(B) The intracellular distribution of exogenously co-expressed DP-3 $\delta$  (i and ii), DP-3 $\delta$ S61A (iii and iv) or DP-3 $\delta$ S75A (v and vi) with HA-E2F-1 was detected by immunofluorescence using a rabbit polyclonal anti-DP-3 antibody (i, iii and v) and a mouse monoclonal anti-HA antibody (ii, iv and vi).

(C) The intracellular distribution of exogenously co-expressed DP-3 $\delta$  (i and ii), DP-3 $\delta$ S61A (iii and iv) or DP-3 $\delta$ S75A (v and vi) with HA-E2F-5 was detected by immunofluorescence using a rabbit polyclonal anti-DP-3 antibody (i, iii and v) and a mouse monoclonal anti-HA antibody (ii, iv and vi).





### Figure 7.4

Mutation of serine 61 or serine 75 of DP-38 to alanine does not block 14-3-3 effects on E2F transcriptional activity

The indicated expression vectors were transfected into U2OS cells and the activity of a luciferase reporter, which carries E2F binding sites from the cyclin E promoter, assessed.

# 7-2.3. 14-3-3 effects on the transcriptional activity of E2F are not mediated through Serine61 or Serinc75 of DP-38

As shown previously (Figure 6.1), 14-3-3 stimulated E2F-dependent transcriptional activity of an E2F-5 heterodimer (Figure 7.4-A compare lanes 2 and 3; Figure 7.4-B compare lanes 2 and 3). Transcriptional activity of the E2F-5 heterodimer with either the Ser75 or the Ser61 mutant DP-3 was similar to that of the wild-type protein (Figure 7.3-A compare lanes 2 and 4; Figure 7.4-B compare lanes 2 and 6). Increased activation of the heterodimer by 14-3-3 was also unaffected by the mutations in DP-3 $\delta$  (Figure 7.4-A compare lanes 3 and 5; Figure 7.4-B compare lanes 3 and 7). Figure 7.4-B, lanes 4 and 5, also show the transcriptional activity of the basic deletion mutant DP-3 $\delta$  And the effect of 14-3-3 on activation of this heterodimer for comparison with the other mutants. These results suggest that neither Ser61 nor Ser75 are required for 14-3-3 effects on E2F transcription factor activity.

### 7-3. Conclusion

As the basic region has been identified as a critical component in the regulation of the interaction between 14-3-3 and DP, it was reasoned that a phosphoserinecontaining 14-3-3 binding motif may be located in that region. However a mutant DP-38 incapable of phosphorylation at either Ser61 within the basic region, or Ser75 just out with the basic region, did not abolish 14-3-3-dependent effects on E2F transcriptional activity. This suggests that the association of 14-3-3 with DP is not dependent upon phosphorylation at either of these two sites.

### Chapter 8. Discussion

### 8-1. Nuclear accumulation of the E2F heterodimer

The E2F heterodimer is known to be regulated on a number of different levels, such as binding and transcriptional repression by pocket proteins (Flemington *et al.*, 1993), phosphorylation by cdk complexes (Krek *et al.*, 1994, 1995) and transcriptional activation by the MDM2 oncoprotein (Martin *et al.*, 1995). Here, I have shown that an additional mechanism of control in regulating the activity of E2F is mediated at the level of intracellular location. Specifically, these data show that nuclear accumulation of the E2F/DP heterodimer is regulated by an alternatively spliced nuclear localisation signal in the DP component and also through subunit composition of the transcription factor (Figure 8.1). Since transcription factors exert their effects on gene expression in the nucleus, control of intracellular location is an important regulatory mechanism.

By investigating their intracellular distribution, it was possible to divide the E2F family into two distinct classes according to their ability to accumulate in nuclei. The first group, exemplified by E2F-1, underwent efficient nuclear accumulation whereas the second, which includes E2F-4 and -5, failed to do so and remained, for the most part, in the cytoplasm. In support of these findings, a NLS has been identified in E2F-1, -2 and -3 in the N-terminal region, a domain absent from E2F-4 and -5 (Krck *et al.*, 1994). The non-NLS containing E2F family members therefore rely on other proteins to provide NLS activity. These observations are in accordance with previous studies indicating that the E2F family can be subdivided into two functionally and structurally



### Figure 8.1

### Three pathways for the nuclear accumulation of E2F

Nuclear accumulation of E2F can occur throught three distinct mechanisms dependent upon either the alternatively spliced E region in DP (i), or the E2F subunit (ii), or an association with a pocket protein (iii)

The pocket protein mediated uptake allows a dominantly repressive form of E2F to enter the nucleus and repress transcription.

During cell cycle progression, the activation of Cdks enables E2F to be released. Where the NLS is supplied by E2F or DP, the heterodimer presumably enters the nucleus in a transcriptionally active state. distinct groups (Krek *et al.*, 1994; Lukas *et al.*, 1996; Mann and Jones, 1996; Zhu *et al.*, 1993). It is conceivable that the mechanism of nuclear accumulation of the two E2F sub-families may account for some of their distinct biological properties.

The DP family, too, can be divided into two sub-families based on their cellular location. Alternative splicing events in the RNA of the DP-3 gene produce four distinct DP-3 proteins. One of these processing events determines whether the E region is incorporated into the protein. DP-3 proteins that possess the E region, DP-3 $\alpha$  and DP-3 $\delta$ , were found in the nucleus when expressed in COS-7 cells, whereas those missing the E region, DP-3 $\beta$  and DP-3 $\gamma$ , were found in the cytoplasm of COS-7 cells. A comparison of the E region of DP-3 with the same region in DP-1 indicated that DP-1 lacks a domain analogous to E and, as expected, the protein was found in the cytoplasm when expressed in COS-7 cells. The conclusion here is that the E region influences the cellular location of the DP family members.

Although these data establish a dependence upon the E region for nuclear accumulation, they do not distinguish between the possibilities that the E region regulates nuclear entry or export. For example, it is possible that the E- variants can enter and exit nuclei, and that the presence of the E region impedes nuclear export, resulting in a net nuclear accumulation, possibly due to the E region being involved in tethering the DP to an insoluble nuclear structure.

DP-1 was observed to be cytoplasmic when expressed in COS-7 cells. However, co-expression of an NLS-containing E2F was sufficient to promote nuclear accumulation of all E- DP variants. This provides an explanation for the small amount of E- DP found in the cytoplasm of transfected COS-7 cells, as endogenous E2F would be able to transport an E- DP into the nucleus. It may also explain why endogenous DP-1 is nuclear.

This study has also shown that E2Fs devoid of an NLS, E2F-4 and E2F-5, are subject to two distinct mechanisms which regulate the levels of nuclear E2F, one in which the NLS is provided by the DP partner and the other where the NLS is supplied by the physical association of a pocket protein. E2F-4 and E2F-5 can accumulate in the nucleus through association with DP-3 $\alpha$  or DP-3 $\delta$  but not with DP-1, DP-3 $\beta$ , or DP-3 $\gamma$ , indicating that in the context of E2F, an E+ DP partner can provide the NLS. But, most of the E2F-4 and E2F-5 present in cell extracts is bound to E- DP-1 (Bandara *et al.*, 1993,1994; Girling *et al.*, 1993; Wu *et al.*, 1995). These studies have shown that this heterodimer is cytoplasmic when expressed in COS-7 cells. However, upon co-expression with the pocket protein p107, the heterodimer underwent nuclear accumulation. Surprisingly, co-expression of E1a did not alter the distribution of the E2F heterodimer even though the pocket protein-E2F complex was dissociated. Considering that E1a is an exclusively nuclear protein, this suggests that E1a may displace the pocket protein from the heterodimer after transporting the heterodimer into the nucleus. This would presumably result in release of a transcriptionally active E2F.

Subsequently, Allen *et al.*, (1997) found that the mechanism of nuclear accumulation had a profound influence on the growth-regulating properties of E2F; when nuclear accumulation was E+DP mediated, cells received a growth stimulus, whereas when it was mediated by the pocket protein complex, cell cycle progression could not ensue and cells accumulated in an arrested state. This suggests that a non-

NLS-containing heterodimer, transported into the nucleus by a pocket protein is a transcriptionally inactive and dominantly repressive form of E2F, which maintains genes regulated by E2F binding sites in a transcriptionally inactive state. In conclusion, the mechanism of nuclear accumulation dictates the properties of the E2F heterodimer and the functional consequences on the cell cycle.

# 8-2. An alternatively spliced bipartite nuclear localisation signal in the E2F transcription factor

Chapter 3 identified a sequence of sixteen amino acid residues, the E region, which was required for efficient nuclear accumulation of DP. Although there was some similarity between the E region and the single-site SV40 Tag NLS, the functional properties of the E region did not satisfy the requirements for this type of NLS: Although necessary for nuclear accumulation of DP-3, the E region, by itself, was not sufficient to target a protein for nuclear accumulation. An additional basic region, located immediately C-terminal to the E region, was necessary to transfer a nuclear phenotype onto a non-nuclear protein.

Comparison of this sequence to previously defined NLSs suggested a closer resemblance to a bi-partite NLS rather than the single-site SV40 Tag- like NLS. Indeed, deletion of the basic region from an E+ DP resulted in a failure in nuclear accumulation, indicating that together, the E region and the basic region constitute a bi-partite NLS in DP. As expected, removal of the basic region from DP also abolished its ability to transport a non-NLS-containing E2F into the nucleus.

Unexpectedly, however, heterodimerisation of the basic deletion mutant DP with a NLS-containing E2F was insufficient to efficiently transport the DP into the nucleus. The data presented here showed that only 50% of co-transfected cells demonstrated colocalisation of the heterodimer in the nucleus, with the other 50% of cells exhibiting nuclear E2F and cytoplasmic DP. This indicates that in 50% of these co-transfected cells, the basic region was required for nuclear co-localisation, i.e. under certain circumstances, the basic region is involved either in nuclear retention of the DP after transport into the nucleus via E2F, or in regulating heterodimerisation in the cytoplasm, or in inhibiting export (Figure 8.2). It should be borne in mind however, that the basic deletion mutant could still heterodimerise with E2F and bind DNA *in vitro*, suggesting that these results propose a physiological regulatory mechanism involving the basic region, and not a mutation-derived irregularity in E2Fs ability to act as a transcription factor.

The theory that the basic region is involved in regulating nuclear accumulation of an E2F heterodimer was reinforced by experiments showing that the basic region in cytoplasmic DPs was still necessary for nuclear co-localisation with a NLS-containing E2F. Surprisingly, though, co-expression of p107 was sufficient to transport the basic deletion mutant DP into the nucleus with E2F-1, implying that downstream E2F functions are regulated differently after pocket-protein mediated nuclear transport compared with free E2F nuclear transport.

Since the COS-7 cell line is transformed with SV40 large T antigen these transfections were also carried out in the parental cell line CV-1. T antigen was in fact



### Figure 8.2

### Possible roles for the basic region of DP

(i) The basic region is required for the DP to be retained in the nucleus. The mutant DP $\Delta b$  will therefore be returned to the cytoplasm.

(ii) The basic region is required for heterodimerisation of E2F in the cytoplasm prior to nuclear translocation. The mutant fails to heterodimerise correctly and is therefore left in the cytoplasm whilst the E2F translocates to the nucleus.

(iii) The basic region is involved in maintaining heterodimerisation of E2F in the nucleus. The mutant DP $\Delta b$  dissociates from the E2F partner and exits the nucleus.

(iv) The basic region is involved in the negative regulation of export of DP. Removal of the basic region results in the export of DP. implicated in the phenotype of the E2F-1/ DP-3 $\Delta$ b since co-localisation of E2F-1 with basic deletion mutant DPs occurred in the nucleus of all double-stained CV-1 cells.

A major role of T antigen in transformation is in sequestering pocket proteins from E2F complexes to release free active E2F transcription factor. Considering that over-expression of the pocket protein p107 in COS-7 cells reverts the phenotype of the mutant heterodimer to that of the wild type heterodimer, and the possibility that p107 may be titrating out T antigen implies a role for pocket proteins in a pathway resulting in nuclear accumulation of E2F.

Data were presented here to show that the E1a-mediated release of free E2F from E2F/pocket protein complexes resulted in nuclear retention of the heterodimer. Perhaps in 50% of the co-transfected cells, pocket proteins were bound to the E2F-1/ DP-3&Ab heterodimer, and once in the nucleus, T antigen released the free heterodimer from the pocket protein. The observed phenotype could then be explained if the basic region was involved in tethering E2F in the nucleus. As only the DP component located to the cytoplasm, the basic region, in this scenario, must also be involved in maintaining an association between the DP and the E2F protein in the nucleus. Presumably then, if this were the case, co-expression of E1a with E2F-1/ DP-3&Ab in CV-1 cells would cause release of the DP component into the cytoplasm. On the other hand, as p107 can titrate out T antigen, another unidentified function of T antigen, perhaps unrelated to pocket proteins, may have become abolished.

Alternatively, these results may show that two pathways exist for nuclear translocation. 50% of cells utilising a path that does not require the basic region and the

other 50% utilising a pathway that relies on the basic region for efficient nuclear accumulation. One of these pathways is a target of T antigen, illuminating possible functions for the basic region in:

(i) Heterodimerisation of DP with E2F in the cytoplasm. T antigen prevents heterodimerisation therefore destroying a nuclear uptake vehicle for the non-NLS-containing DP.

(ii) Heterodimerisation of DP with E2F in the nucleus. T antigen causes the dissociation of the heterodimer once in the nucleus and the DP $\Delta$ b is returned to the cytoplasm.

(iii) Nuclear retention or export. T antigen causes release of DP from the heterodimer and DP $\Delta b$  is returned to the cytoplasm due to removal of the retention signal, or T antigen causes export of the mutant DP once dissociated from an E2F partner.

Pocket proteins mediate the other pathway of nuclear transport, which is not reliant on the basic deletion for import, nuclear retention or export. This alternative nuclear import pathway for the mutant heterodimer is not affected by the presence of T antigen.

In conclusion, these data support the theory that the basic region is involved in the nuclear accumulation of the E2F heterodimer. The basic deletion mutants behave as non-NLS containing components of the E2F transcription factor relying on heterodimerisation with a NLS-containing partner for nuclear accumulation. Assays in SV40 T antigen transformed cells have shown that the basic region plays a role in colocalisation of the E2F heterodimer in the nucleus even when an NLS is supplied by the E2F component, but not if the NLS is supplied *in trans* by a pocket protein

### 8-3. 14-3-3 associates with the E2F transcription factor

The phenotypic characteristics of the basic deletion DP strongly suggested that the basic region was involved in an interaction between DP and another protein. Therefore, a yeast-two hybrid interaction trap assay was used to conduct an *in vivo* search for a gene encoding a protein that interacts with DP-38 but not with DP-38\Deltab.

The epsilon isoform of the 14-3-3 family of signalling molecules was identified as a DP-3 $\delta$ -binding partner, which required the presence of the basic region for the interaction in yeast. This interaction was then confirmed using several different methods. GST-14-3-3 $\epsilon$  was shown to bind to E2F-5 only when DP-3 $\delta$  was also present; no binding was observed with DP-3 $\delta\Delta$ b. When immuno-blotting these pulled-down extracts for DP-3, the amount of DP-3 $\delta$  that was retained compared to the input level was far greater than the amount of DP-3 $\delta\Delta$ b retained. A mammalian two-hybrid interaction assay confirmed the association in mammalian cells. A far greater association was evident between 14-3-3 and DP-3 $\delta$  than was seen between 14-3-3 and DP-1, indicating that specificity of the interaction occurs between members of the DP family. This is presumably due to sequence variations around the basic region in DP-1. Immunoprecipitation assays showed that an interaction between 14-3-3 and DP-3 $\delta$  required the basic region. A semi-endogenous *in vivo* IP showed that endogenous 14-3-3 co-immunoprecipitated with exogenously expressed DP-3 $\delta$  (The anti-DP-3 antibodies were unable to detect endogenous DP-3). Finally, 14-3-3 was shown to accumulate in the nucleus when expressed with E2F-5/DP-3 $\delta$ . When expressed alone, 14-3-3 is a cytoplasmic protein; therefore these data demonstrate that E2F can drive 14-3-3 into the nucleus, a result consistent with 14-3-3 having a physiological functional consequence on E2F.

At present, conventional 14-3-3 binding motifs, such as RSXpSXP (pS indicates a critical phosphoserine), have been identified in most 14-3-3 binding proteins (Yaffe *et al.*, 1997). However, there is no such consensus motif in DP. Crystal structure analysis predicts that 14-3-3 forms an amphipathic groove and its binding partners would possess an amphipathic helix to fit into the groove (Liu *et al.*, 1995; Xiao *et al.*, 1995). Therefore, it may be possible that removal of the basic region is altering the structure of DP-3 sufficiently enough to no longer be able to fit into the amphipathic groove.

Recently, another binding motif has been identified that consists of a serine cluster (Du *et al.*, 1996). Figure 7.1 showed that the basic region of the DP proteins is within a serine-rich region, supporting the idea that the basic region is involved in a direct interaction with 14-3-3. Mutation of more than one serine has been shown to be needed to disrupt the interaction of HDAC4 with 14-3-3 (Grozinger and Schreiber, 2000). HDAC4 contains three 14-3-3 binding sites and mutation of individual or two 14-3-3 binding sites was not sufficient to abrogate binding of 14-3-3, but mutation of all three serine residues to alanine abolished binding. Bearing this in mind, the negative

results of the single-site *in vitro* mutagenesis chapter may not rule out phospho-binding at these sites. Perhaps 14-3-3-dependent effects upon E2F are not abolished when only one serine is mutated to alanine. Moreover, the lack of phosphorylation at the mutated sites may be overcome by the presence of negatively charged residues surrounding the basic region. Testing the binding of DP-3 $\delta$  to 14-3-3 under alkaline phosphatase treatment would be useful in determining whether the interaction is phosphorylation dependent. Nonetheless, several observations have led to the notion that 14-3-3 is also capable of interacting with unphosphorylated ligands, for example Raf-1 (Muslin *et al.*, 1996).

### 8-4. Functional consequences of the 14-3-3-E2F interaction

The 14-3-3 proteins, which constitute a highly conserved family of homo- and heterodimeric molecules, associate with a number of different signalling molecules. They have been proposed to be important in controlling intracellular signalling pathways by acting as molecular scaffolds or platforms that bring together signalling molecules. Considering the broad range of partners for 14-3-3, a specific cellular function for these proteins has not been defined; rather they have been suggested to act as general biochemical regulators. With this in mind, this work set out to realize what aspect of E2F function 14-3-3 modulates

This work has shown that 14-3-3 has the ability to regulate E2F site-dependent transcription. Co-expression of 14-3-3 with E2F5/ DP-3 $\beta$  or E2F5/ DP-3 $\delta$  heterodimers repeatedly caused a three to eight-fold increase in the level of E2F site-dependent

transcription. This effect was not observed in heterodimers containing the basic deletion mutant DP, supporting the previous data showing that the basic region was required for an interaction with 14-3-3. These data also demonstrated that the failure of the mutant beterodimer to respond to 14-3-3 was not due to the lack of a NLS in the heterodimer as both the cytoplasmic heterodimer E2F-5/ DP-3 $\beta$ , and the nuclear heterodimer E2F-5/ DP-3 $\delta$ , were responsive to 14-3-3.

Strikingly, opposite effects were observed for 14-3-3 on an E2F-1 heterodimer. 14-3-3 diminished the transcriptional activity of an E2F-1/DP-38 heterodimer almost to background levels but increased the activity of an E2F-1/ DP-3δΔb heterodimer. These data suggest that 14-3-3 effects on transcription are heterodimer specific. This result may correlate with differences in the functional properties of E2F-1 and E2F-5 (Wang et al 2000). Although both proteins induce proliferation in transgenic models, unlike E2F-1, E2F-5 can not induce apoptosis, and this correlates with the differential abilities of these two species to stimulate p19ARF expression in vivo. Unlike E2F-1 transgenic mice, E2F-4 (and presumably also E2F-5) transgenic mice develop skin tumours with a decreased latency and increased incidence compared to controls. These findings demonstrate that while the effects of E2F-1 and E2F-5 on cell proliferation in vivo are similar, their apoptotic and oncogenes properties are quite different. Assumedly, 14-3-3 provides a growth stimulus by increasing the activity of the E2F-5 heterodimer and concurrently shutting off E2F-1-dependent transcription in order to prevent an apoptotic response.

Surprisingly, higher transcriptional activity was not the result of an increase in heterodimer protein levels. Although the E2F-5/ DP-3 $\delta\Delta$ b heterodimer failed to

activate transcription as highly as the E2F-5/ DP-38 heterodimer did, western blot analysis of the extracts used for the reporter assay showed that removal of the basic region resulted in higher expression levels of E2F-5 and DP proteins. Consistent with this paradox, increased transcriptional activity of E2F-5/DP caused by the expression of 14-3-3 also resulted in a decrease in heterodimer protein levels. These results leads to the conclusion that the binding of DP-38 to E2F-5 causes stabilisation of both protein levels, presumably due to DNA binding, removal of the basic region results in greater stabilisation of the protein levels, and 14-3-3 causes an increase in transcriptional activity of the wild type heterodimer whilst decreasing the heterodimer protein levels.

The greater abundance of protein levels of the mutant heterodimer was shown to be due to an increase in the half-life of both components of the heterodimer. Removing the basic region form both the nuclear DP-3 $\delta$  and the cytoplasmic DP-3 $\beta$  resulted in an increase in the half-life of the DP protein. This demonstrates that the increase in stability was not due to the cytoplasmic location of the mutant DP. Also, the mutant DP had the ability to confer an increase in stabilisation onto an E2F-5 partner. An increase in the half-life of E2F-5 was observed when co-expressed with a basic region-deleted DP-3 compared to when expressed with the wild type DP-3 partner.

Addition of 14-3-3 to the wild type heterodimer resulted in a decrease in halflife of E2F-5 consistent with the theory that 14-3-3 promotes the degradation of the E2F transcription factor. As the 14-3-3 was unable to affect the half-life of E2F-5 in a heterodimer with the mutant DP-3, the basic region must be necessary for 14-3-3 to exert its degradation effects on E2F-5.

Using a proteasome-specific inhibitor, LLnL, showed that DP-38 was subject to degradation via the ubiquitin mediated pathway. Proteins targeted for degradation via this pathway become stabilised in the presence of the inhibitor, allowing quantitative analysis of protein stability changes. Protein levels of the basic deletion mutant DP-3 did not increase in the presence of the inhibitor indicating that it was not subject to degradation. As expected, protein levels of DP-3 $\delta$  rose when expressed with an E2F partner, however, when co-expressed with 14-3-3, the relative abundance of DP-38 protein decreased. This effect of 14-3-3 was shown to be dependent upon the presence of the basic region as the mutant heterodimer protein levels failed to increase in the presence of the proteasome inhibitor. These results indicate that stabilisation of E2F components as a result of heterodimerisation is due to a decrease in ubiquitinproteasome-mediated degradation. They also indicate that the association of the heterodimer with 14-3-3 causes an up-regulation of ubiquitin-proteasome-mediated degradation of the heterodimer. Since the mutant DP3δΔb stability did not change, whether expressed alone, as a heterodimer, or in the presence of 14-3-3, indicates that the basic region is instrumental in 14-3-3-mediated targeting of the heterodimer to the ubiquitin-proteasome-mediated degradation pathway.

Protein degradation within cells is a very tightly regulated multi-step process, believed to be initiated by site-directed ubiquitination of target proteins. Ubiquitinmediated proteolysis is a prominent mechanism regulating transcription factor function that keeps the intracellular levels of these proteins low and responsive to environmental stimuli, e.g. Jun (Treicr *et al.*, 1994), p53 (Chowdary *et al.*, 1994), and Myc (Salghetti *et al.*, 1999). Despite the importance of this process, however, the mechanisms governing substrate recognition are poorly understood. How 14-3-3 targets E2F for degradation is an important question that warrants further investigation. In accordance with the theory that 14-3-3 targets E2F for degradation, it has been shown that nitrate reductase in spinach leaves is also targeted for degradation after associating with 14-3-3 (Weiner and Kaiser, 1999).

A major draw back of this work has been the inability to measure the half-life of the endogenous E2F heterodimers. Also to be considered is whether the E2Fs should be less stable in cell lines lacking members of the pRB family, and whether the E2Fs have shorter half-lives in the S phase of the cell cycle, when the pRB family members are phosphorylated at high stoichiometry.

It is not uncommon for degradation signals to overlap with other functional domains within the target protein. Interestingly, Salghetti *et al.*, (2000) have found that protein destruction elements in transcription factors overlap with transcriptional activation domains. This suggests that cells have evolved a mechanism whereby potent transcriptional activators would be destroyed. Such regulation may be important for rapid reprogramming of transcriptional patterns, to limit activation by any one single activator or to prevent accumulation of excess activator that could lead to 'squelching' of the basal machinery. Activators can stimulate transcription by recruiting components of the ubiquitin-proteasome pathway that subsequently modify histones, basal factors, and ultimately the activators themselves (Salghetti *et al.*, 2000). The basic region, although not part of a transactivation domain, is very close to the DNA binding domain. Although mutation of the basic region did not affect DNA binding or heterodimerisation of E2F, transcriptional activity of the mutant heterodimer was

reduced 50% compared to the wild type heterodimer, signifying a requirement for this region for efficient activation.

Perhaps of significance to these results is the discovery that histone deacetylases have been found to associate with 14-3-3 proteins. HDAC4 and HDAC5 associate with 14-3-3 resulting in sequestration of these proteins in the cytoplasm (Grozinger and Schreiber, 2000). This prevents HDACs translocating to the nucleus where they repress gene expression. This role for 14-3-3 proteins may explain the increase in transcriptional activity if 14-3-3 is brought into the nucleus with DP-3 then transports the HDAC out of the nucleus (Figure 8.3).

The 14-3-3-mediated degradation of E2F was shown to be necessary for cell cycle progression. The E2F/DP-3δ heterodimer caused an increase in S phase with a concomitant decrease in G1, as would be expected from a cell proliferation inducer. Removal of the basic region disrupted the ability of the heterodimer to promote cell cycle progression and cells became arrested in S-phase. This suggests that cells with high levels of DP protein are impaired in their ability to exit S phase. Degradation of the E2F transcription factor, mediated by 14-3-3 through the basic region of DP3, is necessary before cells with high levels of E2F can progress through the cell cycle. This provides an explanation for the apparently paradoxical reporter assay results. High levels of the heterodimer cause cells to arrest and possibly apoptose, this would result in a lowering in E2F-site dependent transcription. Allowing cells to carry on cycling restores E2F transcriptional activity.



### Figure 8.3

# Model showing how 14-3-3 may promote degradation and increase transcriptional activation of E2F

14-3-3 and E2F co-localise to the nucleus. 14-3-3 transports HDAC to the cytoplasm, thus removing transcriptional repression. Excess E2F is targeted for ubiquitin-proteasome mediated degradation to prevent squelching of transcription machinery.

The down-regulation of E2F activity in the S phase of the cell cycle has been shown to be essential in order to allow cells to enter G2 (Krek et al 1995). One possible explanation for this is that certain E2F regulated genes must be down-regulated in order for cells to enter G2, and the constitutive activation of such genes would be deleterious for the cell. In agreement with such a suggestion is the observation that deregulated expression of E2F-1, -2, -3 or nuclearly expressed E2F-4 all induce S phase followed by apoptosis (Helin 1998). The inactivation of E2F-1, -2 and -3, but not E2F-4 and -5, DNA binding activity can be achieved by cyclin A dependent kinase activity (Krek et al., 1995), and this regulatory mechanism is therefore sufficient for down regulating E2F activity generated by E2F-1, -2 and -3 but not by E2F-4 and -5. Expression of E2F-1 occurs late in G1, reaching its maximum levels as cells enter S phase. In contrast, E2F-4 and E2F-5 mRNA is constitutively expressed throughout the cell cycle with up to three-fold higher levels in mid G1, which fall again by S phase (Sardet *et al.*, 1995). Since E2F-4 and E2F-5 do not associate with cyclin A, they must rely on another method for inactivation, 14-3-3-directed ubiquitin-mediated degradation is one such possibility.

14-3-3 proteins often regulate intracellular localisation of their binding partners. For example, phosphorylation of Cdc25 by Chk1 kinase creates a binding site in Cdc25 for 14-3-3 (Peng *et al.*, 1997; Sanchez *et al.*, 1997). The subsequent 14-3-3 binding to Cdc25 markedly reduces the nuclear import rate of Cdc25, allowing nuclear export mediated by an NES present in the N-terminus of Cdc25 to predominate (Yang *et al.*, 1999). Since Cdc25 has a functional NLS that lies adjacent to the site of 14-3-3 binding, 14-3-3 binding might sterically block access of Cdc25 to the nuclear import machinery (Kumagai and Dunphy, 1999; Yang *et al.*, 1999). Although the basic region is involved both in nuclear import, and 14-3-3 association, 14-3-3 does not prevent nuclear accumulation of the heterodimer as is reported for some other 14-3-3 interactions. 14-3-3 did not appear to have any influence on the cellular location of DP-3. In fact, co-expression of the heterodimer with 14-3-3 resulted in nuclear translocation of the 14-3-3 protein. This indicates that either 14-3-3 does not bind directly with the basic region, or both 14-3-3 and the nuclear import machinery can associate with the basic region simultaneously. Recently Seimiya *et al.*, (2000) have reported 14-3-3 mediated nuclear accumulation of hTERT telomerase, thus supporting the idea that 14-3-3 can function in the nucleus as well as in the cytoplasm.

What triggers the association of 14-3-3 with DP-3 is unknown. Many 14-3-3 interactions are induced by extra-cellular stimuli, e.g. DNA damage (Hermeking *et al.*, 1997) or platelet-derived growth factor (Autieri *et al.*, 1996). More efficient binding between E2F and 14-3-3 may require the correct conditions.

### 8-5. Overall conclusions

To fully understand the role of each individual member of the E2F and DP family members in gene expression and cell cycle control, it will be necessary to document which factors associate with one another in the cytoplasm under specific growth conditions, which of the resulting E2F/ DP complexes enter the nucleus and interact with specific subsets of cellular promoters, and how the distribution of each of these distinct complexes changes during the cell cycle. This study aims to move closer to realising these questions by defining a bipartite NLS in DP, which will direct nuclear

accumulation of a non-NLS containing E2F partner. The basic region of the bipartite NLS is also involved in directing the nuclear accumulation of an NLS-containing E2F heterodimerisation partner. This study has also identified 14-3-3 as a regulatory protein that can influence E2F activity. However, in contrast to the pRB family, where the association with E2F is direct and occurs through the C-terminal region in each E2F family member, the interaction of 14-3-3 depends upon the DP family member. This view is supported by a variety of data presented in this study, including the isolation of 14-3-3 with a DP 'bait' in the yeast two-hybrid system, the *in vitro* physical interaction between 14-3-3 and DP-3, the mammalian two hybrid assays, the DP-dependent regulation of E2F activity by 14-3-3 and the DP-directed intracellular localisation of 14-3-3. Moreover the analysis of a mutant derivative of DP-3, lacking the ability to form an interaction with 14-3-3, has defined a region in DP necessary for 14-3-3 directed ubiquitin-proteasome mediated degradation. This pathway has been proposed as a means of lowering abnormally high levels of E2F to allow S phase progression.

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