Rab11-FIP4, its Small GTPase Binding Partners and Their Roles in Cytokinesis

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By

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Abstract

Rab11-FIP4 is a member of the Rab11-Family of Interacting Proteins (FIPs) and is also known as arfophilin 2. It has previously been shown to bind members of two families of small GTPases, namely Rab11 from the Rab family and ARF5 from the ADP-Ribosylation Factor (ARF) family. Rab11-FIP4 has also been shown to have a partially overlapping localisation with the Rab11 recycling endosome. Its function remains undetermined.

Here, three aspects of Rab11-FIP4 are investigated. Firstly, a binding study confirms that FIP4 binds to Rab11 and ARF5 and identifies ARF6 and Rab5 as additional binding partners. It also shows that Rab11-FIP4 can simultaneously bind a Rab and ARF family member. Secondly, the localisation of Rab11-FIP4 and its co-localisation with its binding partners is examined in interphase cells and also in cells at various stages of cell division. This shows that Rab11-FIP4 and its binding partners show various degrees of overlap in interphase endosomes and particularly that they all show striking and similar localisations throughout mitosis and cytokinesis.

The third part of this study examines the possible functional roles which Rab11-FIP4, Rab11 and ARF5 may play in cytokinesis. Although a functional role for Rab11-FIP4 is not established, it is shown that both Rab11 and ARF5 play an important role in cytokinesis.

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Authors Declaration

The research reported within this thesis is my own work, except where otherwise stated, and has not been submitted for any other degree.

Andrew Brian Fielding.

Abbreviations

ADP	Adenosine diophosphate
APP1	ArfGAP-putative, Pix-interacting, paxillin-interacting protein 1
ARFs	ADP-ribosylation factors
BFA	Brefeldin A
cdk	Cyclin-dependant kinase
CHO1	Chinese hamster ovary 1
COOL	cloned out of library
COPI	coat protein I
DAPI	4-6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DTT	dithiothreitol
ECT2	epithelial cell transforming gene 2
EDTA	ethylenediaminetetraacetic acid
EEA1	early endosome antigen 1
Eferin	EF-hands-containing Rab11-interacting protein
EGTA	ethyleneglycol-bis(2-aminoethyl) N,N,N&',N&',-tetraacetic acid)
ER	Endoplasmic Reticulum
FCS	fetal calf serum
FIP	Rab11 Family Interacting Proteins
GAPs	GTPase activating proteins
GDFs	GDI-displacement factors
GDIs	GDP dissociation inhibitors
GDP	Guanosine diphosphate.
GEFs	guanine-nucleotide exchange factors

GFP	green fluorescent protein
GIT	G-protein-coupled receptor kinase interacting protein
GLUT 4	Glucose Transporter 4
GST	glutathione *S*-transferase
GTP	guanosine 5'-triphosphate
GTPase	guanosine 5'-triphosphatase
HA	hemagglutinin
HeLa	Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid)
IgA	Immunoglobulin A
INCEMP	Inner Centromere Protein
IPTG	isopropyl b-D-thiogalactoside
MDCK	Madin-Darby canine kidney
MEM	Minimal Essential Media
MKlp1	Mitoic kinesin like protein 1
MKlp2	Mitoic kinesin like protein 2
Nuf	Nuclear-fallout
OD	Optical Density
PAG	paxillin-associated protein with ARFGAP activity
PAK	p21-activated serine-threonine kinase
PBS	Phosphate Buffered Saline
PBS1	paxillin-binding sub-domain 1
PBS2	paxillin-binding sub-domain 2
PE	phosphatidylethanolamine
PIX	PAK-interacting exchange factor
PKL	paxillin kinase linker protein

PLK1	Polo-like-kinase 1
PMSF	phenylmethylsulfonyl fluoride
PRA1	prenylated rab-acceptor 1
PRC1	protein-regulating cytokinesis 1
Rab11-FIP	Rab11 Family Interacting Proteins
Rid	Rho-inhibitory domain
RNA	ribonucleic acid
RNAi	RNA interference
ROCK	Rho Kinase
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
SNAP	synaptosomal-associated protein
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TEMED	Tetramethylethylenediamine
Tfn	transferrin
VAMP	vesicle-associated membrane protein
Yips	Ypt-interacting proteins

Chapter 1

Introduction

1.1.1 Cytokinesis

Cell division comprises of two processes, namely mitosis and cytokinesis. Mitosis is the division of the cell's chromosomes into spatially distinct areas of the cell. This is generally followed by cytokinesis, which is the process by which the mother cell divides itself into two daughter cells. Although these can be described as two distinct processes, it is clear that several aspects of mitosis overlap with cytokinesis. For example, it is well documented that the mitotic spindle, an array of microtubules, is required for both mitosis and cytokinesis. Below is a brief description of cell division described using the traditional terminology for the various stages of this process. The distinctions between mitosis and cytokinesis are highlighted.

Prophase

This is the first stage of cell division, where the chromosomes start to condense and the nuclear envelope is deconstructed. At this stage the centrosome also divides into two and the two new centrosomes move to opposing side of the nucleus. The mitotic spindle, an array of microtubules radiating from each of the centrosomes has begun to be constructed.

Metaphase

At this stage, the chromosomes are aligned along the centre of the cell, equidistant from each of the centrosomes and the plus ends of microtubules attach themselves to the kinetochores of the chromosomes.

Anaphase

At this stage the chromosomes are pulled apart due to shortening of the microtubules, probably from both the plus and minus ends. Also, cytokinesis begins as actin starts to be recruited to an area just below the plasma membrane at the midpoint between the two mitotic spindles. This will be the site of the cleavage furrow. This actin recruitment is likely dependant on a previous accumulation of myosin II at this point. The actin polymerises into increasing numbers of filaments and, together with the myosin II forms the actomyosin contractile ring, the structure that will provide the force to physically constrict the mother cell at its centre.

Telophase

The chromosomes have now separated to their opposite poles of the cell and a nuclear envelope begins to form around the two daughter nuclei. Therefore, mitosis is complete at this stage Cytokinesis, however, starts to have its first effects. The actomyosin ring constricts and squeezes the cell into a "dumbbell" shape, until just a narrow bridge of cytoplasm remains connecting the two daughter cells. This structure is often referred to as the "midbody". The contractile ring is thought to have now completed its function and in some cases the midbody can persist for several hours before the final event of "abscission", that is the cleaving and sealing of the midbody to form two separate cells occurs. This final step is thought to depend upon delivery of membrane to the abscission site.

This is a highly simplified version of the events that take place in cell division and serves only as a reference for further discussions. It is also important to point out that cell division can vary widely between species and that the events I have described above are only a "consensus" as far as current information will allow. To give just one

example, the actomyosin contractile ring can take different forms between species. In *Dictostelium*, for example, it can be clearly visualised as a tight ring structure. In mammalian cells, however, it tends to merely a concentration of actin and myosin in the area of the cleavage furrow.

Some of the major conclusions to have come from the cytokinesis field are that it is undoubtedly a very complexly integrated process, with many stages relying upon the successful completion of the previous stages, and that, as it is such a vital process to an organism's well-being there is much redundancy within the system. Indeed, even in the case of some major defects within the cytokinesis machinery, the cell often manages to complete divisions successfully, if at a slower rate. This "robustness" was summed up by Ray Rappaport during his keynote address at the ASCB Summer meeting on Cytokinesis (2004); "when I began working on cytokinesis, I thought I was tinkering with a beautifully made Swiss watch, but what I was really working on was an old Maine fishing boat engine: overbuilt, inefficient, never-failed, and repaired by simple measures." This robustness, however, often makes the identification of proteins involved in this process, and at which points their functions are required, difficult to determine.

1.1.2 Positioning of the Cleavage Furrow

The first stage at which cytokinesis can be observed to have its effects is during anaphase when the actomyosin ring is assembled, half way between the two sets of astral microtubules emanating from around the centrosomes, and subsequently starts to constrict, squeezing the cell into a dumbbell shape. It is essential that this furrowing process is tightly regulated in both time and space to ensure that DNA, organelles and the cytosol are equally divided between the two daughter cells. However, the events leading up to the placement and assembly of the actomyosin ring and hence the future cleavage furrow are undoubtedly complex, seem to vary to some extent between

organisms and at the moment are not fully understood. This section provides a brief description of the various current theories behind the positioning of the cleavage furrow.

The search for the initial signal that leads to the correct placement of the cleavage furrow has long be on and the illusive factor required for this process has often been dubbed "stimulin". In *Sachromyces pombe,* fission yeast, this factor may have been identified as the protein Mid1p. A study by Wu et al., 2003, showed that Mid1p was the first known factor to arrive at the site of the cleavage furrow. Mid1p is secreted from the yeast nucleus prior to cytokinesis and has been shown to be essential for the positioning of the cleavage furrow. However, anillin, the closest homologue of Mid1p in higher eukaryotes, does not seem to play such a decisive role in the positioning of the furrow. It can interact with septins, actin and myosin, other proteins which need to be recruited to the cleavage furrow, but it arrives at either the same time as, or later than, other furrow components and is either non-essential or only essential for a much later events in cytokinesis.

Rather than a single protein being responsible for cleavage furrow positioning, there are now three main schools of thought on how the cleavage furrow is positioned in higher eukaryotes. The first two of these are based on the observation that astral microtubules are essential and indeed sufficient for the positioning of the cleavage furrow in sand dollar and sea urchin eggs (Rappaport, 1961; Hamaguchi, 1975; Rappaport, 1985). These results lead to the proposal of two models known as the "astral stimulation" and "astral relaxation" models. They oppose each other, in that they focus on different areas of the astral microtubules as being the important for the positioning of the cleavage furrow. However, they do agree that astral microtubules are essential for this process and in fact do not necessarily negate each other, with a combination of both models being plausible.

The third model is known as "central spindle induction". The central spindle (also known as the spindle midzone) is a group of tightly bundled microtubules that forms directly between the two centrosomes during anaphase. Its molecular components are described in section 1.1.4 below. The central spindle induction model states that signals deriving from this structure are what position the actomyosin ring and hence the cleavage furrow. This model derives from studies in several different organisms. Strong evidence comes from experiments performed in *Drosophila*, where cells that fail to form a central spindle, yet still have apparently normal astral microtubules, do not furrow (Adams et al., 1998; Somma et al., 2002). In addition, *Drosophila* cells lacking astral microtubules, but retaining their central spindle, do go on to furrow successfully (Bonaccorsi et al., 1998). Further experiments in both rat and grasshopper cells support a role for the central spindle in the positioning of the cleavage furrow (Cao and Wang, 1996; Alsop and Zhang, 2003).

However, there is evidence that in *Caenorhabditis elegans* the central spindle is not required for cleavage furrow positioning, although is instead required at a slightly later stage, for the cleavage furrow to maintain ingression and for the successful completion of cytokinesis (Powers et al., 1998; Raich, 1998; Jantsch-Plunger et al., 2000). However, it has since been shown that perturbation of the central spindle does actually cause a slight delay in furrow formation in *C.elegans* and that, in some circumstances, the central spindle does become essential for furrow positioning (Dechant and Glotzer,2003). Therefore it seems that the central spindle plays a major, if not always absolutely essential, role in positioning of the cleavage furrow.

Overall, it seems clear that the contribution of each of the three models described to the positioning of the cleavage furrow varies in different cell types, although the "consensus" process may actually include elements of all three of the proposed models.

1.1.3 RhoA- regulating the contractile ring

The Rho family of small GTPases, consisting of Rho, Rac and cdc42 in human cells are well established regulators of the actin cytoskeleton. In cytokinesis it is Rho that has been shown to be particularly important, playing a major part in regulating the contraction of the actomyosin ring, although the other family members are likely to also play roles in cytokinesis.

Rho is thought to act via at least two downstream kinases, Rho kinase (or ROCK) and citron kinase. ROCK has been shown play an important role at the cleavage furrow ingression stage as it localises to the cleavage furrow and can phosphorylate the light chain of myosin II, a central constituent of the actomyosin ring (Kosako et al., 2000), and also inhibits myosin II light chain phosphatase (Kimura et al., 1996). It therefore activates myosin II, stimulating the actomyosin ring to contract. *Rok* (the *Drosophila* ROCK) RNAi induces the formation of bi-nucleate cells (Hickson et al., reported at American Society for Cell Biology summer meeting on cytokinesis, 2004). Rho also activates citron kinase, although this kinase is thought to play a much later role in cytokinesis, with RNAi knockdown of this kinase leading to destabilisation of the midbody and ultimately bi-nucleate cells in *Drosophila* (Echard et al., 2004).

Rho itself is tightly regulated by a number of processes during cytokinesis, the most important of which are outlined in sections 1.1.4 and 1.1.14 below).

1.1.4 Central Spindle

The central spindle is the central bundle of anti-parallel microtubules that forms directly between the two cell poles during anaphase (Saxton and McIntosh, 1987). Numerous studies have shown that its constituents are essential for cytokinesis and now that many of these components have been identified a complex picture of interactions

and regulations that are necessary for the regulation and progression of cytokinesis is gradually being revealed.

Many of the key proteins that have thus far been identified fall into two groups, one of which is the "passenger proteins". "Passenger proteins" are proteins which reside on the kinetochores of chromosomes at metaphase. Then, upon initiation of anaphase, when the chromosomes segregate, the passenger proteins transfer to the central spindle. The key passenger proteins studied thus far are Auroa-B-kinase, inner centromere protein (INCEMP) and Survivin. These three proteins form a complex that is required for metaphase chromosome alignment, chromosome segregation and cytokinesis (Adams et al., 2000, 2001; Katina et al., 2000 and Wheatly et al., 2001). INCEMP is a large coiled-coil, scaffold-type protein, whilst Survivin is so named as it contains an "inhibitor of apoptosis" motif, although this does not seem to play a function in cytokinesis. Rather, the function of both INCEMP and Survivin is the regulation and targeting of Auroa-B-Kinase, who's phosphorylating activities are essential for both mitotic chromosomal processes and organisation of the central spindle during cytokinesis (Giet and Glover, 2001; Kallio et al., 2002; Kaitna et al., 2002; Murata-Hori et al., 2002; Murata-Hori and Wang, 2002).

A fourth member of the passenger proteins family is Polo-like-kinase 1 (PLK1). This is the human homologue of the *Drosophila* Polo protein and, like the other passenger proteins above, has been shown to be required for a normal metaphase spindle and the successful completion of cytokinesis.

Another important group of proteins that reside on the central spindle are MKlp1 (or CHO1), CYK4 and ECT2. In human cells, it has been shown that MKlp1 binds to CYK4 (Mishima et al., 2002), whilst in *Drosophila* Pebble (the ECT2 homologue) and RacGAP50C (the CYK-4 homologue) may interact (Somers et al., 2003). Therefore it is possible that all three of these proteins are in a complex. Each of these proteins is well

studied and their interactions with each other as well as other proteins and the cytoskeleton quite complex. Briefly, MKlp1 has been shown to organise microtubule bundles of the central spindle (Nislow et al., 1992; Adams et al., 1998; Powers et al., 1998; Raich et al., 1998), whilst CHO1, a splice variant of MKlp1, contains an actin binding motif and may interact with the ingressing cleavage furrow although at present is thought to play a role in the very final stages of cytokinesis (Kuriyama et al., 2002). MKlp1 is discussed in further detail in section 1.1.5 below. ECT2 (Pebble) and CYK4 (RacGAP50C) play complimentary roles in the regulation of the RhoGTPase required for cleavage furrow ingression, RhoA. ECT2 is a Rho GEF (Prokopenko et al., 1999; Tatsumoto et al., 1999), whilst CYK4 is a Rho GAP (Jantsch-Plunger et al., 2000). Therefore, together, these proteins can bind microtubules, bind actin and regulate the Rho GTPase required for cytokinesis. They therefore play a major role in regulation of cleavage furrow ingression.

A final protein which should not be omitted from even a short introduction to the central spindle is PRC1. This protein has been shown to be essential for the formation and maintenance of the central spindle due to its microtubule bundling activity (Mollinari et al.). It is thought to be regulated, at least in part, by cdk1-cyclin B mitotic kinase (Jiang et al., 1998).

1.1.5 MKlp1 and other Kinesins involved in cytokinesis

Mitotic-Kinesin-like-protein 1 (MKlp1) is a member of the kinesin super family and undoubtedly plays a major role in cytokinesis. Both human MKlp1, its splice-variant CHO1 and it's *Drosophila* homologue, Pavarotti, are well studied. Indeed in a family-wide study of kinesins in *Drosophila*, Pavarotti was the only one which was shown to be essential for cytokinesis as when knocked down by RNAi in S2 cells, double-nucleated cells resulted (Goshima and Vale, 2003). From this study it was clear that Pavarotti is

required for formation and maintenance of the central spindle during cytokinesis as when it was knocked down by RNAi the central spindle did not form, resulting in an anaphase to cytokinesis arrest.

Although this study showed that in *Drosophila* S2 cells, Pavarotti was the only one of the 25 *Drosophila* kinesins required for cytokinesis, nine others were identified as being required for the earlier event of mitosis. Of these, 4 were involved in bipolar spindle assembly, 4 were involved in metaphase spindle alignment and Dynein was found to play a role in the metaphase to anaphase transition.

In human cells a second member of the MKlp1 family, MKlp2 (previously known as Rab kineisin-6 or Rab6 KIFL) has also been shown to be required for cytokinesis. This in discussed in the next section.

1.1.6 Rab6A and Rab6-KIFL/MKlp2: Roles in Cytokinesis?

Rab6A is a member of the small GTPase family of Rab proteins which resides primarily in the medial/trans Golgi cisternae in interphase cells (Antony et al., 1992; Martinez et al., 1994). Over-expression of Rab6A results in the complete redistribution of Golgi markers into the Endoplasmic Reticulum (ER) (Martinez et al., 1997; Echard et al., 2000) and it is therefore thought that Rab6A is involved in anterograde traffic from the Golgi to the ER. This movement is thought to be microtubule dependent (Martinez et al., 1997, White et al., 1999).

Rab6-KIFL (also termed Rabkinesin6, MKlp2, KIF20A) was identified as a Rab6A effector through its interaction with a GTP-locked mutant of Rab6A in a Yeast-2-Hybrid screen (Echard et al., 1998). Rab6-KIFL is a member of the kinesin family of proteins, which share a conserved motor domain which is able to bind to microtubules and, through hydrolysis of ATP, able to generate force which results in the movement of kinesins along microtubules. During interphase, Rab6-KIFL is thought to be an effector

of Rab6A and has shown to be involved in the same processes of anterograde membrane traffic through the Golgi and to the ER and is thought to act by providing motility to membrane vesicles (Echard et al., 1998, Allan and Schroer, 1999).

It has since been shown that Rab6-KIFL also plays a role in cytokinesis. In 2000, Hill et al. showed that Rab6-KIFL accumulates in mitotic cells, localises to the central spindle in anaphase and later the midbody during telophase. They also showed that HeLa cells microinjected with Rab6-KIFL specific antibodies showed a cytokinesis defect, resulting in a large proportion of cells becoming bi-nucleate after one cell cycle (Hill et al., 2000). More recently, this data was backed up by siRNA studies which showed that specific knockdown of Rab6-KIFL also resulted in the formation of large numbers of bi-nucleate cells compared to control siRNAs (Neef et al., 2003). In addition, studies on the cell-cycle expression of Rab6-KIFL have shown that its expression is up-regulated at both the mRNA and protein level during cell division, with the Rab6-KIFL gene promoter showing maximal activity and mRNA and protein levels peaking during cell division (Fontijn et al., 2001). This expression pattern was confirmed by immunofluorescence studies showing that ten times higher Rab6-KIFL-specific fluorescence occurred in prophase nuclei than in interphase Golgi (Fontijn et al., 2001). Due to all of this data suggesting that Rab6-KIFL plays a role in cytokinesis and the fact that this kinesin shows some homology to the mitotic kinesin, MKlp1, Barr and co-workers suggested the re-classification of Rab6-KIFL to a mitotic kinesin and therefore re-named Rab6-KIFL MKlp2 (mitotic kinesin like protein 2) (Neef et al., 2003). This group have further investigated the role that MKlp2/ Rab6-KIFL plays in cytokinesis and have found that it interacts with components of the central spindle. Firstly, they found that MKlp2 is phosphorylated by and binds to Polo-like kinase (PLK1, see section 1.1.4), with this phosphorylation and binding essential for the correct localisation of both of these proteins to the central spindle (Neef et al., 2003). Secondly, they have found

that MKlp2 can interact directly and indirectly with the passenger protein Aurora B (see section 1.1.4) and that these interactions are essential for the relocation of Aurora B from the centromeres onto the central spindle at the metaphase to anaphase transition (Gruneberg et al., 2004).

All of the above evidence, then suggests that Rab6-KIFL/MKlp2, originally identified as a Rab6A binding partner, is required for cytokinesis. However, it remains undocumented whether or not Rab6A itself is required for cytokinesis. The specific roles identified for Rab6-KIFL/MKlp2 at the central spindle outlined above are unlikely to require membrane events and therefore Rab6A is unlikely to be required for these processes. Also, it has been shown that the high levels of Rab6-KIFL/MKlp2 which are newly expressed during cell division do not co-localise with Rab6A (Fontijn et al., 2001). However, these pieces of evidence do not rule out the possibility that Rab6A may be required at some stage in cytokinesis, whether in conjunction with Rab6-KIFL/MKlp2 or not. Indeed, as discussed in sections 1.1.7-1.1.8 it is now well established that membrane trafficking, possibly from the Golgi, is required for cytokinesis. Therefore, Rab6A/ Rab6-KIFL/MKlp2 complex, or Rab6A alone, may be required for membrane delivery to either the cleavage furrow for furrow ingression and/or the midbody for abscission (see sections 1.1.8 and 1.1.15). In fact, a recent study of mitotic kinesins in human cells has suggested that Rab6-KIFL/MKlp2 may play a role in the final abscission stage of mitosis (Zhu et al., 2005), at which time the likely requirement for membrane delivery/fusion would also favour the involvement of a Rab protein.

In summary, the Rab6A binding kinesin, Rab6-KIFL/MKlp2 has been shown to be required, possibly at multiple stages, for cytokinesis. However, the potential role of Rab6A itself in cytokinesis has not been studied. Therefore investigations determining the localisation, interactions and possible functional effects of Rab6A in cell

division/cytokinesis need to be carried out to determine if this Rab family member is involved in this process.

1.1.7 Membrane trafficking in cytokinesis

It is clear from studies in several different organisms that membrane trafficking is required for successful cytokinesis (for recent reviews see Bednarek and Falbel, 2002; Finger and White, 2002; Xu et al., 2002; Strickland and Burgess, 2004; Schweitzer and D'Souza-Schorey, 2004).

Perhaps the most striking example of this is in plant cells. These completely lack an actomyosin ring and cytokinesis proceeds by delivery of membrane vesicles along microtubules to the centre of the cell where they accumulate and fuse, forming the "phragmoplast". Continued addition of vesicles eventually leads to the fusion of the phragmoplast with the mother cell membrane, hence dividing the cell into two (reviewed Bednarek and Falebl, 2002).

Of course, in all animal cells currently known, an actomyosin ring is present which physically constricts the cells until the two daughter cells are only connected by a narrow intracellular bridge. However, membrane dynamics are still required at at least two stages of cytokinesis. Firstly, membrane delivery to the surface of the cell is required during cleavage furrow ingression in order to provide the increased surface area necessary to form two new daughter cells. This process will be discussed in section 1.1.8 below. Secondly, once the cleavage furrow has completed its ingression and the cells remain connected by a narrow intracellular bridge, membrane dynamics must take place in order to finally separate the mother cell into two separate daughter cells and to seal the two newly formed cells. This second process will be discussed in section 1.1.15 below.

1.1.8 Membrane trafficking to the cleavage furrow

As mentioned above, extra membrane is needed at the cell surface as the cell divides into two in order to provide the increased surface area necessary for the formation of two new daughter cells. It has been shown in both sea urchin embryos and *Xenopus* eggs that this membrane addition does not happen at random across the cell surface but is delivered specifically to the cleavage furrow. (Shuster and Burgess, 2002; Danilchik et al., 2003) This directed delivery may indicate that, in addition to a simple increase in surface area, the membrane being added to the cleavage furrow contains lipids and proteins that are required at the site of furrow invagination.

The internal source of this membrane remains somewhat controversial and may vary between organisms. However, there are two membrane compartments that are certainly important for cytokinesis in at least some organisms. These are the Golgi, and secretory vesicles deriving from it, and the recycling endosome.

1.1.9 The Golgi in cytokinesis

In some cell types the Golgi and vesicles derived from it do seem to be required for cytokinesis. For example, in both *C.elegans* (Skop et al., 2001) and *Drosophila* cells (Sisson et al., 2000), disruption of anterograde Golgi trafficking by addition of the ARF1 inactivator Brefeldin A (BFA) leads to a disruption in cytokinesis. However, in sea urchin embryos and mammalian cells, the addition of BFA seemingly has no effect on cytokinesis (Shuster and Burgess, 2002; Axelsson and Warren, 2004; Pecot and Malhotra, 2004).

Further support for the involvement of the Golgi in cytokinesis comes from the fact that several Golgi-resident proteins have been shown to be required for cytokinesis. For example, in *Drosophila*, Strabismus is an integral membrane protein which has been shown to be primarily localised to the Golgi. In embryos made mutant for Strabismus

membrane addition at the cleavage furrow is impaired, suggesting a requirement for Golgi-derived embryo for this process. (Lee et al., 2003). Lava lamp, another *Drosophila* Golgi protein has also been shown to be required for cellularisation in *Drosophila* (Sisson et al., 2000).

What has not yet been determined is whether vesicles are derived directly from the Golgi or they are first sorted through the recycling endosome. Indeed, the state of the Golgi during cell division is the subject of a long running debate which is too large and complex a topic to discuss here (for a recent review, see Barr, 2004). Briefly it centres around two models; one suggesting that the Golgi is absorbed into the Endoplasmic reticulum during cell division, whilst the other suggests the Golgi fragments into smaller parts but remains separate from the ER. Interestingly, two recent reports suggest that rather than the Golgi being required to provide membrane for cytokinesis, it is actually the dispersal of the Golgi that is important for mitosis/cytokinesis (Altan-Bonnet et al., 2003; Sutterlin et al., 2002). Altan-Bonnet et al. argue that proteins that are resident in the Golgi during interphase are required elsewhere to carry out their functions in cytokinesis. Therefore in order to carry out their functions in cytokinesis the Golgi must first disassemble. These reports suggest that ultimately the Golgi is required for cytokinesis as a source of certain protein components, so that actually at the time of cytokinesis it is rather the lack of a normal interphase-type Golgi that is important for cell division.

1.1.10 The Recycling Endosome in Cytokinesis

The recycling endosome is the second important compartment from which membrane vesicles are thought to be required for cytokinesis.

Currently, the best evidence for this comes from two studies in *Drosophila*. Pelissier et al. have shown that functional endocytosis is required prior to cellularisation

in the *Drosophila* embryo, therefore implicating recycled endosomes as a source of membrane required for cellularisation (Pelissier et al., 2003). Further to this, they showed that knocking down Rab11 activity before cellularisation results in defects in membrane addition at the cleavage furrow. As Rab11 is the small GTPase that is resident in the recycling endosome and is required for the budding of vesicles from this compartment (Ullrich et al., 1996) they conclude that trafficking through the Rab11 compartment is required for cellularisation.

The second *Drosophila* study implicates the recycling endosome as being required for cellularisation even more strongly. Riggs et al. have studied the role of Rab11 and Nuf in both vesicle trafficking to and actin remodelling at the furrow. As later described in section 1.2.13, Nuf is the *Drosophila* homologue of mammalian Rab11-FIP4/arfophilin2 and Rab11FIP3/arfophilin1, is a known binding partner of Rab11 and has previously been shown to play a role in both membrane delivery to the furrow and in recruitment of actin to the contractile ring (Rothwell et al., 1999; Rothwell et al., 1998). In their latest paper, Riggs et al. show that Nuf and Rab11 co-localise and physically associate with each other at the recycling endosome, that they are each dependent on each other for this localisation and that there are similar defects in both membrane delivery to and actin remodelling at the cleavage furrow when either of them are genetically knocked down (Riggs et al., 2003). They propose two similar models which explain their findings (see figure 1.1). Both of these suggest that Rab11 and Nuf are required for delivery of membrane to the cleavage furrow. The first model proposes that actin filaments are incorporated into the membrane vesicles being delivered to the furrow and that therefore both actin and membrane are delivered to the site at which they are required. The second model suggests that rather than actin itself being included in the vesicles, it is actually an actin remodelling factor that is co-delivered with the

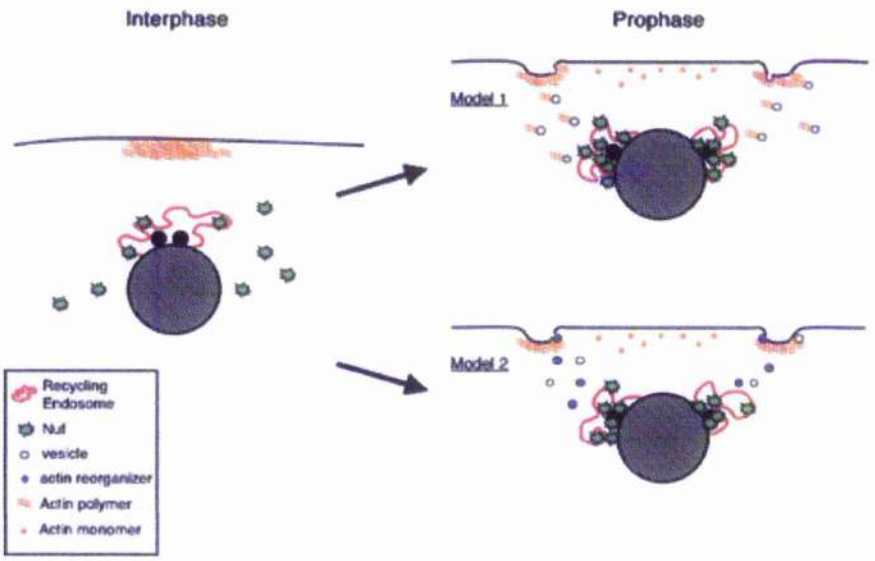


Figure 1.1 Riggs et al's models for recycling endosome/Rab11/Nuf involvement in *Drosophila* cellularisation:-

"Model 1: Nuf localization to the RE during prophase may stimulate vesicle delivery to sites of metaphase furrow formation. Previous reports demonstrated these vesicles are often associated with actin particles, thus membrane and actin are delivered as a unit to the furrows (Rothwell et al., 1999). Model 2: vesicles delivered to the furrow site include potent actin-remodeling factors. This model is based on reports demonstrating that Rac1 is delivered to the membrane through vesicles (Radhakrishna et al., 1999)."

Figure and text taken from Riggs et al, 2003

membrane which then has its effects on the actin cytoskeleton once it has been delivered to the cleavage furrow.

In either case, this work suggests that membrane delivery to and actin remodelling at the cleavage furrow are linked processes and that the compartment providing the driving force for both of these processes is the recycling endosome.

1.1.11 The final stages of cytokinesis

Once the actomyosin ring has contracted and the cleavage furrow has fully ingressed, the two daughter cells remain connected by a narrow intracellular bridge, known as the midbody. The final stages of cytokinesis result in the resolution of this bridge into two separate, sealed daughter cells and can be described as three separate events, outlined in sections 1.1.13-1.1.15 below. Failure of this process leads to several possible phenotypes. The most common of these are bi-nucleate or multinucleated cells due to a regression of the cleavage furrow after the completion of mitosis or cells remaining connected by an intracellular bridge long after normal cells have completed division. Before describing the final three events which lead to the completion of cytokinesis, the midbody itself is first reviewed.

1.1.12 The Midbody

The midbody is the term given to the narrow band of cytoplasm that links the two daughter cells after cleavage furrow ingression. One of its main components is tightly packed, anti-parallel microtubules derived from the midzone microtubules that have been squeezed into a dense bundle. Where these microtubules overlap is the electron dense central "Flemming body". Surrounding the Flemming Body (or possibly the outer, non-microtubule part of this structure) is a little-described ring-like structure

which contains, amongst other proteins, MKlp1 and which here will be referred to as the "midbody ring".

It is known that many proteins reside in the midbody and that these play a number of roles in the final stages of cytokinesis. For example, Skop et al.'s recent functional proteomic approach to identify proteins in the midbody and also what their corresponding functions may be found 172 proteins present in the mammalian midbody, of which 100 homologous proteins showed cytokinesis defects when they were knocked down by RNAi in C.elegans. The proteins present at the midbody carry out a variety of functions, which Schweitzer and D'Souza-Schorey divide into three groups in their recent review on the final stages of cytokinesis (Schweitzer and D'Souza-Schorey, 2004). These functional groups are midbody formation and stabilisation, actin ring disassembly and membrane events leading to abscission.

1.1.13 Stabilisation of the Midbody

For the first of these stages, many proteins which are present on the central spindle, which I have already described, are required. For example, in addition to MKlp1 having been shown to be required for furrow formation and ingression, it has also been shown to be required during the completion of cytokinesis. Specifically, Matuline and Kurigama show that MKlp1 is required for stable midbody formation. This is perhaps not surprising as the midbody microtubules are derived from the central spindle where, as previously described, MKlp1 carries out a microtubule bundling role. Nevertheless, the formation of a stable midbody is essential for the progression through to abscission as in cells failing to form a stable midbody, the cleavage furrow often regresses, leading to bi-nucleated cells.

Echard et al. have also shown that midbody stabilisation is a key step to successful cytokinesis. In their RNAi screen for proteins involved in the final stages of

cytokinesis in *Drosophila* they identify both anilin and citron kinase as being required for the stabilisation of the intracellular bridge. When these proteins were knocked down, cytokinesis failed in a significant proportion of cells (Echard et al., 2004).

1.1.14 Actomyosin ring disassembly

For the second stage of this process, actin ring disassembly, at least two distinct events are required. The first of these is a membrane lipid event, the externalisation of phosphatidylethanolamine (PE) at the cleavage furrow. Emoto et al. have observed that PE, which normally resides in the inner leaflet of the plasma membrane, is exposed on the cell surface just before midbody formation (Emoto et al., 1996). If PE is trapped on the outer leaflet, then a stable midbody forms but then remains indefinitely as the actomyosin ring fails to disassemble (Emoto and Umeda, 2000). Little is known as to the mechanism of this process, but it is thought that the flipping of PE from the inner to the outer leaflet must provide a signal which leads to actomyosin ring disassembly.

The second event required for actomyosin ring disassembly is Rho down-regulation. This is a logical event as Rho activation was the most important signal for actomyosin ring assembly at the beginning of cytokinesis. As for its activation, Rho down-regulation is likely to be a complex event, with several different pathways leading to independent means of inactivating Rho, yet another example of the "belt and braces" theme of cytokinesis. For example, the previously mentioned Rho GAP, CYK-4 (MgcRacGAP, RacGAP50C) is likely to be actively down-regulating Rho at this stage in cytokinesis by driving most of the Rho into a GDP-bound state (Minoshima et al., 2003). Another pathway to ensure inactive Rho involves Nir2, a member of the Nir/retinal degeneration protein B family. This contains a Rid (Rho-inhibitory domain) which has been shown to bind preferentially to Rho-GDP over Rho-GTP (Tian et al., 2000). Nir2

co-localises with Rho at the midbody and is likely to bind the inactive form of Rho and maintain Rho in this state, allowing actomyosin ring disassembly (Litvak et al., 2002).

Cofilin is another protein which may play a key role in actin ring disassembly. It is known to promote actin filament disassembly, is up-regulated by dephosphorylation at a late stage of cytokinesis (Kaji et al., 2003) and when its mutant gene is expressed or its expression is knocked down by RNAi or in *Drosophila*, large actin structures accumulate at the cleavage furrow during telophase and then persist into late cytokinesis, resulting in an inhibition of the completion of cytokinesis (Gunsalus et al., 1995; Somma, 2002).

1.1.14 Membrane events leading to abscission

Sections 1.1.7-1.1.10 have already commented quite extensively on membrane delivery in cytokinesis. However this was mostly concerned with membrane that was required for the ingressing cleavage furrow which is required in dividing cells, above anything else, to simply provide the cells with extra plasma membrane to accommodate the increase in surface area that occurs as the cleavage furrow ingresses. However, as was mentioned in section 1.1.7, membrane events must also be required for the very final stage of cytokinesis, abscission. The cells are now connected by the midbody, the actomyosin ring has disassembled and can therefore constrict the cell no further. It must be membrane events that ultimately divide one cell into two and ensure that these two new cells are sealed. It is not yet clear as to exactly how this event occurs, although two major models can be envisaged. Firstly, division may occur at the centre of the midbody in a process similar to plant cell division i.e. membrane vesicles are delivered along microtubules to the centre of the midbody where they accumulate and fuse with each other and eventually the plasma membrane, thus dividing the cell into two. The second

model is one of endocytosis at the midbody, with endocytosed membrane leading to the division of the cell in to two.

The first, vesicle delivery and fusion model seems to be feasible as microtubules are already in place, terminating in the centre of the midbody. Here they overlap, which would provide an ideal situation for vesicles travelling from opposite sides of the midbody to meet and fuse. In addition, several parts of the membrane fusion machinery have been found to localise to the centre of the midbody and play important roles in cytokinesis. For example, Low et al. have shown that two members of the SNARE membrane fusion machinery, syntaxin 2 and endobrevin/VAMP 8 localise to the midbody and that when mutant forms of these proteins are expressed, bi-nucleate cells result. By using time-lapse microscopy they show that this failure in cytokinesis occurs specifically at the final abscission stage, as cleavage furrow ingression and all other parts of cytokinesis proceed normally up until this stage. Thereby they also confirm that membrane events for abscission are indeed distinct to earlier, cleavage furrow ingression events (Low et al., 2003). Also Gromley et al. have been studying a novel, centrosomal protein, centriolin, which they have shown to be required for the final stage of cytokinesis (Gromley et al., 2003). More recently, they have reported that this protein binds to both sec15, a member of the exocyst complex (a complex which has been shown to be essential for cytokinesis) and to the SNARE protein snapin. Knocking these two proteins down by RNAi produces abscission defects, as for centriolin. They have also shown that these, and other, SNARE and exocyst components co-localise in the "midbody ring" structure which was introduced in section 1.1.12 above. Further to this they have also observed membrane vesicle trafficking into the midbody during cytokinesis. They conclude from all of these observations that membrane is delivered to the "midbody ring" structure at the centre of the midbody where the membrane fusion

and exocytic proteins identified above carry out their functions, leading to abscission of the midbody (Gromley et al., 2004).

The other model is one of endocytosis at the midbody, with endocytosed membrane somehow leading to the division of the cell in to two. Endocytosis has certainly been shown to occur earlier in cytokinesis, during cleavage furrow ingression, in Zebrafish (Feng et al., 2002). It has also shown to be essential for successful cytokinesis in *C.elegans* (Thompson et al., 2002). However, there are some hints which suggest it may be important at a later stage of cytokinesis in mammalian cells. For example dynamin, which is a key endocytic protein, has been shown to localise to the central spindle and later the midbody in both *C.elegans* and mammalian cells. In addition, depletion of dynamin in *C.elegans* has been shown to cause both early and late, possibly abscission, cytokinesis defects (Thompson et al., 2002). It has also been shown that the small GTPase ARF6 plays a role in mammalian cytokinesis (Schweitzer and D'Souza-Schorey, 2002). Wild type ARF6 was seen to accumulate at the cleavage furrow during cytokinesis, whilst a constitutively active mutant of ARF6, ARF6Q67L localises to the midbody late during cytokinesis and when expressed at high levels causes various defects in cytokinesis (Schweitzer and D'Souza-Schorey, 2002). More recently they have suggested that the function that ARF6 is carrying out in the late stages of cytokinesis may be endocytosis, although this has yet to be confirmed (Schweitzer and D'Souza-Schorey, 2004).

1.2.1 ARFs, Rabs and FIPs

Rab11 Family Interacting protein 4 (Rab11-FIP4), originally described as arfophilin 2, is a dual Rab/ADP-Ribosylation Factor (ARF) binding protein (Hickson et al., 2003). Therefore the ARF and Rab families are described below before an introduction to the Rab11-FIP proteins.

1.2.2 ADP-Ribosylation Factors

ADP-ribosylation factors (ARFs) are a family of small guanine-nucleotide binding proteins and are members of the Ras super-family (Welsh et al., 1994a). ARFs were originally identified by their ability to activate the cholera-toxin catalyzed ADP-ribosylation of Gs alpha (Kahn and Gilman, 1984; Kahn and Gilman, 1986), resulting in the permanent activation of adenyl cyclase. ARFs play key roles in membrane trafficking and organelle structure (Donaldson et al., 1995). There are six mammalian ARFs, these being grouped into three classes. Class I contains ARFs 1, 2 and 3, Class II, ARFs 4 and 5 and Class III ARF6 (Welsh et al., 1994b). There are also ARF homologues in both yeast and *Drosophila melanogater*. *Sacchromyces cerevisiae* contains three ARF proteins, *ARFs 1,2* and *3*. Either *ARF 1* or *2* is essential for viability, whilst ARF 3 is not essential for growth. *Drosophila melanogater* has at least one orthologue of each of the three mammalian classes of ARFs (Lee et al., 1994).

Like other members of the Ras super-family, ARFs function as molecular switches, cycling between a GTP-bound active and a GDP-inactive form. The exchange of GDP for GTP on ARFs allows them to associate with membranes, where they carry out their functions. Under physiological conditions, this cycling between the GDP and GTP-bound forms occurs very slowly. *In vivo*, this cycle is massively accelerated by the presence of two families of ARF regulators, the guanine-nucleotide exchange factors (GEFs) and the GTPase activating proteins (GAPs). The GEFs function by catalyzing the exchange of GDP for GTP, therefore activating the ARFs (Jackson and Casanova, 2000). The GAPs (Moss and Vaughan, 1998; Chavrier and Goud, 1999) catalyse the intrinsic GTPase activity of the ARFs to return the GTP-bound from back to the inactive GDP bound form. There is also recent evidence to show that ARF GAPs can interact with a number proteins involved in cytoskeletal remodelling as well as the ARFs. These findings will be discussed in the "ARF GAP signalling" section.

Of the mammalian ARFs, class I ARFs were the first to be extensively studied. These act in the Golgi where, in their activated GTP- bound form, they bind to ARF receptors in the Golgi membranes (Liang and Kornfeld, 1997). Here they act to recruit coat protein I (COPI), which in turn forms non-clathrin coated vesicles by membrane budding (Lippincott-Schwartz et al., 1998). More recently, a role of the class I ARFs, particularly ARF1, in the regulation of the actin cytoskeleton has been discovered. In 1998, Norman et al. showed that ARF1 mediates the recruitment of paxillin to focal adhesions, in a Rho-dependent manner (Norman et al., 1998). It has since been shown that this may occur via the ARF-GAPs, as discussed in section 1.1.4 below.

1.2.3 ARF6

ARF6 is the sole member of the class III ARFs and yet is has a staggeringly wide range of interactors and functions. It is highly conserved through vertebrates, invertebrates and yeast, although an ARF6 homologue does not occur in plants (Donaldson, 2003). Initially it may seem very similar to the other ARFs as its effector domains, Switch regions I and II, are very similar to the other ARFs. However, there are two fairly subtle differences which are thought to be responsible for the unique actions of ARF6. Firstly all of the ARF6 homologues contain a Gln-Ser motif adjacent to the Switch I region which is thought to be important for ARF6's specificity and is required for the actin-re-arrangement properties of ARF6 (Al-Awar et al., 2000). Secondly, all of the ARF6 homologues throughout evolution have a basic pI of 8.5-9.5, whilst other members of the ARF family have pIs of 6.0-7.0 (Donaldson, 2003). This positive charge, in addition to ARF6's myristilation (as for all ARFs) is thought to maintain ARF6's association with membranes, (Cavenagh et al. 1996; Song et al. 1998) even, to a large

extent in the GDP bound form, although the cytosolic GDP bound form may exist (Gaschet and Hsu, 1999).

ARF6 seems to have a diverse range of roles in membrane trafficking, including playing a part in recycling endosomal vesicles (Chavrier and Goud, 1999), receptor-mediated endocytosis (Altschuler et al., 1999; Mostov et al., 2000), GLUT 4 translocation (Millar et al., 1999) and exocytosis (Caumont et al., 1998). Interestingly, like ARF1, it has also been shown to be involved in the organization and remodelling of the cytoskeleton underlying the plasma membrane. For example, it has been shown to be essential for adherens junction turnover and cell migration in MDCK cells (Palacios et al., 2001). Also, several studies also suggest a link to Rac 1 (D'Souza-Schorey, 1997; Franco et al., 1999; Radhakrishna et al., 1999) a member of the Rho family of small Ras-related GTP-binding proteins, which regulates membrane ruffling and lamellipodia formation.

More recently it has also been suggested to play a role in cytokinesis. Schweitzer and D'Souza-Schorey localized ARF6 to the cleavage furrow of dividing cells and showed that expressing a constitutively active form of ARF6, ARF6-Q67L, caused defects in cytokinesis (Schweitzer and D'Souza-Schorey, 2002). However, the function that ARF6 is playing in cytokinesis has yet to be determined, with both membrane recycling (endocytosis/exocytosis) and actin rearrangement events, or both of these possibilities being important.

Current models of ARF6 action suggest that ARF6 cycles between a plasma membrane pool and an intracellular pool, that can be referred to as the ARF6 endosome. From this endosome, a proportion of ARF6 returns directly to the surface whilst the remainder merges with the sorting endosome from where it is trafficked back to the surface by a route which has not yet been fully determined (Radhakrishna and Donaldson, 1997; Donaldson, 2002). This could be directly from the sorting endosome

to the cell surface or, as some reports suggest, it may firstly cycle through the recycling endosome and then return to the cell surface. (Chavrier and Goud, 1999) (see figure 1.2). In this way it may exert its effects on both the vesicle trafficking/recycling system and the actin cytoskeleton underlying the plasma membrane.

1.2.4 Class II ARFs

The class II ARFs, ARFs 4 and 5, have been least studied. They have been shown to have and a similar localisation to, and share at least some functions with, class I ARFs. As they are typically present in cells at 3 to 10 times lower levels than class I ARFs it was originally thought they may only play a supplementary role to the class I ARFs. However, in 1999 a class II specific GEF was reported, (Claude et al., 1999), suggesting that this class of ARFs must have its own, unique functions. These functions have the potential to be both membrane trafficking and cytoskeletaly related, like the class I and III ARFs. This indeed may be the case for ARF5 as this has been shown to localize to regions where membrane trafficking takes place, for example the Golgi, as well as being able to interact with regulatory proteins involved in cytoskeletal regulation, such as a group of GAPs able to bind paxillin (Turner et al., 2001), as discussed in section 1.2.5 below.

Also, both our group and Shin et al. have performed yeast-2-hybrid screens using GTP-bound ARF5 as the bait and in this manner arfophilin (Shin et al., 1999) and arfophilin 2 (Hickson et al., 2003), were identified as class II ARF effector proteins. These will be discussed further in section 1.2.9 below.

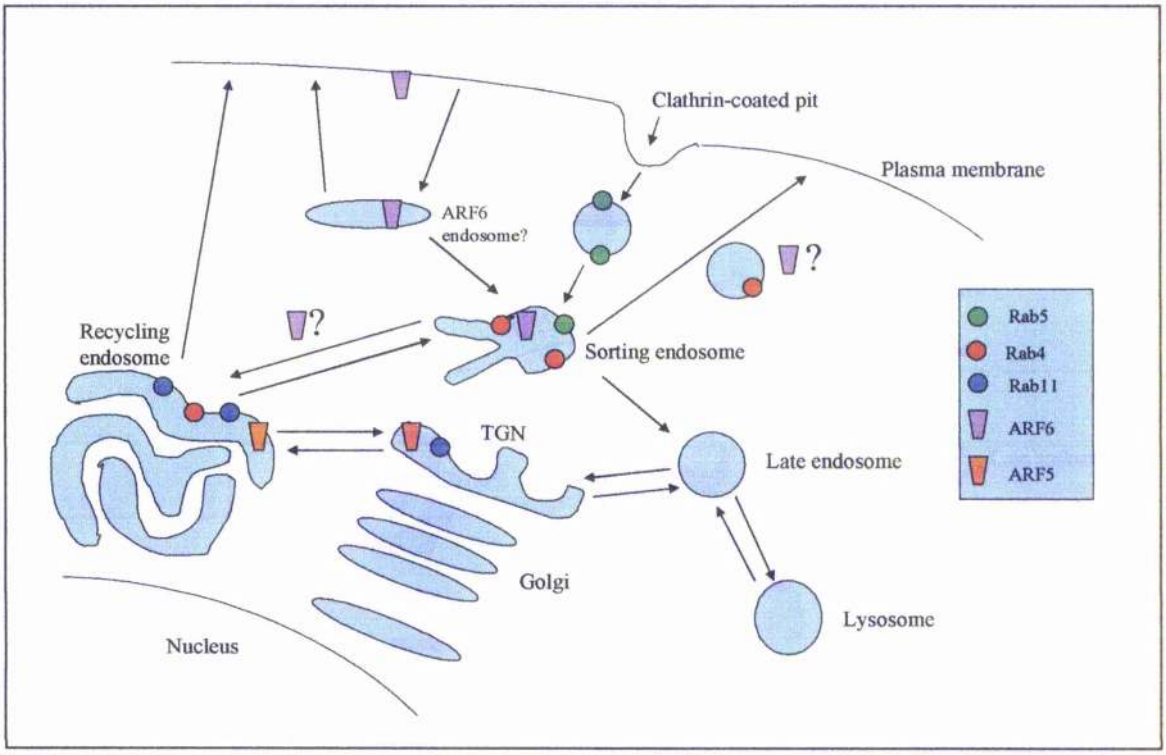


Figure 1.2 Diagram showing probable locations of Rabs 4, 5 and 11 and ARFs 5 and 6. ARF6 has been shown to cycle from the cell surface to a little characterised endosome, via non-clathrin coated endocytosis. This endosome is marked as the "ARF6 endosome" here. From here some ARF6 moves directly back to the surface, whilst a proportion of it traffics to the sorting endosome. From here it is not clear which route it takes to re-join the surface, although either direct trafficking or movement via the recycling endosome are possible, as indicated by the ARF6 symbols followed by question marks.

1.2.5 ARF GAP signalling

As mentioned above, it has recently come to light that a number of ARF GTPase activating proteins (ARF GAPs) not only regulate ARFs, but also interact with a number of proteins involved in the regulation of the cytoskeleton. This supports the evidence for the role of ARFs in these functions. The first direct piece of evidence that ARF GAPs were able to interact with cytoskeletal proteins came from the identification of paxillin kinase linker protein (PKL). This is an ARF GAP which contains two paxillin-binding sub-domains, PBS1 and PBS2 (Turner et al., 1999). It is also binds the PIX (PAK-interacting exchange factor)/COOL (cloned out of library) family of Rac GEFs and the p21-activated serine-threonine kinase (PAK) family. These are both involved in Rho signalling pathways, the role of which in regulating the actin cytoskeleton is well established (Hall, 1998). Since then a number of other GAPs able to bind paxillin, PIX/COOL and PAKs have also been found, including p95-APP1, the GIT (G-protein-coupled receptor kinase interacting protein) family of GAPs and PAG3 (Di Cesare et al., 2000; Premont et al., 2000; Mazaki et al., 2001; Kondo et al. 2000; Turner et al., 2001).

The interactions of these groups of GAPs confirm the convergence between the ARF and Rho family GTPase signalling pathways as suggested by the ARF1 and ARF6 studies mentioned earlier (see Norman et al., 1998 and Takai et al., 2001) and shows the dual (most probably linked) role of ARFs in both membrane trafficking and cytoskeletal re-arrangements).

It is also interesting to note that all of this family of GAPs posses GTPase activating activity for all three classes of ARFs (Turner et al., 2001). Therefore, although the interactions have not been shown *in vivo*, it is quite possible that the class II ARFs also play a dual role in signalling, as has been shown for the class I and III ARFs.

1.2.6 Rab Proteins

Rabs are another, and in fact the largest, family of monomeric small GTPases. The size of the family (11 Rabs in *Sacchromyces cerevisiae* (Lazar et al., 1997) and up to 63 family members in humans (Pereira-Leal and Seabra, 2001; Zerial and McBride, 2001) is indicative of the large range of functions that these molecules regulate. Numerous studies have identified Rabs as regulators of vesicle transport between distinct intracellular membrane bound organelles (reviewed, Novick and Zerial, 1997). It is now clear that this family of proteins regulate at least three stages in vesicle trafficking events, these being membrane tethering/docking/fusion, (e.g. Cao et al., 1998; Allan et al., 2000) , vesicle budding (e.g. Nuoffer et al., 1994; Riederer et al., 1994) and vesicle movement along cytoskeletal filaments (Echard et al., 1998; Nielsen et al., 1999).

1.2.7 The Rab cycle

Like the ARFs, Rab activity is dependent on their bound nucleotide and they cycle between the GTP-bound active and the GDP-bound inactive form. The regulation of this cycle is slightly more complex than that of the ARF cycle, with four groups of proteins being implicated as regulators. Firstly, in their inactive GDP-bound form, Rabs locate to the cytosol where they are bound by GDP dissociation inhibitors (GDIs). These proteins prevent indiscriminate membrane binding by the Rabs (Novick and Zerial, 1997). Then, as the Rabs bind to their donor membrane, the GDIs are replaced by GDI-displacement factors or GDFs (Dirac-Svejstrup et al., 1997). The Rab is then activated by a guanine nucleotide exchange factor (GEF) which catalyses the exchange of bound GDP for GTP (Martinez and Goud, 1998). The Rab, now in its active form can interact with its effector. Once this has occurred, GTPase-activating proteins then act on the Rabs to catalyze the hydrolysis of the bound GTP to GDP, thereby rendering the protein

inactive (Takai et al., 1996). The GDI protein can now again bind the Rab, removing it from the acceptor membrane into the cytosol, thereby allowing the cycle to start again.

1.2.8 YIPs are GDFs

As described above, GDFs (GDI dissociation factors) are thought to be required to catalyse the release of GDP-bound Rabs from the GDIs, hence allowing the Rabs to associate with membranes. A family of proteins recently identified as GDFs are the Yips (Ypt-interacting proteins). Yip1 was identified in yeast as being able to bind to yeast Rabs. A human Yip family, containing 16 proteins has now been identified. Of these, Yip3 (also known as prenylated rab-acceptor 1, PRA1) has been most extensively studied. It has been shown that it can act as a GDF as small quantities of Yip3 can dissociate pure, stable Rab9/GDI complexes. Also, perturbing YIP3 alters the membrane associations of Rab9.

If there are only 16 mammalian Yips, yet more than 60 Rabs, can Yips convey the specificity required to deliver Rabs back to their correct membranes compartments? It is thought that, by a combination of factors that this may be possible. Firstly, although Yips can bind more than one of the Rabs, they typically only bind to a subset of the Rab family. For example, Yip3 seems to be specific for endosomal Rabs, being able to bind Rab9 but not being able to bind Rab1 either in vivo or in vitro. Secondly, the Yips have distinct membrane localisations which will therefore aid the delivery of Rabs back to their correct compartments. For example, Yip3 is present on the late Golgi and endosomal membranes, whereas Yip6a (PRA2) is found on the Endoplasmic Reticulum and Yip1 is localised to the ER/Golgi interface. In addition, there is also evidence that the Yips can hetero-dimerise. This could allow for another level of complexity, further aiding the specificity of interactions available for each of the Rabs to bind to their correct membranes.

1.2.9 Rab Targeting

Whether Yips are the "target" for Rabs to be delivered to their specific membranes or not, it is still not totally clear which regions on the Rab proteins themselves are responsible for their targeting. Early studies suggested that the C-terminal hypervariable domain may be the section of the Rabs responsible for targeting (Chavrier et al., 1991; Stenmark et al., 1994). However, recent studies by Seabra and co-workers suggest that the C-terminal domain is not a general targeting determinant and have identified alternative sequences within the Rabs which are responsible for their correct targeting (Ali et al., 2004). However, the regions identified show only partial overlap between Rab different members of the Rab family, suggesting that targeting may be complex and at least partially species specific.

In summary, although the hypervariable domain may play a role in targeting in some cases (most likely the endosomal Rabs), in general it does not. It is other, more N-terminal regions, which are possibly species specific, which lead to the correct targeting of Rabs to their specific membranes.

1.2.10 Early and Recycling Endosomal Rabs

Rabs 4, 5 and 11 have been identified as the most important Rabs for the internalisation of vesicles and their recycling back to the plasma membrane. In elegant experiments Zerial and co-workers showed that these 3 Rabs occupied partially overlapping, yet subsequent endocytic domains along the vesicle recycling pathway (Sonnichsen et al., 2000): Rab5 was present on primary endosomes and the sorting endosome, Rab4 was present at the sorting endosome and partly on the recycling endosome and Rab11 was predominantly localised to the pericentriolar recycling endosome (see figure1.2).

These localisations reflect their known functions: Rab5, and one of it's principle effectors, EEA1 (early endosome antigen 1), have been shown to regulate the fusion of primary endosomes with the sorting endosome; Rab4 has been shown to be important for trafficking out of the sorting endosome, whilst Rab11 has been shown to be required for the exit of membrane traffic from the recycling endosome, from where vesicles then typically travel back to the cell surface.

1.2.11 Rab11 Subfamily

There are three closely related proteins in the Rab11 subfamily, Rab11a, Rab11b and Rab25 (Pereira-Leal and Seabra, 2001). Rab11a and b are highly homologous, ubiquitously expressed and as of yet, any functional differences remain unclear. Therefore, in this work, as in many other pieces, the term "Rab11" will be used to include both Rab11a and Rab11b. Rab25 is expressed exclusively in epithelial cells and it is therefore thought that it may play a role in membrane trafficking events specific to these cells (Goldenring et al., 1993 and 2003).

1.2.12 Recently Identified Rab effectors

With the large size of the Rab family and the diverse functions these proteins are able to regulate it would be expected that there would be a large number of distinct Rab effectors. Indeed, since the first Rab effector, rabphillin3 was identified in 1993 (Kishida et al., 1993), a great number of effectors have been found. For example, over 20 proteins were identified that bind specifically to the active GTP form of Rab5 in 1999, suggesting a great complexity of interactions downstream of this Rab (Christoforidis et al., 1999).

One of the most recent groups of Rab effectors to have been identified are the Rab11 Family Interacting Proteins (Rab11-FIP family). These are a number of proteins, which, as a family interact with Rab11a and, as has been recently revealed, Rab11b

(Junutula et al., 2004). Some members of the Rab11-FIP family may also interact with other Rabs, for example Rab4 or Rab25 (Hales Henderson et al., 2001; Prekeris et al., 2001; Lindsay et al., 2002)(see figure 1.3). The binding of members of this family to Rab11 seems to be through a conserved domain at the C-terminus of the proteins which has been shown to be an amphiphatic alpha-helix region (Prekeris et al., 2001; Hales et al., 2001).

As mentioned in section 1.2.10 the primary role of Rab11 seems to be in vesicle recycling through the pericentriolar recycling endosome, where it partially co-localises with the transferrin receptor and is responsible for the membrane trafficking out of this compartment (Ullrich et al., 1996; Green et al., 1997). It is also involved in the translocation of secretory vesicles in gastric parietal cells and glands (Calhoun et al., 1998; Duman et al., 1999), the recycling of the IgA receptor in Madin-Darby canine kidney (MDCK) cells (Wang et al., 2000) and in phagocytosis in macrophages (Cox et al., 2000).

Interestingly, *Drosophila* Rab11 has also been shown to play an important role in cellularisation of the *Drosophila* syncytium, a process analogous to mammalian cytokinesis. This is discussed further in section 1.2.8.

At present, it is not clear in which of these processes members of Rab11-FIP family play a role. What has been discovered is that several members of this family of Rab11 effectors co-localise with Rab11 in plasma membrane recycling systems in both non-polarised HeLa cells and polarised MDCK cells (Hales et al., 2001).

It happens that two of the Rab11-FIP family members, Rab11-FIP3 (previously named Eferin, EF-hands-containing Rab11-interacting protein)(Prekeris et al., 2001) and Rab11-FIP4 are identical to arfophilin and arfophilin 2, respectively (see figure 1.3), which as described above were originally described as ARF5 effectors (Shin et al, 1999;

Arfophilins	Rab11-FIPs	Rab binding	ARF binding
Arfophilin 1	Rab11-FIP3	11	5 and 6
Arfophilin 2	Rab11-FIP4	11 (and 5?)	5 (and 6?)
	Rab11-FIP1	11	?
	Rab11-FIP2	11	?
	Rip11a	11	No
	RCP	11 (and 4?)	?

Figure 1.3 Table showing relation of arfophilins to the Rab11-FIPs and the Rab and ARF binding interactions of these proteins. NB. It has been shown for several of the arfophilns/Rab-11FIPs that they can bind all members of the Rab11 subfamily, i.e. Rab11a, Rab11b and Rab25 (Prekeris et al, 2001; Junutula et al, 2004). This is likely to be the case for all the arfophilins/Rab11-FIPs as their Rab11 Binding Domains show a high degree of homology. Therefore, Rab11 in the above table refers to the Rab11 subfamily. (Data collated from shin et al, 1999; Shin et al, 2001; Prekeris et al, 2001; Meyers and Prekeris, 2002; Hickson et al, 2003; Junutula et al, 2004).

Hickson et al, 2003). The next section summarizes what is currently known about these two proteins.

1.2.13 Rab11 Family of Interacting Proteins (FIPs) 3 and 4/ The Arfophilins

As previously discussed, both arfophilin (Rab11-FIP3) and arfophilin 2 (Rab11-FIP4) were originally identified as class II specific ARF effectors. Using the GTP - locked form of ARF5, Shin et al. performed a yeast-2 hybrid screen and identified arfophilin as an 82.4kDa novel ARF5-binding protein (Shin et al., 1999). Similarly, our group identified arfophilin 2, a novel 83KDa protein showing homology to arfophilin 1 (see figure 1.4) (Hickson et al., 2003). Both proteins were shown to bind ARF5 in its active, GTP-bound form and not GTP-bound ARF1. Therefore it was proposed that the arfophilins were downstream effectors of the class II ARFs. Since then, it has been shown that arfophilin also interacts with the GTP-bound form of the class III ARF, ARF6 (Shin et al., 2001).

Binding of the ARF5 and 6 to arfophilin is dependent on two different sequences on the ARF proteins; amino acids 2-17 in the case of ARF5 and amino acids 37-80 in the case of ARF6, which are not homologous to each other. Therefore, although both the ARFs bind to arfophilin within the same region of it's C-terminal domain (amino acids 612-756), it seems that there are two separate binding sites within this region for ARF5 and ARF6 (Shin et al., 2001). It has not yet been determined whether arfophilin2/Rab11-FIP4 can bind ARF6. As both the class II and III ARFs bind arfophilin/Rab11-FIP3, it has been suggested that these ARFs may actually influence the same downstream pathways (Shin et al., 2001).

Arfophilin (Rab11-FIP3) shows co-localisation with ARF5 to intracellular membranes, particularly the Golgi, suggesting it may play a role in vesicle trafficking at this site.

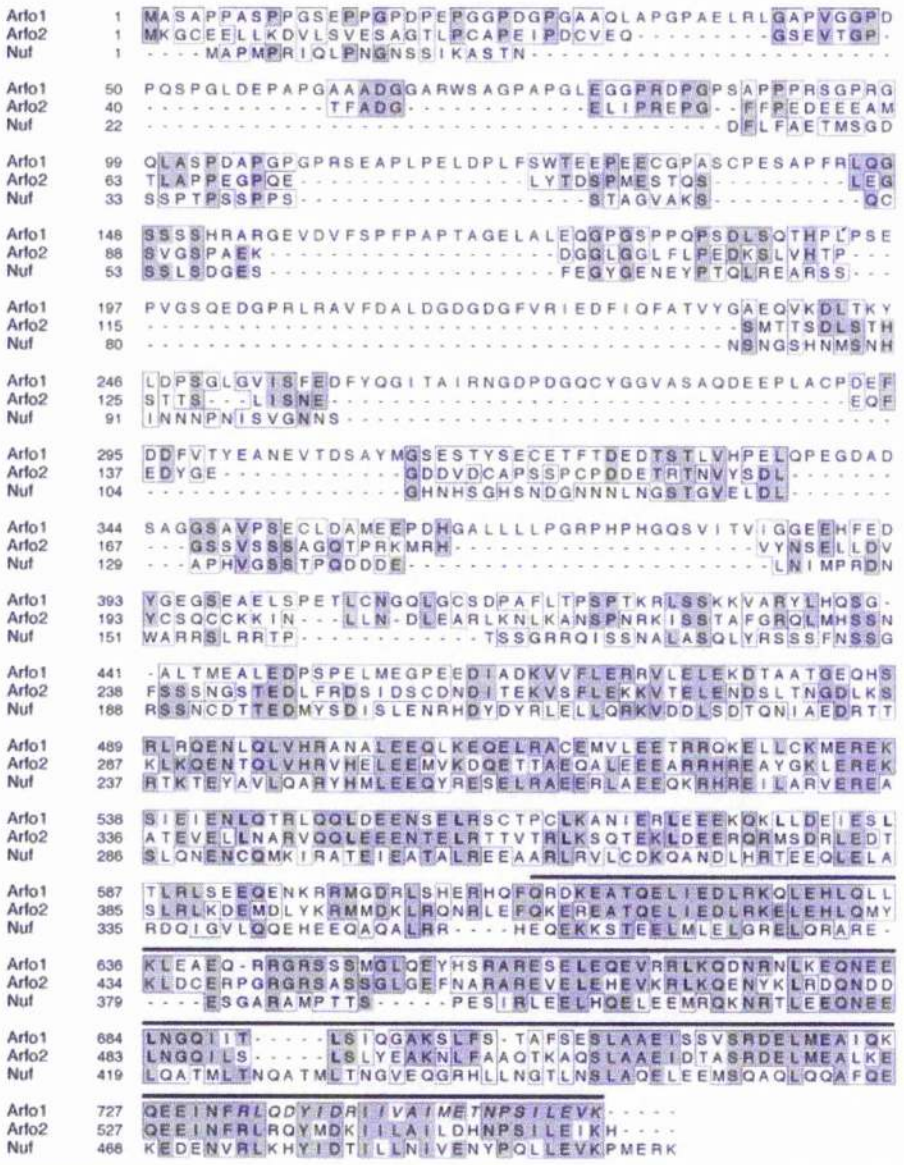


Figure 1.4 Alignment of Arfophilin1/Rab11-FIP3 (Arfo1), Arfophilin 2/Rab11-FIP4 (Arfo2) and *Drosophila* Nuf. These proteins show greatest homology in their C-terminal sections, particularly over their last 146 amino acids (indicated by line). In this area 26% of the sequence is identical across all three proteins, with 63% identity occurring between the Arfo1 and Arfo2 proteins. This is the area thought to bind both the ARF proteins and Rab11 (Shin et al,1999; Prekeris et al, 2001). The residues shown in italics at the extreme C-terminus indicate the minimal "Rab binding domain" (RBD) necessary to bind Rab11 as described by Prekeris et al (Prekeris et al, 2001).
Alignment taken from Hickson et al, 2003.

Studies of arfophilin 2 (Rab11-FIP4), suggest a number of potential roles for this protein. It has been localised to three main areas of the cell. Firstly, it co-localises with Rab11 in the pericentriolar recycling endosome. It is also present in focal adhesions, where it co-localises with paxillin. Thirdly, it is present at the centrosomes, and later the midbody during mitosis/cytokinesis (Hickson et al., 2003).

Over-expression of a GFP-tagged arfophilin 2/Rab11-FIP4 resulted in a tight pericentrosomal localization in a range of cells and this also caused the redistribution of transferrin receptors and Rab11 to the same location, whilst early endosomal markers were unaffected. This suggests a role for arfophilin 2/Rab11-FIP4 in the regulation of the pericentriolar recycling endosomes.

Currently no functional data has been gathered about the potential role of arfophilin 2/Rab11-FIP4 in focal adhesions. However, it is striking to note the known roles of ARFs 1 and 6 in the regulation of the action cytoskeleton through interaction with the Rho family of small GTPases, as discussed previously, and that the Class II ARFs remain unstudied in this area.

Finally, regarding the localization of arfophilin 2/Rab11-FIP4 to the midbody during cytokinesis, there is good evidence to suggest a possible role for this protein in this process. Arfophilin2/Rab11-FIP4 (and arfophilin/Rab11-FIP3) shows close homology to Nuclear-fallout (Nuf) (Hickson et al., 2003) (see figure 1.4). This is a *Drosophila* protein that is required for cellularisation (Riggs et al., 2003), a process analogous to cytokinesis in the syncytium of *Drosophila* embryos (see section 1.2.8).

1.3 Summary and Aims

In summary, studying Rab11-FIP4 should aid our understanding of the co-ordination between the Rab and ARF families, which is currently a novel concept and

also has the exciting possibility of discovering a role for the Rabs, ARFs and Rab11-FIP4 itself in the process of cytokinesis.

The aims of this study are to confirm and further evaluate the interactions of Rab11-FIP4 with the ARF and Rab family members with which it is known to interact, to investigate the localization and co-localization of these proteins in human cells during both interphase and throughout cell division and then to investigate the possible functional roles for Rab11-FIP4 and its binding partners in cytokinesis.

Chapter 2

Methods

2.1 Recombinant Protein Expression

75ml terrific broth (for 1 L; 12 g bacto-tryptone, 24 g bacto-yeast extract, 4 mL glycerol, 2.31 g KH2PO4, 12.54 g K2HPO4, ddH_2O to 1L) was inoculated with BL21 cells transformed with the appropriate plasmid and either Kanamycin or Ampicillin added to 30ug/ml or 100ug/ml respectively. After 16 hours at 37°C, spinning at 200rpm, this culture was added to 1 litre terrific broth plus the appropriate antibiotic. When the OD_{600} reached 0.6, IPTG was added to a final concentration of 1mM and cultures were left spinning at 200rpm, 37°C for a further 2 hours. Cultures were then spun down at 4000rpm, 4°C, for 15 minutes and then the supernatant removed and pellets re-suspended in 20ml/litre of breaking buffer (PBS (138 mM NaCl, 2.7 mM KCl, 8.1 mM $Na2HPO_4$, 1.5 mM $KH2PO_4$, pH 7.4) plus 0.1%Tween, 1mM EDTA, 1mM 2-mercaptoethanol, 4mM PMSF and 1mM Pepstatin). Samples were then French pressed twice at 950psi and subsequently spun at 17,000rpm, 4°C for 25 minutes. The supernatant was added to 0.5ml/litre original culture of Glutathione Sepharose (Amersham Biosciences) if the protein had a GST-tag or Talon Resin (BD Biosciences) if the protein was his-tagged. The supernatant plus the beads were then incubated at 4°C for 1 hour with gentle rotation to allow binding of the protein.

In the case of the GST-tagged proteins, the beads were then washed 4 times with 20 bed volumes of PBS, 0.1% Tween, by centrifugation at 1000rpm for 3 minutes. The beads were then re-suspended in an equal volume of PBS and stored at 4°C. If soluble GST-tagged protein was required then the beads were placed in a filter column and the protein eluted with glutathione in elution buffer (50mM Tris-HCl, 10mM reduced glutathione, pH8.0). If soluble, un-tagged protein was required, thrombin (Amersham Biosciences) was added to the beads and these were then incubated overnight at 4°C, then added to a filter column, and the flow-through collected and stored at -80°C.

In the case of His-tagged proteins, the talon resin was washed four times with 20 bed volumes of His wash buffer (25mM HEPES, pH8.0, 250mM NaCl, 10% glycerol, 5mM Immidazole), before being re-suspended in an equal volume of wash buffer and added to a filter column. The resin was then allowed to settle and then the wash buffer allowed to run through. Proteins were then eluted with elution buffer (25mM HEPES, pH8.0, 250mM NaCl, 10% glycerol, 75-250mM Immidazole) containing increasing concentrations of Immidazole (typically 75mM, 150mM, 250mM).

2.2 GST Binding Experiments

Beads with 5µg of the GST-protein in question attached were taken and the volume of beads made up to 50µl with glutathione beads. 100µl of reaction buffer (PBS plus 200mM NaCl, 1mM $MgCl_2$, 0.2% Triton X-100) was added to each tube. BSA was added to a final concentration of 0.2mg/ml, PMSF to 4mM and GMP-PMP (Roche Molecular Biochemicals) to 0.2mM. 20µg (unless otherwise stated) of the soluble protein under investigation was then added to each tube and the total volume brought to 500µl with reaction buffer. Tubes were then incubated at 4°C for 1 hour whilst rotating. Beads were then washed 4 times with reaction buffer by spinning for 2 minutes, 3000rpm, 4°C and then eluted with 50µl of 1x SDS-PAGE sample buffer (50mM Tris-HCl (pH6.8), 100mM DTT, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol). Tubes were boiled for 5 minutes, then spun at 6000rpm for 1 minute to pellet the beads and 10µl of the supernatant was then run on a gel for western blotting.

2.3 HeLa Cell Transfections

HeLa cells were 75ml flasks (Corning) at 37°C, 5% CO_2 in Minimal Essential Media (Invitrogen Life Technologies) supplemented with 10% Fetal Calf Serum (Invitrogen Life Technologies), 1% Non-Essential Amino Acids (Invitrogen Life

Technologies), and 1% Penicllin/Strepomycin (Invitrogen Life Technologies).HeLa cells were transfected using Lipofectamine 2000 (Invitrogen Life Technologies) and the protocol provided for this product followed. Briefly, 24 hours prior to transfection the HeLa cells were split into 12-well plates (Corning) containing 13mm glass cover slips (BD Biosciences). This was done in normal growth media except that the antibiotic mix, Penicllin/Strepomycin), normally added to the media was excluded as this becomes toxic to the cells if it enters them via the Lipofectamine 2000. At the time of transfection, 1.6µg of plasmid DNA per well is added to 100µl Optimem I Reduced Serum Medium (Invitrogen Life Technologies). In a separate tube, 6µl of Lipofectamine 2000 per well is added to 100µl OptiMEM I. These solutions were left to incubate at room temperature for 5 minutes. The DNA and Lipofectamine 2000 solutions were then combined, gently mixed and allowed to incubate for a further 20 minutes to allow the DNA/ Lipofectamine 2000 complexes to form. Meanwhile, the cells were washed 3 times in OptiMEM I and 0.8ml of OptiMEM I added to each well. After the 20 minute incubation, the solution was then added to the well. Cells were then placed back in their incubator for 5 hours before 1ml of 20% serum media was added back to the cells. Cells were then left 24 or 48 hours before analysis.

2.4 Immunofluorescence Staining of Cells

Growth media was removed and cells gently washed 1x with PBS. Fresh 4% paraformaldehdye was then added and left for 10 minutes at room temperature. Cover slips were then washed 3 times with PBS and then 30mM NH_4Cl added for 5 minutes. Cover slips were again washed 3 times with PBS before 0.1% Triton (in PBS) was added for 10 minutes to permeabilise the cells. Again cells were washed 3 times in PBS, then 3 times in blocking buffer (PBS plus 0.2% Fish Skin Gelatin (Sigma), 0.1% Donkey

Antibody type	Antigen	Source	Species	Dilution for Western Blots	Dilution for immuno-fluorescence
Primary	ARF6	Gift, J.Donaldson	Rabbit	1:1000	1:200
Primary	ARF5	Home made, anti peptide	Sheep	1:1000	1:200
Primary	Rab11	Zymed	Rabbit	1:1000	1:200
Primary	FIP4	Home made (characterised Hickson et al, 2003)	Sheep	1:2000	1:200
Primary	Rab5	Santa-Cruz	Rabbit	1:1000	N/A
Primary	Transferrin Receptor	Zymed	Mouse	N/A	1:200
Primary	MKLP1	AbCam	Rabbit	N/A	1:200
Primary	Survivin	Novus	Rabbit	N/A	1:200
Primary	RhoA	Upstate	Mouse	1:1000	N/A
Primary	Alpha-Tubulin	Sigma	Mouse	N/A	1:3000
Primary	myc	Santa-Cruz	Rabbit	1:1000	1:200
Primary	HA	Santa-Cruz	Rabbit	1:1000	1:200
Secondary, Horse Radish Peroxidase coupled	Sheep	Pierce	Rabbit	1:2000	N/A
Secondary, Horse Radish Peroxidase coupled	Mouse	Amersham Biosciences	Sheep	1:1000	N/A
Secondary, Horse Radish Peroxidase coupled	Rabbit	Amersham Biosciences	Donkey	1:1000	N/A
Secondary, Alexa Fluor® 594 or 488 or 647 coupled	Sheep, Rabbit or Mouse	Molecular Probes	Donkey	N/A	1:400

Table 2.1 Details of antibodies used in this study.

Serum (Sigma). Blocking buffer was freshly prepared and passed through a 0.2μM filter. Cells were washed 3 more times in PBS, then primary antibody added, usually at a 1:200 dilution in blocking buffer for 2 hours (see table 2.1 for full list of antibody details). Cells were then washed again 3 times in blocking buffer followed by 3 times in PBS before the secondary, fluorescent, antibody was added at a dilution of 1:400 in blocking buffer and left for 1 hour. Molecular Probes 488 (green), 594 (red) and 647 (blue) "Alexa Fluor Dyes" secondary antibodies were used (see table 2.1 for full list of antibody details). Cover slips were then washed 3 more times in blocking buffer and PBS. If actin staining was required then phalloidin was added for 30 minutes at this stage. A 1 in 20 dilution (in PBS) of Molecular probes 488, 594 or 647nm labelled phalloidin stock was used as appropriate. Cells were then washed again 3 times with PBS. If nuclear staining was required, then DAPI (Molecular probes), diluted in PBS to a final concentration of 1ug/ml was added for 2 minutes, before a final 3 washes in PBS. Cover slips were then mounted onto glass slides using a drop of "Immunomount" (Shandon Lipshaw, Pittsburgh, PA) and then left overnight in the dark to dry. The exposure of cover slips to light was minimised throughout the procedure to help prevent fading of fluorescence.

2.5 Confocal Microscopy

After staining, cells were observed and data collected on either a confocal microscope, or a Deltavision Microscope, as described in section 2.6 below. The confocal microscope used was a Zeiss LSM 5 Pascal instrument, with a Zeiss 63x or 40x oil immersion objective. 488nm Alexa-Fluor dyes and GFP were excited using a 488 nm argon laser, whilst helium neon lasers were used to excite both the 594 and 647nm Alexa-Fluor dyes. The appropriate long-pass filters were used to detect the fluorescence. Zeiss Pascal software was used to collect the images.

2.6 DeltaVision Microscopy

3D data sets were either acquired using a MicroMax cooled CCD camera (5MHz: Roper Scientific, USA), on a DeltaVision Restoration Microscope (Applied Precision, LLC, WA, USA), built around a Nikon TE200 Eclipse stand, fitted with a 100X/1.4N.A. PlanApo lens, or using a CoolSnap HQ cooled CCD camera on a DeltaVision Spectris Restoration Microscope built around an Olympus IX70 stand fitted with an a 60x/1.4NA lens (Applied Precision, LLC, WA, USA). Optical sections were recorded every 0.2 μm. 3D data sets were deconvolved using the constrained iterative algorithm (Swedlow et al, 1997; Wallace, 2001), implemented in SoftWoRx software (Applied Precision LLC, WA, USA). Image data was saved in TIFF format for presentation.

2.7 Adenovirus Production and Infections

A sample of adenovirus containing a myc-Rab11-S25N vector was kindly donated by Rytis Prekeris. A GFP alone expressing virus was already available in our lab. Viruses were amplified according to standard protocols. Briefly, HEK293 cells were grown in 150ml flasks (Corning) in DMEM (Invitrogen Life Technologies) supplemented with 1% Glutathione (Invitrogen Life Technologies) and 5% Fetal Calf Serum (Invitrogen Life Technologies). Once cells were 60-70% confluent, virus was added. Cells were left under normal growth conditions until the cells floated off the flask base (typically 3-4days). Media and cells were then collected in 50ml tubes and cells spun down at 1000rpm for 5 minutes. Supernatent was removed and then cell pellets re-suspended in an equal volume of PBS. Tube then freeze-thawed 4 times to break open cells. For continued amplification, this was then added back to a larger number of HEK 293 flasks. For purification at this stage, an equal volume of Arklone P added to the freeze-thawed mixture and this was mixed thoroughly. Tubes were left to stand for 10

minutes for the layers to separate and then spun at 1000rpm for 5 minutes. Meanwhile, CsCl gradients were prepared in Beckman 14x95mm tubes. 1.5ml of CsCl 1.2 was added to the tube and then carefully underlain with 3mls of CsCl 1.4. The top, lysed layer from the spun tubes was now carefully added to the top of the gradient and these then spun in a Beckman SW40 swingout rotor at 27000rpm, 8°C, for 90 minutes with 0 deceleration. The viral band was then removed from the tubes with a 19 gauge needle and inserted into a dialysis pack. This was then dialysed overnight at 4°C in 2 Litres of TBS. Dialysed virus was then removed and stored at -80°C until required. To infect HeLa cells with virus media was first replaced with 5% FCS Minimal Essential Media (rather than the normal 10%). Virus was then added at a concentration to give 80-95% infection (typically a 1:500 to 1:1000 dilution of stock). After 3 hours, extra FCS was added to bring the final concentration to 10%. Cells were left for 24 or 48 hours before being processed for immunofluorescence or protein extraction.

2.8 Microinjections

HeLa cells were grown on "Cell Locate" gridded cover slips (Eppendorf). These are marked with a grid and letters to allow identification of particular cells. Prior to microinjection, cells were transferred from their normal growth buffer to a dish containing microinjection buffer. Cells were then transferred to a heated stage and were microinjected using an Eppendorf Microinjector 5171 and Transjector 5246 system with Eppendorf Femtoptips. The FIP4 antibody used was at a concentration of 0.68mg/ml, whilst proteins were diluted to a concentration of 1mg/ml in 1x PBS. Antibody and protein buffers were free from detergents. Antibodies and proteins were centrifuged for 15 minutes at 13000 rpm, transferred to a new tube and kept on ice prior to the injections. In some cases, a small amount of 488 nm fluorescently tagged Bovine Serum

Albumin conjugate (Molecular Probes) was added to the antibody or protein solution to aid identification of micro-injected cells.

2.9 siRNA Transfections

siRNAs were prepared according to the Ambion "Silencer™ siRNA Construction Kit" protocol. They were then transfected into HeLa cells outlined below:-

24 hrs prior to transfection, HeLa cells plated out onto 13mm glass coverslips in 6-well plates in HeLa media without antibiotics. For a final siRNA concentration of 50nm, the following amounts of siRNA and reagents were used. Amounts adjusted as necessary for lower/higher concentrations. For each well (6-well plate, total 1ml) 10ul of 6μM siRNA plus150μl Optimem 1 Reduced Serum Medium (Invitrogen Life Technologies) was mixed. In a separate tube, 9ul Oligpfectamine (Invitrogen Life Technologies) +36μl Optimem 1 Medium were mixed. Both tubes were then left for 5 minutes before being combined, mixed gently and left for a further 20 minutes. Meanwhile, the media in plates was washed twice and replaced with 800μl Optimem I. After 20 mins, the siRNA/Olifectamine mixtures added to cells, giving a final volume of 1ml. After 5hrs, 110μl FCS (Invitrogen Life Technologies) was added to make 10% Serum media. Cells were then left for 24, 48, 72 or 96hrs at 37♯ C, 5%CO_2.

Coversilps were then removed to separate 6-well plate and fixed for immunofluorescence staining as described in section 2.4.

For the cells remaining in the original 6-well plate, protein extracts were prepared as described in section 2.10 below.

2.10 Protein Extracts from HeLa Cells

The whole of the following procedure was performed on ice:-

Firstly, the media was aspirated off and then the cells gently washed twice in ice-cold PBS. 150µl of following protein extract buffer (50mM HEPES, 100mM KCl, 5mM NaCl, 1mM $MgCl_2$, 0.5mM EGTA, 1mM EDTA, Proteinase inhibitor tablet, 0.1% Triton-x-100, 1mM DTT) was added to each 6-well dish. Cells were then scraped and left for 5 minutes, before being passed five times through a 23 gauge needle. The cells were then spun at 14,000rpm for two minutes and then the supernatant transferred to a separate tube and either used immediately or stored at -80°C. To determine the concentration of the samples, 10µl supernatant added to BioRad reagent (BioRad) diluted 1:5 with dH_20. The solution was then mixed and left 5 minutes and then the absorbance read at 595nm against a blank. The protein concentration of the sample could then be gained by comparison to a standard curve (prepared with known concentrations of Bovine Serum Albumin.

2.11 SDS PAGE

SDS Polyacrylamide-Gel-Electrophresis (PAGE) was carried out according to standard protocols. Briefly, a "separation" buffer containing 12-15% Polyacrylamide was poured between glass plates (Bio-Rad), leaving a 1.5cm gap at the top of the plates (For 30mls of 12% separating gel:- 10.2ml ddH_2O, 7.5ml 1.5 M Tris-HCl, pH 8.8, 0.15ml 20% (w/v) SDS, 12.0ml Acrylamide/Bis-acrylamide (30%/0.8% w/v), 0.15ml 10% (w/v) ammonium persulphate (APS)* and 0.02ml TEMED*. *Add these last). 2mls of isopropanol was added to the top of the separation buffer to ensure a level upper surface of the separating gel and the gel was then left to set. Once set, the isopropanol was poured off and the top of the gel rinsed with dH_2O. A "stacking" buffer was then mixed and added up to the top of the glass plates and a comb inserted to create wells

(For 5mls of 4% stacking gel:- 3.075ml ddH_2O, 1.25ml 0.5 M Tris-HCl, pH 6.8, 0.025ml 20% (w/v) SDS, 0.67ml Acrylamide/Bis-acrylamide (30%/0.8% w/v), 0.025ml 10% (w/v) ammonium persulphate (APS)* and 0.005ml TEMED*. *Add these last). Once set, the comb was removed. The plates were then inserted into a SDS-PAGE tank (Bio-Rad) and the tank filled with SDS-PAGE running buffer (0.025 M Tris-HCl, 0.192 M Glycine, 0.1% (w/v) SDS, pH 8.3). Protein samples were then loaded onto the gel alongside a coloured molecular weight marker (Bio-Rad). Gels were then run at 80Volts until the samples had "stacked" along the interface between the "stacking" and "separating" gels. The supply was then turned up to 180Volts and the gel ran until the protein markers were well separated. Gels were then removed from the tank and either placed in Coomassie blue stain (0.025% Coomassie Brilliant Blue R250, 40% (v/v) methanol, 7% (v/v) acetic acid, ddH_20 to required volume) or transferred onto nitrocellulose in preparation for Western blotting.

2.12 Western Blotting

Transfer buffer (25mM Tris, 190mM glycine, 20% (v/v) MeOH) was prepared and a piece of nitrocellulose membrane (Schleicher & Schuell BioScience), plus four pieces of filter paper (Whatman) were soaked in this buffer for 10 minutes. The gel was then placed on the nitrocellulose membrane and sandwiched between two sheets of filter paper and a transfer sponge on either side. This stack was then placed in a transfer cassette and subsequently a transfer tank containing transfer buffer. Gels were transferred to the nitrocellulase by passing a current (250mAMPs) through the tank for 2 hours. Nitrolcelluloase membranes were then carefully removed and placed in PBS plus 5% Marvel dried milk (Premier Brands), 0.05% Tween 20 for 1 hour in order to "block" the membrane. Primary antibody was then added in PBS plus 2% dried milk, 0.05% Tween 20 at an appropriate concentration (see table 2.1). After 2 hours, primary

antibody solution was removed and the membrane washed 3x 10 minutes in PBS plus 2% dried milk, 0.05% Tween 20. Secondary antibody was then added (for concentrations see table 2.1) in PBS plus 2% dried milk, 0.05% Tween 20 for 1 hour. Membranes were then washed 3x 10 minutes in PBS plus 0.05% Tween 20. ECL re-agents were then mixed and added to the membrane for 1 minute before the membrane was placed in a cassette and overlaid with film (Kodak) Film was then developed using a Kodak "Exomat" machine.

NB. Unless otherwise stated, all reagents were supplied by Sigma, UK.

Chapter 3

Binding of Rab11-FIP4 to ARF and Rab Family Members

Chapter 3 – Binding of Rab11-FIP4 to ARF and Rab Family Members

3.1 Introduction

Rab11-FIP4 (FIP4) has previously been shown to bind 2 proteins of different sub-families within the small GTPase superfamily. These are the Class II ADP-Ribosylation factor, ARF5, and Rab11, a member of the Rab family of proteins (Hickson et al., 2003). Other members of the Rab11-FIP family have been shown to bind further proteins in both the ARF and Rab families. For example, Rab11-FIP3/ arfophillin1 has been shown, by yeast-2-hybrid and GST-pull down approaches, to bind both ARF5 and ARF6 (Shin et al., 1999, 2001) and also Rab11 (Prekeris et al., 2001).

Therefore, the aims of this chapter were to further investigate the binding of Rab11-FIP4 (FIP4) to Rab11 and ARF5, to determine whether or not FIP4, like FIP3, can bind to ARF6, and to start to investigate the possibility of FIP4 binding to further members of the ARF and Rab protein families.

3.2 Results

3.2.1 Expression of FIP4, Rab and ARF proteins

In order to carry out pull-down experiments it was first necessary to express and purify the proteins in question. It was chosen to express ARF5, ARF6, Rab11 and Rab5 as N-terminally GST-tagged proteins cloned into the pGEX-5X-1 vector (Pharmacia) in BL21 cells and to purify these proteins over Glutathione sepharose. A GST-alone construct to use as a negative control in the binding experiments was also expressed and purified. By running these purified proteins on Coomassie gels alongside BSA standards it was found that all of these proteins expressed at a good level and that only one major product of the correct size for the intact protein was produced in each case, indicating the proteins had been expressed and purified successfully (Figure 3.1 (a)). A

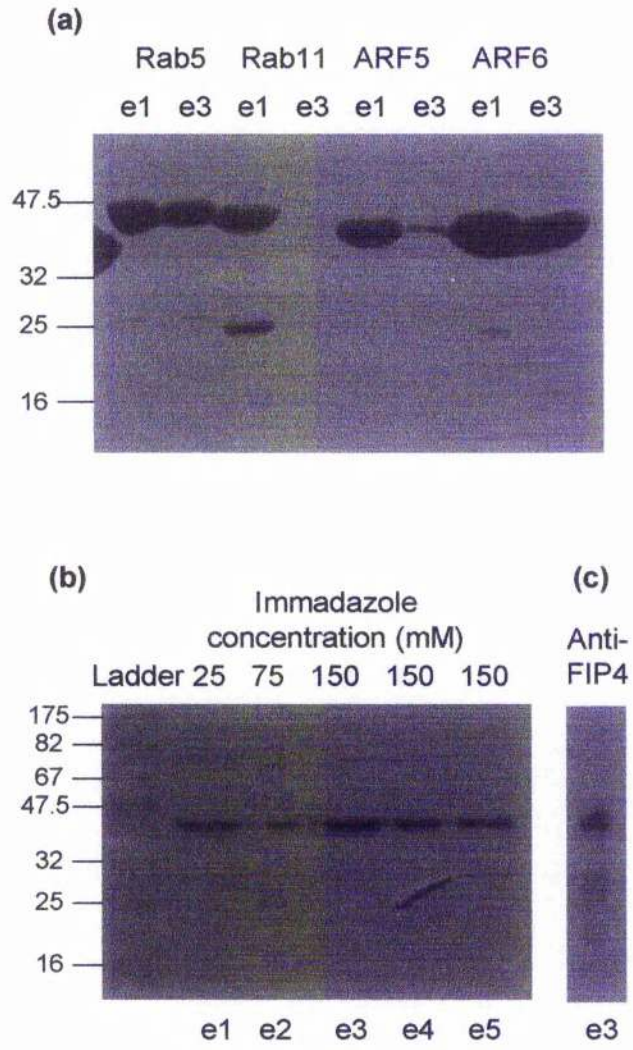


Figure 3.1(a) Purified GST-Rab/ARF proteins were run on a 12% SDS-PAGE gel and Coomassie stained. For each protein, 5µl of each of the first (e1) and third (e3) elutions were run to check their approximate concentration (against BSA standards, not shown) and integrity. **(b)** 5µl of each of elutions 1-5 of purified his-tagged FIP4 330 were run on a 12% SDS-PAGE gel to check their integrity and approximate concentration (e1-e5 below the gel indicate elutions1-5). The concentration of Immadazole used to elute each of the five 1.5ml aliquots of the purification is shown. **(c)** 5µl of a sample of elution 3 diluted 1:200 in elution buffer was run on a 12% SDS-PAGE gel and then transferred to nitrocellulose membrane and immunoblotted for FIP4. Molecular weight of markers in ladder is shown in kDa at the left side of each panel.

more accurate determination of the protein concentration of each sample was made by performing BioRad protein assays, as described in section 2.10.

Initially it was attempted to purify full length, GST-tagged FIP4 using the same methods as for the Rabs and ARFs. However, purification of the full-length product proved difficult, usually resulting in a ladder of protein fragments of several different sizes when run on a protein gel. A possible reason for this mixed population of proteins is due to the possible stalling of the bacterial translational machinery at various points along the GST-FIP4 sequence, resulting in a mixed population of peptides. In normal *E.Coli* cells this problem can occur due to the variability in the preferred codon usage for an amino acid between higher eukaryotes and prokaryotes. Because some common human codons may rarely occur in bacterial DNA, the bacteria will contain few of these tRNAs. Therefore, when attempting to translate a human gene the process may stall due to a lack of the required tRNA. To try and overcome this problem GST-FIP4 was transformed into Rosetta *E.Coli*. This strain of *E.Coli* is engineered to contain supplemental copies of rare *E.Coli*, common higher eukaryote codons to aid translation of higher eukaryote proteins. However, expressing and purifying the GST-FIP4 from Rosetta cells seemed to make little difference to the quality of the purification, with a mixed population of peptides still resulting (data not shown). Various induction times and temperatures were also trialled as these have been reported to have effects on the quality of purifications but these also did not lead to pure preparations (data not shown).

It is also known that the purification of larger recombinant proteins using the tagged approach is generally more difficult than the purification of smaller proteins. This phenomenon is detailed by Frangioni and Neel in their study on the purification of GST-tagged proteins (Frangioni and Neel, 1993). They found that the amount of GST-fusion protein purified per volume of beads used decreased as the size of the fusion protein

increased, more greatly than would be expected than if binding capacity was constant on a molar basis. Indeed they commented that proteins of over 100kDa would be predicted to give extremely low yields. The total size of the GST-FIP4 would be 107 kDa. They also suggested that if the protein is at all liable to proteolytic cleavage during the purification process, then the smaller fragments of your protein will be preferentially bound to the GST-sepharose and purified, making a clean purification of a large protein even more difficult to achieve. This comment may explain the "ladder" of proteins typically visible of a GST-FIP4 purification. For these reasons it was decided to try and purify a smaller part of FIP4 for the binding experiments in this chapter. It has been previously demonstrated that both the ARF5 and Rab11 binding sites on FIP4 are located within the C-terminal 330 amino acids of the protein. Therefore, it was decided to try and purify this part of FIP4 and use this for the binding studies.

3.2.2 Purification of His-tagged FIP4 330

A construct containing the C-terminal 330 amino acids of FIP4 cloned into a pET28b vector (Novagen) with an N-terminal hexa-his tag was already available from within the lab. This was transformed into BL21 cells and expressed and purified over Talon Metal Affinity Resin (BD Biosciences) as described in section 2.1. In this case, providing protease inhibitors were used and the preparation kept cold throughout purification, a single peptide of a satisfactory concentration could be obtained. This is shown in figure 3.1(b) and (c) where a sample of each of the elutions from a typical purification has been run on an SDS-PAGE gel and Coomassie stained. The gel shows one major band in each lane, at approximately the correct size for the C-terminal 330 amino acids of FIP4. Figure 3.1 (c) shows a western blot of one of these elutions which has been probed with an anti-FIP4 antibody. Again, one major band is seen at the same size as on the Coomassie stained gel, with some much fainter bands of smaller size

indicating a very minor pool of breakdown products. Therefore, this was the protein used for the binding experiments performed in this chapter.

3.2.3 Recombinant Protein GST-pull down Experiments

GST-pull downs are now a firmly established method of investigating interactions between two or more proteins. With the inclusion of appropriate controls, they are an ideal method of determining the specificity of binding within a protein family. Pull-downs can be performed with proteins recombinantly expressed and purified from bacteria or from *in vivo* proteins purified from the appropriate cells, or a combination of both approaches. Each method has its own advantages. Here, recombinantly expressed proteins were used as a method which allows known amounts of each of the proteins within the experiments to be used, therefore allowing comparisons of the amount of binding observed of the ARF and Rab family members to FIP4. *In vivo* type experiments to confirm that the interactions shown in this chapter do actually take place in living cells are being carried out by other members of our lab. In order to ask different questions, several different types of experiment were undertaken, although the basic methodology behind each, as described in section 2.2, remained the same. Firstly, a "specificity" experiment was performed which allowed the confirmation of the binding of FIP4 to Rab11 and ARF5, the absence of binding to ARF1, as previously shown by Hickson et al. (Hickson et al., 2003) and to determine whether or not ARF6 binds FIP4. "Affinity" experiments were then carried out to determine the relative strength of binding of FIP4 to each of the small GTPases. Finally, "competition" and "trimeric" experiments were conducted to investigate the possibility that FIP4 can bind more than one of the small GTPases simultaneously.

3.2.4 Specificity Experiments

A specificity experiment was performed as an initial means of confirming the binding status of ARF1, 5 and Rab11 to FIP4 and to ask if ARF6 is also a binding partner for FIP4.

5μg of GST-ARF/Rab or GST alone (as determined by comparing to BSA standards on Coomassie gels of the purified proteins) were added to separate tubes. To each of these, 20μg of FIP4 was added. After incubation and washing, the proteins were eluted from the beads by adding an equal volume of 1x SDS-PAGE buffer and 10μl of each of the eluted solutions was run on a 12% gel. The gels were then transferred to a nitrocellulose membrane, the membranes Ponceau stained and scanned to check for equal loading of the proteins and then the membranes probed with FIP4 antibody. Figure 3.2(a) shows a typical result of this experiment.

As can be seen in figure 3.2(a), the GST- alone lane shows no FIP4 binding, showing that FIP4 does not readily bind GST on it's own and therefore any binding that is seen is a bona-fide result of FIP4 binding to the protein in question. As a further negative control, GST-ARF1 was included. This has previously been shown not to bind FIP4 (Hickson et al., 2003) and lack of a band in this lane confirms that there is little or no binding between ARF1 and FIP4. The GST-ARF5 lane shows a band of FIP4. This is expected as the binding between these two proteins has previously been shown and was indeed how FIP4 was originally identified (Hickson et al., 2003). The GST-ARF6 lane shows a very strong band for FIP4, indicating strong binding of these two proteins, which has not previously been shown. As expected, the Rab11 lane shows a significant band for FIP4, indicating binding between these two proteins, as has previously been shown (Hickson et al., 2003).

It is noted that only a low percentage of the input FIP4 was recovered in this pull down experiment. This can be seen to be the case by comparing the strength of the FIP4

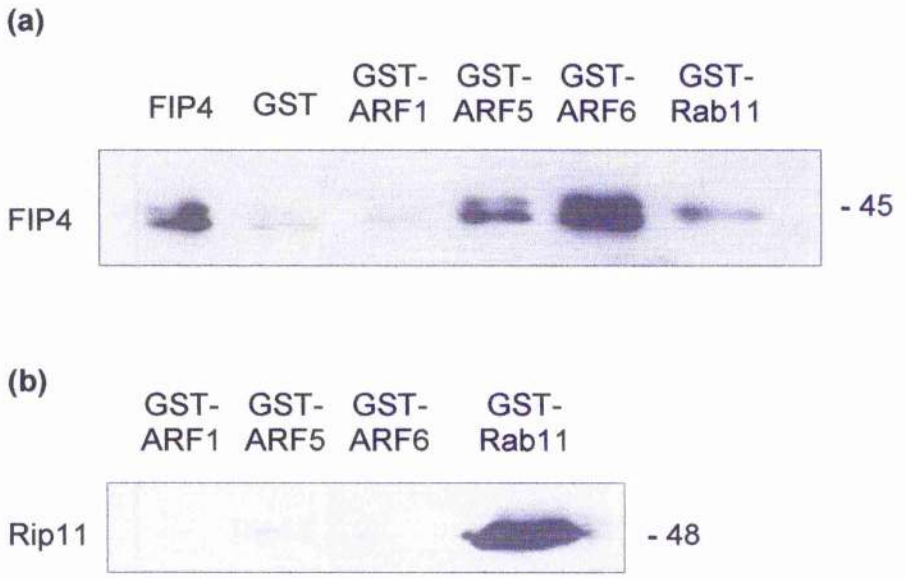


Figure 3.2 (a) Anti FIP4 western blot of specificity pull down experiments. The first lane contained 25ng of purified FIP4 run as a positive control. The subsequent lanes show experiments where 20μg of FIP4 has been added to 5μg of GST alone, GST-ARF1, GST-ARF5, GST-ARF6 and GST-Rab11. The Western blot shows a typical result of three independent experiments. **(b)** Anti-Rip11 western blot of Rip11 specificity, control experiment. The lanes show GST-ARF1, GST-ARF5, GST-ARF6 and GST-Rab11 pull downs. The Western blot shows a result typical of three independent experiments. The numbers on the right of both panels (a) and (b) indicate the approximate molecular mass (in kDa) of the bands shown.

signal in the control lane to the other lanes. The control FIP4 lane contained 25ng of FIP4, whilst the total input to each of the pull down experiments was 200g. It can be seen the strength of the FIP4 signal in, for example the GST-ARF5 lane, is approximately equal to that of the control lane. This suggests that only 25ng out of the 200g, approximately 0.1%, of the added FIP is recovered. There are several possible reasons for this, including that the small GTPases may not all be in their GTP-bound conformations, that not all the proteins are folded correctly or that the binding affinities of these proteins may only be relatively weak, either specifically in this *in vitro* situation or in the *in vivo* situation. All of these possible reasons are discussed in section 3.3. One of the major implications for this experiment is that the comparative strengths of binding as described above should not be over-interpreted as, with such a low recovery of protein, the potential variance in results of experiments of this type could be large.

Due to the somewhat unexpected result of ARF6 seeming to readily bind to FIP4, a control experiment to test the specificity of the ARF6 binding was devised to ensure that the GST-ARF6 was not, for whatever reason, non-specifically "sticky".

3.2.5 Rip11 control experiment

Rip11 is another member of the Rab11-FIP family and therefore has a broadly similar sequence and predicted structure to FIP4. It has previously been shown to bind Rab11 but not to bind any of the ARF family of proteins. Here, an experiment identical to the "specificity" experiment described above was carried out, except that soluble Rip11 rather than FIP4 was added to the beads. In this case, if Rip11 were blotted for, then it would be expected to only see a band in the Rab11 lane. However, if the ARF6 beads are non-specifically "sticky" then a band may also be seen in the ARF6 lane.

The results of this experiment are shown in figure 3.2(b). This blot shows Rip11 has only bound significantly to Rab11 and not to any of the ARFs tested. This therefore

suggests that the ARF6 beads do not non-specifically bind protein of the Rab11-FIP family type and subsequently suggests that the binding of FIP4 to ARF6 seen in figure 3.2(a) is genuine.

3.2.6 Affinity Experiments

To further characterise the binding of FIP4 to the Rabs and ARFs "affinity" experiments were performed for each of the ARFs and Rabs that had been shown to bind FIP4. These experiments were designed to both confirm the pattern of binding observed in the initial "specificity" experiment and to further study the relative strengths of binding of the different binding partners. The experiments were carried out for ARF5, ARF6, Rab11 and, in addition, Rab5. Rab5 was included in these experiments as by this stage of the work it had become clear from experiments performed by others in our lab (unpublished data) that Rab5 was also a potential FIP4 binding partner.

These experiments were performed as follows. For each of the ARFs or Rabs, 8 tubes were assembled. Into each of these was placed 5μg of GST-bound Rab or ARF. The experiments were then carried out as described in the methods section except that varying amounts of FIP4 were added to each tube. These were 0, 0.1, 0.5, 2, 10, 20, 40 and 100μg for each of the 8 tubes. After binding, washing and elution with SDS-PAGE buffer, 10μl from each of the tubes was run on an SDS-PAGE gel. After transferring to nitrocellulose, the membrane was Ponceau stained to verify the equal loading of the ARF or Rab in each tube and then the membrane immunoblotted for FIP4 to determine how much FIP4 had bound in each of the tubes. An example of one of these blots, showing an ARF5 "affinity" experiment, is shown in figure 3.3(a).

Blots were then quantified and the intensity of the bands then plotted as a graph, as shown in figure 3.3(b). If a specific protein-protein interaction was taking

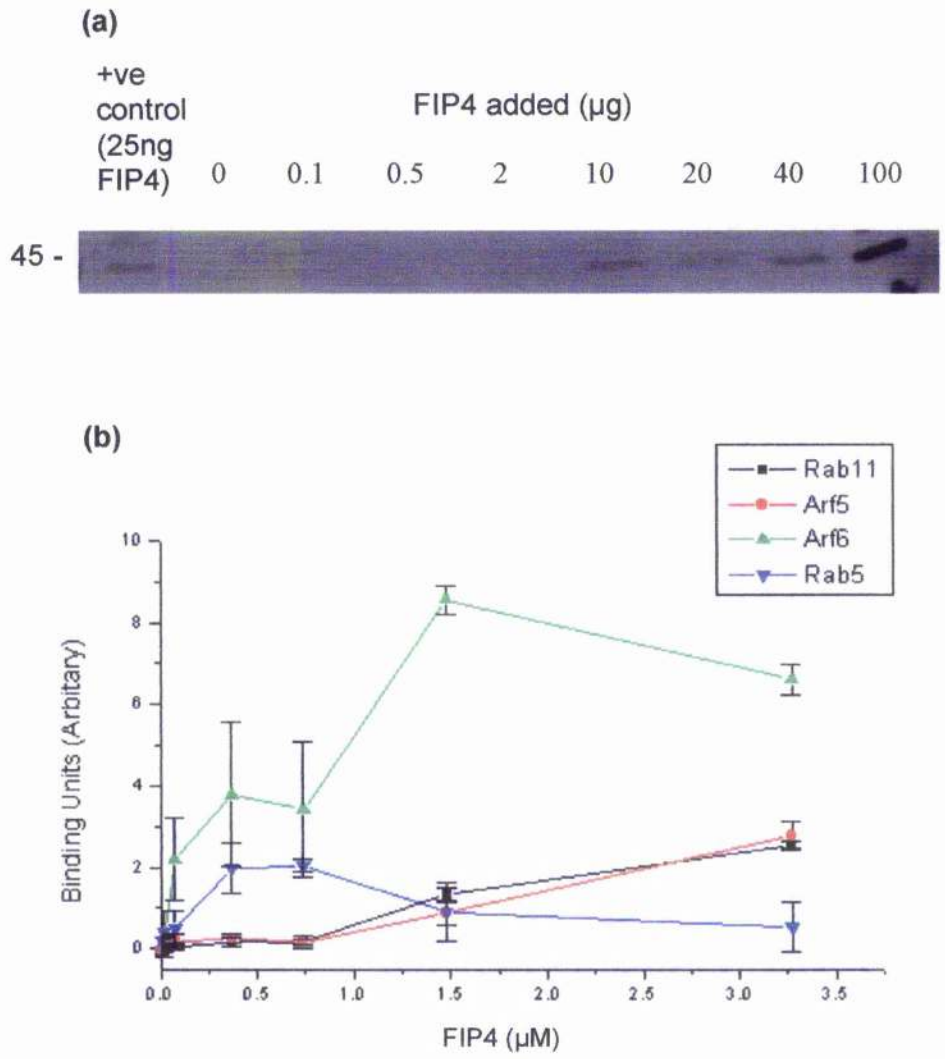


Figure 3.3 (a) Anti-FIP4 western blot of GST-ARF5/FIP4 affinity experiment. The first lane shows 25ng of FIP4 which was loaded as a positive control. Subsequent lanes are 10µl samples from tubes containing 5µg of GST-ARF5 with increasing amounts of added soluble FIP4 (as indicated). The approximate size of the shown band is indicated on the left hand side (in kDa). **(b)** This shows the quantification of the affinity blots for GST-ARF5, GST-ARF6, GST-Rab5 and GST-Rab11, displayed graphically. The x-axis shows the µM concentration of FIP4 whilst the y-axis shows an arbitrary "binding units" scale. Each point is the average of at least three experiments and error bars representing one standard deviation each side of the points are shown.

place and was plotted as a graph as appears in figure 3.3(b) then a specific shape of curve, known as a saturation curve, would be expected. This is a hyperbolic curve an initial steep increase in the amount of binding seen, followed by a gradual decrease in the steepness, leading to a flattening out of the curve as the point at which saturation occurs is approached. Figure 3.3(b) is difficult to interpret as generally the data shown do not appear as saturation curves. Possible reasons for this and a general discussion about the limitations of this experiment can be found in section 3.3. Here, I shall consider each set of data as it appears in figure 3.3(b).

The ARF6 plot shows an initial steep increase in the amount of binding measured, followed by a small decrease at the 0.750M FIP4 point, before increasing to its maximum and then showing a slight decrease in binding at highest concentration of FIP4. This is not the shape of curve expected for two reasons. Firstly, a smooth curve up to the maximum amount of binding would be expected. This makes the point at 0.750M an anomaly. Secondly, as the maximum amount of binding is approached, a flattening of the curve, but not a decrease in binding as observed here, would be expected. However, although the data for ARF6 does not seem to be of high quality, it does show the main characteristics of a saturation curve, i.e. an initial steep increase in binding followed by a flattening out of the curve. Therefore, it does seem likely that there is specific binding between ARF6 and FIP4 in this experiment.

The Rab5 plot shows an initial steep increase and then flattening up to the 0.750M FIP4 point. However, at higher concentrations of FIP4, there is a marked decrease in the amount of binding measured. Therefore, although binding is detected between Rab5 and FIP4 at relatively low concentrations of FIP4, the shape of plot is not what would be expected, which makes this set of data very difficult to analyse.

The Rab11 and ARF5 plots show a similar pattern. At the lower concentrations of FIP4 they show very little binding. They then show a steady increase in binding up to

the last point where they have reached a binding value of approximately 25% of the maximum value seen for ARF6. This straight line increase in binding suggests that, in this experiment, ARF5 and Rab11 are binding only in a non-specific manner to FIP4. This is highly unexpected as FIP4 has previously been shown to interact with both of these proteins (Hickson et al, 2003). Possible reasons for not observing a specific interaction between these proteins in this experiment are discussed in section 3.3.

3.2.7 Competition Experiments

Now that the relative strengths of binding of the small GTPases had been analysed it was interesting to ask another question regarding the binding of the Rab and ARF species. This was "can FIP4 bind both a Rab and an ARF simultaneously?". Answering this question may help to explain why FIP4 was a relatively rare example of a protein which was able to bind small GTPases of two different classes (and indeed the first example of a dual ARF/Rab binder) and would help towards proposing a model for FIP4's function. To address this question two different types of binding experiments were performed. Firstly "competition experiments" were carried out. These involved the binding of FIP4 to the GST-ARF proteins before the addition of increasing amounts of soluble Rab11 protein. Rab5 was not included in these studies as at the time of performing these experiments, the binding of FIP4 to Rab5 had not been confirmed. If the Rabs and ARFs bound to the same or overlapping site on FIP4, thus negating the formation of a trimeric complex, then increasing amounts of soluble Rab11 should compete off the bound GST-ARF and so a decrease in the amount of FIP4 bound to the GST-ARF would be expected. The poor binding of Rab11 and ARF5 seen in figure 3.3 also makes figure 3.4 somewhat difficult to interpret as this also involves Rab11 and ARF5 binding to FIP4. However, here the results are interpreted as they appear, whilst the limitations of the approaches used in this chapter are discussed in section 3.3.

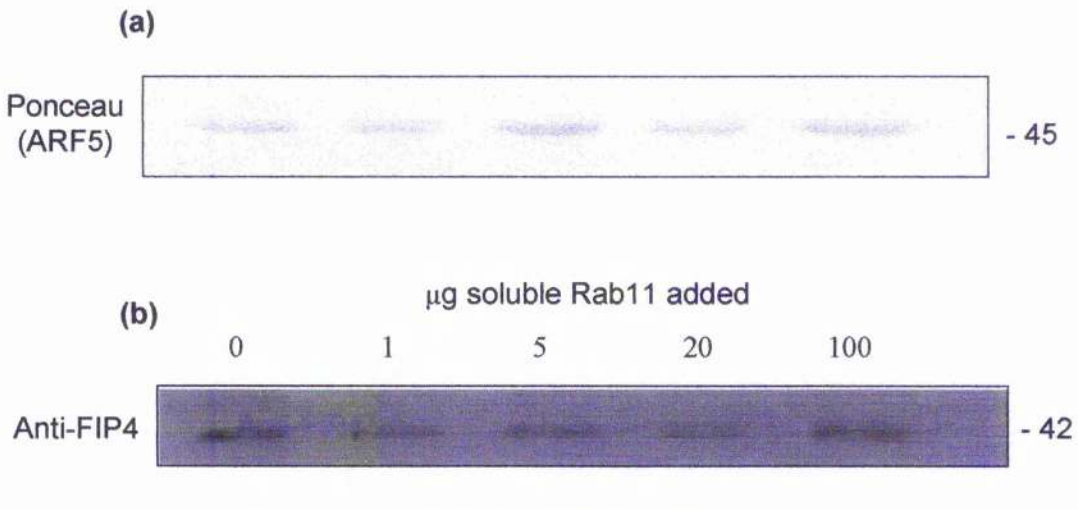


Figure 3.4 "Competition Experiment". 5 tubes, each containing 5μg of GST-ARF5 were taken. To each of these tubes 20μg of FIP4 was added. Then, varying amounts of un-tagged Rab11 were added to each tube (0-100μg) as shown in panel (b) above. After incubation, beads were washed and then eluted in loading buffer. 10ul from each tube was run on a 12% SDS-PAGE gel and this transferred to nitrocellulose. This was then Ponceau stained to verify that equal amounts of GST-ARF5 had been loaded in each lane. **(a)** shows one of these Ponceau stains showing the GST-ARF5 band. The nitrocellulose membrane was then immunoblotted using an anti-FIP4 antibody. The result of one of these blots is shown in **(b)**. The approximate size of the band (in kDa), as deduced by comparison to the molecular weight markers, is indicated on the right hand side. The Western blot shown is a typical result of three experiments.

Experiments were carried out with GST-ARF5 against soluble Rab11 (figure 3.4). It can be seen that the amount of FIP4 that remains bound to the GST-ARF5 at even the highest concentrations of soluble Rab11 was constant (figure 3.4 (b)). The same experiment was carried out with GST-ARF6 and this showed a similar result (data not shown). The fact that the highest concentrations of Rab11 do not seem to have competed with FIP4 for ARF5 binding suggests that the ARFs and the Rab proteins bind at different sites on FIP4. In turn this suggests that a trimeric complex of FIP4 binding both an ARF and Rab simultaneously could be formed.

To further test this hypothesis the "trimeric" experiments described below were carried out.

3.2.8 Trimeric Experiments

These experiments were designed to show that a complex of FIP4, ARF6 and Rab11 could be formed. GST-ARF6 plus soluble FIP4 and Rab11 were all added to the same tube. After binding and washing, the beads were eluted and a gel and transfer performed. The membranes were Ponceau stained to verify the presence of GST-ARF6 and immunoblotted with an anti-FIP4 antibody to show the presence of FIP4. Membranes were then immunoblotted with an anti-Rab11 antibody to determine if Rab11 was present. If Rab11 was present, this would suggest that a trimeric complex of ARF6, FIP4 and Rab11 could be formed. As a control, Rab 11 was added to GST-ARF6 in the absence of FIP4 and the same blotting procedures carried out. This would indicate whether FIP4 was necessary for the binding of the Rab11 to the GST-ARF6. An additional control was to use another member of the Rab11-FIP family, Rip11 in place of FIP4. This has been shown to bind Rab11 but not ARF6. This further ensured that any Rab11 binding seen in the GST-ARF6, Rab11 and FIP4 experiment would be due to the Rab *and* ARF binding properties of FIP4.

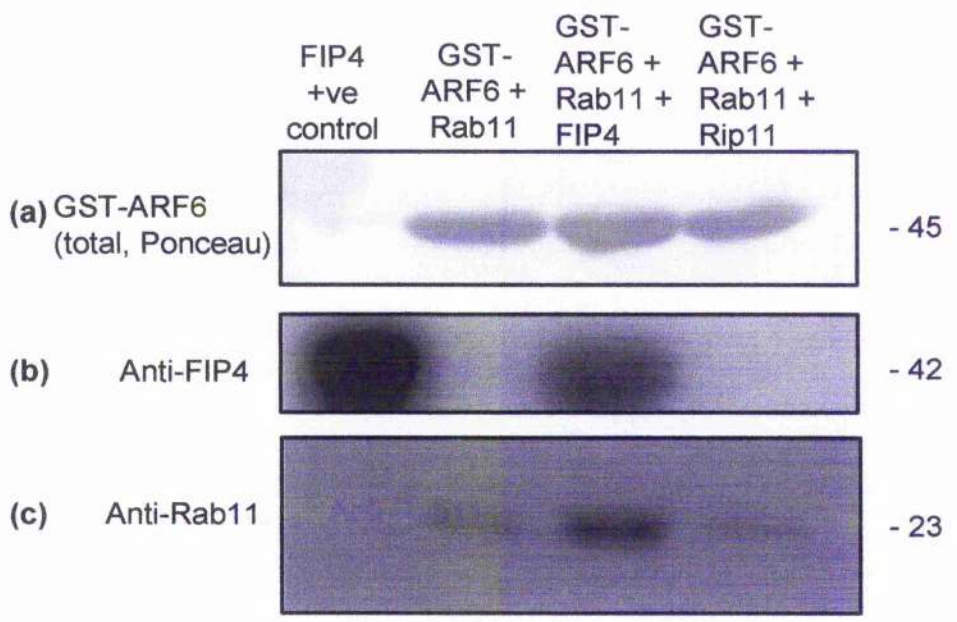


Figure 3.5 "Trimeric" Experiment. 3 tubes, each containing 5µg of GST-ARF6 were taken. To the first of these tubes, 20µg of un-tagged Rab11 was added. To the second, 20µg of un-tagged Rab11 plus 20µg of FIP4 was added. To the third, 20 µg of un-tagged Rab11 plus 20µg of Rip11 was added. After incubation, beads were washed and then eluted in loading buffer. 10µl from each tube was run on a 12% SDS-PAGE gel, next to a FIP4 positive control, and this transferred to nitrocellulose. This nitrocellulose was firstly Ponceau stained. (**a**) shows a Ponceau stain of the area of the membrane containing GST-ARF6. The membrane was then cut in two along the 32kDa marker and the top half immunoblotted for FIP4 whilst the lower half was immunoblotted for Rab11. The results of these two FIP4 and Rab11 immunoblots are shown in **(b)** and **(c)** respectively. The size of the bands, as deduced from comparison to molecular weight markers, is indicated on the right of the panels. The results shown are typical of three independent experiments.

Figure 3.5 (c) shows that significant amounts of Rab11 were only found in the lane including GST-ARF6, Rab11 and FIP4. The very faint band of Rab11 in the GST-ARF6 and Rab11 lane indicates that Rab11 only binds directly to ARF6 either very weakly or not at all. This suggests that FIP4 either greatly enhances or indeed is responsible for the presence of the Rab11 band in the GST-ARF6, Rab11 and FIP4 lane. This is further confirmed by the lack of a Rab11 band in the GST-ARF6, Rab11 and Rip11 lane. The simplest explanation for these results would be that FIP4 can simultaneously bind to ARF6 and Rab11 in a heterotrimeric complex.

3.3 Discussion

In this chapter recombinantly expressed proteins have been used to investigate the binding of FIP4 to members of the ARF and Rab small GTPase families. In summary, it has been shown that FIP4 can bind to Rab5, Rab11, ARF5 and ARF6 but cannot bind ARF1, a closely related small GTPase. The binding of ARF5 and Rab11 to FIP4 has previously been shown (Hickson et al., 2003). However, ARF6 and Rab5 are newly identified binding partners. To verify that the observed binding to ARF6 was not an artefact of the GST-ARF6 preparation, a "specificity" experiment was carried out using Rip11, another member of the Rab11-FIP family, instead of FIP4. This protein has been shown to bind to Rab11 and not to any of the ARFs. The result of this experiment confirmed that binding of ARF6 to FIP4 was indeed specific to FIP4 as ARF6 did not bind Rip11.

It must be stressed that these experiments were all performed using recombinantly expressed proteins in an *in vitro* situation and that therefore the relative strengths of binding discovered may not be true reflections of what is occurring in the cellular environment. This may be the case for a number of reasons, for example the recombinant proteins lack the post-translational modifications they may have in the cell, the cellular concentrations of the proteins in question are not known, and even if these were known, localised concentrations which would be difficult to determine are likely to occur within the cell. There are also other limitations of the experiments performed in this chapter, particularly the "affinity" experiment, shown in figure 3.3. These limitations, their consequences for the conclusions which can be drawn from this data and alternative methods to ask the questions asked here are described below.

3.3.1 Use of Proteins Lacking Post-translational Modifications

One of the major issues for consideration when using recombinant proteins expressed in bacteria is that these proteins are likely to lack the post-translational modifications that they would acquire *in vivo* in a higher eukaryote cell. Post-translational modifications may be important for various aspects of the protein's function. In some cases, the post-translational modifications may be required for the binding of one protein to another. Therefore, using bacterially expressed proteins may result is misleading findings if protein-protein interactions were to be studied, as has been done here. In light of this, the post-translational modifications which occur on the Rabs, ARFs and FIP4 will be discussed here and the likely effects of using these proteins lacking their post-translational modifications for binding studies will be considered.

Rab proteins are singly or doubly geranylgeranylated on C-terminal cysteine residues. This modification is carried out as Rabs are bound to the escort protein REP (Rab escort protein). Once geranylgeranylated the Rab can be delivered to its membrane where the geranylgeranyl groups insert into the lipid bi-layer, providing a membrane anchor for the Rab. Here the Rab is converted to its GTP bound form and can then interact with its effectors. Once it has carried out its function the Rab is converted to its GDP bound form and is then removed from the membrane into the cytosol by a GDI protein (GDP-dissociation inhibitor).

GDI proteins share structural homology with REP proteins in that they both contain a hydrophobic groove to accept the geranylgeranyl groups, which allows these proteins to interact with Rabs in the cytoplasm. Therefore, the geranylgeranyl groups are likely to be important for the binding of Rabs to REPs and GDIs, so if these interactions were to be studied, then bacterially expressed proteins, lacking the post-translational modifications, would be a poor choice. However, Rab11-FIPs have been identified as Rab11 effectors, i.e. they have been shown to interact with only GTP-bound Rab11 at

physiologically relevant concentrations (Junutula et al., 2004; Lindsay et al., 2002; Meyers and Prekeris, 2002). In the cellular environment, GTP bound Rabs are associated with membranes, with their geranylgeranyl groups inserted into the lipid bi-layer. Therefore it is unlikely that the geranylgeranyl groups will be required for Rab/Rab-effector interactions. Therefore, in this case the lack of post-translational modifications on the Rabs would be predicted to not have a major effect on the binding of the Rab to FIP4, suggesting that this aspect of the experimental approaches used here is reasonable.

ARF proteins are myristoylated at their N-termini. This modification, like the Rab geranylgeranyl groups, has been shown to be important for ARF association with membranes, where it inserts into the lipid bi-layer. As ARFs go through a similar regulatory cycle as Rabs, being membrane bound and interacting with their effectors in the GTP-bound form and that FIP4 has been shown to specifically bind the GTP-bound form of ARF5 (Hickson et al., 2003) then the same conclusions can be drawn for ARFs as for the Rabs regarding this issue. This is that the lack of post-translational modifications on bacterially-expressed ARFs would not be expected to significantly effect the binding of the ARF to the FIP4 and that therefore the approaches used in this chapter are reasonable one in this regard.

Currently, no post-translational modifications have been identified on the Rab11-FIP family of proteins. Therefore the effects of using FIPs lacking post-translational modifications cannot be evaluated. However, as interactions between the FIPs and small GTPases have been identified previously using bacterially expressed proteins (Junutula et al., 2004; Lindsay et al., 2002; Meyers and Prekeris, 2002) then it seems unlikely that post-translational modifications on the FIPs are required for their interactions with the Rabs and ARFs.

3.3.2 Are the Rabs and ARFs in their GTP-bound Conformations?

The FIPs have been identified as classic effectors of the Rab and ARF small GTPases in that they have been shown to preferentially bind the GTP bound form of the Rabs and ARFs over the GDP bound forms (Shin et al., 2001, 1999; Junutula et al., 2004; Hickson et al., 2003; Lindsay et al., 2002; Meyers and Prekeris, 2002). Indeed, this has been specifically been shown for FIP4 in the case of ARF5 and Rab11 (Hickson et al., 2003). Therefore studies examining the binding of effector proteins to the small GTPases, as carried out in this chapter, should use, as far as possible, the Rabs and ARFs in their GTP bound forms. Although, as explained below, attempts were made for this to be the case, the method used was not a stringent one. The fact that the Rabs and ARFs used in these experiments are likely to have not all been in their GTP bound forms could be one of the major explanations as to why some of the experiments here, particularly the "affinity experiment" presented in figure 3.3, produced results which were difficult to analyse.

In each of the experiments in this chapter, wild type recombinant proteins were used. In order to try and ensure that the small GTPases were in their GTP bound forms during the experiments, GMP-PMP (a non-hydrolysable analogue of GTP) was added to each of the reaction mixtures at a concentration of 0.2mM. It was envisaged that this excess of a GTP analogue would ensure that the majority of the Rabs or ARFs present would be in their GTP bond conformation. However, this is not a very stringent method of converting the small GTPases to their GTP bound form. This is because, although the GTP analogue was present in excess, the rate of small nucleotide exchange on small GTPases is intrinsically very low. *In vivo* the exchanges are catalysed by protein regulators, the GEFs and GAPs (see section 1.2.2 and 1.2.7). However, these will obviously not be present in bacterially-expressed purified protein preparations. This can

be overcome by performing a "nucleotide exchange" step with bacterially expressed proteins prior to starting a pull-down experiment in order to ensure that the majority of small GTPase present is in its GTP (or GDP) bound conformation. This reaction achieves loading of the small GTPase with GTP, GDP (or an analogue) by making use of the fact that Mg^{2+} is an essential co-factor which is required for nucleotide binding to the small GTPases. Briefly, the small GTPases are incubated in an exchange buffer containing EDTA to remove Mg^{2+} ions, thereby rendering the small GTPases unable to bind any nucleotide and so forcing them into a nucleotide free status. The desired small nucleotide is included in the exchange mixture in excess. The reaction is terminated by adding a high concentration of Mg^{2+} which results in the small GTPases binding the small nucleotides in the solution, the vast majority of which will be the added nucleotide. This is a more stringent method than the one used in the experiments presented here to ensure small GTPases are in the appropriate conformation. Therefore this nucleotide exchange reaction would ideally be carried out in future experiments of this type.

3.3.3 Quantification of Western Blots

Another limitation of the approach used in figure 3.3 is the fact that the quantification of the binding was made by analysing western blots, using computer software. Although western blots can be quantified in this way, the data gained will not be a linear function of the amount of protein that was present on the membrane, making the values gained somewhat unreliable. This is the case because of the method by which the protein is detected inherently involves several steps of amplification: Initially, more than one primary antibody is likely to bind to each protein present in the membrane and in turn more than secondary antibody is likely to bind to each of the primary antibodies. Therefore, although western blotting can be used to gain rough estimates of the relative

amounts of protein present, it is not a good method where more detailed studies are to be made. One example of a method which would allow more accurate quantifications of pull-down type experiments would be to use radio-labelled proteins. Quantification of the radioactivity of a sample would provide a more linear signal than that from a western blot.

3.3.4 Alternative Methods for Assessing Protein-Protein Affinities

The "affinity" experiment described here was simply designed to see the comparative strengths of binding of each of the small GTPases tested to FIP4. However, quantitative values for the strength of binding between two proteins can be deduced.

Perhaps the current method of choice for gaining quantitative values of protein interactions is the technique of surface plasmon resonance, which is probably best developed for asking biological questions in the "Biacore" system. In this technique your protein "bait" of choice is linked to a chip. A solution containing the target protein in question is then flowed over the chip. The amounts of protein bound to the chip at various time points throughout an experiment are detected by the physical surface plasmon resonance technique and then the data can be taken to calculate various values. For example, a value known as the "Equilibrium affinity constant" can be calculated from the data gained. This value gives an indication of the "strength" or "affinity" of an interaction. This Biacore approach was begun to study the interactions of FIP4 and the small GTPases used in this chapter. However, time restraints and the difficulties of obtaining protein samples of the high degree of purity required for this technique meant that this work needs to be continued in the future in order to gain quantitative data about the interactions of these proteins.

3.3.5 Other Considerations

In addition to the points discussed in detail above there are a number of other considerations regarding the design of the experiments in this chapter. Some of these will be briefly discussed here.

In the "specificity" experiment shown in figure 3.2, GST-ARF1 and GST-alone are used as negative controls. These are probably sufficient for the ARF5 and ARF6 experiments. However, ideally a Rab protein which is not thought to bind to FIP4 would be used as a negative control for the Rab11 experiment. Indeed, this leads on to the fact that the binding of FIP4 to other Rabs in addition to Rab11 and Rab5 has not yet been investigated and therefore these are experiments which need to be carried out in order to further investigate FIP4s Rab binding properties. This issue is discussed further in section 6.1.

In these experiments no checks were made as to whether the bacterially expressed proteins had folded correctly. It was assumed that as FIP4 showed binding to ARF5, 6 and Rab11 but not to ARF5 and GST alone that the binding seen was specific, indicating at least some correctly folded proteins. However, not all of the proteins produced may have been folded correctly, which may account for some of the unexpected results that were observed. In order to determine if a protein is folded correctly a number of approaches could be taken. Circular Dichroism Spectroscopy is a biophysical method which can indicate the presence or absence of secondary structure in a sample of protein, therefore indicating if the protein had folded to at least some extent. More specifically for the proteins used here, the nucleotide binding properties of the small GTPases could be assessed using radiolabelled nucleotides. If the Rabs and ARFs were folded correctly they should be able to bind GTP or GDP or one of their analogues. Performing one of these experiments would give an indication of the proportion of folded protein within a purified sample.

3.3.6 Conclusions

Despite the numerous limitations of the experiments in this chapter which have been discussed here and the fact that these findings do not necessarily represent the more complex situation that may be occurring *in vivo,* some conclusions can be drawn from this work which can at least give us a starting point for proposing a model for FIP4 function. Figure 3.2 shows that FIP4 binds GST-ARF5, GST-ARF6 and GST-Rab11 and yet does not bind GST-ARF1 and GST-alone, suggesting that the binding observed is specific. The Rip11 control shown in figure 3.2 (b) further suggest that the GST-ARF6 binding to FIP4 seen in figure 3.2(a) is specific.

In addition, figure 3.3 shows that GST-Rab5 can also bind, to some degree, to FIP4, although further analysis of this interaction is clearly required. As for reasons discussed the data in figure 3.3 is not of high quality and therefore the comparative affinities of the ARFs and Rabs for FIP4 are difficult to deduce. A more powerful approach, using the Biacore system, to assess the interactions of these proteins has been initiated.

Although not an ideal approach, for the reasons discussed, the GST-pull down "trimeric" experiment shown in figure 3.5 does suggest that the presence of FIP4 at least considerably enhances the amount of soluble Rab11 recovered from a GST-ARF6 pull down experiment, in comparison to the amount of soluble Rab11 recovered from a pull down including GST-ARF6 but no FIP4. This suggests that the presence of FIP4 at least enhances, and may be responsible for, the interactions of ARF6 and Rab11. The most likely explanation of this, given that FIP4 has been shown to bind both Rab11 and ARF6, is that FIP4 can bind both of these proteins simultaneously, forming a trimeric complex.

3.3.7 FIP4 and its Binding Partners: Links to Cytokinesis

A major observation made in this chapter relates to the involvement of identified binding partners of FIP4 in cytokinesis. As previously mentioned, FIP4 (and FIP3) are homologues of the *Drosophila* Nuf protein, which has been shown to be required for cellularisation, a process analogous to cytokinesis in the syncitium of a developing drosophila embryo (Riggs et al., 2003). FIP4 has also previously been observed to localise to the midbody during cytokinesis in mammalian cells (Hickson et al, 2003). These observations suggest that FIP4 may play a role in cytokinesis. In this chapter I have confirmed the binding of FIP4 to Rab11 and identified the binding of FIP4 to ARF6. Interestingly, both of these proteins have been implicated in cytokinesis (see sections 1.1.10 and 1.2.3). Rab11 has been shown to be involved in cytokinesis in *Drosophila* and *C.elegans* (Riggs et al., 2003; Pelissier et al., 2003; Skop et al., 2001), although not yet in mammalian cells. ARF6 has been shown to be involved in cytokinesis in mammalian cells (Schweitzer and D'Souza-Schorey, 2002). Therefore the binding of FIP4 to these molecules is further evidence that FIP4 may be involved in cytokinesis. Indeed, the trimeric complex which I have shown can be formed between these three molecules is an exciting progression that suggests that the vesicle trafficking properties of Rab11 may combine with the vesicle trafficking and actin re-arrangement properties of ARF6, through an interaction with FIP4, in order to regulate a membrane trafficking and/or actin remodelling step that is necessary for the completion of cytokinesis.

In the next chapter the co-localisations of FIP4 with the ARFs and Rabs that have been shown to be binding partners here are examined. This is carried out in interphase cells and, due to the possible links with cytokinesis discussed here and during the introduction, in cells at various stages of cell division.

Chapter 4

Localisation of FIP4, Rab11, ARF5 and ARF6 in Interphase and Cytokinesis

Chapter 4 - Localisation of FIP4, Rab11, ARF5 and ARF6 in Interphase and Cytokinesis

4.1 Introduction

In this chapter it was sought to identify the localisation and co-localisation of the proteins studied during the previous chapter, namely FIP4 and the small GTPases ARF5, ARF6, Rab5 and Rab11. As FIP4 has been identified as binding each of these proteins in an *in vitro* setting it was hoped to use co-localisation studies as a method of determining whether this binding could be possible *in vivo*. To do this interphase HeLa cells were stained with antibodies against FIP4 and, where possible, each of the small GTPases. They were then counter-stained with species-specific secondary antibodies conjugated to varying fluorescent probes and then the cells were examined under either fluorescent or confocal microscopy. In some cases cells were also co-stained with fluorescent markers of parts of the cell structure, for example fluorescently labelled phalloidin toxin, which stains the actin cytoskeleton, or DAPI, a dye which stains DNA and fluoresces blue under UV light, therefore marking the chromosomes/nucleus.

In the second part of this chapter the localisation and co-localisation of FIP4, the Rabs and the ARFs in cells at various stages of mitosis are examined. These experiments were carried out due to the possible links that FIP4, Rab11 and ARF5 and 6 may have with cytokinesis, as described in sections 1.1.10, 1.2.3 and 1.2.13.

4.2 Results

4.2.1 Localisation of FIP4 with Rab11 ARF5 and ARF6 During Interphase

FIP4 has previously been shown to localise predominantly to a pericentrosomal recycling endosome compartment and to have a partial overlapping localisation with Rab11, a molecule known to reside in the recycling endosome. However, as ARF6 was a

previously unidentified binding partners of FIP4, their co-localisation within cells has not yet been characterised. Also, the localisation of ARF5 compared to FIP4 within cells has not been previously examined, due to the lack of an appropriate antibody.

4.2.2 ARF6 and FIP4 Localisations

Figure 4.1 (a) shows the localisation of ARF6 identified by a rabbit anti-ARF6 antibody. The cell is counterstained with Alexa-Fluor 488nm labelled phallodin toxin to mark the actin cytoskeleton and with DAPI to mark the nucleus.

As can be seen, ARF6 shows three primary locations within the cell, firstly, a large peri-nuclear pool, secondly a plasma membrane pool where it co-localises with the actin staining and thirdly some nuclear staining, producing a "magenta" nucleus due to co-localisation with the blue DAPI staining.

Although the exact localisation of ARF6 is not yet clear (as discussed in section 1.2.3), the consensus seems to be that there is a plasma membrane pool and a, possibly unique, endosomal pool (Radhakrishna and Donaldson, 1997; Chavrier and Goud, 1999; Donaldson, 2003).

The staining observed agrees with this data, with a clear plasma-membrane pool and an endosomal pool, which in this case is, for the vast majority, in a well defined, peri-nuclear, location. Some nuclear staining was also observed which has not been previously reported. As the nature of this staining is not clear and, as described below, none of the other proteins studied here localise to the nucleus, it was thought to be not within the scope of this work to continue investigation of this localisation.

In figure 4.1 (b), the cell shown is stained for both ARF6 and FIP4. It can be seen that the largest pool of each ARF6 and FIP4 is a peri-centrosomal pool, which co-localise very well as indicated by the yellow patch below the nucleus in the merged

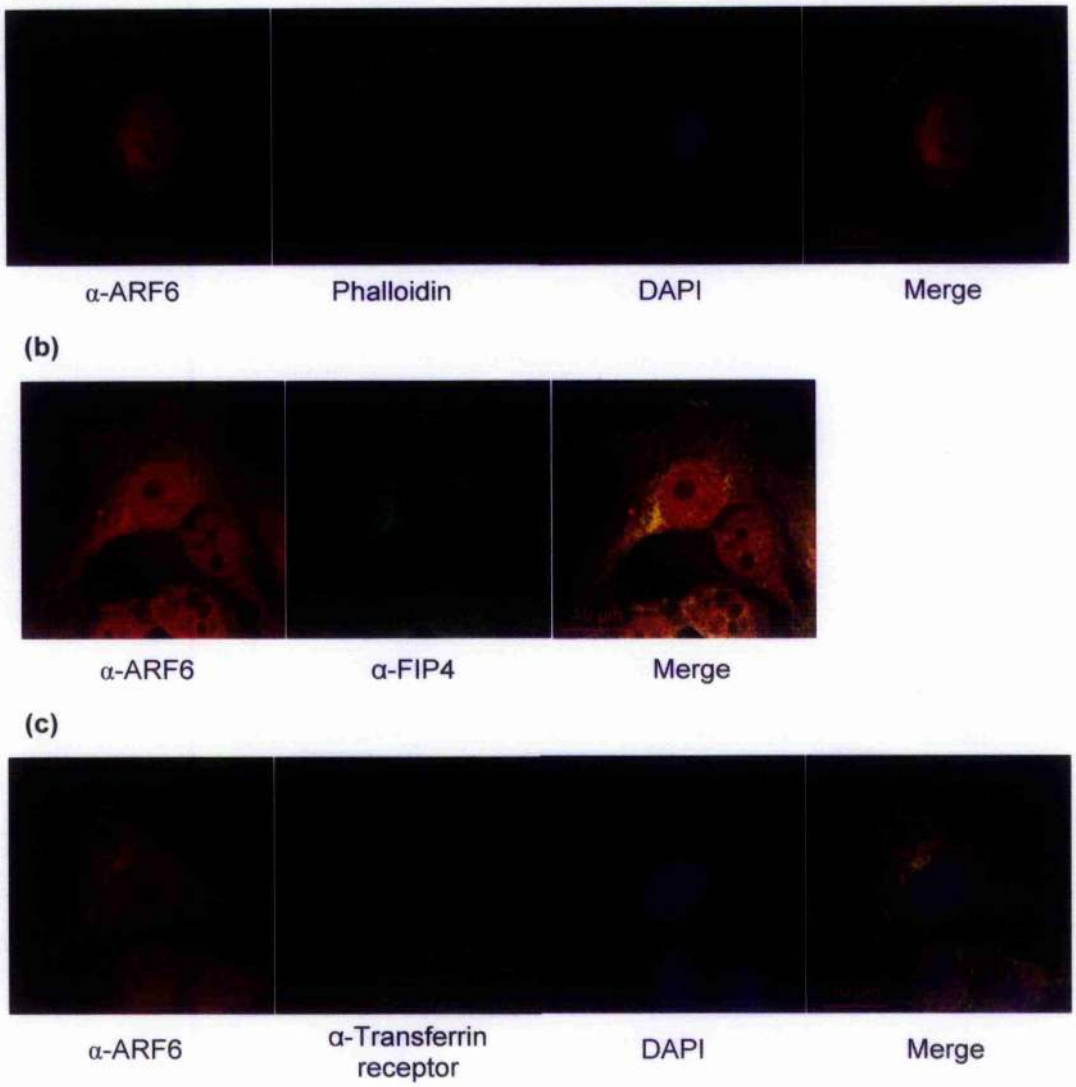


Figure 4.1 (a) HeLa cells were paraformaldehyde fixed onto glass coverslips and then permeablised with Triton-X-100, as described in section 2.4. Cells were then incubated with a rabbit polyclonal anti-ARF6 antibody, followed by an Alexa-Fluor 594nm fluorescent secondary anti-rabbit antibody (for antibody details see table 2.1). The cells were also incubated with Alexa-Fluor 488nm labelled phalloidin and DAPI. Images were taken on a Zeiss Pascal Microscope (see section 2.5) and the 594,488 and DAPI channels are shown separately and then merged together. **(b)** shows HeLa cells fixed, stained and observed in a similar fashion, except that this time an anti-FIP4 sheep polyclonal antibody was stained with an Alexa-Fluor 488nm labelled sheep secondary antibody in addition to the ARF6 594nm staining. **(c)** shows HeLa cells stained with an anti-transferrin receptor antibody (Alexa-Fluor 488nm labelled) in addition to ARF6 594nm and DAPI staining. Scale bars are shown in each of the merged images.

picture. There are also significant amounts of both ARF6 and FIP4 at some areas of the plasma membrane and, where present, they show an at least partially overlapping localisation. The staining of FIP4, seems to concentrate, although not exclusively so, to small spike-like structures. These probably represent focal adhesions as FIP4 has previously been shown to localise to these structures (Hickson et al., 2003). The ARF6 staining occurs a little more broadly at areas underlying the plasma membrane, showing some overlap with FIP4. It is well documented that ARF6 is present at many sites underlying the plasma membrane, usually associated with cortical actin (Song et al., 1998; Donaldson, 2003). It has also been shown to interact with components of focal adhesions (Kondo et al., 2000; Turner et al., 1999, 2001). Figure 4.1 (c) shows cells stained for ARF6, the Transferrin Receptor and DAPI. The Transferrin Receptor is a marker of the recycling endosome (and is used here as a substitute for Rab11 as the ARF6 and Rab11 antibodies available were both raised in the same species). It can be seen that there is some overlap between ARF6 and the Transferrin Receptor in a peri-nuclear patch but elsewhere there is little co-localisation. This suggests partial overlap between ARF6 and the recycling endosome.

Therefore, figure 4.1 shows that the staining seen for ARF6 agrees with other reports in that there is a plasma membrane and endosomal pool of ARF6. In these images it can be seen that the ARF6 endosomal pool is located in a peri-nuclear position. It is also clear that FIP4 co-localises with ARF6 at both the peri-nuclear and plasma membrane sites. This agrees with the finding in chapter 3 that ARF6 binds to FIP4. The partial overlap with the Transferrin receptor agrees with previous findings that show that there is an "ARF6 endosome" which is initially distinct from other endosome compartments but components of this endosome fuse with sorting endosome and may recycle through the conventional recycling endosome (Radhakrishna and Donaldson, 1997; Chavrier and Goud, 1999; Donaldson, 2003).

4.2.3 ARF5 localisation

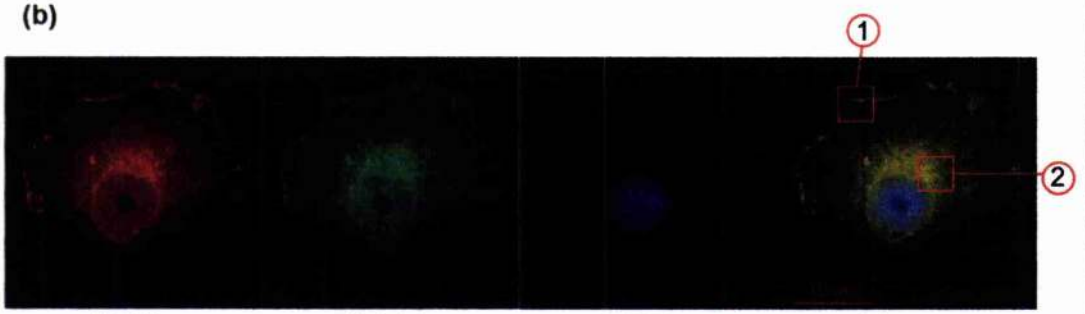
In Figure 4.2(a) a HeLa cell has been stained with a sheep anti-ARF5 antibody followed by a 594nm Alexa-Fluor conjugated anti-sheep secondary antibody. The cell has then been stained with 488nm Alexa-Fluor conjugated phalloidin toxin to label the actin cytoskeleton. It can be seen that the major pool of ARF5 staining is a peri-nuclear patch which looks very similar to that of FIP4 and ARF6 shown in figure 4.1. There is also some staining present at the plasma membrane which, similarly to FIP4, seems to concentrate at (although not exclusively to) small patches which could be focal adhesions. Further study is required to identify the nature of these sites.

Figure 4.2(b) shows cells stained with both the sheep anti-ARF5 antibody and a rabbit anti-Rab11 antibody labelled with 594nm and 488nm Alexa-Fluor conjugated secondary antibodies respectively. Figure 4.2(c) shows a close up view of a plasma membrane area and peri-nuclear area which are indicated in the "merged" panel of figure 4.2(b). Ideally I would liked to have co-stained ARF5 with FIP4 but as I only had one antibody available to me against each of these proteins and these were both raised in the same species this was not possible. As Rab11 has previously been shown to localise to the peri-nuclear recycling endosome and to have at least partial overlap with FIP4 it was thought that this would be an ideal marker to use to localise ARF5. It can be seen in figure 4.2(c) that the peri-nuclear patch of ARF5 is largely, although not completely, co-localised with the major pool of Rab11. The fact that some green and some red patches (rather than all being yellow) are visible indicates that although these proteins seem to reside close to each other they are not necessarily completely co-localised. The sites of ARF5 membrane association show a similar pattern in that, in the same patches there is also Rab11 staining present, although the red and green staining shows partial rather than complete co-localisation.

(a)

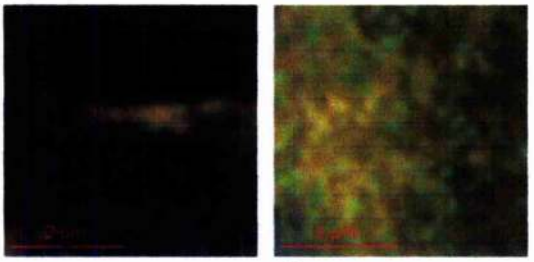
α-ARF5 Phalloidin Merge

(b)



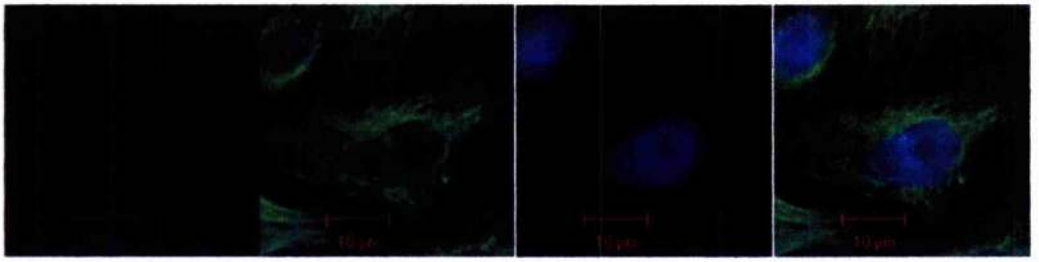
α-ARF5 α-Rab11 DAPI Merge

(c)



1 2

(d)



ARF5 pre-immune | Alpha-tubulin | DAPI | Merge

Figure 4.2 (a) HeLa cells were paraformaldehyde fixed onto glass coverslips and then permeabilised with Triton-X-100, as described in section 2.4. Cells were then incubated with a sheep polyclonal anti-ARF5 antibody, followed by an Alexa-Fluor 594nm labelled secondary anti-sheep secondary antibody (for details of antibodies and concentrations used see table2.1). The cells were also incubated with Alexa-Fluor 488nm labelled phalloidin (Molecular Probes) and DAPI, as described in section 2.4. Images were taken using a Zeiss LSM 5 Pascal Microscope (see section 2.5) and the 594nm and 488nm channels are shown separately and then merged together. **(b)** shows HeLa cells stained with an anti-Rab11 rabbit polyclonal antibody labelled with an Alexa-Fluor 488nm secondary antibody, a sheep polyclonal anti-ARF5 antibody, labelled with an Alexa-Fluor 594nm secondary antibody and stained for DAPI. In the "merged" panel, two boxes, labelled 1 and 2 are shown. **(c)** shows a larger image of the two boxes highlighted in panel (b). **(d)** shows HeLa cells which were incubated with pre-immune serum of the ARF5 antibody and subsequently with a 594nm Alexa-Fluor labelled secondary anti-sheep secondary antibody. In addition, cells were labelled with a anti-alpha tubulin antibody and a 488nm Alexa-Fluor labelled secondary anti-mouse secondary and

As the ARF5 antibody was made in our lab (although designed from a published peptide sequence which had previously been successfully used to make an ARF5-specific antibody (Cavenagh et al.,1996)) and had not previously been used for immunofluorescence, a control experiment was carried out, shown in figure 4.2(d). Cells were incubated with the pre-immune serum of the ARF5 antibody and then labelled with an Alexa-Fluor 594nm sheep secondary antibody. The blank "ARF5 pre-immune" panel in figure 4.2(d) indicates that there was no non-specific staining arising from components of the serum used to make the antibody. Cells were also stained for alpha tubulin and DAPI to show staining had been successful. In addition to this control, blots using the ARF5 antibody were carried out against recombinantly purified ARF4, ARF5 and ARF6 proteins. These blots showed that ARF5 is specifically stained by this antibody (data not shown).

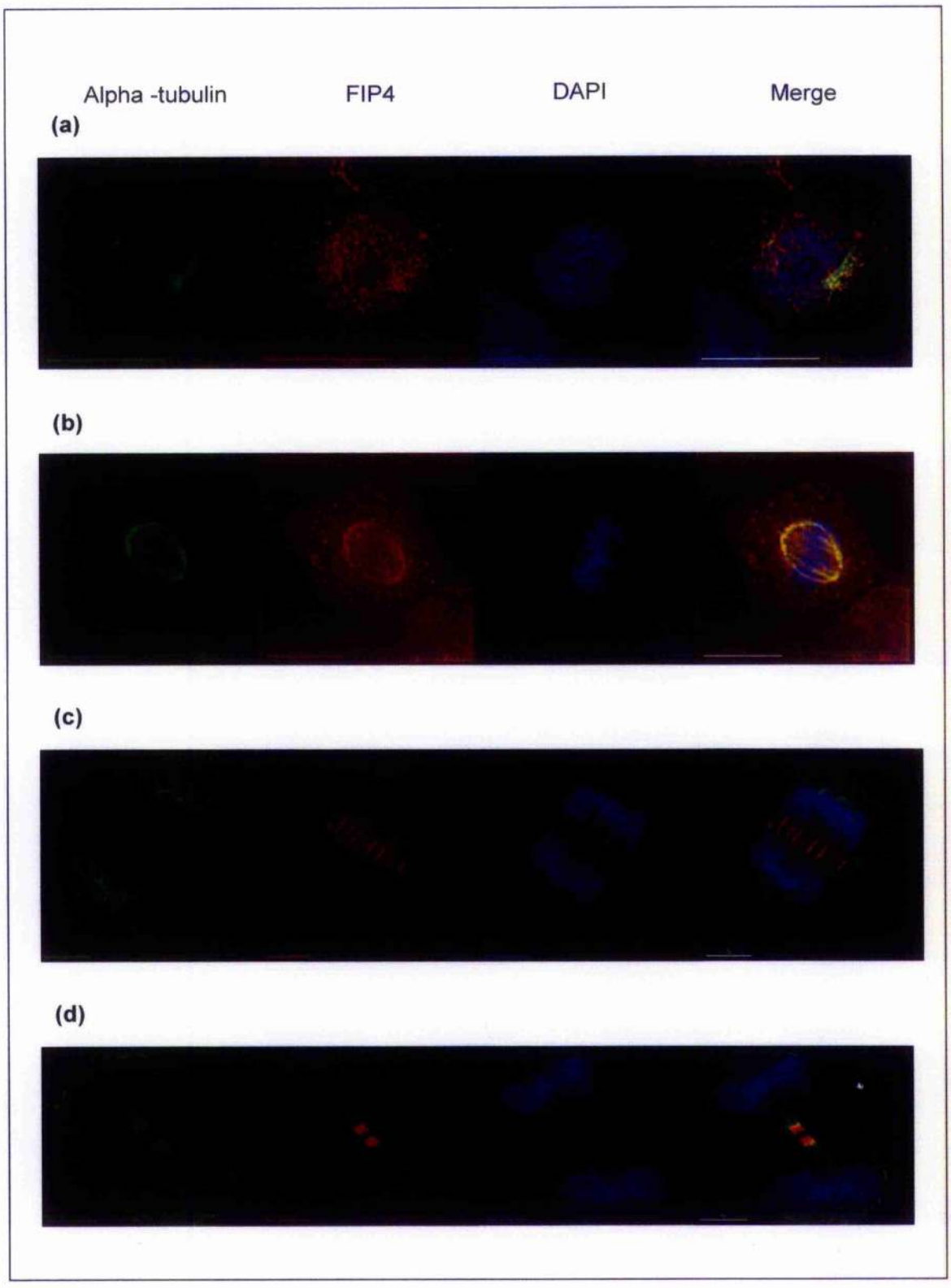
4.2.4 Staining of FIP4 during cell division

The localisation of FIP4 in interphase HeLa cells has been examined, both by myself and previously (Hickson et al., 2003). In the course of making these observations the striking localisation of FIP4 in dividing cells was noted, particularly its localisation to the spindle poles during late prophase/early metaphase and to the midbody and Flemming body in cells connected by just an intracellular bridge in late telophase. Therefore I thought it would be of interest to further study the localisation of FIP4 in dividing cells. A further reason to study FIP's localisation and possible function in cytokinesis is that, as discussed in section 1.2.13, FIP4 (and FIP3) are the mammalian homologues of the *Drosophila* protein Nuclear fallout. This has been shown to localise to the microtubule organising centre and be essential for the completion of cellularisation (Riggs et al., 2003), a process analogous to cytokinesis during the late syncytial divisions of a *Drosophila* embryo.

To examine the location of FIP4 during cell division, HeLa cells were fixed and stained as described in the section 2.4, except that, in order to gain a large number of cells undergoing cell division, cells were "pseudo-synchronised". This is a non-biochemical approach where by normally growing HeLa cells are trypsinised and split to approximately 15% confluency onto glass coverslips. Cells are then left for 17 hours and then fixed. This typically results in a high proportion (20-30%) of cells being fixed during cell division. Cells were then stained with anti-FIP4, anti-alpha tubulin to stain microtubules, DAPI to stain DNA and in some cases Mitotic Kinesin-Like Protein-1 (MKlp1) as a marker for the midbody ring structure (see section 1.1.11). Images of cells in prophase, metaphase, anaphase, telophase (during cleavage furrow ingression) and late telophase (where extended midbody is present) were taken.

4.2.5 FIP4's Localisation in Mitosis and Cytokinesis

Figure 4.3 (a) shows a cell which has just entered prophase, where the DNA, shown in blue, has started to condense into chromosomes and the green alpha-tubulin staining shows that the two microtubule organising centres (MTOCs), have been positioned on opposite sides of the DNA. FIP4 seems to show punctate staining of vesicular and tubulo-vesicular structures which have started to concentrate around the two MTOCs. As this staining still appears to be vesicular (rather than cytoplasmic) a simple hypothesis to explain this localisation would be that the endosomal compartment that FIP4 is largely present in during interphase has been split into two in parallel with the centrosome /MTOC and has now moved with the two separated centrosomes to the opposite MTOCs. This would be supported by interphase observations of FIP4 localisation, where it resides in an endosomal compartment which is associated with the centrosomes, localises to purified centrosomes and, upon overexpression, collapses to a centrosomal location (Hickson et al., 2003).


Alpha -tubulin
FIP4
DAPI
Merge
(a)
(b)
(c)
(d)

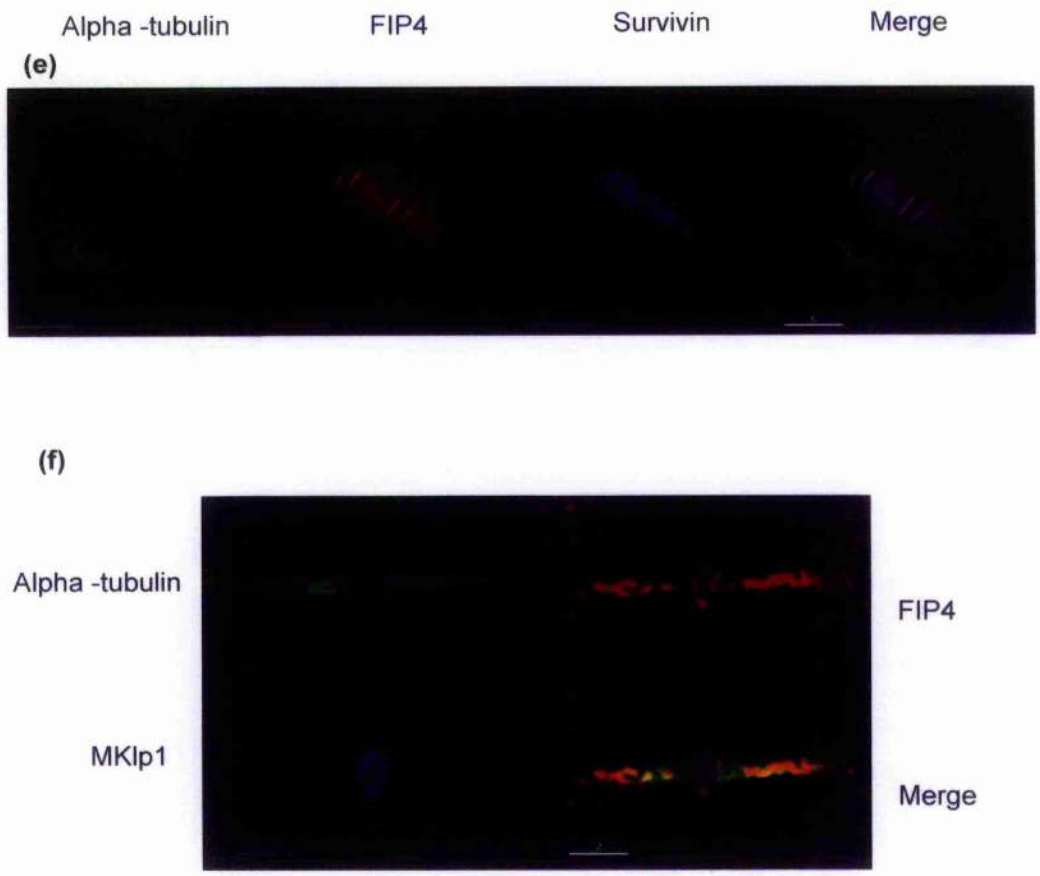


Figure 4.3 Panels **(a)-(d)** show HeLa cells that have been fixed and stained for alpha tubulin (488nm secondary antibody), FIP4 (594nm secondary antibody) and DAPI (for antibody details see table 2.1). Cells shown are at various stages of cell division, namely **(a)** prophase, **(b)** metaphase, **(c)** anaphase and **(d)** early telophase. For each image the 488nm, 594nm and DAPI channels are shown separately and then merged. Panel **(e)** shows the same cell as panel **(c)**, except that here the image shows staining for Survivin (stained with 647 secondary and appearing as blue) in addition to the 488nm aplha tubulin and 594nm FIP4 staining. Panel **(f)** shows a close up view of a midbody forming an intracellular bridge between two, almost separated, daughter cells, in late telophase. Again alpha tubulin is in the 488nm channel and FIP4 in the 594nm channel, but this time MKLP1 is stained with a 647nm secondary and is coloured blue. All of these images were acquired on a Deltavision microscope and de-convolved using the appropriate software. section 2.6).

In figure 4.3 (b) a cell in the next stage of division, metaphase, is shown. Here it can be seen that the chromosomes have aligned halfway between the two MTOCs and that an array of microtubules stretch from each MTOC towards the aligned chromosomes. Here, although there is some punctate, vesicular, FIP4 staining throughout the cell, a large proportion of FIP4 seems to have moved onto the microtubule arrays, where it is co-localising with alpha tubulin. It seems that FIP4 has been loaded onto the microtubules and is being delivered towards the spindle-midzone area of the cell.

Figure 4.3(c) shows a cell in anaphase, where the chromosomes have segregated and are moving towards each of the MTOCs at opposite ends of the cell. FIP4 now seems to be positioned on the spindle midzone microtubules, the area of overlapping microtubules equidistant from each of the two MTOCs. Here it co-localises with Survivin, a protein known to position to the spindle midzone (Wheatley et al., 2001).

A cell in early telophase is shown in figure 4.3 (d). By this stage mitosis is complete, the nuclear envelope has formed and it can be seen the DNA has started to de-condense as individual chromosomes are no longer discernable. The cleavage furrow has largely ingressed and the spindle midzone microtubules are starting to be compacted to form the midbody. Here, the majority of FIP4 localises to towards the ends of the microtubule bundles which will go on to form the midbody structure. In the final late telophase image in figure 4.3(f), the cells are now only connected by a narrow canal of cytoplasm which contains tightly bundled microtubules whose opposite ends overlap in the protein dense central "Flemming Body". Encircling this structure is the "midbody ring", a structure which is thought to be an important site for the delivery of membrane for the final "abscission" of the intracellular canal to form two separate daughter cells (see section 1.1.14). Here, the midbody ring is stained with MKlp1 in blue, a protein which is known to localise to this structure at a relatively early stage after its formation.

As can be seen, FIP4 is present on the microtubules of the midbody as well as on the midbody ring where it co-localises with the MKlp1 staining. It should be noted here that this midbody ring localisation is likely to be a relatively late event as, as can be seen in this image there is still a large amount of FIP4 localised to the midbody microtubules rather than on the ring itself. Also, in some cells FIP4 can only be seen localised to the midbody microtubules and not to the ring structure. It is likely that these cells are at a slightly earlier stage of division where the FIP4 has not yet translocated to the centre of the midbody.

4.2.6 Localisation of FIP4's Binding Partners During Cell Division

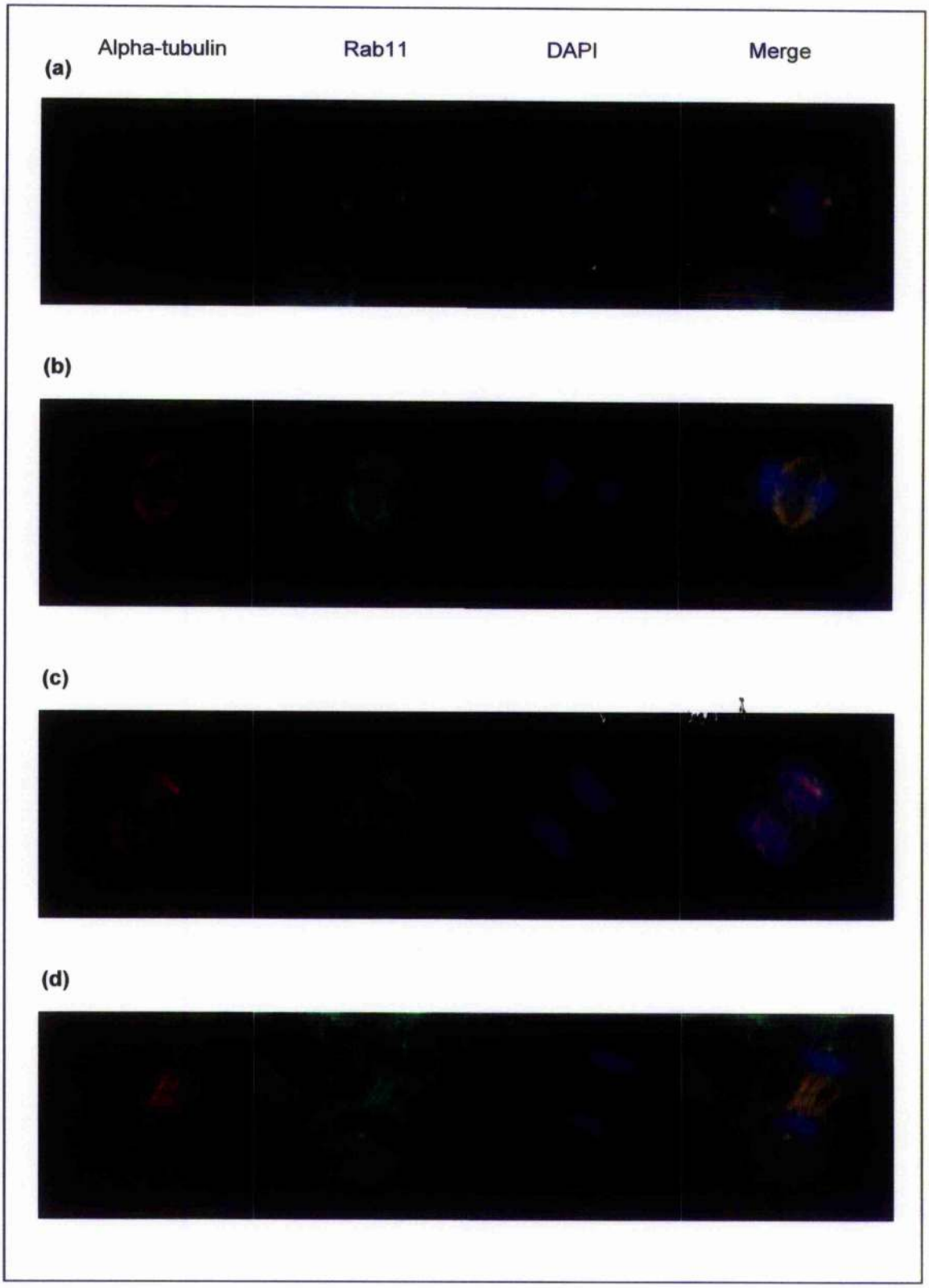
As FIP4 showed a striking localisation throughout mitosis and cytokinesis it was decided to also localise the binding partners of FIP4 which had been identified, i.e. ARF5, ARF6 and Rab11. As the discovery of Rab5 binding to FIP4 was a relatively late event in the work and that the antibody available for Rab5 did not work well for immuno-fluorescence, the localisation of this protein during cell division was not examined.

As for the localisation of ARF5, ARF6 and Rab11, as well as being an interesting question to ask from the results of my work alone, there is also substantial evidence that membrane traffic involving members of the Rab and ARF families of small GTPases is essential for the completion of cytokinesis. Indeed Rab11 has been shown to be required for cytokinesis in *Drosophila* and *C.elegans* (Pelissier et al., 2003; Riggs et al., 2003; Skop et al., 2001) but a function in mammalian cytokinesis has not yet been demonstrated. ARF6 has also been shown to have an involvement in cytokinesis, this time in mammalian cells (Schweitzer and D'Souza-Schorey, 2002). The localisation and possible function of ARF5 in cytokinesis, however, has not previously been studied.

To study their localisation in cells undergoing division, the same approach as for the FIP4 studies was taken.

4.2.7 Rab11's localisation during cell division

A Zymed anti-Rab11 antibody was used to stain for Rab11. Figure 4.4 shows Rab11 staining in prophase, metaphase, anaphase, late anaphase, telophase and late telophase. In figure 4.4(a) a cell in prophase is shown. This cell is probably at a slightly more advanced stage than the FIP4 prophase image as the MTOCs are now well defined, as shown by alpha-tubulin staining radiating from two dense centres and that the chromosomes have begun to align along the centre of the cell. Here, although some Rab11 can be seen throughout the cell, it can be seen strongly concentrated around each of the two MTOCs, with some staining starting to appear on some of the microtubules radiating towards the chromosomes. Like FIP4, the most simple explanation for this strong staining would be that the endosomal compartment that Rab11 resides in during interphase has now divided into two and become concentrated around the centrosomes/MTOCs. In metaphase, shown in figure 4.4(b), a large proportion of Rab11 is seen to have moved onto the microtubules leading towards the aligned chromosomes, whilst some remains at the MTOCs. As for FIP4, a population of the Rab11 seems to remain in punctate structures distributed throughout the cell. By anaphase, shown in figure 4.4(c), Rab11's localisation has become less distinct, although a proportion of the staining remains associated with the MTOCs and there does seem to be a slight concentration of Rab11 on the microtubules close to the centre of the cell, where the midzone microtubules have not yet formed a dense structure. In figure 4.4(d), a cell in late anaphase is shown. By this stage, it can be seen that the midzone microtubules are being bundled together and have started to form the midbody structure. It can be clearly seen that Rab11 is concentrating on the midzone microtubules, as well as distinct


Alpha-tubulin
Rab11
DAPI
Merge
(a)
(b)
(c)
(d)

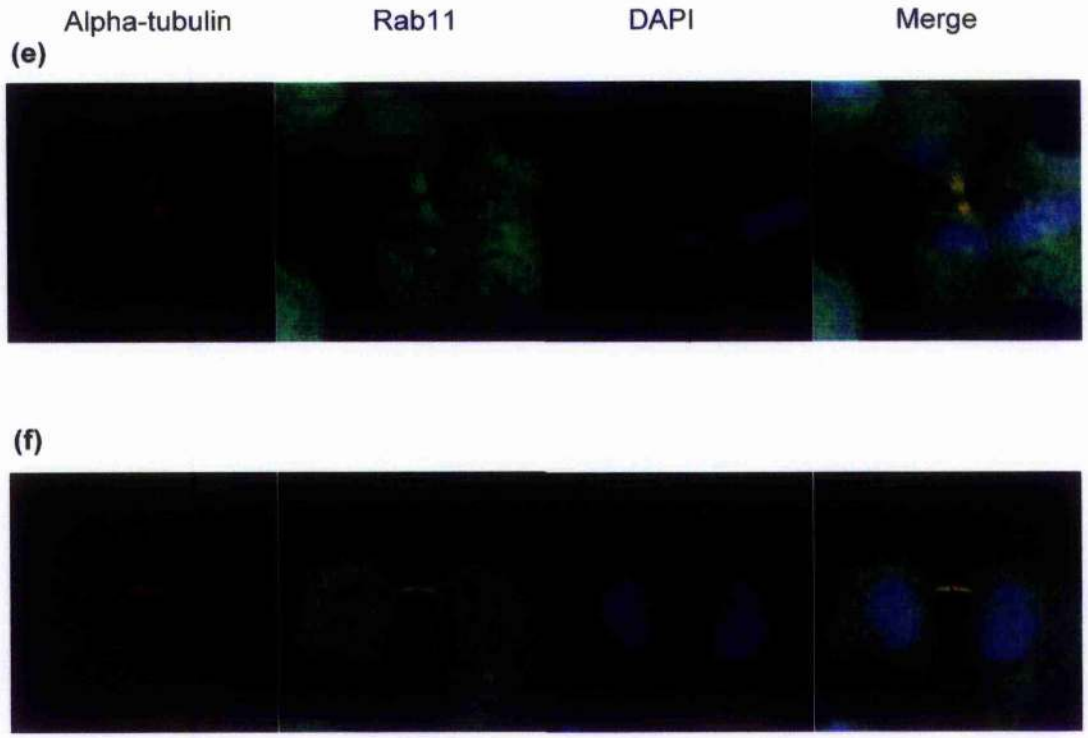


Figure 4.4 HeLa cells at various stages of cell division were fixed and stained (as described in section 2.4) with an alpha tubulin antibody which was labelled with a 593nm Alexa-Fluor conjugated secondary antibody and a Rab11 antibody which was labelled with a 488nm Alexa-Fluor conjugated secondary antibody (for antibody details see table 2.1). In addition the cells were DAPI stained. Images were taken on a Zeiss LSM 5 Pascal microscope (see section 4.5). Cells in **(a)** prophase, **(b)** metaphase, **(c)** early anaphase, **(d)** late anaphase, **(e)** early telophase and **(f)** late telophase are shown. In each case the 593nm, 488nm and DAPI channels are shown separately and then merged.

staining being visible at the two MTOCs. Figure 4.4(e) shows a cell in telophase where the Rab11 staining is now clearly concentrated on the densely packed midzone microtubules adjacent to the cleavage furrow, which has almost completely ingressed. By late telophase, shown in figure 4.4(f) a significant amount of Rab11 has moved onto the midbody microtubules where it is clearly concentrated and some Rab11 has reached the central Flemming body/ midbody ring structure.

4.2.8 Localisation of ARF 6 during cell division

An anti-ARF6 antibody was used to image ARF6 localisation through the various stages of cell division. Figure 4.5 shows panels of images of ARF6 stained in cells at prophase, metaphase, anaphase and late telophase. The images shown were taken in the absence of alpha tubulin staining as a control to verify that high degree of overlap seen between the microtubules and ARF6, Rab11 and FIP4 was indeed genuine.

In figure 4.5(a) a cell with condensed, yet not yet aligned chromosomes is shown, indicating that the cell is in prophase. Here, the ARF6 staining is largely clustered around two points and in addition some staining can be seen on thread-like projections emanating towards the centre of the cell, suggesting that ARF6, like FIP4 and Rab11 is clustered around the two MTOCs and has started to move onto microtubules. In figure 4.5(b) a cell in metaphase is shown, with its condensed chromosomes aligned in the centre of the cell. Here ARF6 can be clearly seen in two dense patches, the MTOCs and now, much more clearly, on the microtubules which are leading towards the condensed chromosomes. Figure 4.5(c) shows a cell in early anaphase where the chromosomes have begun to separate and move to opposite poles of the cell. Here ARF6 is still visible at one of the MTOCs, although less so at the second which may be out of focus. Like FIP4 in anaphase, it is now also clearly visible between the separating chromosomes at the site of the spindle midzone microtubules. A close up

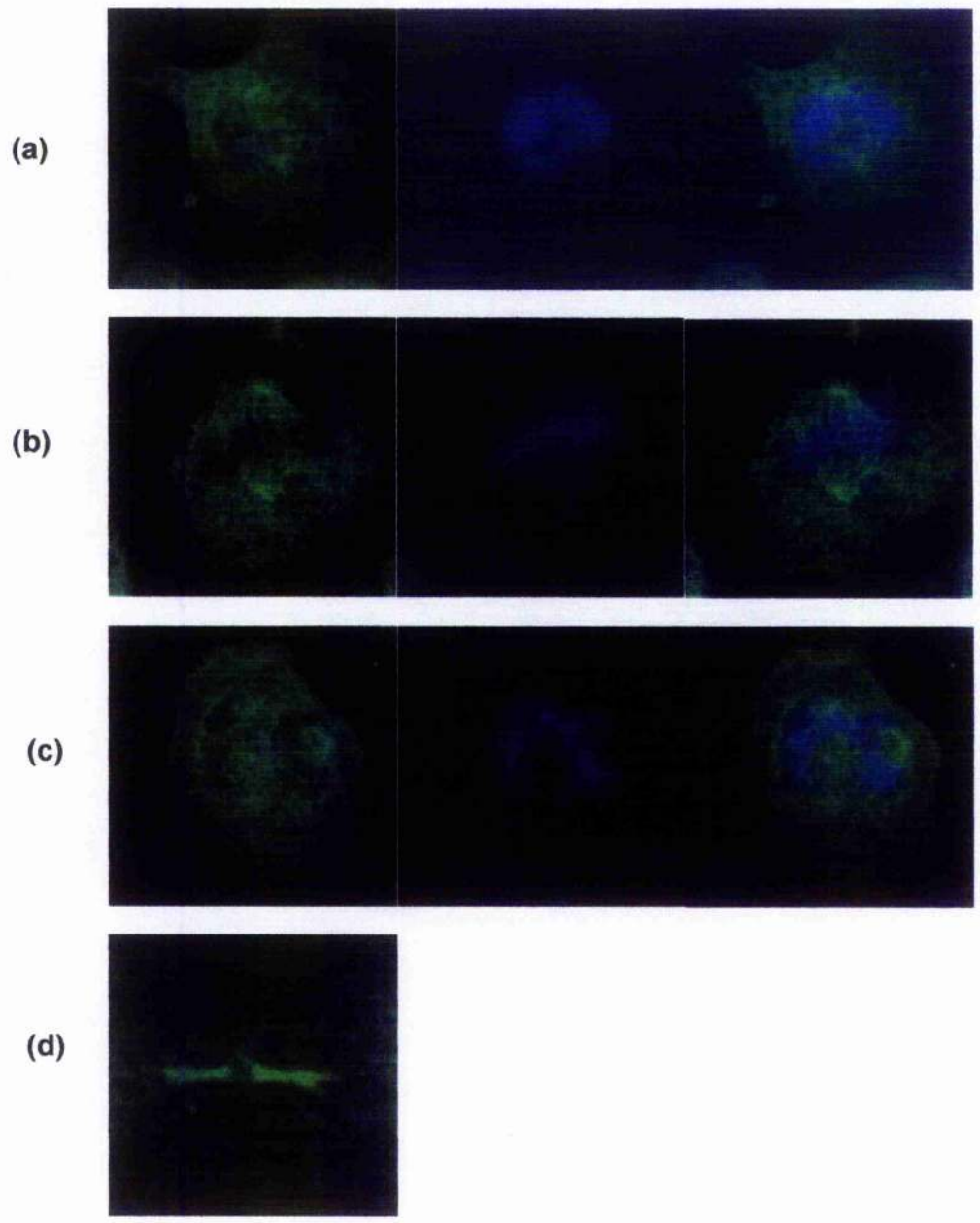


Figure 4.5 HeLa cells at various stages of cell division were fixed and stained (as described in section 2.4) with an anti-ARF6 antibody which was labelled with a 488nm Alexa-Fluor conjugated secondary antibody (for antibody details see table 2.1). In addition the cells were DAPI stained. Images were taken on a Zeiss LSM 5 Pascal microscope (see section 4.5).Panels show cells in **(a)** late prophase, **(b)** metaphase, **(c)** anaphase and **(d)** telophase. The 488nm and DAPI channels are shown separately and merged.

of an intracellular midbody bridge is shown in figure 4.5(d). ARF6 is very clearly concentrated along both arms of this bridge, with only fainter staining at the Flemming Body at the centre of the bridge visible at this stage. Again, this staining is very similar to that seen for both FIP4 and Rab11.

4.2.9 ARF5 localisation at late telophase

The anti-peptide ARF5 antibody described in section 4.2.3 above was used to localise ARF5 at the final stage of cell division, late in telophase where the cells are connected only by a thin intracellular bridge. Figure 4.6 shows two cells at this late stage of division. In both of these cells, ARF5 localises to the "midbody ring" structure where it is co-localised with MKlp1, as described for FIP4. The graphs shown beneath the images verify this localisation and co-localisation with MKlp1. They are a plot of the intensity of fluorescence measured across the midbody structure (as indicated by the red lines on the images) for DAPI, ARF5 and MKlp1. It can be seen that in figure 4.6(a) that ARF5 concentrates at the centre of the midbody with MKlp1. In figure 4.6(b) an enlarged view of the midbody can be seen. The accompanying graph shows not only that ARF5 and MKlp1 are concentrated at the centre of the midbody but both appear as a twin peak in this area, indicative of the "midbody ring" structure that they are both present in.

Experiments are currently being carried out by members of our laboratory to identify the localisation of ARF5 at earlier stages of cell division and cytokinesis. It is predicted that it will show a similar localisation to Rab11 (and ARF6 and FIP4) as it largely co-localises with Rab11 during interphase (see section 4.2.3) and here shares the same localisation as Rab11, ARF6 and FIP4 in late telophase. As can be seen from figures 4.5 and 4.6, the localisation of ARF6 at each stage of cell division and of ARF5 in the late telpohase stage are very similar to that of both Rab11 and FIP4.

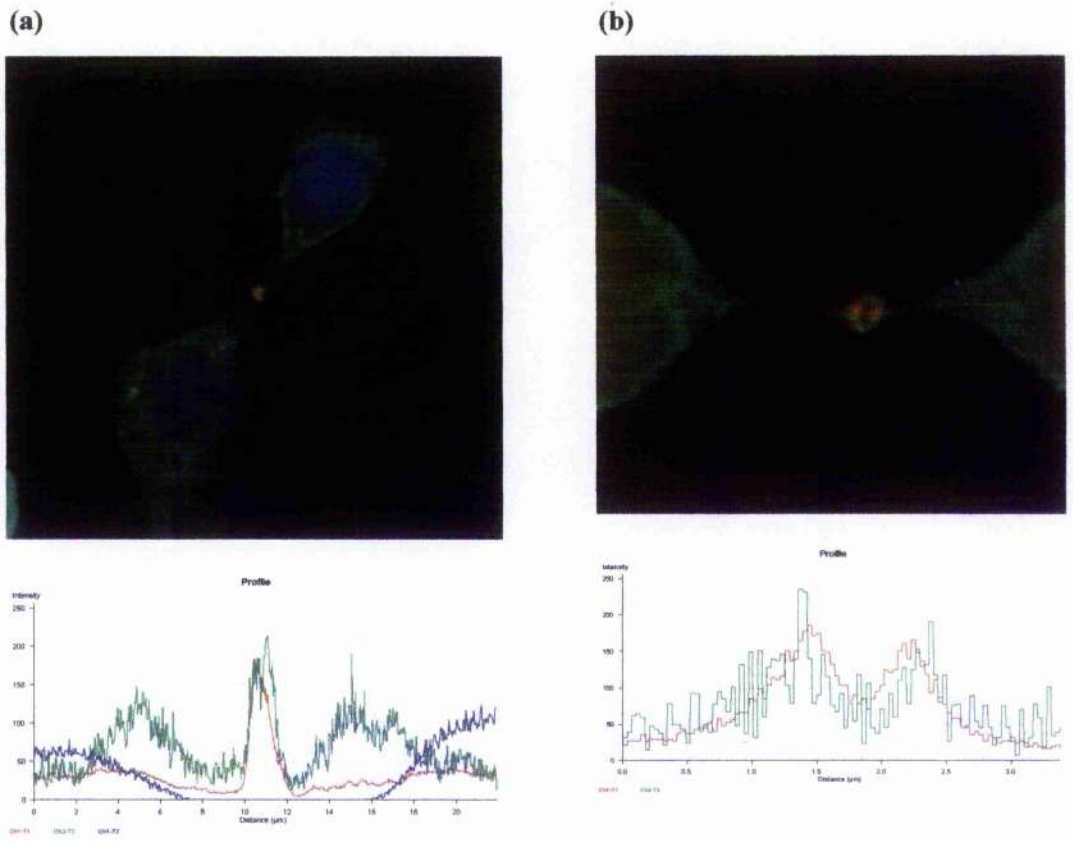


Figure 4.6 HeLa cells were fixed and stained (as described in section 2.4) with an ARF5 antibody which was labelled with a 488nm Alexa-Fluor conjugated secondary antibody and a MKLP1 antibody which was labelled with a 594nm Alexa-Fluor conjugated secondary antibody (for antibody details see table 2.1). In addition the cells were DAPI stained. Images were taken on a Zeiss LSM 5 Pascal microscope (see section 4.5). Two cells in late telophase are shown in (a) and (b). Panel **(a)** shows a picture of a whole cell whilst panel **(b)** shows a view of the midbody of a cell. In both cases a red line has been traced along the centre of the midbody. In both cases the graphs shown beneath the images show representations of the intensity of fluorescence of each of the fluorophores along the traced red line (intensity is in arbitrary units on the Y-axis whilst distance, in µm, is on the X-axis). The colours on the graphs match the fluorophores they represent in the images. The total length of the traced red line in image (a) is 22µm whilst in image (b) is 3.5µm.

4.3 Discussion

4.3.1 Localisation of FIP4 and its binding Partners In Interphase Cells

It has been previously shown that FIP4 has some overlapping with Rab11 and therefore the pericentrosomal recycling endosome (Hickson et al., 2003). However, although the majority of FIP4 does undoubtedly reside in a peri-centrosomal patch, this patch only shares partial co-localisation with Rab11 and the precise location of the remaining FIP4 in interphase cells has, as yet, remained undetermined. Here it is shown that ARF6 staining in HeLa cells occurs mostly at a peri-centrosomal patch. This patch shows partial, but by no means complete localisation with Transferrin Receptor staining, which, like Rab11 is a marker of the recycling endosome. This suggests that ARF6 and FIP4 may share the same localisation, in an endosomal compartment which partially overlaps the recycling endosome compartment. This is confirmed by co-localisation studies of ARF6 and FIP4 which show that these two proteins co-localise almost completely in interphase cells (disregarding the unconfirmed nuclear localisation of ARF6). Therefore I would suggest that FIP4 resides in the "ARF6 endosome" in HeLa cells, as described by Donaldson and co-workers (Radhakrishna and Donaldson, 1997; Donaldson, 2003). This endosome's constituents and properties have not yet been entirely elucidated although the fact that it shows a peri-centrosomal location and some overlap with the Rab11/Transferrin Receptor recycling endosome does not contradict any previous studies on ARF6. A a plasma membrane pool of ARF6 is also seen here, which again is well documented by others (Song et al., 1998; Donaldson, 2003). FIP4 also seems to at least partially co-localise with ARF6 at the plasma membrane.

These suggestions of nearly complete overlapping localisation of FIP4 and ARF6 agree with my findings in chapter 3 which show that FIP4 binds to ARF6. Taking both the localisation and binding results together may suggest that FIP4 shows "constitutive" binding to ARF6 within a cell. Where ARF6/FIP4 and Rab11 localisations overlap may

indicate the presence of an ARF6+FIP4+Rab11 trimeric complex, the formation of which was shown to be possible in chapter 3.

The localisation of ARF5 in interphase HeLa cells has also been studied, using DAPI, phalloidin and Rab11 co-staining to aid identifying its localisation. It was not possible to directly visualise the co-localisation of ARF5 with FIP4 as the antibodies which were available for both of these proteins were raised in the same species. ARF5 showed a very similar co-localisation to Rab11, with both a major peri-centrosomal pool and a plasma membrane pool, which, like Rab11, FIP4 and ARF6, concentrated at spike-like structures which are likely to be focal adhesions.

Several possible theories can be proposed which may explain the function of ARF5 based on the localisation and binding properties that have been discovered here, although this list is obviously not complete. A first possibility is that ARF5 plays a redundant role to ARF6, as it seems to share a similar localisation to ARF6 and yet its binding to FIP4 seems to be considerably weaker. However this seems to be a somewhat naïve idea as weaker binding of FIP4 to an ARF may be required for some processes. Also the possibility that both ARF5 and ARF6 could simultaneously bind to FIP4 was not tested. As the exact binding sites for the ARFs on FIP4 have not yet been identified this would be difficult to predict. Also it may be the case that there are specific localisations of ARF5 and therefore perhaps compartmentalised interactions with FIP4 that have not been able to be identified here due to the resolution of images possible with confocal microscopy.

Another possibility is that ARF5 may be more constitutively present in the Rab11 recycling endosome compartment than ARF6, therefore having a specific role in this compartment. This is a good possibility as ARF5 seems to have almost total co-localisation with Rab11, unlike ARF6 and FIP4 which have been concluded to lie in an "ARF6 endosome", which only has partial co-localisation with the Rab11 recycling

endosome. This could also explain the weaker binding of FIP4 to ARF5 that was found as it may only be desirable for this interaction to take place whilst FIP4 is in the recycling endosome. A transient binding to ARF5 to FIP4 would ensure that once FIP4 moves out of the Rab11 recycling endosome, the localisation of ARF5 could be maintained within this compartment.

4.3.2 FIP4, Rab and ARF localisation during cell division

Here it has been shown that FIP4, Rab11 and ARF6 to share a very distinct localisation at each stage of cell division and in addition shown that in the final stage of division, ARF5 also has the same localisation as the other proteins. At prophase it is clear that FIP4, Rab11 and ARF6 are tightly clustered around the centrosomes/ MTOCs. As the normal interphase localisations of all three of these proteins is lost it seems most likely that cause for this tight peri-centrosomal location at prophase is due to the "collapse" of the ARF6 and/or Rab11 recycling endosome to this point. The cause of this collapse is not clear, although a feasible reason could be a change in the way the endosome interacts with the microtubule network at the onset of mitosis. A candidate for the mediation of this interaction is FIP4 itself as it has previously been shown that if C-terminally truncated constructs of FIP4 are over-expressed in cells then this causes a collapse of the recycling endosome to a tight peri-centrosomal spot, similar to that seen in prophase cells. Therefore it is possible that the C-terminal section of FIP4 mediates an interaction between the endosome and the microtubule network which under normal circumstances anchors it in place. In the event of truncation, or in the case of the onset of mitosis, a modification such as phosphorylation, FIP4 may no longer be able to mediate the endosome's interaction with the microtubule network, therefore causing its collapse to a tight spot. In the case of mitosis, this collapse will have the beneficial function of

brining the contents of the endosomes into close proximity with the MTOC. Once at the MTOC, FIP4, ARF6 and Rab11 are now in a position to be loaded onto microtubules.

By metaphase it is clear that FIP4, Rab11 and ARF6 are associated with the microtubules leading towards the aligned chromosomes at the centre of the cell and that by anaphase they have moved past the chromosomes and are now associated with the spindle midzone microtubules. Again, how this association takes place is not known, but one of the possibilities are described in section 4.3.3, below.

FIP4, Rab11 and ARF6 remain associated with the spindle midzone microtubules through into telophase where the microtubules elongate to from the midbody. Finally, late in telophase FIP4 and at least some ARF6, ARF5 and Rab11 move to the very centre of the midbody and become associated with a midbody ring structure, concurrent with MKlp1 staining. It is maybe here that the FIP4/ARF6/Rab11 complex is having its effect, as it is thought that this ring structure serves as a docking site for the delivery of membrane fusion and remodelling components. For example, both SNAREs and the exocyst, a complex of proteins required for exocytosis, have been shown to localise to this ring structure (Gromley et al., 2004). In addition it has here been shown that ARF6, ARF5 and Rab11 also go to this structure. Obviously these are also proteins known for their membrane interactions, the ARFs for their role in vesicle formation and, at least in the case of ARF6, cortical actin re-arrangements and the Rabs for their multiple roles in vesicle trafficking. Once all components have reached this site it is likely that a complex interaction takes place between the membrane at the centre of the intracellular canal, the entourage of membrane trafficking and remodelling proteins which have been delivered and, possibly, new membrane which has been delivered to this site as vesicles along the midbody microtubules. These interactions will lead to the sealing of the intracellular canal into two separate daughter cells and therefore cytokinesis will be complete.

4.3.3 Association of FIP4+ARF6/ARF5+Rab11 Complexes with the Microtubule Network During Cytokinesis

The descriptions above show that FIP4, ARF6/5 and Rab11 seem to associate with and move along microtubules throughout cell division, firstly moving from the MTOCs to the spindle midzone and later moving along the midbody to the midbody ring structure present at its very centre. How this is achieved is not known but obvious candidates are the kinesin/dynein family of proteins which can both bind cargo and "walk" along microtubules, thereby providing a means of transporting cargos around cells. There are many members of the kinesin/dynein family and it is not currently known which protein interacts with FIP/ARF5/6 and Rab11. However one good candidate is Mitotic Kinesin Like Protein 1 (MKlp1). This protein is known function in cytokinesis and locate to the midzone microtubules, where it serves a microtubule-bundling function and to the "midbody ring" structure, where here it was observed co-localising with FIP4. In addition to its known cytokinesis function and co-localisation with FIP4 during cell division there is a further reason to suspect this proteins specific involvement with the proteins being investigated here. That is that it has been shown to be able to bind to every member of the ARF family of proteins (Boman et al., 1999). Therefore, although at this stage purely speculation, MKlp1 would provide a mechanism for the localisation of an ARF6/FIP4/Rab11 complex that I have observed during cytokinesis.

4.3.4 What is function of ARF5/6+FIP4+Rab11 localisation?

Possibly the simplest hypothesis to explain the localisation of ARF5/6+FIP4+Rab11 throughout cytokinesis is that, as discussed above, Rab11 and ARF5 and/or 6 are required as membrane trafficking proteins at the centre of the midbody during the final stages of cytokinesis. Here they interact with other components

that have been delivered there to make the final abscission of the intracellular bridge and seal the ends to form two separate daughter cells. The localisation seen at the earlier stages of cytokinesis may simply be the most efficient route for the delivery of these proteins to the midbody during telophase. In this model FIP4 would act as a scaffold type protein, binding both an ARF and Rab11 simultaneously and ensuring their delivery to the same place and at the same time where they can then carry out their functions correctly. This idea seems logical as a Rab protein will probably act immediately after an ARF protein in the course of vesicle delivery; i.e. the ARF is required for the formation and budding of the vesicle and once budded, this vesicle now needs to bind a Rab protein to ensure it is tethered to the correct target membrane. Also, as shown by the interphase co-localisation experiments, ARF6, 5 and Rab11 all originate from the same area of the cell, even if the overlap between their localisations is not complete. Therefore it makes sense that FIP4 would bind these proteins here and then the complex be delivered, most probably as constituents of a membrane vesicle, from the same part of the cell to the midbody. This then is the simplest situation, i.e. the ARFs and Rab11 are delivered, coupled by FIP4 to the centre of the midbody, where they have their effect.

A more complex situation may occur in that in addition to the ARF/FIP4/Rab11 complex and membrane vesicles being delivered together, actin would also be part of the "package". This is proposed in parallel with the model for Nuf (the *Drosophila* homologue of FIP4/3) action, which is proposed to load vesicles containing actin and deliver these to the cleavage furrow, thereby providing a source of both the membrane and actin required for furrow invagination (see section 1.1.9 and figure 1.1). However, this hypothesis does not fit as well with the model proposed here as it is envisaged that the ARF/FIP4/Rab11 complex would have its major effect at the midbody abscission stage rather than the furrow ingression stage, due to its localisation to the midbody and midbody ring. At this final stage it is unlikely that actin is still required.

Having discounted this, however, it should also be mentioned that the situation could be more complex still, with the ARF/FIP4/Rab11 complex acting not just at the final abscission event but additionally at other, earlier stages of cytokinesis.

However as there is currently no clear evidence for any of the above hypotheses the most simple hypothesis should first be tested. That is that the ARF/FIP4/Rab11 complex is delivered to the midbody and here it has its effects, at a late stage of cytokinesis.

In the next chapter it was aimed to establish if these proteins are indeed required for cells to divide successfully.

Chapter 5

Do Rab11, ARF5/6 and FIP4 Play a Functional Role in Cytokinesis?

Chapter 5 : Do Rab11, ARF5/6 and FIP4 Play a Functional Role in Cytokinesis?

5.1 Introduction - Bi-nucleate Experiments

In this chapter I wanted to start to investigate whether the striking localisations of FIP4, Rab11, ARF5 and ARF6 that were observed throughout cell division in the previous chapter are indicative of a functional role for these proteins in this process. One of the most striking and therefore easily quantifiable defects observed when cell division fails at a late, post-anaphase, stage is the formation of bi-nucleated cells. These occur when mitosis, i.e., the separation of the chromosomes into the two halves of the cell, has been completed successfully but then cytokinesis fails and therefore the cell fails to divide into two. In this case the end result is a cell containing two nuclei. Therefore an easy way to check whether a protein is involved in cytokinesis is to perturb it's action is some way in living cells, for example by over-expression of a mutant form, knocking down expression using RNAi or prevent the protein from binding to its normal partners by injection of antibody. The cells are then left for enough time for the perturbation to have its effect and the cells to go through the cell cycle at least once (a HeLa cell cycle lasts 15-20 hours on average) and finally fix the cells, stain the nuclei and count the percentage of cells with two or more nuclei. This technique has been used in many studies on cytokinesis and provides a relatively quick and easy approach to investigate whether a protein is required for cytokinesis. However, it does have a number of drawbacks. Firstly, as emphasised in section 1.2.1, cytokinesis seems to be a remarkably resilient process making any type of study on its individual constituents a difficult process. There are many "backups" in place, meaning that perturbing one protein may have little or no effect on the process if its semi-redundant partner is still able to function. In some cases, even when a seemingly essential process is perturbed, the cytokinesis machinery struggles on and eventually successfully divides the cell, although

this may be at a significantly slower rate than normal. Live cell experiments may be able to detect a delay in cytokinesis but the bi-nucleate experiment described above will not. The other limitation of the experiment is that if a bi-nucleate phenotype does occur it cannot be determined at which stage cytokinesis has failed. It may have been an early furrow ingression event or could have been that much later, the final abscission stage has failed and the cleavage furrow has subsequently regressed. A further problem is that bi-nucleate cells are not the only phenotype associated with cells that have failed cytokinesis. In some cases, cells failing cytokinesis remain interconnected by a long, intracellular bridge or in other cases may undergo apoptosis.

Despite its limitations, however, the bi-nucleate experiment is an ideal method to start determining whether a protein is involved in cytokinesis. Therefore, in this chapter this approach has been used, with various methods of protein perturbation, to start an initial investigation into the involvement of FIP4, ARF5 and Rab11 in cytokinesis (as discussed in section 1.2.3, ARF6 has already been shown to play a role in mammalian cytokinesis; Schweitzer and D'Souza-Schorey 2002).

5.2 Results

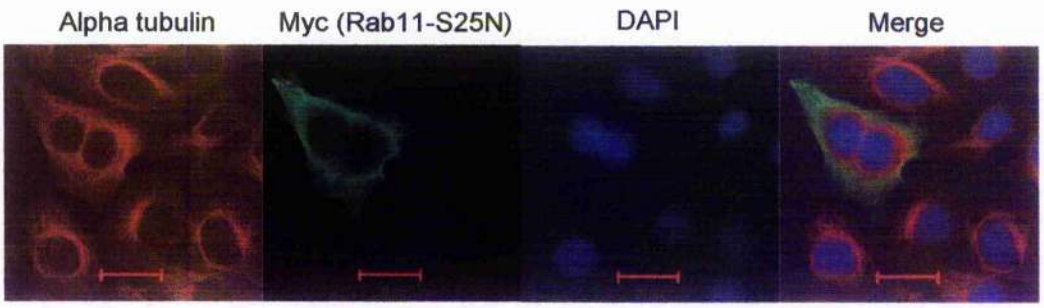
5.2.1 Rab11-S25N Virus Bi-nucleate experiments

Rab11-S25N refers to a point mutation in Rab11 sequence in which an asparagine residue is substituted for serine 25. This amino acid is located in the loop 1 region of the protein, which, together with loop 2, is the region that binds to GTP/GDP. The S25N mutation renders Rab11 in a state unable to bind GTP and therefore is referred to as an "inactive" mutant. This is a well characterised mutant which has been used in many studies on Rab11 and has been shown to block normal recycling through the pericentrosomal recycling endosome compartment (Ullrich et al., 1996; Ren et al., 1998). Therefore, if the vesicle trafficking properties of Rab11 are required for

cytokinesis then upon expression of the inactive mutant it would be expected to see defects in this process.

In this experiment I used the Rab11-S25N construct with an N-terminal myc-tag inserted into an adenovirus vector. A virus with a vector containing GFP-alone was used as a negative control. Use of adenovirus ensures a high transfection efficiency into the HeLa cells I used for the experiment. Cells were grown on glass cover slips in normal media and when they reached ~20% confluency they were infected with the virus at a concentration that gave a 20-30% infection rate. Cells were then left for 48hrs at standard growth conditions (37°C, 5% CO_2) before being fixed for 10 minutes with fresh 4% paraformaldehyde. Cells were then processed for immunofluorescence staining as described in section 2.4 and stained with an anti-myc antibody (Santa-Cruz) to label cells expressing myc-Rab11-S25N, alpha-tubulin in order to help determine individual cells and DAPI stained to show the DNA. Cells were then visualised under a confocal microscope and the number of infected cells with two or more nuclei were counted for both the Rab11-S25N infected cells and the control, GFP-expressing virus. Figure 5.1(a) shows a field of cells from a coverslip infected with the myc-Rab11-S25N virus and stained for myc, alpha tubulin and DAPI. In this field of cells only one of the cells is expressing myc-Rab11-S25N, therefore appearing green. This cell has two nuclei, whilst all of the surrounding, uninfected cells have the normal single nucleus. This illustrates that, whilst the majority of uninfected cells appeared normal, a large proportion of the infected cells had two or more nuclei. This observation was quantified by counting the number of Rab11-S25N expressing cells with two or more nuclei compared to cells infected with and expressing a GFP-alone construct. These results are shown in figure 5.1(b). This shows the results of five independent experiments, where 100 infected cells were counted on each occasion for both the Rab11-S25N and GFP virus.

(a)



(b)

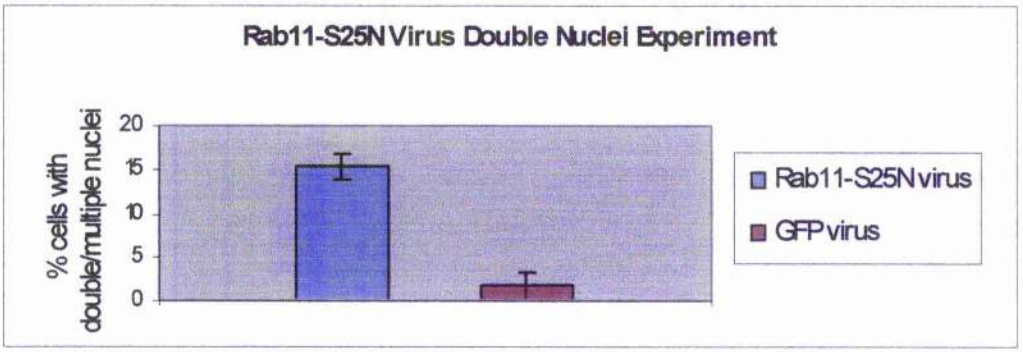


Figure 5.1 HeLa cells were infected with either the myc-Rab11-S25N virus or a virus containing GFP alone as a negative control and cells were left for 48 hours. Cells were then fixed and stained with alpha tubulin which was labelled with an Alexa-Fluor 594nm secondary antibody, anti-myc which was labelled with an Alexa-Flour 488nm secondary antibody and DAPI (as described in section 2.4, see table 2.1 for antibody details). The number of infected cells with double or multiple nuclei were then counted. **(a)** A field of cells stained for alpha tubulin, myc (to identify Rab11-S25N expressing cells) and DAPI. Images were taken on a Zeiss LSM Pascal microscope (see section 2.5). 594nm, 488nm and DAPI channels are shown separately and merged. **(b)** The bar graph shows the percentage of cells with double or multiple nuclei for both Rab11-S25N expressing cells and GFP expressing cells. Each series is the average of 5 independent experiments in which 100 cells were counted for both Rab11-S25N and GFP expressing cells. Plus and minus one standard deviation is shown.

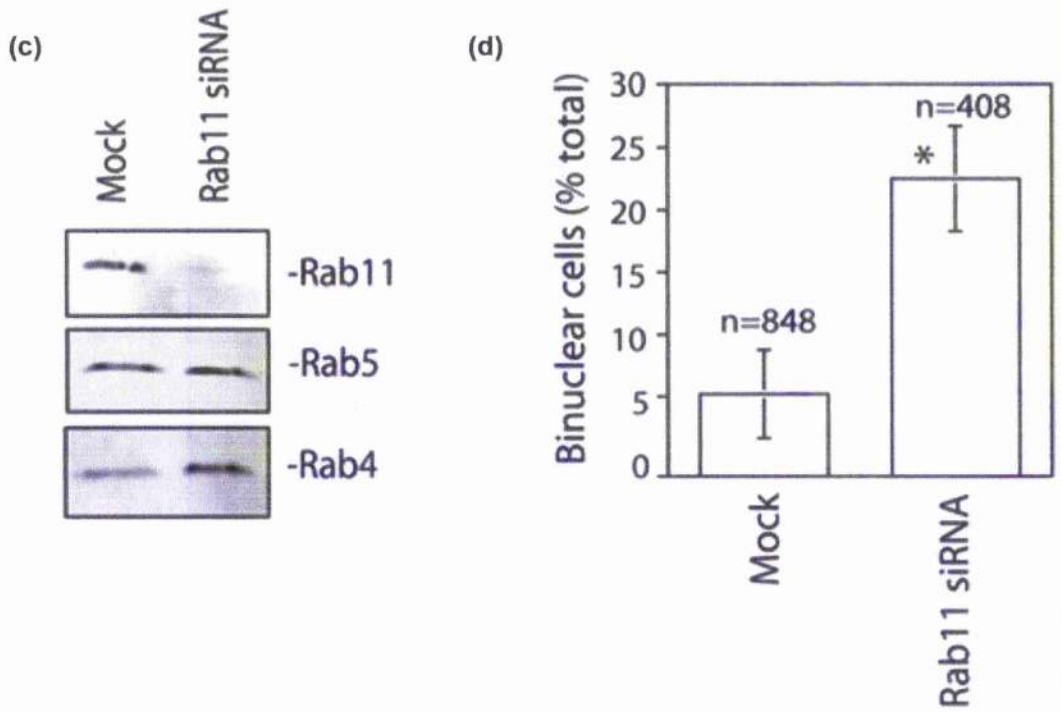


Figure 5.1 (continued) **(c)** Shows immunoblots for Rab11, Rab5 and Rab4 of protein extracts taken from untreated cells (mock) and cells treated with Rab11a and Rab11b siRNAs (Rab11 siRNA) for 72 hours. **(d)** shows the number of binuclear cells counted in mock cells compared to Rab11 siRNA depleted cells. N is the number of cells counted. Data are the means ± SE. *Statistically significant difference at $p < 0.01$. **N.B.** Data shown in Figure 5.1 (c) and (d) was collected by members of the Prekeris Lab and is shown as published in Wilson et al's recent paper (Wilson et al, 2005).

After 48 hours an average of 15.33% of Rab11-S25N infected cells have two or more nuclei compared to the GFP control virus where only an average of 1.67% of cells are bi or multi-nucleate. This shows that there is clearly an increased number of bi or multinucleated cells in the presence of Rab11-S25N, indicating that over-expression of this non-cycling, GDP locked from of Rab11 in some way perturbs cytokinesis, which in turn suggests that the normal functioning of Rab11 is required for cytokinesis.

This data has now been further backed up by our collaborators investigating the possible role for Rab11 by an independent experimental method. They have shown that when Rab11a and Rab11b are simultaneously knocked down by RNAi that a high proportion of cells become bi-nucleate (Wilson et al., 2005). This data is shown in figure 5.1 (c) and (d). 5.1 (c) shows that, compared to control cells, the level of Rab11 in Rab11a and b siRNA treated cells is greatly reduced. This knockdown seems to be specific for Rab11 as levels of Rab5 are not affected. Interestingly, Rab11 depletion resulted in increased levels of Rab4 GTPase (Figure 5.1 (c)). Because Rab4 is also known for its role in endocytic recycling, it is likely that this represents a compensatory increase as a result of Rab11 depletion. The sequences of the siRNAs which they used to knock down Rab11a and Rab11b are shown in appendix 1a.

Figure 5.1 (d) shows that the number of bi-nucleate cells occurring in cells treated with siRNAs targeting Rab11a and Rab11b is significantly increased over untreated cells. Furthermore, transfection of HeLa cells with scrambled Rab11 siRNA did not have any effect on the number of binucleate cells (unpublished data). It is not surprising that both Rab11a and Rab11b have to be knocked down to see an effect as the two isoforms are very similar to each other, reside in the same compartment and therefore it could be easily envisaged that they could be redundant to each other for at least some functions (see section 1.2.11).

5.2.2 ARF5 Bi-nucleate experiments

Studies by Schweitzer and D'Souza-Schorey (Schweitzer and D'Souza-Schorey, 2002) show that over-expression of an ARF6 mutant, ARF6-Q67L an "active", GTP-locked from of ARF6, results in a significant number of cells failing to successfully complete cytokinesis, implicating ARF6 in this process. This supports the striking localisation of ARF6 which I observed in cells undergoing cell division. This also supports the notion that the localisation of ARF5 (and Rab11 and FIP4) seen through cell division, which was very similar to that of ARF6, suggests a function for this protein in cytokinesis. As a functional assay had not been carried out for ARF5, I sought to carry out this experiment by over-expressing the ARF5 equivalent of the ARF6-Q67L mutation, ARF5-Q71L.

I carried out the experiment very similarly to the Rab11-S25N experiment described above except that an HA-tagged ARF5-Q71L plasmid was transfected into the cells using the standard protocol for Lipofectamine 2000 (Invitrogen), as described in section 2.3. A plasmid containing only GFP was transfected into separate cells as a negative control. Again, the cells were left for 48 hours and then fixed and stained for HA to identify transfected cells, alpha-tubulin and either DAPI or MKlp1 (which resides in the nucleus during interphase and the central spindle and later the midbody during cytokinesis).

Figure 5.2(a) shows a cell with green staining, indicating that it has been transfected with the HA-ARF-5Q71L construct. The blue staining is against MKlp1. This staining clearly shows that this cell contains two nuclei. Interestingly, a dense patch of blue staining in between the two nuclei probably represents a failed midbody which has at least partially formed but as, at some point, cytokinesis has failed, it is now within a cell with two nuclei. Anti-FIP4 staining shows two bright patches which partially overlap with the MKlp1 staining in the "failed midbody". This suggests that FIP4 was

(a)

Anti-FIP4 | Anti-HA ARF5Q71L | Anti-MKlp1 | Merge

(b)

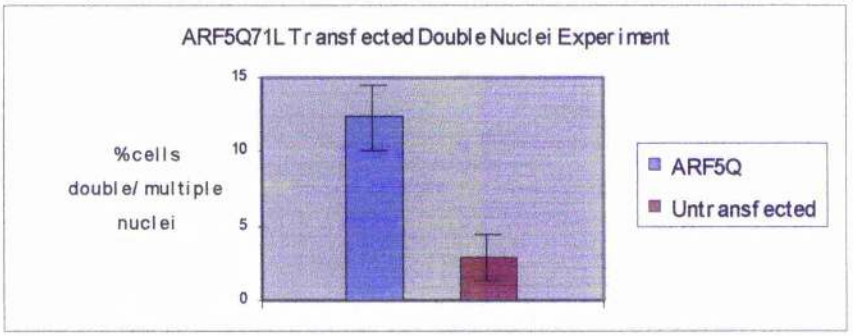


Figure 5.2 (a) HeLa cells were transfected with an ARF5Q71L-HA-tagged plasmid and left for 24 hours. Cells were then fixed with 4% paraformaldehyde and permeabilised with 0.1% Triton. Cells were then incubated with a 488nm fluorescently tagged anti-HA antibody, an anti-FIP4 and an anti-MKlp1 antibody. FIP4 was labelled with a 594nm secondary antibody whilst MKlp-1 was labelled with a 647nm secondary antibody. All antibody concentrations can be found in table 2.1. **(b)** The number of transfected cells with double or multiple nuclei were counted, whilst on the same coverslip the number of untransfected cells (those showing no green staining) with double or multiple nuclei were also counted. Counts were done as sets of 100 cells for either the transfected or untransfected cells. The graph shows the mean of 5 independent sets of experiments (i.e. 5 x 100 cells). The error bars show plus and minus one standard deviation.

able to move to the midbody but in the absence of a functional ARF5, cytokinesis has failed. Figure 5.2 (b)shows the results of 5 independent experiments where, for each experiment 100 transfected cells were counted for both the ARF5-Q71L and the GFP-alone control. On average, 12.33% of ARF5-Q71L transfected cells have two or more nuclei, which is significantly more than the GFP control cells which have an average of 2.83% double or multi-nucleated cells. Therefore, over-expression of a non-cycling, active mutant of ARF5 seems to interfere with cytokinesis, implicating that normal ARF5 function is required for cytokinesis to complete normally.

Further experimental approaches to verify that ARF5 does indeed play a functional role in cytokinesis are currently being carried out by members of our research group.

5.2.3 FIP4 in mitosis

Now that I had established that both Rab11 and ARF5 play at least some role in mammalian cytokinesis (in addition to previous studies implicating ARF6) I hypothesised that FIP4, as a protein which could couple together Rab11 and ARF5 or ARF6 could also be required. Therefore I aimed to carry out similar experiments with FIP4 to that which I had completed for Rab11 and ARF5 to see if a functional role could be elucidated. However the initial difficultly in the design in these experiments was trying to find a method of perturbing FIP4 action. In the case of both Rab11 and ARF5, mutants were already available which had been established in many studies as non-cycling, therefore functionally perturbed forms of the proteins. These mutants could be over-expressed, therefore interrupting the normal function of the proteins within cells. However, no such mutants were available for FIP4. Therefore a number of approaches were taken to try and study the possible role of FIP4 in cytokinesis. These were micro-

injections of both specific antibodies and purified proteins and RNAi knockdown of FIP4.

5.2.4 Microinjection of FIP4 antibody and protein

Microinjection of individual cells with either a specific antibody or recombinantly produced protein is a common method to try to perturb a protein's function. Binding of antibody to the protein in question will prevent it from interacting with its normal partners, therefore preventing its action, whilst large amounts of injected protein may perturb the protein's function by binding to and therefore "soaking-up" all of the protein's normal interactors, leaving the system unable to function.

HeLa cells were grown on gridded coverslips (Eppendorf) and Anti-FIP4 antibody or protein was injected (as described in section 2.8). Cells were left for 48hours and then cells fixed, stained and observed. The number of injected bi-or multi-nucleated cells were counted as compared to a control.

In some cases, bi-nucleated cells were observed in areas of the cover slip that had been injected, as shown in figure 5.3. However, despite numerous attempts, the numbers of bi-or multi-nucleated cells observed did not prove to be significantly different to that of control cells. This could have been due to technical difficulties with the microinjection technique. Initially, the major problem here was that, although the location of cells was noted upon injection, when observing the cells it was difficult to determine exactly which cells had been injected. During the 48hours that the cells were left, they had been undergoing normal processes of replication, migration and, in some cases, apoptosis. This made the identification of injected cells very challenging. To try and overcome this problem, further experiments were carried out where the antibody or protein to be injected was first mixed with a 488nm fluorescent dye to try and aid identification of injected cells (see section 2.8). This worked to some extent, in that in

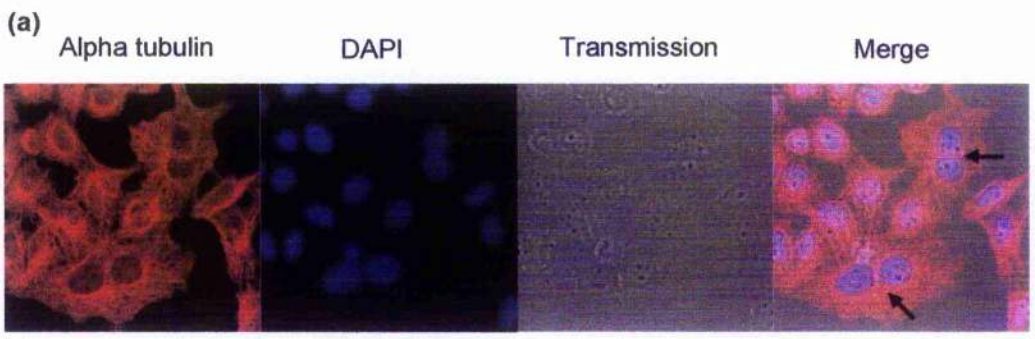


Figure 5.3 HeLa cells were grown on gridded coverslips and injected with anti-FIP4 antibody (see section 2.8). The cells which had been injected were noted on a log sheet. Cells were then left for 48 hours and then fixed and stained for alpha tubulin, labelled with an Alexa-Fluor 594nm secondary anitbody and DAPI (see section 2.4 and table 2.1 for antibody details). Coverslips were examined under a Zeiss LSM Pascal microscope (see section 2.5) and areas of cells that had been injected with antibody were identified using the grids on the coverslips. **(a)** shows an area that had been microinjected, with 594nm, DAPI and transmission channels shown separately and then merged. In the merged image arrows indicate two cells which have double nuclei.

some cases it was possible to observe some green fluorescing cells after fixation. However it showed that on the whole the microinjections being performed were very variable in terms of the amount of antibody/protein/dye injected. Of the cells which I had attempted to inject I would typically see a great range in the brightness of their fluorescence. A very low percentage of the cells would fluoresce brightly, some would fluoresce faintly, whilst in the majority of cells no fluorescence was visible. With varying amounts of antibody or protein being injected, the interpretation of the results became difficult and somewhat qualitative. Therefore, despite some success as illustrated in figure 5.3, other methods of investigating FIP4's potential role in cytokinesis were undertaken.

5.2.5 RNAi of FIP4

RNAi is the current method of choice for perturbing a protein's function in cells. In some cases, once optimised, simply transfecting a short RNA strand into cells can specifically knock down levels of your chosen protein to 10% or less of normal levels. This makes the RNAi approach a potentially very powerful tool for studying the function of your protein.

There are several methods of implementing RNAi, each with their advantages and disadvantages. Here, it was chosen to transcribe short interfering RNAs (siRNAs) *in vitro* and to then transfect these into cells, using the "Silencer™ siRNA Construction Kit" from Ambion, as described in section 2.9. This is a rapid and reasonably economic approach which should allow the analysis of the effects of knocking down your protein for up to 96 hours.

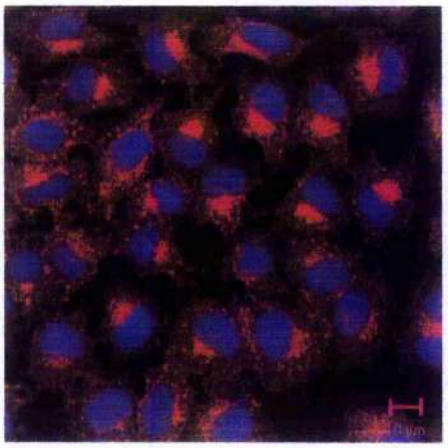
Using programmes and advice from several websites (see appendix 1c for details) three siRNAs were chosen which were targeted against different sections of the FIP4 sequence and were predicted to have a high degree of potency. These were named

436, 1067 and 1654, according to the position in the FIP4 sequence on which they began (see appendix 1b for sequences). A control sequence was also designed, which was a scrambled version of the 1067 siRNA. Once designed, all the sequences were run through a "BLAST" programme (NCBI) to check the sequences did not match any other human sequences too closely (see appendix 1c for parameters used).

Transcription and transfection of the siRNAs was carried out as described in section 2.9 and then cells were left for 24, 48, 72 or 96 hours for the siRNA to have its effect. Various concentrations of transfected siRNAs were tried although typically cells were either transfected with individual siRNAs to a final concentration of 50nM or were transfected with all three FIP4 siRNAs at a concentration of 40nM, giving a total concentration of 120nM. Duplicate sets of cells were then either fixed and prepared for immunofluorescence or harvested for protein extraction.

Figure 5.4 shows a set of cells stained for FIP4 (and DAPI) 72 hours after transfection with 40nm each of 436, 1067 and 1654. Panel (a) shows a typical field of approximately 30 cells, viewed under a Zeiss Pascal confocal microscope. It can be seen that all the cells show significant staining for FIP4, localised to the areas of the cell that would be expected (largely peri-centrosomal). Panel (b) shows another field of cells from the same slide. Again red anti-FIP4 staining can be seen at the expected localisation in all cells, including two cells which are undergoing cytokinesis. This indicates that the knockdown of FIP4 by these siRNAs has not been successful. Blots of FIP4 protein levels in lysates prepared from siRNA treated cells also showed no consistent knockdown of FIP4 compared to a standard protein (RhoA). A problem with this experiment was the lack of a positive control for knockdown. This was not carried out due to the siRNA work being carried out in the very late stages of this work and therefore being constrained by time. However, this issue has now been addressed by other members of the lab using the Ambion "Silencer™ siRNA Construction Kit" and

(a)



(b)

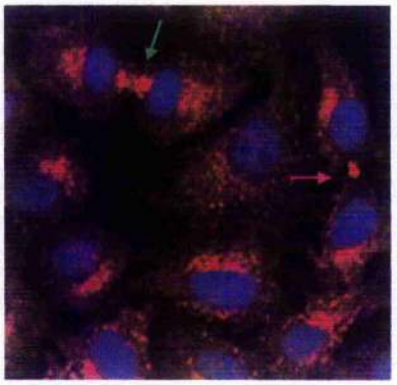


Figure 5.4 (a) HeLa cells were transfected with siRNAs 436, 1067 and 1654 simultaneously (as described in section 2.9). Each siRNA was at a concentration of 40nM, giving a total concentration of 120nM. Cells were then left for 72 hours before being fixed with 4% paraformaldehyde and then stained with anti-FIP4 antibody. This was is turn stained with a 593nM Alexa-Fluor anti-sheep antibody (visualised as red). For antibody details, see table 2.1.The cells were also stained with DAPI to mark the nuclei (visualised as blue). Stained cells were visualised under a Zeiss Pascal confocal microscope. **(a)** shows a typical field of approximately 30 cells is shown. There seems to be significant staining for FIP4 in all cells. **(b)** Cells were treated in an identical manner to panel (a). Here, arrow heads indicate two cells undergoing cytokinesis. The green arrow indicates a cell in late anaphase/ early telophase whilst the pink arrow indicates a cell in late telophase. In both cases is can be seen that the localisation of FIP4 appears normal.

successful knockdown of a control gene has been accomplished. This suggests that this is an effective method for producing siRNAs. Possible explanations for the lack of FIP4 knockdown observed are discussed below.

A number of reasons are possible for the lack of FIP4 knock down. However, several reasons were ruled out: it was verified that the siRNAs had been successfully synthesised and that, by fluorescently labelling some of the siRNAs, they were being successfully transfected into the cells (data not shown). Therefore it did not seem to be a problem of production, degradation or of transfection into the cells. 40nM is a relatively high concentration for a siRNA, with most effective siRNAs being able to knock down protein levels at below 20nM. Therefore, a low concentration of the siRNA also seemed unlikely to be the reason for the failure to knock down FIP4. Therefore there are two main reasons that remain which may be responsible for the failure to significantly knock down this protein. Firstly, the siRNAs used were not from the most effective part of the sequence or were not of optimum design. In this case designing siRNAs against different target sequences may be successful. However, the target sequences chosen were deliberately taken from three different areas of the coding sequence to give the best chance of targeting the most "vulnerable" parts of sequence. Also, each siRNA was designed using all the parameters available from three separate, reliable, sources and each of these sources picked each one of the siRNAs as being particularly well designed. Therefore, although it is possible that simply designing further siRNAs would, in the end, lead to a successful outcome, these siRNAs were rigorously designed to give the best chance of knock down. It is possible, therefore, that FIP4 is a particularly difficult sequence to target by this method. However, it is not known whether this is the case for FIP4 and further attempts to carry out RNAi of FIP4 are currently being carried out by our research group.

5.3 Discussion

5.3.1 Rab11 and ARF5 play a functional role in Mammalian Cytokinesis

For both Rab11 and ARF5 I have shown that introducing a non-guanine-nucleotide-cycling mutant of these proteins, a GDP locked, "inactive" mutation in the case of Rab11 and a GTP-locked, "active" mutation in the case of ARF5, prevents cytokinesis from completing successfully in a significantly higher percentage of cells than control cells. This implicates both proteins to be functionally active in cytokinesis. I have not been able to conclusively show a functional role for FIP4 in cytokinesis. However, my experiments do certainly not rule out a role for FIP4 in this process and its striking localisation and binding to Rab11, ARF5 and 6, along with it's homology to *Drosophila* Nuf certainly suggest it has an important role to play. This and the possible reasons for not observing a phenotype here are discussed in section 5.3.3 below.

This is the first study to show that Rab11 is involved in mammalian cytokinesis. These results complement studies which have been carried out in both *Drosophila* and *C.elegans* which have shown that Rab11 is required for successful cellularisation/ cytokinesis in these organisms (Riggs et al., 2003; Skop et al., 2001). The Rab11S25N over-expression studies carried out here have now also been backed up by RNAi studies in mammalian cells. Together these data convincingly show that Rab11 plays an important role in mammalian cytokinesis (Wilson et al., 2005).

This is the first study of the involvement of ARF5 in cytokinesis in any organism and indeed one of very few studies to have looked at the function of ARF5 in any cellular role. Therefore, to have found that ARF5 plays a functional role in this process is of great importance, both for the cytokinesis field and for the Class II ARFs. As it is not yet clear what function ARF5 may carry out within cells it cannot yet be determined what role it is playing in cytokinesis. However, it is probable that, like the class I and III ARFs, the class II ARFs play roles in both membrane trafficking and cytoskeletal

rearrangements. Therefore, ARF5 could be important for cytokinesis because of membrane delivery events to the cleavage furrow and/or midbody as well as actin-rearrangements at these sites.

5.3.2 "Inactive" Rab11 and "Active" ARF5 Produce The Same Phenotype in Cytokinesis

The experiments here showed that expression of Rab11S25N, a mutant of Rab11 locked in its GDP-bound, "inactive" conformation and ARF5Q71L, a mutant of ARF5 locked in its GTP-bound, "active" conformation, produced the same phenotype of bi-nucleated cells, suggesting a failure of cytokinesis. This may at first seem difficult to explain, particularly in light of the claims made here that PIP4 binding to these proteins is likely to be important for cytokinesis. However, these findings can be explained if the results of these mutations on the small GTPase cycle are considered.

Small GTPases carry out their functions by cycling between their GTP-bound and GDP-bound forms. For example, a Rab could be considered to start its cycle in its GDP bound form, bound to a GDI protein in the cytosol. In this form it is recruited to a specific membrane compartment, which is the "donor" membrane compartment for that Rab. Here, it is converted to its GTP-bound form and can hence bind its effectors, therefore having its effects on vesicle trafficking, involving possibly the budding of a vesicle from the membrane compartment, movement of the vesicle towards another "target" compartment and docking and fusion of the vesicle to this different "target" membrane compartment. Once it has had carried out these functions, the GTP-bound Rab is converted to its GDP-bound form. In this conformation it can be recognised by a GDI protein, which removes the Rab from the target membrane into the cytosol, before delivering the Rab back to its "donor" membrane where the cycle can start again.

If the cycle is considered in this way it can be seen that both mutants locked in their GTP-bound or GDP-bound conformations will become "stuck" at one stage of their cycle and therefore one point in the cell and so neither can effectively carry out their functions. For example, a GDP-locked mutant will be "stuck" in the cytosol or the donor membrane, unable to bind its effectors and therefore unable to have its effects. A GTP-locked mutant may be able to bind its effectors and thereby carry out its functions once. However, it will then be "stuck" on the target membrane. In its GTP-bound form it will be unable to bind its GDI and therefore unable to return via the cytosol to its donor membrane. Therefore, it can be seen that a GTP-locked mutant will only, at best, be able to carry out its functions once (although even this is unlikely as newly synthesised Rabs rely on being delivered to their correct membranes by REP proteins, which only bind Rabs in their GDP-bound forms). So for functional analyses, the terms "active" and "inactive" mutants of the small GTPases, referring to their GTP-bound and GDP-bound conformations respectively are perhaps misleading as it is the cycling between GTP and GDP bound forms which is important for small GTPase function and therefore neither the GTP or GDP-locked forms are able to carry out their functions effectively. Therefore, it could be envisaged how an "active" and "inactive" mutant of a small GTPase could produce the same phenotype, regardless of whether it is able to bind its effectors or not. It can also be envisaged how this may be the case for Rabs, as described here, but also for other small GTPases, including the ARFs, as these undergo a similar cycle. This has indeed been noted and commented on for one member of the ARF family, ARF6. In a review paper, J. Donaldson points out that "observations obtained with these inactive and active mutants should be interpreted with caution as Arf6 function normally depends on its GTPase cycle, and expression of any mutant that blocks the cycle may block Arf6 function". Later in the paper she describes work carried out by several research groups on the actin rearrangement activities of ARF6. These

pieces of work show that if either the GTP-binding defective, dominant negative mutant of Arf6, T27N or Arf6Q67L, the constitutively active mutant are expressed then the same phenotype, a block of the actin rearrangement activities of ARF6, results (Franco et al., 1999; Brown et al., 2001; Zhang et al., 1998). She again concludes that this is the case as it is required "that Arf6 cycles between active and inactive forms to function properly." (Donaldson, 2003).

5.3.3 Weak phenotypes are due to Experimental Limitations and Redundancy in Cytokinesis

It may at first seem surprising that only a relatively low percentage of the cells (15.3% in the case of Rab11S25N and 12.3% in the case of ARF5Q71L) show defects. If these proteins play a role in cytokinesis would it not be expected that a much higher percentage of cells would show defects? I believe the explanation for this anomaly is due to two factors, the design of the experiment and the nature of cytokinesis itself. As mentioned in the introduction to the bi-nucleate experiments, the approach I have used is not sensitive enough to capture all defects in cytokinesis as it measures only the cells which have completely failed to complete cytokinesis. As discussed earlier, there are several examples where defects have been introduced into important cytokinetic machinery and the cells have still been able to divide, although at a slower rate. The bi-nuclear experiment would not sense these defects as, as along as the cells go on to divide, they would appear normal. An obvious experiment that could be carried out to resolve this "timing" issue would be to carry out live-cell microscopy on infected/transfected cells and measure the length of time required for Rab11 or ARF5 mutant expressing cells to complete cytokinesis compared to that of controls.

The other reason why the phenotype I observed after introducing the mutated proteins I used in my experiments may have shown a relatively weak phenotype is the

possibility that Rab11 and ARF5 show partial redundancy, i.e. that their functions in cytokinesis can be partially carried out by alternative proteins. From the experiments I have performed it is not possible to conclude whether redundancy is present for Rab11 and ARF5 but it has been shown for other proteins involved in cytokinesis to be the case. An obvious candidate for a partially redundant partner for ARF5 would be ARF6 as these have both been shown to localise strikingly throughout cytokinesis and both have relatively weak phenotypes in the bi-nucleate experiments described above. However, ARF5 and 6 are not part of the same class of ARFs (ARF5 is a class II ARF, whereas ARF6 is in class III) and both have been shown to have specific effectors. Therefore I think it would be surprising if they were completely redundant to each other. An experiment where both ARF5Q and ARF6Q were transfected into the same cells simultaneously could begin to address these ideas.

Rab11 also has potentially redundant partners, in particular the other members of the Rab11 family, Rab11b and Rab25. As discussed in section 1.2.11 all of these proteins reside in the recycling endosome and share relatively high homology. Therefore they could potentially carry out the same roles as Rab11 during cytokinesis. However, as these proteins have been studied relatively little, have not been localised during cell division and I have not tested them for FIP4 binding, it is difficult to comment here on their possible functions within cytokinesis.

5.3.4 FIP4 Discussion

In this chapter I have attempted to determine whether FIP4 plays a functional role in cytokinesis. Like Rab11 and ARF5 (and ARF6) there are several factors which suggest it does. Firstly, there is the striking localisation of FIP4 throughout cell division, as demonstrated in chapter 4. This evidence alone is suggestive of FIP4 playing a functional role in the process. Also, its localisation is the same as Rab11, ARF5 and

ARF6 throughout cytokinesis. As these proteins have been shown here, and elsewhere, to play a role in cytokinesis and I have shown that FIP4 binds to each of these proteins, this is also strong evidence that FIP4 itself plays a functional role. Lastly, but equally importantly, is the fact that FIP4 (and FIP3) are the mammalian homologues of the *Drosophila* protein, Nuf, which has been shown to play a role in cellularisation, a process analogous to cytokinesis (Riggs et al., 2003).

Here I have not been able to conclusively show that FIP4 is essential for cytokinesis. Some of the possible technical reasons for this are described in the results section above (sections 5.2.4 and 5.2.5). However, it is likely that, as discussed for the Rabs and ARFs above, there are real *in vivo* reasons that could also explain the lack of an observed phenotype. The most likely of these is that FIP4 is semi-redundant, i.e. that another protein can carry out its function in FIP4's absence, bringing us back to Ray Rapparot's "old Maine fishing boat engine" (see chapter1.2.1). FIP3 is an obvious candidate as a semi-redundant partner for FIP4. It shares good homology with FIP4 and is the only other member of the Class III FIPs (Hickson et al., 2003). As mentioned above, it is also a homologue of Nuf. In addition, it also localises similarly to FIP4 in cytokinesis (Wilson et al., 2005). These are all good pieces of evidence that FIPs 3 and 4 could be redundant to each other. Therefore, in order to study the possible roles of FIP4 (and FIP3) in cell division it may be necessary to perturb both of the proteins simultaneously. Unfortunately, these types of experiments were not possible within this work.

Therefore, FIP3 and FIP4 may be at least semi-redundant to each other in terms of cytokinesis. But is the sole reason for having both FIP3 and FIP4, to ensure that they can "stand-in" for each other if the other fails? This is possible, although some evidence points to individual roles for these proteins. One argument is a fairly subtle, but possibly important difference between their binding strengths for the various Rabs and ARFs.

Both FIP3 and FIP4 have been shown to bind Rab11 and ARF6. However, it is possible that the binding affinities of FIP3 and FIP4 vary for these two proteins. Also, FIP4 has been shown to bind ARF5 and Rab5, whereas FIP3 does not seem to bind these proteins. Therefore these potential differences suggest that, at least in interphase cells, they could be associated with subtly different membrane trafficking steps. This may not be the case in cytokinesis, where they may truly play the same role. In addition to these binding partner anomalies, FIP3 and FIP4 show some differences in their tissue distributions (Hickson et al., 2003). Therefore, they may also have tissue-specific roles to play.

5.3.5 Rab11, FIP4 and ARF5 and/or ARF6 complexes co-ordinately deliver membrane and actin rearrangement properties to the cleavage furrow and midbody

In chapter 1, several models were discussed which argued for the importance of membrane trafficking in cytokinesis and how this might be achieved. Section 1.2.8 included the *Drosophila* based models by Riggs et al. which proposed that Rab11 and Nuf were important for the co-ordinated delivery of membrane and either actin or an actin re-arrangement factor to the site of cellularisation (Riggs et al., 2003).

In mammals, Gromley et al. proposed a model suggesting that membrane delivery to the centre of the midbody, the "midbody ring", was essential for the final abscission of the two daughter cells (Gromley et al., 2004, section 1.2.13).

The data collected here suggest a model which incorporates both of the above ideas. FIP4, the mammalian homologue of Nuf, binds Rab11 and ARF5 and/or ARF6. In this way membrane vesicles and an actin rearrangement factor (ARF5 and/or 6) are co-ordinately delivered to the cleavage furrow where they provide both the membrane and the actin rearrangement properties required for furrow ingression.

In addition, taking into account the localisation of all of the proteins studied here in the final stages of cytokinesis, Rab11/FIP4/ARF5 and/or 6 vesicles are delivered to the centre of the midbody where they dock with the "midbody ring" and hence deliver the membrane and possibly the actin re-arrangement capabilities necessary for the final abscission of the cell into two, separate daughter cells.

The evidence gathered here, i.e. that Rab11/FIP4/ARF5 and or 6 can form a complex, that these proteins all localise to the "midbody ring" structure and that perturbing Rab11 or ARF5 causes cytokinesis defects is highly supportive of this model. In addition, several other pieces of evidence support this model. Firstly, ARF6 has been shown to be involved in cytokinesis (Schweitzer and D'Souza-Schorey, 2002). Although these authors propose that its role may be of endocytosis at the midbody, the function it plays has not yet been determined and therefore it could equally as likely play a role in the model proposed here (or even be a part of both models, as they are not mutually exclusive). In addition, Gromley et al. observed that one of the early components to arrive at the midbody ring is MKlp1 (Gromley et al., 2004). In this work, MKlp1 was also observed at the midbody ring (section 4.2.4), where is co-localised with FIP4, Rab11, ARF5 and ARF6. As mentioned in section 4.3.3 it has been previously shown that MKlp1 can bind to all members of the ARF family. This binding would serve as a way for the membrane vesicles containing Rab11/FIP4/ARF5 and or 6 complexes to "dock" at the midbody ring, therefore being held in place in order for an accumulation of membrane to occur at this site.

Chapter 6

Discussion

Chapter 6 – Discussion

Rab11-FIP4 is a human protein which is known to bind ARF5 and Rab11 and shows partial co-localisation with the recycling endosome (Hickson et al., 2003). It also shows homology to Nuclear Fallout, a *Drosophila* protein which has been shown to play an essential role in cellularisation (Hickson et al., 2003; Riggs et al., 2003), a process that is analogous to mammalian cytokinesis.

6.1 FIP4 binds and Co-localises with ARF5, ARF6, Rab5 and Rab11

Here it has been shown that Rab11-FIP4 can bind to further members of the ARF and Rab families, namely ARF6 and Rab5. It has also been shown that Rab11-FIP4 is likely to form multi-protein complexes, simultaneously binding members of the Rab and ARF families. This provides a mechanism by which the actions of the ARF and Rab families of small GTPases could be spatially co-ordinated.

Although it seems that Rab11-FIP4 binds only to ARF5 and ARF6 from the ARF family, the binding to further Rab proteins in addition to Rab11 and Rab5 has not yet been tested. Therefore there is the possibility for further co-ordination between the ARFs and additional Rab family members. However, it is unlikely that Rab11-FIP4 will bind to many other Rabs as its closest homologue in mammals, Rab11-FIP3, has been shown to bind only members of the Rab11 subfamily (i.e. Rab11a, Rab11b and Rab25) and not Rab1a, Rab2, Rab3a, Rab3b, Rab5, Rab8a and Rab17 (Hales et al., 2001; Prekeris et al., 2001). There have been reports that one members of the Rab11-FIP family, RCP, can bind Rab4 in addition to Rab11 (Lindsay et al., 2002) but it has been suggested that this may not be the case *in vivo* (Peden et al., 2004). In either case, due to Rab11-FIP4's homology to other FIPs and also its endosomal location, it seems likely that FIP4 will bind few, if any other Rabs in addition to Rab11 and Rab5.

Although initially it was somewhat surprising to find that FIP4 bound to Rab5, this interaction can be explained well in parallel to the discovery that FIP4 also binds ARF6. ARF6 is thought to cycle from the plasma membrane to its own "ARF6 endosome" (Radhakrishna and Donaldson, 1997). From here a proportion of it returns directly to the cell surface, whilst the remainder moves to the sorting endosome before being recycled back to the surface, possibly via the recycling endosome (Radhakrishna and Donaldson, 1997; Donaldson, 2002; see figure 1.2). Here it has been shown that FIP4 co-localises with ARF6, probably both in the "ARF6 endosome" and whilst ARF6 travels through the sorting and recycling endosomes. As Rab5 localises (at least in part) to the sorting endosome, it makes sense that FIP4 can bind Rab5, therefore providing co-ordination between ARF6 and the sorting endosome. Then, as vesicles leave the sorting endosome and travel to the recycling endosome, FIP4 could cease to bind Rab5 and instead bind Rab11. FIP4's binding to ARF6 provides a link for ARF6 to be targeted through the sorting and recycling endosomes.

ARF5 seems to show partial overlap with Rab11 and, as previously suggested (Hickson et al., 2003), probably cycles between the recycling endosome and Trans-Golgi-Network (see figure 1.2). Evidence from FIP3 suggests that the ARFs bind the FIPs at different regions (Shin et al., 2001). Therefore, although not tested here, it could be the case that FIP4 can bind to ARF5 and ARF6 (and Rab11/Rab5) simultaneously. Exactly what the function of this may be is not clear although ARF5 binding could provide an additional "anchor" for the FIP4 complex at the recycling endosome.

Further studies need to be conducted to explore the possibility of FIP binding to other members of the Rab family. More detailed work on the multi-protein complexes which could form also needs to be carried out. These studies may help in identifying a more precise role for FIP4. Also, *in vivo* binding studies need to be carried out to confirm the new binding partners identified here (ARF6 and Rab5). However, as these

experiments have already been carried out for ARF5 and Rab11 (Hickson et al., 2003) and for ARF6 in the case of FIP3 (Prekeris et al., unpublished data) and that these findings agree with the *in vitro* data here then it is likely that the ARF6 and Rab5 binding shown here does occur *in vivo*. Additional support for these binding events comes from the co-localisation of ARF6 with FIP4 that has been shown here.

6.2 FIP4 and its binding partners in Cytokinesis

Here it has been shown that FIP4, Rab11, ARF6 and ARF5 show a striking and largely overlapping localisation throughout cell division. Initially, at prophase, these proteins become tightly clustered around the centrosomes, suggesting a "collapse" of the Rab11 recycling endosome and possibly the ARF6 endosome to this point. It is possible that this "collapse" is, at least in part, mediated by FIP4. This comes from the observation that expressing N-terminally truncated versions of FIP4 causes it (and Rab11) to collapse to a tight peri-centrosomal spot in interphase cells (Hickson et al., 2003) which looks identical to that observed at prophase. Therefore, in addition to co-ordinating Rab and ARF actions, FIP4 could mediate the interaction of the recycling and ARF6 endosomal compartments with components of the cytoskeleton. Upon onset of mitosis, a modification on FIP4 could be altered, resulting in the collapse of it and its associated endosomes to the tight peri-centrosomal localisation.

FIP4, ARF6 and a proportion of Rab11 then seem to be loaded onto the microtubules radiating from the centrosomes and re-localise to the central spindle during anaphase. Subsequent to this they localise to the midbody and laterally to the midbody ring in the centre of the midbody where they co-localise with MKlp1 at a late stage of cytokinesis. In agreement with observations made by Gromley et al. (Gromley et al., 2004), it is likely that they are co-delivered to the midbody ring with membrane vesicles. An accumulation of membrane and membrane re-modelling components at this point

could then lead to a "phragmoplast" like abscission event to divide and seal the cell into two, separate daughter cells (see figure 6.1 (c)).

Here it has been shown that, as is the case in *C.elegans* and *Drosophila* (Skop et al., 2001; Pelissier et al., 2003; Riggs et al., 2003), Rab11 is required for cytokinesis in mammalian cells. This was performed by over-expressing a non-cycling mutant form of Rab11 in cells which resulted in an increased number of cells with two or more nuclei, a phenotype of failed cytokinesis. These observations were backed up by RNAi studies performed by our collaborators (Wilson et al., 2005).

Here it has also been shown that ARF5 plays a role in cytokineisis. Again, this was performed by over-expression of a non-cycling mutant of ARF5, in a similar manner to experiments performed by Schweitzer and D'Souza-Schorey (Schweitzer and D'Souza-Schorey, 2002) which identified a role for ARF6 in mammalian cytokinesis. Further approaches to verify a role of ARF5 in cytokinesis are currently being carried out in our laboratory.

In this work it was not shown that FIP4 plays a functional role in cytokinesis, despite much evidence suggesting that this may be the case (FIP4's binding partners playing roles in cytokinesis, its localisation during cytokineiss and its homology to Nuclear Fallout). This was probably due to difficulties with the experiments conducted here, as discussed in chapter 5, although partial redundancy of FIP4 with its closest mammalian homologue, FIP3, could also mask a potential role for FIP4. Clearly further experiments need to be carried out to study the function of FIP4. Possible approaches would be to design new siRNA targets, optimising the micro-injection experiments or to design mutant forms of FIP4 which, for example, are unable to bind one or more of its small GTPase partners. All these approaches are currently being pursued in our laboratory.

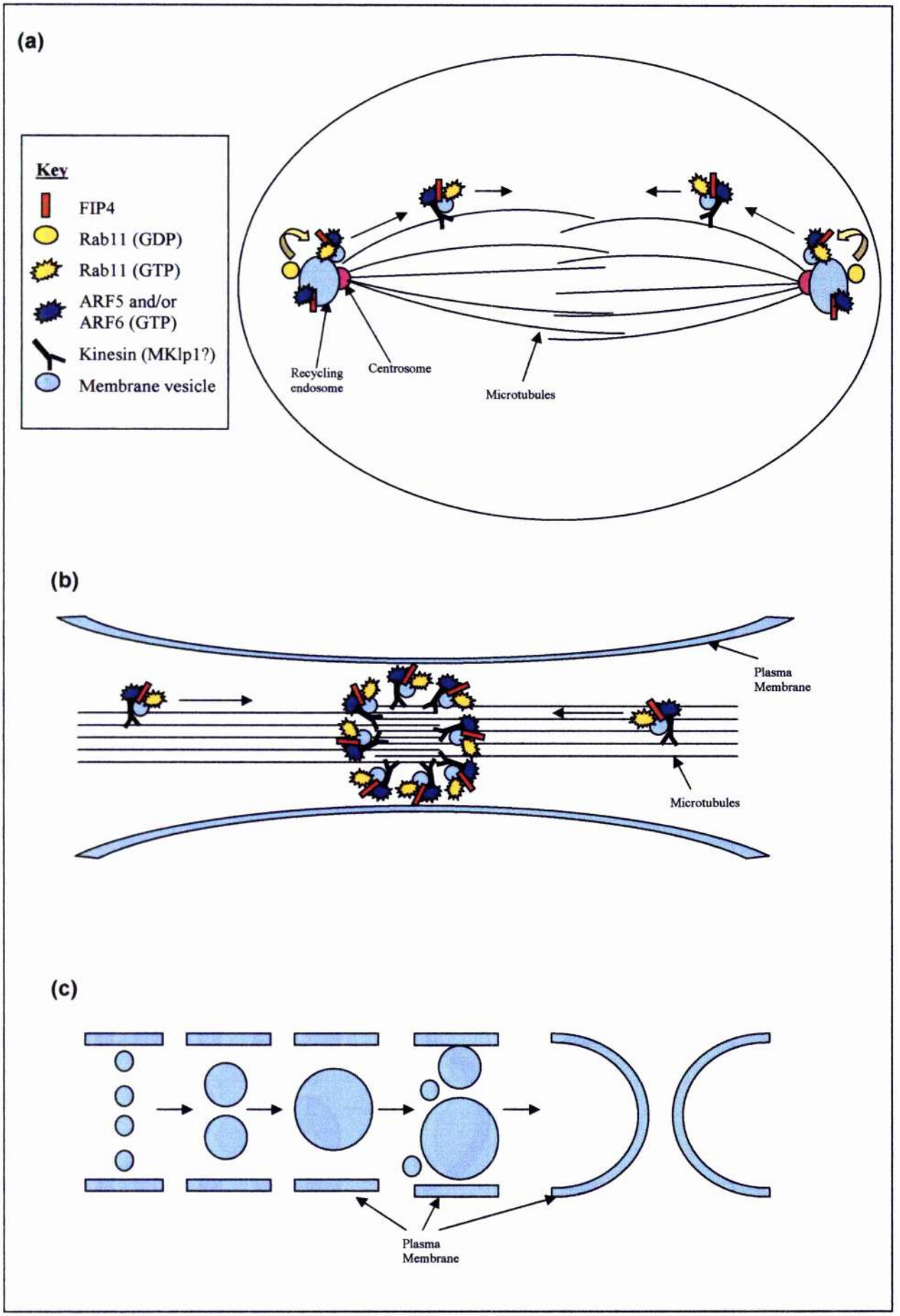

(a)
Key
FIP4
Rab11 (GDP)
Rab11 (GTP)
ARF5 and/or ARF6 (GTP)
Kinesin (MKlp1?)
Membrane vesicle
Recycling endosome
Centrosome
Microtubules
(b)
Plasma Membrane
Microtubules
(c)
Plasma Membrane

Figure 6.1 Model of FIP4/ARF5/6/Rab11 actions during cytokinesis. **(a)** Cartoon of cell at prophase/metaphase. At prophase, the recycling endosome and possibly the "ARF6 endosome" collapse to a tight peri-centrosomal location. This collapse may be a result of an alteration of a modification on FIP4. This brings constitutive FIP4/ARF6 complexes to this point and into close proximity to Rab11. Rab11 regulates the exit of vesicle trafficking from the recycling endosome. Upon activation, during late prophase/early metaphase, Rab11 binds to the FIP4/ARF6 complex and vesicles containing these proteins then bud from the recycling endosome and are loaded onto astral microtubules which lead towards the centre of the cell, through an interaction with a kinesin-like protein, possibly MKlp1 (ARFs bind MKlp1). ARF5/FIP4/Rab11 complexes or possibly ARF5/ARF6/FIP4/Rab11 complexes may also form at the collapsed endosome and become loaded onto the microtubules. The membrane vesicles containing the protein complexes are delivered towards the central spindle during anaphase. **(b)** Cartoon of extended midbody during late telophase. Anti-parallel microtubules overlap at the protein-dense central "Flemming Body". Rab11/FIP4/ARF5/6 complexes on membrane vesicles are delivered along the microtubules to the Flemming Body. Here, as the kinesin reaches the end of its microtubule, the membrane vesicles and protein complexes congregate. Due to the very high protein density at the Flemming Body, antibody staining of proteins at this point may appear as a ring around this central structure. This is known as the "midbody ring". **(c)** Shows the final steps of abscission at the centre of the midbody. For clarity, only membrane vesicles are shown. As the vesicles accumulate at the centre of the midbody, the membrane manipulating proteins which have gathered there along with the vesicles (these include Rab11 and ARF5 and 6 as well as the exocyst and various members of the SNARE protein family which have also been shown to localise to this point) allow the vesicles to fuse with one another, forming larger vesicles, and eventually with the plasma membrane. This results in the abscission of the midbody into two separate, sealed daughter cells. This membrane accumulation and fusion model is analogous to the phragmoplast model of cell division which occurs in plant cells. The binding of FIP4 to both an ARF and Rab11 simultaneously ensures that the delivery of these proteins to the centre of the midbody is both temporarily and spatially co-ordinated. This is likely to be of importance to the success of the membrane abscission events at the midbody as ARFs and Rabs have inter-related roles in membrane trafficking events.

A major question which remains to be answered for Rab11, ARF5 and 6 and FIP4 (should it be found to play a role in cytokinesis) is "at what stage of cytokinesis do these proteins function?" Figure 6.1 presents a model for the roles of these proteins in cytokinesis, based upon the evidence gathered in this thesis. This suggests they will play a role in the final abscission stage of cytokinesis. This is likely due to the localisation of the proteins to the midbody ring. However, it is also possible that these proteins will play additional roles at earlier stages of cytokinesis. The best way to ask these questions would be by studying the effects of perturbing these proteins in live cells, using time-lapse photography. Our laboratory is currently starting to perform these experiments.

The possible functions of FIP4 in interphase cells has not yet been thoroughly examined although, as previously mentioned, it does seem to play a role in maintaining the normal morphology of the recycling endosome (Hickson et al., 2003). The binding properties and localisation of FIP4 presented here suggest that FIP4 is likely to play a role in co-ordinating ARFs and Rabs during the cycling of membrane from the cell surface through the ARF6 endosome, the sorting endosome and the recycling endosome. Its binding to the ARFs and Rabs would ensure spatial and temporal co-ordination of their actions and therefore help ensure a high fidelity of membrane traffic through these compartments.

6.3 Membrane Traffic is Required For Cytokinesis

More generally, this work lends further support to the now widely accepted view that membrane traffic is required for cytokinesis. Specifically, it supports the more recent revelation that membrane events are required in mammalian cells for abscission, the very final stage of cytokinesis. This suggests that although animal and plant cell division differs greatly in that animal cells make use of an actomyosin ring to constrict the cell, whereas plants rely solely on membrane delivery to the central phragmoplast,

the fundamental mechanism by which the cells finally divide themselves into two may actually be conserved from plants to animals, relying upon membrane delivery to a central point between the two divided daughter nuclei.

A model incorporating the binding properties, localisations and functional roles of FIP4 and its small GTPase binding partners discovered in this thesis, in the context of the "phragmoplast like" abscission model which may occur in animal as well as plant cells, is presented in figure 6.1.

Appendices

Appendix 1a Rab11 siRNAs

Rab11a and Rab11b isoforms were depleted with siRNA oligonucleotides based on human Rab11a and Rab11b sequences. These sequences, designed by Prekeris and co-workers (as described in Wilson et al., 2005) are shown below:-

Rab11a

5'-AATGTCAGACAGACGCGAAAA-3'

Rab11b

5'-AAGCACCTGACCTATGAGAAC-3'

Appendix 1b FIP4 siRNAs

The antisense and sense sequences of the FIP4 siRNAs, described as 436, 1067, 1654 and 1067-scrambled are shown below:-

436

Antisense siRNA Oligonucleotide Template:

5'- AACTTCAAGGACTTTTGCCGGCCTGTCTC -3'

Sense siRNA Oligonucleotide Template:

5'- AACCGGCAAAAGTCCTTGAAGCCTGTCTC -3'

1067

Antisense siRNA Oligonucleotide Template:

5'- AAAATCAACCTGCTCAATGACCCTGTCTC -3'

Sense siRNA Oligonucleotide Template:

5'- AAGTCATTGAGCAGGTTGATTCCTGTCTC -3'

1654

Antisense siRNA Oligonucleotide Template:

5'- AAGCGCATGATGGACAAGCTGCCTGTCTC -3'

Sense siRNA Oligonucleotide Template:

5'- AACAGCTTGTCCATCATGCGCCCTGTCTC -3'

1067-Scrambled

Antisense siRNA Oligonucleotide Template:

5'- AATCATGCACGCATACTAAACCCTGTCTC -3'

Sense siRNA Oligonucleotide Template:

5'- AAGTTTAGTATGCGTGCATGACCTGTCTC -3'

Appendix 1c siRNA Websites

The following websites were used to aid the design of the FIP4 siRNAs:-

Ambion

http://www.ambion.com/techlib/misc/siRNA_tools.html

This site allows you to enter the target sequence and then picks suitable siRNA sequences based upon parameters published by Tuschl's group (Elbashir et al., 2001). It also provides a tool to add the necessary sequences to the chosen siRNA so it can be transcribed using the Ambion Silencer™ siRNA Construction Kit.

Whitehead Institute of Biomedical Research at MIT

http://jura.wi.mit.edu/pubint/http://iona.wi.mit.edu/siRNAext/

This is a publicly available site which picks siRNA sequences from your target sequence using both Tuschl's and their own, stricter, additional selection parameters.

Qiagen

http://www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx

This design tool incorporates standard Tuschl-based design, with additional parameters. For example the overall GC content is considered and long stretches of Gs or Cs avoided.

These online tools were used to select the above three sequences which were predicted as good target sequences by the various programmes. The "1067 scramble" sequence contains the same bases as the 1067 siRNA but in a different order. This was used as a negative control.

All sequences were then run through the NCBI's BLAST programme to check they did not contain an unacceptably high degree of homology with any other human sequences. This ensured that the siRNAs that had been designed would be specific for FIP4. The sequences chosen showed a maximum of 15 contiguous base pairs homology to any other coding sequences. The accepted maximum number of base pairs of contiguous sequence allowing a siRNA to still act specifically is 16-17. As the above siRNAs fall within this limit, they should be specific.

The BLAST programme can be found on the NCBI server at:

www.ncbi.nlm.nih.gov/BLAST.

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