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**The translation initiation factor (eIF) 4F complex is compartmentalised in normally growing fibroblasts, but is not directly localised to either the actin or tubulin cytoskeleton.**

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## **Abstract**

Previous observations of association of mRNAs and ribosomes with subcellular structures highlight the importance of localised translation within cells. However, little is known regarding associations between eukaryotic translation initiation factors and cellular structures within the cytoplasm of normally growing cells. Here we have used detergent-based cellular fractionation methods coupled with immunofluorescence microscopy to investigate the subcellular localisation of the eukaryotic initiation factors involved in recruitment of mRNA for translation in NIH3T3 fibroblasts. We have focussed on eIF4E, the mRNA cap-binding protein, the scaffold protein eIF4G and poly(A) binding protein (PABP). Our data suggest that the bulk of these proteins exist in a soluble cytosolic pool, with only a sub-fraction tightly associated with cellular structures. However, translation initiation factors engaged in active eIF4F complexes were more extensively sequestered in association with subcellular structures. Immunofluorescence analysis reveals both a diffuse and a perinuclear distribution of eIF4G, with the perinuclear staining pattern similar to that of the endoplasmic reticulum. eIF4E also shows both a diffuse staining pattern and a tighter perinuclear stain, partly coincident with vimentin intermediate filaments. For all three proteins we observed localisation to the lamellipodia of migrating cells in close proximity to ribosomes, microtubules, microfilaments and focal adhesions, with eIF4G and eIF4E at the periphery showing a similar staining pattern to the focal adhesion protein vinculin.

## **Introduction**

Localised translation is increasingly recognised as an important mechanism of delivering proteins to their sites of function within cells (Carson et al., 1998; Jansen,

2001; Jockusch et al., 2003; Kloc et al., 2002), with complexes of mRNAs and associated proteins shown to interact with cytoskeletal networks to facilitate trafficking within the cell. Examples include mRNAs and other components of the protein synthesis machinery moving as granules in oligodendrocytes (Carson et al., 1998; Jansen, 1999; Jansen, 2001), actin mRNA moving to the leading edge of migrating fibroblasts (Chicurel et al., 1998; Farina et al., 2003), and staufen-mediated transport of *oskar* and *bicoid* mRNA to the appropriate poles of developing *Drosophila* embryos (Micklem et al., 2000). In addition, it has long been known that interaction of translating ribosomes with the endoplasmic reticulum plays a key role in directing proteins into the secretory pathway, and recent studies have elucidated further details of this mechanism (Lerner et al., 2003; Nicchitta et al., 2005).

Early work addressing possible links between protein synthesis and the cytoskeleton involved the fractionation of mammalian cells using detergents to select for free or cytoskeleton-associated components. Gentle lysis of cells in the presence of a non-ionic detergent (e.g. Triton X-100) released a sub-fraction of the cellular ribosomes into the extract, most of which were inactive 80S monomers (Lenk et al., 1977). Considerably more ribosomes were subsequently released if the pellet was extracted with the anionic detergent sodium deoxycholate (DOC). Generally, this fraction contained a much higher proportion of ribosomes in polysomes, suggesting that they were more active in protein synthesis (Bonneau et al., 1985; Cervera et al., 1981; Lemieux and Beaud, 1982; Pramanik et al., 1986; van Venrooij et al., 1981). The prevailing conclusion from these data, that translation *in vivo* was mostly associated with the cytoskeleton, was reinforced by observations that ribosomes were released by treatment of cells with cytochalasin D (Lenk et al., 1977), and that protein synthesis was impaired in unattached cells (Farmer et al., 1983) or when the actin

cytoskeleton was disrupted (Hudder et al., 2003; Ornelles et al., 1986; Stapulionis et al., 1997). Moreover, in vitro translation systems prepared from vertebrate cells under conditions that partially or wholly retained the cytoskeletal structure appear to have higher or more sustained protein synthetic activity than those prepared by conventional lysis (Biegel and Pachter, 1991; Negrutskii et al., 1994; Patrick et al., 1989).

However, there are differing interpretations on the extent to which association of the translational apparatus with the endoplasmic reticulum, rather than with the cytoskeleton, may contribute to these findings (Dang et al., 1983; Lenk et al., 1977; van Venrooij et al., 1981; Hovland et al., 1996; Ramaekers et al., 1983). Indeed, evidence for the direct association of ribosomes and translation factors with cytoskeletal components remains highly variable. Ribosomes have been reported to associate with microtubules in sea urchin embryos (Hamill et al., 1994) and with intermediate filaments in fibroblasts (Traub et al., 1998), with elongation factor eEF1A recognised as a binding partner of both actin (Clare et al., 1996; Liu et al., 2002; Murray et al., 1996; Umikawa et al., 1998) and tubulin (Moore and Cyr, 2000; Moore et al., 1998). eEF2 has also been identified as interacting with actin (Shestakova et al., 1991). Both eEF1A (Munshi et al., 2001; Murray et al., 1996) and the release factor eRF3 (Valouev et al., 2002) have been reported to influence the organisation of the actin cytoskeleton, with eEF1A also involved in maintaining the localisation of  $\beta$ -actin mRNA in protrusions of migrating fibroblasts (Liu et al., 2002).

For translational initiation factors, relatively little is known about their association with cellular structures within the cytoplasm, although in response to severe cellular stress several of them become sequestered with other proteins and 40S ribosomal

subunits in granules ((Cuesta et al., 2000; Kedersha et al., 2005; Kedersha et al., 2001; Kim et al., 2005; Kimball et al., 2003). The largest subunit (eIF3a, p 170, TIF32) of the multimeric initiation factor eIF3 has variously been reported to interact with an actin-associated protein (Palacek et al., 2001), with membranes *via* actin filaments (Pincheira et al., 2001), with microtubules (Hasek et al., 2000) and with intermediate filaments (Lin et al., 2001). A smaller subunit (eIF3g, p44) is suggested to be an anchor between the protein synthesis apparatus and the cytoskeleton in red blood cells (Hou et al., 2000). The poly (A) binding protein (PABP) which associates with the initiation factor eIF4G (Prevot et al., 2003), has been shown to be localised to RNA granules on oligodendrocytes (Barbarese et al., 1995), to stress granules (Kedersha and Anderson, 2002; Kedersha et al., 1999) and, interestingly, has been shown to co-localise with paxillin in the endoplasmic reticulum and at the leading edge of migrating fibroblasts (Woods et al., 2002). In addition, relocalisation of the cap recognition factor eIF4E during platelet activation from the membrane skeleton to the mRNA-rich cytoskeletal core has been shown to occur concomitantly with a stimulation of protein synthesis, an event prevented by disruption of the actin cytoskeleton (Lindemann et al., 2001). Moreover, in neuronal preparations, eIF4E was found to associate with two different actin networks in dendrites; one contained longer filaments easily disrupted by latrunculin A (lat A) while the other, located in dendritic spine heads consisted of a highly branched network of shorter filaments enriched in granules containing mRNA and more resistant to lat A (Smart et al., 2003). Following treatment with brain-derived neurotrophic factor (BDNF), the proportion of eIF4E in the dendritic spine heads was increased, a change suggested to facilitate local translational activity.

These observations raise important questions concerning the topology of protein synthesis. A model whereby cellular translation largely involves localised components associated with cellular structures would be consistent with earlier indications of “channelling” of aminoacyl-tRNAs into protein synthesis (Hudder et al., 2003; Negrutskii and Deutscher, 1991; Negrutskii and Deutscher, 1992; Negrutskii et al., 1994; Stapulionis and Deutscher, 1995; Stapulionis et al., 1997) and with the notoriously low translational activity of extracts derived from adherent cultured cells. To examine this we have focussed on the group of translation initiation factors that interact with the mRNA 5' cap as the first step in the recruitment of mRNAs for translation and examined the association of eIF4E, eIF4G and PABP with the major cytoskeletal networks. Our evidence suggests that, while the main pool of each of these proteins in cells is cytosolic, the majority of the eIF4F complex (eIF4E/4G/PABP) is compartmentalised, but not directly localised to either the actin or tubulin cytoskeletons. Rather, a significant proportion of each of these proteins appears to be localised with the ER, with a smaller proportion observed at the leading edge of migrating cells.

## **Materials and Methods**

**Cell culture and treatments.** NIH3T3 cells were cultured in DMEM (Invitrogen,UK) supplemented with 10% (v/v) foetal bovine serum (Labtech,UK) in a humidified atmosphere containing 5% CO<sub>2</sub>. In some experiments, as specified, microfilaments were disrupted by incubating cells with 2  $\mu$ M Cytochalasin-D in ethanol for 1 hour and stress fibre formation was facilitated by incubating cells with 25  $\mu$ M lysophosphatidic acid (LPA) (Sigma, UK) for 1 hour.

**Immunofluorescence microscopy.** Coverslips were coated with 100 µg/ml poly-L lysine (Sigma,UK) in PBS and allowed to dry overnight. The coverslips were then washed twice in 1 ml PBS and coated with 100 µg/ml bovine fibronectin (Sigma, UK), incubated for 1 hour then washed once in PBS.  $5 \times 10^4$  cells were seeded onto each 22 mm coverslip and allowed to grow for 24 hours. Cells were then washed once in 1 ml PBS at 37°C, then fixed in 4% paraformaldehyde/PBS for 20 minutes and permeabilised with 0.1% (v/v) Triton X-100/PBS for 5 minutes prior to staining. For staining of the endoplasmic reticulum or intermediate filaments, cells were washed once in 1 ml PBS at 37°C followed by fixation in 100% methanol at -20°C for 5 minutes and permeabilised with 200 µg/ml saponin in cytoskeleton buffer (100 mM PIPES.KOH pH 6.9, 1 mM EGTA, 1 mM MgCl<sub>2</sub>) for 5 minutes (Pahlad et al., 1998). For the saponin washout experiments (Fig. 3), the cells were washed in 1ml PBS at 37°C and then permeabilised with 200 µg/ml saponin in cytoskeleton buffer (100 mM PIPES.KOH pH 6.9, 1 mM EGTA, 1 mM MgCl<sub>2</sub>) for 30 seconds followed by immediate fixation in 4% (w/v) paraformaldehyde/PBS for 20 minutes. Following fixation and permeabilisation, non-specific binding was blocked by adding 3% (w/v) BSA in PBS for a minimum of 20 minutes at room temperature. Cells were incubated in the primary antibody solution for 60 minutes, washed extensively and then incubated with the appropriate secondary antibody and/or phalloidin-FITC or anti-tubulin-FITC conjugate for 60 minutes. Following further extensive washing, nuclei were stained with DAPI for 5 minutes. After a further two washes, coverslips were mounted on microscope slides with Mowiol mounting solution (0.2 M Tris pH 8.5, 33%(w/v) glycerol, 13% (w/v) Mowiol, 2.5% (w/v) 1,4-diazobicycol [2,2,2]-octane (DABCO)) and sealed with clear nail polish. Images were collected on a Zeiss Axioscop 2 widefield fluorescence microscope using a 63x objective. Polyclonal



rabbit antibodies were raised against a C-terminal peptide of eIF4GI, RTPATKRSFSKEVEERSR (amino acids 1179-1206) (used at 1 in 200); eIF4E, TATKSGSTTKNRFVV (amino acids 203-217) (1 in 50); and PABP, IPQTQNRAAYYPPSQIAQLRPS (amino acids 413-434) (1:300) (Bushell et al., 2000a; Bushell et al., 2000b; Coldwell et al., 2004). These rabbit antisera were immunopurified from crude serum by affinity chromatography with the corresponding peptide using the SulfoLink kit (Perbio Science, UK) according to the manufacturer's instructions. Monoclonal mouse antibodies included anti  $\alpha$ -tubulin-FITC conjugate clone DM 1A (Sigma, UK) 1:300, anti-calnexin clone 37 (Transduction Laboratories, USA) 1:50, anti-vimentin clone VIM-13.2 (Sigma, UK) 1:100, anti-vinculin clone VIN-11-5 (Sigma, UK) 1:100, anti-paxillin clone 349 (Transduction Laboratories, USA) 1:100. Nuclei were stained with 12.5 ng/ml 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) (Sigma, UK). Actin was visualised using a phalloidin-FITC conjugate (Dako, UK) at a concentration of 100 ng/ml. Secondary antibodies used were goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) at a concentration of 1:100, or porcine anti-rabbit IgG conjugated to rhodamine at 1:300 (Dako, UK).

**Lysis of cells and preparation of “free” and “free + bound” cell extracts.** Cells were seeded onto 10 cm plates and grown to 70% confluency and harvested by scraping in PBS containing 2 mM benzamidine on ice. Cells were then recovered by centrifugation in a microfuge at 15,000 rpm for 1 minute and re-suspended in 200  $\mu$ l lysis buffer (20 mM Mops.KOH pH 7.2, 25 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM benzamidine, 2 mM EGTA, 0.1 mM GTP, 0.5 mM DTT, 10% (v/v) glycerol and protease inhibitor cocktail (Roche, UK). Lysis buffer was either supplemented with

0.2 % (v/v) Igepal to yield the “free” fraction or with 0.2% (v/v) Igepal and 0.4 % (v/v) sodium deoxycholate to release the “free + bound” fraction. After vortexing, the lysates were then centrifuged in a microfuge for 3 minutes at 15,000 rpm, and the supernatant was recovered. For the preparation of the detergent free mechanically lysed “free” extract, cells were scraped as above and resuspended in hypotonic buffer as described by (Lerner et al., 2003) containing (10 mM Mops.KOH pH7.2, 10 mM KAc, 1.5 mM MgAc, 0.5 mM DTT and protease inhibitor cocktail (Roche) and incubated for 5 minutes on ice. The cell suspension was then homogenised by passage six times through a 25 gauge needle using a 5ml syringe and the resulting lysate centrifuged for 3 minutes at 15,000 rpm and the supernatant recovered.

**m<sup>7</sup>GTP-Sepharose Affinity Chromatography.** Protein was bound onto the beads by mixing equal cell equivalents of the S10 cell extracts with 30 µl of a 50% (v/v) slurry of m<sup>7</sup>GTP-Sepharose 4B resin (Amersham Biosciences, UK) in m<sup>7</sup>GTP-Sepharose Wash Buffer (20 mM Mops.KOH pH 7.2, 20 mM KCl, 2 mM benzamidine, 7 mM 2-mercaptoethanol, 1 mM MgAc, 0.1 mM GTP, 0.25 % (v/v) Igepal) in a final volume of 230 µl. The mixture was incubated with gentle agitation for 10 minutes at 4°C. Afterwards the resin was isolated by centrifugation in a microfuge at 15,000 rpm for 3 minutes at 4°C and the supernatant aspirated and discarded. The bulk of non-specifically interacting proteins were removed by 3 washes in 500 µl m<sup>7</sup>GTP-Sepharose Wash Buffer interspersed with centrifugation as above. Finally, eIF4E and associated factors were eluted from the resin into 25µl SDS-PAGE sample buffer and separated on 10% SDS-PAGE gels and resolved by immunoblotting. The primary antisera used were those specified above with detection using donkey anti-rabbit IgG

conjugated to horseradish peroxidase (Amersham Biosciences, UK) (1:2000) and ECL.

## Results

As discussed above, a substantial proportion of ribosomes in mammalian cells is associated with cellular structures that are sedimented when cell extracts obtained by gentle lysis are centrifuged at around 10,000 x g. These data suggest that at least part of the protein synthetic machinery is associated with the cytoskeleton. To address this for the initiation factors that recruit mRNA for translation, we compared extracts obtained from NIH-3T3 fibroblasts by lysis in the presence of a low (0.2% (v/v)) concentration of the non-ionic detergent Igepal (“free”) with those prepared in parallel in the presence of both 0.2% (v/v) Igepal and 0.4% of the anionic detergent sodium deoxycholate (DOC; (“free + bound”)). The latter is commonly used to extract cytoskeletal and membrane-bound proteins into the soluble fraction (Bonneau et al., 1985; Cervera et al., 1981; Lemieux and Beaud, 1982; Pramanik et al., 1986; van Venrooij et al., 1981). Fig.1A shows that, as assessed by direct analysis of the extracts by SDS-PAGE and Western immunoblotting, the supernatants contained similar amounts of eIF4E, eIF4G and PABP. We then subjected each extract to the affinity resin m<sup>7</sup>GTP-Sepharose, which captures the cap-binding protein eIF4E together with proteins associated with it in the eIF4F complex. Fig.1B shows that the eIF4E recovered from cells lysed with Igepal alone was associated with modest levels of its binding partners eIF4G and PABP, whereas a considerably larger amount of these proteins, relative to eIF4E, was recovered in the complexes from the Igepal/DOC-lysed cells. Similar results were obtained when we compared extracts from cells lysed

mechanically in the absence of detergent (Fig.1C) with those lysed in the presence of both Igepal and DOC (Fig.1B). These data suggest that the bulk cellular pool of eIF4G and PABP is easily released from cells, indicating a cytosolic localisation. However, the population associated with eIF4E in the active eIF4F complex is more extensively sequestered in association with cellular structures.

In view of a number of suggestions that the integrity of the actin cytoskeleton is required for optimal translation rates (Hudder et al., 2003; Stapulionis et al., 1997) and that  $\beta$ -actin mRNA is localised in fibroblasts (Chicurel et al., 1998; Farina et al., 2003), we examined the localisation of eIF4G, PABP and eIF4E in NIH-3T3 cells in comparison with actin microfilaments (Fig. 2A). While actin stress fibres can clearly be seen in these cells, there is no obvious co-localisation of these proteins with the actin cytoskeleton. Furthermore, there is also no apparent co-localisation of eIF4E, eIF4G or PABP with microtubules (Fig. 2B). These data were confirmed in studies where microtubules were disrupted with nocodazole; while this treatment had severe effects on overall cell morphology, no specific effect on initiation factor localisation was observed (data not shown). Rather, these initiation factors appear to distribute widely across the cytoplasm, tending to be enriched in the perinuclear area. In agreement with published data (Woods et al., 2002), PABP exhibits a degree of localisation to the cell periphery. Another possibility for functional compartmentalisation of the protein synthetic machinery is association with intermediate filaments, and evidence has been presented for interaction of ribosomes (Traub et al., 1998) and eIF3 (Lin et al., 2001) with these elements. We therefore compared the immunofluorescence patterns of eIF4G, PABP and eIF4E with the major intermediate filament protein in these cells, vimentin (Fig. 1C). For this we had to use methanol, rather than paraformaldehyde fixation, as the latter procedure

resulted in pronounced disruption of intermediate filaments in these cells (data not shown). While there is a similarity in distribution pattern between initiation factors and vimentin (Fig. 2C), particularly in the perinuclear region, these data show that for eIF4G and PABP there is little direct co-localisation with vimentin filaments. However, with eIF4E some co-localisation was more evident (see enlarged section, lower right panels) although the physiological relevance of this is not yet clear.

A potential problem with this type of analysis is that only a proportion of each protein is at any one time involved in the eIF4F complex that forms during mRNA recruitment. Indeed, as seen in Fig.1, the eIF4F complex appears to be associated with cellular structures to a greater degree than any of the free proteins. It is therefore possible that in immunofluorescence studies a sub-population of initiation factors associated with microfilaments or microtubules is obscured by an excess of the free proteins in the cytosol. To address this, we attempted to release some of the bulk cytosolic factors by subjecting the cells to rapid, gentle permeabilisation with saponin prior to fixation with paraformaldehyde (Fig. 3). This procedure presented some difficulty, as the time of exposure to saponin before fixation was critical (typically 30 sec, after which cellular structures were seriously disrupted). The data in Fig. 3 show the distribution of eIF4G and PABP under conditions in which the microfilaments and microtubules were maintained and confirm the results presented in Fig. 2 indicating no co-localisation of either factor with these structures. Whilst the general distribution of eIF4G and PABP in the cytosol appears somewhat more granular than in the conventionally fixed cells, their localisation in the perinuclear region and at the cell periphery was maintained. Interestingly, these staining patterns show a general similarity to that of poly(A)<sup>+</sup> mRNA in human diploid fibroblasts subjected to

permeabilisation with Triton X-100 (Taneja et al., 1992), consistent with association of these proteins with mRNA.

As disruption of microtubules with nocodazole had no clear effect on the localisation of initiation factors (data not shown), we investigated the effect of disrupting microfilaments with cytochalasin D (Fig. 4A-C). Under these conditions, although microfilaments are efficiently disrupted, initiation factors are seen to localise in a similar manner to that observed with the vehicle control. To further probe the dependence of initiation factor localisation on the actin cytoskeleton we investigated the effects of stimulating stress fibre formation with lysophosphatidic acid (LPA). As shown in Fig. 4D, there was no tendency for eIF4E to associate with these fibres. Similar results were obtained with eIF4G and PABP (data not shown).

It has long been known that a substantial proportion of protein synthesis takes place on ribosomes associated with the ER, and recent data from Nicchitta's laboratory (Lerner et al., 2003) have extended previous models to suggest that the initial recruitment of a wide variety of mRNAs may involve ER-bound 40S subunits. In addition, a proportion of cellular PABP has been reported to be indirectly associated with the ER in the perinuclear region of fibroblasts (Woods et al., 2002). Hesketh and colleagues have pointed out that the cell fractionation procedures used by earlier workers to identify cytoskeletal association, on which the procedures used for Fig. 1 were based, would also score as "bound" proteins associated with the ER (Hovland et al., 1996). To address whether eIF4E, eIF4G and PABP are associated with the ER in NHI3T3 cells, we compared their localisation with calnexin, an ER transmembrane protein that acts as a chaperone in protein folding (Trombetta and Helenius, 1998) and interacts directly with ribosomes (Delom and Chevet, 2006). As shown in Fig. 5, while calnexin is enriched in the perinuclear region, it can clearly be seen extending

to the cell periphery. While the overall distribution of eIF4E and PABP are all superficially quite similar to that of calnexin, these data indicate that there is no evidence for direct co-localisation. In contrast, there does appear to be some degree of co-localisation of eIF4G with calnexin and presumably the ER in these cells (Fig. 5). These preliminary findings await further biochemical confirmation.

Finally we investigated the relationship between the localisation of eIF4E and eIF4G and the focal adhesion proteins vinculin and paxillin, as earlier work had demonstrated recruitment of poly(A)+ mRNA and ribosomes to focal adhesions in response to integrin stimulation (Chicurel et al., 1998). In addition, PABP has been shown by a variety of methods to interact with paxillin in these cells (Woods et al., 2002), but the same study did not reveal interaction between PABP-paxillin and eIF4G using co-immunoprecipitation techniques. Fig. 6 shows the localisation of paxillin and vinculin in NIH3T3 cells, clearly showing the presence of focal adhesions. While the localisation of eIF4E did not resemble that of paxillin, the distribution of both eIF4E and eIF4G exhibited quite marked similarities to that of vinculin. For both factors we have frequently observed localisation to distinct sites at the cell periphery close to the focal adhesions, and this appears to be the case particularly in cells undergoing migration (Fig. 7). In general, these initiation factors are seen close to, or surrounded by, cytoskeletal structures but not apparently co-localised with them.

## **Discussion**

In this work we aimed to assess the importance of the interaction of the translational machinery with structures such as the cytoskeleton, the endoplasmic reticulum and focal adhesions by examining the localisation of initiation factors

involved in recruitment of mRNA molecules for translation. These proteins are known to initiate this process by interacting to form a complex known as eIF4F at the 5' cap of mRNA. In order to avoid the possibility of altered localisation behaviour resulting from unbalanced expression we have used immunofluorescence microscopy to detect the endogenous proteins rather than monitoring the localisation of GFP-fusion proteins following transfection. Earlier reports had indicated the association of a substantial proportion of cellular ribosomes and mRNA with cellular components that are not easily released into soluble form when cells are gently lysed with non-ionic detergents. Some studies had suggested selective association of cytoskeletal components with actively translating ribosomes and had found “free” cytosolic ribosome pools to be enriched in inactive 80S particles. Other reports suggested that the integrity of the actin cytoskeleton is essential for active translation in mammalian cells. The data we present in Fig. 1 is consistent with such conclusions, since it suggests that, while free eIF4G and PABP are well represented in cell extracts following gentle lysis, the population of these proteins present in association with eIF4E in the active eIF4F complex (recovered on m<sup>7</sup>GTP affinity resin), is only solubilised efficiently in the presence of the harsher anionic detergent, sodium deoxycholate. We have made similar observations using CHO cells and a *Xenopus* kidney cell line (S. van Wageningen, H. Pollard & V. M. Pain, unpublished observations). An important practical consequence of this is that choice of lysis conditions may have unintended, selective effects on the results of investigations of eIF4F complexes in mammalian cells and that cell-free translation extracts prepared by mechanical lysis (Bergamini et al., 2000; Svitkin et al., 2001; Svitkin and Sonenberg, 2004; Thoma et al., 2004) may be selectively depleted of such complexes.



Many reports of interaction between individual translation factors and either membrane or cytoskeletal structures have been based entirely on cell fractionation studies. Microscopy studies have concentrated particularly on highly specialised cell types such as those of the nervous system or on cells subjected to severe stress. Here we have used immunofluorescence microscopy to examine the localisation of the initiation factors involved in mRNA recruitment in parallel with some of the major cellular networks in normally growing mammalian fibroblasts. In general the factors were widely distributed throughout the cytoplasm and did not appear to co-localise with any of the major cytoskeletal networks or with the ER. Attempts to examine the cytoskeletal core remaining after gentle permeabilisation of the cells prior to fixation did not reveal any more subtle co-localisation patterns (Fig. 3). In addition, the distribution of the factors was not grossly affected by disrupting the microtubules (data not shown) or microfilaments (Fig. 4A-C), or by inducing the formation of more prominent actin stress fibres (Fig. 4D). However, since a significant proportion of these factors, and particularly the eIF4F complex, remain with the structural cellular material that is not solubilised or washed out during gentle detergent treatment (Figs. 1 and 3), it is likely that there is some association of them with cytoskeletal networks. One possibility is suggested by work from Singer's laboratory (Bassell et al., 1994), where electron microscopy was used to follow up earlier immunofluorescence studies (Taneja et al., 1992) investigating the distribution of total poly(A)<sup>+</sup> mRNA in fibroblasts. This work suggested that a high proportion of poly(A)<sup>+</sup> mRNA and polysomes in fibroblasts were closely associated with actin filaments, but restricted to the sites of intersections, such that their localisation did not follow the whole length of the filament. The rather granular appearance of the images of initiation factors in our permeabilised cells (Fig. 3) would be consistent with this. A significant minority of

the mRNA was also shown to be very close to vimentin intermediate filaments (Bassell et al., 1994), and, although we did not see significant co-localisation of eIF4G or PABP with these filaments in our cells, the distribution of eIF4E appeared close to that of vimentin (Fig. 2C). Indeed, we have occasionally observed filamentous patterns of eIF4E in the perinuclear region that could be explained by association of a subpopulation of eIF4E with intermediate filaments. In contrast, eIF4G localisation appeared perinuclear and closer to that of calnexin (Fig. 5), similar to findings of PABP localisation to this compartment in association with paxillin (Woods et al., 2002). As the perinuclear region is enriched in ER, it is likely that this reflects the role for eIF4G and PABP in recruitment of mRNAs encoding secretory or membrane proteins, and possibly some nuclear proteins whose mRNAs have been localised to this area (Levadoux et al., 1999; Mickleburgh et al., 2005).

Finally, our observations of distinct areas of localisation of translation initiation factors to the cell periphery have mainly been made with cells exhibiting migration activity, associated with the extension of protuberances (see Figs. 6 and 7). This is of interest in fibroblasts, where at least one major mRNA ( $\beta$ -actin) is known to move towards the periphery when cells are stimulated to proliferate or migrate (Latham et al., 1994; Hill et al., 1994; Kislauskis et al., 1997). PABP, again in association with paxillin, has also been reported to move from the perinuclear region to the periphery in response to conditions promoting migration (Woods et al., 2002). However, the latter report failed to detect direct association of eIF4G with PABP-paxillin complexes. Interestingly the areas of eIF4E and eIF4G enrichment observed at the cell periphery appeared to localise adjacent to, but not coincident with, microfilaments, microtubules and focal adhesions (Fig. 6). Many of the experiments reported here were performed with cells growing on fibronectin-coated cover-slips,

conditions which promote integrin signalling. The peripheral localisation of initiation factors in migrating cells observed here thus reinforces an earlier observation of recruitment of ribosomes and mRNAs to focal adhesions (Chicurel et al., 1998), suggesting the up regulation of localised translation following integrin engagement.

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## **Figure legends**

**Figure 1. Solubilisation of eIF4F complexes into extracts from mammalian fibroblasts requires disruption of cellular structures by anionic detergent.**

**Panel A.** Cells were lysed either in the presence of Igepal alone (“free”) or in the presence of Igepal and DOC (“free + bound”), as described in Materials and Methods. Equal cell equivalents (approx 10 µg of protein) from the “free” and the “free and bound” S10 supernatants were analysed on SDS-PAGE gels, and total eIF4G, eIF4E and PABP released into the extracts were visualised by Western immunoblotting.

**Panel B.** Equal cell equivalents (approx 60 µg of protein) from the “free” and the “free and bound” fractions were subjected to m<sup>7</sup>GTP affinity chromatography as described in Materials and Methods, followed by SDS-PAGE and Western immunoblotting of the retained material.

**Panel C.** Cells were lysed by mechanical disruption in the absence of detergents, and S10 extracts prepared as described. A parallel “free + bound” extract was prepared as for Panel A. Equal cell equivalents of the resultant extracts were subjected to m<sup>7</sup>GTP affinity chromatography and the retained material analysed by SDS-PAGE and immunoblotting.

**Figure 2. Localisation of eIF4G, PABP and eIF4E in growing fibroblasts in comparison with filamentous actin, tubulin and vimentin.**

Cells were either fixed with paraformaldehyde followed by permeabilisation with Triton X-100 (Panels A and B) or fixed with methanol followed by permeabilisation with saponin (Panel C), as described in Materials and Methods.

**Panel A.** Immunofluorescence microscopy of eIF4G (top), PABP (middle) and eIF4E (bottom; TRITC) co-stained with phalloidin-FITC to detect filamentous actin.

**Panel B.** eIF4G, PABP and eIF4E (TRITC), each co-stained with anti-tubulin FITC conjugate.

**Panel C.** Initiation factors, as described above (TRITC), were co-stained with vimentin. The inset shows a magnification of the marked area demonstrating the similarity between the pattern of eIF4E and vimentin, particularly in the area proximal to the nucleus.

**Figure 3. Pre-permeabilisation of cells with saponin prior to paraformaldehyde fixation to remove the bulk of un-associated proteins does not reveal co-localisation between eIF4G and PABP with the cytoskeleton.**

Cells were briefly treated with saponin prior to fixation with paraformaldehyde, as described in Materials and Methods. The distribution of eIF4G (top two panels) and PABP (bottom two panels) was then compared with those of filamentous actin and tubulin, as described in Fig. 2.

**Figure 4. The localisation of eIF4E, eIF4G and PABP is not markedly affected by drug-induced disassembly or assembly of actin filaments.**

**Panels A-C.** Cells were treated with 2 $\mu$ M cytochalasin-D for 1 hour to disrupt filamentous actin, fixed with paraformaldehyde and permeabilised with Triton-X100, as described in Materials and Methods. Immunofluorescence staining patterns for eIF4E (Panel A), eIF4G (Panel B) and PABP (Panel C; all TRITC) are shown in comparison with phalloidin-FITC to detect filamentous actin. Results for cells treated with ethanol (the vehicle for cytochalasin D) are also shown.

**Panel D.** Cells were treated with 25  $\mu$ M lysophosphatidic acid (LPA) for 1 hour to stimulate the formation of actin stress fibres. The immunofluorescence pattern of eIF4E is shown in comparison to that of filamentous actin.

**Figure 5. eIF4G, PABP and eIF4E show a similar but not identical overall staining pattern to that of the endoplasmic reticulum.**

Cells were fixed and permeabilised with methanol followed by treatment with saponin, as described in Materials and Methods. Immunofluorescence staining patterns of eIF4G (top),

PABP (middle) and eIF4E (bottom) were compared with that of the integral endoplasmic reticulum protein, calnexin (stained with FITC). The inset panel shows a magnification of the marked area demonstrating the similarity between the pattern of eIF4G and the endoplasmic reticulum.

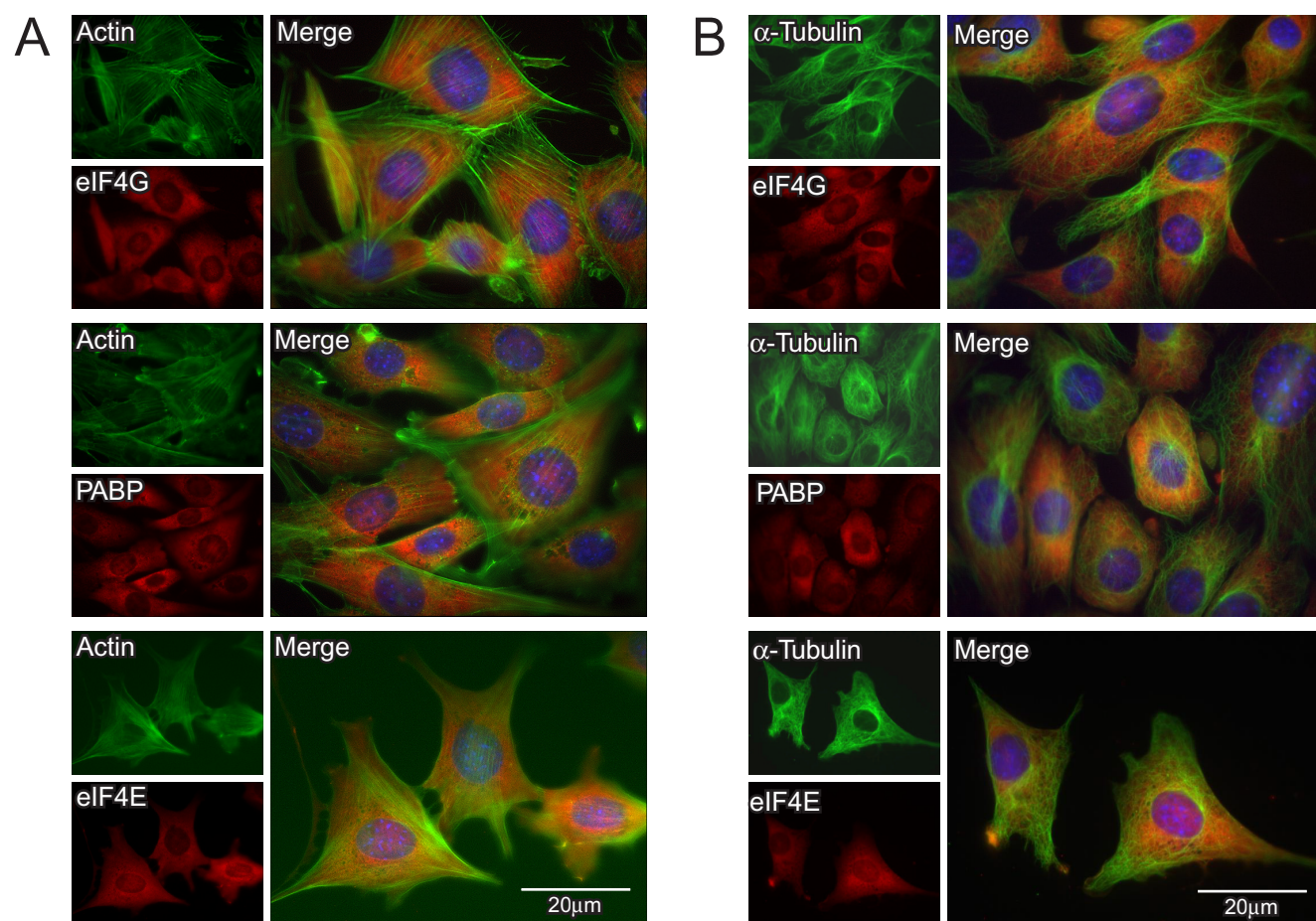
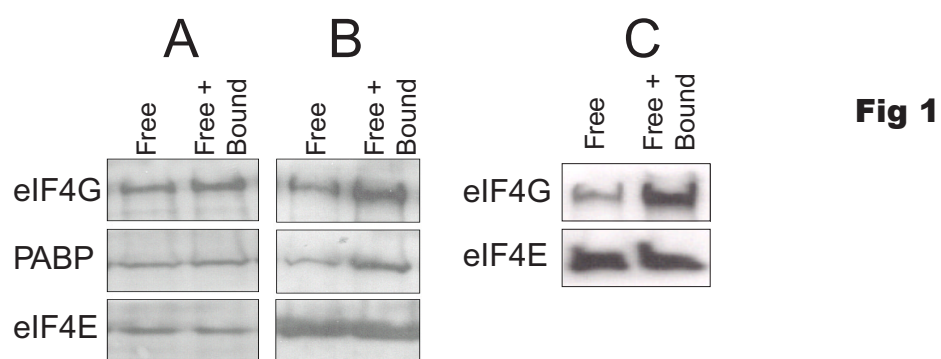
**Figure 6. Similar localisation between vinculin and eIF4G and eIF4E in the perinuclear region and in focal adhesions at the cell periphery.**

Cells were grown on fibronectin-coated coverslips and fixed with paraformaldehyde followed by permeabilisation with Triton X-100 as described in Materials and Methods.

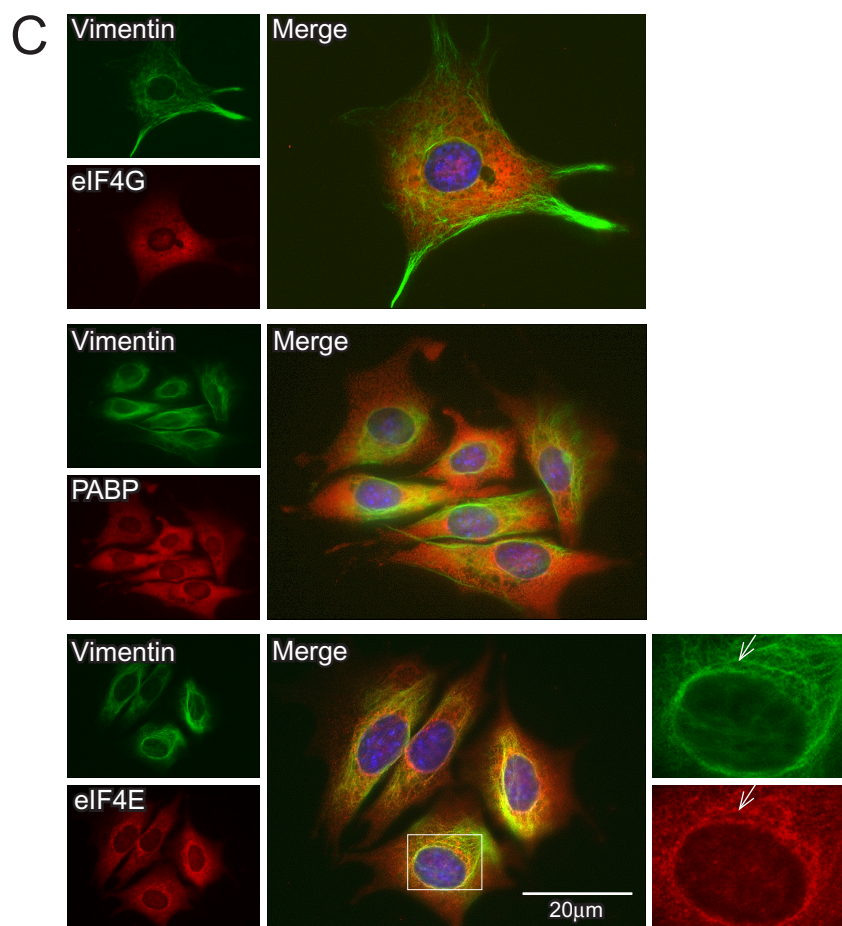
Immunofluorescence staining patterns for eIF4E (top and middle panels) and eIF4G (bottom panel) were compared with FITC staining of paxillin (top panel) and vinculin (middle and bottom panels). The middle and bottom panels show further magnification of the designated areas to demonstrate similarity of patterns between both factors and vinculin at the periphery.

**Figure 7. Local concentration of eIF4E, PABP, eIF4G and the ribosomal protein S6 to the leading edge of migrating cells.**

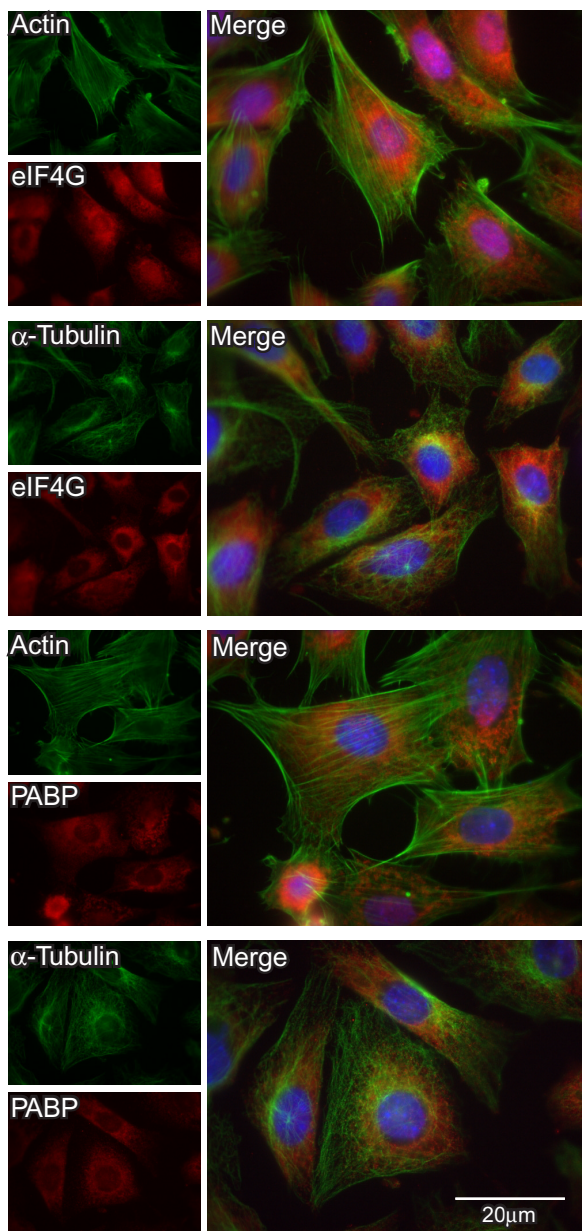
Cells were grown on fibronectin coated coverslips and fixed with paraformaldehyde followed by permeabilisation with Triton X-100 as described in Materials and Methods. Ribosomal protein S6 was detected using a phospho-specific antibody, because the antisera available for total S6 protein were poor at detecting ribosomes by immunofluorescence staining in these cells.



**Fig 2 A-B**

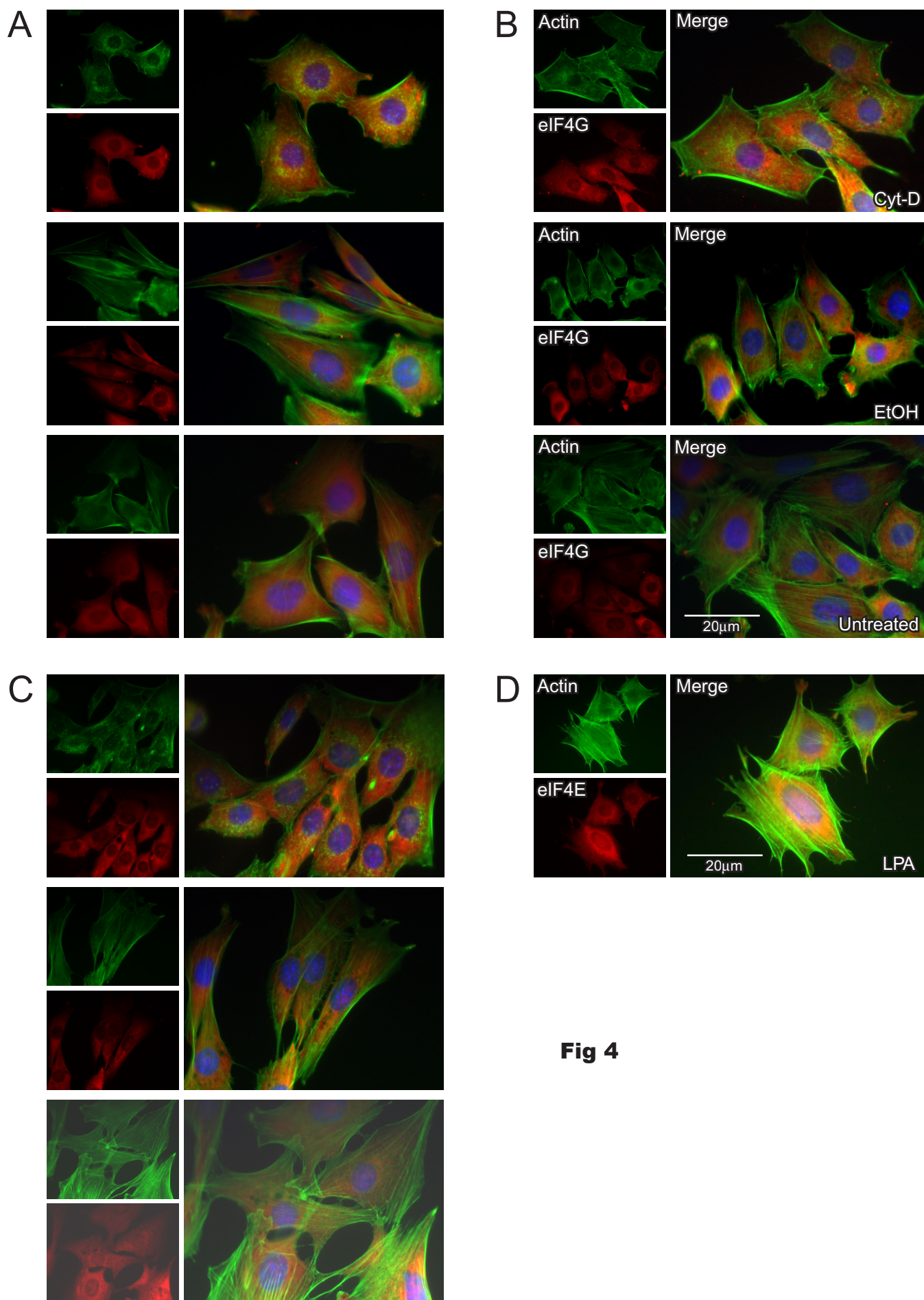


**Fig 2 C**

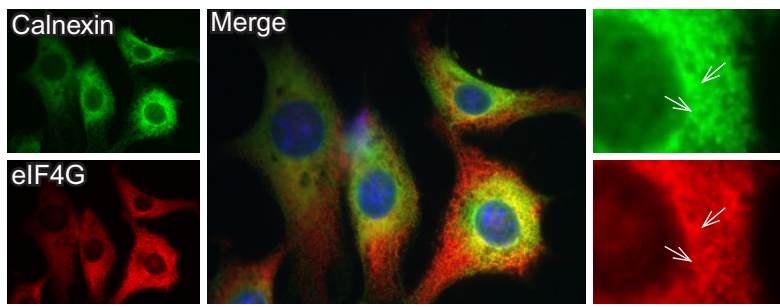


**Fig 3**

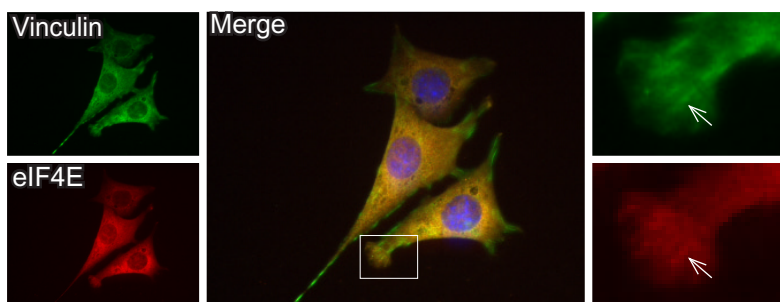
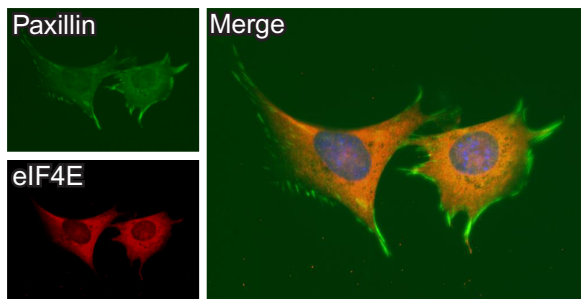
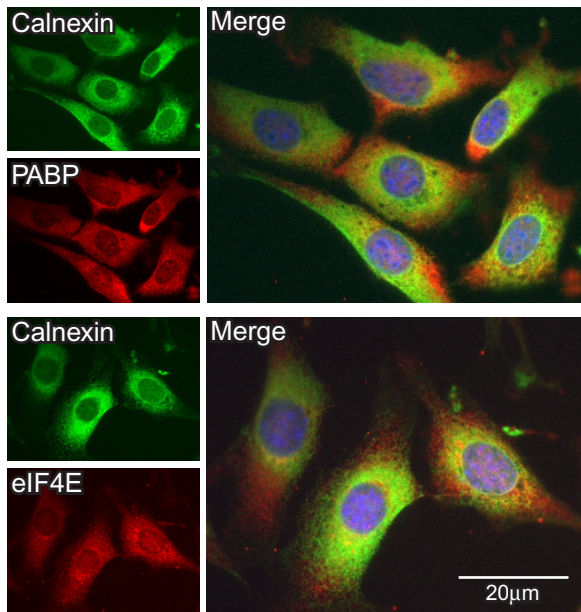




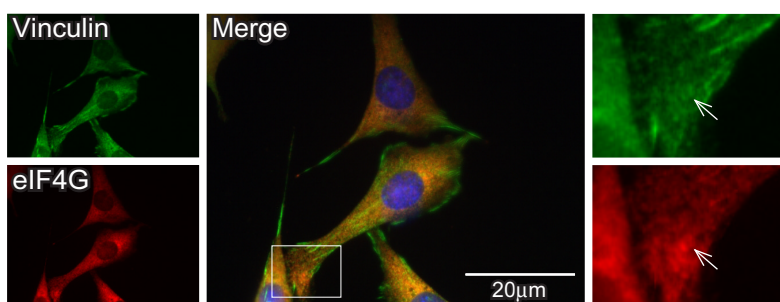
**Fig 4**

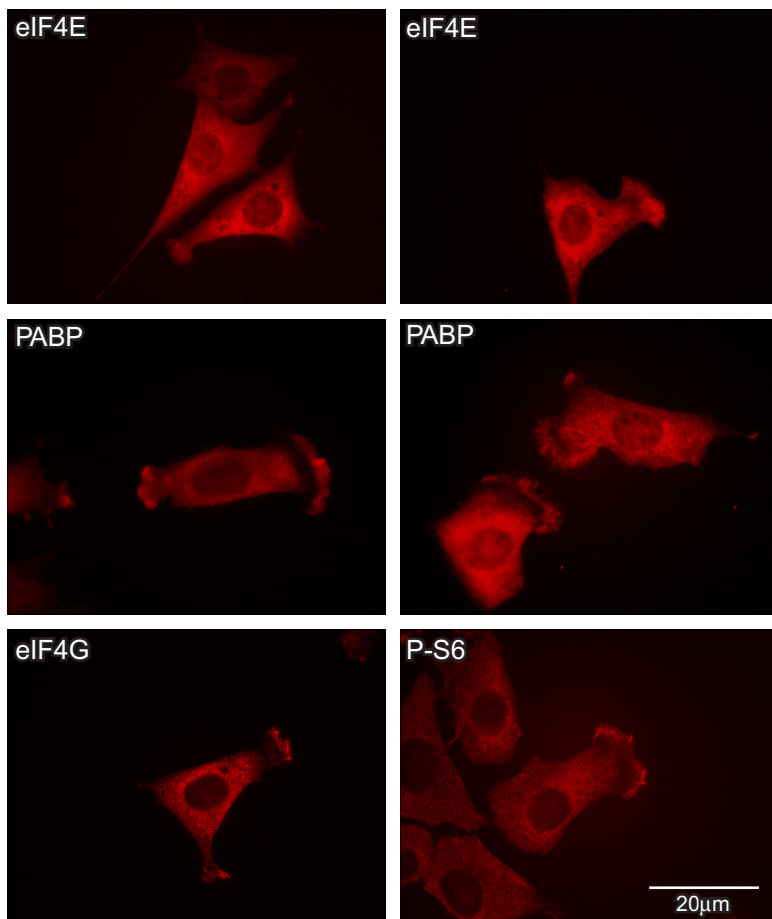


**Fig 5**



**Fig 6**





**Fig 7**