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Sumoylation of eIF4A2 affects stress granule formation

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SUMMARY STATEMENT

In response to stress, proteins required to initiate protein synthesis are modified; we demonstrate that sumoylation of eIF4A2 correlates with its recruitment to stress granules.

ABSTRACT

Regulation of protein synthesis is crucial for cells to maintain viability and to prevent unscheduled proliferation that could lead to tumorigenesis. Exposure to stress results in stalling of translation, with many translation initiation factors, ribosomal subunits and mRNAs being sequestered into stress granules or P bodies. This allows the re-programming of the translation machinery. Many aspects of translation are regulated by post-translational modification. Several proteomic screens have identified translation initiation factors as targets for sumoylation, although in many cases the role of this modification has not been determined. We show here that eIF4A2 is modified by SUMO, with sumoylation occurring on a single residue (K226). We demonstrate that sumoylation of eIF4A2 is modestly increased in response to arsenite and ionising radiation but decreases in response to heat shock or hippuristanol. In arsenite treated cells but not in hippuristanol treated cells, eIF4A2 is recruited to stress granules, suggesting sumoylation of eIF4A2 correlates with its recruitment to stress granules. Furthermore, we demonstrate that inability to sumoylate eIF4A2 results in impaired stress granule formation, indicating a novel role for sumoylation in the stress response.
INTRODUCTION

Protein synthesis is a fundamental cellular process, which needs to be efficiently regulated, particularly in response to environmental stresses. It comprises three stages: initiation, elongation and termination. Of these, the initiation step has a major regulatory role in protein synthesis, and affects not only the level of protein synthesis, but also which mRNAs are translated (Sonenberg, & Hinnebusch 2009; Guertin, & Sabatini 2007; Laplante, & Sabatini 2012; Morley et al 2005; Jackson et al 2010). Translation initiation involves the binding of the preinitiation complex (that comprises the 40S ribosomal subunit, eukaryotic initiation factor 3 (eIF3), eIF1A, eIF1, eIF5 and eIF2 (eIF2/GTP/methionyl initiator tRNA) to capped mRNA that is bound by eIF4F (a complex of eIF4A, eIF4E and eIF4G). The resulting 48S preinitiation complex then scans the 5’ untranslated region of the mRNA for the initiation codon, at which point early initiation factors are released and the large ribosomal subunit is recruited, allowing protein synthesis to begin (reviewed in (Jackson et al 2010)).

In response to stress, most of the protein synthesis within a cell is shut down in order to conserve energy to allow repair of stress-induced damage and reprogramming of the translational machinery e.g. (Spriggs et al 2010; Balagopal, & Parker 2009). Polysomes are disassembled, leading to the stalling of initiation complexes that are then recruited to specialised bodies, termed stress granules. These granules are proposed to be sites where, during stress and recovery, individual mRNAs are sorted for storage, degradation or translation. The granules are highly dynamic and can either fuse with P bodies that contain the mRNA decay machinery, or can release components to allow resumption of translation (Anderson, & Kedersha 2009). Formation of stress granules is induced by phosphorylation of eIF2α (Kedersha et al 1999), or by other factors, such as conditions that inhibit eIF4A e.g. (Dang et al 2006). Also important for induction are the TIA proteins (T-cell internal antigen), TIA-1 and TIA-R, that have prion-related C-terminal domains (Waris et al 2014). In mammalian cells, stress granules comprise numerous proteins including eIF2, eIF3, eIF4A, eIF4E, eIF4G, PABP, the small ribosomal subunit and mRNAs e.g. (Balagopal, & Parker 2009; Anderson et al 2015). Depletion of any one of a number of these proteins can lead to the formation of stress granules (Mazroui et al 2006; Mokas et al 2009).

eIF4G is a large scaffold protein, which possesses domains that interact with eIF4E and eIF4A, to form the eIF4F complex, as well as with eIF3 and the poly(A) binding protein (PABP) (Sonenberg, & Hinnebusch 2009; Laplante, & Sabatini 2012; Morley et al 2005;
Jackson et al 2010). eIF4E is an mRNA cap binding protein, and eIF4A comprises a family of DEAD-box RNA helicases, which are involved in many aspects of RNA metabolism. In mammalian cells there are three highly related eIF4A proteins, eIF4A1, eIF4A2 and eIF4A3, which have diverse and non-overlapping roles in mRNA metabolism (Lu et al 2014). eIF4A3 is nuclear (Chan et al 2004), while eIF4A1 and eIF4A2 are cytoplasmic and although functionally interchangeable in vitro, are not functionally redundant in vivo (Nielsen, & Trachsel 1988; Galicia-Vázquez et al 2012; Yoder-Hill et al 1993). While both eIF4A1 and eIF4A2 interact with eIF4G, eIF4A2, but not eIF4A1 binds cNOT7 (Meijer et al 2013). cNOT7 is a member of the CCR4-NOT complex, that is required for deadenylation of mRNA, and for miRNA-mediated gene regulation, indicating a specific role for eIF4A2 in this process.

The interactions and functions of the proteins in the eIF4F complex are regulated by a number of post-translational modifications. For example, during normal protein synthesis, the activity of eIF4F is regulated by eIF4E binding proteins (4E-BPs) (Matsuo et al 1997). 4E-BPs bind to eIF4E and compete for interaction with eIF4G, thus inhibiting translation initiation. In mammalian cells regulation of the interaction between 4E-BPs and eIF4E occurs via the activation of the mechanistic target of rapamycin (mTORC1) that leads to the multi-site phosphorylation of 4E-BP1 (reviewed in (Sonenberg, & Hinnebusch 2009)). This prevents 4E-BP1 from binding to eIF4E, thereby allowing formation of the eIF4F initiation complex and ribosomal recruitment of mRNA. Another post-translational modification that regulates the interaction of eIF4E with eIF4G is sumoylation of eIF4E by SUMO1 (Xu et al 2010).

SUMO is a small ubiquitin-like modifier that can be covalently attached to target proteins (Hannoun et al 2010). Modification by SUMO affects protein-protein interactions, protein localisation, protein activity or can target proteins for ubiquitin-mediated proteolysis (Enserink 2015; Geiss-Friedlander, & Melchior 2007; Watts 2004; Watts 2007). In many cases it acts by providing an altered binding surface on the target protein. In mammalian cells there are three SUMO proteins, SUMO1, 2 and 3. SUMO2 and 3 are 97% identical and are capable of forming SUMO chains, while SUMO1 is less similar (50% identical) and unable to form chains, but can act as a chain terminator (Matic et al 2008). SUMO is produced as a precursor protein, which is processed to the mature form by one of a number of specific proteases. It is then activated by interaction with an E1 enzyme, SAE (SUMO activating enzyme), from where it is passed to an E2, SUMO conjugating enzyme. From here it can be attached directly to target proteins, while in some cases conjugation requires the activity of
one of a small number of SUMO ligases. Several proteomic screens have identified many of the translation initiation factors as sumoylation targets, e.g. (Matafora et al 2009; Bruderer et al 2011; Blomster et al 2009) and reviewed in (Watts et al 2014). However, in most cases, the effect of sumoylation on individual target protein function remains to be determined.

We have previously demonstrated that fission yeast and mammalian eIF4G are sumoylated (Jongjitwimol et al 2014), and have identified two sumoylation sites in the C-terminus of human eIF4G. Our initial studies suggest that sumoylation may have a role in regulating protein synthesis in response to stress. To further investigate this, we have analysed the effects of arsenite, heat shock, hippuristanol and ionising radiation on the sumoylation of members of the human eIF4F complex. We report here the sumoylation of eIF4A in vivo, by both SUMO1 and SUMO2, and the identification of single sumoylation sites in eIF4A1 and eIF4A2. We demonstrate that modification of eIF4A2 by SUMO1 is increased in response to arsenite and ionising radiation but decreased in response to heat shock and hippuristanol. Furthermore, knocking down endogenous eIF4A2 and re-transfection with an unsumoylatable version of eIF4A2 has a detrimental effect on the formation of stress granules, indicating a role for sumoylation of eIF4A2 in its localisation in stress granules. Possible mechanisms whereby stress granule formation is affected by this modification are discussed.

RESULTS

eIF4A is sumoylated in mammalian cells.

The translation initiation factor eIF4F consists of three proteins: eIF4A, eIF4E and eIF4G. We and others previously demonstrated that both mammalian eIF4E and eIF4G are sumoylated (Xu et al 2010; Jongjitwimol et al 2014). We therefore wished to determine whether eIF4A is also modified by SUMO. To investigate this, we affinity-purified His-tagged SUMO conjugates using Ni²⁺ agarose under denaturing conditions from cell lines stably transfected with either His-SUMO1 or His-SUMO2. Under these conditions, non-covalent interactions are disrupted allowing the identification of post-translational modifications. Western blots of affinity-purified proteins were probed with antibodies against SUMO1 and SUMO2/3 (Figure 1A) and against eIF4A; eIF4E and eIF4G were used as positive controls (Figure 1B).

Western blotting with anti-eIF4A (panel (i)) identified a high Mr species (>250 KDa) following affinity purification of both His-SUMO1 and His-SUMO2 supporting our previous observation (Jongjitwimol et al 2014) that eIF4G is modified by SUMO1 and, to a lesser extent, by SUMO2. Additionally, probing with anti-eIF4E antisera (panel (ii)) identified two minor species of ~50 and 80 kDa and a more abundant species of ~175 KDa following purification
of His-SUMO1 and His-SUMO2. Although the predicted Mr of SUMO is 11 KDa, it migrates on SDS PAGs with an apparent Mr of 15-17 KDa. Thus the minor species (~50 and ~80 KDa) likely represent di- and tri-sumoylated eIF4E, while the 175 KDa species corresponds to poly-sumoylated eIF4E. (Although SUMO1 cannot be incorporated into SUMO chains, it can act as a chain terminator (Ulrich 2008).) These modified forms are similar in size to the previously described SUMO1-modified forms of eIF4E (Xu et al 2010). As well as being modified by SUMO1, the results in Figure 1B (ii) indicate that eIF4E is also modified by SUMO2. This would be consistent with the presence of high Mr poly-SUMO chains that we observe.

Having demonstrated that we are able to identify sumoylated species using denaturing conditions, we probed similar blots with anti-eIF4A antisera (Figure 1B panel (iii)). Following affinity purification of His-SUMO we observe species migrating with approximate Mr of ~70 and 150 KDa after purification of His-SUMO1, and His-SUMO2. Since the anti-eIF4A antisera recognise both eIF4A1 and eIF4A2 proteins (Figure S1), we repeated the affinity purification and probed with anti-eIF4A2 affinity purified antibodies in order to determine whether this isoform of eIF4A is sumoylated. In this case a species of ~150 KDa is observed with both His-SUMO1 and His-SUMO2 (Figure 1B panel (iv)), likely corresponding to the species of around 150 kDa observed with anti-eIF4A antisera. These results indicate that like eIF4E and eIF4G, eIF4A2 is sumoylated in vivo, and that it is modified by both SUMO1 and SUMO2. The levels of sumoylation of these initiation factors are typically in the region of 2-5% total eIF4A2 (similar to sumoylation levels reported for other proteins).

**Sumoylation of eIF4A2 is increased in response to arsenite and ionising radiation**

We have previously demonstrated that *S. pombe* eIF4G is sumoylated in response to 1M KCl (a condition that induces stress granules in yeast) (Jongjitwimol et al 2014). We therefore investigated the effect of stress on sumoylation of eIF4G, eIF4E or eIF4A2 in mammalian cells. We began by investigating the effect of arsenite (AR) that induces the formation of stress granules, and ionising radiation (IR), which causes genotoxic stress. Analysis of global levels of sumoylation in response to AR (1 mM) and IR (3 Gy) (Figure S2A), indicates that exposure to arsenite has minimal effect on the levels of sumoylation by either SUMO1 or SUMO2. In contrast exposure to IR reduces the level of global sumoylation by approximately 50%. To analyse sumoylation of the individual eIFs, His-SUMO1 and His-SUMO2 were affinity-purified from untreated cells (UT) and cells exposed to arsenite or IR. Figure 2A indicates that sumoylation of eIF4G by SUMO1 is somewhat reduced following exposure of cells to arsenite. In contrast, sumoylation by SUMO1 is reduced in response to IR. In
comparison to sumoylation by SUMO1, only low levels of SUMO2-containing species were observed. In the case of eIF4E, sumoylation by both SUMO1 and SUMO2 increases in response to both arsenite and IR (Figure 2A). All four species seen represent sumoylated forms of eIF4E; since the significance of the four different forms is unknown, we have compared the levels of the total amount of sumoylated species here.

We next investigated whether sumoylation of the eIF4A1 and eIF4A2 isoforms was affected by stress. Analysis of the sumoylated species of eIF4A1 indicates that only very low levels of sumoylated eIF4A1 are observed under normal conditions, and that the levels of these species are not altered in response to arsenite or IR (Figure 2A). In contrast, levels of sumoylated eIF4A2 increase in response to both stresses (Figure 2A). This increased sumoylation in response to AR and IR resembles that of eIF4E suggesting that sumoylation of these two factors may be coordinately regulated.

Sumoylation of proteins is known to affect protein localisation, e.g. (Muller et al 1998; Finkbeiner et al 2011). Since exposure of cells to arsenite results in the relocalisation of certain translation initiation factors to stress granules (Kedersha et al 1999) we wished to determine whether sumoylation of eIF4A2 is correlated with stress granule formation. We therefore compared the effects of arsenite and IR on the localisation of SUMO, eIF4G and eIF4A2. In untreated cells, the majority of the SUMO is present in small nuclear speckles in the nucleus, with low levels in the cytoplasm (Figure 3). In cells treated with arsenite, SUMO was relocalised to intra-nuclear foci, known as promyelocytic leukaemia (PML) bodies, as has been demonstrated previously (Muller et al 1998), with low levels remaining in the cytoplasm, while exposure to IR resulted in small nuclear foci. In untreated cells, eIF4G and eIF4A2 are predominantly in the cytoplasm, but with a significant proportion in the nucleus (Figure 3). Arsenite treatment resulted in the relocalisation of both eIF4G and eIF4A2 into cytoplasmic granules. Staining of arsenite-treated cells with antibodies against TIA-1, a known stress granule marker (Kedersha et al 1999), identifies these as stress granules (Figure 4). In contrast to the effect of arsenite on eIF4A2 and eIF4G localisation, exposure to IR had little effect on the localisation of eIF4G and eIF4A2 (Figure 3).

**Sumoylation of eIF4A2 is reduced following exposure to heat shock and hippuristanol**

We next extended our studies to analyse sumoylation of eIF4A2 in response to two other stresses: heat shock and hippuristanol. Hippuristanol is a small molecule inhibitor of eIF4A1 and eIF4A2 that has previously been demonstrated to induce the formation of eIF4A1-
containing stress granules in an eIF2α phosphorylation-independent manner (Mazroui et al 2006). Since modification of eIF4E and eIF4A2 by SUMO1 and SUMO2 appears similar, we focussed here on sumoylation by SUMO1. Analysis of global sumoylation levels of SUMO-1 containing species indicates an increase in response to heat shock, but a slight reduction in response to hippuristanol (Figure S2B). As described previously, His-SUMO1 was purified from cells under denaturing conditions and western blots were probed with anti-eIF4A2 antibodies. Figure 2B indicates that in contrast to the increased levels of sumoylation we observe with arsenite, sumoylation by SUMO1 of eIF4A2 is reduced following exposure to heat shock or hippuristanol.

As shown in Figure 3, increased sumoylation of eIF4A2 correlates with its relocalisation to stress granules. We therefore investigated whether reduced sumoylation of eIF4A2, e.g. as in the case with hippuristanol, has any effect on this repositioning. Further, we wished to confirm that the aggregates we observe are indeed stress granules. We therefore repeated the immunofluorescence studies to compare the effects of arsenite, heat shock, hippuristanol and IR using anti-eIF4A2 antibodies, along with anti-TIA-1 antibodies to identify stress granules. Figure 4 indicates that in response to arsenite, eIF4A2 colocalises with TIA-1, confirming the relocalisation of eIF4A2 into stress granules, an effect not observed with heat shock under our assay conditions. As expected, exposure of cells to hippuristanol resulted in the formation of stress granules: however, these do not contain eIF4A2. This further supports the notion that sumoylation of eIF4A2 correlates with its ability to form stress granules.

Identification of the sumoylation sites on eIF4A1 and eIF4A2

In order to identify the lysine residues used as SUMO-acceptor sites on eIF4A1 and eIF4A2, we used an in vitro sumoylation assay previously established in our lab (Ho et al 2001). Recombinant eIF4A1 and eIF4A2 were incubated with purified sumoylation components, with a modified form of SUMO containing a trypsin cleavage site adjacent to the diglycine motif (Jongjitwimol et al 2014), and products analysed by LC MS/MS. In both cases, a single lysine residue was identified: K225 and K226 respectively (Figure 5A). The eIF4A1 and eIF4A2 proteins are highly conserved, as is the region containing the sumoylation sites (Figure 5B). This is not the case with eIF4A3 that does not have a lysine residue at the equivalent position. Modeling of the sumoylation sites onto the known crystal structures indicates that the sites are in similar positions in the two proteins (Figure 5C) on a highly accessible surface of the protein in an alpha helical domain. These sites face the ATP binding pocket (Oberer et al 2005), suggesting that sumoylation of eIF4A may affect its interaction with ATP.
We previously demonstrated that the C-terminus of mammalian eIF4G is also sumoylated. Two sites were identified: K1386 and K1588. These residues map to eIF4A- and Mnk1- (mitogen-activated protein kinase (MAPK) interacting protein kinase 1) interacting regions of eIF4G (Figure 5D). Molecular modeling indicates that, interestingly K1386 is close to Q1379 (Figure 5E), which when mutated to lysine results in increased binding of eIF4G to eIF4A (Bellsolell et al 2006).

**eIF4A2 is sumoylated on K226 in vivo**

Since eIF4A2 is more highly sumoylated in vivo than is eIF4A1 (Figure 2A), we concentrated our studies on the eIF4A2 isoform, focusing on SUMO1-containing species, as this would cover singly sumoylated species as well as those containing SUMO chains terminating with SUMO1. Having shown that eIF4A2 is sumoylated in vitro on K226, we wished to confirm that this residue is used for sumoylation in vivo. To do this, expression of endogenous eIF4A2 was knocked down using siRNA, and cells were then transfected with wild type or mutant eIF4A2 (Figure 6A). Despite the fact that the knockdown was only partial (eIF4A2 is a very abundant protein making it difficult to achieve 100% knockdown), introduction of siRNA-resistant wild type eIF4A2 into cells clearly results in novel high Mr species following affinity-purification of His-SUMO1 (lane 4). These species are of similar Mr to those observed in Figures 1B and 2B, and are not observed in the untransfected controls (lanes 1-3). In contrast, while it is clear that the eIF4A2-K226 protein is expressed at similar levels to the wt protein (lanes 4 and 5, lower panel), such species are not observed when the eIF4A2-K226R mutant is introduced (lane 5). This indicates that K226 is the main sumoylation site in eIF4A2 that is used in vivo.

**Inability to sumoylate eIF4A2 results in a reduction in stress granule volume**

To determine whether inability to sumoylate eIF4A2 affects either its recruitment to stress granules, or the formation of the granules, we used immunofluorescence with anti-FLAG antibodies to compare the localisation of FLAG-tagged wt and FLAG-eIF4A2-K226R protein expressed in cells as described above (Figure 6B). In unstressed cells the localisation of the wt and mutant proteins were very similar and resembled what we observe for endogenous eIF4A2 (Figure 3B). However, there is a distinct difference in the localisation of the wt and mutant proteins in response to arsenite. As observed with the endogenous protein, FLAG-tagged eIF4A2 is observed in peri-nuclear stress granules, indicating that the tag is not affecting localisation. However, recruitment of FLAG-eIF4A2-K226R to stress granules is significantly less than that of the wt eIF4A2. To confirm the identity of the granules, we repeated these experiments using anti-TIA-1 antibodies in combination with anti-FLAG
antibodies. In arsenite-treated cells where eIF4A2 has been knocked down there is a significant reduction in TIA-1-containing stress granules, particularly in the number of large granules, compared to the situation in mock treated cells (Figure 6C). Transfection with FLAG-eIF4A2 wt restored the ability of cells to produce TIA-1-containing granules in response to arsenite treatment. In cells transfected with FLAG-eIF4A2-K226R, stress granules were also observed, although there were generally fewer granules per cell. In particular there was a significant reduction in the number of large TIA-1-containing stress granules (Figure 6D), similar to the situation observed in eIF4A2 knock down cells. This reduction in large TIA-1-containing stress granules correlated with the reduction in the number of large eIF4A2-containing granules. Furthermore, in cells transfected with either wt or mutant eIF4A2 there was a high degree of colocalisation of eIF4A2 (both wt and mutant) with TIA-1 (Figure 6E).

Despite the inability of eIF4A2 to be sumoylated, low levels of SUMO are still detected in stress granules in cells expressing FLAG-eIF4A2-K226R (Figure 6B, bottom panels). This is likely to be due to the fact that other components in stress granules are also sumoylated e.g. eIF4G (Jongjitwimol et al 2014; Bish et al 2015). These results indicate that it is the inability of eIF4A2 to be sumoylated, rather than the absence of SUMO that is causing the defect in stress granule formation.

**DISCUSSION**

Stress granules have been characterised as cytoplasmic aggregates that contain a number of proteins, including translation initiation factors, ribosomal subunits, polyA binding protein, and TIA proteins. Among the translation initiation factors that are recruited to stress granules are eIF4G and eIF4A, and more specifically eIF4A1 (Mazroui et al 2006). Here we demonstrate, that another isoform of eIF4A, eIF4A2, which is functionally distinct from eIF4A1 (Lu et al 2014), can also be recruited to stress granules.

It has been well documented that post-translational modifications are required for stress granule assembly (Buchan, & Parker 2009). For example, a key event required for this process is phosphorylation of eLF2α, and mutation of HDAC6, a histone deacetylase impairs stress granule formation (Kwon et al 2007), implicating acetylation as being involved. Additionally, stress granules have been demonstrated to contain ubiquitin-modified proteins (Kwon et al 2007). Our studies here suggest that sumoylation also has a role in protein recruitment to stress granules. Specifically, we demonstrate that although arsenite and hippuristanol both induce the formation of stress granules, only in cells exposed to arsenite, which increases the level of sumoylation of eIF4A2, is eIF4A2 recruited to stress granules. In
contrast, in hippuristanol-treated cells where sumoylation of eIF4A2 is decreased, there is decreased recruitment of eIF4A2 to stress granules. Unlike eIF4A2, eIF4A1 is recruited to stress granules in response to hippuristanol (Mokas et al 2009), further supporting the view that the two proteins are functionally distinct (Lu et al 2014).

Using a combination of in vitro and in vivo studies we have demonstrated that eIF4A2 is sumoylated on K226. Mutation of this site results in the impaired formation of TIA-1-containing stress granules, as demonstrated by a decrease in overall stress granule volume after treatment of cells with arsenite, thus indicating a role for sumoylation in the formation of these granules. The sumoylation site on eIF4A2 is on the same face as the ATP binding site suggesting that sumoylation may affect the interaction of eIF4A2 with ATP or its ATPase activity. It is known that ATP binding by eIF4A causes a switch in the protein to a closed conformation that allows ATP hydrolysis (Marintchev et al 2009). This subsequently facilitates the formation of an open conformation, resulting in reduced affinity of eIF4A for mRNA. Thus, inability to sumoylate eIF4A2 may affect its interaction with mRNA, which in turn could disrupt stress granule formation. Interestingly, hippuristanol, which inhibits the recruitment of eIF4A2 to stress granules, is known to interact with the eIF4A helicases at the ATP binding site, and inhibit the ATPase, RNA binding, and helicase activities of the protein (Lindqvist et al 2008). This supports the notion that sumoylation of eIF4A2 may be affecting its interaction with ATP and/or RNA binding.

Molecular modeling indicates that one of the sumoylation sites in eIF4G that we have identified (K1386), maps to a region involved in interacting with eIF4A (Figure 5D). This further suggests that sumoylation may regulate the interaction between eIF4G and eIF4A. Indeed, mutation of a nearby residue (Q1379 to lysine) results in increased binding of eIF4G to eIF4A (Bellsolell et al 2006). Since this lysine residue could potentially be targeted by sumoylation, it would be interesting to determine whether the same result is achieved by mutation to arginine (still a charge reversal mutation, but to a residue which does not act as a SUMO acceptor).

As well as interacting with eIF4G, eIF4A2 interacts with cNOT7, where it is likely involved in miRNA-mediated translational repression. In collaboration with Lu and Bushell (University of Leicester), we have observed that mutation of the eIF4A2 sumoylation site (K226) has no effect on this process (Lu and Bushell, pers. comm). This would be consistent with miRNA-mediated translational repression being a process used under normal growth conditions, rather than a stress response, a condition where we see increased sumoylation of eIF4A2.
Identification of the effect(s) of sumoylation on individual proteins has, in many cases proved problematic. For example, there are a number of well studied cases where several members of multiprotein complexes are all sumoylated (Jentsch, & Psakhye 2013; Nie et al 2015). In such cases, inability to sumoylate a single member of the complex has little affect since sumoylation of other components of the complex is sufficient for function. Thus, while we have shown that sumoylation of eIF4A2 is required for efficient formation of stress granules it is possible, since it is present in eIF4F complexes that contain eIF4G and eIF4E both of which are also sumoylated, there may be further roles for sumoylation of eIF4A2 still to be uncovered. However, now that the sumoylation sites on the members of the eIF4F complex have been identified, it should be possible to obtain a fuller understanding of the role of sumoylation in regulating translation initiation.

MATERIALS AND METHODS

Protein Expression and Purification

Recombinant His-eIF4A1 and GST-eIF4A2 were prepared from E. coli BL21 cells using Ni\textsuperscript{2+} agarose (Novagen) and glutathione sepharose respectively, according to the manufacturer’s instructions. Mammalian whole cell extracts were prepared from 5–10 × 10\textsuperscript{5} mammalian cells. Cells were washed once with PBS and then resuspended in 500 µl of ice-cold 0.24 M NaOH, 1% v/v β-mercaptoethanol. The mixture was incubated on ice for 15 minutes. 75 µl of 50% TCA was then added to the lysate that was further incubated on ice for 10 minutes. Denatured proteins were precipitated by centrifugation at 13,000 g for 20 minutes at 4°C and then resuspended in 30 µl of 1xSDS sample buffer. His-tagged SUMO was affinity purified from HeLa cells under denaturing conditions using Ni\textsuperscript{2+} agarose as described previously (Jongjitwimol et al 2014). Western blotting was carried out as described in (Harlow, & Lane 1988).

Identification of sumoylation sites

The \textit{in vitro} sumoylation assay was carried out as described elsewhere (Ho et al 2001). A modified form of SUMO containing a trypsin cleavable site adjacent to the C-terminal diglycine motif used for mass spectrometric analysis, has been described previously (Jongjitwimol et al 2014). Samples were prepared for mass spectrometry using a modification of the method of (Shevchenko et al 2006), as described in (Jongjitwimol et al 2014).
Molecular modeling

The positions of sumoylation sites on eIF4A and eIF4G crystal structures were located using Pymol.

Tissue Culture, Cell lines and Reagents

HeLa cells (supplied by ATCC, validated 14.5.2015 and demonstrated to be mycoplasma free 27.05.2015) were cultured in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), penicillin, streptomycin, and L-glutamine HeLa cells stably transfected with His-tagged SUMO1 and SUMO2 were gifts from Prof R Hay (University of Dundee) (Girdwood et al 2003; Vertegaal et al 2004). For analysis of stress responses, cells were treated with 1 mM sodium arsenite for 30 min, 3 Gy IR from a $^{137}$Cs source and left to recover for 30 min, heat shock at 42°C for 30 min or 1 µM hippuristanol for 60 min.

siRNA depletion of eIF4A2 and siRNA-resistant expression of eIF4A2

The K226R mutation was introduced into siRNA-resistant eIF4A2 by site-directed mutagenesis using the QuickChange method (Stratagene). siRNA (Silencer Select s4572) was from Life Technologies as described in (Meijer et al 2013) and the siRNA-resistant FLAG-myc-eIF4A2 construct was a gift from M Bushell (Meijer et al 2013).

Antibodies

Antibodies used were as follows with dilutions in parentheses. Rabbit anti-eIF4G (1:10000), anti-eIF4E (1:3000) and anti-eIF4A (1:2000) were as described previously (Coldwell et al 2012; Morley, & Pain 1995). Mouse monoclonal anti-eIF4G (sc-373892) (1:3000 for WB) (1:50 for IF), rabbit polyclonal anti-SUMO1 antibody (sc-9060) (1:2500 for WB) (1:50 for IF), mouse monoclonal anti-SUMO1 (sc-5308) (1:2500 for WB) (1:50 for IF), rabbit polyclonal anti-SUMO2/3 antibody (sc-32873) (1:2500) were from Santa Cruz Biotechnology, Mouse monoclonal anti-FLAG (F1804) (1:1000 for WB) (1:400 for IF) was from Sigma-Aldrich, goat polyclonal anti-eIF4A1 (sc-14211) (1:1000) and mouse anti-eIF4A2 (sc-137147) (1:200 for IF) were from Santa Cruz, rabbit anti-eIF4A2 (Ab194471) (1:3000), and rabbit anti-TIA-1 (Ab40693) (1:400) were from Abcam. Secondary antibodies used were: goat-anti-rabbit-HRP and rabbit anti-mouse-HRP (Dako) both used at 1:2500 for WB, anti-mouse-FITC and anti-
rabbit-TritC (Sigma-Aldrich), Cy5-rabbit (Life Technologies) and anti-goat-TritC (Santa Cruz) all used at 1:200.

**Immunofluorescence**

Cells cultured on coverslips were fixed using 4% (w/v) PFA in PBS for 10 minutes and permeabilised using 0.1% (v/v) Triton-X100 in PBS for 30 seconds. Cells were washed 3 times with PBS before incubation for 1 hour at room temperature with the primary antibodies diluted to the indicated concentration in 4% (w/v) BSA in PBS. Cells were washed a further 3 times with PBS before incubation for 30 minutes with the secondary, fluorophore-coupled, antibodies. Secondary antibodies were diluted as before in 4% (w/v) BSA in PBS. Cells were washed a final 3 times before mounting on glass slides with ProLong Gold with DAPI mounting media (Life Technologies). Cells were visualized using an Olympus IX70 microscope. Stress granules were analysed by taking deconvolved high-resolution z-stack images over 4 μm at 0.05 μm intervals. Imaris software suite was used to render 3-dimensional images. Surface mapping was used to create and calculate stress granule volumes.

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**CONFLICT OF INTEREST**

No competing interests declared

**AUTHOR CONTRIBUTIONS**

REFERENCES


Jentsch, S. & Psakhye, I., 2013, Control of nuclear activities by substrate-selective and protein-group SUMOylation, Annual review of genetics, 47, pp. 167-86.


FIGURE LEGENDS

Figure 1  eIF4A, eIF4E and eIF4G are sumoylated in mammalian cells.
Whole cell extracts (WCE) and affinity purification on Ni$^{2+}$ agarose under denaturing conditions (AP) of His-tagged SUMO from non-tagged HeLa cells (NT) and HeLa cell lines stably transfected with His-SUMO1 (S1) or His-SUMO2 (S2), analysed by SDS PAGE (7.5%) and Western blotted (IB) with anti-SUMO1 and anti-SUMO2 affinity purified antibodies (sc-9060, sc-32873, rabbit, 1:2500) (A), or anti-eIF4G antisera (raised in house, rabbit polyclonal, 1:10000), eIF4E and eIF4A antisera (both raised in house, rabbit polyclonal, 1:3000) and anti-eIF4A2 affinity purified antibodies (Ab194471, rabbit polyclonal, 1:3000) as indicated (B).

Figure 2 Exposure to arsenite and ionising radiation affects sumoylation of eIF4G, eIF4E, eIF4A1, eIF4A2
Whole cell extracts (WCE) and affinity purification under denaturing conditions as in Figure 1 (AP) of His-tagged SUMO1 (S1) or SUMO2 (S2) from stably transfected HeLa cell lines or non-tagged cells (NT), untreated (UT) or subjected to 1 mM arsenite (AR) for 30 min or 3 Gy ionising radiation (IR) with 30 min recovery, analysed by SDS PAGE (7.5%) and western blotted. A. Western blots probed with antisera against eIF4G and eIF4E under conditions used in Figure 1B, as indicated and with affinity purified antibodies against eIF4A1 (Santa Cruz, goat polyclonal, 1:3000) and eIF4A2 (Abcam, rabbit polyclonal, 1:3000) as indicated. B. Whole cell extracts (WCE) and affinity purification (AP) as in Figure 1A from cells exposed to 1 mM arsenite, heat shock 42°C 30 min or 1μM hippuristanol for 60 min. Samples analysed by Western blotting as in A.

Figure 3 Exposure to arsenite affects the localisation of eIF4A2 and eIF4G
HeLa cells were cultured and treated as before, either treated with 1mM arsenite (AR) for 30 minutes or exposed to 3 Gy ionising radiation (IR) and allowed to recover for 30 minutes. Cells were then fixed and stained for SUMO1 and either eIF4A2 or eIF4G. A. Immunofluorescence staining was performed using eIF4G (raised in house, rabbit polyclonal 1:100) and SUMO1 (sc-5308, mouse monoclonal, 1:50). B. eIF4A2 (sc-137147, mouse monoclonal, 1:200) and SUMO1 (sc-9060, rabbit polyclonal, 1:50). Merged images show DAPI - blue, eIF4A2/eIF4G - red and SUMO1
- green. Bar = 10 μm. We have observed at least 100 cells in each of three replicates and clearly see that all cells exposed to arsenite contain stress granules.

**Figure 4**  elf4A2 localises to arsenite-induced stress granules but not to hippuristanol-induced stress granules

HeLa cells were cultured and either left untreated or subjected to the following treatments, 1mM sodium arsenite (AR) for 30 minutes, heat-shock (HS) at 42°C for 30 minutes, 1μM hippuristanol (HP) for 60 minutes or 3 Gy ionizing radiation (IR) and left to recover for 30 minutes. The cells were fixed and immunostained for both elf4A2 (as in Figure 3) and TIA-1 (Ab40693, rabbit polyclonal, 1:400), followed by DAPI. TIA-1 - red, elf4A2 – green, DAPI – blue. Bar = 10 μm

**Figure 5**  elf4A1 and elf4A2 are sumoylated on K225 and K226 respectively

A. Mass spectra of sumoylation products. High Mr species from an in vitro sumoylation assay were excised from SDS PAGs and analysed by LC MS/MS. B. Sequence alignment of human elf4A1, elf4A2 and elf4A3. Sumoylation sites indicated in red. C. Position of sumoylation sites on elf4A1 (i) and elf4A2 (ii). Pymol derived figure indicating sumoylation sites on human elf4A1 (PDB ID 2ZU6) and elf4A2 (PDB ID 3BOR). ABP = ATP binding pocket. D. Schematic indicating organisation of interacting motifs in elf4G. E. Positions of sumoylation site (K1386 - green), and Q1379 (magenta) in the C-terminal fragment of elf4G (PDB IB 1UG3).

**Figure 6**  Mutation of K226 results in loss of sumoylation of elf4A2 in vivo and reduction on stress granule size

A. His-SUMO1 stably transfected cells were reverse transfected with elf4A2 siRNA (lanes 3-5). 48 hours later cells were mock treated (lane 2) or transfected with FLAG-myc-elf4A2 wt or FLAG-myc-elf4A2-K226R mutant as indicated. His-SUMO1 was purified from non-tagged cells (NT, lane 1) or His-SUMO1 stably transfected cells (S1, lanes 2-5) using Ni²⁺ agarose under denaturing conditions as in Figures 1 & 2. Proteins were analysed by SDS PAGE and western blotted with anti-elf4A2 antisera (Ab194471, rabbit polyclonal, 1:3000). B. HeLa cells were depleted of endogenous elf4A2 and transfected with either FLAG-myc-elf4A2 wt or FLAG-myc-elf4A2-K226 and either left untreated (UT) or treated with 1 mM arsenite (AR) for 30 minutes. Cells were immunostained with an anti-FLAG antibody (F1804, mouse
monoclonal, 1:400) - red, and anti-SUMO (sc-9060, rabbit polyclonal, 1:50) - green. Bar = 10 μm. High-resolution images were taken over a z-plane of 4μm at 0.05μm slices. C. HeLa cells were prepared as in B and immunostained for FLAG (eIF4A2) and TIA-1 (ab40693, rabbit polyclonal, 1:400). Bar = 10μm. D. High resolution z-stack images were taken and deconvolved using Huygens deconvolution software. Images were 3-dimensionally rendered using IMARIS software suite. 3D-images were used to calculate the volumes of SGs for both TIA-1 and FLAG (eIF4A2). Box and whisker plots were used to show the distribution of SG volumes. E. Cells processed as in C were analysed for co-localisation of FLAG- and TIA-1 signal. The chart shows the percentage of FLAG (eIF4A2) signal that overlaps with TIA-1 in cells transfected with either FLAG-eIF4A2 WT or the K226R mutant.
Figure 1

(A) Western blot analysis showing the expression of SUMO1 and SUMO2/3 proteins in different samples. WCE (Whole Cell Extract) and AP (Antibody Precipitation) samples are compared.

(B) Immunoblot (IB) analysis showing the expression of various eIF4G, eIF4E, eIF4A, and eIF4A2 proteins in different samples (NT, S1, S2).

1. α-SUMO1
2. α-SUMO2/3
3. α-eIF4G
4. α-eIF4E
5. α-eIF4A
6. α-eIF4A2
Figure 3

A  eIF4G  SUMO1  Merge

UT

AR

IR

B  eIF4A2  SUMO1  Merge
<table>
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<th>TIA-1</th>
<th>eIF4A2</th>
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Figure 5

A

(i) eIF4A1 K225

(ii) eIF4A2 K226

B

C

(i) eIF4A1 K225

(ii) eIF4A2 K226

D

E

eIF4G

Q1379

K1386

ABP