Sumoylation of eIF4E activates mRNA translation

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Eukaryotic translation initiation factor 4E (eIF4E) is the cap-binding protein that binds the 5' cap structure of cellular messenger RNAs (mRNAs). Despite the obligatory role of eIF4E in cap-dependent mRNA translation, how the translation activity of eIF4E is controlled remains largely undefined. Here, we report that mammalian eIF4E is regulated by SUMO1 (small ubiquitin-related modifier 1) conjugation. eIF4E sumoylation promotes the formation of the active eIF4F translation initiation complex and induces the translation of a subset of proteins that are essential for cell proliferation and preventing apoptosis. Furthermore, disruption of eIF4E sumoylation inhibits eIF4E-dependent protein translation and abrogates the oncogenic and antiapoptotic functions associated with eIF4E. These data indicate that sumoylation is a new fundamental regulatory mechanism of protein synthesis. Our findings suggest further that eIF4E sumoylation might be important in promoting human cancers.

Keywords: eIF4E; mRNA translation; oncogenic transformation; sumoylation; translation initiation

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INTRODUCTION

Protein function might be regulated through covalent modification with any of four small ubiquitin-related modifier (SUMO) polypeptides. The sequence identity and expression of these four SUMO molecules is highly variable. Whereas SUMO2 and SUMO3 are 97% identical, they share only 50% identity with SUMO1 and whereas SUMO1, 2 and 3 are expressed ubiquitously, SUMO4 seems to be expressed mainly in the kidney, lymph node and spleen.

Protein sumoylation is mediated by activating (E1), conjugating (E2) and ligating (E3) enzymes (di Bacco & Gill, 2006). Ubc9 is the only identified SUMO E2 conjugating enzyme. However, several sequence-specific SUMO E3 ligases have been identified (reviewed in Geiss-Friedlander & Melchior, 2007).

SUMO modification has emerged as an important regulatory mechanism for protein activity, stability and localization. At a molecular level, sumoylation might alter protein function by masking or adding interaction surfaces and by inducing conformational changes that result in altered protein–protein interactions (Meulmeester & Melchior, 2008). Most SUMO targets identified so far are involved in nuclear events such as transcription, DNA repair, nuclear bodies and nucleocytoplasmic transport (Johnson, 2004; Hay, 2005; Geiss-Friedlander & Melchior, 2007). Sumoylation regulates several aspects of gene expression, including DNA transcription, messenger RNA (mRNA) splicing and mRNA polyadenylation (Johnson, 2004; Geiss-Friedlander & Melchior, 2007; Vethantham *et al*, 2007, 2008). Furthermore, a recent study identified *Drosophila* eukaryotic initiation factor 4E (eIF4E) as a SUMO substrate (Nie *et al*, 2009). However, whether eIF4E sumoylation has a role in regulating mRNA translation and protein synthesis in the cytoplasm is unknown.

In eukaryotes, more than 95% of proteins are synthesized through cap-dependent mRNA translation. A rate-limiting step in cap-dependent translation is the formation of the eIF4F complex containing eIF4E (cap-binding protein), eIF4A (ATP-dependent mRNA helicase) and eIF4G (scaffold protein; reviewed in Gingras *et al*, 1999). Binding of eIF4E to the cap structure of mRNA is inhibited by a small family of eIF4E-binding proteins (4E-BPs). 4E-BP1, the most abundant member of the 4E-BP family, is phosphorylated at many sites; several of these phosphorylations are necessary for dissociation of 4E-BP1 from eIF4E and the subsequent formation of the eIF4F complex and initiation of cap-dependent translation (Gingras *et al*, 2001). eIF4E might also be regulated by phosphorylation. In particular, phosphorylation of eIF4E at Ser209 seems to be important for the initiation of cap-dependent translation (Scheper & Proud, 2002).

Here, we show that eIF4E is modified by SUMO1 conjugation and that phosphorylation of eIF4E at Ser209 promotes sumoylation.

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Fig 1 | *In vivo* and *in vitro* sumoylation of eIF4E. (A) *In vivo* modification of eIF4E in HCT116 cells. For serum starvation and stimulation experiments, the HCT116 cells were starved (0.2% FBS) for 22 h before stimulation with or without 20% FBS for an additional 2 h. For 10% FBS treatment, the cells were cultured in regular complete medium. After harvesting, the cells were lysed in the presence or absence of 20 mM NEM. Sumoylation of eIF4E was evaluated by IP with anti-eIF4E and subsequent IB with anti-eIF4E or anti-SUMO1. (B) Cap-bound eIF4E in HCT116 cells was evaluated by pull-down assay using m⁷GTP Sepharose resin and subsequent IB with anti-eIF4E. (C) Overexpression of Ubc9 enhances the sumoylation of eIF4E in HCT116 cells. Sumoylation of eIF4E was evaluated by IP with anti-eIF4E and subsequent IB with anti-SUMO1. (D) Mutations of lysines 36, 49, 162, 206 and 212 in eIF4E abolish *in vitro* eIF4E sumoylation. *In vitro* synthesis of HA-eIF4E and HA-eIF4E-S5 was conducted by using the rabbit reticulocyte Lysate TNT Coupled Transcription/Translation System (Promega, Madison, WI, USA). A 3 µl volume of *in vitro* translated wild-type eIF4E and its mutant protein was used as a SUMO substrate for the assay. eIF4E-S5 represents eIF4E-KKKKK36/49/162/206/212RRRRR. eIF, eukaryotic translation initiation factor; FBS, fetal bovine serum; HA, haemagglutinin; IB, immunoblotting; IP, immunoprecipitation; NEM, N-ethylmaleimide; shRNA, short hairpin RNA; SUMO, small ubiquitin-related modifier; VC, vector control.

Whereas eIF4E sumoylation does not directly affect cap-binding activity, it promotes the dissociation of eIF4E and 4E-BP1, thereby enhancing the formation of the eIF4F complex and subsequent cap-dependent translation. Disruption of eIF4E sumoylation abrogates the antiapoptotic property of eIF4E and inhibits the eIF4E-mediated transformation of mouse fibroblasts. Together, our findings uncover a new mechanism that has a fundamental role in the regulation of mRNA translation.

RESULTS AND DISCUSSION

eIF4E is sumoylated on lysines 36, 49, 162, 206 and 212

As eIF4E is sumoylated in the early *Drosophila* embryo (Nie *et al*, 2009), we examined the modification of eIF4E in human colon cancer HCT116 cells. After immunoprecipitation with anti-eIF4E in the presence of the isopeptidase inhibitor N-ethylmaleimide (NEM), we detected multiple bands above 25 kDa by immunoblotting with either anti-eIF4E (Fig 1A, left panel) or anti-SUMO1 (Fig 1A, right panel). A similar modification pattern was seen in cap-bound eIF4E (Fig 1B). Whereas overexpression of the SUMO E2 conjugating enzyme Ubc9 dramatically increased the modification of eIF4E (Fig 1C), downregulation of Ubc9 by short-hairpin RNA (shRNA) inhibited the modification of eIF4E

(supplementary Fig S1A online). These findings suggest that, like its *Drosophila* homologue, human eIF4E might be modified by SUMO1 conjugation. eIF4E was not detectably modified by SUMO2/3 conjugation either *in vitro* (supplementary Fig S1B online) or *in vivo* (data not shown). Thus, mammalian eIF4E is modified by SUMO1 conjugation.

Notably, in many cases, only a small fraction—often less than 1%—of the SUMO substrate is sumoylated at any given time (Johnson, 2004; Hay, 2005). The fact that a large portion—more than 20%—of cap-bound eIF4E gets sumoylated strongly suggests that sumoylation might be crucial for the role of eIF4E in translation initiation. The observation that mitogen (20% fetal bovine serum) stimulation dramatically enhanced eIF4E sumoylation (Fig 1A) without affecting total cellular SUMO1 levels (supplementary Fig S2 online) supports the idea that eIF4E sumoylation is associated positively with protein translation.

To identify the amino acid(s) in eIF4E that are covalently bound by SUMO1, we mutated various lysine residues to arginines. We then determined the sumoylation of the mutated eIF4E *in vitro*. Five SUMO sites were identified on eIF4E: Lys 36 (IKHP), Lys 49 (FKND), Lys 162 (DKIA), Lys 206 (TKSG) and Lys 212 (TKNR). Whereas single mutation of these sites did not seem to affect the

Fig 2 | Interplay between eIF4E phosphorylation and sumoylation. (A) Disruption of eIF4E phosphorylation prevents its sumoylation. HCT116 cells were transfected with empty vector, HA-tagged wild-type eIF4E, eIF4E phosphorylation mutant (labelled as Ser 209A), eIF4E phosphorylation mimic (labelled as Ser 209E) or eIF4E SUMO-deficient mutant (labelled as S5). Sumoylation of eIF4E was evaluated by pulldown with anti-SUMO1 and subsequent IB with anti-HA. (B) shRNA knockdown of Ubc9 does not inhibit eIF4E phosphorylation. The HCT116 cell lines expressing shRNA control vector or shRNA Ubc9 were starved (0.2% FBS) for 22 h then stimulated with 20% FBS for 30 min. Whole-cell lysates were used for the IB. The band intensity was analysed by using the UN-SCAN-IT gel-graph digitizing software. Statistical analyses were performed by one-way analysis of variance followed by Tukey's multiple comparison test using the data obtained from three independent experiments. (C) A lack of eIF4E sumoylation does not inhibit eIF4E phosphorylation. The Rat1 cell lines expressing eIF4E or its mutant were first starved with 0.2% FBS for 22 h then stimulated with 20% FBS for 1 h before harvesting. Whole-cell lysates were used for the IB. eIF, eukaryotic translation initiation factor; FBS, fetal bovine serum; HA, haemagglutinin; IB, immunoblotting; IP, immunoprecipitation; shRNA, short hairpin RNA; SUMO, small ubiquitin-related modifier.

in vitro sumoylation of eIF4E (supplementary Fig S3 online), mutation at all five sites abolished eIF4E sumoylation *in vitro* (Fig 1D). Thus, lysines 36, 49, 162, 206 and 212 in eIF4E are sumoylated. It is important to point out that all these lysine residues of eIF4E are conserved across species including human, mouse, rat, rabbit, *Xenopus* and *Drosophila*.

Phosphorylation is required for eIF4E sumoylation

Although a significant proportion of cellular eIF4E is modified by SUMO1 conjugation (Fig 1A,B), the efficiency of eIF4E sumoylation *in vitro* was low (Fig 1D). We considered that eIF4E must be modified before its sumoylation and that this modification is not abundant in eIF4E translated *in vitro*. eIF4E is phosphorylated at Ser209 (Scheper & Proud, 2002). The eIF4E phosphorylation mutant Ser 209A (serine-to-alanine mutation) was not sumoylated *in vivo* (Fig 2A, lane 3; supplementary Fig S4A online, lane 3), but the eIF4E phosphorylation mimic (eIF4E-Ser 209E, serine-to-glutamic acid mutation) was sumoylated as efficiently as wild-type eIF4E (Fig 2A, lanes 2 and 5). These findings thus suggest that phosphorylation on Ser209 is required for sumoylation. If so, one would predict that the *in vivo* sumoylated eIF4E should be phosphorylated. Indeed, the results confirmed that sumoylated eIF4E was also phosphorylated (supplementary Fig S4B online).

Next, we examined whether sumoylation affects eIF4E phosphorylation. Whereas shRNA knockdown of Ubc9 inhibited

sumoylation of eIF4E (supplementary Fig S1A online), it did not inhibit mitogen stimulation-induced eIF4E phosphorylation (Fig 2B). Furthermore, downregulation of eIF4E sumoylation by mutation did not inhibit its phosphorylation (Fig 2C). Therefore, sumoylation of eIF4E is not required for its phosphorylation.

Sumoylation promotes the eIF4F complex formation

Next, we examined the molecular consequences of eIF4E sumoylation. As eIF4E is the mRNA cap-binding factor, we determined whether sumoylation affects the ability of eIF4E to bind to the cap analogue m⁷GTP. Similar amounts of wild-type eIF4E and eIF4E SUMO-deficient mutant protein (eIF4E-S5) bound to m⁷GTP resin (Fig 3A), not only suggesting that the mutant and wild-type proteins have similar structures, but also indicating that sumoylation does not affect the cap-binding capability of eIF4E.

As one of the main molecular consequences of SUMO modification is alteration of inter- or intramolecular interactions of the modified target (Meulmeester & Melchior, 2008), we determined whether eIF4E sumoylation affected the interaction of eIF4E with either eIF4G or 4E-BP1. We used the eIF4E mutant that cannot be sumoylated (eIF4E-S5) as a tool. The association of eIF4G in the cap-bound translation initiation complex was not stable when eIF4E-S5 was expressed in both HCT116 cells and HEK293 cells, and significant binding of eIF4E-S5 and 4E-BP1 was detected (Fig 3B and data not shown). These findings suggest that

Fig 3 | Formation of the active eIF4F complex requires eIF4E SUMO1 modification. (A) A loss of sumoylation does not affect eIF4E cap-binding activity. The HCT116 cells were transfected with HA-tagged wild-type eIF4E or S5 mutant (KKKKK36/49/162/206/212RRRRR). Forty-eight hours after transfection, the cells were lysed and whole-cell lysates were used for the study. The cap-binding affinities of eIF4E and its mutant were evaluated by pull-down assay using m⁷GTP Sepharose resin. To better compare the total cap-bound eIF4E, the cells were lysed in the absence of *N*-ethylmaleimide to avoid spread-out of the sumoylated eIF4E bands. (B) Disruption of eIF4E sumoylation inhibits the formation of the eIF4F complex in HCT116 cells. To enhance SUMO1 signal, plasmids expressing Ubc9 and SUMO1 were co-transfected with wild-type eIF4E and eIF4E-S5 mutant. Forty-eight hours after transfection, the cells were harvested and whole-cell lysates were used for the assay. (C) Sumoylation regulates the expression of eIF4E-responsive genes. The Rat1 cell lines expressing control vector, wild-type eIF4E or eIF4E-S5 mutant were used for the study. Whole-cell lysates were used for IB. (D) The polysome distributions of mRNAs. The Rat1 cell lines expressing control vector, eIF4E or eIF4E S5 mutant were used for the assay. After fractionation, RNAs from each fraction were extracted and mRNAs coding for ODC, c-Myc, survivin, Bcl-2 and β -actin were analysed by reverse transcription-PCR. The band intensity was analysed by using the UN-SCAN-IT gel-graph digitizing software. eIF, eukaryotic translation initiation factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HA, haemagglutinin; IB, immunoblotting; IP, immunoprecipitation; mRNAs, messenger RNAs; SUMO, small ubiquitin-related modifier; VC, vector control; wt, wild type.

eIF4E sumoylation is important for dissociation of eIF4E from 4E-BP1 and for the formation of the eIF4F complex. The binding sites of 4E-BP1 and eIF4G on eIF4E are overlapping (Ptushkina *et al*, 1999). How does sumoylation of eIF4E influence these interactions in diametrically opposed ways? We propose that sumoylation might induce a conformational change of eIF4E. A change in the conformation of eIF4E produces a change in the interaction surfaces. Such change might increase the binding affinity of eIF4E for proteins with a certain type of conformation, such as eIF4G, and reduce the affinity of eIF4E for proteins like 4E-BP1.

To determine whether sumoylation has a role in translation, we examined the expression of eIF4E-responsive targets. Although eIF4E is a general translation factor, overexpression of eIF4E enhances the translation of a subset of mRNAs with complex 5' untranslated regions, rather than increasing global protein synthesis (Graff *et al*, 2007). Many of the eIF4E-regulated genes are related to growth, proliferation and antiapoptosis, and include the oncogenes ODC, c-Myc and the Bcl family, as well as Survivin (de Benedetti & Graff, 2004). Whereas wild-type eIF4E substantially induced the expression of ODC, c-Myc, Survivin and

Bcl-2—which is consistent with previous reports (Graff *et al*, 2007; Mamane *et al*, 2007)—the SUMO-deficient mutant eIF4E-S5 failed to induce the expression of these proteins (Fig 3C). These findings suggest that sumoylation is important for the translation activity of eIF4E. By using polysome fractioning assay, we further found that wild-type eIF4E, but not eIF4E-S5, shifted the mRNA of the above mentioned genes from the monosome/intermediate polysome fraction to the heavy polysome fraction without any detectable change in the level of these mRNAs in the cytoplasm (Fig 3D). These data thus link eIF4E sumoylation and eIF4E translation activity.

Functional relevance of eIF4E sumoylation

Previous studies have shown that activation of the eIF4F translation complex is essential for transformation of human mammary epithelial cells and maintenance of the malignant phenotype (Avdulov *et al*, 2004). Further, eIF4E is a potent oncogene and overexpression of eIF4E transforms rodent fibroblasts and promotes tumorigenesis (Lazaris-Karatzas *et al*, 1990; Ruggiero *et al*, 2004; Wendel *et al*, 2007). The oncogenic function of eIF4E requires its S209 phosphorylation-dependent antiapoptotic activity (Topisirovic *et al*, 2004; Wendel *et al*, 2007).

Earlier studies have shown that overexpression of eIF4E inhibits serum starvation-induced apoptosis in NIH-3T3 fibroblasts (Polunovsky *et al*, 1996). The correlation between mitogen deprivation-induced apoptosis and inhibition of eIF4E sumoylation (Fig 1A) suggests that sumoylation of eIF4E has a role in apoptosis. Using the serum depletion-induced apoptosis model (Polunovsky *et al*, 1996), we examined whether sumoylation is important for the antiapoptotic function of eIF4E. We found that both eIF4E-S5, which cannot be sumoylated, and eIF4E-S209A, which cannot be phosphorylated at this site, were unable to inhibit serum starvation-induced apoptosis (Fig 4A). By contrast, the eIF4E single SUMO mutants (Lys 36R, Lys 49R, Lys 162R, Lys 206R and Lys 212R), which can be sumoylated as efficiently as wild-type eIF4E (supplementary Fig S3 online), were able to prevent serum starvation-induced apoptosis as effectively as wild-type eIF4E (supplementary Fig S5 online). Together, these findings indicate that eIF4E sumoylation is required for its antiapoptotic activity. To test the role of eIF4E sumoylation in oncogenic transformation, we performed focus formation and soft agar assays. Consistent with previous reports (Lazaris-Karatzas *et al*, 1990; Topisirovic *et al*, 2004), overexpression of eIF4E yielded transformation foci (Fig 4B) and anchorage-independent growth in soft agar (Fig 4C), two definitive measures of malignant transformation in Rat1 cells. Whereas eIF4E-Ser209A impaired the eIF4E-induced formation of transformed foci and cell growth in soft agar, eIF4E-S5 totally abrogated the formation of transformed foci (Fig 4B) and the growth of colonies in soft agar (Fig 4C). Thus, sumoylation is required both for the antiapoptotic and oncogenic transformation properties of eIF4E.

In summary, we have shown that sumoylation of eIF4E is a new regulatory mechanism for eIF4E-mediated translation and oncogenic transformation. Given that cap-dependent translation is a fundamental operation in almost all aspects of cell functions, we suggest that regulation of eIF4E sumoylation has the potential to profoundly affect various biological processes and pathogenesis. For instance, our finding that sumoylation activates eIF4E translation activity offers a potential explanation for the observations that Ubc9

Fig 4 | Functional significance of eIF4E sumoylation in apoptosis and oncogenic transformation. (A) A loss of eIF4E sumoylation and phosphorylation abrogates the antiapoptotic function of eIF4E. The NIH-3T3 cell lines were incubated in medium containing 0.1% fetal calf serum for 36 h. After treatment, the cells were harvested for the apoptosis assay. Results are mean $\pm$ s.e. ($n = 3$). *Significantly different ($P < 0.01$) by one-way analysis of variance, followed by Tukey's multiple comparison test. (B) Characteristics of Rat1 cell lines expressing eIF4E (wild type or mutants) in monolayer. Pictures were taken 7 days after plating. Light Giemsa stain and phase contrast, $\times 40$ objective. (C) Anchorage-independent growth of Rat1 cells. Pictures were taken 21 days after plating. Crystal violet stain and phase contrast, $\times 40$ objective. eIF, eukaryotic translation initiation factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VC, vector control; wt, wild type.

is essential for the viability of higher eukaryotic cells (Hayashi *et al*, 2002) and that disruption of Ubc9 or SUMO1 is embryonic lethal (Nacerddine *et al*, 2005; Alkuraya *et al*, 2006).

METHODS

Most of the methods used are described in the supplementary information online.

Evaluation of *in vivo* eIF4E sumoylation. *In vivo* sumoylation of eIF4E was evaluated as described in our previous report (Vatsyayan *et al*, 2008). To reduce interference from other sumoylated proteins that might be associated with eIF4E, 0.1% sodium dodecyl sulphate was added to the lysis buffer (for both immunoprecipitation and immunoblotting). Unless stated in the figure legend or labelled in the figure, 20 mM SUMO isopeptidase inhibitor NEM (Sigma, St Louis, MO, USA) was added to the lysis buffer for immunoprecipitation. For straight western blotting, unless indicated, for the purpose of comparing the input or total amount of the protein, NEM was not added to the lysis buffer. Sumoylation of total cellular eIF4E was evaluated by immunoprecipitation with anti-eIF4E or anti-SUMO1 and subsequent immunoblotting with anti-SUMO1 or anti-eIF4E or anti-haemagglutinin (HA). As input control, a 10% amount of input lysate was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotted with anti-eIF4E.

In vitro sumoylation assay. *In vitro* sumoylation assay was performed by using an *in vitro* sumoylation kit (LAE Biotech International, Rockville, MD, USA). The kit provided the necessary reagents (including recombinant SUMO E1 enzymes SAE-I and SAE-II, SUMO E2 enzyme Ubc9, SUMO1 or SUMO2 and reaction buffer) for the assay except SUMO substrate. Substrate eIF4E was synthesized by using a cell-free system. *In vitro* synthesis of HA-eIF4E and HA-eIF4E-S5 was conducted by using the rabbit reticulocyte Lysate TNT Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to the protocol provided. As SUMO substrate, 3 µl of *in vitro* synthesized HA-eIF4E (wild type and S5 mutant) was used for each assay. The *in vitro* sumoylation was performed according to the protocol provided. After *in vitro* sumoylation reaction, the reaction mixtures were subjected to immunoblotting. Non-sumoylated and sumoylated HA-eIF4E were detected by an antibody recognizing an HA tag.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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