ARE-binding protein ZFP36L1 interacts with CNOT1 to directly repress translation via a deadenylation-independent mechanism

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Abstract

Eukaryotic gene expression can be spatiotemporally tuned at the post- transcriptional level by cis-regulatory elements in mRNA sequences. An important example is the AU-rich element (ARE), which induces mRNA destabilization in a variety of biological contexts in mammals and can also mediate translational control. Regulation is mediated by trans-acting factors that recognize the ARE, such as Tristetraprolin (TTP) and BRF1/ZFP36L1. Although both proteins can destabilize their target mRNAs through the recruitment of the CCR4-NOT deadenylation complex, TTP also directly regulates translation. Whether ZFP36L1 can directly repress translation remains unknown. Here, we used an *in vitro* translation system derived from mammalian cell lines to address this key mechanistic issue in ARE regulation by ZFP36L1. Functional assays with mutant proteins reveal that ZFP36L1 can repress translation via AU-Rich elements independent of deadenylation. ZFP36L1-mediated translation repression requires interaction between ZFP36L1 and CNOT1, suggesting that it might use a repression mechanism similar to either TPP or miRISC. However, several lines of evidence suggest that the similarity ends there. Unlike, TTP, it does not efficiently interact with either 4E-HP or GIGYF2, suggesting it does not repress translation by recruiting these proteins to the mRNA cap. Moreover, ZFP36L1 could not repress ECMV-IRES driven translation and was resistant to pharmacological eIF4A inhibitor silvestrol, suggesting fundamental differences with miRISC repression via eIF4A. Collectively, our results reveal that ZFP36L1 represses translation directly and suggest that it does so via a novel mechanism distinct from other translational regulators that interact with the CCR4-NOT deadenylase complex.

Keywords: ZFP36L1; AU-rich element; CCR4-NOT; deadenylation; translation

1. Introduction

Eukaryotic mRNAs have an m^7G cap structure at the 5′-end and a poly(A) tail at the 3′-end, which serve to promote both translation and block mRNA decay. The 5′cap structure is recognized by the eIF4F cap-binding complex [1]. The eIF4F complex consists of the cap-binding protein eIF4E, the scaffold protein eIF4G and the ATP-dependent RNA helicase eIF4A. This complex is formed during the rate-limiting step of translation and is highly regulated by various pathways [2]. The 3′ poly(A) tail is bound by the poly (A)-binding protein (PABP). Physical interaction between PABP and eIF4G bridges both the 5′- and 3′-ends and circularizes mRNA, and this is the basis for synergistic translational enhancement by the cap and poly(A) tail [3]. mRNA circularization also leads to mRNA stabilization by inhibiting mRNA decay. Generally, mRNA decay starts with deadenylation by a deadenylase complex, such as the CCR4-NOT complex or the PAN2/PAN3 complex. Poly(A)-trimmed mRNAs are subjected to decapping by the Dcp1/Dcp2 complex followed by the 5′ to 3′ decay mediated by the exonuclease, XrnI, and/or 3′ to 5′ decay by the exosome complex [4]. Hence, mRNA circularization can stabilize mRNA by blocking access of the mRNA decay machinery, in addition to its role in enhancing translation efficiency. These dual functions of the cap and poly(A) tail raise the question of how tightly they are coupled and whether they are regulated by the same mechanism.

RNA-binding proteins (RBPs) play a central role in the post-transcriptional control of gene expression, including translation and mRNA decay. This involves binding to specific RNA sequences in their mRNA regulatory targets. AU-rich elements (AREs) are specific cis-regulatory sequences present in mRNAs with short half-lives [5]. ARE-binding proteins (ARE-BPs) recognize AREs and typically promote mRNA destabilization by recruiting the mRNA decay complex [6-11]. Butyrate response factor 1 (BRF1), also known as ZFP36 ring finger protein-like 1 (ZFP36L1), is one of the ARE-BPs belonging to the TPA-inducible sequence 11 (TIS11) family of proteins. It has been shown that another TIS11 family protein, TTP, recruits the CCR4-NOT complex to target mRNAs via direct

binding to its core subunit, CNOT1 [6]. The CNOT1-interacting motif (CIM) of TTP is conserved in ZFP36L1, while other groups have shown that ZFP36L1 interacts with subunits of the CCR4-NOT complex and is involved in mRNA decay [6, 7, 12-14]. Therefore, it has been thought that ZFP36L1 utilizes the CCR4-NOT complex primarily to destabilize target mRNAs.

The miRNA-induced silencing complex (miRISC) also causes deadenylation by the CCR4-NOT complex [15]. Argonaute (Ago) proteins in miRISC bind to the scaffold protein TNRC6, which physically interacts with CNOT1 and CNOT9 [16, 17]. Fukao et al. and Fukaya et al. discovered that miRISC can repress translation by promoting dissociation of eIF4A from the eIF4F complex in mammalian cells and *Drosophila* extracts, and that this is independent of deadenylation [18, 19]. The precise molecular mechanism underlying this capability is still unclear, but other groups demonstrated that miRISC recruits 4EHP through interaction between CNOT1 and DDX6 and 4E-T [20-22]. 4EHP is a homologue of eIF4E and binds to the 5′ cap structure but not eIF4G, thereby repressing translation [23]. TTP also recruits 4EHP to target mRNA via binding to GIGYF2 and represses translation [24, 25]. These findings indicate the possibility that ZFP36L1 also represses translation independently of deadenylation by a mechanism similar to TTP. To test this hypothesis, we constructed an *in vitro* translation system with mammalian cell lines that can analyze both translation activity and mRNA deadenylation by ARE-BPs.

Here, we demonstrate that ZFP36L1 causes translation repression in parallel with deadenylation and that this deadenylation independent repression depends on ZFP36L1 interaction with CNOT1. This dependence is apparently similar to previously described translational repression mechanisms involving TTP and miRISC. However, unlike TTP, we find that ZFP36L1 does not interact efficiently with 4EHP or GIGYF2. Furthermore, ZFP36L1 represses translation in the presence of the pharmacological eIF4A inhibitor silvestrol, which blocks miRNA-mediated translation repression. Thus, ZFP36L1 represses translation in a manner distinct from TTP and miRISC, even though they all utilize the CCR4-NOT complex for deadenylation and translational repression.

2. Materials and methods

2.1. Plasmids

To obtain the expression plasmids encoding FLAG-tagged human ZFP36L1, and TTP, the coding region of each cDNA fragment was inserted into the pFLAG-CMV-2 plasmid (SIGMA). FLAG-tagged ZFP36L1 mutants, C129R and CIM AA mutant were generated from pFLAG-ZFP36L1 by replacing Arg326 and Phe330 with Ala or Cys129 with Arg using the PrimeSTAR Mutagenesis Basal Kit (TAKARA). Reporter constructs, pBSII-Nluc-A114 and pBSII-EMCV-IRES-Nluc-A114 were constructed previously [18]. FLAG-tagged human CNOT7 dominant negative mutant (CNOT7-DN) was generated from a cDNA fragment coding for CNOT7 by replacing Asp40 and Glu42 with Ala (catalytic mutant), Cys67 and Leu71 with Glu (CNOT6 interaction mutant) [30] using the PrimeSTAR Mutagenesis Basal Kit (TAKARA). To make the reporter construct pBSII-Nluc-IL6 3′UTR-A114, the 3′UTR sequence of IL6 from pGL3-IL6 3′UTR [28] was cloned into the XbaI site of pBSII-Nluc-A114 [18] using a restriction enzyme strategy. All constructs were verified by DNA sequencing.

2.2. Cell culture and transfection

HEK293F cells were cultured in FreeStyle 293 Expression Medium (Invitrogen). Cells were transiently transfected using Lipofectamine 2000 transfection reagent (Invitrogen) [18].

2.3. In vitro Translation and in vitro Transcription

In vitro transcription of mRNAs in the presence of either 7mGpppG or ApppG (for IRES-conjugated mRNA) and *in vitro* translation were described previously [37, 38]. A total of 7 µl of standard HEK293F cell extract, 3 µl of HEK293F cell extract expressing FLAG-tagged GFP, ZFP36L1 or ZFP36L1 mutants, and 2.5 µl of 10 ng/µl

reporter mRNAs, and 12.5 µl of reaction buffer mix were incubated at 37 ˚C. HEK293F cell extracts were treated by Micrococcal nuclease before translation reaction [18]. The luciferase reporter assay was performed by Nano-Glo Luciferase Assay Systems (Promega).

2.4. RNase H-mediated poly(A) tail analysis

The poly(A) tail length was analyzed by RNase H digestion and northern blotting as previously described [18]. Briefly, total RNA was extracted from *in vitro* translation reaction at each time point (incubation time is 0, 10, 20 and 30 minutes) with ISOGEN II (Nippon Gene). Total RNA (1.5 µg) was mixed with 25 pmol oligo DNA complementary to the Nluc reporter sequence near the stop codon. Samples were first denatured at 65 ˚C for 5 min and annealed at 37 ˚C for 10 min. Subsequently, they were mixed with 0.5 U RNase H (TAKARA) and 10 U RNase inhibitor (TOYOBO) in RNase H buffer (20 mM HEPES-KOH [pH 7.9], 10 mM MgCl2, 50 mM KCl, and 1 mM DTT), and incubated for 10 min at 37 ˚C. The fragment of Nluc reporter mRNAs containing the IL-6 3′UTR sequence was detected by northern blot using a RNA probe to IL-6 3′UTR sequence made by DIG RNA Labeling Kit (Roche) and visualized by CDP-Star Chemiluminescent Substrate (Roche).

2.5. Immunoprecipitation

HEK293F cells transfected with FLAG-tagged plasmids were lysed in TNE buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). Anti-FLAG M2 monoclonal antibody (SIGMA) was added to the extracts together with protein G-Sepharose beads. Bound proteins were washed with TNE buffer 1 mL x 5 times and after that they were eluted with SDS-PAGE loading buffer and subjected to SDS-PAGE followed by Western blotting.

2.6. Western blotting

Immunoblotting to polyvinylidene difluoride (PVDF) was performed with wet transfer under standard conditions according to the manufacturer's guidelines. Blots were blocked in 10% milk/PBS-T solution and probed with antibodies diluted as indicated. Signals were either visualized using HRP-conjugated secondary antibodies and ImmunoStar LD (Fujifilm) and imaged on a Fujifilm LAS-4000 luminescent image analyzer. Antibodies used in this study were an anti-CNOT1, CNOT6, CNOT6L monoclonal antibody (1:1,000 dilution, gifted from Dr. Yamamoto in OIST), an anti-GIGYF2 polyclonal antibody (1:1,000 dilution, BETHYL).

3. Results

3.1. ZFP36L1 induces deadenylation and translation repression via ARE-binding activity in a HEK293F cell-free system

It has been assumed that ZFP36L1 would promote deadenylation by the CCR4-NOT complex because ZFP36L1 interacts with subunits of the CCR4-NOT complex and is involved in mRNA decay [12-14, 26, 27]. To investigate directly whether ZFP36L1 induces deadenylation of an ARE-containing mRNA and its impact on translation, we utilized *in vitro* transcribed NanoLuc (NLuc) mRNA reporters in a HEK293F-derived cell free translation system. This system reconstitutes the synergism between the 5′cap structure and the 3′poly (A) tail [18]. Our ARE^{+} reporter mRNA contains the IL-6 3′UTR sequence, which bears five AREs [28], inserted between the Nluc-coding sequence and the 114 nt poly(A) sequence (cap-Nluc-IL6 3′UTR-A114). The ARE^{-} control mRNA (cap-Nluc-A114), lacks the IL6-mRNA-derived sequence and has no AREs (Fig. 1A). ARE^{+} and ARE^{-} NLuc reporter mRNAs were incubated in nuclease-treated HEK293F translation extracts supplemented with additional extracts derived from transfected HEK293F cells to deliver either wild type (WT) ZFP36L1 or the RNA-binding mutant C129R [29], both with a FLAG epitope tag. Extracts containing FLAG-GFP were used as a negative control (see Experimental Procedures). Under these conditions, we observed significant repression of translation of the ARE^{+} reporter mRNA by wild type ZFP36L1, as compared to the negative control with GFP. Translational repression by ZFP36L1 was dependent on the presence of AREs, as it was not observed with the control ARE^{-} reporter. Moreover, we found that the RNA-binding mutant of ZFP36L1 (C129R) was unable to support translation repression (Fig. 1B). To determine whether translation repression by ZFP36L1 correlates with deadenylation of the reporter, we extracted total RNA from each reaction of the *in vitro* system at several incubation time points and performed RNaseH analysis to detect poly(A) tail length (Fig. 1C). Deadenylation of the ARE+ reporter mRNA

was detected in the presence of wild type ZFP36L1, but did not occur in the extract supplemented with either GFP or the RNA-binding mutant of ZFP36L1 (C129R) (Fig. 1C). Thus, this *in vitro* system can reconstitute ZFP36L1-specific deadenylation and translation repression.

3.2. ZFP36L1 represses translation in a deadenylation-independent manner

To investigate whether the ZFP36L1-mediated deadenylation of reporter mRNA is induced by the CCR4-NOT complex, we took advantage of a dominant negative form of CNOT7. This mutant lacks deadenylase activity and cannot interact with the other deadenylase subunit CNOT6/6L, resulting in inactivation of deadenylation by the CCR4-NOT complex [30]. We first verified that this reagent would function in the expected dominant-negative manner in our system. Specifically, we transfected HEK293F cells with either FLAG-tagged wild-type or dominant negative CNOT7 and examined interaction of endogenous CNOT complex components with these proteins by co-immunoprecipitation (co-IP) on anti-FLAG beads. We also tested extracts with FLAG-tagged GFP as a negative control. As shown in Figure 2A, CNOT1, CNOT6 and CNOT6L all co-IPd efficiently with CNOT7-WT on anti-Flag beads. As expected, we did not detect any of these proteins in FLAG-GFP control IPs (Fig. 2A). We also found that CNOT7-DN retained its binding activity to CNOT1, but could not interact efficiently with CNOT6/6L. This confirms a dominant negative binding mode for CNOT7-DN in HEK293F cells [30].

Having confirmed that CNOT7-DN was functioning as expected in HEK293F cells, we next checked whether CNOT7-DN impedes ZFP36L1-mediated deadenylation in our *in vitro* system. Deadenylation time course assays were performed with the ARE^{+} reporter mRNA in nuclease-treated HEK293F translation extracts which were supplemented with either GFP as a control or FLAG-tagged ZFP36L1. The potential of these proteins to induce deadenylation was tested in the additional presence of either extracts transfected with an empty FLAG-tag vector (+mock) or FLAG-tag CNOT7-DN vector (+CNOT7 DN). As expected, GFP did not lead to any significant

deadenylation. In contrast, ZFP36L1 efficiently promoted deadenylation of the ARE^{+} reporter mRNA under mock conditions, but this activity appeared to be strongly inhibited in the presence of CNOT7-DN (Fig. 2B). Strikingly, we found that ZFP36L1 repressed translation to the same extent under these conditions (Fig. 2C). These results imply that ZFP36L1 induces deadenylation via the CCR4-NOT complex, but can still repress translation in a deadenylation-independent manner. To obtain further evidence that ZFP36L1 indeed represses translation independently of deadenylation, we employed a modified reporter mRNA with a random 40nt sequence downstream of the poly(A) tail (Fig. 3A), which should be resistant to deadenylation [18]. This "blocked poly(A)" reporter mRNA (cap-Nluc-IL6 3´UTR-A114+N40) was incubated in HEK293F translation extracts supplemented with FLAG-tagged ZFP36L1 and GFP. As shown in Figure 3B, the blocked poly(A) reporter mRNA was completely resistant to ZFP36L1-mediated deadenylation. Nevertheless, ZFP36L1 continued to repress translation of the blocked poly(A) reporter (Fig. 3C).

3.3. ZFP36L1-mediated translation repression requires its CNOT1-binding activity

We next examined how important the CCR4-NOT complex is for ZFP36L1-mediated translation repression. TTP, a homologue of ZFP36L1, directly binds to CNOT1 via its C-terminal domain, CCR4-NOT interaction motif (CIM), and this is disrupted by specific mutations of conserved arginine residues [6]. We therefore developed a ZFP36L1-CIM AA mutant in the conserved-CIM domain of ZFP36L1 (Fig. 4A), reasoning that this would disrupt interaction with CNOT1. This could be confirmed by anti-FLAG Co-IP assays from HEK293F cell-free extracts. As expected, we observed efficient CNOT1 Co-IP with wild type FLAG-ZFP36L1, but not with FLAG-GFP control or the FLAG -ZFP36L1-CIM AA mutant (Fig. 4B). Next, we examined whether ZFP36L1-mediated translation repression requires CNOT1-binding using the ZFP36L1-CIM AA mutant. A blocked poly(A) reporter mRNA was incubated in HEK293F translation extracts supplemented with one of three FLAG-tagged proteins:

GFP, WT ZFP36L1 or the ZFP36L1-CIM AA mutant. This revealed that the ZFP36L1 mutant that cannot interact with CNOT1 was also unable to support translation repression (Fig. 4C).

3.4. ZFP36L1 represses translation through a different mechanism from TTP and miRISC

The observation that ZFP36L1 mediated translation repression requires interaction with CNOT1 suggested that ZFP36L1 might repress translation in a similar manner to TTP and miRISC, both of which recruit 4EHP to target mRNAs via interaction with the CNOT1 subunit of the CCR4-NOT complex [20-22, 24, 25]. To examine whether ZFP36L1 interacts with 4EHP, we performed co-IP analysis with anti-FLAG antibodies from co-transfected cell lysates containing T7-tagged 4EHP and FLAG-tagged GFP, ZFP36L1 or TTP. T7-tagged 4EHP and endogenous GIGYF2, a co-factor, both co-purified efficiently with TTP, but not with GFP or ZFP36L1 (Fig. 5A). These data indicate that interaction of ZFP36L1 with 4EHP and GIGYF2 is much less efficient than with TTP, suggesting that ZFP36L1 is unlikely to recruit these proteins for translational repression in a manner analogous to TTP.

In a previous study, we used two methods to show that miRISC represses translation by dissociating eIF4A from eIF4F [18]: (1) comparing efficiency of miRNA repression of reporter mRNAs undergoing either eIF4A-dependent or -independent translation *in vitro* and (2) comparing responsiveness to pharmacological eIF4A inhibition via silvestrol. To further analyze the mechanism of translation repression by ZFP36L1, we constructed another ARE^{+} reporter whose translation would be driven by the Internal Ribosome Entry Site derived from the encephalomyocarditis virus (EMCV IRES). This reporter was made in a blocked poly(A) context to enable examination of translational repression in the absence of deadenylation and with an A-cap, which stabilizes the mRNA, but is not recognized by the eIF4F cap binding complex [31] (Fig. 5B). Importantly, EMCV IRES-driven translation requires eIF4A. For this reason, miRISC can still repress translation of a reporter mRNA with the EMCV IRES [18]. We examined repression of the EMCV IRES-driven reporter in HEK293F translation extracts

supplemented with FLAG-tagged ZFP36L1 or GFP as a control. Unlike the capped ARE^{+} reporter mRNA, ZFP36L1 did not repress translation from the A-capped EMCV ARE^{+} reporter mRNA (Fig. 5C). Furthermore, we performed *in vitro* translation analysis in the presence of the eIF4A inhibitor silvestrol, which blocks miRNA-mediated translation repression [18]. This revealed that ZFP36L1 can still repress translation of the ARE-containing reporter in the presence of silvestrol (Fig. 5D). Collectively, these results lend support to the notion that ZFP36L1 represses translation of target mRNAs through a molecular mechanism that is distinct from TTP and miRISC.

4. Discussion

In this study, we demonstrate that ZFP36L1 represses translation independently of deadenylation via the CCR4-NOT complex and provide several lines of evidence suggesting that the mechanism of translational repression by ZFP36L1 differs from that of the other ARE regulators, TTP and miRISC. First, we showed by co-IP assay that the affinity of interaction between ZFP36L1 and either 4EHP or GIGYF2 is very weak (Fig. 5A), suggesting it would not be able to recruit them to promote translational repression, as TTP has been shown to do [24, 25]. Moreover, unlike miRISC, ZFP36L1 could not repress translation driven by the EMCV IRES, which requires eIF4A (Fig. 5C), and remained translation repression in the presence of silvestrol at the same dose that is needed to interfere with miRISC (Fig. 5D) [18]. These results suggest that ZFP36L1-mediated translation repression requires another cap binding complex component, presumably eIF4E and/or eIF4G.

ZFP36L1 can interact with PABP indirectly via binding to the BTG/Tob protein as an adapter for the recruitment of the CCR4-NOT complex [32]. PABP is necessary for the mRNA circularization leading to synergistic translational enhancement [3]. On the other hand, PABP is required for the miRISC-mediated deadenylation and BTG/Tob-associated general deadenylation by CCR4-NOT complex [33, 34]. Moreover, recent studies show that PABP coordinates mRNA deadenylation with the CCR4-NOT complex [35, 36]. CAF1a/CNOT7 cannot trim the poly(A) tail if it is bound by PABP, conversely CCR4b/CNOT6L is unable to deadenylate the PABP-free poly(A) tail [35]. These reports indicate that PABP is a key modulator, with dual functions as either an activator or repressor of mRNA deadenylation and translation.

We showed that ZFP36L1 represses the translation from an ARE^{+} reporter mRNA in a CNOT1-binding dependent manner (Fig. 4). However, it is still unclear whether the interaction between ZFP36L1 and CNOT1 is necessary for both deadenylation and translational repression. In Figure 2B, partial activity of ZFP36L1-mediated deadenylation is still observed, even in the presence of CNOT7-DN. This partial deadenylation might be induced

through other deadenylase complex with an unknown mechanism. Our data suggest that we need to re-consider our views of the CCR4-NOT complex. How can it be that ZFP36L1 and miRISC use completely distinct mechanisms for translation repression even though both require the CCR4-NOT complex for repression and deadenylation? It is possible that the CCR4-NOT complex can potentially take on different conformations depending on the specific molecular context, enabling it to use different mechanisms to coordinate specific gene silencing events.

In contrast to TTP and ZFP36L1, Hu proteins are ARE-BPs that stabilize their target mRNAs. We previously showed that HuD, a member of the neuronal Hu family of proteins, stimulates translation via interaction with the poly (A) tail and eIF4A [39]. Moreover, our previous study has shown that HuD attenuates miRNA-mediated translation repression in an eIF4A binding-dependent manner [18]. Interestingly, we found that HuD impedes ZFP36L1-mediated translational repression independently of its capability to bind to eIF4A (A.F. and T.F., unpublished data). This provides additional evidence that ZFP36L1-mediated translation repression is mechanistically distinct from miRISC-mediate repression. Future work will be necessary to identify the specific mechanism used by ZFP36L1 for CNOT1-dependent translational repression.

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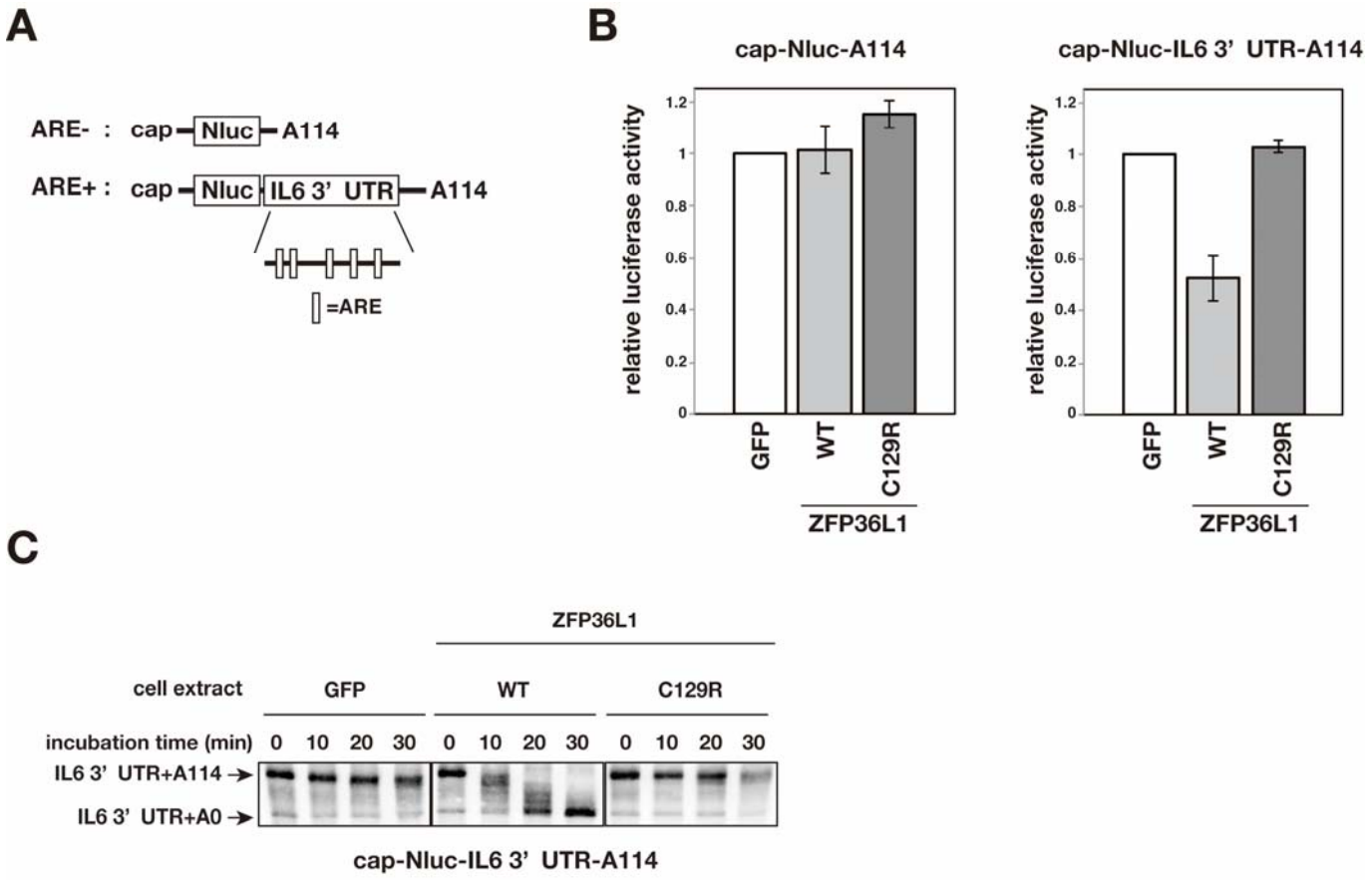

Figure 1. ZFP36L1 induces translation repression and deadenylation via ARE-binding activity in HEK293F cell-free system**.** (A) Schematic representation of reporter mRNAs. Capped and polyadenylated (A114) NLuc reporter mRNAs with (lower) or without (upper) IL-6 3´UTR sequence. (B) *In vitro* translation assay of ZFP36L1-mediated translation repression with polyadenylated (A114) reporter mRNA. Nuclease-treated HEK293F translation extracts were mixed with cell extracts expressing FLAG-GFP, ZFP36L1 or ZFP36L1 (C129R) and the polyadenylated (A114) reporter mRNAs with or without IL-6 3´UTR sequence. After 30 min incubation at 37˚C, luciferase assays were performed. Fold repression was calculated by dividing Nluc counts obtained in the respective ZFP36L1-containing translation reactions by those in GFP-containing translation reactions, which are set as 1. Error bars reflect standard deviation from three independent translation reactions using three independent transfected extracts. (C) Time course analysis of ZFP36L1-mediated deadenylation with polyadenylated (A114) reporter mRNA. Nuclease-treated HEK293F translation extracts were mixed with cell extracts expressing FLAG-GFP, ZFP36L1 or ZFP36L1 (C129R) and the polyadenylated (A114) reporter mRNAs and incubated at 37˚C. Reactions were stopped by flash freezing in liquid N_2 at each incubation time point (0, 10, 20, 30min). Total RNA was extracted from each translation reaction, and poly(A) length of reporter mRNAs was analyzed. The experiment is representative of three independent experiments using three independent transfected extracts.

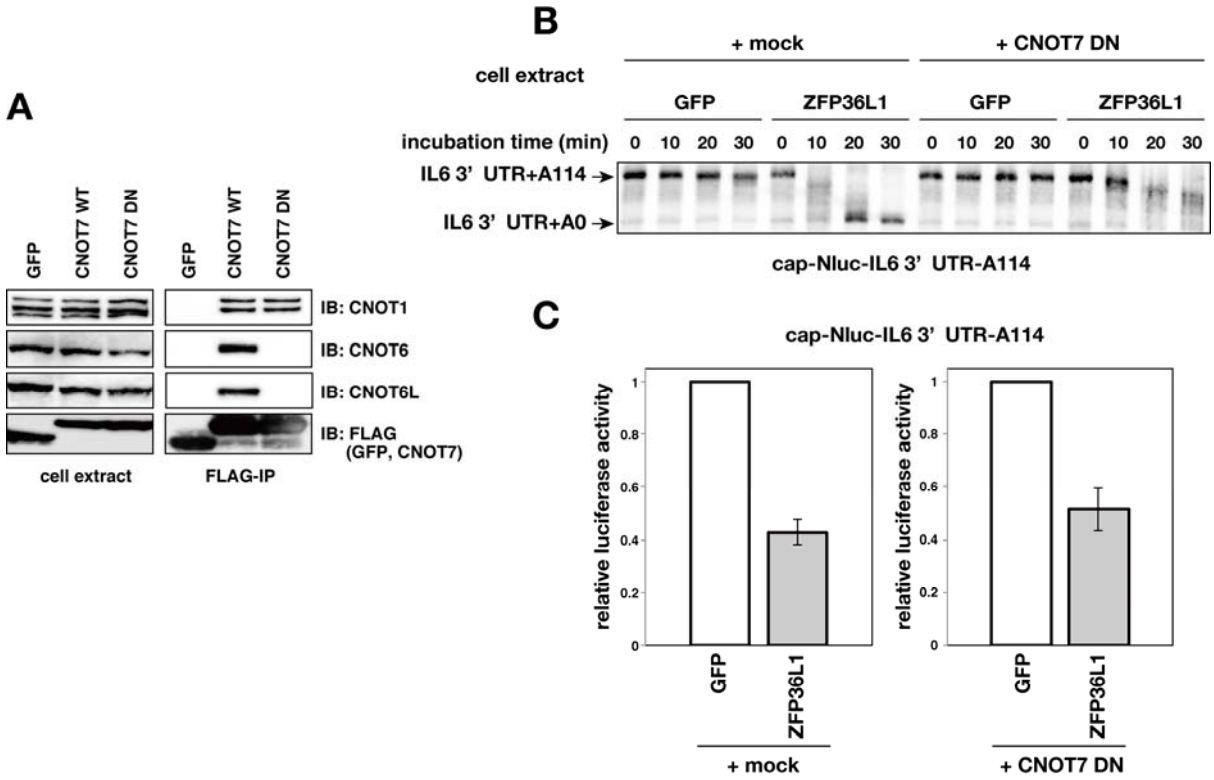

Figure 2. Disrupting CCR4-NOT complex function interferes with deadenylation, but not translational repression by ZFP36L1 (A) Immunoprecipitation analysis using a dominant negative form of CNOT7. HEK293F cell lysates expressing FLAG-tagged constructs of proteins GFP, wild type CNOT7 (WT) or a dominant negative form of CNOT7 (DN) were subject to immunoprecipitation. Copurified CNOT1, CNOT6 and CNOT6L were detected by western blotting. (B) ZFP36L1-mediated deadenylation was inhibited by CNOT7-DN. Nuclease-treated HEK293F translation extracts expressing FLAG-mock or CNOT7-DN were incubated with cell extracts expressing FLAG-GFP or ZFP36L1 and cap-Nluc-IL6 3′UTR-A114 reporter mRNA and incubated at 37˚C. Reaction was stopped by liquid-N_2 at each incubation time point (0, 10, 20, 30min). Total RNA was extracted from each translation reaction, and poly(A) length of reporter mRNAs was analyzed. The experiment is representative of three independent experiments using three independent transfected extracts. (C) ZFP36L1-mediated translation repression is not inhibited by CNOT7-DN. Nuclease-treated HEK293F translation extracts expressing FLAG-mock or CNOT7-DN were incubated with cell extracts expressing FLAG-GFP or ZFP36L1 and cap-Nluc-IL6 3′UTR-A114 reporter mRNA and incubated at 37˚C. Luciferase reporter assays were performed after 30 min. Fold repression was calculated by dividing Nluc counts obtained in the respective ZFP36L1-containing translation reactions by those in GFP-containing translation reactions, which are set as 1. Error bars reflect standard deviation from three independent translation reactions using three independent transfected extracts.

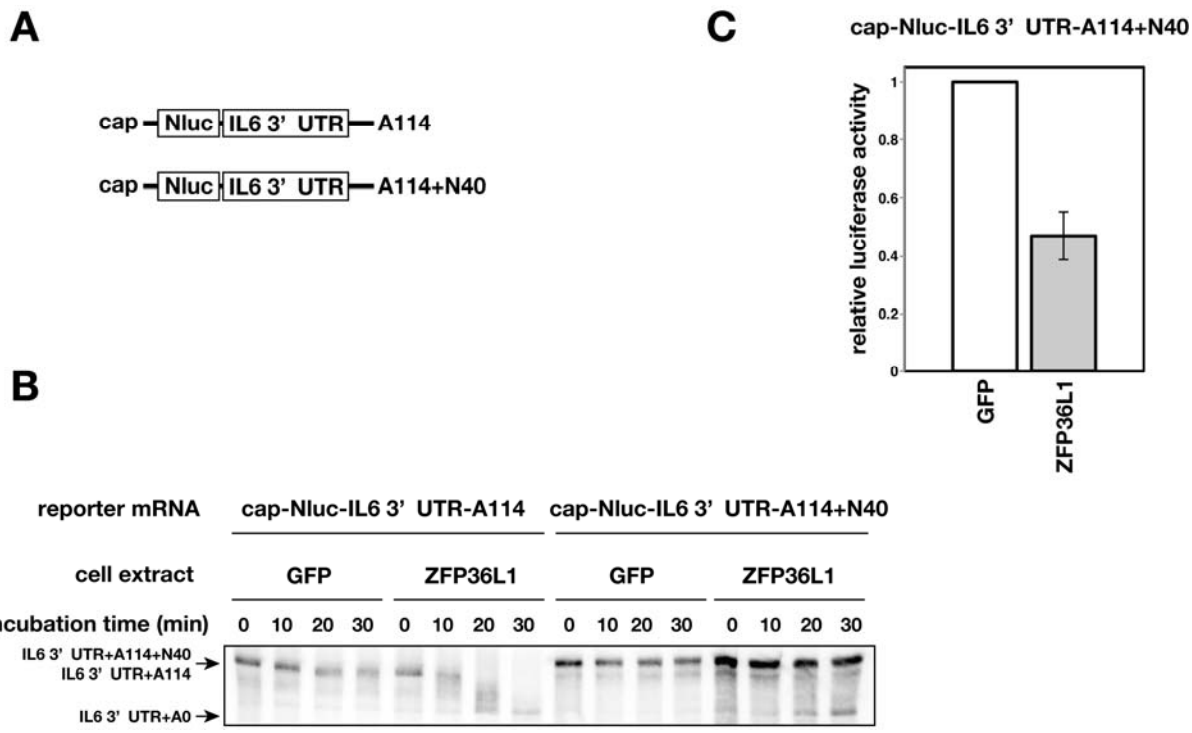

Figure 3. ZFP36L1 represses translation independently of deadenylation. (A) Schematic representation of reporter mRNAs. Capped and polyadenylated (A114) NLuc reporter mRNAs containing IL-6 3′UTR sequence with (lower) or without (upper) random 40nt sequence downstream of the poly(A) tail. (B) Effect of blocked poly(A) reporter mRNA on ZFP36L1-mediated deadenylation. Nuclease-treated HEK293F translation extracts expressing FLAG-GFP or ZFP36L1 and reporter mRNAs (cap-Nluc-IL6 3′UTR-A114 or cap-Nluc-IL6 3′UTR-A114+N40) and incubated at 37˚C. Reactions were stopped by flash freezing in liquid N_2 at each incubation time point (0, 10, 20, 30min). Total RNA was extracted from each translation reaction, and poly(A) length of reporter mRNAs was analyzed. The experiment is representative of three independent experiments using three independent transfected extracts. (C) ZFP36L1 represses translation from a blocked poly(A) reporter mRNA. Nuclease-treated HEK293F translation extracts were incubated with cell extracts expressing FLAG-GFP or ZFP36L1 and cap-Nluc-IL6 3′UTR-A114+N40 reporter mRNA. Luciferase reporter assays were performed at 30 min. Fold repression was calculated by dividing Nluc counts obtained in the respective ZFP36L1-containing translation reactions by those in GFP-containing translation reactions, which are set as 1. Error bars reflect standard deviation from three independent translation reactions using three independent transfected extracts.

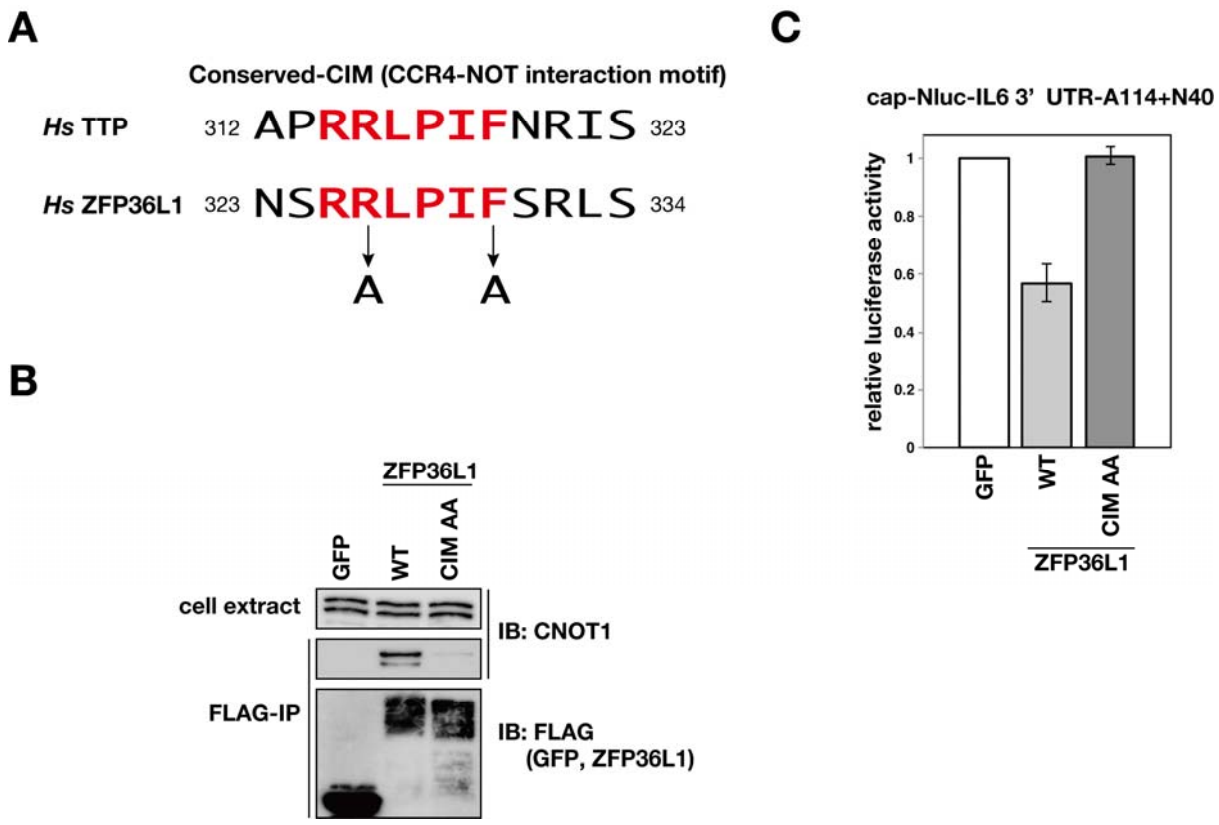

Figure 4. Mutations that compromise interaction between ZFP36L1 and CNOT1 also abolish ZFP36L1-mediated translational repression. (A) Schematic representation of conserved-CIM (CCR4-NOT interaction motif, shown in red) in TTP and ZFP36L1 showing the conserved arginine and phenylalanine residues that are essential for interaction of TTP with CNOT1 [6]. ZFP36L1-CIM AA is a mutant with R326A and F330A. (B) The ZFP36L1-AA mutant does not bind to CNOT1. HEK293F cell lysates expressing FLAG-tagged constructs of proteins GFP, ZFP36L1 or ZFP36L1-CIM AA mutant were subject to immunoprecipitation analysis. Levels of interacting CNOT1 were detected by western blotting. (C) The ZFP36L1-CIM AA mutant does not repress translation. Nuclease-treated HEK293F translation extracts were incubated with cell extracts expressing FLAG-tagged constructs of proteins GFP, ZFP36L1 or ZFP36L1-CIM AA mutant and cap-Nluc-IL6 3′UTR-A114+N40 reporter mRNA. Luciferase reporter assay was performed at 30 min. Fold repression was calculated by dividing Nluc counts obtained in the respective ZFP36L1-containing translation reactions by those in GFP-containing translation reactions, which are set as 1. Error bars reflect standard deviation from three independent translation reactions using three independent transfected extracts.

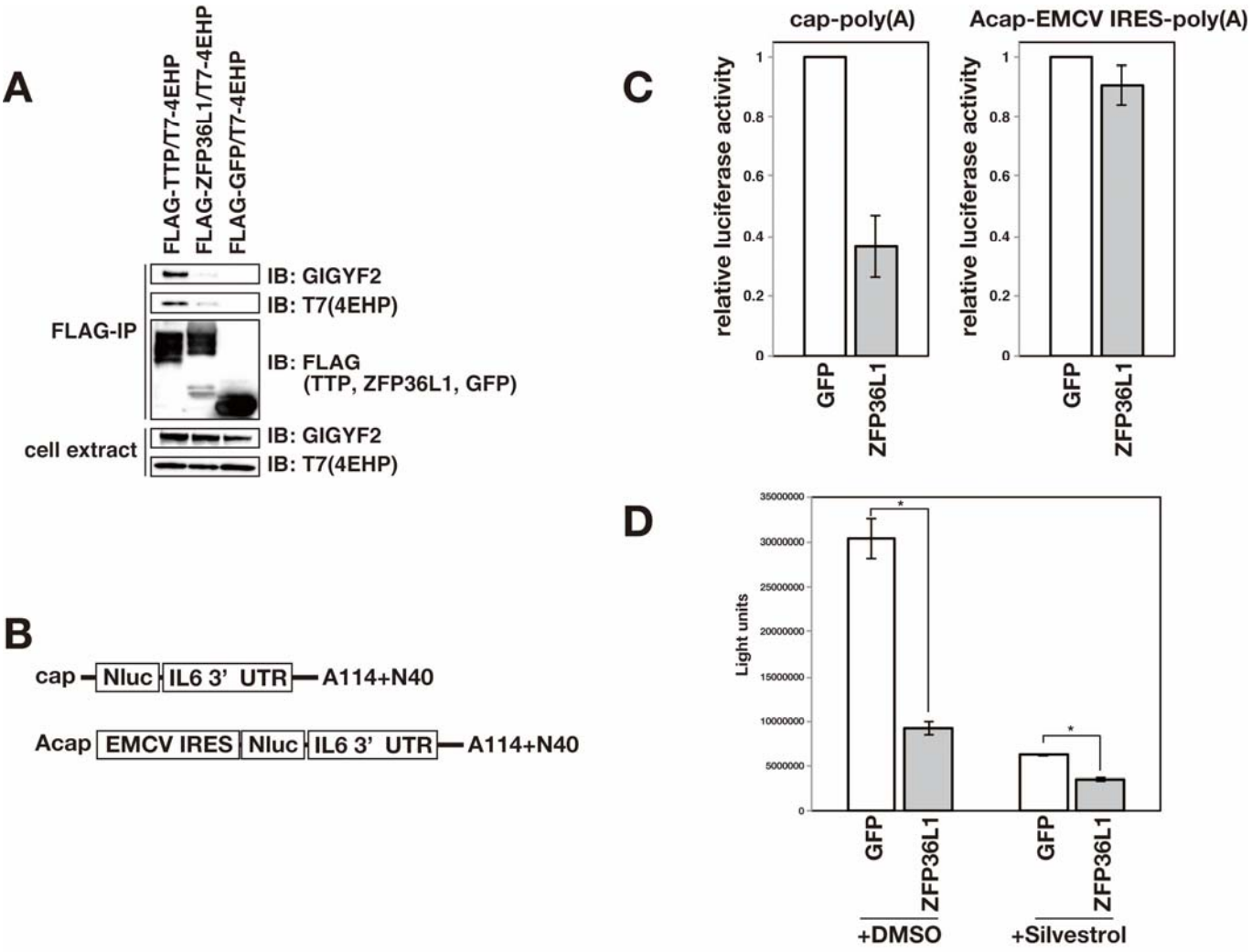

Figure 5. The mechanism of ZFP36L1-mediated translation repression is distinct from TTP and miRISC. (A) ZFP36L1 interacts less efficiently than TTP with 4EHP and GIGYF2. HEK293F cell lysates expressing FLAG-GFP, TTP or ZFP36L1 and T7-4EHP were subject to immunoprecipitation analysis. Copurified endogenous GIGYF2 and T7-4EHP were detected by western blotting. The experiment is representative of three independent experiments. (B) Schematic representation of reporter mRNAs. Cap (7mGpppG) or Acap (ApppG: for IRES-conjugated mRNA) and blocked poly(A) (A114+N40) Nluc reporter mRNAs with IL-6 3′UTR sequence. (C) ZFP36L1 does not repress EMCV IRES-driven translation. Nuclease-treated HEK293F translation extracts were mixed with cell extracts expressing FLAG-GFP or ZFP36L1 and the blocked poly(A) reporter mRNAs with (right) or without (left) EMCV IRES. After 30 min incubation at 37˚C, luciferase reporter assay was performed. (D) Nuclease-treated HEK293F translation extracts were mixed with cell extracts expressing FLAG-GFP or ZFP36L1 and the poly(A)-blocked reporter mRNAs in the presence of 2.5 μM silvestrol (final concentration) or DMSO (vehicle) as a negative control. After 30 min incubation at 37˚C, luciferase reporter assays were performed. (C, D) Fold repression was calculated by dividing Nluc counts obtained in the respective ZFP36L1-containing translation reactions by those in GFP-containing translation reactions, which are set as 1. Error bars reflect standard deviation from three independent translation reactions using three independent transfected extracts. The asterisk in (D) indicates a statistically significant difference ($p < 0.05$).