UNIVERISTY OF CALIFORNIA, SAN DIEGO

Characterizing the interaction between TTP and the 4EHP-GYF2 complex

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

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2015

The thesis of Myanna Teresa Olsen is approved, and is acceptable

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Chair

University of California, San Diego

2015

DEDICATION

I dedicate this to my mother, Maggie, and father, Michael, for your constant love and support.

My sister, Chelsea, and cousin, Melissa, for keeping me sane.

Lastly, I'd like to thank Andres for encouraging me and making me laugh with Arrested Development quotes.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Jens Lykke-Andersen for allowing me to start my journey in research in his lab. His thorough understanding of both molecular biology and public speaking has helped shape the researcher I am today. His genius continues to amaze me.

I would like to thank my advisor and mentor, Rui Fu, for teaching me everything I needed to know to be successful in molecular biology. My thesis would not be what it is without his guidance and support. Many nights Rui stayed late to help me practice for countless presentations, for which I am deeply grateful.

I would also like to thank Dr. Dong-Er Zhang and Dr. Ella Tour for being members of my committee. I sincerely enjoyed the courses I took with both of you and appreciated the compassion you both show to your students.

I would like to thank the Eric Bennett Lab for the data they provided to the project.

Finally, I would like to thank the entire JLA lab for supporting me and giving me helpful comments and feedback throughout the BS/MS program, especially Suzanne Lee, Rea Lardelli, Marcos Arribas-Layton, and Liz Corpuz.

ABSTRACT OF THE THESIS

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Professor Jens Lykke-Andersen, Chair

Precise control of gene expression involves multiple steps beyond transcription, including mRNA turnover and translational regulation. One pathway involves the RNA-binding protein tristetraprolin (TTP), which binds to AU-rich elements (AREs) in the 3' UTR of certain cytokine mRNAs and promotes their decay by recruiting degradation factors. Though TTP's role in mRNA decay is well established, its role in translational repression is poorly understood. Recently, the Lykke-Andersen lab, by mass spectrometry in

collaboration with the Bennett Lab, revealed a novel interaction between TTP and the translational repressor complex 4EHP-GYF2. Aside from the potential insight into the mechanism of TTP-mediated translational repression, this finding also suggests TTP-mediated mRNA decay and translational repression could be interdependent. Bacterially expressed recombinant TTP, 4EHP, and GYF2 in *in vitro* pull-down experiments revealed weak binding of 4EHP to TTP, but strong enrichment for binding upon the addition of GYF2, indicating GYF2 is important in bridging this complex together. The better understand the molecular interaction, the domains of TTP needed for binding GYF2 and 4EHP were mapped out by mutagenesis. Co-immunoprecipitation of wild-type (WT) and deletion mutants of TTP expressed in HEK293T cells revealed the N-terminal domain of TTP to be necessary and sufficient for binding to the 4EHP-GYF2 complex and further mapping revealed conserved PPPPGF motifs, that when mutated, results in almost complete loss of binding to the 4EHP-GYF2 complex. The functional implication of these interactions was tested in *in vitro* translation experiments; however, only modest translational repression by the TTP-4EHP-GYF2 complex was observed under those conditions.

INTRODUCTION

Gene expression is the process in which genes are used to make a functional gene product, such as RNA and protein. The regulation of gene expression is tightly controlled at many levels—including at the DNA, RNA, and protein levels, which collectively are responsible for the proper development and function of all life. Many post-transcriptional processes are involved in gene expression regulation at the RNA level, including mRNA turnover and cap-dependent translational activation and repression.

RNA turnover regulation, in which RNA stability is modified in response to various stimuli, such as environmental changes, development, and immune responses, is one way to control mRNA levels and downstream protein production in the cell (Parker et al., 2007). Many mRNAs encode within themselves regulatory elements that predispose them to instability. For example, mRNAs encoding for proto-oncogenes and cytokines (for example interleukins, TNFalpha, GM-CSF) contain adenosine- and uridine-rich element (ARE) motifs in their 3' untranslated region (3'UTR) (Chen et al., 1995). These cis-acting elements target the mRNAs for rapid decay through interaction with trans-acting RNA-binding proteins, such as tristetraprolin (TTP) (Blackshear, 2002; Lai et al., 2001). TTP directly binds to ARE-containing mRNAs through its two zinc-finger domains and activates decay by recruiting degradation factors, such as the decapping complex subunits, Dcp2 and Dcp1a, the 5'-to-3' exonuclease Xrn1, the CCR4-NOT deadenylase complex, as well as a complex of 3'-5' exonucleases termed

the exosome (Lai et al., 2001; Lykke-Andersen & Wagner, 2005), thereby subjecting certain mRNAs to decay. Both the N- and C-terminal domains of TTP interact with decay factors and are involved in activating decay (Lykke-Andersen & Wagner, 2005; Fabian et al., 2013). Contrary to TTP's well documented activity in ARE-mediated decay (Lai et al., 2001; Lykke-Andersen & Wagner, 2005), TTP has been shown to repress ARE reporter mRNA translation as well as shift these mRNAs to lighter polysome fractions (Qi et al., 2012; Kratochvill et al., 2015), but how exactly TTP is imparting this repression is unclear.

Translational repression is another common way to regulate gene expression at the mRNA level. The rate of translation is often mediated by initiation, which involves the interaction of the 5' methylated guanosine cap of mRNA with the cap binding complex and subsequent recruitment of the pre-initiation complex. The cap binding complex, termed eukaryotic initiation factor (eIF4F), is composed of three subunits: eIF4E, eIF4G, and eIF4A (Gringas et al., 1999). Direct eIF4E binding the 5' cap of mRNAs is the rate-limiting step. eIF4G, a larger scaffold, mediates interactions with the pre-initiation complex and eIF4E, while eIF4A, a helicase, unwinds mRNA 5' secondary structure (Sonenberg et al., 2009). Interfering with the binding potential of these proteins during translation initiation alters translation rates. eIF4E binding proteins (4E-BPs) have been shown to compete with eIF4G in binding eIF4E, thereby inhibiting cap-dependent translation (Gingras et al.,1999). A homolog of eIF4E, eIF4E homologous protein (4EHP) competes with eIF4E for the 5' cap structure, with 100-fold less affinity than eIF4E (Rom et al., 1998), but is unable to bind eIF4G, thus preventing the

recruitment of the pre-initiation complex needed for translation initiation (Rom et al., 1998; Joshi et al., 2004). In *Drosophila*, d4EHP forms a complex with Bicoid to repress translation of *caudal* mRNA and subsequent CAD protein production early in embryonic development (Cho et al., 2009). Bicoid binds to specific 3' UTR elements of the *caudal* mRNAs and acts to tether d4EHP to the 5' cap, thereby increasing d4EHP binding affinity and its ability to outcompete eIF4E for the 5' cap. This complex is thought to form a closed loop structure connecting the 5' end of the mRNA with the 3' UTR, effectively displacing eIF4E from the cap to repress translation of certain mRNAs (Cho et al., 2005).

More recently, mammalian 4EHP has been shown to interact with Grb10-Interacting GYF Protein 2 (GIGYF2 or GYF2), which was given its name due to its interaction with Grb10, an adapter protein for insulin receptors (Giovannone et al., 2003). GYF2 binds 4EHP through a binding motif similar to that of Bicoid (Morita et al., 2012). Deletion of the mouse (m)4EHP gene leads to increased incorporation of radiolabeled methionine into protein and shifted polysome profile toward the heavier end, corresponding to increased translation globally, as well as perinatal lethality in mice (Morita et al., 2012). Although they bind 4EHP through a similar binding motif, GYF2 alone does not appear to completely mimic the function of Bicoid, as it contains no RNA-binding domain (Giovannone et al., 2003). Whether other RNA-binding proteins are actively involved in tethering the 4EHP-GYF2 complex to specific pools of mRNA is currently unknown. Mass spectrometry data from the Lykke-Andersen lab reveals TTP's association with the 4EHP-GYF2 complex, while co-immunoprecipitation

data suggests TTP binds directly to GYF2 through a conserved GYF domain (Fu et al., unpublished). If a parallel is drawn from Bicoid in *Drosophila* to TTP in mammals, TTP may be the link that tethers 4EHP-GYF2 to the 3' UTR, thus promoting circularization of mRNA, eIF4E displacement, and translational repression, in a manner similar to that of Bicoid. The main objective of my thesis was to test the hypothesis that TTP mediates translational repression via its interaction with 4EHP-GYF2. First, we studied the molecular interaction of TTP to the 4EHP-GYF2 complex, and found that TTP directly binds GYF2 through a conserved GYF-domain-binding motif PPPPGF. We then studied TTP's ability to repress translation via its interaction with 4EHP-GYF2 of an ARE-containing reporter *in vitro*, but found only modest effects under the tested conditions.

RESULTS

TTP associates with the translational repression complex 4EHP-GYF2

To identify novel TTP-binding partners during the innate immune response, Rui Fu from the Lykke-Andersen lab performed immunoprecipitation (IP) of endogenous TTP at different time points during a lipopolysaccharide (LPS) treatment of mouse RAW 264.7 macrophage cells. Samples were then subjected to liquid chromatography followed by tandem mass spectrometry (LC/MS-MS) by the Eric Bennett lab. IPs were performed in the presence of RNase A to avoid co-purification of proteins that associate with TTP in an RNA-dependent manner. IP in the absence of LPS treatment where TTP expression is minimal served as a negative control. As expected, well-known binding partners of TTP were observed in the IP/MS, such as components of the CCR4-NOT deadenylase complex, 14-3-3 proteins, and PP2A subunits (Fig. 1). Of interest were the novel protein interactions of TTP to 4EHP and GYF2, a known translational repression complex (Morita et al., 2012). Enrichment of 4EHP and GYF2 were comparable to the levels observed of the CCR4-NOT subunits, whereas no peptides were detected in samples of co-IP pre-LPS treatment (0 hrs), when TTP is not expressed, or co-IP with pre-immune rabbit serum (not shown).

4EHP-GYF2 interacts with the N-terminus of TTP

To further study the association of TTP to 4EHP-GYF2 observed in the IP/MS data, we first studied the molecular interaction of these proteins, namely,

which region or domain of TTP is responsible for association. TTP's domains are well defined, consisting of the RNA-binding zinc-finger domain (RBD), flanked by N-terminal (NTD) and C-terminal (CTD) domains (Lykke-Andersen & Wagner, 2005) (Fig 2a). Recently, within the CTD, a conserved C-not interacting motif (CIM) responsible for binding the CCR4-NOT complex was identified (Fabian et al., 2013), therefore we were also interested in whether the association of 4EHP-GYF2 and CNOT affected each other. To reveal potential binding motifs on TTP, I tested each of these domains for their ability to co-purify with flag-tagged 4EHP and GYF2. RNase-treated lysate from 293T cells co-expressed with domain truncation mutant myc-tagged TTP and flag-tagged 4EHP and GYF2 were coimmunoprecipited using anti-myc-coupled beads. TTP truncated mutants containing the NTD were capable of 4EHP and GYF2 association (NTD, ΔCTD, ΔCIM), while fragments lacking the NTD (CTD, RBD, ΔNTD) failed to co-purify with 4EHP or GYF2 (Fig. 2b). Evidently, 4EHP-GYF2 association to TTP is unaffected by the loss of the CIM, suggesting that TTP binds 4EHP-GYF2 independent of the deadenylase complex.

The N-terminus of TTP contains a conserved predicted GYF-domain-binding motif

Observing that the NTD of TTP is necessary and sufficient for association with 4EHP-GYF2, we analyzed the amino acid sequence of TTP in different species in search of any conserved motifs within the N-terminus that may be a potential binding site for the 4EHP-GYF2 complex. Alignment of the N-terminus

of TTP from various vertebrate species revealed a conserved proline-rich (PPPPGF) motif. TTP also contains 2 additional proline-rich regions, both located in the C-terminal domain, the last of which is the least conserved (Fig. 3). Interestingly, PPPGF/L is a canonical binding target of GYF-domain-containing proteins (Kofler & Freund, 2006). This suggests the GYF domain in GYF2 may preferentially bind this proline-rich motif in TTP.

The first and second proline-rich regions of TTP interact with 4EHP-GYF2 complex

To uncover whether these proline-rich motifs in TTP were responsible for binding the 4EHP-GYF2 complex, Rui Fu generated flag-tagged TTP constructs in which the three proline-rich regions of mouse (m)TTP (P1, P2, and P3), were mutated at the second, third, and fourth proline to serines (Fig 4a). When the 1st proline-rich motif was mutated (P1S), a substantial loss in binding was observed, as assessed by coimmunoprecipitation, performed by Rui Fu (Fig. 4b). Single mutations at the 2nd and 3rd motifs (P2S, P3S) showed little to no effect on binding. The P12S mutant of TTP, in which the first and second proline-rich regions were mutated to serines, showed almost a complete loss of binding to 4EHP and GYF2, whereas the P13S and P23S showed much less of an effect on binding. Combining all three mutations (P123S) did not lead to further loss of binding, suggesting that the 3rd proline-rich region is not involved in this interaction and that the first and second proline-rich regions (P12) are most

important for this binding interaction. These findings may finally ascribe a role to TTP's characteristic name, tris-tetra-prolin.

TTP interacts with the 4EHP-GYF2 complex via a direct interaction with GYF2

Since TTP contains GYF-binding motifs that when mutated results in the loss of binding to the 4EHP-GYF2 complex as seen in co-immunoprecipiation analysis, we hypothesized that TTP may be directly interacting with GYF2, thereby linking 4EHP to TTP. To test direct protein-protein interactions and to rule out the possibility of other co-factors mediating this interaction, I expressed and purified recombinant proteins in *E. coli* and performed *in vitro* GST-pulldown experiments (Fig. 5). GST protein or GST-tagged TTP were coupled to glutathione sepharose beads and incubated with His_6-tagged 4EHP and/or His_6-tagged GYF2. When added alone, His_6-4EHP weakly bound GST-TTP WT, when compared to both proteins added (Fig. 5). His_6-GYF2, however, was pulled down strongly with GST-TTP with and without the presence of His_6-4EHP, indicating a direct protein interaction exists between these two proteins. Moreover, when both His_6-4EHP and His_6-GYF2 were added to GST-TTP WT, there is a great enrichment for His_6-4EHP binding. GST only protein was used as a negative control to ensure His_6-tagged proteins were not binding nonspecifically to the beads or the GST domain.

Rabbit reticulocyte lysate cell-free system exhibits cap-dependent translation

To better understand the functional implications of TTP's interaction with the translational repression complex 4EHP-GYF2, we used nuclease-treated rabbit reticulocyte lysate (RRL) as our cell-free system to measure translational repression by

these proteins on a reporter mRNA. Since 4EHP is known to repress translation by competing with eIF4E for the 5' cap, it was crucial to ensure our system showed cap-dependent translation. I transcribed a Firefly luciferase reporter with AU-rich elements from human GM-CSF in the 3'UTR (F-Luc-ARE) using different ratios of GTP nucleotide to anti-reverse cap analog (ARCA). Higher ARCA-to-GTP ratio is known to produce a higher percentage of capped transcripts, but also less overall yield. I separately added capped and uncapped F-Luc-ARE reporter mRNA to RRL and incubated at 30°C for 1.5 hr (Fig. 6a). Translation of capped mRNA at all tested ratios of GTP to ARCA, as measured by luciferase activity, far exceeded translation of the uncapped mRNA (~10-fold increase) (Fig. 6b). Importantly, these results allowed further testing with our proteins of interest using this particular in vitro system.

Renilla Luciferase can be used as an internal control in our cell-free system

Next, we tested whether *Renilla* luciferase (R-Luc) mRNA could be used in our system as an internal control to which expression of capped F-Luc-ARE reporter values may be normalized. Either capped F-Luc-ARE mRNA alone or capped F-Luc-ARE mixed with uncapped R-Luc reporters were incubated in RRL for 1.5 hr at 30°C. F-Luc-ARE activity was largely unaffected by the presence of R-Luc mRNA in the system, as measured by firefly luciferase activity. R-Luc mRNA was used in an uncapped form to prevent repression of this internal control by 4EHP in the assays below.

4EHP modestly represses translation of capped transcripts

Since 4EHP is the cap-binding protein in the 4EHP-GYF2 complex and competes for cap binding with eIF4E, we sought to first determine an amount of 4EHP that specifically represses translation of our capped reporter mRNA to a moderate degree, predicting that when GYF2 and TTP proteins are added to the system, a complete translational repression complex would form on the ARE-containing reporter and repress translation more strongly. I titrated in purified 4EHP protein mixed with capped F-Luc-ARE reporter mRNA into RRL, and measured luminescence. Normalizing values to the condition with no 4EHP protein added, with a 115 nM (4 ng/µl) concentration of 4EHP there is a slight repression of translation of the capped F-Luc-ARE mRNA, whereas uncapped R-Luc was not affected (Fig. 8b). At a higher concentration of 460 nM (16 ng/µl) of 4EHP, translation of the F-Luc-ARE mRNA was further repressed, but repression was also observed for the R-Luc mRNA that lacks a cap. Thus, we chose 115 nM concentration of 4EHP for following experiments.

4EHP-GYF2-TTP complex represses translation of ARE-containing reporter moderately

Finally, after optimizing the amount of 4EHP protein needed for *in vitro* translation reactions, I added GYF2 and TTP protein (protein amounts were kept at 4 ng/µl each, as in the *in vitro* pull-down assays) to the reaction and translation was measured (Fig. 9a). As seen in Fig. 9b, after normalizing to the no protein added condition, we see that in the presence of only 4EHP and GYF2, there is a very slight repression (6%); a similar amount of repression is seen when TTP and GYF2 are added to the system (10%); in samples combining all three proteins, repression of translation

increases to ~ 26% (n=3), although not reaching statistical significance compared to translation reactions absent of TTP. It was not possible to determine whether the effect seen with all three proteins added is simply an additive effect from individual proteins or a synergistic effect as we would predict when the 4EHP-GYF2-TTP complex is recruited to ARE-containing mRNAs.

Adjusting molar ratios in hopes of forming competent RNP complexes resulted in modest repression of translation

Observing a modest (~26%) repression of translation using 4 ng/ul of each protein, we sought to increase repression by using increasingly higher molar amounts of our proteins in hope of saturating 1) TTP to RNA binding, 2) GYF2 binding to TTP, and 3) 4EHP binding to GYF2 (Fig. 10a). TTP has a peptide-ARE-RNA dissociation constant (K_d) of 10 nM (Blackshear et al. 2003), so in order to form competent RNP complexes, we increased the molar amounts of each corresponding protein, so that the concentrations were: RNA (1.6 nM), TTP (24 nM), GYF2 (60 nM), 4EHP (34, 114, 228 nM). In the presence of 4EHP and GYF2 only, little to no repression is observed (Fig. 10b). The addition of TTP to the lowest concentration of 4EHP (35 nM) and GYF2 showed some repression (20%) when compared to the no protein added condition as well as the 4EHP and GYF2 only condition; however, overall repression was modest. Further optimization of this assay needs to be done to derive solid conclusions regarding a role for 4EHP-GYF2 in TTP-mediated translation repression.

FIGURES

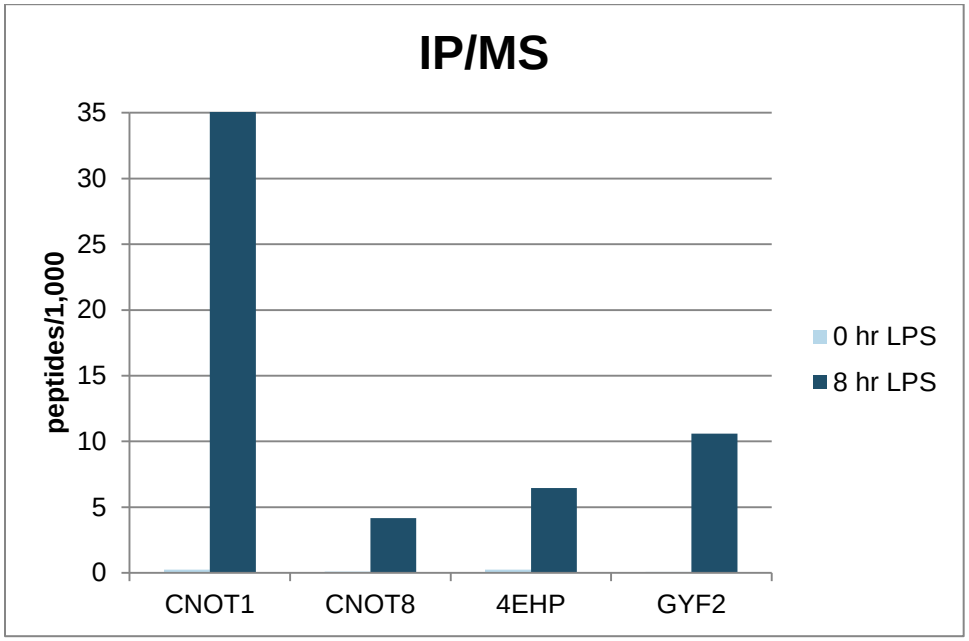
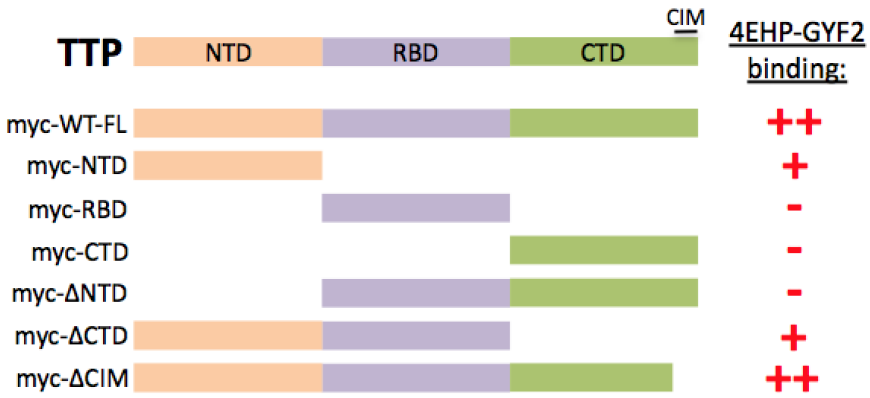


Figure 1. TTP associates with the translational repression complex 4EHP-GYF2. Partial graph of binding partners of TTP identified via IP/MS in LPS-treated RAW 264.7 cells at O hr and 8 hr time points. (Experiment by Rui Fu, analyzed by the Bennett lab).

(a)



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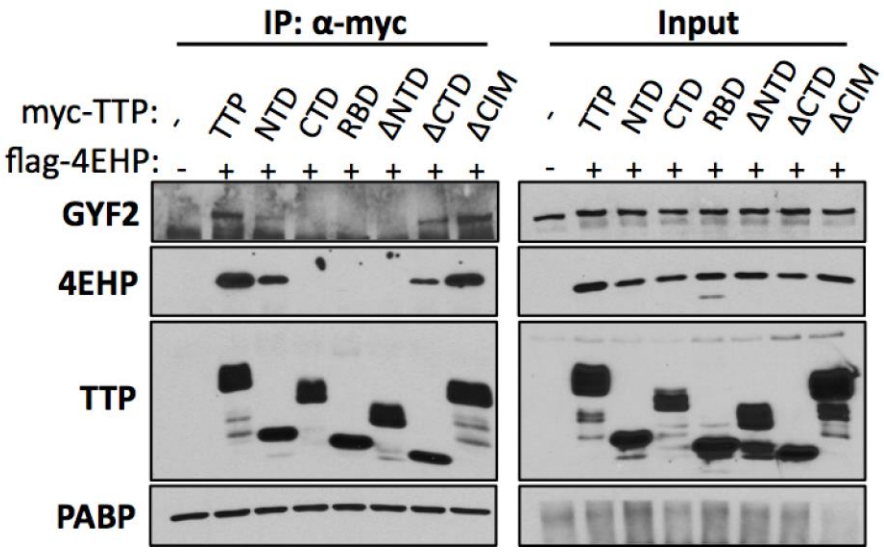


Figure 2. 4EHP-GYF2 interacts with the N-terminus of TTP. (**a**) Schematic diagram of full-length TTP (WT-TTP) and TTP truncation mutants used in co-immunoprecipitation experiments. (**b**) Cell lysate treated with RNase were immunoprecipitated with anti-myc antibody. Immunoblotting was performed with anti-myc, anti-GYF2, anti-Flag, and anti-PABP.

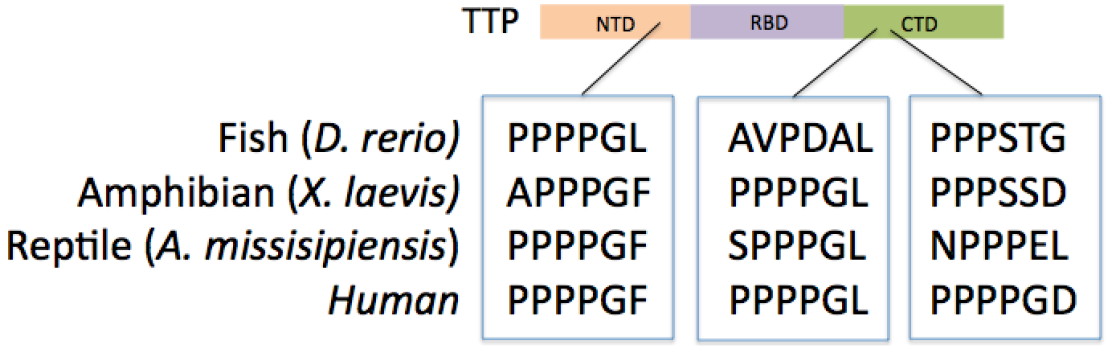


Figure 3. The N-terminus of TTP contains a conserved predicted GYF-domain-binding motif. Alignment of tetraproline motifs of TTP from different species. TTP contains a highly conserved PPPPGF motif in its NTD and two slightly less conserved proline-rich motifs in its CTD.

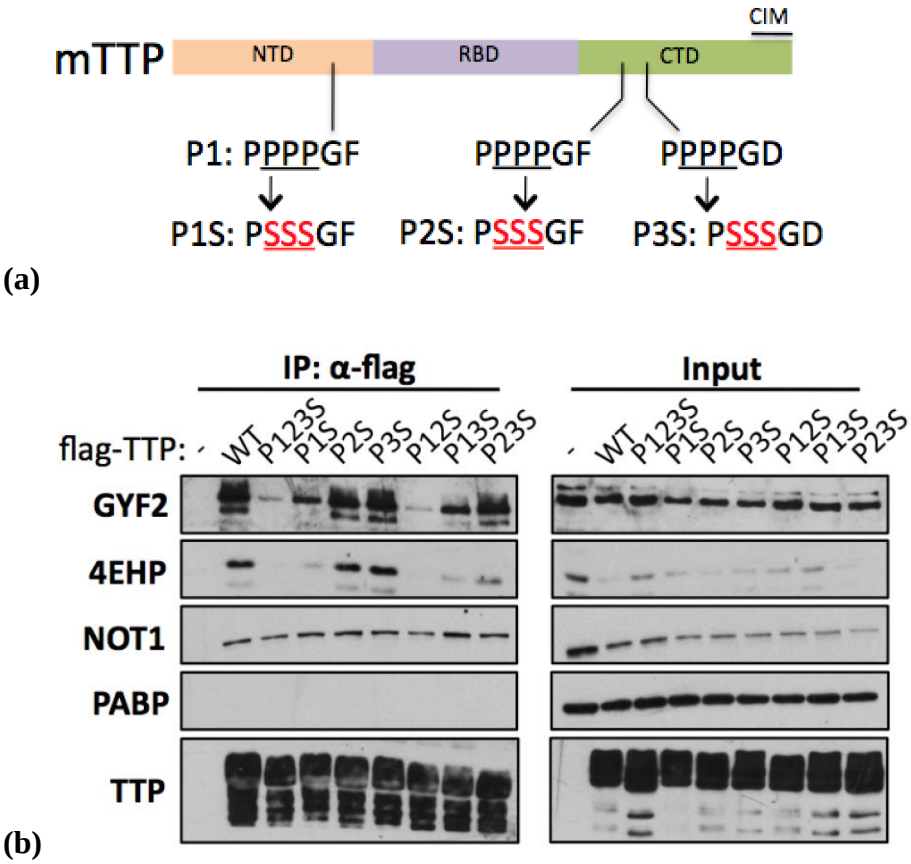
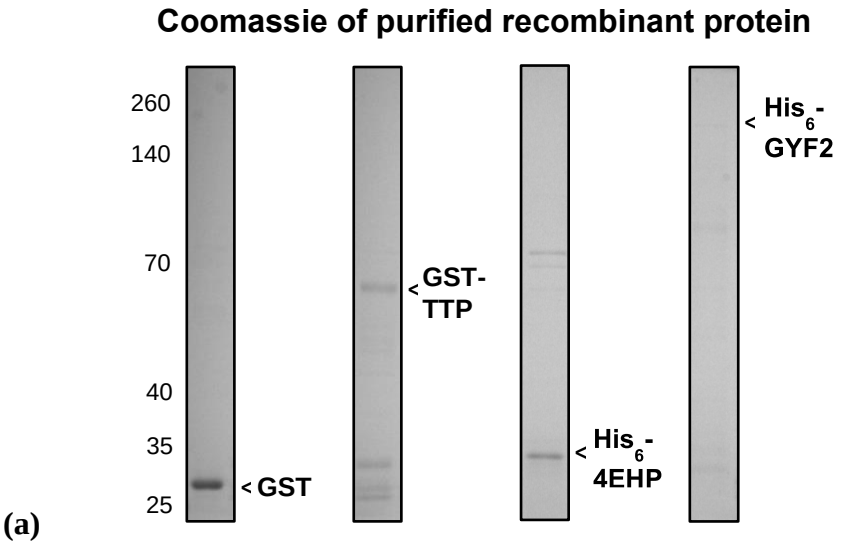


Figure 4. The first and second proline-rich regions of TTP interact with 4EHP-GYF2 complex. (a) Schematic diagram of full-length mouse TTP and its domains with proline to serine mutations made at the 1st (P1S), 2nd (P2S) and 3rd (P3S) proline-rich motifs, used in co-immunoprecipitation experiments. **(b)** Cell lysates treated with RNase were immunoprecipitated with α-flag antibody. Immunoblotting was performed with α-flag (TTP), α-GYF2, α-4EHP, α-CNOT1, and α-PABP.



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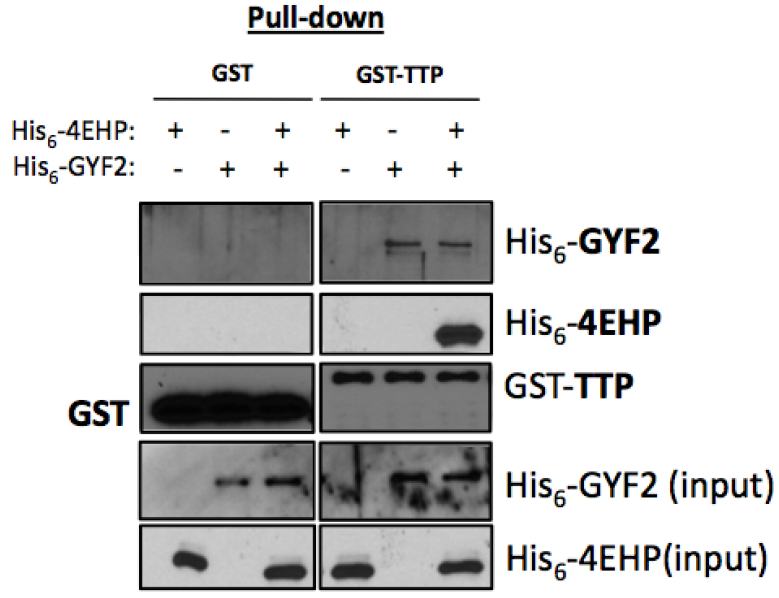
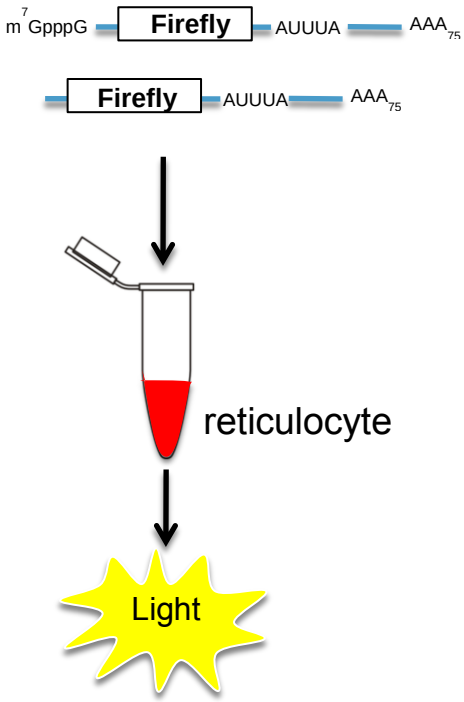


(b)

Figure 5. **TTP interacts with the 4EHP-GYF2 complex via a direct interaction with GYF2. (a)** Coomassie stained gel of recombinant proteins expressed and purified from E. *coli*. **(b)** Glutathione sepharose pull downs were performed to look at interaction of GST-TTP WT with His_6-4EHP and/or His_6-GYF2. His_6-4EHP and His_6-GYF2 were detected by anti-4EHP and anti-GYF2 antibodies respectively. GST-tagged proteins were detected by anti-GST.



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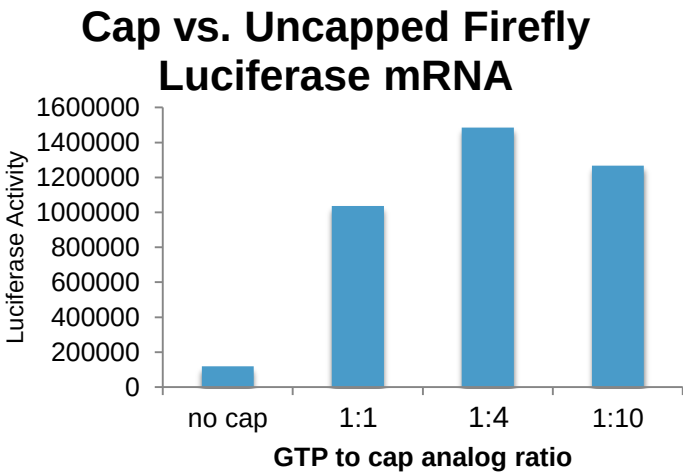
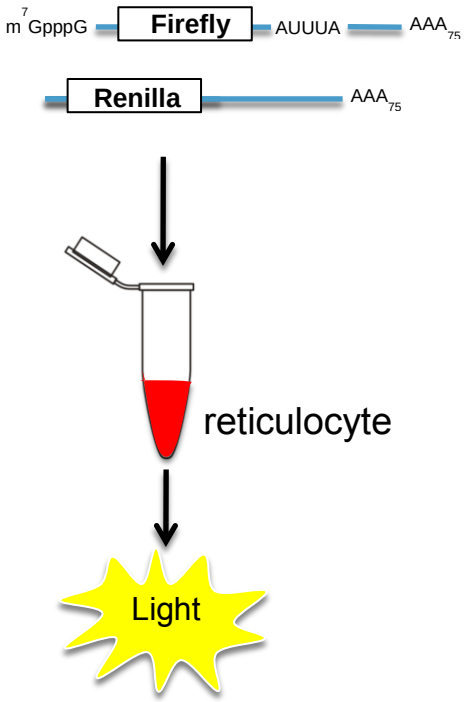


(b)

Figure 6. Rabbit reticulocyte lysate translation is cap-dependent. (a) Schematic of *in vitro* translation reaction performed **(b)** Varying ratios of GTP to anti-reverse cap analog were added in a transcription/capping reaction to generate m7GpppG capped F-Luc-ARE mRNA. Capped or uncapped F-Luc-ARE reporter mRNAs were then combined with RRL and allowed to translate for 1.5 hr at 30°C. Levels of firefly luciferase were determined using a luminescence based detection system.



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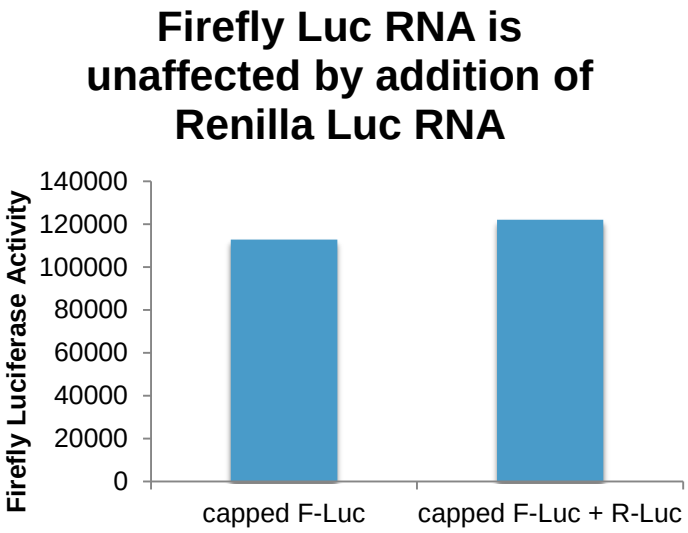
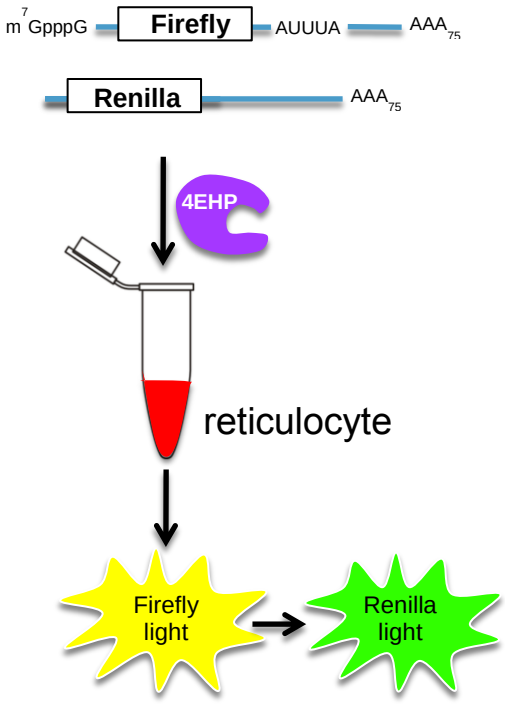


(b)

Figure 7. **F-Luc-ARE mRNA is unaffected by addition of uncapped R-Luc mRNA.** **(a)** Schematic of reaction performed. **(b)** Capped F-Luc-ARE alone or mixed with uncapped R-Luc reporter mRNAs were incubated with RRL and allowed to translate for 1.5 hr at 30C.



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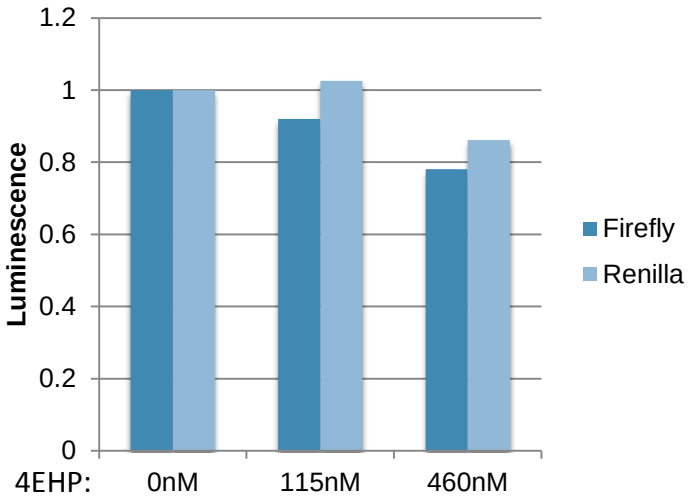
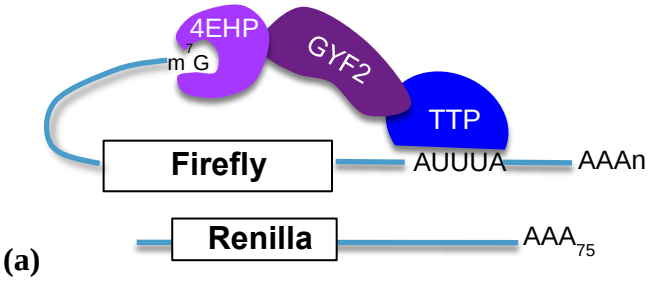
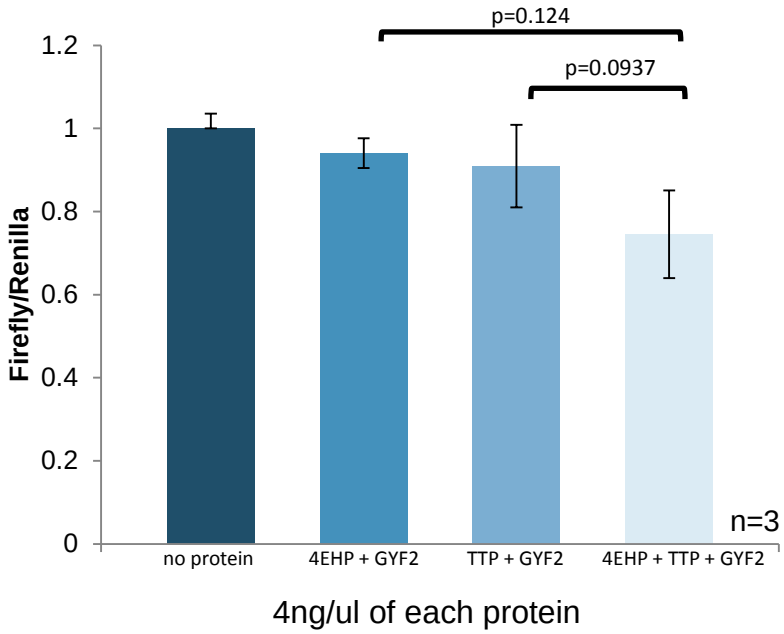


(b)

Figure 8. **4EHP modestly represses translation of capped transcripts**. 115nM of 4EHP specifically represses translation of capped F-Luc-ARE. **(a)** Schematic of reaction set-up. **(b)** RRL and capped F-Luc-ARE and uncapped R-Luc mRNA were combined with a titration of 4EHP protein. The final concentrations of 4EHP were 0, 115, or 460nM; n=1.

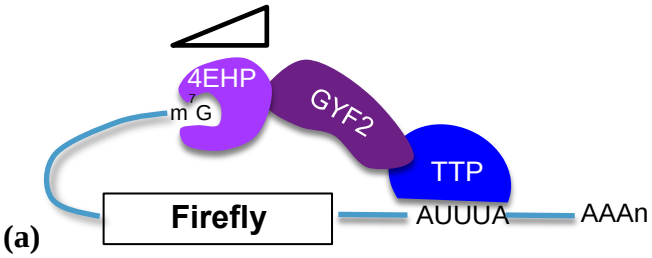


(a)

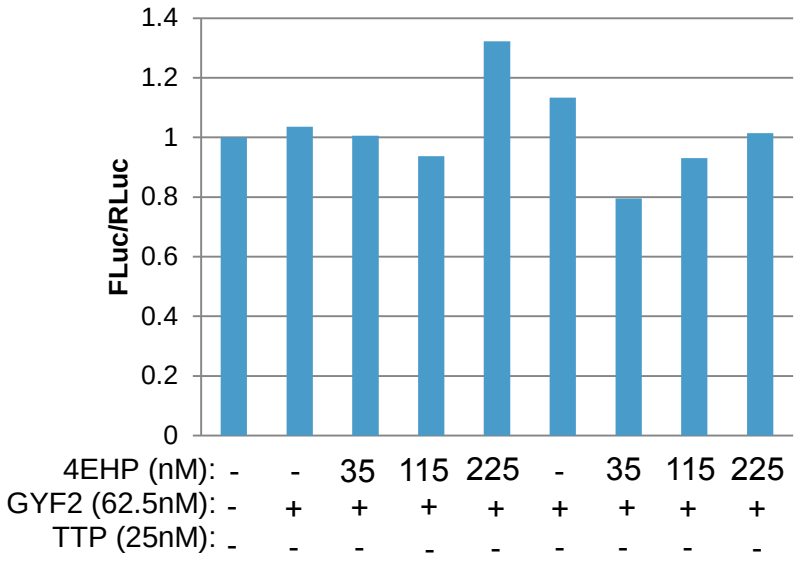


(b)

Figure 9. **4EHP-GYF2-TTP complex represses translation of ARE-containing reporter moderately. (a)** Schematic of reaction set-up. **(b)** F-Luc-ARE reporter mRNA was incubated with purified 4EHP, GYF2, and TTP in RRL and allowed to translate for 1.5 hr at 30°C. A R-Luc reporter lacking ARE-binding sites was included in every reaction as an internal control. All values were normalized to no protein.



(a)



(b)

Figure 10. Increasing molar amounts of TTP, GYF2, and 4EHP protein did not lead to further repression of translation. **(a)** Schematic of reaction set-up. **(b)** Increasing amounts of 4EHP protein were added to GYF2 with or without TTP along with F-Luc-ARE in RRL.

DISCUSSION

Post-transcriptional processes involved in gene expression regulation at the RNA level include mRNA turnover and cap-dependent translational activation and repression. TTP plays a large role in regulating the expression of many unstable mRNAs both at the level of mRNA turnover and translational repression. TTP's role and binding partners in ARE-mediated decay is well established (Lai et. al. 2000, Blackshear 2002, Lykke-Andersen 2005); however, TTP's role in translational repression is less understood. Recently, our lab observed TTP to associate with a translational repression complex 4EHP-GYF2. In this study, I performed experiments aimed at 1) characterizing the interaction of TTP to 4EHP-GYF2, and 2) elucidating the role 4EHP-GYF2 play in TTP directed translational repression. My results indicate GYF2 directly interacts with TTP, thereby bridging TTP to 4EHP. Co-IP analysis revealed the N-terminus of TTP to be needed for strong binding to the 4EHP-GYF2 complex. Further mutational analysis revealed the first and second PPPGF/L motifs in TTP are needed for binding 4EHP-GYF2, presumably through the GYF domain of GYF2.

We hypothesized that 4EHP and GYF2 play a role in TTP-dependent translational repression. To test this and to distinguish translational repression from mRNA decay, I performed *in vitro* translation experiments. Using a cell-free *in vitro* system has the advantages of simplifying an otherwise highly complex pathway seen in cells. Exact control of proteins and target RNA of interest can be added into the system with little concern of interfering pathways. Although much of our system was optimized for our particular proteins and RNA of interest, only moderate (~25%) repression of translation was observed. Many considerations may explain the modest effects observed. A chief

concern was the integrity of the purified proteins, mainly purified TTP. We mapped the key interacting motifs of GYF2 to the NTD of TTP, whereas the 2 zinc-fingers for binding RNA lie ~20 amino acids away (Fig, 2a). Assessing the purity of TTP via silver staining (data not shown) there appears to be a proportion of partially degraded TTP in the protein preparations. Since TTP must bind both RNA and GYF2 to carry out its predicted translation expression role, it is possible that 4EHP-GYF2 or RNA is sequestered away by fragments of TTP capable of binding either GYF2 or RNA, but not both.

Another concern for our recombinant TTP protein was its ability to bind our reporter mRNA in RRL. Although we increased TTP molarity so to saturate TTP-ARE binding, the K_d of TTP to GYF2 is unknown, and hence the concentration of proteins used in our system may have been suboptimal. TTP binds RNA in a zinc-dependent manner in cells (Blackshear, 2002). RRL is usually treated with EGTA to inhibit micrococcal nucleases, which may chelate the zinc ions. We speculated whether adding zinc would increase TTP's RNA-binding ability, but found no added effect on translation repression under these conditions (data not shown). There is also the possibility of other co-factors involved in enhancing repression by 4EHP-GYF2-TTP, that, when missing, leaves a mostly inactive complex.

Although modest effects were observed, they repeated over three experiments, suggesting repression is occurring. The next steps in further optimizing our system would be to establish the K_d for our novel protein complex and to determine optimal molar ratios of our proteins that show the greatest repression of ARE-containing mRNA. Much more optimizing is needed to draw any major conclusions on the role TTP-4EHP-GYF2

is playing on translational repression.

MATERIALS & METHODS

Plasmids

RAW 264.7 mouse macrophage cDNA was used as the template in a polymerase chain reaction (PCR) to amplify GYF2 and 4EHP's open reading frame. A pcDNA3-myc-his-mTTP vector was obtained from previous lab members. Subcloning of 4EHP, GYF2, and TTP and mutagenesis of TTP were performed using PCR. The PCR-amplified open reading frames, flanked by an XbaI site in the 5' and a NotI site in the 3', were subcloned into a pcDNA3-myc vector for TTP and a pcDNA-flag vector for 4EHP and GYF2. The single domain mutants of TTP were also inserted into pcNMS2-myc vector, which contains an upstream in-frame myc-tagged MS2 coat protein. GYF2-specific reverse transcription primers and colony PCR were used to obtain a positive clone of GYF2. For recombinat protein expression plasmids, TTP, GYF2, and 4EHP ORFs were placed into pET-His and pGTevH (GST-Tev cut site-Polylinker-His) plasmids using 5' SalI (TTP) or 5' BamHI (4EHP,GYF2) TTP and 3' NotI sites. Firefly luciferase or *Renilla* luciferase ORF were placed in a pcDNA3-myc WT and mut ARE-containing (GM-CSF) plasmid using a HindIII site.

Cell Culture, transfection, immunoprecipitation and immunoblotting

Plasmid transfections were carried out in HEK 293T cells using Transit 293 reagent in 10-cm plates. Cells were harvested 72 hours after transfection in hypotonic lysis buffer. RNase A-treated lysates were then incubated with

Sepharose preconjugated to anti-myc for 2 hours at 4C. Beads were washed 8 times with NET2 buffer before SDS Loading buffer was added to denature the proteins. For Western blotting, proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. For immunoblotting, membranes were incubated overnight with the following: mouse anti-myc, mouse anti-flag, mouse anti-GST, rabbit anti-TTP, rabbit anti-4EHP, rabbit anti-GYF2, rabbit anti-PABP, or rabbit anti-CNOT. This was followed by a 2- hour incubation with horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibodies at 1:20,000.

Recombinant Proteins

For expression of mouse GST-TTP, His-4EHP, and His-GYF2, appropriate plasmids were transformed into E. coli BL21(DE3) cells. Bacteria were grown in LB medium to OD 600nm of 0.5, and induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG). His-4EHP was induced for 16hr at 15°C in 0.1 mM IPTG. GST-TTP was induced for 3 hr at 25°C in 0.3 mM IPTG. GST-TTP P12S was induced for 16 hr at 15°C in 0.1 mM IPTG. His-GYF2 was purified under denaturing conditions (6M guanidine hydrochloride followed by 8M urea). Cells were harvested, resuspended in TKET buffer (His-tagged proteins contained 20 mM imidizole in lysis buffer) and disrupted by sonication (Misonix XL-2000). After centrifugation of the lysate (15,000g, 5 min), lysates containing GST-tagged proteins were passed over glutathione-sepharose columns 2 times and washed with TKET 3 times and eluted in 100 mM Tris-HCl pH7.5, 100 mM KCl, 20 mM glutathione, while lysates containing His-tagged protein were passed over

Ni-NTA column 2 times, washed with TKET with 20 mM imidazole 3 times and eluted in TKET and 200mM imidazole. Eluates for each respective protein were pooled and dialyzed against PBS over night at 4°C. Protein concentrations were determined using Bradford protein assay.

GST pull-down assay

5μg of the GST-tagged wild-type or mutant TTP or GST only protein were incubated with 25ul glutathione-sepharose 4B (MAKER) in 500 ul TKET buffer (10 mM Tris-HCl pH 7.5, 100 mM KCl, 0.1 mM EDTA, 0.05 % Triton X100) for 2 hrs at 4c. The samples were then spun down at 1000 rpm for 1 min and washed 3 times with TKET buffer. His-tagged wild type 4EHP and/or wild type GYF2 protein were then added to the beads previously incubated with GST-TTP or GST protein in 500 ul TKET for 2hrs at 4c. The samples were then spun down at 1000 rpm for 1 min and washed 4 times with 700ul TKET buffer. The proteins were eluted by boiling at 95°C in SDS loading buffer. Samples were then run on 9% SDS-PAGE and analyzed by western blot.

***In vitro* translation assay**

Commercially available nuclease treated rabbit reticulocyte lysate were used according to manufactures protocol (Promega). Rabbit reticulocyte lysate, 1mM amino Acid Mixture minus Leucine, 1 mM amino acid mixture minus methionine, 40U RNaseOUT, 50ng or 10ng reporter mRNA, recombinant protein at varying concentrations, and RNase-free water were mixed and incubated at 30°C for 1.5 hr. A 3ul sample from the final translation mix was removed and placed in a plate reader. The

luciferase activity, defined as recorded luminescence units (RLU), was measured from the individual wells using the luciferase assay substrate (Dual-Luciferase Reporter Assay System (Promega).

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