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The GCN2-ATF4 signaling pathway activates 4E-BP to bias mRNA translation and boost antimicrobial peptide synthesis in response to bacterial infection

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Summary

Bacterial infection often leads to suppression of mRNA translation, but hosts are nonetheless able to express immune response genes through as yet unknown mechanisms. Here, we use a *Drosophila* model to demonstrate that antimicrobial peptide (AMP) production during infection is paradoxically stimulated by the inhibitor of cap-dependent translation, 4E-BP (encoded by the *Thor* gene). We found that *4E-BP* is induced upon infection with pathogenic bacteria by the stress-response transcription factor, ATF4, and its upstream kinase, GCN2. Loss of *gcn2, atf4* or *4e-bp* compromised immunity. While *AMP* transcription is unaffected in *4e-bp* mutants, AMP protein levels are substantially reduced. The 5' untranslated regions (UTRs) of AMPs score positive in cap-independent translation assays, and this cap-independent activity is enhanced by 4E-BP. These results are corroborated *in vivo* using transgenic 5'UTR reporters. These observations indicate that ATF4-induced *4e-bp* contributes to innate immunity by biasing mRNA translation toward cap-independent mechanisms, thus enhancing AMP synthesis.

eTOC blurb

The cap-dependent translation inhibitor, 4E-BP, is induced during bacterial infection in *Drosophila* and animals lacking 4E-BP are immunocompromised. How an effective innate immune response is mounted in a translation suppressive environment, and why 4E-BP is required in this process was unknown. Vasudevan *et al* demonstrate that antimicrobial peptides, which are key components of

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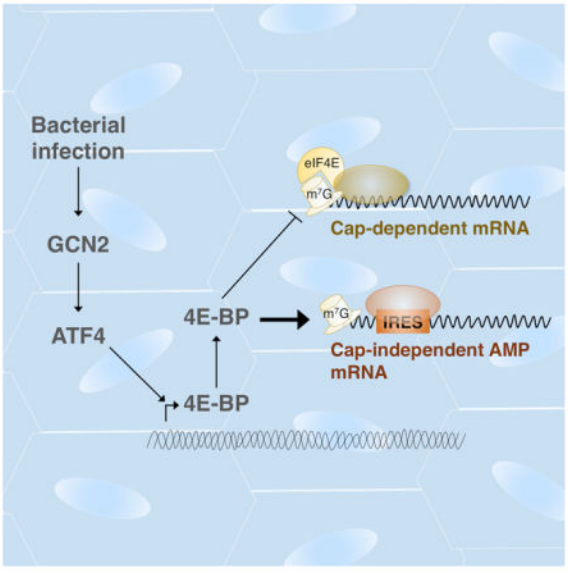
the *Drosophila* innate immune response, are translated cap-independently via IRES-like elements in their 5'UTRs and that their translation is enhanced by 4E-BP-mediated suppression of cap-dependent translation.



Introduction

Various types of stresses dampen protein synthesis by reducing the availability of critical components of translation initiation. One such example is mediated by stress-activated kinases that phospho-inhibit eIF2α, which is part of the ternary complex (eIF2-GTP-Met-tRNAi^{Met}) that delivers initiator methionine to the translation preinitiation complex. Remarkably, even when translation initiation is inhibited by phosphorylation of eIF2α, a subset of transcripts containing overlapping upstream ORFs (uORFs) in their 5'UTR, such as ATF4 (encoded by *cryptocephal* or *crc* in *Drosophila)*, is favorably synthesized. ATF4 responds to cellular stress by transcriptionally inducing various stress responsive transcripts. As metazoans have multiple eIF2α kinases that active this pathway, these signaling pathways are often collectively referred to as the 'integrated stress response' (ISR)(Harding et al., 2003; Ron and Walter, 2007).

Translational inhibition mechanisms associated with viral infection were first observed over fifty years ago in ascites-tumor cells infected with encephalomyocarditis virus and since then this phenomenon has been extended to most viral infections (Mohr and Sonenberg, 2012). Such translational inhibition is mediated in part by PKR, one of the four eIF2α kinases in mammals that are activated by dsRNAs. While not as well recognized, literature reports that pathogenic bacterial infection also causes translation inhibition in various infection models from *C. elegans* to mammals (reviewed comprehensively in (Lemaitre and Girardin, 2013)). In *Drosophila,* GCN2, an eIF2α kinase that responds to amino acid deprivation, triggers an mRNA translational block in the host when infected with *Pseudomonas entomophila* (*P.e)*. Other studies have reported the induction of the cap-dependent translational inhibitor *4e-bp* (*Thor* in *Drosophila)* in response to bacterial

pathogens (Bernal and Kimbrell, 2000; Chakrabarti et al., 2012; Rodriguez et al., 1996). Inhibition of translation initiation by 4E-BP targets at the 5'-cap of eukaryotic mRNAs, where the 7-methylguanosine moiety is recognized by eIF4E (as part of the eIF4F complex) to recruit other translation initiation factors, and eventually the 40S ribosomal subunit. 4E-BP, or eIF4E-binding protein, imposes translation inhibition by binding to eukaryotic initiation factor 4E (eIF4E), thereby specifically inhibiting cap-dependent translation. As *4e-bp* mutant flies are immune compromised (Bernal and Kimbrell, 2000; Rodriguez et al., 1996), it is thought that induction of *4e-bp* in response to bacterial infection is a host adaptation mechanism. The hypothesis that translation inhibition contributes to a host defense mechanism raises two important questions: First, how does translational inhibition aid the host in mounting an immune response that relies on antimicrobial peptide (AMP) expression? Second, what signaling pathways mediate *4e-bp* induction in response to bacterial infection? Here, we identify the GCN2-ATF4 signaling as the mediator of *4e-bp* induction in *Drosophila* during infection. We also show that 4E-BP paradoxically stimulates AMP synthesis as part of the innate immune response to bacterial infection in *Drosophila*. In a broader sense, we demonstrate a mechanism that explains one of the long-standing issues of how *Drosophila* AMPs can be expressed under conditions of translational inhibition.

Results

4e-bp is transcriptionally induced by ATF4 during infection in the fat body and gut

Independent studies have established that bacterial and fungal infection induce *4e-bp* transcription in *Drosophila* (Bernal and Kimbrell, 2000; Levitin et al., 2007). However, the signaling pathway(s) responsible for this induction remains unknown. There are two known transcriptional regulators of *4e-bp*, the best characterized of which is the stress-responsive transcription factor, FOXO (Jünger et al., 2003; Puig et al., 2003). Recently, we reported that the eIF2α-kinase-responsive transcription factor, ATF4, regulates *4e-bp* transcription in *Drosophila* through an intronic element in the *4e-bp* locus (Kang et al., 2017; Yamaguchi et al., 2008). Based on this regulation, we developed the 4E-BP^{intron}-dsRed reporter that faithfully reports ATF4 activity in *Drosophila* tissues (Kang et al., 2017) and this led us to examine the possible role of eIf2α kinases in *4e-bp* induction during infection. We specifically tested if ATF4 is active in the context of infection, by orally infecting 3rd instar larvae with the non-lethal gram-negative bacterial pathogen, *Ecc15*. In response to infection, dsRed expression was elevated in the larval gut (Fig. 1A', B') and also in the fat body (Fig. 1C', D'), which is known to be an auxiliary immune-response tissue. We verified by qPCR that 4E-BP^{intron}-dsRed induction reflected induction of *4E-BP* itself, and found that *4e-bp* mRNA was upregulated in response to *Ecc15* infection (Fig. 1E), as reported previously (Bernal and Kimbrell, 2000). This induction was suppressed in the background of the homozygous *atf4* hypomorphic mutant, *crc*^{1} (Fig. 1E) indicating that ATF4 mediates *4e-bp* induction during infection. *4e-bp* induction was also suppressed in *foxo* mutants (Fig. 1E), indicating that multiple transcription factors can regulate 4E-BP induction during infection.

4e-bp mutants were previously shown to be immune-compromised. Since we identified ATF4 as a regulator of *4e-bp* expression during infection, we predicted that *atf4* mutants would be similarly immune-compromised. We tested this using systemic pathogen load

assays to measure *Ecc15* levels in the larvae after infection. When compared to isogenic controls, crc^{1} homozygotic mutants had higher systemic pathogen load (Fig. 1F). Consistent with previously published data, homozygous null *4e-bp* mutants, $Thor^{2}$, were similarly immune compromised when compared to its respective isogenic control, $Thor^{rev}$ (see materials and methods for details).

GCN2 signaling primarily activates ATF4-mediated 4e-bp induction in the fat body

Upon further examination of the transcriptional induction of *4e-bp* post-infection, we see increased *4e-bp* transcripts both in the fat body and in the gut (Fig. S1). Since the fat body is the primary site of AMP synthesis in response to infection (Imler, 2014), we sought to examine the signaling pathways upstream of *4e-bp* in this tissue. ATF4 can be activated by either of two known eIF2α-kinases in *Drosophila*: the ER stress-responsive kinase PERK, or the amino acid deprivation activated kinase GCN2. To determine which of these two kinases lies upstream of ATF4 during infection, we knocked down *gcn2* and *perk* with the fat body-specific *Dcg-Gal4* driver. Depletion of *gcn2*, but not *perk*, in the fat body resulted in an increased systemic pathogen load upon *Ecc15* infection (Fig. 1G). While depletion of *gcn2* in the fat body led to near complete suppression of *4e-bp* induction during *Ecc15* infection, depletion of *perk* also resulted in a reduction of *4e-bp,* albeit to a lesser extent (Fig. 1H). To further examine the role of the kinases, we tested the effect of *gcn2* and *perk* depletion in the fat body 4E-BP^{intron}-dsRed induction during infection. We observed that knockdown of *gcn2* in the fat body had a stronger effect on 4E-BP^{intron}-dsRed than that caused by the knockdown of *perk* (Fig S1B). Together these data suggest that GCN2 is the major modulator of ATF4 in the context of *4e-bp* induction by pathogenic bacterial infection, with a minor role for PERK. Notably, AMP mRNA levels were also induced when cultured *Drosophila* S2 cells were deprived of amino acids (Fig. S1C). However, this is likely to be mediated through an ATF4-independent mechanism as Tunicamycin treatment, which also stimulates ATF4 induction, did not induce AMP mRNAs (Fig. S1D).

We next examined if ATF4-mediated *4e-bp* induction occurs in response to infection by other bacterial strains. Several *Drosophila* pathogens were tested, including gram-positive bacteria (*Enterococcus faecalis*, *Micrococcus luteus*) and other gram-negative bacteria (*Serratia marcescens*, *Pseudomonas aeruginosa*, *Pseudomonas entomophila*, *Providencia rettgrei*). We observed *4e-bp* induction with all pathogens we tested, albeit to different extents (Fig. 1I). Knockdown of *atf4* with a fat body specific driver, *Dcg-Gal4*, resulted in suppression of *4e-bp* induction with all pathogens tested (Fig. 1I), suggesting that ATF4/4E-BP axis activation occurs as part of a general immune response to pathogenic bacteria.

Levels of AMP proteins, but not transcripts, are reduced in 4e-bp mutants

To gain insight into why *4e-bp* mutants were immune-compromised, we asked whether 4E-BP regulated the canonical innate immune response pathways. In response to infection, two branches of the NF-κB signaling involving the Toll and immune deficiency (IMD) receptors are activated and initiate a signaling cascade which culminates in the induction of transcripts encoding *AMPs* (Buchon et al., 2014). AMPs are required for combating pathogen load with different classes of AMPs targeting different aspects of pathogen physiology. We examined whether 4E-BP affected the Toll or IMD signaling by using *AMP* transcriptional induction

upon infection as readouts for pathway activity. We observed that the transcriptional induction of *AMPs* such as *Drosomycin* (downstream of Toll), *Diptericin A* and *Attacins* (downstream of IMD) is unaffected in $Thor^{2}$ homozygotic mutants (Fig. 2A). Therefore, we concluded that 4E-BP is not required for signaling events leading to *AMP* transcriptional induction upon infection.

However, western blotting of hemolymph collected from infected and uninfected $Thor^{2}$ homozygotic larvae showed a significant reduction in the protein levels of Attacins in comparison to $Thor^{rev}$ control larvae. (Fig. 2B, B', S2A). Consistent with data in Figure 1, knockdown of *gcn2* and *atf4* in the fat body also resulted in reduced attacin protein levels in the hemolymph (Fig. 2C). This was corroborated by mass spectrometric analysis of hemolymph from $Thor^{2}$ larvae, which indicated a reduction in the levels of other AMPs such as Drosocin, Diptericin B and Metchnikowin, and other *Drosophila* immune molecules (DIMs) (Fig. 2D, S2D, D') when normalized to larval serum protein. Thus, *4e-bp* mutant animals display normal induction of AMP mRNA but lower levels of AMP proteins. As these data could not rule out the alternative possibility that reduced Attacin levels were due to proteasomal degradation in $Thor^{2}$ mutants, we fed larvae with 10 μM of a proteasomal inhibitor, bortezomib (Fig. S2B), a condition that readily activates the proteasome inhibition response in flies (Tsakiri et al., 2013). These conditions did not restore the reduced Attacin levels in the hemolymph (Fig. S2C). Together, these data suggested that *4e-bp* mutants were immune-compromised because of reduced AMP synthesis.

The 5'UTRs of Drosomycin, Attacin A and 4e-bp support cap-independent translation

Since 4E-BP is an inhibitor of cap-dependent translation, we expected loss of *4e-bp* to enhance cap-dependent translation but our data shows that AMP synthesis was reduced under these conditions. Moreover, the transcriptional induction of *4e-bp* during infection suggests that AMP mRNAs are capable of being translated in the presence of *4e-bp.* These observations prompted us to examine the possibility that AMPs are translated through an eIF4E-independent mechanism. There are several known mechanisms of translation that can bypass eIF4E requirement. One is through the presence of an internal ribosomal entry site (IRES) element in the 5'UTR of mRNAs (Komar and Hatzoglou, 2011), which allow those transcripts to recruit the translation machinery independent of eIF4E and thus can bypass translational regulation by 4E-BP (Marr et al., 2007). To test the 5'UTRs of the *AMPs* for possible IRES elements we performed bicistronic assays in *Drosophila* S2 cells using Renilla and Firefly luciferase reporters (schematic, Fig. 3A). These two reporters are transcribed as a single mRNA, where translation of Renilla luciferase is dependent on cap-recognition, but Firefly luciferase translation is dependent on the intervening sequence being able to recruit ribosomes independent of cap-recognition (schematic, Fig. 3A). We used the Hepatitis C virus (HCV) IRES as a positive control (Fig. 3A). We did not see a significant change in the Renilla luciferase activities of the reporters tested, thus the ratio of Firefly to Renilla luciferase activity represents the ability of the 5'UTRs tested to support cap-independent translation. S2 cells expressing bicistronic reporters with the 5'UTRs of *Drosomycin* and *Attacin A* inserted in the forward orientation allowed the translation of the Firefly luciferase (Fig. 3A). Interestingly, we also found that the 5'UTR of *4e-bp* itself

scored positively in the bicistronic assay (Fig. 3A). Surprisingly, the cap-independent activities of these 5'UTRs were higher than that of the well-characterized HCV IRES.

To further validate the 5' cap-requirement of the *AMP* transcripts we performed *in vitro* cap competition assays. We measured translation of 5'-capped, monocistronic Firefly luciferase reporter mRNA (schematic, Fig. 3B) *in vitro* using rabbit reticulocyte lysates (RRL) in the presence of excess cap analog (m^7GpppG). Excess cap compound competes with cap-dependent transcripts for eIF4E complexes but should not affect cap-independent transcripts. While translation of the control luciferase reporter mRNA was negatively affected by the addition of excess m^7GpppG, reporter transcripts containing the 5'UTR *of Drosomycin, Attacin A* and *4e-bp* were indifferent to the presence of the excess m^7G (Fig. 3B). We also performed these assays in RRL treated with micrococcal nuclease, to eliminate effects of competing mRNAs (Fig. 3C). *Drosomycin* and *4e-bp* remain resistant to excess cap while the *Attacin A* 5'UTR reporter showed a small but significant decrease in translation. Nevertheless, the *Attacin A* 5'UTR reporter's resistance to excess cap remained higher than the control mRNA (Fig. 3C). To further investigate the requirement for an m7G cap we performed monocistronic assays with uncapped mRNA. We observed that the 5'UTRs of *Drosomycin*, *Attacin A* and *4e-bp* supported translation with similar efficiencies, retaining roughly 75% activity without a cap. By contrast the control transcripts retained only 30% activity when uncapped (Fig. 3D). Together these data support the idea that certain AMP transcripts can be translated independent of eIF4E.

Cap-independent translation bias imposed by 4E-BP drives AMP synthesis

Data from Fig. 2 showing that AMP protein levels are reduced in *4e-bp* mutants, and Fig. 3 showing that *AMPs* can be translated cap-independently together suggest a role for 4E-BP in biasing cellular translation to cap-independent mechanisms. To test the idea that 4E-BP is required for favoring cap-independent translation of AMPs during infection, we examined how its expression affected translation of the bicistronic reporter in S2 cells (Fig. 3A). We used a constitutively active mutant of *4e-bp*, 4E-BP^{LLAA}, which cannot be inactivated by the 4E-BP kinase TOR, and binds tightly to eIF4E therefore inhibiting eIF4E consistently when overexpressed (Miron et al., 2003). The cap-independent translation of Firefly luciferase in bicistronic reporters (schematic, Fig 4A) containing the 5'UTRs of *Drosomycin, Attacin A* and *4e-bp* was enhanced over 5, 4 and 2 fold respectively in the presence of 4E-BP^{LLAA} in comparison to cells expressing GFP as a control (Fig. 4A). Strikingly, the enhancement of AMP translation in the presence of 4E-BP^{LLAA} exceeded that seen with the HCV IRES (over 2 fold). Since in the context of infection, we saw that *4e-bp* is activated by GCN2/ATF4, we asked whether GCN2 activation by amino-acid deprivation was sufficient to enhance cap-independent activity of *AMP* 5'UTRs. We tested this using a second bicistronic reporter with GFP reporting cap-dependent translation and dsRed reporting cap-independent translation (schematic, Fig 4B). S2 cells expressing the *Drosomycin* 5'UTR bicistronic reporter in the forward orientation showed an enhanced dsRed expression when subjected to amino acid deprivation to activate GCN2 (Fig. 4A). While the *Attacin A* 5'UTR supported cap-independent translation of dsRed, this was not enhanced significantly upon GCN2 activation. *Drosomycin* and *Attacin A* 5'UTRs in the reverse orientation did not yield cap-independent translation. GFP levels remained unchanged with both reporters, most likely

due to the persistence of GFP that was synthesized prior to the relatively short amino acid deprivation treatment (4 h). To eliminate the possibility that the reporter may be transcriptionally regulated due to any cryptic promoter activity in the 5'UTR, we measured the ratio of *dsRed* mRNA to *GFP* mRNA in amino acid deprived S2 cells expressing the fluorescent bicistronic reporters (Fig. 4C). We saw no change in relative levels of *GFP* and *dsRed* qPCR signals suggesting that there is no cryptic promoter element that may respond to amino acid deprivation. We also did not detect any change in the transcript levels of the bicistronic reporter itself upon starvation (Fig. 4C). Consistent with previous observations, we saw an induction of *4e-bp* in response to amino acid deprivation (Fig. 4C).

To test the cap-independent features of the AMP 5'UTRs *in vivo*, we generated transgenic flies bearing monocistronic reporters (schematic, Fig. 4D). These reporters utilized the *Drosomycin* promoter ($Drom^P$) to drive the expression of Drosomycin-GFP bearing either the *Drosomycin* 5'UTR or *Tubulin* 5'UTR as a negative control. To allow for a fair comparison of their expression, the two reporters were targeted into the same attP landing site in the genome. As expected, both versions of the reporter were transcriptionally induced in response to *Ecc15* infection, since the *Drosomycin* promoter is driving their expression (Fig. S3A). We then tested the expression of these two reporters in control and infected larva by western blotting for GFP to detect the Drosomycin-GFP fusion protein in the hemolymph. Our data showed that while the level of Drosomycin-GFP bearing the *Drosomycin* 5'UTR increased substantially in response to infection, the reporter bearing the *Tubulin* 5'UTR did not show a significant increase (Fig. 4D). Normalizing the GFP protein levels for each reporter to the respective GFP mRNA levels indicates that while the *Drosomycin* 5'UTR reporter sees a substantial increase in translation upon infection, the translation of the *Tubulin* 5'UTR reporter is relatively suppressed (Fig 4D'). Taken together, these data show that increased 4E-BP levels during infection result in preferential translation of cap-independent transcripts, such as Drosomycin, over cap-dependent transcripts.

Discussion

Much research has focused on the transcriptional response to infection mediated by the innate immune response pathways (Buchon et al., 2014). Although translational inhibitors such as GCN2 and 4E-BP have been implicated in the antibacterial response, the incongruence of translation inhibition and the need for AMP synthesis had not been addressed experimentally. Here, we provide results that resolve this discrepancy and establish that *AMPs* have evolved mechanisms to bypass translation inhibition imposed by pathogenic bacteria via 4E-BP activation. We show that the ISR pathway promotes innate immunity against pathogenic bacteria by mediating *4e-bp* induction, which in turn biases cellular translation towards cap-independent mechanisms that favor AMP synthesis. The newly identified role of the ISR pathway in the innate immune response is likely coordinated with the established roles of other innate immune response pathways, such as those mediated by FOXO, IMD and Dorsal/Dif.

In addition to regulation by GCN2/ATF4 and FOXO, 4E-BP is also famously regulated post-translationally by another amino-acid sensitive kinase, TOR. While under steady state conditions, TOR phospho-inactivates 4E-BP, TOR itself is inactivated in response to amino

acid deprivation. Thus 4E-BP newly synthesized in response to infection-mediated amino acid deprivation will likely not be subject to inactivation by TOR.

Our observations are consistent with the positive effects of *4e-bp* induction against a non-lethal pathogen such as *Ecc15* (Bernal and Kimbrell, 2000). However, infection by a severe pathogen such as *P.e.* results in a GCN2-mediated translation block in the gut and is detrimental to the host immune response (Chakrabarti et al., 2012). Why GCN2 mediates such different outcomes remains unresolved, but it could be due to factors other than ATF4 that are downstream of GCN2.

It is worth noting that GCN2 engages two different translation inhibition mechanisms: 1) phospho-eIF2α, which induces ATF4 and, 2) *4e-bp*, which is transcriptionally induced by ATF4 (Kang et al., 2017; Yamaguchi et al., 2008). We primarily focused on 4E-BP because the effect of phospho-eIF2α on translation may be temporary since ATF4 induces the expression of an eIF2α phosphatase subunit, *GADD34,* thereby stimulating eIF2α dephosphorylation. We speculate that eIF2α phosphorylation may not serve as a long-term deterrent for *AMP* translation, while the second translational block by 4E-BP may persist longer. In addition, our data shows that *4e-bp* itself is synthesized favorably by cap-independent translation conferred by its 5'UTR (Fig. 3, 4A), suggesting that this inhibition mechanism is self-sustaining.

Intriguingly, the 5'UTRs of *Drosomycin* and *Attacin A,* which confer cap-independent translation capabilities to their respective mRNA (Fig. 3, 4A), are relatively small at 63 and 29 bases respectively. To the best of our knowledge, these are the smallest characterized cap-independent translation elements known. While there are no known consensus sequences for IRESes, scoring positively in biochemical assays such as bicistronic assays and cap competition assays has been recognized to be a reliable indicator for them (Dever et al., 1992; Young et al., 2015; Zhou et al., 2008). While both *Drosomycin* and *Attacin A* are likely translated cap-independently via IRES-like elements in their 5'UTRs, they may utilize slightly different mechanisms. Though both 5'UTRs confer cap-independence which is enhanced by 4E-BP (Fig. 3, 4A, B), the *Attacin A* 5'UTR reporter shows basal translation of the reporter even in the absence of 4E-BP while the *Drosomycin* 5'UTR relies heavily on 4E-BP (Fig 4B). Additionally, eliminating competing transcripts in the presence of excess m^7GpppG also had a small but significant effect on *Attacin A* 5'UTR reporter mRNA (Fig 3B, C), suggesting that there is an additional layer of regulatory mechanisms acting on these 5' UTRs.

Since suppression of cap-dependent translation by 4E-BP appears to stimulate the synthesis of AMPs (Fig. 4), we speculate that the *AMP* 5'UTRs do not compete well with other mRNAs for ribosomes and initiation factors. Thus, in addition to the established role of 4E-BP in the inhibition of cap-dependent translation, our data shows a more nuanced role for 4E-BP as a promoter of cap-independent translation required for driving the synthesis of essential immune response proteins. Interestingly, it had been previously suggested that eIF2α-kinases such as GCN2 could promote cap-independent translation (Fernandez et al., 2001, 2002), although the effectors of such regulation were unknown. Based on our work here showing that 4E-BP downstream of GCN2/ATF4 signaling regulates AMP translation,

we can now surmise that these previously observed instances of cap-independent translation downstream of phospho-eIF2α are also likely mediated by ATF4-induced *4E-BP*. Additionally, given the conservation of translation inhibition during infection in mammals, it would be interesting to examine whether the mammalian innate immune response is aided by any of the three known mammalian 4E-BPs and if first-response cytokines are synthesized cap-independently.

Methods

Fly stocks and S2 cell culture

All *Drosophila* stocks were reared on standard cornmeal medium at room temperature. Stocks used are listed in Table S1.

S2 cells were grown on standard Schneider's medium (Life Technologies) supplemented with 10% FBS and 1% penicillin/streptomycin. For luciferase reporter assays, DNA was transfected at a 5:1 ratio of expression plasmid to reporter plasmid using PEI or Effectene (Qiagen).

Larval infection

Bacteria were grown in LB broth in an overnight culture and pelleted. All bacteria tested were grown at 37°C except *Ecc15* and *S.marcescens*, which were grown at 25°C. 4-day old larvae orally infected with food mixed 5:1 by weight with the bacterial pellet for 4 hours.

Systemic pathogen load

After infection, the larvae were briefly washed in 70% ethanol to surface sterilize them. 3–4 larvae per condition were homogenized using a pestle in 100 μl of PBS and serially diluted homogenate was plated on LB agar. Colonies were counted from the same dilution for different conditions and genotypes and normalized to the control.

Immunofluorescence (IF) and Western blotting (WB)

Larval guts and fat body were dissected in PBS and fixed in 4% PFA, PBT (0.1% Tween) for 20 minutes. The tissues were then washed 3x in PBT and stained with respective antibodies. Samples were imaged using a LSM700 Zeiss microscope at 20X magnification unless otherwise specified. Details for antibodies can be found in Supplemental Information.

Hemolymph collection for WB and mass spectrometry (MS)

Hemolymph from larvae was collected by making a small incision in the cuticle and bleeding them into PBS. For WB, hemolymph from 10–20 larvae was mixed with sample buffer and analyzed by SDS-PAGE. For mass spectrometry, hemolymph samples were prepared from 75 larvae (see Supplemental Information for details) and the peptide spectral matches (PSM) for each AMP was normalized to the PSM for controls used in Fig. 2D, S2D and S2D'.

Bicistronic assays

Molecular cloning details for reporters and assay buffer composition can be found in the Supplemental Information. 36 hours after transfection with the luciferase bicistronic reporter, cells were lysed in passive lysis buffer (Promega). Firefly expression was measured in FF assay buffer. Renilla expression was measured by addition of an equal volume of Ren assay buffer.

For experiments in Fig. 4A, cells were co-transfected with an expression construct driven by the mini-Actin5C promoter driving either GFP or 4E-BP^{LLAA} either (pMA6-GFP or pMA6-4E-BP^{LLAA}) cells were harvested for luciferase assays as described above. For experiments in Fig 4B, cells transfected with the fluorescent bicistronic reporter were starved using amino acid-free Schneider's growth medium (US Biologicals) and harvested in lysis buffer containing 1% NP40, 1x TBS, protease inhibitor cocktail (Roche), and subjected to western blotting.

In vitro Transcription and mRNA reporter preparation—Transcription templates for monocistronic reporters were created by PCR with a template specific forward primer containing either a T7 or T3 promoter sequence and a vector specific reverse primer. Templates were transcribed using either T7 or T3 RNA polymerase and purified using LiCl precipitation. Transcripts were capped using Vaccinia Virus Capping enzyme (New England Biolabs) and purified by phenol/chloroform extraction and isopropanol precipitation. Transcripts were poly(A) tailed using *E.coli* poly(A)polymerase (New England Biolabs) and purified by phenol/chloroform extraction and isopropanol precipitation.

In vitro translation assays

Translation assays were performed in rabbit reticulocyte Lysate (Green Hectares, McFarland, WI) (treated with Micrococcal nuclease for experiments in 3C, D) and mRNA reporter prepared as described above (see Supplemental Information for buffer composition details). Translation reactions were incubated at 37°C for 30 min and luc iferase activity was measured as described above. For experiments with excess m^{7}G cap, a cap structure analogue (New England Biolabs, #S1407S) was added to a final concentration of 1 mM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- 4E-BP is transcriptionally induced by GCN2-ATF4 signaling in response to bacterial infection
- 4E-BP mutants show unaltered antimicrobial peptide (AMP) transcript levels, but have reduced AMP translation
- AMP 5’UTRs support cap-independent translation
- Translation bias by 4E-BP drives cap-independent AMP translation

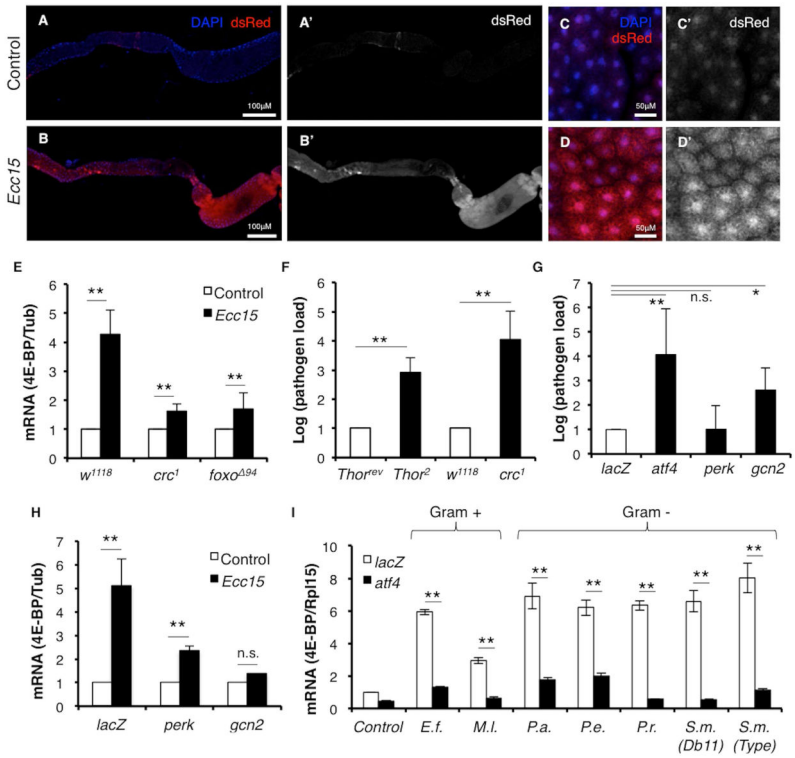

Fig. 1. GCN2-ATF4 signaling induces 4E-BP during infection

1A–D. 4E-BP^{intron}-dsRed expression in uninfected larva (A, A', C, C') and in larva infected with *Ecc15* (B, B', D, D'). Shown are the gut (A, A', B, B') and the fat body (C, C', D, D'). A', B', C', D' are dsRed channel only images.

1E. qPCR analysis of *4E-BP* expression from control larvae (w^{1118}), *atf4* mutants (crc^{1} homozygotes) and *foxo* mutants ($foxo^{\Delta 94}$ homozygotes) infected with *Ecc15*.

1F. Systemic pathogen load assay measuring *Ecc15* levels in *4e-bp* mutants ($Thor^{2}$ homozygotes) with *4e-bp* revertants ($Thor^{rev}$) as control, and in homozygotic crc^{1} with w^{1118} as control.

1G. Systemic pathogen load assay on larvae expressing $UAS\text{-}lacZ^{RNAi}$ (negative control), -$atf4^{RNAi}$, -$perk^{RNAi}$, -$gcn2^{RNAi}$ driven by a fat body specific driver (*Dcg-Gal4*).

1H. qPCR analysis of *4E-BP* induction in larvae expressing $UAS\text{-}lacZ^{RNAi}$ or –$gcn2^{RNAi}$ driven by *Dcg-Gal4*.

1G. qPCR analysis of *4E-BP* induction in larvae expressing $UAS\text{-}lacZ^{RNAi}$ or $atf4^{RNAi}$ driven by *Dcg-Gal4* infected with various gram-positive and -negative pathogens. (*E.f.* = *Enterococcus faecalis, M.l.* = *Micrococcus luteus, S.m.* = *Serratia marcescens, P.a.* = *Pseudomonas aeruginosa, P.e.* = *Pseudomonas entomophila, P.r.* = *Providencia rettgrei*). Error bars indicate standard error from at least 3 independent experiments. *=p<0.05, **=p<.01, n.s.=not significant.

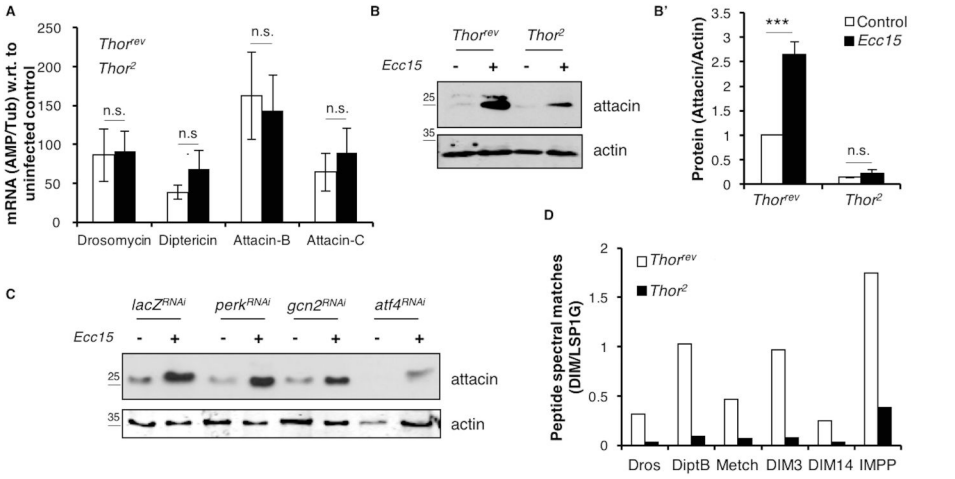

Fig. 2. Levels of AMP protein, but not transcripts, are negatively affected in 4E-BP mutants

2A. qPCR analysis of *AMP* induction in homozygotic $Thor^{rev}$(white) and $Thor^{2}$ (blue) infected with *Ecc15*. Data were normalized to the uninfected control of the respective genotype.

2B. Western blot analysis of hemolymph collected from homozygotic $Thor^{rev}$ and $Thor^{2}$ infected with *Ecc15* with Attacin A antibody (top panel) and a loading control, actin (bottom panel).

2B' shows quantification of the blot with all values normalized to $Thor^{rev}$ control. Fig. S2A shows individual blots used to generate the graph in 2B'.

2C. Western blot analysis of hemolymph collected from control and infected larvae expressing RNAi targeting *perk, gcn2 and atf4* driven by *Dcg-Gal4*.

2D. Mass spectrometry analysis of hemolymph collected from $Thor^{rev}$and $Thor^{2}$ infected with *Ecc15*. Data represent the peptide spectral matches (PSM) for each AMP or DIM normalized to larval serum protein 1G (LSP1G). (Dros = Drosocin, DiptB = Diptericin B, Metch = Metchnikowin, DIM = Drosophila immune molecule, IMPP = Immune induced peptide precursor). Also see Fig. S2D and Table S4.

For 2A, B, error bars indicate standard error from at least 3 independent experiments.. ***=p<0.001. n.s.=not significant.

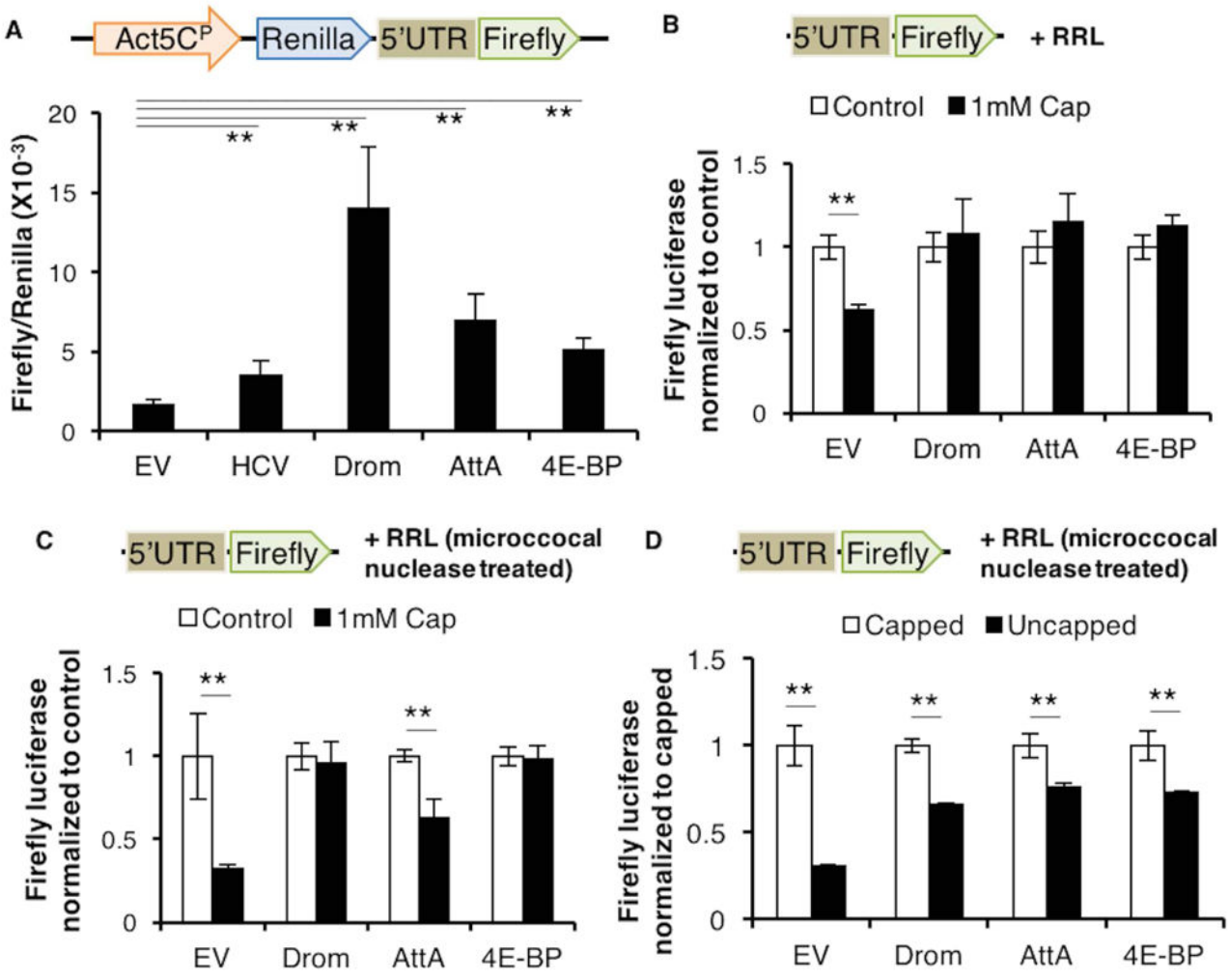

Fig. 3. 5'UTRs of *Drosomycin*, *Attacin A* and *4E-BP* can be translated cap-independently

3A. Schematic showing the bicistronic construct (top). (Bottom) Luciferase activity from S2 cells expressing bicistronic constructs containing the 5'UTRs from *Drosomycin* (Drom), *Attacin A* (AttA) and *4e-bp,* with the empty vector (EV) without any 5'UTR inserted (EV) as a negative control and, Hepatitis C virus (HCV) IRES inserted as a positive control. The mini-Actin 5C promoter ($Act5C^P$) drives reporter expression. Firefly luciferase activity of each sample is normalized to its respective Renilla luciferase activity.

3B. 5'-capped Firefly monocistronic reporter mRNA (schematic on top) translated in control RRL (white) or RRL treated with excess cap-complex (black). Data for each 5'UTR are normalized to respective control samples.

3C. Monocistronic assay as performed in 3B but in RRL treated with microccocal nuclease to eliminate competing capped transcripts.

3D. Comparison of translation efficiency of capped versus uncapped monocistronic mRNA in microccocal nuclease-treated RRL (as performed in 3B).

Error bars indicate standard error from at least 3 independent experiments. **=p<.01. Fold changes are not significant unless indicated otherwise.

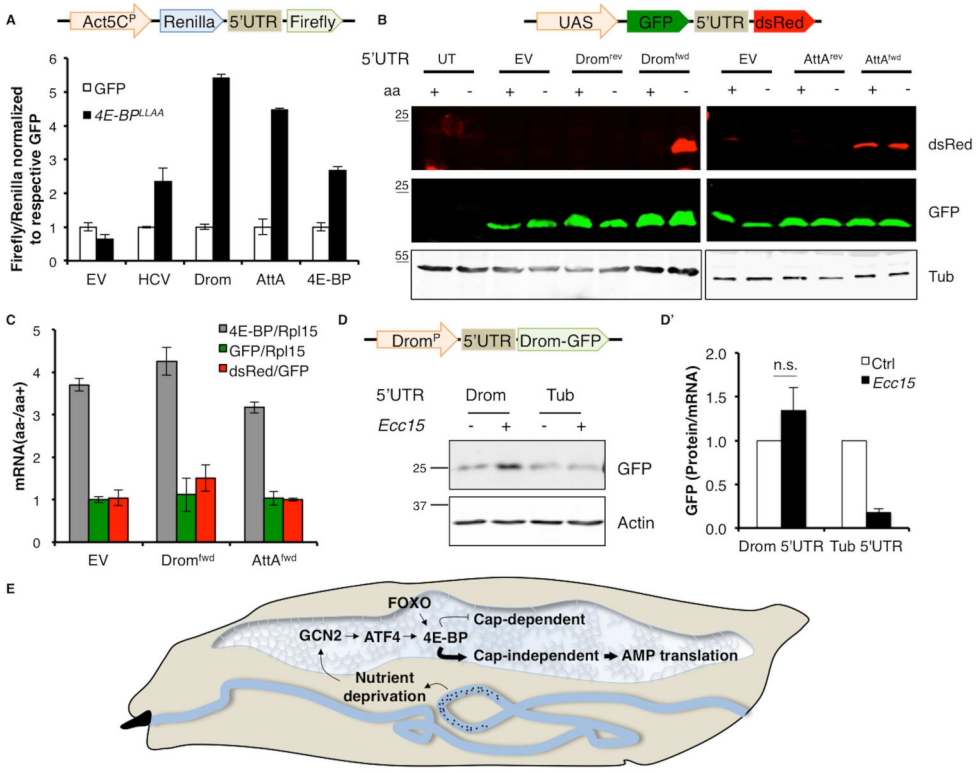

Fig. 4. 4E-BP enhances the translation of AMPs

4A. Bicistronic reporter (schematic on top) assays with 5'UTRs indicated on X-axis in S2 cells overexpressing either *GFP* as a control (white) or *4E-BP*^{LLAA} (black). Firefly luciferase activity of each sample is normalized to Renilla luciferase activity and all samples are normalized to their respective control (GFP). Error bars indicate standard deviation.

4B. Western blot analysis of the fluorescent bicistronic reporter (schematic on top) with Drosomycin (Drom) and Attacin A (AttA) 5'UTR in forward (^{fwd}) or reverse (^{rev}) orientation in S2 cells grown in either complete media (control) or media lacking amino acids (aa). (UT= untransfected control, EV= empty vector).

4C. qPCR analysis of S2 cells expressing the *Drom*^{fwd} and *AttA*^{fwd} 5'UTR fluorescent bicistronic reporter (as in 4B) subjected to amino acid (aa) deprivation. Data represents fold changes in aa deprived cells w.r.t. cells grown in complete media, with mRNA normalized as indicated in legend.

4D. Western blot of hemolymph from transgenic larvae expressing monocistronic reporters (schematic on top) driven by the Drosomycin promoter (Drom^{P}) with indicated 5'UTRs (Drom= Drosomycin, Tub= Tubulin) infected with *Ecc15*. The Drosomycin-GFP (Drom-GFP) fusion protein is detected using an anti-GFP antibody. 4D' quantifies translation of Drom-GFP.relative to Drom-GFP mRNA levels.

4E. **A model for 4E-BP activation and function during enteric infection in larvae.** In response to enteric infections by pathogens (indicated by black dots), GCN2 is activated in the fat body likely due to nutrient deprivation. GCN2 activation in the fat body leads to ATF4 synthesis and subsequent transcriptional induction of *4e-bp*. FOXO also contributes to *4e-bp* induction. Once induced, 4E-BP itself is translated cap-independently and blocks cap-

dependent translation of most cellular transcripts. In doing so, it promotes (indicated by bold arrow) cap-independent translation of AMP transcripts. Such bias in cellular translation by 4E-BP is required to drive AMP synthesis during the infection, thus explaining the role of 4E-BP in the innate immune response.

Error bars indicate standard error from 3 independent experiments.