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Differential Requirement for Translation Initiation Factor Pathways during Ecdysone-Dependent Neuronal Remodeling in *Drosophila*

**Highlights**
- Dendrite pruning in the fly PNS requires eIF4A and eIF3, but not eIF4E/G
- The helicase eIF4A and eIF3 cooperate for expression of the ecdysone target Mical
- Initiation pathway choice depends on signals in the Mical 5’ UTR
- eIF3 and eIF4A seem to be linked to several ecdysone-induced processes

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**In Brief**
Ecdysone regulates developmental neurite pruning in *Drosophila* through the activation of pruning genes, but it also negatively affects global translation rate. Rode et al. find that these effects are coordinated at the level of translation initiation, as pruning genes use the eIF3 initiation complex instead of the ecdysone-sensitive canonical eIF4F complex.

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Differential Requirement for Translation Initiation Factor Pathways during Ecdysone-Dependent Neuronal Remodeling in Drosophila

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SUMMARY

Dendrite pruning of Drosophila sensory neurons during metamorphosis is induced by the steroid hormone ecdysone through a transcriptional program. In addition, ecdysone activates the eukaryotic initiation factor 4E-binding protein (4E-BP) to inhibit cap-dependent translation initiation. To uncover how efficient translation of ecdysone targets is achieved under these conditions, we assessed the requirements for translation initiation factors during dendrite pruning. We found that the canonical cap-binding complex eIF4F is dispensable for dendrite pruning, but the eIF3 complex and the helicase eIF4A are required, indicating that differential translation initiation mechanisms are operating during dendrite pruning. eIF4A and eIF3 are stringently required for translation of the ecdysone target Mical, and this depends on the 5′ UTR of Mical mRNA. Functional analyses indicate that eIF4A regulates eIF3-mRNA interactions in a helicase-dependent manner. We propose that an eIF3-eIF4A-dependent alternative initiation pathway bypasses 4E-BP to ensure adequate translation of ecdysone-induced genes.

INTRODUCTION

Pruning, the developmentally controlled degeneration of synapses and neurites without loss of the parent neuron, is an important mechanism used to specify neuronal connections or to remove developmental intermediates (Luo and O’Leary, 2005; Schuldiner and Yaron, 2015). In holometabolous insects like Drosophila, the nervous system is remodeled during metamorphosis in response to the steroid hormone ecdysone. In the peripheral nervous system (PNS), the sensory class IV dendritic arborization (c4da) neurons completely prune their long and branched larval dendrites at the onset of the pupal phase, while their axons stay intact (Kuo et al., 2005; Williams and Truman, 2005). C4da neuron dendrite pruning involves the specific destabilization of the dendritic cytoskeleton and plasma membrane (Herzmann et al., 2017, 2018) and phagocytosis of severed dendrites by surrounding epidermal cells (Han et al., 2014).

Ecdysone induces c4da neuron dendrite pruning through the hormone receptors EcR-B1 and ultraspiracle (Usp), which activate the transcription of pruning genes (Kuo et al., 2005; Williams and Truman, 2005). Among these are headcase, a pruning gene of unknown function (Loncle and Williams, 2012), and SOX14, an HMG box transcription factor that activates transcription of MICAL, encoding an actin-severing enzyme (Kirilly et al., 2009). Regulation of MICAL expression also involves the ubiquitin-proteasome system at a posttranscriptional level (Rumpf et al., 2014).

In addition to transcriptional activation of target genes, several lines of evidence suggest that ecdysone also regulates global translation rates through activation of the translation inhibitor eukaryotic initiation factor 4E-binding protein (4E-BP). In the Drosophila fat body, this occurs transcriptionally through FOXO (Colombani et al., 2005), while in c4 da neurons, ecdysone inhibits the insulin and Target of Rapamycin (TOR) pathway to activate 4E-BP posttranslationally (Wong et al., 2013).

4E-BP inhibits translation initiation, the rate-limiting step of protein synthesis, by sequestering the cap-binding protein elf4E (Gingras et al., 1999). During canonical translation initiation, elf4E binds to the 7-methylguanosine (m7Gppp) cap of eukaryotic mRNAs and then forms the so-called elf4F complex by recruiting elf4G, an adapter that binds the 43S preinitiation complex (PIC), containing the 40S small ribosomal subunit, and the helicase elf4A, which is thought to resolve hairpin structures in the 5′ UTRs of mRNAs (Svitkin et al., 2001; Wolfe et al., 2014). This enables the 43S complex to scan 5′ UTRs for the initiation
codon, where it is joined by the large ribosomal subunit and translation can start (Hinnebusch, 2014). While eIF4A’s role has been mainly linked to 5’UTR hairpins, it can also stimulate translation of mRNAs with unstructured 5’UTRs (Yourik et al., 2017). Moreover, eIF4A is more abundant than eIF4E (Gingras et al., 1999), suggesting that it has functions beyond the eIF4F complex.

Activated 4E-BP binds to eIF4E and prevents eIF4F assembly, thus inhibiting ribosome recruitment to mRNAs and globally dampening translation rates under stress or during development (Gingras et al., 1999; Richter and Sonenberg, 2005). Interestingly, 4E-BP affects translation of some mRNAs more than others (Gandin et al., 2016; Hsieh et al., 2012). To explain this, eIF4E-independent translation initiation mechanisms have been proposed. One such mechanism could depend on internal ribosome entry sites (IRESs) that bypass the requirement for the m7Gppp cap (Yamamoto et al., 2017). For example, the mRNAs of the Drosophila cell death factors reaper and hid may contain IRES sequences in their 5’UTRs that allow them to be translated under stress (Hernández et al., 2004; Vazquez-Pianzola et al., 2007).

Alternative cap recognition mechanisms have also been proposed under conditions of high 4E-BP activity. In particular, the initiation factor eIF3, a 13-subunit complex, could provide a mechanism for eIF4E-independent initiation (Lee et al., 2015, 2016). It binds to the small ribosomal subunit as part of the 43S PIC, and it is thought to act downstream of eIF4G in mRNA recruitment. However, eIF3 dependence varies between mRNAs, and eIF3 can even suppress translation of some targets (Lee et al., 2015). Importantly, it was recently shown that translation of some 4E-BP-resistant mRNAs depends on an eIF3-based cap recognition activity in the eIF3d subunit that is stimulated by hairpin motifs in the 5’UTR (Lee et al., 2016). Other eIF3 subunits have also been shown to interact with the cap (Kumar et al., 2016).

Given that ecdysone inhibits eIF4E-dependent translation, we asked whether there are mechanisms that ensure the translation of ecdysone target mRNAs. To this end, we assessed the requirements for translation initiation factors during c4da neuron dendrite pruning. We found that the canonical eIF4F components eIF4E and eIF4G are not required for c4da neuron dendrite pruning, while the helicase eIF4A and the eIF3 complex are. Both eIF4A and eIF3 are required for Mical expression, and this specificity is conferred by the 5’UTR of Mical mRNA. Further biochemical analyses suggest that eIF4A regulates the interaction between eIF3 and the Mical 5’UTR. We propose that eIF4A/eIF3 constitute a 4E-BP bypass mechanism that ensures the adequate translation of ecdysone-induced genes in c4da neurons.

RESULTS

**eIF4A, but Not eIF4E, Is Required for Sensory Neuron Dendrite Pruning**

To address the requirements for translation initiation factors during dendrite pruning, we expressed transgenic double-stranded RNA (dsRNA) constructs against the three eIF4F subunits in c4da neurons under the control of ppk-GAL4. C4da neurons have long and branched dendrites at the larval stage (Figure 1A), which are pruned completely at 18 hr after pupariation formation (APF) (Figure 1B). Transgenic RNAi constructs against eIF4E1, eIF4G1, and the eIF4A-activating protein eIF4B did not cause defects in c4da neuron dendrite pruning (Figures S1A–S1G and S1K). Ubiquitous knockdown of eIF4E1 and eIF4G1 under the driver actin-GAL4 caused lethality (Table S1), indicating that the dsRNAs were functional. In contrast, expression of three dsRNA constructs directed against Drosophila eIF4A caused strong pruning defects, with up to 95% of c4da neurons retaining dendrites attached to the cell body at 18 hr APF (Figures S1H–S1J and S1K).

To confirm these results, we next inhibited eIF4F by over-expressing constitutively active 4E-BP (UAS-4E-BP<sup>PP</sup> (Miron et al., 2001). Consistent with previous results (Niehues et al., 2015), 4E-BP<sup>PP</sup> caused a reduction of larval dendrites compared to controls (Figures 1A, 1B, and 1F). However, dendrites of 4E-BP<sup>PP</sup>-expressing c4da neurons were pruned largely normally at 18 hr APF (Figures 1A’, 1B’, 1G, and 1H). C4da neurons homozygous for the previously characterized strong P-element mutation el<sup>f4E</sup>1<sup>1058911</sup> (Lachance et al., 2002) generated by MARCM (Lee and Luo, 1999) also did not show pruning defects (Figures 1C, 1G, and 1H).

To confirm that loss of eIF4A causes pruning defects, we generated a UAS line expressing an eIF4A variant with a mutation in the DEAD box motif (eIF4A<sup>E172Q</sup>). Expression of this ATPase-dead eIF4A in c4da neurons caused strong dominant dendrite-pruning defects (Figures 1D, 1G, and 1H). Further, homozygous eIF4A<sup>E1006D</sup> mutant c4da neurons generated by MARCM also showed strong dendrite-pruning defects (Figures 1E, 1G, and 1H).

As the strong eIF4A manipulations caused a decrease in the number of larval dendrites (Figure 1F), we sought to exclude that the pruning defects were caused by pre-existing defects. To this end, we used a drug-inducible ppk-GeneSwitch driver (Osterwalder et al., 2001) to activate UAS-eIF4A<sup>E172Q</sup> expression in c4da neurons only 24 hr before the pupal stage, at a time when larval dendrites are well elaborated. Short-term induction of eIF4A<sup>E172Q</sup> caused dendrite-pruning defects indistinguishable from those caused by constitutive expression (Figures S1L and S1M). These results suggest that eIF4E-independent translation initiation mechanisms are important for c4da neuron dendrite pruning and that eIF4A is required for dendrite pruning independently of the eIF4F complex.

**eIF4A Requirement during Pruning Cannot Be Explained by an Effect on Global Translation Rate**

To assess translation rates in c4da neurons, we used fluorescent noncanonical amino acid tagging (FUNCAT) (Erdmann et al., 2015). Briefly, we expressed a variant methionyl-tRNA synthetase in c4da neurons to allow incorporation of the noncanonical amino acid azidonorleucine (ANL) into newly translated proteins. Proteins labeled in this way can be fluorescently tagged via click chemistry (Erdmann et al., 2015). Third instar larvae expressing variant methionyl-tRNA synthetase in c4da neurons were placed on ANL-containing medium for 48 hr, dissected, and click-labeled with TAMRA dye. Incorporated TAMRA was then visualized by confocal microscopy in animals.
expressing control or eIF4A dsRNA constructs and quantified (Figures S2A and S2B). Knockdown of eIF4A reduced the amount of newly translated proteins by approximately 40% (Figure S2E). We then expressed constitutively active 4E-BP LL in c4 da neurons to inhibit eIF4E. This had previously been shown to strongly decrease translation rates (Niehues et al., 2015). 4E-BPLL reduced translation rates to a similar degree as eIF4A knockdown (Figures S2C, S2D, and S2F). As the inhibition of eIF4A and eIF4E has very different effects on c4 da neuron dendrite pruning, our data suggest that these two factors regulate different sets of mRNAs.

### The eIF3 Complex Is Required for Dendrite Pruning

The lack of pruning defects upon inhibition of core eIF4F components was surprising because dendrite pruning requires expression of several pruning factors. We therefore hypothesized that alternative translation initiation factors may be required instead.

A candidate component of such a pathway is the initiation factor eIF3, a large 13-subunit complex that is thought to cooperate with eIF4G in ribosome recruitment during canonical translation initiation (Cate, 2017). eIF3 was recently shown to participate in eIF4E-independent modes of translation initiation (Lee et al., 2016; Meyer et al., 2015) (see also Figure 7 for model).

To test whether eIF3 is important for c4da neuron dendrite pruning, we expressed dsRNA constructs directed against its core subunits eIF3c and eIF3e in c4 da neurons. Knockdown of these two factors caused strong c4da neuron dendrite pruning defects at 18 hr APF (Figures 2A–2C’, 2F, and 2G). Furthermore, homozygous eIF3g<sup>-1A</sup> or eIF3b<sup>Y14430</sup> mutant c4da neuron clones generated by MARCM also displayed strong dendrite pruning defects at 18 hr APF (Figures 2D–2G).

eIF3 may also contribute to eIF4E-independent translation initiation through a recently discovered cap-binding activity in its eIF3d subunit (Lee et al., 2016). To address whether this
Figure 2. The eIF3 Complex Is Required for Translation Initiation during c4da Neuron Dendrite Pruning

(A–E) c4da neurons of the indicated genotypes at the third instar larval stage; (A')–(E') show c4da neurons at 18 hr APF. (A and A') Control c4da neurons labeled by CD8::GFP expressed under ppk-GAL4. (B and B') c4da neurons expressing elf3c RNAi. (C and C') c4da neurons expressing elf3e RNAi. (D and D') elf3g1A c4da MARCM clones labeled by the expression of CD8::GFP. (E and E') elf3bEY14430 c4da MARCM clones labeled by the expression of tdTomato.

(F) Penetration of pruning defects in (A')–(D'). ***p < 0.001, Fisher’s exact test (n = 11–30).

(G) Number of primary and secondary dendrites attached to the cell body at 18 hr APF. Data are mean ± SD. ***p < 0.001, Wilcoxon test.

(H–K) Dominant effects of an eIF3d cap-binding mutant on c4da neuron dendrite pruning. Wild-type eIF3d (H) or the cap-binding mutant eIF3d helix11 (I) were overexpressed in c4da neurons, and pruning defects were assessed at 18 hr APF. (J) Penetration of dendrite-pruning defects in (H) and (I) (n = 42–75). **p < 0.01, Fisher’s exact test. (K) Number of primary and secondary dendrites attached to the cell body at 18 hr APF in (H) and (I). Data are mean ± SD. **p < 0.01, Wilcoxon test. Scale bars, 100 μm (A–E) and 50 μm (A’–E’ and H).
activity contributes to eIF3 function during dendrite pruning, we generated a UAS-eIF3d construct containing mutations in the cap-binding domain. This mutant, termed eIF3d<sup>hetix11</sup>, had previously been shown to prevent recruitment of a specific target mRNA to the 43S PIC <em>in vitro</em> (Lee et al., 2016). While overexpression of wild-type eIF3d in c4da neurons did not cause dendrite-pruning defects (Figure 2H), overexpression of eIF3d<sup>hetix11</sup> caused more than 30% of c4da neurons to retain dendrites at 18 hr APF (Figure 2I). This effect was weaker than those of eIF3 knockdowns or mutants (Figures 2J and 2K), likely because such dominant-negative approaches can be ineffective or because additional functions of eIF3 also contribute to pruning. Nevertheless, our phenotypic data suggest that eIF3 is required for translation initiation of crucial dendrite pruning factors in c4da neurons during the early pupal phase. Moreover, the fact that an eIF3 cap-binding mutant caused dominant pruning defects suggests that c4da neuron dendrite pruning relies on cap-dependent translation initiation.

**eIF4A and eIF3 Are Required for Mical Expression**

The above data suggest that eIF4A and eIF3, but not eIF4E/G, are required for translation initiation of dendrite pruning factors during the early pupal phase. To identify such targets, we next assessed the expression of known ecdysone-induced pruning factors during the early pupal stage by immunofluorescence. The ecdysone receptor EcR-B1 is activated at the onset of the pupal stage during the early pupal phase. To identify such targets, we next assessed the expression of known ecdysone-induced pruning factors during the early pupal stage by immunofluorescence. The ecdysone receptor EcR-B1 is activated at the onset of the pupal stage and activates transcription of target genes, such as SOX14 and headcase (Kirilly et al., 2009; Loncle and Williams, 2012). Sox14 expression is not affected by eIF4A downregulation (Figures S3A–S3D). We therefore focused on the Sox14-Mical branch of the transcriptional cascade. At 2 hr APF, Sox14 expression in c4da neurons was affected neither by the expression of 4E-BPLL to inhibit eIF4E (Figures 3A and 3B) nor by eIF4A manipulation (eIF4A RNAi, the <em>eIF4A</em><sup>1066</sup> mutation, or expression of eIF4A<sup>E172Q</sup>) (Figures 3C–3E). Furthermore, Sox14 was also present in c4da neurons expressing dsRNA constructs against eIF3c or eIF3e (Figures 3F and 3G).

We next assessed the effects of translation initiation factor manipulations on Mical protein expression at 2 hr APF. Mical protein expression was not affected by eIF4E inhibition (Figures 3A′ and 3B′), but it was strongly reduced by all tested eIF4A manipulations (eIF4A RNAi, the <em>eIF4A</em><sup>1066</sup> mutation, or expression of eIF4A<sup>E172Q</sup>) (Figures 3C′–3E′) and upon knockdown of eIF3c and eIF3e (Figures 3F′ and 3G′). Thus, eIF4A and eIF3 might be required for c4 da neuron dendrite pruning through a specific role in pruning gene expression.

To explain why Sox14 was unaffected by manipulations of both the eIF4F complex and eIF3, we reasoned that redundant translation initiation pathways might exist for Sox14 mRNA. Indeed, simultaneous inhibition of eIF4E (by 4E-BPLL) and eIF3 (by eIF3e knockdown) abrogated Sox14 expression (Figure 3H). Taken together, eIF4A and eIF3 appear to have similar target preferences during c4da neuron dendrite pruning, as they are both specifically required for Mical expression. Sox14 translation, on the other hand, can be initiated by either an eIF4E- or an eIF3-dependent mechanism.

**Mical Is a Major Target for eIF4A and eIF3 during c4 da Neuron Dendrite Pruning**

Given that eIF4A and eIF3 were stringently required for Mical expression, we wondered whether loss of Mical expression was a major cause for the pruning defects upon loss of eIF4A or eIF3. We therefore tested whether we could rescue these pruning defects by upregulation of Mical. To this end, we attempted to rescue the pruning defects caused by eIF3-eIF4A manipulations by overexpression of a transgenic Mical construct harboring all crucial domains (Terman et al., 2002) (herafter called Mical<sup>FL</sup> for full length) or Mical<sup>H</sup>, a truncated version of Mical insufficient for pruning (Kirilly et al., 2009). Coexpression of eIF4A dsRNA with tdTomato caused approximately 70% of c4da neurons to retain dendrites attached to the soma at 18 hr APF (Figures 4A, 4D, and 4E). Remarkably, overexpression of Mical<sup>FL</sup>, but not Mical<sup>H</sup>, strongly reduced both the penetrance and severity of these pruning defects (Figures 4B–4E). A similar suppression was also seen upon coexpression of a GFP-tagged Mical<sup>FL</sup> transgene (Hung et al., 2010), but not upon coexpression of Mical mutants lacking the calponin homology region or the flavoprotein monoxygenase domain encoding the active site for actin oxidation (Figure S4).

We next assessed the relationship between eIF4A and Mical by the same strategy. Silencing eIF3e expression led to the retention of dendrites attached to the soma in approximately 70% of c4da neurons at 18 hr APF (Figure 4F). Remarkably, Mical<sup>FL</sup> overexpression was again able to rescue the pruning defects induced by eIF3e knockdown, while Mical<sup>H</sup> was not (Figures 4G–4J). Thus, Mical is an important target of both eIF4A and eIF3 during c4da neuron dendrite pruning.

Exogenous Sox14 activates transcription of the endogenous Mical gene and can bypass the pruning defects of several mutants with defects in ecdysone-dependent gene expression (Kirilly et al., 2009; Rumpf et al., 2014). Interestingly, Sox14 overexpression in neurons expressing eIF4A dsRNA caused a decrease in the length of unpruned dendrites at 18 hr APF, but it did not lead to a decrease in the penetrance of the defects, indicating that mere transcriptional upregulation of endogenous Mical could not reduce the severing defects caused by the loss of eIF4A (Figure S4) (see also below).

**eIF4A/eIF3 Dependence Is Conferred by the 5′ UTR of Mical mRNA**

What are the signals that render Mical expression dependent on eIF4A and eIF3? Both eIF4A and eIF3 interact with the 5′ UTRs of their target mRNAs during translation initiation (Hinnebusch, 2014). The cap-binding activity of eIF3d also requires specific secondary structures in the 5′ UTRs of a target mRNA (Lee et al., 2016). An involvement of the Mical 5′ UTR would also be consistent with the above data showing that induction of endogenous Mical mRNA via Sox14 (i.e., containing the endogenous 5′ UTR) cannot rescue the eIF4A knockdown phenotype (Figure S4).

To address whether the Mical 5′ UTR (256 bp) is a determinant of eIF4A/eIF3 dependence, we characterized it in different reporter assays. We first used <em>in vitro</em> transcription to generate a 5′ UTR<sup>Mical</sup>-Luciferase reporter mRNA for <em>in vitro</em> translation experiments. Translation of this reporter mRNA in reticulocyte lysate yielded robust luciferase activity when the mRNA was...
capped with the functional cap analog ARCA (m₇G₃OpppG), but not when the mRNA was capped with the inactive cap analog ApppG (Figure S5A). Thus, 5' UTR-Mical requires a functional cap in order to promote translation initiation.

We next generated a UAS-5' UTR-Mical-GFP reporter for in vivo studies. In control third instar c4 da neurons, UAS-5' UTR-Mical-GFP yielded robust fluorescence (Figure 5A). In keeping with our above results, expression from 5' UTR-Mical-GFP was not affected by 4E-BP LL overexpression to inhibit eIF4E (Figures 5B and 5E). Likewise, expression of a UAS-GFP reporter was not affected by 4E-BP LL (Figures 5C–5G).

We next assessed the effects of downregulation of eIF4A and eIF3. Expression from 5' UTR-Mical-GFP was significantly reduced upon eIF4A knockdown (Figures 5H, 5I, and 5L) and even more so in eIF4A₁₀₀₆ mutant c4 da neuron MARCM clones (Figures S5B and S5C). UAS-GFP expression was also reduced by eIF4A knockdown (Figures 5J, 5K, and 5M), but to a lower degree than UAS-5' UTR-Mical-GFP (Figure 5N).

Knockdown of eIF3e attenuated 5' UTR-Mical-GFP activity even more strongly than eIF4A knockdown (Figures 5O, 5P, and 5S). UAS-GFP expression was also reduced by eIF3e knockdown, but again less severely than UAS-5' UTR-Mical-GFP (Figures 5Q, 5R, 5T, and 5U).

Surprisingly, the fluorescence intensity of the UAS-ttdtomato reporter used to label c4 da neurons in these experiments was consistently upregulated upon knockdown of eIF4A and eIF3e.
This reporter gene carries additional sequences in its 5′ UTR designed to strengthen expression (Han et al., 2014) that might confer a different sensitivity to initiation factors. Indeed, eIF3 has been shown to act inhibitory on some mRNAs (Lee et al., 2015).

Taken together, our data suggest that the 5′ UTRs of the tested reporter genes confer differential dependence on eIF4A and eIF3. Since expression from the 5′ UTRMical reporter was more strongly reduced by eIF4A/eIF3e knockdown than that of the regular UAS-GFP reporter, we conclude that the 5′ UTR of Mical mRNA encodes a signal for an eIF4A/eIF3-dependent initiation pathway.

**eIF4A Regulates eIF3 Interactions with the Mical 5′ UTR**

The phenotypic similarities caused by manipulations of eIF4A and eIF3 suggested that these two initiation factors cooperate closely during translation initiation of Mical mRNA. To explore this possibility further, we performed biochemical experiments in S2 cells. We first asked whether eIF4A and eIF3 are found in a complex. To this end, we transfected S2 cells with FLAG-tagged eIF4A or ATPase-dead eIF4AE172Q, and we performed immunoprecipitations using anti-FLAG antibodies. We then probed the precipitates for the presence of eIF3 using an antibody against eIF3b. eIF3b could be readily detected in precipitates from cells expressing wild-type FLAGeIF4A, but its amount was strongly reduced when cells expressed FLAGeIF4AE172Q (Figure 6 A), suggesting an ATPase-dependent interaction between the two factors. This potential ATPase dependence of the eIF3-eIF4A interaction is in keeping with the previous notion that PIC recruitment of natural mRNAs with folded 5′ UTRs requires the eIF4A ATPase (Yourik et al., 2017; Sokabe and Fraser, 2017).

eIF3 is thought to interact with the 5′ ends of mRNAs in several ways. As part of the 43S PIC, it can bind to 5′ UTRs directly via several subunits (Lee et al., 2015). In addition, eIF3 can bind to the m7Gppp cap via the subunit eIF3d (Lee et al., 2016) and possibly eIF3l (Kumar et al., 2016). However, these cap interactions are likely to be specifically regulated by cis-acting 5′ UTR sequences (Lee et al., 2016). In support of this notion, we did not detect eIF3 subunits or eIF4A in pull-down with m7GTP-agarose, both from control and ecdysone-treated samples (Figure S7). We therefore next assessed in more detail the interactions among eIF3, eIF4A, and their natural target, the...
Mical 5' UTR. To inhibit eIF4A pharmacologically, we used Rocaglamide A (RocA), a compound known to clamp eIF4A on the 5' UTRs of target mRNAs (Iwasaki et al., 2016). S2 cells treated with 0.3 μM RocA for 10 min showed a decrease in translating ribosomes in polysome profiles (Figure 6B). We then used this condition to assess how eIF4A inhibition affects the interactions between eIF3 and 5' UTRMical using an eIF3-RNA co-immunoprecipitation protocol (Gross et al., 2017).

Figure 5. The Mical 5' UTR Confers eIF4A/eIF3 Dependence
The effects of eIF4A and eIF3 manipulation on a GFP reporter gene containing the Mical 5' UTR (UAS-5' UTRMical-GFP) was assessed in third instar c4 da neurons. A regular UAS-GFP reporter served as a control. (A)–(D), (H)–(K), and (O)–(R) show GFP reporter signals, and (A')–(D'), (H')–(K'), and (O')–(R') show tdTomato signal in the same neurons.

(A and B) GFP intensity of UAS-5' UTRMical-GFP in c4da neurons expressing UAS-lacZ as control (A) or c4da neurons expressing 4E-BP LL (B).

(C and D) Intensity of UAS-GFP in c4da neurons expressing UAS-lacZ (C) or expressing 4E-BP LL (D).

(E) GFP intensities of 5' UTRMical-GFP reporters in (A) and (B).

(F) GFP intensities of regular GFP reporters in (C) and (D).

(G) The reduction of GFP intensities of UAS-5' UTRMical-GFP and UAS-GFP by 4E-BP LL was calculated relative to controls.

(H and I) GFP intensity of UAS-5' UTRMical-GFP in c4da neurons expressing a control RNAi (H) or eIF4A RNAi (I).

(J and K) GFP intensity of UAS-GFP in c4da neurons expressing a control RNAi (J) or eIF4A RNAi (K).

(L) GFP intensities of 5' UTRMical-GFP reporters in (H) and (I).

(M) GFP intensities of regular GFP reporters in (J) and (K).

(N) Reduction of GFP intensities of UAS-5' UTRMical-GFP and UAS-GFP by eIF4A knockdown.

(O and P) GFP intensity of UAS-5' UTRMical-GFP in c4da neurons expressing a control RNAi (O) or eIF3e RNAi (P).

(Q and R) GFP intensity of UAS-GFP in c4da neurons expressing a control RNAi (Q) or eIF3e RNAi (R).

(S) GFP intensities of 5' UTRMical-GFP reporters in (O) and (P).

(T) GFP intensities of regular GFP reporters in (Q) and (R).

(U) Reduction of GFP intensities of UAS-5' UTRMical-GFP and UAS-GFP by eIF3e knockdown. Data are mean ± SEM. *p < 0.05, **p < 0.005, ***p < 0.0005, Wilcoxon test; n = 15–18. Scale bars, 10 μm.

See also Figures S5 and S6.
S2 cells with a 5′ UTRMical-GFP reporter plasmid, then immuno-precipitated elf3, and determined the amount of bound reporter mRNA by GFP RT-PCR. GFP mRNA could be detected in elf3b precipitates from untreated cells, supporting the notion that elf3 binds to the 5′ UTRMical-GFP reporter also in S2 cells (Figure 6C).

In samples treated with RocA, the GFP mRNA signal was increased at least 3-fold (n = 3; Figure 6C). This suggests that elf4a activity is required for scanning of elf3-dependent PICs on Mical mRNA.

The above result indicated that elf4a might regulate elf3mRNA interactions through its helicase. To test in vivo whether the elf4a helicase is important for dendrite pruning, we generated a UAS transgene encoding elf4aSAT, an elf4a mutant that still has an active ATPase but that lacks helicase activity (Pause and Sonenberg, 1992). While wild-type elf4a efficiently rescued the c4da neuron dendrite-pruning defects conferred by the elf4a1006 mutant (Figure 6D), elf4aSAT did not (Figures 6E–6G), indicating that the helicase activity of elf4a is required for dendrite pruning. Taken together, we conclude that elf4a regulates the interaction between elf3 and Mical mRNA, likely in a helicase-dependent manner.

Figure 6. elf4a Regulates Interactions between elf3 and the Mical 5′ UTR

(A) elf3 co-immunoprecipitates with eFLAG-tagged elf4a in an ATPase-dependent manner. FLAG-tagged wild-type (WT) or ATPase-dead elf4a constructs were transfected in S2R+ cells and precipitated with FLAG agarose. Precipitates were probed with anti-FLAG and anti-elf3b antibodies. Molecular weight (MW) is indicated in kilodaltons (kDa).

(B) elf4a inhibition with RocA leads to loss of translating ribosomes. Polysome profiles from control S2R+ cells (black line) or S2R+ cells treated with 0.3 μM RocA for 10 min are shown.

(C) elf4a regulates interactions between elf3 and a 5′ UTRMical-GFP reporter mRNA. Control or anti-elf3b immunoprecipitates from S2R+ cells expressing 5′ UTRMical-GFP were analyzed by semiquantitative GFP RT-PCR. A 10-min treatment with 0.3 μM RocA results in increased elf3b/5′ UTRMical-GFP interaction (n = 3).

(D–G) Requirement for elf4a helicase activity during dendrite pruning. Wild-type elf4a or helicase-dead were expressed in elf4a1006 mutant MARCM clones, and the effects on c4da neuron dendrite pruning were assessed at 18 hr APF.

(D) Wild-type elf4a rescues the pruning defects of elf4a1006 c4da neuron MARCM clones at 18 hr APF (n = 10).

(E) Helicase-dead elf4aSAT does not rescue the pruning defects of elf4a1006 mutant c4da neuron MARCM clones (n = 11).

(F) Penetration of dendrite-pruning defects in (D) and (E). *p < 0.05, Fisher’s exact test.

(G) Severity of dendrite-pruning defects in (D) and (E). *p < 0.05, Wilcoxon test. Data are mean ± SD.

See also Figure S7.

elf4a and elf3 Are Broadly Required for Neuronal Remodeling in the Pupal PNS

So far, we have shown that elf4a and elf3 are specifically required for c4 da neuron dendrite pruning. Here, translation of Mical mRNA stringently depends on elf4a and elf3, while Sox14 mRNA can be translated via an elf3- or an elf4e-dependent pathway. As previous data had shown that edcsyne induces 4E-BP activity in tissues as diverse as fat body and neurons (Colombani et al., 2005; Wong et al., 2013), we next asked how broadly elf4a and elf3 were required for edcsyne-dependent neuronal remodeling in the PNS.

Two other types of peripheral sensory neurons undergo neuronal remodeling during the pupal phase: c1da neurons prune their larval dendrites with a similar time course as c4da neurons, whereas c3da neurons undergo apoptosis in an edcsyne-dependent manner around 7 hr APF (Williams and Truman, 2005). The c1da neuron dendrite pruning requires Mical, but c3da neuron apoptosis does not (Kirilly et al., 2009).

We first asked whether elf4a and elf3 were required for edcsyne-induced apoptosis. At 18 hr APF, the dorsal c3da...
neurons ddaA and ddaF had disappeared in control animals (Figure 7A). In contrast, expression of elf4A<sup>E172Q</sup> under a c3da-specific driver led to persistence of a fraction of c3da neurons, mainly ddaF, with intact processes until this stage (Figure 7B). Furthermore, we could detect homozygous elf3g1A mutant ddaF c3da neurons persisting at 18 hr APF (Figure 7C). Thus, elf3 and elf4A are required for c3da neuron apoptosis. Potential translation targets here could include pro-apoptotic factors, as reaper and hid mRNAs do not require elf4E for translation (Hernández et al., 2004; Vazquez-Pianzola et al., 2007).

We next addressed the importance of elf4A and elf3 for c1da neuron dendrite pruning. In controls, ddaD c1da neuron dendrites were completely pruned at 18 hr APF (Figure 7D). In contrast, ddaD neurons lacking elf4A or elf3g1 (in elf4A<sup>1006</sup> or elf3g1A MARCM clones) still had long and branched dendrites attached to the cell body (Figures 7E–7G), indicating that elf4A and elf3 are also required for c1da neuron dendrite pruning. As c1da neuron dendrite pruning requires Mical (Kirilły et al., 2009), it is the most likely elf4A/elf3 target also during c1da neuron dendrite pruning. Taken together, our data indicate that elf4A and elf3 are part of a 4E-BP bypass system that is broadly required for ec dysone-induced neuronal remodeling in the Drosophila PNS.

Figure 7. elf4A/elf3 Are Required Broadly for Ecdysone-Induced Neuronal Remodeling of Peripheral Sensory Neurons

(A–C) elf4A and elf3 are required for c3da neuron apoptosis. C3da neurons were labeled with the c3da neuron driver GAL4<sup>19-12</sup> or by MARCM and imaged at 18 hr APF. (A) Control c3da neurons labeled by GAL4<sup>19-12</sup> have undergone apoptosis (0/18 still detectable). (B) Expression of elf4A<sup>E172Q</sup> inhibits apoptosis in c3da neurons, mostly ddaF (18/48 still detectable). (C) A homozygous elf3g1A mutant c3da neuron failed to undergo apoptosis at 18 hr APF (n = 5).

(D–G) elf4A and elf3 are required for c1da neuron dendrite pruning. Pupae were imaged at 18 hr APF. (D) control c1 da neurons labeled with GAL4<sup>2-21</sup> have completely pruned their larval dendrites. (E and F) ddaD MARCM clones homozygous for elf4A<sup>1006</sup> (E) or elf3g1A (F) still possess long and branched dendrites at 18 hr APF. (G) Severity of c1 da neuron pruning defects in (D–F). Data are mean ± SD. **p < 0.005, Wilcoxon test; n = 6–11.

(H) Model for the regulation of translation initiation during c4 da neuron dendrite pruning. Cap recognition during the pupal stage is likely mediated by elf3 (question mark in right panel). Scale bars, 50 μm.

**DISCUSSION**

Developmental control of translation rate is required under various conditions. One well-characterized regulatory mechanism is through 4E-BP, which inhibits assembly of the cap-binding elf4F complex (Gingras et al., 1999). Despite the obvious need for global translation control during development, it is also clear that there must be exceptions to such regulation. Several lines of evidence suggest that global, elf4E-dependent translation is downregulated by ec dysone during the pupal phase (Colombani et al., 2005; Hoopfer et al., 2008; Wong et al., 2013) and that this is important for c4 da neuron dendrite pruning (Wong et al., 2013). How downregulation of elf4E-dependent translation contributes to dendrite pruning is not clear. TOR activity (and hence elf4E-dependent translation) is associated with neurite regrowth after pruning in a Drosophila model for neuronal remodeling (Yaniv et al., 2012), and beta-actin mRNA was identified as a 4E-BP target in vertebrate neurons (Leung et al., 2006). General suppression of elf4E-dependent translation may, therefore, serve to prevent precocious neurite growth or neurite stabilization through increased actin polymerization.

Despite the need for translation downregulation during dendrite pruning, ec dysone-induced mRNAs must still be efficiently translated. We found that c4 da neuron dendrite pruning does not depend on the elf4F subunits elf4E and elf4G, but instead on elf3 and elf4A (Figure 7H). In keeping with a specific effect on dendrite-pruning genes, we could identify Mical mRNA as the crucial target for elf3 and elf4A. Our data suggest that this specificity is encoded in the 5’ UTR of Mical mRNA, as a
UAS-GFP reporter containing the Mical 5’ UTR showed consistently stronger dependence on eIF4A and eIF3 than a regular UAS-GFP reporter (Figure 5). The important role of the Mical 5’ UTR is also supported by our observation that Sox14 overexpression (which induces endogenous Mical mRNA) did not rescue the pruning defects induced by eIF4A RNAi, while overexpression of Mical from a UAS transgene (and thus lacking the endogenous 5’ UTR) did (Figures 4, S4, and S5). It is tempting to speculate that eIF3-eIF4A recognition signals may be abundant in 5’ UTRs of ecdyson-induced genes.

Several lines of evidence indicate that translation initiation of pupal pruning factors in c4 da neurons is still cap dependent: for one, overexpression of a cap-binding-deficient eIF3d mutant causes dominant dendrite-pruning defects (Figure 2I), and in vitro translation of a 5’ UTRMical reporter mRNA depends on a functional cap (Figure S5). While we observed physical interactions between eIF3 and a 5’ UTRMical reporter mRNA in S2 cells, we could not directly demonstrate cap binding by eIF3 in vivo. eIF3 does not bind to the isolated cap structure (Lee et al., 2016; Figure S7), and a biochemical cap-binding assay for eIF3 would require crosslinking eIF3 with a purified mRNA with a radioactively labeled cap. To further investigate developmental control of translation initiation in the future, it would be interesting to set up such an assay to address whether the Mical mRNA cap is also recognized via eIF3d or another eIF3 subunit.

Sox14 expression seemed resistant to the inhibition of either eIF4E or eIF3, but we found that these pathways can mediate Sox14 expression in a redundant fashion. Sox14 is upstream of the Cul-1 ubiquitin ligase that activates 4E-BP in c4 da neurons (Wong et al., 2013). Its mRNA may be adapted to this position in the pruning pathway, as it could still use the regular eIF4F pathway early during the pupal phase and the eIF3 pathway later. Mical translation may only start when 4E-BP activity is already high, hence explaining its strong eIF3 dependence.

Translation of long mRNAs is sensitive to the eIF4A cofactor eIF4B (Sen et al., 2016), and eIF4A dependence is also in part conferred by sequences in the coding region (Yourik et al., 2017). eIF4B manipulation did not cause dendrite pruning defects (Figure S1), but the Mical construct used to rescue the pruning defects induced by eIF4A knockdown lacks an internal region non-essential for pruning (Krilly et al., 2009). It is, therefore, possible that internal regions of the long Mical mRNA also contribute to its dependence on eIF4A.

The strong similarities between the phenotypes caused by the manipulation of eIF4A and eIF3 suggested that these two factors cooperate functionally. We found that eIF4A and eIF3 can be found in an eIF4A ATPase-dependent complex and that eIF4A clamping on the mRNA prevents eIF3 release from a 5’ UTRMical reporter mRNA (Figure 6). Two recent in vitro studies found functional interactions between eIF4A and eIF3 in the context of canonical eIF4F-dependent translation initiation (Yourik et al., 2017; Sokabe and Fraser, 2017): first, eIF3 stimulates eIF4A ATPase activity via its eIF3g subunit to promote PIC maturation (Yourik et al., 2017); and, second, eIF4A ATPase activity was required to reposition the eIF3j subunit within the PIC during maturation (Sokabe and Fraser, 2017). We now demonstrate genetically that eIF4A has an eIF3-related function independently of eIF4F. Our data showing that eIF3 and eIF4A interact in an ATPase-dependent manner and that eIF4A helicase activity is required for dendrite pruning are consistent with both the above proposals.

Taken together, our data suggest that eIF3-eIF4A are part of a bypass mechanism that ensures translation of crucial ecdyson-induced mRNAs in the absence of an eIF4E-dependent translation initiation during developmental neuronal remodeling in the Drosophila PNS.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.07.074.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.
REFERENCES


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- rev primer for GFP RT-PCR

**Recombinant DNA**

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- pUAST attB<sup>-FL</sup>ΔH2 eIF4A E172Q
- pUAST attB<sup>-5'UTR</sup>ΔMical-GFP
- ActSC-GAL4
- pBS SK(-)5'UTRΔMical-RLuc
- pMRNA mRNAExpress-RLuc
- pGEX4T-1 MicalC245 for recombinant protein and antibody production

**Software and Algorithms**

- Fiji
- Graphpad Prism
CONTACT FOR REAGENT AND RESOURCE SHARING

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EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly Strains
All strains and crosses were kept at 25 °C under standard conditions. For expression in c4 da neurons, we used ppp-GAL4 insertions on the second and third chromosomes or ppp-GeneSwitch (Grueber et al., 2007; Rumpf et al., 2011). C3 da neurons were labeled by GAL419-12 (Xiang et al., 2010), and c1 da neurons with GAL42-21. MARCM clones were induced with SOP-FLP (Matsubara et al., 2011) and labeled by tdTomato expression under nsyb-GAL4109G30 or mCD8GFP expression under GAL4109G30. Mutant alleles used for MARCM were (1) elf4e110568B11, FRT2A, (2) P{lacW}elf4e110568A, FRT40A (Galloni and Edgar, 1999), (3) FRT42D, elf3b11443D0 (Kyoto #114685) and (4) elf3g114, FRT19A (BL # 52344). Other fly lines were UAS-Mical, UAS-MicalΔC (Terman et al., 2002), UAS-GFP::Mical, UAS-GFP::MicalΔCH, UAS-GFP::MicalΔRedox (Hung et al., 2010), UAS-Sox14 (Rumpf et al., 2014), UAS-tomato (Han et al., 2014), UAS-GFP (BL #5441), UAS-4E-BP LL (Miron et al., 2001), UAS-RNAi lines were: elf4e1 (BL #34096, VDRC 7800), elf4g1 (VDRC 17003, BL #33049), elf4b (VDRC 31664, BL #57305), elf4a (#1, VDRC 42202, #2, VDRC 42201, #3, BL #39270), elf3c (VDRC 26667), elf3e (VDRC 27032). UAS-cherry RNAi (BL #35785) or UAS-Orco RNAi (BL #31278) were used as controls. UAS-RNAi lines were used with UAS-dcr2 (Dietzl et al., 2007). UAS-dMetRS1262G was used for fluorescent noncanonical amino acid tagging (FUNCAT) (Erdmann et al., 2015).

METHOD DETAILS

Cloning and transgenes
eIF4A1172C, elf4A3AT and 5’UTR-Mical-GFP were cloned into pUAST attB by standard methods, and point mutations were introduced by Quikchange mutagenesis. Transgenes were injected in flies carrying VK37 or attP2 acceptor sites.

Dissection, Microscopy and Live Imaging
For analysis of pruning defects, pupae were dissected out of the pupal case at 18 h APF and analyzed live using a Zeiss LSM710 confocal microscope. For elf4A4 genetic interactions, candidates were crossed to a second chromosome insertion of ppp-GAL4 combined with UAS-Cd8GFP and UAS-elf4a RNAi #1 (VDRC 42202).

5’UTR-Mical-GFP reporter
Reporter lines were established with UAS-GFP, and UAS-5’UTR-Mical-GFP on the second chromosome and ppp-GAL4, UAS-tomato on the third. These lines were then crossed to UAS-4E-BP LL (and UAS-lacZ as control) or UAS-dcr2; UAS-elf4a RNAi#1 and UAS-dcr2; UAS-elf3e RNAi lines, respectively (and UAS-dcr2; UAS-Orco RNAi as control).

Fluorescent Noncanonical Amino Acid Tagging
Translation rate measurements in c4 da neurons were done as described (Niehues et al., 2015). Briefly, larvae were kept on food containing 4 mM ANL for 48 h. Click chemistry with TAMRA dye was performed after dissection and fixation in 4% formaldehyde.

Antibodies and immunohistochemistry
Pupal body wall files were dissected quickly in PBS and fixed in PBS containing 4% formaldehyde for 20 minutes. Mouse, or chicken anti-GFP antibodies were from Life technologies or Aves labs, respectively, rabbit anti-DsRed from Clontech, and rat anti-mcherry.

In vitro transcription and translation
For the production of Renilla luciferase (RLuc) mRNAs, the following plasmids were used: pMRNA10 mRNAExpress-RLuc (Holstein et al., 2016) (for production of a cap-dependent control mRNA) and pBS KS(-)T7-5’-UTR-Mical-RLuc (for analysis of cap-dependence of the Mical 5’-UTR). In vitro T7 transcription was performed in 25 µl scale in 1x transcription buffer (200 mM Tris-HCl (pH 7.9), 30 mM MgCl2, 50 mM DTT, 50 mM NaCl, 10 mM spermidine using 100 ng DNA template (linearized plasmid), 1 mM ARCA or ApppG cap analog, 30 U Ribolo RNAse Inhibitor, 50 U T7 RNA polymerase and 0.1 U pyrophosphatase for 3 h at 37°C. DNA templates were digested by with 2 U DNase I for 1 h at 37°C and mRNAs were purified using the RNA Clean & Concentrator™-5 Kit (Zymo Research). Digestion of non-capped ppp-RNAs were performed in 20 µl scale in 1x reaction buffer (epicenter) using 2.5 µg purified mRNA and 20 U RNA 5’ polyphosphatase for 30 min at 37°C. Immediately after the incubation, 5 mM MgCl2 and 1 U XRN-1 were added, and the reaction mixture was incubated at 37°C for 1 h. The capped mRNAs were purified using the RNA Clean & Concentrator™-5 Kit (Zymo Research).
RNA purity and integrity was confirmed by RNA gel analysis using 7.5% denatured polyacrylamide gel (25% acrylamide/bisacrylamide 19:1 and 50% urea).

In vitro translation was performed using the Retic Lysate IVT™ Kit (Invitrogen), 0, 6.25, 12.5, 25 and 37.5 ng mRNA were incubated with 8.5 μL rabbit reticulocyte lysate, 0.05 mM L-methionine and 1x translation mix, respectively, in an adjusted volume of 12.5 μL for 90 min at 30 °C, and reactions were stopped on ice. Luminescence measurements were performed using the Gaussia-Juice Luciferase Assay Kit (pjk) on a Tecan Infinite M1000 PRO. The luciferase activity was determined by adding 2 μL of the translation sample to a 96-well plate followed by injection of 50 μL freshly prepared reaction mix (1:50 coelenterazine: reaction buffer, pjk) and the integration time was 3 s. Each translation sample was assayed for luciferase activity in duplicate. Translational efficiency data were normalized to ARCA capped RLuc mRNA.

**Immunoprecipitations and affinity pulldown**

For immunoprecipitation of FLAG-tagged eIF4A versions, 4x10⁶ S2R+ cells each were transfected with ActC5-GAL4 and the corresponding pUAST attB-FLAGeIF4A plasmids (or an empty vector) using Fugene reagent (Promega). After 48-72 hours, cells were washed with ice-cold PBS, lysed in 150 mM NaCl, 50 mM Tris/HCl pH 7.4, 2 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 5% glycerol and complete protease inhibitors for 20 minutes on ice. After clearing, lysates were incubated with anti-FLAG beads for 3 hours, washed three times with lysis buffer, and eluted with SDS loading buffer. Samples were analyzed by western blotting.

For eIF3b-RNA immunoprecipitations, 4x10⁶ S2R+ cells each were transfected with pUAST attB-5'UTRMical-GFP and empty pUAST attB (ratio 1:2 for low expression) coupled to Dynabeads Protein G over night. Beads were washed three times with lysis buffer and once with PBS, 5%–10% of precipitates were removed and dissolved in SDS sample buffer for Western analysis, the rest of the beads was eluted with Trizol reagent to elute bound RNA according to the supplier’s instructions. Bound RNA was reverse transcribed with a GFP-specific primer, and analyzed by GFP PCR.

In order to test for cap interaction of translation initiation factors, 8x10⁶ untransfected S2R+ cells were mock-treated or treated with 20 μM 20-hydroxyecdysone for 1 hour, washed with ice-cold PBS, and lysed in 200 mM NaCl, 25 mM Tris/HCl pH 7.4, 2 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 0.5 mM DTT and complete protease inhibitors for 20 minutes on ice. Lysates were then immunoprecipitated with 3 μL eIF3b serum (gift from M. Hentze, EMBL) or 3 μL control serum (unrelated preimmune) coupled to Dynabeads Protein G over night. Beads were washed three times with lysis buffer and once with PBS, 5%–10% of precipitates were removed and dissolved in SDS sample buffer for Western analysis, the rest of the beads was eluted with Trizol reagent to elute bound RNA according to the supplier’s instructions. Bound RNA was reverse transcribed with a GFP-specific primer, and analyzed by GFP PCR.

**Polysome profile**

For polysome profiles from Drosophila S2R+ cells, 3x10⁷ cells each were grown for 48 hours. After mock treatment or treatment with 0.3 μM RocA, the medium was removed, and cells were first washed with ice-cold PBS and frozen in liquid nitrogen. Frozen cells were then lysed on the plate in 10 mM Tris/HCl pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM DTT, 1% Triton X-100, 0.5% deoxycholate, and 100 μg/ml cycloheximide and collected. After clearing by centrifugation, lysates were flash-frozen in liquid nitrogen. Samples were centrifuged on a 10 – 50% linear sucrose gradient in a TH-641/SW41 rotor (Thermo Scientific/Beckman Coulter), and gradients were fractionated using a density gradient fractionator (Isco) and a SYR-101 syringe pump (Brandel) with continuous monitoring of A254.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Imaging analysis**

Pruning phenotypes in Figures 1, 2, 4, 6 and 7 were analyzed by counting the number of neurons that still had dendrites attached to the soma, these data were analyzed using a two-tailed Fisher’s exact test. To assess severity, we also counted the number of primary and secondary branches still attached to the soma at 18 h APF. These data were analyzed using Wilcoxon’s test.

GFP reporter fluorescence intensity in c4 da neurons (Figure 5; Figure S5) was measured on a Zeiss LSM710 confocal microscope under identical, nonsaturating conditions for each pair of manipulations and background was subtracted. The relative reduction of reporter intensities by experimental manipulations compared to control were calculated as (1-(fluorescenceexp/fluorescencecont))×100. These data were compared by Wilcoxon’s test. For tdtomato fluorescence, reporter fluorescence ratios of experimental and control RNAi was calculated and compared using a Student’s t test.