The DLK-1 Kinase Promotes mRNA Stability and Local Translation in *C. elegans* Synapses and Axon Regeneration

Dong Yan,[1,4] Zilu Wu,[1,4] Andrew D. Chisholm,[1,2] and Yishi Jin[1,3,4,*]
[1]Division of Biological Sciences, Section of Neurobiology
[2]Division of Biological Sciences, Section of Cell and Developmental Biology
[3]Department of Cellular and Molecular Medicine, School of Medicine
University of California, San Diego, La Jolla, CA 92093, USA
[4]Howard Hughes Medical Institute
*Correspondence: yijin@ucsd.edu
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SUMMARY

Growth cone guidance and synaptic plasticity involve dynamic local changes in proteins at axons and dendrites. The *D*ual-*L*eucine zipper Kinase MAPKK*K* (DLK) has been previously implicated in synaptogenesis and axon outgrowth in *C. elegans* and other animals. Here we show that in *C. elegans* DLK-1 regulates not only proper synapse formation and axon morphology but also axon regeneration by influencing mRNA stability. DLK-1 kinase signals via a MAPKAP kinase, MAK-2, to stabilize the mRNA encoding CEBP-1, a bZip protein related to CCAAT/enhancer-binding proteins, via its 3′UTR. Inappropriate upregulation of *cebp-1* in adult neurons disrupts synapses and axon morphology. CEBP-1 and the DLK-1 pathway are essential for axon regeneration after laser axotomy in adult neurons, and axotomy induces translation of CEBP-1 in axons. Our findings identify the DLK-1 pathway as a regulator of mRNA stability in synapse formation and maintenance and also in adult axon regeneration.

INTRODUCTION

Neurons respond to environmental stimuli and insults in a compartmentalized manner. Local protein synthesis in dendrites and axonal growth cones has emerged as a major mechanism allowing compartmentalized responses in growth cone guidance (Piper and Holt, 2004) and neuronal plasticity (Sutton and Schuman, 2005). Several mRNAs are known to be transported and localized to growth cones or axons; transport of such mRNAs often is mediated by their 3′ untranslated regions (3′UTRs) (Lin and Holt, 2008).

However, it remains controversial whether mature neurons employ axonal regulation of mRNA and local protein synthesis. mRNA-binding proteins, such as Zip-code binding proteins (ZBPs) and cytoplasmic polyadenylation element-binding proteins (CPEBs), are abundant in growing neurites but are mostly undetectable in axons and synapses of mature neurons (Brittis et al., 2002; Leung et al., 2006). Polyribosomes are rarely seen in axons (Giuditta et al., 2002). Nonetheless, several reports suggest that axonal mRNA regulation must occur in mature neurons. In *Aplysia* neurons, mRNA for the peptide neurotransmitter Sensorin is concentrated at synapses upon contact with target motor neurons (Lyles et al., 2006), and local translation and secretion of Sensorin promote synapse maturation and plasticity (Hu et al., 2004; Wang et al., 2009). A number of mRNAs are upregulated in axons of injured adult dorsal root ganglion (DRG) neurons (Wang et al., 2007). Regulation of Ran GTPase via local translation of RanBP1 is implicated in retrograde signaling of axon injury (Yudin et al., 2008). Local translation thus can transmit injury signals and initiate local repair processes.

CCAAT/enhancer-binding proteins (C/EBP) are widely expressed basic-leucine-zipper (bZip) domain transcription factors with long-studied roles in cell proliferation, differentiation, and stress (Ramji and Foka, 2002). In neurons the transcriptional roles of C/EBP proteins have been linked to learning and memory (Alberini et al., 1994; Chen et al., 2003). Learning and memory tasks trigger activation of Erk or p38 kinases, leading to phosphorylation of specific C/EBP isoforms. mRNAs of murine and leech C/EBP are also upregulated following axonal injury, and murine C/EBPβ can activate the transcription of an α-tubulin gene associated with injury responses (Korneev et al., 1997; Nadeau et al., 2005). The pathways that induce C/EBP after injury are largely unknown.

We have been studying the function of the conserved ubiquitin E3 ligase RPM-1 in synaptogenesis and axon formation in *C. elegans*. *C. elegans* neurons have simple unipolar or bipolar axon trajectories and form synapses en passant. For example, the ALM and PLM mechanosensory neurons have a long axon that bifurcates into a branch exclusively forming synapses and another branch transducing mechanoreception (Chalfie, 1995). RPM-1 regulates the organization and stabilization of presynaptic terminals and axon termination in both mechanosensory and motor neurons (Schaefer et al., 2000; Zhen et al., 2000). A major target of RPM-1 ubiquitination is the Dual-Leucine zipper

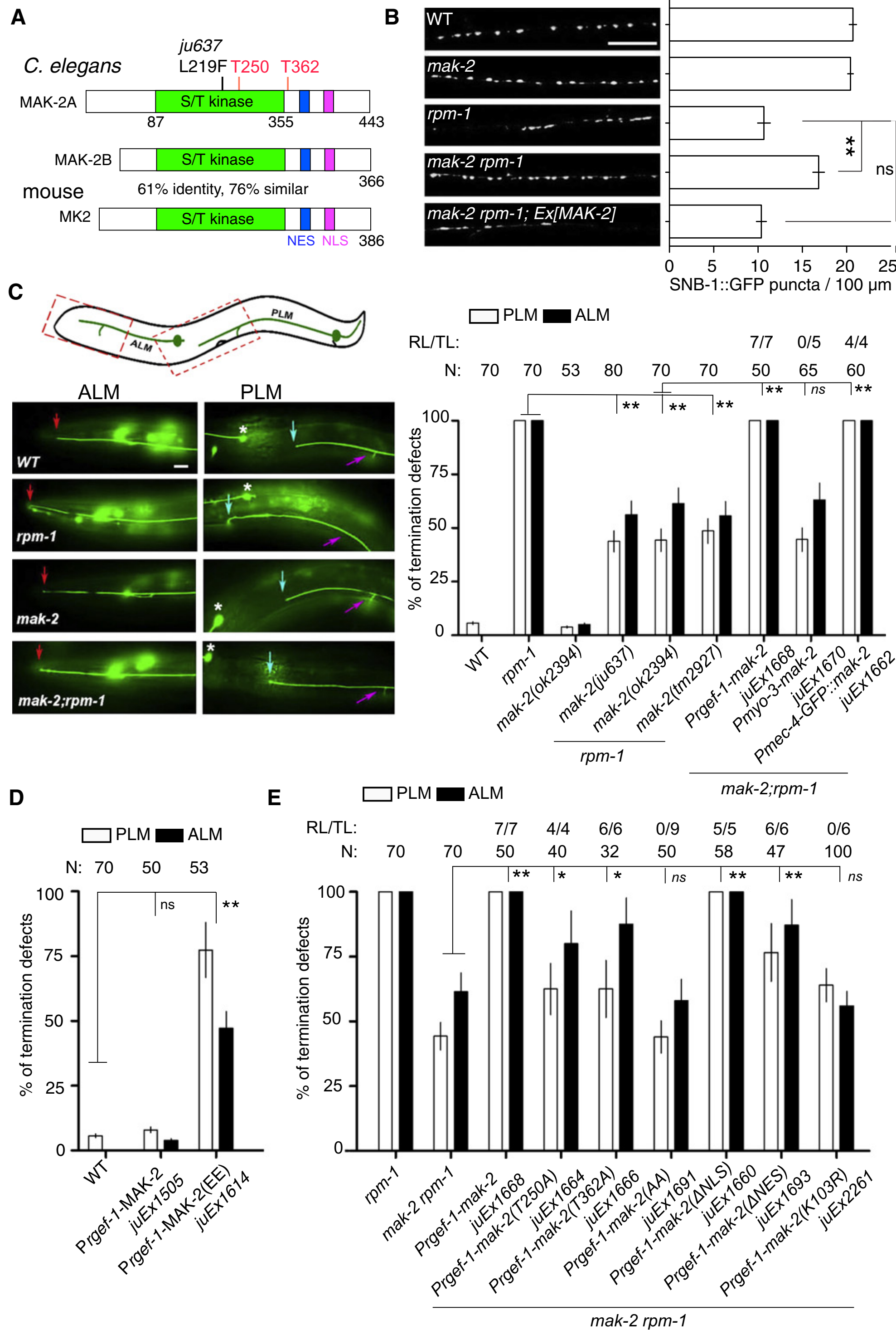

Figure 1. Loss of Function in *mak-2* Suppresses *rpm-1(lf)*

(A) MAK-2 is a member of the MAPKAPK family and has two isoforms that differ at their N termini. Mouse MK2 is NP_032577.

(B) *mak-2(lf)* suppresses motor neuron presynaptic morphology defects of *rpm-1* cell autonomously. Images are *juIs1[Punc-25-SNB-1::GFP]* in the adult dorsal cord. SNB-1::GFP puncta are uniformly shaped and evenly distributed in wild-type and *mak-2* but are irregular and fewer in *rpm-1* mutants. Right panel, quantification of SNB-1::GFP puncta # (n ≥ 12 animals per genotype; data shown as mean ± standard error of the mean [SEM]).

(C) *mak-2* suppresses touch neuron axon termination defects of *rpm-1* cell autonomously. Images are *muIs32[Pmec-7-GFP]* in adults. The ALM axon (red arrows) ends before the tip of the nose in wild-type but overextends and frequently loops in *rpm-1* mutants. The PLM axon (blue arrows) terminates posterior to the ALM

Kinase DLK-1 MAPKKK, which acts in a MAPK cascade consisting of the MAPKK MKK-4 and the p38 kinase PMK-3 (Nakata et al., 2005). By controlling the level of DLK-1, RPM-1 keeps the activity of the DLK-1 cascade at optimal levels. This negative regulation of the DLK pathway by ubiquitin-mediated protein degradation is conserved in *Drosophila* and mammalian neurons (Collins et al., 2006; Jin and Garner, 2008; Lewcock et al., 2007).

The targets of the DLK-1/p38 cascade have not previously been identified. Here we report the identification of MAK-2, a member of the MAPKAP kinase family, and CEBP-1, a member of the C/EBP class of bZip factors, as effectors of the *C. elegans* DLK-1 cascade. MAPKAPKs are conserved Ser/Thr kinases that are direct targets of p38 and Erk kinases (Gaestel, 2006). We find that *cebp-1* mRNA is destabilized by RPM-1 via the DLK-1/MAK-2 cascade, acting on the *cebp-1* 3′UTR. The DLK-1/MAK-2/CEBP-1 pathway is essential for regenerative regrowth of mature axons following laser axotomy, in part by regulating axonal *cebp-1* mRNA stability and translation.

RESULTS

Identification of MAPKAPK MAK-2 as a Suppressor of *rpm-1*

Previous screens for suppressors of *rpm-1(lf)* neuronal defects uncovered many alleles of the MAPK genes *dlk-1, mkk-4,* and *pmk-3* (Nakata et al., 2005). By analyzing a large number of additional *rpm-1* suppressors, we identified two new loci (see Supplemental Data available online). The suppressor mutation *ju637* was mapped to a region containing *mak-2,* which encodes a MAP kinase-activated protein kinase (MAPKAPK) related to murine MAPKAPK2 (MK2) (Figures 1A and S1A). *ju637* results in a replacement of Phe at an invariant Leu (L219) in the kinase domain (Figure 1A). Two deletion alleles of *mak-2* suppressed *rpm-1(lf)* to a similar degree as did *ju637* (Figures 1C and S1A), suggesting that all three alleles eliminate *mak-2* function. A *mak-2* reporter was expressed in the nervous system (Figure S1B). By expressing *mak-2* under the control of neuron-type-specific promoters we found that *mak-2* functions autonomously in neurons (Figures 1B and 1C). These results show that, like *dlk-1, mkk-4*, and *pmk-3*, loss of *mak-2* function suppresses the synaptic and axon defects of *rpm-1* mutants. *mak-2* mutants did not exhibit overt neuronal or behavioral phenotypes (Figures 1B and 1C).

MAPKAPKs are activated by p38-dependent phosphorylation of Thr and Ser residues (Gaestel, 2006), two of which are conserved among all MAPKAPKs and correspond to Thr250 and Thr362 of MAK-2 (Figure 1A). In a yeast two-hybrid assay, we found that MAK-2 interacted with PMK-3 (Figure S2A). This interaction was also observed in coimmunoprecipitation studies in HEK293T cells (Figure S2B), consistent with the idea that MAK-2 is a target of PMK-3. We next tested whether MAK-2 function depends on phosphorylation. Transgenes expressing a predicted nonphosphorylatable form of MAK-2, in which both Thr residues were mutated to Ala, failed to rescue suppression of *rpm-1(lf)* by *mak-2(lf)*; mutation of either Thr residue partially abrogated rescuing activity (Figure 1E). In contrast, a phosphomimetic form of MAK-2, in which both Thr were mutated to Glu, caused gain-of-function defects resembling those of *rpm-1(lf)* mutants (Figure 1D). The AA and EE mutations did not appear to alter MAK-2 expression levels (Figure S1E). Further, a kinase-dead MAK-2(K103R) lacked rescuing activity (Figure 1E). These data suggest that MAK-2 activation via phosphorylation of the two Thr residues is critical for its function in synapse development and axon termination.

Loss of Function in the bZip Protein CEBP-1 Suppresses *rpm-1*

We mapped the *rpm-1* suppressor mutations *ju659* and *ju640* to the left arm of chromosome X (Figure S3A). Transgenes containing cosmid W05H7 rescued suppression of *rpm-1(lf)* by *ju659* (not shown). We localized rescuing activity to a 7.5 kb DNA region containing the gene D1005.3, which encodes a protein of 358 aa containing a bZip domain most closely related to those of the C/EBP family (Figures 2A, S3B, and S3C) (Landschulz et al., 1988). We refer to this gene as *cebp-1*. *ju659* changes Arg290 to Cys and *ju640* changes Ser289 to Leu in the basic region of the bZip domain (Figures 2A and S3B). Both residues are highly conserved among bZip proteins; previous studies have shown that the Arg is critical for DNA binding (Kim et al., 1993). The *cebp-1(tm2807)* deletion allele suppressed *rpm-1(lf)* to a similar degree as did *ju659* (Figures 2C and S3A). Neuronal expression of *cebp-1* rescued the suppression of *rpm-1(lf)* in a cell-autonomous manner (Figure 2C). CEBP-1 lacking the bZip domain lacked rescuing activity (Figure 2D). bZip proteins usually function as dimers, and mutations impairing DNA binding often have dominant-negative effects (Ramji and Foka, 2002). Transgenic overexpression of *cebp-1* containing either the *ju659* (R290C) or *ju640* (S289L) mutation partly suppressed *rpm-1(lf)* defects (Figure 2E). Although *ju659* and *ju640* are recessive to wild-type for suppression of *rpm-1*, these findings suggest that both mutations may have dominant-negative activities by inhibiting either endogenous CEBP-1 or its binding partners. These data indicate that CEBP-1 acts similarly to MAK-2 and the MAP kinases in neurons.

To establish the order of *mak-2* and *cebp-1* function in the *dlk-1* pathway, we performed genetic epistasis analysis. Double mutants between null mutations in *mak-2*, *cebp-1*, or *pmk-3* developed superficially normal synapses and axons (not shown). *pmk-3;mak-2* or *mak-2;cebp-1* double mutants suppressed the neuronal defects of *rpm-1(lf)* to a level comparable to that of

cell body (*) in wild-type but overextends and turns ventrally in *rpm-1.* The PLM synaptic branch (purple arrows) is frequently missing in *rpm-1.* Axon termination defects of PLM and ALM neurons are quantified as % of total # of animals. Quantification of PLM synaptic branch shows similar pattern (not shown).

(D) Expression of MAK-2(EE) induces gain-of-function phenotypes.

(E) MAK-2 rescuing activity is abolished by mutation of Thr250 and Thr262 or of Lys103 in the ATP-binding site but is not affected by deletion of the NLS or NES.

(B–E) Unless noted, alleles are *rpm-1(ju44)* and *mak-2(ok2394)*. Promoters: P*rgef-1* (F25B3.3) for pan-neural; P*myo-3* for body wall muscles; P*mec-4* for touch neurons; P*unc-25* for GABA motor neurons. N, total # of animals; TL, total # of transgenic lines; RL, # of transgenic lines showing rescuing activities. Data shown as mean ± standard error of proportion (SEP) in (C)–(E). Statistics in this and subsequent figures, ANOVA (B) or Fisher exact test (C–E). *p < 0.05; **p < 0.01; ns, not significant. Scales, 10 μm.

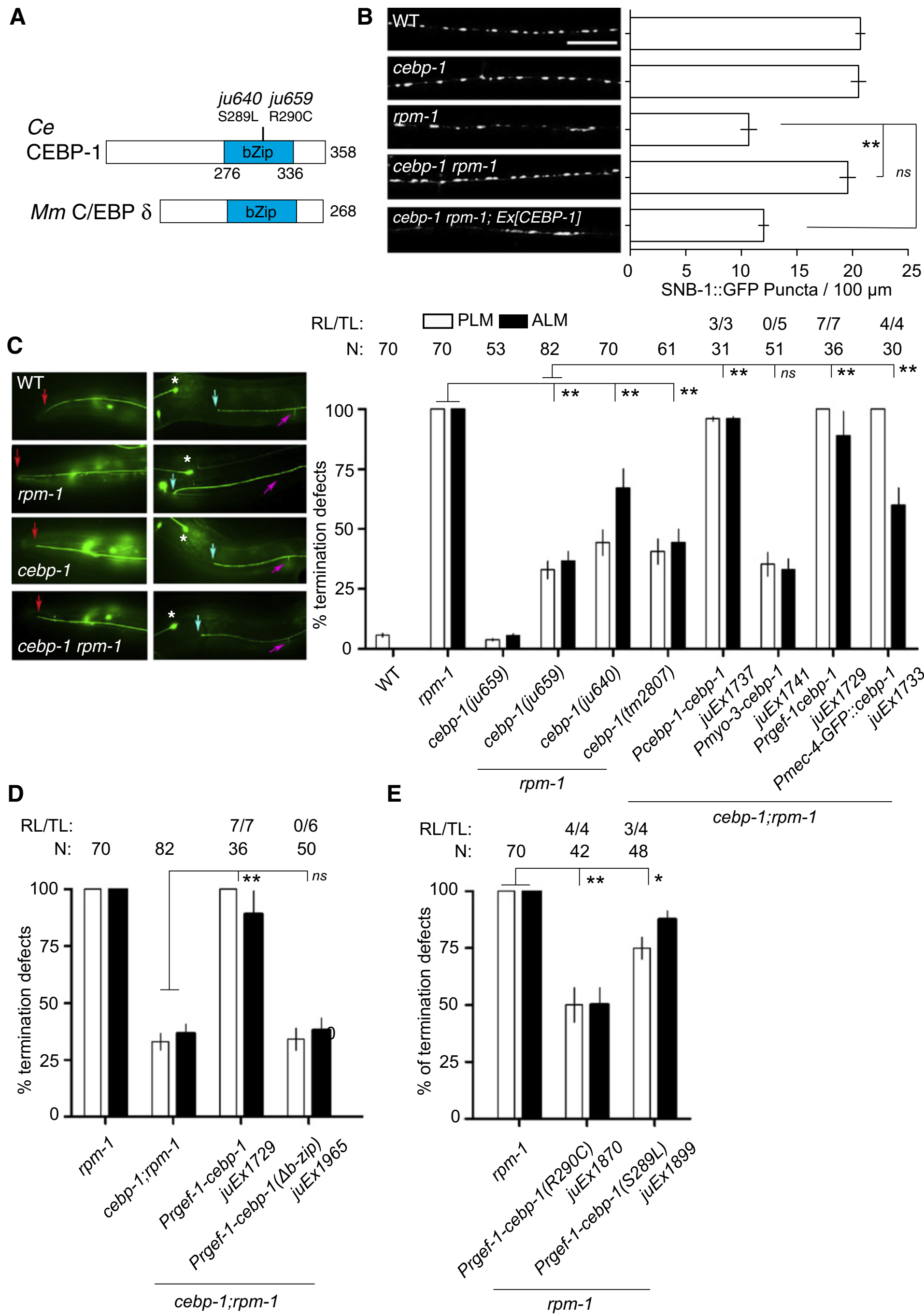

Figure 2. Loss of Function in *cebp-1* Suppresses *rpm-1(lf)*

(A) CEBP-1 contains a bZip domain most similar to those of C/EBP proteins.

(B) *cebp-1(lf)* suppresses motor neuron presynaptic morphology defects of *rpm-1* cell autonomously (*juIs1* marker). Right panel, quantification of SNB-1::GFP puncta (n ≥ 10 animals per genotype; data shown as mean ± SEM).

(C) *cebp-1(lf)* suppresses the touch axon overextension of *rpm-1* cell autonomously (*muIs32* marker).

(D) The bZip domain is required for *cebp-1* function.

(E) CEBP-1(R290C) and CEBP-1(S289L) have dominant-negative activity.

(B–E) Unless noted, alleles are *rpm-1(ju44)* and *cebp-1(ju659)*. Data shown as mean ± SEP in (C)–(E). Quantitation, labels, details of mutant phenotypes, promoters, and statistics as in Figure 1. Scales,10 μm.

each single mutant (Figure S4A). Overexpression of *dlk-1* causes abnormal synapse development and axon termination defects resembling those of *rpm-1(lf)* (Nakata et al., 2005). This gain-of-function effect was largely eliminated in *mak-2(lf)* or *cebp-1 (lf)* mutants (Figure S4B). Additionally, the gain-of-function effect of phosphomimetic *mak-2(EE)* in wild-type animals was suppressed by *cebp-1(lf)* but not by *pmk-3(lf)* (Figure S4B). Thus, both *mak-2* and *cebp-1* act downstream of the *dlk-1/mkk-4/pmk-3* cascade, and *cebp-1* acts downstream of *mak-2* (Figure S4C).

RPM-1 and the MAP Kinases Regulate *cebp-1* mRNA via Its 3′UTR

In the course of transformation rescue of *cebp-1*, we observed that *cebp-1* transgenes lacking the 3′UTR often caused gain-of-function defects such as touch axon overextension and uncoordinated movement, reminiscent of those resulting from constitutive activation of the DLK-1 pathway (Nakata et al., 2005). Analysis of *cebp-1* cDNAs revealed that *cebp-1* transcripts contain a long 3′UTR (Figure S5A). To test whether the DLK-1 pathway might regulate *cebp-1* mRNA levels, we first performed quantitative RT-PCR and found that *cebp-1* mRNA was increased in *rpm-1(lf)* mutants compared to wild-type animals or MAP kinase mutants (Figure 3A). The increase of *cebp-1* mRNA levels in *rpm-1(lf)* mutants was eliminated by loss of function in *dlk-1*, *pmk-3*, or *mak-2* (Figure 3A), indicating that activation of the MAP kinases caused by *rpm-1(lf)* results in upregulation of *cebp-1* mRNA. *cebp-1(ju659)* did not affect the increase of *cebp-1* mRNA level caused by *rpm-1(lf)* (Figure 3A), suggesting that the effect on *cebp-1* mRNA does not involve transcriptional autoregulation of *cebp-1*. Expression of a *cebp-1* promoter reporter was unaltered in *rpm-1(lf)* compared to the wild-type (Figures S6A and S6B). We further compared the stability of *cebp-1* mRNA by qRT-PCR on animals cultured in the presence of α-amanitin to block transcription (Sanford et al., 1983). The half-life of *cebp-1* mRNA was increased 3-fold in *rpm-1* mutants compared to wild-type (Figure S6C). This increase in stability was abolished in *dlk-1;rpm-1* animals but not in *rpm-1;cebp-1* animals. We infer that RPM-1 acts via the DLK-1 pathway to regulate *cebp-1* mRNA stability.

To test whether the 3′UTR of *cebp-1* is required for *cebp-1* mRNA regulation, we expressed mCherry-tagged full-length *cebp-1* with its own 3′UTR or with a heterologous 3′UTR, driven by the *unc-25* promoter (Supplemental Data). In *rpm-1(lf)* mutants the fluorescence intensity of mCherry::CEBP-1 with the *cebp-1* 3′UTR was over 6-fold higher than in wild-type (Figure 3B). This increase in fluorescence intensity reflected a corresponding increase in transgene mRNA levels in *rpm-1* (Figures S6D and S6E). mCherry::CEBP-1 fluorescence intensity was also significantly increased by transgenic expression of MAK-2(EE) but not MAK-2(AA) (Figure 3B). The 3′UTR of the myosin gene *unc-54* is known to stabilize mRNAs (Fire et al., 1990). However mCherry::CEBP-1 transgenes containing the *unc-54* 3′UTR did not show increased fluorescence intensity in *rpm-1(lf)* (Figure 3C). Indeed, the *cebp-1* 3′UTR was sufficient to confer *rpm-1*-dependent regulation on mCherry (Figure 3D). Transgenic expression of *cebp-1* with the *unc-54* 3′UTR consistently caused more severe defects in motor neuron synapses (Figure 3E) and touch neuron morphology (Figure 3F) than did transgenic expression of *cebp-1* with the *cebp-1* 3′UTR. Overexpression of *cebp-1* in muscles did not generate neuronal abnormalities (Figure 3F), suggesting that regulation of *cebp-1* mRNA by *rpm-1* and the MAP kinases occurs in neurons. Thus, activation of the DLK-1 cascade, either by loss of function in *rpm-1* or by constitutive activation of MAK-2, can stabilize the *cebp-1* mRNA via its 3′UTR.

MAK-2 and CEBP-1 Are Present in Presynaptic Termini

We next examined localization of MAK-2 and CEBP-1 proteins in touch and motor neurons. Expression of mCherry-tagged MAK-2 or CEBP-1 in motor neurons fully rescued *rpm-1* suppression phenotypes of *mak-2(lf)* or *cebp-1(lf),* respectively (Figures 1B and 2B), and did not cause overt defects in wild-type animals (Figures 4A and 4B), suggesting that the expression levels were close to physiological. mCherry::MAK-2 was consistently present in cell bodies and synapses (Figure 4A) but rarely seen in axon commissures (not shown). MAK-2 was broadly localized at motor neuron synapses and significantly colocalized with synaptobrevin/SNB-1 but not with RPM-1 (Figures 4A and S7A–S7C). Unexpectedly, mCherry::CEBP-1 was also detected at synapses, in addition to the cytoplasm and nucleus of the soma (Figure 4B). At synapses, mCherry::CEBP-1 colocalized with SNB-1, but not with RPM-1 (Figure S7D and S7E). MAK-2 and CEBP-1 partly colocalized at synapses (Figure S7F). In touch neurons, CEBP-1 was present in the soma, in the synaptic area, and at discrete regions along axons (Figure 5A).

As RPM-1 and DLK-1 are present at synapses (Abrams et al., 2008; Nakata et al., 2005), the synaptic localization of MAK-2 and CEBP-1 suggested that the DLK-1/MAK-2 cascade might act locally to promote *cebp-1* mRNA stability. We first tested this by manipulating MAK-2 localization. As predicted, removing the nuclear localization signal (NLS) caused MAK-2 to accumulate in the somatic cytoplasm, whereas removing the nuclear export signal (NES) confined MAK-2 to the nucleus (Figure S1D). Neither manipulation affected the synaptic localization of MAK-2 (Figure S1D). Both constructs could fully rescue the *rpm-1* suppression phenotype of *mak-2(lf)* (Figure 1E). Phosphomimetic MAK-2(EE), which causes abnormalities in synapses, was also localized at synapses but excluded from the nucleus (Figure S1E). These data suggest that nucleocytoplasmic shuttling may not affect MAK-2 synaptic function and that activated MAK-2 might function at synapses. We therefore attempted to target activated MAK-2(EE) to motor neuron synapses by tagging MAK-2 with the PHR domain from RPM-1, which can target GFP to synapses (Abrams et al., 2008). mCherry::PHR::MAK-2 was concentrated at synapses, adjacent to SNB-1::GFP, and weakly detected in the somatic cytoplasm and excluded from the nucleus (Figure 4C). Synaptic targeting of activated MAK-2(EE), but not of wild-type MAK-2 or inactive MAK-2(AA), disrupted presynaptic morphology to a degree similar to those caused by untargeted MAK-2(EE) (Figures 4D and 4E). These results suggest that, in addition to possible roles in the soma and nucleus, MAK-2 function may be regulated at synapses.

cebp-1 mRNA Is Present in Synapses and Axons

The above observations raised the possibility that *cebp-1* mRNA might be localized at synapses. To test this, we used an in vivo

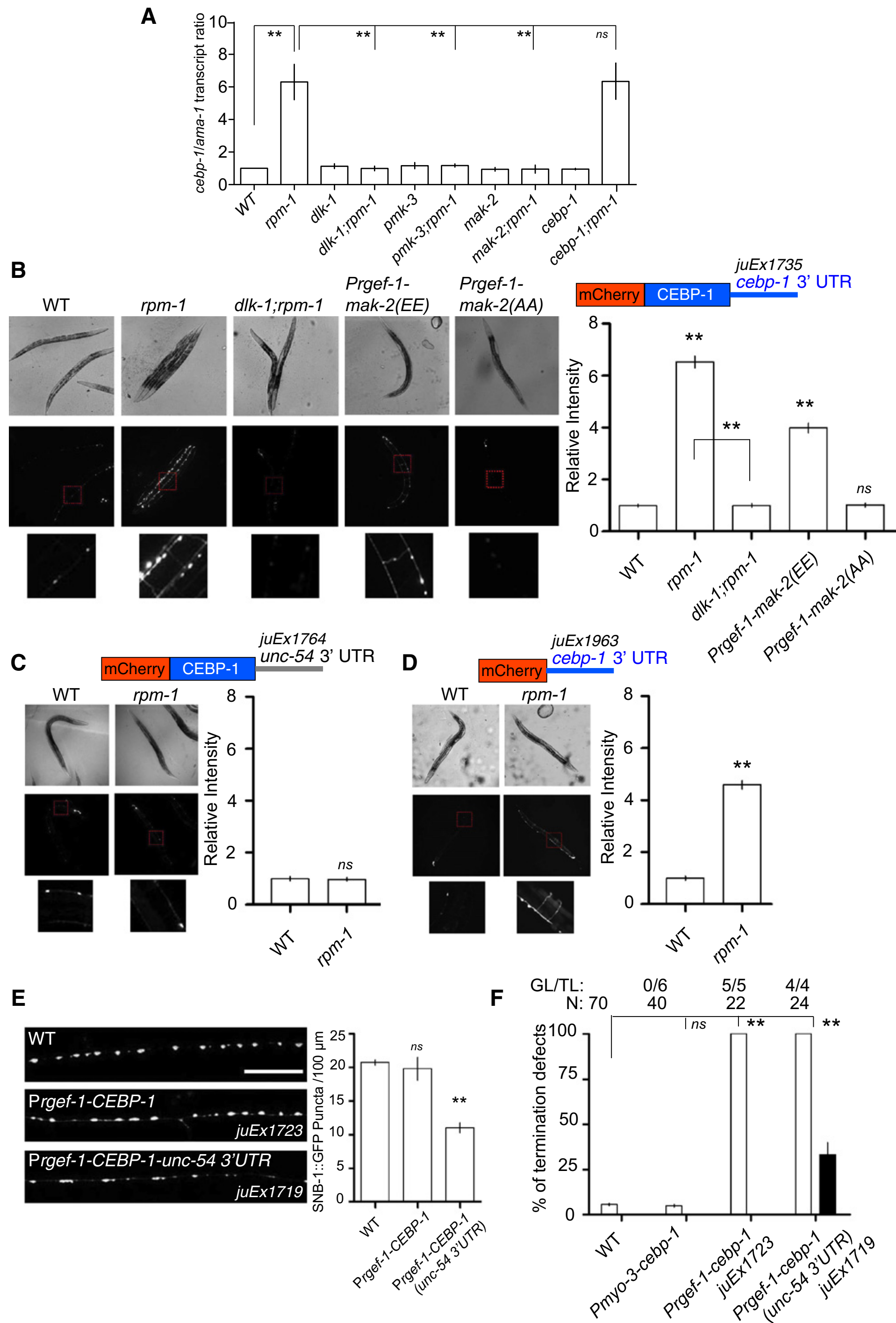

Figure 3. *rpm-1* and the MAP Kinases Regulate Levels of *cebp-1* mRNA via Its 3′UTR

(A) Quantitative RT-PCR analysis shows upregulation of *cebp-1* mRNA in *rpm-1(lf),* which is eliminated in *dlk-1*, *pmk-3*, or *mak-2* mutants (n = 3). The control transcript *ama-1* encodes the large subunit of RNA polymerase II (Sanford et al., 1983).

(B–D) Transgenic expression of mCherry reporters in GABA motor neurons and quantification of fluorescence (n = 35 per genotype). Blue lines and letters denote inclusion of the *cebp-1* 3′UTR in the transgene. (B) CEBP-1 with its own 3′UTR is more highly expressed in *rpm-1(lf)* mutants and *mak-2(EE)* transgenic animals than in wild-type, *dlk-1(lf)*, and *MAK-2(AA)* transgenic animals. (C) A CEBP-1 transgene with the *unc-54* 3′UTR is expressed at similar levels in *rpm-1(lf)* and wild-type. (D) The *cebp-1* 3′UTR confers upregulation of mCherry in *rpm-1(lf)*.

(E) Expression of *cebp-1* with the *unc-54* 3′UTR causes abnormal motor synapses. Data in (A)–(E) shown as mean ± SEM; n = 30 animals per genotype; statistics, Anova.

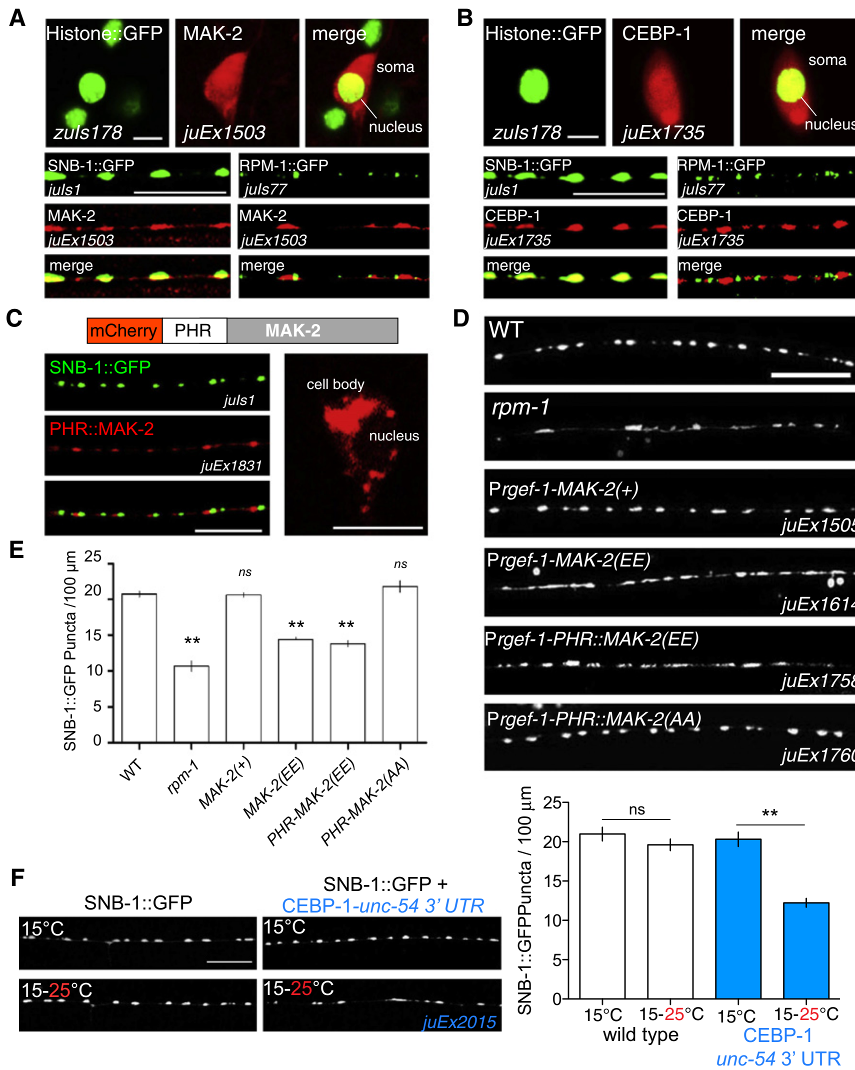

Figure 4. MAK-2 and CEBP-1 Are Present at Motor Neuron Synapses

(A) Functional mCherry::MAK-2 in GABA motor neurons. Top panels are motor neuron soma in animals coexpressing P*unc-25*-mCherry::MAK-2 (red) and Histone::GFP (*zuIs178*). MAK-2 is present both in nucleus and cytoplasm. Bottom panels are motor neuron synapses in the dorsal cord coexpressing mCherry::MAK-2 with P*unc-25*-SNB-1::GFP (*juIs1*, left) or with RPM-1::GFP (*juIs77*, right). MAK-2 partially colocalizes with SNB-1 but not with RPM-1 (quantitation in Figure S7).

(B) Functional mCherry::CEBP-1 in motor neuron cell bodies (top panels) and synapses (bottom panels) using the same markers as in (A). CEBP-1 shows nuclear and cytoplasmic localization in soma and colocalizes with SNB-1 but is excluded from areas of RPM-1 at synapses (Figure S7). Synapse morphology (SNB-1::GFP) is unaltered in these transgenic animals.

(C) mCherry::PHR::MAK-2 is confined to motor neuron synapses (left panels) and is excluded from the nucleus (N) in the soma (right panel).

(D) Synaptic targeting of constitutively active MAK-2(EE), but not wild-type MAK-2(+) or inactive MAK-2(AA), alters synapse morphology and reduces SNB-1::GFP puncta number (*juIs1*).

(E) Quantification of the SNB-1::GFP phenotypes in (D) ($n \geq 30$ per genotype).

(F) Induction of unregulated *cebp-1* in adult neurons alters synapse morphology and number (*juIs1*). Quantification is on the right ($n \geq 30$ for each group). 15°C–15°C indicates animals cultured continuously at 15°C; 15°C–25°C indicates animals grown at 15°C until late L4, then shifted to 25°C for 24 hr. Data shown as mean ± SEM; statistics, Anova. Scales, 10 µm.

mRNA imaging method based on the high-affinity binding of the coat protein of the MS2 bacteriophage to a 19 nucleotide RNA stem-loop structure (Bernardi and Spahr, 1972; Bertrand et al., 1998). GFP::NLS::MS2 fluorescence was observed in cell bodies and only faintly along the axons of touch neurons and motor neurons (Figures 5B, S8A, and S8B). As background fluorescence of GFP::NLS::MS2 in touch neuron axons was much lower than in motor neurons, we focused on *cebp-1* mRNA in the touch neurons. Upon coexpression of *cebp-1* mRNA containing six copies of the MS2-binding site in the 3′UTR, we detected punctate GFP::NLS::MS2 fluorescence along axons and at synapses (Figures 5B, S8A, and S8B). A control reporter containing MS2-binding sites in the *unc-54* 3′UTR did not cause an increase of GFP::NLS::MS2 in axons (Figure 5B). The intensity of GFP::NLS::MS2 in axons was further increased in *rpm-1(lf)* mutants and reduced in *dlk-1(lf)* mutants (Figures 5B and 5C). The half-lives of the transgenic MS2-binding site-tagged *cebp-1* transcripts were similar to those of endogenous *cebp-1* mRNA and were likewise increased in *rpm-1* mutants (Figure S8C). We also saw GFP::NLS::MS2 fluorescence increase in the synaptic regions of the motor neurons upon coexpression with MS2-binding site-tagged *cebp-1* mRNA (Figure S8A). These findings suggest that *cebp-1* mRNAs may be present in axons and presynaptic regions, and that activation of the DLK-1 cascade could locally stabilize *cebp-1* mRNA.

Regulation of the DLK-1 Pathway in Adult Neurons Is Necessary for Maintenance of Synapse Morphology

Our observations that MAK-2 and CEBP-1 are present at synapses of adult neurons raised the possibility that this pathway

(F) Overexpression of *cebp-1* with its own 3′UTR causes PLM axon overextension; transgenes with the *unc-54* 3′UTR cause further defects in ALM. Overexpression of *cebp-1* from muscles does not affect neuronal morphology. Data shown as mean ± SEP. Quantitation, labels, details of mutant phenotypes, promoters, and statistics as in Figure 1; GL, # of transgenic lines exhibiting gain-of-function abnormalities. Scales,10 µm.

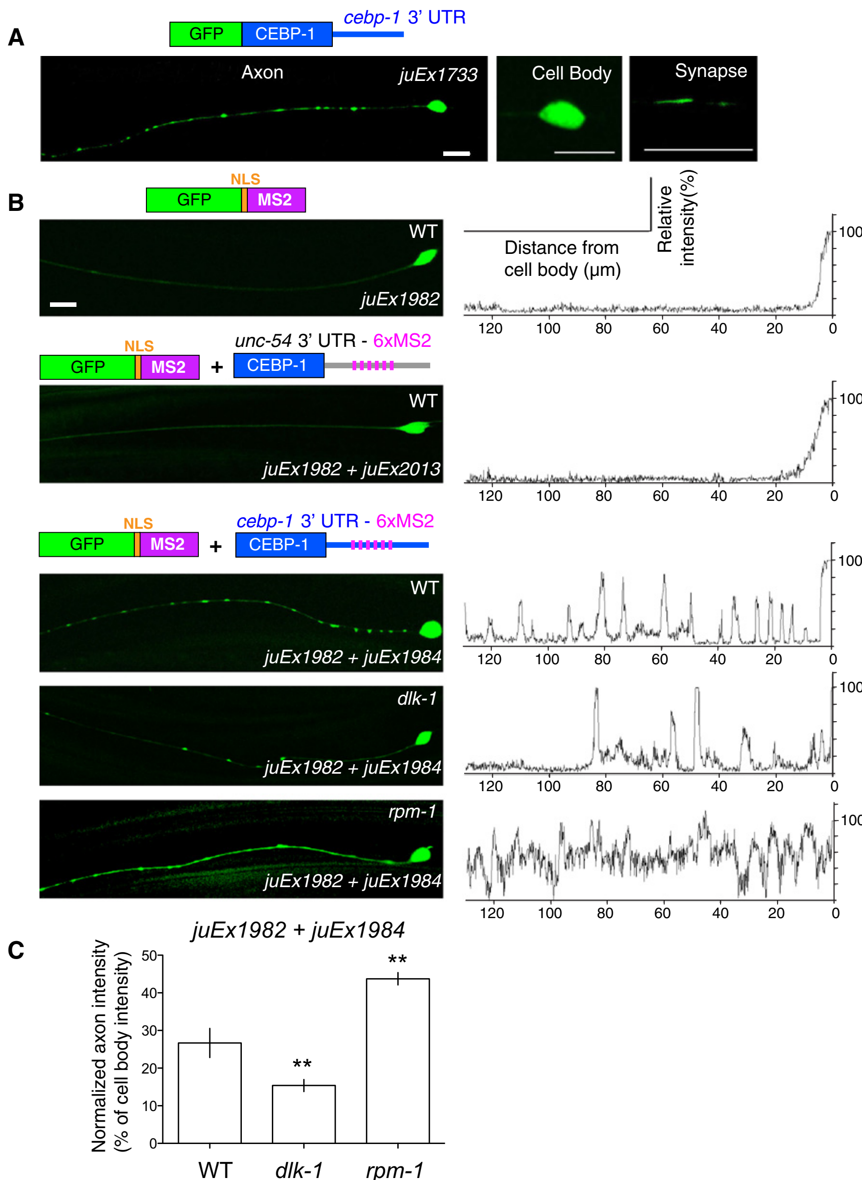

Figure 5. *cebp-1* Protein and mRNA Are Localized in Touch Neuron Axon and Synapses

(A) Expression of functional GFP::CEBP-1 in touch neurons. GFP is present along the axon (left panel), in the cell body (middle) and synapse (right).

(B) GFP::NLS::MS2 reporter detects mRNA of *cebp-1* in touch neuron axons. Illustrations of the constructs are shown above each image. Axonal fluorescence intensity as a fraction of peak cell body intensity is plotted versus distance along the axon from the cell body. GFP::NLS::MS2 alone (*juEx1982*) is concentrated in the cell body and faintly seen along the axon. Coexpression of *juEx1982* with a *cebp-1* transgene containing six MS2-binding sites in the *cebp-1* 3′UTR (*juEx1984*), but not with a control transgene containing six MS2-binding sites in the *unc-54* 3′UTR (*juEx2013*), results in increased punctate GFP in axons. Axonal expression of GFP::NLS::MS2 + CEBP-1-*cebp-1* 3′UTR is decreased in *dlk-1(lf)* animals but significantly elevated in *rpm-1(lf)* animals.

(C) Average GFP::NLS::MS2 fluorescence intensity in axons when coexpressed with *juEx1984* as % of cell body intensity (n = 15 per genotype). Data shown as mean ± SEM; statistics, Anova. Scale, 10 μm.

may also act in adults. To test this, we examined the effects of acute adult overexpression of *cebp-1* on synaptic morphology, exploiting the temperature-sensitive splicing of a *mec-2* intron (Poon et al., 2008; as modified from A. Calixto, C. Ma, and M. Chalfie, personal communications). In control experiments, the *mec-2* intron became permissive for expression within 1 hr of temperature shift (Figure S9A). Transgenic animals expressing *cebp-1* lacking its 3′UTR were cultured at 15°C until late L4 stage, then shifted to 25°C for the next 24 hr to overexpress *cebp-1* in adults. These transgenic animals displayed fewer synapses, of abnormal morphology, compared to controls (Figure 4F). Likewise, PLM neurons showed axon overextension and retraction of the synaptic branch upon induction of *cebp-1* overexpression in adults (Figure S9B). We infer that regulation of the DLK-1/CEBP-1 pathway is required continuously in mature neurons to maintain axon and synapse morphology.

The DLK-1 Pathway and CEBP-1 Are Necessary for Regenerative Growth of Adult Touch Neurons after Laser Axotomy

Animals lacking components of the DLK-1 pathway develop morphologically normal synapses and axon trajectories and show normal locomotion, egg-laying, and touch responses. To test whether this pathway has a later role in the mature nervous system, we assayed regeneration after laser axotomy in adult mechanosensory axons (Wu et al., 2007). In wild-type adults, axotomized PLM axons exhibit robust regrowth of ~100 μm in 24 hr (Figure 6A). In contrast, PLM regrowth was essentially blocked in animals lacking each MAP kinase or CEBP-1 (Figure 6A). In these mutants, axons that failed to regenerate did not display growth cones, unlike wild-type axotomized axons (Figure S10A). ALM neurons also failed to regrow in these mutants (data not shown). The MAPK/CEBP-1 pathway is required in adult axons for regrowth because animals in which we induced expression of a dominant-negative *cebp-1(R290C)* by heat shock in late L4 stage showed reduced regeneration (Figure 6B). Elevating the activity of the DLK-1 pathway by overexpression of *dlk-1(+)* enhanced regeneration in a *cebp-1*-dependent manner (Figure 6A). Moreover, touch neuron-specific expression of *mak-2(+),* but not of the kinase-dead *mak-2(K103R),* rescued the *mak-2(lf)* regrowth defect (Figure 6A), indicating that this pathway acts cell autonomously. We conclude that the DLK-1 pathway is required for regrowth of mature touch neuron axons, and that activity of

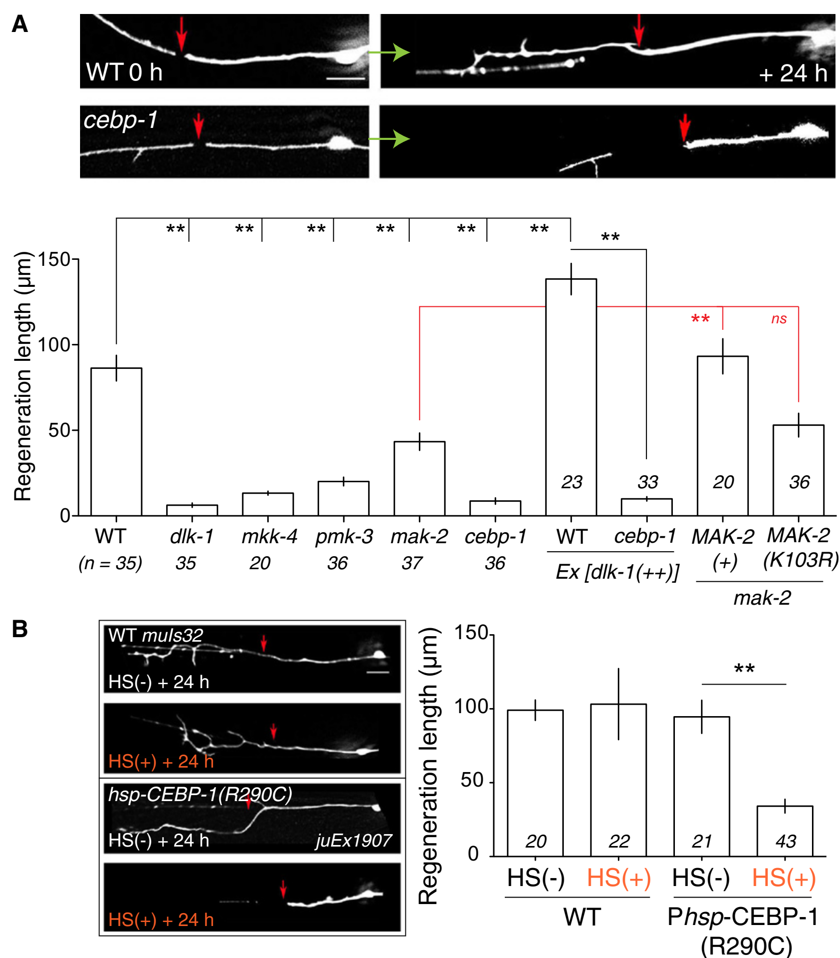

Figure 6. The DLK-1 Pathway and CEBP-1 Are Required for Touch Axon Regeneration in Adults

(A) Regeneration of PLM axon (*muIs32* marker) is reduced in loss-of-function mutants in each MAP kinase and *cebp-1*. *mak-2(lf)* has modest effects on regeneration compared to *dlk-1(lf)*; the defect is rescued by wild-type MAK-2 but not by MAK-2(K103R). Overexpression of DLK-1 enhances regrowth in a CEBP-1-dependent manner. Images are at 0 hr and 24 hr; quantitation at 24 hr. Alleles are *dlk-1(ju476), mkk-4(ju91), pmk-3(ok169), cebp-1(ju659), juEx1301[Prgef-1-dlk-1(+)]* as *dlk-1(++), juEx2111 [Pmec-4-GFP::MAK-2(+)],* and *juEx2262[Pmec-4::MAK-2(K103R)]*. n, # of animals shown below genotype or in column.

(B) Heat-shock-induced expression of *cebp-1(R290C)* transgene in adults blocks PLM regeneration. n, # of animals, in columns. Data shown as mean ± SEM; statistics, Anova. Scales,10 μm.

DLK-1 and CEBP-1 may be rate limiting for regrowth after injury.

Axotomy Induces Local Translation of *cebp-1* mRNA

We next addressed whether the DLK-1 pathway acts via similar mechanisms in regenerative responses in mature neurons as in developing neurons. We tagged CEBP-1 with the photoconvertible fluorescent protein Dendra (Gurskaya et al., 2006) to distinguish newly synthesized CEBP-1 from pre-existing protein. In uninjured neurons, we observed a low level of synthesis of new Dendra::CEBP-1 within 4 hr of photoconversion (Figure 7A). Following axotomy, within 2 hr, the appearance of new Dendra::CEBP-1 was accelerated both in the severed distal fragments and in the soma and proximal axon (Figures 7B and 7D). Animals laser-wounded in adjacent epidermis did not show elevated synthesis of Dendra::CEBP-1 (Figure 7B). The appearance of newly synthesized CEBP-1 in distal axon fragments suggests that the translation machinery is present in axons. Consistent with this interpretation, cycloheximide blocked new synthesis of CEBP-1 in axon fragments (Figures 7B and 7D). *dlk-1* mutants did not display new Dendra within the same time course (Figures 7B and 7D). *mak-2* mutants showed partly reduced synthesis of new Dendra (Figures 7B and 7D), consistent with the partial block of regeneration in *mak-2* mutants (Figure 6A). These data suggest that axotomy induces activation of DLK-1/MAK-2, which in turn promotes local translation of *cebp-1* mRNA. Dendra::CEBP-1 transgenes in which the 3′UTR of *cebp-1* was deleted were not upregulated after axotomy (Figures 7C and 7D). To test if the 3′UTR of *cebp-1* was responsible for the induced local synthesis, we expressed Dendra with the *cebp-1* 3′UTR or the *unc-54* 3′UTR. Axotomy resulted in elevated synthesis of new Dendra from transgenes containing the *cebp-1* 3′UTR, but not from transgenes with the *unc-54* 3′UTR (Figures 7E and 7F). Lastly, to address whether new synthesis of CEBP-1 protein is required for axon regeneration, we induced expression of *cebp-1(+)* with its own 3′UTR in *cebp-1(lf)* mutants following axotomy and observed partial rescue of axon regeneration (Figure S10C). Induced expression of *cebp-1* with the *unc-54* 3′UTR did not rescue the *cebp-1* phenotype (Figure S10C). These observations indicate that axotomy induces local activation of the DLK-1 kinase cascade, which results in local synthesis of CEBP-1 protein.

DISCUSSION

The DLK kinases have been known to be present in axons and synaptic terminals for over a decade (Holzman et al., 1994; Mata et al., 1996), but only recently have their functions in neurons been revealed. Through genetic studies in *C. elegans* and *Drosophila*, these kinases have been identified as substrates of the conserved PHR family E3 ligases (Collins et al., 2006; Nakata et al., 2005). Regulation of the DLK kinases by ubiquitin-mediated degradation also plays roles in axon pathfinding and synapses in vertebrates (Bloom et al., 2007; D'Souza et al., 2005; Lewcock et al., 2007). However the output of this pathway is not well understood. We have shown that in *C. elegans* neurons the DLK-1 pathway acts via the MAPKAP kinase,

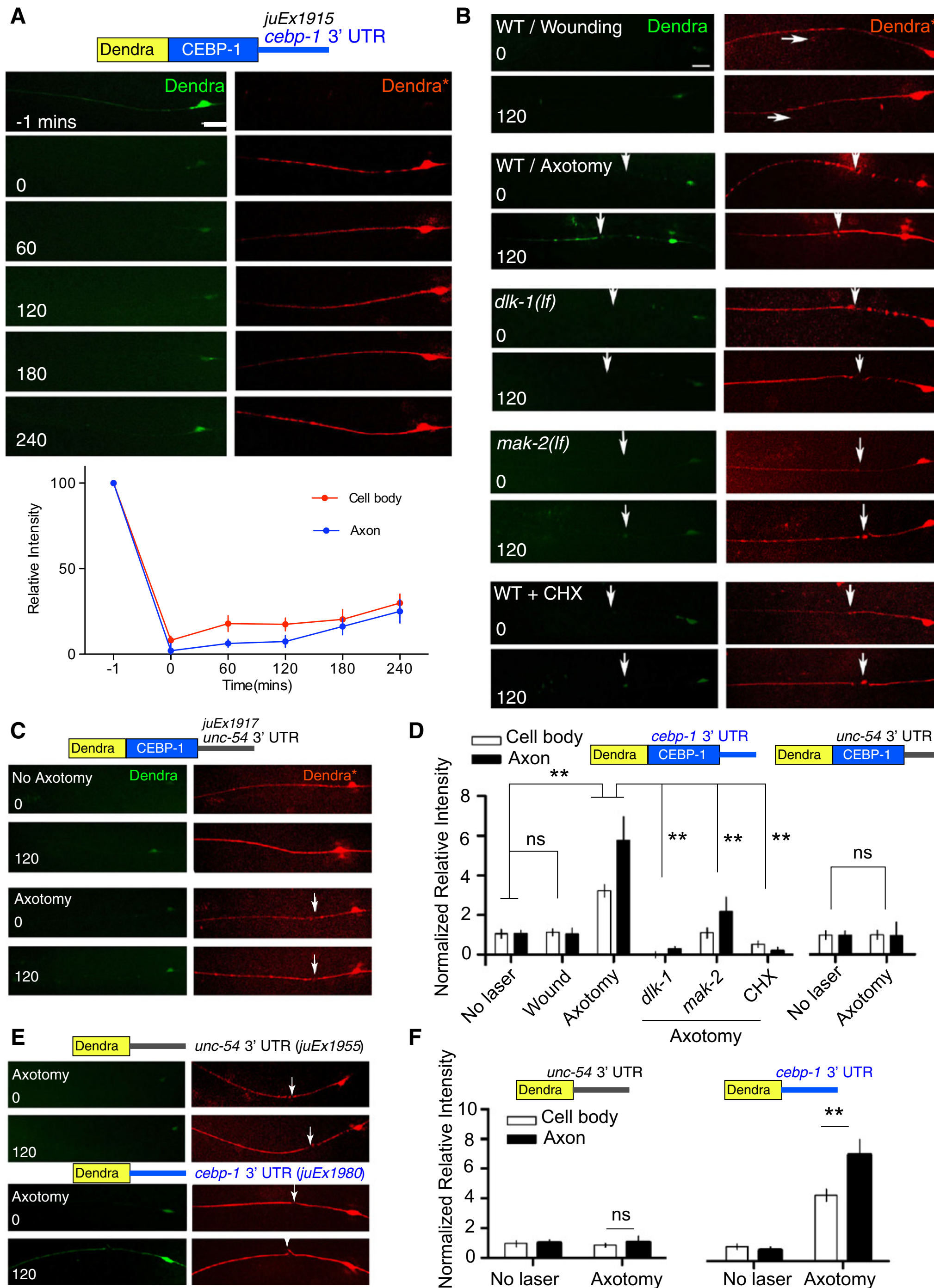

Figure 7. Axotomy Induces Translation of CEBP-1 in a MAP Kinase-Dependent Manner and Involves the 3′UTR of *cebp-1*

Shown are images of Dendra reporter transgenes expressed in touch neurons, with illustrations of constructs above. Green images are unconverted or newly synthesized Dendra; red images show photoconverted Dendra (Dendra*).

(A) Under normal conditions, synthesis of new Dendra::CEBP-1 from transgenes containing the *cebp-1* 3′UTR (*juEx1915*) is slow. Quantification data are collected from soma and axons 60 μm away from soma (n = 10 for each time point).

(B) Axotomy of PLM axons (middle), but not laser wounding of nearby epidermis (top panels), increases synthesis of Dendra::CEBP-1 (*juEx1915*). The increase is blocked by *dlk-1(lf)* or cycloheximide and reduced by *mak-2(lf)*. White arrows mark the laser-cutting sites.

(C) Axotomy of PLM axons does not induce new protein synthesis from a Dendra::CEBP-1 transgene lacking *cebp-1* 3′UTR.

(D) Quantitation of (B) and (C) (n = 12 for each group).

(E and F) *cebp-1* 3′UTR is sufficient to confer axotomy-induced protein synthesis (n = 12 for each group). Data shown as mean ± SEM. Statistics, Anova or t test. Scales, 10 μm.

MAK-2, to promote stabilization of the mRNA of the bZip protein CEBP-1, resulting in enhanced CEBP-1 translation. This mechanism is also required for the regenerative responses of adult neurons.

The DLK-1 Pathway Regulates mRNA Stability

A key outcome of the DLK-1 pathway is to stabilize *cebp-1* mRNA, leading to its increased translation at synapses and in axons. Local protein synthesis is now well established as a mechanism in synaptic plasticity in mature neurons, following the identification of polyribosomes in dendrites (Steward and Levy, 1982; Sutton and Schuman, 2005). Local translation in growth cones of developing neurons has also been linked to growth cone turning, although possibly not in all situations (Lin and Holt, 2008; Roche et al., 2009). Whether protein synthesis occurs in mature axons has been unclear, largely because of a lack of evidence for polyribosomes in axons (Piper and Holt, 2004). Nonetheless, local synthesis of some proteins is reported in axons of select neurons (e.g., Wang et al., 2009), and expression of many mRNAs increases in injured adult axons (Di Giovanni et al., 2005; Willis et al., 2007; Xiao et al., 2002; Wang et al., 2007). Our studies indicate that local regulation of mRNA and protein translation in axons is functionally important in mature neurons under both normal and injured conditions.

How might the DLK-1 pathway regulate *cebp-1* mRNA stability? Studies in non-neuronal cells have shown that murine MK2 can regulate stability of mRNAs such as TNF-α and interleukins; known targets of MAPKAPKs include mRNA-binding proteins such as tristetraprolin and HuR (Gaestel, 2006). Future studies may reveal which components of the RNA turnover machinery are relevant in *cebp-1* mRNA stabilization.

The DLK-1 Pathway and CEBP-1 Are Essential for Axon Regrowth

Although the DLK-1/CEBP-1 pathway has a restricted role in developmental axon outgrowth, we show here that this entire pathway is essential for regenerative regrowth in adult touch neurons, consistent with recent findings in motor neurons (Hammarlund et al., 2009). Our results further suggest a model in which unknown injury signals activate DLK-1, which then triggers regrowth by promoting *cebp-1* mRNA regulation and translation. As *C. elegans* axonal transport occurs at rates comparable to those of much larger neurons in other animals (Zhou et al., 2001), it is unexpected that *C. elegans* neurons use local synthesis for responses to acute localized stimuli such as axotomy. By analogy to our findings at synapses, DLK-1-dependent upregulation of CEBP-1 by axotomy may involve local stabilization of *cebp-1* mRNA, although for technical reasons we have not been able to visualize this directly. Activation of the DLK-1 pathway by axotomy could have additional effects, such as direct stimulation of translation.

In injured *Aplysia* neurons, ApC/EBP is activated by phosphorylation via a MEK-ERK cascade, which involves retrograde transport of the "injury signal" to the nucleus (Sung et al., 2001). bZip proteins of the Jun and ATF subfamilies are also implicated in mammalian axon regeneration (Herdegen et al., 1997; Raivich et al., 2004). C/EBPβ mRNA and protein phosphorylation are elevated after injury, and C/EBPβ is required for injury-induced upregulation of α-tubulin and GAP-43 (Nadeau et al., 2005). However, as yet there has been no demonstration that any of these bZip proteins are required for regenerative growth, apart from Jun, which has a partial requirement (Raivich et al., 2004). The finding that DLK-1, its effector kinases, and CEBP-1 are essential for regeneration suggests that their mammalian homologs may be required for axonal injury responses, and that local mRNA stability and translation could be a conserved axonal response to injury.

Role of CEBP-1 in Axons and Synapses

It is unexpected that CEBP-1 is present at synapses and in axons. A related bZip protein does not appear to be synaptically localized, suggesting that this localization of CEBP-1 is specific (D.Y. and Y.J., unpublished data). CEBP-1 joins an increasing list of nuclear proteins associated with synapses and neurites. For example, Ribeye, an isoform of the transcriptional repressor C-terminal Binding Protein (CtBP2), is found at the ribbon synapse active zone (Schmitz et al., 2000). The homeodomain proteins Emx2 and Engrailed2 localize to presynaptic termini of olfactory neurons and *Xenopus* neuron growth cones, respectively, and interact with the translation factor eIF4E (Nedelec et al., 2004; Brunet et al., 2005). The bZip protein CREB can be locally translated in axons and transported retrogradely (Cox et al., 2008). At this stage, our data do not distinguish whether CEBP-1 has two functions, one locally at the axon and synapses, and the other at the nucleus. The observations that abnormally high levels of CEBP-1 disrupt synapse development and maintenance imply that CEBP-1 is tightly regulated. That this regulation is in part achieved via local regulation of mRNA stability suggests that CEBP-1 could form a link between synapse dynamics and gene expression. Such dual modes of regulation have been long studied in CREB-mediated neural plasticity (Harris, 2008; Lonze and Ginty, 2002).

Improved sensitivity in mRNA detection has led to the identification of an increasing number of axonally localized mRNAs (Lin and Holt, 2008; Wang et al., 2007). However, a mechanistic understanding of how such mRNAs are targeted to axons and activated for translation is only beginning (Kiebler and Bassell, 2006; Krichevsky and Kosik, 2001). Transport and subcellular localization of mRNAs depends on both sequence tags in the mRNA itself and its interactions with carrier proteins. We find that the *cebp-1* 3′UTR is essential both for its axonal delivery and its posttranscriptional regulation in axons and contains multiple adenine/uridine-rich motifs associated with unstable mRNAs (Wilusz et al., 2001). Several mammalian C/EBP mRNAs also contain similar nucleotide motifs in their 3′UTRs (Figure S5B). Future work will address the multiple levels at which CEBP-1 is regulated in synaptogenesis and regeneration.

EXPERIMENTAL PROCEDURES

C. elegans Genetics

We maintained *C. elegans* strains on NGM plates at 20°C–22.5°C as described in Brenner (1974). The characterization of the suppressors is in the Supplemental Data. The mutations and transgenes are as follows: *dlk-1(ju476) I, mkk-4(ju91) X, pmk-3(ok169, ju485) IV, mak-2(tm2927, ju637, ok2394) IV, cebp-1(tm2807, ju659, ju640) X; juIs1[Punc-25-SNB-1::GFP]* for GABA motor neuron synapses (Hallam and Jin, 1998); *muIs32[Pmec-7-GFP]* (Ch'ng et al.,

2003) and *zdIs5[Pmec-4-GFP]* (Clark and Chiu, 2003) for touch neuron morphology. Other transgenes are described in Supplemental Data and listed in Table S2.

Fluorescence Microscopy and Laser Axotomy

We scored fluorescent reporters in living animals using a Zeiss Axioplan 2 microscope equipped with Chroma HQ filters. For quantification of touch neuron morphology, 30–90 animals were analyzed. Images of *juIs1* synapse morphology, *cebp-1* mRNA localization, and MS2::GFP reporters were collected on 1-day-old adults immobilized in 1% 1-phenoxy-2-propanol (TCI America, Portland, OR, USA) in M9 buffer using a Zeiss LSM510 confocal microscope (Supplemental Data).

We cut PLM axons using a near-infrared Ti-Sapphire laser (KMLabs, Boulder, CO, USA) as described (Wu et al., 2007).

Heat Shock and Temperature Shift Experiments

In heat shock experiments, wild-type or P*hsp::cebp-1(R290C)* transgenic worms were grown at 20°C until late L4, heat-shocked at 35°C for 45 min, and recovered at 20°C for 1 hr before axotomy. Regrowth of axons was imaged 24 hr post-axotomy at 20°C. In temperature shift induction experiments, wild-type or P*rgef-1-intron-cebp-1* transgenic animals were cultivated at 15°C until late L4, then half were shifted to 25°C and half kept at 15°C. For effects on axotomy and motor neuron synapse maintenance, worms were cultivated for 24 hr after temperature shift. For effects on touch neuron axon maintenance, animals were grown 60 hr after temperature shift.

Photoconversion of Dendra

We photoconverted Dendra using a 405 nm laser (Blue Sky Research, Milpitas, CA, USA) at 1 mW power and 40× objective for 30 s. Images were captured under the same conditions using 488 nm and 543 nm laser and spinning-disk imaging. We analyzed two independent transgenes for the same construct, n > 6 animals per line in each experiment. We measured fluorescence intensity (ImageJ) at the cell body, in the axon 50–60 μm from the cell body (no axotomy groups), or in the first 10 μm axon distal to cutting point (for axotomy groups). We quantified the normalized fluorescence intensity increase, NIC = $(F_n - F_0)/(F_{-1} - F_0)$, where F_n = fluorescence intensity n hours after photoconversion; F_0 = fluorescence intensity immediately after photoconversion; F_{-1} = fluorescence intensity before photoconversion. We set the average NICs of wild-type worms to 1. Changes are shown as a multiple of the average NIC of wild-type.

Statistical Analysis

In comparisons of measurements such as puncta density, fluorescence intensity, or axonal regrowth we first tested for normality using a D'Agostino-Pearson test. For comparisons of two groups we used a two-tailed Student's t test. Comparisons involving multiple groups used one-way Anova and Bonferroni or Dunnett post tests in Graphpad Prism (GraphPad Software, La Jolla, CA, USA). To compare variables such as axon termination proportions we used the Fisher exact test. To correct for multiple comparisons in 2 × *k* tables we used a Monte Carlo simulation approach followed by the Marascuilo procedure, as implemented in xlstat (Addinsoft, New York, NY, USA). Correlation coefficients in Figure S7 were calculated using GraphPad Prism.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two tables, and ten figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00723-5.

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