Current Biology 22, 1774–1782, October 9, 2012 ©2012 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2012.07.044

Article

Axon Regrowth during Development and Regeneration Following Injury Share Molecular Mechanisms

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Summary

Background: The molecular mechanisms that determine axonal growth potential are poorly understood. Intrinsic growth potential decreases with age, and thus one strategy to identify molecular pathways controlling intrinsic growth potential is by studying developing young neurons. The programmed and stereotypic remodeling of *Drosophila* mushroom body (MB) neurons during metamorphosis offers a unique opportunity to uncover such mechanisms. Despite emerging insights into MB γ-neuron axon pruning, nothing is known about the ensuing axon re-extension.

Results: Using mosaic loss of function, we found that the nuclear receptor UNF (Nr2e3) is cell autonomously required for the re-extension of MB γ-axons following pruning, but not for the initial growth or guidance of any MB neuron type. We found that UNF promotes this process of developmental axon regrowth via the TOR pathway as well as a late axon guidance program via an unknown mechanism. We have thus uncovered a novel developmental program of axon regrowth that is cell autonomously regulated by the UNF nuclear receptor and the TOR pathway.

Conclusions: Our results suggest that UNF activates neuronal re-extension during development. Taken together, we show that axon growth during developmental remodeling is mechanistically distinct from initial axon outgrowth. Due to the involvement of the TOR pathway in axon regeneration following injury, our results also suggests that developmental regrowth shares common molecular mechanisms with regeneration following injury.

Introduction

Neuronal remodeling is an evolutionarily conserved strategy to sculpt the adult nervous systems of both vertebrates and invertebrates. Remodeling includes both degenerative events such as elimination of exuberant connections and regenerative events such as the formation of new synapses and connections [1]. Due to the importance of the remodeling process, improper remodeling in humans has been implicated in various disease states such as autism and schizophrenia [2, 3]; however, our understanding of the molecular mechanisms remains incomplete.

Neuronal remodeling of the *Drosophila* mushroom body (MB) during metamorphosis is an attractive model for studying the mechanisms of both degenerative and regenerative plasticity during a normal developmental program. Remodeling of MB γ-neurons is initiated by the localized fragmentation and clearance of specified regions of axons and dendrites, a process known as pruning. Subsequently, axons re-extend to form new, adult-specific, connections [4]; (Figure 1A). In contrast to the growing understanding of the mechanisms underlying developmental axon pruning (e.g., [5–8]), nothing is yet known about the axon re-extension phase and whether this process is genetically regulated at all. Therefore, the MB offers an attractive system to investigate axon regrowth following pruning and determine whether it is similar to or distinct from axon growth per se.

Here we report that the nuclear receptor (NR) UNF (also known as Hr51 and Nr2e3) regulates MB axon re-extension following pruning. The mammalian Nr2e3 (also known as photoreceptor-specific nuclear receptor, PNR) has been implicated in the development and maintenance of rods and cones in the eye [9]. Mutations in human and mouse *Nr2e3* result in several forms of retinal degeneration [10, 11], whereas mice also display a progressive loss of rods and cones [12]. The *C. elegans Nr2e3*, *fax-1*, is specifically expressed in the nervous system and has been associated with axon pathfinding and neurotransmitter expression [13, 14]. In *Drosophila*, UNF affects wing expansion [15], fertility [15], and multiple aspects of MB anatomy [16, 17], although its precise role is poorly understood.

Here we provide genetic evidence that the NR UNF is specifically required for the developmental regrowth of MB γ axons following pruning but not autonomously for their initial growth or guidance. Our results demonstrate that UNF promotes developmental axon regrowth by activating an axon growth program that is directly mediated via the TSC1-Rheb-TOR pathway while at the same time inducing a late guidance program that is currently unknown. Due to the known role of TOR in axon regeneration following injury [18, 19], our results suggest that developmental axon regrowth is not only distinct from initial axon growth but also shares molecular similarities with regeneration following injury.

Results

UNF Is Cell Autonomously Required for Adult γ-Neuron Elaboration

To identify novel molecules involved in mushroom body (MB) γ-neuron remodeling (Figure 1A), we performed a genetic screen utilizing *piggyBac* insertional mutagenesis combined with MARCM (Mosaic Analysis with a Repressible Cell Marker) analysis [7]. We identified that the homozygous lethal *piggyBac* line LL04325 ($unf^{LL04325}$), inserted into the first intron of *unf* (also known as *Hr51*; Figure 1B), affects the normal morphology of the adult MB γ lobe. γ-neuron axons homozygous for $unf^{LL04325}$ do not extend and elaborate into the adult-specific lobe (compare Figures 1D to 1C, quantified in 1G; for quantification details see Figure S1A and Supplemental Experimental Procedures available online). To determine whether the *piggyBac* insertion $unf^{LL04325}$ is causal for this neurodevelopmental defect as well as for the lethality, we precisely excised the transposon and indeed observed a rescue of both lethality and γ-neuron defects (Figure 1E,

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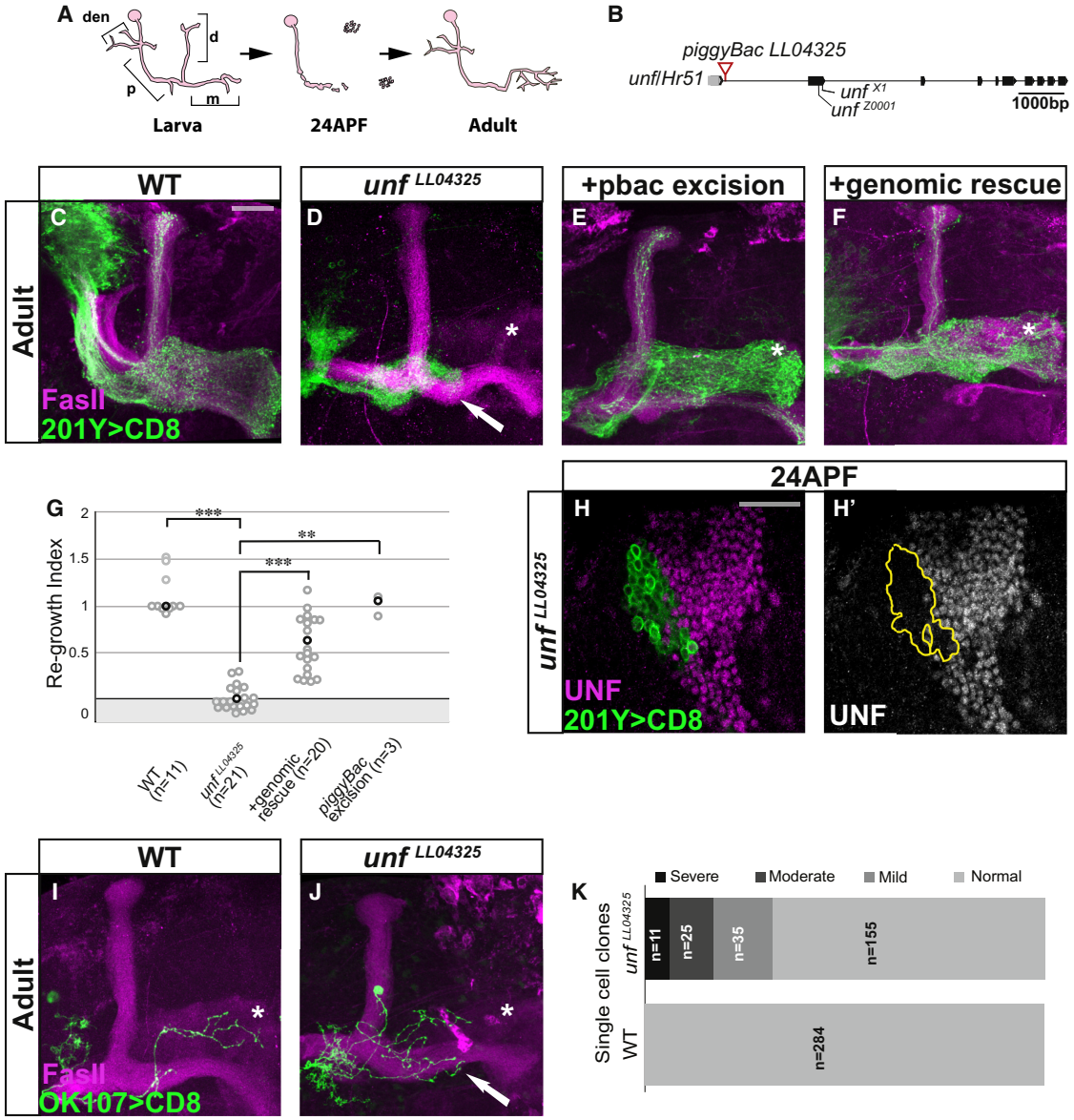

Figure 1. UNF Is Cell Autonomously Required for Developmental Axon Regrowth of MB γ Neurons following Axon Pruning

(A) Scheme of MB γ-neuron remodeling. den, dendrites; CB, cell bodies; p, peduncle; d, dorsal axon branch; m, medial axon branch. 18 hr or 24 hr refer to time after puparium formation.

(B) *unf/Hr51* genomic structure (gray bars depict untranslated exons, black bars translated exons and lines introns). The *piggyBac*^{LL04325} (*unf*^{LL04325}) insertion and the *unf*^{X1} and *unf*^{Z0001} mutations are shown.

(C–F) Confocal Z projections of adult MARCM MB neuroblast clones of the following genotypes: WT (C) *unf*^{LL04325} (D), *unf*^{LL04325} precisely excised (+pbac excision; E) and *unf*^{LL04325} additionally expressing a genomic *unf* rescue transgene (+genomic rescue; F). While adult WT (C) MB γ neurons innervate the adult-specific medial lobe (asterisk), *unf*^{LL04325} (D) γ neurons stall at the branch point (arrow). Excision of the *piggyBac* transposon reverts to normal phenotype (E). Genomic rescue of *unf*^{LL04325} partially rescues the developmental axon regrowth defect (F).

(G) Quantification of developmental axon regrowth defects in neuroblast clones (see Figure S1A and Supplemental Experimental Procedures for more details). Bold shapes represent the median of each group; ***p < 0.001; **p < 0.005.

(H) Single confocal slices of *unf*^{LL0432} MB MARCM neuroblast clone at 24 hr after puparium formation (APF). UNF staining (mangeta in H, white in H') shows lack of protein expression within *unf*^{LL0432} clones (green in H, demarcated by yellow outline in H').

(I and J) Confocal Z projections of WT (I) or *unf*^{LL04325} (J) single cell clones. As in neuroblast clones, the adult WT MB γ (I) neuron extends into the adult specific medial lobe (asterisk) while the *unf*^{LL04325} γ-neuron (J) does not fully innervate the adult lobe (arrow).

(K) Quantification of developmental regrowth defects in single cell clones (see Supplemental Experimental Procedures).

Magenta represents anti-FasII (A–F, I, J) or UNF (H) staining. Green is 201Y-Gal4 (A–F, H) or OK107-Gal4 (I, J) driven mCD8:GFP. The scale bars represent 20 μm.

quantified in 1G). Additionally, expressing an *unf* complementary DNA (cDNA) transgene driven by 3 Kb of its endogenous promoter sequence (genomic rescue; [15]) resulted in a significant rescue of the mutant phenotype in *unf*^{LL04325} MARCM clones (Figure 1F, quantified in 1G). We further investigated UNF by characterizing two additional alleles, *unf*^{X1} and

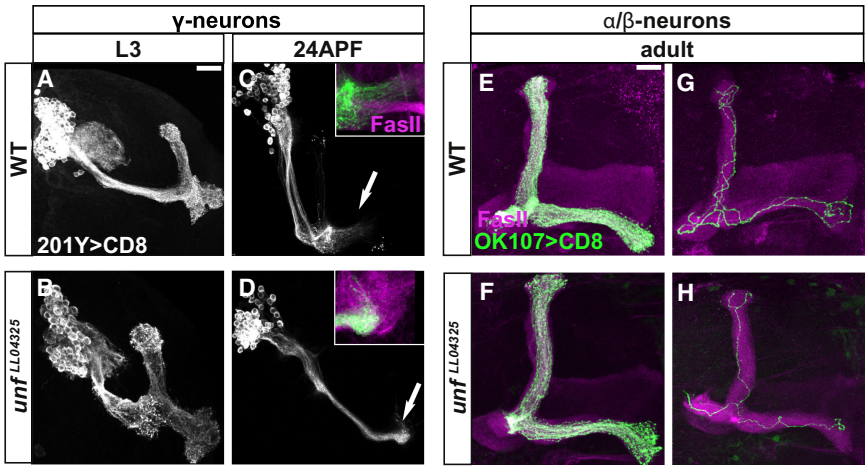

Figure 2. UNF Is Not Required for Initial Axon Growth of MB Neurons

(A–D) Confocal Z projections of WT (A and C) and $unf^{LL04325}$ (B and D) MARCM MB γ neuroblast clones at third-instar larvae (L3; A and B), or 24 hr after puparium formation (APF; C and D). While at L3 $unf^{LL04325}$ γ neurons (B) are indistinguishable from WT neurons (A), at 24 hr APF $unf^{LL04325}$ clonal neurons do not undergo sprouting (arrow in D) as do WT neurons (arrow in C). Insets in (C) and (D) are higher magnification that additionally show anti-FasII staining in magenta that stains nonclonal MB neurons.

(E–H) Confocal Z projections of WT (E and G) or $unf^{LL04325}$ (F and H) MARCM MB α/β neuroblast clones (E and F) or single cell clones (G and H). During MB development γ, α'/β', and α/β neurons are born sequentially from four identical neuroblasts. The α/β neurons are born during the same time frame as γ-neuron developmental regrowth. Mutant α/β neurons are indistinguishable from WT α/β neurons.

White or green, 201Y-Gal4 (A-D) or OK107-Gal4 (E–H) driven mCD8:GFP; magenta, anti-FasII. The scale bars represent 20 μm.

unf^{Z0001}, both mutated in the second exon ([15]; Figure 1B). Although both alleles displayed γ lobe defects similar to $unf^{LL04325}$ (Figures S1B–S1D, quantified in S1E) only unf^{X1} and $unf^{LL04325}$, but not unf^{Z0001}, mutant clones displayed decreased UNF antibody staining (Figure 1H; Figures S1J–S1M). This is consistent with previous work indicating that the unf^{Z0001} is a predicted missense mutation [15]. Taken together, these results show that loss of *unf* results in decreased innervation of the adult γ lobe and that $unf^{LL04325}$ is a strong LOF allele.

To determine whether UNF functions in a cell-autonomous manner, we investigated the phenotypes of single-cell clones. Indeed, one third of single $unf^{-/-}$ cells examined poorly innervated the adult γ lobe (compare Figures 1I and 1J, quantified in Figure 1K; Figures S1F–S1I). The severity of the phenotype in these clones was milder than that of neuroblast clones, most likely due to messenger RNA or protein perdurance (see Supplemental Experimental Procedures for more details). Because in these mosaic animals, nonclonal surrounding cells were heterozygous for the *unf* mutation, we conclude that UNF is cell-autonomously required in γ-neurons for their ability to fully extend to the adult-specific lobe.

UNF Is Required for Developmental Axon Regrowth but Not for Initial Axon Outgrowth

Four alternative mechanisms could explain the defective innervation of the adult γ-lobe: a general axon growth defect, a defect in restricting axon pruning, a specific defect in axon regrowth following pruning, or a maintenance defect resulting in the elimination of adult axons. Each of these hypotheses would predict a different molecular function for UNF. To test these possibilities, we compared the development of WT and mutant MB neurons. Mutant γ neurons appeared normal in third-instar larvae (L3), a time point before axon pruning has begun (compare Figure 2B with Figure 2A), arguing against a general growth or guidance defect. Likewise, our time course analysis of WT and mutant γ neurons indicates that axon pruning occurs with similar dynamics and to the same cellular extent (Figures S2A–S2D), arguing against deregulation or overactivation of the pruning program. However, in contrast to WT neurons, mutant axons failed to initiate axon sprouting at 24 hr after puparium formation (APF; compare Figure 2D with Figure 2C). Nonclonal surrounding neurons, however, initiated axon growth normally (insets in Figures 2D and 2C show clonal neurons in green and surrounding nonclonal neurons in magenta). Finally, we did not observe any signs of degeneration such as blebbed or degenerated axons throughout development or in adult mutant clones (data not shown). Together, these results strongly suggest that UNF is specifically required for MB γ-neuron axon regrowth following pruning.

Although UNF is not required for initial axon growth of γ neurons during larval development, UNF could in principle be important for general axon extension during pupal development. To test this possibility, we focused on the development of the later born MB α/β neurons that extend their initial axons at the same time as γ-axon re-extension [4]. We determined that α/β neurons express UNF using antibody staining (data not shown) and then examined MARCM single-cell or neuroblast clones of α/β neurons homozygous for $unf^{LL04325}$. These α/β neurons exhibited normal morphology and showed no signs of axon stalling (Figure 2, compare 2F to 2E and 2H to 2G). We obtained similar results for axon growth of α/β neurons homozygous mutant for the unf^{X1} and unf^{Z0001} alleles (Figures S2E–S2J) as well as for axon growth of α'/β' neurons which are born during the late larval stage (data not shown). Therefore, we conclude that UNF is not required for the initial axon growth of any MB neuron class and that axon regrowth following pruning is mechanistically distinct from axon growth per se.

UNF Is Not Required for Initial Axon Guidance

Recent studies have reported that homozygous mutant or *unf* knockdown animals display MB axon guidance defects [16, 17]. Our single cell clone analysis of all three available *unf* alleles suggests that UNF is not cell autonomously required for axon guidance of growing γ neurons (Figure 1J; Figures S1G and S1H, quantified in Figure 1K; Figure S1I). We did, however, detect α/β guidance defects in heterozygous animals of unf^{X1} and unf^{Z0001} alleles (Figures S2K and S2L, quantified in S2N) but not in $unf^{LL04325}$ heterozygotes (Figure S2M), suggesting that the unf^{X1} and unf^{Z0001} alleles cause a gain-of-function defect. Previous work has indicated that neither unf^{X1} nor unf^{Z0001} are true null alleles but rather unf^{X1} is splicing defective and unf^{Z0001} contains a missense mutation [15]. Therefore the gain-of-function phenotypes might be a result of the function of a truncated or mutated UNF protein variant. Remarkably, in unf^{X1} and unf^{Z0001} heterozygous

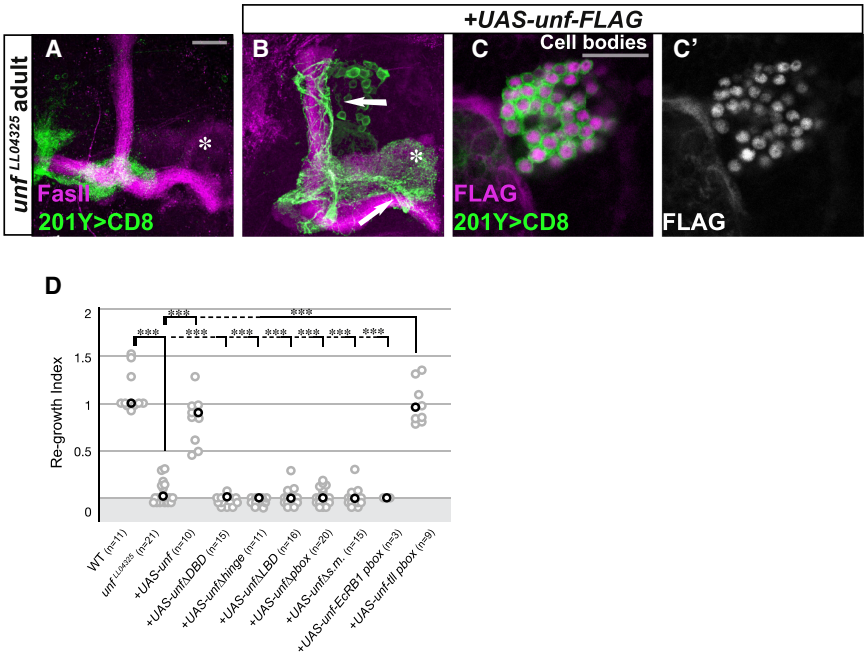

Figure 3. UNF Functions in Postmitotic γ-Neurons to Induce Developmental Regrowth

(A and B) Confocal Z projections of $unf^{LL04325}$ (A) or $unf^{LL04325}$ additionally expressing *UAS-unf-Flag* (B) adult MB MARCM neuroblast clone labeled with 201Y-Gal4 driven mCD8:GFP (green). Expression of *UAS-unf-Flag* within unf^{LL0432} clones rescues the developmental regrowth defect (asterisk in B, compare to A, quantified in G) but also results in unpruned axons (arrows).

(C) Single confocal slice of cell bodies of $unf^{LL04325}$ adult MB MARCM neuroblast clone expressing *UAS-unf-Flag* (magenta in C, white in C') and mCD8:GFP (green) driven by 201Y-Gal4. The scale bars represent 20 μm.

(D) Quantification of developmental regrowth of various *unf* rescue transgenes in which specific NR domains were either mutated or deleted. Bold shapes represent the median of each group; ***p < 0.001. For more information see text and Figure S3.

brains in which the gross MB morphology was normal, homozygous mutant α/β MARCM clones were indistinguishable from WT clones (Figures S2E–S2J). We therefore conclude that UNF is not cell autonomously required for the initial axon guidance of either γ or α/β neurons.

UNF Is Required in Postmitotic Neurons to Induce Developmental Axon Regrowth

In order to better characterize the mechanism of action of UNF in MB neurons, we expressed a FLAG-tagged *unf* transgene driven by a postmitotic MB driver (201Y-Gal4). As expected, transgenic UNF protein localized to nuclei (*UAS-unf-FLAG*; Figure 3C) and rescued the developmental axon regrowth defect of $unf^{LL04325}$ mutant MARCM MB neuroblast clones (compare Figures 3B to 3A, quantified in Figure 3D). These results indicate that UNF functions in postmitotic γ neurons. However, we also observed an additional and unexpected phenotype: overexpression of the *unf* transgene in WT (Figure S3A) or $unf^{LL04325}$ γ neurons (Figure 3B) resulted in a partial inhibition of axon pruning (arrows in Figure 3B and Figure S3A), which we found was due to decreased expression of ecdysone receptor B1 (EcR-B1) levels in clones overexpressing UNF at 0 hr APF (Figures S3B and S3C).

The partial pruning defect resulting from misexpression of UNF suggested that its expression might be temporally regulated. However, we found that UNF expression is ubiquitous throughout development in MB neurons (Figure 1H, Figures 1J and 1K, adult data not shown). Therefore, we propose that the function of UNF in promoting developmental axon regrowth is temporally limited by the presence of its ligand and/or coreceptor.

All Canonical NR Domains Are Required for UNF Function in Developmental Axon Regrowth

In order to determine which domains are necessary for UNF activity in promoting developmental axon regrowth, we performed a structure function analysis in vivo. NRs include distinct domains [20] schematically depicted in Figure S3D. We thus tested the ability of Flag-tagged *unf* transgenes lacking the DNA-binding domain (DBD), ligand-binding domain (LBD), hinge region, P-box or signature motif (*UAS-unfΔdomain*) to rescue the developmental axon regrowth defect of $unf^{LL04325}$ MARCM neuroblast clones (Figure S3E–S3R). In contrast to the full-length transgene (Figure 3B), we found that none of the deletion constructs rescued the regrowth defect (Figure S3, quantified in Figure 3D). Because all of the transgenes were inserted into the same genomic locus and are therefore expressed at similar levels (see Figure S3 for cell body expression levels), these results suggest that all the domains tested are necessary for UNF function during developmental axon regrowth.

The DNA binding specificity of NRs is determined by a subdomain within the DBD that spans a stretch of five amino acids termed the P-box. To better characterize the specific role of the P-box, we engineered two transgenes in which we replaced the *unf* P-box with that of *tailless* (*tll*), the closest homolog of *unf*, or with that of *EcR-B1*. Although *UAS-unf-EcR-B1-P-box* did not rescue the developmental regrowth defect of $unf^{LL04325}$ mutants (Figures S3O and S3P), expression of *UAS-unf-tll-P-box* did (Figures S3Q and S3R). This suggests that UNF promotes developmental axon regrowth by directly binding specific DNA sequences.

The TOR Pathway Is Required for Developmental Axon Regrowth

We next wished to investigate the mechanism of UNF action in promoting developmental axon regrowth. Due to its function as a ligand-dependent transcription factor, we sought to identify the genetic targets of UNF that are required for developmental regrowth. As an alternative to a full-scale genomic search in the fly, we decided to exploit the fact that a genomic screen was already performed to identify genes that are up- or downregulated by the mouse ortholog of *unf*, *Nr2e3*, in the adult mouse eye [21]. We hypothesized that a developmental regrowth program could include genes that promote growth, inhibit degeneration, and those that mediate correct guidance. From the list of NR2E3 regulated genes we decided to focus on *Tsc1* [21] because it is a well-characterized inhibitor of

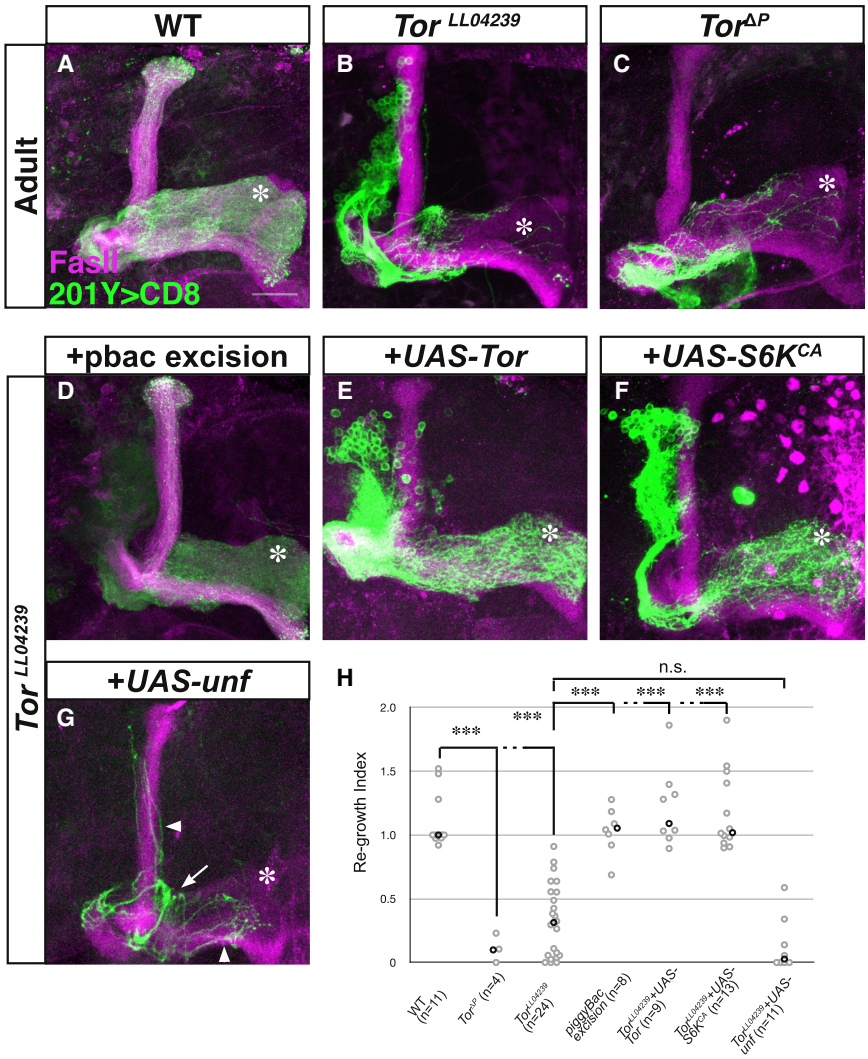

Figure 4. TOR Is Required for Developmental Axon Regrowth of MB γ-Neurons

(A–F) Confocal Z projections of WT (A), $TOR^{LL04239}$ (B), $TOR^{\Delta P}$ (C), $TOR^{LL04239}$ precisely excised (D), $TOR^{LL04239}$ additionally expressing *UAS-TOR* (E), *UAS-S6K*^{CA} (F), or *UAS-unf-Flag* (G) MB MARCM neuroblast clones. *TOR* mutant axons (B and C) display a defect in developmental regrowth that can be rescued by excision of the *piggyBac* insertion (D), expression of *UAS-TOR* (E), or by a constitutive active form of its downstream target, S6K (F). However, *UAS-unf-Flag* cannot rescue the developmental regrowth defect (G). Note that the axons that innervate the medial lobe are unpruned γ-axons due to UNF overexpression (arrowheads) while the adult γ-axons have not re-extended (arrows). Green is 201Y-Gal4 driven mCD8:GFP, magenta is anti-FasII; asterisks represent adult specific γ medial lobe. The scale bars represent 20 μm.

(H) Quantification of developmental regrowth of the genotypes presented in (A)–(G). Bold shapes represent the median of each group; ***p < 0.001.

mammalian target of rapamycin (mTOR) [22], a protein that has been shown to play a key role in promoting axon regeneration following injury [18, 19]. We therefore hypothesized that UNF repression of *tsc1* could disinhibit TOR and thereby promote axon growth.

To test whether *TOR* is required for developmental axon regrowth, we generated MARCM clones for two mutant alleles of *TOR*, a *piggyBac* insertion that we generated, $TOR^{LL04239}$, and a deletion mutant, $TOR^{\Delta P}$. Clones of either allele exhibited developmental axon regrowth defects resembling *unf* mutants (compare Figures 4B and 4C to 4A, quantified in 4H). Also like *unf*, *TOR* was specifically required for developmental regrowth but to our surprise was not necessary for initial axon outgrowth (Figure S4). To confirm that indeed *TOR* is the causal gene in *piggyBac* insertion LL004239, we generated MARCM clones of a precise excision of $TOR^{LL04239}$ (Figures 4D and 4H), which indeed reversed the defective phenotype. Finally, expression of a *TOR* transgene (*UAS-TOR*) within $TOR^{LL04239}$ MARCM clones rescued the developmental regrowth defect (Figures 4E and 4H) proving that *TOR* is required for developmental axon regrowth. TOR's requirement for developmental axon regrowth but not initial axon outgrowth was unexpected and further establishes that these two processes are mechanistically distinct.

mTOR exerts its diverse functions through at least three well characterized targets—S6K, 4E-BP1, and ATG1 [23, 24]. Of these candidates, we hypothesized that positive regulation of S6K is the most plausible mechanism promoting axon growth, because S6K promotes protein synthesis and cell growth [25]. Indeed, overexpression of a constitutively active S6K transgene (*UAS-S6K*^{CA}) within $TOR^{LL04239}$ MARCM clones fully rescued the developmental regrowth defect (Figures 4F and 4H). Taken together, these results indicate that TOR, acting by positive regulation of S6K, promotes developmental axon regrowth. Remarkably, our finding that TOR is required for axon regrowth during development but not initial axon growth, coupled with its previously known role in axon regeneration following injury, suggests that these two processes share molecular similarities.

UNF Promotes Axon Growth via the TOR Pathway

To determine whether UNF and TOR function in the same pathway, we performed genetic interaction experiments. Because genomic analysis has shown that the murine NR2E3 negatively regulates *Tsc1* expression [21], and because TSC1 represses Rheb, a positive activator of mTOR, we predicted that mutating *Tsc1* should suppress the developmental regrowth defect of *unf* mutant neurons. Indeed, we found that the developmental regrowth defect seen in $unf^{LL04325}$ MARCM clones was partially rescued in clones that are doubly mutant for both *unf* and *Tsc1* (compare Figures 5D to 5B, quantified in 5G). To further ascertain whether UNF mediates developmental regrowth via the TOR pathway, we performed two additional epistasis rescue experiments in which we expressed either transgenic *Rheb* (*UAS-Rheb*; Figure 5E) or *S6K* (*UAS-S6K*^{CA}; Figure 5F), in $unf^{LL04325}$ clones. Expression of either Rheb or S6K^{CA} in $unf^{LL04325}$ MARCM clones partially rescued the developmental regrowth defect (quantified in Figure 5G). Although these results suggest that UNF activates

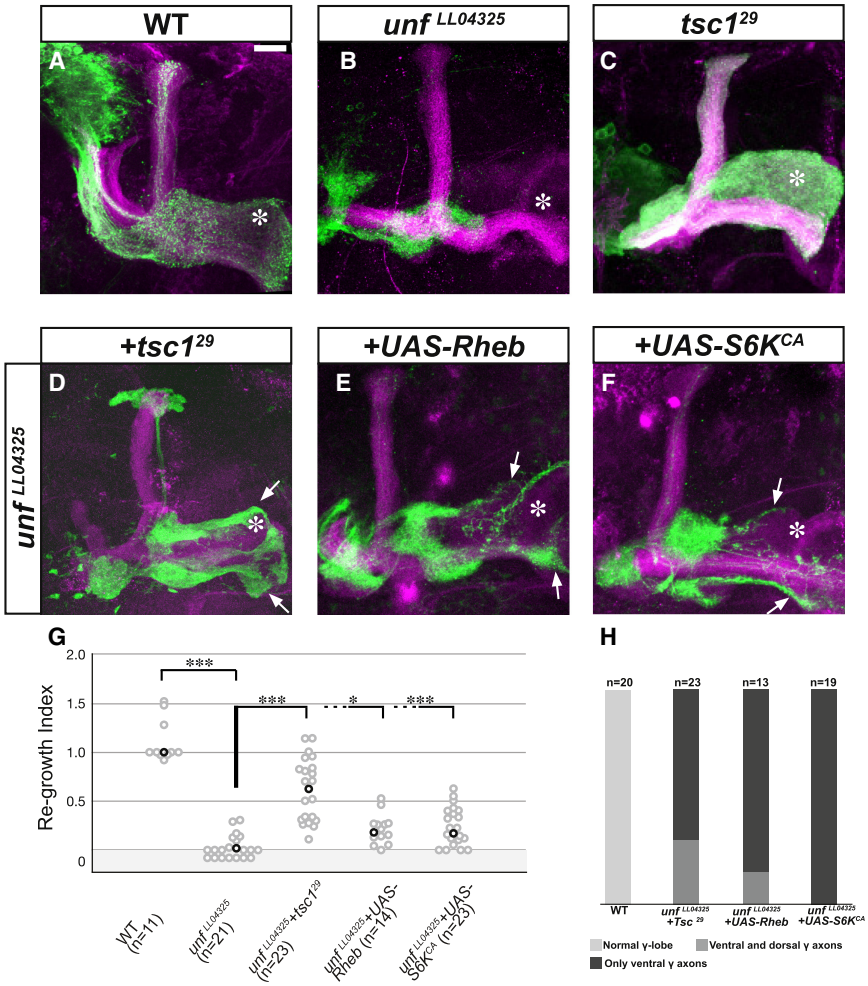

Figure 5. UNF Promotes Axon Growth but Not Guidance via the TOR Pathway

(A–F) Confocal Z projections of WT (A), *unf*^{LL04325} (B), *Tsc1*^{29} (C), double mutant *unf*^{LL04325}; *Tsc1*^{29} (D), *unf*^{LL04325} additionally expressing *UAS-Rheb* (E), or *unf*^{LL04325} additionally expressing *UAS-S6K*^{CA} (F) MB MARCM neuroblast clones. Although a mutation in *Tsc1*, an inhibitor of TOR, in itself shows no effect on developmental regrowth (C), it partially rescues the defect seen in *unf*^{LL04325} clones (D). Overexpression of *UAS-Rheb* (E) or *UAS-S6K*^{CA} (F) within *unf*^{LL04325} MARCM clones partially rescued developmental regrowth (E). Green, 201Y-Gal4 driven mCD8:GFP; magenta, anti-FasII. Asterisks represent adult specific γ medial lobe, arrows show γ neurons that have undergone developmental regrowth but display a guidance defect. The scale bars represent 20 μm.

(G) Quantification of developmental regrowth of genotypes shown in (A) and (B) and (D)–(F). Bold shapes represent the median of each group; ***$p < 0.001$, *$p < 0.05$.

(H) Quantification of the γ-neuron guidance defects seen in (A) and (D–F), see Figure S5 for examples of each category.

the TOR pathway by repressing *Tsc1*, they do not completely rule out the possibility that UNF and TOR function in two parallel pathways and that overactivating one might compensate for defects in the other. In order to examine this possibility, we performed the reciprocal experiment in which we overexpressed UNF in *TOR*^{LL04239} mutant clones. UNF overexpression failed to rescue the regrowth defect apparent in TOR mutant clones (Figures 4G and 4H) consistent with the linear model of the pathway. Ideally, we would like check for alterations in TOR pathway activation in *unf* mutant clones by looking at protein levels of phosphorylated S6K or other activated TOR components during the time point of developmental regrowth. However, there are currently no available antibodies against activated dTOR components that work well in immunofluorescence. Taken together, our results strongly suggest that UNF promotes axon growth at least partially via the TOR pathway.

During the process of quantifying the developmental regrowth of these epistatic rescue experiments, we noticed that many of the axons were misguided. Although overexpression of Rheb or S6K^{CA} alone in MB γ neurons did not affect MB morphology (Figures S5A–S5B), overexpression within *unf* mutant clones resulted in severe guidance defects. In these clones, γ-axons did not occupy the entire adult lobe but instead grew to the dorsal and ventral extremities of the adult γ-lobe and in some cases only to its ventral portion (quantified in Figure 5H, more examples in Figures S5C–S5H). We confirmed that these misguided axons are indeed γ-axons (Figures S5I–S5O, see legend for details; [26]). These results indicate that although UNF is not required for the initial guidance of axons, it does regulate both growth and guidance following pruning. In contrast, the *Tsc/rheb/TOR/S6K* pathway is only required for γ-axon regrowth but not for axon guidance. Thus, our results suggest that UNF regulates axon growth, at least partially, via the TOR pathway and axon guidance during developmental regrowth by a yet uncharacterized pathway.

Discussion

In this study we show that the *Drosophila* nuclear receptor UNF is cell autonomously required for axon re-growth following pruning of MB γ-neurons but not for initial axon outgrowth or guidance of any MB neuron type. Thus, our data suggests that axon growth following pruning is mechanistically distinct from axon growth per se. Furthermore, we found that UNF drives developmental axon regrowth by promoting both axon growth, mediated by the TOR pathway, and axon guidance (Figure 6). Because of the involvement of the TOR pathway in axon regeneration following injury [18, 19], we propose that developmental regrowth is not only distinct from initial axon growth but shares common mechanisms with regeneration following injury.

UNF Is Specifically Required for Developmental Axon Regrowth

The molecular mechanisms underlying MB neuronal remodeling have been the focus of several studies during the last decade. However, up to this point it has not been clear whether the re-extension phase of MB neuronal remodeling involves

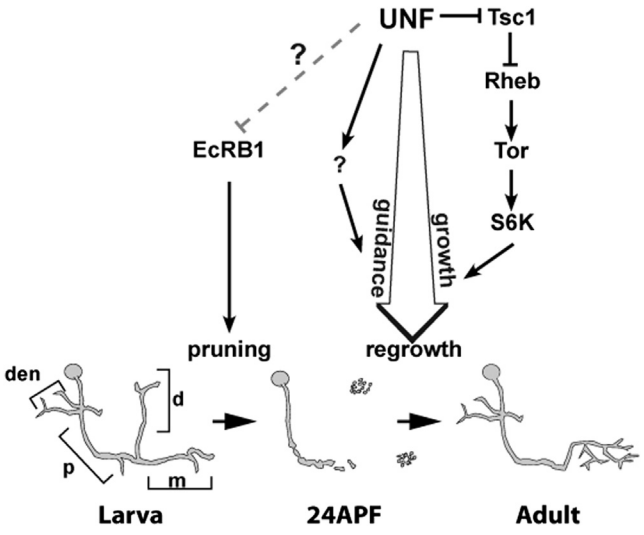

Figure 6. Working Model for UNF Function in Neuronal Remodeling of MB γ-Neurons

Scheme of developing MB γ neurons in which UNF promotes developmental regrowth via at least two separate pathways, the TOR pathway, which promotes axon growth, and another pathway/s, which regulates axon guidance. At the same time overexpressed UNF can also repress EcR-B1 levels.

an instructive genetic program. Here we demonstrate that UNF is required for the effective axon regrowth of MB γ-neurons during development. Because UNF is likely to function similar to other members of the nuclear receptor family, our findings open fresh areas for investigation—identifying the ligand of UNF, its coreceptor, and the direct downstream targets that are involved in promoting developmental axon regrowth.

We explored the role of UNF in MB development using mosaic analysis of three different *unf* alleles. We found that whereas $unf^{LL04325}$ and unf^{X1} are both loss of function alleles resulting in undetectable protein levels, the unf^{Z0001} mutant is not, consistent with previous reports [15]. Moreover, the strong α/β axon guidance defects that are seen in unf^{Z0001} heterozygous animals are likely to be a gain of function phenotype. Nevertheless, mosaic analysis of all three UNF alleles leads us to conclude that UNF is specifically required for developmental regrowth of MB γ-neurons but not for the initial axon growth or early guidance of any MB neuron type. Furthermore, we observed normal expression levels of cell-type-specific markers in *unf* mutant clones during MB development (ElaV, embryonic lethal abnormal vision, a postmitotic neuronal marker; Dac, dachshund, a MB neuron marker; EcR-B1 and 201Y-Gal4, γ-neuron specific markers; data not shown), contrary to previously reported results [16, 17]. Although we cannot completely rule out the possibility that UNF affects other cell-type-specific markers, these results combined with our finding that postmitotic expression of an *unf* transgene is sufficient to rescue the developmental regrowth defect of $unf^{-/-}$ MARCM neuroblast clones (Figure 3B), strongly argues that UNF's effect on developmental regrowth is not likely to involve a change in cell fate.

Our data thus suggest that the differences arising between our results and previous studies [16, 17] stem mainly from the fact that in our study we focus only on the cell-autonomous roles of UNF, whereas in previous studies the whole animal mutant analyses resulted in a combination of cell-autonomous and non-cell-autonomous effects. The allelic differences that we identified, and especially the gain-of-function defects in unf^{Z0001}, likely contribute to some of the guidance defects but do not impact our conclusions on the mechanism of action of UNF because the results using all three alleles were essentially identical. It would therefore be of interest to further explore the non-cell-autonomous functions of the genetic program induced by UNF.

UNF Induces Developmental Axon Regrowth by Promoting Growth and Guidance

Our finding that the TOR pathway acts downstream to UNF to regulate axon regrowth, but not axon guidance, suggests that developmental regrowth involves the activation of at least two separate pathways, growth and guidance. The involvement of the TOR pathway in the intrinsic neuronal growth state is in line with several works implicating the mTOR pathway in cellular growth [27], synapse formation [28], sprouting [29], and axon regeneration [18, 19]. Our inability to completely rescue the UNF induced developmental regrowth defect by overactivation of the TOR pathway may reflect insufficient activation of the pathway, an intrinsic underestimation of the regrowth in the quantification process due to axon guidance defects (see Figure S1A for details on quantification), or a requirement for additional pathways downstream of UNF. Future work should focus on identifying the global genetic targets of UNF and thus uncover additional growth promoting molecules as well as those required for proper guidance.

Overexpression of UNF represses expression of EcR-B1, leading to a defect in axon pruning. This finding suggests that UNF restricts the expression of EcR-B1 following pruning. Despite several attempts, we have not been able to show, in vivo, that a mutation in *unf* increases EcR-B1 levels or prolongs its expression. This may be due to technical issues (such as antibody sensitivity or protein perdurance in MARCM clones), the presence of a backup mechanism to regulate and shut down EcR-B1 expression or may reflect an artificial gain-of-function defect. Interestingly, we have also observed that axotomized *C. elegans* AVK interneurons mutant for *fax-1*, the worm Nr2e3, show both decreased regeneration as well as increased degeneration (Figure S6). These results are consistent with the speculation that UNF/FAX-1 may simultaneously switch off a degeneration program while initiating regeneration.

Developmental Regrowth Shares Mechanistic Similarities with Regeneration Following Injury

γ-axon regrowth after developmental axon pruning is molecularly distinct from initial axon growth because both UNF and TOR only affect developmental regrowth and not initial axon outgrowth of any MB neuron type. Moreover, previous work in adult mice has linked the *mTOR* pathway with efficient central nervous system (CNS) axon regeneration following injury in both corticospinal neurons [18] and in retinal ganglion cells [19]. Our initial observations suggesting reduced regenerative capacity of *fax-1* mutant AVK interneurons (Figure S6) is consistent with the hypothesis that the role of Nr2e3 is conserved beyond development and during evolution. Taken together, we propose that developmental regrowth is not only distinct from initial axon growth but also shares molecular similarities with axon regeneration following injury.

Developmental axon pruning is an evolutionarily conserved process that has been characterized in many animal models, as well as in humans. Improper axon pruning has been linked

to several mental disabilities such as schizophrenia [3], autism [2], and ADHD [30]. During adolescence, massive changes in white brain matter occur with connections being lost in some areas while new connections are formed in other regions [31, 32]. Furthermore, the involvement *TSC1*, a gene implicated in autism [33], in normal MB development raises the possibility that exuberant connections seen in the early stages of autism may reflect increased regenerative capabilities rather than or perhaps in addition to decreased axon pruning. Developmental regrowth may thus similarly represent a conserved process in brain development.

Because UNF plays key roles in both developmental regrowth and injury induced regeneration, it will be interesting to examine whether its orthologs function similarly in other models of regeneration. However, it is quite plausible that different NRs control axon regeneration in different contexts while the downstream genetic targets of these NRs, such as mTOR, rather than the NRs themselves, are more conserved between neurons and among organisms. The finding that the *TOR* pathway regulates axon growth further validates the use of the MB developmental axon regrowth model as a platform to identify key downstream targets. This strategy may also provide new directions for future therapeutic approaches for treatments following CNS injuries.

Experimental Procedures

Construction of UAS Transgenes and Transgenic Flies

At the time of its discovery, the flybase annotation of the *unf* ORF was incomplete. We used 5′Race kit GeneRacer (Invitrogen) to identify the transcription start site. The full-length *unf* cDNA was amplified using the following primers: (5′) ATGAATAAGGAAGAAAATTCCTCC and (3′) GTTCT TATACATGTCACAGAGCACCTTTTCC and cloned into pENTR D/TOPO (Invitrogen). Deletion of the various domains was performed by site directed mutagenesis (Finnzymes) using the *pENTR-unf* plasmid as a template. All constructs were fully sequenced. To allow site-specific integration of the transgene [34], we created a modified Gateway destination vector that we named pTWF-attB. Transgenes were injected into 86Fb (M{3xP3-RFP.attP}ZH-86Fb) landing site using φC31 integration (by BestGene or Genetic Services). For more details, see Supplemental Experimental Procedures.

Drosophila Strains

unf^{X1}, unf^{Z0001}, and genomic rescue flies (*p[unf]3.1*) were provided by S. Robinow [15]. unf^{X1} and unf^{Z0001} alleles were recombined onto FRTG13 containing chromosomes. $TOR^{\Delta P}$, $Tsc1^{29}$, *UAS-Rheb*, *UAS-*TOR^{WT}, *UAS-S6k.STDE*, and *UAS-S6k.STDETE* were obtained from the Bloomington Drosophila Stock Center (Indiana University, USA). *UAS-EcR-B1* on the second chromosome has been previously described [7]. To precisely excise *piggyBac* insertions, we crossed flies with flies containing the *piggyBac* transposase J10, selected against the DsRed marker and rebalanced [7].

For detailed genotypes, see Supplemental Experimental Procedures.

Generation and Imaging MARCM Clones

MARCM clones of MB neurons and single cell clones were generated at NHL (for γ clones) or 24APF (for α/β clones) and examined later, as described previously [5]. Brains were mounted on Slowfade (Invitrogen) and imaged on Zeiss LSM710 confocal microscopes.

Antibody Staining Conditions

Rat monoclonal anti-mouse CD8 α subunit, (Invitrogen), 1:100; mouse monoclonal anti-Flag M2 antibody (Sigma, F1804), 1:100; anti-UNF, 1:4000 [gift of T. Lee; 17]; mouse monoclonal anti-FasII (1D4), 1:50; mouse monoclonal anti-EcR-B1 (AD4.4), 1:25 (Developmental Studies Hybridoma Bank). Alexa488, Alexa546, Alexa568, Alexa633, or Alexa 647 conjugated secondary antibodies were used at 1:300 (Invitrogen).

Statistical Analysis

For developmental regrowth quantification, statistical analysis was performed by a one-way ANOVA including all groups with a Dunnett T3 post hoc. Significance was calculated as $p < 0.05$.

Supplemental Information

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.07.044.

Acknowledgments

We are grateful to S. Graff, M. Shneyder, N. Strasser, and J.M. Levy for technical assistance. We thank T. Schwarz, B. Shilo, E. Peles, M. Schuldiner, A. Yaron, L. Luo, and the members of the Schuldiner laboratory for critical reading and discussions, T. Lee, B. Wightman, S. Robinow, the Bloomington Stock Center, and the Developmental Studies Hybridoma Bank (DSHB) for fly stocks, nematode strains, plasmids, and antibodies. We are indebted to L. Luo for his mentorship and support in whose laboratory this work was initiated with the support of National Institutes of Health grant R37-NS041044. S.P.Y was supported by the Dean of Faculty Postdoctoral Fellowship at the Weizmann Institute. This work was funded by the European Commission, grant FP7-PEOPLE-2008-IRG-239248, Israel Science Foundation grant (ISF) 686/11, and the Candice Appleton Family trust to O.S. and ISF grant 826/08 and BIKURA/FIRST grant 1542/07 to B.P. All the images except Figure S6 were obtained using a Zeiss LSM710 confocal microscope that was purchased with the help of the Adelis Foundation. O.S. is incumbent of the Aser Rothstein Career Development Chair.

Received: June 25, 2012
Revised: July 19, 2012
Accepted: July 19, 2012
Published online: August 23, 2012

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