2018-01-02

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Et al.

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Edited by Liqun Luo, Howard Hughes Medical Institute and Stanford University, Stanford, CA, and approved December 1, 2017 (received for review September 7, 2017)

Genetic studies of Wallerian degeneration have led to the identification of signaling molecules (e.g., dSarm/Sarm1, Axundead, and Highwire) that function locally in axons to drive degeneration. Here we identify a role for the *Drosophila* C_2H_2 zinc finger transcription factor Pebbled [Peb, Ras-responsive element binding protein 1 (RREB1) in mammals] in axon death. Loss of Peb in *Drosophila* glutamatergic sensory neurons results in either complete preservation of severed axons, or an axon death phenotype where axons fragment into large, continuous segments, rather than completely disintegrate. Peb is expressed in developing and mature sensory neurons, suggesting it is required to establish or maintain their competence to undergo axon death. *peb* mutant phenotypes can be rescued by human RREB1, and they exhibit dominant genetic interactions with *dsarm* mutants, linking *peb/RREB1* to the axon death signaling cascade. Surprisingly, Peb is only able to fully block axon death signaling in glutamatergic, but not cholinergic sensory neurons, arguing for genetic diversity in axon death signaling programs in different neuronal subtypes. Our findings identify a transcription factor that regulates axon death signaling, and *peb* mutant phenotypes of partial fragmentation reveal a genetically accessible step in axon death signaling.

Wallerian degeneration | axons | axon death

Neurons are connected over long distances by their axons, which can extend over more than a meter in humans. Maintenance of axon integrity is essential for sustained neural circuit function because axon breakage can block nervous system signal propagation. Axon loss is a hallmark of nervous system injuries, such as traumatic brain injury and spinal cord injury (1–3), is a unifying feature of neurodegenerative diseases (4), and is strongly correlated with functional loss in patients (5).

Wallerian degeneration (WD; axotomy) serves as a useful model to study basic aspects of axon biology, and to identify axon death signaling molecules. Severed axons, after a defined latent phase, undergo explosive fragmentation and are ultimately cleared by surrounding phagocytes (6–8). The discovery of the slow WD (Wld^S) mutant mouse, where severed distal axons survived for weeks after axotomy, radically changed our view of axonal biology (9). The observed long-term survival of distal severed axon fibers in Wld^S animals demonstrated that axon degeneration is a controlled process, and that under some conditions, axons could survive for weeks without a cell body (10–13). A growing number of studies across several species support the notion that the competence to undergo degeneration is likely a genetically programmed event: in the rock lobster, distal severed axons have been found to survive for a year after transection in vivo, and remain capable of evoked release at neuromuscular junctions (14); fragments of *Aplysia* axons can survive in vitro for extended periods of time without degeneration (15); and in *Caenorhabditis elegans*, most distal severed axons never degenerate (16). Despite these surprising observations, to our knowledge, nothing is known about transcriptional mechanisms that regulate the competence of axons to degenerate.

In axons that do undergo WD, the execution of degeneration is driven by axon death signaling molecules. *Drosophila* dSarm (sterile α, ARM, and TIR domain protein) was the first endogenous molecule shown to actively promote axon death (17). Sarm1 functions in a conserved role in mammals (17, 18), where it has been proposed to act as an NAD^+ hydrolase that drives axonal degeneration through promoting metabolic catastrophe (19, 20). *Drosophila* dSarm is similarly capable of NAD^+ hydrolysis (20), but requires signaling downstream through the BTB/BACK domain molecule Axundead to execute axon death in vivo (21). The E3 ubiquitin ligase Highwire/Phr1 also modulates axon death signaling (22, 23) through a mechanism that appears to involve regulating levels of the NAD^+ biosynthetic molecule dNmnat/Nmnat2 (22, 24). In both *Drosophila* and mammals, all neurons tested thus far have been strongly protected by loss-of-function mutations in dSarm/Sarm1 (17, 18, 25), which suggests that axon death signaling molecules are engaged to drive destruction in a wide array of, or perhaps all, neuronal subtypes.

In this study, we present the identification and characterization of a role for Pebbled (Peb), a transcription factor, in axon degeneration. *peb* mutants show two predominant axon death-defective phenotypes: (*i*) severed distal axons are fully preserved morphologically, or (*ii*) the axon shaft breaks into large fragments (partially fragmented axons, PFAs) that fail to disintegrate

Significance

Known regulators of Wallerian degeneration appear to function locally in distal axons after axotomy to drive axon destruction. How competence to undergo axon death is established, and whether different subsets of neurons use alternate genetic programs to execute axon death, remains unclear. In this study, we show the transcription factor Pebbled (Peb) is required primarily in glutamatergic, but not cholinergic neurons, for appropriate axon degeneration after axotomy. Analysis of Peb mutant phenotypes also revealed an axon death phenotype where axon shafts initially broke into large fragments, but then failed to disintegrate. Peb is unique as a transcription factor shown to regulate Wallerian degeneration, and our analysis identifies a genetically accessible step in axon death signaling in vivo.

Author contributions: J.E.F., T.C.B., and M.R.F. designed research; J.E.F., T.C.B., and R.B. performed research; L.J.N. contributed new reagents/analytic tools; J.E.F., T.C.B., K.P.K., and J.E.L. analyzed data; and J.E.F. and M.R.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1715837115/-/DCSupplemental.

further, lingering in the nervous system for weeks. The PFA phenotype in *peb* mutants is not observed in control or axon death mutants, and therefore defines a genetically accessible step in axon death signaling. Surprisingly, while PFAs form in all neurons, the ability of *peb* mutations to completely suppress axon degeneration was only observed in glutamatergic neurons, and not in cholinergic neurons, arguing that Peb functions to differentially modulate competence to undergo axon degeneration in distinct subsets of neurons.

Results

Isolation of a Mutant That Suppresses WD. To identify novel endogenous regulators of WD, we performed an X chromosome-based F_2 forward genetic screen in glutamatergic sensory neurons in the adult *Drosophila* wing using mosaic analysis using a repressible cell marker (MARCM) (26). Flies were bred to generate neuronal MARCM clones using the *OK371-GAL4* driver, and axons were severed by surgical removal of the distal half of the wing. Animals were aged for 7 d postaxotomy (dpa), after which wings were dissected from the animal and axon death was quantified and imaged in the proximal wing vein. Neurons with cell bodies that were proximal to the injury site remained healthy and uninjured, and served as internal controls, represented by "cb" (cell body) in the upper right corner of each figure panel.

In control wings, injured axons typically undergo fragmentation by 12 h postaxotomy (hpa), degenerate fully by 24 hpa, and are cleared by surrounding glia after 5 dpa (26). We screened ~2,000 independent X-chromosome mutant stocks, and identified one, *345x*, that exhibited suppression of WD (Fig. 1*A*). While uninjured *345x* mutant axons were similar in morphology to control, 60% of injured axons in *345x* mutants remained partially or fully preserved at 7 dpa compared with 0% in wild-type control: 22% of severed *345x* mutant axons remained morphologically intact, while 38% initiated fragmentation, but were maintained as PFAs (Fig. 1*B*). We defined PFAs as GFP^+ axon fragments that spanned the imaging field-of-view, and were aligned linearly such that they appeared to represent the remnants of a previously intact axon. We rarely observed PFAs in control animals, and only early in the phase of axon degeneration (Fig. 1*D*), but never in *dsarm*, *axundead*, or *highwire* mutants (17, 21, 22). Therefore, *345x* mutations result in an axon death phenotype, apparently specific to the execution phase of axonal destruction.

To distinguish PFAs from normal cellular debris produced by degenerating axons, we measured the length of all GFP^+ axonal membrane debris from both control and *345x* clones at 7 dpa. The sparse debris in controls averaged 0.9 μm in length, while *345x* mutant axons fragments averaged 3.0 μm, ranging in size from 0.2 to 25.5 μm in length (Fig. 1*C*). Preserved intact axons and PFAs persisted in *345x* mutants over time, as both phenotypes were observed out to 14 dpa (Fig. 1 *D* and *E*), although at reduced levels.

While one portion of a severed axon in *345x* mutants was fully protected, it was plausible that PFAs might be generated at other positions along the axon. To explore this possibility, we scored axon morphological integrity with single-axon resolution along the entire length of individual severed intact axons in the wing vein of *345x* mutants (Fig. 1 *F* and *G*). We found that when axons were preserved in the proximal wing, 100% of those axons remained morphologically intact along their entire length (Fig. 1*H*). We conclude that severed axons in *345x* mutants fall into one of three phenotypic categories: (*i*) they are morphologically preserved along their entire length, (*ii*) they generate PFAs along their entire length, or (*iii*) they degenerate and are cleared normally.

Mitochondrial dysfunction is a hallmark in axon degeneration, and is observed after axotomy and in neurodegenerative diseases (27–29). In Wld^S-expressing axons, mitochondria persist after axotomy long after mitochondria in control animals have been destroyed (30). We therefore examined mitochondrial morphology before and after axotomy in control and *345x* mutant clones. Using a GFP targeted to mitochondria, we found no significant difference in the average mitochondria length in uninjured neurons between control and *345x* mutants (Fig. 1*I*). Just before the explosive fragmentation stage of the distal axon, at 8 h after injury, wild-type mitochondria began to decrease in size, with an average length of 0.36 μm. At 8 hpa, mitochondria of *345x* mutants were significantly longer, with lengths averaging 0.9 μm (Fig. 1*I*). However, when we looked at injured axons 24 h after injury, mitochondria in debris of control axons and severed intact *345x* mutant axons were indistinguishable (Fig. 1*J*). These data show that *345x* mutant mitochondria exhibit a normal, albeit delayed, morphological response to axon injury within axon death-defective axons. This loss of mitochondria in *345x* mutant axons that are morphologically intact is strikingly different from *dsarm*-null mutant (Fig. 1 *I* and *J*) or Wld^S-expressing severed axons (30). Surprisingly, *345x* mutations are capable of preserving axon integrity despite significant depletion of mitochondria.

The *345x* Mutation Maps to the C_2H_2 Zinc Finger Transcription Factor Pebbled/RREB1. *345x* mutants were homozygous-lethal, suggesting the mutation affected an essential gene. This observation was supported by the fact that lethality associated with *345x* cosegregated with the axon death phenotype during backcrossing over five generations. To identify the gene affected by the *345x* mutation, we used genome-wide sequencing to identify mutations in the backcrossed *345x* mutant stock, and found only one mutation that remained in *345x* compared with the original isolate, which was a mutation in the *pebbled* (*peb*) gene that resulted in the loss of a splice donor site in exon 2 and generated a premature stop codon (Fig. 2*A*). In a parallel set of experiments, we screened a collection of X-chromosome deficiency lines that had been recombined onto an FRT chromosome with the MARCM approach. In total, these deficiencies removed 38.7% of X chromosome genes, and two nonoverlapping deficiency lines led to a suppression of axon death (Fig. S1). The first deficiency uncovered the E3 ubiquitin ligase Highwire, a known axon death signaling molecule (22, 23). The second overlapped with the genetic region to which we mapped *345x*, and included the *peb* gene.

Peb [Ras-responsive element binding protein 1 (RREB1) in mammals] is a conserved transcription factor that contains 14 C_2H_2 zinc finger domains (31). In *Drosophila* embryos, Peb is expressed in amnioserosa (AS), anterior and posterior midgut (AM and PM, respectively), trachea, and in the peripheral nervous system and imaginal discs during later stages (32–34). Loss of Peb function leads to defects in embryonic germband retraction and dorsal closure of the embryonic epidermis, resulting in lethality. Peb also plays important roles in axon guidance in photoreceptor cells in the developing *Drosophila* visual system (35, 36), but roles for Peb/RREB1 in axon death have not been previously reported. We performed immunohistochemistry (IHC) of early-stage embryos with α-Peb antibodies and confirmed that Peb was localized to the AS, and both the AM and PM (Fig. 2*B*) (32). Consistent with our prediction that the *345x* mutations would lead to a loss of Peb protein product, we found that embryos homozygous for *345x* lacked α-Peb immunoreactivity (Fig. 2*B*). IHC analyses in wing tissues during later stages of development show Peb is detectable only within the nuclei of developing and mature neurons and not present in the cytoplasm of the cell body and axon (Fig. S3).

To confirm that the mutation of *peb* was responsible for the axon death phenotype, we assayed axon death in adult wing glutamatergic neurons with a second *peb* allele, peb^{E8} (37). We found that peb^{E8} mutant clones phenocopied the *345x* mutant phenotype, although the phenotype was slightly weaker: in peb^{E8}

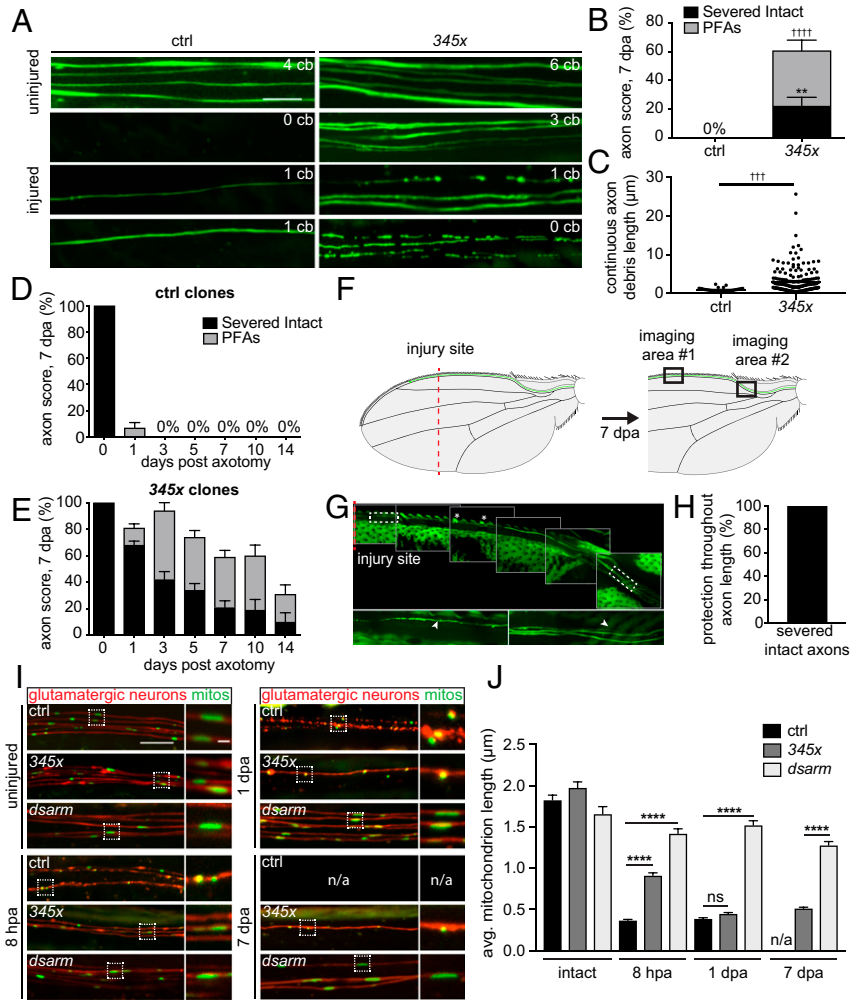

Fig. 1. Mutation *345x* causes defective WD in vivo. (*A*) Glutamatergic clones within the L1 anterior wing vein were labeled with mCD8::GFP. Uninjured and injured (7 dpa) are shown. (Scale bar, 10 μM.) Cell bodies (cb) of uninjured clones within each wing were counted and indicated in the upper right corner of each panel. Mutant *345x* exhibited two axon death-defective phenotypes: either severed intact or PFAs. (*B*) Quantification of *peb* mutant phenotype. PFAs (gray) and severed intact axons (black). Daggers (†) and asterisks (*) represent PFA and severed intact significance, respectively ($n > 30$ wings). Two-way ANOVA, error = SEM ($^{**,\dagger\dagger}P < 0.01$; $^{***,\dagger\dagger\dagger}P < 0.001$; $^{****,\dagger\dagger\dagger\dagger}P < 0.0001$). (*C*) Quantification of total continuous GFP^+ axon debris length, 7 dpa. Unpaired *t* test, error = SEM $^{****}P < 0.0001$. (*D* and *E*) Time-course data for both controls and *345x* mutants over 14 dpa ($n > 30$ wings). (*F*) Schematic of the adult wing showing two imaging fields-of-view, proximal and distal to the injury site. (*G* and *H*) Severed intact axons were imaged and traced along the entirety of the wing (individual images, outlined in grey, stitched together, 63× magnification). Asterisks indicate cell bodies of uninjured clones ($n = 14$ wings, 27 severed intact axons). (*I*) Mitochondria (green) and glutamatergic neurons (red) of control and *peb* mutant clones imaged and indicated time points. (Scale bar, 10 μM; *Insets*, 1 μM.) (*J*) Quantification of mitochondria length at 8 hpa (control: $n = 11$ wings, 185 mitos. *peb:* $n = 13$ wings, 241 mitos. *dsarm:* $n = 11$ wings, 158 mitos.), 1 dpa (control: $n = 10$ wings, 211 mitos. *peb*: $n = 16$ wings 170 mitos. *dsarm*: $n = 10$ wings, 128 mitos), and 7 dpa (*peb*: $n = 15$ wings, 182 mitos. *dsarm*: $n = 12$ wings, 209 measurements). One-way ANOVA, Tukey's multiple comparisons test, error = SEM, $^{****}P < 0.0001$. ctrl, control; n/a, not applicable; ns, not significant.

mutant MARCM clones 12% of severed axons remained morphologically intact, and 21% formed PFAs (Fig. 2 *C* and *D*). We next crossed a bacterial artificial chromosome (BAC) containing the genomic copy of *peb* into *345x* mutants, and found that reintroduction of a wild-type copy of peb^{BAC} into *345x* mutants completely rescued the axon death phenotypes (i.e., both axon degeneration, the production of PFAs, and clearance of axonal debris) to control levels (Fig. 2 *E* and *F*). Addition of peb^{BAC} had no effect on uninjured axons (Fig. S2). Furthermore, we found that the peb^{BAC} rescued the lethality of the *345x* mutation. We conclude that *345x* is a mutation in the *peb* gene, and henceforth refer to it as peb^{345x}.

Human RREB1 Can Functionally Substitute for Pebbled. To explore how Peb regulates axon death, we drove expression of cDNA constructs encoding full-length Peb, truncated Peb containing zinc fingers 1–8 or 10–14, and the human homolog, Ras-responsive element binding protein 1, (Peb, Peb^{1-8}, Peb^{10-14}, and hRREB1, respectively) in control and peb^{345x} clones, and quantified axon degeneration 7 dpa (Fig. 3*A*). Overexpression of full-length Peb using the GAL4/UAS system (and driving expression with the *OK371-GAL4* driver) was sufficient to completely rescue the blockade of axon degeneration observed in peb^{345x} clones: 100% of mutant axons initiated axon fragmentation (Fig. 3 *B* and *C*). Expression of the C-terminal zinc finger domains 10–14 (in Peb^{10-14}) was sufficient to rescue the axon death defect in *peb* mutants. In contrast, expression of N-terminal Peb^{1-8} failed to rescue the axon protective phenotype. This defines the key domains essential for Peb function in prodegenerative signaling, and provides additional support for a transcriptional role for Peb, consistent with previous work demonstrating that C-terminal domains are required for Peb to bind DNA in vitro (31). Expression of hRREB1 was also sufficient to fully rescue the blockade of axon degeneration (Fig. 3 *B* and *C* and Fig. S2), arguing that Peb and hRREB1 exhibit similar properties with respect to target gene regulation. However, while the initiation of axon fragmentation was strongly rescued, 17% of the GFP^+ injured axons persisted as PFAs in peb^{345x} background rescued with Peb. This approximates the percentage of axons that form PFAs in peb^{345x} mutant clones expressing a *UAS-LacZ* control rescue construct, and we observed PFAs in peb^{345x} mutants rescued with hRREB1 expression. Additionally, expression of either Peb or hRREB1 in control animals led to the production of PFAs, which are only ever rarely observed in the earliest phase of normal axon degeneration (Fig. 3 *B* and *C*). These data suggest Peb expression levels may need to be fine-tuned for proper rescue of execution of axon death and destruction of PFAs, with too much or too little Peb resulting in PFA formation.

Peb Loss Can Fully Preserve Severed Axons in Glutamatergic, but Not Cholinergic Neurons. There are ∼250 sensory neurons in the *Drosophila* wing that send axonal projections to the thoracic ganglion in the CNS (38). Most cells fall into two subsets based on neurotransmitter profiles: cholinergic (∼145 cells labeled by *ChAT-GAL4*) and glutamatergic neurons (∼40 cell labeled by *vGlut-QF2* or *OK371-GAL4*) (Fig. 4 *A* and *B*) (26). Because the

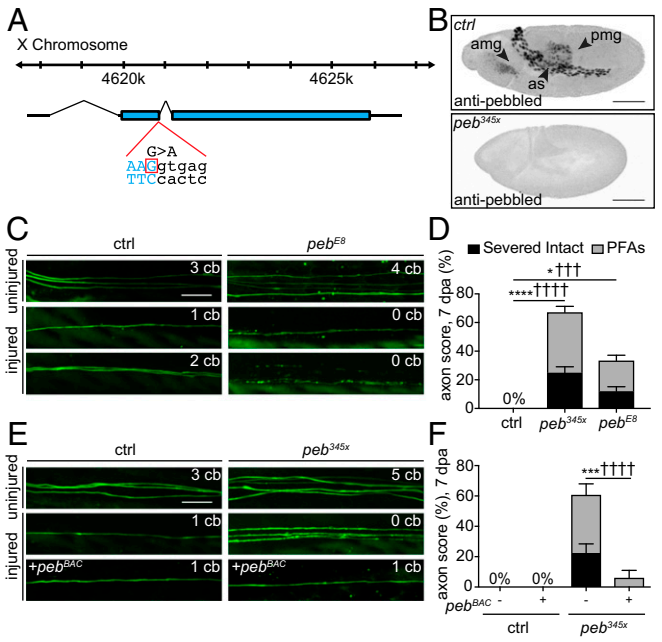

Fig. 2. *345x* encodes the transcription factor *pebbled*. (*A*) The *345x* mutation in exon 2 of the *pebbled* gene. (*B*) Pebbled in control embryos localized to the AS and midgut (anterior AM, posterior PM). peb^{345x} mutant embryos lacked Peb protein. (Scale bars, 100 μm.) (*C* and *D*) Representative images and quantifications showing peb^{E8} mutant allele phenocopied peb^{345x} mutants ($n > 30$ wings). (*E* and *F*) Representative images and quantifications showing genomic peb^{BAC} rescued defective axon death back to control levels ($n > 30$ wings). (Scale bar, 10 μm.) Two-way ANOVA, error = SEM ($^{*,\dagger}P < 0.05$; $^{***,\dagger\dagger\dagger}P < 0.001$, $^{****,\dagger\dagger\dagger\dagger}P < 0.0001$). amg, anterior midgut; as, amnioserosa; ctrl, control; pmg, posterior midgut.

peb^{345x} mutation was identified and characterized in glutamatergic neurons, we wished to determine whether it also regulated axon death signaling in cholinergic neurons. Approximately 23% of severed peb^{345x} glutamatergic clones failed to undergo axon degeneration at 7 dpa, and ∼40% of severed axons formed PFAs (Fig. 4 *C* and *D*). When we assayed cholinergic peb^{345x} clones, we found a complete lack of protection from axon degeneration: 100% of all neurons began fragmenting (Fig. 4 *C* and *D*). Of severed axons, 19% form PFAs, indicating that *peb* also regulates PFA formation at some level in cholinergic neurons compared with control clones that underwent normal axon death (Fig. S4). In axotomy assays with the pan-neuronal driver *nSyb-GAL4*, we observed only 4% of severed mutant clones remaining intact, and there was no change in PFAs compared with peb^{345x} glutamatergic clones. Control pan-neuronal clones had a normal degenerative response to injury. Given that glutamatergic neurons represent ∼20% of the *nSyb-GAL4*$^{+}$ cells, we suspected the only cells protected in this background by the peb^{345x} mutation were the glutamatergic neurons. Indeed, after screening many hundred clones of injured axons where we simultaneously labeled cholinergic neurons (with *ChAT-GAL4*) and glutamatergic neurons (with *vGlut-QF2*), we only found a single severed, intact axon that expressed *ChAT-GAL4*, and it was also positive for *vGlut-QF2* and therefore also glutamatergic (Fig. 4*E*). The observation that only glutamatergic neurons can be fully protected by peb^{345x} mutations, while PFAs appear with equal frequency in both cholinergic and glutamatergic neurons, further supports the notion that the initiation (i.e., fragmentation) and execution (i.e., full degradation) of axons are genetically separable and differentially regulated by Peb levels. Moreover, ability of *peb* mutants to completely block axon death is limited in the wing to glutamatergic sensory neurons.

peb Mutations Do Not Block dSarm-Induced Axon Death, Although *dsarm* Mutations Can Enhance *peb* Mutant Phenotypes in Axon Death.

Activation of Sarm1 signaling potently drives axon death in mammalian and *Drosophila* neurons (19). To determine whether Peb acts downstream of dSarm, we crossed peb^{345x} mutants into a background expressing a gain-of-function version of dSarm ($dSarm^{\Delta ARM}$) (21) and assayed for suppression of axon death. Loss of Peb function was not sufficient to block $dSarm^{\Delta ARM}$-induced axon death (Fig. 5 *A* and *B*), arguing that Peb does not act genetically downstream of dSarm, and demonstrating that constitutive activation of dSarm is sufficient to eliminate the appearance of PFAs. We next sought to determine whether *peb* mutants exhibited any dominant genetic interactions with components of the axon death signaling cascade, including *dsarm* (17), *axundead* (21), or *highwire* (22). We crossed loss-of-function mutations of *dsarm* or *axed* into the peb^{345x} background, and found that loss of a single copy of *dsarm* was sufficient to double the number of intact axons in peb^{345x} animals, while loss of a single copy of *axed* had no effect (Fig. 5C). Removal of one copy of *dsarm* or *axed* in control clones had no effect on axon degeneration (Fig. S4). In addition to the increase in fully protected axons, loss of one copy of *dsarm* also reduced the number of PFAs in peb^{345x} mutants (Fig. 5*C*). Reciprocally, we found that overexpression of dSarm in a peb^{345x} background led to a partial suppression of the ability of peb^{345x} to fully protect axons, and an increase in the number of PFAs. Manipulation of Axed or Hiw had no effect (Fig. 5*D*). Overexpression of dSarm, Axed, and Hiw in control clones had no effect on axon degeneration (Fig. S4). These data indicate the neuroprotective effects of peb^{345x} mutants are sensitive to dSarm levels.

Discussion

Distal axons separated from their cell bodies can survive in a functionally competent state for days to weeks after axotomy in multiple species (14, 21, 39). It therefore seems plausible that some axons are programmed to degenerate while others are not. In this study, we identify the transcription factor Pebbled (Peb)/RREB1 as an essential modulator of axon death in *Drosophila*. Through epistatic analysis, we can place *peb* upstream of *dSarm* or in a separate, parallel pathway, which ultimately converges on axon death. The simplest interpretation of our data is that Peb

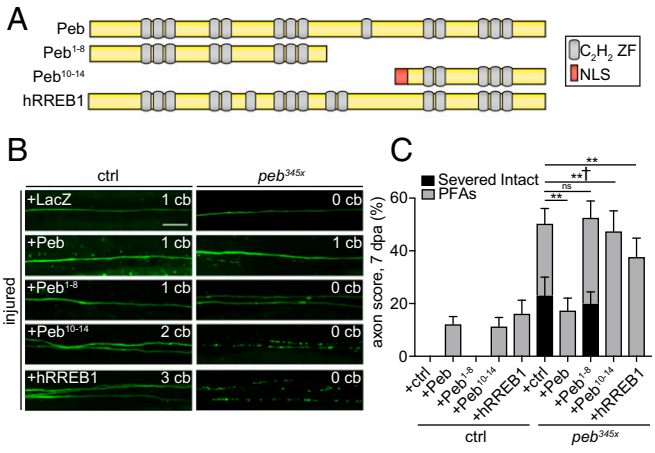

Fig. 3. The Peb C-terminal domain or hRREB1 rescue axon death defects in *peb* mutants. (*A*) Schematic of Pebbled protein. Pebbled contains 14 C_2H_2 zinc finger domains (gray ovals). N-terminal Peb$^{1-8}$ and C-terminal Peb$^{10-14}$ (with NLS added) truncated proteins and hRREB1 are shown. (*B* and *C*) Representative images and quantification of Peb and hRREB1 rescue of *peb* clones, 7 dpa (Scale bar, 10 μm.) ($n > 30$ wings). Two-way ANOVA, Tukey's multiple comparisons test, error = SEM ($^{\dagger}P < 0.05$; $^{**}P < 0.01$; ctrl, control; ns, not significant).

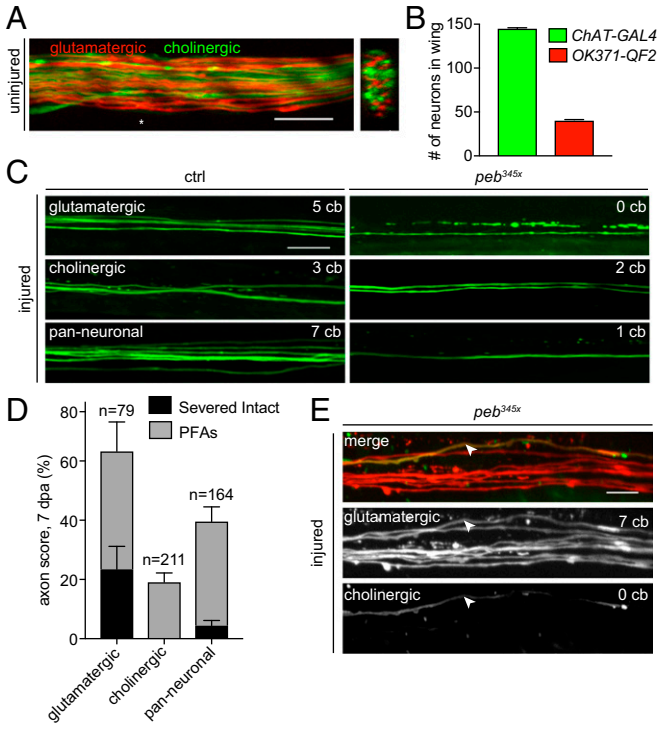

Fig. 4. Pebbled axon death defect observed only in glutamatergic neurons. (*A*) Cholinergic neurons (green) and glutamatergic neurons (red) in the adult wing vein. An asterisk (*) marks position of orthogonal view. (Scale bar, 10 μm.) (*B*) Number of cholinergic and glutamatergic neurons in the wing vein using *ChAT-GAL4* and *vGlut-QF2*. (*C*) Axon death in glutamatergic, cholinergic, or all neurons. (*D*) Axon protection at 7 dpa ($n > 30$ wings per genotype. Glut = 79 injured neurons, chol = 211 injured neurons, all = 164 injured neurons). (*E*) Severed intact *peb* clone double labeled with *OK371-QF2* (red) and *ChAT-GAL4* (green), indicated by arrowhead. (Scale bar, 10 μm.)

regulates axon death signaling in glutamatergic axons at the transcriptional level. Human RREB1 can rescue axon death phenotypes associated with loss of *peb*, arguing for strong conservation of the binding properties of Peb and hRREB1, and implying that RREB1 may play similar roles in axon biology in mammals.

Pebbled appears to identify a step in the axon death signaling cascade. Loss of Pebbled function resulted in the appearance of three axon phenotypes after injury: (*i*) full morphological preservation with a slightly delayed loss of mitochondria; (*ii*) the generation of PFAs that linger in the nervous system for weeks, but which also lose mitochondria after a short delay; or (*iii*) apparently normal axon death signaling and clearance. The cell biology of axon preservation in *peb* mutants is unique, and implies that *peb* mutants identify a genetically accessible step in axon death signaling. PFAs have not been observed in other axon death mutants (i.e., *dsarm*, *hiw*, or *axed*), as these mutants all fully block axon degeneration after axotomy (17, 21, 23). Understanding the nature of PFA production compared with normal axon degeneration is an important goal for future study. In the case of *dsarm* mutants, in addition to the axon shaft maintaining complete integrity, mitochondria also appeared well-preserved. That was not the case in *peb* mutants, where mitochondria degenerated after only a short delay, and preserved axons were severely depleted of mitochondria for the duration of their extended survival. Interestingly, the phenotype of individual *peb* mutant axons is consistent along the entirety of the axon shaft: we never observed an axon that generated PFAs in one portion, but was fully protected elsewhere. Unraveling the molecular basis of this all-or-none type of phenotypic expression is of great interest for the future. Finally, while some PFAs are observed in control animals immediately after the initiation of axon fragmentation, they quickly undergo explosive degeneration and are cleared. From these data, we conclude that Peb functions both at the initial phase of axon breakage into smaller fragments, and subsequently during the phase of explosive degeneration.

To date all known axon death signaling molecules—dSarm, Hiw, and Axed—have been proposed to function locally in the axon to drive destruction, and their neuroprotective effects extend to both glutamatergic and cholinergic neurons (17, 21, 22). Based on its expression in the nuclei of wing sensory neuron precursors and mature neurons, and the fact it is a C_2H_2 zinc finger transcription factor, we propose that Peb functions at the transcriptional level to help establish and maintain competence to undergo axon degeneration. While Peb appears to be expressed broadly in wing sensory neurons, surprisingly, the ability of *peb* mutants to fully block axon fragmentation is restricted to glutamatergic neurons. How Peb selectively protects glutamatergic axons is unclear, but could modify axonal phenotypes through the JNK signaling cascade. During embryogenesis, in AS *peb* mutants show increased levels of AP-1 transcriptional activity downstream of the JNK signaling cascade (40), which in turn inhibits cytoskeletal rearrangements that allow for cell migration (32, 40). We have found a lack of evidence to support a role for JNK signaling in axon death (17, 21), but some data support a neuroprotective role for this pathway by controlling baseline levels of Nmnat (41). Peb has also been shown to negatively regulate *nervy*, the *Drosophila* homolog of mammalian MTG8 proto-oncogene (31); however, we observed no alterations in axon death when we overexpressed nervy in glutamatergic neurons.

The nuclear localization requirement of the carboxy terminal DNA binding zinc finger domains of Peb for rescue suggests that Peb is regulating injury-induced axon degeneration at the transcriptional level. We attempted to identify direct transcriptional targets of Peb by expressing a tagged version of Peb in the *Drosophila* embryonic cell line, GM2, and performing ChIP with antibodies specific to tagged Peb and subsequent deep sequencing (ChIP-seq). This approach successfully identified the

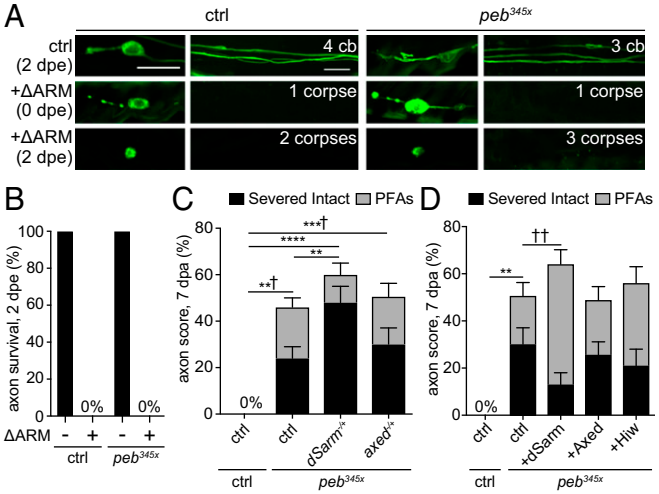

Fig. 5. The neuroprotective effects of peb^{345x} mutants are sensitive to dSarm levels. (*A* and *B*) dSarmΔARM (ΔARM) induced axon death in uninjured control and peb^{345x} clones at 2 d posteclosion (dpe) ($n > 15$ wings). (Scale bar, 10 μm.) (*C*) Quantifications of axon death 7 dpa in control and peb^{345x} clones when one copy of *dsarm* or *axed* was removed ($n > 30$ wings). (*D*) Quantifications of axon death at 7 dpa in control and peb^{345x} clones expressing dSarm, Axed, or Hiw ($n > 30$ wings). Two-way ANOVA, error = SEM ($^{*,\dagger}P < 0.05$; $^{**,\dagger\dagger}P < 0.01$; $^{***,\dagger\dagger\dagger}P < 0.001$; $^{****,\dagger\dagger\dagger\dagger}P < 0.0001$). ctrl, control.

one known target for Peb, the transcriptional regulator Nervy, whose overexpression did not block axon death, and several potential Peb targets (Table S1). We did not find evidence for direct binding of Peb to regions containing known axon death signaling genes (i.e., *dsarm*, *axed*, or *hiw*). This could indicate that Peb does not directly modulate axon death genes in vivo to exert its effects, although it remains unclear how similar Peb transcriptional activity in GM2 cells might be compared with neurons. Finally, it remains unclear why *dsarm*, but not *axed* mutations, modify *peb* mutant phenotypes, given that Axed signals downstream of dSarm in axon death. A deeper understanding of the molecular basis of axon degeneration, and the nature of PFAs, will likely be required to answer this question.

Our analysis of *peb* mutant phenotypes reveals features of the cell biology of axon death. Our discovery of PFAs in *peb* mutants implies that axon degeneration can be genetically dissected into distinct activation and execution phases, and we propose that PFAs represent activation, but failure to execute axon death. We found that either increased or decreased Peb levels could lead to the production of PFAs, arguing that fine-tuning of Peb levels is essential for appropriate execution of axon death. Furthermore, Peb modulation of PFA production is not limited to glutamatergic neurons, since we found PFAs in cholinergic neurons under both loss- and gain-of-function Peb conditions. Interestingly, Peb allows us to genetically separate mitochondrial loss from axon degeneration. We observed mitochondrial degeneration even in fully protected *peb* mutant axons, indicating that mitochondrial destruction must occur through a Peb-independent signaling pathway.

Methods

See *SI Methods* for more information and detailed descriptions of animal genotypes, EMS mutagenesis, transgenic constructs, the wing axotomy assay, subsequent wing microscopy, IHC procedures, antibodies used, cell culture techniques, ChIP methods, and deep sequencing analyses.

ACKNOWLEDGMENTS. We thank all M.R.F. laboratory members and colleagues in the field for feedback, comments, and suggestions; Jaeda Coutinho-Budd for her technical assistance in confocal image acquisition well as her scientific input and skilled editorial assistance; Dr. Maninjay Atianand for assistance with ChIP; and Amy Sheehan for generating the 5XUAS::5XMyc vector. Deficiency lines screened in this study were generated through a collaboration with the laboratories of Dr. Chris Doe and Dr. Tory Herman from the University of Oregon. Sequencing was performed at the Bauer Core Facility at Harvard University. This work was supported by National Institute of Neurological Disorders and Stroke Grant R01 NS053538 (to M.R.F.). During the period of this study, M.R.F. was an Investigator with the Howard Hughes Medical Institute and J.E.L. was a collaborating PI.

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