Regulation of axon regeneration by the RNA repair and splicing pathway

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Mechanisms governing a neuron’s regenerative ability are important but not well understood. We identify Rtca (RNA 3’-terminal phosphate cyclase) as an inhibitor of axon regeneration. Removal of Rtca cell-autonomously enhanced axon regrowth in the Drosophila CNS, whereas its overexpression reduced axon regeneration in the periphery. Rtca along with the RNA ligase Rtcb and its catalyst Archease operate in the RNA repair and splicing pathway important for stress-induced mRNA splicing, including that of Xbp1, a cellular stress sensor. Drosophila Rtca and Archease had opposing effects on Xbp1 splicing, and deficiency of Archease or Xbp1 impeded axon regeneration in Drosophila. Moreover, overexpressing mammalian Rtca in cultured rodent neurons reduced axonal complexity in vitro, whereas reducing its function promoted retinal ganglion cell axon regeneration after optic nerve crush in mice. Our study thus links axon regeneration to cellular stress and RNA metabolism, revealing new potential therapeutic targets for treating nervous system trauma.

Failure of damaged axons to regenerate is the primary cause of permanent disabilities after CNS injury and the irreversible neurologic dysfunction of neurodegenerative diseases. The ability of a neuron to regenerate its axon after trauma is governed by the interaction between its intrinsic growth capacity and the local environment1–4. Notwithstanding the discoveries of extracellular factors and intrinsic pathways that reduce the regenerative capacity of axons5–11, effective therapies have not yet emerged because removing the known inhibitory cues only partially restores regeneration, thus indicating the presence of additional inhibitory machineries that remain to be discovered.

Studies using model organisms such as Caenorhabditis elegans have begun to identify new genes important for axon regeneration12,13, illustrating the power of the genetic approach. To identify more factors that control axon regeneration, we recently established a Drosophila sensory neuron injury model that exhibits class-specific axon regeneration and demonstrated that the class IV dendritic arborization (da) neuron is capable of regenerating its axon in the periphery but exhibits limited regrowth inside the CNS, resembling its mammalian counterpart at the phenotypic and molecular levels14. Using this model, we have performed a candidate-based genetic screen focusing on axotomy-regulated genes from several organisms15–20 and identified Drosophila Rtca (CG4061), a cellular RNA-processing enzyme with unknown biological function21, as an inhibitor of CNS axon regeneration. Furthermore, we find that Drosophila Archease, a RNA ligase cofactor, functions downstream of Rtca as a pro-regeneration factor. Rtca and Archease are components of the RNA repair and splicing pathway, and they regulate the unconventional mRNA splicing of Xbp1, a stress sensor. Thus, Xbp1 acts as a substrate, readout and downstream effector for the regulation of axon regeneration by the RNA repair and splicing pathway.

RESULTS

Drosophila Rtca loss of function enhances axon regeneration

To assess axon regeneration, we used a previously described protocol14. Briefly, with a two-photon laser, we severed the axons of class IV da neurons (labeled with pickpocket (ppk)-CD4tdGFP) in the ventral nerve cord (VNC) (Supplementary Fig. 1) of second-instar larvae 48 h after egg laying (AEL), confirmed the degeneration of the remaining axons after 1 d (72 h AEL) and assessed their regeneration after 2 more days (120 h AEL) (Fig. 1a–g). Using this model, we tested the effect of RtcaNP5057 (Fig. 1b), an insertional loss of function (LOF) allele with a P-element inserted in the 5’-UTR, disrupting mRNA splicing and reducing transcript expression (Supplementary Fig. 2a). Compared to wild types, which showed limited regrowth (Fig. 1a), new axons regrew extensively from the retracted axon stems and extended into the commissure region, forming elaborate branches and reconnected commissure segments in RtcaNP5057 larvae. Similar phenotypes were seen in transheterozygotes of RtcaNP5057 over a deficiency line, Df(1)BSC718, that lacks the Rtca locus (Fig. 1c) and in a Rtca deletion allele, Rtcaab, generated from imprecise excision of RtcaNP5057 (Fig. 1d). Even stronger phenotypes were seen in Rtcaabmat, in which both the zygotic and maternal transcripts were removed (Fig. 1e). RtcaNP5057 is homozygous viable and fertile, so these larvae were derived from homozygous mutant

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mothers. The mothers of *Rtca<sup>NP5057</sup>/Df(1)BSC718* transheterozygotes and *Rtca<sup>A</sup>* mutants were heterozygous for the wild-type allele and may provide maternal wild-type *Rtca* transcripts. The fact that *Rtca<sup>Amat</sup>* mutants, in which both the zygotic and maternal transcripts were removed, showed a stronger phenotype than *Rtca<sup>A</sup>* zygotic mutants confirmed the maternal effect. Thus, the phenotype of *Rtca<sup>NP5057</sup>* mutants compared to *Rtca<sup>NP5057</sup>/Df(1)BSC718* transheterozygotes and *Rtca<sup>A</sup>* mutants is likely stronger because no wild-type maternal transcripts were provided to *Rtca<sup>NP5057</sup>* mutants. The function of *Drosophila* *Rtca* is cell autonomous, as its RNA interference knock-down in class IV da neurons (*ppk-Gal4>RtcaRNAi*) (Fig. 1f) but not in glial cells (*repo-Gal4>RtcaRNAi*) (Fig. 1g) recapitulated the enhancement of regeneration. The regeneration phenotype was further quantified by assessing the following metrics, as described previously<sup>14</sup>: regeneration percentage, terminal branching and commissure regrowth (Fig. 1k,l, Supplementary Fig. 3a and Online Figure 1)

**Figure 1** *Drosophila* *Rtca* loss of function enhances axon regeneration in the CNS and PNS. (a–g) *Rtca* removal increases class IV da neuron axon regrowth in the VNC. The injury site is demarcated by the dotted circle. Limited regrowth is seen in wild type (WT) (a, arrows). Increased regeneration is seen with *Rtca* LOF mutation (b, arrowheads), *Rtca* mutation over deficiency (c), *Rtca* zygotic deletion (d), *Rtca* zygotic and maternal deletion (e) and class IV–specific *Rtca* RNAi (f), but not glia RNAi (g, arrows). The regenerating axons are illustrated in schematic diagrams with terminal branching marked in red, commissure regrowth in blue and other regrowing axons in black. (h–j) *Rtca* removal promotes class III da neuron axon regeneration in the periphery. Dotted circle marks the lesion site. Whereas WT class III da neurons fail to regenerate their axon in the PNS (h, arrow), *Rtca* zygotic and maternal deletion (i) and class III specific *Rtca* RNAi (j) elicit substantial regrowth (arrowheads). (k) *Rtca* LOF increases regeneration percentage of class IV da neuron axons in the VNC (*N* = 46, 16, 18, 13, 14, 14 lesioned segments for each genotype; **P < 0.01, ***P < 0.001, one-way ANOVA followed by Dunnnett’s multiple comparisons test). (m–o) *Rtca* LOF increases the regeneration percentage (**P < 0.01, one-way ANOVA followed by Dunnnett’s multiple comparisons test) and regeneration length (**P < 0.05, one-way ANOVA followed by Holm-Sidak’s multiple comparisons test; *N* = 19, 10, 17 neurons for each genotype) of class III da neuron axons in the PNS. Scale bars, 20 μm. Data are expressed as mean ± s.e.m.
Methods). The enhancement of regeneration is unlikely to be due to developmental defects of axon outgrowth because, first, the overall axon patterning of class IV da neurons in the uncut VNC is grossly normal (Supplementary Fig. 2b) and second, reducing Rta function in Rta mutant or transheterozygotes of Rta over D(f1)BSC718 or via Rta RNAi in class IV da neurons did not result in obvious defects of axon terminal patterning in the VNC (Supplementary Fig. 2c).

We next tested whether reducing Drosophila Rta function would trigger a regenerative response in neurons normally incapable of regeneration by severing their axons in Rta mutants (Fig. 1h–j). Indeed, Rta removal in class III da neurons (labeled with 19-12-Gal4>CD4tdGFP, repo-Gal80), which unlike class IV da neurons did not regrow their axons that were severed in the periphery (Fig. 1h), elicited substantial regeneration in Rta mutants (Fig. 1i) and after RNAi knockdown of Rta specifically in class III da neurons (19-12-Gal4>RtaRNAi) (Fig. 1j), leading to significant increases in the regeneration percentage, regeneration index and regeneration length (Fig. 1m–o, Supplementary Fig. 3b and Online Methods).

Drosophila Rta gain of function reduces axon regeneration

Conversely, overexpression of Rta in class IV da neurons (ppk-Gal4>Rta) mildly reduced their regenerative potential in the peripheral nervous system (PNS). In wild-type class IV da neurons, which regenerated about 74% of the time, new axons extended beyond the lesion site and followed the axonal track (Fig. 2a). In contrast, Rta overexpression caused the incidence of regeneration to be reduced to 48% (Fig. 2b) and the length of the new axons to be significantly shortened as well (Fig. 2c–e). These data indicate that Drosophila Rta is an inhibitor of axon regeneration: not only does its removal cell-autonomously enhance axon regeneration in the CNS and enable regeneratively incompetent neurons such as class III da neurons to regrow their axons in the PNS, its overexpression in regeneratively competent neurons impedes axon regeneration in the periphery.

The inhibitory function of Drosophila Rta is, furthermore, not limited to sensory neurons. Rta overexpression in motor neurons also suppressed motor axon regeneration after nerve crush, as demonstrated by the reduced elaboration of growth cones (Fig. 3 and Online Methods).

The expression pattern of Drosophila Rta

We next examined the expression pattern of Drosophila Rta via two approaches. First, the P-element inserted in Rta'5'-UTR (Rta contains Gal4 in the same orientation as Rta and thus can allow us to infer Rta expression via a UAS reporter. We found that the Rta-Gal4>CD4tdGFP reporter colocalized in the class IV da neuron marker ppp-CD4tdTomato (Fig. 4a), confirming its presence in class IV da neurons. Although Rta-Gal4 expression was observed in other tissues in the PNS and VNC (Fig. 4a,b), our analyses indicate that Drosophila Rta functions cell autonomously in neurons to inhibit axon regeneration.

Second, we generated a polyclonal antibody against Drosophila Rta. The protein was present in wild-type but not in Rta null class IV and class III da neurons, and was enriched in the nucleus (Fig. 4c). Drosophila Rta was also present in other types of multidendritic neurons (Fig. 4c).

Interaction of Rta with known regeneration regulators

To begin to understand the mechanisms underlying Drosophila Rta’s role in regeneration, we first sought to determine how it genetically interacts with the known axon regeneration regulators Pten (phosphatase and tensin homolog) and the cytoskeletal regulator Rac1 GTPase. Deletion of Pten, a negative regulator of the mammalian target of rapamycin (mTOR) pathway, has been shown to increase CNS axon regeneration in both mammals and flies.

Figure 2 Drosophila Rta overexpression reduces axon regeneration in the periphery (arrowheads; dotted circle marks lesion site). (b) Rta overexpression in class IV da neurons mildly reduces their regeneration percentage (P < 0.05, Fisher’s exact test, P = 0.0398) and regeneration length (P < 0.05, two-tailed unpaired Student’s t-test, P = 0.0458; N = 43, 27 neurons for each genotype) of class IV da neuron axons in the PNS. Scale bars, 20 µm. Data are expressed as median with whiskers representing minimum and maximum values and box limits representing interquartile range.

Figure 3 Drosophila Rta overexpression reduces motor axon regeneration. Right after crush, the growth cones of the segmental motor nerves (labeled by m12-Gal4>CD4tdGFP, a GAL4 driver for muscle-12) display a simple morphology with very few filopodia. 14.5 h after crush, motor axons in wild-type (WT) larvae regenerates substantially, with elaborated growth cones. Rta overexpression (OE) reduces their regeneration. The total area of the growth cones at 14.5 h after crush is significantly reduced by Rta overexpression (N = 34, 33 nerves, ***P < 0.001, two-tailed unpaired Student’s t-test). Scale bar, 20 µm. Data are expressed as median with whiskers representing minimum and maximum values and box limits representing interquartile range.
Overexpression of Rtca in a Pten hypomorphic mutant background (Pten\textsuperscript{\text{MAT}}; ppk-Gal4>\textit{Rtca}) or overexpression of Pten in \textit{Rtca} null mutants (\textit{Rtca}\textsuperscript{\text{A}}, ppk-Gal4>Pten) largely abolished the enhancement of axon regeneration as seen in the VNC in \textit{Pten}\textsuperscript{\text{MGH6}} or \textit{Rtca}\textsuperscript{\text{A}} mutants (Fig. 5a,b). This suggests that \textit{Rtca} and \textit{Pten} are likely to function in parallel pathways. Notably, double mutation of \textit{Rtca} and \textit{Pten} (\textit{Rtca}\textsuperscript{\text{NP5057}}, \textit{Pten}\textsuperscript{\text{MGH6}}) did not further improve regeneration, as compared to \textit{Rtca} mutation alone (Supplementary Fig. 4a,b), indicating the presence of additional brakes on regeneration. The regeneration phenotype in \textit{Rtca} mutants appeared to be comparable to if not stronger than that in \textit{Pten}\textsuperscript{\text{MGH6}} mutants or that seen with \textit{Akt} overexpression (ppk-Gal4>Akt) (Supplementary Fig. 4c,d).

Because Rac is required for regenerative axon outgrowth in \textit{C. elegans}\textsuperscript{23}, we overexpressed \textit{Rac1} in class IV da neurons (ppk-Gal4>Rac1). We found an increase in the number of axons initiating the regenerative response in the VNC but not in terminal branching or commissure regrowth; that is, there was a partial improvement in regeneration (Fig. 5a–c). Conversely, overexpressing a dominant negative (DN) form of \textit{Rac1} abolished the enhancement of CNS axon regeneration seen in \textit{Rtca} null mutants (\textit{Rtca}\textsuperscript{\text{A}}; ppk-Gal4>\textit{Rac1DN}) (Fig. 5a, b), whereas \textit{Rac1DN} overexpression alone in class IV da neurons did not result in obvious axon regeneration defects in the PNS (Supplementary Fig. 4e–g). It thus seems likely that \textit{Rac1} functions downstream of \textit{Rtca} in a pathway that converges on regulation of the cytoskeleton.

**RNA repair and splicing pathway regulates axon regeneration**

\textit{Rtca} is a RNA processing enzyme that possesses RNA-3’-phosphate cyclase activity and catalyzes the ATP-dependent conversion of a 3’ phosphate to a 2’,3’-cyclic phosphodiester at the end of RNA molecules\textsuperscript{21}. The RNA 2’,3’-cyclic phosphate ends are important in RNA metabolism—for example, as intermediates during RNA repair by ligases\textsuperscript{24,25}. \textit{Rtcb} (RNA 2’,3’-cyclic phosphate and 5’-OH ligase) represents a new type of RNA ligase that joins 2’,3’-cyclic phosphate and 5’-OH RNA ends to yield a 3’-5’ phosphodiester splice junction\textsuperscript{24,26–28}. Specifically, \textit{Rtcb} is known to possess cyclic phosphodiesterase activity, which hydrolyzes the 2’,3’-cyclic phosphate to a 3’-phosphate, as well as ligase activity, which then joins...
the RNA 3′-phosphate to a 5′-OH RNA end. In addition, the specificity and efficacy of RtcB’s ligase activity can be enhanced by Archease29,30, which is a small acidic protein conserved among Eukarya, Bacteria and Archaea31. In Escherichia coli, RtcA and RtcB are encoded in a single operon, suggesting that they might cooperate to provide a healing and sealing function in an RNA repair pathway27. In one scenario, healing would refer to the restoration of ligatable 2′,3′-cyclic phospho- 
exs in the event of the inciting RNA damage directly generating RNA 3′-phosphates, or of the 2′,3′-cyclic phosphate products of RNA transesterification being further processed to a 3′-phosphate by a 2′,3′-cyclicphosphodiesterase22. However, this model cannot readily be reconciled with the subsequent finding that RtcB readily joins 3′-phosphate to 5′-OH ends or 2′,3′-cyclic phosphate to 5′-OH ends33,34. Therefore, the exact relationship between RtcA and RtcB remains undecided. Notably, the RtcBA operon in E. coli is regulated by the σ54 coactivator RtcR, suggesting that the RNA repair functions are induced in response to cellular stress21. Although the biological function of RtcA remains unknown, the enzyme is speculated to act in some aspect of cellular RNA processing22. Taking into account these findings and our observation that loss of RtcA function enhances axon regeneration, we hypothesized that the RtcA-Archease-dependent RNA repair and splicing pathway regulates axon regeneration. Specifically, we speculated that axon injury triggers a type of cellular stress leading to RNA damage and splicing, producing RNA 3′-phosphates that need to be processed and rejoined by the RtcB ligase, which is catalyzed by Archease. Because RtcA converts RNA 3′-phosphate to 2′,3′-cyclic phosphate, it can slow the ligation process and impede regeneration. Consequently, silencing RtcA promotes axon regeneration (Supplementary Fig. 5). Following this reasoning, we then investigated the role of Archease in axon regeneration.

To determine the role of the Drosophila Archease in axon regeneration, we first examined regeneration of class IV da neuron axons in the periphery. To maximize the phenotype, we modified our PNS axon injury protocol as described previously14, axotomy was induced at 72 h AEL, degeneration was confirmed at 96 h AEL and regeneration was assayed at 120 h AEL. We used the Archease (CG6353) LOF mutant allele ArcheasePko1013, which is an insertional allele with a P-element inserted into the 5′-UTR disrupting its mRNA splicing and eliminating Archease transcripts (Supplementary Fig. 6). Unlike in wild-type neurons, which exhibited substantial regrowth of their severed axons, axon regeneration was significantly impaired in ArcheasePko1013 neurons, as revealed by a significant drop of the regeneration percentage, regeneration index and regeneration length (Fig. 6a–c). This phenotype was confirmed in transheterozygotes of ArcheasePko1013 over either of the two deficiency lines, Df(3R)ED6076 or Df(3R)BSC678, that lack the Archease locus (Fig. 6a–c). The ArcheasePko1013 mutation is larval lethal. ArcheasePko1013 mutants and ArcheasePko1013/Df(3R)ED6076 and ArcheasePko1013/Df(3R)BSC678 transheterozygotes showed similar phenotypes, suggesting that ArcheasePko1013 is likely an amorphic allele. The function of Archease is required cell-autonomously, as class IV da neuron–specific knockdown of Archease (ppk-Gal4>ArcheaseRNAi) but not glial cell knockdown (repo-Gal4>ArcheaseRNAi) was sufficient to phenocopy the regeneration failure (Fig. 6a–c). Moreover, loss of function of both RtcA and Archease (RtcaNPs5075; ArcheasePko1013) completely abolished the axon regeneration–promoting effect in the VNC seen in RtcaNPs5075 mutants (Fig. 6d,e), producing many retracted or stalled axon stems (Fig. 6f). This epistasis analysis indicates that Archease is a pro-regeneration factor downstream of RtcA and that they act in opposing ways to regulate axon regeneration (Supplementary Fig. 5).

Xbp1 is a substrate of RtcA-Archease in regeneration

What might be the RNA substrates processed by this Drosophila RtcA-Archease–dependent RNA repair and splicing pathway for the regulation of axon regeneration? We investigated X-box binding protein 1 (Xbp1) as a candidate substrate for three reasons. First, cellular stress such as endoplasmic reticulum (ER) stress triggers an adaptive intracellular signaling cascade known as the unfolded protein response (UPR)35. One main branch of the UPR is the activation of Ire1, which cleaves Xbp1 pre-mRNA in the cytoplasm, converting the unspliced Xbp1µ, a putative transcriptional repressor, into the unconventionally spliced Xbp1β by eliminating an intron (26 nucleo- 
tides in mammals, 23 in flies) that changes the open reading frame of the third exon, resulting in a new protein that acts as a transcriptional activator36. Xbp1β directly activates ER stress target genes to facilitate refolding and also degradation of misfolded proteins35. Second, the RNA ligase RtcB and its cofactor Archease are involved in the unconventional splicing induced by the UPR, and Archease is required for the splicing of the Xbp1 mRNA37. Third, loss of xbp1 function in C. elegans results in severely reduced axon regeneration13. To determine the function of Xbp1 in axon regeneration in the PNS and CNS, we used a mutant allele, Xbp1k13801, that has a P-element inserted into its 5′-UTR, thus reducing transcripts38. Axon regeneration in the periphery was mildly reduced in these mutants (Fig. 6a–c). This defect
was stronger in transheterozygotes of \( Xbp1^{k13803} \) over a deficiency line, \( Df(2R)BSC484 \), that lacks the \( Xbp1 \) locus (Fig. 6a–c), suggesting that \( Xbp1^{k13803} \) is likely a hypomorphic allele. Class IV da neuron–specific (\( ppk-Gal4>\text{Xbp1RNAi} \)) but not glia–specific (\( repo-Gal4>\text{Xbp1RNAi} \)) RNAi of \( Xbp1 \) reproduced the impairment of regeneration (Fig. 6a–c), indicating it functions cell-autonomously. Moreover, double mutation of \( Rtca \) and \( Xbp1 \) (\( Rtca^{NP5057}; \ Xbp1^{k13803} \)) dampened the enhancement of CNS axon regeneration seen in \( Rtca^{NP5057} \) mutants (Fig. 6d,e), indicating that \( Xbp1 \) is indeed a pro-regeneration factor downstream of \( Drosophila \) Rtca. Consistent with these lines of evidence, overexpression of the spliced form \( Xbp1s \) in class IV da neurons significantly enhanced axon regeneration in the VNC.

**Figure 6** The \( Drosophila \) Rtca pathway in regulating axon regeneration. (a–c) \( Archease \) and \( Xbp1 \) are required for class IV da neuron axon regeneration in the PNS. \( Archease \) (left) or \( Xbp1 \) (right) deficiency in LOF mutants, mutant over deficiency and class IV da neuron–specific RNAi, but not glia RNAi, impede class IV da neuron axon regeneration in the periphery, as quantified by regeneration percentage (a), regeneration index (b) and regeneration length (c) (\( N = 26, 16, 26, 18, 25, 20, 18, 15, 16, 17 \) neurons). (d,e) Epistasis analysis of \( Rtca, Archease \) and \( Xbp1 \) indicates that \( Archease \) and \( Xbp1 \) function downstream of \( Rtca \). Double mutants of \( Rtca \) and \( Archease \) completely eliminate the enhancement of CNS axon regeneration in \( Rtca \) single mutants, while double mutants of \( Rtca \) and \( Xbp1 \) show a milder attenuation (\( N = 46, 16, 15, 16, 18 \) lesioned segments in d; \( N = 29, 8, 7, 8, 9 \) larvae in e). (f) A representative image of the stalled axon regeneration in the VNC of \( Rtca \) and \( Archease \) double mutants. (g,h) Heat shock induces the splicing of the active form of \( Xbp1 \) (\( Xbp1s \)), which is enhanced by \( Rtca \) LOF but inhibited in \( Archease \) LOF mutants and in double mutants of \( Rtca \) and \( Archease \). (g) Semiquantitative RT-PCR. Gels were cropped; all full-length images are presented in Supplementary Figures 12–15. (h) Quantification of \( Xbp1s/Xbp1\mu \) ratio (\( N = 3 \) experiments). (*) \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \); Fisher’s exact test in a,d, \( P = 0.004, P = 0.0023, P = 0.0049, P = 0.0217, P = 0.7406, P = 0.0581, P = 0.0025, P = 0.004, P = 0.7244 \) in a, \( P = 0.0001, P = 0.4831, P = 0.2178, P < 0.0001, P = 0.0008, P = 0.004 \) in d; one-way ANOVA followed by Dunnett’s, Tukey’s, Holm-Sidak’s or Dunn’s multiple comparisons test in b,c,e,h). Scale bars, 20 \( \mu \). Data are expressed as mean ± s.e.m.
Rtca is an inhibitory factor for axon growth in vitro and RGC regeneration in vivo in rodents. (a) An antibody to human RTCA (HPAO27982) recognizes endogenous Rtca in cultured hippocampal neurons. Rtca is present in the soma and neuronal processes (arrowheads), which are labeled with anti-Tau staining. Scale bar, 20 μm. (b) The expression of Rtca transcripts in whole DRGs in vivo is upregulated throughout development, with the highest level in adults (N = 3 experiments; the expression level of each data point was normalized to the average value at embryonic day (E) 18; *P < 0.05, one-way ANOVA followed by Dunnett’s multiple comparisons test). (c) Rtca transcript level in the DRG is significantly reduced after a peripheral sciatic nerve lesion, whereas lesioning the central axon branch of DRG neurons by spinal cord hemisection does not alter its expression (N = 3 experiments; for peripheral lesion, expression level of the lesioned DRG is normalized to the corresponding control expression level of the lesioned DRG is significantly reduced; *P < 0.001, two-way ANOVA). Scale bars, 200 μm. (d–h) Two weeks after optic nerve crush in P35 adult Rtca+/+ and RtcaIns/Ins mice and RtcaIns/Ins mutant siblings, regenerating fibers were labeled by intravitreal injection of Alexa 555–labeled cholera toxin B (CtB555). (f,g) Tissue sections through the optic nerve from a RtcaIns/Ins mutant mice show regenerating axons more than 800 μm distal to the lesion site, whereas no obvious axon regrowth beyond the crush site is seen in similar sections from a sibling control (RtcaIns/+; asterisk marks the crush site). (h) Regenerating fibers were counted at specified distances from the lesion site. More fibers regenerated in RtcaIns/Ins mice than in siblings (N = 5 RtcaIns/+; 5 RtcaIns/Ins and 6 RtcaIns/Ins mice; ***P < 0.001, two-way ANOVA). Scale bar, 200 μm. Data are expressed as mean ± s.e.m.

(Fig. 6d,e), and it also promoted axon regeneration in the periphery when overexpressed in class III da neurons (Supplementary Fig. 7). The observations that Rtca; Xbp1 double mutation did not completely eliminate the enhanced regeneration phenotype in Rtcp mutants and that Xbp1s overexpression led to milder enhancement of regeneration as compared to Rtca LOF, suggest that additional substrates contribute to regeneration regulation.

To directly assess the unconventional splicing of Xbp1 mRNA in vivo, we used a heat-shock model. Fly larvae of various genotypes underwent a 40 °C heat shock and the abundance of Xbp1 splice variants was assessed using semiquantitative RT-PCR. The Xbp1s/Xbp1 ratio was then quantified. Heat-shock induced the expression of the spliced form, Xbp1s. In contrast to the enhanced expression of Xbp1s in Rtcp mutants, Xbp1s levels were greatly reduced in Archease LOF mutants. Double mutants of Rtcp and Archease resembled Archease mutants in the reduction of Xbp1 splicing (Fig. 6g,h). Taken together, these data indicate that Drosophila Rtcp and Archease in the RNA repair and splicing pathway negatively and positively regulate the stress-induced Xbp1 mRNA splicing, respectively, so that Xbp1 acts as a readout and effector for the regulation of axon regeneration (Supplementary Fig. 5).

Mammalian Rtcp inhibits CNS axon regeneration

Having established the role of Rtcp in axon regeneration in Drosophila, we went on to determine whether its function is evolutionarily conserved in mammals. We started by examining the expression pattern of the mammalian ortholog of Drosophila Rtcp in vitro. Antibodies raised against human RTCA recognized rat Rtcp in the cell bodies and processes of cultured hippocampal neurons (Fig. 7a). Moreover, the expression of Rtcp transcripts in the dorsal root ganglion (DRG) in vivo increased progressively throughout development, reaching the highest level in adults (Fig. 7b). Using quantitative RT-PCR, we found that Rtcp transcript levels in the DRG were significantly reduced following lesion of the sciatic nerve peripherally, but not lesion of the
central axon branch of DRG neurons with a spinal cord hemisection (Fig. 7c). Since the peripheral processes of DRG neurons are capable of regeneration, whereas their central axons that project into the spinal cord fail to regrow after injury39, the selective suppression of Rtca following peripheral injury supports the hypothesis that the persisting expression of Rtca is inhibitory to axon regeneration in the CNS. Furthermore, in agreement with the overexpression phenotype in flies, overexpression of Rtc in cultured hippocampal neurons reduced axon complexity and markedly reduced proximal axonal branching (Fig. 7d,e) without affecting total axon length, indicating an inhibitory function of Rtc.

We next asked whether knocking out Rtc during development enhances axon regeneration of adult mouse retinal ganglion cells (RGCs) in vivo. For this purpose, we generated a mutant allele with a lacZ cassette inserted after the third exon (Supplementary Fig. 8a) to disrupt splicing and reduce transcription (to ~19%; Supplementary Fig. 8b), thereby generating RtcαlacZloxP (RtcαloxIns) mutant mice. Rtcα protein level was also reduced to ~18% in the mutants (Supplementary Fig. 8c), suggesting this is a hypomorphic allele. Homozygous RtcαloxIns mice were born, although at less than the Mendelian ratio. By adulthood, there were no obvious differences in RGC number or RGC axon morphology among mutant (RtcαloxIns), heterozygous (Rtcαlox+/+) and wild-type (Rtcαlox+/+) animals (Supplementary Fig. 9a,c). Since the lacZ cassette is inserted into the Rtcα locus, it can be used as a reporter for examining Rtcα expression. β-Galactosidase staining was observed in the RGC layer of Rtcαlox+/+ mice (Supplementary Fig. 10a–c) but not in Rtcαlox+/+ littermates (Supplementary Fig. 10d–f). Moreover, β-galactosidase immunostaining in RtcαloxIns/+ mice showed distinct expression of the lacZ reporter in NeuN+ neurons in the retina, which was absent in Rtcαlox+/+ littermates (Supplementary Fig. 10g), indicating that Rtcα is indeed expressed in RGCs. To assess RGC axon regeneration, we performed optic nerve crush in wild-type (Rtcαlox+/+) and Rtcαlox+/+ littermate mice at two developmental time points, postnatal day (P) 35 and 2–3 months old, and measured the extent of axon regeneration in the optic nerve after 2 weeks or 3 weeks, respectively. RtcαloxIns/+ and Rtcαlox+/+ mice injured at P35 did not exhibit substantial axon regrowth beyond the crush site (Fig. 7f), whereas RtcαloxIns/Ins mutant mice showed a substantial increase in the number of regenerating axons at various distances from the injury site, with some regenerating axons extending over 1.5 mm beyond the crush site (Fig. 7g,h). Mice operated on at 2–3 months old showed a milder regeneration enhancement phenotype (Supplementary Fig. 11a–d).

In these animals, curving, turning and looping of axons were observed, indicative of new axon growth (Supplementary Fig. 11b), and the furthest distance that axons traveled beyond the injury site was about 3.5 times longer in the mutants (Supplementary Fig. 11c). The crush site was further marked by the presence of ED1 staining, which labels infiltrating macrophages40. Whereas axons rarely penetrated beyond the ED1+ region in sibling controls, a large number of axons were seen hundreds of microns beyond the ED1+ region in RtcαloxIns/Ins mutants (Supplementary Fig. 11e). Reducing Rtcα function did not affect RGC survival after injury (Supplementary Fig. 9b), confirming that this increase in regenerating axons was not secondary to an increase in RGC numbers. Our finding that reducing Rtcα expression increased the regenerative potential of adult RGCs thus provides evidence for a potentially conserved role of Rtcα as an anti-regeneration factor.

**DISCUSSION**

Our findings reveal an important role of the RNA repair and splicing pathway in regulating the intrinsic axon regeneration potential in response to PNS and CNS injury in Drosophila. Rtcα and Archease integrate the injury signals triggered by axotomy and lead to the activation of downstream effectors such as the stress response cascade involving Xbp1 splicing, affecting the ability of a neuron to regenerate. Axon injury has been suggested as a cellular stress, and the mTOR pathway, a potential determinant of neuronal regeneration competence, could be inactivated under stress conditions such as hypoxia or DNA damage41. Notably, Xbp1 splicing has been observed in RGCs after optic nerve injury and forced activation of Xbp1 promotes RGC survival42. Our work implicates proper splicing of Xbp1 as also important for axon regeneration in Drosophila.

Moreover, recent work in C. elegans also suggests the involvement of stress response pathways, such as heat-shock, hypoxia and UPR, in axon regeneration13. However, how the injury signal is relayed to the stress response is unclear. Our work identifies a missing link and implicates the Rtcα-Archease–dependent RNA metabolism machinery as a regeneration regulator. A priori, axonal injury could either signal directly to the stress pathways, which then recruit Rtcα-Archease, or alternatively, Rtcα and Archease may represent injury response elements upstream of the stress pathways. Our results showed that the Xbp1-dependent UPR pathway acts downstream of Rtcα-Archease in controlling axon regeneration, and the remaining question is whether and how it impinges on other stress pathways, such as hypoxia or DNA damage. It will be important in future studies to identify other substrates, in addition to Xbp1, that are modified by Rtcα-Archease, and to search for response genes downstream of Xbp1. Our study raises the prospect of manipulating Rtcα, Archease and Xbp1 as potential therapeutic interventions for treating nervous system injury.

As a first step to determining whether the Rtcα pathway may have an evolutionarily conserved function in axon regeneration, we have examined CNS axon regeneration after optic nerve crush in a hypomorphic mouse mutant allele of Rtcα and have evidence suggesting that this is indeed the case. The enhancement of RGC axon regeneration phenotype in the Rtcα mutant is modest as compared to that seen in Pien, Kif4 or Socs3 knockouts. This may be due to the residual Rtcα function in this hypomorphic allele or to developmental compensation. Future experiments using mammalian injury models to examine the Rtcα null allele and to assess other components of the RNA repair and splicing pathway are therefore warranted to further define its potential role in axon regeneration.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
Y.S. carried out most of the experiments and performed the data analysis. D.S. performed the optic nerve crush experiment; E.A.S. performed β-galactosidase staining; I.B. performed RtcA expression analysis; X.H. contributed to the neuronal culture experiment; T.C. contributed to the RGC axon regeneration analysis; X.X. performed the motor axon regeneration assay; S.M. contributed to the stress culture experiment; T.C. contributed to the RGC axon regeneration analysis; and Y.S., L.Y.J. and Y.N.J. together conceived the research and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Fly stocks. ppk-CD4-tdGFP (ref. 43), ppk-CD4-tomato (ref. 43), ppk-Ga4 (ref. 44), repo-FLP (ref. 45), 19-12-Gal4 (ref. 46), repo-Gal80 (ref. 47), 21-7-Gal4 (ref. 48), hs-FLP; ppk-Gal4; UAS-CD2-mCD8-GFP (ref. 49), UAS-Pten (ref. 50) (T. Xu laboratory), PtenG126H (ref. 14) (I. Hariharan laboratory) and UAS-Xbpl (UAS-Xbpl-RB) (ref. 38) (H.D. Ryoo laboratory) have been previously described. UAS-Dcr-2, Df(1)RSC718, Df(3R)Exel6186, Df(2R)BSC484, Dpl(1;3)DCA13, UAS-Rac1, UAS-Rac1DN, Xbp1c12803, UAS-Xbp1RNAi, m12-Gal4 (ref. 51) (P(Gal4)tey5053A) and hs-FDLP05 were from the Bloomington stock center. RtcadPN5057 from Kyoto DCGUR. UAS-RtcaRNAi and UAS-ArcheaseRNAi were from VDRC. ArcheasePrk01013 was from Exelixis. Rtcad was generated by improved precise excision of RtcadPN5057, in which the entire coding sequence of Rtcad3’ to the NPS507 insertion site together with the neighboring gene CG4045 was replaced by a 3kb fragment from the P element. To generate RtcadΔ5, because RtcadΔ5 males were sterile, the duplication stock Dp(1;3)DCA13, which has a duplication of the region covering Rtcad on the third chromosome, was used. Specifically, RtcadΔ5; Dpl(1;3)DCA13/Tm6B males were mated with RtcadΔ5/FM6 females to get the RtcadΔ5 homozygous females. To generate the UAS-Rtcad stock, the entire coding sequence of Drosophila Rtcad was cloned into the PUAST vector using 5’-tgctcgagattgttctgacctac-3’ and 5’-atcttccggcgcctttag-3’. For experiments in Drosophila, both male and female larvae were used.

Mice. We used an embryonic stem (ES) cell from the European Conditional Mouse Mutagenesis Program (EUCOMM Project 82885), a knock-out first-line with a lacz cassette inserted into the Rtcad locus, to generate the Rtcad mutant mouse (UCSF ES Cell Targeting Core). The resulting mouse, RtcadlacZ_loxP (RtcadΔ5/Δ5), has a lacZ cassette inserted between the third and fourth exons of Rtcad; meanwhile, the fourth exon is flanked by loxP sites (Supplementary Fig. 8a). The lacZ insertion disrupted Rtcad splicing and dramatically reduced transcript and protein levels (Supplementary Fig. 8b,c), and it thus resulted in a hypomorphic allele. All mice were housed in an animal room and maintained in a temperature-controlled and light-controlled environment with an alternating 12-h light/dark cycle. Up to 5 mice of the same sex and similar age were housed in a cage. The animals had no prior history of drug administration, surgery or behavioral testing. All protocols were approved by the UCSF Institutional Animal Care and Use Committee.

Sensory axon lesion in Drosophila. da neuron axon lesion and imaging in the PNS or within the VNC were performed in live fly larvae as previously described44.

Quantitative analyses of sensory axon regeneration. Quantification was performed as previously described44. Briefly, for axon regeneration in the VNC, we used the following metrics: regeneration percentage, which depicts the percent of regenerating commissure segments among all the segments that were severed; terminal branching, which counts the number of axons that regrew, reached the commissure bundle and appeared to form elaborate branches; and commissure regrowth, which counts the number of axons that had regenerated to connect the boundaries of commissure segments, longitudinally or laterally (Supplementary Fig. 3a). For axon regeneration in the PNS, we used the following metrics: regeneration percentage, which depicts the percent of regenerating axons among all the axons that were lesioned; regeneration length, which measures the increase of axon length; and regeneration index, which is calculated as the increase of (axon length)/(distance between the cell body and the axon converging point) (Supplementary Fig. 3b).

Live imaging. Live imaging was performed as described42,53. Embryos were collected for 2 h on yeast-dextrose agar plates and were aged at 25 °C or room temperature. At the appropriate time, a single larva was mounted in 90% glycerol under a coverslip sealed with grease, imaged using a Leica SP5 microscope, and returned to grape-juice agar plates between imaging sessions.

Generation of clones. FLP-out clones were produced as previously described49.

Drosophila motor nerve crush assay and quantification. As described previously54, the segmental nerves of third instar larvae were pinched tightly through the cuticle for 5 s with Dumostar #5 forceps, with the larvae under CO2 anesthesia. Larvae were then transferred to a grape-juice plate and kept alive for varying periods of time at 25 °C. For quantification of motor axon regeneration, the total area of the growth cone at 14.5 h after crush was measured.

Neuronal cell culture, transfection and analysis. Hippocampal neurons were cultured from E19 Long-Evans rats at 150,000 per coverslip and maintained in serum-free B27-containing medium. Plasmid transfections were done using Lipofectamine2000 (Invitrogen) at the indicated times (DIV 0–3). For over-expression experiments, neurons were transfected with 0.75 μg of the Rtcad expression construct (Open Biosystems) together with 0.5 μg GFP-expressing plasmid (pLentilox 3.7). For axon analysis, we restricted all our morphological analysis to Ctip2-negative CA3 pyramidal neurons55 and used Neurulocide (MBF Biosciences).

Optic nerve crush. For all in vivo experiments, optic nerve crush, tissue processing and imaging were performed in a blind manner. P35 or 8–12 weeks old mice (male and female) were used, and the surgery was done by crushing the optic nerve behind the globe with forceps. The mice were then kept for 2 or 3 weeks, respectively, until perfusion. For the procedure performed at P35, RGC axons were anterogradely labeled through intravitreal injections of 1 μl cholera toxin subunit B (CB555, 10 μg/μl; Molecular Probes) 2 d before perfusion. Longitudinal sections (20 μm) were prepared and stained with anti-NF200 (labeling the neurofilament in axons) and anti-ED1 (labeling the infiltrating macrophages). The crush site was determined by the presence of ED1 staining, the clustering of NF200 staining, and/or the physical pinching or narrowing of the nerve. For quantification, the distance between the crush site and the tip of the longest axon was measured and the number of axon fibers 50, 100, 150, 200, 250, 300, 350, 500, 750, 1,000 and 1,500 μm from the crush site was counted for each section.

Quantification of RGC survival. Retina sections were immunostained with anti-Tuj1 (β3 tubulin) to visualize RGCs and DAPI to detect nuclei. The number of RGCs were counted and normalized to the length of the corresponding retinal region to calculate the RGC density. The RGC density from each sample was then normalized to the average value of the sibling controls.

Sciatic nerve lesion and spinal cord hemisection for expression analysis. For sciatic nerve lesion, mice were anesthetized (isoflurane) and the sciatic nerve was exposed at mid-thigh level, then sutured and transected distal to the ligature and the wound closed. To lesion central processes of peripheral neurons, mice were anesthetized (ketamine (100 mg/kg) and xylazine (10 mg/kg)) and a laminectomy was performed at the thoracic level. The T6–T7 dorsal columns were then lesioned bilaterally using Dumont #5 fine forceps. In the cases of both peripheral and central nerve injury, L4–L6 DRGs were collected 7 d after lesion for expression analysis. For each condition, DRGs from sham-operated mice (exposed but unlesioned sciatic nerve and thoracic laminectomy without dorsal column transaction) were used as controls.

Immunohistochemistry and β-galactosidase staining. Immunohistochemistry analyses were performed on whole-mount fly larvae, on cultured neurons and on mouse tissue sections collected from OCT-embedded tissues. Primary antibodies used were anti-Tuj1 (Covance, MMS-435P, 1:1,000), anti-Tau (Millipore, MAB3420, 1:1,000), anti-RTCA (Sigma, SAB2102059 and HPA027982, 1:200), anti-CTIP2 (Abcam, ab18465, 1:2,000), anti-NF200 (Sigma, N4142, 1:200), anti-ED1 (Abcam, ab31630, 1:200), anti-β-galactosidase (MP Biomedicals cappel, 55976, 1:5,000) and anti-NeuN (Millipore, MAB377, 1:200). A custom-made anti-body against the entire Drosophila Rtcad protein was generated (Thermo, 1:200). β-Galactosidase staining was performed using the Senescence β-Galactosidase Staining Kit (Cell Signaling) according to manufacturer’s instructions.

Heat-shock studies. To assess the stress-induced splicing of Xbp1, third-instar larvae were heat-shocked at 40 °C for 2 h.

Quantitative RT-PCR. Semi-quantitative RT-PCR was done for Rtcad, α-tubulin (αTub84B), Archease and Xbp1 in flies and for Rtcad and β-actin (Actb) in mice, according to the manufacturer’s protocols. The sequences were as follows:
two regions of the Rtxa transcripts were amplified with primers 5′-gaggac- 
caacggctgagtg-3′ and 5′-caacagggctctggtgga-3′ and 5′-ctgcttcgcgcttc-3′. rt- 
inhb and 5′-ttctggaatggtcagtc-3′. Two regions of the Archease transcripts were 
amplified with primers 5′-acaagggatgtcctggg-3′ and 5′-gggtacaggtcttgcttc-3′; 
5′-agcttcctcagtcttg-3′ and 5′-gcaagggtaagcaagaa-3′. Xhp1 was amplified 
with primers 5′-aggctcaacaggtcagtc-3′ and 5′-cttccagagggctgag-3′. Two 
regions of the mouse Rtxa transcripts were amplified with primers 5′-gggatc 
cacctacagagggctgag-3′ and 5′-ggggaggtaagcaagaa-3′.

Real-time quantitative RT-PCR was done for mouse Rtxa. 2 µg of RNA was 
reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). All 
RNA concentrations were measured with a NanoDrop 1000 spectrophotometer. 
Real-time detection and quantification of cDNAs were performed with the 
iCycler instrument (Bio-Rad). Quantitative PCR was performed in a 20-µL 
reaction mixture using SYBR GreenER qPCR Supermixes (Invitrogen). 50 cycles 
of amplification were performed according to manufacturer’s instructions. 
Fluorescence data were collected at annealing stages and real-time analysis 
was performed with iCycler iQ Optical System Software V3.0a. Serial dilutions 
of cDNAs were used for construction of the standard curve. Ct values were 
determined with automatically set baseline and manually adjusted fluorescence 
threshold. Gene expressions levels were normalized to that of Actb and 

Statistical analysis. No statistical methods were used to predetermine sample 
sizes, but our sample sizes are similar to those reported in previous publica-
tions27,29,42 and the statistical analyses were done afterwards without interim 
data analysis. Data are expressed as mean ± s.e.m. in bar and dot plots or as 
median and maximum with minimum values in box-and-whisker plots. No data 
points were excluded. For the experiments done in Drosophila, data collection 
and analyses were not performed blind to the conditions of the experiments. For in vivo experiments in mice, optic nerve crush, tissue processing and imaging 
were performed in a blind manner. Two-tailed unpaired Student’s t-tests were 
performed for comparison between two groups of samples. One-way ANOVA 
followed by multiple comparison test was performed for comparisons among 
three or more groups of samples. Two-way ANOVA analyses were used to assess 
significance of multiple data points. Fisher’s exact test was used to compare per-
centages. The data meet the assumptions of the tests. The variance was tested 
in each group of the data and the variance was similar among genotypes. Data 
distribution was assumed to be normal, but this was not formally tested. The data 
were collected and processed randomly. Each experiment has been successfully 
reproduced at least three times and was performed on multiple days. Statistical 
significance was assigned as *P < 0.05, **P < 0.01, ***P < 0.001.

A Supplementary Methods Checklist is available.

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