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Doublecortin-like kinases promote neuronal survival and induce growth cone reformation *via* distinct mechanisms

Homaira Nawabi[1,*], **Stephane Belin**[1,*], **Romain Cartoni**[1,*], **Philip R. Williams**[1], **Chen Wang**[1], **Alban Latremolière**[1], **Xuhua Wang**[1], **Junjie Zhu**[1,2], **Daniel G Taub**[3], **Xiaoqin Fu**[4], **Bin Yu**[1,2], **Xiaosong Gu**[2], **Clifford J. Woolf**[1], **Judy S. Liu**[4], **Christopher V. Gabel**[3], **Judith A. Steen**[1,5], **Zhigang He**[1,5]

[1]F.M. Kirby Neurobiology Center, Children's Hospital, and Department of Neurology, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA.

[2]Jiangsu Key Laboratory of Neuroregeneration, Co-innovation Center of Neuroregeneration, Nantong University, Nantong, 226001, China

[3]Departments of Physiology and Biophysics, Boston University School of Medicine, Boston, MA 02118, USA

[4]Center for Neuroscience Research, Children's National Medical Center, Washington, DC 20010, USA.

Abstract

After axotomy, neuronal survival and growth cone re-formation are required for axon regeneration. We discovered that doublecortin-like kinases (DCLKs), members of the doublecortin (DCX) family expressed in the adult retinal ganglion cells (RGCs), play critical roles in both processes, through distinct mechanisms. Over-expression of DCLK2 accelerated growth cone re-formation *in vitro* and enhanced the initiation and elongation of axon re-growth after optic nerve injury. These effects depended on both the microtubule (MT)-binding domain and the serine-proline-rich (S/P-rich) region of DCXs *in-cis* in the same molecules. While the MT-binding domain is known to stabilize MT structures, we show that the S/P-rich region prevents F-actin destabilization in injured axon stumps. Additionally, while DCXs synergize with mTOR to stimulate axon regeneration, alone they can promote neuronal survival possibly through regulating the retrograde propagation of injury signals. Multifunctional DCXs thus represent potential targets for promoting both survival and regeneration of injured neurons.

[5]To whom correspondence should be addressed. zhigang.he@childrens.harvard.edu, judith.steen@childrens.harvard.edu.
[*]These authors contributed equally to this work.

AUTHOR CONTRIBUTIONS

H.N., S.B., X.G., C. J.W., J.S.L., C.V.G., J.A.S., and Z.H. planned the experiments. H.N., S.B., R.C., P.R.W., C.W., A.L., X.W., X.F., J.Z., D.G.T., B.Y. performed the experiments and analyzed the data. C.W. helped with optic nerve crush. H.N., S.B., P.R.W., and Z.H. wrote the paper with input from all authors.

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INTRODUCTION

After axotomy, a neuron faces two critical decisions: survival or death and regeneration or no-regeneration. For a survived neuron to regenerate its injured axon, a required step is the assembly of a growth cone at the leading edge (Bradke et al., 2012; Eva et al., 2012; Hur et al., 2012; Cregg et al., 2014). Based on the correlation between growth cone reformation and successful axon regeneration (Bradke et al., 2012; Verma et al., 2005), it has been proposed that growth cone formation might be a limiting process for initiating axon regeneration. For example, following a crush injury to peripheral nerves in adult rodents, many axons assemble a new growth cone within hours (Erturk et al., 2007; Pan et al., 2003). However, in the adult CNS, injured axonal terminals often appear as dystrophic end bulbs or retraction bulbs, possibly representing abortive attempts of growth cone formation (Erturk et al., 2007; Kerschensteiner et al., 2005; Windle, 1980).

Although growth cone formation is relatively rapid, it involves the coordination of multiple processes, such as re-organization of the membrane, various cytoskeletal components and subcellular organelles. Previous studies suggest that disorganized microtubules occupy the retraction bulbs in the adult CNS where regeneration is abortive (Erturk et al., 2007), highlighting the importance of proper cytoskeletal re-organization for growth cone formation. In support of this, application of taxol, a MT-stabilizing agent, facilitates growth cone formation and promotes axon regeneration (Hellal et al., 2011; Sengottuvel et al., 2011; Chen et al., 2011; Ruschel et al., 2015). However, axon growth involves coordination of microtubule and actin structures (Gomez and Letourneau, 2014; Vitriol and Zheng, 2012; Coles and Bradke, 2015). How actin contributes to growth cone reformation is less understood.

At a molecular level, recent studies suggest that the dual leucine zipper-bearing kinase (DLK) MAP kinase pathway, an evolutionarily conserved pathway that regulates axon regeneration, is a positive regulator of growth cone formation and axon regeneration (Hammarlund et al., 2009; Watkins et al., 2013; Yan et al., 2009). As an injury signal sensor, DLK regulates, *via* p38 MAP kinase activated kinase, at least two pathways, one involving MT post-translational modification factors and another involving the up-regulation of a CCAAT/enhancer binding protein CEBP-1. In addition, kinesin family members such as KIF3C have also been implicated in MT regulation and appear to be required for axon growth and regeneration (Gumy et al., 2013). However, the molecular pathways regulating such cytoskeletal rearrangements during axon regeneration are still poorly defined.

Doublecortin like kinase 1 and 2 (DCLK1/2) are members of the doublecortin (DCX) family, which includes DCX, DCLK1/2 and a few other isoforms expressed in non-neuronal cells. DCX was initially identified as one of the causative genes of lissencephaly (Gleeson et al., 1998) and has been implicated in regulating neuronal migration and axon growth during development (Gleeson et al., 1999; Schaar et al., 2004). DCX is expressed only in newly differentiated neurons (Gleeson et al., 1999), but DCLK1/2 are expressed in both immature and mature neurons (Deuel et al., 2006, Reiner et al., 2006). Previous studies have suggested that, similar to DCX, DCLK1/2 regulate neuronal migration and axon growth during development (Deuel et al., 2006; Friocourt et al., 2007; Koizumi et al.,

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2006) and over-expression of DCX or DCLKs enhances neurite growth *in vitro* (Blackmore et al., 2010). In the adult, these proteins might act in dendritic remodeling and synapse maturation (Shin et al., 2013). However, their function in injured mature neurons has not been characterized. In this study, we describe our results implicating DCLK1/2 regulate growth cone regeneration as well as neuronal survival after injury by different mechanisms.

RESULTS

DCLK1 and DCLK2 are down-regulated in injured RGCs

In our recent efforts to analyze neuronal injury responses, quantitative proteomics was used to profile protein abundance changes in intact and injured RGCs (Belin et al., 2015). Among all identified proteins, DCLK2 was one of the most dramatically down-regulated proteins (Belin et al., 2015). Similar to DCX, both DCLK1 and 2 have an N-terminal MT-binding module and a separate serine-proline rich (S/P-rich) region (Bielas et al., 2007; Fu et al., 2013; Reiner, 1999). Different from DCX, DCLK1/2 have a unique kinase domain at their C-terminal regions.

To verify the results from the proteomics analysis, we assessed the expression of these different DCX members in both intact and injured RGCs. By fluorescent *in situ* hybridization (FISH) with TSA amplification, we found that the levels of both DCLK1 and DCLK2 were significantly reduced in RGCs 72 hr after optic nerve crush (Figure 1A, B), which was verified by immunohistochemistry (Figure S1A). The absence of DCLK1 from our proteomic profiling may reflect incomplete proteomic coverage. Together, the present results suggest that both DCLK1/2 are rapidly down regulated after axotomy.

DCLK2 over-expression promotes RGCs survival and axon regeneration after injury

To assess whether forced DCLK2 over-expression might alter the injury response of RGCs, we injected AAV2 vectors expressing DCLK2 (AAV-DCLK2), or a control AAV encoding placental alkaline phosphatase (PLAP), into the vitreous body of wild type mice. Consistent with our previous results of highly efficient transduction of RGCs by AAV2 (Park et al., 2008, Belin et al., 2015), we showed that most RGCs have DCLK2 expression as examined before or after injury by in situ hybridization (Figure S1B). Using the established method (Park et al., 2008), we performed an optic nerve crush in these mice and analyzed both RGC survival by immunostaining the whole mount retina with an anti-Tuj antibody, and axon regeneration by anterograde tracing using cholera Toxin B conjugated to Alexa-555 (CTB-555). In contrast to controls, in which few injured axons sprout across the injury site, DCLK2 over-expression resulted in a modest yet significant increase in axon regeneration. Approximately 200 axons grew 500 μm beyond the lesion site (Figure 1C-D), consistent with the notion that the down-regulation of DCLK2 might contribute to regenerative failure after optic nerve injury.

Interestingly, we found that DCLK2 over-expression led to a significant protective effect on RGC survival. As shown in Figure 1E & 1F, in contrast to 20% survival in the control group, about 50% of RGCs survived after DCLK2 over-expression, an effect comparable to that observed with PTEN deletion (Figure 2C, Park et al., 2008). Considering its relatively weak

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effect on promoting axon regeneration compared to PTEN deletion (Figure 1C &1D vs 2A &2B), such a strong effect of DCLK2 on neuronal survival was unexpected, suggesting a possible role of cytoskeletal dynamics in regulating injury-induced neuronal survival.

As retrogradely propagated injury signals have been proposed as an important mechanism for axotomy-triggered neuronal death (Watkins et al., 2013; Rishal and Fainzilber, 2014), we examined whether DCLK2 expression might affect injury-induced expression of phospho-c-Jun in RGCs (Figure 1G-H). In wild type mice, an optic nerve crush rapidly induces the appearance of phospho-c-Jun in the cell bodies of most RGCs. However, DCLK2 over-expression significantly reduced such injury-induced phospho-c-Jun in RGCs at both 24 hr and 72 hr post-injury (Figure 1G-H), suggesting that DCLK2 expression may protect injured RGCs by inhibiting the generation and/or propagation of injury-induced signals.

DCLK2 substantiates the survival and regeneration effects of PTEN deletion

We reasoned that the direct effects of DCLK2 over-expression might be mainly limited to axon cytoskeleton and it might have functional interactions with other identified regeneration regulators. As our previous studies indicated that manipulating the mTOR pathway could enhance neuronal regeneration (Liu et al., 2010; Park et al., 2008; Sun et al., 2011; Zukor et al., 2013), we tested the combinatorial outcomes of DCLK2 over-expression and PTEN deletion. $PTEN^{f/f}$ mice received successive intravitreal injections of AAV2-Cre and AAV2-DCLK2 or control (AAV2-PLAP) viruses (Figure 2A-C). Two weeks later, the mice were subjected to an optic nerve crush and neuronal survival and axon regeneration were analyzed two weeks post-injury. As shown in Figure 2C, DCLK2 over-expression further enhanced neuronal survival (from approximately 50% to 70%) when compared to the PTEN deletion alone. Importantly, DCLK2 resulted in a significant enhancement of axon regeneration induced by PTEN deletion (Figure 2A and 2B). In the $PTEN^{-/-}$ AAV2-DCLK2 mice, the numbers of regenerating axons were significantly increased at all distances from the injury site measured, with many axons seen reaching the optic chiasm (Figure 2A). In $PTEN^{-/-}$ RGCs, although DCLK1/2 underwent an injury-induced down-regulation (Figure S1C and S1D), some residual DCLK1/2 still remained (Figure S1C and S1D), possibly as the result of the effects of PTEN deletion on protein translation.

DCLK2 increases axonal initiation and elongation

To assess whether DCLK2 affects the initiation and/or elongation of axon regeneration, we analyzed axon regeneration at shorter time points after injury in PTEN-deleted mice with or without DCLK2 over-expression (Figure 2D and 2E). At 3 days, while few regenerating axons projected beyond the lesion site in $PTEN^{-/-}$ mice, DCLK2 over-expression resulted in significantly more and longer regenerating axons (Figure 2D and 2F), suggesting that DCLK2 over-expression promotes the initiation of axon regeneration. This phenotype was further intensified at 7 days after the injury (Fig 2E and 2G). Based on these results, we estimated the average growth rates of regenerating axons (Figure 2H). These results suggest that DCLK2 over-expression allows regenerating axons to grow faster at all time points, consistent with the notion that DCLK2 promotes both the initiation and the elongation of optic nerve regeneration.

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DCLK2 promotes growth cone initiation

As growth cone formation is a key step for the initiation of axonal regeneration (Bradke et al., 2012; Edwards and Hammarlund, 2014; Hammarlund et al., 2009), we next examined whether DCLK2 is involved in this process. It is difficult to visualize growth cone morphology *in vivo*, we thus adapted an *ex vivo* explant culture system in which retinal tissues dissected from different groups of mice were maintained in vitro for two weeks.

Because of the poor survival of RGCs in explants made from wild type postnatal mice, we used explants with RGCs carrying the PTEN deletion ($PTEN^{f/f}$ mice with prior AAV-Cre injection) to maintain survival of RGCs, which served as controls for further investigating the effects of DCLK2 over-expression. $PTEN^{f/f}$ mice at the age of P21 first received an intravitreal injection of AAV2-Cre and either AAV2-PLAP or AAV2-DCLK2 (Figure 3A). Two weeks later, retinal explants were dissected and kept in culture. After another 2 weeks, we observed a significant difference in the number and length of extended neurites in these two different groups (Figure S2A & S2B). By co-immunostaining with antibodies against Tuj1 and DCLK2, we found that while some residual DCLK2 immunoreactivity remains in the distal segments of the neurites in $PTEN^{-/-}$/AAV2-PLAP explants, significantly more immunoreactivity could be seen in both the axon terminals and shafts from $PTEN^{-/-}$/ AAV2-DCLK2 explants (Figure 3B & 3C), confirming that DCLK2 protein is present in the neurites of RGCs from these explants.

We then used a pulsed femtosecond laser to lesion individual neurites from these cultured explants and imaged them for additional 60 min using bright field microscopy. In all cases from both groups, we found that axotomy triggered a rapid retraction. However, the retracted neurites from these two groups behaved noticeably differently. Representative images and videos from both groups are shown in Figure 2D and Supplemental Movies 1, 2 and 3. In the control $PTEN^{-/-}$/ AAV2-PLAP group, most cut neurites (15 out of 18 from at least 10 independent explants) remained stationary and failed to form growth cones within 60 min (Figure 3D-F; Supplemental Movie 1). In the other three cases, filopodia-bearing growth cones formed, but without subsequent extension. However, in the $PTEN^{-/-}$/ AAV2-DCLK2 group, the majority (12 neurites out of 16 from at least 10 independent explants) reformed growth cone structures within the 60 min imaging period (Figure 2D-F). Among these, 50% of neurites with reformed growth cone extended (Supplemental Movie 2), while the others 50% elaborated filopodia but with minimal extension (Supplemental Movie 3). These results provide direct evidence for a role of DCLK2 in accelerating growth cone initiation.

DCLKs are required for axon regeneration in adult RGCs

Despite the significant down-regulation of DCLK1/2 found in injured RGCs, some residual DCLK1/2 may still remain in axotomized neurons, which may contribute to their limited growth capacity. This is especially obvious for RGCs with PTEN deletion (Figure S1C and S1D). To provide a definitive assessment to their role in growth cone and axon regeneration, we performed optic nerve injury in PTEN-deleted mice with deletion of DCLK1 and/or DCLK2 (AAV2-Cre vitreal injection to the floxed mice) (Figure 4A). In single mutants, the extent of optic nerve regeneration was significantly reduced (Figure S3A &S3B). Strikingly, co-deletion of DCLK1 and DCLK2 abolished nearly all axon regeneration usually observed

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with PTEN deletion (Figure 4B, 4C, S3A, S3B). The lack of axon regeneration is not secondary to compromised neuronal survival because co-deletion of DCLK1 and DCLK2 did not significantly alter either the survival or the mTOR levels of PTEN-deleted RGCs (Figures 4D, 4E, S3C and S3D). These results suggest that DCLK1 and 2 might be functionally redundant and their collective activities are required for optic nerve regeneration induced by PTEN deletion.

Similar to what was observed in DCLK1 and DCLK2 double mutants, single deletion of DCLK1 or DCLK2 did not affect neuronal survival in these PTEN deleted mice (Figure S4A-C). This might be due to the high survival rates induced by PTEN deletion. To assess this, we performed optic nerve injury in wild type mice with deletion of DCLK1 or DCLK2 (AAV2-Cre injection to the floxed mice). As shown in Figure S4, deletion of DCLK1 or DCLK2 did not affect axon regeneration (Figure S4D & S4E), but significantly reduced neuronal survival (Figure S4A-C). Together with the protective effects of DCLK2 over-expression (Figure 1E and 1F), these loss-of-function results support an important role of DCLKs in promoting neuronal survival after injury.

DCLK1/2 are required for peripheral axon regeneration

To assess whether DCLK1/2 are important for other types of axon regeneration, we next investigated whether DCLK1/2 are required for axon regeneration in PNS neurons. Consistent with a previous report (Parikh et al., 2011), an intrathecal injection of AAV8-GFP-IRES-Cre vectors into Rosa-STOP-tdTomato mice resulted in high transduction efficiency in adult DRG neurons, as indicated by co-expression of tdTomato and GFP (Figure S5A). Thus, we used this procedure to inject AAV8-GFP-IRES-Cre or AAV8-GFP (as a control) into $PTEN^{f/f}/DCLK1^{f/f}/DCLK2^{f/f}$ mice to induce the co-deletion of DCLK1 and DCLK2 in DRG neurons. Because DCLK1 and DCLK2 genes are on the same chromosome (only 10cM a part), it was difficult to obtain $DCLK1^{f/f}/DCLK2^{f/f}$ mice. In fact, we failed to obtain $DCLK1^{f/f}/DCLK2^{f/f}$ mice. However, we were lucky enough in obtaining the triple $PTEN^{f/f}/DCLK1^{f/f}/DCLK2^{f/f}$ in crosses between $PTEN^{f/f}/DCLK1^{f/f}$ and $PTEN^{f/f}/DCLK2^{f/f}$. As PTEN deletion has only a modest promoting effect on sciatic nerve regeneration (Abe et al., 2010; Christie et al., 2010; Saijilafu et al., 2013), we do not expect a major impact of PTEN deletion on our results. Thus, we used the triple mutant for this study. The animals were subjected to a sciatic nerve crush injury (Figure 5A) and axon regeneration was assessed by staining sagittal sections of the sciatic nerve with an anti-SCG10 antibody, a marker of regenerating sciatic nerve axons (Shin et al., 2012; Cho et al., 2013).

In the control animals many regenerating axons grew up to 3 mm from injury site at 3 days post-injury (Figure 5B and 5C). However the numbers and length of regenerating axons were significantly reduced in the mice with co-deletion of DCLK1 and DCLK2, suggesting that most injured axons failed to initiate regeneration in the absence of DCLK1/2. Together with the results from optic nerve injury experiments (Figure 4A-F), these data suggest that the requirement of DCLK1/2 for axon regeneration might be a shared mechanism across different types of mature neurons in the adult PNS and CNS. Both DCLK1 and DCLK2, but not DCX, are expressed in adult DRG neurons (Figure S5B). In addition, their expression

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levels in DRG neurons were not significantly altered by sciatic injury (Figure 5D), in contrast to the down-regulation of DCLK1/2 in axotomized RGCs (Figure 1A-B). These results suggest that differences in the injury-induced alterations of DCLK1/2 expression might be a contributing mechanism of the differential regenerative ability of CNS and PNS neurons.

DCX mimics the function of DCLK2

We next attempted to assess the structural basis of DCLK2's function. Similar to DCLK1/2, DCX has a MT binding domain and an S/P-rich region (Bielas et al., 2007; Fu et al., 2013). However, DCX lacks the kinase domain found in DCLK1 and 2. To examine a possible role of the kinase domain, we asked whether DCX could functionally mimic DCLK1/2 by examining the effects of DCX over-expression in RGCs on neuronal survival and axon regeneration after optic nerve injury. Indeed, similar to DCLK2, DCX expression significantly increased RGCs survival (Figure S6A & S6B), suggesting a general role for DCX members in protecting injured RGCs.

In addition, DCX over-expression also exhibited similar effects as DCLK2 in the other functional aspects. As shown in Figure S6C and S6D, DCX induced modest but significant axon regeneration in wild type mice. In a PTEN deleted background, DCX further enhanced axon regeneration assessed at different time points after optic nerve injury (Figure S6E-S6I). Consistently, the neurites from DCX-overexpressed retinal explants had significantly increased growth cone formation after laser cut (Figure S6J). These results suggest that the DCX family members are functionally redundant, implying that the kinase domain of DCLK1/2 is dispensable for their activities on neuronal survival and axon regeneration.

The MT-binding domain of DCX partially promotes optic nerve regeneration

Based on the indistinguishable effects of DCX and DCLK1/2, we next attempted to dissect out the mechanisms by which DCX promotes axon regeneration by a series of structure function analyses. In light of ample evidence demonstrating the importance of proper MT structural regulation in axon regeneration (Hellal et al., 2011; Sengottuvel et al., 2011; Chen et al., 2011), we first examined whether the MT-binding domain of DCX might be sufficient to mimic the effects of DCX and DCLK2. Thus, we generated an AAV2 to over-express the MT-binding domain of DCX (DCX-270; Figure 6A, Fu et al., 2013) and tested its effects on axon regeneration after optic nerve injury in the background of PTEN deletion. As shown in Figure 6C-D, DCX-270 only partially mimicked the effects of DCX on promoting axon regeneration. In retinal explants, we also found that DCX-270 significantly promoted growth cone formation, but not to the extent of full-length DCX (Figure 6E). In contrast, this truncated form of DCX exhibited a protective effect indistinguishable from that of full-length DCX (Fig. 6B).

In an independent experiment, we examined whether DCX-270 over-expression could rescue axon regeneration in the triple deleted mice ($PTEN^{f/f}$/$DCLK1^{f/f}$/$DCLK2^{f/f}$ with AAV2-Cre injection). As shown in Figure 6F and 6G, while DCLK2 and DCX expression resulted in a full rescue of axon regeneration, DCX-270 had only a partial effect. By monitoring the subcellular localization of the over-expressed DCX-270-GFP protein in the neurties

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from retinal explants, we verified that the DCX-270 protein is mostly co-localized with anti-tubulin-stained MT structures (Figure S8B). On the other hand, full-length DCX is concentrated in the transition zone between MT-rich axonal shafts and F-actin-rich growth cone, similar to what shown previously (Bielas et al., 2007; Fu et al., 2013). These results suggest that although the MT-binding domain could mediate the survival effects of DCX, it contributes, but cannot fully account for, the effects of DCX in promoting axon regeneration.

Because of the existence of multiple neuronal MT-associated proteins (MAPs), we next examined whether over-expression of other MAPs could mimic the effects of DCX-270. As shown in Figure S7, AAV-mediated expression of Tau and EB3, two well-characterized MT-binding proteins (Goedert et al., 1991; Straube and Merdes, 2007), had no significant effects on neuronal survival and axon regeneration after optic nerve injury in wild type or PTEN$^{-/-}$ backgrounds, suggesting that the unique MT-binding property of DCX isoforms (Fourniol et al., 2013) might be important for their effects on axon regeneration. In this regard, it has been suggested that different from other MAPs that interact directly on the surface of the MT protofilaments, DCXs bind in the recess between the protofilaments, which might be uniquely suitable for MT stabilization (Moore et al., 2004).

The S/P-rich region of DCX is important for promoting axon regeneration

We next examined the effects of the S/P-rich region of DCX (DCX-S/P) on neuronal survival and axon regeneration. Surprisingly, we found that over-expressed DCX-S/P promoted axon regeneration to a similar extent as DCX-270 in the PTEN-deleted background (Figure 6C-D), but without any significant effect on neuronal survival (Figure 6B). In addition, we also observed a partial effect of DCX-S/P on rescuing the PTEN deletion-induced axon regeneration that was prevented by DCLK1/2 co-deletion (Figure 6F-G). In the retinal explant assay, DCX-S/P could also partially mimic DCLK2's effects on promoting growth cone formation (Figure 6E), consistent with *in vivo* regeneration results (Figure 6C, D, E, and F). As expected, over-expressed DCX-S/P-GFP was co-localized with phalloidin-stained F-actin structure (Figure S8C), which might be relevant to its proposed effects on actin dynamics (Fu et al., 2013). These results suggest that the S/P-rich region of DCX possesses distinct functional capacity: similar to the MT-binding domain, it can enhance growth cone formation and axon regeneration. However, different from the MT-binding domain, it does not affect neuronal survival.

With the partial effects of DCX-270 or DCX-S/P expression, we next examined whether co-injection of AAVs expressing DCX-270 and DCX-S/P to PTEN$^{-/-}$ mice could reconstitute the effects of full-length DCX. Surprisingly, as shown in Figure S8D and S8E, co-expression of these two fragments abolished the partial promoting effects of the individual domains, resulting in similar, or even less, axon regeneration than in the PTEN$^{-/-}$ controls. These results support the possibility that the unique dual activity of DCX domains that regulate MT and actin structures in the same proteins might be required for coordinating cytoskeleton rearrangements for axon regeneration.

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DCX prevents axotomy-triggered actin destabilization at the axon terminal

To investigate the functional mechanisms of the DCX-S/P, we expressed Lifeact-tdTomato fusion protein, an F-actin indicator, together with DCLK2 or its mutant form, in RGCs and prepared retinal explants to monitor the dynamics of actin structures in neurites. Lifeact, a 17 amino-acid peptide, which can bind F-actin (Figure S2C-D), does not interfere with endogenous actin dynamics and shows fast recovery after photobleaching (Riedl et al. 2008; Flynn et al 2012). Thus we used it to monitor F-actin structures by time-lapse imaging (Fig. 7A). We found that in control conditions, immediately after axotomy there was a rapid decrease of Lifeact-tdTomato fluorescence in the axonal terminal, suggesting an injury-triggered F-actin destabilization (Figure 7B-D). Strikingly, this could be prevented by DCLK2 over-expression, suggesting a role of DCLK2 in stabilizing F-actin structures in injured axon terminals (Figure 7B-D). Interestingly, the DCX-S/P, but not DCX-270, could mimic the effects of DCLK2 in this assay (Figure 7B-C). These results suggest that DCXs might stabilize the actin cytoskeleton during growth cone regeneration.

To assess this further, we cultured retinal explants from these conditions (with the expression of GFP, as a volume marker, and one of the following proteins: PLAP, DCLK2, DCX-270 or DCX-S/P), but without Lifeact-tdTomato. The axons were subjected to laser cut 100 μm from the tip. 5-10 min after cut, the explants were fixed and stained with phalloidin. In these experiments, we found that in control explants the laser cut triggers a decline in phalloidin-stained signals in the terminals of the cut neurites, relative to respective uncut controls. As shown in Supp Fig. 2F and Fig. 7E, injured axons show significant differences in the samples expressing PLAP (control) or DCX-270, but not DCLK2 or DCX-SP. Thus these results render additional supports for the notion that DCLK, perhaps by its SP-rich domain, can prevent injury-triggered F-actin destabilization.

Differential mechanisms involved in DCX's activities on neuronal survival and axon regeneration

To further investigate the functional mechanisms of DCXs in these different aspects of injury responses, we took advantage of several characterized DCX mutants (Liu et al 2012; Fu et al 2013) and tested their effects on our optic nerve injury model. First, it has been shown that the protein-phosphatase 1 (PP1) can activate DCX by dephosphorylating DCX on the Ser 297 and DCX-S297A is a constitutive active form (Bielas et al., 2007). After over-expression of DCX-S297A in RGCs, we found that both neuronal survival and axon regeneration were promoted similar to what was observed after full-length DCX or DCLK2 expression (Figure 8A-C). While these results are consistent with the observed effects of DCX, we cannot rule out a modulatory activity of the phosphorylation event.

Second, it has been reported that in mature neurons, DCLK proteins are located in axons as well as cell bodies and dendrites (Shin et al 2013). The protein encoded by a lissencephaly-associated mutant DCX-S47R was previously shown to be sequestered in the neuronal cell bodies (Liu et al., 2012). We thus used this mutant to assess the contribution of the DCX protein in non-axonal compartments. As shown in Figure 8A-C, this mutant failed to improve either neuronal survival or axon regeneration, suggesting the possibility that the axonal localized DCX proteins might account for the observed effects. However, the S47

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residue has also been reported to be a phosphorylation site of PKA and might be involved in orchestrating MT and actin dynamics (Tanaka et al., 2004; Schaar et al., 2004; Tsukada et at al., 2005; Toriyama et al., 2012). We thus cannot rule out the contribution of this mechanism to the observed effects of DCX-S47R.

Third, it has been recently shown that two human disease-associated mutations in DCX's linker sequence between two MT binding domains (e.g. W146C, K174E) compromise axonal anterograde transport by disrupting the interaction between DCX and Kif1a, a kinesin-3 motor protein, but without affecting DCX's MT binding (Liu et al., 2013). By over-expressing each of them in RGCs, we found that both of these mutants retained the activity of promoting neuronal survival, but lost the effects on axon regeneration (Figure 8A-C). Together with the observation that DCX-270, but not DCX-S/P, could mimic the survival effects of DCX (Figure 6B), these results suggest that different mechanisms might mediate the action of DCXs on neuronal survival and axon regeneration (Figure 8D): its effects on axon regeneration might depend on its interaction with Kif1a and axon anterograde transport. However, the survival effects are MT-dependent, but independent of its effects on anterograde transport (Figure 8D and discussion).

DISCUSSION

While past studies suggested that global regulators, such as transcription factors and signaling components, dictate neuronal intrinsic regenerative capacity (Lu et al., 2014), the results presented here implicate the DCX family members localized in the axonal compartment as critical regulators of neuronal injury responses, with strikingly diverse effects on neuronal survival, growth cone formation and perhaps axonal extension. It appears that distinct mechanisms underlie these different roles of DCXs (Figure 8D). The effects on neuronal survival might be mediated by the activity of their MT-binding domains in regulating retrograde transport. However, their effects on growth cone formation and axon regeneration require the anterograde transport activity of their MT-binding domain, as well as the actin-stabilizing activities of their S/P-rich regions. Our results suggest that it is the dual regulatory function (for regulating both MT and actin structures) of DCXs that enable them to coordinate cytoskeletal rearrangements in injured stumps necessary for growth cone formation and perhaps extension.

DCXs prevent injury-triggered actin destabilization

Like MTs, actin-based structures constitute key cytoskeleton components of growth cones during development and after injury (Dent et al., 2011; Bradke et al., 2012). During development, genetic deletion of a single family of actin-regulating proteins, ADF and cofilin, results in a failure of neurite formation due to profound cytoskeleton aberrations, such as a blockade of F-actin retrograde flow and irregular MT growth (Flynn et al., 2012). After injury, while a number of studies point to a critical role of MT stability in growth cone and axon regeneration (Bradke et al., 2012; Erturk et al., 2007), how the actin cytoskeleton responds to axotomy is much less understood. We found that axotomy triggers a F-actin destabilization in the injured axonal stump, which could be prevented by over-expressing DCXs or their actin-regulatory domains.

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Mechanistically DCXs might regulate actin dynamics by direct interaction and/or indirect modulation of actin-binding proteins. Although DCX could co-sediment with F-actin in vitro, in transfected cells DCX does not co-recruit F-actin unless the actin-binding protein spinophilin is present (Tsukada et al., 2005). However, full-length DCX, but not its S-P-rich domain, has been shown to bind spinophillin (Bielas et al., 2007). Thus, in addition to spinophilin other actin-binding proteins might be involved. In fact, a recent study demonstrated that in Dcx/Dclk1 double mutants, there is a significantly reduced distribution of F-actin and actin-binding proteins such as α-actinin-4 and actin-related protein 2/3 complex in the axons (Fu et al., 2013). Future studies are needed to define the involvement of these and other mechanisms.

Coordinated MT and actin cytoskeleton rearrangements regulated by DCLKs

Actin and MTs are tightly coordinated during neuronal growth cone navigation. After injury, how their reorganization is coordinated should be crucial for growth cone formation and axon extension. In principle, this might be achieved by either single molecules that have the dual ability to bind both MT and actin structures or protein complexes that together possess these functions. DCXs can interact with both MT and actin, serving as good candidates for coordinating MT/actin dynamics. In fact, previous studies suggested that DCX could bridge actin and MT structures at least *in vitro* (Tsukada et al., 2005). In this line, the DCX protein has been shown to be enriched in the wrist area of the growth cone, at the interface between the F-actin-rich peripheral area and the central domain with tightly bundled MTs, fitting with the model for DCXs as coordinators of MT and actin dynamics (Bielas et al., 2007). Strikingly, we found that while over-expression of either the MT-binding domain or the actin-regulatory domain of DCX could partially promote axon regeneration, simultaneous expression of both domains abolished such regeneration-promoting effects, suggesting that the presence of both domains in the same proteins is required for its full activity. It would be interesting to monitor how this is achieved temporally and spatially in the future.

Separable mechanisms of the unique MT-binding domains of DCXs in neuronal survival and axon regeneration

In addition to stabilizing and bundling MTs in injured stumps, the MT-binding domains of DCXs also interact with molecular motors and regulate the transport of different cargos. Indeed it has been shown that DCXs interacts with Kif1a, a kinesin-3 motor protein, and enhance motor function by increasing run length (Liu et al., 2012). We found that two human disease-associated mutant DCXs (W146C and K174E) disrupting the interaction with Kif1a, but not MT binding, lose the ability to promote axon regeneration, but still retain the activity of increasing neuronal survival. These results provide direct evidence for separable mechanisms involved in DCXs' role in these processes: DCX/Kif1a interaction-mediated effects on axon anterograde transport might specifically act in axon regeneration, but not neuronal survival. On the other hand, our results suggest that DCXs' effects on neuronal survival are mediated by its MT-binding domain, and are correlated with reduced retrograde axonal transport, including the propagation of injury signals (Rishal and Fainzilber, 2014). Thus, we propose that DCXs might regulate axon regeneration and neuronal survival by enhancing anterograde transport and inhibiting retrograde transport mechanisms respectively.

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Potential implications for neuronal repair

In addition to promoting the initiation of axon regeneration, DCX members also greatly enhance axon elongation induced by PTEN deletion, suggesting a new set of targets for promoting axon regeneration after injury. Furthermore, forced expression of DCX members alone resulted in remarkably increased neuronal survival, but with only a modest promotion of axon regeneration. This is in contrast to the dual leucine kinase (DLK), which is linked to the RGC loss associated with glaucoma (Welsbie et al., 2013). Together, these results suggest that these axonally localized proteins might act as key sensory hubs in coordinating different aspects of neuronal injury responses and could be a set of important targets for potential interventions towards neuronal protection and repair under disease conditions.

EXPERIMENTAL PROCEDURES

Mice lines

DCLK1$^{f/f}$ (*Dclk1tm1.2Jgg*/J- (Koizumi et al., 2006)) and DCLK2$^{f/f}$ (*Dclk2tm1Jgg*/J- (Kerjan et al., 2009)) were obtained from Jackson laboratories. PTEN$^{f/f}$/DCLK1$^{f/f}$ and PTEN$^{f/f}$/DCLK2$^{f/f}$ mice lines were obtained by crossing PTEN$^{f/f}$ (Park et al., 2008) mice line with DCLK1$^{f/f}$ and DCLK2$^{f/f}$ mice lines. Breeding PTEN$^{f/f}$/DCLK1$^{f/f}$ and PTEN$^{f/f}$/DCLK2$^{f/f}$ generated PTEN$^{f/f}$/DCLK1$^{f/f}$/DCLK2$^{f/f}$ mice line by crossing over (10cM; 3 pups out of 33).

Optic nerve Injury and further analysis

All experimental procedures were performed in compliance with animal protocols approved by the IACUC at Boston Children's Hospital. All surgical procedures were performed as described in Park et al., 2008. For optic nerve injury, two weeks following AAV2 injection, the optic nerve was exposed intraorbitally and crushed with forceps (Dumont #5 FST) for 5 seconds approximately 1mm behind the eye-ball. We used the following viruses: AAV2-Cre; AAV2-PLAP; AAV2-DCLK2; AAV2-DCX; AAV2-DCX270; AAV2-DCX-SP; AAV2-EB3-GFP, AAV2-Tau, AAV2-lifeact-tdTomato, AAV2-DCX-S47R; AAV2-DCX-W146C; AAV2-DCX-K174E; AAV2-DCX-S297A; AAV2-DCX-GFP; AAV2-DCX270-GFP and AAV2-DCX-SP-GFP. The titers of all viral preparations were at least $1x10^{12}$ gc/mL. The methods of quantifying axon regeneration and neuronal survival are described in Supplementary Methods.

Explant culture, axotomy and time lapse imaging

Explant culture: PTEN$^{f/f}$ mice at P21 were injected with AAV2-Cre first and 24h later with AAV2-PLAP as a control or other AAV2 vectors. 2 weeks after, retinas were dissected out in Hibernate-A (Brain Bits). Retinal explants were then plated onto Poly-L-Lysin and Laminin (Sigma) coated glass bottom dishes (MatTek) in Neurobasal-A (Life Technology) supplemented with B-27, L-Glutamine (Life Technology) and Penicillin/Streptomycin. After 2 weeks, explants were fixed in PFA 4%/Sucrose 1.5% in PBS and labeled with primary antibodies, anti-Tuj1 (1/400), anti-DCLK2 (1/200-AbCAM) and secondary antibodies, Alexa-488, Alexa-674, and Phalloidin-TRITC (1/800-Sigma).

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Laser axotomy.—Laser severing of single axons in explant preparations was performed using a Ti:sapphire laser system, Mantis PulseSwitch Laser (Coherent Inc., Santa Clara CA) which generated a 1 MHz train of ~100 fs pulses in the near infrared, ~800 nm, with 13.5 nJ/pulse. The beam was focused to a diffraction limited spot using Nikon 60X, 1.4 N.A. microscope objective employed for imaging. Visual inspection of the targeted neuron immediately following brief laser exposure (~100-500 ms) confirmed successful axotomy. In some cases multiple laser exposures were necessary to generate a visual break in the axon.

Time-lapse imaging: Before axotomy, explants were transferred into pre-warmed hibernate medium without phenol red, which buffers CO_2 (Brain Bits) and kept for 15 min prior to time lapse experiment. Single axons in explant cultures were imaged on an inverted Nikon Eclipse Ti-U microscope using DIC imaging techniques. Axons were imaged at 0.25 Hz for 20 minutes before laser cut and for 60min after laser cut. A neurite was considered to have successfully initiated growth formation as soon as a filopodia extended from the retraction bulb.

Lifeact imaging: In addition to the other treatments, $PTEN^{f/f}$ mice were infected with AAV2 expressing Lifeact-tdTomato. After 2 weeks in culture, laser severing of single neurites in explant preparations was performed using zeiss LSM 710 system with a Ti:sapphire laser tuned to 720-750nm. Neurites were then imaged 1 minute after the cut (this delay was necessary to confirm the axotomy and initiate image acquisition), for 10 min using the laser scanning confocal and a 63x water-dipping objective. Lifeact intensity was measured using imageJ from the site of the cut to 20μm away. Values were normalized according to the value of the first frame post cut to account for bleaching of the tdTomato caused by the laser axotomy. To represent the decrease in F-actin stability, the slope of the Lifeact-tdTomato intensity decrease was determined for each axon and averaged across all replicates. One-way ANOVAs were performed and specific differences between groups were confirmed with Bonferroni.

Statistical analysis

One-way ANOVAs were performed using PRISM software and specific differences between groups were confirmed with Bonferroni t-test. For two group comparisons, Student’s t-tests were used. For the growth cone formation experiments, where multiple conditions are compared to the same controls, axons were pooled before analysis to each individual condition. Using N-1 Chi-2 test compared the growth cone formation data (Campbell, 2007). Error bars represent the standard error of mean (SEM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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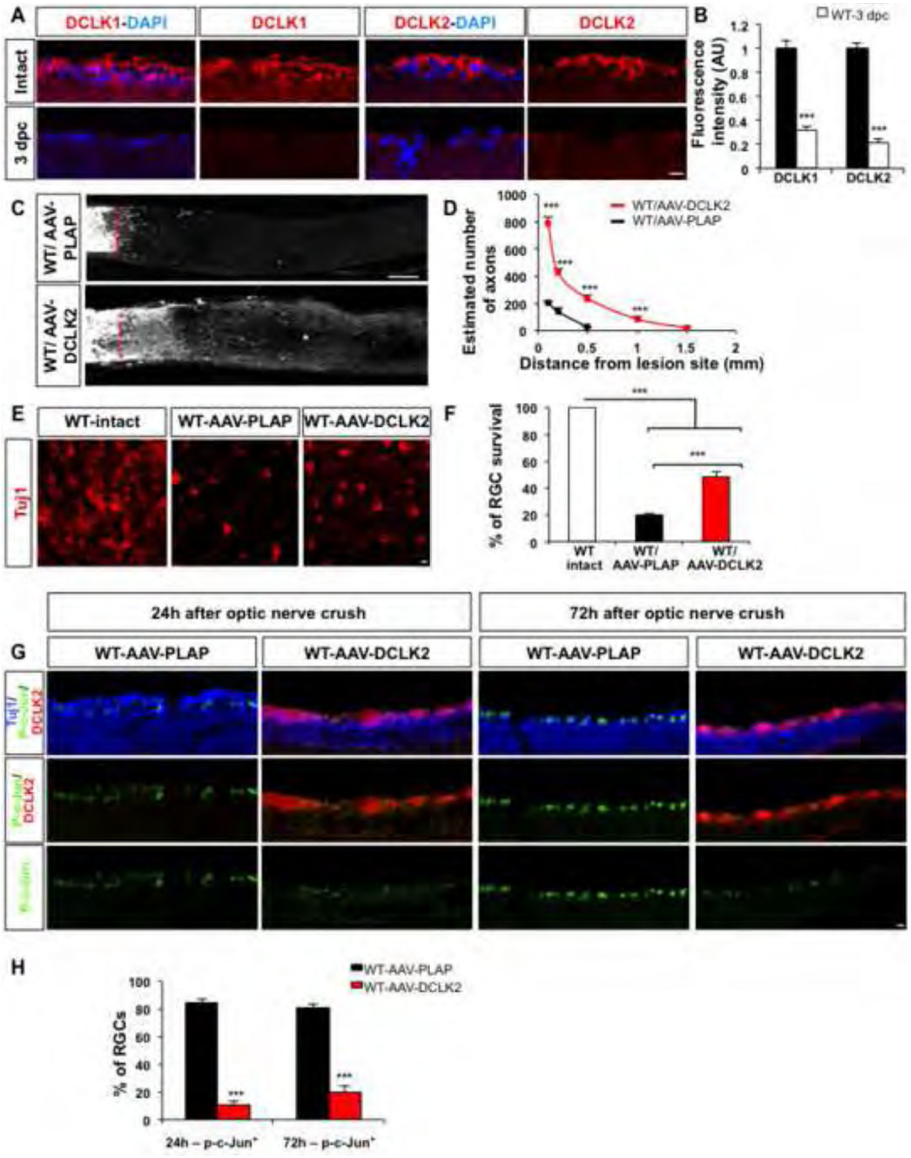

Figure 1: DCLK2 expression is down-regulated by axotomy and DCLK2 overexpression promotes neuronal survival.

(A) Representative confocal images showing mRNA signal of DCLK1 and DCLK2 (red) in intact retina or those at 3 days post crush (dpc). Nuclei are labeled with DAPI (blue). Scale bar: 20μm. (B) Quantification of fluorescence intensity of DCLK1 and DCLK2 signals from (A). ***: $p<0.001$ (n=6). (C) Representative confocal images of optic nerve sections from wild type (WT) mice injected with AAV2-PLAP or AAV2-DCLK2. Axons are labeled with CTB. Scale bar: 100μm. (D) Quantification of the number of regenerating axons presented in (C). ***: $p<0.001$ (n=5). (E) Representative images of Tuj1-immunostained whole mount retinas 2 weeks post injury from wild type mice injected with AAV2-PLAP or AAV2-DCLK2. Scale bar: 20μm (F) Quantification of RGC survival as measured by Tuj1 staining. ***: $p<0.001$. (G) Representative confocal images of retinal sections taken at 24h or 72h post injury stained with antibodies against phospho-c-Jun (green), Tuj1 (blue) and

probed by FISH with antisense probes of DCLK2 (red). Scale bar: 20μm. (H) Quantification of percentage of phospho-c-Jun-positive RGCs from (G). ***: p<0.001.

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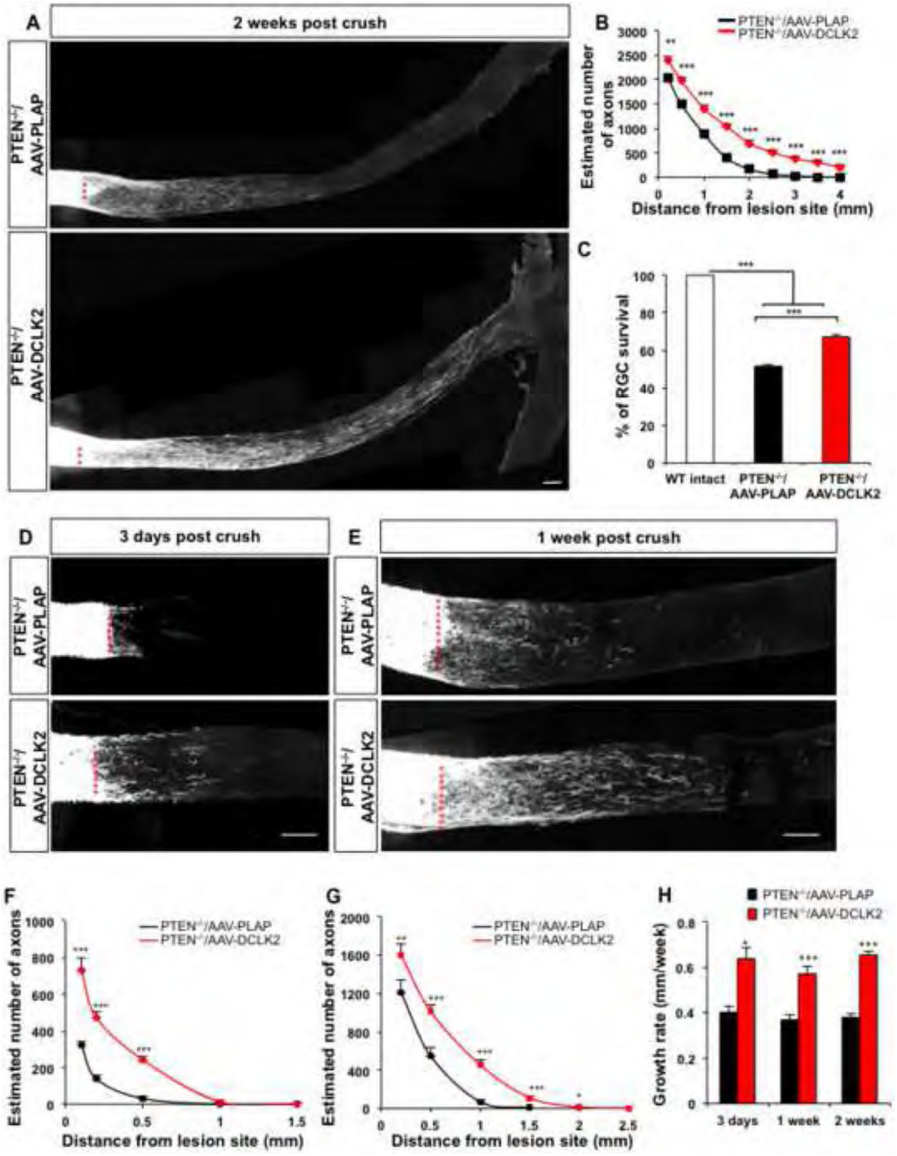

Figure 2: DCLK2 enhances the initiation and elongation of optic nerve regeneration in PTEN-deleted mice.

(A) Representative confocal images of optic nerve sections taken 2 weeks after injury from PTEN$^{f/f}$ mice injected with AAV2-Cre as well as AAV2-PLAP or AAV2-DCLK2. The red stars indicate the crush site. (B) Quantification of regenerative axons in the two groups. ***p<0.001 (n=7). (C) Quantification of RGC survival at 2 weeks after injury in PTEN$^{f/f}$ mice injected with AAV2-Cre as well as AAV2-PLAP or AAV2-DCLK2. ***: p<0.001 (n=3). (D, E) Representative confocal images of optic nerve sections taken from 3 days (D) or 7 days (E) after injury from PTEN$^{f/f}$ mice injected with AAV2-Cre as well as AAV2-PLAP or AAV2-DCLK2. (F, G) Quantification of the number of regenerative axons 3 days (F) and 1 week after injury (G). *p<0.05, ***p<0.001. At least 3 animals for each group. (H) Estimated axon regrowth rates (mm/week) in the two groups. ***: p<0.001, *: p<0.05. Scale bars: 100μm.

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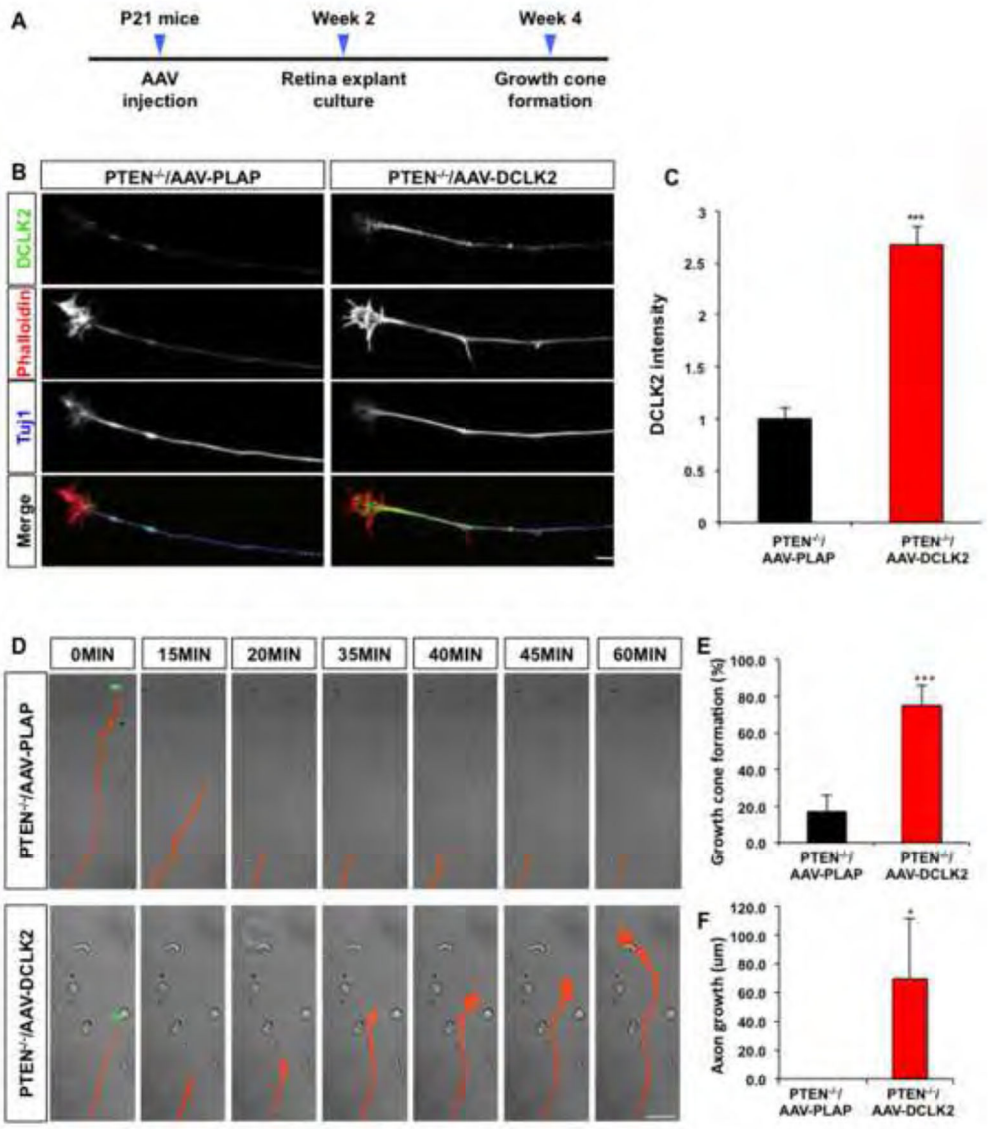

Figure 3: DCLK2 promotes growth cone formation.

(A) Time line of the experimental procedure. (B) Micrographs of neurites from retinal explants from the PTEN$^{f/f}$ mice injected with AAV2-Cre and AAV2-PLAP or AAV2-DCLK2 stained with anti-DCLK2 (green), anti-Tuj1 (blue) and phalloidin (red). Scale bar: 5μm. (C) Quantification of DCLK2 fluorescence intensity normalized by volume of the neurite determined by phalloidin staining. ***: p<0.001. (D) Time-lapse micrographs of post-cut behavior of neurites in two groups for 1h after laser axotomy. Neurites are pseudo-colored in orange and the cut site is indicated by green arrowhead. Scale bar: 5μm. (E) Percentage of growth cone-forming neurites within 60 min after cut in explants from the two groups. ***: p<0.001 (n= 16-18 per group). (F) Quantification of distances navigated by the growth cones from the retraction site in the two groups during the 60 min following laser cut. *: p<0.05 (n= 16-18 per group).

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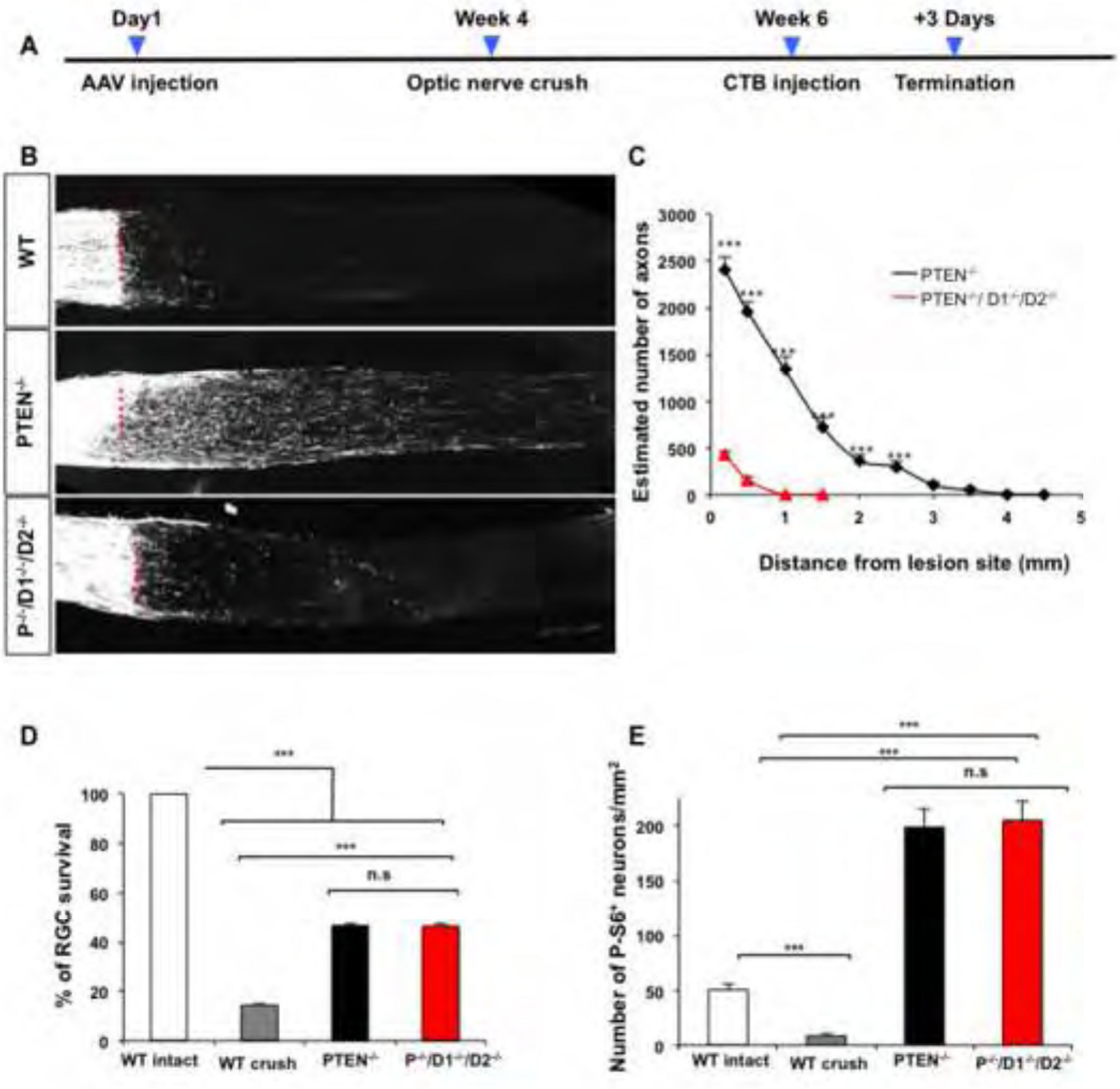

Figure 4: DCLK1/2 are required for optic nerve regeneration induced by PTEN deletion.

(A) Experimental scheme. (B) Representative confocal images of optic nerve sections at 2 weeks post-injury from PTEN$^{f/f}$, or PTEN$^{f/f}$/DCLK1$^{f/f}$/DCLK2$^{f/f}$ ($P^{-/-}D1^{-/-}D2^{-/-}$) mice with AAV2-Cre injection. Scale bar: 100μm. (C) Quantification of regenerative axons from (B). ***: p<0.001 (n=5 per group). (D, E) Quantification of RGC survival (D) and phospho-S6 positive neurons (E) in the four groups. ***p<0.001 (n=5).

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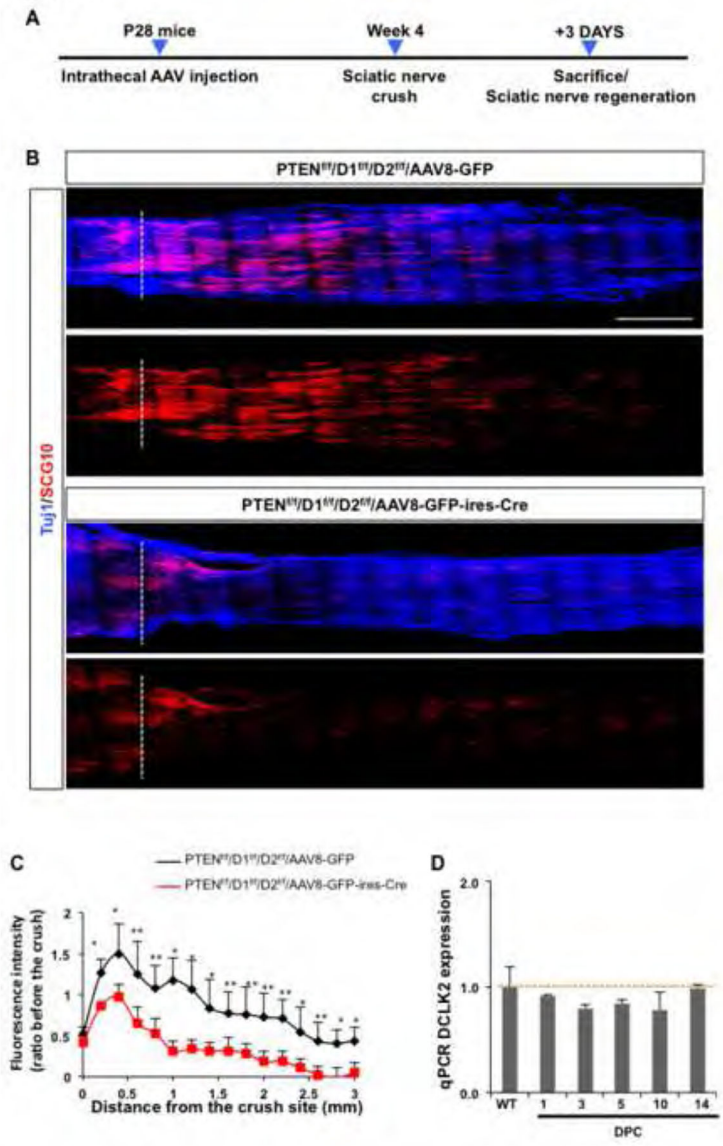

Figure 5: DCLK1/2 are required for sciatic nerve regeneration.

(A) Time line of the experimental procedure to study sciatic nerve regeneration. (B) Representative confocal images of the sciatic nerve sections 3 days post-injury from PTEN$^{f/f}$DCLK1$^{f/f}$/DCLK2$^{f/f}$ mice with AAV8-GFP (control) or AAV8-GFP-IRES-Cre intrathecal injection. Regenerative axons are labeled with anti-SCG10 antibody (red) and sections are co-stained with anti-Tuj1 antibody (blue). White dashed line indicates the crush site. Scale bar: 500μm. (C) Quantification of regenerative axons from (B). ***: p<0.001, **: p<0.01 and *: p<0.05 (n=4-5 per group). (D) Histograms showing the expression of *dclk2* by RT-qPCR on RNA extracted from whole DRG after sciatic nerve crush as indicated. *dclk2* expression is normalized to *GADPH*.

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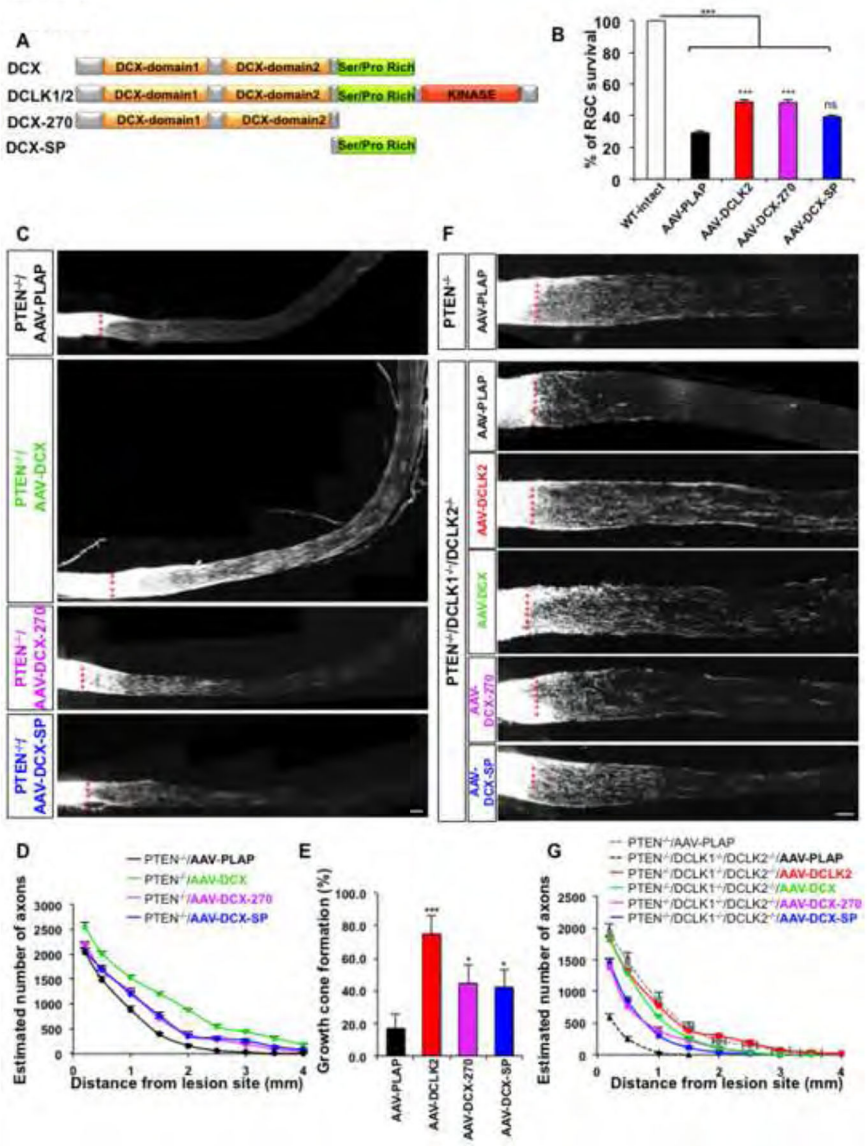

Figure 6: Both MT-binding and S/P-rich domains of DCX can promote axon regeneration after optic nerve injury.

(A) Structures of DCX, DCLK1/2, DCX-270 and DCX-S/P. (B) Quantification of RGC survival in wild type mice infected by individual AAV2 vectors. ***: p<0.001. At least 3 animals per group. (C) Representative confocal images of optic nerve sections from $PTEN^{f/f}$ mice with intravitreal injection of AAV2-Cre and one of the following: AAV2-PLAP, AAV2-DCX, AAV2-DCX270 or AAV2-DCX-S/P. The red stars indicate the crush site. Scale bar: 100μm. (D) Quantification of the number of regenerating axons in different groups. The number of regenerating axons seen at 0.5 mm from the crush site in the mice with DCX-270 or DCX-S/P are statistically different from those with AAV2-PLAP or AAV2-DCX. ANOVA test corrected with Bonferroni's post-test. At least 5 animals per group. (E) Percentage of growth cone-forming neurites within the 60 min following laser cut in the explants from $PTEN^{f/f}$ mice with intravitreal injection of AAV2-Cre and one of the following: AAV2-PLAP, AAV2-DCLK2, AAV2-DCX270 or AAV2-DCX-S/P.

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Chi-2 test, ***: $p<0.001$, *: $p<0.05$. The data in control and DCLK2 groups are the same as presented in Figure 3E. (F) Representative confocal images of optic nerve sections of $PTEN^{f/f}$ mice injected with AAV2-Cre and AAV2-PLAP or $PTEN^{f/f}/DCLK1^{f/f}/DCLK2^{f/f}$ mice injected with AAV2-Cre and one of the following: AAV2-DCLK2, AAV2-DCX, AAV2-DCX-270 or AAV2-DCX-S/P. The red stars indicate the crush site. Scale bar: 100μm. (G) Quantification of the number of regenerating axons in the groups shown in (D). The numbers of regenerating axons seen in the mice with DCX-270 or DCX-S/P are statistically different at all points measured from those with AAV2-PLAP or AAV2-DCX or AAV2-DCLK2. ANOVA test corrected with Bonferroni's post-test.

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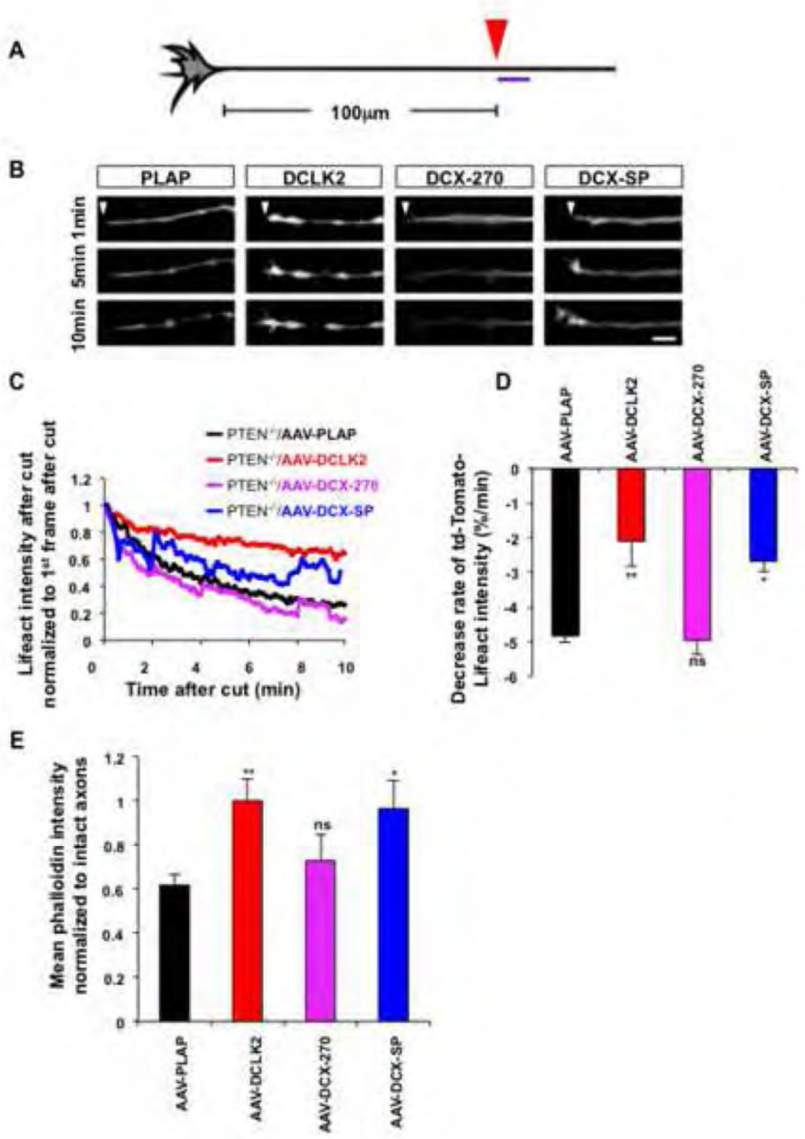

Figure 7: DCX prevents axotomy-triggered F-actin destabilization in injured axon terminals.

(A) Experimental scheme. The red arrowhead indicates the laser cut site (100 µm away from the axonal tip). The blue line indicates the region used for quantification. (B) Micrograph of Lifeact-tdTomato fluorescence in neurites after laser cut in retinal explants from the PTEN$^{f/f}$ mice injected with AAV2-Cre, AAV2-tdtomato-lifeact and one of the following: AAV2-PLAP, AAV2-DCLK2, AAV2-DCX-270-GFP or AAV2-DCX-SP-GFP. Time lapses began 1min post axotomy due to technical restraints of the imaging system (see Methods). Scale bar: 5µm. White arrowheads indicate laser cut site. (C) Quantification of Lifeact-tdTomato intensity from (B) during 10 min after laser cut. Lines correspond to the average of 8 samples per condition. (D) Decrease rate of F-actin intensity over time from (A). ANOVA test corrected with Bonferroni's post-test ***p<0.001. (n=8). (E) Graph showing the quantification of phalloidin intensity after laser ablation and normalized to the

precut phalloidin intensity (at least 10 axons per condition) ANOVA test corrected with Bonferroni's post-test **p<0.01, *p<0.05.

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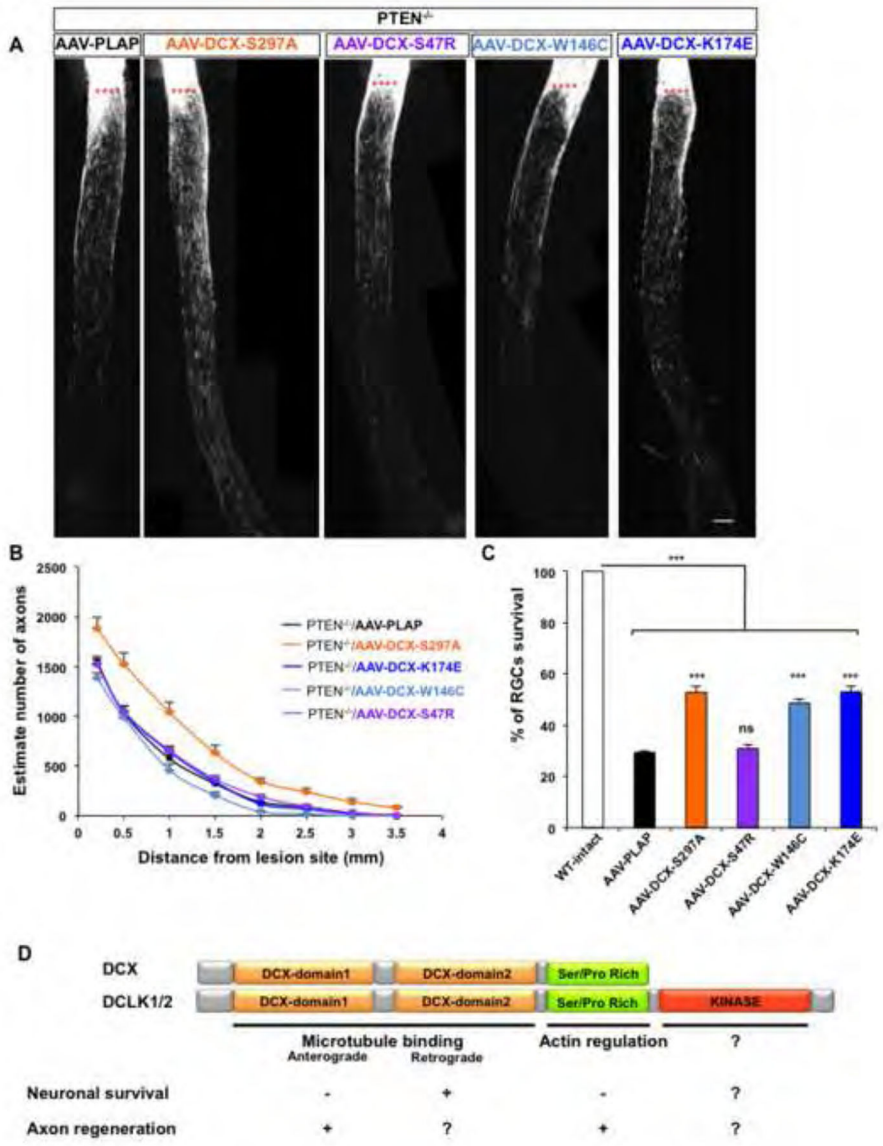

Figure 8: The effects of DCX mutants on neuronal survival and axon regeneration.

Representative confocal images of optic nerve sections of PTEN$^{f/f}$ mice injected with AAV2-Cre and AAV2-PLAP, AAV2-DCX-S297A, AAV2-DCX-S47R, AAV2-DCX-K174E, or AAV2-DCX-W146C. The red stars indicate the crush site. Scale bar: 100μm. Quantification of the number of regenerating axons in the groups (at least 4 animals per group) shown in (A). ANOVA test corrected with Bonferroni's, post-test *p<0.01, ***p<0.001. The number of regenerating axons seen in the mice with DCX-S297A are statistically different at all points measured from those with AAV2-PLAP. (C) Quantification of RGC survival in wild type mice with intravitreal injection of AAV2-PLAP, AAV2-DCX-S297A, AAV2-DCX-S47R, AAV2-DCX-K174E, or AAV2-DCX-W146C. ANOVA test corrected with Bonferroni's, post-test ***p<0.001. Error bars indicate SEM, at least 3 animals per group. (D) Model illustrating different domains and their functions associated with neuronal survival and axon regeneration. While their neuronal survival activity is

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relevant to the retrograde propagation activity of the MT-binding domain, their activity on axon regeneration requires both the anterograde transport activity of MT-binding domain and the F-actin-stabilizing activity of the S/P-rich actin-regulatory domain. The retrograde activity of the MT-binding domains in axon regeneration remains undetermined. The kinase domain of DCLKs is not required for these activities, but we cannot rule out a possible modulatory effect.