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Mst3b, an Ste20-like kinase, regulates axon regeneration in mature CNS and PNS pathways

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Mammalian sterile 20-like kinase-3b (Mst3b, encoded by *Stk24*), regulates axon outgrowth in embryonic cortical neurons in culture, but its role *in vivo* and in neural repair is unknown. Here we show that Mst3b mediates the axon-promoting effects of trophic factors in mature rat retinal ganglion cells (RGCs) and dorsal root ganglion (DRG) neurons, and is essential for axon regeneration *in vivo*. Reducing Mst3b levels using short hairpin RNA prevented RGCs and DRG neurons from regenerating axons in response to growth factors in culture, as did expression of a kinase-dead Mst3b mutant. Conversely, expression of constitutively active Mst3b enabled both types of neurons to extend axons without growth factors. *In vivo,* RGCs lacking Mst3b failed to regenerate injured axons when stimulated by intraocular inflammation. DRG neurons regenerating axons *in vivo* showed elevated Mst3b activity, and reducing Mst3b expression attenuated regeneration and p42/44 MAPK activation. Thus, Mst3b regulates axon regeneration in both CNS and PNS neurons.

Axon regeneration occurs readily after injury in the mature PNS (peripheral nervous system) but not within the CNS. The optic nerve is a classic model of a CNS pathway that does not regenerate when injured. Mature retinal ganglion cells (RGCs) show an abortive sprouting reaction following nerve injury but no long-distance axon growth[1]. Unexpectedly, however, significant regeneration can be achieved by inducing an inflammatory response in the eye. Implanting a peripheral nerve fragment into the vitreous[2], injuring the lens[3–5], or injecting zymosan[6,7] or ciliary neurotrophic factor[8] into the eye all lead to an influx of macrophages and enable RGCs to extend lengthy axons beyond the site of nerve injury. Macrophages release a growth factor, oncomodulin, that stimulates optic nerve regeneration *in vivo* provided cyclic AMP levels are elevated[9,10]. In the PNS, sensory neurons of the dorsal root ganglia (DRG) regenerate their peripherally directed axons readily after injury. The signals that initiate this process remain uncertain, but may include disruption of retrograde signaling, calcium influx, exposure of the proximal stump to an inflammatory environment[11,12] or generation of novel retrogradely transported signals at the injury site[13].

Several lines of evidence suggest that axon outgrowth involves a purine-sensitive protein kinase. The purine analog 6-thioguanine blocks the neurite-promoting effects of trophic agents[14–16] and inhibits the activity of a 45–50 kDa protein kinase[17]. Inosine, a naturally occurring purine nucleoside, reverses the inhibitory effects of 6-thioguanine and can promote axon outgrowth in several types of neurons on its own[15,16,18]. On the basis of these observations, we isolated Mst3b as a purine-sensitive protein kinase and showed that it mediates the axon-promoting effects of inosine and neurotrophic factors in cell culture[15,16]. Mst3b is rapidly activated when embryonic cortical neurons or PC12 cells are exposed to neurotrophins, and inhibiting the expression or activity of Mst3b prevents these cells from extending axons in response to inosine or trophic factors[16]. *In vivo*, inosine stimulates the growth of axon collaterals from intact neurons if other brain pathways have been injured[19–21]. This observation suggests the possibility that Mst3b might play a role in axon growth in the mature nervous system.

In the present study, we have investigated whether Mst3b is required for axon growth in mature neurons. Our results show that Mst3b plays an essential role in the response of both RGCs and DRG neurons to neurotrophic factors through changes in its kinase activity. In addition, suppressing Mst3b expression attenuates axon regeneration in the mature optic nerve and radial nerve. These findings place Mst3b as a key regulator of axon regeneration in the CNS and PNS, and as a potential therapeutic target for improving outcome after axonal injury.

RESULTS

As a model for CNS regeneration, we examined the ability of retinal ganglion cells to extend axons past the site of injury in the mature rat optic nerve when stimulated by intraocular inflammation[3,6]. For the PNS, we used the radial nerve, in which axon regeneration occurs readily after injury in the absence of exogenous stimulation[22]. In both cases, we investigated whether reducing Mst3b expression suppresses the ability of the relevant projection neurons to extend axons in cell culture, whether Mst3b regulates axon growth through changes in its kinase activity and whether altering Mst3b abundance suppresses axon regeneration *in vivo*.

Mst3b is essential for axon outgrowth in RGCs

We transfected RGCs with plasmids expressing a short hairpin RNA to *Stk24* isoform a (subsequently referred to as *Mst3b* shRNA)

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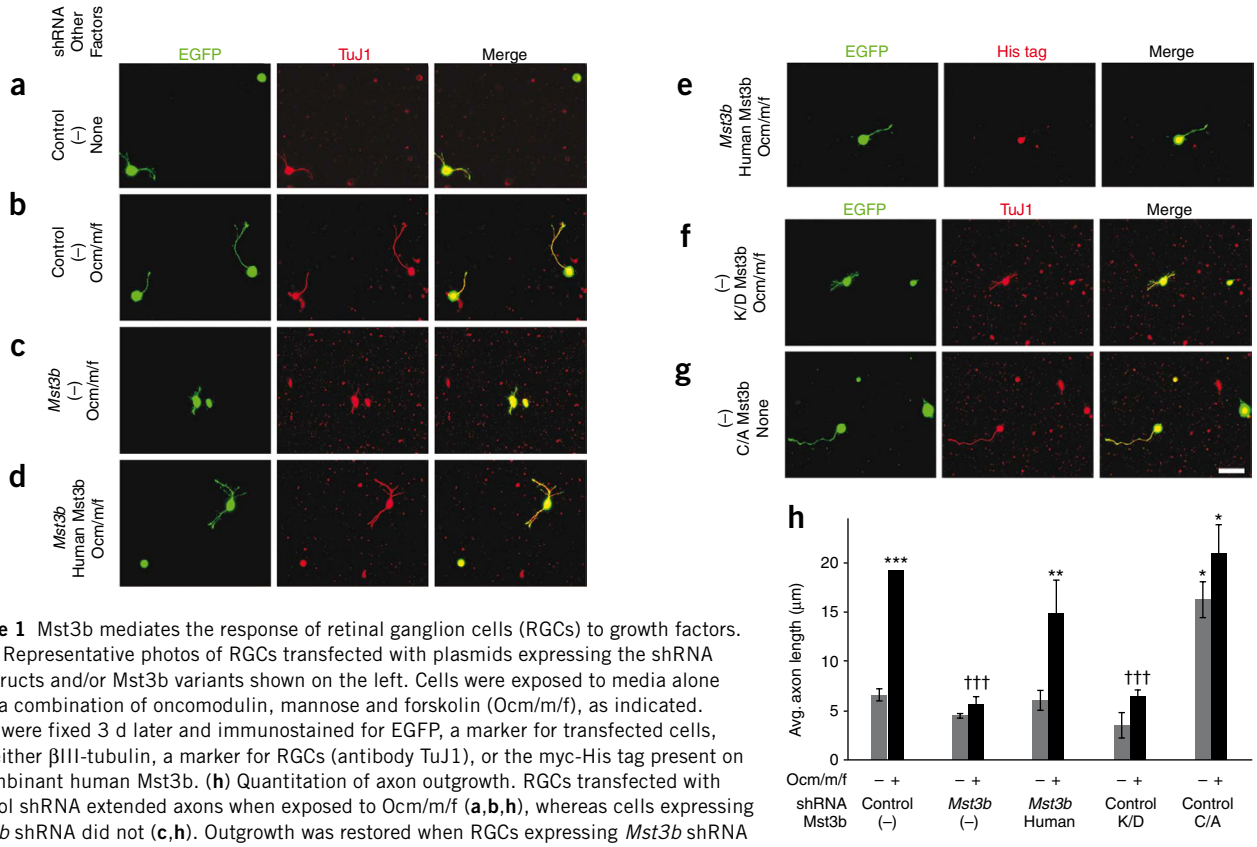

Figure 1 Mst3b mediates the response of retinal ganglion cells (RGCs) to growth factors. (**a**–**g**) Representative photos of RGCs transfected with plasmids expressing the shRNA constructs and/or Mst3b variants shown on the left. Cells were exposed to media alone or to a combination of oncomodulin, mannose and forskolin (Ocm/m/f), as indicated. Cells were fixed 3 d later and immunostained for EGFP, a marker for transfected cells, and either βIII-tubulin, a marker for RGCs (antibody TuJ1), or the myc-His tag present on recombinant human Mst3b. (**h**) Quantitation of axon outgrowth. RGCs transfected with control shRNA extended axons when exposed to Ocm/m/f (**a**,**b**,**h**), whereas cells expressing *Mst3b* shRNA did not (**c**,**h**). Outgrowth was restored when RGCs expressing *Mst3b* shRNA were transfected with a plasmid expressing His-tagged human Mst3b (**d**,**h**). Expression of human Mst3b was verified by immunostaining for the His tag (**e**). Expression of kinase-dead (K/D) Mst3b blocked the effects of growth factors (**f**,**h**), whereas expression of constitutively active (C/A) Mst3b resulted in outgrowth even with growth factors absent (**g**,**h**). Scale bar, 40 μm. Each experiment included four blinded, independent observations (10–100 cells per well), and each experiment was repeated three times. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, increase relative to untreated controls. $^{\dagger\dagger\dagger}P < 0.001$, decrease relative to control cells treated with growth factors. Error bars, s.e.m.

to investigate whether these cells require Mst3b to regenerate axons in response to appropriate growth factors in culture (**Fig. 1**). We dissociated retinas from normal, intact rats, placed these cells into culture, and transfected them with plasmids expressing enhanced green fluorescent protein (EGFP) and either a control shRNA or an *Mst3b*-specific shRNA that was previously shown to knock down Mst3b expression[16]. The control shRNA differs from *Mst3b* shRNA in two nucleotide positions and does not affect axon outgrowth[16]. After 3 d of treatment with the combination of oncomodulin, mannose and forskolin, RGCs expressing the control shRNA showed triple the axon outgrowth of untreated RGCs ($P < 0.001$; **Fig. 1a**,**b**,**h**). In contrast, RGCs expressing *Mst3b* shRNA showed little response to these factors (**Fig. 1**,**c**,**h**).

To investigate whether the reduced outgrowth seen in this study is specifically related to a reduction in Mst3b levels, we cotransfected cells with two plasmids, one expressing *Mst3b* shRNA and the other expressing His-tagged human Mst3b. The mRNA sequence of *STK24* isoform a, encoding human Mst3b, differs considerably from that of the rat gene, and its expression is not blocked by the shRNA construct used here[16]. We verified the presence of exogenous human Mst3b by staining cells with an antibody (anti-His) that recognizes His-tagged Mst3b (**Fig. 1e**). Cotransfecting RGCs with the two plasmids restored RGCs' ability to extend axons in response to growth factors ($P < 0.01$; **Fig. 1d**,**e**,**h**). Thus, the effect of *Mst3b* shRNA in blocking outgrowth depended specifically on its ability to knock down Mst3b expression, and not on off-target effects.

Mst3b regulates growth through changes in kinase activity

To determine whether Mst3b exerts its effects through its kinase activity, we transfected RGCs with a plasmid expressing a mutant form of the protein with an amino acid change in the ATP-binding site[16]. Expression of this 'kinase-dead' Mst3b mutant eliminated the response of RGCs to growth factors (**Fig. 1f**,**h**).

To investigate whether the kinase activity of Mst3b plays a causative role in axon growth, we next transfected RGCs with a plasmid expressing a constitutively active mutant of Mst3b. To create constitutively active Mst3b, we mutated Thr190 to aspartate, a change that has been shown to activate Mst3, a splice variant of Mst3b[23]. Expression of constitutively active Mst3b enabled RGCs to grow axons even with growth factors absent (**Fig. 1g**,**h**). Thus, the kinase activity of Mst3b is necessary and sufficient for axon outgrowth in RGCs. Low-magnification photographs illustrating the transfection efficiency and outgrowth in these cultures are shown in **Supplementary Figure 1**.

Mst3b depletion blocks optic nerve regeneration *in vivo*

To investigate the role of Mst3b *in vivo*, we injected adult rats intravitreally with adeno-associated virus-2 (AAV2) expressing *EGFP* from a cytomegalovirus promoter and, from a U6 promoter, either the shRNA that blocks Mst3b expression or the control shRNA. Rats survived for 4 weeks after viral infections before undergoing surgery to injure the optic nerve and induce intravitreal inflammation. The 4-week delay allowed high levels of shRNA expression and degradation of preexisting Mst3b. In conformity with earlier reports on the high infection

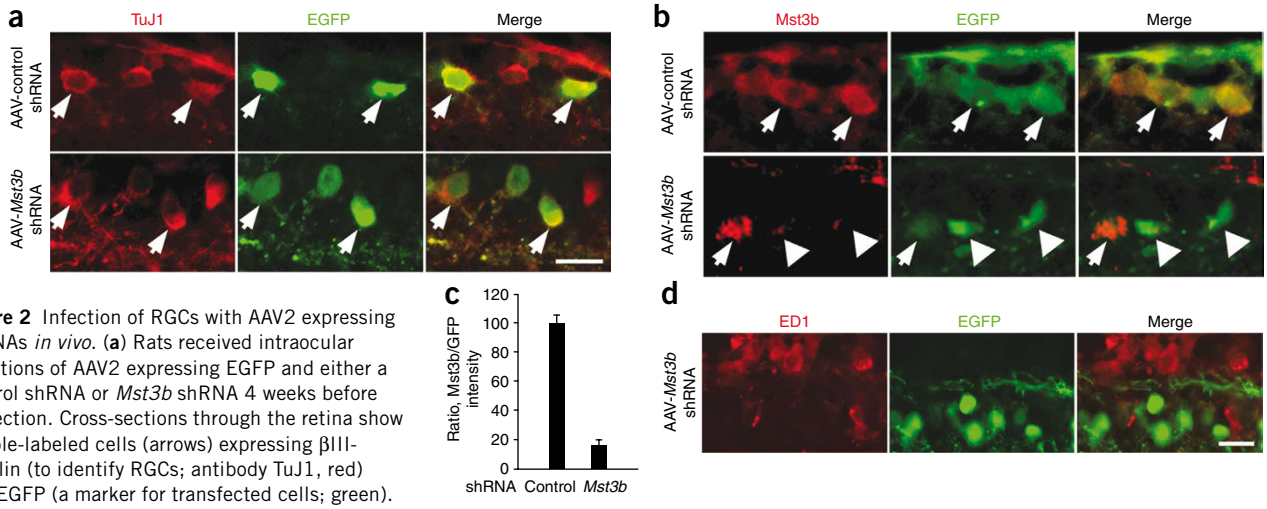

Figure 2 Infection of RGCs with AAV2 expressing shRNAs *in vivo*. (**a**) Rats received intraocular injections of AAV2 expressing EGFP and either a control shRNA or *Mst3b* shRNA 4 weeks before dissection. Cross-sections through the retina show double-labeled cells (arrows) expressing βIII-tubulin (to identify RGCs; antibody TuJ1, red) and EGFP (a marker for transfected cells; green). Transfection efficiencies averaged 58% for the virus expressing control shRNA and 68% for the virus expressing *Mst3b* shRNA. (**b**) Suppression of Mst3b expression. Cross-sections through the retinas of rats injected 4 weeks earlier with AAV2 expressing EGFP and either control shRNA (top) or *Mst3b* shRNA (bottom). Double immunostaining with antibodies to Mst3b (red) and EGFP (green) shows coexpression of the two markers in cells expressing control shRNA (arrows, top) but a loss of Mst3b in RGCs expressing *Mst3b* shRNA (arrowheads, bottom). Scale bar for **a**,**b**, 20 μm. (**c**) Quantitation of Mst3b expression. EGFP$^+$ cells expressing either control or *Mst3b* shRNA were analyzed for intensity of Mst3b immunostaining in cross-sections through all retinas. (**d**) Lack of macrophage infection: 4 weeks after intravitreal injections of AAV2 expressing *Mst3b* shRNA and EGFP, the lens was injured to induce an inflammatory response. Double immunostaining shows ED1$^+$ macrophages to be uninfected (EGFP$^-$; the ED1 antibody recognizes activated macrophage). Scale bar, 40 μm. Error bars, s.e.m.

efficiency of AAV2 (refs. 24–26), we found that AAV2 expressing EGFP and either of the two shRNAs enabled us to visualize EGFP in up to 68% of RGCs 4 weeks after infection (**Fig. 2a**). Infecting RGCs with AAV2 expressing *Mst3b* shRNA strongly suppressed Mst3b expression. Using an antibody that recognizes the N-terminal region of Mst3b[16], we detected the protein in essentially every EGFP-positive RGC expressing control shRNA, but observed an ~85% reduction in average staining intensity in EGFP-positive RGCs expressing *Mst3b* shRNA (**Fig. 2b**,**c**). As expected from the known tropism of AAV2 and the 4-week separation between viral injections and macrophage induction, intraocular injections of AAV2 expressing EGFP and *Mst3b* shRNA did not infect macrophages nor block their activation (**Fig. 2d**).

To verify that virally mediated Mst3b reduction still suppressed axon growth after the time delay used in our *in vivo* studies, we waited

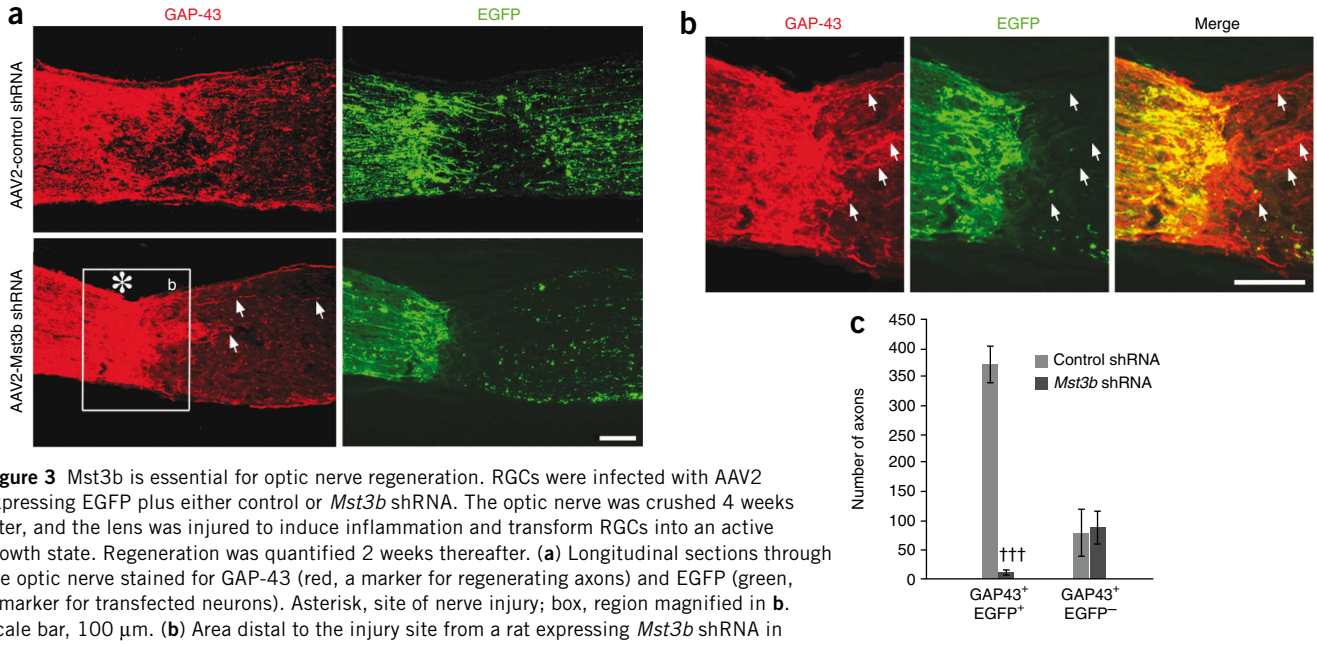

Figure 3 Mst3b is essential for optic nerve regeneration. RGCs were infected with AAV2 expressing EGFP plus either control or *Mst3b* shRNA. The optic nerve was crushed 4 weeks later, and the lens was injured to induce inflammation and transform RGCs into an active growth state. Regeneration was quantified 2 weeks thereafter. (**a**) Longitudinal sections through the optic nerve stained for GAP-43 (red, a marker for regenerating axons) and EGFP (green, a marker for transfected neurons). Asterisk, site of nerve injury; box, region magnified in **b**. Scale bar, 100 μm. (**b**) Area distal to the injury site from a rat expressing *Mst3b* shRNA in RGCs. GAP-43$^+$ axons that arise from uninfected RGCs (red), but not double-labeled axons (yellow) that arise from transfected neurons, extend distal to the injury site. Arrows, regenerating axons from uninfected RGCs. Scale bar, 100 μm. (**c**) Mean number of axons that regenerated ≥500 μm beyond the injury site. Left pair of bars, axons arising from transfected RGCs. The number of regenerating axons was corrected by the percentage of transfected cells. Right pair of bars, axons of uninfected cells, corrected by the percentage of uninfected cells. Results are based on seven rats per group, 4–6 sections per rat, and 0–24 axons per section. $^{\dagger\dagger\dagger}P < 0.001$ decrease relative to rats expressing control shRNA in their RGCs. Error bars, s.e.m.

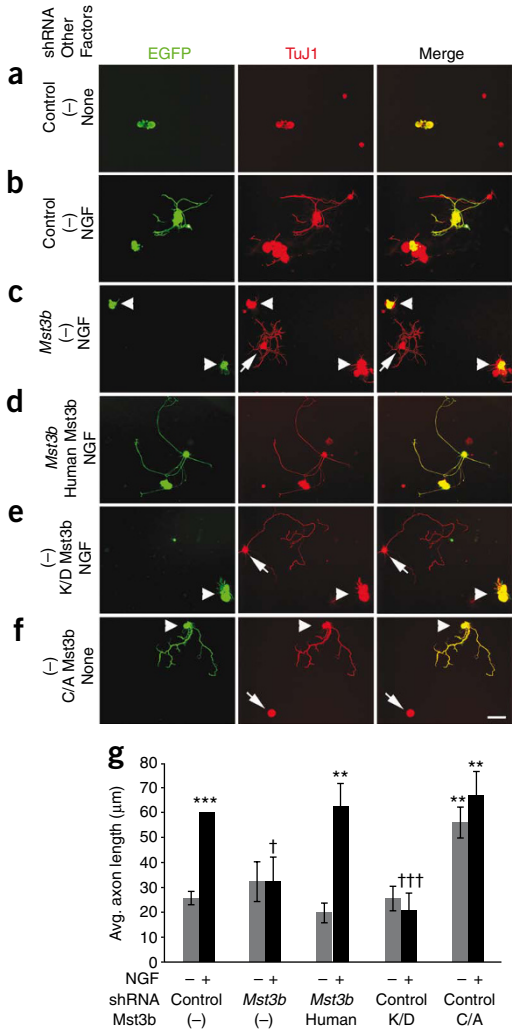

Figure 4 Mst3b mediates the response of DRG neurons to NGF. (**a**–**f**) Representative photos of DRG neurons transfected with plasmids expressing shRNA constructs and/or variant forms of Mst3b, as labeled. Cells were exposed to NGF (50 ng ml^{-1}) or media alone. Cells were fixed 3 d later and immunostained to detect EGFP, a marker for transfected cells, and βIII-tubulin to identify neurons (antibody TuJ1). (**g**) Quantitation of axon growth. Cells expressing the control shRNA (**a**,**b**,**g**) extended neurites when treated with NGF (**b**,**g**). Cells expressing *Mst3b* shRNA (arrowheads; **c**,**g**) did not respond to NGF; an untransfected cell in the same field showed outgrowth (arrow). (**d**,**g**) NGF-induced outgrowth was restored when cells expressing *Mst3b* shRNA (green) were transfected with a plasmid expressing human Mst3b. (**e**,**g**) Cells expressing kinase-dead (K/D) Mst3b (arrowheads) fail to extend neurites when treated with NGF; a untransfected cell (arrows) in the same field shows outgrowth. (**f**,**g**) expression of constitutively active (C/A) Mst3b (arrowhead) is sufficient to induce outgrowth in the absence of NGF; arrow, an untransfected cell that is not growing. Scale bar, 100 μm. Each experiment included four blinded, independent observations of about ten cells per well, and each experiment was performed three times. $^{**}P < 0.01$ and $^{***}P < 0.001$, increase relative to baseline (control shRNA-transfected cells without NGF). $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$, decrease relative to control shRNA-transfected cells treated with NGF. Error bars, s.e.m.

4 weeks after injecting AAVs, dissected and dissociated retinas, and cultured the cells[6] in the presence or absence of oncomodulin, mannose and forskolin[6,10,27]. In RGCs expressing the control shRNA, the addition of oncomodulin, forskolin and mannose caused a doubling in average axon length ($P < 0.001$; **Supplementary Fig. 2**). RGCs expressing *Mst3b* shRNA extended much shorter axons with or without growth factors present (difference from control-infected RGCs significant at $P < 0.001$; **Supplementary Fig. 2**). Thus, the effect of Mst3b knockdown remained strong several weeks after viral infection and resulted in a reduction of both baseline and growth factor-stimulated axon outgrowth. The lower overall outgrowth seen in **Figure 1h** compared to that in **Supplementary Figure 2** may reflect a general decrease in RGCs' ability to extend axons after being exposed to the reagents used for the transient transfections.

In vivo, regenerating axons that arise from infected RGCs were distinguished by virtue of staining positively for both EGFP and GAP-43. The membrane phosphoprotein GAP-43 is normally undetectable in the mature optic nerve, but is strongly induced in axons undergoing regeneration[2,3,28]. Profiles that are GAP-43^{+}EGFP^{-}, by contrast, are expected to reflect regenerating axons that arise from uninfected neurons. Remnants of degenerating axons that already contained EGFP before injury, but that are not regenerating, would be GAP-43^{-}EGFP^{+}. Rats infected with control shRNA ($N = 7$) showed many GAP-43^{+}EGFP^{+} axons regenerating ≥0.5 mm past the lesion site (**Fig. 3a**, top). In contrast, rats with Mst3b knocked down ($N = 7$) showed 97% fewer GAP-43^{+}GFP^{+} regenerating axons (**Fig. 3b**) (difference significant at $P < 0.001$; **Fig. 3a** bottom and **3c**). In all cases, we normalized the number of axons by the percentage of RGCs found to be EGFP-positive to account for individual variations in infection rates. As an internal control, we also quantified the number of regenerating axons arising from uninfected RGCs (GAP-43^{+}EGFP^{-}) and found this number to be similar between rats infected with AAV2 expressing control versus *Mst3b* shRNA (**Fig. 3c**). Thus, Mst3b suppression essentially eliminated axon regeneration in the mature optic nerve, but did not alter the growth capacity of neighboring uninfected neurons.

The effect of Mst3b knockdown on axon regeneration was not due to changes in cell survival. RGCs begin to undergo apoptotic cell death a few days after optic nerve injury, and by 2 weeks ~80% will have died[29,30]. Lens injury enhances survival to allow 40%–50% of RGCs to remain alive after 2 weeks. To investigate whether the loss of axon regeneration after Mst3b knockdown *in vivo* might reflect a selective loss of the infected RGCs, we examined the survival of these cells after infection with AAV2 carrying either control or *Mst3b* shRNA. The number of surviving RGCs was the same in both cases (8.8 ± 0.3 TuJ1^{+} cells per 200 microns per section with *Mst3b* shRNA, and 7.3 ± 0.4 TuJ1^{+} cells per 200 microns per section with the control shRNA). We also calculated the percentage of RGCs that were EGFP positive with and without surgery. In rats expressing *Mst3b* shRNA, the percentage of RGCs that was EGFP-positive was 61.1 ± 3.3% in rats that survived 6 weeks and did not have surgery ($N = 4$) and 68.3 ± 1.9% after optic nerve crush plus lens injury ($N = 7$; difference not significant: $t = 1.89$, $P \approx 0.09$). Rats infected with the control shRNA showed similar results, although the infection rates were lower. In these cases, 16.2 ± 2.2% of RGCs were EGFP positive in rats that did not have surgery ($N = 4$) compared to 12.1 ± 1.6% 2 weeks after optic nerve crush plus lens injury ($N = 7$; difference not significant: $t = 1.51$, $P \approx 0.16$).

Mst3b knockdown blocks NGF-induced outgrowth in DRG neurons

In contrast to the optic nerve, axon regeneration occurs readily in peripheral nerves after injury. Axons that arise from neurons in cervical DRGs 7 and 8 begin to regenerate past a lesion site in the radial nerve within a day or two, with full recovery of function taking place over a few weeks[22,31]. As with RGCs, we first used transient

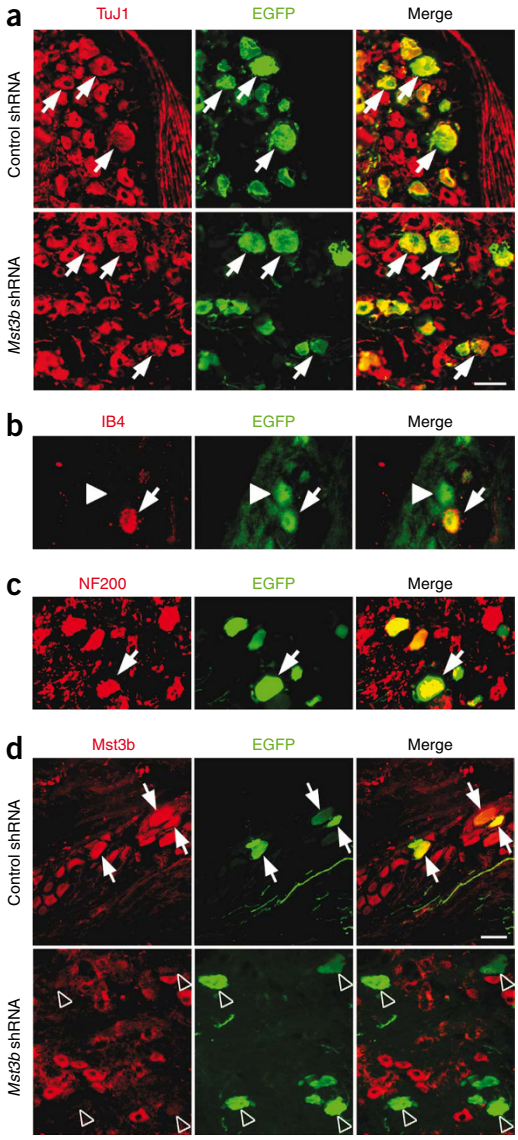

Figure 5 Mst3b knockdown in DRG neurons. Sections through rat DRG 4 weeks after injecting AAV2 expressing EGFP and either control or *Mst3b* shRNA. (**a**) Infection of DRG neurons is demonstrated by immunostaining (arrows) with antibodies to the neuronal marker βIII tubulin (TuJ1, red) and to virally expressed EGFP (green). Overall transfection rates were 21% for the control shRNA group ($n = 10$) and 35% for the *Mst3b* shRNA group ($n = 13$). Scale bar, 40 μm. (**b**) AAV2 expressing EGFP and *Mst3b* shRNA infects small IB4$^+$ (red) non-peptidergic neurons (arrows) as well as small IB-4$^-$ neurons (arrowheads). (**c**) AAV2 also infects large neurons (arrow) that stain with antibodies to the neurofilament protein NF200 (red). (**d**) Knockdown of Mst3b in DRG neurons. Ganglia were immunostained with antibodies to Mst3b (red) and EGFP (green) 4 weeks after infecting DRGs with AAV2 expressing EGFP and either control (top) or *Mst3b* shRNA (bottom). Mst3b is visible in cells expressing control shRNA (arrows, top) but not in cells expressing *Mst3b* shRNA (arrowheads, bottom). Scale bar, 40 μm. **b** and **c** at same scale as **a**.

transfection assays to evaluate whether Mst3b mediates the response of adult DRG neurons to trophic factors, in this case nerve growth factor (NGF) (**Fig. 4**). We dissociated DRG neurons from uninfected, unlesioned rats and transfected these with plasmids expressing EGFP and either control or *Mst3b*-specific shRNA. After being exposed to NGF for 3 d, DRG neurons expressing the control shRNA showed twice the average axon length of untreated controls (difference significant at $P < 0.001$: **Fig. 4a**,**b**,**g**), whereas DRG neurons expressing *Mst3b* shRNA showed no response (**Fig. 4c**,**g**). To investigate whether this failure to respond to NGF was due specifically to reduced Mst3b expression, we cotransfected DRG neurons with plasmids expressing *Mst3b* shRNA and a cDNA encoding the homologous human protein MST3b/STK24, as above. Coexpression of the shRNA and the human cDNA restored DRG neurons' ability to respond to NGF ($P < 0.01$; **Fig. 4d**,**g**). Thus, the effect of *Mst3b* shRNA in blocking outgrowth was due specifically to knockdown of Mst3b expression. These studies show that Mst3b is essential for DRG neurons' ability to extend axons in response to NGF.

As in RGCs, we next investigated whether Mst3b regulates axon outgrowth through changes in its kinase activity. To do this, we transfected DRG neurons with either the kinase-dead or constitutively active form of Mst3b described above. Expression of kinase-dead Mst3b eliminated the ability of DRG neurons to extend axons in response to NGF (**Fig. 4e**,**g**). In contrast, transfection with constitutively active Mst3b caused these cells to extend long axons even in the absence of NGF (**Fig. 4f**,**g**). Thus, the axon-promoting effect of NGF on DRG neurons seems to be mediated by changes in the kinase activity of Mst3b.

Mst3b knockdown attenuates peripheral nerve regeneration

Four weeks after injecting AAV2 expressing EGFP and either control shRNA or *Mst3b* shRNA into cervical ganglia, the percentage of TuJ1$^+$ neurons found to be transfected was 21.4 ± 2.9% and 34.9 ± 2.1%, respectively. Transduction was specific to neurons (**Fig. 5a**) and included small peptidergic (IB4$^-$) and non-peptidergic (IB4$^+$) neurons, as well as large neurons positive for the neurofilament protein NF200 (**Fig. 5b**,**c**). Compared to cells expressing control shRNA, average Mst3b immunostaining was ~85% lower in DRG neurons transfected with AAV2 expressing *Mst3b* shRNA (**Fig. 5d**).

As with RGCs, we examined whether virally mediated knockdown of Mst3b would affect DRG neurons' ability to regenerate axons when tested several weeks later. Four weeks after infecting cervical DRGs *in vivo* with AAV2 expressing either control or *Mst3b*-specific shRNA, we dissociated and cultured DRG neurons on poly-D-lysine-coated glass coverslips. In the absence of NGF, EGFP-positive DRG neurons showed similar outgrowth irrespective of which shRNA was being expressed. However, whereas neurons expressing the control shRNA on average tripled their axon length when exposed to NGF, neurons expressing *Mst3b* shRNA showed no detectable response (**Supplementary Fig. 3**; difference between groups significant at $P < 0.02$).

To investigate the role of Mst3b in peripheral nerve regeneration *in vivo*, we prepared a separate group of rats in which cervical DRG neurons were injected with AAV2 expressing EGFP and either the control or *Mst3b*-specific shRNA. We then crushed the radial nerve 4 weeks later and evaluated axon regeneration after 3 d, a time point at which injured axons have normally grown several millimeters beyond the injury site (**Fig. 6**). As in the optic nerve, we assessed regeneration arising from infected neurons as the number of axons beyond the injury site that were positive for both GAP-43 and EGFP, and then corrected this number by the percentage of cells that were transfected to account for individual variations in transfection efficiency. In DRGs injected with AAV2 expressing the control shRNA, many EGFP$^+$GAP-43$^+$ axons extended 1–2 mm distal to the injury site, and some extended as far as 5 mm (**Fig. 6a**,**b**,**e**,**f**; $N = 8$). Rats expressing *Mst3b* shRNA showed an 80%–85% reduction in axon growth

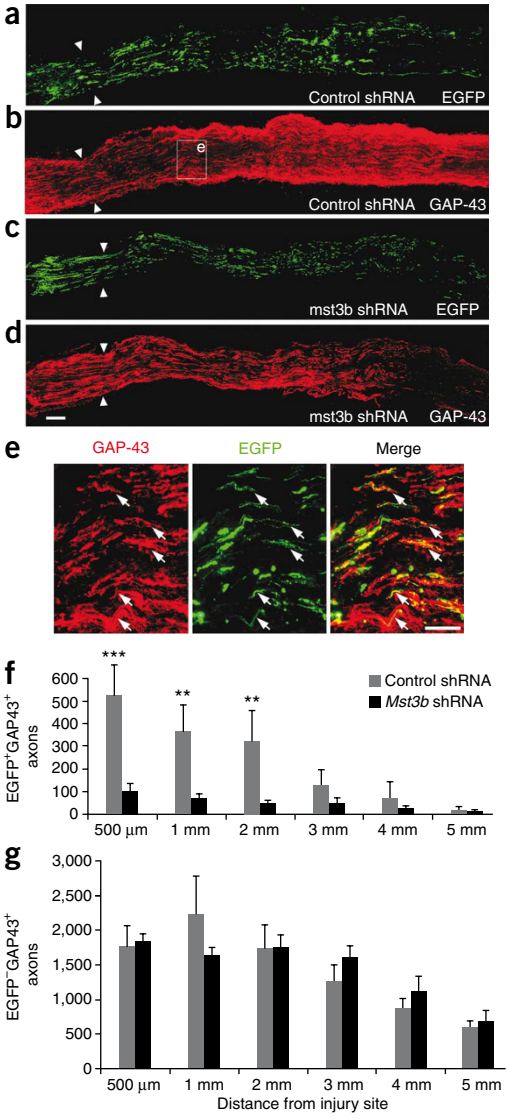

Figure 6 Mst3b knockdown attenuates peripheral nerve regeneration. (**a**–**d**) The radial nerve was crushed 4 weeks after infecting cervical DRG neurons with AAV2 expressing EGFP and either *Mst3b* or control shRNA. Axons extending beyond the injury site (arrowheads) were visualized 3 d later in sections stained with antibodies to GAP-43 (red) and EGFP (green). The box in **b** is magnified in **e**. Only profiles labeled with both markers were considered as regenerating axons arising from infected neurons (arrowheads). Scale bar for **a**–**d**, 200 µm. (**e**) High-power images of regenerating axons arising from neurons expressing control shRNA: many axons are positive for both GAP-43 (red) and EGFP (green) (arrows). Scale bar, 100 µm. (**f**) Quantitation of GAP-43$^+$EGFP$^+$ axons. The number of regenerating axons was corrected by the percent of infected cells. Note the marked attenuation of axon growth in cells expressing *Mst3b* shRNA. (**g**) Quantitation of GAP-43$^+$EGFP$^-$ axons from uninfected neurons shows similar amounts of regeneration irrespective of the virus infecting neighboring neurons. Data show mean number of axons at the indicated distances from the injury site (8 or 9 rats per group, 4–6 sections per rat, 0–10 axons per section). $^{**}P < 0.01$, $^{***}P < 0.001$, differences between number of axons arising from cells expressing control versus *Mst3b* shRNA. Error bars, s.e.m.

($N = 9$; difference from controls significant at $P < 0.001$ for 0.5 mm, $P < 0.01$ for 1 and 2 mm; not significant at 3, 4 and 5 mm: **Fig. 6c**,**d**,**f**). We also evaluated regeneration in uninfected DRG neurons in these same rats—that is, the number of axons that were GAP-43$^+$EGFP$^-$, normalized by the percentage of cells that were uninfected. Axons arising from uninfected DRG neurons regenerated to a similar extent regardless of whether their neighbors expressed the control or *Mst3b* shRNA (**Fig. 6g**). In sum, knocking down Mst3b expression in DRG neurons strongly attenuated, but did not eliminate, the regeneration of injured axons *in vivo*.

The number of DRG neurons (TuJ1$^+$) that survived infection with AAV carrying *Mst3b* shRNA (142.8 ± 6.0 per mm^2) was similar to that seen after infection with AAV carrying control shRNA (139.5 ± 8.8 per mm^2). Thus, knocking down Mst3b expression did not differentially impair DRG neuron survival.

Mst3b kinase activity changes during regeneration

To determine whether the activity of Mst3b in DRG neurons increases during regeneration, we isolated ganglia 3 d after peripheral nerve injury and compared their Mst3b kinase activity with that of control DRG neurons using immunoprecipitation-kinase (IP-kinase) assays as described[16]. We precipitated Mst3b from DRG lysates using the antibody to the N-terminal region of the protein and evaluated kinase activity using histone protein HF1 as an experimental substrate. DRG neurons undergoing axon regeneration showed 2.5-fold more Mst3b kinase activity than control neurons ($P < 0.01$). 6-Thioguanine, an inhibitor of Mst3b, attenuated this increase ($P < 0.05$: **Fig. 7a**,**b**).

The MAP kinase pathway contributes to the development and regeneration of peripheral axons[32,33] and to the effect of NGF in inducing outgrowth in PC12 cells[34,35]. The observation that knocking down Mst3b inhibits outgrowth in both sensory neurons and PC12 cells[16] is consistent with the possibility that it acts through a MAPK pathway. To examine how Mst3b fits into the signal transduction cascade that leads to axon outgrowth, we compared p42/44 MAPK phosphorylation in neurons expressing control versus *Mst3b* shRNA. In sections through DRGs expressing *Mst3b* shRNA, we detected immunostaining for phospho-p42/44 MAPK in 11% of neurons, compared to 87% of neurons expressing the control shRNA (**Fig. 7c–e**). The total p42/44 MAPK immunostaining did not differ between the two groups.

DISCUSSION

Mst3b was recently shown to be a neuron-specific protein kinase that is essential for axon outgrowth in embryonic cortical neurons and PC12 cells in culture[16]. The present study demonstrates that Mst3b also mediates the effects of trophic factors in stimulating axon outgrowth in adult RGCs and DRG neurons, that it exerts these effects through changes in its kinase activity and that this kinase is essential for mature RGCs and DRG neurons to regenerate injured axons *in vivo*.

To study the role of Mst3b in the CNS, we used the adult rat optic nerve as an accessible and widely studied example of a CNS pathway in which some regeneration can be achieved by inducing an inflammatory reaction in the eye[3,6]. A macrophage-derived growth factor, oncomodulin, can mimic the effect of intravitreal inflammation when introduced into the eye together with an agent to elevate intracellular cAMP; mannose, which is also required for the effects of oncomodulin, is normally abundant in the eye[6,10,27]. As shown here, depletion of Mst3b greatly reduced the ability of adult rat RGCs to extend axons in response to oncomodulin, mannose and forskolin in culture, and eliminated axon regeneration *in vivo*. The effect of *Mst3b* shRNA on axon outgrowth can be attributed specifically to the loss of Mst3b protein, as responsiveness to growth factors was rescued when RGCs expressing *Mst3b* shRNA were cotransfected with plasmids

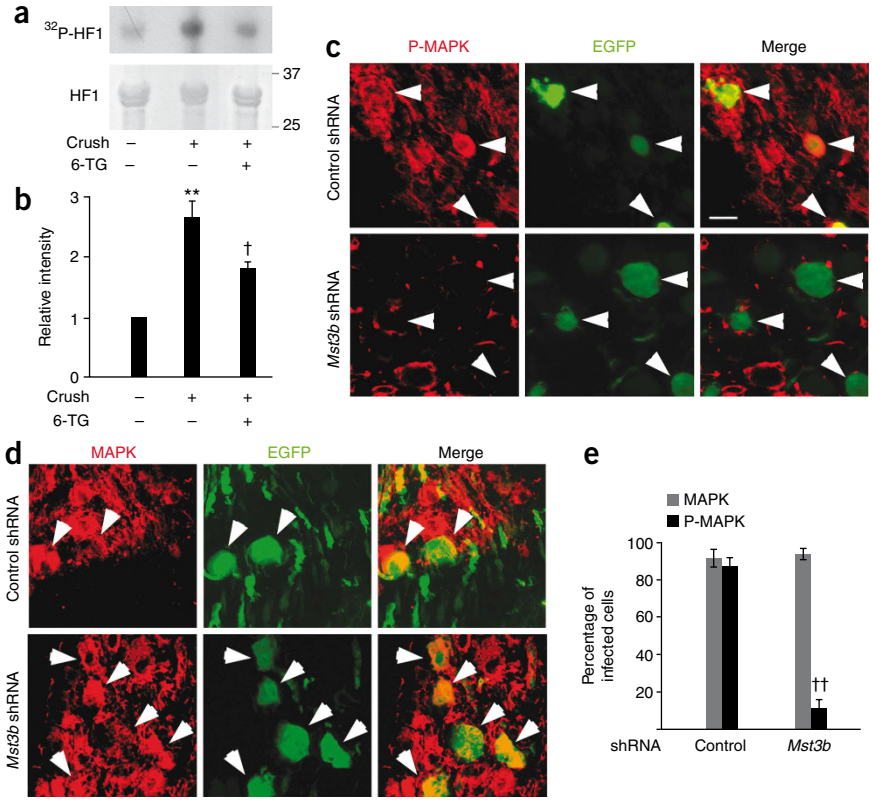

Figure 7 Mst3b activation and regulation of p42/44 MAPK phosphorylation *in vivo*. (**a**) Mst3b was immunoprecipitated from lysates of DRG neurons 3 d after peripheral nerve crush or sham surgery. Kinase assays were performed using HF1 as a pseudosubstrate and γ-[^{32}P]-ATP, with or without 6-thioguanine (6-TG) present. Reaction mixes were separated by SDS-PAGE and kinase activity visualized by autoradiography (top). Protein loading was visualized by Coomassie blue staining (bottom). Right, positions of molecular weight markers. (**b**) Quantitation of Mst3b kinase activity based on densitometry of autoradiograms averaged from three separate experiments. (**c**,**d**) Cross-sections through the DRG of rats injected 4 weeks earlier with AAV2 expressing EGFP and either control shRNA (top) or *Mst3b* shRNA (bottom). Arrowheads, infected (EGFP$^+$) neurons. (**c**) Double immunostaining with antibodies to phospho-p42/44 MAPK (red) and EGFP (green) reveals a selective reduction of phospho-MAPK in DRG neurons expressing the *Mst3b* shRNA. (**d**) Double immunostaining with antibodies to p42/p44 MAPK (red) and EGFP (green). Mst3b knockdown did not alter overall p42/44 MAPK levels. (**e**) Quantitation of changes in the overall expression and phosphorylation of p42/p44 MAPK in 4–6 sections from three rats per condition. Scale bar, 40 μm. $^{**}P < 0.01$, increase relative to controls; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, decrease relative to induced state. Error bars, s.e.m.

expressing human Mst3b (ref. 16). The role of Mst3b in axon growth was further demonstrated by the inability of RGCs expressing a kinase-dead Mst3b mutant to extend axons in the presence of growth factors. Conversely, expression of a constitutively active form of Mst3b caused RGCs to extend axons even in the absence of growth factors. Thus, changes in Mst3b kinase activity are key in controlling axon outgrowth in RGCs.

Mst3b is also important for axon regeneration in the peripheral nervous system. Depletion of Mst3b eliminated the ability of DRG neurons to respond to NGF in culture and attenuated axon regeneration after radial nerve crush *in vivo*. As with RGCs, the role of Mst3b in DRG neurons was shown to require its kinase activity. Expression of a kinase-dead mutant form of Mst3b blocked NGF-induced outgrowth in culture, and, notably, expression of a constitutively active Mst3b mutant enabled DRG neurons to extend axons even in the absence of growth factors.

In vivo, DRG neurons expressing *Mst3b* shRNA showed 80% less axon regeneration 3 d after nerve injury than neurons expressing the control shRNA or than untransfected neurons in the same ganglia. Nonetheless, the continued occurrence of some regeneration even when Mst3b abundance was reduced suggests either that there was enough Mst3b remaining to allow some regeneration or that axon regeneration in the PNS may use both Mst3b-dependent and Mst3b-independent mechanisms. DRG neurons extend processes in response to trophic factors and, through integrin signaling, in response to substrate molecules[36]. In culture, DRG neurons expressing *Mst3b* shRNA continued to show basal outgrowth when cultured on poly-D-lysine, but they were unable to respond to NGF. Peripheral nerves, but not optic nerves, are rich in laminin, and this may contribute to the ability of DRG neurons expressing *Mst3b* shRNA, but not similarly infected RGCs, to show some regeneration *in vivo*. The different amounts of regeneration in the two systems may also be due in part to the marked differences in inhibitory signals between the optic nerve and peripheral nerve[37,38].

Ste20, the prototypic member of the kinase family that includes Mst3b, functions as an upstream activator of a MAP kinase cascade that regulates budding in yeast. Ste20 relays the signal from a pheromone-sensitive, G protein–coupled receptor to Ste11; Ste11 in turn phosphorylates the MAPK kinase Ste7, which then phosphorylates Fus3, a MAPK[39,40]. MAP kinase pathways are known to contribute to the development and regeneration of peripheral axons[32,33] and to the effect of NGF in inducing outgrowth in PC12 cells[34,35]. Thus, the observation that Mst3b depletion inhibits outgrowth in sensory neurons and PC12 cells[16] is consistent with the possibility that Mst3b activates a MAPK pathway in neurons. In conformity with this idea, we found that the kinase activity of Mst3b was elevated in DRG neurons undergoing axon regeneration *in vivo*, and that reduction of Mst3b expression diminished p42/44 MAPK phosphorylation in these neurons. Together, these data support the idea that Mst3b controls axon outgrowth through changes in its kinase activity and subsequent activation of a MAP kinase signaling cascade.

When stimulated to go into an active growth state *in vivo*, RGCs show marked changes in the expression of genes associated with axon growth and cell survival[41], and these changes resemble those seen in DRG neurons undergoing axon regeneration in the PNS[42–44]. The results of the present study suggest that Mst3b could be part of a common signaling pathway that mediates the effects of trophic factors in controlling the expression of axon-outgrowth-related genes in CNS and PNS neurons.

In summary, we have shown that Mst3b, through its kinase activity, is a key regulator of axon regeneration in the adult optic nerve and radial nerve. It will be important to investigate whether Mst3b regulates axon regeneration in other parts of the CNS and PNS, and whether expression of a constitutively active form of Mst3b can augment the limited amount of growth that is at present achievable

after CNS injury. These and further studies into the molecular mechanisms by which Mst3b functions may open up new avenues for the treatment of CNS injuries.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

B.L. helped design some of the experiments, carried out most *in vivo* and *in vitro* studies, drafted much of paper and did most of the data analysis. M.L.H. assisted with many cell culture studies and *in vivo* work. L.I.B. helped conceive the overall structure of the study, supervised parts of it, carried out some of the data analysis and wrote parts of the manuscript. N.I. generated the core idea of the study, helped conceive the overall structure of the study, performed preliminary optic nerve studies, constructed the viral vectors, made the mutations in *Mstb3*, supervised parts of the study, did some of the data analysis and wrote parts of the manuscript.

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1. Ramon y Cajal, S. *Degeneration and Regeneration of the Nervous System* (Oxford University Press, New York, 1991).
2. Berry, M., Carlile, J. & Hunter, A. Peripheral nerve explants grafted into the vitreous body of the eye promote the regeneration of retinal ganglion cell axons severed in the optic nerve. *J. Neurocytol.* **25**, 147–170 (1996).
3. Leon, S., Yin, Y., Nguyen, J., Irwin, N. & Benowitz, L.I. Lens injury stimulates axon regeneration in the mature rat optic nerve. *J. Neurosci.* **20**, 4615–4626 (2000).
4. Fischer, D., Heiduschka, P. & Thanos, S. Lens-injury-stimulated axonal regeneration throughout the optic pathway of adult rats. *Exp. Neurol.* **172**, 257–272 (2001).
5. Pernet, V. & Di Polo, A. Synergistic action of brain-derived neurotrophic factor and lens injury promotes retinal ganglion cell survival, but leads to optic nerve dystrophy in vivo. *Brain* **129**, 1014–1026 (2006).
6. Yin, Y. *et al.* Macrophage-derived factors stimulate optic nerve regeneration. *J. Neurosci.* **23**, 2284–2293 (2003).
7. Lorber, B., Berry, M. & Logan, A. Lens injury stimulates adult mouse retinal ganglion cell axon regeneration via both macrophage- and lens-derived factors. *Eur. J. Neurosci.* **21**, 2029–2034 (2005).
8. Cen, L.P. *et al.* Chemotactic effect of ciliary neurotrophic factor on macrophages in retinal ganglion cell survival and axonal regeneration. *Invest. Ophthalmol. Vis. Sci.* **48**, 4257–4266 (2007).
9. Yin, Y. *et al.* Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. *Nat. Neurosci.* **9**, 843–852 (2006).
10. Yin, Y. *et al.* Oncomodulin links inflammation to optic nerve regeneration. *Proc. Natl. Acad. Sci. USA* (in the press).
11. Makwana, M. & Raivich, G. Molecular mechanisms in successful peripheral regeneration. *FEBS J.* **272**, 2628–2638 (2005).
12. Barrette, B. *et al.* Requirement of myeloid cells for axon regeneration. *J. Neurosci.* **28**, 9363–9376 (2008).
13. Yudin, D. *et al.* Localized regulation of axonal RanGTPase controls retrograde injury signaling in peripheral nerve. *Neuron* **59**, 241–252 (2008).
14. Greene, L.A., Volonte, C. & Chalazonitis, A. Purine analogs inhibit nerve growth factor-promoted neurite outgrowth by sympathetic and sensory neurons. *J. Neurosci.* **10**, 1479–1485 (1990).
15. Benowitz, L.I. *et al.* Axon outgrowth is regulated by an intracellular purine-sensitive mechanism in retinal ganglion cells. *J. Biol. Chem.* **273**, 29626–29634 (1998).
16. Irwin, N., Li, Y.M., O'Toole, J.E. & Benowitz, L.I. Mst3b, a purine-sensitive Ste20-like protein kinase, regulates axon outgrowth. *Proc. Natl. Acad. Sci. USA* **103**, 18320–18325 (2006).
17. Volonte, C. & Greene, L.A. Nerve growth factor-activated protein kinase N. Characterization and rapid near homogeneity purification by nucleotide affinity-exchange chromatography. *J. Biol. Chem.* **267**, 21663–21670 (1992).
18. Zurn, A.D. & Do, K.Q. Purine metabolite inosine is an adrenergic neurotrophic substance for cultured chicken sympathetic neurons. *Proc. Natl. Acad. Sci. USA* **85**, 8301–8305 (1988).
19. Chen, P., Goldberg, D.E., Kolb, B., Lanser, M. & Benowitz, L.I. Inosine induces axonal rewiring and improves behavioral outcome after stroke. *Proc. Natl. Acad. Sci. USA* **99**, 9031–9036 (2002).
20. Smith, J.M. *et al.* Inosine promotes recovery of skilled motor function in a model of focal brain injury. *Brain* **130**, 915–925 (2007).
21. Zai, L. *et al.* Inosine alters gene expression and axonal projections in neurons contralateral to a cortical infarct and improves skilled use of the impaired limb. *J. Neurosci.* **29**, 8187–8197 (2009).
22. Bontioti, E.N., Kanje, M. & Dahlin, L.B. Regeneration and functional recovery in the upper extremity of rats after various types of nerve injuries. *J. Peripher. Nerv. Syst.* **8**, 159–168 (2003).
23. Lu, T.J. *et al.* Inhibition of cell migration by autophosphorylated mammalian sterile 20-like kinase 3 (MST3) involves paxillin and protein-tyrosine phosphatase-PEST. *J. Biol. Chem.* **281**, 38405–38417 (2006).
24. Martin, K.R., Klein, R.L. & Quigley, H.A. Gene delivery to the eye using adeno-associated viral vectors. *Methods* **28**, 267–275 (2002).
25. Sapieha, P.S., Peltier, M., Rendahl, K.G., Manning, W.C. & Di Polo, A. Fibroblast growth factor-2 gene delivery stimulates axon growth by adult retinal ganglion cells after acute optic nerve injury. *Mol. Cell. Neurosci.* **24**, 656–672 (2003).
26. Fischer, D., He, Z. & Benowitz, L.I. Counteracting the Nogo receptor enhances optic nerve regeneration if retinal ganglion cells are in an active growth state. *J. Neurosci.* **24**, 1646–1651 (2004).
27. Li, Y., Irwin, N., Yin, Y., Lanser, M. & Benowitz, L.I. Axon regeneration in goldfish and rat retinal ganglion cells: differential responsiveness to carbohydrates and cAMP. *J. Neurosci.* **23**, 7830–7838 (2003).
28. Schaden, H., Stuermer, C.A. & Bahr, M. GAP-43 immunoreactivity and axon regeneration in retinal ganglion cells of the rat. *J. Neurobiol.* **25**, 1570–1578 (1994).
29. Kermer, P. *et al.* Caspase-9: involvement in secondary death of axotomized rat retinal ganglion cells in vivo. *Brain Res. Mol. Brain Res.* **85**, 144–150 (2000).
30. Berkelaar, M., Clarke, D.B., Wang, Y.C., Bray, G.M. & Aguayo, A.J. Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. *J. Neurosci.* **14**, 4368–4374 (1994).
31. Ygge, J. Central projections of the rat radial nerve investigated with transganglionic degeneration and transganglionic transport of horseradish peroxidase. *J. Comp. Neurol.* **279**, 199–211 (1989).
32. Markus, A., Zhong, J. & Snider, W.D. Raf and akt mediate distinct aspects of sensory axon growth. *Neuron* **35**, 65–76 (2002).
33. Zhong, J. *et al.* Raf kinase signaling functions in sensory neuron differentiation and axon growth *in vivo*. *Nat. Neurosci.* **10**, 598–607 (2007).
34. Cowley, S., Paterson, H., Kemp, P. & Marshall, C.J. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77**, 841–852 (1994).
35. Kaplan, D.R. & Miller, F.D. Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* **10**, 381–391 (2000).
36. Tucker, B.A., Rahimtula, M. & Mearow, K.M. A procedure for selecting and culturing subpopulations of neurons from rat dorsal root ganglia using magnetic beads. *Brain Res. Brain Res. Protoc.* **16**, 50–57 (2005).
37. Schwab, M.E. Repairing the injured spinal cord. *Science* **295**, 1029–1031 (2002).
38. Aguayo, A.J. *et al.* Degenerative and regenerative responses of injured neurons in the central nervous system of adult mammals. *Phil. Trans. R. Soc. Lond. B* **331**, 337–343 (1991).
39. Dan, I., Watanabe, N.M. & Kusumi, A. The Ste20 group kinases as regulators of MAP kinase cascades. *Trends Cell Biol.* **11**, 220–230 (2001).
40. Herskowitz, I. MAP kinase pathways in yeast: for mating and more. *Cell* **80**, 187–197 (1995).
41. Fischer, D., Petkova, V., Thanos, S. & Benowitz, L.I. Switching mature retinal ganglion cells to a robust growth state in vivo: gene expression and synergy with RhoA inactivation. *J. Neurosci.* **24**, 8726–8740 (2004).
42. Costigan, M. *et al.* Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. *BMC Neurosci.* **3**, 16 (2002).
43. Bonilla, I.E., Tanabe, K. & Strittmatter, S.M. Small proline-rich repeat protein 1A is expressed by axotomized neurons and promotes axonal outgrowth. *J. Neurosci.* **22**, 1303–1315 (2002).
44. Fan, M., Mi, R., Yew, D.T. & Chan, W.Y. Analysis of gene expression following sciatic nerve crush and spinal cord hemisection in the mouse by microarray expression profiling. *Cell. Mol. Neurobiol.* **21**, 497–508 (2001).

ONLINE METHODS

Viral transfections. We inserted cDNAs encoding either an shRNA that blocks Mst3b expression or a slightly altered, control shRNA[16] into the SpeI site of an AAV plasmid that expresses enhanced green fluorescent protein (EGFP), described on the website of the Harvard Gene Therapy Initiative (http://hgti.med.harvard.edu/pp/AAV-GFP.png). A U6 promoter drives expression of the shRNA and a cytomegalovirus promoter drives EGFP expression. Virus was produced at the Harvard Gene Therapy Institute core facility.

Intravitreal AAV virus injections and optic nerve surgery. All surgical procedures were approved by the Institutional Animal Care and Use Committee of Children's Hospital Boston. We anesthetized adult male Fischer rats (220–250 g) by intraperitoneal injections of ketamine (75 mg per kilogram body weight; Hospira) and medetomidine hydrochloride (Domitor) (0.5 mg kg^{-1}; Pfizer) and injected ~10^9 AAV particles (2×10^5 infectious units) in 10 µl PBS into the vitreous through the lateral aspect of the eyeball using a glass capillary. Withdrawing fluid from the anterior chamber of the eye alleviated increases in intraocular pressure. We took care to avoid injury to the lens, as evidenced by fundoscopic examination. After allowing 4 weeks to achieve high transgene expression, we performed optic nerve surgery in approximately half of the rats as described[3]. We injured the lens at the time of the optic nerve crush by inserting the tip of a 25-gauge needle into the eye 2 mm anterior to the nerve head, perpendicular to the sclera, and puncturing the lens to a depth of about 2 mm. Lens injury initiates an inflammatory reaction in the eye and stimulates RGCs to enter an active growth state[3,41]. Buprenorphine (0.1 mg kg^{-1}; Reckitt Benckiser) was administered for 48 h after surgery.

DRG injections and radial nerve crush. We anesthetized adult male Sprague-Dawley rats (250–300 g) by intraperitoneal injections of ketamine (75 mg kg^{-1}) and medetomidine hydrochloride (0.5 mg kg^{-1}), exposed the C7 and C8 DRGs through laminectomy, and injected ~10^8 AAV particles (2×10^4 infectious units) in 1 µl of PBS per DRG using a glass capillary. We then closed the muscle in layers, sutured the skin, and administered buprenorphine (0.1 mg kg^{-1}) for 72 h. After allowing 4 weeks to achieve high levels of transgene expression, we crushed the radial nerve in approximately half of the rats. We made a 1–1.5 cm incision, exposed the radial nerve, and crushed it twice for 40 s each using fine forceps, then administered analgesic (buprenorphine, 0.1 mg kg^{-1}) for 72 h after surgery.

Sciatic nerve crush and Mst3b activity. We anesthetized adult male Sprague-Dawley rats (180–200 g) as above, exposed the sciatic nerve, and crushed it three times for 10 s each, 1 cm above the point where it bifurcates into the common peritoneal nerve and the tibial nerve. We verified the nerve injury by the clearing of the nerve at the lesion site, closed the muscles and skin using 2-0 silk sutures, and administered buprenorphine as above. After 3 d, we overdosed rats with isoflurane, traced the sciatic nerves back to the L4 and L5 dorsal root ganglia and excised these ganglia. We homogenized ganglia in RIPA buffer with sodium vanadate as a phosphatase inhibitor, then carried out immunoprecipitation with anti-Mst3b and performed kinase assays with or without 6-thioguanine inhibition as described[16].

Preparation for histology. We killed rats under deep anesthesia 2 weeks after optic nerve crush plus lens injury or 3 d after radial nerve crush, by perfusion through the heart with saline plus heparin followed by 4% paraformaldehyde. In the case of the radial nerve studies, we conducted pilot experiments to establish an appropriate survival time at which to visualize a substantial front of regenerating axons distal to the injury site and clear differences between rats expressing the control versus mst3b shRNA. These criteria were met at 3 d, whereas at 7 d many axons had regenerated considerable distances past the injury site in both groups. We removed tissue (eyes, optic nerves, C7 and C8 DRG, radial nerves), postfixed in 4% paraformaldehyde (4 °C overnight), transferred to 15% and then 30% sucrose solution (in PBS, each overnight at 4 °C) and embedded samples in OCT medium (Miles). We then cut frozen sections (15 µm thick) longitudinally on a cryostat, thaw-mounted these onto glass slides (Superfrost plus; Fisher), and stored the slides at −20 °C until further use.

Immunohistochemistry on tissue sections. We carried out immunohistochemistry to visualize GAP-43-positive axons in optic nerve and radial nerve sections as described[3]. For this, we used the IgG fraction from an antiserum to GAP-43 prepared in sheep (1:2,500)[45] followed by an Alexa Fluor 594–conjugated secondary antibody to sheep IgG (1:1,000, Molecular Probes). Sections were double-labeled with an anti-GFP made in rabbit (1:2,000; Abcam) followed by an Alexa Fluor 488–conjugated secondary antibody to rabbit IgG (1:500, Molecular Probes).

To visualize transfected cells in the retina and DRG, we incubated rehydrated sections in blocking solution (PBS containing 0.3% Triton X-100, PBST) and 4% goat serum (60 min, 20 °C), and double-labeled these with a rabbit anti-GFP (1:2,000; Abcam) and mouse anti–βIII tubulin (1:500, TuJ1, Babco) overnight at 4 °C in a PBS solution containing 4% goat serum. βIII-tubulin is a selective marker for RGCs in the ganglion cell layer[6,46] and is also expressed in DRG neurons[47]. In some cases, we double-labeled sections with a rabbit anti-Mst3b[16] (1:10 for eye sections, 1:500 for DRG sections) and mouse anti-GFP (1:100 for eye sections, 1:200 for DRG sections; Molecular Probes) to examine whether Mst3b expression was suppressed in transfected cells.

In other cases, we double-labeled cells with a mouse ED1 antibody to CD68 (1:200, Serotec) and rabbit anti-GFP (1:2,000, Abcam). In some cases, we stained DRG sections with fluorescent isolectin IB4 (Sigma) or mouse anti-NF200 (1:100; Serotec).

On the second day, we washed slides three times, 10 min each, in PBST and applied secondary antibodies (Alexa Fluor 488–conjugated goat anti–rabbit or anti–mouse IgG, 1:500, and Alexa Fluor 594–conjugated goat anti–rabbit or anti–mouse IgG, 1:500, Molecular Probes) for 1 h at 4 °C in a PBS solution containing 4% goat serum. Following two 10-min washes in PBST and a 10-min wash in PBS, slides were mounted in Fluorsave (Calbiochem). To study intracellular signaling pathways, we double-labeled DRG sections with anti–p42/44 MAPK or anti–phospho-p44/42 MAPK (made in rabbit; 1:100, Cell Signaling) and mouse anti-EGFP (1:200; Molecular Probes). Secondary antibodies included Alexa Fluor 594–conjugated goat anti–rabbit IgG (1:200) and Alexa Fluor 488–conjugated goat anti–mouse IgG (1:500, both Molecular Probes).

Quantitation of axon regeneration and cell survival. We quantified $GAP\text{-}43^+EGFP^+$ axon growth in the optic nerve and radial nerve using published methods[3], with 7–9 rats per condition. An observer blinded to sample identity quantified axon growth by counting the total number of GAP-43-positive and EGFP-positive axons arising from transfected RGCs or DRG neurons at specified distances past the lesion site (500 µm for the optic nerve; 500, 1,000, 2,000, 3,000, 4,000 and 5,000 µm for the radial nerve) in 4–6 longitudinal sections per nerve. The cross-sectional width of the nerve was measured at the point at which the counts were taken, and was used to calculate the number of axons per mm of nerve width. We then averaged the number of axons per millimeter over the sections and corrected this value to the transfection efficiency of the *Mst3b* shRNA infections (68% in the optic nerve and 35% in the radial nerve). Σa_d, the total number of axons extending distance d in a nerve having a radius r, was estimated by summing over all sections, having a thickness t of 15 µm):

$$\sum_{a_d} = \pi r^2 \times (\text{average axons mm}^{-1}),$$

To evaluate cell survival and infection efficiency, we counted both the total number of $\beta III\text{-tubulin}^+$ RGCs and the number that were positive for both EGFP and βIII tubulin in 4–6 sections through the center of the retina of each rat, as previously described[6]. Similar methods were used to evaluate cell survival in DRGs. Results are expressed as mean ± s.e.m. of 7–9 rats per condition for each of the experimental groups. The significance of intergroup differences was evaluated by Student's *t*-test.

RGC cultures. We dissociated and cultured retinas from adult Fischer rats that had received virus injection 4 weeks earlier (**Fig. 1**) or from naive (**Supplementary Fig. 2**) adult Fisher rats (220–250 g) as described[6]. For transient transfection experiments, we transfected neurons from naive adult Fisher rats 24 h after plating with plasmids expressing EGFP and (i) an shRNA against Mst3b or (ii) a control shRNA, identical to the sequences used in the present *in vivo* experiments and in our previous study[16]. In some cases, we cotransfected RGCs with plasmids expressing *Mst3b* shRNA and wild-type myc-His-tagged human MST3b/STK24, which differs significantly in its nucleotide sequence from rat *Mst3b* RNA and is unaffected by the shRNA[16]. In some cases, RGCs were transfected with plasmids expressing a mutant human Mst3b in which Lys65, which lies in the

ATP-binding region of the protein, was mutated to a methionine to produce a kinase-dead mutant[16]. In other cases, we transfected cells with a plasmid encoding a constitutively active form of Mst3b in which Thr190 was changed to aspartate, a change that has been reported to activate Mst3, an isoform of Mst3b that differs only at its amino-terminal end outside of the kinase domain. We transfected neurons with Optimem and Lipofectamine 2000 (both from Invitrogen). Eight hours after transfection, we added complete defined medium (Medium E)[48] to the cultures. In some cases, RGCs were transfected with the same plasmids described above using Program G-13 of the Nucleofector (Amaxa Biosystems) as previously described[49].

For growth factor treatment, we added oncomodulin (200 ng ml^{-1}, gift from M.T. Henzl), mannose (250 μM, Sigma) and forskolin (15 μM, Alomone Labs), either on the day of culturing in the case of RGCs from virus-injected rats, or 24 h after transfection, then maintained RGCs for 3 d more. Each experiment included four blinded, independent observations (10–100 cells each), and each experiment was performed three times.

DRG cultures. We derived DRG neurons from adult Sprague-Dawley rats that were either naive or had received virus injection 4 weeks beforehand. We dissected C7 and C8 ganglia, prepared single-cell suspensions as described[50], and cultured approximately 750 DRG neurons per well in RPMI 1640 medium containing L-glutamine and antibiotics (Sigma) for 72 h on sterile coverslips precoated with 100 μg ml^{-1} poly-D-lysine (Sigma) in four-well tissue culture plates (Nunc; 37 °C, humidified atmosphere containing 5% CO_2). For rescue, kinase-dead mutant, and constitutively active Mst3b experiments, we transfected dissociated DRG neurons from naive adult Sprague-Dawley rats with the same plasmids as described for the RGC culture experiments, using Program G-13 of the Nucleofector[49]. Neurons were either exposed to NGF (50 ng ml^{-1}, Alomone Labs) for 3 d or left untreated. Each experiment included four blinded, independent observations (about ten cells per well), and each experiment was performed three times.

Immunostaining of cells. Cells were fixed with 4% paraformaldehyde at 20 °C for 10 min. For RGCs, we applied a blocking solution containing 3% BSA (Sigma) and 0.1% Triton X-100 in PBS for 10 min, then applied primary antibodies. These included a mouse anti–βIII tubulin (clone SDL.3D10; 1:300; Sigma) and a polyclonal rabbit anti-EGFP (1:1,000; Molecular Probes) diluted in PBS containing 3% BSA, and were applied overnight at 4 °C. The next day, cells were exposed to secondary antibodies (Alexa Fluor 488–conjugated anti–rabbit IgG and Alexa Fluor 594–conjugated anti–mouse IgG, both made in goat (1:500, Molecular Probes)) in PBS containing 3% BSA, 1 h, 20 °C. In some cases, we double-labeled RGCs that had been transfected with either a plasmid expressing *Mst3b* shRNA alone or also with a plasmid expressing myc-His tagged human MST3b/STK24 with a rabbit anti-His-tag (1:500; New England Biolabs) and mouse anti-EGFP (1:200; Molecular Probes). Secondary antibodies included an Alexa Fluor 594–conjugated goat anti–rabbit IgG (1:500) and Alexa Fluor 488–conjugated goat anti–mouse IgG (1:200, both Molecular Probes).

To immunostain DRG neurons, we blocked cells for 30 min in PBS containing 10% goat serum and 0.1% Triton and then applied primary antibodies (mouse TuJ1, 1:5,000, Babco; and rabbit anti-EGFP, 1:1,000, Molecular Probes) overnight at 4 °C in 2% goat serum in PBS. The next day we treated cells with secondary antibodies (Alexa Fluor 488–conjugated goat anti–rabbit IgG and Alexa Fluor 594–conjugated goat anti–mouse IgG (Molecular Probes: 1:500 in 2% goat serum in PBS, 1 h at 20 °C), rinsed, and then mounted using Fluorsave (Calbiochem).

We evaluated axon outgrowth in quadruplicate samples (RGCs, 10–100 cells per well; DRG neurons, ~10 cells per well) in a blinded fashion and repeated all experiments at least three separate times. The significance of intergroup differences was evaluated by Student's *t*-test.

Image acquisition and processing. Fluorescent images were captured using a Nikon Eclipse 80i microscope equipped with a SPOT RT cooled CCD camera and SPOT software. We imported images into Photoshop (Adobe), cropped these for composites and, in some cases, adjusted contrast, taking care to treat all related images similarly. No portions of the images were otherwise edited. Fluorescence intensity was analyzed using Image J (US National Institutes of Health).

45. Benowitz, L.I., Apostolides, P.J., Perrone-Bizzozero, N., Finklestein, S.P. & Zwiers, H. Anatomical distribution of the growth-associated protein GAP-43/B-50 in the adult rat brain. *J. Neurosci.* **8**, 339–352 (1988).
46. Cui, Q., Yip, H.K., Zhao, R.C., So, K.F. & Harvey, A.R. Intraocular elevation of cyclic AMP potentiates ciliary neurotrophic factor-induced regeneration of adult rat retinal ganglion cell axons. *Mol. Cell. Neurosci.* **22**, 49–61 (2003).
47. Moskowitz, P.F. & Oblinger, M.M. Sensory neurons selectively upregulate synthesis and transport of the beta III-tubulin protein during axonal regeneration. *J. Neurosci.* **15**, 1545–1555 (1995).
48. Schwalb, J.M. *et al.* Two factors secreted by the goldfish optic nerve induce retinal ganglion cells to regenerate axons in culture. *J. Neurosci.* **15**, 5514–5525 (1995).
49. Leclere, P.G. *et al.* Effective gene delivery to adult neurons by a modified form of electroporation. *J. Neurosci. Methods* **142**, 137–143 (2005).
50. Gavazzi, I., Kumar, R.D., McMahon, S.B. & Cohen, J. Growth responses of different subpopulations of adult sensory neurons to neurotrophic factors in vitro. *Eur. J. Neurosci.* **11**, 3405–3414 (1999).

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