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

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Abstract

Mammalian sterile 20-like kinase-3b (Mst3b/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 shRNA 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.

Introduction

Axon regeneration occurs readily after injury in the mature peripheral nervous system (PNS) but not within the central nervous system (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

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growth¹. Surprisingly, however, significant regeneration can be achieved by inducing an inflammatory response in the eye. Implanting a peripheral nerve fragment into the vitreous², injuring the lens³⁻⁵, or injecting zymosan^{6,7} or ciliary neurotrophic factor^{8,9} 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 (Ocm), that stimulates optic nerve regeneration *in vivo* provided cAMP levels are elevated¹⁰. 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 site¹³.

Several lines of evidence suggest that axon outgrowth involves a purine-sensitive protein kinase. The purine analog 6-thioguanine (6-TG) blocks the neurite-promoting effects of trophic agents¹⁴⁻¹⁶ and inhibits the activity of a 45-50 kDa protein kinase¹⁷. Inosine, a naturally occurring purine nucleoside, reverses the inhibitory effects of 6-TG and can promote axon outgrowth in several types of neurons on its own^{15,16,18}. Based on 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 was found to be 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 factors¹⁶. *In vivo*, inosine stimulates the growth of axon collaterals from intact neurons if other brain pathways have been injured¹⁹⁻²¹. 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 essential 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 attenuated 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 utilized the radial nerve, in which axon regeneration occurs readily after injury in the absence of exogenous stimulation²². In both cases, we investigated whether reducing Mst3b expression suppressed the ability of the relevant projection neurons to extend axons in cell culture, whether Mst3b regulated axon growth via changes in its kinase activity, and whether altering Mst3b levels suppressed axon regeneration *in vivo*.

Mst3b is essential for axon outgrowth in RGCs

We transfected RGCs with plasmids expressing *mst3b* shRNA to investigate whether these cells require Mst3b to regenerate axons in response to appropriate growth factors in culture. We dissociated retinas from normal, intact animals, 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 expression¹⁶. The control shRNA differs from *mst3b* shRNA in 2 nucleotide positions and does not affect axon outgrowth¹⁶. After 3 days of treatment with the combination of oncomodulin (Ocm), mannose, and forskolin, RGCs expressing the control shRNA showed a 3-fold increase in axon outgrowth relative to untreated controls (difference significant at $P < 0.001$; 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 co-transfected cells with 2 plasmids, one expressing *mst3b* shRNA and the other expressing his-tagged human Mst3b. The mRNA sequence of human *mst3b* differs significantly from that of the rat and its expression is not blocked by the shRNA construct used here¹⁶. We verified the presence of exogenous human Mst3b by staining cells with an anti-his antibody that recognizes his-tagged Mst3b (Fig. 1e). Co-transfecting 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 depends specifically on its ability to knock down Mst3b expression and not on off-target effects.

Mst3b regulates growth via 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 site¹⁶. Expression of this kinase-dead (k/d) Mst3b mutant eliminated the response of RGCs to growth factors (Fig. 1f,h).

To investigate whether the kinase activity of Mst3b plays an *active* role in axon growth, we next transfected RGCs with a plasmid expressing a constitutively active (c/a) mutant of Mst3b. To create c/a Mst3b, we mutated threonine¹⁹⁰ to aspartate, a change that has been shown to activate Mst3, an isotype of Mst3b²³. Expression of c/a 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 Fig.1 online.

Mst3b depletion blocks optic nerve regeneration *in vivo*

To investigate the role of Mst3b *in vivo*, we injected adult rats intravitreally with AAV2 expressing EGFP from a CMV promoter and, from a U6 promoter, either the shRNA that blocks Mst3b expression or the control shRNA. Animals survived for 4 weeks after viral infections prior to undergoing surgery to injure the optic nerve and induce intravitreal inflammation. The 4-week delay allowed for high levels of shRNA expression and

degradation of preexisting Mst3b. In conformity with earlier reports on the high infection efficiency of AAV224-26, 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 Mst3b16, we detected the protein in essentially every EGFP-positive RGC expressing control shRNA, but observed an ~85% reduction 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 4 weeks after injecting AAVs, euthanized animals, dissected and dissociated retinas, and cultured cells in the presence or absence of Ocm, mannose, and forskolin^{6,10,27}. In RGCs expressing the control shRNA, the addition of Ocm, forskolin and mannose caused a two-fold increase in average axon length over baseline (difference significant at $P < 0.001$: Supplementary Fig. 2 on line). 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 online). Thus, the effect of Mst3b knockdown remains strong several weeks after viral infection and results in a reduction of both baseline- and growth factor-stimulated levels of axon outgrowth. The lower overall outgrowth seen in Fig. 1h compared to that in Supplementary Fig. 2e 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⁻, on the other hand, are expected to reflect regenerating axons that arise from uninfected neurons. Remnants of degenerating axons that already contained EGFP before injury, but which are not regenerating, would be GAP-43⁻/EGFP⁺. Animals 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, animals with Mst3b knocked down (N = 7) showed a 97% reduction in GAP-43⁺/GFP⁺ regenerating axons (Fig. 3b) (difference significant at $P < 0.001$: Fig. 3a, bottom and 3c). In all cases, the number of axons was normalized 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 *non-infected* RGCs (GAP-43⁺/EGFP⁻) and found this number to be similar between animals infected with AAV2 expressing control *vs.* *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 non-infected 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, ca. 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 found to be 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 animals expressing *mst3b* shRNA, the percentage of RGCs that was EGFP-positive was $61.1 \pm 3.3\%$ in animals that survived 6 weeks and did not have surgery (N = 4) and $68.3 \pm 1.9\%$ after optic nerve crush plus lens injury (ONC/LI: N = 7: difference not significant: $t = 1.89$, $df = 10$, $P \sim 0.09$). Animals infected with the control shRNA showed similar results, although the infection rates were lower. In these cases, $16.2 \pm 2.2\%$ of RGCs were EGFP-positive in animals that did not have surgery (N = 4) compared to $12.1 \pm 1.6\%$ two weeks after ONC/LI (N = 7: difference not significant: $t = 1.51$, $df = 10$, $P \sim 0.16$).

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 transfection assays to evaluate whether Mst3b mediates the response of adult DRG neurons to trophic factors, in this case nerve growth factor (NGF). We dissociated DRG neurons from non-infected, non-lesioned animals and transfected these with plasmids expressing EGFP and either control or *mst3b*-specific shRNA. After being exposed to NGF for 3 days, DRG neurons expressing the control shRNA showed a 2-fold increase in average axon length relative to 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 co-transfected DRG neurons with plasmids expressing *mst3b* shRNA and human Mst3b, as above. Co-expression of the two plasmids restored DRG neurons' ability to respond to NGF ($P < 0.01$; Fig. 4d,g). Thus, the effect of *mst3b* shRNA in blocking outgrowth is 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 participates in 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 k/d Mst3b eliminated the ability of DRG neurons to extend axons in response to NGF (Fig. 4e,g). In contrast, transfection with c/a 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 appears to be mediated via changes in the kinase activity of Mst3b.

Mst3b knockdown attenuates peripheral nerve regeneration *in vivo*

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 neuron-specific (Fig. 5a) and included small peptidergic (IB4⁻) and non-peptidergic (IB4⁺) neurons, as well as large NF200⁺ neurons (Fig. 5b,c). Compared to cells expressing control shRNA, Mst3b levels were reduced by ~ 85% 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 levels of baseline outgrowth irrespective of which shRNA was being expressed. However, whereas neurons expressing the control shRNA showed a 3-fold increase in average axon length when exposed to NGF, neurons expressing *mst3b* shRNA showed no detectable response (Supplementary Fig. 3 online: 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 animals 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 three days, 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 normalized this number to the percentage of cells that were transfected to account for individual variations in transfection efficiency. In cases 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). Animals expressing *mst3b* shRNA showed an 80 – 85% reduction in axon growth (N = 9; difference from controls significant at $P < 0.001$ at 0.5 mm, $P < 0.01$ at 1, and 2 mm; N.S. at 3, 4, and 5 mm: Fig. 6c,d,f). We also evaluated regeneration in non-infected DRG neurons in these same cases, that is, the number of axons that were GAP-43-positive and EGFP-negative, normalized by the percentage of cells that were uninfected. Axons arising from non-infected 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 mm²) was similar to that seen after infection with AAV carrying control shRNA (139.5 ± 8.8 per mm²). 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 days after peripheral nerve injury and compared their Mst3b kinase activity with that of control DRG neurons using immunoprecipitation-kinase (IP-kinase) assays as described¹⁶. 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 a 2.5-fold increase in Mst3b kinase activity compared with 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 cells¹⁶ is consistent with the possibility that it acts via 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 vs. *mst3b* shRNA. In sections through DRGs expressing *mst3b* shRNA, immunostaining for phospho-p42/44 MAPK was detected in 11% of neurons, compared to 87% of neurons expressing the control shRNA (Fig. 7c–e). The level of total p42/44 MAPK 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 culture¹⁶. 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 plays an essential role in enabling 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, Ocm, 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 Ocm, 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 Ocm, 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, in that responsiveness to growth factors was rescued when RGCs expressing *mst3b* shRNA were co-transfected with plasmids expressing human Mst3b¹⁶. 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 play a key role 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 following 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 strikingly, 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 days after nerve injury than neurons expressing the control shRNA or than nontransfected neurons in the same ganglia. Nonetheless, the fact that some regeneration continued to occur even when Mst3b levels were reduced suggests that there is enough Mst3b remaining to allow some regeneration or that axon regeneration in the PNS may utilize both Mst3b-dependent and Mst3b-independent mechanisms. DRG neurons extend processes in response to trophic factors and, via integrin signaling, in response to substrate molecules³⁶. In culture, DRG neurons expressing *mst3b* shRNA continued to show basal outgrowth when cultured on poly-D-lysine, but 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 exhibit 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 a MAP4K to regulate budding in yeast. Ste20 relays the signal from a pheromone-sensitive G-protein coupled receptor to Ste11, a MAP3K; Ste11 in turn phosphorylates the MAP2K 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 cells¹⁶ 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 survival⁴¹, and these changes resemble those seen in DRG neurons undergoing axon regeneration in the PNS⁴²⁻⁴⁴. 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 currently achievable following 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AAV	adeno-associated virus
CNS	central nervous system
DRG	dorsal root ganglion
EGFP	enhanced green fluorescent protein
forsk	forskolin
Mst3b	mammalian serine-threonine kinase-3b
NGF	nerve growth factor
Ocm	oncomodulin
ON/LI	optic nerve crush plus lens injury

PNS	peripheral nervous system
RGCs	retinal ganglion cells
shRNA	short hairpin RNA
6-TG	6-thioguanine

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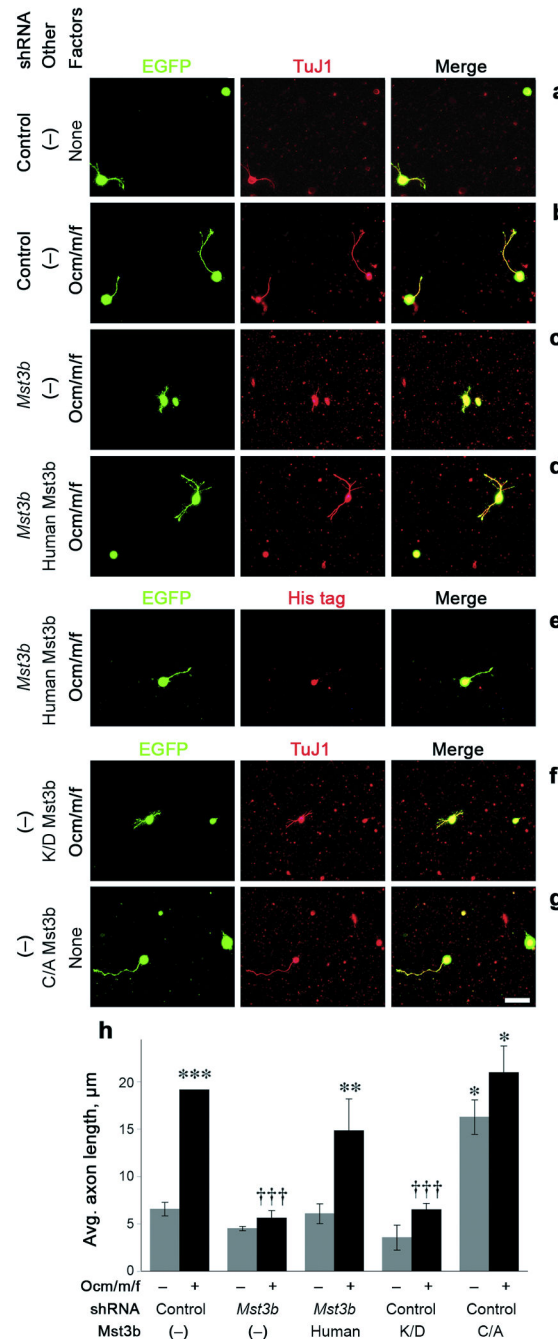
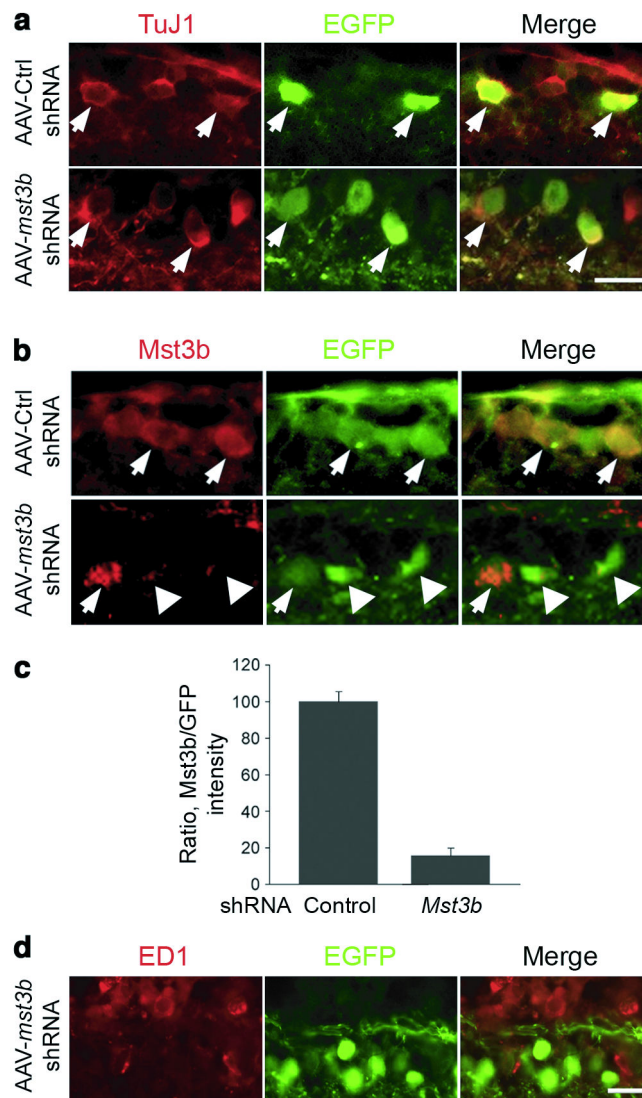


Figure 1.

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 days later and immunostained for EGFP, a marker for transfected cells, and either β III-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 co-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 for **a–g**: 40 μm . Each experiment included 4 blinded, independent observations (10–100 cells per well), and each experiment was repeated 3 times. *, **, ***increase relative to untreated controls significant at $P < 0.05$, $P < 0.01$, or $P < 0.001$, respectively. †††decrease relative to control cells treated with growth factors significant at $P < 0.001$. Error bars represent s.e.m.

**Figure 2.**

Infection of RGCs with AAV2 expressing shRNAs *in vivo*. **(a)** Animals received intraocular injections of AAV2 expressing EGFP and either a control shRNA or *mst3b* shRNA 4 weeks prior to dissection. Cross-sections through the retina show double-labeled cells (*arrows*) expressing TuJ1 (to identify RGCs: 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 animals 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 co-expression 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: 20 μ m. **(c)** Quantitation of Mst3b expression. EGFP-positive cells expressing either control or *mst3b* shRNA were analyzed for intensity of Mst3b immunostaining in cross-sections through all cases using Image J. **(d)** Lack of

macrophage infection: Four weeks after intravitreal injections of AAV2 expressing *mst3b* shRNA and EGFP, the lens was injured to induce an inflammatory response. Double immunostaining shows ED-1-positive macrophages to be uninfected, as evidenced by an absence of EGFP. Scale bar: 40 μ m. Error bars represent s.e.m.

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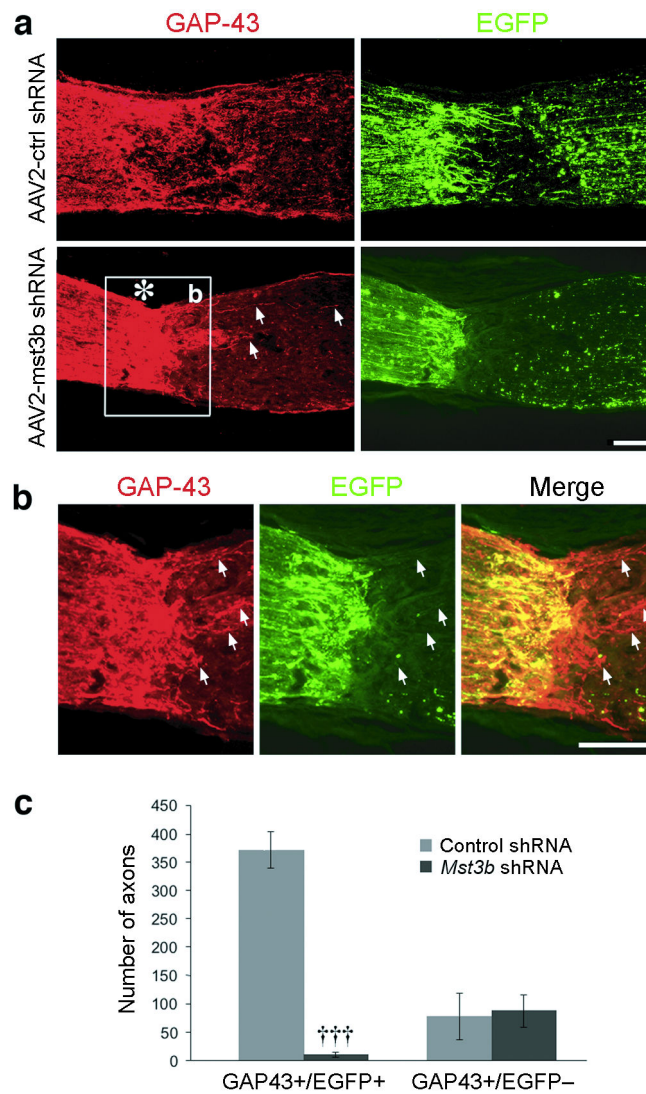


Figure 3. Mst3b is essential for optic nerve regeneration. RGCs were infected with AAV2 expressing EGFP plus either control or *mst3b* shRNA. Four weeks later, the optic nerve was crushed and the lens was injured to induce inflammation and transform RGCs into an active growth state. Regeneration was quantified 2 weeks later. (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 denotes the site of nerve injury; box shows the region magnified in b. Scale bar: 100 μ m. (b) Area distal to the injury site from a case expressing *mst3b* shRNA. Merged image shows that GAP-43-positive 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 indicate regenerating axons from uninfected RGCs. Scale bar: 100 μ m. (c) Mean number of axons that regenerate 500 μ m beyond the injury site. The set of bars on the left shows axons that arise from transfected RGCs. The number of regenerating axons was normalized by the percentage of transfected cells to account for differences in infection efficiency. The set of bars on the right shows axons of *noninfected*

cells, normalized by the percentage of noninfected cells. Results are based on 7 animals per group, 4-6 sections per animal, and 0-24 axons per section. ^{†††}decrease relative to cases transfected with control shRNA significant at $P < 0.001$. Error bars represent s.e.m.

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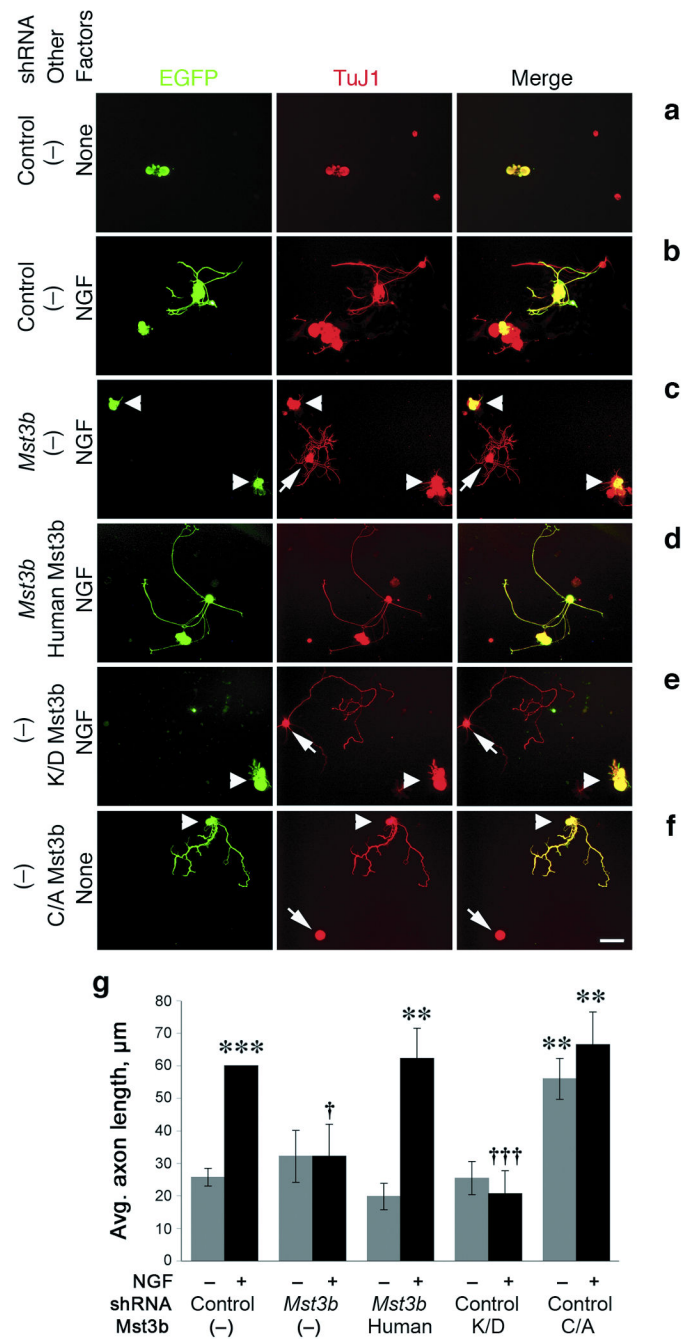


Figure 4.

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 shown on the left. Cells were exposed to NGF (50 ng/ml) or media alone. Three days later, cells were fixed and immunostained to detect EGFP, a marker for transfected cells, and β III-tubulin to identify neurons (antibody TuJ1). (g) Quantitation of axon growth. (a,b,g) Cells expressing the control shRNA extend neurites when treated with NGF (b,g). (c,g) Cells expressing *mst3b* shRNA (arrowheads) fail to respond to NGF; an nontransfected

cell in the same field shows outgrowth (*arrow*). (**d,g**) NGF-induced outgrowth is restored when cells expressing *mst3b* shRNA (green) are co-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 non-transfected 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* points to an untransfected cell in the same field that is not growing. Scale bar in **a–f**: 100 μm . Each experiment included 4 blinded, independent observations of about 10 cells per well, and each experiment was performed three times. **,***increase relative to baseline (control shRNA-transfected cells without NGF) significant at $P < 0.01$ or $P < 0.001$, respectively. †, ††† decrease relative to control shRNA-transfected cells treated with NGF significant at $P < 0.05$ or $P < 0.001$, respectively. Error bars represent s.e.m.

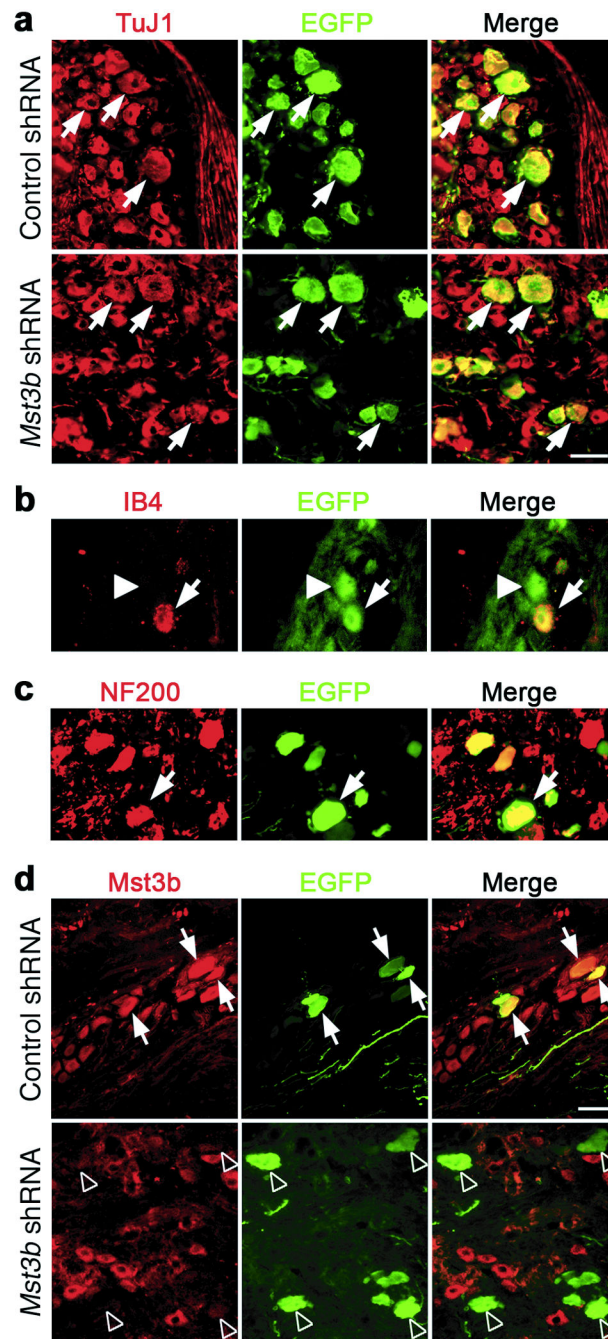


Figure 5. Mst3b knockdown in DRG neurons. Sections through the rat DRG four weeks after injecting AAV2 expressing EGFP and either control or *mst3b* shRNA. (a) Infection of DRG neurons is demonstrated by co-immunostaining (arrows) with antibodies to the neuronal marker, β III tubulin (TuJ1, red), and 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). (b) AAV2 expressing EGFP and *mst3b* shRNA infects small IB4-positive (red) non-peptidergic neurons (arrows) as well as small IB-4-negative neurons (arrowheads). (c)

AAV2 also infects large neurons (*arrow*) that stain positively with antibodies to the neurofilament protein NF200 (*red*). Scale bar: 40 μm . (**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 can be visualized in cells expressing control shRNA (*arrows*) (*top*), but not in cells expressing *mst3b* shRNA (*arrowheads*) (*bottom*). Scale bar: 40 μm .

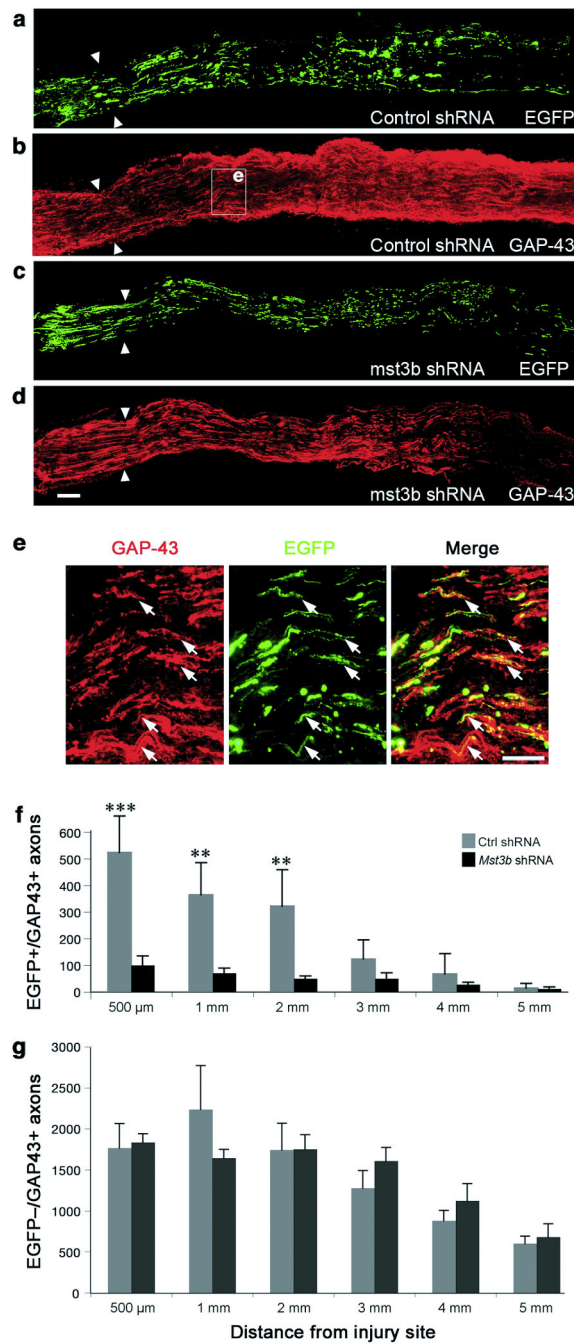


Figure 6. Mst3b knockdown attenuates peripheral nerve regeneration. (**a–d**) The radial nerve was crushed 4 weeks after infecting cervical DRG neurons with AAV2 expressing EGFP and either *mst3b*- or control shRNA. Axons extending beyond the injury site (*arrowheads*) were visualized 3 days later in sections stained with antibodies to detect GAP-43 (red) and EGFP (green). The box in **b** is magnified in panel **e**. Only profiles that are labeled with both markers are considered as regenerating axons that arise from infected neurons (arrows). Scale bar for **a–d**: 200 μ m. (**e**) High-power images show regenerating axons that arise from

neurons expressing control shRNA: many axons are positive for both GAP-43 (red) and EGFP (green) (*arrows*). Scale bar: 100 μ m. **(f)** Quantitation of GAP-43⁺/EGFP⁺ axons. The number of regenerating axons was normalized by the percent of infected cells to account for differences in infection efficiency. Note the marked attenuation of axon growth in cells expressing *mst3b* shRNA. **(g)** Quantitation of GAP-43⁺/EGFP⁻ axons from non-infected neurons shows similar levels of regeneration irrespective of the virus infecting neighboring neurons. Data show mean number of axons at the indicated distances from the injury site (8-9 animals per group, 4-6 sections per animal, 0-10 axons per section). **,***Differences between number of axons arising from cells expressing control vs. *mst3b* shRNA are significant at $P < 0.01$, $P < 0.001$, respectively. Error bars represent s.e.m.

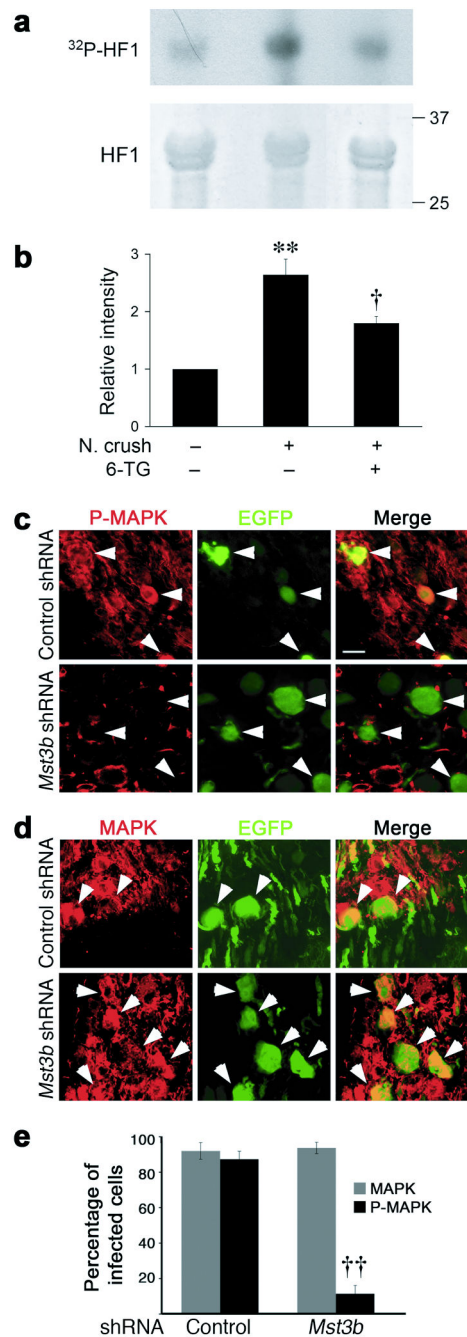


Figure 7. Mst3b activation and regulation of p42/44 MAPK phosphorylation *in vivo*. **(a)** Mst3b was immunoprecipitated from lysates of DRG neurons 3 days after peripheral nerve crush or sham surgery. Kinase assays were performed using HF1 as a pseudo-substrate and γ -[^{32}P]-ATP, with or without 6-thioguanine present. Reaction mixes were separated by SDS-PAGE and kinase activity was visualized by autoradiography (*top*). Protein loading was visualized by Coomassie Blue staining (*bottom*). Position of molecular weight markers are shown on the right. **(b)** Quantitation of Mst3b kinase activity based on densitometry of autoradiograms

using Image J (NIH) averaged from 3 separate experiments. **(c,d)** Cross-sections through the DRG of animals injected 4 weeks earlier with AAV2 expressing EGFP and either control shRNA (top) or *mst3b* shRNA (bottom). Arrows point to infected, EGFP-positive neurons. **(c)** Double-immunostaining with antibodies to phospho-p42/44 MAPK (red) and EGFP (green) reveal 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) reveal that *Mst3b* knock-down does not alter overall p42/44 MAPK levels. **(e)** Quantitation of changes in the overall expression and phosphorylation of p42/p44 MAPK based on 4-6 sections from 3 animals per condition. Scale bar: 40 μm . **Increase relative to controls significant at $P < 0.01$; †, †† Decrease relative to induced state significant at $P < 0.05$ or $P < 0.01$, respectively. Error bars represent s.e.m.