Limited availability of ZBP1 restricts axonal mRNA localization and nerve regeneration capacity

Christopher J Donnelly^{1,9},
Dianna E Willis^{2,9}, Mei Xu^{3,9,10},
Chhavy Tep⁴, Chunsu Jiang³,
Soonmoon Yoo⁵, N Carolyn Schanen^{1,5},
Catherine B Kirn-Safran²,
Jan van Minnen^{6,7}, Arthur English³,
Sung Ok Yoon⁴, Gary J Bassell³ and
Jeffery L Twiss^{5,8,*}

¹Department of Biological Sciences, University of Delaware, Newark, DE, USA, ²Department of Neurology and Neuroscience, Burke Medical Research Institute, Weill Medical College of Cornell University, White Plains, NY, USA, ³Department of Cell Biology, Emory University Medical School, Atlanta, GA, USA, ⁴Department of Molecular and Cellular Biochemistry, Ohio State University, Columbus, OH, USA, ⁵Nemours Biomedical Research, A.I. duPont Hospital, Wilmington, DE, USA, ⁶Department of Molecular and Cellular Neurobiology, Center for Neurogenetics and Cognitive Research, VU University, Amsterdam, The Netherlands, ⁷Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta, Canada and ⁸Department of Biology, Drexel University, Philadelphia, PA, USA

Subcellular localization of mRNAs is regulated by RNA–protein interactions. Here, we show that introduction of a reporter mRNA with the 3'UTR of β -actin mRNA competes with endogenous mRNAs for binding to ZBP1 in adult sensory neurons. ZBP1 is needed for axonal localization of β -actin mRNA, and introducing GFP with the 3'UTR of β -actin mRNA depletes axons of endogenous β -actin and GAP-43 mRNAs and attenuates both *in vitro* and *in vivo* regrowth of severed axons. Consistent with limited levels of ZBP1 protein in adult neurons, mice heterozygous for the ZBP1 gene are haploinsufficient for axonal transport of β -actin and GAP-43 mRNAs and for regeneration of peripheral nerve. Exogenous ZBP1 can rescue the RNA transport deficits, but the axonal growth deficit is only rescued if the transported mRNAs are locally translated. These data support a direct role for ZBP1 in transport and translation of mRNA cargos in axonal regeneration *in vitro* and *in vivo*.

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Introduction

Localized protein synthesis is a mechanism used by many, if not all polarized cells to modulate protein composition in subcellular domains. Locally generated proteins play a role in cellular migration, adherence, polarization, and metabolism (Holt and Bullock, 2009). For neurons, early ultrastructural studies suggested that only dendrites have protein synthetic capacity (Steward and Levy, 1982). However, it is now clear that neurons can also translate mRNAs in their axons (Donnelly *et al*, 2010). The axonally synthesized proteins appear to fulfil several different functions. For example, proteins synthesized in the axon, such as β -actin, can be used for growth cone turning (Leung *et al*, 2006; Yao *et al*, 2006; Lin and Holt, 2008), and locally translated Par3, a protein involved in cell polarization, supports axon elongation (Hengst *et al*, 2009). Injury-induced synthesis of Importin β 1 and RanBP1 in axons allows these distal processes to communicate with their cell bodies (Hanz *et al*, 2003; Perlson *et al*, 2005; Yudin *et al*, 2008), and other locally synthesized proteins contribute to growth cone formation after axotomy (Verma *et al*, 2005).

Based on mRNA profiles of neuronal processes and fibroblasts' pseudopodia, it is clear that neither all mRNAs are targeted for distal transport nor are only the most abundant mRNAs transported (Willis *et al*, 2007; Mili *et al*, 2008; Vogelaar *et al*, 2009; Andreassi *et al*, 2010). For example, β -actin mRNA is transported into axons and dendrites, while the near equally abundant γ -actin mRNA remains in the cell body (Bassell *et al*, 1998; Zheng *et al*, 2001; Tiruchinapalli *et al*, 2003). Delivery of mRNAs to subcellular sites requires mRNA–protein interactions. The 3'UTR of β -actin mRNA contains a conserved 'zipcode' that is essential for its localization in cultured cells through interaction with the zipcode binding protein 1 (ZBP1; also known as the insulin-like growth factor II mRNA binding protein 1 (IMP1)) (Kislauskis *et al*, 1993, 1994; Farina *et al*, 2003). Despite evidence for locally generated β -actin's role in axonal guidance *in vitro* (Zhang *et al*, 2001; Yao *et al*, 2006; Sasaki *et al*, 2010), only a small fraction of its mRNA pool localizes to axons (Eng *et al*, 1999). While there is clearly enrichment of some neuronal mRNAs in neuronal processes (Poon *et al*, 2006; Andreassi *et al*, 2010), many transcripts behave similar to β -actin showing only a small localizing fraction. The mechanisms underlying how much of a given mRNA localizes to axons and whether those low levels can influence axonal growth have not been investigated. Moreover, it is not clear if any RNA binding proteins (RBPs) are needed to drive axonal mRNA localization and influence axon growth *in vivo*. Here, we have used neuronal β -actin mRNA as a tool to ask if limited availability of the *trans*-acting protein machinery needed for its localization could account for the limited levels of this mRNA in axons. We show that ZBP1 is a critical determinant of how much β -actin and GAP-43 mRNAs localize to axons. This in turn contributes to the axons' growth potential both *in vitro* and *in vivo*.

*Corresponding author. Department of Biology, Drexel University, 3141 Chestnut Street, Stratton 118, Philadelphia, PA 19104, USA. Tel.: +1 215 895 2624; Fax: +1 215 895 1273; E-mail: jeff.twiss@drexel.edu

⁹These authors contributed equally to this work

¹⁰Present address: Department of Anatomy, Philadelphia College of Osteopathic Medicine, Philadelphia 19131, PA, USA

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Results

Exogenous 3'UTR of β -actin decreases axonal levels of endogenous β -actin mRNA

To determine if the machinery needed to transport β -actin into sensory axons is limiting, we first used adenoviral (AV) preparations for expressing a myristoylated, destabilized GFP (dzGFP^{myr}) with the 3'UTR of β -actin or γ -actin (AV-GFP-3' β -actin and AV-GFP-3' γ -actin, respectively). Since the 3'UTR of γ -actin has no localizing activity in dorsal root ganglion (DRG) neurons (Willis *et al*, 2007), the AV-GFP-3' γ -actin provided a rigorous control for any non-specific effects of virus transduction. Fluorescent *in situ* hybridization (FISH) probes to the coding region of *actin* allowed us to distinguish endogenous β -actin mRNA in neuronal processes from the GFP + β -actin 3'UTR (GFP-3' β -actin) mRNA (Supplementary Figure S1A). We refer to the processes of cultured DRG neurons as 'axons' throughout the remainder of the text since MAP2 mRNA and protein are excluded from these processes (Zheng *et al*, 2001). These DRG processes also show enrichment of an axonally localizing kinesin isoform by live cell imaging (Vuppalachchi *et al*, 2010) and microtubule polarity typical of axons (Baas *et al*, 1987).

The AV-GFP-3' β -actin transduced neurons showed a significant reduction in endogenous axonal β -actin mRNA signals (Figure 1A and B). There was no corresponding

difference in total endogenous β -actin mRNA levels between AV-transduced and control cultures by standard and quantitative RT-PCR; the mRNA levels from different AV-derived GFP constructs were also comparable (Supplementary Figure S1B). β -Actin is also expressed by the Schwann cells (Zheng *et al*, 2001) and this could mask any changes in β -actin in the DRG neurons in these RT-PCR analyses. Thus, we used quantitative FISH so that we could assess the cell body *actin* mRNA levels; the AV-GFP-3' β -actin transduced neurons showed a slight increase in *actin* mRNA levels in the cell body but this did not reach statistical significance (Figure 1C). Thus, the exogenous β -actin 3'UTR can compete with endogenous mRNAs for the localization machinery.

Depletion of axonal β -actin mRNA decreases axonal outgrowth from adult sensory neurons

Antisense oligonucleotides to the zipcode region of β -actin have been shown to prevent its localization, alter growth cone dynamics in neurons, and alter the polarity of migration in fibroblasts (Shestakova *et al*, 2001; Zhang *et al*, 2001). To determine if the depletion of endogenous β -actin mRNA from the axons of DRG neurons alters their growth, we analysed the morphology of the AV-transduced cultures. DRGs expressing GFP-3' β -actin showed significantly fewer and shorter axons, less branched axons, and smaller growth

Figure 1 Expression of reporter mRNA with 3'UTR of β -actin squelches axonal localization of endogenous β -actin mRNA and attenuates axon outgrowth. (A) FISH for actin mRNA and immunofluorescence for neurofilament and peripherin (NF + P) in DRGs transduced with AV-GFP-3' γ -actin or AV-GFP-3' β -actin (250 multiples of infectivity (MOI)) after 2 DIV and then cultured for additional 1.5 DIV. The FISH probe ('actin') detects the endogenous β -actin mRNA and not the AV-encoded GFP-3' β -actin mRNA (see Supplementary Figure S1), since γ -actin mRNA is restricted to the cell body in the DRG neurons (Zheng *et al*, 2001; Willis *et al*, 2005). Top row shows GFP (green) merged with NF + P (blue) with only GFP signals shown in the inset panel (scale bar = 100 μ m). The middle two rows show NF + P (blue) and actin mRNA (red) in the axon shaft and growth cones, respectively (scale bars = 5 and 10 μ m, respectively). The bottom row shows the corresponding DIC images for the growth cones panels (scale bar = 10 μ m). (B, C) Quantitative FISH signal intensity for axonal and cell body actin mRNA from DRG cultures as in (A) is shown as average pixels/ μ m² $\pm$ s.e.m. Axonal actin mRNA was significantly decreased in the AV-GFP-3' β -actin transduced cultures compared with both the control and AV-GFP-3' γ -actin transduced DRGs. Though cell body actin levels were increased in the AV-GFP-3' β -actin transduced cultures relative to control and AV-GFP-3' γ -actin transduced cultures, this did not reach statistical significance ($N \geq 30$ neurons/condition over three separate experiments; **** $P \leq 0.001$ by one-way ANOVA with Tukey's HSD *post hoc* test for indicated samples).

cones than control or *GFP + γ -actin 3'UTR* (*GFP-3' γ -actin*) mRNA expressing DRGs (Figure 2A and B; Supplementary Table S1). Axon length also decreased as cultures were exposed to increasing amounts of AV-GFP-3' β -actin, but not to AV-GFP-3' γ -actin (Supplementary Figure S2). Thus, β -actin's 3'UTR can function as a dominant-negative agent by preventing endogenous β -actin localization and attenuating axon outgrowth in these adult DRG neurons.

GFP-3' β -actin transgene expression decreases axon outgrowth in vitro and nerve regeneration in vivo

To determine if introducing *GFP-3' β -actin* mRNA into DRG neurons might also impair axonal RNA localization and regeneration *in vivo*, we examined DRG neurons from mice expressing *GFP-3' β -actin* or *GFP-3' γ -actin* transgenes under control of the neuronal-specific *T α 1* tubulin promoter (*T α 1-GFP-3' β -actin* and *T α 1-GFP-3' γ -actin*, respectively). The *T α 1-GFP-3' β -actin* mice show axonal localization of GFP mRNA in sciatic nerves but the GFP mRNA does not localize to axons in the *T α 1-GFP-3' γ -actin* mice (Willis *et al*, 2011). We initially tested *in vitro* axonal growth after an *in vivo* conditioning sciatic nerve crush lesion in these mice. In contrast to the naive cultures used in Figure 2A

and B, here we used injury-conditioned DRG neurons that show a characteristically rapid and translation-dependent axonal outgrowth during the first 24 h *in vitro* (Smith and Skene, 1997; Twiss *et al*, 2000). This translation-dependent growth also includes robust intra-axonal protein synthesis (Willis *et al*, 2005). The injury-conditioned L4-5 DRGs from the *T α 1-GFP-3' β -actin* mice showed decreased axon outgrowth at 1 day *in vitro* (DIV) compared with DRGs from the *T α 1-GFP-3' γ -actin* mice (Figure 2C and D). Moreover, DRGs from the four copy *T α 1-GFP-3' β -actin* mice were also significantly shorter than those from the two copy *T α 1-GFP-3' β -actin* mice, suggesting a dosage effect for the transgene. Note that naive DRGs cultured from these mice expressing *GFP-3' β -actin* mRNA also showed reduced axonal growth over 3 DIV (see Figure 4B below).

To determine if expression of *GFP-3' β -actin* mRNA might also alter *in vivo* axonal regeneration, we tested the ability of the transgenic mice to regenerate transected axons by grafting a wild-type nerve segment into their common fibular nerve (English *et al*, 2005). The *T α 1-GFP-3' β -actin* mice showed significantly decreased regeneration into the nerve graft compared with the *T α 1-GFP-3' γ -actin* mice (Figure 3A). Since axonal regeneration is more robust after crush

Figure 2 DRG neurons expressing GFP with 3'UTR of β -actin show decreased axonal growth *in vitro*. (A) Representative images of control, AV-GFP-3' γ -actin transduced and AV-GFP-3' β -actin transduced DRG neurons as indicated (AV transduction at 1.5 DIV with 3.5 DIV total). Upper row shows immunofluorescence images for low magnification views of entire neurons and lower row shows DIC images of growth cones (scale bars: upper row = 100 μ m; lower row = 5 μ m). (B) Quantification of axon growth in AV-GFP-3' γ -actin transduced and AV-GFP-3' β -actin transduced DRG cultures as in (A). Values represent mean $\pm$ s.e.m. for $N \geq 30$ neurons/condition over three separate experiments (**** $P \leq 0.001$ for AV-GFP-3' β -actin versus AV-GFP-3' γ -actin and control by one-way ANOVA with Tukey's HSD *post hoc* test). (C) Representative images of injury-conditioned L4-5 DRG cultures from the *T α 1-GFP-3' γ -actin* and *T α 1-GFP-3' β -actin* mice are shown at 1 DIV as indicated. (D) Axon length for injury-conditioned cultures as in (C) is reported as mean $\pm$ s.e.m. ($N \geq 30$ over at least three separate experiments; ** $P \leq 0.01$, *** $P \leq 0.005$, **** $P \leq 0.001$ for indicated samples by one-way ANOVA and Tukey's *post hoc* tests) (scale bar = 100 μ m).

Figure 3 Expression of GFP with 3'UTR of β -actin attenuates nerve regeneration. (A) Regeneration in $\text{T}\alpha 1\text{-GFP-3}'\gamma$ -actin and $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin transgenic mice after common fibular nerve transection with wild-type nerve graft is shown at 14 days after transection/grafting. X axis details the distance of GAP-43 immunoreactive profiles from the proximal host-graft junction proceeding distally as indicated. The average number of axons identified at each point in longitudinal sections is illustrated as the mean $\pm$ s.e.m. ($N \geq 5$; $P \leq 0.05$ at all distances by Student's *T*-test). Mice expressing the transgene with 3'UTR of β -actin mRNA have significantly fewer regenerating axons at each point measured than those expressing transgene with 3'UTR of γ -actin mRNA. (B) Representative exposure matched montage confocal images of longitudinal section of crushed sciatic nerve from transgenic mice are shown for PGP9.5 immunoreactivity and DAPI staining; injury site is indicated by the dashed line (scale bar = 200 μm). (C) The number of PGP9.5-positive axons as in (B) was evaluated at 7 days post-nerve crush at 0, 0.5, and 1.0 mm proximal and distal to the injury site. The graph represents the mean $\pm$ s.e.m. of the total number of NF-positive axons at each distance ($N = 8$; $**P \leq 0.01$, $***P \leq 0.005$ by Student's *T*-test). Supplementary Figure S3 shows additional axonal marker proteins used to evaluate regeneration in these animals. (D) Representative images from toluidine blue-stained epon sections of osmicated nerve tissues from transgenic mice are shown at 14 days post-crush; sections were taken at 0.5 cm distal to the crush site (scale bar = 20 μm). (E) G-ratios for myelinated axons of crushed nerves as in (D) are shown as relative frequency histograms ($N = 12$ over three separate experiments). The nerves from $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin mice show a right shift in the distribution in G-ratio histogram consistent with decreased regeneration of sensory axons due to selective transgene expression in the DRGs. By Wilcoxon's rank-sum test of the G-ratio frequency distribution, the G-ratios for $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin nerves are statistically different than the $\text{T}\alpha 1\text{-GFP-3}'\gamma$ -actin nerves ($P \leq 0.0001$). In contrast, histograms of G-ratios in uninjured nerves from these transgenic lines showed no significant difference ($P = 0.8371$; data not shown). Analysis of G-ratio and axon density in uninjured mice is detailed in Supplementary Table S2.

compared with transection injury, we asked if expression of $\text{GFP-3}'\beta$ -actin might also affect regeneration of crushed peripheral nerve. The $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin mice again showed significantly fewer axons at points distal from the crush site than the $\text{T}\alpha 1\text{-GFP-3}'\gamma$ -actin mice (Figure 3B and C). We saw similar decreased regeneration in the $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin mice when analysed by a threshold method with multiple axonal markers (Supplementary Figure S3A). To be certain that these differences were not due to lower levels of axonal proteins in the $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin mice, we determined the ratio of axon diameters to total diameters of the myelinated fibres (i.e., 'G-ratio') in both transgenic lines. G-ratios in the crushed nerves for the $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin mice were different than those for the $\text{T}\alpha 1\text{-GFP-3}'\gamma$ -actin mice (Figure 3D and E). Since the diameter and G-ratio of sensory axons is overall less than motor axons, the right shift in the G-ratio histogram for the $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin mice towards larger axon diameters is consistent with decreased regeneration in the

sensory neurons that selectively express the $\text{GFP-3}'\beta$ -actin transgene. Finally, to determine if differences in axon retraction accounted for the decreased regeneration in the $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin mice, we directly compared retraction after severing axons in cultured DRGs. There were no significant differences in distances of axon retraction in DRGs from $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin and $\text{T}\alpha 1\text{-GFP-3}'\gamma$ -actin mice (Supplementary Figure S3B). We also compared neuron numbers in the L4-5 DRGs at 0 and 14 days after crush injury with no differences between the $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin and $\text{T}\alpha 1\text{-GFP-3}'\gamma$ -actin mice (data not shown).

Considering that developmental abnormalities in the $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin mice could lead to the appearance of decreased nerve regeneration in the above studies, we analysed several parameters of sensory neuron development in these mice. Nerve morphology was assessed to determine if there is a selective loss of sensory fibres in the $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin mice. The G-ratios and axon diameters showed no significant

differences in uninjured T α 1-GFP-3' β -actin and T α 1-GFP-3' γ -actin sciatic nerves (Supplementary Table S2). Unmyelinated axons showed similar density in the T α 1-GFP-3' β -actin and T α 1-GFP-3' γ -actin mice (data not shown). The lines showed no significant differences in numbers of neurons in the L4-5 DRGs and footpads showed similar densities of sensory fibres (Supplementary Table S2). Finally, the transgenic lines showed no statistical differences in temperature sensitivity or mechanosensation (data not shown). Thus, developmental differences between

the T α 1-GFP-3' β -actin and T α 1-GFP-3' γ -actin mice do not account for the differences in their axon regeneration.

Limited levels of ZBP1 account for the dominant-negative effect of GFP-3' β -actin mRNA

Since the GFP-3' β -actin mRNA could be competing for limiting quantities of ZBP1 or other components of the RNA transport machinery, we asked if increasing availability of ZBP1 might rescue the growth deficit in DRG neurons cultured from the T α 1-GFP-3' β -actin mice. Consistent with previous work in cortical neurons (Zhang *et al*, 2001), the ZBP1 fusion protein appeared granular compared with native mCherry, suggesting that it aggregates into ribonucleoprotein particles in these DRG neurons (Figure 4A). At 3 DIV for expression of transfected constructs, the ZBP1 transfection completely reversed the growth deficit in T α 1-GFP-3' β -actin DRGs, with axon lengths comparable to mock- and mCherry-transfected T α 1-GFP-3' γ -actin DRGs ($P=0.238$; Figure 4B). T α 1-GFP-3' β -actin DRGs showed no rescue in axon length with expression of ZBP1 mutants lacking the KH domains needed for RNA binding or tyrosine phosphorylation site (Y396) needed for its translation derepressing activity (Figure 4B). ZBP1 represses translation of its mRNA cargo and, upon phosphorylation at Y396, it releases its cargo for translation (Huttelmaier *et al*, 2005). Thus, the ZBP1^{Y396F} mutant transports mRNAs but cannot release the mRNAs for translation. Together, these data suggest that ZBP1 cargo mRNAs need both localization to and translation within axons to optimally support axon outgrowth. Interestingly, DRG neurons cultured from T α 1-GFP-3' γ -actin mice also showed a significant increase in their axon lengths when transfected with ZBP1 (Figure 4B), which suggests that availability of ZBP1 is limited in adult sensory neurons. Consistent with this, transfection of DRG neurons from adult rats with full-length ZBP1 generated a clear increase in axonal outgrowth (Figure 4C).

The human ZBP1 orthologue appears to bind many different mRNA species in co-immunoprecipitation assays (Jonson *et al*, 2007). To determine if the growth deficit in the GFP-3' β -actin expressing neurons is solely from decreased axonal

Figure 4 Increasing availability of ZBP1 increases axon outgrowth. (A) Representative images of mCherry versus mCherry-ZBP1 proteins in transfected DRG neurons are shown. The upper row shows average projection of Z-stacks with inset showing a single optical plane approximately through the centre of the cell body. The lower two rows show single planes of mid-axon shaft with clear granular profiles for mCherry-ZBP1 (arrows) versus diffuse signals for mCherry (scale bars: upper row = 25 μ m; lower two rows = 10 μ m). (B) Dissociated DRGs from T α 1-GFP-3' γ -actin and T α 1-GFP-3' β -actin mice were co-transfected with ZBP1-mCherry + empty GFP, ZBP1^{Y396F}-GFP + empty mCherry, ZBP1 Δ KH1-4-GFP + empty mCherry vectors, or empty mCherry + empty GFP (control). Neurons were scored for axon growth from NF + P-stained cultures at 3 DIV by a blinded observer. Data are expressed as average percent change compared with corresponding mock transfection $\pm$ s.e.m. (** $P \leq 0.01$, *** $P \leq 0.005$ for T α 1-GFP-3' γ -actin and $^{\dagger\dagger\dagger}P \leq 0.001$ for T α 1-GFP-3' β -actin versus corresponding mCherry-transfected culture by one-way ANOVA with Tukey's HSD *post hoc* test). Average axon length $\pm$ s.e.m. in μ m is shown parenthetically for T α 1-GFP-3' γ -actin and T α 1-GFP-3' β -actin, respectively. (C) Montage of images for NF + P (green) stained 3 DIV adult rat DRG cultures transfected with mCherry versus mCherry-ZBP1. The inset panels show the corresponding mCherry signals (red) (scale bar = 200 μ m; inset = 50 μ m).

β -actin translation, we reasoned that introducing the β -actin coding sequence plus its 3'UTR should compete with GFP-3' β -actin mRNA. Transfecting neurons from T α 1-GFP-3' β -actin mice with β -actin coding sequence fused to mCherry $\pm$ β -actin 3'UTR did not rescue the axon outgrowth deficit despite that the encoded β -actin-mCherry was clearly incorporated into the cytoskeleton (Supplementary Figure S4). Thus, loss of other mRNAs from the DRG axons in addition to β -actin is responsible for the axon growth deficit in the GFP-3' β -actin expressing neurons. This is consistent with recent observations that motor axons can develop and regenerate in complete absence of murine β -actin (Cheever *et al*, 2011).

ZBP1 is needed for axonal localization of GAP-43 mRNA

To determine if other mRNAs might show altered transport with GFP-3' β -actin expression, we quantified axonal levels of several axonal mRNAs in DRG neurons transduced with

AV-GFP-3' β -actin versus AV-GFP-3' γ -actin. Axonal levels of GAP-43 and *Neuritin/cpg15* mRNAs showed significant reduction in AV-GFP-3' β -actin compared with AV-GFP-3' γ -actin transduced cultures (Figure 5A). Axonal *amphotericin* mRNA levels were not affected by AV-GFP-3' β -actin transduction, while axonal *calreticulin* mRNA levels were increased by transduction with either AV preparation (Figure 5A). By RT-qPCR, transduction with AV-GFP-3' β -actin did not affect overall levels of any of these transcripts studied (Supplementary Figure S1B), but FISH analyses showed increased levels GAP-43 and *Neuritin/cpg15* mRNAs in the cell bodies of the AV-GFP-3' β -actin transduced cultures (Figure 5A). Cell body levels of *calreticulin* mRNA were increased with both AV preparations (Figure 5A), suggesting that the increases in axons reflect an overall increase in neuronal *calreticulin* mRNA, possibly as a stress response to the viral transduction.

Figure 5 ZBP1 is needed for axonal localization of GAP-43 mRNA. (A) Quantitative FISH analyses for the indicated mRNAs in axons and cell bodies of cultured rat DRG transduced with AV-GFP-3' γ -actin and AV-GFP-3' β -actin are shown as a heat map relative to corresponding control ($N \geq 30$; * values $\pm$ s.e.m. are shown in Supplementary Table S3). (B) RT-qPCR for indicated mRNAs in proximal sciatic nerves of wild-type, T α 1-GFP-3' γ -actin, and T α 1-GFP-3' β -actin mice at 7 days after mid-thigh sciatic nerve crush is shown as a heat map based on average fold change relative to wild-type nerves. Fold change was calculated from $\Delta\Delta C_t$ using 18S rRNA for normalization between samples ($N = 12$; values $\pm$ s.e.m. are shown in Supplementary Table S4). Note that the *actin* product is detected with coding sequence primers that amplify both β -actin and γ -actin mRNAs to distinguish from transgene GFP-3' β -actin mRNA. (C) Rat DRG neurons were transfected with mCherry-ZBP1 fusion or empty mCherry constructs and analysed by quantitative FISH for axonal levels of *actin* and GAP-43 mRNAs. Mean values $\pm$ s.e.m. are presented relative to empty mCherry vector ($N \geq 30$ neurons over three separate transfection experiments; ** P < 0.01, *** P < 0.005 by Student's *T*-test). (D) Representative immunoprecipitation (IP) for ZBP1 from P5 brain + spinal cord lysates is shown; lanes with 20% input and IgG control IP are for comparison. ZBP1 IPs from P5 T α 1-GFP-3' γ -actin and T α 1-GFP-3' β -actin mouse tissues were used for extraction of RNA. RT-qPCR values for β -actin and GAP-43 mRNAs from the ZBP1 IPs are shown as mean value $\pm$ s.e.m. from three experiments (* P < 0.05, *** P < 0.005 by Student's *T*-test). ΔC_t values for inputs for the T α 1-GFP-3' γ -actin versus T α 1-GFP-3' β -actin preparations showed no significant differences (1.0 ± 0.09 and 0.86 ± 0.13 for *actin* and 0.98 ± 0.11 and 1.0 ± 0.10 for GAP-43 mRNAs, respectively).

We next asked if transport of these mRNAs into the peripheral nerve might be affected in the transgenic mice. After sciatic nerve crush to activate the $T\alpha 1$ promoter, less *GAP-43* mRNA was detected in $T\alpha 1$ -GFP-3' β -actin compared with $T\alpha 1$ -GFP-3' γ -actin nerves, and *GAP-43* levels were also lower in the four copy versus two copy $T\alpha 1$ -GFP-3' β -actin nerves (Figure 5B; Supplementary Table S3). Levels of *peripherin*, which encodes a neuronal-specific intermediate filament that localizes to DRG axons (Willis *et al*, 2005), were not affected and *Neuritin/cpg15* levels were decreased but this did not reach significance. These data suggest that the exogenous 3'UTR of β -actin may also compete *in vivo* with *GAP-43* mRNA. PCR products for *actin* showed a significant decrease in the $T\alpha 1$ -GFP-3' β -actin compared with $T\alpha 1$ -GFP-3' γ -actin nerve (Figure 5B; Supplementary Table S3).

To directly test whether limited availability of ZBP1 determines the axonal levels of β -actin and *GAP-43* mRNAs, we transfected wild-type rat DRG neurons with ZBP1-mCherry and assessed axonal mRNA levels. The ZBP1-mCherry expression significantly increased axonal levels of both β -actin and *GAP-43* mRNAs (Figure 5C). Similar was seen with transfection of wild-type mouse DRGs with ZBP1 (see 7C below). We further asked if GFP-3' β -actin displaces endogenous mRNAs from ZBP1 *in vivo*. Immunoprecipitation of ZBP1 from lysates of neonatal brain plus spinal cord showed displacement of β -actin and *GAP-43* from ZBP1 immunocomplexes in the $T\alpha 1$ -GFP-3' β -actin but not in the $T\alpha 1$ -GFP-3' γ -actin tissues (Figure 5D). RT-qPCR for *amphoterin*, *calreticulin*, and *neuritin/cpg15* gave high cycle threshold values ($C_t \geq 35$); C_t values in this range are generally indicative of noise in the RT-qPCR assay and suggest that these transcripts were not present in these immunoprecipitates. The levels of these transcripts, as well as *actin* and *GAP-43*, were equivalent in the input lysates from $T\alpha 1$ -GFP-3' β -actin and $T\alpha 1$ -GFP-3' γ -actin tissues, and ZBP1 was equally expressed in these tissues (data not shown).

ZBP1 levels limit regeneration capacity in the adult nervous system

Previous studies have shown that ZBP1 is expressed at much higher levels in embryonic compared with adult tissues (Nielsen *et al*, 1999). Consistent with this, ZBP1 mRNA in adult DRGs was at lower levels than seen in the neonatal tissues, and immunoblotting showed much higher ZBP1 protein levels in lysates from E12.5 compared with those from P20 or adult DRG plus spinal cord (Figure 6A and B).

Since these low levels of ZBP1 appeared to limit axonal growth in the adult DRG neurons, we took advantage of previously published ZBP1 knockout mice to determine if further loss of ZBP1 would similarly decrease axonal regeneration potential. Although multiple developmental abnormalities are seen in ZBP1^{-/-} mice, ZBP1^{+/-} mice are viable and reportedly normal (Hansen *et al*, 2004), but ability of ZBP1^{+/-} mice to respond to injury has not been tested. By RT-qPCR for ZBP1, we see that the adult DRGs of ZBP1^{+/-} mice have only 40 ± 9% ZBP1 mRNA levels of ZBP1^{+/+} littermates (data not shown). Similarly to the $T\alpha 1$ -GFP-3' β -actin mice, DRG neurons cultured from adult ZBP1^{+/-} mice showed decreased axonal outgrowth (Figure 6C and D). Transfecting the ZBP1^{+/-} DRGs with ZBP1-mCherry

rescued the axonal growth deficit at 3 DIV, but the ZBP1^{Y396F}-mCherry mutant did not (Figure 6E; Supplementary Figure S5). ZBP1-mCherry expression increased and ZBP1^{Y396F}-mCherry decreased overall axon lengths in the wild-type mouse neurons.

The ZBP1^{+/-} mice also showed decreased axonal regeneration *in vivo*. With sciatic nerve crush, there was a significant decrease in axon extension beyond the injury site at 7 days post-injury (Figure 6F). Similar was seen after common fibular nerve transection/grafting (Supplementary Figure S3C). To determine if these morphological changes also resulted in decreased functional regeneration, we measured evoked responses (M response) of gastrocnemius and tibialis anterior (TA) muscles to stimulation with an electrode inserted into the proximal sciatic nerve. Prior to injury, the M responses of ZBP1^{+/-} and wild-type mice were comparable; however, the ZBP1^{+/-} showed a clear decrease in M responses compared with wild type at 14 days after nerve crush (Figure 6G). Quantifying the M responses showed statistically decreased amplitude in ZBP1^{+/-} compared with wild-type mice (Figure 6H). Taken together, these studies indicate loss of a ZBP1 allele results in haploinsufficiency for axonal regeneration from adult neurons.

ZBP1^{+/-} neurons are haploinsufficient for axonal mRNA transport

Since axonal growth is decreased in the ZBP1^{+/-} neurons and this is rescued by transfection with ZBP1, we asked if axonal mRNA transport would also be affected with loss of a ZBP1 allele. We first examined RNA levels in the nerve transection/graft model. There were no significant differences in the levels *actin*, *amphoterin*, *calreticulin*, *neuritin/cpg15*, and *peripherin* mRNAs in the L4-5 DRGs of ZBP1^{+/-} versus wild-type mice (Figure 7A; Supplementary Table S4). However, *actin* and *GAP-43* mRNA levels were reduced in the proximal nerve stump and the grafted nerve tissue from the ZBP1^{+/-} versus ZBP1^{+/+} littermates. *Neuritin/cpg15* showed a significant decrease in the proximal nerve but not in the graft and *amphoterin*, *calreticulin*, and *peripherin* mRNA levels were not significantly different in the proximal nerve and graft of ZBP1^{+/-} versus ZBP1^{+/+} (Figure 7A; Supplementary Table S4). This suggests that axonal transport of β -actin and *GAP-43* mRNAs decreases *in vivo* with decreases in ZBP1 levels. To further test this possibility, we evaluated these mRNAs in acellular allografts from mice expressing the GFP-3' β -actin mRNA that competes with endogenous mRNAs for binding to ZBP1. *Actin* and *GAP-43* mRNAs also showed significantly decreased levels in allografts of $T\alpha 1$ -GFP-3' β -actin compared with $T\alpha 1$ -GFP-3' γ -actin mice (Figure 7B).

Using quantitative FISH, we analysed axonal levels of mRNAs in DRG neurons cultured from the ZBP1^{+/-} versus ZBP1^{+/+} mice at 3 DIV after transfection with mCherry, ZBP1, or ZBP1^{Y396F}. The mCherry-transfected ZBP1^{+/-} DRGs showed decreased axonal levels of *actin* and *GAP-43* mRNAs compared with DRGs from ZBP1^{+/+} littermates (Figure 7C; Supplementary Table S5). Transfecting with ZBP1-mCherry increased the axonal β -actin and *GAP-43* mRNA levels in the ZBP1^{+/-} DRGs and again increased these axonal mRNA in the wild-type neurons. Though transfecting ZBP1^{+/-} DRGs with the ZBP1^{Y396F} mutant did not rescue their axonal growth deficit (Figure 6D), this did restore the axonal β -actin and *GAP-43* mRNAs to levels comparable to wild-type neurons

Figure 6 ZBP1 haploinsufficiency compromises axonal regeneration. **(A)** Representative RT-PCR result for ZBP1 from P2 brain and adult DRG from wild-type mice is shown as signals from ethidium bromide-stained agarose gel. **(B)** Representative immunoblot for ZBP1 from the DRG + spinal cord of wild-type mice at indicated ages is shown with GAPDH immunoblotting as a loading control. With longer exposures, ZBP1 protein was detected in the P20 and adult DRGs (data not shown). **(C)** Axonal outgrowth in naive DRG cultures prepared from 7-week-old ZBP1^{+/-} versus ZBP1^{+/+} mice was scored after 2 and 3 DIV. Mean axon lengths $\pm$ s.e.m. are shown ($N \geq 60$ over four separate cultures; $*P \leq 0.05$ by one-way ANOVA with Tukey's HSD *post hoc* test). **(D)** Representative images of ZBP1^{+/+} and ZBP1^{+/-} neurons are shown for three DIV cultures (scale bar = 100 μ m). **(E)** ZBP1^{+/-} versus ZBP1^{+/+} mouse DRGs were transfected with mCherry (control), ZBP1-mCherry (ZBP), or ZBP1^{Y396F}-mCherry (ZBP^{Y396F}) constructs and scored for axon outgrowth at 3 DIV. Axon length is expressed relative to control wild-type neurons as average $\pm$ s.e.m. ($N \geq 30$ over three separate experiments; $*P \leq 0.05$, $***P \leq 0.005$ versus corresponding mCherry-transfected samples by one-way ANOVA with Tukey's HSD *post hoc* test). Representative images for these cultures are included in Supplementary Figure S5. **(F)** Regeneration after sciatic nerve crush in ZBP1^{+/-} mice crossed with Thy1-YFP transgenic mice was assessed 7 days post-injury and compared with ZBP1^{+/+} littermates. The number of YFP-positive (YFP+) axons was calculated at the injury site, 0.5 mm and 1.0 mm proximal ($N = 8$; $*P \leq 0.05$ by Student's *T*-test). These mice also showed delayed regeneration after common fibular nerve transection/grafting (see Supplementary Figure S3C). **(G)** Representative evoked EMG activity for the gastrocnemius muscle of wild-type and ZBP1^{+/-} mice following sciatic nerve stimulation is shown prior to (Intact) and 7 and 14 days following crush of the tibial branch of the sciatic nerve. **(H)** Mean response amplitudes $\pm$ s.e.m. from **(F)** are expressed as percentage of the response amplitude recorded prior to nerve crush ($N = 4$ for ZBP1^{+/+} and $N = 6$ for ZBP1^{+/-}; $*P \leq 0.05$ by Student's *T*-test). At both times, the response amplitudes were relatively larger in wild-type mice than in ZBP1^{+/-} mice.

and increased the axonal levels of these mRNAs in wild-type littermate DRGs (Figure 7C; Supplementary Table S5). These ZBP1 rescue studies distinguish mRNA transport and

translational activities of ZBP1, and again emphasize that the translation of ZBP1 cargo mRNAs is needed for optimal axonal outgrowth.

Figure 7 Loss of ZBP1 compromises axonal mRNA localization. **(A)** mRNA transport into the regenerating axons of the ZBP1^{+/-} versus wild-type mice *in vivo* was assessed using the common fibular transection/grafting approach. The levels of indicated mRNAs in L4/5 DRG, sciatic nerve (~1 cm proximal to origin of common fibular nerve), and an acellular graft in ZBP1^{+/-} relative to ZBP1^{+/+} are shown as a heat map ($N = 5$; values $\pm$ s.e.m. are included in Supplementary Table S5). **(B)** RT-qPCR was used to evaluate levels of mRNAs in acellular grafts to the common fibular nerve of Tα1-GFP-3'β-actin versus Tα1-GFP-3'γ-actin mice. There is a significant depletion of *actin* and *GAP-43* mRNAs in the Tα1-GFP-3'β-actin nerve grafts ($N = 8$; $*P \leq 0.05$ calculated by Student's *T*-test). **(C)** Quantitative FISH was used to determine the axonal and cell body levels of the indicated mRNAs in wild-type and ZBP1^{+/-} mice transfected with mCherry (mCh), ZBP1-mCherry (ZBP1-mCh), or ZBP1^{Y396F}-mCherry (ZBP1^{Y396F}-mCh). Average fold change relative of ZBP1^{+/-} versus ZBP1^{+/+} DRGs transfected with mCherry control for each mRNA is shown as a heat map ($N \geq 30$; values $\pm$ s.e.m. are included in Supplementary Table S6). In each case where axonal levels are altered, the cell body values show a reciprocal change. **(D)** DRG neurons from Tα1-GFP-3'γ-actin or Tα1-GFP-3'β-actin mice were transfected with empty mCherry (control), ZBP1-mCherry (ZBP1), or ZBP1^{Y396F}-mCherry (ZBP1^{Y396F}-mCh). Average axonal signals from FISH are shown as mean % change relative to neurons expression GFP-3'γ-actin mRNA transfected with control vector $\pm$ s.e.m. ($*P \leq 0.05$, $***P \leq 0.005$, $****P \leq 0.001$ for indicated samples by one-way ANOVA with Tukey's *post hoc* test).

Since axonal *neuritin/cpg15* mRNA was not depleted to the same degree as β -actin or *GAP-43* mRNAs with expression of GFP plus β -actin 3'UTR (Figure 6A) and it did not show any significant change with ZBP1 depletion (Figure 7A and B), we reasoned that this mRNA might not be a direct target for ZBP1 binding. To test this possibility, we returned to the GFP-3'β-actin expressing neurons as these showed the most consistent depletion of axonal *neuritin/cpg15*. As with the ZBP1^{+/-} neurons, transfecting Tα1-GFP-3'β-actin DRGs with ZBP1^{Y396F} rescued the axonal levels of β -actin and *GAP-43* mRNAs (data not shown); however, transfecting ZBP1^{Y396F} into the Tα1-GFP-3'β-actin DRGs did not rescue the axonal *neuritin/cpg15* levels (Figure 7D). Since ZBP1^{Y396F} does not restore axonal growth in the ZBP1^{+/-} or GFP-3'β-actin expressing neurons (Figures 6D and 4B), these data indicate that *neuritin/cpg15* is not a ZBP1 cargo.

Discussion

Our findings reveal an essential role for ZBP1-mRNA interactions in axonal regeneration, both in cultured neurons and

in vivo. Previous work in non-neuronal cells showed that ZBP1-bound β -actin mRNA is translationally inactive and requires ZBP1's phosphorylation at tyrosine 396 for derepressing this translational inhibition (Huttelmaier *et al*, 2005). In neurons, ZBP1 is locally phosphorylated by Src in growth cones, and overexpression of ZBP1^{Y396F} impairs growth cone turning towards BDNF (Sasaki *et al*, 2010). Here, we show that wild-type ZBP1, but not ZBP1^{Y396F}, reverses the axon outgrowth deficits in GFP-3'β-actin mRNA expressing and ZBP1^{+/-} neurons. However, the ZBP1^{Y396F} mutant rescued deficits in axonal localization of both β -actin and *GAP-43* mRNAs in neurons from the ZBP1^{+/-} mice and those expressing the GFP-3'β-actin mRNA. Thus, both ZBP1-mRNA transport and localized translation of ZBP1-mRNA cargos contribute to axonal growth capacity. This is the first observation that clearly links function of a specific RBP to transport and translation of mRNA cargos for axonal outgrowth *in vitro* and *in vivo*.

These data also emphasize the need to develop means to functionally assess RNA localization mechanisms. Though it is not clear from our studies if *GAP-43* mRNA is bound

directly by ZBP1 or requires additional proteins, there clearly is a functional interaction between this mRNA and ZBP1. Previous studies have shown that the Elav protein HuD binds to and stabilizes *GAP-43* mRNA (Perrone-Bizzozero and Bolognani, 2002). Both *GAP-43* mRNA and HuD have been localized to axons and they colocalize (Smith *et al*, 2004; Willis *et al*, 2007), although a role for HuD in mRNA localization has not been established. However, HuD and IMP-1 (ZBP1) have been shown to interact within a RNP complex associated with *Tau* mRNA (Atlas *et al*, 2004). Additional studies will be needed to define the composition of the *GAP-43* mRNA transport complex. It is intriguing that axonal depletion of *GAP-43* mRNA was never to the same extent as β -actin mRNA, which may suggest additional proteins contribute to transport of *GAP-43* into axons or the affinity of ZBP1 for binding β -actin and *GAP-43* may differ.

The UTR competition approach used here provides a direct test for co-transport of mRNAs in living cells. Previous co-immunoprecipitation analyses for IMP1 (ZBP1) detected *neurtin/cpg15* mRNA as a ZBP1 target (Jonson *et al*, 2007). Here, the decrease in axonal levels of *neurtin/cpg15* was restored by transfection with ZBP1 but not with ZBP1^{Y396F} that has RNA binding activity but cannot be phosphorylated to efficiently release mRNAs for translation. Although we cannot exclude the possibility that the *GFP^{myr}-3'* β -actin mRNA competes with endogenous mRNAs by binding to proteins beyond ZBP1, our data show that axonal localization of *neurtin/cpg15* is not linked to the availability of ZBP1. These data indicate that, like *calreticulin* and *amphoterin*, other RBPs are undoubtedly used to transport *neurtin/cpg15* mRNA into axons. This study emphasizes the need for studies to identify other RBPs that localize mRNAs to axons *in vivo*. Interestingly, two recent reports have shown that HuD binds to *neurtin/cpg15* mRNA (Akten *et al*, 2011; Wang *et al*, 2011) and HuD is known to bind to *GAP-43* mRNA (Mobarak *et al*, 2000).

Although initially generated as a model to visualize sites of axonal translation *in vivo* (Willis *et al*, 2011), the transgenic mice carrying diffusion-limited *GFP^{myr}* with 3'UTR of β -actin proved to be an extremely useful resource for testing *in vivo* functional significance for axonal mRNA transport. This approach for localizing reporter mRNAs through 3'UTRs has previously been used to visualize protein synthesis *in vivo* in dendrites and developing axons (Brittis *et al*, 2002; Meyer-Luehmann *et al*, 2009). However, our studies add a new facet by showing that increasing levels of the UTR elements can interfere with endogenous RNA trafficking *in vivo*. Such a squelching of mRNA transport has been demonstrated *in vitro* for UTR elements from *Integrin α 3* and *COXIV* mRNAs, but potential effects on the localization of other mRNAs or their function in axon growth was not reported (Adereth *et al*, 2005; Aschrafi *et al*, 2010). The ability of ZBP1 to rescue axonal localization of select mRNAs and axon growth defects provides proof for mRNAs utilizing a common RBP for localization.

A critical advance in the studies here is that the levels of an RBP can limit regeneration capacity. Although translation of localized mRNAs has been shown to play a role axon outgrowth, pathfinding, and growth cone formation *in vitro* (Holt and Bullock, 2009), this is the first clear evidence that transport of mRNAs from the neuronal cell body into distal axons contributes to regenerative axonal growth *in vivo*.

By both morphological and electrophysiological measures, PNS axonal regeneration decreased when ZBP1 levels decreased. ZBP1 is expressed at high levels during embryonic development (Nielsen *et al*, 1999), which likely explains why measures of sensory development appear normal in the mice expressing *GFP-3'* β -actin transgene. The decreased regeneration in the $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin and ZBP1^{+/-} mice is undoubtedly the result of low expression of ZBP1 in adults. This raises the possibility that altering expression of ZBP1 in adult neurons may help to increase axonal regeneration by increasing the fraction of mRNAs delivered into the axonal compartment.

Materials and methods

All animal work was approved by Institutional Animal Care and Use Committees (IACUC) at Drexel University, Alfred I. duPont Hospital, Emory University, or Ohio State University.

Cell culture

PC12 cells were maintained as described (Twiss *et al*, 2000). Naive or 7 day injury-conditioned DRGs from 4- to 12-week-old Sprague Dawley rats or mice were cultured as described with minor modifications (Twiss *et al*, 2000). The $\text{T}\alpha 1\text{-GFP-3}'\beta$ -actin and $\text{T}\alpha 1\text{-GFP-3}'\gamma$ -actin lines are on FVN background (Willis *et al*, 2011); ZBP1 gene-trap mice are on C57Bl/6 background (Hansen *et al*, 2004). ZBP1^{+/-} lines were crossed with Thy1-YFP transgenic mice (Feng *et al*, 2000) to facilitate axon visualization. Surgery for injury conditioning is outlined below. In some experiments, ganglia were stored overnight at 4°C in *Hibernate* medium (BrainBits). Neurons were enriched by Percoll gradient (Hanz *et al*, 2003). All transfections were performed with the *Amara Nucleofector* (Lonza).

Plasmid and viral expression constructs

AV expressing *GFP^{myr}* with 3'UTRs of β - and γ -actin mRNAs have been previously described (Willis *et al*, 2007). The *Tg-T α 1-dzGFP^{myr}-3'* β -actin and *Tg-T α 1-dzGFP^{myr}-3'* γ -actin transgene constructs have recently been described (Willis *et al*, 2011). Briefly, the transgenic construct consisted of a destabilized *GFP^{myr}* with 5'UTR of *CamKII α* (Aakalu *et al*, 2001) and 3'UTRs of mouse β -actin or γ -actin mRNAs. Transgene expression was driven by the neuronal-specific $\text{T}\alpha 1$ tubulin promoter (Gloster *et al*, 1994; Bamji and Miller, 1996).

Fusion constructs for ZBP1, ZBP1^{Y396F}, and ZBP1^{AKH1-4} have been described (Farina *et al*, 2003; Huttelmaier *et al*, 2005). For β -actin-mCherry fusion protein, mCherry was amplified from mCherry-ZBP, cloned into pTOPO (Invitrogen), and then used to replace DsRed in pDsRed-Actin (Clontech). β -Actin 3'UTR was amplified and directly cloned into the *Bam*H1 and *Mfe*I sites directly downstream of mCherry- β -actin. All cloned PCR products were sequenced prior to use.

Animal surgeries and procedures

For injury-conditioning and morphological studies of regeneration, rodents were subjected to sciatic nerve crush at mid-thigh as described (Smith and Skene, 1997). The contralateral (control) nerve was exposed but not manipulated. Nerve grafting procedure for common fibular nerve is published (English *et al*, 2005). For acellular grafts, the donor nerve segment was frozen in liquid nitrogen for 2 min and then placed in normal saline at 37°C for 2 min; this freeze-thaw cycle was repeated twice prior to grafting (English *et al*, 2007). For the physiological studies of nerve regeneration, recordings were obtained prior to nerve injury (see below) and then the tibial branch of the sciatic nerve, which innervates gastrocnemius, was isolated and crushed 15 mm distal to the emergence of the sciatic nerve from the pelvis using No. 5 forceps. The common fibular branch of the sciatic nerve, which innervates the TA muscle, was left intact as an internal control.

To harvest tissues for immunofluorescence (IF), deeply anaesthetized mice were perfused transcardially with PBS followed by 4% paraformaldehyde. Tissues were post-fixed with 3% paraformaldehyde for 2–4 h, and then cryoprotected at 4°C overnight in

30% sucrose. The tissues were processed for cryosectioning or used directly for whole mount IF.

For analyses of axon size, perfusion fixative consisted of 3% formaldehyde, 1.5% glutaraldehyde, and 2.5% sucrose in 100 mM cacodylate buffer (pH 7.4). Nerves were then immersion fixed for 60 min, post-fixed in 1% OsO_4 , stained with uranyl acetate (Ted Pella), dehydrated through graded ethanol series, and embedded in Epon. In all, 1 μm sections were used for the morphometry of myelinated axons (i.e., G-ratios, axon diameter). In all, 70 nm sections were contrasted with 1% uranyl acetate and lead citrate and viewed with a Hitachi H7650 electron microscope for unmyelinated axons.

Compound muscle action potentials (M responses) were recorded in response to sciatic nerve stimulation in ZBP1^{+/-} and wild-type littermates. All recordings were made in pentobarbital anaesthetized animals. Bipolar fine wire EMG electrodes were constructed from single-stranded enamel-coated stainless steel wire (California Fine Wire) and inserted into the gastrocnemius and TA muscles using a 25-G hypodermic needle. A monopolar needle electrode was placed next to the sciatic nerve near the sciatic notch and used to deliver short (0.05 ms duration) cathodal stimulus pulses. An indifferent anodal electrode was placed into the adjacent lumbar epaxial musculature. Isolated constant voltage stimuli were applied at a rate of 0.3 Hz at increasing amplitudes ranging from subthreshold to supramaximal for evoking a twitch in innervated muscles. Direct muscle (M) responses evoked by this stimulation were acquired at a sampling rate of 10 kHz and recorded to disc. Activity was recorded for 20 ms prior to and 30 ms after each stimulation. All activity in these records was full wave rectified before analysis. Average voltages in the pre-stimulus period were considered background activity and subtracted from each of the points sampled after stimulation to adjust for background. The average adjusted activity in a defined M response time window, generally 0.5–4.5 ms after stimulation, was determined and expressed in mV. The onset and duration of this window needed to be adjusted slightly over the course of muscle re-innervation, as we have noted previously (English *et al*, 2007). All procedures were performed bilaterally and recordings were repeated on days 7 and 14 after nerve crush. On each day, the maximal M response amplitude was measured, and expressed as a proportion of the maximal M response amplitude recorded prior to transection. Averages of these relative amounts of recovery of the M response at different times were compared between the two mouse groups using an unpaired *t*-test.

Thermal sensation and mechanosensation were assessed in uninjured mice using plantar infrared analgesia meter and Dynamic Plantar Aesthesiometer (Ugo Basile), respectively. After mice were habituated to the apparatus, withdrawal latency to heat source and thresholds to pressure were recorded ≥ 5 times for each hind paw ($n = 15$ mice).

Analyses of RNAs by RT-PCR

DRGs and sciatic nerves were placed in lysis buffer (RNAqueous kit, Ambion) and homogenized using a motorized homogenizer (Omni International). RNA was isolated from cultured cells with *microRNAqueous* kit (Ambion). RNA samples were treated with DNase prior to analysis. In all, 100 ng of total RNA was used as template for RT with *iScript* (Bio-Rad). RT reactions were diluted five-fold for PCR. Standard PCR (≤ 30 cycles) was analysed by agarose gel electrophoresis and ethidium bromide staining. qPCR was performed as above with all analyses in quadruplicate. See Supplementary Table S7 for qPCR primers.

For analyses of RNA binding to ZBP1, brain + spinal cord tissue from P5 mice was homogenized at 4°C in 50 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 150 mM NaCl, 1% NP-40, 5 mM MgCl_2 , 10 U/ml *SUPERase-In*TM (Ambion), plus protease inhibitors. RNA was isolated from an aliquot of each lysate and processed for RT-qPCR to test for input RNA levels. Remainder of each lysate was pre-cleared with protein A agarose (Roche), and then incubated for 3 h at 4°C with protein A agarose loaded with 4 μg of anti-ZBP1 or IgG control (Jackson ImmunoRes). Immunocomplexes were collected and stringently washed. One third of the immunocomplex was used for SDS/PAGE and immunoblotting for ZBP1; the remainder of the immunocomplex was used for RNA extraction with *Trizol* (Gibco) and processed for RT-qPCR. For each ZBP1 immunoprecipitation, C_t values were normalized to the 10% input for the corresponding mRNA.

Immunostaining

Unless otherwise indicated, all steps were performed at room temperature. For tissue sections, samples were immersion fixed in 4% paraformaldehyde for 2–4 h, cryoprotected overnight in 30% sucrose at 4°C, and then cryosectioned. Sections were warmed to room temperature, washed in PBS, and incubated in 20 mM glycine for 30 min followed by 0.25 M NaBH_4 for 30 min. For cultures, coverslips were fixed in 4% paraformaldehyde for 20 min and then rinsed in PBS. IF was then preformed as described (Willis *et al*, 2005). The following antibodies were used: rabbit anti-Peripherin (1:600; Millipore); chicken anti-NFH (1:1000; Millipore); mouse anti-NF (1:1500 each NFL, NFM and NFH; Sigma); rabbit anti-CGRP (1:1000; Millipore/Chemicon); mouse anti-GAP-43 (1:100; Millipore); rabbit anti-GAP43 (1:1000; Millipore); rabbit anti-PGP9.5 (1:1500; Millipore/Chemicon); rabbit anti- $\text{Na}_v1.8$ (1:500; Millipore); *Alexa*⁵⁴⁶ goat anti-chicken (1:1000; Invitrogen); and *Alexa*⁵⁵⁵ goat anti-rabbit (1:1000; Invitrogen). In some experiments, 1:300 dilution of the *FluoroMyelin Red* (Invitrogen) was used per manufacturer's instructions.

In situ hybridization

FISH/IF to detect axonal mRNAs and proteins was performed with digoxigenin-labelled oligonucleotide probes as described (Willis *et al*, 2007). The probe sets are listed in Supplementary Table S7. Fluorescent signals were detected by epifluorescence. All images sets from individual experiments were matched for exposure, gain, and offset. *ImageJ* was used to quantify FISH signals (pixels/ μm^2) in the cell body or the distal 200 μm of the axon. Negative controls for these FISH analyses consisted of scrambled digoxigenin-labelled oligonucleotides; images for individual experiments were normalized to the negative control and reported as pixels/ μm^2 above this threshold level. For cell body analyses, DRG cultures were depleted of Schwann cells by Percoll gradient.

Quantitation of neuronal and axonal morphology

Morphology of the axons was analysed in immunostained cultures. Images of random neurons were collected and blinded for analyses. The mean axon length was obtained by measuring the longest axon per neuron using *ImageJ*. Axon branching and number of processes were manually scored. Growth cone area was calculated using *ImageJ* from merged NFH + peripherin and DIC images. For injury-conditioned DRG cultures, morphology was analysed at 1 DIV. For naive DRG cultures, morphology was assessed at ≥ 3 DIV to allow for expression of AV or plasmid constructs.

For analyses of crushed nerves, sections were immunostained for axonal proteins and then co-stained with *Fluoromyelin Red* and DAPI. For ZBP1^{+/-}/Thy1-YFP mice, YFP was used to identify axons. Matched exposure images were captured proximal and distal to the crush site. Montages of each nerve were constructed to encompass ± 1.5 mm from injury site. PGP9.5- or YFP-positive axons were counted in every fourth longitudinal section of the sciatic nerve using NeuroLucida (MBF Biosciences) and total counts were calculated using the line transect method at -1.0, -0.5, 0, 0.5, and 1.0 mm from the centre of the crush site (Hill *et al*, 2001). Some images were evaluated using a 'thresholding method'. For this, montage images for axonal markers were processed for thresholding (0–50) using *ImageJ* and mean pixel intensity per area was then measured in 100 μm bins. 'Regeneration front' was determined as bin relative to crush where signal thresholds were 50% of the proximal intact nerve.

For analysis of regeneration in nerve transection/graft experiments, whole mount tissues were used. For the transgenic mice, GAP-43 immunostaining was used to detect axons; negative controls consisted of a GAP-43-stained naive nerve and grafts processed without addition of primary antibody. For the ZBP1^{+/-}/Thy1-YFP mice, linear YFP profiles in the acellular graft were counted. Whole mount nerves were imaged confocally with Z-stacks captured at 10 μm intervals. Montages of the nerve/graft were then analysed using *Imaris X64* (Bitplane) at designated intervals from the proximal host-graft junction.

G-ratio and axonal diameters were calculated from images of toluidine blue-stained epon sections using *Image Track* software (<http://www.ucalgary.ca/styslab/imagetrack>). Average neurons/ganglion were calculated from every fourth section of L4/5 DRGs in NF + peripherin-stained sections using *Velocity* software (Improvision). Density of CGRP-positive nerve fibres in the

footpads of transgenic mice was determined from transverse sections of hindlimb footpad over five sections per mouse.

Statistical analyses

Student's *T*-tests were used to compare the means of two independent groups. When three or more independent groups were present, an one-way ANOVA, followed by a Tukey's *post hoc* test was performed. To compare non-parametric distributions, a Wilcoxon's rank-sum test of frequency distributions was used. To compare differences between the linear distributions for axonal length and GFP intensity, an analysis of covariance (ANCOVA) was performed.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: CJD, DEW, and MX performed the analyses of RNA transport and axonal regeneration. CT and SOK performed behavioural analyses of transgenic mice. CJ and AE performed physiological analysis of mice. SY analysed axotomy responses. NCS and CBK-S provided guidance on mouse cross-breeding and analyses. JvM performed morphometric analysis of peripheral nerves. GJB and JLT oversaw experimental design and implementation.

Conflict of interest

The authors declare that they have no conflict of interest.

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