ATF3 promotes regeneration of the central axon branch of sensory neurons but the addition of Smad1, c-Jun and STAT3 does not exert synergistic effects.

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Abstract
Successful axon regeneration after nerve injury is accompanied by the upregulation of hundreds of regeneration-associated genes (RAGs), including a number of transcription factors (TFs) which may be key regulators of the RAG program. We aimed to stimulate the RAG program in rat dorsal root ganglion (DRG) neurons by adeno-associated viral (AAV) vector-mediated expression of TFs, in the absence of a peripheral nerve lesion. Of the known RAG TFs, ATF3, Smad1, STAT3 and c-Jun have been functionally linked to successful axonal regeneration in the peripheral nervous system, and are known to interact functionally and physically. We hypothesised that TF expression would promote regeneration of the central axon branch of DRG neurons and that simultaneous overexpression of multiple regeneration-associated TFs would lead to greater effects than delivery of a single TF. We overexpressed either the combination of ATF3, Smad1, STAT3 and c-Jun with farnesylated enhanced green fluorescent protein (eGFPf), ATF3 only with eGFPf or eGFPf only in DRG neurons and assessed axonal regeneration after dorsal root transection or dorsal column injury and functional improvement after dorsal root injury. Histological analysis shows that both ATF3 alone and the combination of TFs promoted faster regeneration in the injured dorsal root. Surprisingly, however, the combination did not perform better than ATF3 alone. Neither treatment was able to induce functional improvement on sensory tests after dorsal root injury. Neither ATF3 nor the combination of factors promoted sprouting or regeneration in a dorsal column injury model. In conclusion, overexpression of the regeneration-associated TF ATF3 in DRG neurons promotes regeneration of injured dorsal root axons in the absence of a conditioning lesion, but a combination of four TFs, including ATF3, used here did not result in synergistic effects.

Introduction
The primary sensory neuronal cell bodies of the PNS reside in the dorsal root ganglia (DRG) and have one peripheral axonal branch and another that extends to the spinal cord. Spontaneous regeneration takes place after injury to the peripheral axons, while regeneration of the central axon branches is limited after injury. Interestingly, a lesion of the peripheral branch potentiates regeneration of the central axon branches of the injured ganglia (Chong et al., 1999; Neumann and Woolf, 1999; Oudega et al., 1994; Richardson and Issa, 1984; Richardson and Verge, 1987). This so-called conditioning lesion effect is thought to be caused by the upregulation of a wide variety of RAGs in the DRG neuronal cell bodies, for example GAP43 and Cap23 [reviewed in (Hoffman, 2010)]. The cell body response also includes the up-regulation or post-translational activation of a number of regeneration-associated transcription factors (TFs), such as ATF3 (Seijffers et al., 2006; Seijffers et al., 2007), c-Jun (Broude et al., 1997; Raivich et al., 2004), STAT3 (Qiu et al., 2005), Smad1 (Parikh et al., 2011; Zou et al., 2009), SOX11 (Jankowski et al., 2006; Jankowski et al., 2009), CREB (Gao et al., 2004), P53 (Di Giovanni et al., 2006; Di Giovanni et al., 2007), and others. These transcription factors are known to promote axonal regeneration and functionally interact, providing a mechanism for the combination effect observed in the conditioning lesion model.
2005; Di Giovanni et al., 2006), NFIL3 (Macgillavry et al., 2009; Stam et al., 2007), C/EBPbeta (Nadeau et al., 2005), C/EBPdelta (de Heredia and Magoulas, 2013), NFkB (Ma and Bisby, 1998; Pollock et al., 2005), NFATs (Nguyen et al., 2009), and several KLF family members (Blackmore et al., 2012; Moore et al., 2009). These regeneration-associated TFs are likely to be key molecules that coordinate the expression of the RAG program and therefore are interesting candidates to activate the regeneration program in injured CNS neurons targeting long distance axon regeneration (Moore and Goldberg, 2011; Van Kesteren et al., 2011; Quadrato and Di Giovanni, 2013).

The expression of ATF3, c-Jun, STAT3, and Smad1 has been linked to axon growth in a number of studies. Upregulation of ATF3, c-Jun, STAT3, and Smad1 has been observed in regenerating neurons after peripheral nerve injury, in contrast to neurons of the central nervous system that are unable to regenerate (Broude et al., 1997; Macgillavry et al., 2009; Stam et al., 2007; Zou et al., 2009; Schwaiger et al., 2000). Furthermore, intervention studies where the expression of these TFs is manipulated suggest that ATF3, c-Jun, STAT3, and Smad1 play a role in the regenerative response of neurons in vivo and in vitro (Bareyre et al., 2011; Parikh et al., 2011; Qiu et al., 2005; Raivich et al., 2004; Seijffers et al., 2006; Seijffers et al., 2007; Zou et al., 2009).

ATF3 is a member of the ATF/CREB family of TFs and binds to AP-1 and ATF/CRE motif on promoters of its target genes (Hashimoto et al., 2002; Hai et al., 1999; Wolfgang et al., 1997) which potentially include RAGs such as Heat shock protein 27 (Hsp27), Small proline-rich repeat protein 1A (SPRR1A) and c-Jun (Nakagomi et al., 2003; Seijffers et al., 2007). ATF3 expression levels are usually low in adult neurons but is strongly upregulated after axotomy (Isacsson et al., 2005; Seijffers et al., 2006; Takeda et al., 2000; Tsujino et al., 2000) [reviewed in (Hunt et al., 2012)]. Subpopulations of DRG neurons that fail to regenerate express ATF3 at much lower level than neurons that do regenerate (Reid et al., 2010). In vitro, knockdown of ATF3 reduces neurite outgrowth (Macgillavry et al., 2009) and viral delivery of ATF3 was shown to boost neurite outgrowth (Nakagomi et al., 2003; Pearson et al., 2003; Seijffers et al., 2006). Constitutive expression of ATF3 in transgenic mice resulted in enhanced sprouting of injured peripheral sensory axons, but no regeneration was observed in the spinal cord after dorsal column injury (Seijffers et al., 2007).

c-Jun forms a part of the AP-1 transcription factor complex and is partly regulated by phosphorylation by MAP kinases (Angel et al., 1988). Upregulation of c-Jun has been observed in regenerating neurons after peripheral nerve injury (Broude et al., 1997; Greer et al., 2011; Hull and Bahr, 1994; Jenkins and Hunt, 1991; Herdegen et al., 1991), and axotomy also leads to c-Jun phosphorylation (Lindwall et al., 2004; Kenney and Kocsis, 1998; Leah et al., 1991). In response to peripheral nerve injury, c-Jun drives the transcription of RAGs such as CD44, galanin, and a7b-1 integrin (Raivich et al., 2004). Defects in target re-innervation and functional recovery were seen in knock-out mice lacking c-Jun expression (Raivich et al., 2004).
Upregulation and activation by phosphorylation of STAT3 has been observed in regenerating neurons after peripheral, but not central axotomy and might be involved in retrograde signalling after injury (Lee et al., 2004; Qiu et al., 2005; Schwaiger et al., 2000). Blocking phosphorylation of STAT3 results in impairment of the conditioning lesion effect, decreased GAP-43 expression and reduced neurite outgrowth (Qiu et al., 2005). Repression of STAT3 by expression of SOCS3 inhibits axon growth (Miao et al., 2006). Another possible target gene of STAT3 is SPRR1A that has been shown to be upregulated upon delivery of CNTF, an upstream molecule of STAT3, in DRG neurons (Wu et al., 2007). Finally, overexpression of STAT3 in mice has been shown to enhance sprouting of ascending DRG axons after a dorsal column lesion (Bareyre et al., 2011).

Smad1 is a member of the Smad family of TFs which modulate the TFGβ/BMP signalling pathway (Heldin et al., 1997). It is phosphorylated upon stimulation by bone morphogenetic protein 2 (BMP2) and BMP4 and together with Smad4 forms a transcriptional regulator complex [reviewed in (Massague et al., 2005)]. Upregulation of Smad1 has been observed in regenerating neurons after peripheral nerve injury (Okuyama et al., 2007; Zou et al., 2009). Absence of Smad1 and inhibition of Smad1 signalling inhibits axonal growth in vitro, whereas activation of Smad1 signalling by BMP4 overexpression not only increased neurite outgrowth in vitro, but also stimulated axon sprouting and GAP-43 expression in the injured spinal cord in vivo (Parikh et al., 2011).

A convincing volume of evidence exists indicating that ATF3, c-Jun, STAT3, and Smad1 contribute to the intrinsic growth ability of injured neurons, yet alone they are not sufficient for long distance axonal regeneration. ATF3 and c-Jun can form heterodimers (Hai and Curran, 1991; Chu et al., 1994; Hsu et al., 1992) that bind to AP1 and CRE sites (Cai et al., 2000). c-Jun enhanced neurite elongation in PC12 and Neuro-2a cells when ATF3 is co-expressed (Pearson et al., 2003). c-Jun can also interact with STAT3 (Zhang et al., 1999) and synergistic promoter activation occurs when both STAT3 and c-Jun are present (Schuringa et al., 2001; Yoo et al., 2001). In addition, STAT3, ATF3 and c-Jun are cooperatively recruited in order to up-regulate damage-induced neuronal endopeptidase in a synergistic manner (Kiryu-Seo et al., 2008). Smad family members, in turn, have been shown to interact with AP1 complexes to synergistically regulate c-Jun promoter activity (Liberati et al., 1999; Wong et al., 1999; Zhang et al., 1998) and Smad1 can interact with STAT3 to form a transcriptional complex with CBP (Nakashima et al., 1999). These data suggest that ATF3, c-Jun, STAT3, and Smad1 may cooperate to control the expression of RAGs.

We hypothesized that combined overexpression of ATF3, c-Jun, STAT3, and Smad1 would induce a greater regenerative response upon injury than expression of a single TF (ATF3) or eGFPf control only. To test this hypothesis, we used a dual promoter AAV vector (Fagoe et al., 2013) to co-express each TF (ATF3, c-Jun, STAT3 or Smad1) and farnesylated enhanced GFP (eGFPf) in rat DRG. We examined regeneration of injured axons after dorsal root and dorsal column injury in the absence of a conditioning lesion.
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A

[Images showing fluorescence microscopy of different markers including ATF3, eGFP, βIII-tubulin, and merged images.]

B

Graph showing percentage of eGFP-positive neurons per dish at 10 days.

C

Graph showing percentage of eGFP-positive neurons per dish at 20 days.
Results

The left L4 and L5 DRG of adult Fisher 344 rats were injected with AAV vectors containing a dual promoter construct expressing eGFPf and one transcription factor, or eGFPf only. In group 1 an equal titre mixture of 4 AAV dual promoter vectors expressing ATF3, c-Jun, STAT3, or Smad1 and eGFPf was injected. In group 2 a vector expressing ATF3 and eGFPf, and in group 3 a dual vector expressing only eGFPf. In all cases the total titre of injected virus was matched at 4x10^{12} GC/ml. Animals were sacrificed 10 days, 20 days or eight weeks after dorsal root injury.

Overexpression of ATF3, Smad1, c-Jun and STAT3 in DRG

Double-labelling immunohistofluorescence was performed for each TF and eGFPf in the injected DRG. Expression levels of ATF3, c-Jun, STAT3, Smad1, and eGFPf were quantified and the number of eGFPf-positive cells co-expressing each TF determined. At the 10 day time point, co-expression of all four TFs occurred in the majority (65-75\%) of the eGFPf positive neurons in the TF combination group, compared to 15-30\% in the eGFPf control group (figure 1A). Both the ATF3-only and the combination group showed more TF co-expression of ATF3 (One Way ANOVA, overall p-value= 5.50E-05, with Dunnett’s posthoc test: combination vs eGFPf p=3.80E-05; ATF3 vs eGFPf p=2.33E-03) than the eGFPf-controls. The combination group showed also increased expression of c-Jun (p=1.11E-03, unpaired t-test), STAT3 (p=3.71E-02, unpaired t-test), and Smad1 (p=9.74E-10, unpaired t-test) compared to the eGFPf control group (figure 1B).

Figure 1 Overexpression and quantification of ATF3, Smad1, c-Jun and STAT3 in dorsal root ganglion neurons. Sections of dorsal root ganglia (DRG) from animals that received viral vector injections and dorsal root injury were used to quantify transduction rates and overexpression of TFs. (A) sections of DRG injected with the vector combination expressing ATF3/c-Jun/Smad1/STAT3 or vector expressing eGFPf-only processed for immunohistochemistry for TFs (red), eGFPf (green) and βIII-tubulin (blue). Clear overexpression of ATF3, c-Jun, Smad1 and STAT3 was observed 10 days after injury. Some endogenous expression was seen in the eGFPf controls. Scale bar: 50 µm. (B) Percentages of eGFPf-positive neurons that express each TF at 10 days after injury. For all four TFs there was significant overexpression observed. The ATF3-only groups also showed significant overexpression of ATF3. Error bars are SEM, n=10 DRG for combination group, n=10 DRG for ATF3-only group and n=12 DRG for eGFPf group representing five and six animals respectively, * P<0.05, *** P<0.001; ATF3: One Way ANOVA, overall p-value= 5.50E-05, with Dunnett’s posthoc test: combination vs eGFPf p=3.80E-05; ATF3 vs eGFPf p=2.33E-03; c-Jun: p=1.11E-03, unpaired t-test; STAT3: p=3.71E-02, unpaired t-test; Smad1: p=9.74E-10, unpaired t-test. (C) Percentages of eGFPf-positive neurons that express a TF at 20 days after injury. Expression rates of all 4 TFs was similar to at 10 days, and for STAT3 and Smad1 significant overexpression was observed. The ATF3-only group showed a significant increase ATF3 expression. However high levels of endogenous expression of ATF3 and c-Jun were observed in the eGFPf only group. Error bars are SEM, n=12 DRG representing six animals per group, ** P<0.01, *** P<0.001, ATF3: One Way ANOVA, overall p-value= 3.80E-03, with Dunnett’s posthoc test: ATF3 vs eGFPf p=1.80E-03; combination vs eGFPf n.s.; STAT3: p=7.19E-03, unpaired t-test; Smad1: p=6.79E-04, unpaired t-test.
At the 20 day time point, co-expression rates of the TFs were similar in the combination injected group, albeit slightly lower (55-60%), than at 10 days, but c-Jun and ATF3 expression were unexpectedly increased in the eGFPf-only group to similar levels. The ATF3-only injected DRG displayed significantly increased ATF3 co-expression in 75% of the eGFPf-positive neurons and in the TF combination a non-significant increase of 58% co-expression for ATF3 was observed (One Way ANOVA, overall p-value= 3.80E-03, with Dunnett’s posthoc test: ATF3 vs eGFPf p=1.80E-03; combination vs eGFPf n.s.). Co-expression of STAT3 (p=7.19E-03, unpaired t-test) and Smad1 (p=6.79E-04, unpaired t-test) in the TF combination group were again significantly greater than in the eGFPf only group (figure 1C).

**Transduction efficiency**
eGFPf is rapidly transported along the axon, which, while making it preferable to eGFP for long-distance axon labelling (Fagoe et al., 2013), means that it is harder to detect expression in the cell body. To reliably determine transduction rates we measured the percentage of eGFPf-positive fibres in the dorsal root we quantified the number of axons positive for eGFPf as a percentage of neurofilament-positive fibres in transverse sections of the proximal part of the L4 and L5 dorsal roots (figure 2A). Transduction efficiencies ranged between 23% and 35% and did not differ significantly between groups (figure 2B).

**Figure 2 Transduction rates of AAV dual promoter vectors in DRG neurons.** Transverse sections of dorsal roots from animals that received viral vector injections and dorsal root injury were used to quantify transduction rates (A) Transverse section of the proximal part of a dorsal root processed for immunohistochemistry for GFP (green) and neurofilament (red). Scale bar: 100 µm. (B) Quantification of eGFPf positive fibres in the proximal dorsal root for each time point. Percentages of neurofilament-positive axons that are eGFPf-positive are shown. Error bars are SEM, 10 days; n=10 roots for combination group and n=12 roots for eGFPf and ATF3 groups representing five and six animals respectively, 20 days; n=12 roots per group representing six animals per group, eight weeks; n=14 roots for combination group representing seven animals, n=16 roots for eGFPf group representing eight animals and n=10 roots for ATF3 group representing five animals.
Regeneration in the dorsal root

Lesion site
Four weeks after viral vector delivery the L3-L6 dorsal roots were transected and the L4 and L5 roots were re-ligated (figure 3A). Longitudinal sections of the lesion sites of the L4/L5 roots were immunostained for eGFPf (figure 3B) and neurofilament and regenerating axons were quantified at 10 and 20 days post lesion. As shown in figure 3C, at the 10 day time point overall significantly more axons were growing through the lesion site in both the ATF3 group and TF combination group compared to the eGFPf group (Linear Mixed Model, overall p-value p=0.01, with Tukey post-hoc test: ATF3 vs eGFPf p=0.02; combination vs eGFPf p=0.02). No difference was seen between the ATF3 group and the combination group. At 20 days post injury there were no significant differences observed between groups (figure 3D). As a control for the vector injection we included a group of non-injected animals and quantified the number of regenerating neurofilament-positive axons as we did for the eGFPf-positive axons at 10 days after dorsal root lesion. We observed no differences in neurofilament-positive axons compared to the eGFPf-control group, indicating that the vector injection itself has no effect on axon regeneration (figure 3E).

Distal dorsal root
Regeneration was also examined in transverse sections of the dorsal roots, which were taken at 3.5 mm and 7.5 mm distal and 2.5 mm proximal to the lesion and immunostained for eGFPf and neurofilament. Fibres positive for eGFPf were counted manually. As shown in figure 3F, at the 10 day time point, although the mean percentages of eGFPf-positive fibres reaching the distal nerve segments appeared higher in both groups that received TFs compared to the eGFPf control group, this was only significant in the overall test at 7.5mm (Generalized Least Squares; at 3.5mm distal n.s.; at 7.5mm p=0.02; Tukey post-hoc tests were n.s.). At 20 days and 8 weeks post injury there are no significant differences between groups (figures 3G, 3H).

Dorsal root entry zone (DREZ)
In GFAP immunostained sections of the DREZ at the L4/L5 level a clear astrocytic border was visible. Furthermore, eGFPf-positive axons were seen to reach and enter the DREZ in all groups, however none successfully entered the spinal cord. As shown in figure 4A, a quantification grid was laid over the DREZ based on the inner and outer border determined by GFAP labelling in order to quantify the number of eGFPf-positive axons entering the DREZ. The number of axons crossing the central line was normalized to the total number of eGFPf-positive axons counted in the proximal dorsal root. At the eight week time point, also the number of CTB-positive axons was counted. The number of CTB-positive axons was
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[Image of diagrams and graphs related to lesion and axon regeneration in spinal cord]

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normalized to the total number of 2H3 positive axons found in transversal sections of the proximal dorsal root. At the 10 day time point, there were no significant differences (by Generalized Least Squares) in the number of eGFPf-positive axons per section crossing the DREZ between the groups (figure 4B). Figure 4C shows that the number of eGFPf-positive fibres at the 20 day time point was significantly increased in the ATF3 and the TF combination groups compared to the eGFPf control group (Generalized Least Squares, overall test p=0.001, with Tukey post-hoc tests: ATF3 vs eGFPf, p<0.001; combination vs eGFPf, p=0.025; combination vs ATF3 n.s.). However, at 8 weeks, there were no significant differences observed in both eGFPf-positive (figure 4D) and CTB-positive (figure 4E) axon counts.

Functional testing

Recovery of sensory function was examined weekly during eight weeks following dorsal root injury, using tests of nociception. Directly after injury, sensitivity to nociceptive stimulation was severely reduced in all groups that received an injury, while sham animals remained at baseline levels. There were no differences between viral-vector treated groups in both response upon electrical (figure 5A) and heat stimulus (figure 5B). Autotomy was observed in some animals treated with TFs (figure 5C), and this was significantly more prevalent in the ATF3-only treated group while a non-significant increase was seen for animals in the TF combination-treated group (Kruskall-Wallis test for three independent groups, p=0.02; pairwise comparisons: ATF3 vs eGFPf, p=0.003, other comparisons n.s.).

Figure 3 Quantification of regeneration after dorsal root injury. (A) The L4 and L5 DRG were injected with AAV dual promoter vectors titre matched at 4 x 10^{12} GC/ml expressing both a TF (ATF3, c-Jun, STAT3, or Smad1) and eGFPf, ATF3 and eGFPf, or eGFPf alone. Four weeks later the L3-L6 dorsal roots were transected and the L4 and L5 dorsal roots were re-ligated. Animals were sacrificed for histology 10 days, 20 days or eight weeks after injury. (B) Quantification method for determining regeneration in longitudinal sections of the injured dorsal root. Grid lines are drawn at the specified distances and eGFPf fibres crossing the lines are counted. Scale bar: 250 µm. (C) At 10 days after injury the percentages of eGFPf-positive axons distal to the lesion in longitudinal sections of the dorsal root was significantly higher in the ATF3 group and combination group compared to the eGFPf control group. *P<0.05, Linear Mixed Model, overall p-value p=0.01, with Tukey post-hoc test: ATF3 vs eGFPf p=0.02; combination vs eGFPf p=0.02, error bars are SEM, n=5 animals for the combination group, n=6 animals for the ATF3/eGFPf and eGFPf-only groups. (D) No significant differences in the percentage of eGFPf-positive axons distal to the lesion in longitudinal sections of the dorsal root were observed 20 days after injury. Error bars are SEM, n=6 animals per group. (E) No differences in regenerating neurofilament-positive axons were found in the eGFPf group compared to a non-injected control group at 10 days after dorsal root injury. (F) At 10 days after injury the percentages of eGFPf-positive axons in transverse sections of the dorsal root at 7.5mm an overall difference was found. *P<0.05, Generalized Least Squares; at 3.5mm distal n.s.; at 7.5mm p=0.02; Tukey post-hoc tests were n.s.; error bars are SEM, n=10 roots for combination group and n=12 roots for eGFPf and ATF3 groups. (G) At 20 days after injury, a trend was observed for increased percentages of eGFPf-positive axons at both 3.5 mm and 7.5 mm distal. Error bars are SEM, n=12 roots for combination group, n=9 roots for eGFPf group and n=11 roots for ATF3 group. (H) At eight weeks post injury, there were no differences in eGFPf-positive fibres observed in cross sections of the distal dorsal root. Error bars are SEM, n=14 roots for combination group, n=16 roots for eGFPf group and n=10 roots for ATF3 group.
Figure 4 Quantification of regenerating axons at the dorsal root entry zone (DREZ). (A) A horizontal section of the L4/L5 spinal cord containing the DREZ processed for immunohistochemistry for GFAP (blue), CTB (red) and eGFPf (green). Quantification lines to count eGFPF-positive (10 day, 20 day and eight week time point) and CTB-positive (at eight weeks) axons. Scale bar:250 µm. (B) There were no differences observed in normalized percentages of eGFPF-positive axons at 10 days post injury. Error bars are SEM, n=6 animals for eGFPF group and n=5 for ATF3 and combination groups. (C) At 20 days after injury the normalized percentages of eGFPF-positive fibres were significantly increased in the ATF3 and combination group. * P< 0.05, Generalized Least Squares, overall test p=0.001, with Tukey post-hoc tests: ATF3 vs eGFPF, p<0.001; combination vs eGFPF, p=0.025; combination vs ATF3 n.s.; error bars are SEM, n=6 animals for eGFPF group and n=5 for ATF3 and combination groups. (D-E) At eight weeks post injury there were no differences observed in the normalized percentages of (D) eGFPF-positive and (E) CTB-positive axons. Error bars are SEM, n=8 animals for eGFPF group, n=5 animals for ATF3 group and n=7 animals for combination group.
Dorsal column injury
The effects of overexpression of ATF3 and the combination of TFs were also assessed in regeneration of ascending spinal axons after dorsal column injury. The left L4 and L5 DRG of adult Fisher 344 rats were injected with the AAV vector mix (expressing ATF3, c-Jun, STAT3, or Smad1 and eGFPf), AAV expressing ATF3 and eGFPf, or AAV expressing eGFPf alone. Animals received a dorsal column transection four weeks after vector injection. Transganglionic tracing of injured dorsal column axons using CTB was performed after a survival time of four weeks post injury.

The lesion site was visualized with GFAP immunolabelling and the ascending fibres were immunolabelled with eGFPf and CTB (figure 6A). We observed no differences in the maximum penetration distance of regenerating dorsal column axons (figure 6B). Quantification of double labelled eGFPf- and CTB-positive fibres showed that there were also no differences in die-back of injured axons caudal to the lesion (figure 6C).

**Figure 5** Recovery of function during eight weeks following dorsal root injury and delivery of viral vectors expressing transcription factors or eGFPf. (A) Functional assessment using the foot-flick test (an electric nociceptive stimulus). After dorsal root injury animals did not react upon the maximum electrical stimulus of 0.5 mA. There were no differences observed between treatment groups. Error bars are SEM, n=9 per group at start of experiment. (B) Functional assessment using the Hargreaves test (thermal nociceptive stimulus). The reaction delay upon noxious heat stimulus was also not significantly different between groups. Error bars are SEM, n=9 per group at start of experiment. (C) Autotomy scores for ATF3-only treated animals were significantly increased compared to eGFPf-only treated animals, while scores in the combination group were higher but not significantly so. * P< 0.05, Kruskall-Wallis test for three independent groups, p=0.02; pairwise comparisons: ATF3 vs eGFPf, p=0.003, other comparisons n.s.; error bars are SEM, n=9 per group.
Figure 6 Quantification of regenerating ascending spinal axons after dorsal column injury. The L4 and L5 DRG were injected with AAV dual promoter vectors titre matched at $4 \times 10^{12}$ GC/ml expressing both a TF (ATF3, c-Jun, STAT3, or Smad1) and eGFP, ATF3 and eGFP, or eGFP alone. Four weeks later the dorsal column was transected and animals were traced using CTB four weeks after injury and sacrificed for histology three days later. (A-C) Sections were processed for immunohistochemistry for GFAP (blue), CTB (red) and eGFP (green). Representative section of the dorsal column of (A) an eGFP control animal, (B) an ATF3 treated animal and (C) an animal treated with the combination of TFs. Scale bar: 250 µm. (D) Quantification of the maximum distance of lesion penetration by regenerating axons, measured from the lesion border, show no differences between groups. Error bars are SEM, n=4 for eGFP, n=5 for ATF3 and combination groups. (E) Quantification of double labelled eGFP- and CTB-positive axons in the dorsal column. There were no differences between groups observed in the number of dorsal column axons caudal to the lesion. Error bars are SEM, n=4 for eGFP, n=5 for ATF3 and combination groups.
**Discussion**

In this study we aimed to enhance the regenerative response of injured DRG neurons upon injury by delivery of regeneration-associated TFs. We injected AAV5 dual promoter vectors expressing eGFPf alone, eGFPf and ATF3 or a mixture of vectors expressing eGFPf with ATF3, c-Jun, STAT3, and Smad1 into rat DRG. Regenerative growth of the central branch of DRG neurons and ascending dorsal columns was assessed after dorsal root or dorsal column injury in the absence of a conditioning lesion. We hypothesized that combined overexpression of multiple regeneration-associated TFs would be more efficient at enhancing axon growth compared to ATF3 alone. We observed increased numbers of regenerating axons in animals that expressed ATF3 or multiple regeneration-associated TFs (ATF3, c-Jun, STAT3 and Smad1) compared to control animals at 10 days post injury, while at 20 days after injury these differences occurred more distally, namely in the number of axons reaching the DREZ. Finally, eight weeks after injury there were no differences between groups. Taken together, our results demonstrate that overexpression of ATF3 and combinatorial overexpression of ATF3, c-Jun, STAT3 and Smad1 leads to an increase in speed of regeneration of injured dorsal root axons. Surprisingly, however, delivery of multiple regeneration-associated TFs was not more effective than expressing ATF3 alone.

As mentioned, ATF3, c-Jun, STAT3, and Smad1 have individually been shown to contribute to regenerative axon growth of injured DRG neurons. Improved regeneration of the peripheral branch of DRG neurons by ATF3 was reported in transgenic mice overexpressing ATF3 (Seijffers et al., 2007). In our study we show for the first time that ATF3 overexpression also leads to accelerated regrowth of the central branch of DRG neurons. ATF3 expression was insufficient to promote regeneration of injured dorsal column axons in the transgenic mouse model, consistent with our findings. Both Smad1 and STAT3 individually have previously been shown to enhance axon sprouting of injured ascending sensory axons (Bareyre et al., 2011; Miao et al., 2006; Parikh et al., 2011). Overexpression of STAT3 in DRG neurons resulted in a rapid but short-lived increase of axonal sprouting of ascending dorsal column axons (Bareyre et al., 2011). Bareyre and colleagues report differences in speed of axon growth at a very early time point (2 to 4 days after injury), while no continuation of this effect was seen at a later time point of 4 to 10 days post injury. We have looked at relatively late time points after injury, by which time such short-lived effects on axon growth would no longer be visible. Activation of Smad1 by AAV-mediated BMP4 overexpression was shown to increase regeneration of injured ascending sensory axons four weeks after dorsal column transection (Parikh et al., 2011). In our study we have used a constitutively activated form of Smad1 (Smad1-EVE), which is active independent of BMP signalling (Fuentealba et al., 2007). However, we did not observe increased axon growth of injured dorsal column axons by overexpression of the combination of regeneration-associated TFs, which included this constitutively active form of Smad1. Delivery of BMP4 leads to Smad1 activation in transduced DRG neurons.
but may also have additional beneficial effects. For example, BMP4 has shown to promote increased survival of embryonic motor neurons (Chou et al., 2013) and enhances plasticity of sensory axons in the adult female reproductive tract (Bhattacherjee et al., 2013). In addition AAV-based delivery of the secreted ligand BMP4 may also influence surrounding non-transduced neurons in a paracrine fashion (Parikh et al., 2011), thereby enhancing its beneficial effect.

Conversely to our expectations, we did not find synergistic effects upon delivery of multiple regeneration-associated TFs compared to overexpression of a single TF, for which there may be several explanations. In order to get titre-matched vector injections between groups the setup of this experiment was such that the TF combination group contained a quarter of the titre of ATF3-expressing vector compared to the ATF3 only group. This may have resulted in somewhat lower expression of each TF compared to the ATF3 only group. However, delivery of ATF3 alone or the combination of ATF3, c-Jun, STAT3, and Smad1 clearly resulted in increased expression of these factors in the single-ATF3 and the combination group respectively, as compared to eGFP-only controls measured at 10 days post injury. Interestingly, we observed a delayed endogenous upregulation of ATF3, c-Jun in the eGFP control group at 20 days after dorsal root injury. Previous studies of gene expression after dorsal root injury indicate that the expected response is a weak and short-lived upregulation of these factors which declines by 1 week after injury. (Broude et al., 1997;Macgillavry et al., 2009;Stam et al., 2007) so it is not clear why we saw a delayed increase in expression in our model. This endogenous expression in response to dorsal root injury may have diminished potential differences in axonal growth between the eGFP group and the TF combination group. Another reason for the lack of synergistic effects of multiple TF overexpression could be that mixing four viruses leads to suboptimal co-transduction of DRG neurons leading to a mixed population of neurons expressing one, two, three or all four TFs. We observed that the majority of eGFP-positive neurons co-expresses a TF, suggesting that many transduced neurons express all four TFs. A solution may be to use the 2A-peptide system, which enables efficient multicistronic vector design and co-delivery of multiple transgenes (Ryan et al., 1991;Ryan and Drew, 1994;Donnelly et al., 2001). However, this was not possible for these four TFs as the transgenes together with eGFP would be too large to fit in an AAV vector.

Many TFs need to be activated by co-factors to become functional. While we have overexpressed activated forms of STAT3 and Smad1, wild type c-Jun was used in this study. As mentioned, c-Jun can be activated by phosphorylation which occurs after axotomy (Lindwall et al., 2004;Kenney and Kocsis, 1998;Leah et al., 1991). In vitro studies suggest that phosphorylation of c-Jun might be important for neurite outgrowth of DRG neurons (Lindwall et al., 2004). Therefore overexpression of wild type c-Jun in this study could possibly be a reason why we did not see synergistic effects of simultaneous overexpression of ATF3, c-Jun, Smad1 and STAT3. This seems unlikely, however, since phosphorylation of c-Jun
has not shown to be functionally crucial for axon regeneration in vivo (Ruff et al., 2012; Brecht et al., 2005).

Finally, the combination of ATF3, c-Jun, STAT3, and Smad1 may not be the key regulators of the RAG program. As mentioned, many other TFs are associated with successful axon regeneration, including CREB, SOX11, P53, NFIL3, C/EBPbeta, C/EBPdelta, NFkB, NFATs, and several KLF family members. For example, SOX11 promotes nerve regeneration through activation of the RAG Sprr1a (Jing et al., 2012). CREB activation was shown to be sufficient to overcome inhibitors in myelin and promotes spinal axon regeneration in vivo (Gao et al., 2004). Blackmore and colleagues have shown that transcriptional activation of Kruppel-like Factor 7 (KLF7) promotes axon regeneration in the adult corticospinal tract (Blackmore et al., 2012). It is clear that there is a wide variety of potential key TFs that may be in control of the RAG program. Thus it may be that other TFs are in control of the RAG program.

Some potential target genes of ATF3, c-Jun, STAT3 and Smad1 are known RAGs, for example ATF3 drives expression of Hsp27 and SPRR1A (Nakagomi et al., 2003; Seijffers et al., 2007), c-Jun may induce CD44, galanin, a7b-1 integrin (Raivich et al., 2004), STAT3 may regulate SPRR1A and galanin (Bacon et al., 2007; Wu et al., 2007) and smad1 may be upstream of galanin and possibly GAP43 (Bacon et al., 2007; Parikh et al., 2011). Therefore it could be that overexpression of ATF3, c-Jun, STAT3 and Smad1 induces merely partial activation of the RAG program, leading to faster initiation of axon regeneration, however combinatorial expression fails to maintain this regenerative response needed for long distance axon growth. Another possibility is that these TFs are important for supporting more general processes that occur in an injured neuron and that do not directly influence axon regeneration, perhaps by boosting overall gene expression or increasing cell metabolism in response to stressful stimuli. For example, STAT3 is activated upon brain ischemia [reviewed in (Dziennis and Alkayed, 2008)]; c-Jun expression is observed following traumatic events such as brain ischemia and seizures, exposure to neurotoxic chemicals like MPTP and in a number of neurodegenerative diseases [reviewed in (Raivich, 2008)]; Smad1 is activated upon DNA damage by genotoxic drugs in mouse embryonic fibroblasts and stem cells to regulate cell proliferation and survival (Chau et al., 2012). Therefore these TFs might be involved in activating more general compensatory molecular mechanisms to cope with stressful stimuli and thereby support axon regeneration.

Animals from the ATF3 group displayed an increased tendency towards autotomy. It is unclear what molecular mechanisms are responsible for the development of autotomy in our study, but since autotomy has been associated with pain behaviour one explanation could be that ATF3 stimulates the expression of substance P and other neuropeptides resulting in inflammation. The development of inflammation, which was observed in the majority of animals with autotomy, could then be a stimulus for autotomy behaviour in ATF3 treated animals in this experiment.
In this study, no axons were found to successfully grow into the spinal cord. However, AAV-mediated overexpression of α9 integrin in DRG neurons in vivo promoted injured dorsal root axons to grow into the spinal cord (Andrews et al., 2009). Furthermore, forced expression of Kindlin-1, a downstream molecule of integrins, in DRG neurons also enhanced outgrowth of injured dorsal root axons and sprouting into the spinal cord (Tan et al., 2012). A study in which retinoic acid receptor β2 was expressed in DRG neurons showed that injured dorsal root axons were able to cross the DREZ and resulting in functional improvement of sensorimotor tasks (Wong et al., 2006). These studies show that regrowth of injured dorsal root axons into the spinal cord certainly is possible using neuron-intrinsic approaches.

In conclusion, this study shows for the first time that ATF3 overexpression enhances axon regeneration of the central branch of DRG neurons. Although, no synergistic effects were found after simultaneous overexpression of ATF3, c-Jun, STAT3 and Smad1 compared to ATF3 alone, we demonstrate that regeneration of injured dorsal root axons can be enhanced by expression of regeneration-associated TFs in the absence of a conditioning lesion.

Materials & methods

Experimental animals and surgical procedures

In this study, a total of 102 Fisher 344 rats (180-250 g, Harlan, Horst, The Netherlands) were used. Animals were housed under standard conditions with food and water ad libitum, and a 12-hour: 12-hour light/dark cycle. All experimental procedures and postoperative care were carried out with approval from the local animal experimentation ethical committee. The direct DRG injection procedure was performed as described previously (Mason et al., 2010). Viral vectors based on AAV serotype 5 were injected into the left L4 and L5 DRG of six animals per group for the early time points and nine animals per group for the eight week time point. The ATF3-group and the eGFPf-group received DRG injections with dual vectors containing ATF3 and eGFPf (4.0x10^{12} GC/ml) or eGFPf only (4.0x10^{12} GC/ml), respectively. One group received DRG injections containing a mixture of four vectors expressing ATF3 (1.33x10^{12} GC/ml), c-Jun (1.33x10^{12} GC/ml), Smad1 (1.33x10^{12} GC/ml) and STAT3 (1.33x10^{12} GC/ml). All vectors, except STAT3, are dual promoter vectors co-expressing eGFPf. The total titre of eGFPf-expressing vectors in this group is therefore 4.0x10^{12} GC/ml. At the short time points we included a non-injected control group and for the eight week time point, which was used for functional testing, a sham rhizotomy control group with no viral vector injection was added. Four weeks later, animals were anaesthetized with isoflurane and the left L4 and L5 dorsal roots were exposed at L3 level. The dorsal roots were transected and immediately re-ligated using 10-0 sutures. The muscles overlying
the spinal cord were loosely sutured together and the wound was closed. For the dorsal column injury experiment a laminectomy was performed after which a C4 lesion was made using microscissors. Animals were allowed to recover at 37 °C and received postoperative analgesia (Temgesic 0.1mg/kg body weight s.c.; Schering-Plough). Survival times after injury were 10 days, 20 days, or eight weeks. Three days before perfusion, animals of the eight week time point and the dorsal column injury group received 1% unconjugated CTB (103B, List Laboratories Inc., Campbell, CA) into the sciatic nerve to transganglionically label dorsal root axons. Animals were injected with a lethal dose of pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 mol/l phosphate buffer pH 7.4. Tissue was post-fixed for 3–4 hours, transferred to 30% sucrose in phosphate-buffered saline, and frozen in Tissue-Tek OCT (4583; Sakura Finetek Holland, Zoeterwoude, the Netherlands) the following day.

Plasmids
The dual promoter plasmid pAGLWFI has been described (Fagoe et al., 2013). The MCS of pAGLWFI was used to insert each of ATF3, cJun and Smad1r. For AAV-dual-eGFPf/ATF3 the rat full length coding sequence of ATF3 was used from IMAGE clone 7100767; for AAV-dual-eGFPf/c-Jun the rat full length coding sequence of c-Jun was used from IMAGE clone 7124370; for AAV-dual-eGFPf/SMAD1 the constitutively active form of human Smad1 was used from pCS2-hSmad1-EVE (22993; Addgene); For AAV-sCAG-eGFPf/STAT3 the constitutively active form of STAT3 was used from pMXs-Stat3-C (13373; Addgene). Because the STAT3 ORF was too large fit in pAGLWFI a STAT3 construct was used containing the ITRs of AAV2 flanking the sCAG promoter, followed by STAT3, a WPRE and the BgH polyadenylation signal.

Production of viral particles
The production of AAV serotype 5 particles was performed as described (Fagoe et al., 2013). All vector stocks were kept at −80 °C until use. Titres were determined by quantitative PCR for viral genomic copies extracted from DNase-treated viral particles. Titres were in the range of 2.2 x 10^{12} GC/ml to 1.1 x 10^{13} GC/ml.

Immunohistochemical procedures
DRG were cut 20 µm thick on a cryostat and placed onto Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany). Rabbit polyclonal anti-ATF3 (1:400; SC-188, Santa Cruz), rabbit polyclonal anti-c-Jun (1:200; SC-1694, Santa Cruz), rabbit polyclonal anti-STAT3 (1:400; SC-482; Santa Cruz) or rabbit monoclonal anti-Smad1 (1:1000; clone EP565Y, Millipore) primary antibodies were used to visualize ATF3, c-Jun, STAT3 or Smad1, respectively, in every twelfth section of the DRG. Sections were co-stained for eGFPf and βIII-tubulin using chicken anti-GFP (1:1000; AB16901; Millipore) and mouse anti-βIII-tubulin (1:500; clone TuJ1; Covance),
followed by donkey anti-rabbit-Alexa594 (1:600; Jackson Immunoresearch), biotinylated goat anti-chicken (1:300; Vector Labs) and donkey anti-mouse DyLight649 (1:600; Jackson Immunoresearch), and finally streptavidin-Alexa488 (1:400; Jackson Immunoresearch).

Dorsal roots were cut into segments based on the location of the surgical suture at the lesion side. Longitudinal sections of the lesion site were cut 20 μm thick and placed onto Superfrost Plus slides (Menzel-Gläser). These segments comprised -2 mm to 3 mm from the lesion site. One millimetre pieces of dorsal root were taken from -3 to -2 mm, 3 to 4 mm and 7 to 8 mm and transverse sections taken at 20 μm thickness onto Superfrost Plus slides (Menzel-Gläser). Lumbar spinal cords were also cut 20 μm thick and placed onto Superfrost Plus slides (Menzel-Gläser). For detection of eGFPf every sixth section of the dorsal root lesion sites and every second section of the lumbar spinal cords was stained with rabbit anti-GFP (1:15,000; ab290; Abcam), followed by biotinylated horse anti-rabbit (1:300; Vector Labs), ABC reagent (1:200; Vector Labs), then washed in TBS containing 0.05% Tween 20 (P137-9; Sigma Aldrich) and incubated in biotinyl tyramide reagent (1:400; NEL700A001KT; PerkinElmer) in TBS containing 0.001% H2O2, followed by streptavidin-Cy3 (1:400; Jackson Immunoresearch). Dorsal root and lumbar spinal cord sections were co-immunostained with mouse anti-neurofilament (1:500; 2H3; Dev. Stud. Hybridoma Bank, Univ. of Iowa) and mouse anti-GFAP (1:4000; G3893; Sigma), respectively, followed by donkey anti-mouse-Cy3 (1:600; Jackson Immunoresearch).

For the 8 week time point and the dorsal column experiment, CTB-traced spinal cords were sectioned at 20 μm thickness and stained with goat anti-CTB (1:80.000; 703, List Laboratories Inc.), followed by biotinylated horse anti-goat (1:300; Vector Labs), ABC reagent (1:200; Vector Labs), then washed in TBS containing 0.05% Tween 20 (P137-9; Sigma Aldrich) and incubated in biotinyl tyramide reagent (1:400; NEL700A001KT; PerkinElmer) in TBS containing 0.001% H2O2, followed by streptavidin-Cy3 (1:400; Jackson Immunoresearch). The sections were then blocked with Avidin/Biotin solution and co-immunostained with rabbit anti-GFP (1:15.000; ab290; Abcam) and mouse anti-GFAP (1:4000; G3893; Sigma), followed by horse anti-rabbit biotin (1:300; Vector Labs), and donkey anti-mouse Alexa647 (1:600; Jackson Immunoresearch). The sections were washed again in TBS containing 0.05% Tween 20 (P137-9; Sigma Aldrich) and incubated in biotinyl tyramide reagent (1:400; NEL700A001KT; PerkinElmer) in TBS containing 0.001% H2O2, washed and finally incubated with streptavidin-Alexa488 (1:400; Jackson Immunoresearch).
**Histological analysis**

Every section was photographed at fixed exposure settings at ×10 magnification on an Axioplan microscope (Zeiss). Image analysis and quantification of DRG based on nuclear fluorescence intensity after immunostaining for a TF and GFP were performed in ImagePro Plus (Media Cybernetics) as previously described (Mason et al., 2010).

Longitudinal sections of the lesion site in dorsal roots were stained for eGFPf and neurofilament as described above. Using ImagePro Plus software a grid with counting lines at fixed intervals was laid over the photomicrographs by a blinded experimenter and either eGFPf or neurofilament positive axons that crossed each grid line were counted. Regenerated axon counts distal to the lesion were expressed as percentages of the proximal axon counts.

Transverse sections of the dorsal root were stained for eGFPf and neurofilament as described above. With an algorithm in ImagePro Plus software axons in the red channel were identified based on roundness and size. For classification as a neurofilament positive axon, a threshold of 2× the background level was chosen. For quantification of eGFPf positive axons a counting grid was laid over the photomicrographs and eGFPf positive axons were manually counted by a blinded experimenter at 400% zoom. Regenerated axon counts distal to the lesion were expressed as percentages of the proximal axon counts.

The dorsal root entry zone (DREZ) was visualized in sections of the lumbar spinal cord by staining for GFAP. Two lines were drawn to delineate the DREZ, one to mark the furthest continuous extent into the dorsal root of GFAP-positive astrocytes and one to mark the furthest continuous extent towards the spinal cord of GFAP-negative Schwann cells. A third line for measurement of the crossing fibres was placed equidistant between them (see figure 4B). eGFPf labelled axons crossing the middle line, were counted by a blinded experimenter. For the eight week time point, eGFPf and CTB labelled axons in the DREZ were counted separately and axon counts in the DREZ were expressed as percentages of the proximal axon counts in dorsal roots. Some images of the DREZ showed eGFPf positive astrocytes. These astrocytes had likely been transduced due to virus that travelled along the dorsal root. Animals that showed eGFPf positive astrocytes were excluded from the analysis.

In images of spinal cord sections with a dorsal column injury we visualized the lesion border by GFAP staining. We assessed the maximum distance of regenerating axons that were double positive for eGFPf and CTB from the lesion border. Furthermore, a counting grid with 250 µm spacing starting from the lesion border was laid over the images to assess the number of double labelled axons caudal to the lesion. The number of axons at each distance was normalized to the count at 750µm caudal to the lesion border.
Functional tests
Recovery of sensory function was tested weekly for eight weeks by blinded experimenters using the Hargreaves plantar test (Hargreaves et al., 1988) and the footflick test (De Koning et al., 1986) in animals that received a dorsal root injury. Animals were tested for two weeks prior to lesion to habituate them to the tests. The Hargreaves plantar test measures the withdrawal time of a paw after a noxious heat stimulus. Animals were unrestrained and placed in a box on top of a glass plate. An infra-red source is applied to the hind paw until withdrawal, and the time to withdrawal automatically recorded. The heat source intensity was adjusted so that withdrawal time was approximately 10 seconds for uninjured animals. The stimulus was applied for a maximum of 20 seconds. Three measurements were taken for each animal. In the foot flick test animals were restrained and an increasing electrical stimulus applied to the left hind paw from 0.1 to 0.5 mA until a withdrawal reflex occurred. Autotomy scores were assessed weekly for each animal, which ranged from 1 to 5 with increasing severity (1 - one toe partially affected; 2 - one toe fully affected; 3 - multiple toes affected; 4 - foot pad affected; 5 - complete paw affected).

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CHAPTER 5


