# Krox-20 inhibits Jun-NH₂-terminal kinase/c-Jun to control Schwann cell proliferation and death

**David B. Parkinson, Ambily Bhaskaran, Anna Droggiti, Sarah Dickinson, Maurizio D'Antonio, Rhona Mirsky, and Kristjan R. Jessen**

Department of Anatomy and Developmental Biology, University College London, London WC1E 6BT, UK

The transcription factor Krox-20 controls Schwann cell myelination. Schwann cells in Krox-20 null mice fail to myelinate, and unlike myelinating Schwann cells, continue to proliferate and are susceptible to death. We find that enforced Krox-20 expression in Schwann cells cell-autonomously inactivates the proliferative response of Schwann cells to the major axonal mitogen β–neuregulin-1 and the death response to TGFβ or serum deprivation. Even in 3T3 fibroblasts, Krox-20 not only blocks proliferation and death but also activates the myelin genes periaxin and protein zero, showing properties in common with master regulatory genes in other cell types. Significantly, a major function of Krox-20 is to suppress the c-Jun NH₂-terminal protein kinase (JNK)–c-Jun pathway, activation of which is required for both proliferation and death. Thus, Krox-20 can coordinately control suppression of mitogenic and death responses. Krox-20 also up-regulates the scaffold protein JNK-interacting protein 1 (JIP-1). We propose this as a possible component of the mechanism by which Krox-20 regulates JNK activity during Schwann cell development.

## Introduction

Myelination in rodent nerves starts around birth, when unidentified axonal signals trigger a program of differentiation in Schwann cells that results in the generation of the myelin sheath, one of the most highly specialized cellular structures in the body (Mirsky and Jessen, 1996, 2001; Zorick and Lemke, 1996; Scherer and Arroyo, 2002). Here, we investigate the molecular signaling underlying two key components of the myelination program, namely cell cycle exit and appearance of death resistance.

There is a strong temporal correlation between the onset of myelination and the cessation of cell division, and analysis with differentiation markers shows that although individual cells still proliferate in the earliest stages of myelin differentiation, they fall out of division before they start forming myelin sheaths, as detected by elevation in expression of the myelin protein Po (Friede and Samorajski, 1968; Brown and Asbury, 1981; Stewart et al., 1993). Similarly, apoptotic Schwann cell death declines in postnatal nerves as myelination advances and apoptosis is largely restricted to nonmyelinating cells (Grinspan et al., 1996; Syroid et al., 1996; Nakao et al., 1997). In line with this, TGFβ, a factor implicated as a death signal in developing nerves, selectively induces apoptosis in nonmyelinating cells, and spares cells expressing myelin proteins (Parkinson et al., 2001).

Thus, Schwann cell myelination involves, first, escape from the cell cycle and developmental death signaling, followed by strong up-regulation of myelin proteins and formation of compacted membrane wraps.

The transcription factor Krox-20 is essential for myelination. It is strongly up-regulated by axon associated signals only in cells destined to myelinate and, in Krox-20 null mice, although myelin differentiation starts (Parkinson et al., 2003), myelin sheaths do not form and Schwann cells continue to proliferate and remain susceptible to death (Topilko et al., 1994; Zorick et al., 1999). In humans, Krox-20 (Egr2) mutations are associated with Charcot-Marie-Tooth, Dejerine-Sottas, and hereditary sensory and motor neuropathies, underlining the pivotal role of this protein in myelin formation (Wrabetz et al., 2001).

Here, we define the function of Krox-20 in Schwann cell proliferation and death, and analyze the molecular signaling that enables Krox-20 to control these events. We show that expression of Krox-20 is sufficient to cell autonomously alter the response of Schwann cells to the major axonal mitogen, β-neuregulin-1 (NRG-1), so that NRG-1 no longer stimulates DNA synthesis. Similarly, Krox-20 inactivates TGFβ
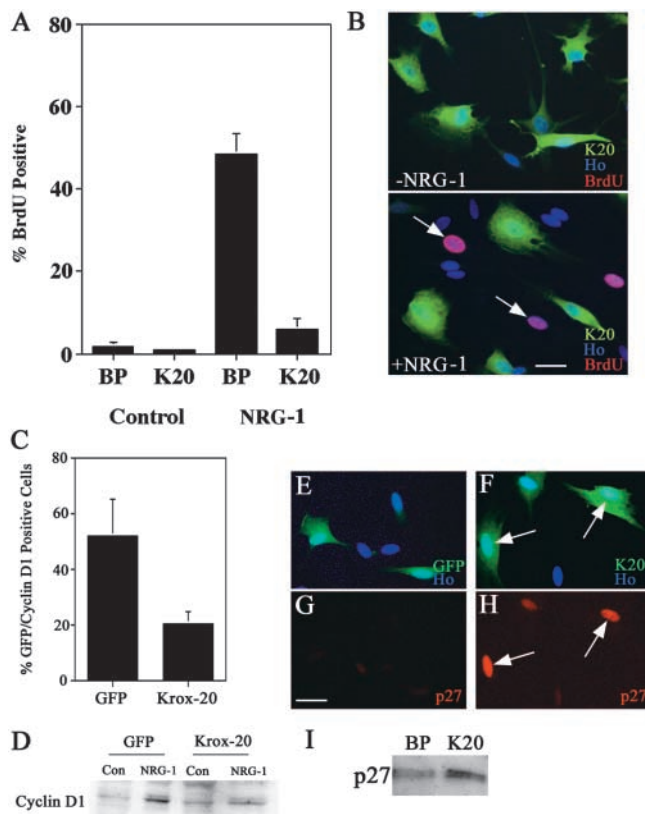
Figure 1.  **Krox-20 inhibits DNA synthesis in Schwann cells.** (A) The proliferation of Schwann cells, as measured by BrdU incorporation, in response to NRG-1 is inhibited by Krox-20. Schwann cells retrovirally infected with empty vector (BP) or vector expressing Krox-20 (K20) were treated with medium alone (Control) or with medium supplemented with 20 ng/ml NRG-1. The error bars represent one SD of the mean. (B) Inhibition of BrdU incorporation in cells expressing Krox-20. Cells infected with adenovirus coexpressing GFP and Krox-20 in control (−NRG-1) and 20 ng/ml NRG-1–treated cells. Note that GFP/Krox-20–positive cells are BrdU negative. Conversely, BrdU-positive cells are GFP negative (arrows). Bar, 20 μm. (C and D) Krox-20 expression inhibits cyclin D1 induction in response to NRG-1. (C) The graph shows percentage of adenovirally infected GFP/cyclin D1–positive cells 6 h after addition of 20 ng/ml NRG-1 in GFP control (GFP) and GFP/Krox-20 (Krox-20)–expressing cells. The error bars represent one SD of the mean. (D) Western blot of cyclin D1 in GFP control (GFP) and GFP/Krox-20 (Krox-20) infected Schwann cells exposed to DM alone (Con) or DM containing 20 ng/ml NRG-1 for 6 h. (E–I) Krox-20 expression increases levels of p27 protein in Schwann cells. (E–H) Immunolabeling of GFP (E and G) and GFP/Krox-20 (F and H) infected Schwann cells with p27 antibody. Arrows indicate GFP/Krox-20 cells with elevated p27 protein. Bar, 20 μm. (I) Western blot using p27 antibody of Schwann cells infected with either empty vector (BP) or Krox-20 (K20)–expressing retrovirus.



Figure 2.  **Krox-20 inhibits apoptosis of Schwann cells.** (A and B) Krox-20 inhibits TGFβ-dependent apoptosis of Schwann cells. Survival assays for immunopanned Schwann cells infected with GFP or GFP/Krox-20 (Krox-20)–expressing adenovirus. Cells were treated for 24 h with TGFβ + TNFα (A) or TGFβ alone (B) for 48 h and the number of surviving GFP-positive cells counted. (C) Krox-20 inhibits Schwann cell death after serum withdrawal. Survival assay of cells infected with GFP or GFP/Krox-20 (Krox-20)–expressing adenovirus. Surviving GFP-positive cells were counted 72 h after serum withdrawal. The error bars represent one SD of the mean.

Schwann cells and that JNK/c-Jun activity is required for proliferation and death. Importantly, Krox-20 suppresses JNK/c-Jun signaling. This provides Krox-20 with a mechanism for coordinated control of mitogenic and death signaling in developing Schwann cells. Finally, we show that Krox-20 regulates levels of JNK-interacting protein 1 (JIP-1), a scaffold protein that controls the activity of JNK-mediated signaling (Davis, 2000). We propose this as a possible component of the mechanism by which Krox-20 regulates the activity of JNK during Schwann cell development.
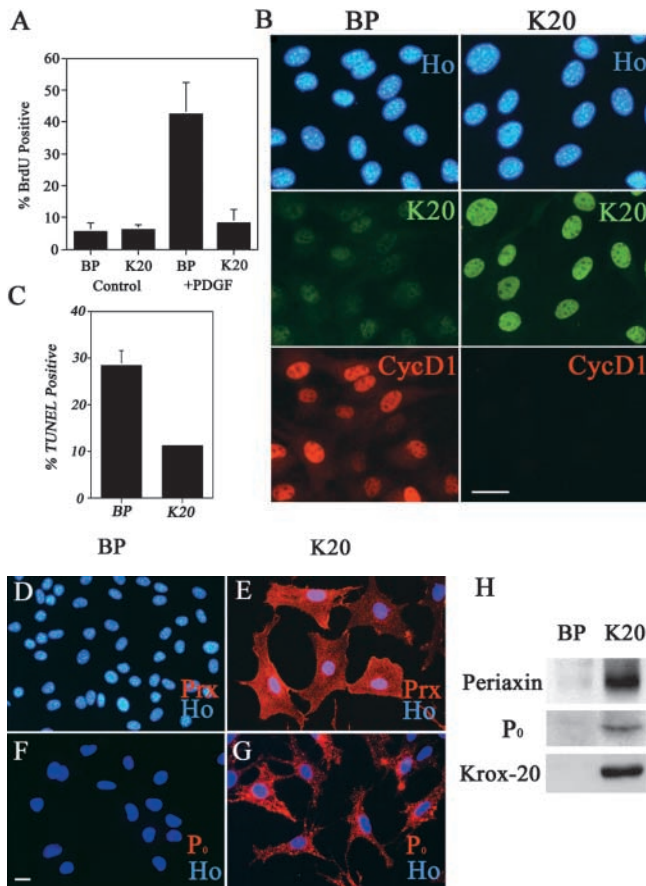
# Results

## Krox-20 blocks NRG-1–induced Schwann cell proliferation

In the absence of Krox-20, Schwann cells arrest at the earliest stage of myelin differentiation and remain proliferating and death susceptible. To test whether expression of Krox-20 alone is sufficient to remove cells from the cell cycle in the face of NRG-1 stimulation, we infected Schwann cell cultures with retroviral or adenoviral constructs expressing full-length Krox-20 or the appropriate empty vector controls. Krox-20 strongly reduced Schwann cell proliferation in response to NRG-1 (Fig. 1, A and B) and caused a significant reduction in the induction of the early cell cycle marker cyclin D1 after NRG-1 addition (Kim et al., 2001) both by immunolabeling ($P < 0.0001$) and Western blot (Fig. 1, C and D).

Krox-20–expressing cells also had elevated levels of the cell cycle inhibitor p27 in immunolabeling experiments, an observation confirmed by two different p27 antibodies, and by Western blotting (Fig. 1, E–I) in line with in vivo observations (Tikoo et al., 2000).

## In the presence of Krox-20, Schwann cells are resistant to killing by TGFβ and growth factor deprivation

TGFβ acts as a death signal in immature Schwann cells (Parkinson et al., 2001) and in mice without functional TGFβ type II receptors, developmental Schwann cell death

death signals and protects cells from death triggered by growth factor deprivation. Notably, Krox-20 also blocks proliferation and death in 3T3 fibroblasts, although these cells are unrelated to Schwann cells. Moreover, in 3T3 fibroblast, Krox-20 triggers expression of the myelin genes periaxin and protein zero ($P_0$), a function previously thought to be a specific for Krox-20 in Schwann cells. We show a significant relationship between Krox-20 signaling and the c-Jun NH$_2$-terminal kinase (JNK)–c-Jun pathway. We show that this pathway is activated by both NRG-1 and TGFβ in

Figure 3.   **Krox-20 regulates proliferation, survival and myelin gene expression in 3T3 fibroblasts.** (A) Krox-20 expression inhibits PDGF-induced proliferation of Swiss 3T3 cells. Shown are the percentage of BrdU-positive cells in empty vector (BP) and Krox-20–expressing (K20) cells in the absence (Control) and presence (+PDGF) of 5 ng/ml PDGF. The error bars represent one SD of the mean. (B) Krox-20 expression in Swiss 3T3 cells inhibits cyclin D1 induction in response to PDGF. Shown are control (BP) and Krox-20 (K20) infected cells, treated for 6 h with 5 ng/ml PDGF. The cells were double immunolabeled with Krox-20 (K20) and cyclin D1 (CycD1) antibodies, and counterstained with Hoechst dye (Ho). Bar, 20 $\mu$m. (C) Krox-20 expression inhibits apoptosis of NIH 3T3 cells in serum-free medium. The graph shows the percentage of TUNEL-positive cells in control (BP) and Krox-20 (K20)–expressing cells 48 h after serum withdrawal. The error bars represent one SD of the mean. (D–G) Krox-20 activates periaxin and $P_0$ myelin protein expression in Swiss 3T3 cells. Swiss 3T3 cells were retrovirally infected with either empty vector (BP; D and F) or Krox-20 (K20; E and G), and immunolabeled with antibody against periaxin (Prx; D and E) or $P_0$ (F and G). Cells were counterstained with Hoechst dye (Ho) to reveal nuclei. Bar, 20 $\mu$m. (H) Western blot of control (BP) or Krox-20 (K20) infected cells showing elevated periaxin and $P_0$ protein levels.



Figure 4.   **Krox-20 does not inactivate NRG-1 or TGF$\beta$ signaling.** (A) Western blot analysis of control (GFP) and Krox-20 adenovirally infected Schwann cells, showing levels of ErbB2 and ErbB3 protein, together with activation (i.e., phosphorylation) of ERK1/2 and Akt in DM only and 10 min after addition of 20 ng/ml NRG-1. Shown also are the corresponding levels of periaxin in control cells and in cells expressing Krox-20. (B) NRG-1 receptor levels are not altered in Krox-20−/− nerves. Western blot of ErbB2 and ErbB3 in sciatic nerve from P10 wild-type (+/+), Krox-20 heterozygous (+/−) and Krox-20 null (−/−) animals. (C–F) SMAD2 and SMAD4 proteins translocate normally to the nucleus in response to TGF$\beta$ in Krox-20–expressing Schwann cells. Note intense nuclear labeling in cells infected with Krox-20–expressing adenovirus, treated for 1 h with 10 ng/ml TGF$\beta$1 and immunolabeled with antibodies to either SMAD2 (C and D) or SMAD4 (E and F). Bar, 20 $\mu$m.

is strongly reduced (unpublished data). The escape from death vulnerability and TGF$\beta$-induced apoptosis that accompanies myelin sheath formation does not take place in Krox-20−/− mice. To test whether presence of the Krox-20 protein is sufficient to induce death resistance and, in particular, to uncouple TGF$\beta$ stimulation from induction of apoptosis, we performed survival assays on immunopanned Schwann cells from newborn animals infected with Krox-20/GFP adenovirus or GFP control virus. First, we found that expression of Krox-20 strongly increased survival in re-

sponse to TGF$\beta$ plus TNF$\alpha$ or TGF$\beta$ alone (P < 0.002 and P < 0.001, respectively; Fig. 2, A and B). Second, we found that Krox-20–expressing cells survived 10% serum withdrawal much better than control cells (Fig. 2 C; Cheng et al., 2001). For both the TGF$\beta$ and serum withdrawal assays, Schwann cell death was confirmed to be apoptotic by immunolabeling for the activated form of caspase-3 and TUNEL analysis (Parkinson et al., 2001; unpublished data).

## Krox-20 regulates proliferation, survival and myelin genes in 3T3 fibroblasts

Krox-20 expression induces myelin-differentiation genes in Schwann cells (Nagarajan et al., 2001; Parkinson et al., 2003). We show in a previous section (Krox-20 blocks NRG-1–induced Schwann cell proliferation) that Krox-20 is also sufficient to organize cell cycle withdrawal and confer death resistance. This spectrum of activity has been associated with master regulatory or selector genes. Because capacity to act in a heterologous cell is a feature of some master regulators, we tested whether Krox-20 could act in similar ways in cells not related to Schwann cells, i.e., 3T3 fibroblasts.

We found that retrovirally sustained Krox-20 expression blocked (P < 0.005) DNA synthesis of Swiss 3T3 cells in response to PDGF-BB and prevented cyclin D1 induction (Fig. 3, A and B). Krox-20 expression also suppressed the proliferation of Swiss 3T3 and NIH 3T3 fibroblasts in donor calf serum (unpublished data). Furthermore, Krox-20 inhibited apoptosis (P < 0.001) of NIH and Swiss 3T3 cells after withdrawal of serum when measured by TUNEL anal-

Figure 5.  **Krox-20 inhibits the JNK–c-Jun pathway in vitro and in vivo.** (A) Western blot (long exposure) showing suppression of phospho–c-Jun (P-cJun), c-Jun protein (cJun), and phospho-JNK1/2 (P-JNK1/2) in cells infected with Krox-20/GFP adenovirus for 48 h and maintained in DM alone. GFP indicates control cells infected with empty vector. (B–E) Krox-20 expression in Schwann cells inhibits NRG-1– (B and C) and TGFβ-dependent (D and E) activation of the JNK–c-Jun pathway. (B and D) Western blot of Schwann cells, 20 h after adenoviral infection, treated with 20 ng/ml NRG-1 (B) or 10 ng/ml TGFβ (D). Note inhibition of c-Jun phosphorylation by Krox-20 after growth factor addition compared with GFP control, whereas c-Jun protein levels are unaffected. (C and E) Western of Schwann cells 48 h after infection, treated with NRG-1 (C) or TGFβ (E). At this time point, both phospho–c-Jun and c-Jun proteins are suppressed by Krox-20 as compared with control cells. Either β-tubulin or GAPDH were used as loading controls. (F–M) c-Jun and Ser63 phospho–c-Jun immunolabeling of Schwann cells infected with either GFP (F, H, J, and L) or GFP/Krox-20 (G, I, K, and M) and treated 48 h after infection with NRG-1 for 30 min. Both c-Jun and phospho–c-Jun levels are reduced in response to NRG-1 in Krox-20–expressing cells compared with GFP control infected cells (arrows). (N and O) Teased preparation of sciatic nerve at E17, before myelination double labeled with c-Jun and Krox-20 antibodies. Hoechst nuclear dye (Ho) is used to visualize nuclei. Note that most/all nuclei contain c-Jun, whereas Krox-20 is not yet present. (P and Q) Teased preparation of sciatic nerve at P1 when many cells are myelinating. The nerves are double immunolabeled with Krox-20 (K20) and c-Jun (P) or phospho–c-Jun (PSer63c-Jun; Q) antibodies. Note that nuclei expressing Krox-20 do not contain c-Jun or phospho–c-Jun. Bar, 20 μm.
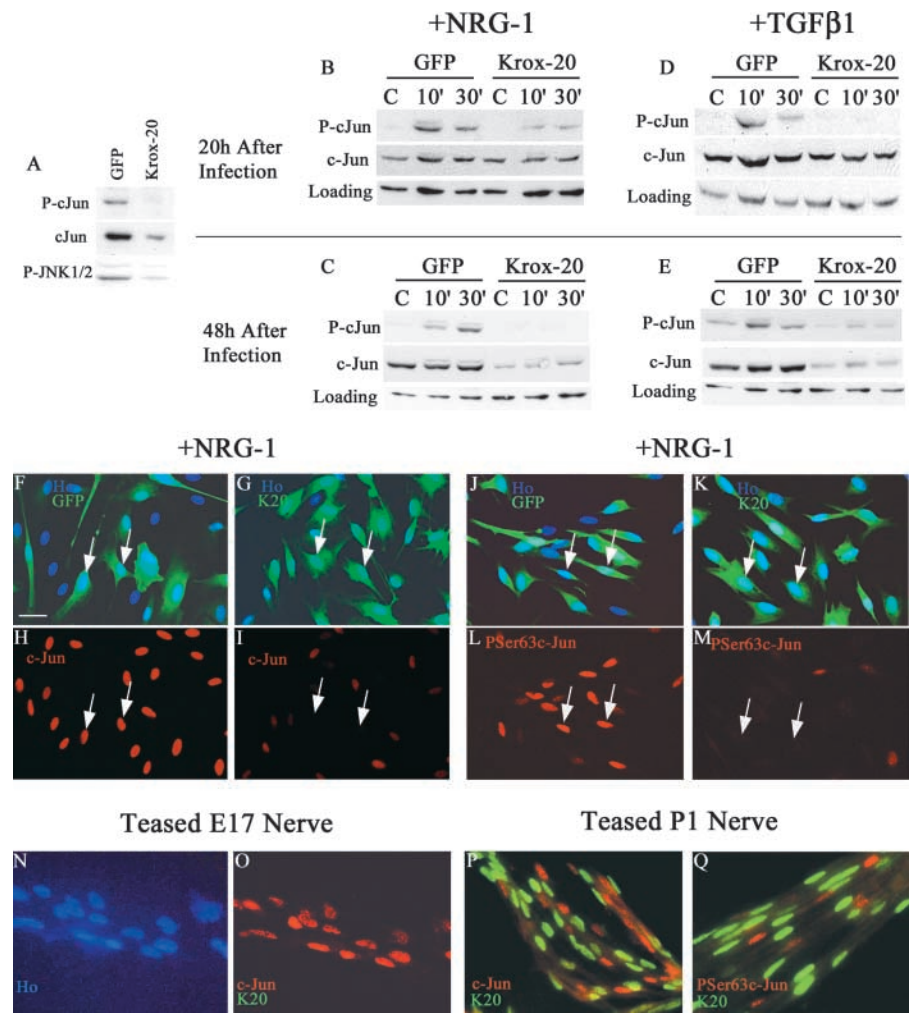
ysis (Fig. 3 C; and not depicted). To our surprise, we found that Krox-20 expression was also sufficient to up-regulate the myelin genes periaxin and P$_0$ in Swiss 3T3 cells. This induction was unambiguous and seen both by immunohistochemistry and Western blotting (Fig. 3, D–H).

These experiments show that Krox-20 can execute some of its key Schwann cell functions in an unrelated cell. This, together with the ability to induce a broad spectrum of differentiation genes and withdraw cells from the cell cycle, is reminiscent of master regulatory genes in other systems.

## Krox-20 causes a selective modulation, rather than general inactivation of NRG-1 or TGFβ signaling in Schwann cells

The experiments in Figs. 1–3 provide a plausible explanation for the increase in cell numbers, proliferation, and death in Krox-20 mutants (Topilko et al., 1994; Zorick et al., 1999) by showing that expression of Krox-20 is sufficient to alter the response of Schwann cells to specific proliferation and death factors present in peripheral nerves. We now set out to examine the underlying molecular mechanisms.

We found that ErbB2 and ErbB3 NRG-1 receptor levels were unchanged in cells infected with Krox-20/GFP adenovirus, and in nerves of Krox-20 null animals (Fig. 4, A and B). Phosphorylation of the ERK1/2 MAPKs, both in unstimulated Schwann cells and in response to NRG-1, was also unchanged by Krox-20 expression, at least in the conditions used in our assay (Fig. 4 A). In addition, basal and NRG-1–induced PI3-kinase activity, measured by phosphorylation of Akt, was not significantly affected by Krox-20 expression (Fig. 4 A).

We also showed that two major members of the TGFβ signaling pathway, SMAD2 and SMAD4, translocated normally to the nucleus in response to TGFβ in Krox-20–expressing Schwann cells (Fig. 4, C–F; and not depicted).

In conclusion, the power of Krox-20 to inactivate mitogenic and death signaling by NRG-1 and TGFβ, respectively, appears to be due to a selective modulation, rather than general inactivation of the signaling pathways activated by these factors. It is also noteworthy that, in Krox-20–expressing cells, NRG-1 still activates two of the major kinases involved in NRG-1–mediated proliferation, although the

cells do not divide. The lack of mitogenic response must therefore derive from interference with other intracellular signaling molecules or pathways.

## Krox-20 suppresses basal JNK/c-Jun activity, and inhibits NRG-1– and TGFβ-stimulated JNK/c-Jun activation

The JNK–c-Jun pathway regulates proliferation and/or death in several cell types (Leppa and Bohmann, 1999; Ham et al., 2000), and in Schwann cells this pathway is required for TGFβ-induced apoptosis (Parkinson et al., 2001).

Therefore, we looked for a linkage between Krox-20 and JNK/c-Jun signaling. First, we showed by double label immunohistochemistry that most/all Schwann cell nuclei expressed c-Jun and serine (Ser)63 phospho–c-Jun before myelination (E17), whereas neither antibody bound the Krox-20–positive nuclei of early myelinating cells in neonatal nerves, although they continued to label the nonmyelin cells (Fig. 5, N–Q; and not depicted). This is consistent with the absence of c-Jun in adult myelinating cells and the observed fall in c-Jun levels during myelination in vivo and myelin induction by cAMP elevation in vitro (Monuki et al., 1989; De Felipe and Hunt, 1994; Stewart, 1995; Shy et al., 1996; Awatramani et al., 2002).

Second, we found that expression of Krox-20 strikingly suppressed basal JNK/c-Jun activity in cultured Schwann cells, as judged by levels of phospho–c-Jun, c-Jun protein and phospho-JNK1/2 (Fig. 5 A), and a reduction in the number of c-Jun–positive nuclei in immunohistochemical tests from 88.4 ± 7% to 12.5 ± 2% (P < 0.0001).

Third, in experiments involving short exposure to Krox-20 (tests performed 20 h, rather than the usual 48 h, after onset of adenoviral infection), we found that NRG-1– and TGFβ-stimulated phosphorylation of c-Jun was suppressed, whereas levels of c-Jun protein still remained unchanged (Fig. 5, B and D). At a later time point (48 h), both phospho–c-Jun and c-Jun levels are reduced by Krox-20 (Fig. 5, C and E). These findings were confirmed by immunolabeling of cells expressing Krox-20 48 h after infection (Fig. 5, F–M). These experiments show that Krox-20 expression first blocks growth factor induced c-Jun phosphorylation, whereas prolonged Krox-20 expression also reduces c-Jun protein levels in Schwann cells.

Together, these experiments indicate that the JNK–c-Jun pathway is inactivated in individual cells as they start myelination and that this takes place as a consequence of Krox-20 activation. We now asked whether this JNK/c-Jun inactivation was, in turn, sufficient to explain the effects of Krox-20 on NRG-1–driven proliferation and TGFβ-induced death.

## Down-regulation of the JNK–c-Jun pathway is sufficient to uncouple NRG-1 receptor activation from mitogenesis

To test whether NRG-1 stimulation of Schwann cell proliferation depended on the JNK–c-Jun pathway, we blocked JNK activity in cultured Schwann cells using two methods. First, we used SP600125, a selective inhibitor of JNK1 and JNK2 (Bennett et al., 2001). Having confirmed that 30 μM SP600125 completely prevented JNK1/2 and c-Jun phosphorylation even in the presence of 20 ng/ml NRG-1 (Fig. 6 A), we found that SP600125 blocked Schwann cell DNA
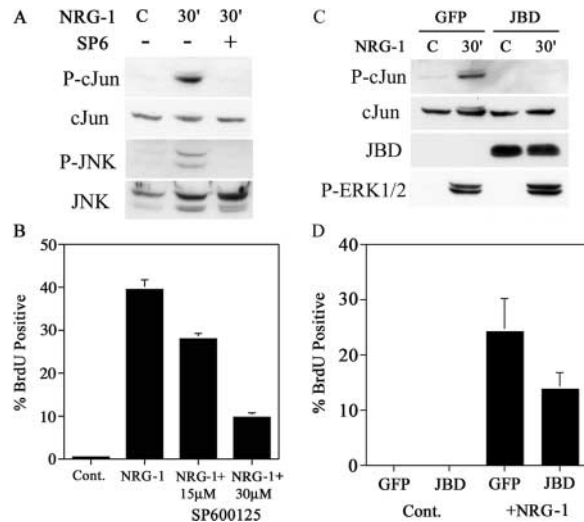


Figure 6.   **Inhibition of the JNK pathway inhibits NRG-1–dependent proliferation of Schwann cells.** (A) Western blot showing inhibition of NRG-1–induced JNK and c-Jun phosphorylation by 30 μM SP600125 (SP6). (B) Percentage of BrdU-positive Schwann cells in DM only (Cont.) and in medium containing 20 ng/ml NRG-1 in the absence or presence of increasing concentrations of the JNK inhibitor SP600125 as indicated. The error bars represent one SD of the mean. (C) Expression of the JBD of JIP-1 inhibits NRG-1–induced c-Jun phosphorylation, but not ERK1/2 activation. (D) Percentage of BrdU-positive Schwann cells infected with control virus expressing GFP only (GFP) or JBD-expressing adenovirus in the absence (Cont.) or presence of 20 ng/ml NRG-1. The error bars represent one SD of the mean.

synthesis in response to 20 ng/ml NRG-1 in a dose-dependent manner (Fig. 6 B). Second, we performed experiments expressing the JNK binding domain (JBD) of the JNK binding protein JIP-1, which specifically inhibits JNK activity and c-Jun phosphorylation (Harding et al., 2001). In confirmation of this, JBD expression blocked c-Jun phosphorylation, but not ERK1/2 activation, in response to NRG-1 (Fig. 6 C). We found that JBD expression significantly (P < 0.001) inhibited 20 ng/ml NRG-1–stimulated BrdU incorporation (Fig. 6 D). Furthermore, JBD expression inhibited Schwann cell death in response to TGFβ or serum withdrawal (unpublished data).

Although Krox-20 is likely to have numerous effects on intracellular signaling in Schwann cells, these observations argue that the Krox-20–mediated inactivation of the JNK–c-Jun pathway is sufficient to cause the proliferation arrest seen in Krox-20–positive Schwann cells.

## Death prevention by Krox-20 depends on Krox-20–mediated inactivation of JNK

Killing of Schwann cells by TGFβ depends on the activation of the JNK–c-Jun pathway (Parkinson et al., 2001), an event that is inhibited by Krox-20 (Fig. 5). This provides a plausible explanation for the protective effects of Krox-20 against TGFβ-induced cell death. It also predicts that enforced activation of this pathway in Krox-20–expressing cells should restore their vulnerability to cell death. To reactivate the JNK pathway in Krox-20–expressing cells, we used activated forms of either MEKK1 (Olson et al., 1995; Whitfield et al.,
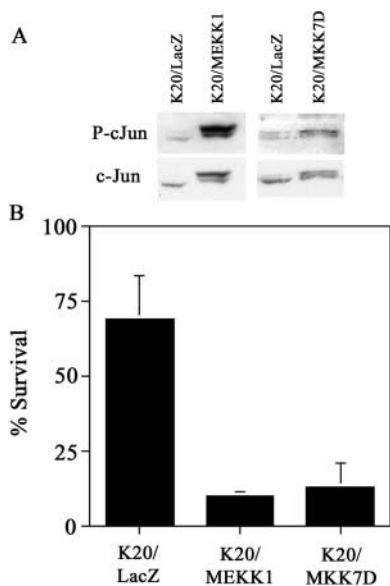
Figure 7. **Expression of MEKK1 or MKK7D and the resulting activation of c-Jun phosphorylation restores vulnerability to death in cells expressing Krox-20.** (A) Western blot showing induction of c-Jun phosphorylation in Schwann cells coexpressing Krox-20 and either MEKK1 (K20/MEKK1) or MKK7D (K20/MKK7D) as compared with control (K20/LacZ) cells. (B) Serum withdrawal survival assay of Krox-20/LacZ, Krox-20/MEKK1 or Krox-20/MKK7D–expressing Schwann cells. The death protection offered by Krox-20 alone (Fig. 2 C) is not seen in Krox-20/MEKK1 or Krox-20/MKK7D–expressing cells. The error bars represent one SD of the mean.

2001) or MKK7 (MKK7D; Wang et al., 1998) kinases. Cultured Schwann cells were coinfected with the Krox-20/GFP and either myc-tagged MEKK1- or MKK7D-expressing adenoviruses. Immunolabeling of infected cells showed that >90% of GFP-positive (Krox-20–expressing) Schwann cells also expressed MEKK1 (unpublished data). In these double infected cells, MEKK1 and MKK7D reversed the Krox-20–mediated inhibition of c-Jun phosphorylation both by Western blot and immunolabeling (Fig. 7 A; and not depicted). To test the effects of MEKK1 or MKK7D on cell death in Krox-20 cells, double infected Krox-20/MEKK1 or Krox-20/MKK7D cells were subjected to the serum withdrawal test (Fig. 2). This showed that most of the cells expressing Krox-20 and a control LacZ adenovirus were protected from death as expected. In contrast, the cells expressing Krox-20/MEKK1 or Krox-20/MKK7D died as expected for uninfected native cells (Fig. 7 B).

Thus, reactivation of the JNK–c-Jun pathway in cells expressing Krox-20 overrides the protective effect of Krox-20. This strengthens our conclusion that the death protection by Krox-20 that we have described in Fig. 2 can be attributed to Krox-20–mediated inactivation of JNK.

### JIP-1, a potential suppressor of JNK activity, is up-regulated by Krox-20

There is evidence that up-regulation of the JNK scaffolding protein JIP-1 (IB1) may in some circumstances inactivate JNK signaling (Bonny et al., 2000; Tawadros et al., 2002). A number of observations were consistent with the idea that JIP-1 is involved in mediating the inhibitory effect of Krox-20 on JNK. First, expression of Krox-20 strongly increased JIP-1 protein levels in Schwann cells in vitro, and we also observed a higher migrating band, which may represent a phosphorylated form of the JIP-1 protein (Fig. 8 A; Meyer et al., 1999). Second, in Krox-20 null mice the levels of JIP-1 protein were substantially reduced, whereas c-Jun levels were elevated (Fig. 8 B). Third, JIP-1 and Krox-20 mRNAs were up-regulated with a similar time course during nerve development, and the JIP-1 mRNA and protein that localized to paranodal regions decreased after nerve cut (Fig. 8, C, F, and G; and not depicted; Nagarajan et al., 2002). A
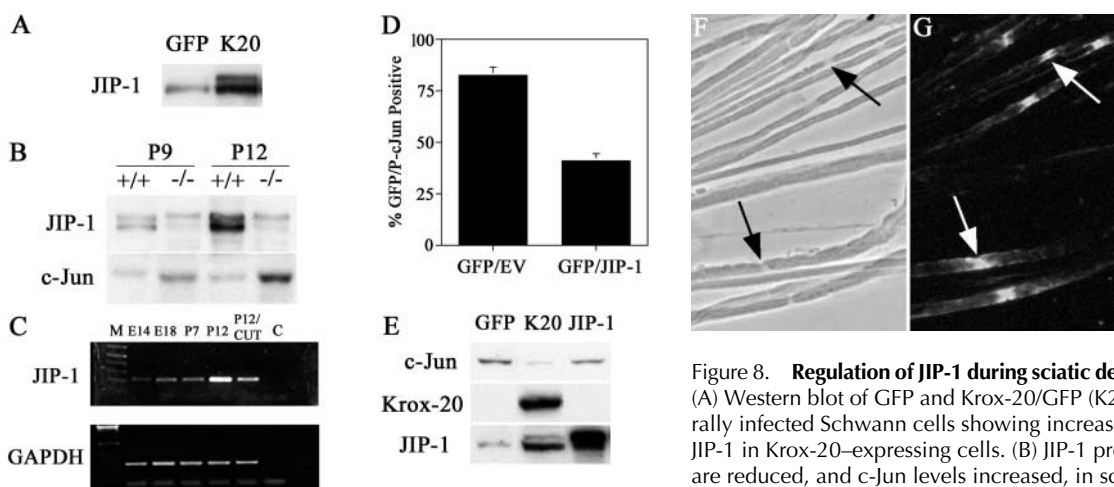


Figure 8. **Regulation of JIP-1 during sciatic development.** (A) Western blot of GFP and Krox-20/GFP (K20) adenovirally infected Schwann cells showing increased levels of JIP-1 in Krox-20–expressing cells. (B) JIP-1 protein levels are reduced, and c-Jun levels increased, in sciatic nerve from P9 and P12 Krox-20 null animals (−/−) compared with wild type (+/+). (C) Semi-quantitative PCR analysis of JIP-1 mRNA expression in sciatic nerve during development from embryonic day 14 (E14) to P12 and at P12, 5 d after nerve cut (P12/CUT). (D) Expression of JIP-1 inhibits c-Jun phosphorylation in Schwann cells. Shown are the percentage of Ser63 phospho–c-Jun–positive cells for cells cotransfected with either GFP-expressing plasmid, for identification of transfected cells, plus empty vector (GFP/EV) or GFP plus JIP-1 (GFP/JIP-1)–expressing plasmids. The error bars represent one SD of the mean. (E) Western blot showing that, unlike Krox-20, JIP-1 expression does not reduce c-Jun protein levels in Schwann cells. (F and G) Immunolabeling of P12 sciatic nerve showing phase (F) and JIP-1 immunofluorescence (G). Arrows indicate localization of JIP-1 in the paranodal loops of myelinating Schwann cells.

comparable profile of JIP-1 mRNA expression was found using Affymetrix gene arrays (unpublished data). Lastly, expression of JIP-1 selectively reduced ($P < 0.0001$) the percentage of Ser63 phosphorylated c-Jun–positive Schwann cells maintained under basal conditions in vitro, whereas leaving c-Jun protein levels unchanged (Fig. 8, D and E). Together, these experiments suggest that JIP-1 may act as a link in the chain that leads from Krox-20 to inhibition of JNK and reduced phospho–c-Jun.

# Discussion

## Krox-20 controls the outcome of NRG-1 and TGFβ signaling in Schwann cells

The first report on Krox-20 inactivation in Schwann cells showed that Krox-20 nerves contained an increased number of Schwann cells (Topilko et al., 1994). Further work revealed that the rates of DNA synthesis and apoptosis were increased in these nerves, and that levels of the transcription factor Oct-6 (SCIP) remained high, although they fall in normal nerves (Zorick et al., 1999). It was plausibly suggested that the ongoing Oct-6 expression might be responsible for the high proliferation rate, and that the increased death could be a downstream consequence of this, because a larger Schwann cell population would result in increased competition for limited amounts of axon-associated survival signals (Zorick et al., 1999). In the first part of this paper, we provide an alternative explanation for the changes in proliferation and death seen in Krox-20−/− nerves. We show that Krox-20 cell autonomously alters the response of Schwann cells to NRG-1, the major axonal mitogen in neonatal nerves, so that NRG-1 no longer stimulates DNA synthesis. Krox-20 is activated as cells start to myelinate and this is correlated with cessation of proliferation. We suggest that this is due to the ability of Krox-20 to signal cell cycle exit, even in the presence of mitogens. In Krox-20−/− nerves, this mechanism is compromised, resulting in ongoing proliferative response to mitogens such as NRG-1.

The increased apoptosis in nerves of Krox-20 mutants can also be related to cell-autonomous effects of Krox-20, because we find that Krox-20 expression provides cells with strong protection against apoptotic death. This effect is broad based, in the sense that Krox-20 expression protects Schwann cells from TGFβ killing and safeguards both Schwann cells and 3T3 fibroblasts from death by growth factor deprivation. Therefore, it is likely that the death resistance that cells acquire as they start to myelinate (Grinspan et al., 1996; Parkinson et al., 2001) is due to the presence of Krox-20 in these cells. We suggest that in Krox-20−/− nerves, Schwann cells are bereft of this protection and consequently die in large numbers (Zorick et al., 1999).

As to the alternative possibility that increased death in nerves of P12 Krox-20 mutants is due to enhanced competition for axonal survival signals, this is now less attractive, because it is realized that postnatal Schwann cells support their own survival by autocrine signaling, and that death of these cells is not increased even when axons are cut (Grinspan et al., 1996; Cheng et al., 1998; Dowsing et al., 1999; Meier et al., 1999).

## Krox-20 shares similarities with master regulatory genes

Unexpectedly, constitutive expression of Krox-20 caused the myelin proteins periaxin and $P_0$ to be expressed in 3T3 fibroblasts, cells that do not normally express these proteins, and are unrelated to Schwann cells. Periaxin and $P_0$ show extremely restricted distribution in vivo, being largely confined to myelinating Schwann cells in the adult peripheral nervous system (Scherer et al., 1995; Lee et al., 1997).

We also observed that Krox-20 induces growth arrest and a decrease in cyclin D1 levels not only in Schwann cells but also in fibroblastic Swiss 3T3 cells treated with PDGF, a classical mitogen for these cells (Withers et al., 1995). This agrees with previous data showing c-Jun transactivates the cyclin D1 promoter (Sabbah et al., 1999; Wisdom et al., 1999). Furthermore, Krox-20 protects 3T3 cells, like Schwann cells, from death induced by growth factor deprivation.

The present experiments and previous work (Nagarajan et al., 2001; Parkinson et al., 2003) show that Krox-20 activates a large range of myelin differentiation genes and proteins, organizes cell cycle exit and protects from death. It can also execute some of its key actions in a heterologous cell. Therefore, Krox-20 acts in many ways in a manner expected of a master regulator for myelin Schwann cells, and has several of the properties of master regulatory genes such as MyoD, the neural basic helix-loop-helix genes, or PPARγ (Davis et al., 1987; Tontonoz et al., 1994; Lee et al., 1995; Lo et al., 1998; Zorick et al., 1999; Sabourin and Rudnicki, 2000).

## Krox-20 suppresses the JNK–c-Jun pathway

We find that constitutive expression of Krox-20 in cultured Schwann cells results in down-regulation of the JNK–c-Jun pathway under basal conditions, and suppression of JNK/c-Jun activation by NRG-1 and TGFβ. This inhibitory interaction between Krox-20 and c-Jun is likely to reflect the action of Krox-20 in vivo. The principal evidence is the excellent negative correlation we see between expression of Krox-20 on the one hand and c-Jun and phosphorylated c-Jun on the other in the nuclei of individual Schwann cells in neonatal nerves. Earlier work also supports this conclusion. Adult myelinating cells express Krox-20 but not c-Jun, although it is detectable in nonmyelinating cells (Shy et al., 1996). After nerve transection, c-Jun levels rise as Schwann cells dedifferentiate and Krox-20 levels fall, whereas during regeneration c-Jun falls and Krox-20 expression increases as cells establish new myelin sheaths (De Felipe and Hunt, 1994; Stewart, 1995; Shy et al., 1996; Topilko and Meijer, 2001).

## Suppression of the JNK–c-Jun pathway reduces proliferation and death in Schwann cells

We find that inhibition of JNK in two unrelated ways, i.e., by the small molecular blocker SP600125 or by the JBD of JIP-1, uncouples NRG-1 stimulation from DNA synthesis. The JNK–c-Jun pathway has not previously been implicated in Schwann cell proliferation. The expression pattern of c-Jun is, however, consistent with such a role. c-Jun is high in embryonic nerves before myelination when proliferation is ongoing, low in mature nerves where proliferation is absent and high in transected nerves (previous paragraph). Although our experiments indicate that JNK/c-Jun activity is necessary for Schwann cell proliferation, at least in response

to NRG-1, it is not sufficient. We see this in Schwann cells maintained in medium without mitogens in vitro and in the distal stump of nerves several weeks after transection. In both cases, proliferation is low or absent but c-Jun expression is relatively high.

The observation that the JNK–c-Jun pathway is required for NRG-1–driven Schwann cell proliferation indicates that the antiproliferative effect of Krox-20 can be attributed to the ability of this transcription factor to inactivate this pathway, as evidenced by down-regulation of c-Jun protein, phosphorylated c-Jun, and phosphorylated JNK. Although the data indicate that suppression of the JNK–c-Jun pathway is sufficient to account for the effects of Krox-20 on proliferation, it is possible that Krox-20 has additional actions in Schwann cells that also contribute to cell cycle exit. This is suggested by the Krox-20–mediated induction of the cell cycle inhibitor p27.

We have shown previously that TGFβ-induced death involves phosphorylation of c-Jun, that an active form of c-Jun kills Schwann cells and that a dominant negative form of c-Jun inhibits TGFβ-induced death (Parkinson et al., 2001). Therefore, as in some other cell types (Kyriakis and Avruch, 2001; Shaulian and Karin, 2002), the JNK–c-Jun pathway is a component of at least two signaling cascades in Schwann cells, those that promote death and those that promote proliferation. This dual function allows Krox-20 to suppress both cell division and death through a single action, namely inactivation of the JNK–c-Jun pathway.

### Regulation of JIP-1 by Krox-20 during Schwann cell development

JNK signaling is regulated by the balance between upstream activating kinases such as MKK4/7 and protein phosphatases, which act to turn off JNK activity (Camps et al., 2000; Weston and Davis, 2002). Another layer of JNK regulation has been revealed with the identification of JIP-1, which acts as a scaffold protein and binds the mixed lineage kinase MLK3, the MAPK MKK7, and JNK1/2 (Dickens et al., 1997; Whitmarsh et al., 1998).

JIP-1 potentiates the activation of JNK by MKK7. However, JIP-1 can also inhibit JNK–c-Jun interactions (Dickens et al., 1997), which would lead to reduced c-Jun phosphorylation, and there are a number of reports that increased levels of JIP-1 suppress JNK signaling (Dickens et al., 1997; Bonny et al., 2000; Tawadros et al., 2002).

Our finding that JIP-1 is present and developmentally regulated in peripheral nerve and a target of Krox-20 is of great interest, because this may be part of the mechanism by which Krox-20 suppresses JNK/c-Jun activation. Notably, neither expression of the JBD region or full-length JIP-1, both of which reduce c-Jun phosphorylation, affected c-Jun protein levels in Schwann cells. The mechanism by which Krox-20 suppresses c-Jun protein therefore remains to be discovered. The localization of JIP-1 in the paranodal region of the myelinating Schwann cells is also of interest. JIP-1 binds the RhoA exchange factor, RhoGEF190 (Meyer et al., 1999), and a similar localization was observed for RhoA in Schwann cells (Scherer and Gutmann, 1996), raising the possibility that JIP-1 may function in regulation of Rho activity in Schwann cells.

## Materials and methods

### Materials
Antibody to BrdU and FuGene6 transfection reagent were purchased from Roche Diagnostics Ltd. Antibody against c-Jun and SMAD2 were purchased from BD Biosciences. Antibodies against ERK1/2, phosphorylated JNK1/2 (Thr183/Tyr185), JNK1/2, and Ser473 phospho-Akt were purchased from Cell Signaling Technology (New England Biolabs, Inc.). Antibodies against phosphorylated ERK1/2 and the FLAG tag were purchased from Sigma-Aldrich. mAb against cyclin D1, SMAD4, and pAbs against ErbB2, ErbB3, and p27 were purchased from Santa Cruz Biotechnology, Inc. mAb against JIP-1 and JIP-1b expression construct were gifts from A. Whitmarsh (University of Manchester, Manchester, UK; Yasuda et al., 1999). Adenoviral constructs expressing EGFP and EGFP/Krox-20 were a gift from J. Milbrandt (Washington School of Medicine, St. Louis, MO; Nagarajan et al., 2001). Adenovirus expressing β-galactosidase and activated MEKK1, and antibody against the Ser63 phosphorylated form of c-Jun were gifts from J. Ham (University College London; Lallemand et al., 1998; Whitfield et al., 2001). Adenovirus expressing the FLAG-tagged JBD of JIP-1 (Harding et al., 2001) was a gift from J. Uney (University of Bristol, Bristol, UK); adenovirus expressing active MKK7 (MKK7D) was a gift from Y. Wang (University of California Los Angeles, CA; Wang et al., 1998); and adenovirus expressing JIP-1 was a gift from J.-A. Haefliger (University Hospital, Lausanne, Switzerland; Tawadros et al., 2002). Recombinant TGFβ1 and heregulin β1 (referred to as NRG-1) were purchased from R&D Systems. Krox-20 null mice were a gift from P. Charnay (Ecole Normale Superieure, Paris, France). Sources of other reagents have been detailed elsewhere (Morgan et al., 1991, 1994; Archelos et al., 1993; Gillespie et al., 1994; Jessen et al., 1994; Dong et al., 1995; Stewart, 1995; Meier et al., 1999; Parkinson et al., 2001, 2003).

### Cell culture
Schwann cells were prepared from the sciatic nerve and brachial plexus from newborn or 3-d-old rats (Brockes et al., 1979; Morgan et al., 1991). Schwann cells for TGFβ-induced apoptosis studies were prepared by immunopanning and used directly (Dong et al., 1999). Schwann cells were cultured unless otherwise stated in serum-free supplemented medium (Jessen et al., 1994) containing $10^{-6}$ M insulin, referred to as defined medium (DM). For adenoviral infection experiments, serum-purified Schwann cells were plated at a density of 5,000 cells in a 15-µl drop on poly-D-lysine/laminin–coated coverslips. For preparation of adenovirally infected cells for Western blot, serum-purified, or immunopanned Schwann cells were infected as described previously (Parkinson et al., 2001). For retroviral infection of Schwann cells, cells were cultured in DME supplemented with 3% FCS/2 µM forskolin/20 ng/ml NRG-1, and infected using retroviral supernatant from GP+E packaging cells as described previously (Parkinson et al., 2001, 2003). NIH 3T3 and Swiss 3T3 fibroblasts were cultured in DME/10% donor calf serum for infection using retroviral supernatants. Transient transfections and Krox-20 genotyping were prepared as described previously (Schneider-Maunoury et al., 1993; Parkinson et al., 2001, 2003).

### Cell survival assays
Schwann cells were infected with GFP control, Krox-20/GFP, or JBD-expressing adenovirus. 24 h later, cells were changed into DM. 6 h later, time 0 controls were fixed, whereas remaining cells were changed into DM or medium containing a combination of 20 ng/ml TGFβ1 plus 40 ng/ml TNFα or 20 ng/ml TGFβ1 alone in DM. The relationship between cell survival and death in this assay has been extensively characterized and is reciprocal and cell survival was assessed as described previously (Meier et al., 1999; Parkinson et al., 2001). For 24-h survival assays with TGFβ1 plus TNFα, percentage of survival is shown relative to time 0 controls. For TGFβ1 assays, percentage of survival is relative to 48 h untreated controls. For survival assays after serum withdrawal, Schwann cells were adenovirally infected in DME containing 10% FCS. 24 h after infection, time 0 controls were fixed, whereas remaining cells were changed into DME alone; cells were fixed after 72 h. Results shown are pooled from three independent experiments. TUNEL analysis and immunolabeling for activated caspase-3 were performed as described previously (Gavrieli et al., 1992; Parkinson et al., 2001).

### Immunocytochemistry
Immunolabeling with BrdU, P₀, and JIP-1 antibodies was performed as described previously (Morgan et al., 1991; Stewart et al., 1993; Willoughby et al., 2003). For all other antibodies, cells were fixed in 4% PFA in PBS, pH 7.5, for 10 min. For immunolabeling with Ser63 phospho-Jun antibod-

ies, cells were permeabilized in 0.5% Triton X-100/PBS for 5 min followed by a block of 50% goat serum/1% BSA/PBS for 30 min; the antibody was diluted in 1% BSA and left overnight at 4°C. For other antibodies, after fixing cells were permeabilized and blocked in antibody diluting solution (PBS containing 10% calf serum, 0.1 M lysine, 0.2% sodium azide) supplemented with 0.2% Triton X-100 for 30 min. Primary and secondary antibodies were diluted and applied in antibody diluting solution, with the secondary antibody conjugated to either FITC or Cy3. Coverslips were mounted in Citifluor (Citifluor Ltd.), and examined at RT with a fluorescence microscope (model Eclipse E800; Nikon). Images were captured at 40× magnification, using a digital camera (model DXM1200; Nikon), and ACT-1 acquisition software (Nikon). Pictures were digitalized using UMAX PowerLookIII scanner and figures were prepared using Adobe Photoshop version 5.0.

## Western blot analysis

40 μg of protein extracts were electrophoresed on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Hybond ECL; Amersham Biosciences), blocked with 5% fat-free milk in TBS/0.1% Tween 20, and incubated with primary antibodies in this block solution. Membranes were washed in TBS/Tween 20 and secondary antibody added in this solution. Specific protein complexes were revealed using ECL Plus chemiluminescent reagent (Amersham Biosciences).

## PCR

For PCR analysis, RNA was isolated from sciatic nerves of rats at E14, E18, P7, P12, and P12 cut at P7, using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized from total RNA using oligo dT primers (Promega) and Avian Myeloblastosis Virus reverse transcriptase (Promega). Oligonucleotide primers (Invitrogen) were used to amplify sequences containing the JIP-1 (5'-CGACTGTCTGT-CATCCCCAG-3' and 5'-CATAGACAGTGGCAGAGTCG-3') and GAPDH (5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCT-GTA-3'). The PCR amplification program consisted of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min for 35 cycles.

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