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Supplemental Data

Serum Response Factor mediates NGF-dependent target innervation by embryonic DRG sensory neurons.

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Supplemental Experimental Procedures

In situ hybridization and fluorescent immunohistochemistry

Digoxigenin (DIG)-labeled *cRNA* probes were generated by PCR and used for in situ hybridization as described (Luo et al., 2007). For immunohistochemistry of fresh frozen DRG sections, the antibodies used in this study were as follows: rabbit anti-TrkA (a gift from Dr Louis Reichardt, UCSF, 1:2000), mouse and rabbit anti-peripherin (Chemicon, 1:1000), rabbit anti-CGRP (Chemicon, 1:1000), rabbit anti-PGP9.5 (Chemicon, 1:1000), rabbit anti-βIII Tubulin/Tuj1 (Covance, 1:1000), rabbit anti-Neurofilament-200 (Chemicon, 1:500), and mouse anti-Neurofilament 165 (2H3, Developmental Studies Hybridoma Bank, 1:150). Secondary antibody incubations were performed with Alexafluor-546 or Alexafluor-488 conjugated secondary antibodies (Molecular Probes, 1:500). Wholemount immunofluorescent staining of embryos was performed using E12 or E14 limb/tissue pieces of embryos that were fixed in 4% paraformaldehyde (PFA) in PBS for 4 hours at 4^oC, bleached overnight in 15% H_2O_2, 1.5% DMSO in methanol and fixed again overnight in 10% DMSO in methanol. Primary antibodies were diluted in blocking solution (5% normal goat serum, 20% DMSO in PBS) and incubated for 5 days at room temperature. Following 3-5 washes at 10 min each in PBS, secondary antibodies were incubated overnight in blocking solution at room temperature. Embryos were cleared using

BABB (1:2 benzyl alcohol:benzyl benzoate) and mounted in the same clearing solution. Confocal stacks were acquired at 10-15 µm intervals and digitally collapsed. For anti-SRF and anti-MAL immunohistochemistry, embryos were immersion fixed in 4% paraformaldehyde overnight, followed by cryoprotection in 30% sucrose for 2 days. Embryos were embedded and 14 µm thick cryostat sections were mounted on slides. Slides were postfixed in 4% paraformaldehyde for 30 min followed by antigen retrieval by incubation at 80°C in a solution of 10 mM sodium citrate buffer, pH 8.9 for 30 min. Sections were then preblocked with 3 % milk in PBS + 0.3% Triton X (PBT) and blocked in 5% normal goat serum in PBT. Primary antibodies for SRF (Santa Cruz, 1:1000) and MAL/MRTF-A (Santa Cruz, 1:500) were diluted in Dako Diluent (Dako) overnight at 4°C. Biotinylated goat secondary antibodies were diluted (1:250) in Dako Diluent and sections were incubated in this solution for 30 minutes at room temperature. Following ABC amplification (Vector Labs), DAB chromogenic reaction was performed to visualize immunostaining. For immunohistochemistry to visualize hindpaw skin innervation in adult animals, mice were processed and immunohistochemistry performed and target innervation was quantitated as previously described (Luo et al., 2007)

Constructs and Inhibitors

FLAG tagged dominant negative MAL (lacking C-terminal region 1-630, dnMAL), full length MAL (wtMAL), SRE and SRE(ΔETS)-luciferase reporters have been previously described and were generous gifts from Drs. Ron Prywes and Jay Baraban. LY294002, PD98059, Latranculin B, and U0126 were purchased from Sigma or Calbiochem (La Jolla, CA).

Supplemental References

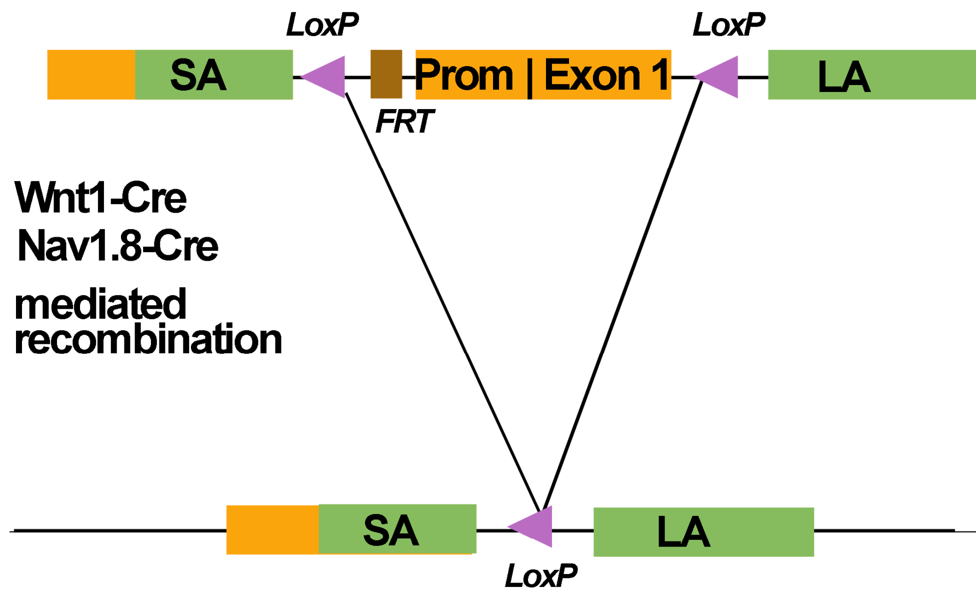
Luo, W., Wickramasinghe, S.R., Savitt, J.M., Griffin, J.W., Dawson, T.M., and Ginty, D.D. (2007). A hierarchical NGF signaling cascade controls Ret-dependent and Ret-independent events during development of nonpeptidergic DRG neurons. Neuron *54*, 739-754.

Meijering, E., Jacob, M., Sarria, J.C., Steiner, P., Hirling, H., and Unser, M. (2004). Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cytometry A *58*, 167-176.

Nassar, M.A., Stirling, L.C., Forlani, G., Baker, M.D., Matthews, E.A., Dickenson, A.H., and Wood, J.N. (2004). Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. Proceedings of the National Academy of Sciences of the United States of America *101*, 12706-12711.

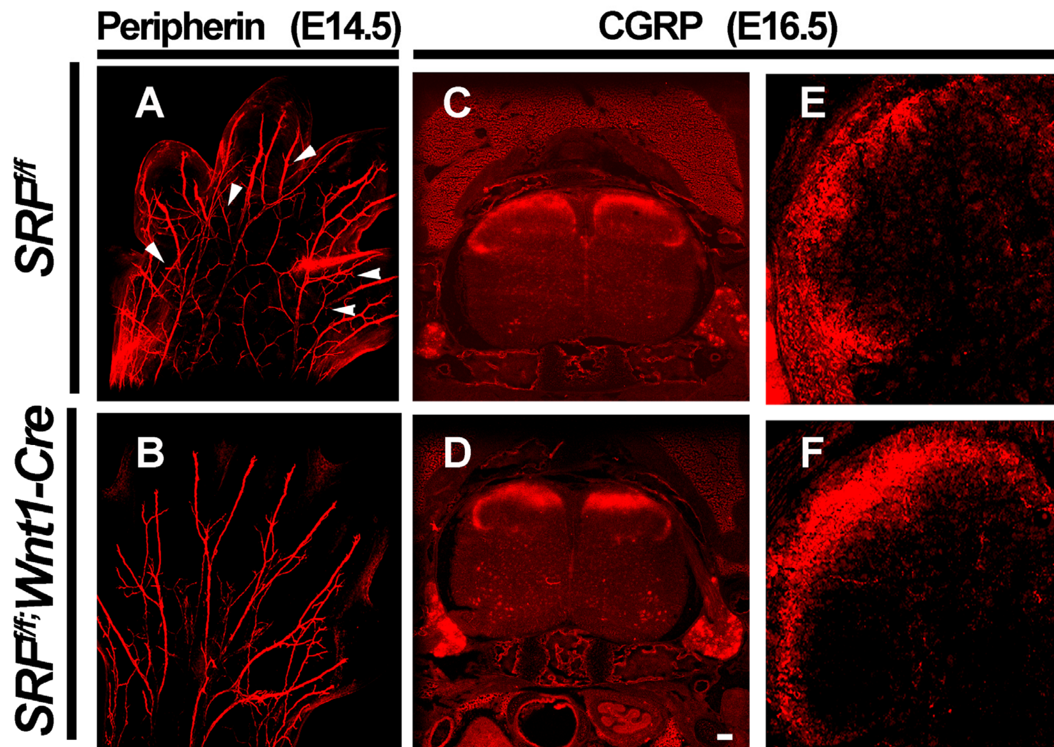
Ramanan, N., Shen, Y., Sarsfield, S., Lemberger, T., Schutz, G., Linden, D.J., and Ginty, D.D. (2005). SRF mediates activity-induced gene expression and synaptic plasticity but not neuronal viability. Nature Neuroscience *8*, 759-767.

Figure S1 : SRF conditional knockout mice



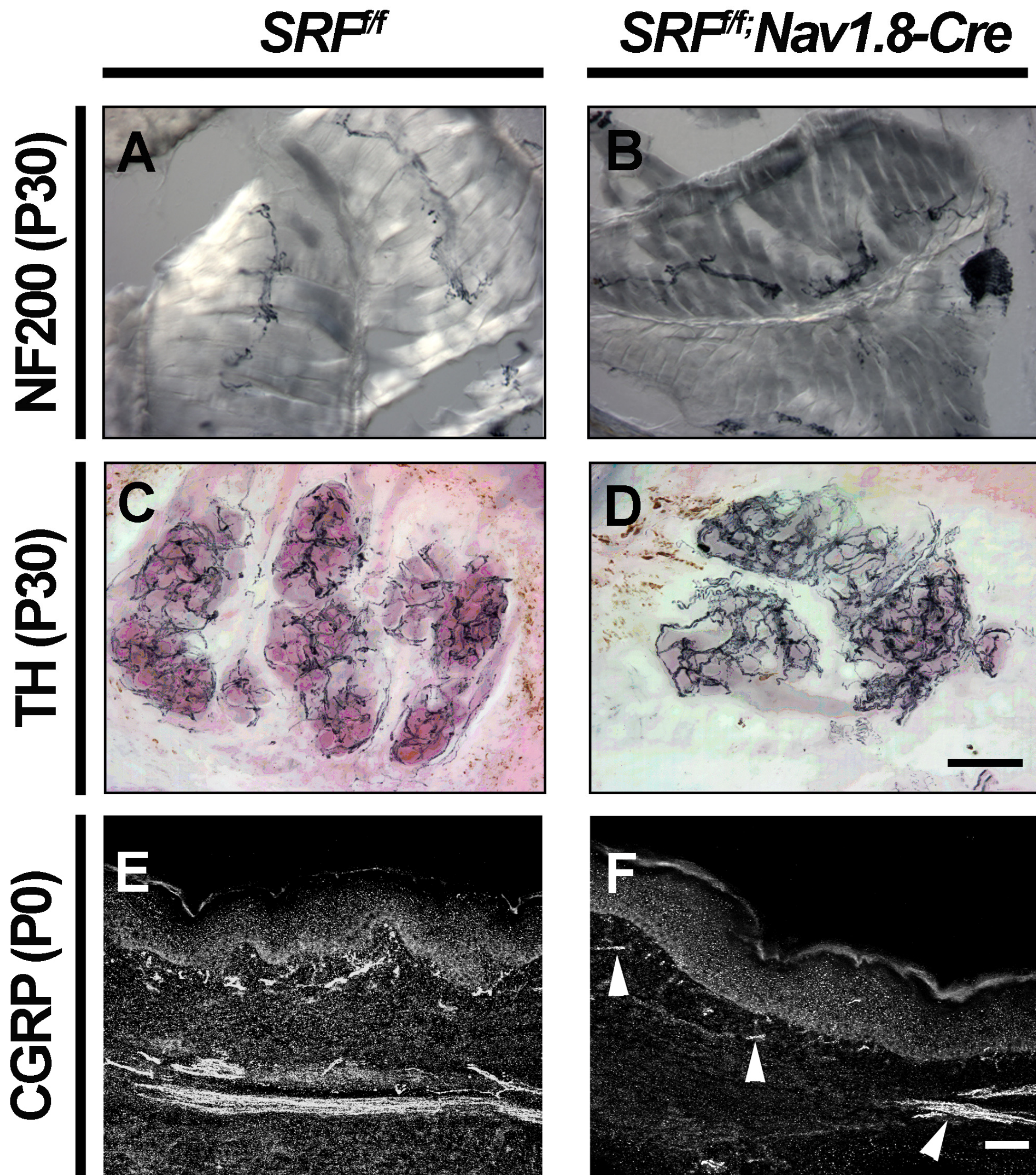
(A) Diagram of targeting vector used to generate $SRF^{f/f}$ mice (Ramanan et al., 2005) and strategy for *Wnt1-Cre* and *Nav1.8-Cre* mediated recombination.

Figure S2 : Peripheral and Central axonal projections in SRF mutant mice



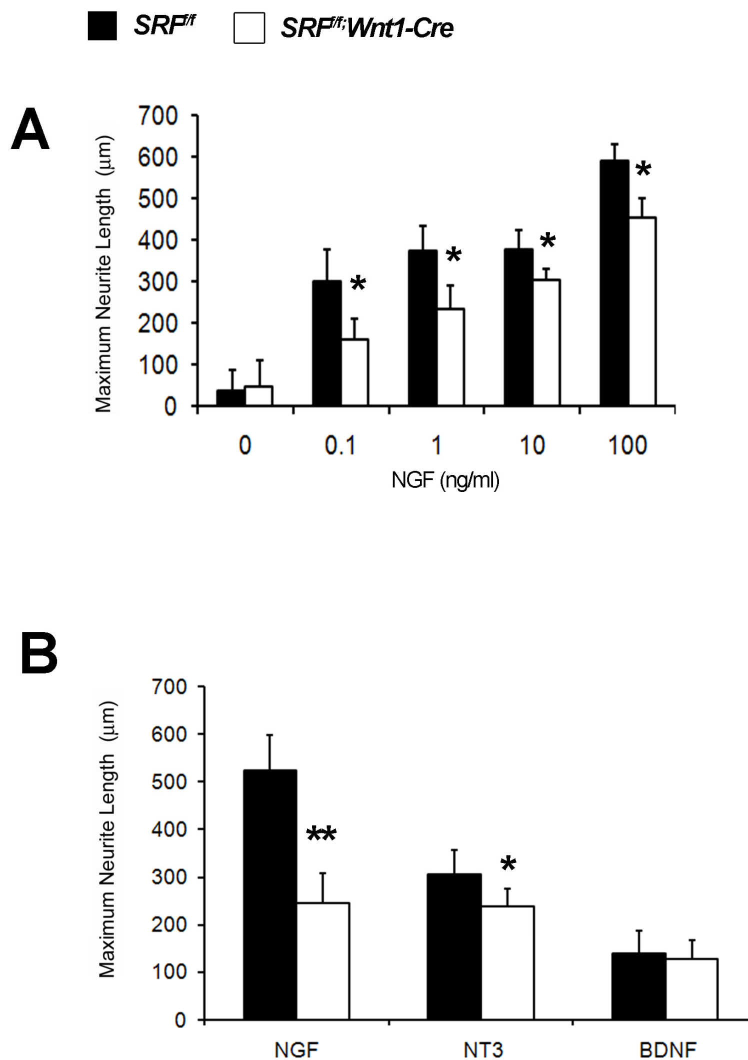
(A-B) High magnification images of wholemount immunofluorescent staining of embryonic limbs from *SRF*$^{f/f}$ and *SRF*$^{f/f}$*;Wnt1-Cre* mice using an antibody against Peripherin at E14.5. Arrowheads point to main trunks that are seen branching extensively in the control limb.
(C-D) Spinal cord and DRG immunohistochemistry using an antibody against CGRP reveals that *SRF*$^{f/f}$ and *SRF*$^{f/f}$*;Wnt1-Cre* mice exhibit robust central projections into the spinal cord at E16.5 (n=5 for each genotype) Scale bar, 80 μm.
(E-F) High magnification view of spinal central projections immunostained for CGRP from *SRF*$^{f/f}$ and *SRF*$^{f/f}$*;Wnt1-Cre* embryos at E16.5.

Figure S3 : Peripheral Projection Defects in adult *SRF^{f/f};Nav1.8-Cre* mice



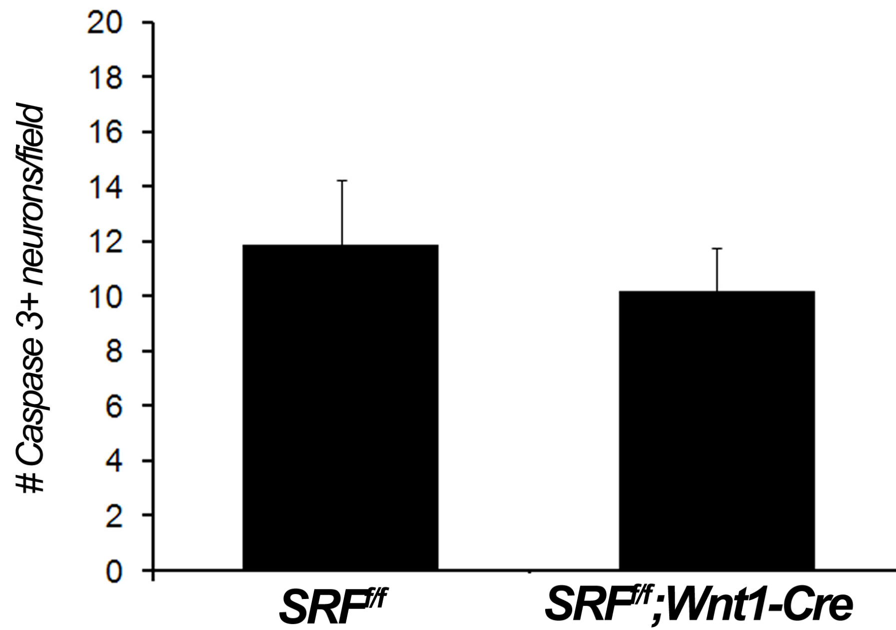
(A-B) Wholemount immunohistochemistry from adult *SRF^{f/f}* and *SRF^{f/f};Nav1.8-Cre* mice reveal that $NF200^{+}$ large diameter, myelinated axonal projections are intact in the absence of SRF. Shown are segments of muscle fibers with myelinated axons forming neuromuscular junctions and innervating muscle spindles.
(C-D) Tyrosine hydroxylase immunohistochemistry labels all sympathetic fibers to the sweat glands in the footpads of adult *SRF^{f/f}* and *SRF^{f/f};Nav1.8-Cre* mice. No difference in the TH^{+} innervation of the sweat glands was detected in the absence of SRF. Scale bar, 40 μm.
(E-F) Immunohistochemistry using an antibody against CGRP in *SRF^{f/f}* and *SRF^{f/f};Nav1.8-Cre* mice at P0 shows that these mice have substantially reduced epidermal innervation even at this early timepoint. Scale bar, 60 μm.

Figure S4 : Axonal outgrowth defects in mice lacking SRF *in vitro*.



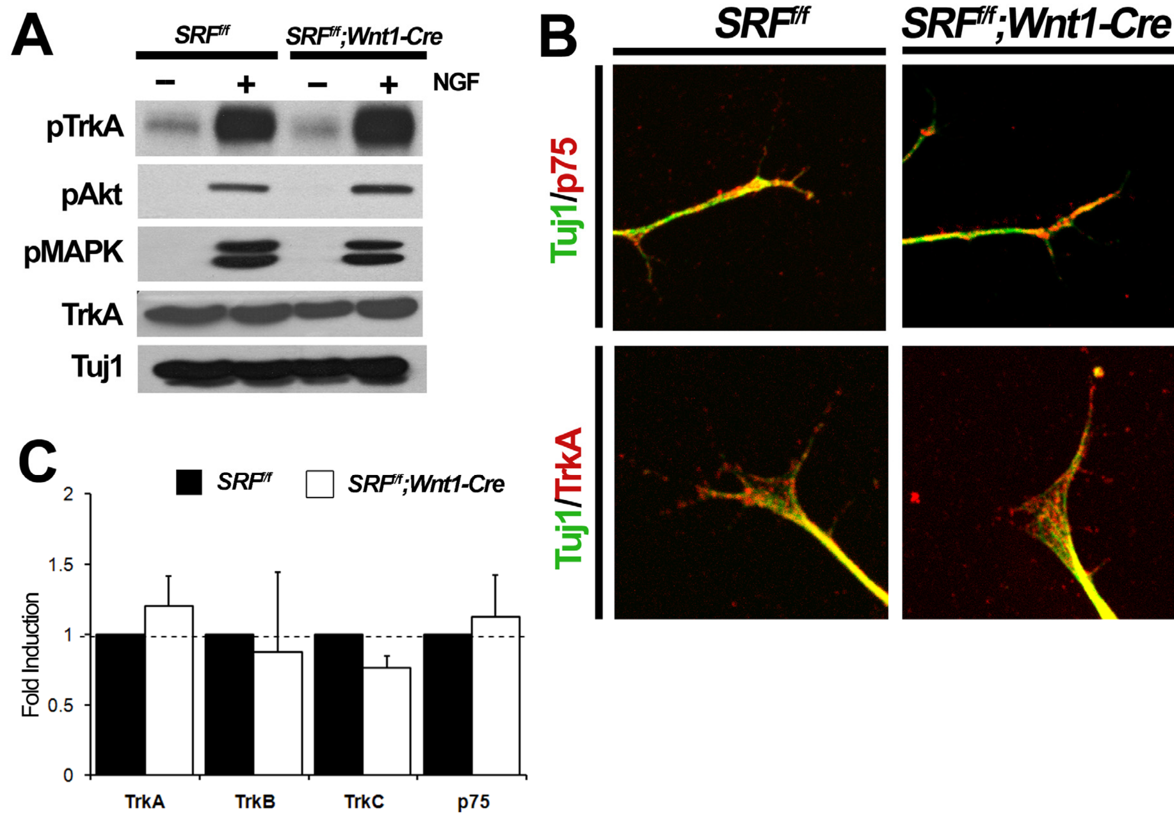
(A) NGF-dependent defects in axonal outgrowth seen in DRG neurons from *SRF^f/f;Wnt1-Cre* embryos occur over a wide range of concentrations *in vitro*. Although DRG neurons from littermate control animals show a dose-dependent effect in axon length, neurons from SRF mutant embryos at E14 consistently demonstrated shorter axon length at all concentrations of NGF tested. (n=3 for each genotype, *=p<0.05)
(B) Effects of different neurotrophins on axonal outgrowth in SRF mutant mice. E14 DRG neurons from *SRF^f/f* and *SRF^f/f;Wnt1-Cre* mice were grown in the presence of different neurotrophins – NGF, NT3 and BDNF *in vitro*, and effects of each of these neurotrophins on axonal outgrowth is shown after 24 hours in culture. Loss of SRF impaired NGF-dependent axonal outgrowth, whereas NT3-dependent axonal outgrowth was only modestly impaired. In contrast, BDNF-dependent axonal outgrowth was not affected. (n=3 for each genotype, **=p<0.01, *=p<0.05).

Figure S5 : SRF does not mediate NGF-dependent survival of DRG neurons *in vitro*.



Dissociated DRG neurons from *SRF*$^{f/f}$ and *SRF*$^{f/f}$*;Wnt1-Cre* embryos at E14 were plated on coverslips for 24 hrs in the presence of NGF. Immunocytochemistry was then performed for activated Caspase-3 and Tuj1 to specifically label apoptotic DRG neurons. The number of Caspase-3^{+}/Tuj1^{+} neurons were counted and the results are shown. (n=3 for each genotype).

Figure S6 : Loss of SRF does not affect expression or trafficking of NGF receptors, or signaling downstream of NGF

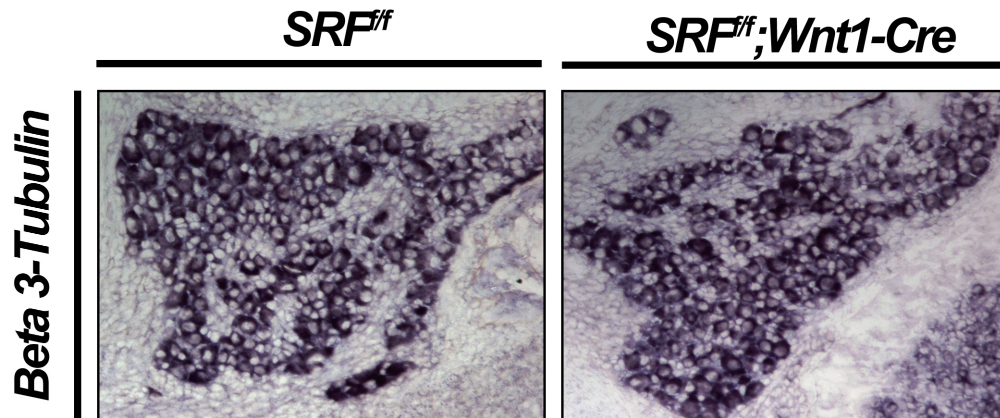


(A) Western blots performed using antibodies against pTrkA, pAkt and pERK (MAPK) from lysates of DRG neurons from SRF mutant and littermate control mice stimulated for 20 minutes with NGF.

(B) Trafficking of NGF receptors TrkA and p75 in SRF mutant and littermate control mice to the leading edge of growth cones of DRG neurons. Immunocytochemistry using antibodies against TrkA, p75 and Tuj1 was performed and growth cones were visualized by confocal microscopy.

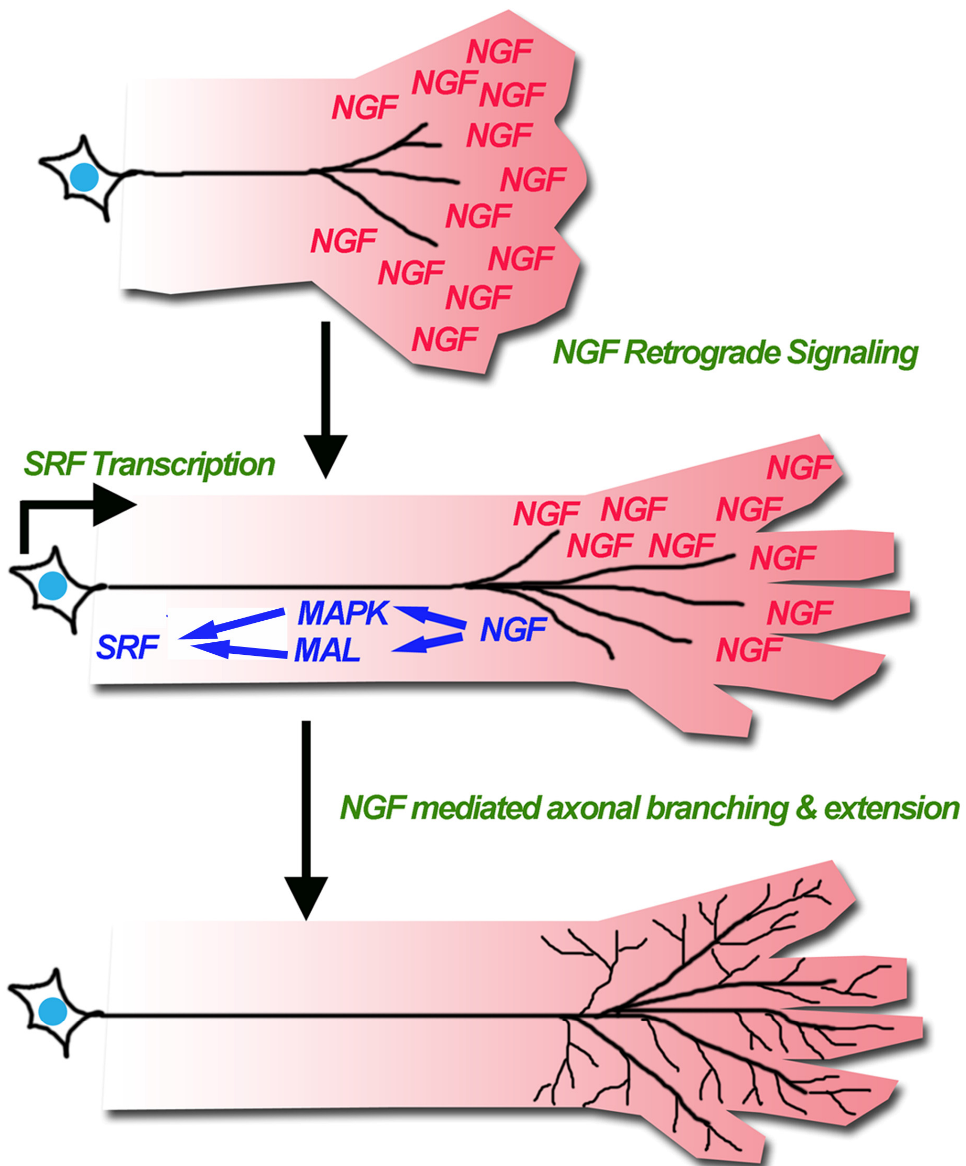
(C) Quantitative real-time PCR for *TrkA, TrkB, TrkC* and *p75* using mRNA isolated from whole DRGs from *SRF$^{f/f}$* and *SRF$^{f/f}$;Wnt1-Cre* embryos at E14.5 demonstrates no difference in the expression of these receptors. (n=3 for each genotype).

Figure S7 : Expression of *beta-3-tubulin* is unaffected in DRG neurons in *SRF$^{f/f}$;Wnt1-Cre* embryos.



In situ hybridization using probes against *β3 Tubulin* demonstrate that this cytoskeletal gene is unaffected in the absence of SRF in DRGs from E14.5 *SRF$^{f/f}$* and *SRF$^{f/f}$;Wnt1-Cre* DRGs.

Figure S8 : A Model for NGF-dependent Target Innervation through SRF



Early embryonic axon outgrowth in DRG neurons occurs independently of NGF. Once axons have grown into the target area, target-derived NGF signals locally, within the growth cone, and retrogradely, through MAPK and MAL signaling pathways to mediate SRF-dependent transcription. Expression of SRF target genes, in turn, is required for the completion of target innervation by supporting distal extension and branching, and penetration of the epidermis.

Table S1 : Analysis of SRF mutant embryo viability *in utero*.

Age	*SRF*$^{f/+}$*; Cre*$^{-}$	*SRF*$^{f/+}$*; Cre*$^{+}$	*SRF*$^{f/f}$*; Cre*$^{-}$	*SRF*$^{f/f}$*; Cre*$^{+}$	Total	Percentage
P0 (32 lit)	**66**	**54**	**72**	**0**	**192**	**0%**
E17.5 (5 lit)	**12**	**9**	**14**	**3**	**38**	**7.89%**
E15.5 (7 lit)	**15**	**11**	**19**	**1**	**46**	**10.8%**
E13.5 (15 lit)	**33**	**21**	**35**	**7**	**96**	**7.3%**
E11.5 (14 lit)	**22**	**28**	**21**	**19**	**73**	**21.1%**

Shown is the distribution of genotypes after analyzing a total of 445 embryos from continuous matings of *SRF*$^{f/f}$ and *SRF*$^{f/+}$*;Wnt1-Cre* animals. Note that continuous record keeping of the distribution of genotypes from matings was suspended after reaching the values shown above, although for the experiments in this study, many additional matings were performed (and not reported in this census).

Table S2 : List of Genes/Proteins examined by Immunohistochemistry and In situ hybridization

CGRP[*]	**Neurofilament M (165 kDa)**[*]
c-Ret[*,†]	**Neurofilament H (200 kDa)**[*]
TrkA [*,†]	**Substance P**[†]
TrkB[†]	**P2X3**[*]
TrkC[†]	**Peripherin**[*]
TRPA1[†]	**Nav1.8**[†]
TRPV1[†]	**Nav1.9**[†]
TRPC3[†]	**ER81**[†]
TRPM8[†]	**Latexin**[†]
HoxD1[†]	**NCAM**[†]
GFRα1[†]	**MrgD**[†]
GFRα2[†]	**NFATc4**[†]

A list of the genes/proteins analyzed by immunohistochemistry and/or in situ hybridization that are markers of differentiation of embryonic DRG sensory neurons. Genes listed in this table were detected but found to exhibit no expression level difference in DRG neurons from $SRF^{f/f}$ and $SRF^{f/f}$*;Wnt1-Cre* mice at E16.5. [*] = by immunohistochemistry, [†] = by *in situ* hybridization.