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Differential Phosphorylation of Smad1 Integrates BMP and Neurotrophin Pathways through Erk/Dusp in Axon Development

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SUMMARY

Sensory axon development requires concerted actions of growth factors for the precise control of axonal outgrowth and target innervation. How developing sensory neurons integrate different cues is poorly understood. We demonstrate here that Smad1 activation is required for neurotrophin-mediated sensory axon growth in vitro and in vivo. Through differential phosphorylation, Smad1 exerts transcriptional selectivity to regulate the expression and activity of Erk1 and Erk2—two key neurotrophin effectors. Specifically, bone morphogenetic proteins (BMPs) signal through carboxy-terminal phosphorylation of Smad1 (pSmad1^{C}) to induce *Erk1/2* transcription for enhanced neurotrophin responsiveness. Meanwhile, neurotrophin signaling results in linker phosphorylation of Smad1 (pSmad1^{L}), which in turn upregulates an Erk-specific dual-specificity phosphatase, Dusp6, leading to reduced pErk1/2 and constituting a negative-feedback loop for the prevention of axon overgrowth. Together, the BMP and neurotrophin pathways form a tightly regulated signaling network with a balanced ratio of Erk1/2 and pErk1/2 to direct the precise connections between sensory neurons and peripheral targets.

INTRODUCTION

During sensory circuit wiring, embryonic neurons need to integrate various extracellular cues for the precise control of initiation, elongation, and branching of their axons. It remains to be clarified how multiple signaling pathways converge to collaboratively mediate axon development.

The neurotrophin family plays a central role in stimulating axonal outgrowth. Nerve growth factor (NGF), a prototypic neurotrophin, is target derived and modulates terminal sensory axon branching. In *NGF* knockout mice on a *Bax*$^{-/-}$ background that prevents neuronal cell death, terminal arborization and epidermal innervation are impaired (Patel et al., 2000). Neurotrophins bind to receptor tyrosine kinases of the Trk family and signal through well-conserved effectors: mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk) and phosphatidylinositol-3-kinase/Akt (PI3K/Akt; Reichardt, 2006). In cultured neurons, both pathways are required for peripheral axon outgrowth, whereas PI3K/Akt is more involved in cell survival (Atwal et al., 2000; Kaplan and Miller, 2000). Double deletion of either *Braf/Raf1* or *Erk1/Erk2* results in aberrant terminal branching of sensory axons in the NGF-expressing target field in vivo (Newbern et al., 2011; Zhong et al., 2007). Although the critical roles of NGF/TrkA pathways in sensory axon outgrowth have been amply demonstrated, it is less clear how the expression and activity of individual signaling components are regulated to ensure proper neurotrophin responsiveness in the developing neurons. Furthermore, safeguard mechanisms to prevent axon overgrowth remain to be uncovered.

Other growth factors involved in axonal outgrowth include the bone morphogenetic proteins (BMPs). BMPs, members of the transforming growth factor β (TGF-β) superfamily, play a variety of roles in neural development (Liu and Niswander, 2005). BMPs activate receptor serine/threonine kinases (BMPR) and signal through C-terminal phosphorylation of Smads (pSmad^{C}; Feng and Derynck, 2005; Shi and Massagué, 2003). Previously, we showed that Smad1, one of the Smads that mediate BMP signaling, is developmentally regulated (Zou et al., 2009). pSmad1^{C} accumulates in the nuclei of embryonic sensory neurons in the dorsal root ganglia (DRGs) from embryonic day 10.5 (E10.5) to E15.5 during active axonal outgrowth. Blocking BMP signaling by pharmacological inhibition of its receptors or by RNA interference (RNAi)-mediated *Smad1* knockdown results in an arrest of axonal outgrowth in cultures (Parikh et al., 2011). In vivo evidence confirming the role of Smad1 in axonal growth has yet to be established. Importantly, when adult DRG neurons are triggered into a regenerative state, Smad1 is induced and activated, and enhances the regeneration of sensory axons after spinal cord injury (Parikh et al., 2011).

An intriguing phenomenon observed in previous studies (Parikh et al., 2011) is that when BMP signaling is blocked, axonal outgrowth is arrested even though neurotrophin is present in the culture media, suggesting that BMP signaling is required for neurotrophin-mediated axonal outgrowth. However, the question remains as to whether the two pathways converge on

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a common node or function in parallel with distinct downstream targets. Precedent exists for a collaboration between BMP and neurotrophin (for example, BMP7 requires NGF as a cofactor to optimally induce dendritic growth in sympathetic neurons; Lein et al., 1995), but mechanistic understanding is lacking. One possible convergence point is Smads. In addition to C-terminal phosphorylation sites, Smads also have a linker area between the MH1 and MH2 domains. This area contains four conserved MAPK phosphorylation sites that can be targeted by members of the MAPK family (Erk, p38, and c-Jun N-terminal kinase [JNK]; Hough et al., 2012; Pera et al., 2003) and several glycogen synthase kinase (GSK) phosphorylation sites. Integration of dorsoventral (BMP) and anteroposterior (Wnt/GSK3) patterning gradients reportedly occurs through differential phosphorylation of Smad1 in *Xenopus* embryos (Fuentealba et al., 2007). The in vivo function of linker phosphorylation of Smad1 (pSmad1^{L}) in the mammalian nervous system is not known.

Here, we tested the hypothesis that Smad1 functions as a convergence node of the BMP and neurotrophin pathways during sensory axon development. We show that Smad1 is specifically expressed in the developing peripheral sensory neurons and that it is activated at both its C terminus and linker region. By studying cultured embryonic DRG neurons and in vivo axon growth patterns in three *Smad1* mutant alleles (two conditional deletions and one linker mutation), we demonstrate that Smad1 activation is required for NGF-mediated terminal axon branching. We identify *Erk1* and *Erk2* as pSmad1^{C} transcriptional targets and reveal a pSmad1^{L}-Dusp-based negative-feedback loop that regulates the intensity of Erk1/2 signaling, thus establishing distinct in vivo roles for pSmad1^{C} and pSmad1^{L} during sensory neuron development. Together, the molecular events of the BMP and neurotrophin pathways contribute to a tightly regulated signaling network that directs the precise connections between sensory neurons and their targets.

RESULTS

Neurotrophin-Induced Axonal Outgrowth Specifically Requires BMP Signaling

To investigate how the neurotrophin and BMP pathways collaboratively mediate sensory axon development, we isolated E12.5 DRGs and monitored axonal outgrowth in explant cultures in media containing NGF and an increasing concentration of dorsomorphin (DM), an inhibitor of the type I BMP receptor that blocks activation of Smad1/5/8 (Yu et al., 2008). Axonal outgrowth was inhibited by DM treatment in a dose-dependent manner (Figures 1A and 1B). At higher doses of DM, axonal outgrowth was completely arrested, confirming the requirement of BMP signaling for NGF-mediated axonal outgrowth. The inhibitory effect of DM was reversible, since DRG neurons resumed growth after DM washout (Figure 1B), indicating no permanent adverse effect from DM treatment. Similar DM-mediated inhibition of axonal outgrowth was observed in dissociated E12.5 DRG neurons plated at low density to minimize the effect of glial cells (Figure 1C).

To examine whether the requirement for NGF-mediated axonal outgrowth is specific to the BMP branch of the TGF-β superfamily, we treated E12.5 DRG explants with SB431542, a specific TGF-β signaling inhibitor. No inhibitory effect was observed (Figures 1A and 1B). Application of a protein kinase A (PKA) inhibitor also did not affect axonal length (Figure S1A), as previously reported (Liu and Snider, 2001).

pSmad1^{C} Is Present Specifically in Embryonic DRG Neurons and Is Required for Neurotrophin-Mediated Axonal Outgrowth

We next investigated whether it is the canonical BMP pathway signaling through pSmad1^{C} that is involved in NGF-dependent axonal outgrowth. The axonal growth arrest with DM treatment occurs in parallel with a marked reduction of pSmad1^{C} in embryonic DRG neurons (Figure 1D), suggesting the involvement of pSmad1^{C} in axonal outgrowth. To rule out nonspecific effects of DM and further confirm the requirement of pSmad1^{C} for NGF-mediated axon growth, we decided to repeat the study using E12.5 DRGs from mutant mice with a genetic deletion of *Smad1*. Because of the early lethality of *Smad1*$^{-/-}$ embryos (Lechleider et al., 2001), we generated conditional knockout (cKO) mice (*Smad1*$^{fl/-}$*;Nestin-Cre*; hereafter referred to as *Smad1* cKO^{Nes}). *Nestin-Cre* induces recombination in neural progenitor cells and is expected to ablate *Smad1* specifically in DRG sensory neurons as early as E11.5 while sparing Schwann cells (Dubois et al., 2006; Kao et al., 2009; Tronche et al., 1999), which we confirmed using the Rosa26-EYFP^{STOP} reporter line (Figures S1C–S1E). First, western blotting confirmed that Smad1 was markedly reduced in E12.5 mutant DRGs as compared with *Smad1*$^{fl/+}$*;Nestin-Cre* control littermates (Figure 1E). Immunostaining showed that pSmad1^{C} was abundantly present in the nuclei of 95.6% ± 5.5% of the sensory neurons in wild-type (WT) DRGs at postnatal day 1 (P1), but not in *Smad1* cKO^{Nes} DRGs (Figure 1F). Notably, colabeling with pSmad1^{C} and β-tubulin III (Tuj1), a pan-neuronal marker, demonstrated that the pSmad1^{C} immunosignals were specifically detected in sensory neurons, but not in satellite glial cells in DRGs or in developing Schwann cells populating the peripheral nerves (Woodhoo et al., 2009; Figures 2A and 2B), thus supporting a role for Smad1 in the development of sensory neurons but not Schwann cells. We then compared the axonal outgrowth of E12.5 DRG explants from *Smad1* cKO^{Nes} and control littermates in NGF-containing media. Mutant DRGs with *Smad1* deletion grew shorter axons than the WT DRGs, with average axonal lengths of 346.6 μm ± 29.0 SEM and 431.7 μm ± 16.2 SEM, respectively, after 24 hr in culture (Figure 1G). We therefore conclude that pSmad1^{C}-mediated BMP signaling is required for NGF-dependent axonal outgrowth.

Smad1 Deletion Hinders Terminal Branching of Peripheral Axons

Next, we investigated the in vivo role of pSmad1^{C} in sensory neuron development by first examining sensory axon projections in *Smad1* cKO^{Nes} mice. We focused on cutaneous innervation, a well-characterized NGF-dependent process, in the mystacial pads of newborn pups by immunostaining for a pan-axonal marker, protein gene product 9.5 (PGP9.5), and a small-caliber axon marker, calcitonin gene-related peptide (CGRP). The *Smad1* cKO^{Nes} newborn pups displayed a mild but significant

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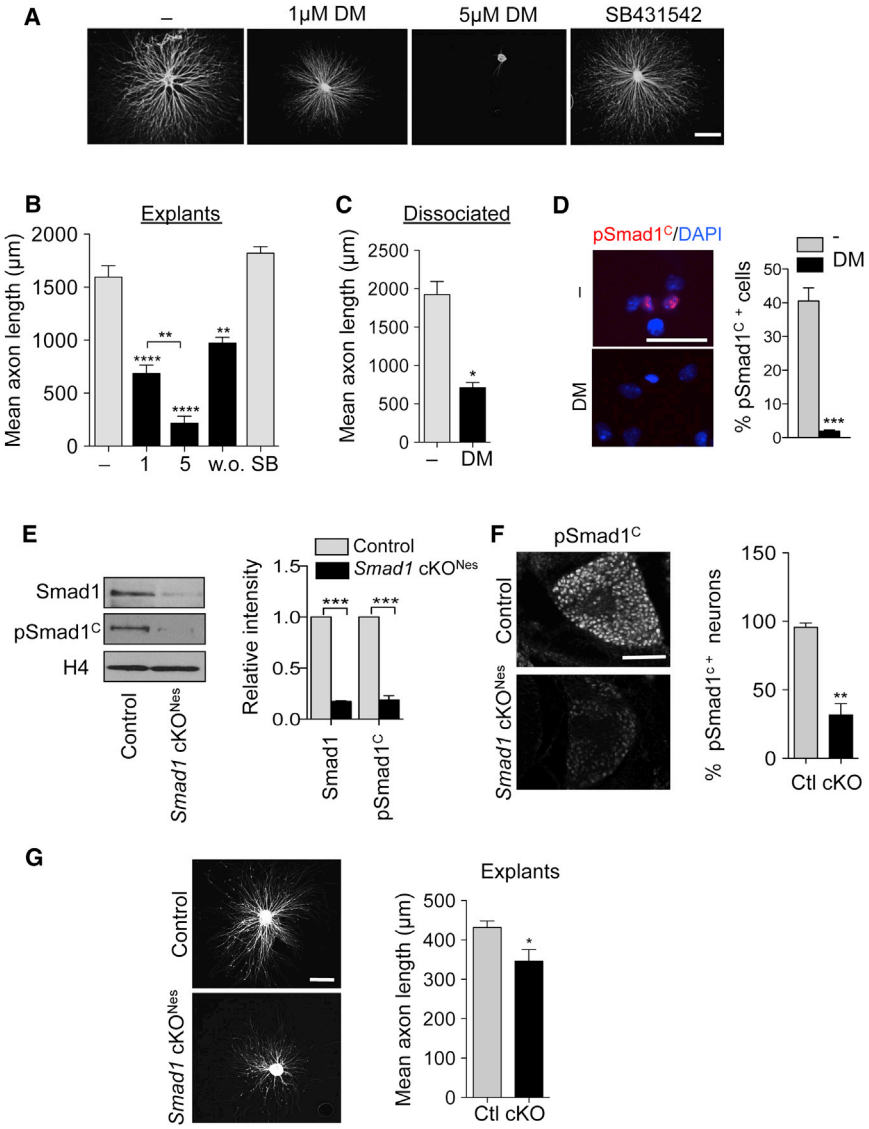

Figure 1. BMP/Smad Signaling Is Required for NGF-Dependent Axon Growth

(A and B) E12.5 DRG explants were cultured in NGF-containing media for 48 hr with DM (1 or 5 μM) or SB431542 (5 μM) and axonal length was determined by Tuj1 immunostaining and averaged over six independent experiments (n = 6). w.o., DM washout.

(C and D) Low-density dissociated cultures of E12.5 DRG neurons were treated with DM (1 μM) for 48 hr.

(C) Quantification of the average axonal length.

(D) pSmad1^C immunostaining. DM treatment reduced pSmad1^C nuclear levels (n = 3).

(E) Western blotting of DRG extracts from E12.5 embryos with antibodies indicated (histone 4 [H4] served as the loading control).

(F) Immunohistochemistry of P1 DRGs and quantification showed that pSmad1^C was present specifically in WT DRGs but absent in *Smad1* cKO^{Nes} DRGs.

(G) E12.5 DRG explants from *Smad1* cKO^{Nes} or control embryos were cultured in NGF-containing media for 48 hr, and axonal lengths were determined by Tuj1 immunostaining and averaged over three independent experiments.

Scale bar: 200 μm (A and G), 50 μm (D), and 100 μm (F). Error bars represent SEM. See also Figure S1.

truncated distal network of axons and diminished terminal arborization in the upper dermis as compared with littermate controls (Figures 2C and S2). The average number of axonal branches at the junction of the upper dermis and epidermis over a similar field examined was 8.8 in control pups and 6.0 in *Smad1* cKO^{Nes} pups (a 32% decrease; Figure 2D). Similarly, the number of CGRP$^+$-terminal axonal branches per similar field examined was reduced from 7.8 in control pups to 6.1 in *Smad1* cKO^{Nes} pups (a significant 22% decrease; Figures 2C and 2D). It is unlikely that the decrease in terminal axon branches was due to a reduced expression of PGP9.5 or CGRP in mutant pups, as their immunoreactivity in sensory neuron soma appeared comparable and the total numbers of PGP9.5$^+$ or CGRP$^+$ neurons in P1 DRGs from the same thoracic spinal segment were also similar regardless of the genotypes (Figures 3D and 3E). Because the terminal axon defects were detected in P1 mice, they more likely originated from stunted distal axonal branching than from axonal retraction. The phenotypes are reminiscent of those observed in *NGF*$^{-/-}$;Bax$^{-/-}$ or *TrkA*$^{-/-}$;Bax$^{-/-}$ mice, although they are milder, which may be attributed to compensatory mechanisms from different Smads or other molecules involved in sensory axon growth (Patel et al., 2000).

We also examined whether Smad1 is required for directing central projections of sensory axons. In *Smad1* cKO^{Nes} P1 pups, the small-caliber CGRP$^+$ nociceptive axons entered the dorsal spinal laminae in a normal pattern, and the large-caliber parvalbumin (PV)$^+$ proprioceptive afferent axons also projected toward the ventral motor pools in a normal fashion (Figure 3A). Therefore, Smad1 appears to be dispensable for central axon projection in vivo. Taken together, these findings indicate that Smad1 signaling is specifically required for proper NGF-mediated target innervation of sensory axons.

Smad1 Is Not Required for Neurotrophin-Mediated Survival and Differentiation of Sensory Neurons

We next asked whether BMP/Smad1 is required for two other key processes that are neurotrophin dependent: survival and differentiation of sensory neurons. The survival of TrkA$^+$ DRG neurons is dependent upon successful competition for target-derived NGF (White et al., 1996), whereas the survival of TrkC$^+$ neurons depends on NT3 (Wyatt et al., 1999). We first assessed the extent of cell death in embryonic DRGs by immunostaining

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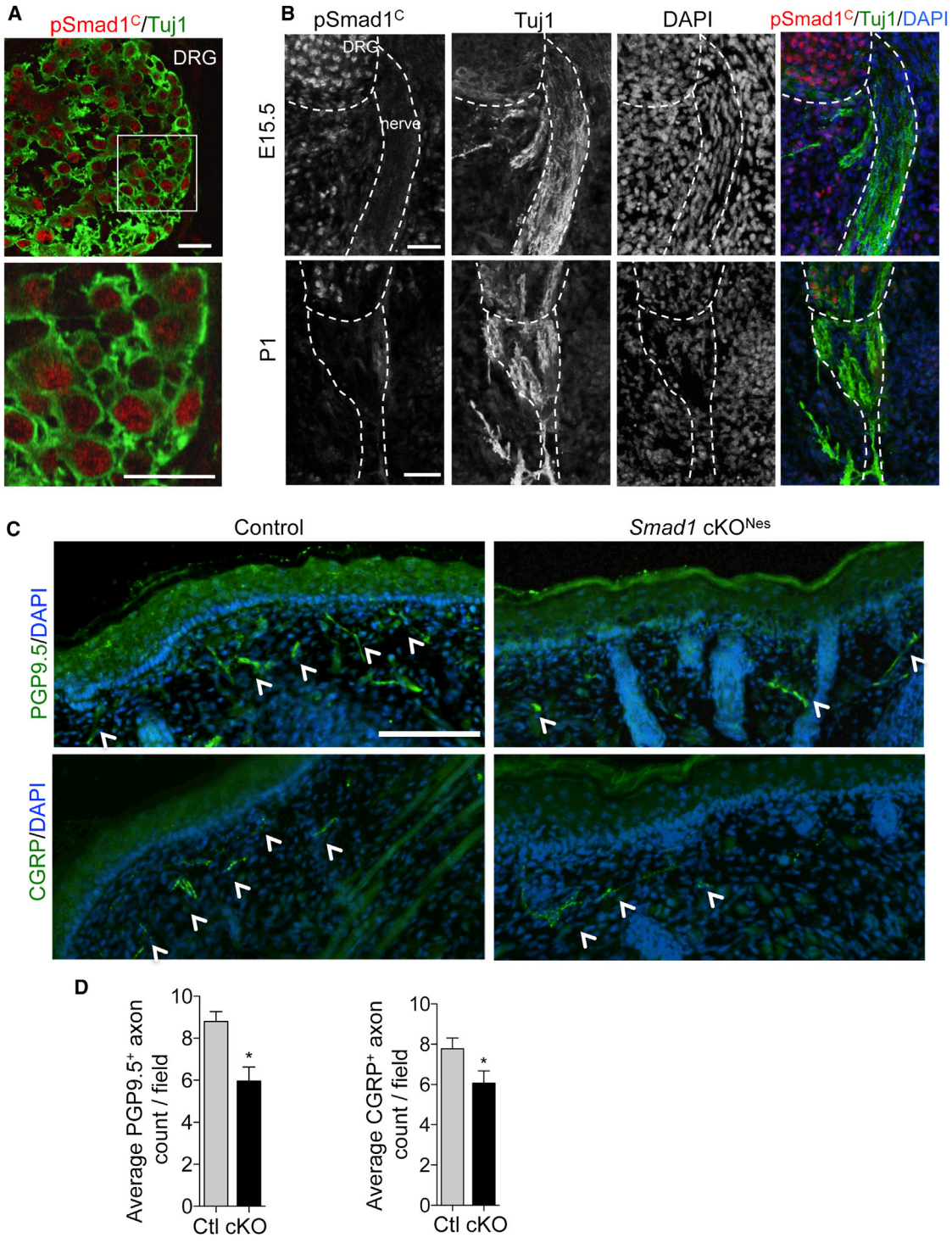

Figure 2. pSmad1^C Is Present Specifically in DRG Sensory Neurons and Is Required for Terminal Axon Branching

(A and B) Immunostaining of E16.5 DRGs (A, confocal images) or E15.5 and P1 DRGs and peripheral nerves (B) from WT mice shows that immunosignals of pSmad1^C were specifically detected in the majority of neurons, but not in satellite glial cells (A) or in developing Schwann cells populating the nerves (Tuj1, green, B).

(C and D) Representative images and quantification of cutaneous innervation of mystacial pads of P1 pups with immunostaining for PGP9.5 (green, arrows, top panel) or CGRP (green, arrows, bottom panel) and DAPI counterstaining.

(D) The average number of axon terminal branches in the upper dermis per field examined was calculated from five independent 20× images from each pup and then averaged over five pairs of littermates.

Scale bars: 50 μm (A), 100 μm (B), and 50 μm (C). Error bars represent SEM. See also Figure S2.

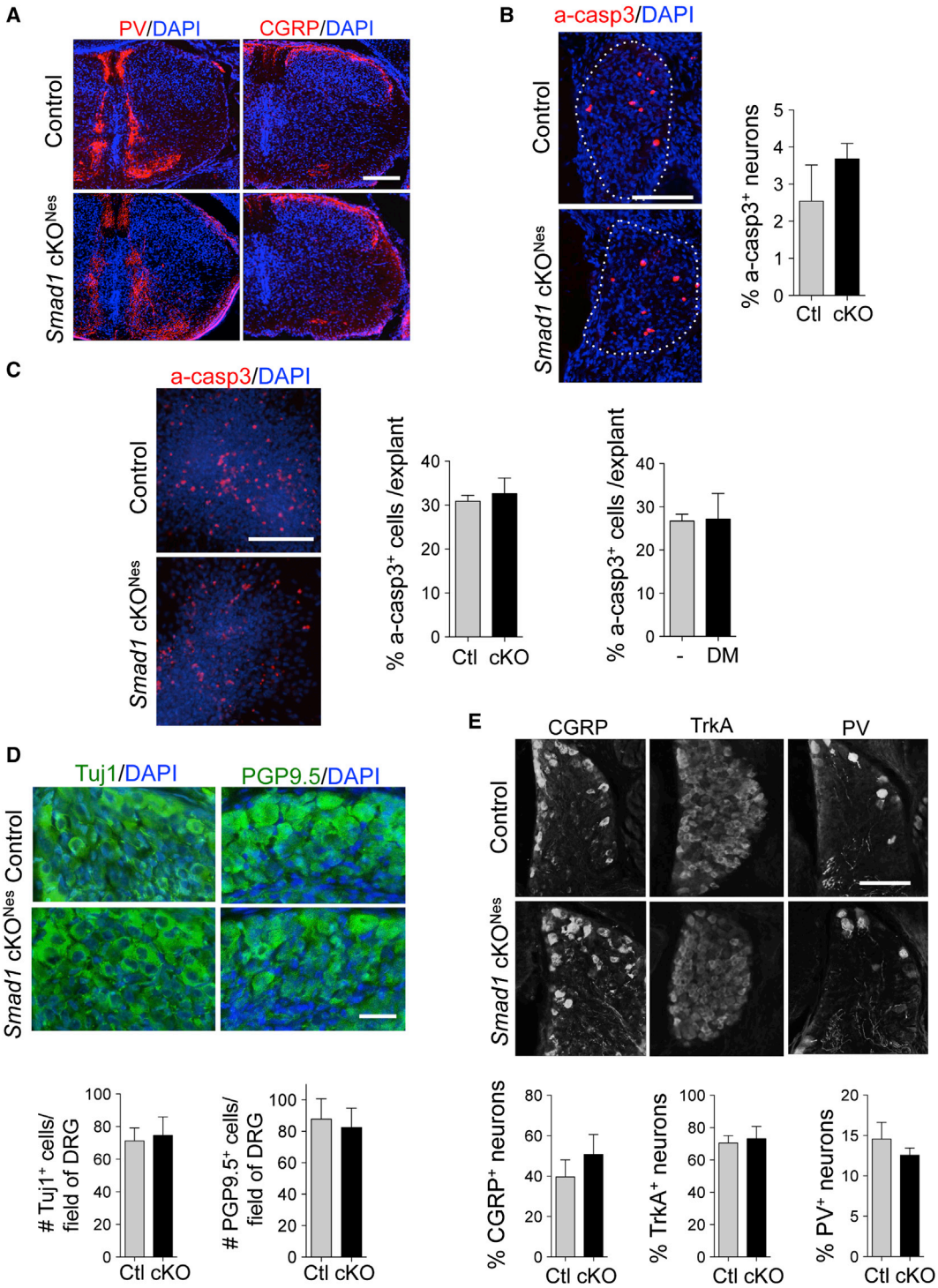

Figure 3. BMP/Smad1 Is Not Required for NGF-Mediated Cell Survival or Differentiation of Peripheral Sensory Neurons

(A) Images of spinal cords of P1 *Smad1* cKO^{Nes} or control pups immunostained for PV or CGRP (red) and counterstained with DAPI (blue).

(B) Apoptotic sensory neurons in E15.5 DRGs were revealed by antibody against activated caspase-3 (a-casp3) and quantified.

(C) E12.5 DRG explants were cultured for 48 hr and immunostained for a-casp3. Quantification was averaged over duplicate DRGs cultures from three littermate pairs (n = 3, left). Quantification of E12.5 DRG explants cultured for 48 hr with or without DM was shown in graph on the right (n = 6).

(D and E) Sensory neuron differentiation in P1 DRGs was determined by immunostaining for the indicated markers and quantified in randomly selected fields of similar sizes from thoracic DRGs of the same spinal segments.

Scale bars: 200 μm (A), 50 μm (B, D, and E), and 100 μm (C). Error bars represent SEM. See also Figure S3.

for activated caspase 3, a marker of apoptosis. Loss of Smad1 did not have an impact on neuronal survival at E15.5, one of the peak times of apoptosis in developing DRGs (White et al., 1996; Figure 3B). The numbers of total DRG neurons (Tuj1$^+$ or PGP9.5$^+$) from the same thoracic spinal segment of P1 mice were also comparable regardless of the genotypes (Figure 3D). Additionally, TrkA$^+$ small-diameter neurons and PV$^+$ large-diameter neurons were similar in number in P1 DRGs (Figure 3E). To rule out a compensatory mechanism by other Smads, E12.5 WT DRG explants were cultured with NGF for 2 days with or without DM treatment, and no change in the percentage of apoptotic cells in DRG explants was found (Figure 3C). Similar results were demonstrated in E12.5 DRG explants from *Smad1* cKO^{Nes} or control embryos after 2 days of culture (Figure 3C).

We then investigated whether loss of Smad1 might affect neurotrophin-dependent neuronal differentiation. We did not detect a difference in the number of TrkA$^+$, CGRP$^+$, or PV$^+$ sensory neurons in P1 thoracic DRGs from mutant or control pups (Figure 3E). Together, these findings indicate that Smad1 is specifically required for neurotrophin-mediated axonal outgrowth, but not for the survival or differentiation of sensory neurons in vivo.

In further support of our conclusion, we confirmed all our findings in another conditional mutant mouse, *Smad1$^{fl/+}$;Wnt1-Cre* (*Smad1* cKO^{Wnt1}). *Wnt1-Cre* induces recombination in premigratory pluripotent neural crest cells at approximately E8.5, and thus is expected to affect both Schwann cells and DRG sensory neurons (Danielian et al., 1998; Figures S1C–S1E). We observed no changes in central branch innervation, cell death, or differentiation of DRG neurons in the *Smad1* cKO^{Wnt1} mice (Figure S3), whereas the average number of terminal axon branches per field inspected in mystacial pads was significantly decreased by ~43% in *Smad1* cKO^{Wnt1} pups as compared with control littermates (Figures S3B and S3C).

Next, we investigated whether a delay or deficit in the initial phase of axon outgrowth could explain the aberrant terminal branching in mutants. Notably, early outgrowth of sensory axons in vivo is neurotrophin independent (Davies et al., 1987; O'Connor and Tessier-Lavigne, 1999). We postulated that BMP might regulate this early process. To test this hypothesis, we performed whole-mount immunostaining for neurofilament marker 2H3 on E11.5–E12.5 embryos. Because *Nestin-Cre* induces recombination starting from E11.5, we focused on *Smad1* cKO^{Wnt1} to evaluate early peripheral axonal projections. In *Smad1* cKO^{Wnt1} embryos, peripheral trigeminal sensory axons initiated in the correct directions and with axonal lengths similar to those of control embryos (Figure S4A). The axonal projections to face, trunk, and limb buds also exhibited similar length, fasciculation, and branching patterns (Figures S4B–S4D). Thus, the initial axon growth did not appear to be affected in the *Smad1* cKO mice. Note that even though in vivo exposure to NGF occurs much later in DRG neurons, E12.5 DRG neurons are already capable of responding to NGF in vitro. Therefore, whereas blocking BMP signaling in E12.5 DRG explants attenuated axon growth stimulated by exogenous NGF, the early phase of axon growth in vivo appeared normal in E12.5 *Smad1* cKO mutants.

pSmad1^C Directly Regulates *Erk1/2* Transcription in Embryonic Sensory Neurons

We next investigated the mechanism by which Smad1 is involved in neurotrophin-dependent sensory axonal outgrowth. Because Smad1 is a transcription factor, we searched for transcriptional targets of Smad1 that may play a role in neurotrophin signaling. We took a candidate approach by analyzing messenger RNA (mRNA) levels of neurotrophin effectors. After they were normalized to *Gapdh* (a housekeeping gene), *Erk1*, *Erk2*, and *TrkA* were significantly downregulated in DM-treated E12.5 DRGs, whereas transcription of another Trk effector, *Akt*, was not changed (Figure 4A). Transcripts of *Rpl13a* (another housekeeping gene) remained unchanged, indicating that BMP inhibition does not lead to global transcriptional repression. A time-course study showed that mRNAs of *Erk1* and *Erk2* began to decline 6 hr after DM treatment and remained downregulated after 48 hr (Figure 4B). Consistent with the DM treatment results, in freshly collected DRGs from WT or *Smad1* cKO^{Nes} embryos, both *Erk1* and *Erk2* were reduced in mutant DRGs (Figure 4C). Conversely, BMP stimulation led to induction of *Erk1/2* in cultured E12.5 DRG neurons (Figure 4D).

We subsequently focused on *Erk1/2* regulation. Western blotting confirmed a marked decrease of Erk1/2 in DM-treated E12.5 DRG neurons and a concordant depletion of phosphorylated Erk1/2 (pErk1/2; Figure 4E). Erk1/2 was similarly downregulated after DM treatment in brain-derived neurotrophic factor (BDNF)- and NT3-dependent DRG neurons (Figure 4F). In contrast, inhibiting TGF-β or PKA did not alter Erk1/2 or pErk1/2 levels (Figures 4G and S1B), in agreement with the above-mentioned findings that axonal outgrowth was unaffected by these two inhibitors. In addition, we confirmed a decrease of Erk1/2 and pErk1/2 in E12.5 DRGs from both *Smad1* cKO^{Nes} and *Smad1* cKO^{Wnt1} embryos as compared with controls by western blotting (Figures 4H–4I), albeit to a smaller extent than in cultured DM-treated DRG neurons. This may be explained by in vivo compensatory mechanisms that maintain a tight transcriptional regulation of *Erk1/2*. Also notable is that the magnitude of reduction of Erk1/2 or pErk1/2 was smaller in E12.5 DRGs from *Smad1* cKO^{Nes} than in those from *Smad1* cKO^{Wnt1} embryos, which may be related to an earlier *Smad1* deletion in the latter.

To establish a direct link between pSmad1^C and *Erk1* and *Erk2* transcription, we analyzed the *Erk1* and *Erk2* promoter regions, and identified multiple conserved GC-rich Smad-binding elements (SBEs) within 1 kb upstream of the transcription start site (TSS; Figures 5A and S5A; Morikawa et al., 2011). Chromatin immunoprecipitation (ChIP) on freshly dissected E12.5 DRG neurons showed preferential binding of pSmad1^C to the SBE-containing region of the *Erk2* promoter as compared with the internal coding or promoter regions that do not contain SBEs (Figure 5B). pSmad1^C occupancy on the SBE-containing *Erk2* promoter is a direct result of BMP stimulation, as we observed an 8-fold increase of pSmad1^C binding in a ChIP assay performed in BMP-treated Neuro-2A cells and a concomitant 3-fold increase in *Erk2* expression (Figures 5C and 5D). We did not detect any changes of pSmad1^C binding in the internal coding region of *Erk2* or on the promoter of a housekeeping gene, *Gapdh* (Figure 5C). Similar findings were obtained for

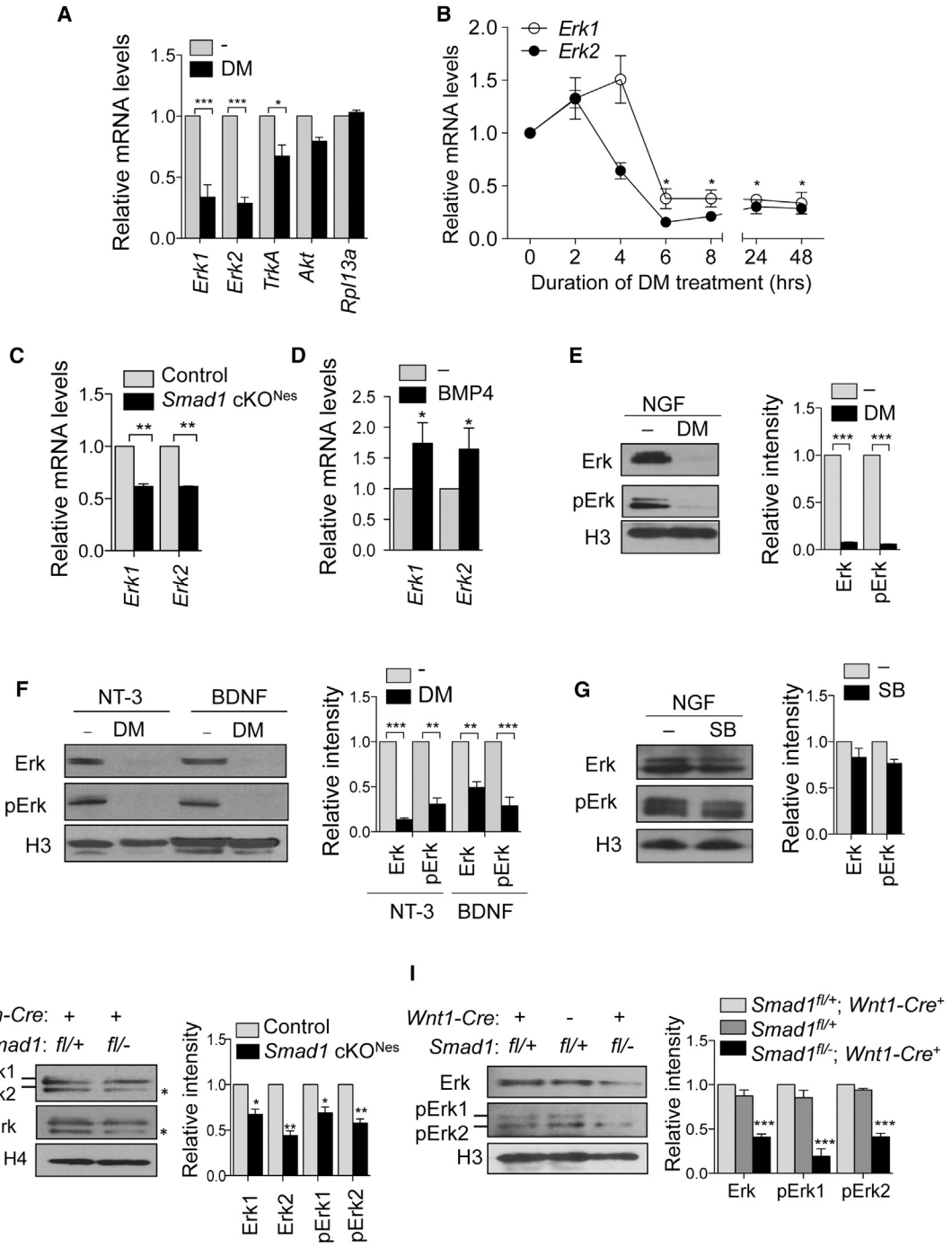

Figure 4. The BMP Pathway Is Involved in Regulating *Erk1/2* Transcription

(A) qRT-PCR of mRNA extracts from dissociated E12.5 DRG neurons cultured for 48 hr with or without DM (1 μM). Data were normalized to *Gapdh* (n = 6).

(B) Time-course analysis of *Erk1* and *Erk2* transcripts by qRT-PCR from E12.5 dissociated DRG neurons cultured with DM (1 μM) for the indicated period as compared with no DM treatment (at 0 hr; n = 6).

(C) qRT-PCR of mRNA extracts from DRGs of E12.5 *Smad1* cKO^{Nes} or control littermates.

(D) mRNA levels of *Erk1/2* from dissociated DRG treated with or without BMP (10 ng/ml) for 24 hr.

(E–G) Western blots of protein extracts from dissociated E12.5 DRG neurons cultured for 48 hr in the specific neurotrophin-containing media (E and F) with or without DM (1 μM), or (G) with SB431542 (SB, 5 μM), with antibodies against the indicated proteins (H3 as loading control).

(H and I) Western blots of protein extracts of DRGs from E12.5 *Smad1* cKO^{Nes} (H) or *Smad1* cKO^{Wnt1} (I) littermates with antibodies against the indicated proteins show a decrease in Erk1/2 and pErk1/2 in both mutant DRGs (H3 and H4 as loading control). Asterisks in (H) denote the Erk2 band.

Error bars represent SEM. See also Figure S4.

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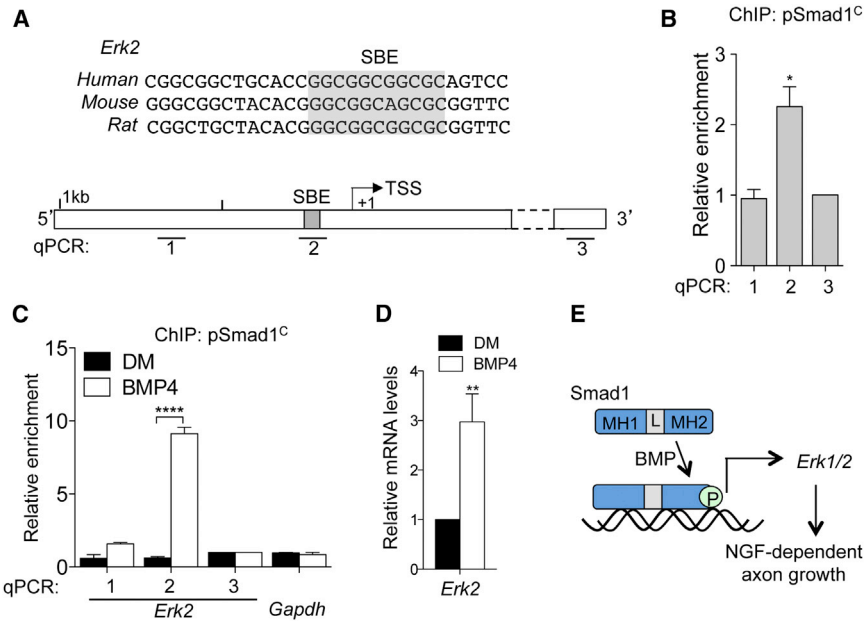

Figure 5. pSmad1^C Regulates *Erk1/2* Transcription

(A) *Erk2* contains conserved SBEs in its promoter region. Human, mouse, and rat *Erk2* promoter sequences were aligned and one GC-rich SBE is highlighted in gray. The schematic representation of the *Erk2* gene shows the location of SBEs in the *Erk2* promoter. For clarity, only one SBE is shown here. The locations of the qPCR primer sets covering specific promoter or coding regions are shown at the bottom.

(B) Relative enrichment of ChIP values. The pSmad1/5/8^C-immunoprecipitated DNA from freshly dissected E12.5 DRGs was amplified by qPCR using primer sets shown in (A) (n = 3).

(C) ChIP assays with antibody against pSmad1/5/8^C using Neuro-2A cells treated with BMP (50 ng/ml) or DM (2 μM) for 1 hr before crosslinking (n = 3). *Gapdh* is a housekeeping gene.

(D) qRT-PCR for *Erk2* expression in Neuro-2A cells treated with BMP4 (50ng/ml) or DM (2 μM).

(E) Schematic model of pSmad1^C, activated by BMP, drives *Erk1/2* transcription, leading to enhanced NGF responsiveness of developing sensory axons.

Error bars represent SEM. See also Figure S5.

Erk1 (Figures S5C–S5E). Collectively, our data support the notion that BMP/pSmad1^C plays a direct role in regulating *Erk1* and *Erk2* transcription, thereby sensitizing developing axons to neurotrophins (Figure 5E).

Neurotrophin Signaling Results in Linker Phosphorylation of Smad1 in Developing Sensory Neurons

So far, we have established that pSmad1^C-mediated transcriptional regulation of *Erk1/2* serves to integrate BMP and neurotrophin signaling. To identify additional convergence points, particularly in light of the multiple MAPK sites in the Smad1 linker region, we investigated the functional roles of pSmad1^L during sensory axon development. To this end, we studied a mouse mutant that harbors phosphorylation-resistant mutations in all four MAPK phosphorylation sites (but not GSK phosphorylation sites) in the *Smad1* linker region. *Smad1$^{L/L}$* mutant mice are viable and exhibit subtle defects in gastric epithelial homeostasis (Aubin et al., 2004), but their neural development has never been studied.

We first examined whether the Smad1 linker region is phosphorylated in developing sensory neurons. Immunostaining demonstrated a persistent presence of pSmad1^L in the nuclei of developing DRG neurons from E12.5 to P1, but its absence in developing Schwann cells (Figures 6A and S6F–S6H). The pSmad1^L antibody was specific because its immunoreactivity was absent in either *Smad1$^{L/L}$* or *Smad1* cKO^{Nes} mutant DRGs (Figures 6A and 6B).

To establish a direct link between neurotrophin stimulation and linker phosphorylation of Smad1, we cultured dissociated E12.5 DRG neurons in media with or without NGF for 24 hr, with caspase inhibitor included to prevent cell death. Neurotrophin stimulation by NGF, BDNF, or NT3 indeed led to a marked increase in pSmad1^L in WT, but not *Smad1$^{L/L}$*, neurons (Figure 6C).

Linker Phosphorylation of Smad1 Functions to Attenuate Neurotrophin/Erk Signaling

We next investigated the functional role of pSmad1^L in sensory neuron development. Previous studies in non-neuronal cell cultures reported conflicting results with regard to whether Smad1 linker phosphorylation antagonizes BMP activity by tethering pSmad1^C in the cytoplasm (Aubin et al., 2004; Kretzschmar et al., 1997). We found that linker mutation did not impact the nuclear accumulation of pSmad1^C at baseline or upon BMP stimulation in E12.5 DRG neurons (Figure 6D). Additionally, pSmad1^C stability was not altered, as the steady-state levels of pSmad1^C were comparable in *Smad1$^{L/L}$* and control E12.5 DRGs by western blotting or immunostaining (Figures 6E and 6G).

We then considered the possibility that Smad1 requires phosphorylation at both the linker region and C terminus for optimal transcriptional activity of pSmad1^C, as shown previously in non-neuronal cell lines (Alarcón et al., 2009; Gao et al., 2009). Since we had identified *Erk1* and *Erk2* as pSmad1^C target genes, we expected that *Erk1/2* mRNA levels would serve as a readout of pSmad1^C transcriptional activity. We therefore compared the mRNA levels of *Erk1* or *Erk2* in E12.5 *Smad1$^{L/L}$* and control DRGs, and found no significant difference (Figure 6F). In addition, when pSmad1^C activation was blocked with DM, both *Erk1* and *Erk2* were downregulated by a similar magnitude in *Smad1$^{L/L}$* and control DRG neurons (Figure S6A). Thus, linker phosphorylation of Smad1 does not affect pSmad1^C transcriptional activity, at least for *Erk1/2*. Consistently, Erk1/2 protein levels remained unaltered in *Smad1$^{L/L}$* DRGs (Figure 6G).

Unexpectedly, we found that pErk1/2 was elevated in E12.5 *Smad1$^{L/L}$* DRGs compared with controls (Figure 6G), suggesting that linker phosphorylation of Smad1 may affect the balance between Erk1/2 and pErk1/2, favoring a decrease in pErk1/2. Because Erk can activate linker phosphorylation of Smad1, we

hypothesize that Erk/pSmad1^{L} may be part of a negative-feedback loop that dampens the signaling intensity of the neurotrophin/Erk signaling (Figure 6H). To evaluate this model in vivo, we examined terminal axon branches in *Smad1*$^{L/L}$ newborn pups. If pSmad1^{L} functions as a brake for neurotrophin stimulation, the lack of such a brake, as observed in *Smad1*$^{L/L}$ DRGs, would lead to exaggerated growth of terminal branches. Indeed, in the mystacial pads of newborn *Smad1*$^{L/L}$ pups, even though the number of terminal axon branches at the upper dermis remained the same, a significantly larger number of axonal branches extended beyond the upper dermis and into the epidermal layer, resulting in longer average axonal lengths in the cutaneous axon network (Figures 7C and S7A). This change was not due to alterations in the expression level of PGP9.5 or an increased number of PGP9.5^{+} neurons, as cell differentiation and survival were unaffected (Figures S6B–S6E).

The Smad1 linker mutation is present in both neurons and glial cells, but the absence of pSmad1^{L} in developing Schwann cells suggests that the overgrowth of terminal axon branches is more likely associated with the enhanced growth state of *Smad1*$^{L/L}$ mutant DRG neurons, perhaps due to the elevated ratio of pErk1/2 over Erk1/2. We thus conducted a neurite outgrowth assay in DRG explant cultures. With stimulation of exogenous NGF (12.5 ng/ml), axonal outgrowth appeared similar regardless of the genotypes (Figure 7A). However, when the total Erk1/2 was reduced with a low dose of DM (1 μM) to ensure that pErk1/2 would not reach saturating levels, *Smad1*$^{L/L}$ DRG neurons grew longer axons as compared with controls (Figure 7A). These results provide a direct link between the linker mutation and the enhanced growth state of developing sensory neurons. Together, our data suggest that pSmad1^{L} is involved in reducing pErk1/2 levels, leading to attenuated axonal outgrowth.

pSmad1^{L} Induces Erk1/2-Specific Dusps to Modulate the Intensity of Erk1/2 Signaling

We next sought to address the cause of elevated pErk1/2 in *Smad1*$^{L/L}$ mutant DRGs. The level of Erk1/2 phosphorylation is a result of balanced activities between Erk-specific kinases and phosphatases. Because components of the NGF signaling cascade, such as TrkA, Akt, and Erk, remained unchanged in *Smad1*$^{L/L}$ DRGs (Figure 6F), we examined Erk-specific phosphatases. Dual-specificity (threonine/tyrosine) phosphatases (Dusps) are a subclass of phosphatases that specifically dephosphorylate MAPKs (Jeffrey et al., 2007). Among the 16 mammalian Dusps that show catalytic activity for MAPK, we examined the Dusps that have substrate preference for Erk1/2 over JNK or p38. The mRNA levels of *Dusp2*, *Dusp4*, and *Dusp6* were significantly decreased in E12.5 *Smad1*$^{L/L}$ DRGs, whereas the mRNA levels of *Dusp5* and *Dusp9* remained the same (Figure 7D). *Dusp3* and *Dusp8* do not act upon Erk1/2, and their mRNA levels were not affected (Figure 7D). Consistently, *Dusp4* and *Dusp6*, but not *Dusp3*, were also downregulated in *Smad1* cKO^{Nestin} DRGs compared with controls (Figure 7E). Taken together, our data suggest that pSmad1^{L} is involved in the regulation of Erk1/2-specific Dusp levels.

We subsequently focused on *Dusp6* because it functions as a negative-feedback regulator of FGF/Erk signaling during mouse development (Li et al., 2007). We first investigated whether neurotrophin/Erk signaling induces *Dusp6*. Neurotrophin stimulation of E12.5 DRGs led to a 4-fold induction of *Dusp6*. Importantly, the induction was abolished in *Smad1*$^{L/L}$ DRGs (Figure 7F). *Dusp6* induction was evident by increased immunoreactivity in WT, but not *Smad1*$^{L/L}$, DRG neurons treated with NGF (Figure 7B). Taken together, our data suggest that *Dusp6* is specifically regulated in embryonic DRGs by neurotrophin-activated pSmad1^{L} and acts as a brake to attenuate neurotrophin/Erk signaling (Figure 7G).

DISCUSSION

By studying two *Smad1* cKO and *Smad1*$^{L/L}$ mutants, we have revealed in vivo roles of two phosphorylated forms of Smad1 during sensory axon development: pSmad1^{C} mediates *Erk1/2* transcription to sensitize neurotrophin response, and pSmad1^{L} regulates *Dusp6* induction to dampen neurotrophin signaling through a negative-feedback loop mechanism. BMP and neurotrophin pathways are thus tightly integrated into a signaling network to ensure proper responses to the growth stimulation from growth factors.

A Balanced Ratio of Erk1/2 and pErk1/2 in Developing Sensory Neurons Ensures a Proper Response to Neurotrophin Stimulation

Sensory neurons in DRGs are exposed to neurotrophins throughout development that mediate their survival, differentiation, and axonal outgrowth (White et al., 1996). Early axonal outgrowth is NGF independent, whereas terminal arborization is regulated by target-derived NGF. En route to target fields, developing axons also encounter other members of the neurotrophin family, such as BDNF and NT3. Along the axon trajectory, developing axons may encounter fluctuating concentrations of neurotrophins. How a proper level of neurotrophin responsiveness is ensured is an important yet unanswered question. Here, we demonstrate that in developing DRG neurons, transcription of *Erk1* and *Erk2*, two key neurotrophin effectors, is regulated by Smad1. Furthermore, activity of Erk1/2 is also modulated by Smad1 through Dusp-mediated dephosphorylation of Erk. Notably, the former process is controlled by pSmad1^{C} and the latter by pSmad1^{L}. The pSmad1^{L}/Dusp6-based negative-feedback loop mechanism may prevent large fluctuations of the signaling intensity of neurotrophin by maintaining a balanced ratio of Erk1/2 and pErk1/2 (Figure 7G). Of note, both BMP4 and BMP receptors are expressed in E12.5 DRGs (Figure S7B). Our working model thus reveals the molecular basis of how growth stimulation from neurotrophins can be constantly modulated and swiftly turned off once the target innervation approaches completion.

Erk1/2 also play critical roles in developing Schwann cells (Newbern et al., 2011); however, since the immunoreactivity of pSmad1^{C} or pSmad1^{L} is not detectable in developing Schwann cells, regulation of Erk1 and Erk2 in these cells may rely on entirely different transcription factors. For a similar reason, a neuronal cell-autonomous effect is more likely for the phenotypes observed in *Smad1* mutant mice. Two additional observations further favor a neuronal rather than a glial defect in *Smad1* mutant mice: First, the *Smad1* cKO^{Nes} and *Smad1* cKO^{Wnt-1}

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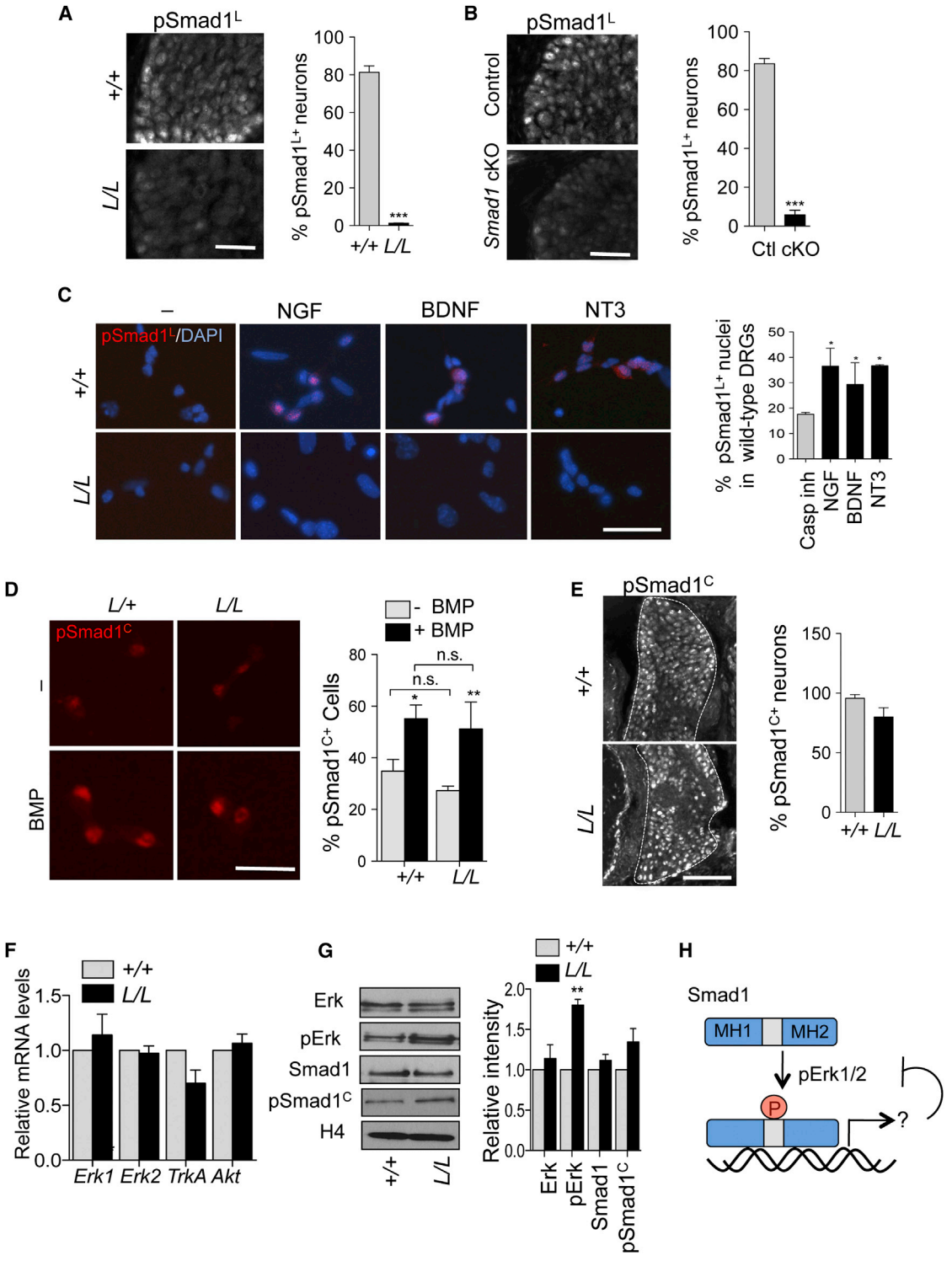

Figure 6. pSmad1^{L} Is Activated by Neurotrophins and Dampens Its Signaling Intensity

(A and B) Immunohistochemistry of pSmad1^{L} in DRGs from P1 pups of the indicated genotypes and quantification.

(C) Immunocytochemistry of pSmad1^{L} in dissociated E12.5 WT DRG neurons cultured for 24 hr with or without the indicated neurotrophin. Caspase inhibitor was included in all conditions to prevent cell death. The percentage of nuclei that were pSmad1^{L+} was quantified from WT DRGs only, because no pSmad1^{L} immunosignals were detected in *Smad1*$^{L/L}$ DRGs.

(D) Immunocytochemistry of pSmad1/5/8^{C} in dissociated E12.5 DRG neurons cultured with or without BMP (10 ng/ml) for 24 hr. The percentage of DRG neurons with strong nuclear pSmad1^{C} immunosignals remained similar in *Smad1*$^{L/L}$ and *Smad1*$^{L/+}$ DRGs (n = 3). n.s., not statistically significant.

mutants have similar phenotypes. Because *Nestin-Cre* leads to recombination more specifically in neurons in the DRGs, it is more likely that the sensory neurons are the cellular components that are responsible for the phenotypes. Second, in cultured explants of DRGs from *Smad1* cKO^{Nes} mice, axon outgrowth is affected. An indirect effect from glial cells remains a possibility.

Besides the well-studied role of NGF in peripheral nervous system development, other members of the neurotrophin family (BDNF in particular) play a number of roles in the CNS, ranging from axonal and dendritic growth to synaptogenesis, learning, and memory (Aguado et al., 2003; Cheng et al., 2011; Yamada et al., 2002). Whether a similar Smad1-based regulatory mechanism functions in the CNS to maintain a balanced ratio of Erk1/2 and pErk1/2 remains to be determined in future studies.

pSmad1^{C}-Mediated *Erk1/2* Transcription Sensitizes Sensory Neurons to Neurotrophins

This study extends previous findings regarding a modulatory role of BMP in the functions of neurotrophin by revealing that pSmad1^{C}-mediated *Erk1/2* transcription is the basis for this collaboration in developing sensory neurons. Along with *Erk1/2*, blocking of Smad1^{C} activation results in a decrease in the transcription of other neurotrophin signaling components, such as TrkA, suggesting a broader role for Smad1 in regulating neurotrophin signaling. The functional link between Smad1 and Erk1/2 is highly significant given the prominent role of Erk1/2 in the growth and differentiation of a wide variety of cells, and the observation that their aberrant regulation contributes to neoplastic transformation (Roberts and Der, 2007).

Here, we establish that BMP/Smad1 signaling is specifically required for NGF-mediated axon growth, but not for cell survival or differentiation of developing sensory neurons. CGRP expression in sensory neurons is reportedly dependent on TrkA (Snider and McMahon, 1998) and can be induced by BMP in cultured DRGs in vitro (Ai et al., 1999). However, we detected no significant change in the percentage or the total number of CGRP^{+} DRG neurons in *Smad1* cKO pups, arguing against an indispensable role for Smad1 in mediating CGRP expression in DRG neurons in vivo, although compensatory effects from Smad5 or Smad8 remain a possibility. Indeed, Smad1, Smad5, and Smad8 are all expressed in the trigeminal sensory neurons (Ji and Jaffrey, 2012).

The phenotype of stunted terminal axonal branches is milder in *Smad1* cKO pups than in *Erk1/2* knockout mice (Newbern et al., 2011), which may be attributed to a modest reduction of Erk1/2 in *Smad1* cKO DRGs as compared with the dramatic reduction in cultured DM-treated DRG neurons. The difference between the in vivo and in vitro results may be related to (1) a compensatory increase in the transcriptional activity of other Smads or additional transcriptional factors to maintain a tight transcriptional regulation of *Erk1/2* in vivo, and (2) the fact that *Smad1* deletion results in not only Erk1/2 reduction but also compromised Dusp induction, leading to a smaller reduction in pErk1/2 levels. It is also worth noting that although trigeminal sensory neurons are derived from both neural crest progenitors and epithelial placodal progenitors (Streit, 2011), *Wnt1-Cre* affects only the former, which may explain the partial decrease in terminal axon branches in the mutants.

A Negative-Feedback Loop of Neurotrophin/Erk Signaling Relies on pSmad1^{L}-Mediated Expression of Erk-Specific Dusp

Once the sensory axons complete target innervation, growth signals need to be effectively switched off to avoid overgrowth. Previous studies have focused mostly on activation of the neurotrophin-Ras-Raf-MEK-MAPK cascade, and paid little attention to inactivation mechanisms. Our study uncovers a pSmad1^{L}/Dusp-based negative-feedback mechanism that enables developing sensory neurons to attenuate neurotrophin/Erk signaling intensity. Phosphatases are powerful negative regulators. In fact, the enzymatic power of a phosphatase is estimated to be 100–1,000 times greater than that of a kinase (Reth, 2002), and computational analyses confirmed a dominant role of Dusps over kinases with regard to the extent of MAPK phosphorylation (Bhalla et al., 2002). Our results are in agreement with the notion that Dusp is induced upon MAPK activation to dampen mitogenic signaling (Patterson et al., 2009). Moreover, a similar functional link between Smad1 and Dusp has been identified in murine embryonic stem cells, which sets appropriate Erk activity levels to maintain pluripotency (Li et al., 2012). Different Dusps have distinct substrate specificities, subcellular localizations, and functions (Patterson et al., 2009), and the present study suggests that Dusp6 is involved in attenuating axonal outgrowth in developing sensory neurons. Future studies will uncover the roles of each individual Dusp during neural development.

Distinct Roles of pSmad1^{C} and pSmad1^{L} during Sensory Axon Development

In this study, we have assigned distinct in vivo roles to the two phosphorylated forms of Smad1. Thus, differential posttranslational modifications of the same transcription factor can lead to distinct transcription programs. This has been demonstrated for other transcription factors, such as Nkx2-1 (Silberschmidt et al., 2011), Sap-1a (Janknecht and Hunter, 1997), and NF-κB (Anrather et al., 2005). In the case of NF-κB, differential phosphorylation of p65, one of the subunits of NF-κB, results in distinct gene-expression profiles.

We demonstrate that pSmad1^{C} binds to SBE on *Erk1* and *Erk2* promoters upon BMP stimulation. It remains to be determined whether *Dusp6* induction is regulated directly by pSmad1^{L}, and if so, whether pSmad1^{L} and pSmad1^{C} bind to the same or different SBEs, and collaborate with the same or distinct

(E) pSmad1^{C} levels were not affected in P1 DRGs from *Smad1$^{L/L}$* as shown by immunohistochemistry.
(F) qRT-PCR for expression of components of the neurotrophin pathway from E12.5 DRGs from *Smad1$^{+/+}$* and *Smad1$^{L/L}$* embryos.
(G) Western blot of protein extracts from E12.5 DRGs from a linker mutant or control littermate shows that the levels of pErk1/2 were increased in *Smad1$^{L/L}$* DRGs.
(H) Schematic illustrating how pSmad1^{L} is involved in regulating pErk levels.
Scale bars: 50 μm (A–C and E) and 100 μm (D). *p < 0.05, **p < 0.01, ***p < 0.001. Error bars represent SEM. See also Figure S6.

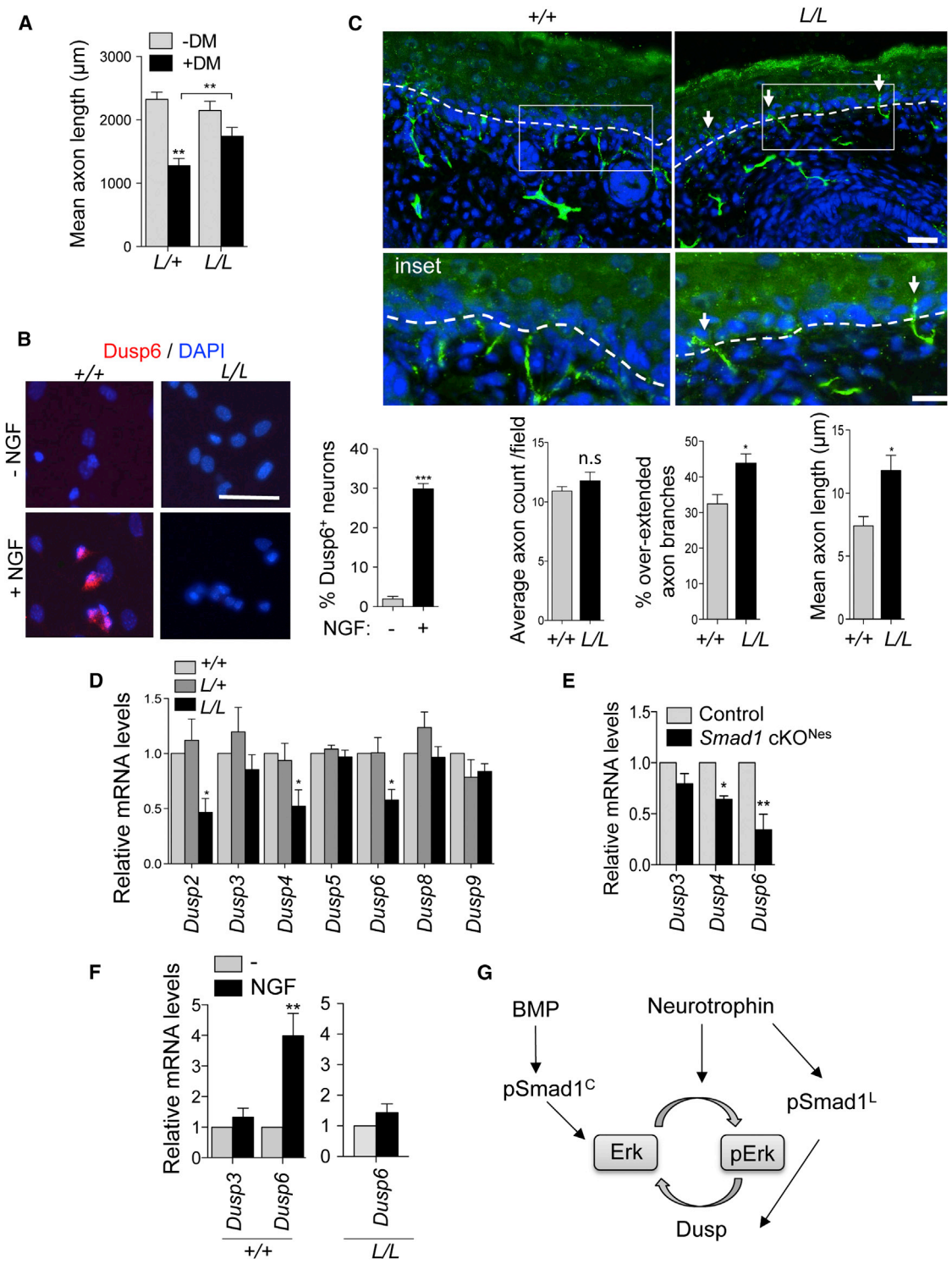

Figure 7. pSmad1^{L} Mediates *Dusp6* Expression

(A) Neurite outgrowth assay on E12.5 DRG explants. The axonal lengths of *Smad1*$^{L/L}$ neurons were compared with those of control DRGs at 24 hr in the presence of 12.5 ng/ml of NGF with or without DM treatment (n = 4).

(B) Immunocytochemistry and quantification of dissociated E12.5 DRG neurons cultured with caspase inhibitor alone or with NGF for 48 hr showed that the increased Dusp6 after NGF treatment was observed in WT but not *Smad1*$^{L/L}$ DRGs (n = 3).

(C) Immunostaining of mystacial pads from P1 pups for PGP9.5 (green) demonstrated exaggerated growth of terminal axons beyond the upper dermis and into the epidermis (white arrows point to several examples) in *Smad1*$^{L/L}$ compared with *Smad1*$^{+/+}$ pups. Dotted white lines depict the dermis/epidermis boundary.

cofactors to mediate transcription. Indeed, precedent exists for specific recruitment of a transcriptional coactivator, yes-associated protein (YAP), to the phosphorylated linker region of Smad1 (Alarcón et al., 2009). YES acts downstream of the Hippo pathway to control organ size (Zhao et al., 2008). Our findings suggest that Smad1 plays versatile roles in regulating different sets of genes through differential phosphorylation, thereby enabling neurons to respond to combinations of instructive cues in a context-specific manner.

Conclusions

We have identified collaborative signaling events of BMP and NGF pathways during sensory axon development. Through differential phosphorylation, Smad1 potentiates neurotrophin signaling by pSmad1^{C}-dependent transcription of *Erk1/2*, and then dampens its signaling intensity by pSmad1^{L}-mediated *Dusp* expression. The concerted integration of the BMP and neurotrophin pathways forms a tightly regulated signaling network that is of key importance for sensory axon development.

EXPERIMENTAL PROCEDURES

Generation of Mice

Smad1$^{flox/flox}$ mice (Huang et al., 2002) were used to generate a heterozygous germline deletion of *Smad1* (*Smad1$^{+/-}$*). *Smad1$^{fl/-}$* mice were then mated to *Nestin-Cre* mice (The Jackson Laboratory; Tronche et al., 1999) to generate *Smad$^{fl/-}$;Nestin-Cre* conditional mutant mice, or to *Wnt1-Cre* mice (Danielian et al., 1998) to generate *Smad$^{fl/-}$;Wnt1-Cre* mice. The *Smad1$^{L/L}$* mutant (Aubin et al., 2004) and Rosa26-EGFP^{STOP} reporter line (Srinivas et al., 2001) were obtained from The Jackson Laboratory. At least five littermate pairs were used for each phenotype studied.

DRG Cultures and Neurite Outgrowth Assay

DRGs from E12.5 embryos were dissected for explant cultures or low-density (50,000 cells/cm^2) dissociated cultures in Neurobasal media supplemented with B27 (GIBCO) and NGF (12.5 ng/ml). The lengths of at least 20 axons from each explant, or >100 dissociated neurons, were measured and averaged for each experiment. A minimum of three replicates were performed for each condition.

ChIP

The ChIP-IT enzymatic kit (Active Motif) was used per the manufacturer's instructions. Briefly, crosslinked chromatin from Neuro-2A cells or freshly dissected E12.5 DRGs was enzymatically digested to yield 150–200 bp fragments and incubated overnight with magnetic beads plus 5 μg of pSmad1/5/8^{C} (Cell Signaling; see Table S1) antibody or water at 4°C. This was followed by crosslinking reversal, proteinase K digestion, DNA extraction (PCR purification kit; QIAGEN), and quantitative PCR (qPCR) with the primer sets listed in Table S2. The specific binding of pSmad1/5/8^{C} to promoters was calculated by subtracting the value of the no-antibody control and then normalized to the diluted input (1:10). The relative enrichment was then calculated as arbitrary units relative to the ChIP value at the coding region.

Statistical Analysis

Prism GraphPad software was used for Student's t test, one-way ANOVA, or two-way ANOVA, followed by Bonferroni's post hoc test for multiple comparisons. Data are presented as mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

For further details about the materials and methods used in this work, see Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.04.011.

LICENSING INFORMATION

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The average number of axon branches per field of inspection, the percentage of cutaneous axons extending beyond the upper dermis, and the mean axonal length in the terminal network of axons were quantified from five independent 20× images for each pup and averaged over four littermate pairs.
(D and E) qRT-PCR of *Dusp* transcript levels in E12.5 DRGs from embryos of the indicated genotypes (n = 3).
(F) qRT-PCR of *Dusp6* and *Dusp3* transcript levels in E12.5 DRGs from *Smad1$^{+/+}$* or *Smad1$^{L/L}$* embryos cultured for 48 hr in caspase-inhibitor-containing media with or without NGF. *Dusp6* was induced by NGF only in control DRGs.
(G) Working model. During DRG sensory neuron development, BMP activates pSmad1^{C} to induce *Erk1/2* transcription, which enhances neurotrophin responsiveness, while neurotrophin activates pSmad1^{L} to regulate Erk-specific Dusp, which dampens the NGF/Erk signaling intensity.
Scale bars: 50 μm (C) and 100 μm (A, B, D, and E). Error bars represent SEM. See also Figure S7.

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