Axon growth is essential for the establishment of a functional nervous system as well as for the restoration of neuronal connectivity after injury or disease. It has long been hypothesized that reactivation of developmental growth mechanisms might help to achieve axon regeneration in the injured adult nervous system (Filbin, 2006). The role of MAP kinases in axon growth signaling has been much studied and discussed (Markus et al., 2002; Hanz and Fainzilber, 2006; Agthong et al., 2009; Hollis et al., 2009). However, depending on the model systems used, the outcomes have been controversial or even contradictory (Pernet et al., 2005; Sapieha et al., 2006; Hollis et al., 2009). We have shown that RAF–MEK signaling robustly promotes axon growth in primary sensory neurons in vitro (Markus et al., 2002). In vivo, conditional gene targeting studies have shown that RAF signaling is necessary for developing sensory neurons to arborize in their target fields in the skin (Zhong et al., 2007). However, it remains unknown whether RAF signaling is sufficient to enable axon growth in vivo or whether concomitant activation of other signaling pathways is necessary to drive long-range axon projections. Furthermore, it is unclear whether this pathway can promote axon growth in neuronal populations beyond the sensory neurons and the extent to which it can be harnessed to promote regeneration in the injured central nervous system (CNS). To address these questions, we have used conditional B-RAF gain-of-function mouse models to show that cell-intrinsic RAF signaling is a crucial pathway promoting developmental and regenerative axon growth in the peripheral and central nervous systems.

Activation of intrinsic growth programs that promote developmental axon growth may also facilitate axon regeneration in injured adult neurons. Here, we demonstrate that conditional activation of B-RAF kinase alone in mouse embryonic neurons is sufficient to drive the growth of long-range peripheral sensory axon projections in vivo in the absence of upstream neurotrophin signaling. We further show that activated B-RAF signaling enables robust regenerative growth of sensory axons into the spinal cord after a dorsal root crush as well as substantial axon regrowth in the crush-lesioned optic nerve. Finally, the combination of B-RAF gain–of–function and PTEN loss–of–function promotes optic nerve axon extension beyond what would be predicted for a simple additive effect. We conclude that cell–intrinsic RAF signaling is a crucial pathway promoting developmental and regenerative axon growth in the peripheral and central nervous systems.
activation of intraneuronal RAF–MEK signaling is sufficient to promote robust axon growth in developing and regenerating neurons in the peripheral nervous system and CNS.

RESULTS
Activation of B-RAF signaling alone is sufficient to promote sensory axon extension during early development
In vivo, the neurotrophin nerve growth factor (NGF) signals through its receptor kinase TrkA to promote developmental extension of dorsal root ganglion (DRG) nociceptive TrkA⁺peripheral axons into the epidermis. To examine whether RAF signaling alone is sufficient to promote long-range axon extension of TrkA-positive neurons, we set out to selectively activate RAF kinase signaling in these neurons in a TrkA-null background.

To this end, we first conditionally activated RAF signaling in a WT background using a genetically modified loxP-STOP-loxP-Braf<sup>V600E</sup> (LSL-kaBraf) knock-in mouse line (Mercer et al., 2005), in which a kinase-activated B-RAF (kaB-RAF) mutant is expressed from the endogenous B-RAF locus upon Cre recombination. We next bred LSL-kaBraf mice with a neuronal
(Fig. 1, C and D). Note that compared with MEK1/2, ERK1/2 activation appears minor in the kaB-RAF–expressing DRGs; this is because of relatively high levels of pERK1/2 in the DRG at baseline. B-RAF activation did not affect mTOR phosphorylation (Fig. 1 E). Levels of pAKT, pS6K, and pGSK3β were not changed significantly in the DRG of LSL-kaBraf:nes-Cre mice (Fig. 1 F), indicating minimal cross talk between the MAP kinase and PI3-kinase–AKT pathways. Because the expression of kaB-RAF is under the control of endogenous Braf promotor, the expression level of B-RAF protein is not changed in nestin promoter–driven Cre deleter (nes-Cre; Tronche et al., 1999). In DRG neurons, nes-Cre–mediated recombination occurs as early as embryonic day (E) 11.5 (Galabova-Kovacs et al., 2008). Embryos heterozygously expressing kaB-RAF progressively developed macrocephaly from E13.5 onwards (Fig. 1 A) but appeared otherwise normal, including normally sized DRGs (Fig. 1 B). The known RAF effectors were activated in neuronal tissues expressing Cre recombinase, as indicated by elevated phospho-MEK1/2 (pMEK1/2) and pERK1/2 in the neocortex and spinal cord of E12.5 LSL-kaBraf:nes-Cre mice (Fig. 1, C and D). Note that compared with MEK1/2, ERK1/2 activation appears minor in the kaB-RAF–expressing DRGs; this is because of relatively high levels of pERK1/2 in the DRG at baseline. B-RAF activation did not affect mTOR phosphorylation (Fig. 1 E). Levels of pAKT, pS6K, and pGSK3β were not changed significantly in the DRG of LSL-kaBraf:nes-Cre mice (Fig. 1 F), indicating minimal cross talk between the MAP kinase and PI3-kinase–AKT pathways. Because the expression of kaB-RAF is under the control of endogenous Braf promotor, the expression level of B-RAF protein is not changed in...
the DRG and spinal cord at E12.5 (Fig. 1 G). At E13.5, the branching pattern of sensory nerves in the skin was not changed by kaB-RAF expression (Fig. 1, H and I).

**B-RAF activation rescues nociceptor axon extension in embryos lacking TrkA**

To test whether kaB-RAF is sufficient to drive nociceptor axon growth in the absence of TrkA signaling, we next mated the LSL-kaBraf:nes-Cre line with available TrkA<sup>taulacZ</sup> and Bax<sup>−/−</sup> lines to generate LSL-kaBraf:TrkA<sup>taulacZ/taulacZ</sup>:Bax<sup>−/−</sup>:nes-Cre mice. In TrkA<sup>taulacZ</sup> mice, the WT TrkA gene is replaced by a <i>taulacZ</i> expression cassette, such that the axonal morphology of putative TrkA<sup>+</sup> neurons can be visualized by β-gal staining (Moqrich et al., 2004). Because TrkA expression is absent in homozygous TrkA<sup>−/−</sup> mice, we refer to the "TrkA<sup>taulacZ/taulacZ</sup>" as "TrkA<sup>−/−</sup>" in the text below. Removal of the Bax gene blocks apoptosis in embryonic DRG neurons, rescuing them from cell death that is otherwise observed in the absence of TrkA signaling. The Bax<sup>−/−</sup> background thus allows for the molecular dissection of signaling pathways that specifically affect axon growth (Knudson et al., 1995; Lentz et al., 1999; Patel et al., 2000; Markus et al., 2002; Kuruvilla et al., 2004; Moqrich et al., 2004). In TrkA<sup>−/−</sup>:Bax<sup>−/−</sup> mice, DRG neurons survive, but sensory afferent innervation in the skin is completely abolished (Fig. 2, A and B). Compared with control littermates (which include LSL-kaBraf:TrkA<sup>−/−</sup>:Bax<sup>−/−</sup>, nes-Cre:TrkA<sup>−/−</sup>:Bax<sup>−/−</sup>, and TrkA<sup>−/−</sup>:Bax<sup>−/−</sup> genotypes), in which we detected no LacZ-positive fibers in the skin at E16.5, expression of kaB-RAF partially rescues nociceptive innervation in the TrkA<sup>−/−</sup> background. The dashed lines indicate the dermal-epidermal border. Bar, 100 µm. (B) Quantification of axon innervation in footpad (Luo et al., 2007; Hancock et al., 2011). Data are from three fetuses per genotype. Error bars indicate SEM. One-way ANOVA with post-hoc Tukey’s HSD test: **, P < 0.01.

**Figure 3. Axon terminal innervation of E18.5 footpad.** (A, top) Normal innervation. (middle) In the absence of TrkA, innervation is diminished overall, and the CGRP-positive nociceptor endings are completely absent. Red arrowheads indicate the CGRP-positive axon terminals in the epidermis. (bottom) kaB-RAF expression partially rescues nociceptive innervation in the TrkA<sup>−/−</sup> background. The dashed lines indicate the dermal-epidermal border. Bar, 100 µm. (B) Quantification of axon innervation in footpad (Luo et al., 2007; Hancock et al., 2011). Data are from three fetuses per genotype. Error bars indicate SEM. One-way ANOVA with post-hoc Tukey’s HSD test: **, P < 0.01.
Specifically, nociceptive TrkA+ fibers terminate in the superficial lamina I and II of the dorsal horn, and proprioceptive parvalbumin-positive afferents project to intermediate laminae or to the ventral spinal cord.

In B-RAF gain-of-function mice, we observed excessive growth of both nociceptive and proprioceptive afferents (Fig. 5). Nociceptive axons normally restricted to superficial dorsal horn extended ectopically into deeper layers of dorsal spinal cord, and many axons aberrantly crossed the midline (Fig. 5 A). This kaB-RAF–driven overgrowth was substantially rescued by concomitant elimination of MEK1/2, the canonical down-stream kinases of RAF (Fig. 5 C), suggesting that the effect of kaB-RAF expressed from the endogenous Braf locus depends strictly on canonical signaling.

In WT mice, the central proprioceptive afferents enter the cord medially at tightly circumscribed dorsal root entry zones (DREZs; Fig. 5 B, left). kaB-RAF expression caused the proprioceptive sensory axons to enter the spinal cord all across its surface and to aberrantly terminate some branches in the superficial dorsal laminae (Fig. 5 B, right). Proprioceptive axons in the DREZs normally are subject to repulsive guidance from Semaphorin 6C/D (Sema6) expressed in the spinal cord, acting on PlexinA1 on the sensory axons (Yoshida et al., 2006). kaB-RAF expression did not detectably alter the protein (Fig. 5 D) or transcript levels (RNAseq; not depicted).
of these factors in E12.5 DRG and spinal cord. The overgrowth phenotype for both nociceptive and proprioceptive afferents was observed at all levels of the spinal cord (Fig. 5, E–J). This phenotype suggested that reactivation of the B-RAF pathway in injured adult neurons might be exploited to promote regeneration.
Figure 6. Activation of B-RAF signaling in mature DRG neurons elevates their growth competency. (A, top) Schematic of the brn3a-CreER<sup>T2</sup> construct used to generate the brn3a-CreER<sup>T2</sup> deleter mouse line. (bottom) A cross section of the spinal cord of a 10-wk-old Rosa26-lacZ:brn3a-CreER<sup>T2</sup> mouse treated with tamoxifen. Blue LacZ staining indicates CreER<sup>T2</sup>-mediated recombination in the DRG neurons. (B) Representative DRGs from adult LSL-kaBraf:TdTom:brn3a-CreER<sup>T2</sup> mice without (top left) and with (bottom left) tamoxifen treatment. TdTom expression indicates recombination in DRG neurons. Cells were counterstained with Draq5 (Dq5) to label nuclei. (C) ATF3 is induced by preconditioning lesion. Blue shows nuclear stain Draq5. (D) Representative images of adult DRG neurons derived from intact brn3a-CreER<sup>T2</sup>:TdTom (left), LSL-kaBraf:brn3aCreER<sup>T2</sup>:TdTom (right), and pre-conditioning lesion (right). The graphs represent (E) the percentage of axons bearing cells and (F) the average length of longest neurite from WT and B-Raf-treated neurons at 20h and 24h. **p < 0.01, *p < 0.05 compared to WT. (G) The average length of longest neurite in axons bearing cells 24h post-conditioning lesion. **p < 0.01 compared to WT. (H) The average length of longest neurite from WT and B-Raf-treated axons bearing cells.
As expected, in T2 (E–H) Quantitation of axon extension in adult DRG cultures at 24 h in vitro. Data were collected from three independent experiments from three animals, and WT preconditioning lesioned mice (right) after 24 h in vitro. TdTom is shown in green to improve contrast. Bars: (A–C) 100 µm; (D) 20 µm.

Numbers of axon-bearing neurons and increased total axon length in DRG central axons across the DREZ

To test whether activation of B-RAF signaling can drive mature sensory axon regeneration, we generated LSL-kaBraf: TdTom:brn3a-CreERT2 mice to inducibly express kaB-RAF in adult DRG neurons. The brn3a-CreERT2 deleter mouse line was generated using a brn3a promoter (Eng et al., 2001), which mediates expression selectively in sensory neurons (Fig. 6 A). We first assessed B-RAF gain-of-function in cultured adult neurons. 12-wk-old mice were treated with tamoxifen for a consecutive 5 d to induce kaB-RAF expression, as indicated by TdT om expression (Fig. 6 B). Dashed yellow lines indicate the DREZ, dashed gray lines indicate the border between gray and white matter, and arrowheads indicate the extent of axon growth across the DREZ (B) and into gray matter (D). DH, dorsal horn; DR, dorsal root; PNS, peripheral nervous system; SC, spinal cord. n = 2 DRGs from each of three animals per genotype. Bars, 200 µm.

kaB-RAF enables regeneration of injured adult DRG central axons across the DREZ

To test whether activation of B-RAF kinase signaling can promote axon regeneration of injured mature CNS neurons, we used an optic nerve regeneration model (Fig. 8, A and B; Park et al., 2008; Benowitz and Yin, 2010). 8–12-wk-old LSL-kaBraf: Bax−/− mice and Bax−/− controls were injected intravitreally with AAV2-Cre to induce kaB-RAF expression in retinal ganglion cells (RGCs) and then subjected to optic nerve crush. The Bax−/− background was used to minimize apoptotic death of retinal ganglion neurons triggered by optic nerve injury, which may amount to 80% at 2 wk after optic nerve crush (Li et al., 2000). 2 wk after the injury, we observed robust regenerative axon growth up to 3 mm past the lesion site in the kaB-RAF–expressing neurons (Fig. 8, D and G), with very limited regrowth of adult DRG neurons and, importantly, renders the axons capable of overcoming growth–inhibitory signals that are abundant at the DREZ and within the spinal cord.

kaB-RAF enables regenerative axon growth in the injured optic nerve through an MEK-dependent pathway

To test whether activation of B-RAF kinase signaling can promote axon regeneration of injured mature CNS neurons, we used an optic nerve regeneration model (Fig. 8, A and B; Park et al., 2008; Benowitz and Yin, 2010). 8–12-wk-old LSL-kaBraf: Bax−/− mice and Bax−/− controls were injected intravitreally with AAV2-Cre to induce kaB-RAF expression in retinal ganglion cells (RGCs) and then subjected to optic nerve crush. The Bax−/− background was used to minimize apoptotic death of retinal ganglion neurons triggered by optic nerve injury, which may amount to 80% at 2 wk after optic nerve crush (Li et al., 2000). 2 wk after the injury, we observed robust regenerative axon growth up to 3 mm past the lesion site in the kaB-RAF–expressing neurons (Fig. 8, D and G), with very limited regrowth in the control Bax−/− littermates (Fig. 8 C), consistent with previous observations that survival alone is not sufficient to promote growth of adult RGC axons (Goldberg et al., 2002).

Combined deletion of the canonical RAF effector kinases MEK1 and MEK2 substantially suppressed the regenerative axon growth caused by kaB-RAF (Fig. 8 E), indicating that kaB-RAF drives axon growth through the canonical MEK effectors. Whereas the length of axon extension induced by
B-RAF gain-of-function is comparable with the maximal axon growth reported in PTEN deletion mice (Fig. 8 F; Park et al., 2008), in a direct comparison, we found up to a 3.9-fold higher density of regenerating axons in the LSL-kaBraf:Bax<sup>−/−</sup> mice 1.5 mm distal to the crush site than is seen in the crushed Pten<sup>−/−</sup>:Bax<sup>−/−</sup> optic nerve (Fig. 8, D, F, and G).
DISCUSSION

An understanding of the mechanisms that drive axon growth is important, both to decipher how connectivity develops in the nervous system and to develop therapeutic strategies for nervous system repair after injury or disease. We show that the RAF–MEK axis plays a key role in axon growth signaling. Activation of B-RAF in neurons is sufficient to drive sensory axon growth in the embryo, to enable adult sensory axons to regenerate across the DREZ and further into the spinal cord, and to induce robust regeneration of adult retinal axons in the injured optic nerve. Both developmental DRG axon overgrowth in the spinal cord and mature RGC axon regeneration in the optic nerve were abrogated by concomitant ablation of MEK1 and MEK2. We thus establish classical cell-autonomous RAF–MEK signaling as a fundamental driver of axon growth. We should note that this pathway seems to be selective to axon growth signaling because we have never observed that B-RAF activation supports neuronal survival (unpublished data).

In vitro work has long suggested a potential role for RAF–MEK signaling in axon growth. Previous in vivo data, however, have been scarce and controversial. In the retina, for example, pharmaceutical inhibition of MEK–ERK signaling abrogated optic nerve regeneration supported by FGF2 (Sapiha et al., 2006). Two putative intracellular activators of RAF signaling, BAG1 and Mst3b, have been shown to promote regenerative axon growth in the optic nerve (Planchamp et al., 2008; Lorber et al., 2009), but the expression of a constitutively active MEK1 did not drive any regeneration in the optic nerve (Pernet et al., 2009). Others have concluded that ERK activity promotes RGC axon regeneration via an indirect mechanism dependent on glial cells (Müller et al., 2009). Although it is likely that multiple mechanisms, direct as well as indirect, will contribute to axon regeneration in the inhibitory environment of the CNS, the current cacophonous state of the field is likely caused by the mainly indirect approaches of incomplete penetrance that have been taken by various laboratories. When using small molecule inhibitors or transient viral overexpression of interfering or activating constructs, it is difficult to accurately titrate the dose for the entire duration of an experiment. We believe that we have applied a stringent approach toward activation of RAF signaling in RGCs, and our data argue strongly for a direct positive effect of RAF–MEK signaling on axon growth and regeneration of RGCs, as well as in DRG neurons. Possible downstream mechanisms beyond the MEK kinases remain speculative at this point. Stabilization of microtubules improves axon regeneration in a spinal cord injury (SCI) model through both neuron-intrinsic and -extrinsic mechanisms (Hellal et al., 2011), and it is likely that activation of RAF–MEK signaling will directly affect microtubule stability in injured axons via its effects on microtubule-regulating enzymes such as HDAC6 (Williams et al., 2013). Furthermore, B-RAF has been shown to directly interact with tubulin (Bonfiglio et al., 2011). Activation of B-RAF signaling is also likely to trigger the expression of axon growth-enhancing gene sets in injured neurons. The elucidation of exact mechanisms awaits further study.
The developmental phenotypes we observed in the B-RAF gain-of-function embryos were generally complementary to those previously observed in B-RAF/C-RAF loss-of-function mice (Zhong et al., 2007). In contrast to nociceptors’ peripheral projections, the development of their central projections does not depend on NGF/TrkA signaling (Patel et al., 2000; Harrison et al., 2004; Zhong et al., 2007). Notably, we found that activation of B-RAF resulted in overgrowth of both proprioceptive and nociceptive axons in the spinal cord, whereas the expression of two known repulsive signaling molecules in the dorsal cord and DRG; Semaphorin and PlexinA1, remained unaltered. Thus, kaB-RAF appears to activate a normally quiescent axon growth signaling pathway in the central nervous system that is uncoupled to neurotrophic factors in the adult nervous system and that may generally translate to SCI models. Here, we show that expression of PI3-kinase–mTOR via PTEN deletion also enhances regeneration above the level reported for deletion of either gene alone (Sun et al., 2011). Combined deletion of endogenous inhibitors of these two pathways enhanced regeneration above the level reported for deletion of either gene alone (Sun et al., 2011).

The importance of this effect, the lack of response to repulsive or inhibitory cues, becomes clear in the context of regeneration of central sensory branches after dorsal root crush injury. Sensory axons expressing kaB-RAF robustly regenerated into the DREZ and spinal cord.

The regeneration failure of DRG axons after dorsal root avulsion injuries has been variously attributed to the lack of intrinsic growth capacity, to extrinsic growth barriers such as glia-associated growth inhibitors at the DREZ, and to premature synaptic differentiation (Han et al., 2012; Smith et al., 2012). Application of neurotrophic factors acting via tyrosine kinase receptors has shown substantially enhanced regeneration (Ramer et al., 2000; Wang et al., 2008; Harvey et al., 2010), even functional recovery with the systemic administration of artemisin (Wang et al., 2008), although these results await independent replication. Future studies will test whether a combination of RAF activation with trophic growth factors can further enhance axon regeneration and reinnervation of presumptive targets in the spinal cord.

Compared with spinal cord lesions, the optic nerve’s simpler structure allows for clear evaluation of both lesion and regeneration. In recent years, the optic nerve model has revealed several intracellular signaling pathways that can drive CNS axon regeneration, most prominently the PI3-kinase–mTOR and the JAK–STAT pathways, engaged by growth factor tyrosine kinase receptors and cytokines (Park et al., 2008; Smith et al., 2009; Buchser et al., 2012; Leibinger et al., 2013; Pernet et al., 2013). Combined deletion of endogenous inhibitors of these two pathways enhanced regeneration above the level reported for deletion of either gene alone (Sun et al., 2011). Activation of PI3-kinase–mTOR via PTEN deletion also enhanced regenerative growth in an SCI model (Liu et al., 2010), suggesting that results obtained in the optic nerve crush model may generally translate to SCI models. Here, we show that the classic growth factor signaling module RAF–MEK enables axon regeneration in the optic nerve at least as powerfully as any previously reported single molecule manipulation and that the combination of kaB-RAF with activation of PI3-kinase–mTOR via PTEN deletion enhances optic nerve axon regeneration even more strongly than would be expected for a simple additive effect.
phospho-mTOR and p70S6K (9206), and pS6 (9062) were obtained from Cell Signaling Technology. BII-Tubulin (AA10) was purchased from Invitrogen; Sena6D (S-16) was purchased from Santa Cruz Biotechnology, Inc.; C-Raf antibody (610151) was purchased from BD. Parvalbumin antibody (PV26) was obtained from Swant. CGRP antibodies (AB9290 and AB5705) were obtained from EMD Millipore. All Western blot and immunohistochemical experiments were repeated with tissue from at least three embryos for each genotype, and these embryos were obtained from a different litter for each experiment. Littermate controls were used throughout.

LacZ staining. E16.5 embryos were fixed in 4% paraformaldehyde and stained with X-gal using EMD Millipore’s Tissue Base staining solution according to the manufacturer’s protocol. After imaging of axon skin innervation, embryos were dehydrated using a methanol in PBS dilution series (25–50%, 75–95%, and 100%), followed by incubation in 50% methanol: 50% benzyl alcohol/benzyl benzoate (BABB), and subsequently cleared in 100% BABB (Sigma–Aldrich). Specimens were imaged with a M205A stereomicroscope equipped with a DFC310FX color digital camera system (Leica).

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