A CONVERGENCE OF EXTRINSIC AND INTRINSIC SIGNALS FOR POSTMITOTIC DIFFERENTIATION OF NOCICEPTORS

by

Siyi Huang

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

December, 2013

Abstract

Diverse neuronal subtypes are the building blocks of functional neural circuits that underlie behaviors. The generation of correct types of neurons at appropriate times and positions is therefore fundamental to the development of the nervous system. Specification of neuronal subtypes is a multistep process that extends beyond the initial specification of neural progenitors and continues as postmitotic neurons differentiate further. The postmitotic aspect of neuronal subtype specification, although important for generation of neuronal subtype diversity, remains understudied. Here, using nociceptors, a class of primary sensory neurons in the dorsal root ganglion (DRG) that detect painful stimuli, as a model system and a combination of *in vivo* and *in vitro* approaches, we uncover a novel mechanism by which NGF, the prototypic neurotrophic factor and Runx1, a Runx family transcription factor, coordinate the specification of nonpeptidergic nociceptors, a major, well-characterized nociceptor subtype. We show that NGF promotes Runx1-dependent transcription that confers molecular and morphological identity of nonpeptidergic nociceptors through transcriptional upregulation of *Cbfb*. The protein product of *Cbfb*, CBFβ, is an integral component of the heterodimeric Runx1/CBFβ complex in DRGs, since conditional deletion of *Cbfb* in DRGs produces the same spectrum of phenotypes in nonpeptidergic nociceptors as observed in *Runx1* mutants. NGF is necessary for *Cbfb* expression prior to the onset of NGF dependence of Runx1, implicating CBFβ as a critical link between NGF signaling and Runx1 function. NGF activates *Cbfb* expression through a MEK/ERK pathway. On the other hand, transcriptional initiation of *Runx1* requires Islet1, a LIM-homeodomain transcription factor, while *Cbfb* expression is largely Islet1-independent. These findings together reveal a novel NGF/TrkA–MEK/ERK–Runx1/CBFβ axis that promotes gene expression and maturation of nonpeptidergic nociceptors and provide a common principle by which a convergence of extrinsic and intrinsic signals instructs postmitotic neuronal subtype specification.

Advisor: David D. Ginty, Ph.D.

Acknowledgments

I thank Dr. David Ginty for being a terrific mentor scientifically and personally; Dr. Xinzhong Dong for helpful suggestions and comments on this dissertation; my thesis committee members: Dr, Xinzhong Dong, Dr. Jeremy Nathans, and Dr. Seth Blackshaw for scientific insights on my thesis project; my collaborators: Dr. Alan Friedman, Dr. Jian Zhong, and Dr. Eric Turner for sharing valuable mouse lines; my rotation hosts: Dr. King-Wai Yau, Dr. Hongjun Song, and Dr. Craig Montell for the wonderful first-year experience; and my labmates for moral support and stimulating discussions.

Lastly, I want to dedicate this thesis to my family who is everything to me.

Table of contents

List of tables

List of figures

Chapter 1. Neuronal subtype specification in DRG sensory neurons

1.1. The question of neural cell-fate determination

Neuroscientists have been fascinated by the remarkably high degree of cell type diversity in the nervous system for over a century (Ramon y Cajal, 1899). Since different types of neurons serve as the building blocks of myriad functional neuronal circuits, which underlie all complex behaviors in the animal kingdom, studying the process of cell-fate determination during neural development has proven central to understanding brain function. Moreover, recent success in directing differentiation of embryonic stem cells into neuronal subtypes *in vitro* demonstrates the translational value of studying normal neuronal specification in the development of cell replacement therapies for diseases characterized by a loss of specific neuronal types (Robertson et al., 2008).

Neuronal cell fate specification is a multistep process that can be broadly divided into two stages, early specification of neural progenitor cell identity and postmitotic differentiation of neuronal subtypes. Vertebrate studies over the past 20 years, primarily in the retina, cerebral cortex and developing spinal cord, have provided insights into this process. Most notably, there is general agreement on the importance of the interplay between intrinsic determinants and extrinsic signals in controlling neural cell-fate decisions (Fishell and Heintz, 2013). Examples of coordinate regulation of cell-fate choice by both extrinsic and intrinsic factors are particularly well documented in the context of specification of neural progenitor cells. Conceptually, at least two different modes of interaction have been observed for defining progenitor cell identity. First, it is widely accepted that as development progresses, neural progenitors pass through a series of intrinsically determined competence states, during which they can only produce a subset of cell types. In other words, cell-fate choices at a given time are restricted by limitations in progenitor competence, which is largely intrinsically defined. Extrinsic factors have the most influence on progenitors within a given competence state to alter the relative proportions of each cell type generated within the confines set by the intrinsic state of progenitors. In the retina, for instance, it was shown that retinal progenitors obtained from the embryonic rat retina and cultured in an environment mimicking the postnatal retina, fail to produce the main cell type that postnatal progenitors generate, indicating intrinsic limitations in the competence of retinal progenitors at different times. Yet, extrinsic factors characteristic of the postnatal retina alter the

relative abundance of each cell type that can be generated within the confines of the intrinsic competence state of embryonic progenitors (Belliveau and Cepko, 1999; Livesey and Cepko, 2001). Similarly, during cortical neurogenesis, neocortical progenitors become progressively restricted in their competence states, such that early progenitors are multipotent and are able to give rise to pyramidal neurons across layers II-VI, while late progenitors have very limited fate potential, even when transplanted into the niches of early progenitors (Frantz and McConnell, 1996; Reid and Walsh, 2002). Unlike retinal progenitors, under appropriate conditions, the competence state of neocortical progenitors can be overridden by extrinsic factors, suggesting an even greater contribution of extrinsic factors to cell-fate decisions in the neocortex (Fukumitsu et al., 2006; McConnell and Kaznowski, 1991).

A second paradigm where extensive interaction between extrinsic and intrinsic factors has been intimately linked to neuronal identity is in the context of specification of positional identities of progenitor domains. It is well known from seminal work in the developing spinal cord that the position that a progenitor cell assumes in the neural tube is a critical determinant of the neural fate of its progeny. The steps linking spatial patterning of progenitor domains and generation of specific neuronal subtypes in the spinal cord have been well defined, especially along the dorsoventral (D-V) axis. There, initially identical progenitors are segregated into different domains based on their distinct positions along the D-V axis, where they are exposed to different combinations of spatially restricted extrinsic morphogens at different concentrations. For instance, the morphogen Sonic hedgehog (Shh) emanating from the notocord and floor plate is critical for the patterning of all five ventral progenitor domains that each give rise to one of the five neuronal subtypes in the ventral spinal cord (Briscoe and Ericson, 2001; Briscoe and Novitch, 2008; Patten and Placzek, 2000). Shh signaling acts in a concentration-dependent manner to specify each ventral progenitor domain by regulating the expression profiles of homeodomain HD transcription factors that provide positional identity along the D-V axis in the form of a combinatorial transcription factor code (Briscoe and Ericson, 2001; Briscoe and Novitch, 2008). In broader terms, these findings describe a common way in which extrinsic signaling factors and intrinsic transcription factors coordinate the specification of neuronal progenitors. That is, extrinsic signaling regulates the expression of intrinsic transcription factors.

Although it is generally believed that cell identity becomes fixed when cells exit the cell cycle, diversification of neuronal subtypes continues in postmitotic neurons (Fishell and Heintz, 2013). Indeed, it has been increasingly appreciated that postmitotic differentiation of neuronal subtypes is the major source of neuronal subtype diversity. For instance, three broad subtypes of neocortical projection neurons defined based on their axonal projection patterns, namely, subcerebral projection neurons, corticothalamic projection neurons and callosal projection neurons, are specified postmitotically (Greig et al., 2013). Specification of motor neuron columnar identity along the rostrocaudal (R-C) axis and even finer segregation of motor neurons into motor pools based on the specific muscle they innervate are all processes that take place following the initial generation of motor neurons (Shirasaki and Pfaff, 2002). Studies of postmitotic specification of neuronal fate in motor neurons have so far demonstrated dependence of subtype identity on both extrinsic and intrinsic regulators much like what was described for specification of motor neuron progenitors (Dasen et al., 2003; Liu et al., 2001; Shirasaki and Pfaff, 2002; Sockanathan et al., 2003). It appears that at least in some cases, similar mechanisms are used. For example, convergent activities of extrinsic signals, including FGFs, Gdf11 and retinoid signals, impose motor neuron columnar fate by establishing the pattern of *Hox* gene expression along the R-C axis (Liu et al., 2001). However, in most cases, extrinsic and intrinsic requirements have not been defined, preventing a comprehensive assessment of the relative roles of extrinsic and intrinsic signals in the late phase of cell-fate determination. Further investigation of molecular mechanisms underlying postmitotic specification of neuronal subtypes in neural systems is therefore of great importance to close the gap between neurogenesis and neuronal subtype specification.

1.2. Development of primary sensory neurons in the DRG

The somatosensory system is the part of the nervous system that generates the conscious perception of diverse sensory stimuli from the external environment and internal organs, informing us of painful or harmful cues, temperature, itch, touch, limb movement and body position. The ability to detect such diverse sensory modalities arises from the existence of functionally specialized primary sensory neurons that are the first order neurons of the somatosensory system. Primary sensory neurons that carry most sensory information from the trunk and limbs have their cell bodies located in the dorsal root ganglion (DRG), hence are termed DRG neurons. DRG neurons are pseudounipolar in that each neuron extends one axon that bifurcates to innervate both a peripheral target, for example the skin, and the central target, the dorsal spinal cord. Notably, depending on the functional type, DRG neurons not only have unique molecular characteristics, but they also establish stereotyped modality-specific contacts with peripheral and central targets. DRG neurons are therefore broadly divided into three functionally, molecularly and morphologically distinct classes, namely, nociceptors that preferentially respond to painful stimuli, mechanoreceptors mediating mechanical sensations, and proprioceptors that sense body position and limb movement (Figure 1). Due to this heterogeneity, the DRG has long been an attractive model system for studying various developmental processes leading up to the generation of diverse neuronal subtypes.

DRG neurons together with sympathetic and enteric neurons of the peripheral nervous system (PNS) and cells of many other nonneuronal lineages are derived from multipotent trunk neural crest cells (NCCs). The neural crest which arises at the border between the nonneural ectoderm and the neural plate is specified as early as gastrulation (Basch et al., 2006). Neural crest induction is driven by extrinsic signaling that culminates in profound changes in cell-intrinsic properties. BMPs and Wnts are both involved in this process. Specifically, Wnt molecules are both necessary and sufficient to induce neural crest cells in avian embryos, whereas BMPs are necessary for maintaining Wnt expression (Garcia-Castro et al., 2002). Under the influence of these signals, cells of the dorsal neural tube undergo an epithelial-to-mesenchymal transition, which involves downregulation of N-cadherin and cadherin 6, to become NCCs that are much more motile (Bronner-Fraser et al., 1992; Nakagawa and Takeichi, 1998; Newgreen and Gooday, 1985). Between embryonic day E8.5 and E10 in the mouse, and between stage 11 and stage 21 in the chick, NCCs

delaminate from the neural tube and migrate along a ventral pathway to produce DRG neurons as well as sympathetic and enteric neurons that belong to the autonomic lineage (Bronner-Fraser and Fraser, 1988; Frank and Sanes, 1991; Serbedzija et al., 1990).

Migration of NCCs is largely temporally coupled with sensory neurogenesis which occurs in three successive waves. The first two waves are initiated either during or immediately after migration, while the third wave, which was recently described, emerges much later (Marmigere and Ernfors, 2007; Maro et al., 2004). Using clonal analysis with a retroviral approach, the first two waves of neurogenesis were characterized in detail and their respective neuronal progeny were also examined (Frank and Sanes, 1991). Notably, early migrating NCCs, which contribute to the first wave of neurogenesis, undergo limited rounds of cell division, only generating an average of 3.1 neurons each, as opposed to 35.9 neurons each that NCCs produce during the second wave. Distinct cellular behaviors can be explained by a combination of duration of neurogenesis and the rate of proliferation determined by the balance between proneural genes and multipotency genes. Another major distinction between these two waves is the subtype distribution of their progeny. The first wave of neurogenesis preferentially generates neurons that in chick populate the ventrolateral region of the DRG where large mechanoreceptive and proprioceptive neurons expressing the neurotrophin receptors, TrkB and/ or TrkC (TrkB/TrkC) reside. By contrast, the second wave gives rise to all principle sensory subtypes, which in chick include both dorsomedially-situated small nociceptive neurons that express the neurotrophin receptor TrkA and ventrolateral TrkB/TrkC neurons (Frank and Sanes, 1991; Rifkin et al., 2000). Corresponding waves were also observed in the mouse where it was shown that the first and second waves are mediated by basic helix-loop-helix (bHLH) transcription factors neurogenin 2 (Ngn2) and neurogenin 1 (Ngn1), respectively (Ma et al., 1999).

For NCCs, neurogenesis and specification of sensory neuron fate are intimately related. In fact, the same proneural genes *Ngn1* and *Ngn2* that are required for neurogenesis also direct NCCs to the sensory lineage as opposed to the autonomic lineage which depends on a different bHLH transcription factor, mammalian achaete-scute homologue 1 (Mash1) (Bertrand et al., 2002; Lo et al., 2002; Ma et al., 1999). Gain-of-function studies *in vivo* in chick and in cultured neuronal progenitor cells in which sensory markers are induced by overexpression of Ngns further suggest a sensory-specifying role for Ngns (Lo et al., 2002;

Perez et al., 1999). Analysis of knockout mice nicely complements the studies in the chick showing that the first, Ngn2-mediated wave of neurogenesis, which occurs largely during migration, produces only TrkB/TrkC mechanoreceptive and proprioceptive neurons, while the second Ngn1-mediated wave, which is initiated and continues for a long time in the DRG, contributes to both small $TrkA^+$ nociceptive neurons and large TrkB/TrkC neurons. Indeed, in *Ngn2* mutant animals, Ngn1 can compensate for loss of Ngn2, such that large TrkB/TrkC neurons develop normally. Conversely, *Ngn1* mutant mice develop with an almost complete depletion of $TrkA^+$ neurons (Ma et al., 1999). Neurogenesis is further facilitated by gradual extinction of the high-mobility group transcription factor SRY (sex determining region Y) box 10 (SOX10), which functions in NCCs to maintain multipotency and inhibits neurogenesis (Kim et al., 2003; Montelius et al., 2007). In addition to intrinsic transcription factors, extrinsic signals that either promote or inhibit sensory neuron fate have also been identified. Interestingly, Wnt and BMP signaling which are required for neural crest induction during early development, also play a late instructive role in cell-fate decisions between sensory and autonomic lineages. Loss-of-function and gain-of-function studies in the mouse or in cultured NCCs that manipulated either the Wnt ligand or β-catenin, the key downstream mediator of the canonical Wnt signaling pathway, show bidirectional regulation of sensory neuron fate by altered Wnt signaling (Hari et al., 2002; Kleber et al., 2005; Lee et al., 2004). Moreover, in gain-of-function paradigms, there is evidence that sensory neuron and autonomic neuron fates represent alternative choices in a lineage decision that are regulated in opposite directions by Wnt signaling, further confirming that canonical Wnt signaling instructs the sensory neuron fate (Lee et al., 2004). It is at least in part achieved through the ability of Wnt to regulate *Ngn2* expression (Hari et al., 2002; Lee et al., 2004). The lineage-specific function of BMP is concentration-dependent in that at high concentrations, it directs NCCs to the autonomic lineage, while low levels of BMP promote the sensory neuron identity instead (Lo et al., 2002; Reissmann et al., 1996; Shah et al., 1996). Thus, as in other systems, lineage decisions of progenitor cells are controlled by the interplay between extrinsic signals and intrinsic determinants.

Although the first wave mediated by Ngn2 produces large TrkB/TrkC neurons, Ngn2-expressing NCCs do not appear to be restricted in their fate potential, suggesting that neither Ngn1 nor Ngn2 are able to specify sensory subtypes (Zirlinger et al., 2002). Therefore, specification of neuronal subtypes in the DRG is a stepwise process that takes place immediately after sensory neurogenesis. The initial differentiation of

sensory neuron subtypes into distinct principle functional types is characterized by establishment of largely non-overlapping patterns of expression of the neurotrophic receptors TrkA, TrkB, TrkC and Ret, the receptor for glial-derived neurotrophic factor (GDNF) family ligands (GFLs). Trk receptors are a family of receptor tyrosine kinases that mediate the majority of biological effects of neurotrophins in both the central nervous system (CNS) and the PNS. In the PNS, in particular, Trk receptors and their cognate neurotrophins function to support survival, axonal growth, modality-specific target innervation, phenotypic maturation (Harrington and Ginty, 2013). A detailed account of neurotrophins and their intracellular signaling pathways will be presented in a separate section. Furthermore, a gain-of-function study where *TrkC* is expressed in place of *TrkA* from the endogenous *TrkA* locus suggests that Trk receptors may contribute to the specification of principle sensory subtypes in an instructive manner (Moqrich et al., 2004). Thus, mechanisms governing expression of Trk receptors and neurotrophin signaling are expected to contribute significantly to the process of sensory neuronal subtype specification. For instance, the characterization of tissue-specific enhancers in the *TrkA* promoter has led to the identification of intrinsic regulators of *TrkA* expression, such as Kruppel-Like factor 7 (KLF7) and the POU homeodomain transcription factor Brn3a (Lei et al., 2005; Lei et al., 2001; Ma et al., 2003; Ma et al., 2000). Although there seems to be incomplete consensus, dynamic regulation of neurotrophin receptors by Runx transcription factors has been described (Abdel Samad et al., 2010; Inoue et al., 2007; Kramer et al., 2006; Marmigere et al., 2006; Nakamura et al., 2008; Yoshikawa et al., 2007). In at least one such case, the regulation is mediated by direct binding of Runx3 to the cis-regulatory element within the *TrkB* gene (Inoue et al., 2007). Lineage-specific functions of neurotrophin signaling have been most well-established for TrkA and TrkC in the context of specification of nociceptors and proprioceptors, respectively. In particular, loss-of-function studies of NGF, the preferred ligand for TrkA, or TrkA reveal a failure to acquire nociceptor-specific molecular and morphological characteristics which is independent of massive death of nociceptors as result of NGF deprivation (Guo et al., 2011; Luo et al., 2007; Patel et al., 2000; Wickramasinghe et al., 2008). Likewise, ablating NT3, the preferred ligand for TrkC, selectively disrupts specification of proprioceptors, including axonal projections both centrally and peripherally as well as expression of proprioceptor molecular markers (Genc et al., 2004; Patel et al., 2003). In some cases, the specific intracellular mediators of these lineage-specific effects of neurotrophins have been identified. For

instance, serum response factor (SRF), a member of the MADS-box transcription factor family, mediates NGF-dependent axonal growth, branching, and target innervation by embryonic $TrkA^+$ DRG sensory neurons (Wickramasinghe et al., 2008). Hoxd1, a Hox transcription factor, mediates NGF-dependent nociceptive axonal projections in the spinal cord (Guo et al., 2011). The ETS transcription factor ER81 acts downstream of NT3 signaling to establish proprioceptive afferent-motor neuron monosynaptic connections (Patel et al., 2003). More recently, it was shown that the cytoplasmic Ser/Thr kinases SAD kinases transduce NT3 signals to regulate central axonal arborization of NT3-dependent DRG neurons (Lilley et al., 2013). Interestingly, in all cases, extrinsic signaling regulates the expression of intrinsic mediators. As described previously, this strategy appears to be a common mechanism for the specification of neuronal subtypes.

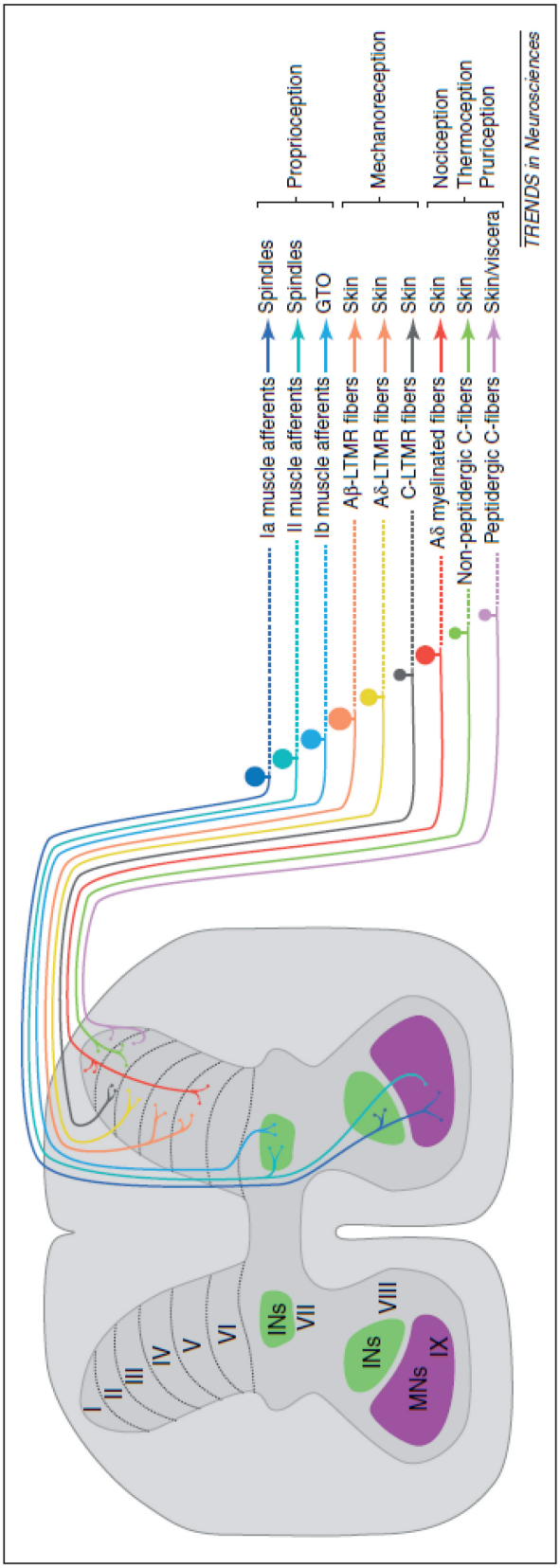
In addition to extrinsic signals exemplified by neurotrophins, intrinsic determinants of specific sensory neurons subtypes have been defined. Among them, the Runx family of transcription factors has emerged as key intrinsic regulators of specific lineages. In fact, Runx family members, including Runx1, Runx2 and Runx3 in mammals, have long been implicated in specification of diverse lineages during hematopoiesis, immune function and osteogenesis (Banerjee et al., 1997; de Bruijn and Speck, 2004; Ducy et al., 1997). Since Runx transcription factors, particularly Runx1, will be discussed in more detail in the context of diversification of nociceptors in a separate section, here only the common principles regarding how Runx proteins function in the DRG are described. Consistent with their non-overlapping subtype-specific expression patterns, Runx1 and Runx3, the only Runx members expressed in the DRG, act non-redundantly to promote specific sensory subtype identity. Runx3 is required for acquisition of the proprioceptive identity, whereas Runx1 is important for differentiation of nociceptors into one subclass of mature nociceptors, nonpeptidergic nociceptors. Regardless of the specific lineage requirement, in the absence of Runx, there is, in addition to a block in normal differentiation into a specific lineage, general expansion of alternative cell types, suggesting that part of the lineage-promoting activity of Runx results from suppression of alternative cell-fate decisions, a notion further confirmed by gain-of-function studies (Abdel Samad et al., 2010; Chen et al., 2006b; Inoue et al., 2007; Kramer et al., 2006). Both activator and repressor activities of Runx proteins have been described and they both contribute to Runx functions in sensory neuronal subtype specification (Liu et al., 2008; Marmigere et al., 2006; Yarmus et al., 2006). The

endogenous targets of either activator or repressor activity of Runx proteins, however, remain unknown. There is also increasing evidence for a general effect of Runx on axonal growth and branching (Chen et al., 2006a; Kramer et al., 2006; Lallemend et al., 2012; Marmigere et al., 2006). As observed both *in vivo* and *in vitro*, the length of axons is positively correlated with the level of Runx activity, raising the possibility that Runx regulates modality-specific central terminations of DRG neurons, and more generally, axonal growth by a cell-intrinsic mechanism, such that differential axonal growth rates are encoded by different levels of *Runx* expression (Lallemend et al., 2012; Marmigere et al., 2006). Thus, Runx transcription factors play key roles in the generation of sensory neuron diversity by coordinating subtype-specific gene programs and modality-specific central axonal projections. In light of the critical requirement for extrinsic signaling during sensory neuron subtype specification, an important question arises: How do extrinsic signals, such as neurotrophins, and intrinsic genetic programs, including Runx activities, interact to coordinate subtype specification?

Figure 1 Diverse types of DRG neurons that subserve different sensory modalities

A schematic summarizing morphological, physiological and functional characteristics of various subtypes of DRG neurons. (Diagram from Lallemend and Ernfors, 2012) (Lallemend and Ernfors, 2012)

Figure 1


Ia muscle afferents
II muscle afferents
Ib muscle afferents
Aβ-LTMR fibers
Aδ-LTMR fibers
C-LTMR fibers
Aδ myelinated fibers
Non-peptidergic C-fibers
Peptidergic C-fibers
Spindles
Spindles
GTO
Skin
Skin
Skin
Skin
Skin
Skin/viscera
Proprioception
Mechanoreception
Nociception
Thermoception
Pruriception
TRENDS in Neurosciences
I
II
III
IV
V
VI
INs
VII
INs
VIII
MNs
IX

1.3. Runx transcription factors in diversification of DRG neurons

The metazoan Runt-related (Runx) gene family encodes evolutionarily conserved sequence-specific DNA binding transcription factors that play pivotal roles during development and adult tissue homeostasis. The defining feature of this protein family is the presence of a conserved 128-amino acid DNA binding motif, termed the Runt domain, which recognizes the consensus sequence, RCCRCA (R=purine) (Levanon and Groner, 2004; Otto et al., 2003) . In fact, mammalian Runx proteins were independently identified as the DNA binding subunit of heterodimeric transcription factor complexes, polyoma enhancer binding protein 2 and core-binding factor (PEBP2/CBF), through characterization of proteins that bind to murine viral enhancers that confer permissivity for viral infection or tissue specificity of viral replication (Ito, 2008; Speck and Terryl, 1995). It is known from initial purification that PEBP2/CBF has two subunits, the alpha subunit which corresponds to Runx proteins, and the beta subunit PEBP2β/CBFβ, a structurally unrelated protein that does not have intrinsic DNA-binding ability (Ito, 2008; Speck and Terryl, 1995). In mammals, CBFβ acts as a common cofactor for all three Runx proteins, Runx1,Runx2 and Runx3 by enhancing their DNA binding affinity and protein stability (Adya et al., 2000). CBFβ is indispensable for Runx protein function, as determined by the phenocopy between knockout mice for *Cbfb* and various *Runx* genes (Okuda et al., 1996; Wang et al., 1996a; Wang et al., 1996b). Like Runx proteins, CBFβ and its functions are conserved across species (Adya et al., 2000).

Runx proteins are known to undergo complex transcriptional regulation and posttranslational modifications (Bae and Lee, 2006; Levanon and Groner, 2004). In addition, they are frequently found to interact with a variety of transcription factors as well as transcriptional coactivators and corepressors (Durst and Hiebert, 2004; Miyazono et al., 2004). These features greatly diversify Runx activity, such that Runx proteins mediate both transcriptional activation and repression in a context-dependent manner (Durst and Hiebert, 2004). What is generally true about the function of Runx proteins is that they play key roles in regulating the balance between cell proliferation and differentiation in various developing and adult tissues (Coffman, 2003). This function has been particularly well appreciated in the context of cancer biology, where dysregulation of Runx activity is not only correlated with but also causally associated with several forms of cancers (Blyth et al., 2005; Ito, 2008). In the mammalian system, for instance, Runx1 is well known for both being a critical regulator of fetal and adult hematopoiesis and being responsible for various forms of

leukemia (de Bruijn and Speck, 2004; Speck and Gilliland, 2002). Runx2 is a master regulator of bone development, and its haploinsufficiency is one of the causes of the hereditary bone disease Cleidcranial dysplasia (Komori, 2006; Lian et al., 2004; Mundlos, 1999). Runx3 is essential for the generation of the T-cell sublineage and gastric system development, and is implicated in a number of human cancers as a tumor suppressor (Collins et al., 2009; Ito, 2004; Li et al., 2002; Puig-Kroger and Corbi, 2006). Thus in general, Runx proteins promote cell differentiation in a tissue-specific manner by controlling lineage-specific gene expression.

Tissue-specific requirements for various Runx proteins reflect and largely result from distinct tissue-specific expression patterns of the *Runx* genes (Levanon and Groner, 2004). Notably, in various tissues, where both Runx1 and Runx3 are expressed, they are localized to different cell types resulting in largely non-overlapping and complementary expression patterns (Levanon et al., 2001; Levanon and Groner, 2004). In the nervous system in particular, Runx1 is expressed in both mitotic neuronal precursors, such as immediate neuronal precursors of olfactory sensory neurons, and postmitotic neurons, such as hindbrain cholinergic branchiovisceral motor neurons and select spinal cord motor neurons (Levanon et al., 2001; Simeone et al., 1995; Theriault et al., 2005; Theriault et al., 2004). Runx3 on the other hand is exclusively expressed in postmitotic neurons, such as sensory neurons in DRGs and select cranial ganglia (Levanon et al., 2001). In the DRG, the expression of Runx1 and Runx3 is largely confined to separate sensory subtypes, marking nociceptors and proprioceptors, respectively (Levanon et al., 2001).

The subtype-specific expression pattern of Runx1 and Runx3 in the DRG is functionally relevant, because it ensures that Runx1 and Runx3 each function in a specific sensory sublineage for finer diversification.

Consistent with the segregation of *Runx3* expression with $TrkC^+$ neurons during early development, Runx3 is most well-known for its role in the segregation of a transient population that coexpresses TrkB and TrkC, the TrkB/C lineage, into the $TrkC^+$ proprioceptive and $TrkB^+$ mechanoreceptive populations (Kramer et al., 2006). Specifically, *Runx3* expression that is initiated at E10.5 is both necessary and sufficient for establishing a solitary TrkC phenotype from the TrkB/C lineage by repressing *TrkB* expression and maintaining *TrkC* expression (Inoue et al., 2007; Kramer et al., 2006). Therefore, in the absence of Runx3, there is a loss of $TrkC^+$ proprioceptors and a concomitant increase in the number of $TrkB^+$ neurons due to

derepression of *TrkB* in prospective TrkC$^+$ proprioceptors (Inoue et al., 2007; Kramer et al., 2006). Conversely, overexpression of *Runx3* after neurogenesis in all DRG neurons increases *TrkC* expression and completely eliminates *TrkB* expression even in lineages that do not normally express Runx3 (Kramer et al., 2006). Runx3 acts as a transcriptional repressor for *TrkB* both directly and indirectly. A cluster of Runx binding sites within an evolutionarily conserved intron 7 sequence of the *TrkB* gene was shown to mediate the repressor activity of endogenous Runx3 on the *TrkB* promoter in cultured DRG neurons (Inoue et al., 2007). In addition, Runx3 represses the transcription factor short stature homeobox 2 (Shox2), which is necessary for *TrkB* expression, thereby repressing *TrkB* expression in prospective TrkC$^+$ proprioceptors (Abdo et al., 2011). On the other hand, *Shox2* expression is maintained primarily in neurons with extinguished *Runx3* expression, where Shox2 promotes specification of TrkB$^+$ mechanoreceptors by repressing *TrkC* and activating *TrkB* expression (Abdo et al., 2011; Scott et al., 2011). Since *Runx3* extinction in prospective TrkB$^+$ neurons does not depend on Shox2, it appears that loss of *Runx3* expression is the signal that initiates the specification of TrkB$^+$ mechanoreceptors (Abdo et al., 2011; Scott et al., 2011). Therefore, Runx3 acts instructively in the TrkB/C lineage to promote segregation of TrkC$^+$ proprioceptors from TrkB$^+$ mechanoreceptors. It is possible that the same mechanisms that control spatial and temporal expression of *Runx3* in the DRG initiate the diversification of the TrkB/C lineage.

Analogous functions of Runx1 have been described in the context of diversification of the embryonic TrkA lineage. Here, the embryonic TrkA population specifically refers to those TrkA neurons that are born during the Ngn1-mediated second wave, since the TrkA population that is born by E10.5 during the first wave of neurogenesis appears to have never expressed Runx1 (Bachy et al., 2011; Kramer et al., 2006). It is known that *Runx1* expression undergoes dynamic changes, as the embryonic TrkA precursor is progressively specified into distinct mature subtypes. Specifically, Runx1 goes from being expressed in the majority of TrkA$^+$ neurons at E12.5 in lumbar DRGs to being expressed in a pattern that is almost complementary to that of *TrkA* in adult DRGs (Chen et al., 2006b). This gradual downregulation of *Runx1* in select populations leads to segregation of immature TrkA$^+$/Runx1$^+$ precursors into Runx1-persistent and Runx1-transient populations (Abdel Samad et al., 2010). This process is tightly coupled with the well characterized divergence of peptidergic and nonpeptidergic populations from the immature TrkA$^+$ precursor. These two populations are molecularly, morphologically and functionally distinct (Cavanaugh et

al., 2009; Zylka et al., 2005). The most notable difference of all is that the peptidergic population maintains *TrkA* expression into adulthood, while its nonpeptidergic counterpart switches from expressing TrkA to expressing Ret, the receptor for GFLs in a stereotyped process involving *Ret* upregulation starting from E15.5 and postnatal *TrkA* extinction (Bennett et al., 1996a; Luo et al., 2007; Molliver et al., 1997). Interestingly, the subdivision of Runx1-persistent and Runx1-transient populations largely aligns with the classical nonpeptidergic and peptidergic classification, in that *Runx1* expression is preferentially maintained in the majority of nonpeptidergic neurons, while its expression in the peptidergic population is either transient or weak (Abdel Samad et al., 2010; Chen et al., 2006b; Gascon et al., 2010).

Consistent with its apparent nonpeptidergic-specific expression pattern, Runx1 is generally considered as a master regulator of the nonpeptidergic fate by both promoting the nonpeptidergic phenotype and inhibiting the peptidergic phenotype. As a result, in *Runx1* knockout animals, the expression of nonpeptidergic-specific genes including *Ret* and a wide range of functionally important nociceptive ion channels and receptors is severely impaired, along with a concomitant expansion of the peptidergic phenotype, such as the expression of genes coding for the neuropeptide calcitonin gene-related peptide (CGRP), and Met, a receptor tyrosine kinase for hepatic growth factor (HGF) (Chen et al., 2006b; Gascon et al., 2010; Yoshikawa et al., 2007). Conversely, overexpression of *Runx1* in all DRG neurons during early or late development leads to a selective impairment of Runx1-transient populations, such as suppression of classical markers of peptidergic nociceptors, without significantly altering molecular characteristics of Runx1-persistent populations, which are mostly nonpeptidergic neurons (Abdel Samad et al., 2010; Kramer et al., 2006). Therefore, downregulation of *Runx1* in prospective peptidergic nociceptors is necessary for emergence of peptidergic phenotypes such as expression of *CGRP* and *Met*. Importantly, HGF-Met signaling has recently been shown to repress *Runx1* expression and consolidate *CGRP* expression, thereby promoting late maturation of a subset of peptidergic nociceptors (Gascon et al., 2010). However, it remains unclear whether Met signaling can promote peptidergic maturation independent of regulation of Runx1extinction. Thus, as in the case of the diversification of the TrkB/C lineage, cross-repressive interactions between lineage regulators of alternative cell fates appear to be a common mechanism for consolidating cell-fate decisions.

After specifying the generic nonpeptidergic fate, Runx1 continues to be expressed in developing nonpeptidergic populations where it further contributes to sensory subtype diversity. For instance, Runx1 directs the identity of a distinct Ret^+ nonpeptidergic population that, based on electrophysiological properties, represents C-fiber low threshold receptors (C-LTMRs) (Lou et al., 2013). C-LTMRs normally diverge from the rest of the nonpeptidergic population at two weeks postnatally, when they can then be reliably identified based on morphological and molecular traits, such as expression of tyrosine hydroxylase (*TH*) and vesicular glutamate transporter type 3 (*Vglut3*) (Li et al., 2011; Seal et al., 2009). However, In *Runx1* mutant mice where *Runx1* is conditionally removed from neurons that express *Vglut3* sometime in their lifetime, C-LTMRs failed to develop as evidenced by the inability to acquire the molecular, morphological and electrophysiological identity of C-LTMRs (Lou et al., 2013). Besides C-LTMRs, the nonpeptidergic population differentiates into at least three more subtypes, each expressing a unique profile of the Mas-related G protein-coupled receptors (*Mrg*) class of receptors, which contains four main subclasses, *MrgA-D*. They are the $MrgD^+$ polymodal nociceptor and/or pruriceptor, the $MrgA3/MrgC11^+$ pruriceptor and the $MrgB4^+$ putative stroking-sensitive neuron (Han et al., 2013; Lewin and Moshourab, 2004; Liu et al., 2012; Liu et al., 2009; Liu et al., 2007; Rau et al., 2009; Vrontou et al., 2013). It is known that the mature compartmentalized expression of *MrgA/B/C* versus *MrgD* genes arises from a transient population where their expression largely overlaps. Runx1 is initially required for the expression of all *Mrg* genes presumably due to its transcriptional activator activity (Abdel Samad et al., 2010; Chen et al., 2006b; Liu et al., 2008). During postnatal development, however, Runx1 appears to switch to a repressor for *MrgA/B/C* but not for *MrgD*. Furthermore, the repressive activity of Runx1 on *MrgA/B/*C requires the C-terminal repression domain which is known to recruit the corepressor Groucho/transducin-like Enhancer-of-split (TLE). Therefore, persistent *Runx1* expression allows the *MrgD*-only compartment to emerge from the intermediate population with mixed *Mrg* expression by repressing the expression of other *Mrg* genes and maintaining *MrgD* expression (Liu et al., 2008). Conversely, maintenance of expression of *MrgA/B/C* in the future *MrgA/B/C* compartment depends on downregulation of *Runx1*, as constitutive *Runx1* expression was shown to completely eliminate *MrgA/B/C* expression (Abdel Samad et al., 2010). Thus, differential regulation of *Runx1* expression, together with distinct responses of *Mrg* genes to *Runx1* expression, generates additional diversity within the nonpeptidergic lineage.

The evidence so far is consistent with the notion that Runx transcription factors are a main drive of neuronal subtype diversity in the DRG. However, important details are still missing. For example, although dynamic regulation of *Runx* expression emerges as a common mechanism for initiating segregation of different subtypes, its mechanistic detail remains to be elucidated. Moreover, understanding the molecular basis of the diverse and dynamic nature of Runx activities and identifying additional molecular mechanisms for sensory neuronal subtype specification represent key future directions.

1.4. Neurotrophins in neural development and function

1.4.1. Neurotrophins and their receptors

Neurotrophins are important regulators of many aspects of neuronal development and function, including survival, differentiation, axon growth and synapse formation and synaptic plasticity (Harrington and Ginty, 2013; Huang and Reichardt, 2003). Neurotrophins, as prototypic target-derived trophic factors, have substantial influences on developmental neurobiology. Most notably, the experiments leading to the discovery of neurotrophins form the basis of a central tenet in developmental neuroscience, namely the neurotrophic factor hypothesis (Oppenheim, 1989). The neurotrophic factor hypothesis posits that proper balance between the size of a target tissue and the amount of innervation it receives is achieved through neuronal competition for limiting amounts of target-derived survival factors. In fact, the first neurotrophin, nerve growth factor (NGF), was identified in a search for such factors that can support the survival of motor and sensory neurons (Levi-Montalcini, 1987). Subsequently three other neurotrophins were found to be expressed in mammals: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). These four genes have been proposed to be a product of successive duplication of an ancestral genomic segment based on high homology in structure and sequence at both the protein and genomic level (Hallbook, 1999). Neurotrophins generally function as non-covalently bound homodimers, although some neurotrophins can form heterodimers *in vitro*. Structural studies of NGF, NT-3, NT-4, NT-3/BDNF and NT-4/BDNF dimers revealed highly homologous structures with shared features, such as a tertiary fold and cystine knot, which can be found in several other growth factors, including platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) (Butte et al., 1998; McDonald et al., 1991; Robinson et al., 1995).

Neurotrophins interact with two distinct classes of receptors. The p75 neurotrophin receptor (p75NTR), the first receptor to be discovered, is a low affinity receptor for all neurotrophins (Rodriguez-Tebar et al., 1990, 1991). p75NTR, as a distant member of the tumor necrosis factor receptor family, lacks intrinsic catalytic activity and functions by interacting with other proteins (Frade and Barde, 1998). Although it is not the main endogenous receptor for mature neurotrophins, it plays important modulatory roles in neurotrophin signaling, and more recently, it has received increasing attention as the high affinity receptor for

proneurotrophins, the uncleaved proforms of neurotrophins, to be discussed in detail later (Lee et al., 2001). The second class of neurotrophin receptors is the tropomyosin-receptor kinase (Trk) subfamily of receptor tyrosine kinases (Huang and Reichardt, 2003). In mammals, there are three Trk receptors, namely TrkA, TrkB and TrkC. Each receptor is a single-pass transmembrane receptor with a large extracellular domain containing various protein-interaction domains and an intracellular region consisting of a tyrosine kinase domain surrounded by several tyrosines that serve as phosphorylation-dependent docking sites for cytoplasmic adaptors and enzymes. Neurotrophins activate Trk receptors by inducing receptor dimerization and subsequent transphosphorylation of the kinases present in their cytoplasmic domains. Generally speaking, the binding between the four neurotrophins and three Trk receptors is specific with NGF activating TrkA, BDNF and NT-4 activating TrkB, and NT-3 activating TrkC and, less efficiently, the other Trk receptors. Therefore, neuronal responsiveness to neurotrophins is usually dictated by the type of Trk receptors expressed. The specificity of neurotrophin responsiveness is however strongly influenced by alternative splicing of extracellular exons of the Trk receptor genes (Clary and Reichardt, 1994; Strohmaier et al., 1996). The presence of short amino acid sequences encoded by a small exon that is alternatively spliced in the juxtamembrane domains of TrkA or TrkB has been shown to promote the interaction between the receptor and its non-preferred ligands reducing binding specificity (Clary and Reichardt, 1994; Strohmaier et al., 1996). Conversely, the presence of p75NTR promotes the specificity both *in vivo* and *in vitro* (Benedetti et al., 1993; Bibel et al., 1999; Brennan et al., 1999). Additional mechanisms contribute to regulation of neurotrophin binding and actions. For instance, alternative splicing generates TrkB and TrkC isoforms that lack tyrosine kinase domains (Kaplan and Miller, 2000). Within neurons, these truncated receptors can block normal responses of full-length receptors to neurotrophins by interfering with productive dimerization between full-length receptors (Eide et al., 1996). More recent work has also demonstrated the ability of these truncated receptors to signal directly, further diversifying cellular responses to neurotrophins (Esteban et al., 2006; Rose et al., 2003). Additionally, in some CNS neurons, surface expression of Trk receptors, hence accessibility to neurotrophins, can be modulated by neuronal activity, second messengers such as cAMP and Ca^{2+} and most recently, epidermal growth factor (EGF) (Du et al., 2000; Meyer-Franke et al., 1998; Puehringer et al., 2013). Thus, there are multiple modes of regulation of neurotrophin specificity and responsiveness at the receptor level.

On the ligand side, neurotrophins are regulated at both the transcriptional and posttranscriptional level. Using sensitive two-site ELISA and mRNA blot assays, pioneering studies of the sites of NGF expression in developing and adult tissues have provided direct evidence that neurotrophins are target-derived, a key component in the neurotrophic factor hypothesis (Thoenen et al., 1987). For instance, not only is NGF expressed in target tissues of NGF-dependent sympathetic and sensory neurons, but also its level of expression is positively correlated with the amount of innervation that a target tissue receives (Davies et al., 1987; Korsching and Thoenen, 1983; Shelton and Reichardt, 1984). This observation implies that neurotrophins act on distal axons where they initiate a retrograde signal that promotes neuronal survival a distance away. This idea has been validated by a series of seminal studies using compartmentalized culture systems that are designed to mimic the endogenous distribution of neurotrophins by fluidic isolation of distal axons and cell bodies/proximal axons (Campenot, 1977; Park et al., 2006; Zweifel et al., 2005). In these studies, NGF applied exclusively to axon terminals acts both locally to regulate target innervation and axon terminal function, and distally in cell bodies by engaging a specialized retrograde transport system, the signaling endosome, to promote neuronal survival and differentiation (Campenot, 1977; Harrington and Ginty, 2013; Zhang et al., 2005).

It is important to note that neurotrophins are also expressed outside the final targets of responsive neurons. The expression of neurotrophins in regions invaded by axons en route to their final targets is particularly interesting, as these sites of expression may be crucial to support neuronal survival before final target innervation (Farinas et al., 1998; Farinas et al., 1996). Moreover, after peripheral nerve injury, *NGF* expression is induced in fibroblasts and Schwann cells within the nerve by infiltrating macrophages (Heumann et al., 1987). This response may be beneficial for neuronal survival and axon regeneration after injury. Interestingly, some neurotrophins, such as BDNF and NT3, have been found to be expressed in neurons (de Nooij et al., 2013; Luo et al., 2001; Wetmore and Olson, 1995). For example, sensory-derived BDNF can act as a central modulator of pain in various pathological pain states (Mannion et al., 1999; Pezet and McMahon, 2006). Although the transcriptional control of neurotrophin expression remains to be better characterized, it is clear that cellular interaction and activity play critical roles in determining initial sites and levels of expression and fine-tuning expression in response to changing conditions, respectively (Patapoutian et al., 1999; Zheng et al., 2012).

After protein synthesis, neurotrophins are further modulated during secretion and proteolytic processing. Recent evidence suggests that neurotrophins can be sorted into either the regulated or constitutive secretory pathway depending on the specific neurotrophin. In hippocampal neurons, for example, BDNF but not NGF or NT3 is preferentially sorted into the regulated pathway so that its exocytosis is regulated by specific signals (Farhadi et al., 2000; Mowla et al., 1999). This regulated trafficking and secretion of BDNF have been linked to normal brain function, as a single nucleotide polymorphism in the *BDNF* gene that disrupts this process leads to impairments in hippocampal function and hippocampal-based memory in humans (Egan et al., 2003; Hariri et al., 2003). Since neurotrophins are synthesized as proforms, termed proneurotrophins, which were long considered biologically inert, neurotrophin activity is dependent on intracellular and extracellular proteases that mediate proteolytic processing (Pang et al., 2004; Seidah et al., 1996). These proteases and proneurotrophins have recently attracted growing interest, in light of the provocative finding that biologically active proneurotrophins are secreted into some tissues and their intriguing association with pathological conditions such as neurodegenerative disorders (Fahnestock et al., 2001; Lee et al., 2001). p75NTR, the high affinity receptor for proneurotrophins, is thought to mediate their ability to promote apoptosis in many cells (Lee et al., 2001). Thus, signaling consequences of a specific neurotrophin heavily depend on the state of proneurotrophin processing, as proneurotrophin and neurotrophin-mediated signaling for the most part have opposite effects, partly due to the type of receptor that they preferentially activate (Lu et al., 2005).

1.4.2. Functions of neurotrophins during nervous system development

Diverse functions of neurotrophins in both the PNS and the CNS during normal development and pathological conditions have been extensively reviewed previously (Chao, 2003; Huang and Reichardt, 2001; Lu et al., 2005; Pezet and McMahon, 2006). Here the focus will be on roles for neurotrophins as exemplified by NGF in the development of the PNS.

Since the pioneering work of Hamburger, Levi-Montalcini and others demonstrating dependence of sympathetic and sensory neurons on NGF for survival *in vivo* and *in vitro*, great advances have been made towards a complete understanding of neurotrophin requirements for survival of various neuronal

populations due to availability of gene knockout animals for all neurotrophins and their receptors (Levi-Montalcini, 1987; Northcutt, 1989; Snider, 1994).

In general, the neurotrophin and Trk receptor knockout phenotypes are consistent with *in vitro*-established specificity between neurotrophins and Trk receptors, in that select populations of neurons that express a specific Trk receptor generally depend on the neurotrophins that it binds for survival. The simplest scenario is a one-to-one relationship between ligand and receptor as exemplified by the specific interaction between NGF and TrkA. In the absence of either NGF or TrkA, there is a similar degree of neuronal loss in both sympathetic ganglia and sensory ganglia, resulting from increased apoptosis of TrkA-expressing neurons (Crowley et al., 1994; Smeyne et al., 1994). There are also cases where survival deficits in receptor-deficient mice are greater than those in neurotrophin-deficient mice. In one example, it is due to the ability of different neurotrophins to activate the same Trk receptor in different populations as observed in the nodose-petrosal ganglion. In this ganglion, almost all neurons express TrkB and are eliminated by *TrkB* deletion (Silos-Santiago et al., 1997). By contrast, mice deficient in either BDNF or NT4, the preferred ligands for TrkB, exhibit only partial deficits (Brady et al., 1999; Erickson et al., 1996). The phenotypes are additive, because removing both ligands results in a full blown phenotype much like that in *TrkB* mutant mice (Conover et al., 1995). This together with the observation that BDNF and NT4 are expressed in separate target fields suggests the existence of two populations of TrkB neurons in the nodose-petrosal ganglion that depend on BDNF or NT4 alone for survival. The *in vivo* behavior of NT3 is not as well defined as the other neurotrophins. In both DRGs and trigeminal ganglia, *NT3* deficiency eliminates many more neurons than those lost in *TrkC*-deficient animals. This NT3-dependent, TrkC-independent population includes TrkA- and TrkB-expressing neurons, suggesting that NT3 can directly activate the other Trk receptors *in vivo* (Farinas et al., 1998; Huang et al., 1999). This is consistent with the *in vitro* promiscuous binding of NT3 to Trk receptors (Davies et al., 1995; Ip et al., 1993). Furthermore, when BDNF is expressed from the *NT3* locus, neuronal loss due to NT3 ablation can be partially rescued (Coppola et al., 2001). Additionally, NT3 seems to activate TrkA to support the survival of a subset of sympathetic neurons that also require NGF for survival (Francis et al., 1999; Wyatt et al., 1997). For neurons that coexpress different Trk receptors, their neurotrophin requirement for survival *in vivo* is determined by the spatial and temporal pattern of expression of different neurotrophins that act through the receptors being expressed. In

the case of trigeminal mesencephalic neurons that express both TrkB and TrkC, individual neurons require either BDNF or NT3 for survival, depending on which neurotrophin is present in the muscle spindles that they innervate (Fan et al., 2000). For reasons that are yet unclear, unlike neurons in the PNS, CNS neurons that are responsive to neurotrophins both *in vivo* and *in vitro*, such as basal forebrain cholinergic neurons, generally survive in the absence of any single one of neurotrophins *in vivo* (Chen et al., 1997; Crowley et al., 1994; Muller et al., 2012; Smeyne et al., 1994). Thus, unique spatial and temporal patterns of expression of neurotrophins and their receptors determine specific neurotrophin requirements of different neuronal populations for survival.

In addition to their classical prosurvival activity, neurotrophins have recently been shown to play an increasing number of non-survival functions during later stages of neuronal development. The ability of neurotrophins to regulate the differentiation process is particularly well documented. For instance, even before the advent of gene targeting technology, NGF was shown to direct the differentiation of sympathoadrenal precursors into sympathetic neurons as opposed to chromaffin cells both *in vivo* and *in vitro* (Anderson, 1993; Levi-Montalcini, 1987). Moreover, in sensory neurons, postnatal non-survival functions of neurotrophins were revealed by neurotrophin neutralization with function-blocking antibodies after the critical period of neurotrophin-dependent survival (Lewin and Mendell, 1993; Mendell, 1999). Postnatal deficiency of NGF results in impairments of molecular properties specific to NGF-dependent sensory neurons, which are mainly nociceptors, such as expression of CGRP, specific nociceptive-specific sensory receptors and ion channels (Fjell et al., 1999; Tonra and Mendell, 1998). Physiological recordings also revealed a dramatic change in the physiological phenotype of nociceptors. Specifically, thinly myelinated Aδ fibers that are classified as high threshold mechanoreceptors (HTMRs) undergo a change in phenotype to take on the physiological properties of D-hair fibers that are low threshold mechanoreceptors (Ritter et al., 1991). Furthermore, either NGF overexpression in skin using a transgenic mouse strategy or exogenous administration of NGF to neonatal or adult animals alters the molecular and physiological phenotypes of nociceptors in a direction that is generally opposite to that caused by the lack of NGF (Lewin and Mendell, 1993; Pezet and McMahon, 2006).

More recently, the development of a genetic strategy to assess the non-survival function of neurotrophins unmasks additional requirements for neurotrophins during the differentiation of sensory neuronal subtypes. In those experiments, *Bax*, a proapoptotic gene required for trophic factor deprivation-induced neuronal death, is codeleted with individual neurotrophins or Trk receptors to keep sympathetic and sensory neurons alive in the absence of neurotrophins (Deckwerth et al., 1996). Consistent with a central role of NGF in the differentiation of nociceptors, *NGF/Bax* double animals lack the vast majority of molecular characteristics of nociceptors in DRGs. In fact, the classical process of diversification of nociceptors in nonpeptidergic and peptidergic subpopulations is never initiated in these mutant animals (Luo et al., 2007; Patel et al., 2000). These dramatic effects of the loss of NGF in part reflect the ability of NGF to activate expression of nociceptive-specific genes either directly or indirectly. It is known that for a large subset of nonpeptidergic-specific genes, NGF acts through transcriptional upregulation of *Ret*, which is critical for late maturation of nonpeptidergic nociceptors, to indirectly promote their expression (Luo et al., 2007). However, the identity of transcription factors that meditate the profound effect of NGF on gene expression remains largely unknown and has recently become a topic of active research. Likewise, analysis of *NT3/Bax* double mutants has revealed a similar subtype-specific role of NT3 signaling in determining the molecular and morphological phenotype of proprioceptors, which require NT3 for survival (Genc et al., 2004; Patel et al., 2003). Conversely, ectopic expression of *TrkC* in neurons that normally express TrkA confers a proprioceptive phenotype on a subset of neurons that would otherwise develop into nociceptors (Moqrich et al., 2004). Like neurotrophin-regulated survival, the majority, if not all, of the effects of neurotrophins on the differentiation process described thus far, are mediated by their cognate Trk receptors.

Neurotrophins have also been extensively studied as factors to promote axonal and dendritic growth by responsive neurons. Seminal work using compartmentalized cultures provided some of the early evidence for a local action of neurotrophins on distal axons to promote axonal extension (Campenot, 1977). Specifically, application of NGF to an axonal compartment is necessary and sufficient for axons to extend to that compartment. Moreover, the continuous presence of NGF at distal axons is required for maintaining axon growth and preventing axon degeneration. The potent effect of neurotrophins on axon growth has also been demonstrated by a series of studies that characterized changes in innervation patterns of sensory and sympathetic neurons in response to elevation of neurotrophins either systemically or locally (Edwards et al.,

1989; Guidry et al., 1998; Levi-Montalcini, 1987; Ringstedt et al., 1999; Stucky et al., 1999). In general, elevated expression of neurotrophins in a specific region results in increased innervation by neurons that normally innervate that region and sometimes it even leads to ectopic innervation by responsive neurons that do not normally project there. These findings were complemented by gene knockout studies, where, as described above, survival requirement for neurotrophins is bypassed through codeletion of *Bax*. Although the initial axon extension appears largely intact, *NGF/Bax* mutant animals display severe deficits in final target innervation by sensory and sympathetic neurons in the periphery (Glebova and Ginty, 2004; Kuruvilla et al., 2004; Patel et al., 2000; Wickramasinghe et al., 2008). Similarly, in *NT3/Bax* mutant animals, proprioceptive axon projections to both central and peripheral targets are defective (Genc et al., 2004; Patel et al., 2003). At least three different classes of transcription factors have been identified to mediate neurotrophin-dependent axon outgrowth, namely, cyclic AMP responsive element-binding protein (CREB), serum response factor (SRF) and nuclear factor of activated T-cells (NFAT) (Graef et al., 2003; Lonze et al., 2002; Riccio et al., 1999; Wickramasinghe et al., 2008). The specific transcriptional program dependent on each factor and the mechanism by which these factors cooperate to mediate the profound effect of neurotrophins on axon outgrowth remain to be elucidated. The role of neurotrophins in regulating dendritic growth has also been appreciated. Some of the best examples include NGF-dependent dendritic arborization of sympathetic neurons and spatially distinct effects of BDNF on dendritic arborization of retinal ganglion cells (Lom et al., 2002; Voyvodic, 1989). Taken together, neurotrophins robustly regulate axonal and dendritic behaviors through activation of local signaling at nerve terminals and initiation of proper transcriptional responses in the cell soma.

A large body of evidence has also shown that neurotrophins regulate synapse formation and influence synaptic strength and plasticity. In postganglionic sympathetic neurons, for example, retrograde NGF-TrkA signaling is required for development of postsynaptic specializations with preganglionic sympathetic neurons. Remarkably, in compartmentalized cultures, TrkA endosomes that are formed at distal axons can be detected in dendrites, where they may function as specialized machinery essential for NGF-dependent assembly of postsynaptic densities, which is readily reversible and largely independent of protein synthesis (Sharma et al., 2010). Compared to NGF, NT3 and BDNF appear to regulate synaptic function more broadly. At the synapses formed between Ia afferents and motor neurons, endogenous BDNF controls the

relative contribution of monosynaptic and polysynaptic input onto motor neurons (Seebach et al., 1999). Acute NT3 application during a critical postnatal time window potentiates the monosynaptic strength between Ia afferents and motor neurons in a long-lasting fashion (Arvanov et al., 2000). Neurotrophins such as BDNF, NT3 and NT4 have also been shown to acutely stimulate neurotransmitter release at the CA1 synapse in hippocampal cultures and slices as well as Xenopus neuromuscular synapses (Kang and Schuman, 1996; Wang and Poo, 1997). These *in vitro* effects are likely specific and physiologically relevant, because mice lacking BDNF or NT4 have defects in long-lasting long-term potentiation (LTP) in the hippocampus, a form of synaptic plasticity thought to underlie formation of long-term memory (Korte et al., 1995; Korte et al., 1998; Patterson et al., 1996; Xie et al., 2000). Further analysis revealed that BDNF primarily acts presynaptically through TrkB to facilitate LTP. Consistently, both *BDNF* and *TrkB* mutants show deficits in memory acquisition and consolidation in many learning paradigms (Gorski et al., 2003; Linnarsson et al., 1997; Liu et al., 2004; Minichiello et al., 1999; Mizuno et al., 2000). Interestingly, recent data suggest that proBDNF-p75NTR signaling enhances hippocampal long term depression (LTD) (Woo et al., 2005). Thus, neurotrophins especially BDNF almost certainly regulate a wide range of higher order brain functions which have just begun to be unveiled.

1.4.3. Neurotrophin-mediated signaling pathways

Although the intracellular signaling pathway that links receptor activation to a specific neurotrophin-induced functional consequence remains incompletely characterized, activation of different downstream signaling pathways following ligand engagement of receptors especially the Trk receptor almost certainly contributes to diverse neuronal responses to neurotrophins. Trk receptors have historically been the focus of mechanistic studies of neurotrophin-regulated singling pathways, since they mediate the majority of neurotrophin function *in vivo* and *in vitro*.

Trk receptors, like other receptor tyrosine kinases, are activated by neurotrophin-mediated dimerization and transphosphorylation of activation loop tyrosines (Huang and Reichardt, 2003). Subsequent to activation of Trk tyrosine kinase activity by phosphorylation of activation loop tyrosines, additional tyrosines outside the kinase activation loop in the cytoplasmic domain are phosphorylated to create docking sites for proteins containing phosphotyrosine-binding (PTB) or Src-homology-2 (SH2) domains, which differentially engage

downstream signaling cascades. The major effector pathways activated by Trk receptors are mitogen-activated protein (MAP) kinase cascades, phosphatidyl inositol-3 (PI3)-kinase-Akt and phospholipase C γ (PLC-γ) pathways (Huang and Reichardt, 2003). These pathways are coupled to phosphorylation of two tyrosine sites, Y490 and/or Y785, outside the activation loop, via specific adaptor proteins. Phospho-Y490 serves as a recruitment site for adaptor proteins such as Shc and Frs2, which provide links to MAP kinases, PI3-kinase and other pathways. Phospho-Y785 on the other hand directly recruits the enzyme PLC-γ1 for phosphorylation-dependent activation by Trk receptors (Obermeier et al., 1993b).

MAP kinase cascades, especially the extracellular signal-regulated kinase 1 and 2 (Erk1/2)-mediated pathway, are best known for their roles in normal neuronal differentiation and axon growth (Newbern et al., 2011; Zhong et al., 2007). Broadly speaking, there are two ways to activate these cascades, depending on the duration of activated signaling cascades. Transient activation of MAP kinase signaling is predominately mediated by a signaling cascade initiated by activation of Ras. One of the best characterized adaptors that mediate Ras activation is Shc, which as described earlier, is recruited to phospho-Y490 on Trk receptors (Obermeier et al., 1993b). Subsequent phosphorylation of Shc by Trk receptors creates a binding site for the adaptor Grb2, which is constitutively associated with the Ras exchange factor son of sevenless (SOS) (Grewal et al., 1999). Active Ras then stimulates Erk1/2-mediated signaling through sequential activation of a series of protein kinases, Raf, Mek1/Mek2, which ultimately phosphorylate and activate Erk1/Erk2 (English et al., 1999). Erk5 is activated by Ras through an analogous protein kinase cascade involving Wnk1, MEKK2 and Mek5 (Wang et al., 2005; Xu et al., 2004). The MAP kinase cascades negatively regulate their own activity through phosphorylation of upstream components and activation of phosphatases and are therefore generally transient. Prolonged activation of MAP kinases requires the recruitment of a different adaptor, Frs2, to phospho-Y490 on Trk receptors (Meakin et al., 1999). Similar to Shc, Frs2 is phosphorylated by Trk receptors and phosphorylated Frs2 subsequently recruits adaptor proteins such as Crk and various enzymes (Meakin et al., 1999). These intermediates synergistically promote sustained activation of MAP kinases. For example, Crk is known to bind and activate the Rap1 exchange factor C3G, and hence Rap1 which stimulates B-Raf, an upstream activator of the Erk kinase cascade (York et al., 1998). Based on experiments with PC12 cells that show proliferation and differentiation responses associated with EGF and NGF respectively, sustained but not transient activation

of Erk is associated with and sufficient for a differentiation response suggesting that the duration of Erk activation determines cellular responses to growth factors such as neurotrophins that activate the Erk kinase cascade (Marshall, 1995).

The PI3-K-Akt pathway on the other hand mediates the neurotrophin-dependent survival response. PI3-K can be activated through Ras-dependent and -independent pathways (Reichardt, 2006; Vaillant et al., 1999). For the independent pathway, the recruitment and activation of PI3-K is mediated by Trk receptor-associated adaptor proteins Shc-Grb2 or insulin receptor substrate-1 (IRS1) (Holgado-Madruga et al., 1997; Yamada et al., 1997). In the former case, recruitment of Gab1 by the Shc-Grb2 complex is essential for subsequent PI3-K activation (Holgado-Madruga et al., 1997). PI3-K through production of P3-phosphorylated phosphoinositides activates the protein kinase Akt, thereby promoting cell survival. Akt elicits a survival response by controlling the activity of both prosurvival and proapoptotic proteins through phosphorylation (Brunet et al., 2001; Yuan et al., 2003). For instance, BAD, a proapoptotic Bcl-2 family member, is inactivated by Akt phosphorylation, since the phosphorylated form is sequestered by 14-3-3 proteins (Datta et al., 1997). Conversely, Akt phosphorylation of IκB results in disinhibition of the transcription factor NF-κB, which is known to activate a prosurvival transcriptional program (Datta et al., 1999). Interestingly, a recent study of retrograde neurotrophin signaling in compartmentalized sympathetic neuronal cultures not only demonstrated a critical dependence of survival supported by retrogradely transported NGF on PI3-K, but also identified a novel requirement for PI3-K in distal axons during retrograde NGF signaling. Since inhibition of PI3-K activity in distal axons but not in proximal axons attenuates retrograde transport of NGF initiated in distal axons, PI3-K activity is necessary for initiation, but not propagation, of retrograde transport of NGF in sympathetic neurons (Kuruvilla et al., 2000). A role for PI3-K in Trk receptor-mediated endocytosis has recently been proposed, along with the possibility that it is mediated by P3-phosphorylated phosphoinositide-dependent regulation of several proteins implicated in endocytosis (Harrington and Ginty, 2013). Further studies are needed to directly address that possibility. Thus, both canonical PI3-K-Akt signaling in cell bodies and novel PI3-K signaling in distal axons contribute to survival of neurons supported by NGF acting exclusively on distal axons.

PLC-γ1, as described earlier, is recruited to phospho-Y785 on Trk receptors via its Src homology domains (Obermeier et al., 1993a; Obermeier et al., 1993b). Trk-mediated phosphorylation activates its lipase activity, resulting in generation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP2). IP3 and DAG are important signaling molecules that stimulate mobilization of Ca^{2+} stores and activation of Ca^{2+} and DAG-regulated isoforms of protein kinase C, respectively. Since Ca^{2+} and PKC exert widespread effects on many proteins and enzymes, the PLC-γ pathway controls expression and/or activity of many proteins, including many intracellular enzymes, ion channels and transcription factors (Klein et al., 2005; Minichiello et al., 2002; Toledo-Aral et al., 1995). Compared with the other two signaling pathways that are associated with phosphorylation of the Shc site on Trk receptors, the relative importance of the PLC-γ and PLC-γ binding sites in neurotrophin-dependent neuronal responses is less well defined and deserves further investigation. Interestingly, recent studies of mice carrying point mutations on specific docking sites of TrkB receptors suggest a link between the PLC-γ site downstream of TrkB and learning-associated synaptic changes and LTP in the hippocampus (Gruart et al., 2007; Minichiello et al., 2002).

As mentioned earlier, p75NTR binds proneurotrophins and mature neurotrophins with differential affinity. Proneurotrophins bind with high affinity to a complex of p75NTR and Sortilin, a Vps10-domain containing protein, where both constituents directly participate in binding (Nykjaer et al., 2004; Teng et al., 2005). Neurotrophins, on the other hand, bind p75NTR with much lower affinity (Rodriguez-Tebar et al., 1990). Activation of p75NTR by ligand engagement is known to trigger opposite cellular responses. For example, p75NTR has been shown to promote survival or apoptosis following neurotrophin engagement in a context-dependent manner (Roux and Barker, 2002). p75NTR-induced apoptosis is primarily mediated by the Jun kinase-signaling cascade (Aloyz et al., 1998; Casaccia-Bonnefil et al., 1996). Neurotrophin signaling through this pathway promotes apoptosis by activation of p53 and induction of the extrinsic apoptotic pathway (Aloyz et al., 1998; Le-Niculescu et al., 1999). Several intermediate components of this p75 NTR apoptotic pathway have been identified. Neurotrophin receptor-interacting factor (NRIF) is one of the key mediators sitting at the convergence of multiple regulatory pathways (Linggi et al., 2005). In particular, its nuclear translocation, a prerequisite for p75NTR-mediated apoptosis, is regulated by Traf6-mediated ubiquitination and γ-secretase-mediated release of the intracellular domain of p75NTR (Geetha et al., 2005;

Kenchappa et al., 2006). Induction of NF-κB-mediated prosurvival signaling is required for the survival response as a result of neurotrophin engagement of p75NTR (Hamanoue et al., 1999). NF-κB is effectively activated following p75NTR activation due to the formation of a complex of Traf6, interleukin-1 receptor-associated kinase (IRAK), atypical protein kinase C-ι (aPKC-ι) and the aPKC-interacting protein p62 with p75NTR (Wooten et al., 2001). Although the mechanism by which activation of p75NTR exerts these dichotomous biological actions remains unclear, the specificity of cellular responses in part results from the specific form of neurotrophins and the type of coreceptors that p75NTR associates with.

Considering that neurotrophin signaling through Trk receptors and p75NTR frequently results in opposing biological consequences, a challenging and important question in the field is to understand how these two types of receptors convert a specific neurotrophin signal to a unified cellular response that is appropriate for the cellular context (Patel et al., 2000; Teng et al., 2005; Woo et al., 2005; Zakharenko et al., 2003). Importantly, emerging evidence has supported that the proapoptotic activity of p75NTR is suppressed by Trk-mediated signaling in most cases, while in other cases, the presence of p75NTR potentiates the efficacy of Trk signaling (Curtis et al., 1995; Esposito et al., 2001; Yoon et al., 1998).

Chapter 2. Extrinsic and intrinsic factors coordinate the development of nociceptive subtypes by converging on Runx1/CBFβ.

2.1. Segregation and development of nonpeptidergic and peptidergic nociceptors

Nociceptors are a heterogeneous neuronal population that can be subdivided by a wide array of cytochemical, anatomical and physiological criteria (Gold and Gebhart, 2010). Most commonly, they are classified into nonpeptidergic and peptidergic nociceptors primarily based on their neuropeptide profile. While peptidergic nociceptors are defined by expression of neuropeptides, such as CGRP and substance P, nonpeptidergic nociceptors, at least the majority of them, are conventionally labeled by the lectin IB4 (Mulderry et al., 1988; Silverman and Kruger, 1990). These two molecularly distinct subtypes are also segregated by their central and peripheral projections. Centrally, nonpeptidergic afferents occupy a deeper lamina, inner lamina II, than their peptidergic counterparts that terminate in lamina I and outer lamina II (Molliver et al., 1995; Zylka et al., 2005). Peripherally, unlike peptidergic nociceptors, which have a substantial visceral component, nonpeptidergic nociceptors almost exclusively innervate cutaneous structures (Bennett et al., 1996b; Raybould et al., 1992). Even within the same target, such as the epidermis, axonal terminals from nonpeptidergic and peptidergic populations are targeted to different stratums (Zylka et al., 2005). The existence of these spatially segregated nociceptive endings suggests functional distinctions between the nonpeptidergic and peptidergic populations which were unequivocally shown by various cell ablation studies (Cavanaugh et al., 2009; McCoy et al., 2013; Mishra and Hoon, 2010; Vulchanova et al., 2001). Regardless of the specific strategy for cell ablation, what has emerged from that series of analyses is a selective requirement for nonpeptidergic and peptidergic nociceptors during mechanical and thermal nociception, respectively (Cavanaugh et al., 2009; McCoy et al., 2013; Mishra and Hoon, 2010; Vulchanova et al., 2001).

Despite their remarkable differences, nonpeptidergic and peptidergic nociceptors derive from the same TrkA-expressing precursors, and therefore depend on NGF-TrkA signaling for survival (Ruit et al., 1992; Silos-Santiago et al., 1995). In contrast to peptidergic nociceptors that continue expressing TrkA into maturity, nonpeptidergic nociceptors gradually extinguish *TrkA* expression during the first three weeks of

postnatal development, and instead express Ret and GFRαs, receptor components for GDNF signaling during late embryonic and postnatal development, resulting in a switch from NGF to GDNF for trophic support (Bennett et al., 1996a; Bennett et al., 1998; Molliver and Snider, 1997; Molliver et al., 1997). Several lines of evidence have convincingly shown that NGF-TrkA and GDNF-Ret signaling are both required for normal expression of the nonpeptidergic phenotype (Luo et al., 2007; Patel et al., 2000). Note that in order to study survival-independent functions of NGF, nociceptors are kept alive in the absence of NGF by codeletion of a proapoptotic gene *Bax* (Patel et al., 2000). Hereafter *NGF/Bax* double mutants will be referred to as NGF mutants, unless indicated otherwise. Consistent with the sequential expression of *TrkA* and *Ret*, NGF signaling is required for acquisition of almost all nonpeptidergic-specific features, including expression of *Ret* and *GFRα*s, while Ret signaling plays a critical role in postnatal maturation of nonpeptidergic nociceptors, such as expression of a subset of genes characteristic of a more mature state of nonpeptidergic nociceptors (Luo et al., 2007; Patel et al., 2000). However, little is known about the mechanisms by which these two growth factor signaling pathways support the characteristic nonpeptidergic gene program.

Runx1, a Runx family transcription factor that is heavily studied as a lineage regulator in the hematopoietic system, introduced above, has taken center stage in the field of neuroscience in recent years due to its essential role in establishment of the nonpeptidergic identity. Most notably, genetic removal of *Runx1* from the neural crest lineage or all but the hematopoietic lineage leads to a selective defect in the molecular identity and axonal projection of nonpeptidergic nociceptors and a concomitant expansion of peptidergic-like phenotypes (Chen et al., 2006b; Kramer et al., 2006; Yoshikawa et al., 2007). The effect of *Runx1* deficiency on nonpeptidergic-specific gene expression closely resembles that of *NGF* deletion, in that expression of almost all the nonpeptidergic-specific genes, including those previously defined as Ret-independent is disrupted in Runx1-deficient DRGs, as seen in *NGF* mutants (Chen et al., 2006b; Luo et al., 2007). Therefore, Runx1 normally functions as a master regulator of the nonpeptidergic lineage by directly or indirectly driving the nonpeptidergic-specific transcriptional program and suppressing the alternative differentiation program, analogous to its well-established actions in the hematopoietic lineage (de Bruijn and Speck, 2004).

The fact that both Runx1 and NGF are required for specification of nonpeptidergic nociceptors suggests a potential interaction between them, a possibility that was first articulated and explored by Luo and colleagues. Those authors identified a role for NGF in maintaining *Runx1* expression at the mRNA level (Luo et al., 2007). Although this regulatory event is undoubtedly functionally relevant, the discrepancy between the dramatically disrupted nonpeptidergic development and the relative weak and late *Runx1* mRNA deficit implies the existence of additional modes of interaction between Runx1 and NGF during the development of nonpeptidergic nociceptors. Anecdotally, it was recently shown that the ability of overexpressed Runx1 to activate a subset of nonpeptidergic-specific genes in a heterologous system required the presence of NGF, further supporting the notion that NGF facilitates Runx1 function in ways other than transcriptional regulation of *Runx1* (Lopes et al., 2012). Since an interplay between NGF and Runx1 appears central to normal specification of nonpeptidergic nociceptors, we sought to elucidate the mechanism by which NGF and Runx1 intersect in the hope of defining common principles for the general process of neuronal subtype specification.

2.2. Runx1 mediates NGF-dependent expression of a great majority of nonpeptidergic nociceptor-specific genes.

2.2.1. Nearly all Runx1-dependent genes are also NGF-dependent *in vivo.*

To assess the extent of genetic interaction between NGF and Runx1, beyond the phenocopy between *NGF* and *Runx1* mutants with respect to nonpeptidergic phenotypes which was based on analysis done at different time points and in different labs, we first compared expression patterns of nonpeptidergic-specific genes in DRGs between *NGF* mutants and previously described neural crest derivative-specific *Runx1* conditional knockouts, *Wnt1-Cre;Runx1*$^{f/f}$ (*Runx1 CKO*) mice, side by side at the same developmental stages by in situ hybridization analysis (Chen et al., 2006b). Since *NGF* mutants die at birth, our expression analysis was conducted earlier and at P0. Nonpeptidergic-specific genes that are Ret-dependent according to earlier work, e.g. *MrgA1, MrgA3,* were excluded from the analysis, for the interpretation of potential phenocopy for those genes would be confounded by the *Ret* expression deficit previously described in both *NGF* and *Runx1* mutants (Chen et al., 2006b; Luo et al., 2007; Patel et al., 2000). To determine the extent to which nonpeptidergic-specific genes require NGF and/or Runx1, a few genes that were identified through a microarray screen for differentially expressed genes in E16.5 control and *Runx1CKO* DRGs were also examined (Table 1). *Myo1a*, one of the class I myosins, *Kif21b*, a member of the kinesin superfamily and *Ptprt,* a member of the protein tyrosine phosphatase (PTP) family were selected mainly due to their robust dependence on Runx1. Strikingly, although each gene has its own unique expression pattern, the nonpeptidergic-specific genes that we examined except *Ret* (discussed in Chapter 2.3), including canonical markers of nonpeptidergic nociceptors, *MrgD* and *GFRα2* and novel Runx1-dependent genes, *Ptprt*, *Myo1a* and *Kif21b*, showed marked deficits in expression that were comparable in *NGF* and *Runx1* mutant DRGs at P0 (Figure 2.1). In the case of *GFRα2*, *Myo1a* and *Kif21b*, the neurons that retained expression in both mutants were not nociceptors (data not shown). Similar results were observed at E16.5 (Figure 2.2), which is when the early wave of nonpeptidergic-specific genes, including *MrgD*, *Ptprt*, *Myo1a* and *Kif21b* normally start to be expressed, suggesting that NGF and Runx1 are both required for expression of the majority of nonpeptidergic-specific genes prior to their normal onset of expression.

2.2.2. NGF activates transcription of nonpeptidergic-specific genes in a Runx1-dependent way *in vitro*.

To exclude the possibility that the observed expression deficit *in vivo* was secondary to defects in target innervation that had been previously reported for both *NGF* and *Runx1* mutants (Chen et al., 2006b; Patel et al., 2000; Yoshikawa et al., 2007), dissociated DRG neurons from P0 control and *Runx1 CKO* animals were cultured in the presence or absence of NGF and the level of expression of select subtype-specific genes was measured by real-time PCR analysis. For a subset of nonpeptidergic-specific genes, i.e. *MrgD*, *GFRα2* and *Ptprt*, NGF application robustly induced their expression in wildtype neurons (Figure 2.3 A-C). However, the same treatment failed to increase their expression in Runx1-deficient neurons (Figure 2.3 A-C). This Runx1 dependence was specific to nonpeptidergic-specific genes, since NGF promoted expression of *CGRP*, a peptidergic marker, irrespective of Runx1 (Figure 2.3 D). Therefore, rather than being generally required for NGF signaling, Runx1 most likely acts downstream of NGF to elicit nonpeptidergic-specific transcriptional responses.

Table 1. Microarray analysis of genes that were differentially expressed in E16.5 DRGs of control and *Runx1 CKO* animals. Only genes with a fold change greater than or equal to 1.5, and a p-value less than or equal to 0.05 are listed. Genes that exhibited reduced or increased expression in *Runx1 CKO* DRGs relative to control are shown in table 1.1 and 1.2, respectively.

Table 1.1

Probe Set ID	Gene Symbol	Gene Title	Fold Change	P-value
1443392_at	Trpv1	transient receptor potential cation channel, subfamily V, member 1	3.855363	0.000364
1418723_at	Lpar3	lysophosphatidic acid receptor 3	3.150196	0.017528
1424633_at	Camk1g	calcium/calmodulin-dependent protein kinase I gamma	2.943805	0.003527
1448459_at	Kcnip1	Kv channel-interacting protein 1	2.265259	0.03317
1443959_at	Tmem72	transmembrane protein 72	2.163699	0.003249
1438112_at	9430021M05Rik	RIKEN cDNA 9430021M05 gene	2.14351	0.007824
1436100_at	Sh2d5	SH2 domain containing 5	2.11848	0.03425
1441363_at	Frmpd3	FERM and PDZ domain containing 3	2.078189	0.039236
1435772_at	Kif21b	kinesin family member 21B	2.015971	0.000204
1416456_a_at	Chia	chitinase, acidic	2.005046	0.028087
1416785_at	Kcnip1	Kv channel-interacting protein 1	1.996721	0.002743
1419654_at	Tle3	transducin-like enhancer of split 3, homolog of Drosophila E(spl)	1.934638	0.049115
1428074_at	Tmem158	transmembrane protein 158	1.902171	0.041199
1420564_at	Insrr	insulin receptor-related receptor	1.868479	0.009468
1452263_at	Slc35f4	solute carrier family 35, member F4	1.823904	0.016667

1451062_a_at	Pex5l	peroxisomal biogenesis factor 5-like	1.819641	0.029577
1434635_at	Rph3a	rabphilin 3A	1.803495	0.036091
1455000_at	Gpr68	G protein-coupled receptor 68	1.779183	0.033036
1450224_at	Col4a3	collagen, type IV, alpha 3	1.762817	0.021838
1438160_x_at	Slco4a1	solute carrier organic anion transporter family, member 4a1	1.745016	0.043898
1425483_at	LOC100044677 /// Tox	similar to thymus high mobility group box protein TOX /// thymocyte selection-as	1.744519	0.026119
1419655_at	Tle3	transducin-like enhancer of split 3, homolog of Drosophila E(spl)	1.716283	0.026226
1429805_at	Myo1a	myosin IA	1.714405	0.000569
1433988_s_at	C230098O21Rik	RIKEN cDNA C230098O21 gene	1.707104	0.044154
1440056_at	---	---	1.672219	0.001931
1424923_at	Serpina3g	serine (or cysteine) peptidase inhibitor, clade A, member 3G	1.65806	0.002164
1421037_at	Npas2	neuronal PAS domain protein 2	1.654117	0.027774
1445941_at	---	---	1.650902	0.02853
1417542_at	Rps6ka2	ribosomal protein S6 kinase, polypeptide 2	1.646436	0.020774
1422710_a_at	Cacna1h	calcium channel, voltage-dependent, T type, alpha 1H subunit	1.629623	0.024766
1436013_at	Gsg1l	GSG1-like	1.602431	0.002997
1417392_a_at	Slc7a7	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	1.598559	0.048681
1453801_at	Them5	thioesterase superfamily member 5	1.595386	0.021385

1431852_at	A730035I17Rik	RIKEN cDNA A730035I17 gene	1.584555	0.000854
1441298_at	---	---	1.565934	0.039893
1450174_at	Ptprt	protein tyrosine phosphatase, receptor type, T	1.557751	0.027241
1430159_at	5830408C22Rik	RIKEN cDNA 5830408C22 gene	1.520074	0.039318
1456047_at	LOC433466 /// Pla2g4b	phospholipase A2, group IVB (cytosolic)	1.517543	0.049284
1438055_at	Rarres1	retinoic acid receptor responder (tazarotene induced) 1	1.511225	0.02733
1457128_at	AL024213	expressed sequence AL024213	1.508931	0.040555

Table 1.2

Probe Set ID	**Gene Symbol**	**Gene Title**	**Fold Change**	**P -Value**
1455931_at	Chrna3	cholinergic receptor, nicotinic, alpha polypeptide 3	4.589469	0.030475
1417256_at	Mmp13	matrix metallopeptidase 13	3.542615	0.019814
1433607_at	Cbln4	cerebellin 4 precursor protein	3.482604	0.011625
1435424_x_at	---	---	3.416324	0.001092
1433551_at	Vat1l	**vesicle amine transport protein 1 homolog-like (T. californica)**	3.278425	0.018548
1440484_at	Unc5d	unc-5 homolog D (C. elegans)	3.169972	0.002508
1441329_at	Galr1	galanin receptor 1	3.067817	0.000843
1452010_at	Chrna3	cholinergic receptor, nicotinic, alpha polypeptide 3	2.952662	0.000112

1451263_a_at	Fabp4	fatty acid binding protein 4, adipocyte	2.837796	0.035
1417023_a_at	Fabp4	fatty acid binding protein 4, adipocyte	2.716484	0.034395
1439272_at	Lcorl /// LOC100046011	ligand dependent nuclear receptor corepressor-like /// hypothetical protein LOC1	2.610205	0.04198
1416468_at	Aldh1a1	aldehyde dehydrogenase family 1, subfamily A1	2.508498	0.046711
1437695_at	Prokr2	prokineticin receptor 2	2.473296	0.045341
1443365_at	Htr4	5 hydroxytryptamine (serotonin) receptor 4	2.377891	0.043301
1430107_at	Acbd7	acyl-Coenzyme A binding domain containing 7	2.213863	0.006569
1417680_at	Kcna5	potassium voltage-gated channel, shaker-related subfamily, member 5	2.065996	0.033749
1439887_at	---	---	2.048765	0.007273
1449254_at	Spp1	secreted phosphoprotein 1	2.038316	0.044844
1418304_at	Pcdh21	protocadherin 21	2.020208	0.011017
1457008_at	Chrnb4	cholinergic receptor, nicotinic, beta polypeptide 4	1.957697	0.016487
1419756_at	Dgkg	diacylglycerol kinase, gamma	1.914247	0.039183
1436761_s_at	1200015N20Rik	RIKEN cDNA 1200015N20 gene	1.891189	0.01325
1456233_at	---	---	1.871748	0.026841
1440531_at	Rbm11	RNA binding motif protein 11	1.870702	0.045852
1456609_at	Camk2n1	calcium/calmodulin-dependent protein	1.868836	0.002939

		kinase II inhibitor 1		
1443322_at	---	---	1.868507	0.018301
1437883_s_at	---	---	1.84781	0.047311
1452004_at	Calca	calcitonin/calcitonin-related polypeptide, alpha	1.846593	0.034994
1442379_at	EG574403	predicted gene, EG574403	1.834135	0.049862
1436444_at	6030405A18Rik	RIKEN cDNA 6030405A18 gene	1.819695	0.01243
1436493_at	BB181834	expressed sequence BB181834	1.814729	0.001058
1437262_x_at	Bcas2	breast carcinoma amplified sequence 2	1.813717	0.043032
1424679_at	Mab21l1	mab-21-like 1 (C. elegans)	1.8063	0.012424
1438698_at	Tmem132c	transmembrane protein 132C	1.80257	0.012791
1426222_s_at	Vwa5a	von Willebrand factor A domain containing 5A	1.799035	0.008401
1434150_a_at	Higd1c /// Mettl7a1 /// Mettl7a2	HIG1 domain family, member 1C /// methyltransferase like 7A1 /// methyltransferase	1.793921	0.0031
1456397_at	Cdh4	cadherin 4	1.789226	0.006951
1437781_at	Insm2	insulinoma-associated 2	1.785904	0.043102
1445247_at	C530044C16Rik	RIKEN cDNA C530044C16 gene	1.77605	0.008473
1460244_at	Upb1	ureidopropionase, beta	1.764131	0.005432
1451033_a_at	Trpc4	transient receptor potential cation channel, subfamily C, member 4	1.75628	0.038596
1449251_at	Ndp	Norrie disease (pseudoglioma) (human)	1.749063	0.020795
1460033_at	C030002C11Rik	RIKEN cDNA C030002C11 gene	1.73966	0.035207
1437800_at	Edaradd	EDAR (ectodysplasin-A receptor)-	1.738899	0.008914

		associated death domain		
1423571_at	S1pr1	sphingosine-1-phosphate receptor 1	1.718975	0.040307
1423016_a_at	Gypa	glycophorin A	1.709037	0.048106
1429123_at	Rab27a	RAB27A, member RAS oncogene family	1.70457	0.022827
1434297_at	E130304F04Rik /// LOC100040601	RIKEN cDNA E130304F04 gene /// hypothetical protein LOC100040601	1.69251	0.003604
1448421_s_at	Aspn	asporin	1.656201	0.028697
1419599_s_at	Ms4a6d	membrane-spanning 4-domains, subfamily A, member 6D	1.654013	0.043855
1445838_at	---	---	1.646932	0.046888
1449033_at	Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	1.644202	0.034106
1421854_at	Fgl2	fibrinogen-like protein 2	1.640142	0.000127
1427313_at	Ptgir	prostaglandin I receptor (IP)	1.636612	0.018072
1437989_at	Pde8b	phosphodiesterase 8B	1.624623	2.04E-05
1426937_at	6330406I15Rik	RIKEN cDNA 6330406I15 gene	1.601092	0.049248
1418454_at	Mfap5	microfibrillar associated protein 5	1.593589	0.021051
1442082_at	C3ar1	complement component 3a receptor 1	1.57566	0.042136
1418511_at	Dpt	dermatopontin	1.573438	0.026282
1429197_s_at	Rabgap1l	RAB GTPase activating protein 1-like	1.557097	0.020342
1419468_at	Clec14a	C-type lectin domain family 14, member a	1.548326	0.012029
1417012_at	Sdc2	syndecan 2	1.538801	0.032822
1435616_at	Cyp20a1	cytochrome P450, family 20, subfamily A, polypeptide 1	1.534345	0.048825

1457137_at	---	---	1.531957	0.04647
1424229_at	Dyrk3	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	1.524185	0.037066
1426514_at	4631426J05Rik	RIKEN cDNA 4631426J05 gene	1.50781	0.047725
1427319_at	A230046K03Rik	RIKEN cDNA A230046K03 gene	1.506691	0.036402
1445767_at	Ptprd	protein tyrosine phosphatase, receptor type, D	1.501627	0.001025

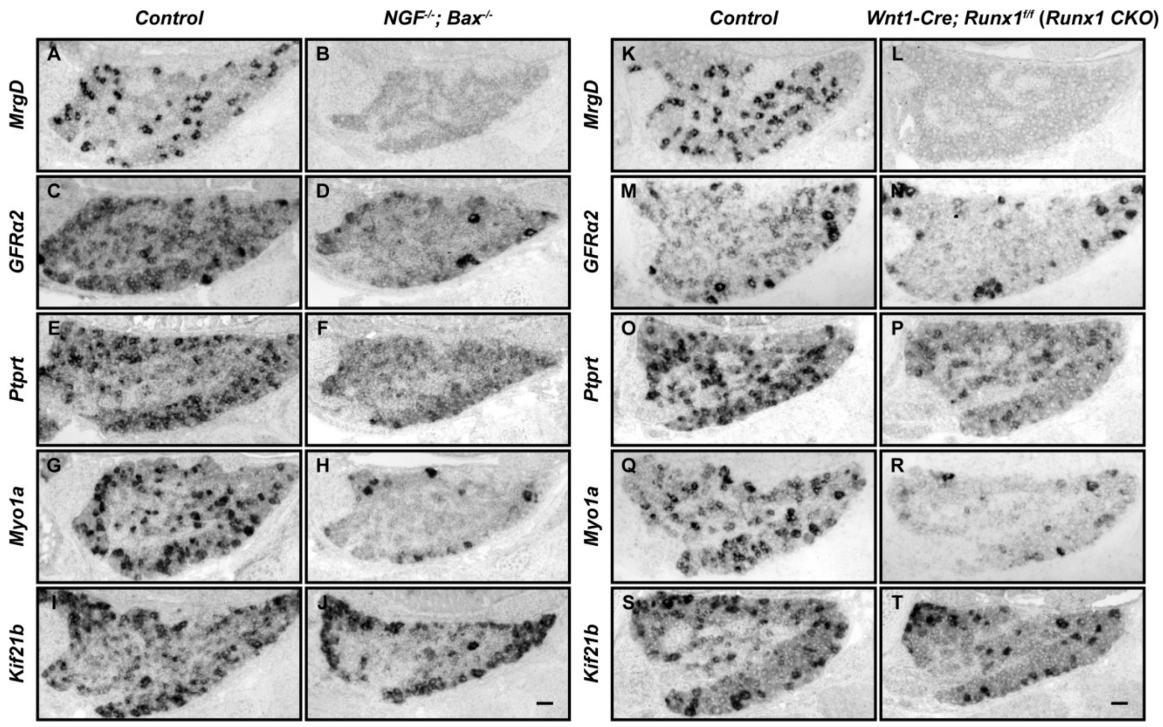


Figure 2.1 Nonpeptidergic-specific genes depend on both NGF and Runx1 for expression *in vivo.*

(**A-J**) In situ hybridization analysis of expression of *MrgD* (Control, 12.7%±2.4%; *NGF*$^{-/-}$*Bax*$^{-/-}$, 0%), *GFRα2* (Control, 28.7%±2.5%; *NGF*$^{-/-}$*Bax*$^{-/-}$, 14.3%±1.1%), *Ptprt* (Control, 19.1%±0.3%; *NGF*$^{-/-}$*Bax*$^{-/-}$, 10.3%±2.1%), *Myo1a* (Control, 26.4%±0.9%; *NGF*$^{-/-}$*Bax*$^{-/-}$, 5.8%±1.5%), and *Kif21b* (Control, 27.2%±2.7%; *NGF*$^{-/-}$*Bax*$^{-/-}$, 14.05%±1.8%) in DRGs of P0 control and *NGF*$^{-/-}$*Bax*$^{-/-}$ animals. (**K-T**) In situ hybridization analysis of expression of *MrgD* (Control, 26.3%±1.0%; *Runx1 CKO*, 0.3%±0.2%), *GFRα2* (Control, 40.0%±3.7%; *Runx1 CKO*, 9.6%±0.7%), *Ptprt* (Control, 36.0%±3.3%; *Runx1 CKO*, 10.4%±2.3%), *Myo1a* (Control, 31.1%±2.3%; *Runx1 CKO*, 5.1%±1.8%), and *Kif21b* (Control, 24.8%±1.7%; *Runx1 CKO*, 10.9%±0.8%) in DRGs of P0 control and *Runx1 CKO* animals. Note that expression of all five genes is severely impaired in DRGs of both *NGF* and *Runx1* mutants. Shown is average±SEM for the percentage of DRG neurons expressing indicated genes based on counts from a total of at least 9 sections from three independent animals per genotype. The total number of neurons per section was counted based on combined NeuN immunostaining, which was not shown. Scale bar, 50μm.

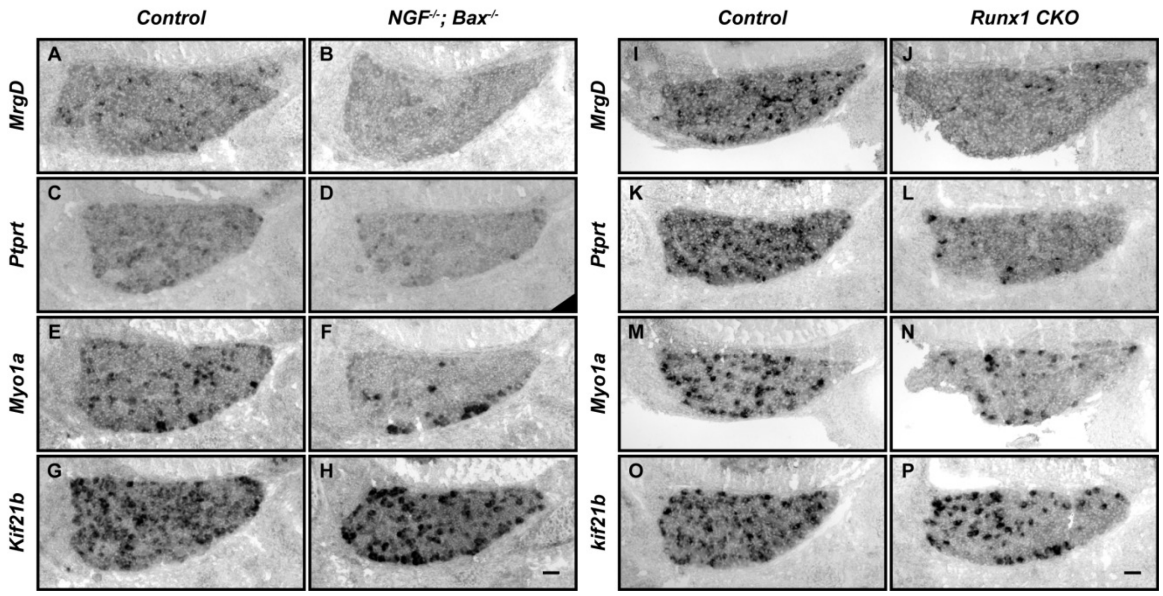


Figure 2.2 Nonpeptidergic-specific genes depend on both NGF and Runx1 for initiation of expression *in vivo.*

(**A-H**) In situ hybridization analysis of expression of *MrgD*, *Ptprt*, *Myo1a* and *Kif21b* in DRGs of E16.5 control and *NGF* mutant animals. (**I-P**) In situ hybridization analysis of expression of *MrgD*, *Ptprt*, *Myo1a* and *Kif21b* in DRGs of E16.5 control and *Runx1 CKO* animals. Note that defects in expression are already evident in DRGs of *NGF* and *Runx1* mutants at E16.5, which is when those genes normally start to be expressed, suggesting a requirement of both NGF and Runx1for initiation of expression. Shown are results representative of at least two independent animals per genotype. Scale bar, 50μm.

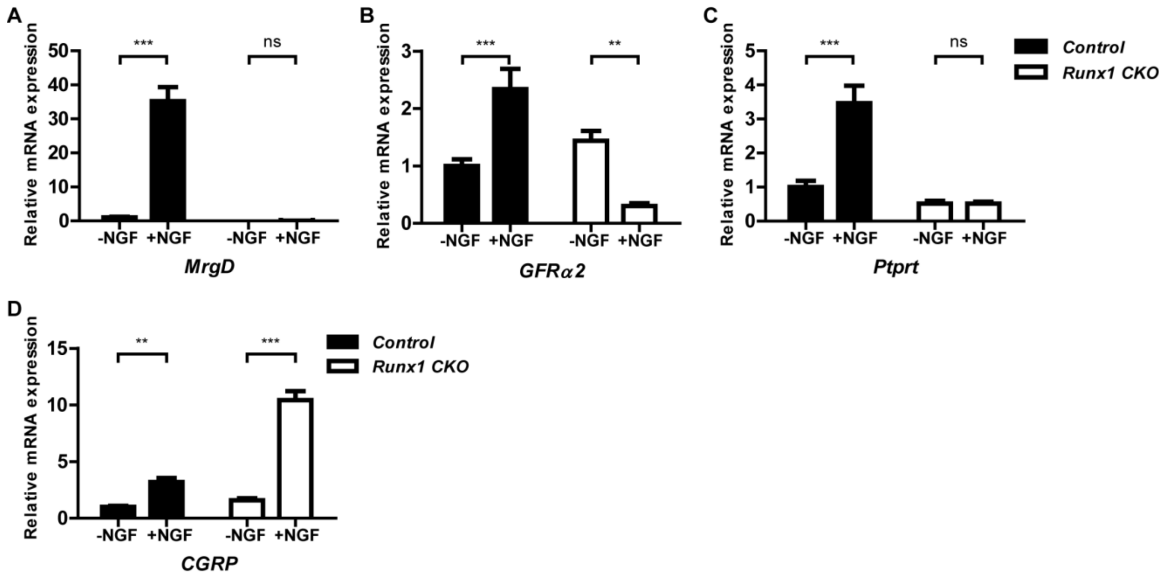


Figure 2.3 NGF is sufficient to promote expression of nonpeptidergic-specific genes in a Runx1-dependent way *in vitro*.

(**A-D**) Real-time PCR analysis of expression of nonpeptidergic-specific genes, including *MrgD* (A), *GFRα2* (B) and *Ptprt* (C) and a peptidergic gene *CGRP* (D) in dissociated DRG neurons from P0 control and *Runx1 CKO* animals cultured in the presence or absence of NGF. Note that the ability of NGF to stimulate expression of nonpeptidergic-specific genes but not the peptidergic gene is abolished in the absence of Runx1, suggesting a specific role for Runx1 in regulating expression of nonpeptidergic-specific genes downstream of NGF. Statistical analyses were done using two-way ANOVA with a Bonferroni post-test, N=5 for A, N=7 for the rest. ** $p \leq 0.01$, *** $p \leq 0.001$, ns non-significant.

2.3. Runx1 potentiates activity of TrkA signaling, thereby indirectly maintaining expression of *Ret*.

As mentioned in Chapter 2.1, the expression of *Ret* as examined by in situ hybridization analysis did not conform to the general rule that defines all the other nonpeptidergic-specific genes, that is, a simultaneous requirement of NGF and Runx1 for expression. The observation that at P0, *Ret* expression was grossly intact except for a slightly reduced level per cell in *Runx1 CKO* DRGs was in stark contrast to the well-documented *Ret* deficiency in P0 *NGF* mutant DRGs (Figure 2.4 A,B,E and F). Expression analysis by in situ hybridization and real-time PCR during late embryonic and postnatal development further confirmed the perinatal onset of Runx1 dependence for *Ret* expression (Figure 2.4. C-I). Therefore, NGF and Runx1 control *Ret* expression in different ways. While NGF is involved early during the initiation of *Ret* expression, Runx1 is not required until later stages for maintenance of expression.

The sequential requirement of NGF and Runx1 for *Ret* expression suggests an intriguing possibility. That is, Runx1 is required for establishing a level of NGF signaling that is critical for postnatal *Ret* expression. To directly test this hypothesis, the level of NGF signaling in control and *Runx1 CKO* DRGs at P0 was assessed by immunohistochemistry with phospho-Trk (pTrk) antibodies that only recognize active forms of Trk receptors that are phosphorylated at SHC or/and PLCγ sites. Only pTrk signal in neurons that lacked neurofilament–H (NFH), a marker for large-diameter neurons, was considered a true reflection of TrkA activity and compared between genotypes. The activity of TrkA receptors as defined by the fluorescence intensity of pTrk immunoreactivity (IR) per cell was significantly lower in *Runx1 CKO* DRGs relative to control, consistent with a role for Runx1 in maintaining a high level of NGF signaling (Figure 2.5 A-F). It is worth emphasizing that Runx1 is not required for the level of receptor activation that supports survival of nociceptors and *CGRP* expression, as the number of neurons as well as $CGRP^{+}$ neurons was not reduced in *Runx1 CKO* DRGs (Chen et al., 2006b; Yoshikawa et al., 2007). Since *TrkA* expression in control and *Runx1 CKO* DRGs was almost indistinguishable by immunostaining, Runx1 mostly likely regulates TrkA activity without affecting its expression (Figure 2.5 G and H).

To determine the contribution of this defect in NGF signaling to deficits in expression of *Ret* and other nonpeptidergic-specific genes in *Runx1 CKO* animals, we asked whether exogenous administration of NGF

by intraperitoneal (IP) injection right after birth can rescue nonpeptidergic expression deficits in *Runx1 CKO* DRGs. Strikingly, *Ret* expression underwent a significant increase following two injections of NGF at P0 and P1 in *Runx1 CKO* animals, suggesting that the *Ret* expression deficit in *Runx1 CKO* DRGs was at least in part an indirect consequence of impaired NGF signaling (Figure 2.6. A-C). By contrast, the same treatment regime had no detectable effect on expression of all the other nonpeptidergic-specific genes which were shown to require both NGF and Runx1 in a similar manner (Figure 2.6. D-L). Therefore, in addition to the majority of genes that require both NGF and Runx1 for expression, these findings reveal a second, novel mode of interaction between NGF and Runx1, in which Runx1, through a yet unidentified mechanism, supports a high level of TrkA activity, which is in turn essential for postnatal *Ret* expression.

Figure 2.4 *Ret* that is strongly NGF-dependent requires Runx1 for maintenance of expression.

(**A and B**) In situ hybridization analysis of expression of *Ret* in DRGs of P0 control and *NGF* mutants. (**C-H**) In situ hybridization analysis of expression of *Ret* in DRGs of control and *Runx1 CKO* animals at E16.5 (C and D), P0 (E and F) and P10 (G and H). Note that while *Ret* expression is almost completely eliminated in *NGF* mutant DRGs at P0, its expression in *Runx1 CKO* DRGs at the same time point is only mildly affected, indicating differential temporal requirements of NGF and Runx1 for *Ret* expression. Only the small-diameter Ret^+ population is relevant, since the remaining large-diameter Ret^+ neurons represent mechanoreceptors. Shown are results representative of at least two independent animals per genotype at each time point. (**I**) Real-time PCR analysis of expression of *Ret* in DRGs of control and *Runx1 CKO* animals at P0 and P14 confirms the progressive nature of *Ret* deficit in *Runx1 CKO* DRGs. Statistical analyses were done using paired t test, N=3 for each time point, **$p \leq 0.01$. Scale bar, 50μm.

Figure 2.4

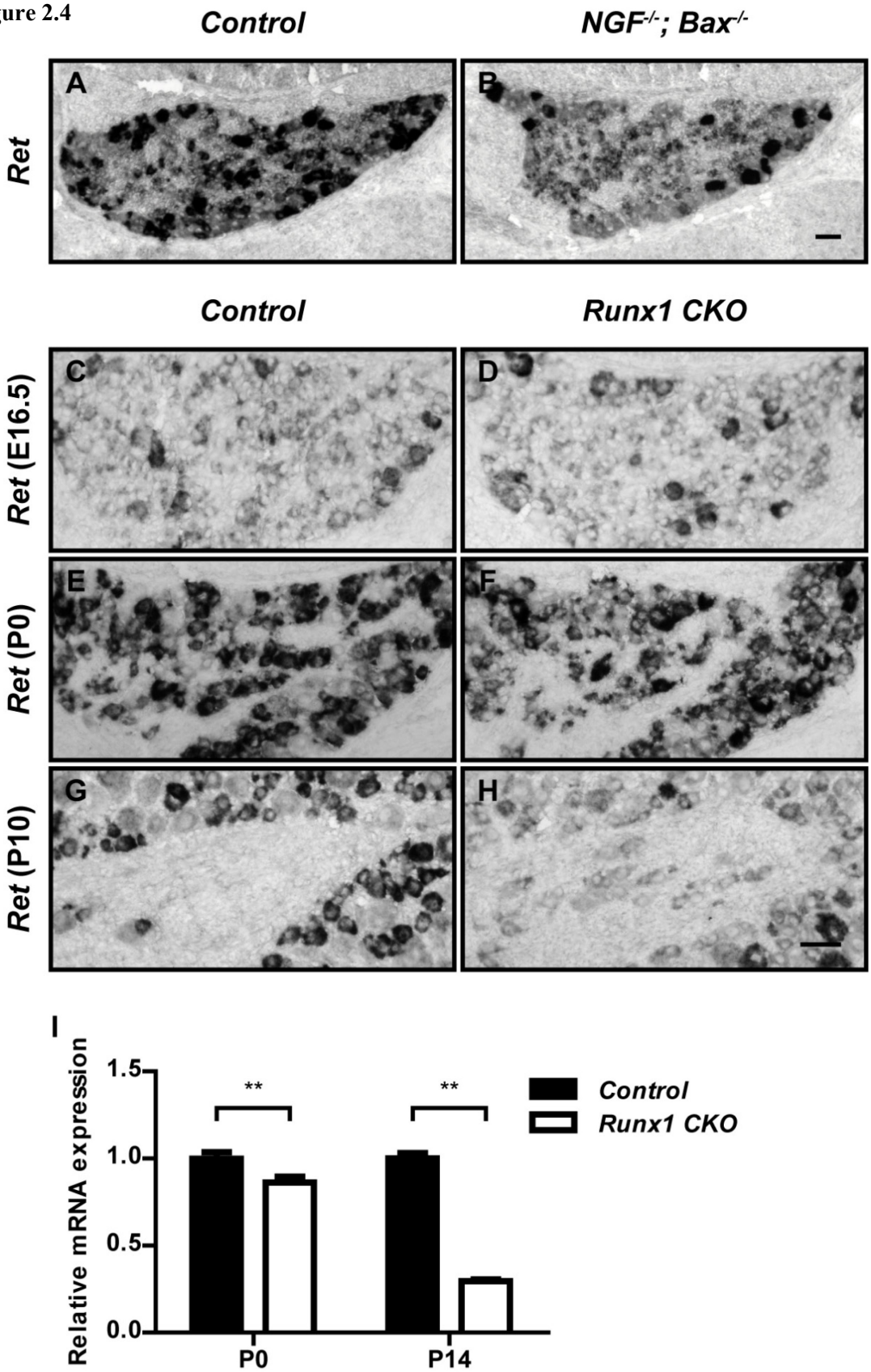
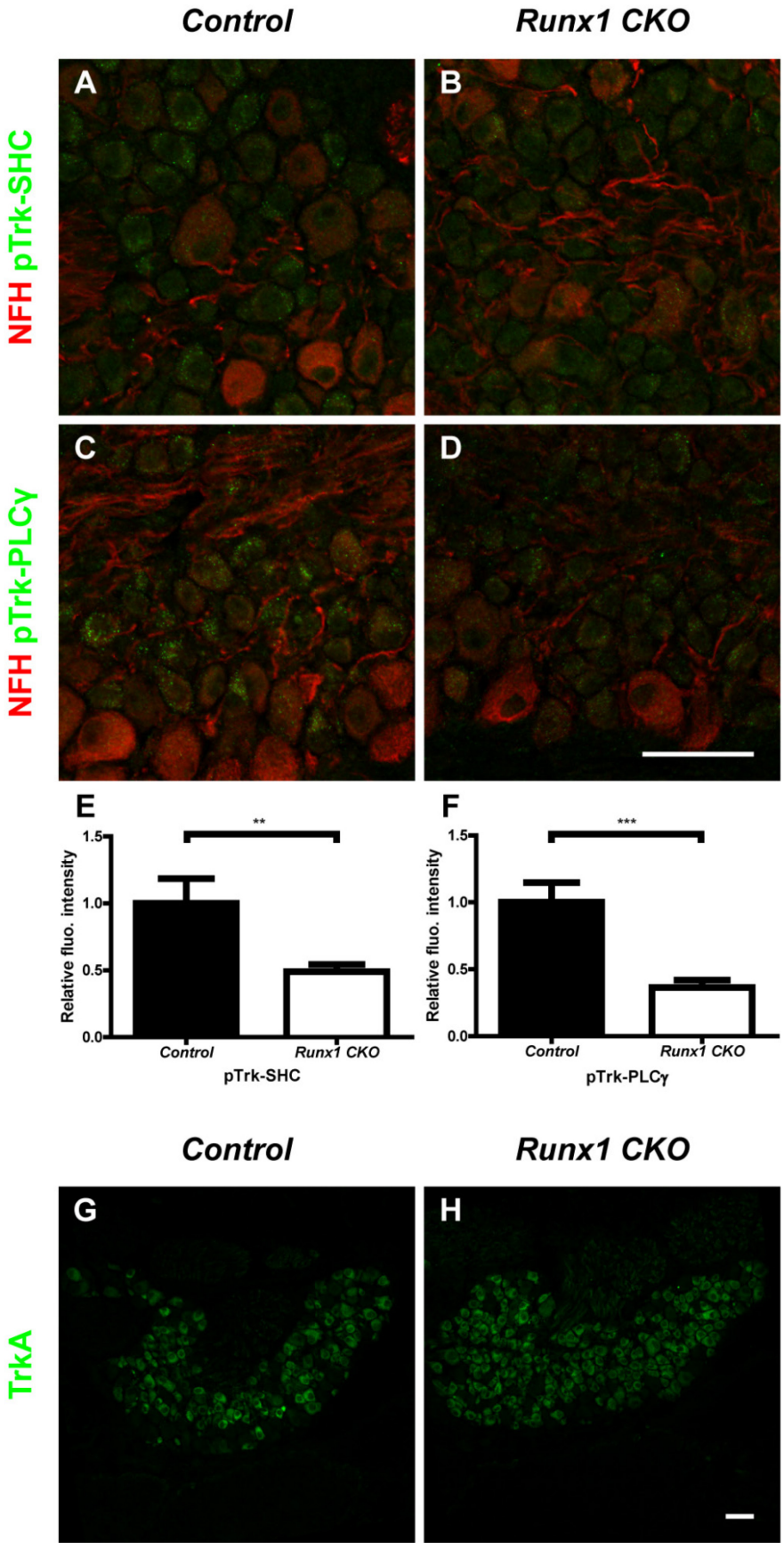

Control
NGF-/-; Bax-/-
A
B
Ret
Control
Runx1 CKO
C
D
Ret (E16.5)
E
F
Ret (P0)
G
H
Ret (P10)
I
Relative mRNA expression
1.5
1.0
0.5
0.0
**
**
Control
Runx1 CKO
P0
P14

Figure 2.5 Runx1 potentiates TrkA activity without regulating *TrkA* expression.

(**A-D**) Double staining of NFH and pTrk-SHC (A and B) or NFH and pTrk-PLCγ (C and D) in DRGs of P0 control and *Runx1 CKO* animals shows greatly attenuated pTrk IR in NFH-negative neurons on a single cell level in *Runx1 CKO* DRGs compared to control, suggesting a deficit in NGF signaling. (**E and F**) Quantification of NGF signaling deficit based on average fluorescence intensity of pTrk-SHC or pTrk-PLCγ IR per cell further confirms reduced TrkA activity in *Runx1 CKO* DRGs. Unpaired t test was performed on data from three independent pairs of control and mutant animals, ** $p \leq 0.01$, *** $p \leq 0.001$. (**G and H**) TrkA immunostaining in DRGs of P0 control and *Runx1 CKO* animals shows comparable expression in both genotypes. Shown are results representative of two independent animals per genotype. Scale bar, 50μm.

Figure 2.5


Control
Runx1 CKO
A
B
NFH pTrk-SHC
C
D
NFH pTrk-PLCγ
E
**
Relative fluo. intensity
1.5
1.0
0.5
0.0
Control
Runx1 CKO
pTrk-SHC
F
***
Relative fluo. intensity
1.5
1.0
0.5
0.0
Control
Runx1 CKO
pTrk-PLCγ
Control
Runx1 CKO
G
H
TrkA

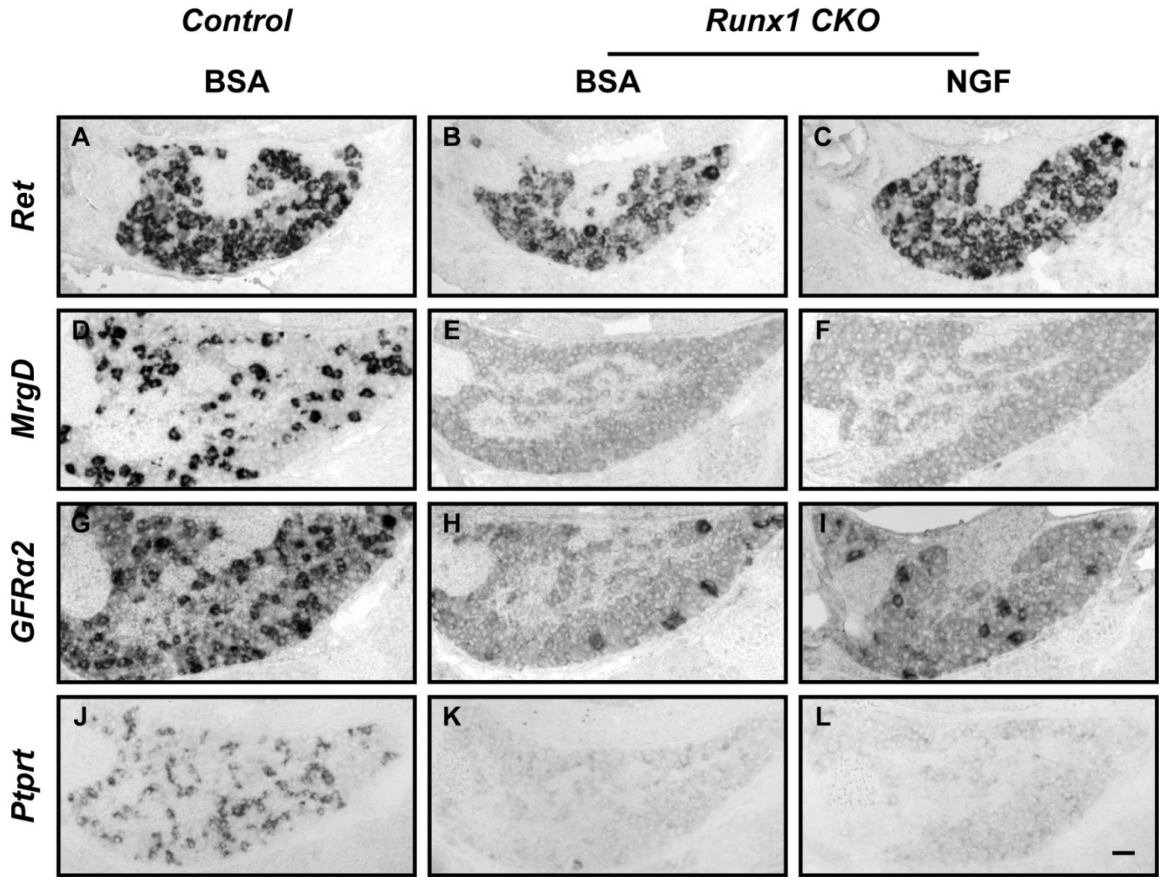


Figure 2.6 Diminished NGF signaling contributes to the postnatal *Ret* expression deficit in *Runx1 CKO* mice.

(**A-C**) In situ hybridization analysis of expression of *Ret* in DRGs of P2 control animals that received BSA injections, *Runx1CKO* animals that received BSA injections and *Runx1 CKO* animals that received NGF injections. Note that overall level of *Ret* expression per cell is considerably higher in *Runx1 CKO* animals that received NGF than mutant littermates that received BSA instead, suggesting *Ret* requires a relatively high level of NGF signaling for normal postnatal expression. (**D-L**) In situ hybridization analysis of expression of *MrgD* (D-F), *GFRα2* (G-I) and *Ptprt* (J-L) in DRGs of P2 control animals that received BSA injections, *Runx1CKO* animals that received BSA injections and *Runx1 CKO* animals that received NGF injections. Importantly, exogenous NGF administration fails to activate expression of those genes in the absence of Runx1, suggesting that Runx1 plays a more direct role in regulating their expression. Shown are results representative of at least three independent injection experiments. Scale bar, 50μm.

2.4. Runx1 and CBFβ are both required for the development of nonpeptidergic populations by forming a heterodimeric transcription factor complex.

2.4.1. Runx1 and CBFβ form a complex in the DRG.

As described above, for the large majority of nonpeptidergic-specific genes, NGF and Runx1 are required simultaneously as opposed to sequentially, suggesting a linear model in which NGF acts upstream of Runx1 to promote Runx1-dependent transcription of nonpeptidergic-specific genes. In order to elucidate the mechanism by which NGF facilitates Runx1 function, we sought to better understand how Runx1 activity is normally regulated in the DRG.

Outside the nervous system, the Runx family transcription factors are thought to function as heterodimers composed of a DNA-binding subunit, which in mammals can be Runx1, Runx2 or Runx3, and a common non-DNA-binding subunit CBFβ. CBFβ is indispensable for Runx activity in cells of the hematopoietic lineage due to its ability to enhance the DNA-binding ability and protein stability of associated Runx proteins (Adya et al., 2000). The functional importance of both components is best illustrated by the fact that *Runx1* and *Cbfb* knockout animals both die of massive CNS hemorrhage due to a block in definitive hematopoiesis (Okuda et al., 1996; Wang et al., 1996a; Wang et al., 1996b). Considering the requirement for CBFβ during Runx1 function in the hematopoietic system, we next asked whether CBFβ plays a similar role in the DRG.

First, the expression pattern of *Cbfb* both at the mRNA level and the protein level was characterized during development. In situ hybridization analysis revealed that *Cbfb* is expressed very broadly in DRG neurons over the entire time course of our analysis, E13.5, E16.5 and P14 (Figure 2.7 D-F). The level of *Cbfb* expression varied considerably among DRG neurons. To visualize endogenous CBFβ proteins, we generated a $Cbfb^{Flag}$ knockin mouse line in which N-terminally Flag-tagged CBFβ protein is produced from the endogenous *Cbfb* locus (Figure 2.7 A). In animals harboring this allele, a custom-made Flag antibody specifically detects endogenous Flag-CBFβ (Figure. 2.7 B and C). Double labeling of Flag and Runx1 showed extensive overlap between Runx1 and CBFβ even at the subcellular level throughout development suggesting a potential for interaction (Figure2.7 B, C, G-I). Note that while virtually all $Runx1^{+}$ neurons

expressed CBFβ, there were a considerable number of CBFβ^{+} neurons that did not express Runx1, many of which represented Runx3^{+} proprioceptors (discussed in Chapter2.8).

To test for a direct physical interaction between Runx1 and CBFβ in the DRG, co-immunoprecipitation (co-IP) experiments were performed using a Flag antibody from DRG homogenates of P0 *Cbfb*$^{Flag/Flag}$ animals. Co-IPs from wildtype DRG lysates served as a negative control. Runx1 was enriched together with Flag-CBFβ in the IP fraction from *Cbfb*$^{Flag/Flag}$ animals but not wildtype animals, providing strong evidence for existence of a Runx1/CBFβ complex in DRG neurons (Figure 2.7 J).

2.4.2. CBFβ is required for the development of Runx1-dependent nonpeptidergic populations.

To assess the function of CBFβ in DRG development, we generated a conditional *Cbfb* allele by targeting the putative promoter sequence and the first two exons of this gene (Figure 2.8 A). Gene ablation in the DRG was achieved by crossing mice harboring the conditional *Cbfb* allele to a *Wnt1-Cre* line that drives recombination specifically in the dorsal neural tube and neural crest, the same Cre line that was used to generate the *Runx1 CKO* animal (Figure 2.8 B and C)(Danielian et al., 1998). Therefore, the contribution of CBFβ if any to DRG development can be directly compared with that of Runx1.

As described before, Runx1 is integral to acquisition of the molecular identity of nonpeptidergic nociceptors as assayed by expression of a select panel of nonpeptidergic-specific genes (Figure 2.1 and Figure 2.2). Strikingly, CBFβ is required for expression of the same set of genes in a manner essentially identical to Runx1 (Figure 2.8 D-M). Furthermore, in both *Runx1 CKO* and *Wnt1-Cre; Cbfb*$^{f/f}$ (*Cbfb CKO*) animals, there was clear evidence of impaired peripheral innervation by nonpeptidergic nociceptors. In particular, sensory fibers in the epidermis, which are known to primarily originate from nonpeptidergic nociceptors, were dramatically decreased in number in both *Runx1* and *Cbfb* mutant animals at P0 (Figure 2.8 N-S). This occurred independent of any change in the subepidermal plexus indicating a developmental defect in the final stage of peripheral target innervation, epidermal penetration. Therefore, morphological characteristics of nonpeptidergic nociceptors as part of the differentiation program critically depend on both Runx1 and CBFβ.

To study CBFβ function in postnatal development, which was precluded by perinatal lethality of *Cbfb CKO* animals due to craniofacial deficits, *Cbfb* was deleted postnatally with the use of a $Runx1^{CreER}$ knockin allele (Samokhvalov et al., 2007). A similar strategy was employed to generate a *Runx1* conditional knockout mouse model with postnatal onset of excision for direct comparison. To identify neurons with functional Cre protein which, depending on genotype, represented either knockout or control cells, Tau^{mGFP}, a previously described neuronal specific Cre-dependent GFP reporter, was also incorporated (Hippenmeyer et al., 2005). This postnatal gene ablation system was particularly well suited for the study of TH^+ C-low threshold mechanoreceptors (C-LTMRs), a unique population of nonpeptidergic small-diameter neurons that is implicated in the pleasurable, affective component of touch and injury-induced mechanical hypersensitivity, because of their late emergence after the second postnatal week (Li et al., 2011; Olausson et al., 2010; Seal et al., 2009). Remarkably, postnatal deletion of either *Runx1* or *Cbfb* disrupted both molecular and morphological characteristics of C-LTMRs. Specifically, within the GFP^+ population which is largely devoid of Runx1 and CBFβ, there are fewer TH^+ neurons in DRGs and far fewer longitudinal lanceolate endings that are typical of C-LTMRs in the periphery compared to control, although phenotypes following *Cbfb* deletion were typically less dramatic and more variable than those following *Runx1* deletion, which mostly likely resulted from less efficient depletion of CBFβ protein compared to Runx1 (Figure 2.9 A-H). Importantly, the phenotypes described here closely resemble those observed in a different *Runx1* conditional mutant (Lou et al., 2013). This set of analysis therefore demonstrates a postnatal requirement for both Runx1 and CBFβ during development of C-LTMRs.

Mechanistically, the apparent phenocopy between *Runx1* and *Cbfb* mutants may be in part explained by a dramatic defect in Runx1 protein expression in *Cbfb CKO* DRGs as shown by immunostaining and western blot analysis (Figure 2.10 A-C). The fact that *Runx1* mRNA expression based on in situ hybridization and real-time PCR analysis remained unchanged, if not increased, in *Cbfb CKO* DRGs, suggested a specific role for CBFβ in posttranscriptional regulation of *Runx1* expression, which is in line with earlier work describing a critical CBFβ dependence of Runx1 protein stability (Figure 2.10 D-F)(Huang et al., 2001). Together, these findings strongly argue that CBFβ is as important as Runx1 for the development of various nonpeptidergic populations, in part due to its ability to regulate *Runx1* expression at a posttranscriptional level.

Figure 2.7 Runx1 and CBFβ form a complex in the DRG.

(**A**) Schematic showing the targeting strategy for the *Cbfb*^{Flag} allele. Following germ-line transmission, the *Neo* selection cassette was removed by crossing the carrier to an animal expressing the FlpE recombinase in germ line. A Bstz171 restriction site was introduced immediately downstream of the *Flag* sequence to facilitate southern screening of embryonic stem (ES) cells. *Flag* tags, *LoxP* and *FRT* sites are shown as red filled triangles, open and filled triangles respectively. (**B and C**) Double staining of Runx1 and Flag in DRGs of P0 *Cbfb*$^{+/+}$ and *Cbfb*$^{Flag/+}$ animals shows extensive colocalization between Runx1 and Flag/CBFβ even at the subcellular level in DRG neurons. Note that CBFβ is expressed in more than just Runx1^{+} neurons in the DRG. (**D-F**) In situ hybridization analysis of expression of *Cbfb* in wildtype DRGs at E13.5, E16.5 and P14 shows a broad expression pattern throughout development. *Cbfb*$^{+}$ neurons can be either low or high expressors. (**G-I**) Double staining of Runx1 and Flag in DRGs of *Cbfb*$^{Flag/+}$ animals at E13.5, E16.5 and P14 shows a pattern of CBFβ protein similar to its mRNA distribution. (**J**) Co-immunoprecipitation experiments by Flag immunoprecipitation using DRGs lysates from P0 *Cbfb*$^{Flag/Flag}$ animals and wildtype controls show Runx1 co-immunoprecipitate with Flag-CBFβ from DRGs of *Cbfb*$^{Flag/Flag}$ animals, indicating the formation of a Runx1/CBFβ complex in the DRG. Scale bar, 50μm.

Figure 2.7

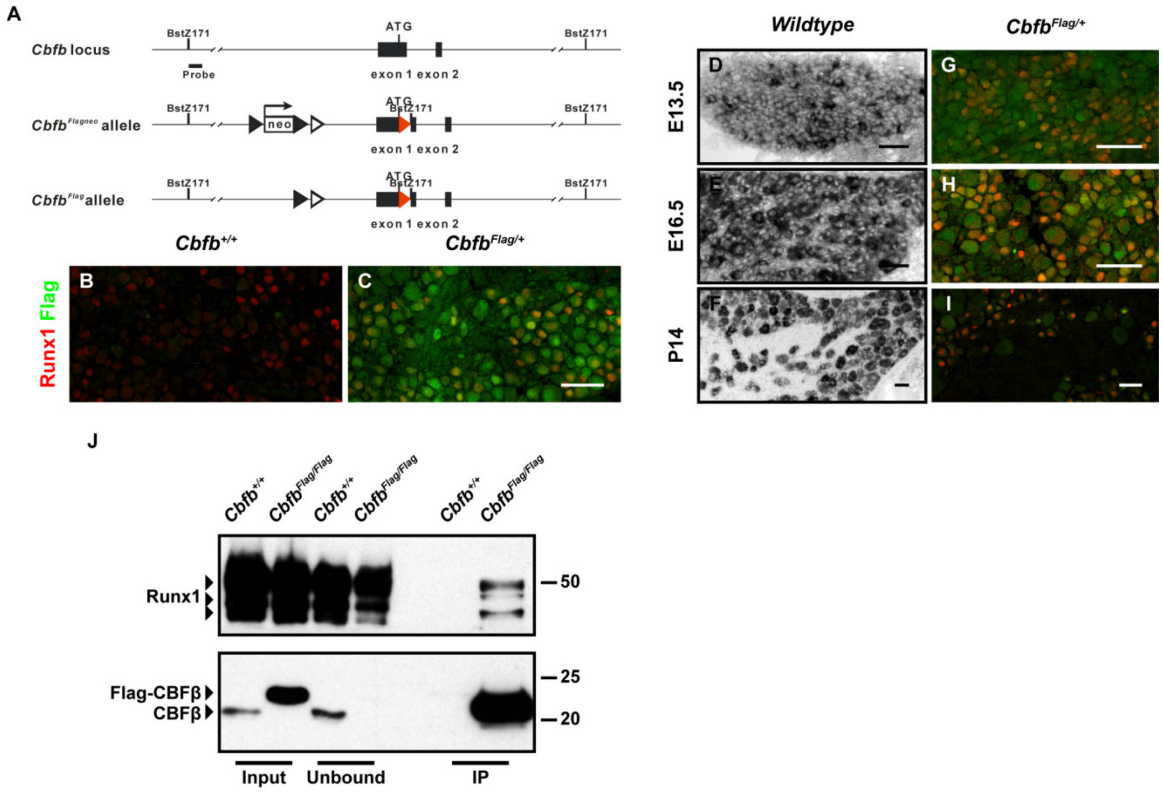

A
Cbfb locus
BstZ171
ATG
Probe
exon 1 exon 2
Cbfb^Flagneo allele
neo
Cbfb^Flag allele
Cbfb+/+
Cbfb^Flag/+
Runx1 Flag
B
C
Wildtype
Cbfb^Flag/+
E13.5
E16.5
P14
D
E
F
G
H
I
J
Cbfb+/+
Cbfb^Flag/Flag
Runx1
Flag-CBFβ
CBFβ
50
25
20
Input
Unbound
IP

Figure 2.8 CBFβ is required for acquisition of Runx1-dependent molecular and morphological features of nonpeptidergic nociceptors.

(**A**) Schematic showing the targeting strategy for generation of the *Cbfb* conditional allele. Following germ-line transmission, the *Neo* selection cassette was removed by crossing the carrier to an animal expressing FlpE recombinase in the germ line. A Bstz171 restriction site was introduced immediately downstream of the 3' *loxP* site to facilitate southern screening of ES cells. *LoxP* and *FRT* sites are shown as open and filled triangles respectively. (**B and C**) In situ hybridization analysis of expression of *Cbfb* in DRGs of P0 control and *Cbfb CKO* animals verifies the knockout strategy. (**D-M**) In situ hybridization analysis of expression of *MrgD* (Control, 26.9%±2.8%; *Cbfb CKO*, 0%), *GFRα2* (Control, 38.8%±2.8%; *Cbfb CKO*, 11.7%±1.9%), *Ptprt*, (Control, 31.9%±3.2%; *Cbfb CKO*, 7.1%±2.8%), *Myo1a* (Control, 26.9%±3.2%; *Cbfb CKO*, 5.6%±0.6%), *Kif21b* (Control, 20.2%±0.1%; *Cbfb CKO*, 2.4%±0.5%) in DRGs of P0 Control and *Cbfb CKO* animals. Note that all those genes exhibit severe deficits in expression which is reminiscent of the phenotype in *Runx1 CKO* animals. The discrepancy between CBFβ dependence and Runx1 dependence of *Kif21b* expression reflects *Kif21b* expression in proprioceptors that depends on Runx3 and CBFβ. Shown is average±SEM for the percentage of neurons expressing indicated genes based on counts from a total of at least 9 sections from three independent animals per genotype. The total number of neurons per section was counted based on combined NeuN immunostaining, which was not shown. (**N-Q**) GFP immunostaining of P0 hairy skin to visualize sensory innervation of the epidermis in control and *Runx1 CKO* animals (N and O) or control and *Cbfb CKO* animals (P and Q) in which all the sensory neurons are labeled by GFP. Note that there is a dramatic reduction in the fiber density specifically in the epidermis in both *Runx1 CKO* and *Cbfb CKO* animals relative to their littermate controls. The yellow dotted line denotes the epidermal-dermal junction. (**R and S**) Quantification of sensory innervation of the epidermis in P0 control and *Runx1 CKO* animals (R) or P0 control and *Cbfb CKO* animals (S) shows remarkably similar reduction in the innervation density in both mutants. The innervation density is defined as the fraction of area occupied by GFP^+ fibers in the epidermis. Unpaired t test was performed on data from three independent animals per genotype. ***$p \leq 0.001$. Scale bar, 50μm.

Figure 2.8

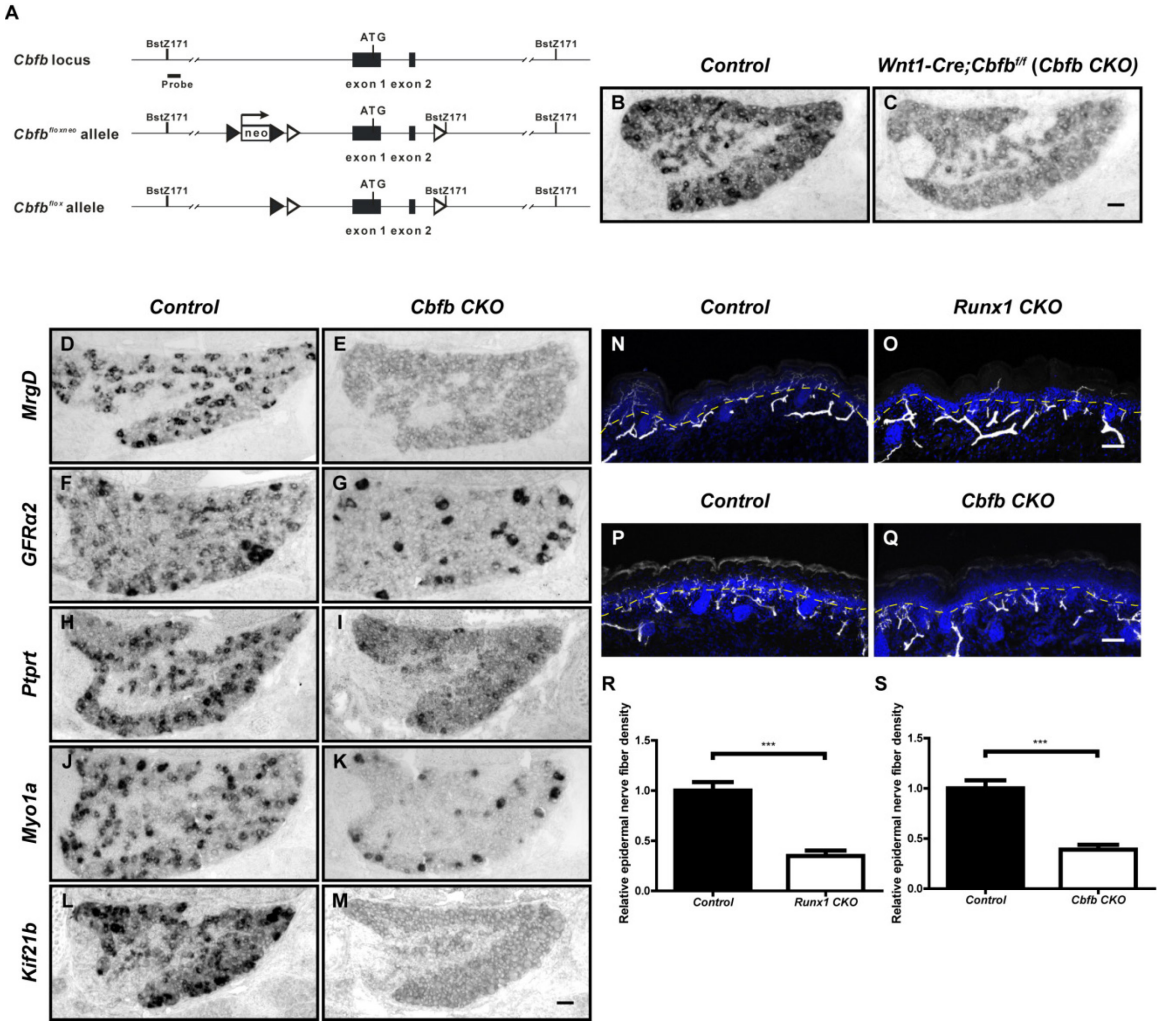
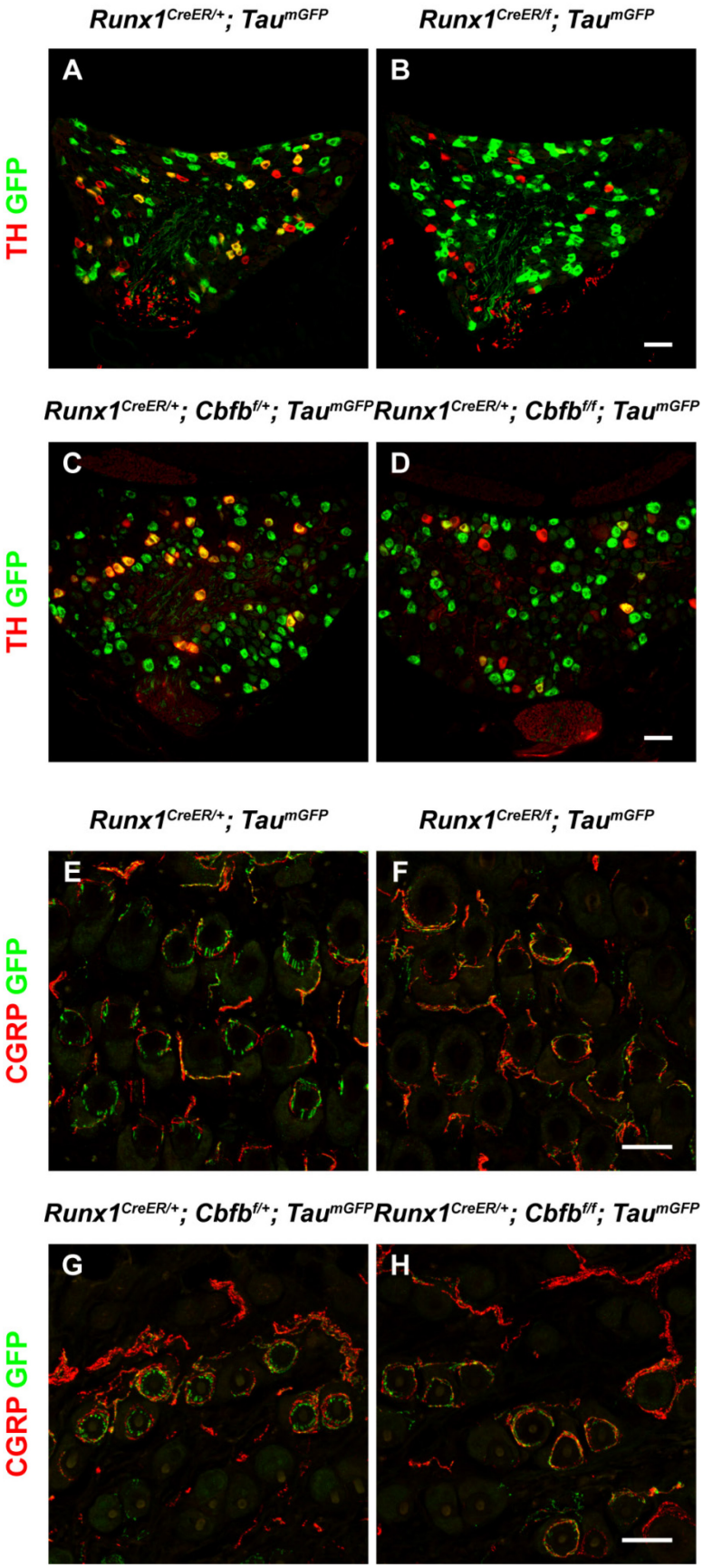

A
Cbfb locus
BstZ171
ATG
BstZ171
Probe
exon 1 exon 2
Cbfb flox neo allele
neo
Cbfb flox allele
Control
Wnt1-Cre;Cbfb f/f (Cbfb CKO)
B
C
Control
Cbfb CKO
D
E
MrgD
F
G
GFRα2
H
I
Ptprt
J
K
Myo1a
L
M
Kif21b
Control
Runx1 CKO
N
O
Control
Cbfb CKO
P
Q
R
Relative epidermal nerve fiber density
***
Control
Runx1 CKO
S
Relative epidermal nerve fiber density
***
Control
Cbfb CKO

Figure 2.9 Runx1 and CBFβ are both required postnatally for acquisition of molecular and morphological features of C-LTMRs.

(**A-D**) Double staining of TH and GFP in DRGs of P21 *Runx1*$^{CreER/+}$*; Tau*$^{mGFP/+}$ and *Runx1*$^{CreER/f}$*; Tau*$^{mGFP/+}$ animals (A and B)(Control, 23.2%±1.6%; *Runx1* mutant, 3.8%±2.7%) or *Runx1*$^{CreER/+}$*;Cbfb*$^{f/+}$*; Tau*$^{mGFP/+}$ and *Runx1*$^{CreER/+}$*; Cbfb*$^{f/f}$*; Tau*$^{mGFP/+}$ animals (C and D)(Control, 21.4%±1.9%; *Cbfb* mutant, 8.9%±2.0%) that received IP injections of tamoxifen at P2. Note that there is a substantial reduction in the number of GFP, TH double positive neurons due to a selective loss of TH expression in the GFP$^+$ population in both *Runx1* and *Cbfb* mutant DRGs. Shown is average±SEM for the percentage of GFP$^+$ neurons that express TH based on counts from a total of at least 9 sections from three independent animals per genotype. (**E-H**) Double staining of CGRP and GFP in back hairy skin of P21 *Runx1*$^{CreER/+}$*; Tau*$^{mGFP/+}$ and *Runx1*$^{CreER/f}$*; Tau*$^{mGFP/+}$ animals (E and F) or *Runx1*$^{CreER/+}$*;Cbfb*$^{f/+}$*; Tau*$^{mGFP/+}$ and *Runx1*$^{CreER/+}$*; Cbfb*$^{f/f}$*; Tau*$^{mGFP/+}$ animals (G and H) that received IP injections of tamoxifen at P2. Note that in both mutant animals, there is a marked decrease in the number of GFP$^+$ longitudinal lanceolate endings characteristic of C-LTMRs, which is accompanied by an increased frequency of GFP$^+$ endings that assume more peptidergic-like morphology. Shown are representative images from more than 3 independent experiments. Scale bar, 50μm.

Figure 2.9


Runx1CreER/+; TaumGFP
Runx1CreER/f; TaumGFP
A
B
TH GFP
Runx1CreER/+; Cbfbf/+; TaumGFP
Runx1CreER/+; Cbfbf/f; TaumGFP
C
D
TH GFP
Runx1CreER/+; TaumGFP
Runx1CreER/f; TaumGFP
E
F
CGRP GFP
Runx1CreER/+; Cbfbf/+; TaumGFP
Runx1CreER/+; Cbfbf/f; TaumGFP
G
H
CGRP GFP

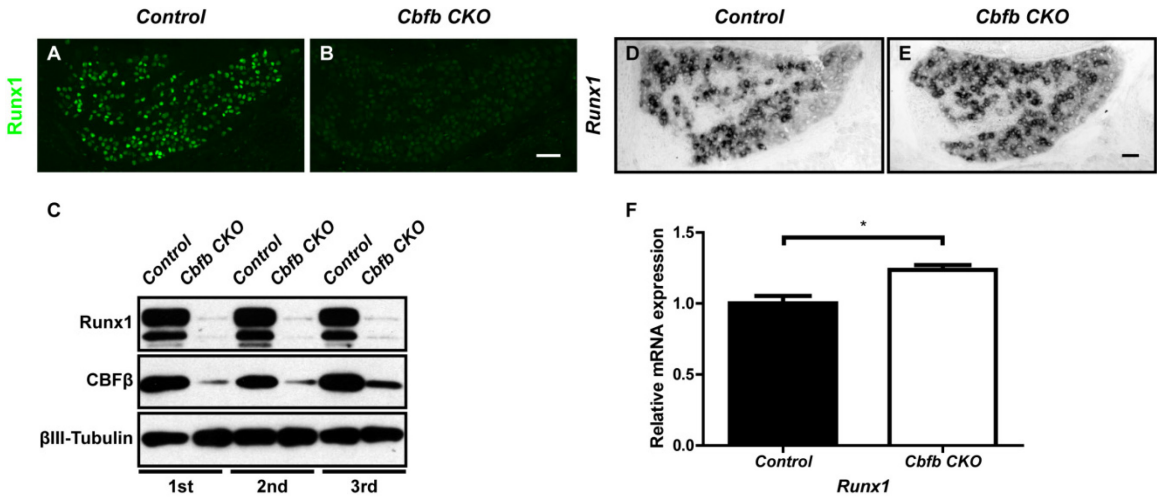


Figure 2.10 CBFβ promotes *Runx1* expression at a posttranscriptional level.

(**A and B**) Runx1 immunostaining in DRGs of P0 control and *Cbfb CKO* animals shows almost complete loss of Runx1 proteins in the absence of CBFβ. Shown are representative images from at least three independent experiments. (**C**) Immunoblot analysis of expression of *Runx1* and *Cbfb* in DRGs of P0 control and *Cbfb CKO* animals shows a dramatic loss of Runx1 proteins as a result of CBFβ depletion. βIII-Tubulin serves as a loading control. Shown are results from three independent experiments. (**D and E**) In situ hybridization analysis of *Runx1* expression in DRGs of P0 control and *Cbfb CKO* animals shows comparable levels of *Runx1* transcripts in control and mutant animals. (**F**) Real-time PCR analysis of *Runx1* expression in DRGs of P0 control and *Cbfb CKO* animals shows increased *Runx1* mRNA expression in *Cbfb CKO* DRGs compared to control, which partly reflects an increased ratio of nociceptors to proprioceptors. *p≤0.05. Scale bar, 50μm.

2.5. NGF regulates *Cbfb* expression at the transcriptional level, thereby facilitating the function of the Runx1/CBFβ complex.

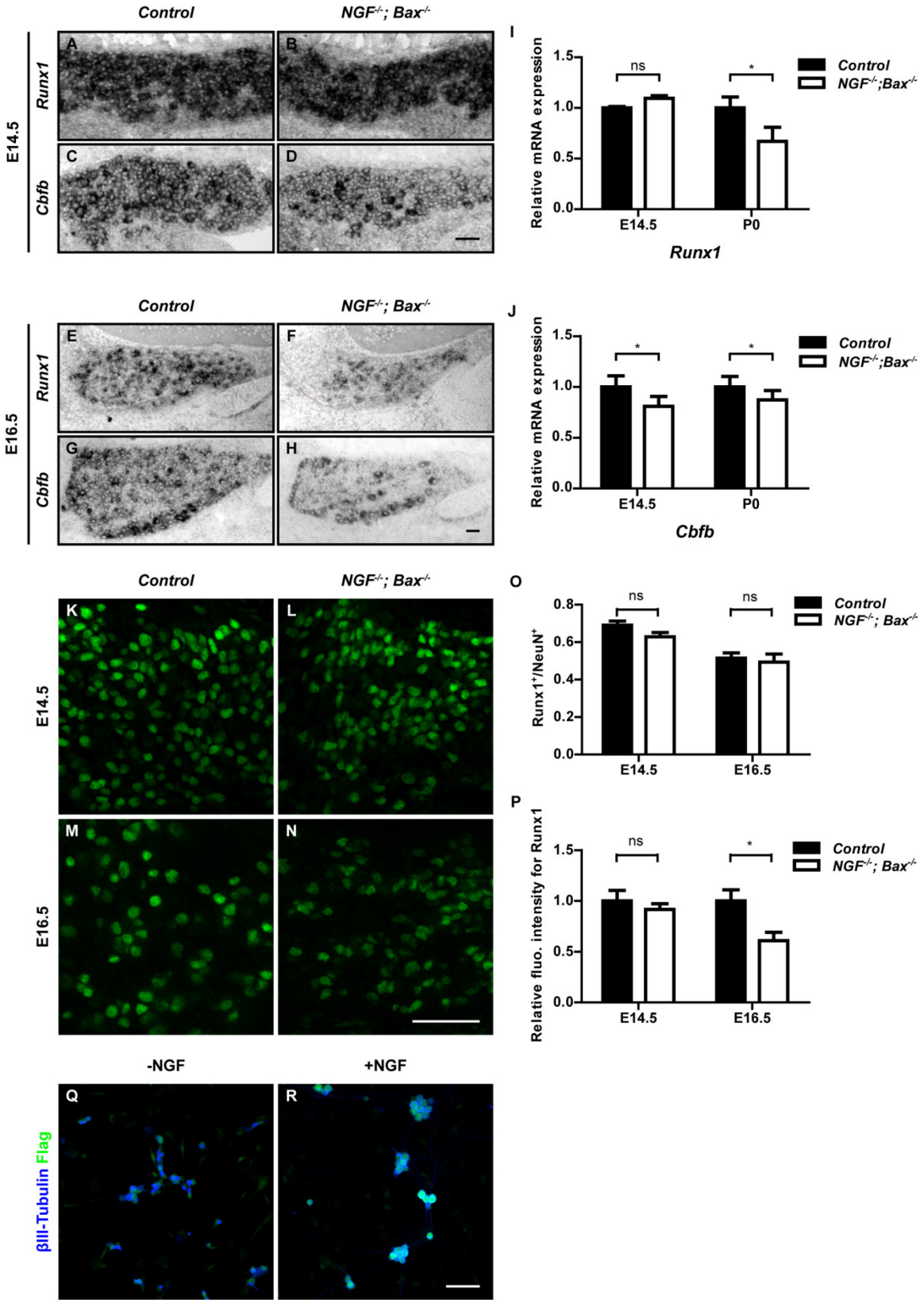
Having established that Runx1 and CBFβ are both required for Runx1 function in the development of nonpeptidergic populations, we explored the possibility that NGF regulates expression of *Runx1* and/or *Cbfb*, thereby promoting Runx1-dependent nonpeptidergic neuron fate. To this end, expression of both *Runx1* and *Cbfb* was examined in DRGs of control and *NGF* mutant animals by in situ hybridization analysis both before and during the onset of deficits in nonpeptidergic-specific gene expression. Consistent with our previous findings, at E14.5, before the differentiation of nonpeptidergic nociceptors, *Runx1* expression was relatively normal in *NGF* mutant DRGs (Figure 2.11 A and B). *Cbfb* expression, on the other hand, was significantly impaired at the same time point in *NGF* mutant DRGs relative to control, specifically in small-diameter neurons that correspond to nociceptors (Figure 2.11 C and D). At later time stages, such as E16.5, *Runx1* expression began to be affected, while the *Cbfb* expression deficit became much more pronounced in *NGF* mutant DRGs (Figure 2.11 E-H). The differential temporal requirement for NGF for expression of *Runx1* and *Cbfb* was corroborated by real-time PCR analysis (Figure 2.11 I and J). Consequently, the level of Runx1 proteins was significantly attenuated at E16.5, which coincided with the earliest deficits in nonpeptidergic nociceptors in *NGF* mutant DRGs (Figure 2.11 K-P). The early onset of NGF dependence of *Cbfb* expression suggested that *Cbfb* expression is directly activated by NGF. Indeed, incubation of DRG cultures with NGF led to a robust increase in CBFβ protein expression as examined by Flag immunostaining in $Cbfb^{Flag/+}$ neurons (Figure 2.11 Q and S). Thus, CBFβ represents a crucial nexus for NGF regulation of Runx1 function.

Figure 2.11 NGF activates *Cbfb* expression at the transcriptional level, before it regulates *Runx1* expression.

(**A and B**) In situ hybridization analysis of *Runx1* expression in DRGs of E14.5 control and *NGF* mutant animals shows comparable levels of transcripts in control and mutant DRGs. Average±SEM for the relative intensity of in situ signals after normalization to the level in control DRGs is as follows: control, 1.00±0.16; *NGF* mutant, 0.73±0.12. p=0.3007, based on paired t test. (**C and D**) In situ hybridization analysis of *Cbfb* expression in DRGs of E14.5 control and *NGF* mutant animals shows a significant reduction in the level of transcripts in small diameter neurons that correspond to prospective nociceptors in *NGF* mutants compared to control. Note that *Cbfb* in situ hybridization was combined with Runx3 immunostaining to exclude the $Runx3^+$ *Cbfb* population from the analysis. Average±SEM for relative intensity of in situ signal after normalization to the level in control DRGs is as follows: control, 1.00±0.20; *NGF* mutant, 0.60±0.18. p=0.0125, based on paired t test. (**E and F**) In situ hybridization analysis of *Runx1* expression in DRGs of E16.5 control and *NGF* mutant animals shows a reduction in the level of signal per cell in *NGF* mutant DRGs compared to control. Control, 1.00±0.07; *NGF* mutant, 0.49±0.06. p=0.0016, based on paired t test. (**G and H**) In situ hybridization analysis of *Cbfb* expression in DRGs of E16.5 control and *NGF* mutant animals shows more pronounced *Cbfb* mRNA deficit in *NGF* mutant DRGs. Control, 1.00±0.19, *NGF* mutant, 0.49±0.11. p=0.0403, based on paired t test. (**I and J**) Real-time PCR analysis of expression of *Runx1* and *Cbfb* in DRGs of control and *NGF* mutant animals at E14.5 and P0 reveals differential temporal requirements for NGF during expression of *Runx1* and *Cbfb*. Pair t test was performed on data collected from three independent animals per genotype. *$p \leq 0.05$, ns non-significant. (**K-N**) Runx1 immunostaining in DRGs of control and *NGF* mutant animals at E14.5 (K and L) and E16.5 (M and N) shows a Runx1 protein deficit at E16.5, which coincides with the onset of nonpeptidergic deficits in *NGF* mutant DRGs. (**O and P**) Quantification of Runx1 protein expression in DRGs of control and *NGF* mutant animals at E14.5 and E16.5 based on the percentage of $Runx1^+$ neurons and the fluorescence intensity of Runx1 IR. Note that the Runx1 protein deficit becomes evident in *NGF* mutant DRGs at E16.5, when the level of expression is diminished without any change in the number of $Runx1^+$ neurons. Pair t test was performed on data collected from three independent animals per genotype. *$p \leq 0.05$, ns non-significant. (**Q and R**) Double staining of βIII-Tubulin and Flag in dissociated DRG neurons from P0 $Cbfb^{Flag/+}$ animals that were

cultured without or with NGF. Note that NGF application robustly stimulates CBFβ protein expression as indicated by increased Flag IR. Average±SEM for average fluorescence intensity of Flag IR per cell normalized to the –NGF condition is as follows: –NGF, 1.00±0.07; +NGF, 2.72±0.11. $p < 0.0001$, based on unpaired t test. Scale bar, 50μm.

Figure 2.11


Control
NGF-/-; Bax-/-
E14.5
Runx1
Cbfb
A
B
C
D
I
Relative mRNA expression
ns
*
Control
NGF-/-;Bax-/-
E14.5
P0
Runx1
E16.5
E
F
G
H
J
Cbfb
K
L
M
N
O
Runx1+/NeuN+
E16.5
P
Relative fluo. intensity for Runx1
-NGF
+NGF
Q
R
βIII-Tubulin Flag

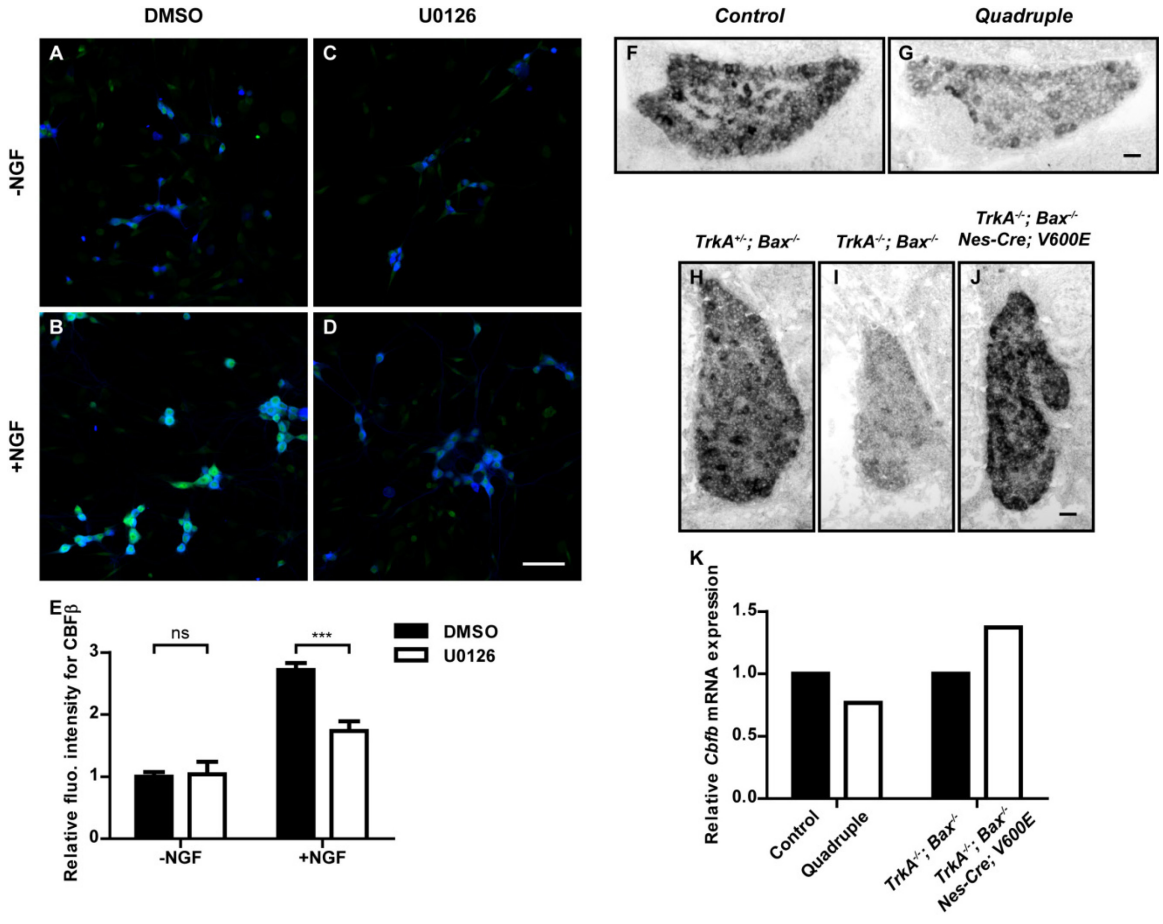
2.6. NGF activates *Cbfb* expression in a MAPK-dependent manner.

To better understand the process of NGF-dependent *Cbfb* expression, which appears to be a crucial step during the specification of nonpeptidergic nociceptors, we sought to identify the downstream signaling cascades that mediate NGF-dependent *Cbfb* expression. We decided to focus on canonical ERK1/2-mediated MAPK signaling, as animals deficient in various components of this signaling pathway exhibited among many other phenotypes, defects in nonpeptidergic nociceptors, such as reduced *Ret* expression and impaired innervation of the epidermis (Newbern et al., 2011; Zhong et al., 2007). In one case, a link to CBFβ was suggested based on a severe deficit in CBFβ protein expression shown by western blot analysis at P30 (Zhong et al., 2007). Nevertheless, a direct test of the involvement of ERK1/2-mediated MAPK signaling in *Cbfb* expression, especially NGF-dependent *Cbfb* expression is lacking. We therefore directly addressed the necessity of MAPK signaling for NGF dependence of *Cbfb* expression in two ways. First, we found *in vitro* that pharmacological inactivation of MEK1/2, the direct activators of ERK1/2, significantly attenuated the ability of NGF to promote CBFβ protein expression (Figure 2.12 A-E). Second, using a conditional loss of function mouse model where the activity of MAPK signaling in the nervous system was expected to be effectively eliminated due to simultaneous deletion of *Erk1/2* and *Mek1/2*, we found a strong dependence of *Cbfb* expression on MAPK signaling *in vivo*, as in 4 out of 5 P0 *Nes-Cre; Mek1*$^{f/f}$*; Mek2*$^{-/-}$*; Erk1*$^{-/-}$*; Erk2*$^{f/f}$ mutants (*Quadruple*), *Cbfb* mRNA expression was severely disrupted (Figure 2.12 F and G). The noted phenotypic variation most likely reflected incomplete excision of all four *flox* alleles. We next asked whether MAPK signaling is sufficient to promote *Cbfb* expression, in the absence of NGF or activation of other NGF signaling pathways. To do this *in vivo*, a constitutively active form of B-raf, an upstream activator of ERK1/2, here referred to as V600E, was expressed specifically in the nervous system in an animal that was also null for both *TrkA* and *Bax*. While *Cbfb* expression in *TrkA*$^{-/-}$*; Bax*$^{-/-}$ DRGs was almost completely abolished, as seen in *NGF* mutant DRGs, constitutive activation of MAPK signaling restored *Cbfb* expression to a near normal level (Figure 2.12 H-J). Real-time PCR analysis was carried out as an independent measure of *Cbfb* expression to further demonstrate the necessity and sufficiency of MAPK signaling for *Cbfb* expression *in vivo* (Figure 2.12 K). Thus, NGF regulates *Cbfb* expression through activation of the MAPK signaling pathway.

Figure 2.12 NGF activates *Cbfb* expression through activation of the MAPK signaling pathway.

(**A-D**) Double staining of Flag (green) and βIII-Tubulin (blue) in DMSO or U0126-treated dissociated DRG neurons from P0 $Cbfb^{Flag/+}$ animals that were cultured without or with NGF. U0126 is a selective inhibitor for MEK1/2, the direct activators of ERK1/2. Note that CBFβ protein expression as defined by Flag IR is greatly diminished in neurons treated with U0126 compared to control-treated neurons in the presence of NGF. (**E**) Quantification of the effect of U0126 treatment on CBFβ protein levels in dissociated DRGs neurons from P0 $Cbfb^{Flag/+}$ animals that were cultured without or with NGF. CBFβ protein abundance was quantified as average fluorescence intensity of Flag IR per cell. Statistical analysis was done using two-way ANOVA with a Bonferroni post-test, based on data from four independent experiments. ***$p \leq 0.001$, ns non-significant. (**F and G**) In situ hybridization analysis of *Cbfb* expression in DRGs of P0 control and *quadruple* mutant animals reveals a severe deficit in *Cbfb* mRNA expression in DRGs when MAPK signaling is disrupted in the nervous system. The same phenotype of varied severity was observed in 4 out of 5 mutant animals. (**H-J**) In situ hybridization analysis of *Cbfb* expression in DRGs of E18.5 $TrkA^{+/-}$; $Bax^{-/-}$, $TrkA^{-/-}$; $Bax^{-/-}$ and $TrkA^{-/-}$; $Bax^{-/-}$; *Nes-Cre; V600E* shows that constitutive activation of MAPK signaling leads to a dramatic increase in *Cbfb* expression in TrkA-deficient animals. Shown are representative images from two independent experiments. (**K**) Real-time PCR analysis of *Cbfb* expression in the same set of loss-of-function and gain-of-function mouse models as described above further demonstrates necessity and sufficiency of MAPK signaling in *Cbfb* expression *in vivo*. Shown are averages from two independent experiments after normalization to littermate control. Scale bar, 50μm.

Figure 2.12


DMSO
U0126
-NGF
+NGF
A
B
C
D
E
Relative fluo. intensity for CBFβ
ns
***
DMSO
U0126
-NGF
+NGF
Control
Quadruple
F
G
TrkA+/-; Bax-/-
TrkA-/-; Bax-/-
TrkA-/-; Bax-/- Nes-Cre; V600E
H
I
J
K
Relative Cbfb mRNA expression
Control
Quadruple
TrkA-/-; Bax-/-
TrkA-/-; Bax-/- Nes-Cre; V600E

2.7. Islet1, a LIM-homeodomain transcription factor, is specifically required for induction of *Runx1* expression.

The apparent NGF-independent *Runx1* expression during early development left the question of transcriptional regulation of *Runx1* expression unanswered, especially the initiation of expression. To explore the possibility that some intrinsic factors control initiation of *Runx1* expression, we tested the involvement of Islet1, a LIM-homeodomain transcription factor, because in a neural crest derivative-specific *Islet1* mutant (*Isl1 CKO*), Runx1 protein deficit was observed as early as E12.5, which is when Runx1 proteins can be first detected in lumbar DRGs (Sun et al., 2008). To directly address at what level *Runx1* expression is regulated by Islet1, *Runx1* expression was assessed by in situ hybridization analysis in DRGs of E12.5 control and *Isl1 CKO* animals. Consistent with a central role for Islet in activating *Runx1* expression at the transcriptional level, *Runx1* transcripts were nearly undetectable in *Isl1 CKO* DRGs (Figure 2.13 A and B). In contrast, *Cbfb* expression was only minimally affected by the same genetic ablation (Figure 2.13 C and D). The differential dependence of *Runx1* and *Cbfb* expression on Islet1was further confirmed by microarray analysis of control and *Isl1 CKO* DRGs at E12.5 (Figure 2.13 E). Thus, Runx1 and CBFβ, obligatory components of a transcription factor complex, appear to be regulated at the expression level by different mechanisms. While *Runx1* expression relies more on intrinsic factors, such as Islet1, *Cbfb* expression depends heavily on extrinsic cues, such as NGF. The significance and general implications of this finding will be discussed in Chapter 3.

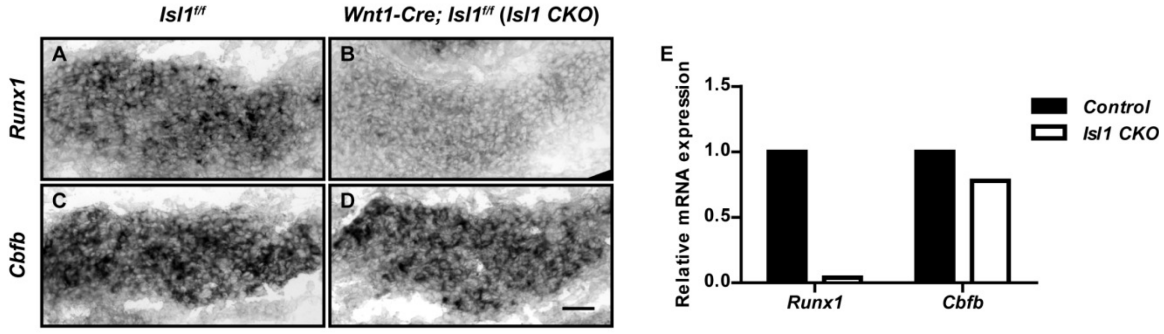


Figure 2.13 Islet1 is required for initiation of *Runx1* expression, however, less so for *Cbfb* expression.

(**A-D**) In situ hybridization analysis of expression of *Runx1* (A and B) and *Cbfb* (C and D) in DRGs of E12.5 control and *Isl1 CKO* animals shows that *Islet1* deficiency abolishes expression of *Runx1* but not *Cbfb* at an early time stage when *Runx1* expression is normally initiated. Shown are representative images from two independent experiments. (**E**) Microarray analysis of E12.5 control and *Isl1 CKO* DRGs further confirms differential dependence of expression of *Runx1* and *Cbfb* on Islet1. Shown are average expression levels from two independent experiments that are further normalized to the control level for each gene. Expression levels have been normalized using globe scaling. Scale bar, 50μm.

2.8. The importance and regulation of the Runx3/CBFβ complex during development of proprioceptors is fundamentally similar to that of Runx1/ CBFβ, with important differences.

It is well established that an analogous role for Runx proteins in the development of proprioceptors is played by Runx3, another member of the Runx protein family. In fact, various *Runx3* knockout animals that were independently generated and analyzed all exhibit the same set of phenotypes indicative of disrupted specification of $TrkC^+$ proprioceptors, such as complete loss of $TrkC^+$ neurons during early embryonic stages, which was attributed to cell death or/and downregulation of TrkC protein expression, derepression of *TrkB* expression in presumptive $TrkC^+$ neurons, and lack of molecular marker and axonal projections characteristic of proprioceptors (Inoue et al., 2007; Inoue et al., 2003; Inoue et al., 2002; Kramer et al., 2006; Levanon et al., 2002; Nakamura et al., 2008). Since it is generally believed that the transcriptional activity of all three Runx proteins is dependent on CBFβ outside the nervous system, we asked what role if any, CBFβ plays in the development of proprioceptors that are Runx3-dependent.

As expected, *Cbfb* was expressed at varying degrees in all $Runx3^+$ neurons during the time window when proprioceptors are being specified, e.g. at E14.5 (Figure 2.14 A-C). As described in *Runx3* knockout animals, in *Cbfb CKO* mice there was an almost complete loss of TrkC protein expression at E13 and a severe reduction in expression of *Parvalbumin* (*PV*), a marker of proprioceptors, at P0 and also throughout embryonic development (data not shown) (Figure 2.14 D-G). Furthermore, both PV and DiI labeling revealed a selective loss of proprioceptive projections to the intermediate and ventral spinal cord in *Cbfb CKO* animals (Figure 2.14 H-K). Proprioceptive innervation of muscle spindles was also completely eliminated in *Cbfb CKO* animal, as visualized by immunostaining for PV or PGP9.5, a panneuronal marker (Figure 2.14 L-O). Thus, similar to its function in nonpeptidergic nociceptors, CBFβ is an essential component of the Runx3 transcription factor complex in proprioceptors.

Mechanistically, however, CBFβ appears to regulate Runx3 function in a way different from what was shown for Runx1. Unlike *Runx1*, whose mRNA level was CBFβ-independent, *Runx3* mRNA expression was defective in *Cbfb CKO* DRGs as early as E13 (Figure 2.14 P-S). Furthermore, a similar *Runx3* expression deficit was observed independent of cell death in *Cbfb/Bax* double mutant DRGs at P0, strongly arguing for a requirement for CBFβ during transcriptional regulation of *Runx3* (Figure 2.15 A-D). However,

whether this represents a requirement for initiation or maintenance of expression remains unclear. In any event, this novel role of CBFβ suggests autoregulation of *Runx3* expression or crossregulation between *Runx* genes, which has long been proposed due to the identification of Runx-binding sites in promoter regions of all three *Runx* genes (Levanon and Groner, 2004). Consistent with this idea, Runx2 can positively regulate its own promoter activity in osteoblasts (Ducy et al., 1999). It is, however, important to note that our data did not distinguish between autoregulation and crossregulation, since ablation of *Cbfb* presumably inactivated all three Runx proteins. A previous finding seemed to favor the crossregulation model, as the promoter activity of *Runx3* indicated by a knockin *LacZ* reporter did not differ between E13.5 control and *Runx3* mutant animals (Lallemend et al., 2012).

Since NT3-TrkC signaling in proprioceptors is equivalent to NGF-TrkA signaling in nociceptors in many respects, we sought to ask whether there is a NT3-CBFβ connection that is analogous to what we described for NGF-CBFβ signaling in nonpeptidergic nociceptors. To our surprise, in situ hybridization analysis revealed little change in *Cbfb* expression in $NT3^{-/-}$; $Bax^{-/-}$ DRGs at P0 (data not shown). This lack of effect however can be explained by the relatively low abundance of Runx3-expressing $Cbfb^{+}$ neurons. To assess *Cbfb* expression more reliably and definitively, we examined *Runx3* expression instead, as its expression was CBFβ-dependent at both mRNA and protein levels in DRGs (Figure 2.15 A-D). Although $Runx3^{+}$ neurons appeared atrophic as a result of *NT3* deficiency at P0, *Runx3* expression, as shown by both in situ hybridization and immunostaining, remained grossly intact, further suggesting that NT3 is not required for *Cbfb* expression in the proprioceptive lineage (Figure 2.15 E-H). One plausible explanation for the difference in the nociceptor and proprioceptor populations is that other trophic factors, such as BDNF, can act either redundantly or compensate for the loss of NT3 to support *Cbfb* expression. In fact, during the time when Runx3-dependent processes are underway, there is large overlap in expression among various Trk receptors, especially between TrkB and TrkC (Kramer et al., 2006). Thus, NT3-TrkC signaling, at least in terms of regulation of *Cbfb* expression, diverges from NGF-TrkA signaling, and the extrinsic cue(s) that are responsible for activating *Cbfb* expression in developing proprioceptors still remain elusive.

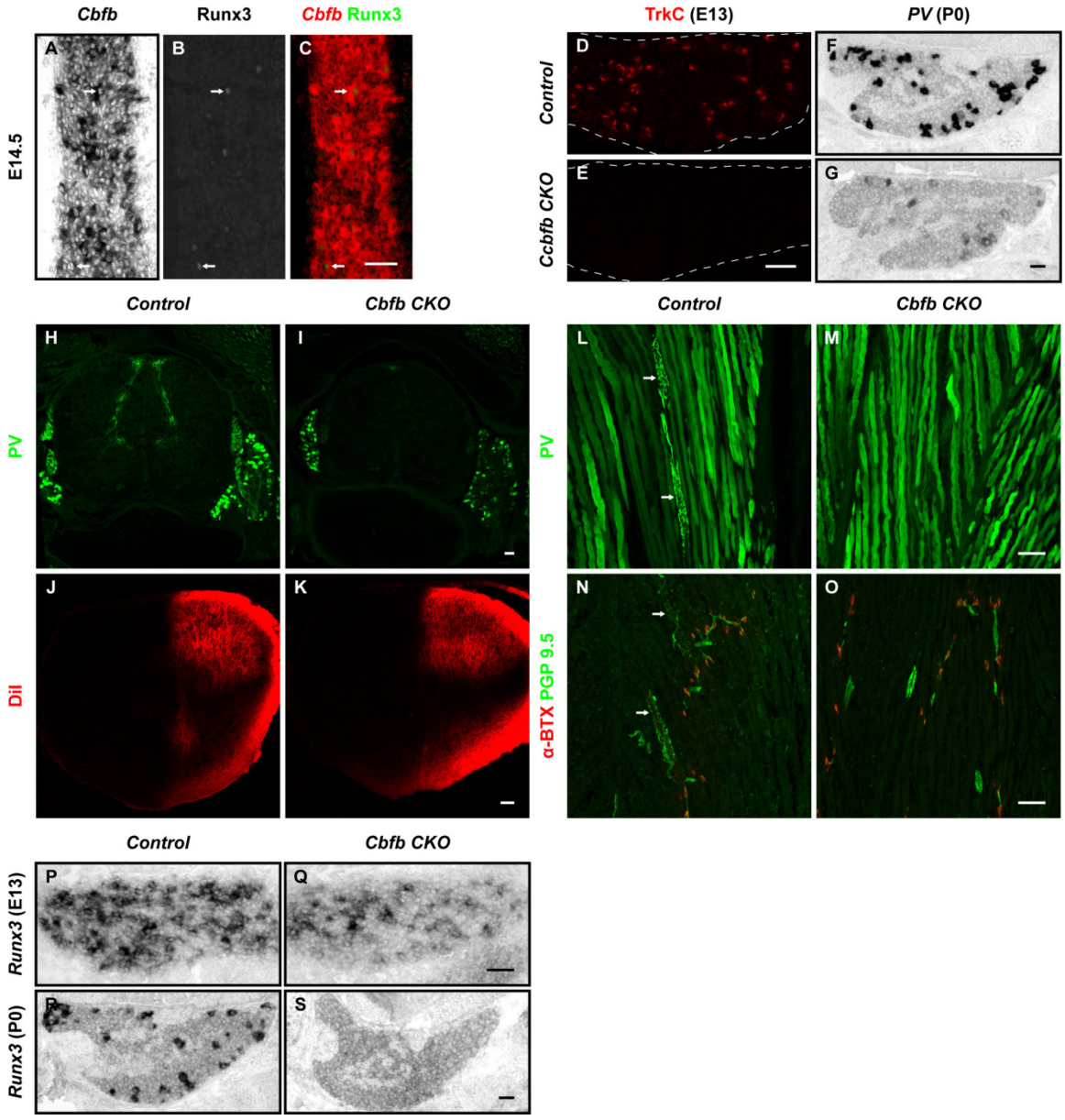
Therefore, on one hand, the Runx3/CBFβ complex acts as a master regulator of proprioceptors, similar to the function of the Runx1/CBFβ complex in nonpeptidergic nociceptors. On the other hand, there are

fundamental differences in the way the complexes are controlled by NGF-TrkA and NT3-TrkC signaling, which are required for analogous Runx1-dependent or Runx3-dependent processes, respectively. While the Runx1/CBFβ complex acts as a key mediator of NGF-TrkA signaling for the specification of the nonpeptidergic fate, the Runx3/CBFβ complex and NT3-TrkC signaling most likely function in parallel pathways.

Figure 2.14 CBFβ is indispensable for the specification of proprioceptors in part by regulating *Runx3* mRNA expression.

(**A-C**) Combined *Cbfb* in situ hybridization and Runx3 immunostaining in E14.5 DRGs shows that all $Runx3^+$ neurons express *Cbfb* mRNA, although at varying levels. Examples of $Runx3^+$ neurons expressing a low or high level of *Cbfb* mRNA are marked by arrows. (**D and E**) TrkC immunostaining in DRGs of E13 control and *Cbfb CKO* animals shows complete loss of $TrkC^+$ neurons in mutant DRGs. Shown are representative images from two independent experiments. (**F and G**) In situ hybridization analysis of *PV* expression in DRGs of P0 control and *Cbfb CKO* animals shows almost complete abolition of *PV* expression in mutant DRGs. Shown are representative images from at least three independent experiments. (**H-K**) Proprioceptive afferents to the spinal cord as shown by PV immunostaining (H and I) and DiI labeling (J and K) in P0 control and *Cbfb CKO* animals. Note that proprioceptive axons fail to innervate their proper targets in the spinal cord which include intermediate laminae and the ventral horn. Shown are results representative of three independent experiments. (**L-O**) Proprioceptive innervation of muscle spindles in hindlimbs of P0 control and *Cbfb CKO* animals as examined by PV immunostaining (L and M) and PGP9.5 staining (N and O). Note that stereotypical terminal structures of Ia afferents, a major type of proprioceptors, marked by arrows, are readily identified in control muscles, but are absent in muscles of mutant animals. $PGP9.5^+$ nerve terminals closely apposed to α-BTX-labeled AChR represent the motor innervation. Shown are results representative of two independent experiments. (**P-S**) In situ hybridization analysis of expression of *Runx3* in DRGs of control and *Cbfb CKO* animals at E13 (P and Q) and P0 (R and S) shows a profound defect in *Runx3* mRNA expression in *Cbfb CKO* DRGs as early as E13 which persists throughout development. Shown are representative images from at least two independent experiments for each time point. Scale bar, 50μm.

Figure 2.14


Cbfb
Runx3
Cbfb Runx3
E14.5
A
B
C
TrkC (E13)
PV (P0)
Control
Ccbfb CKO
D
E
F
G
Control
Cbfb CKO
PV
H
I
L
M
Dil
J
K
α-BTX PGP 9.5
N
O
Control
Cbfb CKO
Runx3 (E13)
Runx3 (P0)
P
Q
R
S

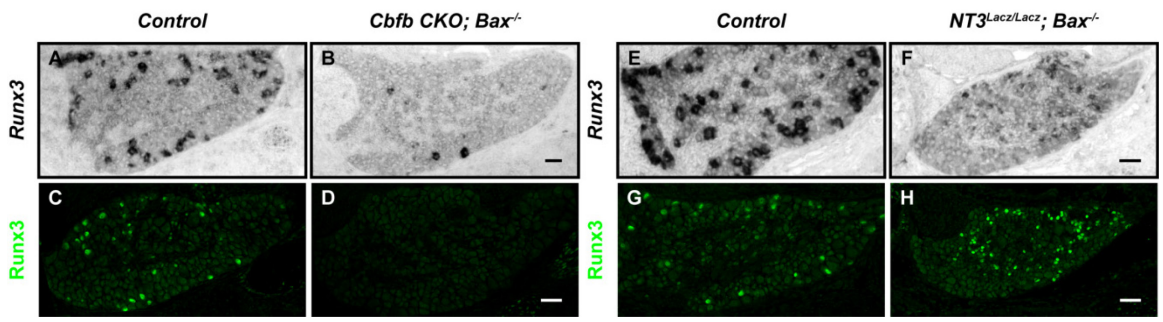


Figure 2.15 NT3 is not required for *Cbfb* expression in proprioceptors.

(**A-D**) Analysis of expression of *Runx3* mRNA (A and B) and Runx3 protein (C and D) in DRGs of P0 control and *Cbfb CKO; Bax*$^{-/-}$ animals shows a severe deficit in *Runx3* expression independent of cell death as a result of *Cbfb* ablation. (**E-H**) Analysis of expression of *Runx3* mRNA (E and F) and Runx3 protein (G and H) in DRGs of P0 control and *NT3*$^{-/-}$*; Bax*$^{-/-}$ animals shows grossly intact *Runx3* expression in atrophic presumptive proprioceptors in *NT3* mutant DRGs, arguing against severely impaired *Cbfb* expression in the absence of NT3. Shown are representative images from two independent experiments. Scale bar, 50µm.

2.9. The site(s) of NGF synthesis required for the specification of nonpeptidergic nociceptors

2.9.1. The spatio-temporal patterns of *NGF* expression

Not only does this newly ascribed role of NGF in regulating *Cbfb* expression add to the growing list of NGF functions, it also illustrates a fundamental problem that the remarkable functional versatility of NGF poses. That is, how the same extrinsic factor NGF elicits physiological responses as diverse as survival, target innervation and subtype specification, in the same population of sensory neurons within a relatively short time window (Harrington and Ginty, 2013; Pezet and McMahon, 2006). Studies of downstream mediators of various NGF effects on sensory neurons indicate engagement of different signaling cascades for different cellular responses (Luo et al., 2007; Riccio et al., 1999; Wickramasinghe et al., 2008). However, it is not known whether or not these distinct biological responses have different activation requirements, such as the threshold level of NGF for activation and the spatial distribution of NGF. Interestingly, there was already *in vitro* evidence showing distinct cellular responses to NGF, depending on the location of NGF application (Campenot, 1977).

A systematic study of the spatial requirement of NGF for its various biological functions relies on extensive information on *NGF* expression which has not been characterized at high resolution due to technical limitations in detection methods. Nonetheless, it is generally believed that NGF is expressed in a wide variety of tissues in both developing and adult animals (Thoenen et al., 1987). Importantly, expression of *NGF* in developing or adult target tissues is correlated with the amount of innervation that they receive from NGF-dependent neuronal populations, including sensory neurons, sympathetic neurons and basal forebrain cholinergic neurons (Davies et al., 1987; Korsching et al., 1985; Korsching and Thoenen, 1983; Shelton and Reichardt, 1984, 1986). This classical view that target-derived NGF is target-derived was however challenged by a series of observations made in rat iris suggesting that Schwann cells rather than smooth muscle cells were a source of NGF for incoming sympathetic and sensory nerves (Finn et al., 1986; Rush, 1984). Therefore, there is a real need for a systematic survey of the temporal and spatial pattern of *NGF* expression at a single cell resolution.

To this end, the endogenous promoter activity of *NGF* was monitored by two different reporters knocked into the *NGF* locus. An NGF^{lacz} reporter was generated for optimal detection sensitivity, whereas an $NGF^{flox\text{-}IRES\text{-}GFP}$ reporter, a variant of a previously characterized NGF^{lx} allele, which also acts as an NGF^{flox} allele, was particularly useful for defining the cellular identity of NGF^{+} cells (Muller et al., 2012). These two lines were characterized with special focus on the trunk region of the animal, which primarily depends on DRG neurons to transmit somatosensory information. Whole-mount X-gal staining revealed a steady increase in *NGF* expression in terms of the level of expression as well as the spatial expanse of NGF^{+} cells from E12.5 to E15.5, which is when an adult-like pattern was mostly established (Figure 2.16 A). Sagittal sections through $NGF^{flox\text{-}IRES\text{-}GFP}$ embryos that were one day apart showed that *NGF* expression in the epidermis lagged behind that in the mesoderm by approximately a day (Figure 2.16 B and C). Notably, the onset of expression in developing epidermis, a major target of nociceptors, coincided with the arrival of $TrkA^{+}$ fibers underneath the developing epidermis, which paralleled earlier observations in the trigeminal system (Davies et al., 1987). It is however important to note that although the general pattern of *NGF* expression was comparable in $NGF^{Lacz/+}$ and $NGF^{flox\text{-}IRES\text{-}GFP/+}$ animals, expression was always detected in $NGF^{lacz/+}$ earlier than in $NGF^{flox\text{-}IRES\text{-}GFP/+}$ animals. Since the developing skin represents the best characterized target of nonpeptidergic nociceptors, we further characterized the cell types that produce NGF in the skin of the hindlimb of both reporter mice at P0. In addition to being expressed relatively diffusely and broadly in what seemed to be dermal fibroblasts, *NGF* was most prominently expressed in three discrete domains: the basal keratinocytes of the epidermis, cells associated with a subset of blood vessels, and cells along nerve bundles (Figure 2.16 D and E). Colabeling with cell type-specific markers established blood vessel-associated cells as pericytes (Figure 2.16 F and G). Furthermore, it nicely illustrated the close association between nerve fibers and GFP^{+} cells, which can be either Schwann cells or perineural fibroblasts based on the tight coupling with the nerve (Figure 2.16 F and G). Although data were not shown here, those three discrete domains of *NGF* expression were already established at E14.5, suggesting important developmental roles. In particular, the expression of *NGF* in pericytes only along a subset of blood vessels that did not appear to belong to a certain type may be physiologically relevant, considering well-documented variations in vascular sympathetic innervation (Birch et al., 2008; Fleming et al., 1989; Grasby et al., 1999; Ruffolo et al., 1991; Tan et al., 2007). Since skin blood vessels were shown

to be simultaneously innervated by both sensory and sympathetic neurons, it is conceivable that vascular-derived NGF in the skin acts to coordinate innervation of skin blood vessels by sensory and sympathetic neurons (Ruocco et al., 2002). The expression of *NGF* along developing nerves is most provocative, in that if biologically active NGF is indeed produced from these transcriptionally active cells, the classic view of NGF as a target-derived trophic factor will have to be modified. However, since this analysis did not distinguish between NGF and proNGF, the functionally distinct precursor form of NGF, the biological function of this expression domain of *NGF*, if it is indeed actively translating, may be difficult to predict. Thus, while this set of expression analyses represents the first attempt at generating a high resolution spatio-temporal map of the promoter activity of *NGF*, it does not address the functional significance of any of the identified sources, which requires phenotypic analysis of tissue-specific *NGF* conditional mutants.

2.9.2. Lineage-specific requirement of NGF for development of nociceptors, in particular, NGF-dependent *Cbfb* expression

Due to the early onset and the wide distribution of *NGF* expression, which inevitably hampered our ability to dissect the functional role of individual sources of NGF defined based on cell type, the spatial requirement for NGF was instead studied by removing NGF from specific lineages from which NGF^+ cell types are derived. That includes the neural crest lineage, the epidermal lineage, and the mesodermal lineage, for specifically targeting the expression in Schwann cells, keratinocytes and fibroblasts, respectively.

The validity of the NGF^{flox} allele was previously demonstrated and further confirmed here with the use of a *T-Cre* line, which drives recombination predominately but not exclusively in a pan-mesodermal pattern (Perantoni et al., 2005). Careful analysis of the pattern of Cre activity using a Cre-dependent tdTomato reporter at both E11.5 and P0 revealed extensive labeling throughout, as well as outside mesodermal lineages (data not shown). Overall, Cre activity was detected in all the previously defined *NGF* expression domains at the time when *NGF* expression was barely detectable. Therefore, as expected, *T-Cre;* $NGF^{f/-}$ conditional mutants ($NGF^{T\text{-}Cre}$) exhibited marked loss of DRG neurons as a result of selective depletion of $TrkA^+$ nociceptors, reminiscent of *NGF* straight knockouts (Figure 2.17 A-D). The remaining nociceptors, although reduced in number, appeared to undergo subtype specification normally, resulting in a normal complement of $CGRP^+$ peptidergic and $MrgD^+$ nonpeptidergic nociceptors (Figure 2.17 E-H). Quantitative

differences in deficits in nociceptors between $NGF^{T\text{-}Cre}$ and straight knockouts likely reflected incomplete or late *NGF* ablation in $NGF^{T\text{-}Cre}$.

In light of the possibility that nerve-associated cells represent Schwann cells, which are of neural crest origin, we examined the biological consequence of *NGF* deletion in the neural crest lineage mediated by *Wnt1-Cre*.

At first glance, *Wnt1-Cre; NGF*$^{f/-}$ mutants ($NGF^{Wnt1\text{-}Cre}$) did not display gross abnormalities in nociceptors with respect to the total neuronal number, or expression of generic and subtype-specific markers of nociceptors, or epidermal innervation, suggesting *NGF* expression in Schwann cell is not an important source of NGF (Figure 2.18 A-J). Interestingly, there was a trend towards increased total neuronal number in $NGF^{Wnt1\text{-}Cre}$ animals (Figure 2.18 A), which most likely resulted from increase in all different classes of DRG neurons, since the relative proportions of TrkA, TrkB and TrkC neurons remained unchanged (data not shown). This paradoxical observation, if later verified, may suggest a specific role for Schwann cell-derived NGF, most likely in the form of proNGF, in fine-tuning the number of DRG neurons.

About three-quarters of epidermal fibers arise from $MrgD^+$ neurons which exclusively terminate in the epidermis, making an epidermal-specific *NGF* conditional mutant a particularly attractive model to rigorously test the neurotrophic hypothesis at least for a major population of nonpeptidergic nociceptors. Epidermal-specific *Cre* expression was achieved through the use of a well-characterized *K5-Cre* line (Ramirez et al., 2004). So far, deleting *NGF* specifically in the epidermis has resulted in modest but consistent defects in nociceptors, including a small reduction in the number of total DRG neurons, $TrkA^+$, $CGRP^+$, $MrgD^+$ neurons and a relatively bigger reduction in epidermal innervation density (Figure 2.18 K-T). Given that the epidermis is the preferred target of nonpeptidergic but not peptidergic nociceptors, $MrgD^+$ nonpeptidergic nociceptors were, as expected, affected more than $CGRP^+$ peptidergic nociceptors (Figure 2.18 N-Q). Therefore, for the most part, our analysis of the *K5-Cre; NGF*$^{f/-}$ mutant ($NGF^{K5\text{-}Cre}$) demonstrates that $MrgD^+$ nonpeptidergic nociceptors depend on target-derived NGF for survival. Interestingly, reduced sensitivity to mechanical stimulation at a behavioral level was reported in a different epidermal-specific *NGF* deficient line, which could be explained by the reduction in the number of $MrgD^+$ neurons and their epidermal endings described here (Davis et al., 1993). However, the apparent

heterogeneous requirement for target-derived NGF, among many other possibilities, some of which might be attributed to the late onset of the *K5-Cre* relative to *NGF* expression in the epidermis (data not shown), seems to suggest a substantial contribution of NGF produced in cells other than the final target, such as numerous fibroblasts that nerve fibers encounter en route to the final target.

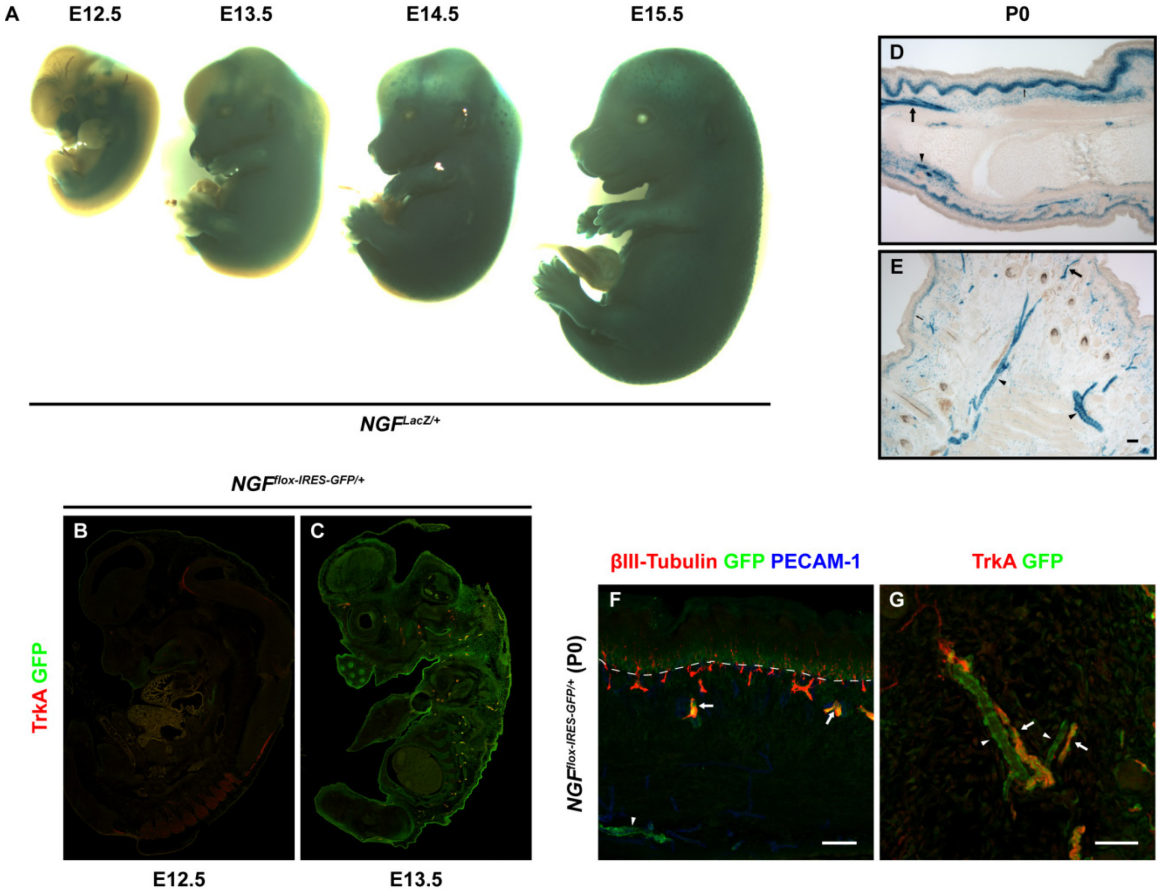
Therefore, it is crucial to directly assess the mesoderm-specific requirement of NGF for the development of nociceptors, especially nonpeptidergic nociceptors. Preliminary analysis of $Twist2^{Cre/+}; NGF^{f/-}$, a mesoderm-specific *NGF* conditional mutant, so far, has shown only limited deficits in nociceptors, which appeared to preferentially affect peptidergic nociceptors (Sosic et al., 2003). Since it was difficult to determine to what extent and how early *NGF* in mesodermal lineages was removed by $Twist2^{Cre/+}$, an alternative mesoderm-specific conditional strategy based on $Mesp1^{Cre/+}$ is currently being developed to better dissect the function of mesoderm-derived NGF (Saga et al., 1999).

Since none of the lineage-specific *NGF* conditional mutants we have analyzed so far exhibit dramatic phenotypes, the possibility that different sources of NGF act redundantly and significantly compensate for each other is worth exploring. Moreover, we have yet to find the source(s) of NGF responsible for *Cbfb* expression, because none of those conditional mutants showed altered *Cbfb* expression in DRGs (data not shown). It is possible that the nonpeptidergic nociceptors require the same source(s) of NGF for both survival and maturation. In that case, a defect in *Cbfb* expression can only be revealed by *Bax* codeletion.

Figure 2.16 The spatial and temporal pattern of *NGF* expression during development

(**A**) Whole-mount X-gal staining of $NGF^{Lacz/+}$ embryos from E12.5 to E15.5 shows widespread *NGF* expression that gradually increases until an adult-like pattern is established at E15.5. (**B and C**) Double staining of TrkA and GFP on sagittal sections of $NGF^{flox\text{-}IRES\text{-}GFP/+}$ embryos at E12.5 and E13.5 shows that *NGF* starts to be expressed in the epidermis, when TrkA+ fibers reach the base of epidermis. Shown are representative images from at least two independent experiments for each timepoint. (**D and E**) X-gal staining on sections through the hindlimb (D) and the back hair skin (E) of P0 $NGF^{Lacz/+}$ animals. Three prominent domains of *NGF* expression are noted, namely, basal keratinocytes in the epidermis (thin arrows), blood vessel-associated cells (arrowheads) and nerve bundle-associated cells (thick arrows). Shown are representative images from at least two independent experiments. (**F and G**) Immunostaining of βIII-Tubulin, GFP and PECAM1 (F) or TrkA and GFP (G) on sections through the hindlimb of P0 $NGF^{flox\text{-}IRES\text{-}GFP/+}$ animals. Note that GFP IR encircling PECAM-1^{+} endothelial cells in (F) reflects expression in pericytes. Examples of *NGF* expression in pericytes and nerve-associated cells are marked by arrowheads and arrows respectively. Shown are representative images from more than three experiments. Scale bar, 50μm.

Figure 2.16


A
E12.5
E13.5
E14.5
E15.5
P0
D
E
NGF LacZ/+
NGF flox-IRES-GFP/+
B
C
TrkA GFP
E12.5
E13.5
βIII-Tubulin GFP PECAM-1
TrkA GFP
F
G
NGF flox-IRES-GFP/+ (P0)

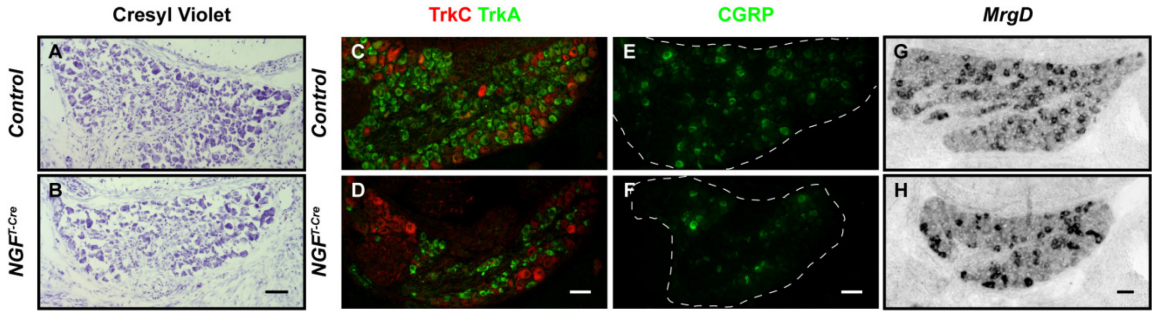


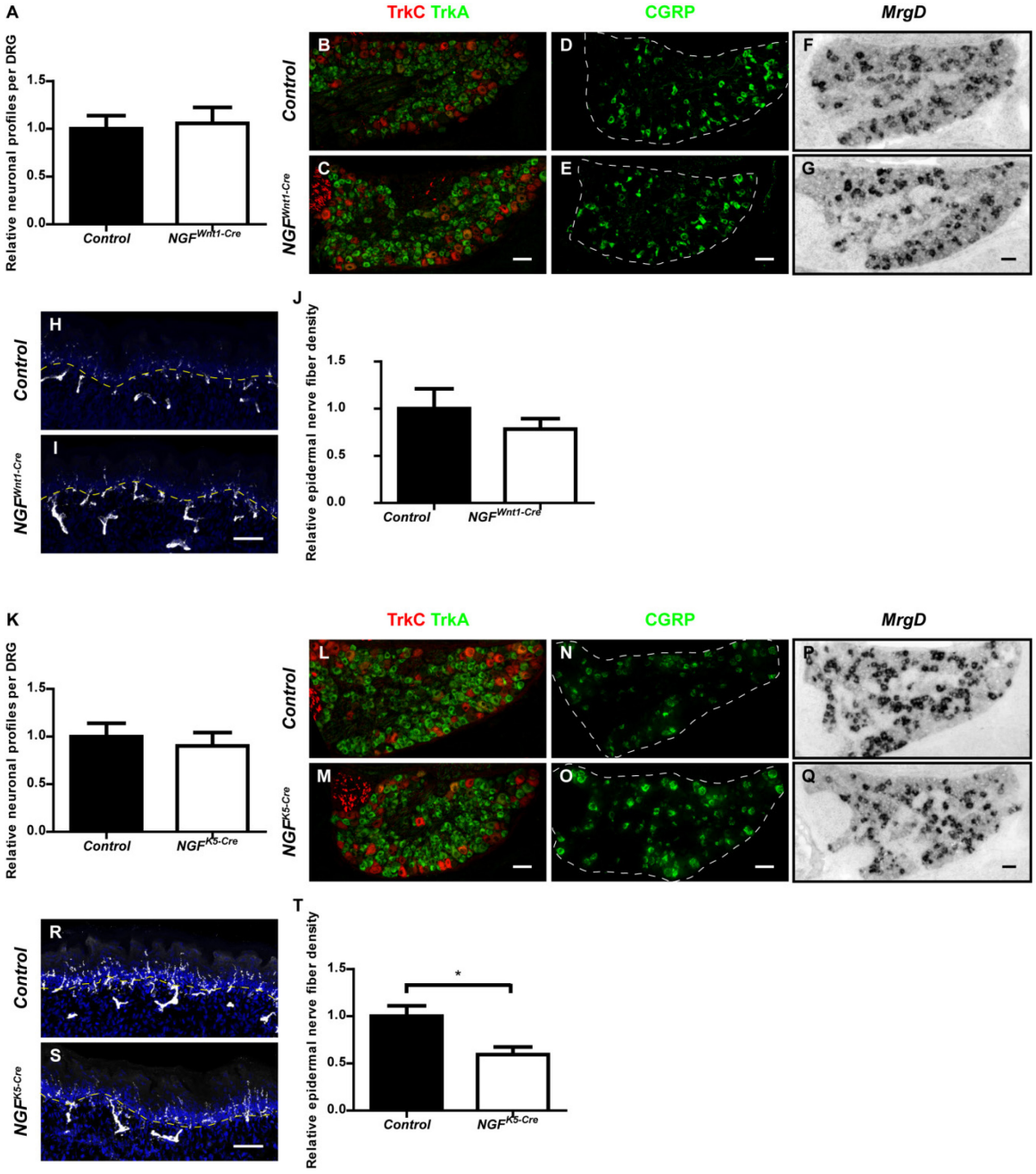
Figure 2.17 Confirmation of the validity of the *NGF^{flox}* allele

(**A and B**) Cresyl violet staining of total DRG neurons in T12 DRGs of P0 control and *NGF$^{T\text{-}Cre}$* animals reveals substantial loss of neuronal profiles in mutant DRGs relative to control. (**C and D**) Double staining of TrkA and TrkC in DRGs of P0 control and *NGF$^{T\text{-}Cre}$* animals shows a selective loss of TrkA^{+} neurons in mutant DRGs relative to control. (**E and F**) CGRP staining in DRGs of P0 control and *NGF$^{T\text{-}Cre}$* animals shows a dramatic reduction in the number of CGRP^{+} neurons in mutant DRGs relative to control. (**G and H**) In situ hybridization analysis of *MrgD* expression in DRGs of P0 control and *NGF$^{T\text{-}Cre}$* animals reveals a similarly dramatic reduction in the number of *MrgD*$^{+}$ neurons in mutant DRGs relative to control. Shown from A-H are representative images from two independent experiments. Scale bar, 50μm.

Figure 2.18 Lineage-specific contributions of NGF to survival and subtype specification of nociceptors

(**A**) Total neuronal counts in T12 DRGs of P0 control and $NGF^{Wnt1\text{-}Cre}$ animals by cresyl violet staining shows a paradoxical increase in total neuronal number in mutant DRGs relative to control. Data from three independent experiments are compiled and shown as mean with SEM. (**B and C**) Double staining of TrkA and TrkC in DRGs of P0 control and $NGF^{Wnt1\text{-}Cre}$ animals. (**D and E**) CGRP staining in DRGs of P0 control and $NGF^{Wnt1\text{-}Cre}$ animals. (**F and G**) In situ hybridization analysis of *MrgD* expression in DRGs of P0 control and $NGF^{Wnt1\text{-}Cre}$ animals. (**H-J**) Sensory innervation of the epidermis of P0 control and $NGF^{Wnt1\text{-}Cre}$ animals as shown by βIII-Tubulin immunostaining. The extent of sensory innervation is quantified based on the fraction of the epidermis that is occupied by βIII-Tubulin^{+} fibers. Yellow doted lines demarcate the epidermal-dermal junctions. From B to J, there are no noticeable differences between control and mutants which is consistent across three independent experiments. (**K**) Total neuronal counts in T12 DRGs of P0 control and $NGF^{K5\text{-}Cre}$ animals by cresyl violet staining shows a modest but consistent decrease in total neuronal number in mutant DRGs relative to control. Data from three independent experiments are compiled and shown as mean with SEM. (**L and M**) Double staining of TrkA and TrkC in DRGs of P0 control and $NGF^{K5\text{-}Cre}$ animals shows a slight reduction in the number of TrkA^{+} neurons in mutant DRGs compared to control. (**N and O**) CGRP staining in DRGs of P0 control and $NGF^{K5\text{-}Cre}$ animals shows a comparable number of CGRP^{+} neurons per section. (**P and Q**) In situ hybridization analysis of *MrgD* expression in DRGs of P0 control and $NGF^{K5\text{-}Cre}$ animals shows a noticeable reduction in the number of *MrgD*$^{+}$ neurons in mutant DRGs relative to control. (**R-T**) Sensory innervation of the epidermis of P0 control and $NGF^{K5\text{-}Cre}$ animals as shown by βIII-Tubulin immunostaining reveals reduced innervation density in the epidermis in the mutant relative to control. The extent of sensory innervation is quantified based on the fraction of the epidermis that is occupied by βIII-Tubulin^{+} fibers. Yellow doted lines demarcate the epidermal-dermal junctions. Unpaired t test was performed on data collected from three independent experiments. * $p \leq 0.05$. Scale bar, 50μm.

Figure 2.18


A
Relative neuronal profiles per DRG
1.5
1.0
0.5
0.0
Control
NGF Wnt1-Cre
TrkC TrkA
CGRP
MrgD
B
D
F
Control
C
E
G
NGF Wnt1-Cre
H
Control
I
NGF Wnt1-Cre
J
Relative epidermal nerve fiber density
1.5
1.0
0.5
0.0
Control
NGF Wnt1-Cre
K
Relative neuronal profiles per DRG
1.5
1.0
0.5
0.0
Control
NGF K5-Cre
TrkC TrkA
CGRP
MrgD
L
N
P
Control
M
O
Q
NGF K5-Cre
R
Control
S
NGF K5-Cre
T
Relative epidermal nerve fiber density
*
1.5
1.0
0.5
0.0
Control
NGF K5-Cre

2.10. Discussion

This study uncovers a gene regulatory mechanism underlying the specification of nonpeptidergic nociceptors. At the core of this process is the formation of the Runx1/CBFβ complex that depends on both components for directing the nonpeptidergic-specific transcriptional program. Importantly, the expression of each component is to a large degree independently regulated at the transcriptional level. On one hand, *Cbfb* expression is strongly activated by NGF, a functionally important extrinsic signal. This transcriptional regulation is mediated by the MAPK signaling pathway. On the other hand, initiation of *Runx1* expression is critically dependent on Islet1, a central intrinsic factor during early development of sensory neurons. Furthermore, the Runx1/CBFβ complex, through an unknown mechanism, maintains a high level of NGF-TrkA signaling, which is central to an important characteristic of nonpeptidergic nociceptors, namely postnatal upregulation of *Ret*. Thus, the initiation of a lineage-specific differentiation program is tightly controlled by a convergence of extrinsic and intrinsic factors at the level of a heterodimeric lineage-specific transcription factor complex. The bidirectional regulation between growth factor signaling and a lineage-specific transcription factor complex in the form of a positive feedback loop confers robustness on that particular lineage identity (Figure 2.19).

The Runx1/CBFβ complex, a coincidence detector for permissive extrinsic and intrinsic cues for specification of nociceptor subtypes

In this study, we found that the activity of the Runx1/CBFβ complex is regulated at the level of expression of *Runx1* and *Cbfb* by Islet1 and NGF, respectively. The Runx1/CBFβ complex therefore acts as a coincidence detector for extrinsic and intrinsic conditions that are conducive to the specification of nonpeptidergic nociceptors. The requirement for NGF as an example of such extrinsic conditions ensures that only nociceptors that survive the competition for NGF during naturally occurring cell death undergo further differentiation. Consistent with this idea, *Cbfb* expression exhibits NGF dependence at E14.5, immediately after the onset of the developmental cell death. The identification of putative cAMP-response element (CRE) sites in an evolutionally conserved 458 bp enhancer-like element upstream of the *Cbfb* gene (data not shown) suggests an interesting possibility that CREB family members coordinate these two NGF dependent processes by mediating the upregulation of both pro-survival genes, such as *Bcl-2*, and *Cbfb*

sequentially (Liu et al., 1999; Riccio et al., 1999). In broader terms, this model we propose represents one way in which the same growth factor can be reused in different developmental processes. Thus, activation of the same transcription factor effector mediates expression of different target genes depending on developmental stages. Therefore, it is important to directly test this model by addressing the requirement of CREB family members for *Cbfb* expression. Islet1 dependence of *Runx1* expression on the other hand ensures that the specification of nonpeptidergic nociceptors takes places after sensory neurogenesis, because Islet1was previously shown to coordinate the orderly transition from pan-sensory neurogenesis to subtype specification (Sun et al., 2008). In fact, in the absence of Islet1, there was in addition to a profound defect in *Runx1* expression, abnormal persistence of expression of genes that are key regulators of sensory neurogenesis, such as *Neurog1* (Sun et al., 2008). Therefore, the fact that induction of Runx1 expression, and hence the activation of the Runx1/CBFβ complex, is temporally coupled to sensory neurogenesis at least in part reflects their common dependence on Islet1.

Overall, both NGF and Islet1 impose important constraints on the timing of activation of the Runx1/CBFβ complex. The importance of precisely timed activation of the Runx1/CBFβ complex remains largely unaddressed. Nonetheless, a detrimental effect of precocious *Runx1* expression was described in one extreme case, where ectopic expression of *Runx1* in neural crest cells (NCCs) prior to sensory neurogenesis suppressed the multipotency of NCCs (Marmigere et al., 2006).

Given that NGF-dependent Islet1^{+} sympathetic neurons do not express Runx1 (data not shown), we expect to identify additional regulatory mechanisms of expression of *Runx1* and *Cbfb*, resulting in a better understanding of intrinsic and extrinsic environments essential for the generation of nociceptors subtypes.

Sufficiency of the Runx1/CBFβ complex for the specification of nonpeptidergic nociceptors

Although in the present study we provide evidence that the Runx1/CBFβ complex mediates NGF-dependent development of nonpeptidergic nociceptors, it is not known to what extent this NGF effect is mediated by a Runx1/CBFβ-independent mechanism. In other words, we have yet to address the sufficiency of the Runx1/CBFβ complex for the specification of nonpeptidergic nociceptors in a heterologous system in order to determine whether a lineage-specific transcription factor plays an

instructive role or a permissive role in lineage commitment. Two lines of evidence from previous studies argue against an instructive role for Runx1 in promoting the nonpeptidergic nociceptor lineage. First, constitutive expression of Runx1 in most nociceptors using *Nav1.8*^{Cre} selectively impaired the molecular features characteristic of Runx1-transient populations such as peptidergic nociceptors, but failed to promote expression of nonpeptidergic-specific genes that define Runx1-persistent nonpeptidergic nociceptors (Abdel Samad et al., 2010). This apparent inability to redirect peptidergic nociceptors to nonpeptidergic nociceptors together with the dramatic effect on nonpeptidergic nociceptors in *Runx1 CKO* animals seems to suggest a permissive role for Runx1 in specifying the nonpeptidergic lineage by suppressing the alternative peptidergic fate. The basic principle of this proposed mechanism was previously observed in other developing tissues, most notably in the ventral spinal cord for progenitor domain specification (Muhr et al., 2001). It is important to note however that this argument suffers from at least two caveats. First, the potential instructive role of Runx1 might be stage-dependent. Considering that forced *Runx1* expression driven by *Nav1.8*^{Cre} is considerably late compared to normal onset of *Runx1* expression, an instructive role, if there is one, might only be revealed by constitutively expressing *Runx1*from an early time point. Second, since we showed that both CBFβ and Runx1 are required for Runx1activity by forming a heterodimeric complex, the amount of complexes and therefore Runx1 activity would be expected to be influenced by the expression level of each component. Therefore, the lack of effect on nonpeptidergic-specific gene expression might be attributed to ineffective activation of overexpressed Runx1 in spite of an apparent increase in *Runx1* expression due to an imbalance between Runx1 and CBFβ. The second piece of evidence came from the observation that overexpression of *Runx1* in the ventricular zone of E12.5 spinal cord failed to bypass the NGF requirement for induction of nonpeptidergic-specific genes (Lopes et al., 2012). This finding, among many other possibilities, suggests Runx1 *per se* is not a primary mediator of NGF-dependent nonpeptidergic-specific gene expression, which is consistent with a permissive role for Runx1 in specification of the nonpeptidergic fate. For the reason that was stated before, it would be premature to rule out an instructive role for Runx1, before a similar experiment is performed where both Runx1 and CBFβ are expressed in the presence or absence of NGF. However, considering the prolonged delay between the onset of *Runx1* expression and that of expression of most of the Runx1-dependent nonpeptidergic-specific genes *in vivo*, it is more than likely that at least for some of these nonpeptidergic-specific genes, their

expression requires a yet-unidentified mediator of NGF for expression. Thus, whether or not Runx1 plays an instructive or permissive role in promoting commitment of the nonpeptidergic fate remains an open question. In fact, work outside the nervous system has provided support for both roles of Runx proteins suggesting that Runx functions are highly context-dependent (Kappes, 2010; Zhao et al., 2005).

The mechanism underlying diversification of nociceptors

As alluded to previously, the extrinsic and intrinsic events that we describe in the study are permissive conditions for the nonpeptidergic cell fate. In other words, they themselves do not instruct lineage choice, as neither of the requirements, be it cell-intrinsic or cell-extrinsic, distinguish between nonpeptidergic and peptidergic nociceptors. Specifically, NGF-TrkA signaling is required for survival, target innervation and normal phenotypic development of both peptidergic and non-peptidergic nociceptors (Harrington and Ginty, 2013). Due to persistent *TrkA* expression in peptidergic nociceptors in adulthood, peptidergic nociceptors continue to respond to NGF and contribute to hyperalgesia induced by NGF or inflammation (Lewin and Mendell, 1993; Pezet and McMahon, 2006). As for Islet1, its widespread expression during development, and the dramatic loss of virtually all nociceptors in its absence, indicate a general requirement for Islet1 during nociceptor development (Sun et al., 2008). Therefore, the specific cues governing divergence of the two main nociceptive lineages remain to be elucidated.

Considering that Runx1is the only lineage-specific transcription factor known to be important for differentiation of nociceptive lineages, and the expression level of *Runx1* represents the earliest known property of nociceptors that distinguishes future nonpeptidergic and peptidergic lineages, differential regulation of the level of *Runx1* expression seems to be an attractive mechanism to drive the segregation of nonpeptidergic and peptidergic nociceptors. In fact, in the hematopoietic system, there is compelling evidence that expression levels of transcriptional regulators of specific lineages represent a crucial determinant of cell-fate decisions (Rosenbauer and Tenen, 2007). Since differential expression of *Runx1* at both mRNA and protein levels, which were correlated, was observed by us and others as early as E14.5, and in a most recent study even at E12.5, the mechanism underlying differential levels of *Runx1* expression seems to operate at the transcriptional level prior to that time (Chen et al., 2006b; Hadjab et al., 2013). The regulation of *Runx1* expression by islet1 is unlikely to directly contribute to differences in the level of

Runx1 expression, since there was little correlation between the level of expression of *Islet1* and that of *Runx1* at the single cell level (data not shown). The late dependence of *Runx1* expression on NGF-TrkA signaling also fails to explain the difference in *Runx1* expression at E14.5, since at that time *Runx1* expression is still NGF-independent. Despite exhibiting varying levels of expression itself, CBFβ is unlikely to contribute to differential expression levels of *Runx1*significantly, considering the lack of effect of *Cbfb* ablation on *Runx1* mRNA level. Varying levels of *Cbfb* expression may instead reflect differences in Runx1 activity as a result of different levels of *Runx1* expression, since based on genetic evidence, *Cbfb* expression is normally suppressed by Runx1(data not shown).

During the search of additional regulators of *Runx1*mRNA expression in particular those that can confer graded expression levels of *Runx1*, a few signaling pathways including fibroblast growth factor (FGF), transforming growth factor-beta (TGF-beta)/bone morphogenic protein (BMP) signaling stand out as possible candidates based on evidence from non-neural systems (Levanon and Groner, 2004). Most relevant of all is FGF signaling. In light of overlapping expression patterns of *Runx1* and *TrkA* in the DRG, it seems particular interesting that basic FGF-induced *Runx1* expression was associated with *TrkA* induction in an olfactory neuroblastoma cell line (Nibu et al., 2000). Indeed, a role for a local source of FGF in initiating *Runx1* expression was recently identified (Hadjab et al., 2013). Further study is needed to explore the possibility that graded activities of FGF signaling generate the initial difference in level of *Runx1* expression, thereby initiating the process of nociceptor diversification.

A common mechanism of integrating intrinsic and extrinsic signals for postmitotic differentiation of neuronal subtypes

In broader terms, the mechanism that we uncovered in this study illustrates the importance of the interplay between extrinsic and intrinsic factors in postmitotic specification of neuronal subtypes. It is important to make a clear distinction between this relatively late process and the specification of progenitor domains which takes place before cell cycle exit. While it has been well established in vertebrate systems, based on work in the developing spinal cord, cerebral cortex and retina, that specification of progenitor domains is coordinately regulated by both the intrinsically defined competence state and a combination of spatially and temporally controlled extrinsic signals, much less is known about the relative roles of extrinsic cues,

intrinsic factors and their interaction in postmitotic specification of a given neuronal subtype, despite increasing knowledge about specific molecular players essential for specification of various neuronal subtypes(Briscoe and Novitch, 2008; Livesey and Cepko, 2001; Molyneaux et al., 2007) . Thus, our study represents one of a few examples where the interaction between extrinsic and intrinsic regulators is shown to be crucial for postmitotic differentiation of a functionally important neuronal population. Furthermore, the gene regulatory mechanism described in this research allows us to propose a simple model for coordinate control of a lineage-specific differentiation program by a convergence of extrinsic and intrinsic signals. That is, to subject expression of the two subunits of a lineage-specific heterodimeric transcription factor complex to transcriptional control of extrinsic and intrinsic factors, respectively. Considering its simplicity and adaptability, this model may prove relevant for a broad range of neuronal subtypes.

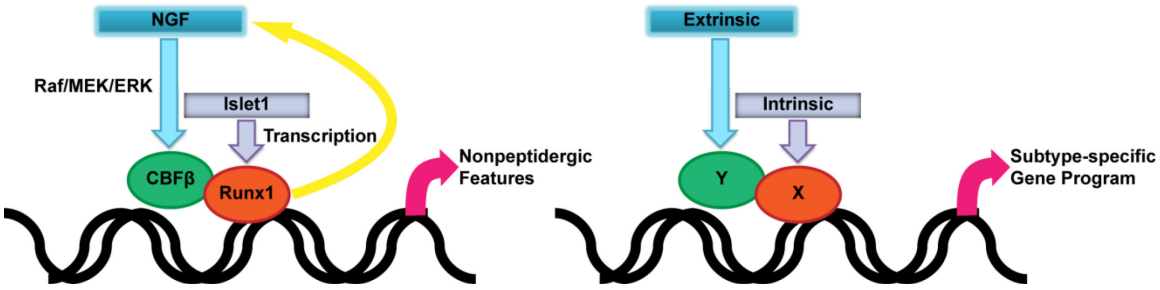


Figure 2.19 Schematics illustrating the molecular mechanism underlying specification of nonpeptidergic nociceptors and its general implication in the context of subtype specification

Runx1 and CBFβ, which function as a heterodimeric transcription factor complex, are both required for expression of Runx1-dependent genes that defines nonpeptidergic features. Importantly, the transcriptional regulation of expression of *Cbfb* and *Runx1* involves fundamentally different mechanisms. While *Cbfb* expression is strongly activated by NGF, an extrinsic cue, in a MAPK-dependent manner, the induction of *Runx1* expression critically depends on Islet1, an intrinsically determined transcription factor. Furthermore, the Runx1/CBFβ complex, through an unknown mechanism, enhances the level of NGF-TrkA signaling, resulting in a positive feedback loop between NGF-TrkA signaling and the Runx1/CBFβ complex. In broader terms, this gene regulatory mechanism not only underscores the importance of the interplay between extrinsic and intrinsic factors during multilineage differentiation, but also illustrates how such interplay controls a cell-fate decision- through a convergence of extrinsic and intrinsic signals at the level of a lineage-specific heterodimeric transcription factor complex.

Materials and methods

Embryo stages

Noon of the day when a vaginal plug was observed in mated females was designated as E0.5. The stages of embryos were further confirmed based on their external features after dissection.

Mouse lines

The mouse lines including *NGF*$^{-}$, *Bax*$^{-}$, *Runx1*^{f}, *Runx1*^{CreER}, *Tau*^{mGFP}, *Wnt1-Cre*, *T-Cre*, *K5-Cre*, *Twist2*^{Cre}, *Mesp1*^{Cre}, *NGF*$^{flox\text{-}IRES\text{-}GFP}$, *Nes-Cre*, *Mek1*^{f}, *Mek2*$^{-}$, *Erk1*$^{-}$, *Erk2*^{f}, *TrkA*^{Lacz}, *Braf*^{V600E} and *NT3*^{Lacz} have been described before (Belanger et al., 2003; Bissonauth et al., 2006; Chen et al., 2006b; Crowley et al., 1994; Danielian et al., 1998; Farinas et al., 1994; Hippenmeyer et al., 2005; Knudson et al., 1995; Mercer et al., 2005; Moqrich et al., 2004; Muller et al., 2012; Nekrasova et al., 2005; Perantoni et al., 2005; Ramirez et al., 2004; Saga et al., 1999; Samokhvalov et al., 2007; Samuels et al., 2008; Sosic et al., 2003; Tronche et al., 1999). The *NGF*^{Lacz} mouse line was generated from a targeting vector (PG00138_X_3_C11) that was designed with a conditional potential obtained through EUCOMM. The linearized vector was electroporated into mouse 129S6SvEvTac ES cells. ES clones were screened by PCR and correctly targeted ES clones were confirmed by southern blot hybridization using both 5' and internal probes following SpeI digestion. After germ-line transmission, animals heterozygous for the mutant allele were crossed with female *Sox2-Cre* mice that express Cre in germ cells to remove the *Neo* cassette (Hayashi et al., 2002). For the *Cbfb*^{f} allele, a 2 kb sequence containing a1 kb sequence immediately upstream of the transcriptional start site as well as exon 1 and exon 2 of the *Cbfb* locus was flanked by two *loxP* sites. A two-step recombineering protocol was used to generate the targeting vector (Copeland et al., 2001; Liu et al., 2003). Briefly, a 129/SvJ BAC clone containing the targeted region of the *Cbfb* gene was obtained from Geneservice. An 11.5 kb region with homology arms that were 1.5 kb and 8 kb long each was inserted into a PBS-DTA plasmid, the backbone for the final targeting vector, via the first recombineering step. The 3' *loxP* site and the *FRT-Neo-FRT*-5' *loxP* cassette were then introduced sequentially during subsequent recombineering steps. A Bstz171 restriction site was engineered 3' to the 3' *loxP* site to facilitate southern screening of ES cells. The targeting construct was linearized with KpnI, electroporated into mouse

129S6SvEvTac ES cells. ES clones were screened by PCR and correctly targeted ES clones were confirmed by southern blot hybridization using both 5' and internal probes following Bstz171 digestion (WT 9.8 kb and Mutant 6.8 kb, data not shown). Chimeric *Cbfb*^{f} mice were produced by injection of the positive ES cells into C57Bl/6 blastocysts. Mice carrying the *Cbfb*^{f} allele were subsequently generated by mating chimeric mice to germ-line *FlpE* mice to remove the *Neo* cassette (Rodriguez et al., 2000). *Cbfb*^{f} mice were genotyped using a 2-primer PCR reaction with the following primers: 5'-GCGCGCCAGTCACTTGTT-3' and 5'- AAACCATCCCACGAACCGAACCAT-3'. The sizes of PCR products from wildtype and mutant alleles are 219 bp and 324 bp, respectively. For the *Cbfb*^{Flag} allele, the targeting vector which was almost identical to that for the *Cbfb*^{f} allele was built using a combination of recombineering and traditional cloning strategies. The same targeted genomic region was engineered to include the *FRT-Neo-FRT-loxP* cassette at the position identical to that in the *Cbfb*^{f} allele using recombineering technology. The sequence coding for one Flag epitope was introduced into the vector immediately upstream of the translational start site of the *Cbfb* gene by replacing a 1.1 kb NotI/AvrII fragment containing the translational start site with the fragment carrying the insertion using standard cloning techniques. A Bstz171 restriction site was inserted immediately downstream of the *Flag* sequence for the purpose of southern screening. Subsequent steps till the generation of *Cbfb*^{flag} mice were exactly the same as described for the *Cbfb*^{f} allele. *Cbfb*^{flag} mice were genotyped using a 2-primer PCR reaction with the following primers: 5'-TGAGAGCTGTCTATGGCAAAC-3' and 5'-TCAGTTCAAGGATGGCAGGTA-3'. The sizes of PCR products from wildtype and mutant alleles are 232 bp and 336 bp, respectively.

In situ hybridization

Digoxigenin (DIG)-labeled *cRNA* probes were used for in situ hybridization. Target sequences for in situ hybridization probes for *Ptprt*, *Myo1a*, *Kif21b* and *Runx3* were amplified with gene specific sets of PCR primers from either *cDNA* templates prepared from P0 mouse DRGs or genomic DNA from wildtype ES cells to generate corresponding plasmids for in situ hybridization. Primer sequences are available upon request. In situ hybridization probes for *MrgD*, *GFRα2*, *Ret* and *Runx1* were previously described (Luo et al., 2007). The in situ hybridization probe for *Cbfb* was directly produced from a commercially available cDNA clone (GenBank: BC026749.1). The in situ hybridization probe for *PV* was kindly provided by Ling

Bai (unpublished, Harvard Medical School). In situ hybridization was carried out on 14 μm fresh frozen DRG sections as described previously (Luo et al., 2007). For combined in situ hybridization and immunostaining, regular BCIP/NBT-based in situ hybridization was performed prior to the standard immunostaining procedure. Bright field and fluorescence images were taken under the same setting. The bright field image was later pseudocolored and merged with the fluorescence image.

Immunohistochemistry

Protocols for immunohistochemistry were described previously (Li et al., 2011). Briefly, all embryonic and neonatal tissues except for the hindlimb and back hairy skin from neonates were processed for cryosectioning without fixation. The skin specimens from neonates were fixed with 4% paraformaldehyde (PFA) in PBS (pH 7.4, 4°C) for 2.5-4 hrs depending on the age of the animal. Fixed tissues were required for PV immunostaining. For adult animals, mice (P14-P21) were anesthetized by CO_2 inhalation and transcardially perfused with PBS (pH 7.4, 4°C) followed by 4% PFA in PBS (pH 7.4, 4°C). Vertebral columns and hairy skin were dissected from the perfused mice, post-fixed at 4°C for 2 hr and overnight, respectively. Tissues were cryoprotected in 30% sucrose in PBS at 4°C overnight, embedded in OCT (Tissue Tek). For immunostaining of pTrk-SHC, pTrk-PLCγ and Flag, fresh frozen tissues were used. Immunostaining on tissue sections was performed using the following protocol, which was shown to be compatible with all the tissues and antibodies that have been tested. 14-20 μm sections were dried on slides at room temperature overnight, and fixed with 4% PFA in PBS for 10 min (fixed sections) or 15 min (fresh frozen sections). The slides were washed with PBS containing 0.1% Triton X-100 (0.1% PBST) and blocked with 5% normal serum (goat or donkey) in PBS containing 0.3% Triton X-100 (0.3% PBST) at room temperature for 1 hr. Tissue sections were incubated with primary antibodies diluted in 0.3%PBST containing 1% normal serum at 4°C overnight. The next day, sections were washed extensively with 0.1% PBST, and incubated with secondary antibodies diluted in blocking solution at room temperature for 1 hr, washed again with 0.1% PBST, and mounted with Fluoromount-G (Southern Biotech). The primary antibodies used for this study were: rabbit anti-Runx1 (a gift from Dr. Thomas Jessell, Columbia University, 1:10000), rabbit anti-Runx3 (a gift from Dr. Thomas Jessell, Columbia University, 1:50000), guinea pig anti-Flag (an affinity purified antibody raised against a C-terminally KLH-conjugated peptide that

corresponds to the N terminal tag of Flag-CBFβ encoded by the *Cbfb*^{flag} allele, 1:500), rabbit anti-CGRP (Immunostar, 24112, 1:1000), chicken anti-GFP (Aves Labs, GFP-1020, 1:500), rabbit anti-GFP (Invitrogen, A11122, 1:1000), chicken anti-NF200 (Aves Labs, NFH, 1:500), rabbit anti-parvalbumin (Swant, PV25, 1:1000), rabbit anti-Tyrosine Hydroxylase (Millipore, AB152, 1:1000), rabbit anti-TrkA (Millipore, AB1577, 1:1000), goat anti-TrkC (R & D system, AF1404, 1:500), rat anti-PECAM-1 (BD biosciences, 557355, 1:500), mouse anti- NeuN (Milllipore, MAB377MI, 1:500), rabbit anti-PGP9.5 (Millipore, AB1761ASR, 1:500), rabbit anti-βIII-Tubulin (Covance, PRB-435P, 1:1000)**.**

The secondary antibodies used were: Alexa 488 or 546 conjugated goat anti-chicken antibody, Alexa 488, 546 or 647 conjugated goat anti-rabbit antibodies, Alexa 647 conjugated goat anti-rat antibodies, Alexa 546 conjugated donkey anti-goat antibody, Alexa 488 conjugated goat anti-guinea pig antibody, Alexa 488 or 546 conjugated goat anti-mouse antibody. All secondary antibodies were purchased from Invitrogen. Alpha-Bungarotoxin, Alexa Fluor 555 conjugate from Invitrogen was used together with secondary antibodies at 1:1000.

Dissociated DRG neuronal cultures and immunocytochemistry

Dissociated DRG cultures from neonatal mice were performed using a method that was adapted from a previously described protocol for sympathetic neuronal cultures (Deckwerth et al., 1996). Briefly, neurons were obtained by sequential steps of enzymatic digestion and mechanical dissociation of DRGs of P0 animals. These neurons were plated on Poly-D-lysine and laminin coated coverslips at a density of ~50000 neurons per 24 well and cultured for 2 days in DMEM supplemented with 10% FBS, penicillin/streptomycin (1 U/ml) and 100 ng/ml of NGF(purified from mouse salivary glands or purchased through Millipore), or an NGF antibody (Sigma) at 1:2000. In addition, Ara-C (Sigma) at 5 μm and Boc-aspartyl (OMe)-fluoromethylketone (BAF) (MP Biomedicals) at 50 μg/ml were added to culture media for the entire culture period to inhibit proliferation of mitotic cells and NGF-deprivation induced apoptosis, respectively. For the set of experiments that addressed the *in vitro* requirement of MAPK signaling, cultures were treated with U0126 (Calbiochem) at 50 μm or an equal amount of DMSO the morning after plating. At the end of culture, neurons were fixed with 4% PFA for 5 min and subsequently stained in the

same way as described before for immunohistochemistry. For Flag immunostaining, the IgG fraction of a home-made guinea pig anti-Flag antiserum was used at 1 to 1000.

Real-time PCR

RNA was extracted from acutely isolated DRGs using the RNeasy micro kit (Qiagen) according to the manufacturer's instructions. First strand cDNA was synthesized using the oligo dT primer and the SuperScript III system (Invitrogen). Real-time PCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen) using 7300 Real-Time PCR System (Applied Biosystems). The abundance of individual transcripts was normalized to that of *PGP9.5*, a panneuronal marker, unless the comparison was between control and *NGF* mutants. In that case, GAPDH served as a better internal control. Primers that were used are as follows:

Cbfb	F-TCGAGAACGAGGAGTTCTTCAGGA	R-AGGCGTTCTGGAAGCGTGTCT
Runx1	F-GCAGGCAACGATGAAAACTACT	R-GCAACTTGTGGCGGATTTGTA
MrgD	F-TGCTGCTGGAAACACTTCTAGGGA	R-GCTGCTGTCAAGAGTGGAGTTCAT
GFRα2	F-TCGTACAGACCACTTGTGCC	R-ATCAAACCCAATCATGCCAG
Ptprt	F-ACCTGCTTCAACACATCACCCAGA	R-TTCATCTTCCTTGGCTGTGTCCCA
Myo1a	F-ACAGGTGCTTCAACACAGCCAATC	R-GCCCTTAAACAGTTCACTGGCACA
Ret	F-TCAACCTTCTGAAGACAGGCCACA	R-ATGTCAGCAAACACTGGCCTCTTG
CGRP	F-AAGAGTCACCGCTTCGCA	R-GAGCAAGATGCTGACAACCA
PGP9.5	F-CAGACCATCGGAAACTCCTG	R-CACTTGGCTCTATCTTCGGG
GAPDH	F-ATGCCTGCTTCACCACCTTCTT	R-ATGTGTCCGTCGTGGATCTGA

Immunoblotting

Acutely dissected DRGs were lysed in ice-old FA-M2 Lysis Buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, PH 7.5) supplemented with a protease inhibitor cocktail at 1:100 (Sigma) by sonication on ice. After clarifying lysates by centrifugation at 4°C for 20 min, boiling 5x SDS- Laemmli buffer was added to a final concentration of 1x SDS-Laemmli buffer. Lysates were boiled for another 5 min

before they were ready for immunoblot analysis. Immunoblotting was performed using antibodies against Runx1 (Abcam, 1:5000), CBFβ (1:1000, Santa Cruz), and βIII-Tubulin (1:1000, Covance) as described (Kuruvilla et al., 2000). When quantifying band intensity, optical density for βIII-Tubulin was used for normalization.

Co-Immunoprecipitation

Immunoprecipitation for Flag was performed using the anti-Flag M2 affinity gel (Sigma) according to the manufacturer's instructions. Briefly, lysates were made from acutely dissected DRGs of P0-P1 animals by gentle sonication in FA-M2 lysis buffer supplemented with protease inhibitor and phosphatase inhibitor cocktails (Sigma) that was described in the previous section for immunoblotting. Clarified lysates were precleared by incubation with protein A/G resin (Pierce) at 4°C for 1 hr. 5% of precleared lysates were saved as input for estimating the efficiency of IP and co-IP. The remainder of lysates was incubated with Flag-M2 resin at 4°C for 1.5 hr. 5% of the unbound fraction was saved for immunoblot analysis. The resin was extensively washed twice with FA-M2 buffer, twice with high salt FA-M2 buffer (500mM NaCl). Proteins were eluded from the resin with boiling for 3 min in equal volume of 2x SDS-Laemmli buffer with no reducing agent. Samples were then subjected to SDS-PAGE and immunoblot for Runx1 and CBFβ.

Nissl Staining and Cell Counts

Thoracic segments of vertebral columns of P0 animals were fixed in 4% PFA at 4°C for 1-2.5 hr. 10 μm cryosections were stained with 0.5% cresyl violet and cell counting was performed as described previously (Sakai et al., 2000), except that sections were taken every 50 μm through T12 DRGs, and total neuronal profiles were determined by counting the number of cells with visible nuclei on all sections and multiplying it by five. Percentages of neurons expressing various molecular markers were calculated by dividing the number of neurons expressing a particular marker by the total number of neurons on the same section which was determined by NeuN immunostaining.

NGF administration via intraperitoneal injections

Mouse pups of desired genotype were given a single intraperitoneal injection of either NGF (2 μg reconstituted at 100 μg/ml in 1% BSA in PBS) or equal volume of 1% BSA in PBS at both P0 and P1. Animals were sacrificed at P2 and vertebral columns were dissected and processed for in situ hybridization analysis.

DiI labeling

Vertebral columns of P0 pups were isolated and fixed with 4% PFA at 4°C overnight. DiI (Molecular Probes) crystals were inserted into T9 DRGs, and preparations were incubated in 4% PFA at 37°C for 10 days. 20 μm cryosections through the entire span of T9 spinal cord were collected and mounted with Fluoromount-G (Southern Biotech) for confocol imaging with a rhodamine filter.

Tamoxifen Injections

Tamoxifen (Toronto Research Chemicals) was dissolved in ethanol (20 mg/ml). 50 μl (~1 mg) of tamoxifen in ethanol was mixed with 50 μl of sunflower seed oil (Sigma), vortexed for 20 min and centrifuged under vacuum for 45 min to remove the ethanol. The tamoxifen solution was delivered once at P2 via intraperitoneal injection to animals harboring the *Runx1*^{CreER} allele.

X-gal staining

Embryos and hindlimbs of neonatal animals were fixed with a glutaraldehyde solution (0.2% glutaraldehyde, 2 mM MgCl2 in PBS) overnight at 4°C. For whole-mount staining, fixed embryos were washed with detergent rinse buffer (0.01% sodium deoxycholate, 0.02% NP40, 2 mM MgCl2 in phosphate buffer, pH 7.4), and stained with staining buffer (0.01% sodium deoxycholate, 0.02% NP40, 2 mM MgCl2, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranosidein phosphate buffer, pH 7.4) at room temperature until the signal intensity was desirable. After staining, embryos were post-fixed with 4% PFA at 4°C for 2 hr and rinsed extensively with PBS. If needed, stained embryos were further processed for cryosectioning by following the standard procedure of tissue processing for immunostaining. For staining on sections, 20 μm cryosections were collected from

fixed tissues or embryos and dried on slides at room temperature overnight. Tissue sections were post-fixed with the same glutaraldehyde solution as described above at room temperature for 10 min. The rest of the staining protocol was exactly the same as that for whole-mount preparation.

Microarray analysis

A total of 6 RNA samples (~1 μg each) were prepared using Trizol and the RNeasy micro kit from DRGs of three pairs of E16.5 control and *Runx1 CKO* animals from different litters. Samples were labeled and hybridized to Affymetrix mouse 430 2.0 chips and microarray data were analyzed with Spotfire software. Only genes with a fold change greater than or equal to 1.5, a p-value less than or equal to 0.05 were considered differentially expressed in control and *Runx1 CKO* DRGs and were reported.

Antibody production and purification

A guinea pig polyclonal antiserum was raised against a MDYKDDDDKLVY peptide that corresponds to the N terminus of Flag-CBFβ encoded by the $Cbfb^{Flag}$ allele as a service provided by Covance. The peptide was synthesized and conjugated at its C-terminus to KLH, so the N-terminus is freely exposed to mimic the endogenous state of the Flag tag in Flag-CBFβ. Exsanguination bleeds were enriched for IgG by binding with Protein A Agarose. Part of the IgG fraction was further affinity purified with a Flag-conjugated column prepared using the Sulfolink immobilization kit for peptides (Pierce).

Quantification of epidermal innervation and the intensity of fluorescent or colorimetric signal

Three randomly selected regions of the epidermis were imaged for each animal. For each image, the epidermal region was outlined and defined as a region of interest. The image was then thresholded based on βIII-Tubulin or GFP immunostaining, and the area fraction, i.e. the percentage of pixels above threshold in the region of interest was returned by Image J as a measure of epidermal innervation density. Area fraction for each image was considered an individual data point for statistical analysis. For quantifying the intensity of fluorescence images, images were thresholded and regions of interest were defined either on a cell-by-cell basis or as populations of cells depending on the purpose of comparison. The mean or total intensity of

pixels above threshold was measured. For quantifying the signal intensity for in situ hybridization, the same procedure was performed, except that images were first converted to grayscale.

Statistical Analysis

Statistical differences for mean values between two groups and among multiple groups were analyzed using GraphPad Prism 5. The type of test used was specified for each statistics analysis in the figure legend. The criterion for statistical significance was set at $p \leq 0.05$.

References

Abdel Samad, O., Liu, Y., Yang, F.C., Kramer, I., Arber, S., and Ma, Q. (2010). Characterization of two Runx1-dependent nociceptor differentiation programs necessary for inflammatory versus neuropathic pain. Mol Pain 6, 45.

Abdo, H., Li, L., Lallemend, F., Bachy, I., Xu, X.J., Rice, F.L., and Ernfors, P. (2011). Dependence on the transcription factor Shox2 for specification of sensory neurons conveying discriminative touch. Eur J Neurosci 34, 1529-1541.

Adya, N., Castilla, L.H., and Liu, P.P. (2000). Function of CBFbeta/Bro proteins. Semin Cell Dev Biol 11, 361-368.

Aloyz, R.S., Bamji, S.X., Pozniak, C.D., Toma, J.G., Atwal, J., Kaplan, D.R., and Miller, F.D. (1998). p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. The Journal of cell biology 143, 1691-1703.

Anderson, D.J. (1993). Cell fate determination in the peripheral nervous system: the sympathoadrenal progenitor. Journal of neurobiology 24, 185-198.

Arvanov, V.L., Seebach, B.S., and Mendell, L.M. (2000). NT-3 evokes an LTP-like facilitation of AMPA/kainate receptor-mediated synaptic transmission in the neonatal rat spinal cord. J Neurophysiol 84, 752-758.

Bachy, I., Franck, M.C., Li, L., Abdo, H., Pattyn, A., and Ernfors, P. (2011). The transcription factor Cux2 marks development of an A-delta sublineage of TrkA sensory neurons. Dev Biol 360, 77-86.

Bae, S.C., and Lee, Y.H. (2006). Phosphorylation, acetylation and ubiquitination: the molecular basis of RUNX regulation. Gene 366, 58-66.

Banerjee, C., McCabe, L.R., Choi, J.Y., Hiebert, S.W., Stein, J.L., Stein, G.S., and Lian, J.B. (1997). Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. Journal of cellular biochemistry 66, 1-8.

Basch, M.L., Bronner-Fraser, M., and Garcia-Castro, M.I. (2006). Specification of the neural crest occurs during gastrulation and requires Pax7. Nature 441, 218-222.

Belanger, L.F., Roy, S., Tremblay, M., Brott, B., Steff, A.M., Mourad, W., Hugo, P., Erikson, R., and Charron, J. (2003). Mek2 is dispensable for mouse growth and development. Mol Cell Biol 23, 4778-4787.

Belliveau, M.J., and Cepko, C.L. (1999). Extrinsic and intrinsic factors control the genesis of amacrine and cone cells in the rat retina. Development 126, 555-566.

Benedetti, M., Levi, A., and Chao, M.V. (1993). Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc Natl Acad Sci U S A 90, 7859-7863.

Bennett, D.L., Averill, S., Clary, D.O., Priestley, J.V., and McMahon, S.B. (1996a). Postnatal changes in the expression of the trkA high-affinity NGF receptor in primary sensory neurons. Eur J Neurosci 8, 2204-2208.

Bennett, D.L., Dmietrieva, N., Priestley, J.V., Clary, D., and McMahon, S.B. (1996b). trkA, CGRP and IB4 expression in retrogradely labelled cutaneous and visceral primary sensory neurones in the rat. Neurosci Lett 206, 33-36.

Bennett, D.L., Michael, G.J., Ramachandran, N., Munson, J.B., Averill, S., Yan, Q., McMahon, S.B., and Priestley, J.V. (1998). A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. J Neurosci 18, 3059-3072.

Bertrand, N., Castro, D.S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. Nat Rev Neurosci 3, 517-530.

Bibel, M., Hoppe, E., and Barde, Y.A. (1999). Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J 18, 616-622.

Birch, D.J., Turmaine, M., Boulos, P.B., and Burnstock, G. (2008). Sympathetic innervation of human mesenteric artery and vein. Journal of vascular research 45, 323-332.

Bissonauth, V., Roy, S., Gravel, M., Guillemette, S., and Charron, J. (2006). Requirement for Map2k1 (Mek1) in extra-embryonic ectoderm during placentogenesis. Development 133, 3429-3440.

Blyth, K., Cameron, E.R., and Neil, J.C. (2005). The RUNX genes: gain or loss of function in cancer. Nat Rev Cancer 5, 376-387.

Brady, R., Zaidi, S.I., Mayer, C., and Katz, D.M. (1999). BDNF is a target-derived survival factor for arterial baroreceptor and chemoafferent primary sensory neurons. J Neurosci 19, 2131-2142.

Brennan, C., Rivas-Plata, K., and Landis, S.C. (1999). The p75 neurotrophin receptor influences NT-3 responsiveness of sympathetic neurons in vivo. Nat Neurosci 2, 699-705.

Briscoe, J., and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. Curr Opin Neurobiol 11, 43-49.
Briscoe, J., and Novitch, B.G. (2008). Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. Philosophical transactions of the Royal Society of London Series B, Biological sciences 363, 57-70.
Bronner-Fraser, M., and Fraser, S.E. (1988). Cell lineage analysis reveals multipotency of some avian neural crest cells. Nature 335, 161-164.
Bronner-Fraser, M., Wolf, J.J., and Murray, B.A. (1992). Effects of antibodies against N-cadherin and N-CAM on the cranial neural crest and neural tube. Dev Biol 153, 291-301.
Brunet, A., Datta, S.R., and Greenberg, M.E. (2001). Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol 11, 297-305.
Butte, M.J., Hwang, P.K., Mobley, W.C., and Fletterick, R.J. (1998). Crystal structure of neurotrophin-3 homodimer shows distinct regions are used to bind its receptors. Biochemistry 37, 16846-16852.
Campenot, R.B. (1977). Local control of neurite development by nerve growth factor. Proc Natl Acad Sci U S A 74, 4516-4519.
Casaccia-Bonnefil, P., Carter, B.D., Dobrowsky, R.T., and Chao, M.V. (1996). Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature 383, 716-719.
Cavanaugh, D.J., Lee, H., Lo, L., Shields, S.D., Zylka, M.J., Basbaum, A.I., and Anderson, D.J. (2009). Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc Natl Acad Sci U S A 106, 9075-9080.
Chao, M.V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 4, 299-309.
Chen, A.I., de Nooij, J.C., and Jessell, T.M. (2006a). Graded activity of transcription factor Runx3 specifies the laminar termination pattern of sensory axons in the developing spinal cord. Neuron 49, 395-408.
Chen, C.L., Broom, D.C., Liu, Y., de Nooij, J.C., Li, Z., Cen, C., Samad, O.A., Jessell, T.M., Woolf, C.J., and Ma, Q. (2006b). Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. Neuron 49, 365-377.
Chen, K.S., Nishimura, M.C., Armanini, M.P., Crowley, C., Spencer, S.D., and Phillips, H.S. (1997). Disruption of a single allele of the nerve growth factor gene results in atrophy of basal forebrain cholinergic neurons and memory deficits. J Neurosci 17, 7288-7296.
Clary, D.O., and Reichardt, L.F. (1994). An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3. Proc Natl Acad Sci U S A 91, 11133-11137.
Coffman, J.A. (2003). Runx transcription factors and the developmental balance between cell proliferation and differentiation. Cell Biol Int 27, 315-324.
Collins, A., Littman, D.R., and Taniuchi, I. (2009). RUNX proteins in transcription factor networks that regulate T-cell lineage choice. Nature reviews Immunology 9, 106-115.
Conover, J.C., Erickson, J.T., Katz, D.M., Bianchi, L.M., Poueymirou, W.T., McClain, J., Pan, L., Helgren, M., Ip, N.Y., Boland, P., and et al. (1995). Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. Nature 375, 235-238.
Copeland, N.G., Jenkins, N.A., and Court, D.L. (2001). Recombineering: a powerful new tool for mouse functional genomics. Nature reviews Genetics 2, 769-779.
Coppola, V., Kucera, J., Palko, M.E., Martinez-De Velasco, J., Lyons, W.E., Fritzsch, B., and Tessarollo, L. (2001). Dissection of NT3 functions in vivo by gene replacement strategy. Development 128, 4315-4327.
Crowley, C., Spencer, S.D., Nishimura, M.C., Chen, K.S., Pitts-Meek, S., Armanini, M.P., Ling, L.H., McMahon, S.B., Shelton, D.L., Levinson, A.D., and et al. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76, 1001-1011.
Curtis, R., Adryan, K.M., Stark, J.L., Park, J.S., Compton, D.L., Weskamp, G., Huber, L.J., Chao, M.V., Jaenisch, R., Lee, K.F., and et al. (1995). Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. Neuron 14, 1201-1211.
Danielian, P.S., Muccino, D., Rowitch, D.H., Michael, S.K., and McMahon, A.P. (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Current biology : CB 8, 1323-1326.
Dasen, J.S., Liu, J.P., and Jessell, T.M. (2003). Motor neuron columnar fate imposed by sequential phases of Hox-c activity. Nature 425, 926-933.

Datta, S.R., Brunet, A., and Greenberg, M.E. (1999). Cellular survival: a play in three Akts. Genes Dev 13, 2905-2927.
Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91, 231-241.
Davies, A.M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H., and Thoenen, H. (1987). Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. Nature 326, 353-358.
Davies, A.M., Minichiello, L., and Klein, R. (1995). Developmental changes in NT3 signalling via TrkA and TrkB in embryonic neurons. EMBO J 14, 4482-4489.
Davis, B.M., Lewin, G.R., Mendell, L.M., Jones, M.E., and Albers, K.M. (1993). Altered expression of nerve growth factor in the skin of transgenic mice leads to changes in response to mechanical stimuli. Neuroscience 56, 789-792.
de Bruijn, M.F., and Speck, N.A. (2004). Core-binding factors in hematopoiesis and immune function. Oncogene 23, 4238-4248.
de Nooij, J.C., Doobar, S., and Jessell, T.M. (2013). Etv1 inactivation reveals proprioceptor subclasses that reflect the level of NT3 expression in muscle targets. Neuron 77, 1055-1068.
Deckwerth, T.L., Elliott, J.L., Knudson, C.M., Johnson, E.M., Jr., Snider, W.D., and Korsmeyer, S.J. (1996). BAX is required for neuronal death after trophic factor deprivation and during development. Neuron 17, 401-411.
Du, J., Feng, L., Yang, F., and Lu, B. (2000). Activity- and Ca(2+)-dependent modulation of surface expression of brain-derived neurotrophic factor receptors in hippocampal neurons. The Journal of cell biology 150, 1423-1434.
Ducy, P., Starbuck, M., Priemel, M., Shen, J., Pinero, G., Geoffroy, V., Amling, M., and Karsenty, G. (1999). A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev 13, 1025-1036.
Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L., and Karsenty, G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89, 747-754.
Durst, K.L., and Hiebert, S.W. (2004). Role of RUNX family members in transcriptional repression and gene silencing. Oncogene 23, 4220-4224.
Edwards, R.H., Rutter, W.J., and Hanahan, D. (1989). Directed expression of NGF to pancreatic beta cells in transgenic mice leads to selective hyperinnervation of the islets. Cell 58, 161-170.
Egan, M.F., Kojima, M., Callicott, J.H., Goldberg, T.E., Kolachana, B.S., Bertolino, A., Zaitsev, E., Gold, B., Goldman, D., Dean, M.*, et al.* (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell 112, 257-269.
Eide, F.F., Vining, E.R., Eide, B.L., Zang, K., Wang, X.Y., and Reichardt, L.F. (1996). Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. J Neurosci 16, 3123-3129.
English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S., and Cobb, M.H. (1999). New insights into the control of MAP kinase pathways. Experimental cell research 253, 255-270.
Erickson, J.T., Conover, J.C., Borday, V., Champagnat, J., Barbacid, M., Yancopoulos, G., and Katz, D.M. (1996). Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. J Neurosci 16, 5361-5371.
Esposito, D., Patel, P., Stephens, R.M., Perez, P., Chao, M.V., Kaplan, D.R., and Hempstead, B.L. (2001). The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to nerve growth factor. J Biol Chem 276, 32687-32695.
Esteban, P.F., Yoon, H.Y., Becker, J., Dorsey, S.G., Caprari, P., Palko, M.E., Coppola, V., Saragovi, H.U., Randazzo, P.A., and Tessarollo, L. (2006). A kinase-deficient TrkC receptor isoform activates Arf6-Rac1 signaling through the scaffold protein tamalin. The Journal of cell biology 173, 291-299.
Fahnestock, M., Michalski, B., Xu, B., and Coughlin, M.D. (2001). The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. Mol Cell Neurosci 18, 210-220.
Fan, G., Copray, S., Huang, E.J., Jones, K., Yan, Q., Walro, J., Jaenisch, R., and Kucera, J. (2000). Formation of a full complement of cranial proprioceptors requires multiple neurotrophins. Developmental dynamics : an official publication of the American Association of Anatomists 218, 359-370.

Farhadi, H.F., Mowla, S.J., Petrecca, K., Morris, S.J., Seidah, N.G., and Murphy, R.A. (2000). Neurotrophin-3 sorts to the constitutive secretory pathway of hippocampal neurons and is diverted to the regulated secretory pathway by coexpression with brain-derived neurotrophic factor. J Neurosci 20, 4059-4068.
Farinas, I., Jones, K.R., Backus, C., Wang, X.Y., and Reichardt, L.F. (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature 369, 658-661.
Farinas, I., Wilkinson, G.A., Backus, C., Reichardt, L.F., and Patapoutian, A. (1998). Characterization of neurotrophin and Trk receptor functions in developing sensory ganglia: direct NT-3 activation of TrkB neurons in vivo. Neuron 21, 325-334.
Farinas, I., Yoshida, C.K., Backus, C., and Reichardt, L.F. (1996). Lack of neurotrophin-3 results in death of spinal sensory neurons and premature differentiation of their precursors. Neuron 17, 1065-1078.
Finn, P.J., Ferguson, I.A., Renton, F.J., and Rush, R.A. (1986). Nerve growth factor immunohistochemistry and biological activity in the rat iris. J Neurocytol 15, 169-176.
Fishell, G., and Heintz, N. (2013). The neuron identity problem: form meets function. Neuron 80, 602-612.
Fjell, J., Cummins, T.R., Fried, K., Black, J.A., and Waxman, S.G. (1999). In vivo NGF deprivation reduces SNS expression and TTX-R sodium currents in IB4-negative DRG neurons. J Neurophysiol 81, 803-810.
Fleming, B.P., Gibbins, I.L., Morris, J.L., and Gannon, B.J. (1989). Noradrenergic and peptidergic innervation of the extrinsic vessels and microcirculation of the rat cremaster muscle. Microvascular research 38, 255-268.
Frade, J.M., and Barde, Y.A. (1998). Nerve growth factor: two receptors, multiple functions. BioEssays : news and reviews in molecular, cellular and developmental biology 20, 137-145.
Francis, N., Farinas, I., Brennan, C., Rivas-Plata, K., Backus, C., Reichardt, L., and Landis, S. (1999). NT-3, like NGF, is required for survival of sympathetic neurons, but not their precursors. Dev Biol 210, 411-427.
Frank, E., and Sanes, J.R. (1991). Lineage of neurons and glia in chick dorsal root ganglia: analysis in vivo with a recombinant retrovirus. Development 111, 895-908.
Frantz, G.D., and McConnell, S.K. (1996). Restriction of late cerebral cortical progenitors to an upper-layer fate. Neuron 17, 55-61.
Fukumitsu, H., Ohtsuka, M., Murai, R., Nakamura, H., Itoh, K., and Furukawa, S. (2006). Brain-derived neurotrophic factor participates in determination of neuronal laminar fate in the developing mouse cerebral cortex. J Neurosci 26, 13218-13230.
Garcia-Castro, M.I., Marcelle, C., and Bronner-Fraser, M. (2002). Ectodermal Wnt function as a neural crest inducer. Science 297, 848-851.
Gascon, E., Gaillard, S., Malapert, P., Liu, Y., Rodat-Despoix, L., Samokhvalov, I.M., Delmas, P., Helmbacher, F., Maina, F., and Moqrich, A. (2010). Hepatocyte growth factor-Met signaling is required for Runx1 extinction and peptidergic differentiation in primary nociceptive neurons. J Neurosci 30, 12414-12423.
Geetha, T., Kenchappa, R.S., Wooten, M.W., and Carter, B.D. (2005). TRAF6-mediated ubiquitination regulates nuclear translocation of NRIF, the p75 receptor interactor. EMBO J 24, 3859-3868.
Genc, B., Ozdinler, P.H., Mendoza, A.E., and Erzurumlu, R.S. (2004). A chemoattractant role for NT-3 in proprioceptive axon guidance. PLoS biology 2, e403.
Glebova, N.O., and Ginty, D.D. (2004). Heterogeneous requirement of NGF for sympathetic target innervation in vivo. J Neurosci 24, 743-751.
Gold, M.S., and Gebhart, G.F. (2010). Nociceptor sensitization in pain pathogenesis. Nat Med 16, 1248-1257.
Gorski, J.A., Balogh, S.A., Wehner, J.M., and Jones, K.R. (2003). Learning deficits in forebrain-restricted brain-derived neurotrophic factor mutant mice. Neuroscience 121, 341-354.
Graef, I.A., Wang, F., Charron, F., Chen, L., Neilson, J., Tessier-Lavigne, M., and Crabtree, G.R. (2003). Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. Cell 113, 657-670.
Grasby, D.J., Morris, J.L., and Segal, S.S. (1999). Heterogeneity of vascular innervation in hamster cheek pouch and retractor muscle. Journal of vascular research 36, 465-476.
Greig, L.C., Woodworth, M.B., Galazo, M.J., Padmanabhan, H., and Macklis, J.D. (2013). Molecular logic of neocortical projection neuron specification, development and diversity. Nat Rev Neurosci 14, 755-769.

Grewal, S.S., York, R.D., and Stork, P.J. (1999). Extracellular-signal-regulated kinase signalling in neurons. Curr Opin Neurobiol 9, 544-553.
Gruart, A., Sciarretta, C., Valenzuela-Harrington, M., Delgado-Garcia, J.M., and Minichiello, L. (2007). Mutation at the TrkB PLC{gamma}-docking site affects hippocampal LTP and associative learning in conscious mice. Learning & memory 14, 54-62.
Guidry, G., Landis, S.C., Davis, B.M., and Albers, K.M. (1998). Overexpression of nerve growth factor in epidermis disrupts the distribution and properties of sympathetic innervation in footpads. J Comp Neurol 393, 231-243.
Guo, T., Mandai, K., Condie, B.G., Wickramasinghe, S.R., Capecchi, M.R., and Ginty, D.D. (2011). An evolving NGF-Hoxd1 signaling pathway mediates development of divergent neural circuits in vertebrates. Nat Neurosci 14, 31-36.
Hadjab, S., Franck, M.C., Wang, Y., Sterzenbach, U., Sharma, A., Ernfors, P., and Lallemend, F. (2013). A Local Source of FGF Initiates Development of the Unmyelinated Lineage of Sensory Neurons. J Neurosci 33, 17656-17666.
Hallbook, F. (1999). Evolution of the vertebrate neurotrophin and Trk receptor gene families. Curr Opin Neurobiol 9, 616-621.
Hamanoue, M., Middleton, G., Wyatt, S., Jaffray, E., Hay, R.T., and Davies, A.M. (1999). p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. Mol Cell Neurosci 14, 28-40.
Han, L., Ma, C., Liu, Q., Weng, H.J., Cui, Y., Tang, Z., Kim, Y., Nie, H., Qu, L., Patel, K.N.*, et al.* (2013). A subpopulation of nociceptors specifically linked to itch. Nat Neurosci 16, 174-182.
Hari, L., Brault, V., Kleber, M., Lee, H.Y., Ille, F., Leimeroth, R., Paratore, C., Suter, U., Kemler, R., and Sommer, L. (2002). Lineage-specific requirements of beta-catenin in neural crest development. The Journal of cell biology 159, 867-880.
Hariri, A.R., Goldberg, T.E., Mattay, V.S., Kolachana, B.S., Callicott, J.H., Egan, M.F., and Weinberger, D.R. (2003). Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. J Neurosci 23, 6690-6694.
Harrington, A.W., and Ginty, D.D. (2013). Long-distance retrograde neurotrophic factor signalling in neurons. Nat Rev Neurosci 14, 177-187.
Hayashi, S., Lewis, P., Pevny, L., and McMahon, A.P. (2002). Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. Mech Dev 119 Suppl 1, S97-S101.
Heumann, R., Korsching, S., Bandtlow, C., and Thoenen, H. (1987). Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection. The Journal of cell biology 104, 1623-1631.
Hippenmeyer, S., Vrieseling, E., Sigrist, M., Portmann, T., Laengle, C., Ladle, D.R., and Arber, S. (2005). A developmental switch in the response of DRG neurons to ETS transcription factor signaling. PLoS biology 3, e159.
Holgado-Madruga, M., Moscatello, D.K., Emlet, D.R., Dieterich, R., and Wong, A.J. (1997). Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. Proc Natl Acad Sci U S A 94, 12419-12424.
Huang, E.J., and Reichardt, L.F. (2001). Neurotrophins: roles in neuronal development and function. Annual review of neuroscience 24, 677-736.
Huang, E.J., and Reichardt, L.F. (2003). Trk receptors: roles in neuronal signal transduction. Annual review of biochemistry 72, 609-642.
Huang, E.J., Wilkinson, G.A., Farinas, I., Backus, C., Zang, K., Wong, S.L., and Reichardt, L.F. (1999). Expression of Trk receptors in the developing mouse trigeminal ganglion: in vivo evidence for NT-3 activation of TrkA and TrkB in addition to TrkC. Development 126, 2191-2203.
Huang, G., Shigesada, K., Ito, K., Wee, H.J., Yokomizo, T., and Ito, Y. (2001). Dimerization with PEBP2beta protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation. EMBO J 20, 723-733.
Inoue, K., Ito, K., Osato, M., Lee, B., Bae, S.C., and Ito, Y. (2007). The transcription factor Runx3 represses the neurotrophin receptor TrkB during lineage commitment of dorsal root ganglion neurons. J Biol Chem 282, 24175-24184.
Inoue, K., Ozaki, S., Ito, K., Iseda, T., Kawaguchi, S., Ogawa, M., Bae, S.C., Yamashita, N., Itohara, S., Kudo, N., and Ito, Y. (2003). Runx3 is essential for the target-specific axon pathfinding of trkc-expressing dorsal root ganglion neurons. Blood Cells Mol Dis 30, 157-160.

Inoue, K., Ozaki, S., Shiga, T., Ito, K., Masuda, T., Okado, N., Iseda, T., Kawaguchi, S., Ogawa, M., Bae, S.C., *et al.* (2002). Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. Nat Neurosci 5, 946-954.
Ip, N.Y., Stitt, T.N., Tapley, P., Klein, R., Glass, D.J., Fandl, J., Greene, L.A., Barbacid, M., and Yancopoulos, G.D. (1993). Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells. Neuron 10, 137-149.
Ito, Y. (2004). Oncogenic potential of the RUNX gene family: 'overview'. Oncogene 23, 4198-4208.
Ito, Y. (2008). RUNX genes in development and cancer: regulation of viral gene expression and the discovery of RUNX family genes. Advances in cancer research 99, 33-76.
Kang, H., and Schuman, E.M. (1996). A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. Science 273, 1402-1406.
Kaplan, D.R., and Miller, F.D. (2000). Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10, 381-391.
Kappes, D.J. (2010). Expanding roles for ThPOK in thymic development. Immunological reviews 238, 182-194.
Kenchappa, R.S., Zampieri, N., Chao, M.V., Barker, P.A., Teng, H.K., Hempstead, B.L., and Carter, B.D. (2006). Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. Neuron 50, 219-232.
Kim, J., Lo, L., Dormand, E., and Anderson, D.J. (2003). SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. Neuron 38, 17-31.
Kleber, M., Lee, H.Y., Wurdak, H., Buchstaller, J., Riccomagno, M.M., Ittner, L.M., Suter, U., Epstein, D.J., and Sommer, L. (2005). Neural crest stem cell maintenance by combinatorial Wnt and BMP signaling. The Journal of cell biology 169, 309-320.
Klein, M., Hempstead, B.L., and Teng, K.K. (2005). Activation of STAT5-dependent transcription by the neurotrophin receptor Trk. Journal of neurobiology 63, 159-171.
Knudson, C.M., Tung, K.S., Tourtellotte, W.G., Brown, G.A., and Korsmeyer, S.J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270, 96-99.
Komori, T. (2006). Regulation of osteoblast differentiation by transcription factors. Journal of cellular biochemistry 99, 1233-1239.
Korsching, S., Auburger, G., Heumann, R., Scott, J., and Thoenen, H. (1985). Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. EMBO J 4, 1389-1393.
Korsching, S., and Thoenen, H. (1983). Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. Proc Natl Acad Sci U S A 80, 3513-3516.
Korte, M., Carroll, P., Wolf, E., Brem, G., Thoenen, H., and Bonhoeffer, T. (1995). Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc Natl Acad Sci U S A 92, 8856-8860.
Korte, M., Kang, H., Bonhoeffer, T., and Schuman, E. (1998). A role for BDNF in the late-phase of hippocampal long-term potentiation. Neuropharmacology 37, 553-559.
Kramer, I., Sigrist, M., de Nooij, J.C., Taniuchi, I., Jessell, T.M., and Arber, S. (2006). A role for Runx transcription factor signaling in dorsal root ganglion sensory neuron diversification. Neuron 49, 379-393.
Kuruvilla, R., Ye, H., and Ginty, D.D. (2000). Spatially and functionally distinct roles of the PI3-K effector pathway during NGF signaling in sympathetic neurons. Neuron 27, 499-512.
Kuruvilla, R., Zweifel, L.S., Glebova, N.O., Lonze, B.E., Valdez, G., Ye, H., and Ginty, D.D. (2004). A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell 118, 243-255.
Lallemend, F., and Ernfors, P. (2012). Molecular interactions underlying the specification of sensory neurons. Trends Neurosci 35, 373-381.
Lallemend, F., Sterzenbach, U., Hadjab-Lallemend, S., Aquino, J.B., Castelo-Branco, G., Sinha, I., Villaescusa, J.C., Levanon, D., Wang, Y., Franck, M.C., *et al.* (2012). Positional differences of axon growth rates between sensory neurons encoded by Runx3. EMBO J 31, 3718-3729.
Le-Niculescu, H., Bonfoco, E., Kasuya, Y., Claret, F.X., Green, D.R., and Karin, M. (1999). Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. Mol Cell Biol 19, 751-763.

Lee, H.Y., Kleber, M., Hari, L., Brault, V., Suter, U., Taketo, M.M., Kemler, R., and Sommer, L. (2004). Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. Science 303, 1020-1023.
Lee, R., Kermani, P., Teng, K.K., and Hempstead, B.L. (2001). Regulation of cell survival by secreted proneurotrophins. Science 294, 1945-1948.
Lei, L., Laub, F., Lush, M., Romero, M., Zhou, J., Luikart, B., Klesse, L., Ramirez, F., and Parada, L.F. (2005). The zinc finger transcription factor Klf7 is required for TrkA gene expression and development of nociceptive sensory neurons. Genes Dev 19, 1354-1364.
Lei, L., Ma, L., Nef, S., Thai, T., and Parada, L.F. (2001). mKlf7, a potential transcriptional regulator of TrkA nerve growth factor receptor expression in sensory and sympathetic neurons. Development 128, 1147-1158.
Levanon, D., Bettoun, D., Harris-Cerruti, C., Woolf, E., Negreanu, V., Eilam, R., Bernstein, Y., Goldenberg, D., Xiao, C., Fliegauf, M*., et al.* (2002). The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. Embo J 21, 3454-3463.
Levanon, D., Brenner, O., Negreanu, V., Bettoun, D., Woolf, E., Eilam, R., Lotem, J., Gat, U., Otto, F., Speck, N., and Groner, Y. (2001). Spatial and temporal expression pattern of Runx3 (Aml2) and Runx1 (Aml1) indicates non-redundant functions during mouse embryogenesis. Mech Dev 109, 413-417.
Levanon, D., and Groner, Y. (2004). Structure and regulated expression of mammalian RUNX genes. Oncogene 23, 4211-4219.
Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. Science 237, 1154-1162.
Lewin, G.R., and Mendell, L.M. (1993). Nerve growth factor and nociception. Trends Neurosci 16, 353-359.
Lewin, G.R., and Moshourab, R. (2004). Mechanosensation and pain. Journal of neurobiology 61, 30-44.
Li, L., Rutlin, M., Abraira, V.E., Cassidy, C., Kus, L., Gong, S., Jankowski, M.P., Luo, W., Heintz, N., Koerber, H.R*., et al.* (2011). The functional organization of cutaneous low-threshold mechanosensory neurons. Cell 147, 1615-1627.
Li, Q.L., Ito, K., Sakakura, C., Fukamachi, H., Inoue, K., Chi, X.Z., Lee, K.Y., Nomura, S., Lee, C.W., Han, S.B*., et al.* (2002). Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell 109, 113-124.
Lian, J.B., Javed, A., Zaidi, S.K., Lengner, C., Montecino, M., van Wijnen, A.J., Stein, J.L., and Stein, G.S. (2004). Regulatory controls for osteoblast growth and differentiation: role of Runx/Cbfa/AML factors. Critical reviews in eukaryotic gene expression 14, 1-41.
Lilley, B.N., Pan, Y.A., and Sanes, J.R. (2013). SAD kinases sculpt axonal arbors of sensory neurons through long- and short-term responses to neurotrophin signals. Neuron 79, 39-53.
Linggi, M.S., Burke, T.L., Williams, B.B., Harrington, A., Kraemer, R., Hempstead, B.L., Yoon, S.O., and Carter, B.D. (2005). Neurotrophin receptor interacting factor (NRIF) is an essential mediator of apoptotic signaling by the p75 neurotrophin receptor. J Biol Chem 280, 13801-13808.
Linnarsson, S., Bjorklund, A., and Ernfors, P. (1997). Learning deficit in BDNF mutant mice. Eur J Neurosci 9, 2581-2587.
Liu, I.Y., Lyons, W.E., Mamounas, L.A., and Thompson, R.F. (2004). Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning. J Neurosci 24, 7958-7963.
Liu, J.P., Laufer, E., and Jessell, T.M. (2001). Assigning the positional identity of spinal motor neurons: rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. Neuron 32, 997-1012.
Liu, P., Jenkins, N.A., and Copeland, N.G. (2003). A highly efficient recombineering-based method for generating conditional knockout mutations. Genome research 13, 476-484.
Liu, Q., Sikand, P., Ma, C., Tang, Z., Han, L., Li, Z., Sun, S., LaMotte, R.H., and Dong, X. (2012). Mechanisms of itch evoked by beta-alanine. J Neurosci 32, 14532-14537.
Liu, Q., Tang, Z., Surdenikova, L., Kim, S., Patel, K.N., Kim, A., Ru, F., Guan, Y., Weng, H.J., Geng, Y*., et al.* (2009). Sensory neuron-specific GPCR Mrgprs are itch receptors mediating chloroquine-induced pruritus. Cell 139, 1353-1365.
Liu, Q., Vrontou, S., Rice, F.L., Zylka, M.J., Dong, X., and Anderson, D.J. (2007). Molecular genetic visualization of a rare subset of unmyelinated sensory neurons that may detect gentle touch. Nat Neurosci 10, 946-948.
Liu, Y., Yang, F.C., Okuda, T., Dong, X., Zylka, M.J., Chen, C.L., Anderson, D.J., Kuner, R., and Ma, Q. (2008). Mechanisms of compartmentalized expression of Mrg class G-protein-coupled sensory receptors. J Neurosci 28, 125-132.

Liu, Y.Z., Boxer, L.M., and Latchman, D.S. (1999). Activation of the Bcl-2 promoter by nerve growth factor is mediated by the p42/p44 MAPK cascade. Nucleic acids research 27, 2086-2090.
Livesey, F.J., and Cepko, C.L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. Nat Rev Neurosci 2, 109-118.
Lo, L., Dormand, E., Greenwood, A., and Anderson, D.J. (2002). Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. Development 129, 1553-1567.
Lom, B., Cogen, J., Sanchez, A.L., Vu, T., and Cohen-Cory, S. (2002). Local and target-derived brain-derived neurotrophic factor exert opposing effects on the dendritic arborization of retinal ganglion cells in vivo. J Neurosci 22, 7639-7649.
Lonze, B.E., Riccio, A., Cohen, S., and Ginty, D.D. (2002). Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. Neuron 34, 371-385.
Lopes, C., Liu, Z., Xu, Y., and Ma, Q. (2012). Tlx3 and Runx1 act in combination to coordinate the development of a cohort of nociceptors, thermoceptors, and pruriceptors. J Neurosci 32, 9706-9715.
Lou, S., Duan, B., Vong, L., Lowell, B.B., and Ma, Q. (2013). Runx1 controls terminal morphology and mechanosensitivity of VGLUT3-expressing C-mechanoreceptors. J Neurosci 33, 870-882.
Lu, B., Pang, P.T., and Woo, N.H. (2005). The yin and yang of neurotrophin action. Nat Rev Neurosci 6, 603-614.
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.
Luo, X.G., Rush, R.A., and Zhou, X.F. (2001). Ultrastructural localization of brain-derived neurotrophic factor in rat primary sensory neurons. Neuroscience research 39, 377-384.
Ma, L., Lei, L., Eng, S.R., Turner, E., and Parada, L.F. (2003). Brn3a regulation of TrkA/NGF receptor expression in developing sensory neurons. Development 130, 3525-3534.
Ma, L., Merenmies, J., and Parada, L.F. (2000). Molecular characterization of the TrkA/NGF receptor minimal enhancer reveals regulation by multiple cis elements to drive embryonic neuron expression. Development 127, 3777-3788.
Ma, Q., Fode, C., Guillemot, F., and Anderson, D.J. (1999). Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia. Genes Dev 13, 1717-1728.
Mannion, R.J., Costigan, M., Decosterd, I., Amaya, F., Ma, Q.P., Holstege, J.C., Ji, R.R., Acheson, A., Lindsay, R.M., Wilkinson, G.A., and Woolf, C.J. (1999). Neurotrophins: peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. Proc Natl Acad Sci U S A 96, 9385-9390.
Marmigere, F., and Ernfors, P. (2007). Specification and connectivity of neuronal subtypes in the sensory lineage. Nat Rev Neurosci 8, 114-127.
Marmigere, F., Montelius, A., Wegner, M., Groner, Y., Reichardt, L.F., and Ernfors, P. (2006). The Runx1/AML1 transcription factor selectively regulates development and survival of TrkA nociceptive sensory neurons. Nat Neurosci 9, 180-187.
Maro, G.S., Vermeren, M., Voiculescu, O., Melton, L., Cohen, J., Charnay, P., and Topilko, P. (2004). Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. Nat Neurosci 7, 930-938.
Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80, 179-185.
McConnell, S.K., and Kaznowski, C.E. (1991). Cell cycle dependence of laminar determination in developing neocortex. Science 254, 282-285.
McCoy, E.S., Taylor-Blake, B., Street, S.E., Pribisko, A.L., Zheng, J., and Zylka, M.J. (2013). Peptidergic CGRPalpha primary sensory neurons encode heat and itch and tonically suppress sensitivity to cold. Neuron 78, 138-151.
McDonald, N.Q., Lapatto, R., Murray-Rust, J., Gunning, J., Wlodawer, A., and Blundell, T.L. (1991). New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor. Nature 354, 411-414.
Meakin, S.O., MacDonald, J.I., Gryz, E.A., Kubu, C.J., and Verdi, J.M. (1999). The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. A model for discriminating proliferation and differentiation. J Biol Chem 274, 9861-9870.
Mendell, L.M. (1999). Neurotrophin action on sensory neurons in adults: an extension of the neurotrophic hypothesis. Pain Suppl 6, S127-132.

Mercer, K., Giblett, S., Green, S., Lloyd, D., DaRocha Dias, S., Plumb, M., Marais, R., and Pritchard, C. (2005). Expression of endogenous oncogenic V600EB-raf induces proliferation and developmental defects in mice and transformation of primary fibroblasts. Cancer research 65, 11493-11500.
Meyer-Franke, A., Wilkinson, G.A., Kruttgen, A., Hu, M., Munro, E., Hanson, M.G., Jr., Reichardt, L.F., and Barres, B.A. (1998). Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. Neuron 21, 681-693.
Minichiello, L., Calella, A.M., Medina, D.L., Bonhoeffer, T., Klein, R., and Korte, M. (2002). Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron 36, 121-137.
Minichiello, L., Korte, M., Wolfer, D., Kuhn, R., Unsicker, K., Cestari, V., Rossi-Arnaud, C., Lipp, H.P., Bonhoeffer, T., and Klein, R. (1999). Essential role for TrkB receptors in hippocampus-mediated learning. Neuron 24, 401-414.
Mishra, S.K., and Hoon, M.A. (2010). Ablation of TrpV1 neurons reveals their selective role in thermal pain sensation. Mol Cell Neurosci 43, 157-163.
Miyazono, K., Maeda, S., and Imamura, T. (2004). Coordinate regulation of cell growth and differentiation by TGF-beta superfamily and Runx proteins. Oncogene 23, 4232-4237.
Mizuno, M., Yamada, K., Olariu, A., Nawa, H., and Nabeshima, T. (2000). Involvement of brain-derived neurotrophic factor in spatial memory formation and maintenance in a radial arm maze test in rats. J Neurosci 20, 7116-7121.
Molliver, D.C., Radeke, M.J., Feinstein, S.C., and Snider, W.D. (1995). Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. J Comp Neurol 361, 404-416.
Molliver, D.C., and Snider, W.D. (1997). Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurons. J Comp Neurol 381, 428-438.
Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q., and Snider, W.D. (1997). IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron 19, 849-861.
Molyneaux, B.J., Arlotta, P., Menezes, J.R., and Macklis, J.D. (2007). Neuronal subtype specification in the cerebral cortex. Nat Rev Neurosci 8, 427-437.
Montelius, A., Marmigere, F., Baudet, C., Aquino, J.B., Enerback, S., and Ernfors, P. (2007). Emergence of the sensory nervous system as defined by Foxs1 expression. Differentiation 75, 404-417.
Moqrich, A., Earley, T.J., Watson, J., Andahazy, M., Backus, C., Martin-Zanca, D., Wright, D.E., Reichardt, L.F., and Patapoutian, A. (2004). Expressing TrkC from the TrkA locus causes a subset of dorsal root ganglia neurons to switch fate. Nat Neurosci 7, 812-818.
Mowla, S.J., Pareek, S., Farhadi, H.F., Petrecca, K., Fawcett, J.P., Seidah, N.G., Morris, S.J., Sossin, W.S., and Murphy, R.A. (1999). Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J Neurosci 19, 2069-2080.
Muhr, J., Andersson, E., Persson, M., Jessell, T.M., and Ericson, J. (2001). Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. Cell 104, 861-873.
Mulderry, P.K., Ghatei, M.A., Spokes, R.A., Jones, P.M., Pierson, A.M., Hamid, Q.A., Kanse, S., Amara, S.G., Burrin, J.M., Legon, S., and et al. (1988). Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. Neuroscience 25, 195-205.
Muller, M., Triaca, V., Besusso, D., Costanzi, M., Horn, J.M., Koudelka, J., Geibel, M., Cestari, V., and Minichiello, L. (2012). Loss of NGF-TrkA signaling from the CNS is not sufficient to induce cognitive impairments in young adult or intermediate-aged mice. J Neurosci 32, 14885-14898.
Mundlos, S. (1999). Cleidocranial dysplasia: clinical and molecular genetics. Journal of medical genetics 36, 177-182.
Nakagawa, S., and Takeichi, M. (1998). Neural crest emigration from the neural tube depends on regulated cadherin expression. Development 125, 2963-2971.
Nakamura, S., Senzaki, K., Yoshikawa, M., Nishimura, M., Inoue, K., Ito, Y., Ozaki, S., and Shiga, T. (2008). Dynamic regulation of the expression of neurotrophin receptors by Runx3. Development 135, 1703-1711.
Nekrasova, T., Shive, C., Gao, Y., Kawamura, K., Guardia, R., Landreth, G., and Forsthuber, T.G. (2005). ERK1-deficient mice show normal T cell effector function and are highly susceptible to experimental autoimmune encephalomyelitis. Journal of immunology 175, 2374-2380.

Newbern, J.M., Li, X., Shoemaker, S.E., Zhou, J., Zhong, J., Wu, Y., Bonder, D., Hollenback, S., Coppola, G., Geschwind, D.H., *et al.* (2011). Specific functions for ERK/MAPK signaling during PNS development. Neuron 69, 91-105.
Newgreen, D.F., and Gooday, D. (1985). Control of the onset of migration of neural crest cells in avian embryos. Role of Ca++-dependent cell adhesions. Cell and tissue research 239, 329-336.
Nibu, K., Li, G., Kaga, K., and Rothstein, J.L. (2000). bFGF induces differentiation and death of olfactory neuroblastoma cells. Biochemical and biophysical research communications 279, 172-180.
Northcutt, R.G. (1989). Body and Brain. A Trophic Theory of Neural Connections. Dale Purves. Harvard University Press, Cambridge, MA, 1988. viii, 231 pp., illus. $35. Science 244, 993.
Nykjaer, A., Lee, R., Teng, K.K., Jansen, P., Madsen, P., Nielsen, M.S., Jacobsen, C., Kliemannel, M., Schwarz, E., Willnow, T.E., *et al.* (2004). Sortilin is essential for proNGF-induced neuronal cell death. Nature 427, 843-848.
Obermeier, A., Halfter, H., Wiesmuller, K.H., Jung, G., Schlessinger, J., and Ullrich, A. (1993a). Tyrosine 785 is a major determinant of Trk--substrate interaction. EMBO J 12, 933-941.
Obermeier, A., Lammers, R., Wiesmuller, K.H., Jung, G., Schlessinger, J., and Ullrich, A. (1993b). Identification of Trk binding sites for SHC and phosphatidylinositol 3'-kinase and formation of a multimeric signaling complex. J Biol Chem 268, 22963-22966.
Okuda, T., van Deursen, J., Hiebert, S.W., Grosveld, G., and Downing, J.R. (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell 84, 321-330.
Olausson, H., Wessberg, J., Morrison, I., McGlone, F., and Vallbo, A. (2010). The neurophysiology of unmyelinated tactile afferents. Neuroscience and biobehavioral reviews 34, 185-191.
Oppenheim, R.W. (1989). The neurotrophic theory and naturally occurring motoneuron death. Trends Neurosci 12, 252-255.
Otto, F., Lubbert, M., and Stock, M. (2003). Upstream and downstream targets of RUNX proteins. Journal of cellular biochemistry 89, 9-18.
Pang, P.T., Teng, H.K., Zaitsev, E., Woo, N.T., Sakata, K., Zhen, S., Teng, K.K., Yung, W.H., Hempstead, B.L., and Lu, B. (2004). Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 306, 487-491.
Park, J.W., Vahidi, B., Taylor, A.M., Rhee, S.W., and Jeon, N.L. (2006). Microfluidic culture platform for neuroscience research. Nature protocols 1, 2128-2136.
Patapoutian, A., Backus, C., Kispert, A., and Reichardt, L.F. (1999). Regulation of neurotrophin-3 expression by epithelial-mesenchymal interactions: the role of Wnt factors. Science 283, 1180-1183.
Patel, T.D., Jackman, A., Rice, F.L., Kucera, J., and Snider, W.D. (2000). Development of sensory neurons in the absence of NGF/TrkA signaling in vivo. Neuron 25, 345-357.
Patel, T.D., Kramer, I., Kucera, J., Niederkofler, V., Jessell, T.M., Arber, S., and Snider, W.D. (2003). Peripheral NT3 signaling is required for ETS protein expression and central patterning of proprioceptive sensory afferents. Neuron 38, 403-416.
Patten, I., and Placzek, M. (2000). The role of Sonic hedgehog in neural tube patterning. Cellular and molecular life sciences : CMLS 57, 1695-1708.
Patterson, S.L., Abel, T., Deuel, T.A., Martin, K.C., Rose, J.C., and Kandel, E.R. (1996). Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 16, 1137-1145.
Perantoni, A.O., Timofeeva, O., Naillat, F., Richman, C., Pajni-Underwood, S., Wilson, C., Vainio, S., Dove, L.F., and Lewandoski, M. (2005). Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. Development 132, 3859-3871.
Perez, S.E., Rebelo, S., and Anderson, D.J. (1999). Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. Development 126, 1715-1728.
Pezet, S., and McMahon, S.B. (2006). Neurotrophins: mediators and modulators of pain. Annual review of neuroscience 29, 507-538.
Puehringer, D., Orel, N., Luningschror, P., Subramanian, N., Herrmann, T., Chao, M.V., and Sendtner, M. (2013). EGF transactivation of Trk receptors regulates the migration of newborn cortical neurons. Nat Neurosci 16, 407-415.
Puig-Kroger, A., and Corbi, A. (2006). RUNX3: a new player in myeloid gene expression and immune response. Journal of cellular biochemistry 98, 744-756.

Ramirez, A., Page, A., Gandarillas, A., Zanet, J., Pibre, S., Vidal, M., Tusell, L., Genesca, A., Whitaker, D.A., Melton, D.W., and Jorcano, J.L. (2004). A keratin K5Cre transgenic line appropriate for tissue-specific or generalized Cre-mediated recombination. Genesis 39, 52-57.
Ramon y Cajal, S. (1899). Histology of the Nervous System of Man and Vertebrates (Oxford: Oxford University Press).
Rau, K.K., McIlwrath, S.L., Wang, H., Lawson, J.J., Jankowski, M.P., Zylka, M.J., Anderson, D.J., and Koerber, H.R. (2009). Mrgprd enhances excitability in specific populations of cutaneous murine polymodal nociceptors. J Neurosci 29, 8612-8619.
Raybould, H.E., Sternini, C., Eysselein, V.E., Yoneda, M., and Holzer, P. (1992). Selective ablation of spinal afferent neurons containing CGRP attenuates gastric hyperemic response to acid. Peptides 13, 249-254.
Reichardt, L.F. (2006). Neurotrophin-regulated signalling pathways. Philosophical transactions of the Royal Society of London Series B, Biological sciences 361, 1545-1564.
Reid, C.B., and Walsh, C.A. (2002). Evidence of common progenitors and patterns of dispersion in rat striatum and cerebral cortex. J Neurosci 22, 4002-4014.
Reissmann, E., Ernsberger, U., Francis-West, P.H., Rueger, D., Brickell, P.M., and Rohrer, H. (1996). Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons. Development 122, 2079-2088.
Riccio, A., Ahn, S., Davenport, C.M., Blendy, J.A., and Ginty, D.D. (1999). Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science 286, 2358-2361.
Rifkin, J.T., Todd, V.J., Anderson, L.W., and Lefcort, F. (2000). Dynamic expression of neurotrophin receptors during sensory neuron genesis and differentiation. Dev Biol 227, 465-480.
Ringstedt, T., Ibanez, C.F., and Nosrat, C.A. (1999). Role of brain-derived neurotrophic factor in target invasion in the gustatory system. J Neurosci 19, 3507-3518.
Ritter, A.M., Lewin, G.R., Kremer, N.E., and Mendell, L.M. (1991). Requirement for nerve growth factor in the development of myelinated nociceptors in vivo. Nature 350, 500-502.
Robertson, M.J., Gip, P., and Schaffer, D.V. (2008). Neural stem cell engineering: directed differentiation of adult and embryonic stem cells into neurons. Frontiers in bioscience : a journal and virtual library 13, 21-50.
Robinson, R.C., Radziejewski, C., Stuart, D.I., and Jones, E.Y. (1995). Structure of the brain-derived neurotrophic factor/neurotrophin 3 heterodimer. Biochemistry 34, 4139-4146.
Rodriguez-Tebar, A., Dechant, G., and Barde, Y.A. (1990). Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4, 487-492.
Rodriguez-Tebar, A., Dechant, G., and Barde, Y.A. (1991). Neurotrophins: structural relatedness and receptor interactions. Philosophical transactions of the Royal Society of London Series B, Biological sciences 331, 255-258.
Rodriguez, C.I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A.F., and Dymecki, S.M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nature genetics 25, 139-140.
Rose, C.R., Blum, R., Pichler, B., Lepier, A., Kafitz, K.W., and Konnerth, A. (2003). Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. Nature 426, 74-78.
Rosenbauer, F., and Tenen, D.G. (2007). Transcription factors in myeloid development: balancing differentiation with transformation. Nature reviews Immunology 7, 105-117.
Roux, P.P., and Barker, P.A. (2002). Neurotrophin signaling through the p75 neurotrophin receptor. Progress in neurobiology 67, 203-233.
Ruffolo, R.R., Jr., Nichols, A.J., Stadel, J.M., and Hieble, J.P. (1991). Structure and function of alpha-adrenoceptors. Pharmacological reviews 43, 475-505.
Ruit, K.G., Elliott, J.L., Osborne, P.A., Yan, Q., and Snider, W.D. (1992). Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development. Neuron 8, 573-587.
Ruocco, I., Cuello, A.C., Parent, A., and Ribeiro-da-Silva, A. (2002). Skin blood vessels are simultaneously innervated by sensory, sympathetic, and parasympathetic fibers. J Comp Neurol 448, 323-336.
Rush, R.A. (1984). Immunohistochemical localization of endogenous nerve growth factor. Nature 312, 364-367.

Saga, Y., Miyagawa-Tomita, S., Takagi, A., Kitajima, S., Miyazaki, J., and Inoue, T. (1999). MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. Development 126, 3437-3447.
Sakai, R., Henderson, J.T., O'Bryan, J.P., Elia, A.J., Saxton, T.M., and Pawson, T. (2000). The mammalian ShcB and ShcC phosphotyrosine docking proteins function in the maturation of sensory and sympathetic neurons. Neuron 28, 819-833.
Samokhvalov, I.M., Samokhvalova, N.I., and Nishikawa, S. (2007). Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. Nature 446, 1056-1061.
Samuels, I.S., Karlo, J.C., Faruzzi, A.N., Pickering, K., Herrup, K., Sweatt, J.D., Saitta, S.C., and Landreth, G.E. (2008). Deletion of ERK2 mitogen-activated protein kinase identifies its key roles in cortical neurogenesis and cognitive function. J Neurosci 28, 6983-6995.
Scott, A., Hasegawa, H., Sakurai, K., Yaron, A., Cobb, J., and Wang, F. (2011). Transcription factor short stature homeobox 2 is required for proper development of tropomyosin-related kinase B-expressing mechanosensory neurons. J Neurosci 31, 6741-6749.
Seal, R.P., Wang, X., Guan, Y., Raja, S.N., Woodbury, C.J., Basbaum, A.I., and Edwards, R.H. (2009). Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. Nature 462, 651-655.
Seebach, B.S., Arvanov, V., and Mendell, L.M. (1999). Effects of BDNF and NT-3 on development of Ia/motoneuron functional connectivity in neonatal rats. J Neurophysiol 81, 2398-2405.
Seidah, N.G., Benjannet, S., Pareek, S., Savaria, D., Hamelin, J., Goulet, B., Laliberte, J., Lazure, C., Chretien, M., and Murphy, R.A. (1996). Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases. The Biochemical journal 314 (Pt 3), 951-960.
Serbedzija, G.N., Fraser, S.E., and Bronner-Fraser, M. (1990). Pathways of trunk neural crest cell migration in the mouse embryo as revealed by vital dye labelling. Development 108, 605-612.
Shah, N.M., Groves, A.K., and Anderson, D.J. (1996). Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. Cell 85, 331-343.
Sharma, N., Deppmann, C.D., Harrington, A.W., St Hillaire, C., Chen, Z.Y., Lee, F.S., and Ginty, D.D. (2010). Long-distance control of synapse assembly by target-derived NGF. Neuron 67, 422-434.
Shelton, D.L., and Reichardt, L.F. (1984). Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. Proc Natl Acad Sci U S A 81, 7951-7955.
Shelton, D.L., and Reichardt, L.F. (1986). Studies on the expression of the beta nerve growth factor (NGF) gene in the central nervous system: level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several distinct populations of neurons. Proc Natl Acad Sci U S A 83, 2714-2718.
Shirasaki, R., and Pfaff, S.L. (2002). Transcriptional codes and the control of neuronal identity. Annual review of neuroscience 25, 251-281.
Silos-Santiago, I., Fagan, A.M., Garber, M., Fritzsch, B., and Barbacid, M. (1997). Severe sensory deficits but normal CNS development in newborn mice lacking TrkB and TrkC tyrosine protein kinase receptors. Eur J Neurosci 9, 2045-2056.
Silos-Santiago, I., Molliver, D.C., Ozaki, S., Smeyne, R.J., Fagan, A.M., Barbacid, M., and Snider, W.D. (1995). Non-TrkA-expressing small DRG neurons are lost in TrkA deficient mice. J Neurosci 15, 5929-5942.
Silverman, J.D., and Kruger, L. (1990). Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. J Neurocytol 19, 789-801.
Simeone, A., Daga, A., and Calabi, F. (1995). Expression of runt in the mouse embryo. Developmental dynamics : an official publication of the American Association of Anatomists 203, 61-70.
Smeyne, R.J., Klein, R., Schnapp, A., Long, L.K., Bryant, S., Lewin, A., Lira, S.A., and Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature 368, 246-249.
Snider, W.D. (1994). Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 77, 627-638.
Sockanathan, S., Perlmann, T., and Jessell, T.M. (2003). Retinoid receptor signaling in postmitotic motor neurons regulates rostrocaudal positional identity and axonal projection pattern. Neuron 40, 97-111.
Sosic, D., Richardson, J.A., Yu, K., Ornitz, D.M., and Olson, E.N. (2003). Twist regulates cytokine gene expression through a negative feedback loop that represses NF-kappaB activity. Cell 112, 169-180.

Speck, N.A., and Gilliland, D.G. (2002). Core-binding factors in haematopoiesis and leukaemia. Nat Rev Cancer 2, 502-513.
Speck, N.A., and Terryl, S. (1995). A new transcription factor family associated with human leukemias. Critical reviews in eukaryotic gene expression 5, 337-364.
Strohmaier, C., Carter, B.D., Urfer, R., Barde, Y.A., and Dechant, G. (1996). A splice variant of the neurotrophin receptor trkB with increased specificity for brain-derived neurotrophic factor. EMBO J 15, 3332-3337.
Stucky, C.L., Koltzenburg, M., Schneider, M., Engle, M.G., Albers, K.M., and Davis, B.M. (1999). Overexpression of nerve growth factor in skin selectively affects the survival and functional properties of nociceptors. J Neurosci 19, 8509-8516.
Sun, Y., Dykes, I.M., Liang, X., Eng, S.R., Evans, S.M., and Turner, E.E. (2008). A central role for Islet1 in sensory neuron development linking sensory and spinal gene regulatory programs. Nat Neurosci 11, 1283-1293.
Tan, A.Y., Chen, P.S., Chen, L.S., and Fishbein, M.C. (2007). Autonomic nerves in pulmonary veins. Heart rhythm : the official journal of the Heart Rhythm Society 4, S57-60.
Teng, H.K., Teng, K.K., Lee, R., Wright, S., Tevar, S., Almeida, R.D., Kermani, P., Torkin, R., Chen, Z.Y., Lee, F.S.*, et al.* (2005). ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J Neurosci 25, 5455-5463.
Theriault, F.M., Nuthall, H.N., Dong, Z., Lo, R., Barnabe-Heider, F., Miller, F.D., and Stifani, S. (2005). Role for Runx1 in the proliferation and neuronal differentiation of selected progenitor cells in the mammalian nervous system. J Neurosci 25, 2050-2061.
Theriault, F.M., Roy, P., and Stifani, S. (2004). AML1/Runx1 is important for the development of hindbrain cholinergic branchiovisceral motor neurons and selected cranial sensory neurons. Proc Natl Acad Sci U S A 101, 10343-10348.
Thoenen, H., Bandtlow, C., and Heumann, R. (1987). The physiological function of nerve growth factor in the central nervous system: comparison with the periphery. Reviews of physiology, biochemistry and pharmacology 109, 145-178.
Toledo-Aral, J.J., Brehm, P., Halegoua, S., and Mandel, G. (1995). A single pulse of nerve growth factor triggers long-term neuronal excitability through sodium channel gene induction. Neuron 14, 607-611.
Tonra, J.R., and Mendell, L.M. (1998). Effects of postnatal anti-NGF on the development of CGRP-IR neurons in the dorsal root ganglion. J Comp Neurol 392, 489-498.
Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R., and Schutz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nature genetics 23, 99-103.
Vaillant, A.R., Mazzoni, I., Tudan, C., Boudreau, M., Kaplan, D.R., and Miller, F.D. (1999). Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. The Journal of cell biology 146, 955-966.
Voyvodic, J.T. (1989). Peripheral target regulation of dendritic geometry in the rat superior cervical ganglion. J Neurosci 9, 1997-2010.
Vrontou, S., Wong, A.M., Rau, K.K., Koerber, H.R., and Anderson, D.J. (2013). Genetic identification of C fibres that detect massage-like stroking of hairy skin in vivo. Nature 493, 669-673.
Vulchanova, L., Olson, T.H., Stone, L.S., Riedl, M.S., Elde, R., and Honda, C.N. (2001). Cytotoxic targeting of isolectin IB4-binding sensory neurons. Neuroscience 108, 143-155.
Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A.H., and Speck, N.A. (1996a). Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc Natl Acad Sci U S A 93, 3444-3449.
Wang, Q., Stacy, T., Miller, J.D., Lewis, A.F., Gu, T.L., Huang, X., Bushweller, J.H., Bories, J.C., Alt, F.W., Ryan, G.*, et al.* (1996b). The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo. Cell 87, 697-708.
Wang, X., Merritt, A.J., Seyfried, J., Guo, C., Papadakis, E.S., Finegan, K.G., Kayahara, M., Dixon, J., Boot-Handford, R.P., Cartwright, E.J.*, et al.* (2005). Targeted deletion of mek5 causes early embryonic death and defects in the extracellular signal-regulated kinase 5/myocyte enhancer factor 2 cell survival pathway. Mol Cell Biol 25, 336-345.
Wang, X.H., and Poo, M.M. (1997). Potentiation of developing synapses by postsynaptic release of neurotrophin-4. Neuron 19, 825-835.

Wetmore, C., and Olson, L. (1995). Neuronal and nonneuronal expression of neurotrophins and their receptors in sensory and sympathetic ganglia suggest new intercellular trophic interactions. J Comp Neurol 353, 143-159.
Wickramasinghe, S.R., Alvania, R.S., Ramanan, N., Wood, J.N., Mandai, K., and Ginty, D.D. (2008). Serum response factor mediates NGF-dependent target innervation by embryonic DRG sensory neurons. Neuron 58, 532-545.
Woo, N.H., Teng, H.K., Siao, C.J., Chiaruttini, C., Pang, P.T., Milner, T.A., Hempstead, B.L., and Lu, B. (2005). Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. Nat Neurosci 8, 1069-1077.
Wooten, M.W., Seibenhener, M.L., Mamidipudi, V., Diaz-Meco, M.T., Barker, P.A., and Moscat, J. (2001). The atypical protein kinase C-interacting protein p62 is a scaffold for NF-kappaB activation by nerve growth factor. J Biol Chem 276, 7709-7712.
Wyatt, S., Pinon, L.G., Ernfors, P., and Davies, A.M. (1997). Sympathetic neuron survival and TrkA expression in NT3-deficient mouse embryos. EMBO J 16, 3115-3123.
Xie, C.W., Sayah, D., Chen, Q.S., Wei, W.Z., Smith, D., and Liu, X. (2000). Deficient long-term memory and long-lasting long-term potentiation in mice with a targeted deletion of neurotrophin-4 gene. Proc Natl Acad Sci U S A 97, 8116-8121.
Xu, B.E., Stippec, S., Lenertz, L., Lee, B.H., Zhang, W., Lee, Y.K., and Cobb, M.H. (2004). WNK1 activates ERK5 by an MEKK2/3-dependent mechanism. J Biol Chem 279, 7826-7831.
Yamada, M., Ohnishi, H., Sano, S., Nakatani, A., Ikeuchi, T., and Hatanaka, H. (1997). Insulin receptor substrate (IRS)-1 and IRS-2 are tyrosine-phosphorylated and associated with phosphatidylinositol 3-kinase in response to brain-derived neurotrophic factor in cultured cerebral cortical neurons. J Biol Chem 272, 30334-30339.
Yarmus, M., Woolf, E., Bernstein, Y., Fainaru, O., Negreanu, V., Levanon, D., and Groner, Y. (2006). Groucho/transducin-like Enhancer-of-split (TLE)-dependent and -independent transcriptional regulation by Runx3. Proc Natl Acad Sci U S A 103, 7384-7389.
Yoon, S.O., Casaccia-Bonnefil, P., Carter, B., and Chao, M.V. (1998). Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. J Neurosci 18, 3273-3281.
York, R.D., Yao, H., Dillon, T., Ellig, C.L., Eckert, S.P., McCleskey, E.W., and Stork, P.J. (1998). Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 392, 622-626.
Yoshikawa, M., Senzaki, K., Yokomizo, T., Takahashi, S., Ozaki, S., and Shiga, T. (2007). Runx1 selectively regulates cell fate specification and axonal projections of dorsal root ganglion neurons. Dev Biol 303, 663-674.
Yuan, J., Lipinski, M., and Degterev, A. (2003). Diversity in the mechanisms of neuronal cell death. Neuron 40, 401-413.
Zakharenko, S.S., Patterson, S.L., Dragatsis, I., Zeitlin, S.O., Siegelbaum, S.A., Kandel, E.R., and Morozov, A. (2003). Presynaptic BDNF required for a presynaptic but not postsynaptic component of LTP at hippocampal CA1-CA3 synapses. Neuron 39, 975-990.
Zhang, X., Huang, J., and McNaughton, P.A. (2005). NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. EMBO J 24, 4211-4223.
Zhao, Z., Zhao, M., Xiao, G., and Franceschi, R.T. (2005). Gene transfer of the Runx2 transcription factor enhances osteogenic activity of bone marrow stromal cells in vitro and in vivo. Molecular therapy : the journal of the American Society of Gene Therapy 12, 247-253.
Zheng, F., Zhou, X., Moon, C., and Wang, H. (2012). Regulation of brain-derived neurotrophic factor expression in neurons. International journal of physiology, pathophysiology and pharmacology 4, 188-200.
Zhong, J., Li, X., McNamee, C., Chen, A.P., Baccarini, M., and Snider, W.D. (2007). Raf kinase signaling functions in sensory neuron differentiation and axon growth in vivo. Nat Neurosci 10, 598-607.
Zirlinger, M., Lo, L., McMahon, J., McMahon, A.P., and Anderson, D.J. (2002). Transient expression of the bHLH factor neurogenin-2 marks a subpopulation of neural crest cells biased for a sensory but not a neuronal fate. Proc Natl Acad Sci U S A 99, 8084-8089.
Zweifel, L.S., Kuruvilla, R., and Ginty, D.D. (2005). Functions and mechanisms of retrograde neurotrophin signalling. Nat Rev Neurosci 6, 615-625.
Zylka, M.J., Rice, F.L., and Anderson, D.J. (2005). Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. Neuron 45, 17-25.

CURRICULUM VITAE FOR Ph.D. CANDIDATES

The Johns Hopkins University School of Medicine

Siyi Huang 12/12/2013

Educational history:

Ph.D. expected	2013	Program in Neuroscience Mentor: David Ginty, PhD	Johns Hopkins School of Medicine
B.S.	2007	Biochemistry	Hong Kong University of Science and Technology

Other professional experiences:

Research rotations	2007-2008	Labs of Craig Montell, Hongjun Song, David Ginty, and King-Wai Yau, Johns Hopkins School of Medicine
Undergraduate final year project	2006-2007	Lab of Mingjie Zhang, Hong Kong University of Science and Technology Topic: Structural and Functional Characterization of Beclin1
Summer research	2006	Undergraduate Research Opportunities Project, Lab of Mingjie Zhang, Hong Kong University of Science and Technology Topic: Biochemical and Biophysical Characterization of the Interaction between Bcl-2 and Beclin1
Summer research	2005	Undergraduate Research Opportunities Project, Lab of Mingjie Zhang, Hong Kong University of Science and Technology Topic: Construction of the Molecular Marker for the Analytical Gel Filtration Column
Undergraduate research training	2004-2005	Lab of Mingjie Zhang, Hong Kong University of Science and Technology

Academic awards:

June 17-22, 2012	Best poster award	2012 Molecular & Cellular Neurobiology, Gordon Research Conferences, Hong Kong
June 2007	First Class Honor & the Outstanding Student Award	Hong Kong University of Science and Technology
2005-2006	the Honor of Academic Excellence	Hong Kong University of Science and Technology
2005-2006	HSBC Scholarship for Mainland Students	Hong Kong University of Science and Technology
2004-2006	The Dean's List	Hong Kong University of Science and Technology

Publications:

Nam, J., Onitsuka, I., Hatch, J., Uchida, Y., Ray, S., **Huang, S.**, Li, W., Zang, H., Ruiz-Lozano, P., Mukouyama, Y. S. (2013). Coronary veins determine the pattern of sympathetic innervation in the developing heart. Development 140, 1475-1485.

Liu, Y., Rutlin, M., **Huang, S.**, Barrick, C. A., Wang, F., Jones, K. R., Tessarollo, L., Ginty, D. D. (2012). Sexually dimorphic BDNF signaling directs sensory innervation of the mammary gland. Science 338, 1357-1360.

Wu, H., Feng, W., Chen, J., Chan, L. N., **Huang, S.**, Zhang, M. (2007). PDZ domains of Par-3 as potential phosphoinositide signaling integrators. Molecular cell 28, 886-898.

Feng, W., **Huang, S.**, Wu, H., Zhang, M. (2007). Molecular basis of Bcl-xL's target recognition versatility revealed by the structure of Bcl-xL in complex with the BH3 domain of Beclin-1. Journal of molecular biology 372, 223-235.

Manuscript in preparation:

Huang, S., O'Donovan, K. J., Turner, E. E., Zhong, J., Ginty, D. D. A convergence of extrinsic and intrinsic signals for postmitotic differentiation of nociceptors. in preparation.

Poster presentations:

Huang, S., O'Donovan, K. J., Turner, E. E., Zhong, J., Ginty, D. D. (2013). Mediation of NGF-dependent maturation of nonpeptidergic nociceptors by a Runx1/CBFβ transcription factor complex. the Assembly and Function of Neuronal Circuits, Ascona, Switzerland, September 29- October 4, 2013.

Huang, S., O'Donovan, K. J., Zhong, J., Ginty, D. D. (2012). Mediation of NGF-dependent maturation of nonpeptidergic nociceptors by a Runx1/CBF β transcription factor complex. Molecular & Cellular Neurobiology, Gordon Research Conferences, Hong Kong, China, June 17-22, 2012.

Teaching service:

Teaching Assistant for Neuroscience and Cognition II, January-May 2009

The Johns Hopkins University, School of Medicine, Neuroscience Graduate Program