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Runx Transcription Factors: Lineage-Specific Regulators of Neuronal Precursor Cell Proliferation and Post-Mitotic Neuron Subtype Development

Chrissandra J. Zagami, Morena Zusso, and Stefano Stifani*

Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada H3A 2B4

ABSTRACT

runt-related (*RUNX*) genes encode evolutionarily conserved transcription factors that play essential roles during development and adult tissue homeostasis. RUNX proteins regulate the transition from proliferation to differentiation in a variety of cell lineages. Moreover, they control the diversification of distinct cellular phenotypes in numerous tissues. Alterations of RUNX functions are associated with several cancers and other human pathologies, underscoring the vital roles of these transcription factors in adult organs. Insights into the functions and regulations of mammalian RUNX proteins have been provided mostly by studies of RUNX involvement in mechanisms of hematopoietic and skeletal development and disease. A growing number of recent investigations are revealing new functions for RUNX family members during the development of the mammalian nervous system. This review will discuss recent progress in the study of RUNX protein involvement in mammalian neural development, with emphasis on the differentiation of olfactory, sensory, and motor neuron lineages. J. Cell. Biochem. 107: 1063–1072, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: ACUTE MYELOID LEUKEMIA; DORSAL ROOT GANGLION; MOTOR NEURONS; NERVOUS SYSTEM; PAIN; PROLIFERATION; RUNX; SENSORY NEURONS; SPINAL CORD

The *runt*-related gene family encodes a number of evolutionarily conserved DNA-binding transcription factors that control lineage-specific gene expression in both invertebrate and vertebrate species [Ito, 2004; Levanon and Groner, 2004; Lian et al., 2004; de Bruijn and Speck, 2004; Kagoshima et al., 2007; Nimmo and Woollard, 2008; Coffman, 2009]. RUNX proteins mediate both transcriptional activation and repression, depending on specific contexts, and interact with a variety of nuclear effectors of several important signaling pathways [Durst and Hiebert, 2004; Miyazono et al., 2004; Bae and Lee, 2006; Katoh, 2007]. As a result, they participate in the regulation of numerous developmental mechanisms. For instance, the *C. elegans runt*-related gene, *rnt-1*, is a key regulator of lateral hypodermal stem cell (seam cell) divisions [Kagoshima et al., 2007; Nimmo and Woollard, 2008]. *Drosophila runt* regulates several developmental processes including embryonic segmentation, sex determination, and neuronal differentiation. Another *Drosophila runt*-family member, *Lozenge*, is important for eye development and hematopoiesis [Canon and Banerjee, 2000]. Among other functions, the three mammalian *runt*-related genes (hereafter designated as *RUNX* when referring to both human and mouse genes or *Runx* when specifically referring to mouse family members) play vital roles in the regulation of cell proliferation and differentiation in a number of developing and adult organs, including tissues such as blood or epithelia where persistent cell replenishment from stem cells occurs throughout life [Ito, 2004, 2008; Lian et al., 2004; de Bruijn and Speck, 2004; Whiteman and Farrell, 2006; Coffman, 2009; Collins et al., 2009]. Deregulated RUNX activity is correlated with cancer and other diseases. More specifically, RUNX proteins are causally associated with several malignancies, where they exhibit context-dependent oncogenic or tumor suppressor activities, underscoring the importance of these proteins in the regulation of the balance between proliferation and differentiation [Speck and Gilliland, 2002; Alarcon-Riquelme, 2004; Blyth et al., 2005; Ito, 2008; Coffman, 2009].

EVOLUTIONARY CONSERVATION OF RUNX PROTEINS

runt-related genes define an ancient family present in animal phyla ranging from sponge to humans [Sullivan et al., 2008; Coffman, 2009]. There are four *runt*-related genes in certain metazoan species

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*Correspondence to: Dr. Stefano Stifani, Montreal Neurological Institute, 3801 rue University, Montreal, Quebec, Canada H3A 2B4. E-mail: stefano.stifani@mcgill.ca
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such as *Drosophila* and *Fugu*, while mammals have three *RUNX* genes and most other species have single orthologs. All Runt-related proteins contain a conserved 128-amino acid DNA binding motif, termed the Runt domain, which recognizes the consensus sequence, RCCRCA (R = purine) [Otto et al., 2003; Durst and Hiebert, 2004; Ito, 2004; Levanon and Groner, 2004]. DNA binding sites for Runt-related proteins can be located within both proximal and distal regulatory regions, as well as within introns and even coding regions. The prototypical Runt-domain binding site was revealed by the characterization of a mammalian protein complex that binds to specific viral enhancers. The polyomavirus enhancer-binding protein 2 (PEBP2) and the Moloney murine leukemia virus enhancer core binding factor (CBF) were characterized as identical hetero-dimers composed of α and β subunits. RUNX proteins correspond to the α subunit of these complexes [Speck and Gilliland, 2002; Ito, 2004]. The β subunit, termed PEBP2β/CBFβ (CBFβ), is a structurally unrelated protein that has no intrinsic DNA binding ability and binds to the Runt domain of RUNX [Adya et al., 2000; Nimmo and Woollard, 2008; Ito, 2008]. CBFβ is thought to act by enhancing RUNX affinity for DNA, as well as protecting RUNX from proteasome-mediated degradation. CBFβ is evolutionarily conserved and Runt-related orthologs in invertebrates and vertebrates share the ability to interact, and regulate transcription, with CBFβ [Nimmo and Woollard, 2008; Sullivan et al., 2008]. *CBFβ* expression does not always overlap with *RUNX* expression, suggesting that both RUNX and CBFβ might have functions independent of each other, although this possibility remains to be proven [Coffman, 2009].

ROLES OF RUNX PROTEINS DURING ORGAN DEVELOPMENT AND HOMEOSTASIS

RUNX family members form complexes with a variety of other transcription factors, including several DNA-binding proteins, as well as transcriptional coactivators or corepressors with no intrinsic DNA-binding ability [Ito, 2004; Durst and Hiebert, 2004; Miyazono et al., 2004; Katoh, 2007]. The capacity to engage numerous transcriptional regulators enables RUNX proteins to mediate transcriptional activation or repression mechanisms depending on the specific context in which they bind to DNA and the properties of their transcription partners. As a result, RUNX proteins sit at the crossroad of various signaling pathways and are involved in the regulation of a large number of genes. The transcriptional functions of RUNX proteins play essential roles in regulating the balance between cell proliferation and differentiation in a variety of developing organs and during adult tissue homeostasis. For instance, mammalian RUNX1 (AML1/PEBP2αB/CBFA2) is a critical regulator of fetal and adult hematopoiesis and alterations of *RUNX1* function in humans are associated with various forms of leukemia [Speck and Gilliland, 2002; Ito, 2004, 2008; de Bruijn and Speck, 2004; Whiteman and Farrell, 2006]. RUNX2 (AML3/PEBP2αA/CBFA1) regulates bone development and its haploinsufficiency results in a form of human bone disease termed cleidocranial dysplasia. Human *RUNX2* is expressed at high levels in breast and prostate tumors and cells that aggressively metastasize to bones [Lian et al., 2004; Pratap et al., 2006; Komori, 2008]. RUNX3 (AML2/PEBP2αC/CBFA3) is required for thymopoiesis and gastric system development and acts as a tumor suppressor in a number of tissues [Puig-Kroger and Corbi, 2006; Ito, 2008; Collins et al., 2009]. The demonstration that *RUNX* family members are expressed in a variety of tissues suggests that these genes might play important roles in several organs, both during development and in the adult. Here, we shall review studies that are revealing key functions for Runx1 and Runx3 during the development of the mammalian nervous system.

RUNX GENE EXPRESSION IN THE DEVELOPING NERVOUS SYSTEM

Expression and mutant analysis in *Drosophila* demonstrated for the first time that *runt*-related genes are involved in neuronal development. In the embryonic insect central nervous system (CNS), *runt* is expressed in selected neuronal precursor cells and their progeny, a specific subset of neurons termed *even-skipped*-expressing lateral (EL) neurons. *runt* inactivation using a temperature-sensitive allele caused a selective loss of EL neurons, whereas ectopic *runt* expression resulted in the formation of supernumerary EL neurons that extended axons along the normal trajectory used by EL neurons [Canon and Banerjee, 2000]. *Drosophila runt* is also expressed in specific types of photoreceptor cells in the developing eye, where it is involved in the control of the axon target choices made by those neurons [Kaminker et al., 2002]. These observations provided the first suggestions that *runt*-related genes participate in context-restricted mechanisms underlying the emergence of specific neuron subtypes and the establishment of precise axonal connections.

A restricted activation of *runt*-related gene expression in selected populations of neural cells was subsequently observed in the developing nervous system of the zebrafish [Kataoka et al., 2000; Kalev-Zylinska et al., 2002] and the mouse [Simeone et al., 1995; Levanon et al., 2001, 2002; Inoue et al., 2002; Theriault et al., 2004, 2005]. In the developing mouse nervous system, *Runx1* and *Runx3* are expressed in essentially non-overlapping patterns marking a few selected neuronal lineages. *Runx1* is expressed in mitotic neuronal precursors in certain lineages or in post-mitotic neurons in others. In the former, *Runx1* expression is mostly correlated with cells undergoing the final rounds of division before differentiation [Theriault et al., 2005]. In the latter, *Runx1* is generally expressed after neuronal generation and concomitant with developmental maturation events, such as the acquisition of specialized phenotypes and axonal targeting [Theriault et al., 2004; Kramer et al., 2006; Marmigere et al., 2006; Chen et al., 2006a; Stifani et al., 2008]. *Runx3* appears to be exclusively expressed in post-mitotic neurons. Similar to *Runx1*, *Runx3* is expressed during later stages of post-mitotic development, following the initial generation of the cells in which it is expressed [Inoue et al., 2002, 2008; Levanon et al., 2002; Kramer et al., 2006]. Little is known about the expression of *Runx2* during neural development. Recent RT-PCR analysis has revealed the presence of *Runx2* transcripts in adult mouse brain [Takarada and Yoneda, 2009]. In agreement with that observation, *Runx2*$^{LacZ/+}$ knock-in mice exhibit restricted expression of β-galactosidase in

the postnatal hippocampus and frontal lobe area [Jeong et al., 2008]. *RUNX2* expression was also detected in the adult human hippocampus. Interestingly, hippocampal *RUNX2* expression is decreased in bipolar disorder patients [Benes et al., 2007]. However, the phenotype of *RUNX2*-expressing cells in the brain, as well as the in vivo role of *RUNX2* in those brain cells, remain to be determined. Thus, this review will focus on our current understanding of the neural expression and function of *Runx1* and *Runx3*.

ROLE OF RUNX PROTEINS IN SELECTED MITOTIC NEURON PRECURSOR CELLS

Runx1 is expressed in a limited number of selected cells in the developing mouse nervous system. The site of most robust neural expression of *Runx1* is the olfactory epithelium in the nasal cavity. *Runx1* expression is first observed in the olfactory placodes and then persists in the olfactory epithelium throughout embryogenesis and into postnatal stages [Simeone et al., 1995; Theriault et al., 2005]. *Runx1*-expressing cells are preferentially localized to the basal side of the olfactory epithelium, where mitotic olfactory sensory neuron progenitor and precursor cells reside. Some *Runx1*-expressing cells correspond to rapidly proliferating transit-amplifying progenitors, but most of them correspond to immediate neuronal precursors, which are cells that undergo only a small number of cell divisions before differentiating into olfactory sensory neurons [Theriault et al., 2005]. In summary, *Runx1* expression in the developing olfactory epithelium is correlated with proliferating cells approaching terminal mitosis and neuronal differentiation.

Analysis of *Runx1*-deficient mouse embryos showed that *Runx1* inactivation caused immediate neuronal precursors of the olfactory epithelium to prematurely exit from the cell cycle, resulting in olfactory sensory neuron differentiation. Conversely, forced *Runx1* expression in cultured neuronal precursors prolonged cell proliferation. This latter effect was not correlated with reduced neuronal differentiation, suggesting that the main role of Runx1 in neuronal precursor cells is to promote proliferation and not to inhibit differentiation [Theriault et al., 2005]. The finding that Runx1 was able to repress transcription from the promoter of the cell cycle inhibitory gene $p21^{Cip1}$ in neuronal precursor cells suggested further that Runx1 might regulate cell proliferation in those cells at least in part by antagonizing mechanisms that promote cell cycle exit [Theriault et al., 2005]. Taken together, these results strongly suggest that Runx1 acts in olfactory sensory neuron precursor cells to promote proliferation.

Whether or not Runx1, and/or other Runx family members, play a similar role in other types of neural progenitor cells remains to be determined. Studies to date have not reported expression of *Runx2* or *Runx3* in mitotic neuronal precursor cells. However, recent investigations have revealed the presence of *Runx2* transcripts in cultured rat forebrain astrocytes and astrocytic C6 glioma cells [Takarada and Yoneda, 2009]. Moreover, *RUNX2* is expressed in primary human brain tumors of astrocytic origin [Vladimirova et al., 2008]. Although the functional significance of these observations remains to be determined, they raise the possibility that RUNX2 might be involved in mechanisms regulating the proliferation of glial-restricted progenitors and/or neoplastic glioma cells.

IMPLICATIONS OF RUNX1 FUNCTIONS IN OLFACTORY NEURON PRECURSORS FOR CELL PROLIFERATION AND CANCER

RUNX1 mutations are associated with leukemogenesis in humans. At least two mechanisms could underlie the leukemogenic effects of mutated RUNX1 proteins. Oncogenic mutants could act by preventing pluripotent progenitors from differentiating, thereby forcing them to remain in a default proliferative state. Such a mechanism would be consistent with the demonstration that RUNX1 promotes the differentiation of several hematopoietic cell types and that at least some oncogenic RUNX1 mutant proteins have dominant-inhibitory effects [de Bruijn and Speck, 2004; Blyth et al., 2005; Ito et al., 2008; Collins et al., 2009]. Alternatively, at least some of those RUNX1 mutants could actively promote progenitor cell proliferation, resulting in a deregulated expansion of the progenitor pool. The observation that Runx1 can promote neuron precursor cell proliferation but does not appear to inhibit neuron precursor differentiation suggests that Runx1 is able to positively regulate cell proliferation. This possibility is consistent with the function of the sea urchin *runt*-family member, *SpRunt-1*, which is essential for cell proliferation in blastula-stage embryos at the time when cells in two of the embryonic territories (aboral ectoderm and skeletogenic mesoderm) are entering their final rounds of division prior to terminal differentiation. Sea urchin embryos depleted of SpRunt-1 exhibited reduced expression of mitotic genes such as *cyclinD* and *Wnt* pathway components [Coffman, 2009]. *C. elegans rnt-1* is also required for cell proliferation as it promotes both symmetric and asymmetric divisions of seam cells, a self-renewing stem cell-like population that gives rise to differentiated epidermal cells during larval development [Kagoshima et al., 2007; Nimmo and Woollard, 2008]. Consistent with these observations in developmental model systems, increased dosage of human *RUNX1* has oncogenic consequences in hematopoietic lineages. Moreover, *Runx2* is a frequent target for proviral insertion in murine leukemia virus-induced T cell tumors [Blyth et al., 2005; Ito, 2008]. These results provide evidence suggesting that Runt-related proteins are able to promote cell proliferation in a number of contexts.

A mitotic function for RUNX proteins is suggested further by the demonstration that they can regulate the expression and/or activity of several cell cycle regulatory factors. For instance, RUNX1 overexpression transformed mammalian fibroblasts and shortened the progression from G1 to S phase of the cell cycle in myeloid progenitors, while inducing expression of *cyclin D2* and *D3*. Conversely, RUNX1 repressed transcription of the cell cycle inhibitory gene $p21^{Cip1}$ in the same cells [Strom et al., 2000; Bernardin-Fried et al., 2004; Durst and Hiebert, 2004]. Similarly, exogenous RUNX2 expression promoted the proliferation of endothelial cells, possibly by mediating repression of $p21^{Cip1}$ [Galindo et al., 2005; Qiao et al., 2006]. Interestingly, RUNX1 and RUNX2 levels are low in G1 and maximal at G2 and G2/M phases in

proliferating hematopoietic or endothelial cells, respectively [Bernardin-Fried et al., 2004; Qiao et al., 2006]. Moreover, RUNX factors were shown to interact with cell cycle control proteins such as pRb and E1A, and to regulate the expression of genes required for protein synthesis [Kagoshima et al., 2007; Nimmo and Woollard, 2008; Coffman, 2009]. Taken together, these observations support the notion that, at least in certain contexts, RUNX proteins might be oncogenic not simply by preventing the differentiation of proliferating progenitors, but by directly promoting cell proliferation.

The roles of RUNX proteins in the control of cell proliferation are, however, context-dependent. The observation that certain leukemogenic *RUNX1* translocations give rise to mutated proteins with proposed dominant-negative effects suggests that RUNX1 normally acts as a tumor-suppressor in hematopoietic progenitor cells. This possibility is consistent with the demonstration that *Runx3*-deficient mice exhibit increased epithelial cell proliferation and hyperplasia in the gastric mucosa, and that deletion of the human *RUNX3* gene or hypermethylation of its promoter are associated with several cancers [Ito, 2004, 2008; Blyth et al., 2005]. In that regard, it is worth mentioning that *RUNX3* is frequently hypermethylated and down-regulated in human glioblastoma brain tumors [Mueller et al., 2007]. This situation is in contrast to the up-regulation of *RUNX2* in glioblastoma, compared to normal brain tissue [Vladimirova et al., 2008]. Together, these observations suggest that RUNX proteins perform context-specific functions associated with both suppression and development of several types of cancer, including malignant brain tumors.

ROLE OF RUNX PROTEINS IN SELECTED POST-MITOTIC NEURONS

EXPRESSION OF *Runx1* AND *Runx3* IN SENSORY NEURONS

Runx1 and *Runx3* are expressed in peripheral nervous system dorsal root ganglion (DRG) sensory neurons, which convey somatosensory stimuli. Both genes are exclusively expressed in post-mitotic sensory neurons and not their proliferating progenitors. During embryonic development, *Runx1* is specifically expressed in DRG nociceptive neurons involved in pain transduction ("nociceptors") [Levanon et al., 2002; Kramer et al., 2006; Marmigere et al., 2006; Chen et al., 2006a]. Nociceptive neurons generally have peripheral cutaneous targets and send afferent axons to superficial layers of the dorsal region of the spinal cord. *Runx3* expression is restricted to separate DRG sensory neurons, termed proprioceptive neurons [Inoue et al., 2002; Levanon et al., 2002; Kramer et al., 2006; Chen et al., 2006b]. 'Proprioceptors' are connected to peripheral muscle spindles, send afferent axons to medial and ventral regions of the spinal cord, and mediate sense of position. In summary, *Runx1* and *Runx3* display mostly non-overlapping and complementary patterns of expression during the development of separate DRG sensory neuron lineages.

EXPRESSION OF *Runx1* DURING DORSAL ROOT GANGLION NOCICEPTIVE SENSORY NEURON DEVELOPMENT

During mouse embryonic development, most if not all nociceptive neurons initially express the gene encoding the nerve growth factor receptor, TrkA. *Runx1* is first expressed in the majority of *TrkA+* nociceptors at E12.0–E12.5 in lumbar DRG [Levanon et al., 2002; Kramer et al., 2006; Chen et al., 2006a]. As development proceeds, *TrkA+/Runx1+* neurons gradually disappear and become replaced by at least two neuronal subtypes in postnatal animals. One type remains *TrkA+* and loses *Runx1* expression ("*Runx1*-transient nociceptors"). The second group retains *Runx1* expression, switches off *TrkA*, and activates *Ret*, the gene encoding the receptor for glial-derived neurotrophic factor ("*Runx1*-persistent nociceptors") [Kramer et al., 2006; Chen et al., 2006a; Yoshikawa et al., 2007] (Fig. 1A). Most *Runx1*-transient nociceptors acquire a "peptidergic" phenotype characterized by the expression of the neuropeptide calcitonin-gene-related peptide (CGRP) and specific cell surface proteins. In contrast, the majority of *Runx1*-persistent nociceptors acquire a 'non-peptidergic' phenotype characterized by the lack of CGRP expression and the expression of *Ret* [Kramer et al., 2006; Marmigere et al., 2006; Chen et al., 2006a; Yoshikawa et al., 2007].

REGULATION OF DORSAL ROOT GANGLION NOCICEPTIVE SENSORY NEURON DEVELOPMENT BY *Runx1*

Conditional *Runx1* inactivation in DRG neurons of *Runx1*-deficient mice caused increased numbers of *TrkA+* neurons and a decrease in *Ret+* cells in postnatal animals. This alteration was correlated with a derepression of peptidergic genes such as *CGRP* [Chen et al., 2006a] (Fig. 1A). A similar effect was also observed in transgenic *Runx1*-deficient mice in which the embryonic lethality associated with *Runx1* inactivation was rescued by the specific reactivation of *Runx1* expression in hematopoietic, but not DRG neuron, lineages [Yoshikawa et al., 2007]. Conversely, *Runx1* overexpression or misexpression in DRG neurons of transgenic mice caused a suppression of peptidergic differentiation genes like *CGRP* [Kramer et al., 2006] (Fig. 1A). Importantly, *Runx1* inactivation also resulted in the loss of many nociceptive ion channels and receptors, including ATP channels, sodium channels, G protein-coupled receptors, and TRP channels, showing that Runx1 is directly or indirectly required for their expression [Chen et al., 2006a; Liu et al., 2008]. Taken together, those results show that Runx1 is essential for the separation of *TrkA+* and *Ret+* nociceptor phenotypes during late phases of DRG development and for the regulation of a large cohort of nociceptive genes.

It should be noted that in ovo electroporation-mediated expression into pre-migratory neural crest cells of a mutated form of Runx1 predicted to act as a dominant-negative inhibitor resulted in a loss of *TrkA*, but not *TrkC*, expression in embryonic chicken DRG neurons. Conversely, forced expression of full-length Runx1 induced *TrkA* expression in migratory neural crest cells, without promoting neuronal differentiation [Marmigere et al., 2006]. Those findings suggest the possibility that Runx1 might also participate in mechanisms that promote/sustain *TrkA* expression in DRG nociceptors at early embryonic stages, prior to its involvement in switching off *TrkA* expression during the separation of peptidergic and non-peptidergic phenotypes. In that regard, Runx1 is not required for de novo DRG *TrkA* induction during embryogenesis [Yoshikawa et al., 2007]. However, a *TrkA* minimal enhancer contains putative Runx1 binding sites to which Runx1 was shown to bind in vitro [Marmigere et al., 2006]. Because *Runx1* is necessary

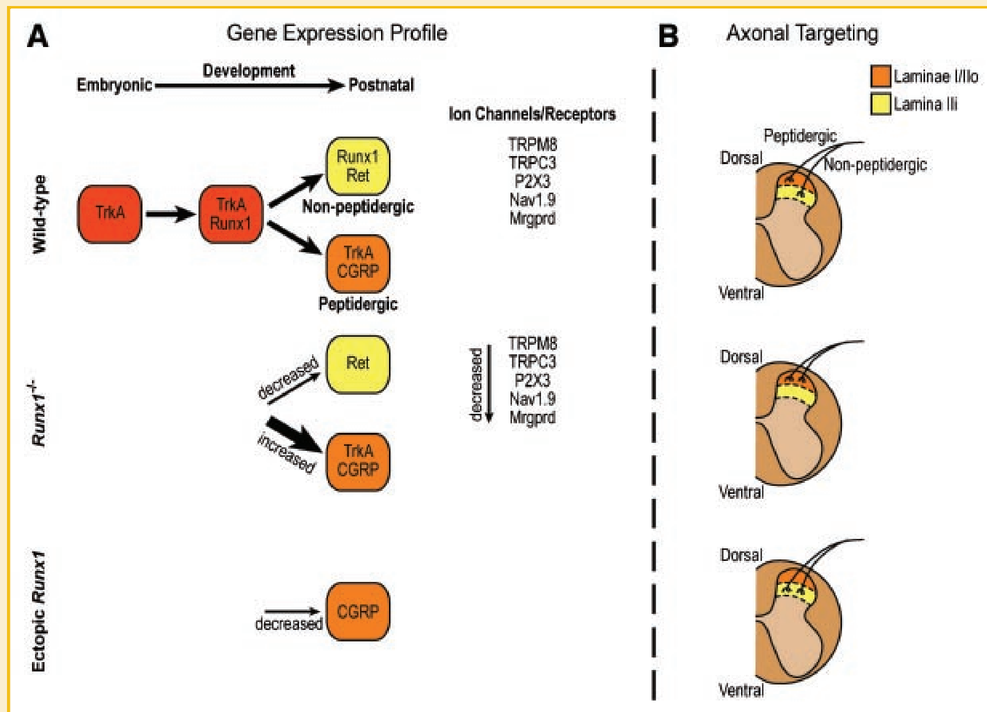

Fig. 1. Regulation of dorsal root ganglion nociceptor development by Runx1. A: During mouse embryonic development, *Runx1* is initially expressed in the majority of *TrkA*+ DRG nociceptors, where it might be involved in maintaining *TrkA* expression. By postnatal stages, however, *Runx1* and *TrkA* are no longer coexpressed and are found in two separate neuronal populations. One population retains the expression of *Runx1* ("*Runx1*-persistent"), switches off *TrkA*, and develops a "non-peptidergic" phenotype characterized by the lack of *CGRP* expression and the expression of *Ret*. The other population retains the expression of *TrkA* but not *Runx1* ("*Runx1*-transient") and acquires a "peptidergic" phenotype characterized by the expression of *CGRP*. *Runx1* knockout mice display decreased *Ret* expression and increased *TrkA* and *CGRP* expression in nociceptors, implicating Runx1 in the regulation of the expression of these genes. Conversely, *CGRP* expression is reduced if *Runx1* is ectopically expressed in developing DRG neurons. Moreover, *Runx1* inactivation causes a loss/attenuation of numerous nociceptive ion channels and sensory receptors. B: Peptidergic and non-peptidergic neurons project to either superficial ("I and IIo") or deeper ("IIi") layers of the dorsal spinal cord, respectively. *Runx1* inactivation causes a mistargeting of the nociceptive neurons in which *Runx1* would have been expressed resulting in innervation of more superficial laminae. Conversely, ectopic *Runx1* expression in all nociceptors leads to increased innervation of deeper layers of the dorsal spinal cord.

for the activation of ion channel/receptor expression at prenatal stages, before it is required for switching off *TrkA* expression postnatally, it is possible that Runx1 can participate in trans-activation mechanisms during embryonic DRG development, including events that consolidate *TrkA* expression. These observations suggest a model where Runx1 would have no role in the initial induction of *TrkA* expression in DRG but would be important for sustained *TrkA* activation during prenatal development, and would then switch to a repressor of *TrkA* expression at postnatal stages. This switch could be an indirect effect mediated by Runx1-induced Ret signaling [Chen et al., 2006a; Luo et al., 2007] and/or the result of changes in Runx1 transcriptional activity due to developmentally regulated changes in the composition of Runx1-containing transcription complexes, or other mechanisms.

In vivo studies in both mouse and chicken experimental systems showed further that *Runx1* is also important for the target innervation pattern of nociceptive neurons. *Ret*+ non-peptidergic nociceptors normally send their axons to targets in the dorsal spinal cord that are deeper than the targets of *TrkA*+ peptidergic neurons. This situation was perturbed in *Runx1*-deficient animals, where the majority of nociceptor axons projected to more superficial laminae [Chen et al., 2006a; Yoshikawa et al., 2007] (Fig. 1B). A consistent result was obtained in gain-of-function studies in which ectopic Runx1 expression in DRG neurons was shown to be sufficient to drive TrkA+ axons to deeper layers of the spinal cord [Kramer et al., 2006]. These results are in agreement with the demonstrated involvement of *Drosophila* Runt in the regulation of axon targeting choices in the insect visual system [Kaminker et al., 2002]. Although little is currently known about the mechanisms underlying the role of Runx1 in regulating target innervation, in vitro studies suggests that it might participate in mechanisms promoting axon growth and branching [Inoue et al., 2002; Marmigere et al., 2006]. In summary, Runx1 is required for the establishment of nociceptive DRG neuron identity and target connectivity during embryonic and early postnatal development.

EXPRESSION OF *Runx3* DURING DORSAL ROOT GANGLION PROPRIOCEPTIVE SENSORY NEURON DEVELOPMENT

Several other types of DRG neurons, in addition to *TrkA*+ cells, are present during early DRG development, including *TrkB*+, *TrkC*+, and *TrkB*+/*TrkC*+ cells. The later cell population is transient and disappears by E14.5, resulting in the appearance of separate *TrkC*+ (proprioceptive) and *TrkB*+ (mechanoreceptive) neuron lineages. *Runx3* is initially expressed only in *TrkC*+ proprioceptors. More

importantly, *Runx3* activation is correlated with the switch from *TrkB*+/*TrkC*+ to *TrkC*+ neurons [Kramer et al., 2006]. Thus, *Runx3* expression is negatively correlated with *TrkB* expression, suggesting that Runx3 is involved in the suppression of *TrkB* expression during DRG development. In agreement with this possibility, studies in cultured cells showed that Runx3 can bind to, and represses transcription from, a *TrkB* intronic gene regulatory element containing consensus Runx-binding sites [Inoue et al., 2007]. It should be mentioned that the correlation between *Runx3* expression and *TrkC*+ proprioceptive neurons appears to be lost at postnatal stages, when *Runx3* expression was also observed in certain *TrkA*+ and *TrkB*+ DRG neurons. An overlap of *Runx3* and *Runx1* expression was also observed at postnatal stages [Nakamura et al., 2008]. The physiological significance of these observations remains to be defined, but it is possible that Runx3 participates in specific aspects of non-proprioceptive neuron functions in certain postnatal DRG cell populations.

REGULATION OF DORSAL ROOT GANGLION PROPRIOCEPTIVE SENSORY NEURON DEVELOPMENT BY Runx3

The roles of *Runx3* during DRG proprioceptive neuron development were examined using a combination of loss- and gain-of-function studies. *Runx3* inactivation in knockout mice resulted in a loss of DRG *TrkC*+ proprioceptive cells, with a parallel increase in the number of *TrkB*+ neurons [Inoue et al., 2002, 2007; Levanon et al., 2002; Kramer et al., 2006]. Conversely, ectopic *Runx3* expression in all DRG neurons caused a loss of *TrkB* expression and increased numbers of *TrkC*+ neurons [Kramer et al., 2006] (Fig. 2A). Together, these results demonstrated that Runx3 is required for the *TrkC*+ proprioceptive neuron phenotype and inhibits the *TrkB*+ mechanoreceptor fate. This role is similar to the involvement of Runx1 in the generation of *Runx1*+ or *TrkA*+ nociceptors from *Runx1*+/*TrkA*+ neurons. Thus, different Runx proteins seem to share a common ability to act in selected types of bipotential post-mitotic neurons to promote the development of specialized phenotypes.

TrkC+ proprioceptive neurons normally send afferents to intermediate and ventral regions of the spinal cord, where they make contacts with motor neurons to establish the stretch reflex circuit that mediates information about muscle length and tension. In *Runx3*-deficient mice, *TrkC*+ neurons failed to project to the intermediate/ventral spinal cord and instead innervated more dorsal positions [Inoue et al., 2002; Levanon et al., 2002; Kramer et al., 2006; Chen et al., 2006b; Nakamura et al., 2008]. Conversely, ectopic expression of Runx3 in *TrkA*-positive DRG neurons biased their axon targeting choices towards more ventral zones of the spinal cord, similar to *TrkC*-positive neurons [Chen et al., 2006b] (Fig. 2B). Together, these results demonstrate a key role for Runx3 in directing proprioceptive axon targeting to the spinal cord. Thus, Runx3 also shares with Runx1 the ability to regulate sensory axon targeting choice, in addition to controlling specific neurotrophin receptor

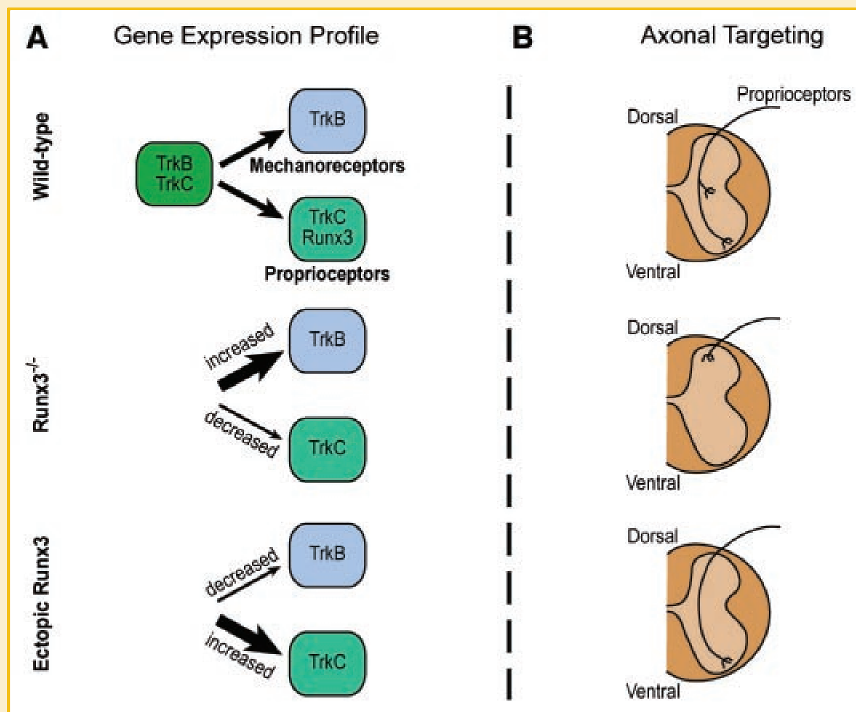

Fig. 2. Regulation of dorsal root ganglion proprioceptor development by Runx3. A: During mouse embryonic development, transient *TrkB*+/*TrkC*+ neuron populations give rise to separate *TrkB*+ mechanoreceptive and *TrkC*+ proprioceptive neuron populations. *Runx3* is exclusively expressed in proprioceptors, where it represses *TrkB* expression. *Runx3* inactivation in knockout mice causes an increase in *TrkB*+ neurons and a loss of *TrkC*+ neurons. Conversely, ectopic *Runx3* expression in DRG neurons results in reduced *TrkB* expression and increased numbers of *TrkC*+ neurons. B: *TrkC*+ proprioceptors normally send afferents to intermediate and ventral regions of the spinal cord. In *Runx3* null mice, DRG neuron afferents fail to reach the more ventral regions of the spinal cord. In contrast, ectopic expression of *Runx3* in *TrkA*+ DRG neurons results in their afferents projecting mostly to ventral zones of the spinal cord, similar to *TrkC*+ neurons.

expression and sensory neuron phenotype. In summary, Runx1 and Runx3 act during DRG neuronal development to specify separate sensory neuron fates and establish precise axonal connections with targets in the spinal cord.

EXPRESSION OF *Runx1* IN SELECTED SPINAL CORD MOTOR NEURONS

Runx1 is also expressed in post-mitotic neurons in the mouse CNS, in addition to peripheral sensory neurons. The majority of CNS *Runx1*+ cells correspond to motor neurons in the hindbrain and spinal cord [Theriault et al., 2004; Stifani et al., 2008]. In the latter, *Runx1*-expressing motor neurons are present only in the cervical region, where they comprise two main groups. The first group is made of a subpopulation of spinal accessory column (SAC) motor neurons, which are cells that innervate branchial arch-derived muscles in the neck and have axons that leave the spinal cord through lateral exit points roughly located midway along the dorsoventral axis of the spinal cord. SAC motor neurons are developmentally and functionally related to hindbrain branchial motor neurons, which innervate muscles in the face and jaw. *Runx1* is also expressed in hindbrain branchial motor neurons, where its function is required for the proper embryonic development of those cells [Theriault et al., 2004].

The second group of *Runx1*+ motor neurons in the cervical spinal cord comprises selected populations of motor neurons that send their axons out of the ventral root to innervate somatic forelimb and axial muscles ("ventrally exiting" motor neurons). *Runx1* is expressed in those motor neurons when they are presumably starting to make axonal connections with their peripheral targets [Stifani et al., 2008]. The cervical ventrally exiting motor neurons that express *Runx1* define restricted groups of cells that are part of either of two main types of spinal motor neuron columns, termed median motor column (MMC) or lateral motor column (LMC). MMC motor neurons innervate axial muscles, while medial LMC (LMCm) and lateral LMC (LMCl) motor neurons innervate muscles in the ventral or dorsal limb, respectively. *Runx1*+ MMC neurons are found mostly at cervical levels C3–C6 in the medial portion of the column. Within the LMC, *Runx1* expression is correlated with both LMCm and LMCl motor neuron subpopulations [Stifani et al., 2008] (Fig. 3). A similar, but seemingly more restricted, situation was observed in the developing chicken spinal cord, where *Runx1* expression marks a selected group of LMCl motor neurons at forelimb level [Dasen et al., 2005]. In summary, *Runx1* is expressed in selected post-mitotic motor neurons that are progressing toward their terminal phenotype and establishing axonal connections.

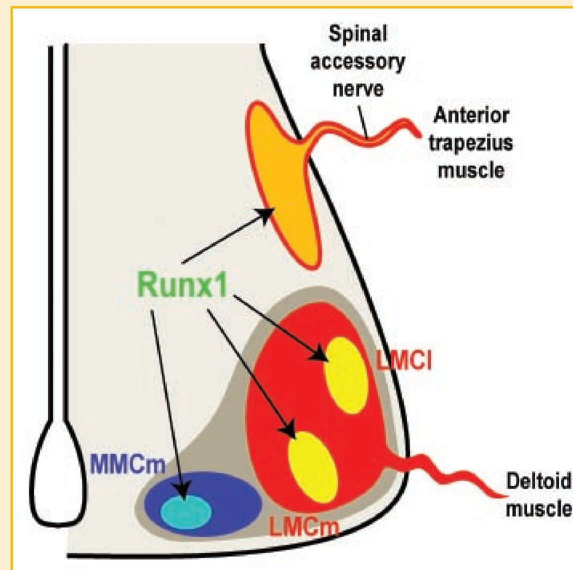

Fig. 3. Schematic representation of Runx1 expression in spinal cord motor neurons. In the cervical spinal cord of E13.5 mouse embryos, *Runx1* is expressed in selected subpopulations of both dorsally and ventrally exiting motor neurons. At levels C1–C4, *Runx1* is expressed in specific SAC motor neurons that project axons along the spinal accessory nerve and innervate the anterior trapezius muscle (orange). At levels C4–C8, *Runx1* is expressed in ventrally exiting motor neurons that comprise a medial subpopulation of the MMCm (light blue), as well as in selected LMCm and LMCl motor neuron subtypes (yellow). At this level of the spinal cord, LMC motor neurons innervate forelimb muscles and a group of *Runx1*-expressing motor neurons are known to innervate the deltoid muscle.

REGULATION OF THE DEVELOPMENT OF SELECTED SPINAL CORD MOTOR NEURONS BY *Runx1*

Loss-of-function studies to examine the role of *Runx1* in spinal motor neurons were performed using both *Runx1* null embryos (which die at ~E12.5) and "reactivated" null embryos in which *Runx1* expression was conditionally reestablished in hematopoietic, but not neuronal, cells to circumvent the embryonic lethality associated with complete *Runx1* inactivation [Stifani et al., 2008]. In both cases, loss of Runx1 did not cause a detectable loss of the specific motor neurons in which *Runx1* is normally expressed, suggesting that Runx1 is not important for motor neuron generation or survival [Stifani et al., 2008]. However, those studies revealed that ventrally exiting motor neurons in which *Runx1* would have been expressed had it not been inactivated displayed decreased expression of both general and specific motor neuron markers compared to control conditions. This situation was correlated with a derepression of genes characteristic of interneurons and not postmitotic motor neurons [Stifani et al., 2008]. Gain-of-function studies in which Runx1 was exogenously expressed in the developing spinal cord of chicken embryos by in ovo electroporation showed an opposite effect characterized by decreased numbers of neurons expressing interneuron genes and increased expression of motor neuron-specific genes. These effects were phenocopied by exogenous expression of the leukemogenic fusion protein AML1/ETO [Stifani et al., 2008]. AML1/ETO contains the DNA-binding domain of human RUNX1 fused to the eight-twenty one protein, a strong transcriptional repressor [Speck and Gilliland, 2002; Ito, 2004; Blyth et al., 2005]. ETO replaces the C-terminal transcription activation and repression domains of RUNX1 and AML1/ETO is hypothesized to act as a constitutive transcriptional repressor. These results strongly suggest that Runx1 acts to maintain specific motor neuron differentiation programs at least in part by repressing the development of alternative interneuron fates after motor neuron differentiation has been activated. The latter activity is similar to the essential role of Runx1, as well as Runx3, in establishing terminal

sensory neuron phenotypes through repression of genes that mediate alternative developmental programs.

IMPLICATIONS OF RUNX FUNCTIONS DURING POST-MITOTIC NEURON DEVELOPMENT FOR HEMATOPOIETIC DEFECTS AND OTHER PATHOLOGICAL CONDITIONS

The study of *Runx1* and *Runx3* involvement in mammalian sensory neuron development has provided informative examples of the important roles of *RUNX* genes in the regulation of cell differentiation and how perturbations of those functions can result in pathological conditions. More specifically, the molecular and axon targeting defects caused by *Runx1* inactivation in conditional *Runx1* knockout mice were correlated with impaired responses to noxious stimuli such as heat and cold, but not mechanical pain. They also decreased response to neuropathic pain, a heightened pain sensitivity experienced following peripheral nerve injury [Chen et al., 2006a]. It will be important to determine whether those phenotypes are the consequence of perturbation of either the role of Runx1 in promoting the expression of proteins that mediate nociceptive responses or its role in controlling nociceptor axon connectivity (or both). This information will facilitate future studies aimed at testing the relevance of Runx1-targeted therapeutic strategies for the treatment of pain.

Another remarkable example of how perturbation of neural Runx activity causes pathological conditions is the demonstration that *Runx3* null mice display a limb ataxia phenotype characterized by uncoordinated gait and abnormal positioning of the legs at rest [Inoue et al., 2002; Levanon et al., 2002]. This phenotype is likely the result of the failure of the stretch reflex circuit mediated by *Runx3*+ proprioceptive neurons caused by the abnormal targeting choices made by those cells when *Runx3* is inactivated. The finding that alterations of *Runx1* function also cause sensory neurons to make targeting errors suggests the possibility that *Runx1* regulates the target innervation choices made by other *Runx1*+ neuronal populations, like the spinal motor neurons in which it is expressed. In the future, it will be important to determine whether mice carrying a conditional *Runx1* inactivation in motor neurons exhibit locomotion defects.

It should be emphasized that the involvement of Runx1 and Runx3 in the control of bipotential cell fate decisions during sensory neuron subtype development is remarkably similar to their functions during T-cell lineage development. In the latter, thymocyte precursors coexpressing CD4 and CD8 give rise to either CD4+ or CD8+ T cells [Collins et al., 2009]. Both RUNX1 and RUNX3 act to repress the development of the alternative T-cell fate when one of the two lineages has been specified. More specifically, they are required for the silencing of *CD4* expression at sequential phases of T-cell development, with RUNX1 performing this function first in CD4/CD8-double-negative thymocyte precursors and RUNX3 in CD8+ thymocytes at later stages [Collins et al., 2009]. *Runx3* inactivation in mice leads to derepression of *CD4* expression in CD8+ thymocytes, while *Runx3*/*Runx1* double mutation causes a complete loss of those cells [Collins et al., 2009]. The picture emerging from the regulation of T-cell lineage is one where RUNX1 and RUNX3 are components of a transcription factor network involving a number of cross-regulatory proteins [Collins et al., 2009]. It is therefore possible that these proteins might also functionally interact with several cell type-specific transcription complexes in sensory (and motor) neurons. The identity of the transcription factors that work together with RUNX1 and RUNX3 to regulate neuron subtype development is currently unknown.

CONCLUSIONS AND FUTURE DIRECTIONS

Runx proteins are emerging as critical regulators of selected cell proliferation and differentiation processes in the developing nervous system. The remarkably specific expression of *Runx1* and *Runx3* in sensory and motor neuron lineages has identified these genes as ideal markers of selected post-mitotic neuron subtypes. This information will enable a number of future studies. For instance, the isolation and whole-genome analysis of specific motor neuron cell populations might be facilitated by the use of transgenic animals expressing fluorescent proteins under the control of *Runx1* gene regulatory sequences. In addition to their usefulness as specific neuron subtype markers, *Runx1* and *Runx3* have established themselves as key regulators of important developmental mechanisms in the nervous system, thereby providing investigators with powerful experimental tools to study those events. As an example, the continued in vivo analysis of *Runx1* involvement in motor neuron development is expected to enable the characterization of specific motor neuron-to-muscle connections. In mice, *Runx1*+ SAC motor neurons innervate the anterior trapezius muscle in the neck, whereas certain *Runx1*-expressing LMC neurons project to the deltoid muscle in the forelimb [Stifani et al., 2008] (Fig. 3). Future studies are expected to characterize the identity of other muscles innervated by *Runx1*-expressing MMC and LMC motor neurons, as well as the molecular mechanisms regulating the targeting choices of those cells. More generally, it will be important to determine whether *Runx1* and/or *Runx3* are expressed in additional neuronal populations in the central and peripheral nervous system and, if so, whether they perform roles similar to their functions in sensory and motor neuron lineages. This will also be the case for *Runx2*, which is expressed in restricted regions of the postnatal and adult brain in cells that remain to be defined.

Although significant progress has already been made in characterizing the neuron subtypes in which *Runx1* and *Runx3* are expressed, little is known about the upstream mechanisms that regulate their expression in those cells. In the developing chicken spinal cord, *Runx1* is expressed in a group of rostral LMCl motor neurons at forelimb (brachial) level. Brachial LMC neurons express specific types of homedomain proteins and *Runx1* expression overlaps specifically with Hox5 (Hoxa5 and Hoxc5) expression [Dasen et al., 2005]. The position of the brachial LMC is initially determined by FGF signaling and increased FGF8 expression at brachial level caused a rostral shift in the expression of *Hox5*. This shift was accompanied by a similar rostral shift of *Runx1* expression. Moreover, a caudal expansion of *Hox5* expression resulted in

ectopic caudal expression of *Runx1*. Those extra *Runx1*+ motor neurons projected to the muscle normally targeted by more anterior *Runx1*+ neurons [Dasen et al., 2005]. These findings suggest that mechanisms involving FGF and Hox5 are involved in promoting *Runx1* gene expression in motor neurons. In that regard, it is worth mentioning that the analysis of human olfactory neuroblastoma, a malignant tumor arising from olfactory neuronal precursors, showed that *RUNX1* expression is induced by FGF in those tumors, suggesting that FGF signaling might be important for mechanisms regulating *RUNX* expression in different regions of the nervous system [Nibu et al., 2000]. In peripheral sensory neurons, *Runx1* expression is initially not perturbed in *TrkA*-deficient embryos, showing that its onset does not depend on TrkA [Yoshikawa et al., 2007]. At later developmental stages, however, the absence of NGF-mediated TrkA signaling results in diminished *Runx1* expression, implicating TrkA signaling in maintaining *Runx1* expression [Luo et al., 2007]. Little is known about the mechanisms involved in the initial activation of *Runx1* and *Runx3* in sensory neurons, but the expression of both of these genes is reduced in mice lacking the transcription factor Brn-3a, consistent with the presence of Brn-3a-binding sites in their promoters [Eng et al., 2007]. No direct evidence for Brn-3a-mediated regulation of *Runx1* and/or *Runx3* expression has thus far been presented, however, and the key upstream regulators of neuronal *Runx* expression remain to be defined.

The analysis of Runx1 activity in nociceptive neurons has revealed that most nociceptive ion channels and receptors are directly or indirectly regulated by Runx1 [Chen et al., 2006a]. The identification of this specific cohort of downstream targets will enable further characterization of the roles of Runx1 in nociceptor subtype specification and pain. More specifically, it will be important to determine whether or not Runx1 regulates those target genes throughout DRG development and how their regulated expression contributes to the perception of specific types of pain. By analogy, it is reasonable to speculate that Runx3 might regulate the expression of proprioceptive-specific sensory determinants, in addition to repressing mechanoreceptive genes such as *TrkB*. Genes encoding axon guidance molecules are also likely downstream targets of Runx1 and Runx3, and this may be true in both sensory and motor neurons. However, virtually nothing is known about the identity of those possible targets. Regardless of the precise identity of the neuron-specific targets of RUNX proteins, it seems likely that only a fraction of them will be common to the different neuron subtypes in which each *RUNX* gene is expressed. This might not be the case in mitotic neural precursor cells, because available evidence suggests that Runx1 might regulate neuron precursor cell proliferation by regulating the expression of cell cycle control genes [Theriault et al., 2005], like RUNX proteins do in a number of other cell types [Strom et al., 2000; Bernardin-Fried et al., 2004; Qiao et al., 2006; Nimmo and Woollard, 2008; Coffman, 2009]. Thus, non-neural and neural targets of RUNX proteins might be similar in proliferating cells, suggesting that RUNX proteins might interact with a few common coregulators of cell cycle gene expression in different cellular contexts.

In conclusion, RUNX proteins play important roles during central and peripheral nervous system development. Increased knowledge of those roles will facilitate our understanding of how neural cell diversity is generated. This information is also expected to contribute to the study of the mechanisms controlling the generation of many non-neural cell types in which RUNX proteins perform similar molecular functions.

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