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Hepatocyte Growth Factor-Met Signaling Is Required for *Runx1* Extinction and Peptidergic Differentiation in Primary Nociceptive Neurons

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Nociceptors in peripheral ganglia display a remarkable functional heterogeneity. They can be divided into the following two major classes: peptidergic and nonpeptidergic neurons. Although RUNX1 has been shown to play a pivotal role in the specification of nonpeptidergic neurons, the mechanisms driving peptidergic differentiation remain elusive. Here, we show that hepatocyte growth factor (HGF)-Met signaling acts synergistically with nerve growth factor-tyrosine kinase receptor A to promote peptidergic identity in a subset of prospective nociceptors. We provide *in vivo* evidence that a population of peptidergic neurons, derived from the RUNX1 lineage, require Met activity for the proper extinction of *Runx1* and optimal activation of *CGRP* (calcitonin gene-related peptide). Moreover, we show that RUNX1 in turn represses Met expression in nonpeptidergic neurons, revealing a bidirectional cross talk between Met and RUNX1. Together, our novel findings support a model in which peptidergic versus nonpeptidergic specification depends on a balance between HGF-Met signaling and *Runx1* extinction/maintenance.

Introduction

Sensory neurons of the dorsal root ganglia (DRG) detect and transduce somatosensory stimuli from the periphery and convey this information to the spinal cord. Most sensory neurons arise from neural crest cells that migrate ventrally to form the DRG (White et al., 1996; Raible and Ungos, 2006; Marmigère and Ernfors, 2007). Neuronal precursors in the ganglion undergo the first specification process [embryonic day (E)10.5–E13.5 in the mouse] that gives rise to the three principal neuronal subtypes: nociceptive-thermoceptive, mechanoreceptive, and proprioceptive neurons. Each neuronal population requires a specific neurotrophic support for proper survival and expresses distinct neurotrophin receptors of the Trk family (Lewin, 1996; Patapoutian and Reichardt, 2001). After this early diversification event, a second maturation step further expands

the heterogeneity within each population. In nociceptive neurons, this phase is characterized by the progressive segregation of tyrosine kinase receptor A (TrkA) neurons into two subpopulations: peptidergic neurons that retain the expression of TrkA receptor and signal through nerve growth factor (NGF); and nonpeptidergic neurons that extinguish TrkA and respond to glial cell-derived neurotrophic factor-Ret signaling (Molliver et al., 1997; Ibáñez and Ernfors, 2007; Luo et al., 2007; Woolf and Ma, 2007).

Recent work has given new insights into the molecular control of late nociceptive maturation. A number of studies provided genetic evidence that persistent expression of the transcription factor RUNX1 is the key event for prospective nociceptors to undergo nonpeptidergic differentiation (Chen et al., 2006; Kramer et al., 2006b; Marmigère et al., 2006; Woolf and Ma, 2007; Yoshikawa et al., 2007). However, it is still unclear whether the peptidergic phenotype is the default differentiation pathway in prospective nociceptive neurons devoid of RUNX1 or, rather, an active process of cell fate choice. Given that the population of RUNX1⁻ prospective nociceptors (~10% of the total population) cannot account for the number of peptidergic neurons observed in the adult DRG (25% of the total population) (Chen et al., 2006), it has been proposed that a subset of RUNX1⁺ progenitors actively turn off *Runx1* expression and acquire peptidergic identity (Woolf and Ma, 2007). In agreement with this hypothesis, it has been shown that expression of some ion channels of the transient receptor potential (TRP) family, normally expressed in adult peptidergic neurons, is lost in the absence of *Runx1*. These observations strongly suggest that peptidergic neurons (or at least, a subset of them) derive from *Runx1*⁻-expressing precursors.

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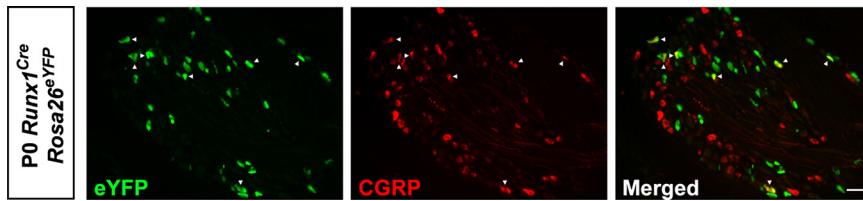


Figure 1. A population of CGRP⁺ peptidergic neurons arises from RUNX1⁺ precursors. Fate-mapping analysis of RUNX1 lineage using tamoxifen-inducible *Runx1*^{Cre}. Representative examples of CGRP and YFP immunofluorescence in newborn DRG of *Runx1*^{Cre} *Rosa26*^{eYFP} injected with 4-OHT at E12.5. These lineage experiments clearly demonstrated that a number of CGRP⁺ peptidergic neurons (arrowheads) derive from cells expressing RUNX1 at early developmental stages. Scale bars, 20 μ m.

How is *Runx1* expression controlled during nociceptive segregation? Recent work demonstrated that NGF signaling is necessary for the maintenance of *Runx1* in nonpeptidergic nociceptive neurons (Luo et al., 2007). Since both peptidergic and nonpeptidergic neurons require NGF for proper development (Smeyne et al., 1994), these observations strongly suggest that additional factor(s), in concert with NGF, ensure the extinction of *Runx1* in a subset of prospective peptidergic neurons. However, the molecular identity of such factor(s) has remained elusive.

Here, we provide the first *in vivo* evidence that a subset of peptidergic nociceptive neurons do derive from the RUNX1 lineage. We also identify a novel regulatory mechanism involving an active repressive loop between Met and RUNX1 that specifies the molecular identity of this subpopulation of nociceptive neurons. We show that, in conditional Met knock-out mice, a subset of prospective peptidergic neurons is no longer able to extinguish *Runx1*, leading to incomplete segregation of RUNX1 from TrkA⁺ neurons. The consequence of this RUNX1 persistence is the loss of several peptidergic markers, including *CGRP*, *TRPV1*, and *TRPA1*, as well as the expansion of the intermediate population of neurons expressing both TrkA and Ret. Conversely, disruption of *Runx1* expression in the peripheral nervous system resulted in a massive deregulation of Met and impaired maturation of nonpeptidergic neurons, arguing that persistent expression of RUNX1 is necessary to repress Met and instruct nonpeptidergic differentiation. Collectively, our findings indicate that Met/RUNX1 cross talk is a key event in the molecular logic of primary sensory neuronal diversification, thereby extending our understanding of nociceptive diversification.

Materials and Methods

Animals. We first introduced the *Met*^d allele [in which the mutation of two key tyrosine residues renders Met receptor functionally inactive (Ponzetto et al., 1994)] into a mouse strain expressing Cre recombinase under nestin promoter (*Nestin-Cre* mice) (Isaka et al., 1999; Kramer et al., 2006b; Zhong et al., 2007) to obtain *Met*^{d/+}; *Nes-Cre*⁺ animals. These animals were then crossed to previously described *Met*^{flx/flx} mice [in which two loxP sites flank *Met* exon 16 (Huh et al., 2004)], kindly provided by Dr. Snorri S. Thorgeirsson (National Cancer Institute, National Institutes of Health, Bethesda, MD). Mice obtained from that breeding were tail genotyped using *Cre*-, *Met*^d-, and *Met*^{flx}-specific primers. Conditional *Met* knock-out animals (*Met*^{flx/d}; *Nes-Cre*⁺, called *Nes-Met*) and control littermates (*Met*^{flx/d}; *Nes-Cre*⁻, called control) were used for all analysis. *Runx1* mutant animals were a generous gift from Dr. Ma (Dana Farber Cancer Institute, Boston, MA) and have been previously characterized (Chen et al., 2006). *Runx1*^{Cre} mice were provided by Dr. I.M. Samokhvalov (RIKEN Kobe, Kobe, Japan) and have been described previously (Samokhvalov et al., 2007). *Runx1-LacZ* animals were a generous gift from Dr. Nancy A. Speck (University of Pennsylvania, Philadelphia, PA) and have been previously described (North et al., 1999). Animals were maintained under standard housing conditions (25°C, 40% humidity, 12 h light cycles, and free access to food and water). Special effort was made to minimize the number as well as the

stress and suffering of mice used in this study. All protocols are in agreement with European Union recommendations for animal experimentation.

In situ hybridization and immunofluorescence. *In situ* hybridization and immunofluorescence were performed following standard protocols (Moqrich et al., 2004). To obtain adult tissues, animals were deeply anesthetized with a mix of ketamine/xylazine and then transcardially perfused with an ice-cold solution of paraformaldehyde 4% in PBS (PAF). After dissection, they were postfixed for at least 24 h in the same fixative. Embryos were collected in ice-cold PBS, gently washed, and fixed for 48 h in 4% PAF. For skin immunofluorescence, trunk skin was excised from anesthetized animals and fixed directly in 15% (v/v) acid picric–2% formaldehyde for 16 h. Tissues were then transferred into a 30% (w/v) sucrose solution for cryoprotection before being frozen and stored at -80°C . Samples were sectioned using a standard cryostat (Leica).

RNA probes were synthesized using gene-specific PCR primers and cDNA templates from embryonic or adult mouse DRG. Double-fluorescent *in situ* hybridization was performed using a combination of digoxigenin and fluorescein/biotin-labeled probes. Probes were hybridized overnight at 55°C, and the slides incubated with the horseradish peroxidase anti-digoxigenin/fluorescein/biotin antibody (Roche). Final detection was achieved using fluorescein/cy3/cy5 TSA plus kit (PerkinElmer). For double-fluorescent *in situ* experiments, the first antibody was inactivated using H₂O₂ treatment.

For immunofluorescence, primary antibodies were diluted in PBS–10% donkey serum (Sigma)–3% bovine albumin (Sigma)–0.4% Triton X-100 and incubated overnight at 4°C. Corresponding donkey anti-rabbit or anti-goat Alexa 488, 555, or 647 (Invitrogen) were used for secondary detection. Primary antibodies used in this study are as follows: rabbit anti-TrkA 1:1000 (generous gift from Dr. L. Reichardt, University of California, San Francisco, CA), rabbit anti-Runx1 1:4000 (generous gift from Dr. T. Jessell, New York University School of Medicine, New York, NY), rabbit anti-activated caspase-3 1:2000 (Cell Signaling Technology), goat anti-TrkC 1:500 (R&D Systems), goat anti-Ret 1:500 (R&D Systems), rabbit anti-calcitonin gene-related peptide (CGRP) 1:1000 (Millipore Bioscience Research Reagents), chicken anti-green fluorescent protein (GFP) 1:1000 (Aves Labs), and goat anti-parvalbumin 1:2000 (Swant).

DRG cultures, transfection, and growth factors. DRG cultures were prepared following standard protocols. Briefly, DRG from embryonic day (E) 14.5 embryos were collected in cold DMEM. After trypsinization (10 min at 37°C), tissue was triturated using a fire-polished Pasteur pipette. Cells were then rinsed in Roswell Park Memorial Institute (RPMI) medium and counted before transfection.

DRG neurons were transfected using Amaxa Nucleofection. For each transfection reaction, 5×10^5 cells were used. To limit cell death after transfection, a recovery step (15 min in RPMI medium) was performed. Cells were then plated onto 35 mm dishes coated with poly-L-lysine (Sigma) containing pre-equilibrated culture medium. Cells were grown in Neurobasal (Invitrogen) supplemented with 2% B27 (Invitrogen) and 100 μM sodium pyruvate. Culture medium was replaced 2 and 24 h after transfection.

Plasmid containing the human Met was provided by Professor C. Ponzetto (University of Turin, Turin, Italy). For nucleofection, 1 μg of plasmid DNA/reaction was used.

Exogenous growth factors were added to the culture medium immediately after transfection. Recombinant NGF was purchased from Alomone Labs, and hepatocyte growth factor (HGF) from R&D Systems.

Reverse transcription-PCR. RNA was extracted (embryonic or adult DRG, PC12, and DRG cultures) using TriZol reagent (Invitrogen) according to manufacturer's protocol. Total RNA (1 μg) was converted to cDNA using oligodT₁₅ as primer and ImPromII reverse transcriptase (Promega). Reverse transcription was performed for 1 h at 42°C.

For PCR, GoTaq polymerase (Promega) and a standard thermal cycler (Applied Biosystems) were used. Real-time PCR was performed in an iCycler system (Bio-Rad). PCRs were performed in triplicate using SYBR Green ER reagent system (Invitrogen). Each SYBR Green reaction (25 μl total volume)

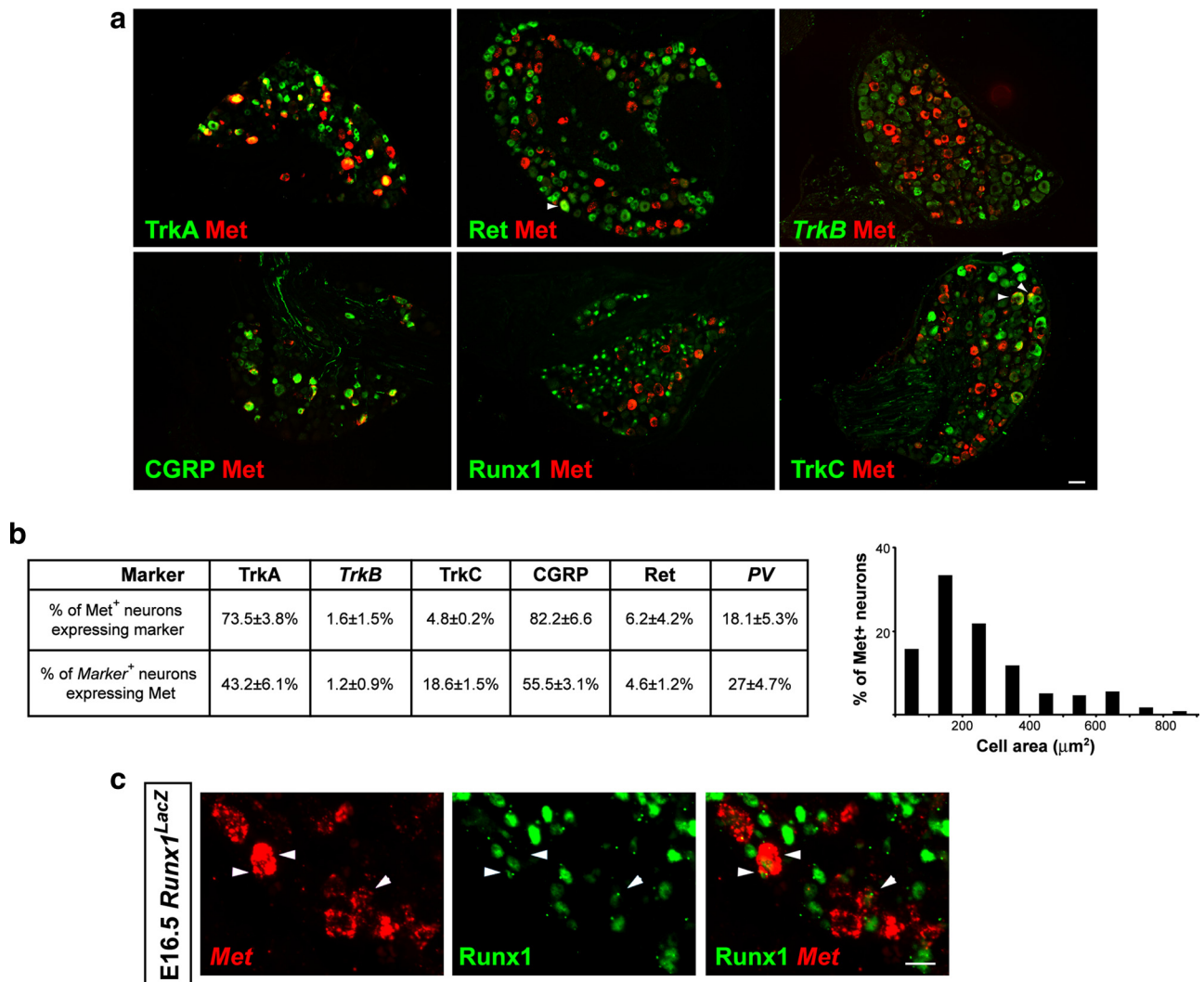


Figure 2. *Met* is expressed in a subset of adult DRG neurons. **a**, Pictures of adult DRG illustrating double labeling of *Met* together with different markers of specific neuronal populations. As shown, most *Met*⁺ neurons are TrkA⁺/CGRP⁺ peptidergic neurons. It is also expressed in some TrkC⁺ neurons (arrowheads). In contrast, *Met* is largely excluded from Ret⁺/RUNX1⁺ population of nociceptive neurons and TrkB⁺ mechanoceptive neurons. Immunofluorescence for CGRP, TrkA, TrkC, Ret, and RUNX1. *In situ* hybridization for *Met* and *TrkB*. **b**, Proportions of Met⁺ cells expressing different markers (data obtained from three different animals) and size distribution of Met⁺ neurons. **c**, Pictures illustrating coexpression of *Met* and RUNX1 at E16 in *Runx1*^{LacZ} reporter embryos. Immunofluorescence for LacZ. *In situ* hybridization for *Met*. Scale bars: **a**, 50 μ m; **b**, 30 μ m.

contained 1 μ l of cDNA as template and 0.3 μ M each primer. Controls without template DNA (water and reverse transcription reaction) were always negative. The reactions were incubated at 95°C for 10 min to activate the HotStar Taq polymerase followed by 40 cycles at 95°C for 15 s (denaturation) and at 60°C for 1 min (annealing and extension). β -Actin was used as internal control. The oligonucleotide primers used for PCR were shown in supplemental Table 1 (available at www.jneurosci.org as supplemental material).

Because SYBR Green indiscriminately binds to double-stranded DNA, other products in the PCR such as primer dimers may be detected along with the target gene. To verify that the SYBR Green dye detected only one PCR product, the samples were subjected to the heat dissociation protocol after the final cycle of the PCR.

Cell counts and statistical analysis. For adult tissues, we adopted a strategy that has been previously validated for DRG cell counts (Chen et al., 2006). Briefly, serial sections of thoracic DRG were distributed on six slides, which were subjected to different markers including the pan-neuronal marker *SCG10*. This approach allowed us to refer all countings to the total number of neurons (*SCG10*⁺). For each genotype, two to four DRG were counted in at least three independent animals. For embryonic tissues, after counting the total number of neurons expressing a certain marker, we related that figure to the number of sections. All cell counts were conducted by an individual who was blind to the experimental

condition of the animals. Statistical significance was set to $p < 0.05$ and assessed using one-way ANOVA followed by unpaired *t* test (all other experiments).

Cell lines and transfection. PC12 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and 5% horse serum. The day before transfection cells were trypsinized and cells plated onto 5 cm dishes [for quantitative reverse transcription (RT)-PCR; 5×10^5 cells] or six well plates (for luciferase assays; 2×10^5 cells) coated with polyornithine. Transfection was performed using Lipofectamine reagent and Opti-MEM medium (Invitrogen) following the manufacturer's instructions. After 6 h, transfection complexes were removed and cells cultured overnight in DMEM with no serum.

To test *Met* overexpression, HEK cells were cultured in DMEM-10% FCS. The day before transfection, they were plated onto 10 cm dishes. This cell line was transfected using Promofectin reagent (PromoKine) following the manufacturer's instructions.

For treatment with exogenous growth factors, cells were starved overnight in serum-free medium and the next day recombinant NGF (Alomone Labs), HGF (R&D Systems), EGF (R&D Systems), and FGF8 (R&D Systems) were added to the medium.

Western blot. HEK cells were first homogenized in ice-cold disruption buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 10 mM KCl, 3 mM

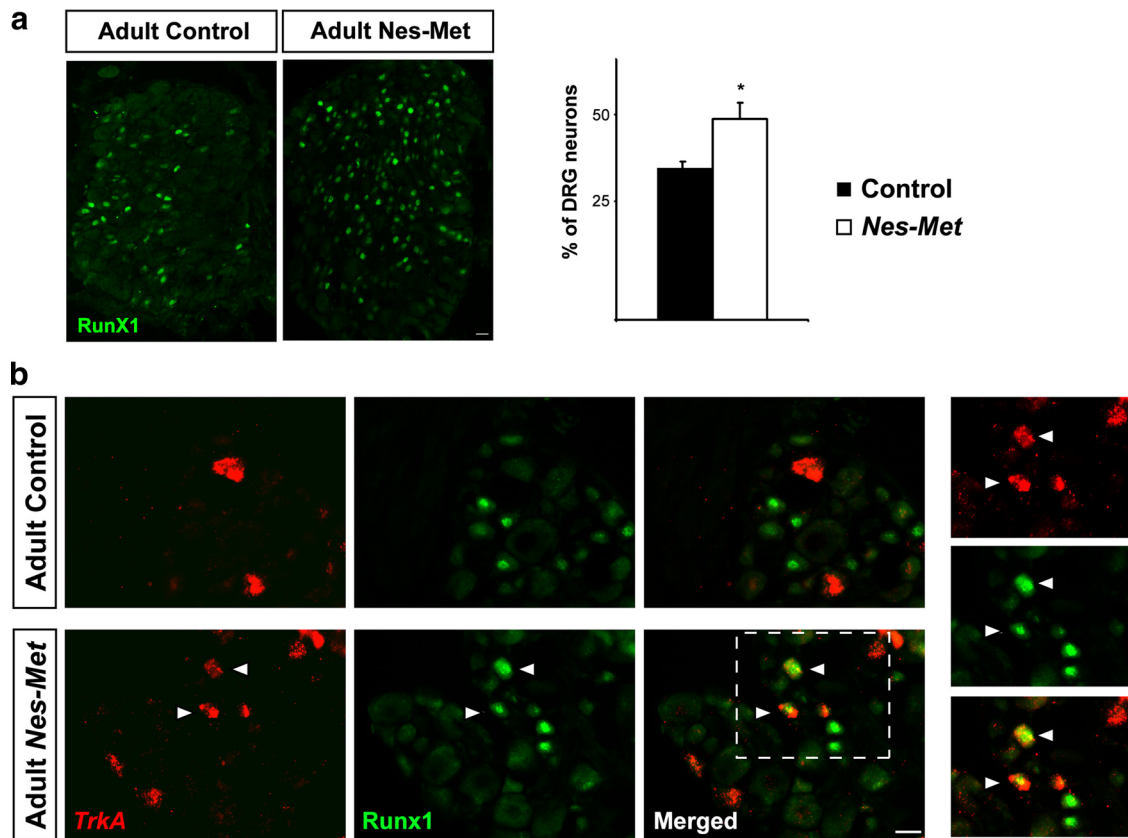


Figure 3. Met controls *Runx1* extinction *in vivo*. **a**, Increased number of RUNX1 neurons in *Nes-Met* mutant animals. Pictures of RUNX1 immunofluorescence show a moderate increase of RUNX1⁺ neurons in *Nes-Met* mice. Quantitative analysis revealed a significant difference in these animals compared with control ($*p < 0.05$; control, $n = 3$; *Nes-Met*, $n = 3$). **b**, Persistent expression of RUNX1 in *TrkA*⁺ neurons in adult *Nes-Met* mice. *TrkA in situ* hybridization followed by immunofluorescence for RUNX1 revealed that RUNX1 is completely excluded from peptidergic neurons in control animals (top). In contrast, a number of *TrkA*⁺ neurons maintains RUNX1 expression in adult *Nes-Met* mice (bottom) suggesting that Met is necessary for proper *Runx1* extinction. Immunofluorescence for RUNX1. *In situ* hybridization for *TrkA*. Scale bars, 20 μ m.

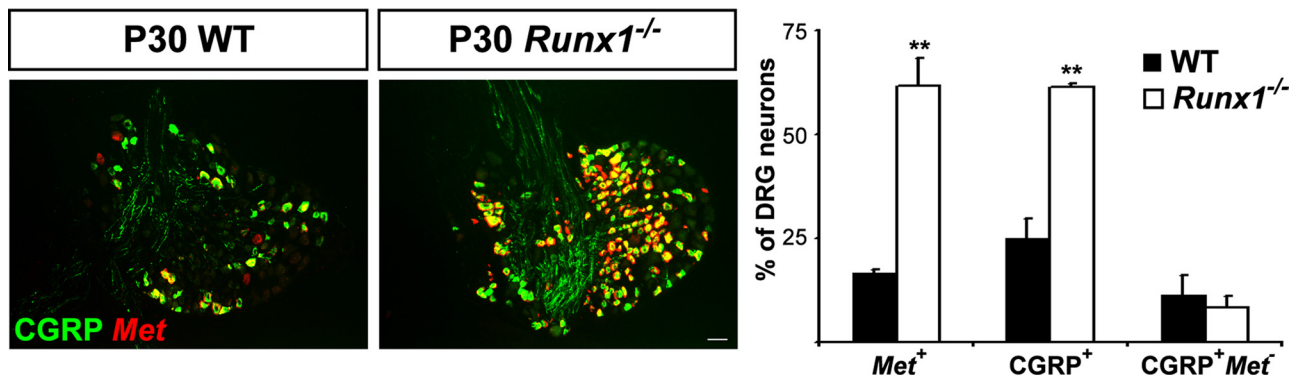


Figure 4. RUNX1 represses *Met* expression *in vivo*. *Met* is derepressed in *Runx1*^{-/-} DRG neurons. Representative pictures illustrating the increase of *Met* expression in DRG neurons in the absence of RUNX1. Quantitative analysis demonstrated that *Met* increase in *Runx1*^{-/-} mice was parallel to the expansion of CGRP population. Interestingly, the proportion of CGRP⁺Met⁻ neurons was not affected (** $p < 0.001$, control, $n = 3$; *Runx1*^{-/-}, $n = 3$). Immunofluorescence for CGRP. *In situ* hybridization for *Met*. Scale bar, 50 μ m.

MgCl₂, 0.5% NP-40) containing a protease inhibitor mix. After sonication, samples were centrifuged for 10 min at 10,000 \times *g* at 4°C. The supernatant was removed and stored at -80°C. Fifty micrograms of protein was electrophoresed and then transferred to a nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% ethanol. After blocking (for 1 h at room temperature in Tris buffer containing 5% nonfat dried milk and 0.05% Tween-20), blots were incubated in primary mouse monoclonal antibody anti- α -tubulin 1/10,000 (Sigma) or rabbit polyclonal anti-human Met 1/500 (Santa Cruz Biotechnology) overnight at 4°C. Membranes were rinsed three times, incubated with the appropriate horseradish peroxidase-linked sec-

ondary antibody at 1/5000 (Jackson ImmunoResearch Laboratories) for 1 h at room temperature and developed using ECL reagents (PerkinElmer).

Results

A subpopulation of peptidergic neurons arise from the *Runx1* lineage

Previous studies have proposed that *Runx1* downregulation is essential for a subpopulation of prospective nociceptors to undergo peptidergic differentiation (Chen et al., 2006; Woolf and Ma, 2007).

However, experimental evidence showing that peptidergic CGRP⁺/TrkA⁺ neurons derive from the initial population of nociceptive precursors coexpressing RUNX1 and TrkA is still lacking. To examine this possibility, we performed a fate-mapping analysis using a recently developed tamoxifen-inducible *Runx1-Cre* knock-in mouse strain (*Runx1^{Cre}*) (Samokhvalov et al., 2007) crossed with reporter mice (*Rosa26-loxP-STOP-loxP-eYFP*) (Srinivas et al., 2001). To induce recombination, pregnant females received a single injection of 4-hydroxytamoxifen at three developmental time points: E11.5, E12.5, or E13.5. Using this system, and knowing that ~88% of TrkA⁺ neurons coexpress Runx1 at E14.5 (Chen et al., 2006), we first evaluated the efficiency of recombination by monitoring the proportion of DRG neurons coexpressing TrkA and enhanced yellow fluorescent protein (eYFP) at birth. We found that pregnant mothers injected at E12.5 yielded the highest proportion of recombination in TrkA⁺ neurons compared with the two other time points ($1.61 \pm 0.19\%$ at E11.5, $n = 2$; $16.25 \pm 1.93\%$ at E12.5, $n = 5$; and $10.2 \pm 2.03\%$ at E13.5, $n = 4$). More importantly, we found a moderately high proportion of CGRP⁺ neurons coexpressing eYFP ($10.3 \pm 1.9\%$; $n = 5$) (Fig. 1). Taking into account the recombination efficiency, the percentage of CGRP⁺ neurons coexpressing eYFP rose to $50.0 \pm 6.7\%$. These data provide the first *in vivo* evidence showing that a subset of CGRP⁺ neurons (about one half) derives from TrkA⁺/RUNX1⁺ progenitors (Fig. 1).

Met is expressed in a subset of DRG neurons

Lineage-mapping experiments indicate that a subset of peptidergic neurons extinguish *Runx1* during development. In the search for factors that could participate in *Runx1* extinction, the HGF–Met system was a particularly interesting candidate. Previous studies have reported the presence of Met in DRG (Maina et al., 1997; Funakoshi and Nakamura, 2001). More importantly, HGF has been shown to act synergistically with NGF both in DRG and superior cervical ganglia (SCG) neurons (Maina et al., 1997).

The expression pattern of Met in the somatosensory system has not hitherto been examined in detail. Using *in situ* hybridization, we found in adult thoracic DRG that *Met* is present in $\sim 19.4 \pm 2.6\%$ of all DRG neurons. We then sought to determine whether *Met* expression was restricted to precise neuronal populations. We observed that $73.5 \pm 3.8\%$ of *Met* neurons coexpress TrkA, indicating that *Met*⁺ neurons mainly encompass peptidergic nociceptors (Fig. 2*a,b*). In support of this hypothesis, we found an extensive overlap between *Met* and the neuropeptide CGRP. Indeed, $>80\%$ of *Met*⁺ neurons coexpress CGRP and, conversely, $55.5 \pm 3.1\%$ of the total number of CGRP⁺ neurons coexpress *Met* (Fig. 2*a,b*).

We have also observed that *Met* is expressed in relatively few Ret⁺ neurons ($4.6 \pm 1.2\%$ corresponding to $6.2 \pm 4.2\%$ of *Met* neurons). Several lines of evidence suggest that *Met*⁺/Ret⁺ neurons are not nociceptive but proprioceptive neurons. First, they are large-diameter neurons and represent $<5\%$ of the total number of Ret-expressing neurons. Second, a significant proportion of *Met*⁺ neurons are positive for proprioceptive markers such as parvalbumin (PV) ($18.1 \pm 5.3\%$) or TrkC ($4.8 \pm 0.2\%$) (Fig. 2*a,b*). Third, and more importantly, it seems that *Met* and *Runx1* have mutually exclusive expression patterns and are never expressed in the same neurons in the adult DRG (Fig. 2*a*). Finally, we showed that most, if not all, *TrkB*⁺ putative mechanoreceptive neurons are devoid of *Met* ($1.2 \pm 0.9\%$).

We next examined the temporal expression pattern of *Met* in DRG. At E14.5, *Met* is mainly expressed in large neurons (supplemental Fig. 1*a*, available at www.jneurosci.org as supplemental material) and is coexpressed with PV (supplemental Fig. 1*b*, available at www.jneurosci.org as supplemental material). As de-

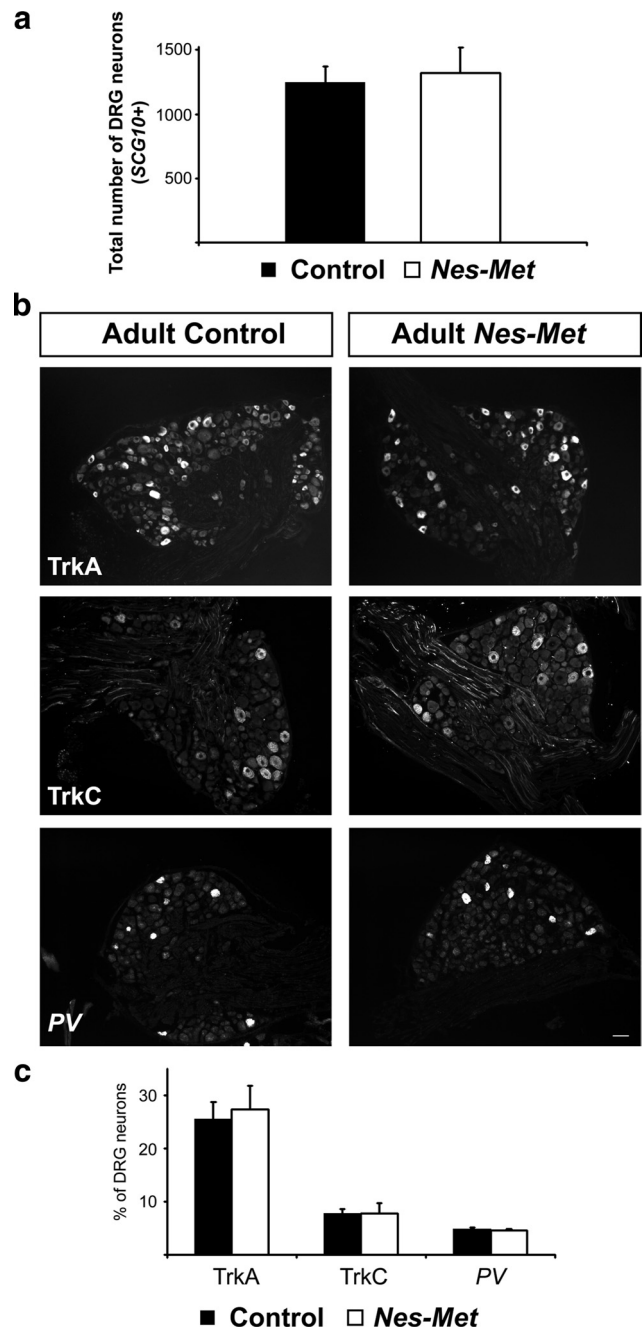


Figure 5. Conditional *Nes-Met* mutants do not show defects in the number of neurons or early specification of nociceptive and proprioceptive neurons. **a**, Quantification of DRG neurons using the pan-neuronal marker *SCG10* revealed no differences between *Nes-Met* and their control littermates (control, $n = 3$; *Nes-Met*, $n = 3$). **b**, Representative pictures illustrating the distribution of TrkA, TrkC, and PV-expressing neurons in control and *Nes-Met* DRG. Immunofluorescence for TrkA and TrkC. *In situ* hybridization for PV. **c**, Quantitative analysis of the numbers of DRG neurons positive for TrkA, TrkC, and PV showed no significant differences in *Nes-Met* mutants compared with control mice (control, $n = 4$; *Nes-Met*, $n = 4$). Scale bars, 50 μm .

velopment proceeds, *Met* expression expands and starts to be detected in a subset of small-diameter CGRP⁺ neurons (supplemental Fig. 1*c,d*, available at www.jneurosci.org as supplemental material).

We hypothesized that progressive expression of *Met* in a subset of CGRP⁺ neurons might be the key event for *Runx1* extinction *in vivo*. An absolute prerequisite to validate this hypothesis is

peptidergic fate. To explore this possibility, DRG cultures were transfected with a GFP-reporter vector alone or together with a plasmid driving human Met overexpression (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Immediately after transfection, neurons were treated for 24 h with no growth factors, NGF (20 ng/ml), HGF (50 ng/ml), or a combination of NGF (20 ng/ml) and HGF (50 ng/ml), and the levels of *Runx1* were assessed by quantitative RT-PCR (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). We found that although HGF alone did not modify *Runx1* transcription, the combination of HGF and NGF led to a significant reduction of *Runx1* transcripts compared with NGF-treated cultures ($p < 0.05$) (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Overexpressing human Met in DRG neurons resulted in a more pronounced decrease of *Runx1* levels in response to HGF ($p < 0.05$) (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Similar results were obtained in PC12 cells (data not shown). Together, these *in vitro* studies indicated that activation of the Met-signaling cascade interferes with TrkA-triggered *Runx1* transcriptional regulation.

We next sought to examine whether repression of *Runx1* by Met signaling also occurs *in vivo*. For that purpose, we followed *Runx1* expression in *Nes-Met* mutants and control mice. We found that, at E15.5 and E18.5, the number of RUNX1⁺ neurons was similar in *Nes-Met* and control embryos (data not shown). In contrast, there was a significant increase in RUNX1 in the adult DRG ($34.3 \pm 1.4\%$ in control and $48.1 \pm 4.8\%$ in *Nes-Met*; $p = 0.019$) (Fig. 3a). These observations strongly argue for a repressive effect of Met on *Runx1* expression *in vivo*.

We hypothesized that if Met is necessary for *Runx1* extinction, then RUNX1 might persist in adult peptidergic TrkA⁺ neurons in the *Nes-Met* mice. As expected, TrkA and RUNX1 are largely segregated in non-overlapping populations in control animals (Fig. 3b). In contrast, a number of TrkA⁺ neurons coexpress RUNX1 in *Nes-Met* animals, demonstrating that *Runx1* extinction is impaired in the absence of Met. Altogether, these findings demonstrate that, *in vivo*, Met activity is required for proper extinction of *Runx1* in a subset of DRG sensory neurons.

RUNX1 suppresses Met expression *in vivo* and *in vitro*

We next sought to examine whether RUNX1 exerts a similar repressive effect on *Met* to ensure the generation of nonpeptidergic neurons. To verify this bidirectional regulation of Met and RUNX1, we examined *Met* expression in DRG of conditional *Runx1* knock-out mice (*Runx1*^{-/-}) (Chen et al., 2006). We found a dramatic expansion of *Met*⁺ neurons in *Runx1*^{-/-} DRG ($16.8 \pm 0.7\%$ in control and $61.6 \pm 6.7\%$ in *Runx1*^{-/-}; $p < 0.001$) (Fig. 4), which correlates with the previously described increase in the proportion of CGRP nociceptive neurons in *Runx1*^{-/-} mice. These findings demonstrate that RUNX1 also represses *Met* *in vivo* and further supports the role of Met in regulating CGRP expression. Moreover, the proportion of CGRP neurons expressing *Met* was found to be significantly increased in *Runx1*^{-/-} compared with controls ($54.7 \pm 10.1\%$ in control and $86.1 \pm 4.5\%$ in *Runx1*^{-/-}, $p < 0.01$). Interestingly, the number of CGRP⁺*Met*⁻ neurons was not significantly altered ($11.5 \pm 4.6\%$ in control and $8.4 \pm 2.7\%$ in *Runx1*^{-/-}, $p > 0.35$) (Fig. 4), suggesting that the subset of peptidergic neurons that does not express *Met* (representing about one half of the CGRP⁺ neurons in control mice) is not regulated by RUNX1. Finally, PC12 cells transfected with a plasmid driving human *Runx1* expression exhibited significantly lower levels of *Met* transcript compared with

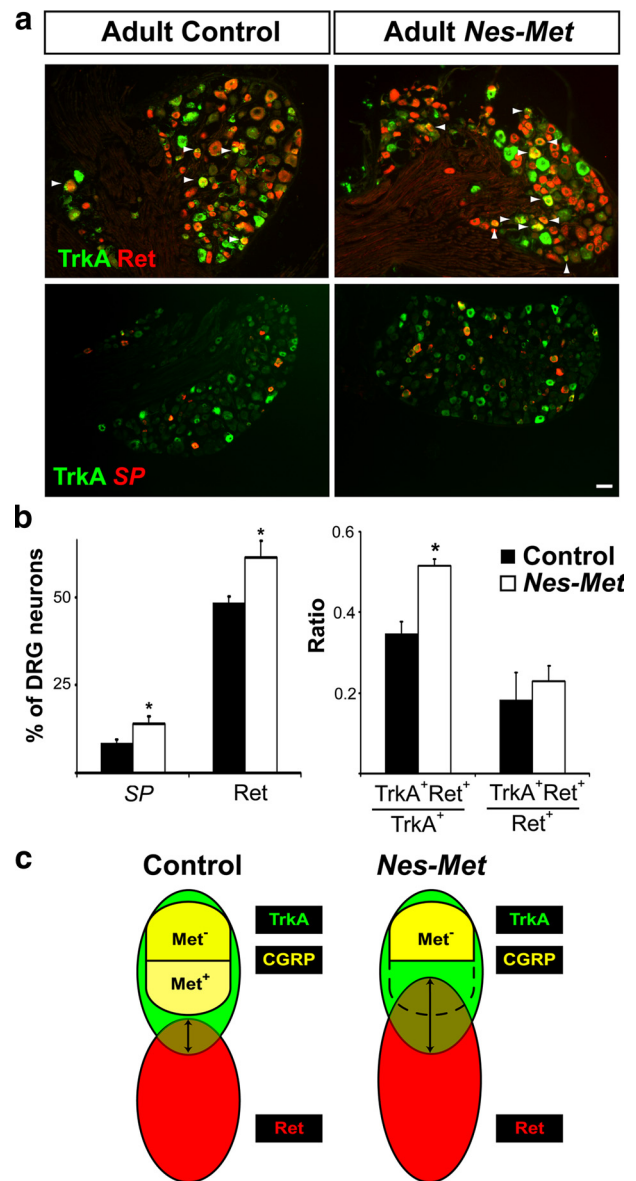


Figure 7. Phenotypic switch of nociceptive neurons in *Nes-Met* mice. **a**, Double staining of TrkA/Ret and TrkA/SP showed an increase in the number of DRG neurons positive for Ret, SP, and TrkA/Ret (arrowheads) in *Nes-Met* mice. Conversely, TrkA remained unchanged. Immunofluorescence for TrkA and Ret. *In situ* hybridization for SP. **b**, Quantitative analysis of the percentage of Ret and SP-expressing neurons (left) demonstrated a significant increase in *Nes-Met* DRG. The proportion of double-labeled TrkA⁺/Ret⁺ neurons is also augmented (right) (control, $n = 4$; *Nes-Met*, $n = 5$; $p < 0.05$). **c**, Schematic representation of the observed defects of nociceptive maturation in *Nes-Met* mice. Briefly, a subset of prospective peptidergic CGRP⁺ neurons is lost (dotted line) and switch their fate to become TrkA⁺/Ret⁺ neurons expressing SP. Scale bar, 50 μ m.

GFP-transfected or naive PC12 cells (data not shown). Altogether, these findings demonstrate the impact of RUNX1 repressor activity on *Met* expression and the importance of a proper balance between RUNX1 and Met for the correct development of nonpeptidergic nociceptive neurons.

Impaired maturation of a subset of peptidergic neurons in *Nes-Met* mice

We next investigated the impact of Met loss of function on the molecular diversification of DRG neurons. We found similar DRG neuronal counts in adult *Nes-Met* and control mice (Fig. 5a), confirming that the trophic effect elicited by the HGF/Met

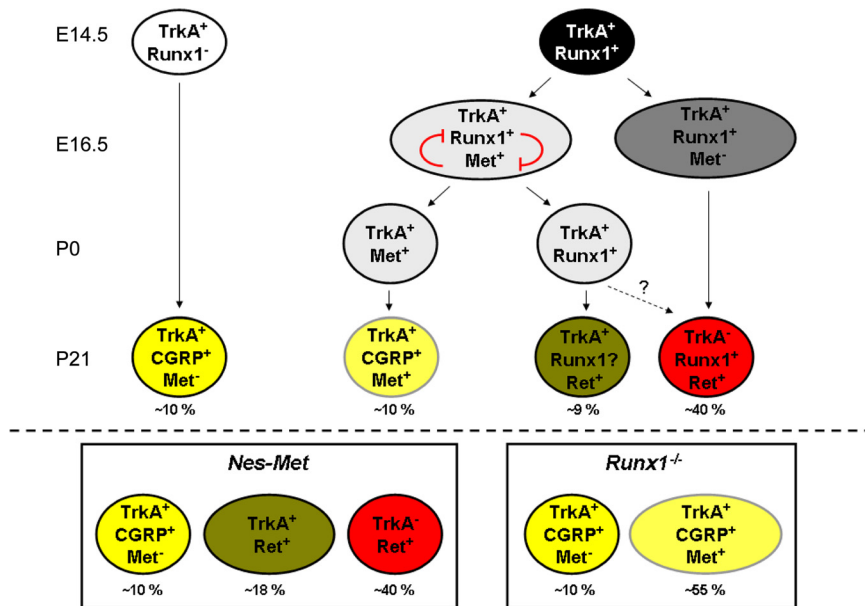


Figure 8. Proposed model for late maturation of nociceptive neurons. As described previously, prospective nociceptive neurons are $TrkA^+$ at E14.5. At this stage, most of these cells (88%) coexpresses $Runx1$ (right column), whereas only a minor population remains $TrkA^+ Runx1^-$ (left column). Our findings suggest that this latter population give rise to a subset of $CGRP^+$ nociceptive neurons independently from $Runx1$ and Met . According to our hypothesis, $Runx1$ high expressors would give rise to Ret^+ nonpeptidergic neurons. Finally, low levels of $Runx1$ might allow initial Met expression and the apparition of a transitory population of $Met^+ Runx1^+$ cells. Due to mutually repressive activity, this population would segregate and become either $Met^+ CGRP^+$ or $TrkA^+ Ret^+$ neurons. Boxes in the lower part of the scheme represent the final distribution of different subsets of nociceptive neurons in the absence of Met (left) or $Runx1$ (right).

system is only confined to early DRG neurons developmental stages (Maina et al., 1997). We then assessed the different subpopulations of DRG neurons and found that expression of PV and $TrkC$ remained unaffected (Table 1, Fig. 5*b,c*). Similarly, markers of nonpeptidergic nociceptive neurons such as $IB4$, $Mrgpra1$, $Mrgpra3$, or $Mrgprd$ (Table 1) were not altered in *Nes-Met* animals. These results indicate that Met inactivation does not affect either proprioceptive or nonpeptidergic nociceptive neurons differentiation.

When we examined $CGRP$ expression, on the other hand, we found it to be markedly reduced in *Nes-Met* mutants (Fig. 6*a,b*). In the adult thoracic DRG, $CGRP$ is expressed in $21.2 \pm 3.1\%$ of neurons, whereas this percentage is reduced to $11.1 \pm 2.3\%$ in *Nes-Met* mice ($p < 0.01$). Accordingly, the dorsal horn of the spinal cord and the trunk skin showed decreased immunoreactivity to $CGRP$ staining in *Nes-Met* mice, suggesting that less central and peripheral projections express this neuropeptide (supplemental Fig. 4*a,b*, available at www.jneurosci.org as supplemental material). However, staining with $IB4$ and the pan-neuronal marker $PGP9.5$ showed no differences between control and *Nes-Met* animals, suggesting that both central and peripheral axonal pathfinding occurs normally when Met is lost at late developmental stages.

We also found that the expression of other proteins specific to $CGRP^+$ nociceptive neurons is perturbed in *Nes-Met* animals. Previous studies have shown that $TRPV1$ is expressed in both peptidergic and nonpeptidergic neurons (Price and Flores, 2007), with peptidergic neurons being more responsive to capsaicin than nonpeptidergic neurons (Dirajlal et al., 2003). When we quantified the total number of $trpv1^+$ neurons, we found no difference between control and *Nes-Met* mice (Table 1). Importantly, the percentage of neurons expressing high levels of $trpv1$ was significantly reduced in mutant mice (control $6.5 \pm 1\%$;

Nes-Met $3.8 \pm 0.6\%$; $n = 3$; $p = 0.02$) (Fig. 6*a,b*, Table 1). Similarly, the total number of $trpa1^+$ neurons was found to be only moderately decreased in *Nes-Met* mice (Table 1), whereas the difference was significant for neurons expressing high levels of $trpa1$ (control, $4.7 \pm 0.4\%$; *Nes-Met*, $2.2 \pm 0.4\%$; $n = 4$; $p < 0.001$) (Fig. 6*a,b*, Table 1). In agreement with these observations, we found that Ca^{2+} transients in response to capsaicin ($1 \mu M$) or allyl-isothiocyanate ($1 \mu M$) had significantly smaller amplitudes in DRG neurons from *Nes-Met* compared with control mice (data not shown). Interestingly, $TrkA^+$ neuronal counts were not perturbed in the absence of Met ($25.6 \pm 3.2\%$ in control vs $27.3 \pm 4.4\%$ in *Nes-Met*). Together, these results indicate that the late maturation of peptidergic neurons is specifically impaired in *Nes-Met* mice.

A subset of peptidergic neurons switches fate in the absence of Met

We examined the molecular phenotype(s) adopted by the subset of presumptive $TrkA^+/CGRP^+$ peptidergic neurons. Previous studies have shown that adult nociceptive neurons can be divided into the following three classes (Molliver et al., 1997; Woolf and Ma, 2007): $TrkA^+$ peptidergic neurons; Ret^+ nonpeptidergic neurons; and a poorly characterized subtype of neurons expressing both $TrkA$ and Ret receptors. Unexpectedly, quantitative analyses showed that *Nes-Met* mutant animals displayed a significant increase in the proportion of Ret -expressing neurons ($48.6 \pm 1.6\%$ in control and $61.5 \pm 4.6\%$ in *Nes-Met*; $p = 0.02$) (Fig. 7). Given that the proportion of $TrkA^+$ neurons was unchanged in *Nes-Met* mice, we next investigated whether a lack of Met signaling led to an expansion of the neuronal population coexpressing $TrkA$ and Ret . Importantly, we found that the number of $TrkA^+/Ret^+$ neurons was doubled in *Nes-Met* mutants compared with control mice (control, $9.1 \pm 0.7\%$, $n = 3$; *Nes-Met*, $18.4 \pm 3.7\%$, $n = 3$). Moreover, when this proportion of $TrkA^+/Ret^+$ neurons was normalized to the total number of $TrkA^+$ or Ret^+ neurons, only the $TrkA$ ratio was significantly increased ($p < 0.05$) (Fig. 4*b*). Thus, these results reveal a potential role of Met in nociceptive maturation before $TrkA$ - Ret segregation. In addition, they indicate that loss of Met signaling drives a subset of peptidergic neurons from a $TrkA^+/CGRP^+$ to a $TrkA^+/Ret^+$ molecular phenotype.

The population of $TrkA^+/Ret^+$ neurons has not hitherto been described in detail. We therefore performed a number of triple-staining experiments to define molecularly this neuronal subtype and found the neuropeptide substance P (SP) to be of particular interest. SP has been previously described as a marker of peptidergic neurons (Marmigère and Ernfor, 2007). Nonetheless, our quantitative analysis demonstrated that $43.1 \pm 5.5\%$ of $TrkA^+/Ret^+$ neurons also express SP . As would be expected, we found that SP -expressing neurons were markedly increased in *Nes-Met* mutants compared with controls (Fig. 7*b*, Table 1).

The molecular switch from $TrkA^+/CGRP^+$ to $TrkA^+/Ret^+$ in *Nes-Met* mice suggests that $CGRP$ expression may be regulated by Met in a subset of peptidergic neurons. To test this hypothesis, we

followed the temporal expression pattern of CGRP in control and *Nes-Met* mice. At E15.5, a time point at which Met is not yet fully expressed in nociceptive neurons, we found no difference in the number of CGRP⁺ neurons in *Nes-Met* compared with control embryos (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). It is worth noting that at this same time point, CGRP just begins to be expressed in a subset of nociceptive neurons (Funakoshi et al., 2003; our unpublished observations). In contrast, a small, albeit significant, reduction of CGRP-expressing neurons was detected in *Nes-Met* embryos at E18.5 (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). These findings perfectly correlate with the late onset and confinement of *Met* expression to a subset of CGRP⁺ neurons and raise the possibility that HGF-Met signaling regulates CGRP expression exclusively in these neurons.

Discussion

Here we report a novel mechanism for peptidergic specification involving a cross talk between RUNX1 and HGF-Met signaling. We provide compelling *in vivo* evidence that a cohort of nociceptive neurons acquires a peptidergic identity by using HGF-Met signaling in concert with NGF-TrkA to extinguish *Runx1* and activate CGRP expression. Conversely, we also demonstrated that a second subset of peptidergic neurons mature independently of HGF-Met and RUNX1. Finally, we observed that persistent expression of RUNX1 is required for proper *Met* repression in prospective nociceptors undergoing nonpeptidergic differentiation. These data indicate that a balance between RUNX1 and Met is essential to determine nonpeptidergic versus peptidergic neuronal fate.

While intensive work has unraveled the transcriptional program driving nonpeptidergic maturation (Chen et al., 2006; Marmigère et al., 2006; Luo et al., 2007; Yoshikawa et al., 2007), little is known about the generation of peptidergic nociceptors. Our findings, obtained using four complementary mice models (*Nes-Met*, *Runx1*^{-/-}, *Runx1*^{Cre}, and *Runx1*^{LacZ}), indicate that peptidergic nociceptive neurons have a dual origin. Fate-mapping analysis using *Runx1*^{Cre} mice (Samokhvalov et al., 2007) provides strong *in vivo* evidence that a class of peptidergic neurons derive from a RUNX1 lineage. Data obtained from *Nes-Met* mice show that a subset of presumptive peptidergic nociceptors loses CGRP expression in the absence of Met. More importantly, a thorough examination of *Runx1*^{-/-} DRG demonstrates that a fraction of peptidergic neurons (CGRP⁺ Met⁻) remains unaffected in the absence of RUNX1 (representing ~50% of CGRP⁺ neurons in the wild type) arguing that half of the TrkA⁺CGRP⁺ neurons might be generated independently from Met and Runx1 (i.e., from TrkA⁺Runx1⁻Met⁻ precursors). Conversely, the other half could be modified in the absence of Met or RUNX1, indicating that it might arise from TrkA⁺RUNX1⁺Met⁺ precursors. Finally, use of *Runx1*^{LacZ} transgenic mice allowed us to confirm the existence of a transient population of TrkA⁺RUNX1⁺Met⁺ precursors, lending further support to the emerging theory of the dual origin of peptidergic neurons. Collectively, these data provide an additional aspect that highlights the exquisite refinement of nociceptive maturation.

Mechanisms controlling *Runx1* expression are still poorly understood (Marmigère and Ernfors, 2007; Woolf and Ma, 2007). Pioneering work from David Ginty's group has begun to unravel the molecular pathways responsible for RUNX1 maintenance in nonpeptidergic neurons (Luo et al., 2007). This study provided compelling evidence that NGF signaling is crucial for this process. In agreement with these results, we observed that NGF treatment seems to be required for *Runx1* expression in PC12 cells

(data not shown). The findings reported here demonstrated not only that *Runx1* extinction is an active process necessary for peptidergic specification but also demonstrated that Met signaling is an essential player in these events. We showed that deletion of Met in the DRG resulted in a significant increase of RUNX1⁺ neurons and therefore in a deficient peptidergic maturation. More importantly, we showed that RUNX1 is retained in TrkA⁺ neurons (Fig. 3*b*) from adult *Nes-Met* animals, indicating that Met is essential for proper extinction of *Runx1*. Additionally, primary cultures of DRG neurons treated with NGF and HGF exhibit lower levels of *Runx1*. Thus, our finding that HGF-Met contributes to *Runx1* extinction extends our understanding of the molecular control of *Runx1* expression.

Based on our *in vivo* and *in vitro* results, we propose the following model of late nociceptor specification (Fig. 8). Depending on *Runx1* expression, prospective nociceptive neurons can be divided into the following two classes (Chen et al., 2006): RUNX1⁻ neurons and RUNX1⁺ neurons. According to our *in vivo* data, the first population of peptidergic nociceptors most likely derives from TrkA⁺RUNX1⁻ neurons (Fig. 8, left column). The absence of RUNX1 repressor activity would allow NGF-TrkA signaling to drive peptidergic differentiation as previously shown (Lindsay and Harmar, 1989; Freeland et al., 2000). This population probably accounts for the remaining CGRP⁺ neurons in the *Nes-Met* mice, as well as for the population of CGRP⁺Met⁻ neurons that is not modified in *Runx1*^{-/-} mice. Regarding neurons expressing RUNX1, several scenarios could be envisaged. In the first one, the final phenotype of these neurons might depend on RUNX1 levels. The large majority of these neurons express high levels of RUNX1 and would repress *Met* expression, becoming therefore nonpeptidergic neurons (Fig. 8, right column). In contrast, a small proportion of these same neurons will express RUNX1 at a level that is permissive for *Met* expression (Fig. 8, middle column). Given the mutually repressive activity of Met and RUNX1, nociceptors coexpressing these two factors might be transient and rapidly segregate either into Met⁺ peptidergic (if Met signaling is strong enough to extinguish *Runx1* expression) or into TrkA⁺Ret⁺ neurons (if RUNX1 activity is strong enough to repress *Met* expression). An alternative hypothesis is that RUNX1 might change its transcriptional activity during development; at early stages, it might act as an activator (e.g., positively controlling Trp channels, Ret, and Met expression), whereas, later on, it might switch to a repressive state (e.g., negatively controlling CGRP), as previously shown for Mrg receptors (Liu et al., 2008). Finally, one could imagine that additional factors might participate in this process. Thus, access to HGF might limit Met activation to certain neurons and/or expression of distinct cofactors might differentially modulate RUNX1 activity.

Why is *Runx1* expression activated only in a subset of TrkA⁺ neurons? Prior work has revealed that nociceptive neurons are generated in three sequential waves (Ma et al., 1999; Maro et al., 2004; Kramer et al., 2006b; Marmigère and Ernfors, 2007). The first arises from neural crest progenitor cells expressing the transcription factor Neurogenin2 (Ngn2). The second one is dependent on Ngn1 cells and gives rise to the majority of nociceptive neurons. Finally, the third wave derives from boundary cap cells known to be Krox20⁺ (Maro et al., 2004). It is well established that RUNX1 is restricted to Ngn1-derived neurons (Ma et al., 1999; Kramer et al., 2006b). Therefore, one attractive hypothesis is that nociceptive neurons arising from other sources (Ngn2⁺ progenitors and/or boundary cap cells) might give rise to the population of TrkA⁺/RUNX1⁻ neurons. Analysis of RUNX1 in

previously described *Ngn* mutants (Ma et al., 1999) might provide new insights into this issue.

Is this developmental diversification of peptidergic neurons functionally relevant? Electrophysiological recordings showed that DRG neurons exhibiting strong responses to capsaicin and cinnamaldehyde mainly belong to the peptidergic class of nociceptive neurons (Dirajlal et al., 2003; Story et al., 2003). Interestingly, absence of *RUNX1* leads to the expansion of a peptidergic population, accompanied by the selective loss of *Trpa1* and high levels of *Trpv1* (Chen et al., 2006). These molecular findings nicely correlate with the decreased behavioral responses to cold stimulation and noxious heat in *Runx1*^{-/-} knock-out mice. Our observation that high levels, but not intermediate levels, of *TRPV1/TRPA1* are also affected in an independent mouse model (*Nes-Met*) gives further support to this hypothesis.

Collectively, the data presented in this study indicate that Met signaling is crucial for at least two elements of peptidergic maturation: the extinction of *Runx1* and the activation of CGRP expression. In this context, the reciprocal repression described here for Met and *RUNX1*, as well as their subsequent segregation, provide an attractive mechanism for the late diversification of nociceptive neurons.

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