Development/Plasticity/Repair

Mechanisms of Compartmentalized Expression of Mrg Class G-Protein-Coupled Sensory Receptors

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Mrg class G-protein-coupled receptors (GPCRs) are expressed exclusively in sensory neurons in the trigeminal and dorsal root ganglia. Pharmacological activation of Mrg proteins is capable of modulating sensory neuron activities and elicits nociceptive effects. In this study, we illustrate a control mechanism that allows the Runx1 runt domain transcription factor to generate compartmentalized expression of these sensory GPCRs. Expression of *MrgA*, *MrgB*, and *MrgC* subclasses is confined to an "A/B/C" neuronal compartment that expresses Runx1 transiently (or does not express Runx1), whereas *MrgD* expression is restricted to a "D" compartment with persistent Runx1 expression. *Runx1* is initially required for the expression of all *Mrg* genes. However, during late development Runx1 becomes a repressor for *MrgA/B/C* genes. As a result, *MrgA/B/C* expression persists only in the Runx1$^-$ "A/B/C" compartment. In Δ*446* mice, in which Runx1 lacks the C-terminal repression domain, expression of *MrgA/B/C* genes is dramatically expanded into the Runx1$^+$ "D" compartment. *MrgD* expression, however, is resistant to Runx1-mediated repression in the "D" compartment. Therefore, the creation of Runx1$^+$ and Runx1$^-$ compartments, in conjunction with different responses of *Mrg* genes to Runx1-mediated repression, results in the compartmentalized expression of *MrgA/B/C* versus *MrgD* genes. Within the *MrgA/B/C* compartment, *MrgB4*-expressing neurons innervate exclusively the hairy skin. Here we found that Smad4, a downstream component of bone morphological protein-mediated signaling, is required selectively for the expression of *MrgB4*. Our study suggests a new line of evidence that specification of sensory subtypes is established progressively during perinatal and postnatal development.

Key words: **Runx1; nociceptors; Mrg class G-protein-coupled receptors; nociceptive ion channels and receptors; cell type specification; dorsal root ganglia**

Introduction

The mouse genome encodes 50 Mrg (also named Mrgpr/SNSR) class G-protein-coupled receptors (GPCRs), 12 of which are expressed exclusively in the somatic sensory neurons located in the trigeminal ganglia and dorsal root ganglia (DRG) (Dong et al., 2001; Lembo et al., 2002; Choi and Lahn, 2003; Zylka et al., 2003; Zhang et al., 2005; Burstein et al., 2006). These *Mrg* genes are divided into four subclasses: A (*MrgA1-A8*), B (*MrgB4* and *B5*), C (*MrgC11*), and D (*MrgD*) (Zylka et al., 2003; Zhang et al., 2005). In adult mice, these *Mrg* subclasses exhibit compartmentalized expression, as indicated by the nonoverlapping expression of *MrgD* with other *Mrg* genes (Dong et al., 2001; Zylka et al., 2003, 2005) (see below). Most interestingly, *MrgD*$^+$ and *MrgB4*$^+$ neurons innervate distinct peripheral targets, skin epidermis and the hairy skin, respectively (Zylka et al., 2005; Liu et al., 2007). Pharmacological activation of Mrg proteins is able to modulate neuronal activities and evoke painful responses (Grazzini et al., 2004; Cai et al., 2007; Crozier et al., 2007). However, how compartmentalized expression of *Mrg* genes is established during development is poorly understood.

A series of recent studies has shown that the runt domain transcription factor Runx1 plays a pivotal role in controlling the development of sensory neurons, particularly those involved with nociception (Theriault et al., 2005; Chen et al., 2006; Kramer et al., 2006; Marmigere et al., 2006; Zhong et al., 2006; Woolf and Ma, 2007; Yoshikawa et al., 2007). Runx1 is initially expressed in most nociceptors (Levanon et al., 2002; Chen et al., 2006). Subsequently, Runx1 expression is extinguished in most peptidergic nociceptors (Chen et al., 2006), and persists primarily in nonpep-

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tidergic nociceptors (Chen et al., 2006; Kramer et al., 2006). Genetic studies demonstrate that Runx1 is required for the expression of nearly two-dozen ion channels and receptors, including the whole family of *Mrg* genes (Chen et al., 2006) (see Fig. 1).

In this study, we will illustrate a control mechanism that allows Runx1 to establish compartmentalized expression for Mrg class sensory GPCRs. Accordingly, expression of *MrgA*, *MrgB*, and *MrgC* subclasses is confined to an "A/B/C" neuronal compartment that expresses Runx1 transiently (or does not express Runx1), whereas *MrgD* expression is restricted to a "D" compartment with persistent Runx1 expression.

Materials and Methods

Animals. The generation of *Runx1* conditional mutant mice, *Wnt1-Cre* transgenic mice, Δ*446* mice, *Smad4* conditional null mice, *MrgD-GFP* mice and *SNS-Cre* transgenic mice has been described previously (Jiang et al., 2000; Yang et al., 2002; Agarwal et al., 2004; Nishimura et al., 2004; Growney et al., 2005; Zylka et al., 2005). The morning that vaginal plugs were observed was considered as E0.5. PCR-based genotyping for conditional null mice has been described previously (Chen et al., 2006). The following primers were used for the *MrgD-GFP* mutant allele, 5′-ATG GTG AGC AAG GGC GAG GAG-3′ and 5′-TCG CGC TTC TCG TTG GGG TCT TTG-3′; for the *MrgD-GFP* wild-type allele, 5′-ATG AAC TCC ACT CTT GAC AG-3′ and 5′-CAC TGG TGT TTG TTG GGA TG-3′; for the Δ*446* mutant allele, 5′-TCG CTT TCA AGG TGG TGG CA-3′ and 5′-TCC GGA GCC GTT GAG AGT C-3′; and for the wild-type *Runx1* allele, 5′-TGT CTC TGC ATC GCA GGA CT-3′ and 5′-TGT GCG TTC CAA GTC AGT TGT-3′.

In situ *hybridization and immunostaining.* The *in situ* hybridization (ISH) procedure and the probes used in this study have been described previously (Chen et al., 2006). For ISH combined with anti-GFP fluorescent immunostaining, GFP was detected before the ISH procedure. Frozen sections were dried at room temperature for 10 min, postfixed in 4% paraformaldehyde for 15 min, washed 3 times with PBS for 5 min each, incubated with anti-GFP primary antibody (1:500 in PBT; Invitrogen, Carlsbad, CA) for 30 min, washed three times with PBS for 5 min each, and incubated with Alexa-488-conjugated secondary antibody (1:200 in PBT; Invitrogen) for 30 min. Note that all solutions were prepared under RNase-free conditions. To avoid the masking of fluorescent signal by the subsequent ISH dye signal, all sections were photographed by fluorescence microscopy, followed by regular ISH procedure using an alkaline phosphatase (AP)-conjugated antibody (1:2000; Roche, Indianapolis, IN) and 5-bromo-4-chloro-indolyl-phosphate/nitroblue–tetrazolium (BCIP/NBT) substrates for development. The bright-field images of opaque *in situ* signals were inverted into pseudo-fluorescent images, and then merged with GFP fluorescent images.

ISH combined with anti-Runx1 (from Dr. Thomas Jessell, Columbia University, New York, NY) or IB4 fluorescent staining has been described previously (Chen et al., 2006), with the following modification. Fluorescent signals of Runx1 and IB4 were photographed first, followed by color development of ISH signals with BCIP/NBT substrates. The bright-field images of ISH signals were inverted and then merged with fluorescent images. As noted above, this sequential photographing avoids the masking of low-level fluorescent signals by nonfluorescent ISH signals, leading to a more sensitive detection of the coexpression of Runx1 or IB4 with genes of interest. For example, in this study, we were able to detect high or medium levels of Runx1 protein in virtually all $MrgD^+$ cells, whereas the previous procedure (in which ISH signals developed first, followed by Runx1 immunostaining) failed to show medium levels of Runx1 expression in a fraction of $MrgD^+$ neurons (Chen et al., 2006).

For double color ISH, two probes were labeled with fluorescein- or digoxigenin-UTP. The probes were detected with peroxidase (POD)-conjugated anti-digoxigenin antibody (1:400; Roche) and AP-conjugated anti-fluorescein antibody (1:1000; Roche). The fluorescent signal development for POD consists of three sequential amplification steps: (1) TSA Biotin System (1:100; PerkinElmer, Wellesley, MA), (2) Vectastain ABC Kit (Vector Laboratories, Burlingame, CA), and (3) TSA Fluorescein System (1:50; PerkinElmer). The fluorescent signals were photographed, followed by development for AP with BCIP/NBT substrates. The bright-field images of nontransparent purple signals were inverted, and then merged with fluorescent images. Because the signal of the second probe was developed with nonfluorescent BCIP/NBT substrates, this modified double color ISH procedure is more sensitive than the procedure that involves fluorescent substrates for both probes (Dong et al., 2001; Zylka et al., 2003). For example, the colocalization of *MrgA3* and *MrgB4* shown in this study was not detected in the previous study (Zylka et al., 2003).

Cell counting. To compare total DRG neurons, T12 thoracic and L4/L5 lumbar DRG were dissected from three pairs of mutant and control mice, fixed, embedded, sectioned at 12 μm thickness. One of six adjacent sets of sections was hybridized with the pan-neuronal probe *SCG10*, and the number of $SCG10^+$ neurons was counted. Only cells containing nuclei were counted. To determine the percentages of neurons expressing molecular markers, six adjacent sets of sections were prepared from each T12 DRG and probed separately with six different probes, one of which was the pan-neuronal marker *SCG10* so that percentages of DRG neurons expressing a gene can be calculated. Four or more independent T12 DRG or L4/L5 lumbar DRG were used for each counting. The difference between wild-type and mutant samples was subjected to a Student's *t* test, with $p < 0.05$ considered significant.

Results

Loss of *Mrg* gene expression in conditional Runx1 null mutants

We recently reported that Runx1 is necessary for the expression of two *Mrg* subclasses, class B (*MrgB4* and *MrgB5*) and class D (*MrgD*) (Chen et al., 2006). To determine whether Runx1 controls the expression of class A (*MrgA1-A8*) and class C (*MrgC11*) genes, we analyzed *Runx1* conditional knock-out mice by crossing floxed *Runx1* mice ($Runx1^F$) with *Wnt1-Cre* transgenic mice (Jiang et al., 2000; Growney et al., 2005). We have previously reported that in $Runx1^{F/F}$;*Wnt1-Cre* mice [referred here to as $Runx1^{-/-}$(*Wnt1*)], Runx1 is removed in sensory precursors, therefore representing a complete null mutation in nociceptors (Chen et al., 2006). We found that expression of *MrgA1-A8* was eliminated in $Runx1^{-/-}$(*Wnt1*) mice at every stage examined, from embryonic day 16.5 (E16.5) to postnatal day 30 (P30) (Fig. 1*A–F*) (data not shown). High levels of *MrgC11* expression were also eliminated in adult $Runx1^{-/-}$(*Wnt1*) mice (Fig. 1*H* vs *G*, arrow), but low levels of expression were independent of Runx1 (Fig. 1*H* vs *G*, arrowheads). We have previously shown that neuronal survival is not affected in $Runx1^{-/-}$(*Wnt1*) mice (Chen et al., 2006). Therefore, the loss of *Mrg* gene expression is unlikely to be caused by a loss of neuronal cells. Altogether, these data imply a requirement of Runx1 for the expression of all *Mrg* class GPCR genes.

Compartmental expression of *Mrg* genes in adult DRG

To understand how *Mrg* gene expression is regulated, we examined in detail the cellular compartments that express *Mrg* subclasses by using double color *in situ* hybridizations. As reported previously, adult $MrgD^+$ neurons did not coexpress *MrgA3* (Fig. 2*A*) or *MrgB4* (Fig. 2*B*) (Dong et al., 2001; Zylka et al., 2005). Here we found that the expression of two class *A* members, *MrgA3* and *MrgA4*, overlapped extensively with each other (Fig. 2*C*, arrows). Expression of *MrgB4* was confined to neurons that exhibited a low level of *MrgA3* (Fig. 2*D*, arrows); neurons with elevated *MrgA3* expression did not coexpress *MrgB4* (Fig. 2*D*, arrowheads). It was reported that in adult DRG, elevated *MrgC11* expression is excluded from $MrgD^+$ neurons (also see Fig. 3*C*), but overlaps with $MrgA3^+$ neurons (Zylka et al., 2005). Consistent with the restriction of *MrgB4* expression to a subset of

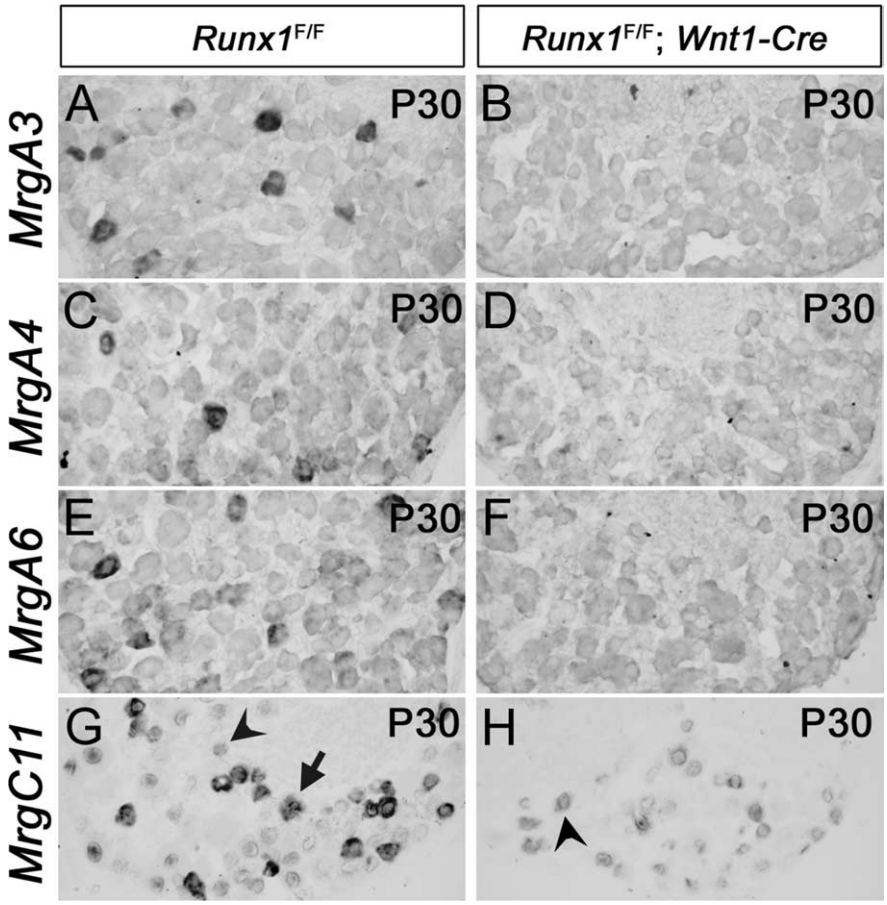

Figure 1. Loss of *Mrg* gene expression in *Runx1*$^{F/F}$; *Wnt1-Cre* mice [mentioned as *Runx1* $^{-/-}$(*Wnt1*) mice in text]. *In situ* hybridizations with indicated *Mrg* probes were performed on sections through P30 T12 thoracic DRG.

MrgA3$^+$ neurons, *MrgB4* was expressed in a subset of *MrgC11*$^+$ neurons (Fig. 2*E*, arrows). Previous studies failed to detect a coexpression of *MrgB4* with *MrgA3* or *MrgC11* (Zylka et al., 2005); the discrepancy may be attributable to a difference in sensitivity between using nonfluorescent substrates and fluorescent substrates (see Materials and Methods)

In summary, neurons expressing *Mrg* genes in adult DRG can be divided into two compartments: the "D" compartment that expresses *MrgD* and the "A/B/C" compartment that shows a partially overlapping expression of *MrgA*, *MrgB*, and *MrgC* genes (Fig. 2*F*).

Progressive segregation of *Mrg* expression compartments

We next asked when the segregation of the "A/B/C" and "D" neuronal compartments emerges, by examining their expression at early developmental stages. We found that robust expression of *MrgC11* and *MrgD* was already detected at E16.5, whereas expression of *MrgA3* and *MrgB4* was initiated at P0 and P2, respectively (Fig. 3*A*). To further examine the relationship among *Mrg*$^+$ neurons, we performed a transient fate-mapping experiment by using the *MrgD*^{GFP} knock-in mice, in which the *MrgD* coding region is replaced by the gene for green fluorescent protein (GFP) (Zylka et al., 2005). In adult *MrgD*$^{GFP/+}$ heterozygous mice, double staining detected a complete overlap between GFP and *MrgD* mRNA, suggesting that GFP expression can be used to faithfully mark *MrgD*$^+$ neurons (Zylka et al., 2005) (data not shown).

In P2 *MrgD*$^{GFP/+}$ heterozygous mice, ~96.3% (734 of 762) of *MrgD*$^+$ neurons, marked by GFP expression, coexpressed *MrgC11* (Fig. 3*B*). We also found that at P2 and P4, GFP protein was detected in 79.4% (131 of 165) and 82.3% (51 of 62) of *MrgA3*$^+$ and *MrgB4*$^+$ neurons, respectively (Fig. 3*D*,*F*, arrows). Because adult *MrgA3*$^+$ and *MrgB4*$^+$ neurons coexpress *MrgC11* (Fig. 2), we concluded that most *Mrg*$^+$ neurons at early developmental stages coexpress *MrgD* and *MrgC11*. By P30, GFP protein, however, can no longer be detected in *MrgA3*$^+$, *MrgB4*$^+$ or *MrgC11*$^+$ neurons in *MrgD*$^{GFP/+}$ heterozygous mice (Fig. 3*C*,*E*,*G*). These data suggest that the "D" compartment retains *MrgD* expression and extinguishes *MrgC11* expression, and the "A/B/C" compartment retains *MrgC11* expression, extinguishes *MrgD* expression, and acquires the expression of *MrgA* and *MrgB* genes (summarized in Fig. 3*H*).

Dynamic Runx1 expression marks two *Mrg* expression compartments

To gain insights into how Runx1 regulates *Mrg* genes, we examined Runx1 expression in *Mrg*$^+$ neurons at multiple developmental stages. Expression of *MrgD* was confined predominantly to Runx1$^+$ neurons, from P7 to P30 (Fig. 4*A*,*B*). Unexpectedly, despite the loss of expression of *MrgA3* and *MrgB4* in *Runx1* $^{-/-}$(*Wnt1*) mice, Runx1 protein was not detected in *MrgA3*$^+$ or *MrgB4*$^+$ neurons at neonatal and adult stages, including P2 and P4 when the expression of these two genes is actively being established (Fig. 4*C*–*F*). It was reported previously that Runx1 is expressed in most, if not all, nociceptors at E12.5-E14.5, but is extinguished in ~50% of nociceptors during perinatal and postnatal development (Chen et al., 2006). The simplest interpretation is that Runx1 is expressed transiently in immature nociceptors that are fated to become *MrgA3*$^+$ or *MrgB4*$^+$ neurons.

In summary, among *Mrg*$^+$ neurons, persistent Runx1 expression marks the "D" neuronal compartment that expresses *MrgD*, whereas Runx1 is probably expressed transiently in the "A/B/C" compartment that expresses *MrgA*, *MrgB*, and *MrgC* subclasses (Fig. 4*G*). However, it is possible that Runx1 might never be expressed in some *MrgA3*$^+$ or *MrgB4*$^+$ neurons, thereby nonautonomously controlling the expression of these sensory GPCRs (Fig. 4*G*). Previous studies show that *Mrg*$^+$ neurons express Ret, but not TrkA (Dong et al., 2001; Zylka et al., 2003). The lack of Runx1 expression in *MrgA/B/C*$^+$ neurons implies that Runx1-negative cells include both TrkA$^+$ neurons (Chen et al., 2006) and Ret$^+$ neurons.

Δ*446*/Δ*446* mice

Runx1 protein contains an N-terminal DNA-binding runt domain, a middle transcriptional activation domain, and a C-terminal peptide VWRPY that is capable of binding the Groucho class transcriptional repressor complex (Fig. 5*A*). Runx1 therefore can act as either a transcriptional repressor or activator (Durst and Hiebert, 2004). To determine whether Runx1 repressor activity contributes to nociceptor phenotype

specification, including compartmentalized expression of *Mrg* genes, we analyzed Δ*446* mice (Nishimura et al., 2004). Δ*446* encodes a truncated Runx1 protein that lacks the C-terminal repression motif, the VWRPY peptide, but retains the activation domain (Fig. 5*A*) (Nishimura et al., 2004). In the Δ*446* mutant allele, the DNA sequence that encodes the truncated Δ446 protein was inserted into the *Runx1* locus. As a result, the expression of Δ*446* is under the control of the endogenous *Runx1* promoter. Homozygous Δ*446*/Δ*446* mutant mice survive to adulthood (Nishimura et al., 2004). Total neuron numbers in T12 thoracic DRG, detected by the expression of the pan-neuronal marker *SCG10* (Stein et al., 1988), were not affected by this *Runx1* mutation, with 1244 ± 53 per set of T12 DRG sections in Δ*446*/Δ*446* mice versus 1276 ± 35 in wild-type mice ($p = 0.27$) (see Materials and Methods). The number of DRG neurons that were labeled by the isolectin B4 (IB4) of *Griffonia simplicifolia* (Silverman and Kruger, 1988) were also not changed, with 645 ± 15 per set of T12 DRG sections in Δ*446*/Δ*446* mice versus 643 ± 16 in wild-type mice ($p = 0.45$).

Expression of the truncated Δ446 protein can still be detected by the anti-Runx1 antibody (Fig. 5*B*). Double staining of Δ446 and IB4 in T12 thoracic DRG revealed that ~64.0 ± 3.6% of $IB4^+$ neurons expressed Δ446 in Δ*446*/Δ*446* mice, which was not different from 65.4 ± 2.3% of $IB4^+$ neurons that expressed the full-length Runx1 protein in wild-type mice ($p = 0.18$). The percentages of $\Delta446^+$ or $Runx1^+$ neurons that were labeled by IB4 were also comparable, with 50.3 ± 6.9% in Δ446/Δ446 mice versus 46.4 ± 2.7% in wild-type mice ($p = 0.15$). These data suggest that deletion of the C-terminal repression domain affects neither neuronal survival nor Runx1 expression.

Runx1 is required to suppress *TrkA* and the precursor gene encoding the α-CGRP protein in prospective nonpeptidergic neurons (marked by the staining of IB4), as indicated by the derepression of these two genes in $Runx1^{-/-}$ (*Wnt1*) mice (Chen et al., 2006; Yoshikawa et al., 2007). Expression of *TrkA* and *α-CGRP*, however, was not expanded in $IB4^+$ neurons in Δ*446*/Δ*446* mice (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). These data suggest that the C-terminal repression domain is not involved with Runx1-mediated suppression of *TrkA* and *α-CGRP*.

Expression of a set of ion channels and receptors, which is eliminated in conditional *Runx1* null mice (Chen et al., 2006), was also not affected in Δ*446*/Δ*446* mice, including TRP channels (TRPA1, TRPM8, and TRPC3), the ATP-gated channel $P2X_3$, the sodium channel (SNS2/Nav1.9), and the GDNF receptor Ret (supplemental Figs. 1, 2, available at www.jneurosci.org as supplemental material), implying that Runx1-mediated activation of these nociceptive molecules does not operate through the C-terminal repression domain.

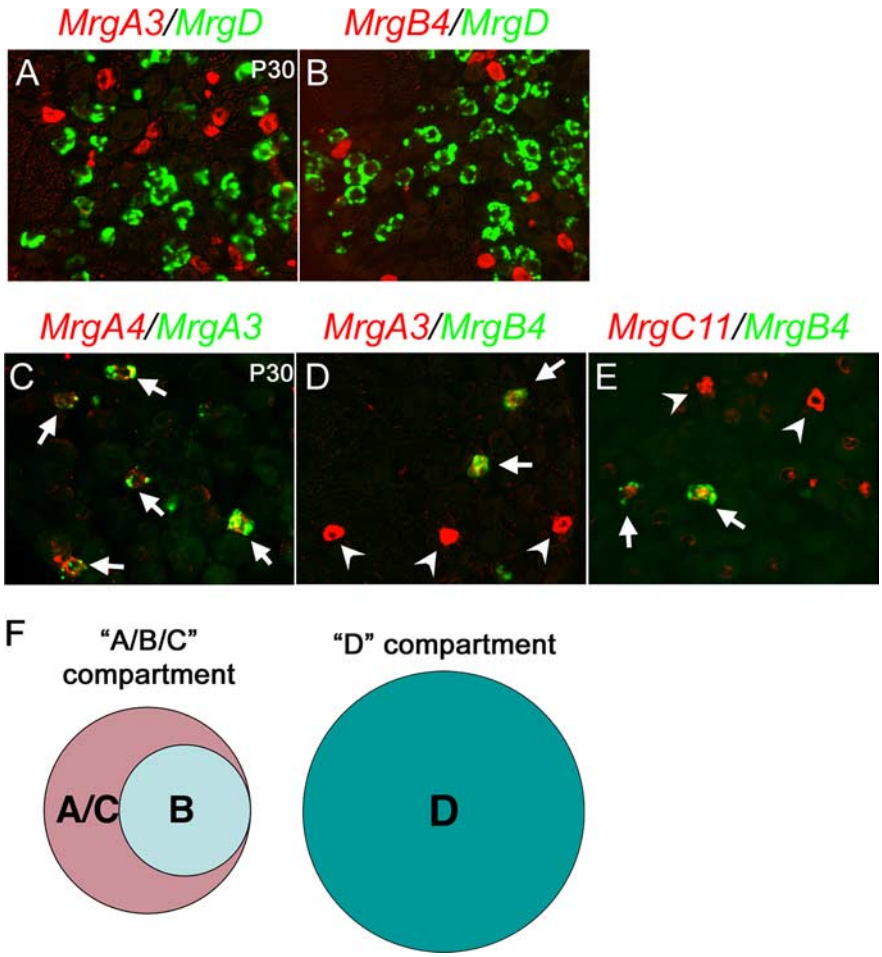

Figure 2. Compartmental expression of *Mrg* genes. ***A–E***, Double color *in situ* hybridizations with indicated probes on sections through P30 T12 thoracic DRG of wild-type mice. ***F***, Schematics of two *Mrg* compartments. The "A/B/C" compartment expresses *MrgA3/A4* ("A"), *MrgB4* ("B"), and *MrgC11* ("C") in a partially overlapping manner, and the "D" compartment expresses *MrgD* ("D").

Expansion of *MrgA/B/C* gene expression in Δ*446*/Δ*446* mice

We next examined the expression of *Mrg* genes in Δ*446*/Δ*446* mice. We found that the expression of class A, B and C *Mrg* genes was dramatically expanded in adult thoracic DRG of Δ*446*/Δ*446* homozygous mice, including *MrgA2-A7*, *MrgB4*, *MrgB5*, and *MrgC11* (Fig. 6*A*) (data not shown). The percentage of $MrgA3^+$ neurons in P30 T12 thoracic DRG increased from 4.2 ± 0.6 in wild-type mice to 30.1 ± 1.5 in Δ*446*/Δ*446* mice, a 7.2-fold increase ($p < 0.0006$). Similarly, the percentage of $MrgA4^+$ neurons increased from 4.0 ± 0.1–28.0 ± 3.0, a 7.0-fold increase ($p < 0.003$), the percentage of $MrgB4^+$ neurons increased from 2.9 ± 0.8–19.4 ± 1.7, a 6.7-fold increase ($p < 0.004$), and the percentage of $MrgC11^+$ neurons increased from 4.2 ± 0.6–32.3 ± 1.2 ($p < 0.0002$) (Fig. 6*B*). An expansion of *MrgA3* and *MrgB4* expression was also observed in lumbar DRG (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). In contrast, we did not observe increased expression of *MrgD*, the most abundantly expressed family member. In fact, the percentage of $MrgD^+$ neurons was slightly but significantly reduced, from 31.1 ± 1.4 in wild-type mice to 23.9 ± 0.4 in Δ*446*/Δ*446* mice ($p < 0.005$) (Fig. 6*A*,*B*). These data suggest that, during removal of the Runx1 repressor domain, there is a selective expansion of neurons that express *MrgA/B/C* genes.

We next determined when the derepression of *MrgA3*, *MrgB4* and *MrgC11* expression was established, by examining their ex-

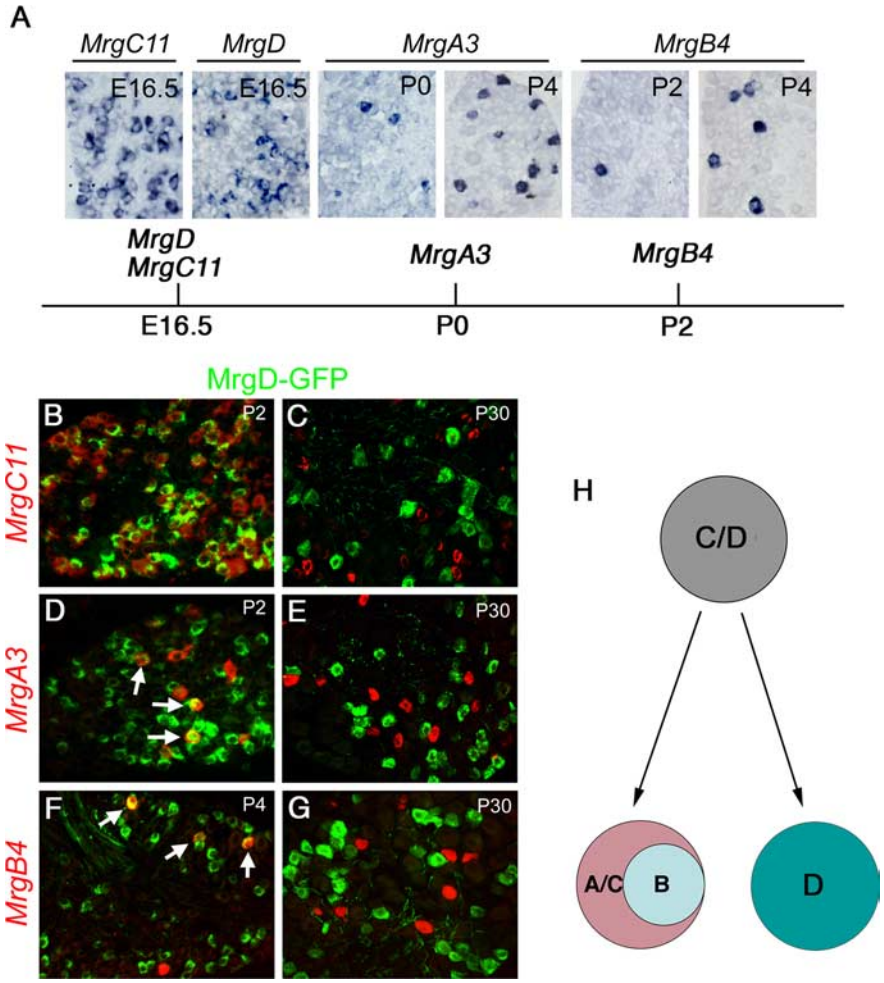

Figure 3. Progressive segregation of *Mrg* compartments. ***A***, *In situ* hybridizations on sections through T12 thoracic wild-type DRG at indicated stages and the schematics for the onset of expression of *Mrg* genes. ***B–G***, Double staining of GFP protein (green) and *Mrg* mRNA (red) on sections through thoracic DRG at various stages of $MrgD^{GFP/+}$ heterozygous mice. ***H***, Schematics for progressive compartmental segregation of *Mrg* gene expression.

pression at multiple developmental stages. In wild-type mice, expression of *MrgA3* and *MrgB4* started at P0 and P2, respectively, and reached peak levels at approximately P4 (Fig. 6*C*). In Δ*446*/Δ*446* mice, onset of *MrgA3* and *MrgB4* expression was also established at P0 and P2, respectively (Fig. 6*C*). Expansion of *MrgA3* expression occurred from P2 to beyond P7, and from P4 to beyond P7 for *MrgB4* (Fig. 6*C*), implying a progressive activation of these GPCR genes. The lack of precocious expression in Δ*446*/Δ*446* mice also suggests that the late onset of *MrgA3* and *MrgB4* expression in wild-type mice is not attributable to Runx1-mediated repression at embryonic stages. Instead, the activator activity for these GPCR genes is established progressively during neonatal development.

In wild-type mice, *MrgC11* expression is extinguished from $MrgD^+$ neurons from P2 to adulthood (Fig. 3). In Δ*446*/Δ*446* mice, the numbers of $MrgC11^+$ neurons in T12 thoracic DRG did not exhibit gross reduction at stages from P2 to P7 to P30 (Fig. 6*C*), implying that Runx1 repression domain is required for postnatal extinguishment of *MrgC11* expression (see also below).

Expansion of *MrgA/B/C* expression in Δ*446*/Δ*446* mice is confined to the $IB4^+$;$MrgD^+$ neuronal compartment

We next asked which population of neurons shows derepression of these GPCR genes in Δ*446*/Δ*446* mice. In wild-type thoracic DRG, *MrgA3* and *MrgC11* were expressed in both $IB4^+$ and IB4− neurons (Fig. 7*A*,*E*, arrows vs arrowheads), whereas *MrgB4* (Fig. 7*C*) and *MrgD* (Dong et al., 2001) are expressed exclusively in $IB4^+$ neurons. In Δ*446*/Δ*446* homozygous mice, double staining of IB4 and *Mrg* mRNAs showed that expansion of *MrgA3*, *MrgB4* and *MrgC11* expression was confined to $IB4^+$ neurons (Fig. 7*B*,*D*,*F*). The percentage of $IB4^+$ neurons expressing *MrgA3* increased from 8.2 ± 1.4 in wild-type mice to 74.7 ± 1.2 in Δ*446*/Δ*446* mice ($p < 0.0001$), that of $MrgB4^+$ neurons increased from 6.9 ± 1.2–37.4 ± 8.0 in Δ*446*/Δ*446* mice ($p < 0.02$), and that of $MrgC11^+$ neurons increased from 9.7 ± 1.2–81.4 ± 3.5 ($p < 0.02$) (Fig. 7*G*). However, the numbers of $MrgA3^+$, $MrgA4^+$, or $MrgC11^+$ neurons in the IB4− compartment were not significantly changed (data not shown). Expansion of *MrgA3* and *MrgB4* expression in lumbar DRG was also confined to $IB4^+$ neurons (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). The percentage of $MrgD^+$ neurons in T12 thoracic $IB4^+$ neuronal population, however, is slightly but significantly reduced, from 81.6 ± 1.0 in P30 wild-type mice to 68.5 ± 1.7 in Δ*446*/Δ*446* mice ($p < 0.005$) (Fig. 7*G*).

In wild-type DRG, the majority of $IB4^+$ neurons express *MrgD*, and these $MrgD^+$ neurons normally do not coexpress *MrgA3*, *MrgB4* or *MrgC11* (Fig. 2) (Zylka et al., 2003). In adult Δ*446*/Δ*446* mice, nearly all $MrgD^+$ neurons in thoracic DRG coexpressed *MrgA3* (Fig. 7*H*) and *MrgA4* (data not shown). Similarly, a portion of $MrgD^+$ neurons coexpressed *MrgB4* (Fig. 7*I*). In addition, because 81.4% and 68.5% of $IB4^+$ neurons express *MrgC11* and *MrgD*, respectively, we concluded that at least 49.9% [81.4-(100−68.5) = 49.9] of $IB4^+$ neurons coexpress *MrgC11* and *MrgD* in adult Δ*446*/Δ*446* mice. These results suggest that Runx1 actively suppresses the expression of *MrgA/B/C* genes in $MrgD^+$ neurons. However, we noted that a small number of $MrgA3^+$ or $MrgB4^+$ cells lacked *MrgD* expression in Δ*446*/Δ*446* mice (Fig. 7*H*,*I*, arrowheads). The simplest interpretation is that in Δ*446*/Δ*446* mice, *MrgD* expression is not expanded into the prospective "A/B/C" compartment that normally lack persistent Runx1 expression.

In summary, removal of the C-terminal Runx1 repression domain leads to a unidirectional expansion of *MrgA/B/C* genes into the "D" neuronal compartment.

Discussion

Mechanism of compartmentalized expression of Mrg class sensory GPCRs

Our studies suggest a model that leads to compartmentalized expression of *Mrg* genes. This model contains two key components. First, Runx1 is initially expressed in most embryonic nociceptors at E12.5-E14.5, but is extinguished in ~50% of cells during perinatal/postnatal development (Chen et al., 2006), leading to the creation of $Runx1^+$ and $Runx1^-$ neuronal compart-

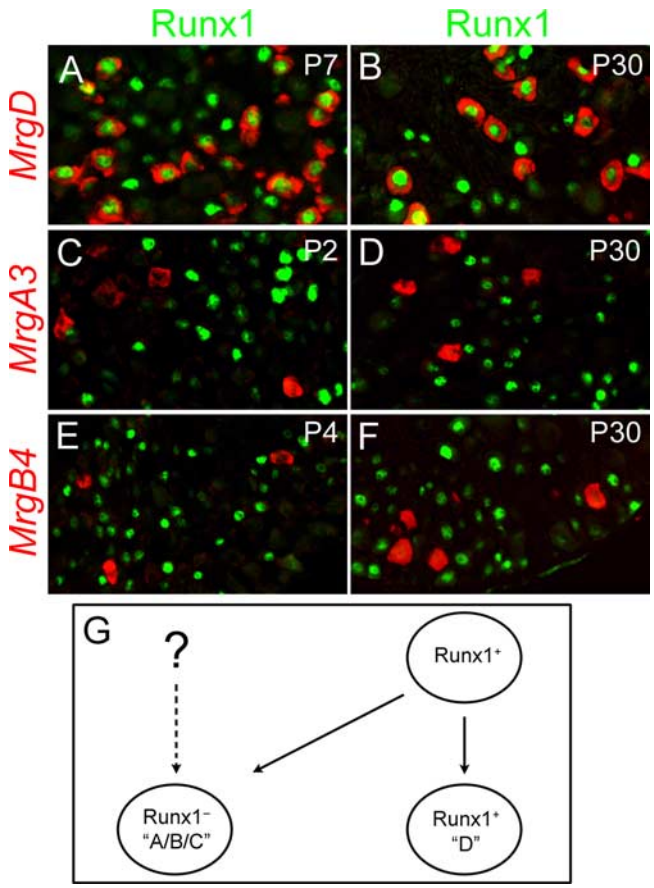

Figure 4. Dynamic Runx1 expression marks distinct *Mrg* compartments. ***A–F***, Double staining of Runx1 protein (green) and *Mrg* mRNAs (red) on sections through thoracic wild-type DRG at various stages. ***G***, Schematics for the origin of *Mrg* compartments. Runx1 expression in immature *MrgA/B/C*$^+$ neurons is inferred from the fact that Runx1 is expressed in most, if not all, TrkA$^+$ neurons at early embryonic stages (Chen et al., 2006), and TrkA signaling is required for the expression of most *Mrg* genes (Luo et al., 2007). However, it remains a possibility that Runx1 is never expressed in some *MrgA/B/C*$^+$ neurons (the dashed line).

ments. Second, different *Mrg* family members exhibit different responses to Runx1-mediated repression. Runx1 is initially required for the expression of all *Mrg* genes, but at an undefined developmental stage Runx1 becomes a repressor of *MrgA/B/C*. Accordingly, expression of *MrgA/B/C* is confined to the Runx1$^-$ "A/B/C" compartment, and is actively suppressed in the Runx1$^+$ "D" compartment. During removal of the Runx1 repressor domain in Δ*446*/Δ*446* mice, the truncated Runx1 protein is converted into an activator, leading to a dramatic expansion of *MrgA* and *MrgB* expression in the "D" compartment. The truncated Δ446 protein is required for the expansion because a null mutation in *Runx1*$^{-/-}$(*Wnt1*) mice leads to a loss, rather than an expansion, of *MrgA* and *MrgB* expression. *MrgC11* is initially expressed in the "D" compartment. Subsequent extinguishment of *MrgC11* expression in this compartment relies on Runx1-mediated repression. Expression of *MrgD*, however, is essentially insensitive to Runx1-mediated repression, thereby allowing sustained *MrgD* expression in the Runx1$^+$ "D" compartment. In summary, the selective extinguishment of Runx1 is critical for the establishment of the "A/B/C" compartment (by avoiding Runx1-mediated suppression of *MrgA/B/C* genes at late developmental stages), whereas persistent Runx1 expression allows a singular *MrgD* expression in the "D" compartment (by actively suppressing *MrgA/B/C* expression and possibly maintaining *MrgD* expression).

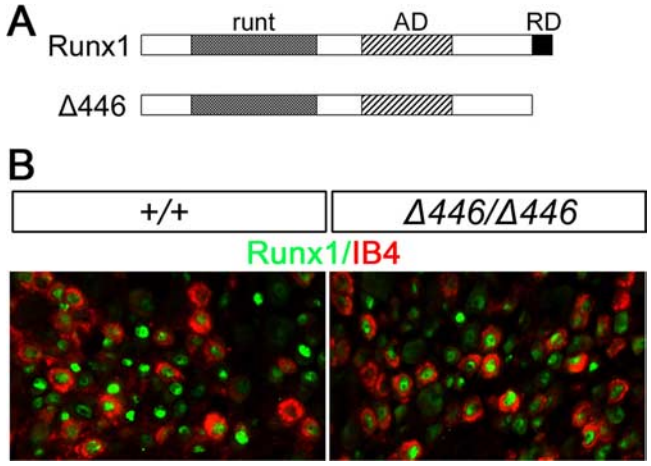

Figure 5. Sensory neuron development in Δ*446*/Δ*446* mice. ***A***, Schematics for full-length Runx1 protein and the truncated Δ446 protein. The wild-type Runx1 protein ("Runx1") contains the DNA-binding motif "runt," the activation domain "AD," and the C-terminal repression domain "RD." Δ446 is a truncated Runx1 protein that lacks the RD. ***B***, Double staining of IB4 (red) and full-length or truncated Runx1 protein (green) on sections through T12 thoracic DRG of wild-type and Δ*446*/Δ*446* mice.

A few outstanding issues remain to be solved. First, Runx1 protein is not detected in *MrgA3*$^+$ or *MrgB4*$^+$ neurons at neonatal stages, raising the question about whether Runx1 indirectly or even non-autonomously controls the expression of these GPCR genes. Second, the signal that is responsible for Runx1 extinguishment in the "A/B/C" compartment is unknown. Third, it should be noted that in rat DRG, *MrgA* and *MrgD* gene expression overlaps extensively (Zylka et al., 2003), mimicking the expression pattern present in Δ*446*/Δ*446* mice. The mechanism for species-specific expression pattern is unclear. One attractive possibility is that the rat *MrgA* promoter loses the capacity to bind to the Runx1 repressor complex, leading to a concurrent Runx1-mediated activation of *MrgA* and *MrgD* genes.

Runx1 uses distinct pathways to suppress gene expression in nociceptors

Runx1 is required to suppress several peptidergic neuron markers in IB4$^+$ nonpeptidergic nociceptors, including *TrkA* and *CGRP* (Chen et al., 2006; Yoshikawa et al., 2007). Here we found that Runx1 also actively suppresses *MrgA/B/C* genes in these neurons. Interestingly, Runx1 uses distinct mechanisms to suppress these two categories of genes. Runx1-mediated suppression of *MrgA/B/C*, but not *TrkA/CGRP*, is dependent on the C-terminal repression domain that is known to interact with the Groucho-repressor complex (Durst and Hiebert, 2004; Nishimura et al., 2004). Removal of this repression domain in Δ*446*/Δ*446* mice leads to a derepression of *MrgA/B/C* (Figs. 6, 7), without causing a concurrent derepression of *TrkA/CGRP* in IB4$^+$ neurons (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In the immune system, Runx1 is able to use multiple repression domains to suppress T cell receptor expression (Durst and Hiebert, 2004; Telfer et al., 2004). Runx1 could in principle use a different repression domain to suppress *TrkA*/*CGRP*. Alternatively, Runx1 may activate a downstream pathway that indirectly suppresses peptidergic neuron markers. A support for the latter scenario is the finding that Ret-mediated signaling is required for postnatal suppression of *TrkA* in IB4$^+$ neurons (Luo et al., 2007). Accordingly, the loss of *Ret* expression after conditional *Runx1* knock-out may explain the derepression of *TrkA*

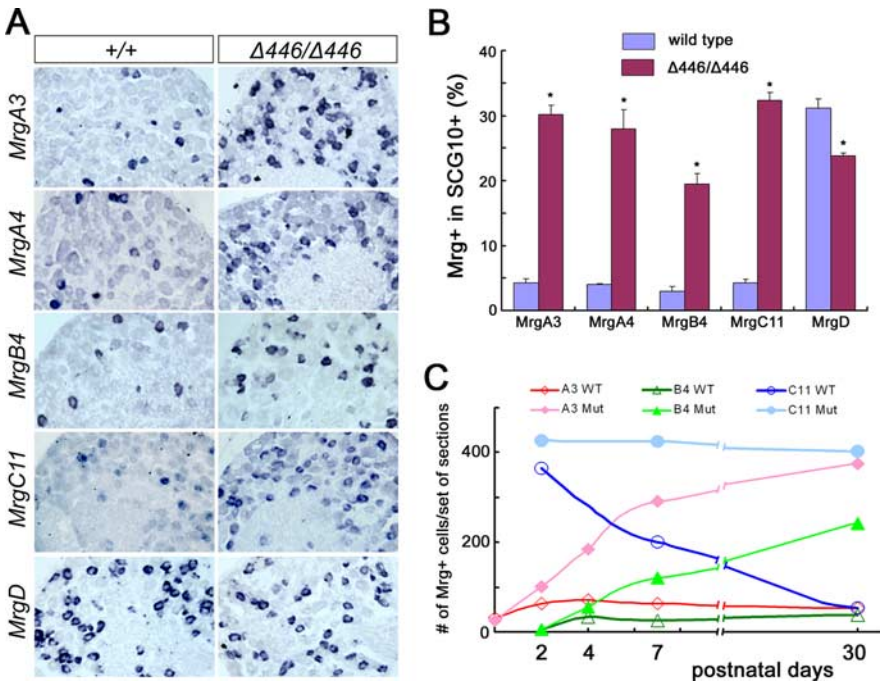

Figure 6. Expansion of *Mrg* genes in Δ*446*/Δ*446* mice. ***A***, *In situ* hybridizations with indicated *Mrg* probes on sections through T12 thoracic DRG of P30 wild-type and Δ*446*/Δ*446* mice. ***B***, Percentages of *Mrg*$^+$ neurons in T12 thoracic DRG of P30 wild-type and Δ*446*/Δ*446* mice (*$p < 0.005$). ***C***, *MrgA3*$^+$, *MrgB4*$^+$, and *MrgC11*$^+$ neuron numbers in T12 thoracic DRG of wild-type and Δ*446*/Δ*446* mice at multiple developmental stages. Six adjacent sets of sections were prepared from each DRG, and numbers of positive neurons in one of the six sets were presented.

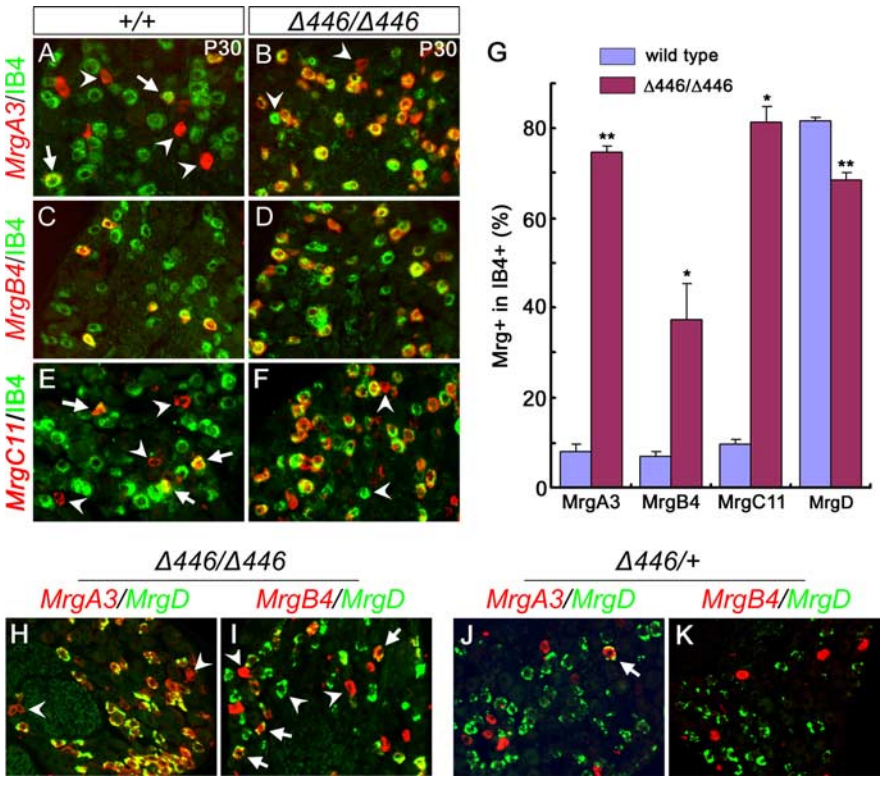

Figure 7. Expansion of *MrgA3*, *MrgB4*, and *MrgC11* expression in IB4$^+$;*MrgD*$^+$ neurons in Δ*446*/Δ*446* mice. ***A–F***, Double staining of IB4 (***A–F***, green) and *MrgA3* mRNA (***A***, ***B***, red), *MrgB4* mRNA (***C***, ***D***, red) or *MrgC11* mRNA (***E***, ***F***, red) on sections through thoracic DRG of P30 wild-type and Δ*446*/Δ*446* mice. ***G***, The percentage of IB4$^+$ neurons that express *MrgA3*, *MrgB4*, *MrgC11*, or *MrgD* in T12 thoracic DRG of P30 wild-type and Δ*446*/Δ*446* homozygous mice (*$p < 0.02$; **$p < 0.005$). ***H–K***, Double color *in situ* hybridization on sections through T12 thoracic DRG of P30 Δ*446*/Δ*446* homozygous mice (***H***, ***I***) and P30 Δ*446*/+ heterozygous mice (***J***, ***K***).

and *CGRP* in *Runx1*$^{-/-}$*(Wnt1)* mice (Chen et al., 2006; Yoshikawa et al., 2007), whereas a normal expression of *Ret* in Δ*446*/Δ*446* mice (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) may explain the lack of *TrkA* derepression. Regardless, Runx1 operates through distinct pathways to suppress peptidergic differentiation and *MrgA/B/C* expression.

Progressive specification of sensory neuron subtypes

Our studies, combined with a series of recent studies, suggest that specification of sensory subtypes is progressively established during perinatal and postnatal development. Most embryonic nociceptors (and thermoceptors) initially express both Runx1 and TrkA (Chen et al., 2006). To our knowledge, there are no known molecular markers that are able to divide TrkA$^+$ neurons into distinct subgroups at E12.5. It is from E14.5 to postnatal stages that nociceptors are progressively segregated into Ret$^+$, TrkA$^+$ and Ret$^+$;TrkA$^+$ subclasses, with persistent Runx1 expression is confined to a portion of Ret$^+$ neurons (Bennett et al., 1996, 1998; Molliver et al., 1997; Chen et al., 2006). Nociceptors that express distinct profiles of TRP class thermal receptors also emerge during perinatal/postnatal development (Hjerling-Leffler et al., 2007). Here we further found that *Mrg*$^+$ sensory neurons initially coexpress *MrgD* and *MrgC11* at embryonic stages. During postnatal development, future *MrgC11*$^+$ neurons extinguish *MrgD* and activate *MrgA* and/or *MrgB* genes. Conversely, future *MrgD*$^+$ neurons switch off *MrgC11*, leading to a mutually exclusive expression of *MrgA/B/C* and *MrgD*.

Intriguingly, *MrgD*$^+$ afferents innervate skin epidermis (Zylka et al., 2005), whereas *MrgB4*$^+$ afferents innervate exclusively the hairy skin (Liu et al., 2007). Such topographically distinct innervation raises the hypothesis that segregation of *MrgD*$^+$ and *MrgB4*$^+$ nociceptors might partly depend on specific target-derived signals. Indeed, Ret signaling is required for the expression of *MrgB4* (and *MrgA3*), but not *MrgD* (Luo et al., 2007). Hair follicles also release other signaling molecules, such as bone morphological proteins or BMPs (Blanpain and Fuchs, 2006). Indeed, a conditional knock-out of *Smad4*, encoding a key component of BMP-mediated signaling (Yang et al., 2002), is required selectively for the expression of *MrgB4* (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Future studies will be directed to investigate how

target-derived signals interface with intrinsic factors, such as Runx1, in generating sensory cell diversity.

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