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RESEARCH ARTICLE

# RGMB and neogenin control cell differentiation in the developing olfactory epithelium

**Joseph Wai Keung Kam[1,2], Emilie Dumontier[1,2], Christopher Baim[1,2], Alexandra C. Brignall[1,2], David Mendes da Silva[1,3], Mitra Cowan[4], Timothy E. Kennedy[1,2,5] and Jean-François Cloutier[1,2,5,*]**

## ABSTRACT

Cellular interactions are key for the differentiation of distinct cell types within developing epithelia, yet the molecular mechanisms engaged in these interactions remain poorly understood. In the developing olfactory epithelium (OE), neural stem/progenitor cells give rise to odorant-detecting olfactory receptor neurons (ORNs) and glial-like sustentacular (SUS) cells. Here, we show in mice that the transmembrane receptor neogenin (NEO1) and its membrane-bound ligand RGMB control the balance of neurons and glial cells produced in the OE. In this layered epithelium, neogenin is expressed in progenitor cells, while RGMB is restricted to adjacent newly born ORNs. Ablation of *Rgmb* via gene-targeting increases the number of dividing progenitor cells in the OE and leads to supernumerary SUS cells. Neogenin loss-of-function phenocopies these effects observed in *Rgmb*$^{-/-}$ mice, supporting the proposal that RGMB-neogenin signaling regulates progenitor cell numbers and SUS cell production. Interestingly, *Neo1*$^{-/-}$ mice also exhibit increased apoptosis of ORNs, implicating additional ligands in the neogenin-dependent survival of ORNs. Thus, our results indicate that RGMB-neogenin-mediated cell-cell interactions between newly born neurons and progenitor cells control the ratio of glia and neurons produced in the OE.

**KEY WORDS: Neurogenesis, Gliogenesis, Cellular differentiation, Neogenin, Olfactory epithelium, RGM, Mouse**

## INTRODUCTION

Organogenesis relies on the production of distinct cell types from stem/progenitor cells during embryogenesis. The survival, proliferation and differentiation of these cell types are tightly regulated and depend on both positive and negative signals from the extracellular milieu. Although there is increasing evidence that cell-cell communication in developing tissues plays an important role in regulating cell fate determination, the molecular mechanisms underlying this communication remain poorly understood (Artavanis-Tsakonas et al., 1999; Eisen, 1992; Liu et al., 2010; Ohlstein and Spradling, 2007; Reh, 1987; Zhang et al., 2013).

The layered spatial organization of cells in the developing olfactory epithelium (OE) greatly facilitates the study of mechanisms that orchestrate the production of various cell populations from stem/progenitor cells. Progenitor cells located in the basal region of the OE give rise to two main populations of cells, namely the olfactory receptor neurons (ORNs) and the sustentacular (SUS) cells, which are glial-like support cells that populate the apical region of the OE (reviewed by Kam et al., 2014). ORNs detect odorants and relay information to the central nervous system, whereas SUS cells regulate ionic balance and participate in the active phagocytosis of dead cells (Carter et al., 2004; Graziadei and Graziadei, 1979; Iwai et al., 2008; Schwob et al., 1994). The production of these two types of cells relies on a complex transcriptional regulatory network involving OE stem cells that express SOX2 and give rise to ASCL1 (MASH1)-expressing progenitor cells. These progenitor cells give rise to ORN precursors that express neurogenin 1 and NEUROD1, which regulate gene expression required for ORN commitment and differentiation. Newly born immature ORNs, which reside in the intermediate layer of the OE, turn off expression of proneural genes and turn on NCAM and βIII-tubulin expression, eventually maturing into olfactory marker protein (OMP)-expressing ORNs that reside in the apical region of the OE (Fig. 1A) (Avilion et al., 2003; Cau et al., 2002, 1997; Donner et al., 2007; Murray et al., 2003; Packard et al., 2011).

In addition to producing neurons, ASCL1-expressing progenitor cells also give rise directly to SUS cells, which form a monolayer in the apical region of the OE, indicating that signaling mechanisms must be in place to control cell fate choice in these bipotential progenitors (Gokoffski et al., 2011; Krolewski et al., 2012). While several members of the TGFβ family of molecules have been implicated in the generation and survival of ORNs, much less is known about the signals that regulate the production of SUS cells (Gokoffski et al., 2011; Kawauchi et al., 2009, 2005; Wu et al., 2003).

Cellular interactions that take place between newly born neurons and progenitor cells in the OE are likely to play a role in modulating the differentiation of progenitor cells into ORNs and SUS cells. Expression of cell surface proteins on immature ORNs could serve to signal to and control various characteristics of progenitor cells, including their proliferation, survival and differentiation. During phases of neurogenesis in the OE, newly born ORNs may inhibit the proliferation of progenitor cells to promote the differentiation of ORNs or prevent the overproduction of SUS cells through cellular interactions with progenitor cells. For example, in the ventricular zone of the brain, contact-mediated cell-cell signaling by multiple ligand-receptor pairs, including Ephrin-EphR and Delta-Notch, play crucial roles in the regulation of the stem/progenitor cell pools (Corbin et al., 2008; Jiao et al., 2008). In our efforts to examine the role of cell-cell interactions in the differentiation of OE cell populations, we have identified the receptor neogenin (NEO1) and its membrane-bound ligand RGMB as key candidates to regulate olfactory neurogenesis.

[1]Montreal Neurological Institute, 3801 University, Montréal, Québec, Canada H3A 2B4. [2]Department of Neurology and Neurosurgery, McGill University, 3801 University, Montréal, Québec, Canada H3A 2B4. [3]Center for Neuroscience and Cell Biology and Department of Life Sciences, University of Coimbra, Rua Larga, Coimbra 3004-517, Portugal. [4]Centre de Recherches du Centre Hospitalier de l'Université de Montréal, 900 rue Saint-Denis, Montréal, Canada H2X 0A9. [5]Department of Anatomy and Cell Biology, McGill University, 3640 University, Montréal, Québec, Canada H3A 0C7.

*Author for correspondence (jf.cloutier@mcgill.ca)

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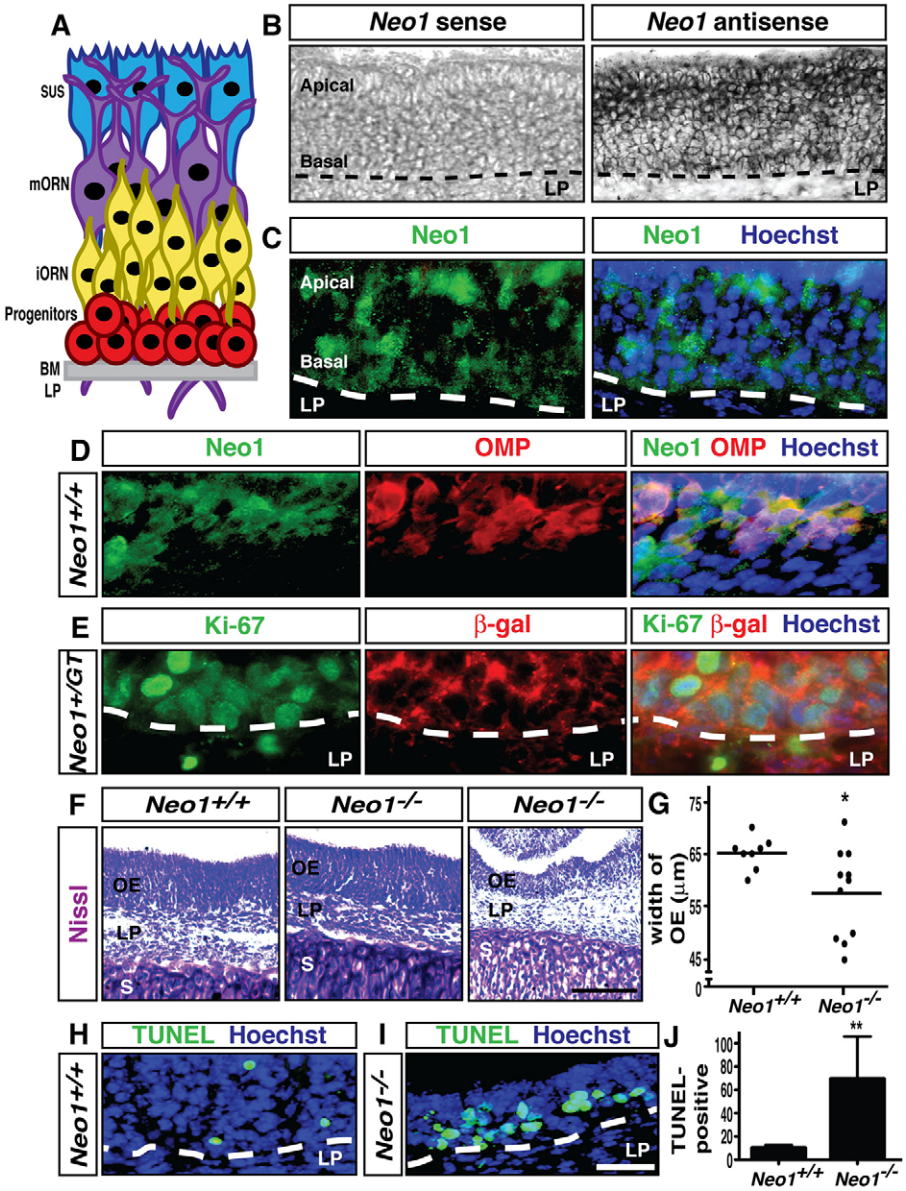


**Fig. 1. Neogenin expression is spatially regulated in the OE and its ablation leads to increased apoptosis.** (A) Layered organization of cells in the mouse olfactory epithelium (OE) during late embryonic development. SUS, sustentacular cells; iORN, immature olfactory receptor neurons; mORN, mature olfactory receptor neurons; BM, basement membrane; LP, lamina propria. (B,C) Neogenin expression in OE sections examined by ISH (B) and immunolabeling (C). The dashed line indicates the basement membrane. (D) Immunolabeling of OE sections from E16.5 embryos for neogenin and OMP. (E) Immunolabeling with β-galactosidase and Ki-67 antibodies on OE sections from E16.5 *Neo1*$^{+/GT}$ embryos. (F) Nissl staining of OE sections in control and *Neo1*$^{-/-}$ E16.5 embryos. Whereas some *Neo1*$^{-/-}$ embryos (middle) show comparable OE width to the control mice (left), a subset of *Neo1*$^{-/-}$ embryos (right) have a severely reduced OE width. S, septum. (G) Quantification of OE width for individual embryos. *Neo1*$^{+/+}$, $n$=8; *Neo1*$^{-/-}$, $n$=11. (H-J) TUNEL analysis revealed a significant increase in cell death in the OE of *Neo1*$^{-/-}$ embryos. *Neo1*$^{+/+}$, $n$=7; *Neo1*$^{-/-}$, $n$=9. Cell counts (mean±s.e.m.) represent cell number per 2.5 mm of OE. Student's unpaired $t$-test, *$P$<0.05, **$P$<0.01. Scale bars: 100 μm.

Neogenin is a member of the immunoglobulin superfamily of receptors that shares high homology with the netrin receptor DCC and is expressed in the developing and adult nervous system (De Vries and Cooper, 2008; Keeling et al., 1997; van den Heuvel et al., 2013; Vielmetter et al., 1994; Zhou et al., 2010). Members of three different families of molecules, namely the netrins, the repulsive guidance molecules (RGMs) and the bone morphogenetic proteins (BMPs), have been reported to bind to neogenin (Hagihara et al., 2011; Rajagopalan et al., 2004; Srinivasan et al., 2003; Wang et al., 1999). RGMs are GPI-anchored proteins widely expressed in the nervous system that can repel axons in a neogenin-dependent fashion (Conrad et al., 2010; Monnier et al., 2002; Oldekamp et al., 2004; O'Leary et al., 2013; Rajagopalan et al., 2004; Tassew et al., 2012; Wilson and Key, 2006). Furthermore, RGM-neogenin signaling has been implicated in the regulation of neuronal differentiation, neural tube closure and neuronal survival (Kee et al., 2008; Koeberle et al., 2010; Matsunaga et al., 2006, 2004; O'Leary et al., 2015; Shin and Wilson, 2008).

Here, we demonstrate that neogenin plays a key role in regulating progenitor numbers and cell fate decisions in the mouse OE. We have generated mouse mutants for neogenin and its ligand RGMB and show that loss of this ligand-receptor pair perturbs the balance of cell types produced in the OE. Ablation of neogenin expression *in vivo* results in altered progenitor cell cycle kinetics and in an accumulation of progenitor cells, which is associated with the generation of supernumerary SUS cells. Moreover, loss of RGMB function also results in the overproduction of progenitor cells and SUS cells. These findings establish RGMB and neogenin as a crucial ligand-receptor pair modulating OE development and homeostasis.

## RESULTS

### Neogenin regulates cell survival in the developing OE

The OE is organized as layers of cells that can be identified based on their location and their expression of specific molecular markers. Proliferating progenitor cells located in the basal region of the OE give rise to ORNs that mature and populate the apical region of the

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OE. In addition, the progenitor cells also produce glial-like SUS cells situated in the apical lining of the OE (Fig. 1A). Using *in situ* hybridization (ISH) and immunohistochemistry, we found that neogenin expression is detected throughout the OE, but higher levels were observed in the most apical and basal layers of the OE where mature ORNs and proliferating progenitor cells are located, respectively (Fig. 1B,C). To confirm the expression of neogenin in these two populations of cells, we compared its expression with that of specific markers of mature ORNs and proliferating cells. Double immunohistochemistry experiments using a neogenin antibody (characterized in Fig. S1) and OMP confirmed the expression of neogenin in mature ORNs (Fig. 1D). Unfortunately, we could not identify experimental conditions for specific colabeling of neogenin and a basal proliferating cell marker. We therefore took advantage of an existing gene-trap neogenin (*Neo1*$^{GT}$) mouse line that expresses a neogenin–β-galactosidase fusion protein as a surrogate for expression of neogenin in proliferating cells of the OE (Bae et al., 2009; Leighton et al., 2001). Labeling with a β-galactosidase antibody revealed that neogenin is highly expressed in Ki-67 (MKI67)-positive basal dividing cells (Fig. 1E). The spatially defined expression of neogenin in the OE strongly suggests that it may regulate OE development, possibly by controlling differentiation of progenitor cells or by regulating the maturation or survival of ORNs.

To distinguish these possibilities, we ablated neogenin expression using a gene targeting approach. The gene-trap *Neo1* allele described previously does not fully ablate neogenin expression (Bae et al., 2009; Lee et al., 2010). Instead, we generated a novel *Neo1* floxed allele by introducing loxP sites around the first exon of the *Neo1* gene, which harbors the start codon and the signal sequence that allows neogenin to reach the cell surface (Fig. S1). Germline excision of the floxed *Neo1* allele (*Neo1*$^{-/-}$) by crossing this line with mice expressing Cre under the control of the CMV promoter resulted in the complete loss of *Neo1* transcript and protein (Fig. S1B-D). Following three generations of backcrossing (F3) into the C57BL/6J strain background, normal Mendelian ratios of homozygous animals were observed throughout embryogenesis, but no homozygotes survived past birth (Fig. S1E).

To examine the morphology of the OE in *Neo1*$^{-/-}$ mice, we first performed Nissl staining on OE from E16.5 embryos, when the cell layers are well defined and neurogenesis remains robust. Ablation of neogenin expression resulted in a significant decrease in the width of the OE (Fig. 1F,G). This effect was particularly strong in approximately one-third of the mutants analyzed, whereas in the remaining two-thirds the OE was similar in width to, or slightly narrower than, that of wild type (Fig. 1F,G). TUNEL staining revealed a significant increase in the number of cells undergoing apoptosis in the OE of *Neo1*$^{-/-}$ mice, and the significantly thinner OEs exhibited massive cell death (Fig. 1I,J). These results indicate that neogenin plays an important role in cell survival in the OE.

### Increased cell death and proliferation in the OE of *Neo1*$^{-/-}$ mice

The robust cell death and thinner OE phenotypes observed in a subset of F3 *Neo1*$^{-/-}$ embryos reached full penetrance once these mice were further backcrossed into the C57BL/6J strain background (data not shown). However, owing to the large amount of cell death observed in these OEs, immunostaining with marker antibodies for cell populations of the OE resulted in faint and diffuse staining (data not shown). This made it difficult to examine a potential role for neogenin during neurogenesis in the OE of these embryos. We therefore focused all our future analyses on the development of various populations of cells in the OE of F3 *Neo1*$^{-/-}$ embryos showing milder defects in OE thickness, in which each population of cells could be reliably identified. In these embryos, we observed a modest but significant increase in the number of TUNEL-positive cells in the OE (Fig. 2A,B). To determine whether the increased cell death affects neuronal populations in the OE, we quantified the number of immature and mature ORNs in the OE of the *Neo1*$^{-/-}$ embryos by immunostaining for βIII-tubulin and OMP, respectively. These analyses revealed a significant decrease in the number of mature ORNs, whereas the number of immature ORNs appeared unchanged, in *Neo1*$^{-/-}$ embryos (Fig. 2C-F), suggesting that neogenin might regulate the maturation of ORNs or their survival.

The high levels of expression of neogenin in mature ORNs, combined with our observation that its ablation leads to increased cell death and reduced numbers of mature ORNs, suggest that neogenin might be required for the survival of mature ORNs. To examine whether neogenin expression in mature ORNs is necessary for their survival, we ablated neogenin expression specifically in these cells. Mice carrying the *Neo1* floxed allele were crossed with OMP-Cre mice, which express Cre recombinase exclusively in mature ORNs; this led to the ablation of neogenin expression in these cells but not in basal progenitor cells (Fig. S2A). Immunohistochemical labeling for TUNEL, Ki-67 and OMP revealed no significant change in cell death, the number of dividing cells or the number of mature ORNs in *Neo1*$^{lox/lox}$*;Omp-Cre* embryos (Fig. S2C-H). These results demonstrate that neogenin expression is not required in mature ORNs for ORN production and survival, raising the possibility that its expression in progenitor cells might contribute to their proper differentiation into neurons and to their subsequent survival.

An important step in the generation of neurons from progenitor cells is the silencing of proneural gene expression in differentiated neurons (Cai et al., 2000). ORNs that fail to turn off expression of proneural or glial genes have been proposed to undergo apoptosis (Cau et al., 1997; Murray et al., 2003). To determine whether dysregulation of proneural gene expression is associated with the increased apoptosis observed in the OE of *Neo1*$^{-/-}$ embryos, we examined the expression of the transcription factor RUNX1, which is expressed in progenitor cells and in some immature ORNs, but is downregulated during the differentiation process (Fig. 2J) (Theriault et al., 2005). Our analyses revealed an increased number of RUNX1-positive cells in the basal region of the OE in *Neo1*$^{-/-}$ embryos, suggesting that there is an increased number of progenitor cells in these OEs (Fig. 2G,H). Interestingly, the number of RUNX1-expressing cells located in the neuronal layer of the OE also increased significantly in *Neo1*$^{-/-}$ embryos (Fig. 2G,I). These RUNX1-positive cells located in the intermediate layer also express βIII-tubulin, a marker of ORNs, indicating that a portion of differentiated neurons retain expression of RUNX1 in the OE of *Neo1*$^{-/-}$ embryos (Fig. 2K). Furthermore, some RUNX1-positive cells located in the intermediate layer of the OE are also labeled with TUNEL, indicating that they are undergoing apoptosis (Fig. 2L). Taken together, these results implicate neogenin in the survival of ORNs and suggest that maintained proneural gene expression in differentiated ORNs might underlie the increased cell death observed in the OE of *Neo1*$^{-/-}$ embryos.

### Neogenin regulates cell cycle progression and cell fate decisions in the OE

Since neogenin is highly expressed in the basal progenitors of the OE, and because we observed increased numbers of RUNX1-

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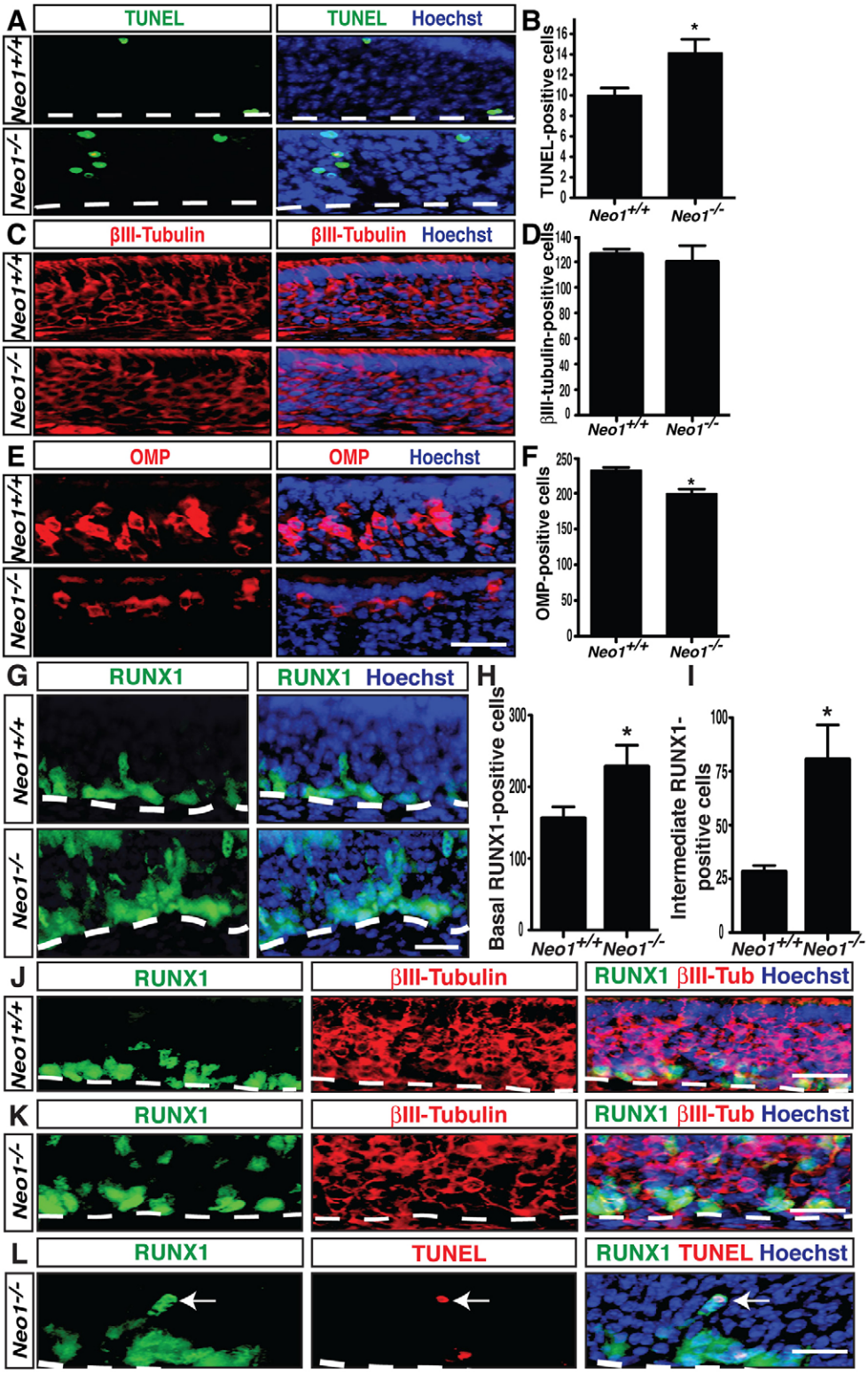


**Fig. 2. Loss of neogenin alters cell survival in the OE.** (A-F) OE sections from E16.5 *Neo1*$^{-/-}$ embryos displaying comparable OE width to wild-type embryos were immunolabeled to visualize cell death (A,B) (*Neo1*$^{+/+}$, *n*=4; *Neo1*$^{-/-}$, *n*=6), βIII-tubulin (C,D) (*Neo1*$^{+/+}$, *n*=4; *Neo1*$^{-/-}$, *n*=5) and OMP-positive ORNs (E,F) (*Neo1*$^{+/+}$, *n*=5; *Neo1*$^{-/-}$, *n*=7). (G-I) Immunolabeling for RUNX1 in OE sections from E16.5 *Neo1*$^{+/+}$ and *Neo1*$^{-/-}$ embryos. An increased number of RUNX1-positive cells in the basal (G,H) and intermediate (G,I) layers of the OE was observed in *Neo1*$^{-/-}$ embryos (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4). (J,K) Immunolabeling for RUNX1 and βIII-tubulin shows that RUNX1-positive cells located in the intermediate layer of the OE co-express βIII-tubulin in *Neo1*$^{-/-}$ embryos. (L) Double labeling for RUNX1 and TUNEL in *Neo1*$^{-/-}$ embryos shows that some RUNX1-positive cells in the intermediate layer undergo cell death (arrow). Counts (mean±s.e.m.) represent cell number per 1 mm (D) or 2.5 mm (B,F,H,I) of OE. Student's unpaired *t*-test, **P*<0.05. Scale bars: 100 µm.

positive cells in the basal OE of *Neo1*$^{-/-}$ embryos (Fig. 2G,H), we considered whether neogenin might regulate progenitor cell numbers. We counted the number of dividing cells labeled with the cell cycle marker Ki-67 in the basal region of the OE. In *Neo1*$^{-/-}$ embryos the number of Ki-67-positive cells was significantly increased when compared with control embryos (Fig. 3A,C). Interestingly, the number of SOX2-positive stem cells in the basal region of the OE was similar in *Neo1*$^{+/+}$ and *Neo1*$^{-/-}$ embryos, and so the generation of stem cells appears unaffected in the absence of neogenin (Fig. 3B,D).

The supernumerary Ki-67-positive cells observed in the basal region of the OE from *Neo1*$^{-/-}$ embryos could be a consequence of the increased cell death observed among ORNs in these OEs. Alternatively, the loss of neogenin from OE progenitor cells might

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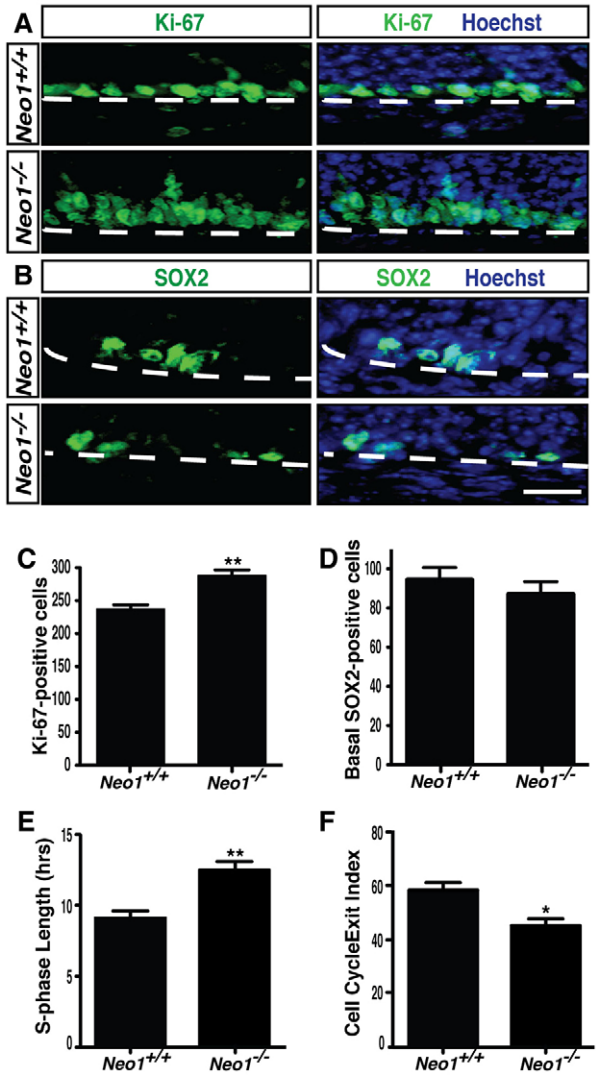


**Fig. 3. Ablation of neogenin leads to altered cell cycle kinetics and cell cycle exit.** (A-D) Immunolabeling and quantification of Ki-67-positive (A,C) (*Neo1*$^{+/+}$, *n*=5; *Neo1*$^{-/-}$, *n*=8) or SOX2-positive (B,D) (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4) cells in OE sections from E16.5 *Neo1*$^{+/+}$ and *Neo1*$^{-/-}$ embryos. (E) Increased S-phase length in progenitor cells in OE from *Neo1*$^{-/-}$ embryos (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4). (F) Reduced cell cycle exit index (the percentage of dividing cells exiting the cell cycle over a 3 h period) (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4). Counts in C and D represent cell number per 2.5 mm of OE. Values are mean±s.e.m. Student's unpaired *t*-test, **P*<0.05, ***P*<0.01. Scale bar: 100 µm.

affect cell cycle progression, leading to an accumulation of proliferating progenitor cells. To examine cell cycle progression in the OE, control and *Neo1*$^{-/-}$ embryos were harvested 24 h after an injection of EdU in pregnant dams at E15.5. OE sections were stained by the Click-iT reaction and with Ki-67 antibodies (see Materials and Methods). Under these conditions, cells that exited the cell cycle after injection of EdU should be EdU positive and Ki-67 negative, whereas cells that are still cycling should be positive for both markers. The proportion of cells that exited the cell cycle was reduced in *Neo1*$^{-/-}$ compared with control embryos (Fig. 3F).

To assess whether altered cell cycle kinetics may be associated with the decreased cell cycle exit, we determined the length of S-phase in progenitor cells in the OE of *Neo1*$^{-/-}$ embryos. Pregnant dams were injected with EdU, followed by an injection of BrdU 2 h later, as a means of determining S-phase duration (see Materials and Methods) (Alexiades and Cepko, 1996; Huard and Schwob, 1995). In control embryos, the length of S-phase was estimated to be 9.03±0.5 h, which is consistent with that previously reported in adult rat (Huard and Schwob, 1995). By contrast, S-phase duration was significantly increased in *Neo1*$^{-/-}$ embryos (12.49±0.5 h), indicating that cell cycle kinetics is altered in these progenitors (Fig. 3E). Interestingly, increased S-phase duration has been correlated with self-renewing proliferative divisions of progenitor cells in the developing cortex (Arai et al., 2011). Hence, the increased S-phase duration, combined with reduced cell cycle exit, is likely to result in the increased number of Ki-67-positive progenitor cells observed in embryos lacking neogenin expression.

Although the number of dividing cells is increased in the OE (Fig. 3C), the same number of immature ORNs as in wild-type embryos is produced in *Neo1*$^{-/-}$ embryos (Fig. 2C), suggesting that these dividing cells might eventually differentiate into another cell type. Since OE progenitor cells can give rise to both ORNs and SUS cells, we examined whether ablation of neogenin expression affects the generation of SUS cells. To assess this in *Neo1*$^{-/-}$ embryos, we counted the number of SOX2-positive cells in the apical region of the OE after confirming that these cells express the SUS cell marker cytokeratin 18 (CK18, or KRT18), as previously described (Fig. 4B) (Gokoffski et al., 2011; Packard et al., 2011). We observed a significant increase in the number of SOX2-positive cells in *Neo1*$^{-/-}$ embryos (Fig. 4A,C). Furthermore, the CK18 staining signal appeared more intense throughout the OE in *Neo1*$^{-/-}$ embryos, which might reflect an increased number of SUS cell basal processes in the OE of these mice (Fig. 4A).

Even though SUS cells are self-renewing, EdU labeling experiments revealed that the proliferation index of SUS cells is the same in control and *Neo1*$^{-/-}$ embryos, suggesting that neogenin does not control self-renewal of SUS cells (Fig. 4D). We therefore examined whether the supernumerary basal progenitor cells observed in *Neo1*$^{-/-}$ embryos may give rise to SUS cells, using an EdU pulse-chase experiment (Fig. 4E). If supernumerary progenitor cells migrate to the apical region to give rise to SUS cells, we would expect to detect an increased number of EdU-labeled SUS cells 24 h following an EdU pulse. As shown in Fig. 4, a similar number of EdU-labeled SUS cells were observed in the apical region of the OE from control and *Neo1*$^{-/-}$ embryos 2 h following a pulse of EdU, a time point chosen to exclude EdU-labeled cells migrating from the basal region to the apical membrane. However, 24 h following an EdU pulse, we observed a 20% increase in the number of EdU-positive SUS cells, indicating that basal progenitor cells have given rise to the supernumerary SUS cells. Taken together, our results indicate that neogenin plays a role in regulating SUS cell production either by inhibiting their generation from progenitor cells or by regulating cell cycle kinetics of these progenitor cells.

### The neogenin ligand RGMB is highly expressed in immature ORNs

Neogenin is likely to control olfactory neurogenesis by interacting with one or more of its identified ligands. Since neogenin binds to members of the netrin, RGM and BMP families of proteins, we examined their expression by ISH. Our results showed that netrin 1 (*Ntn1*), *Rgma*, *Rgmb*, *Bmp4*, *Bmp6* and *Bmp7* are all expressed in the OE at E16.5. Although *Ntn1* was most highly expressed in the apical region of the OE, reducing its expression did not affect the development of OE cell populations *in vivo* (Fig. 5A, Fig. S3). *Bmp4* and *Bmp7* were widely expressed in the OE, whereas *Bmp6* was restricted to the apical layer of the OE (Fig. 5A). BMPs have

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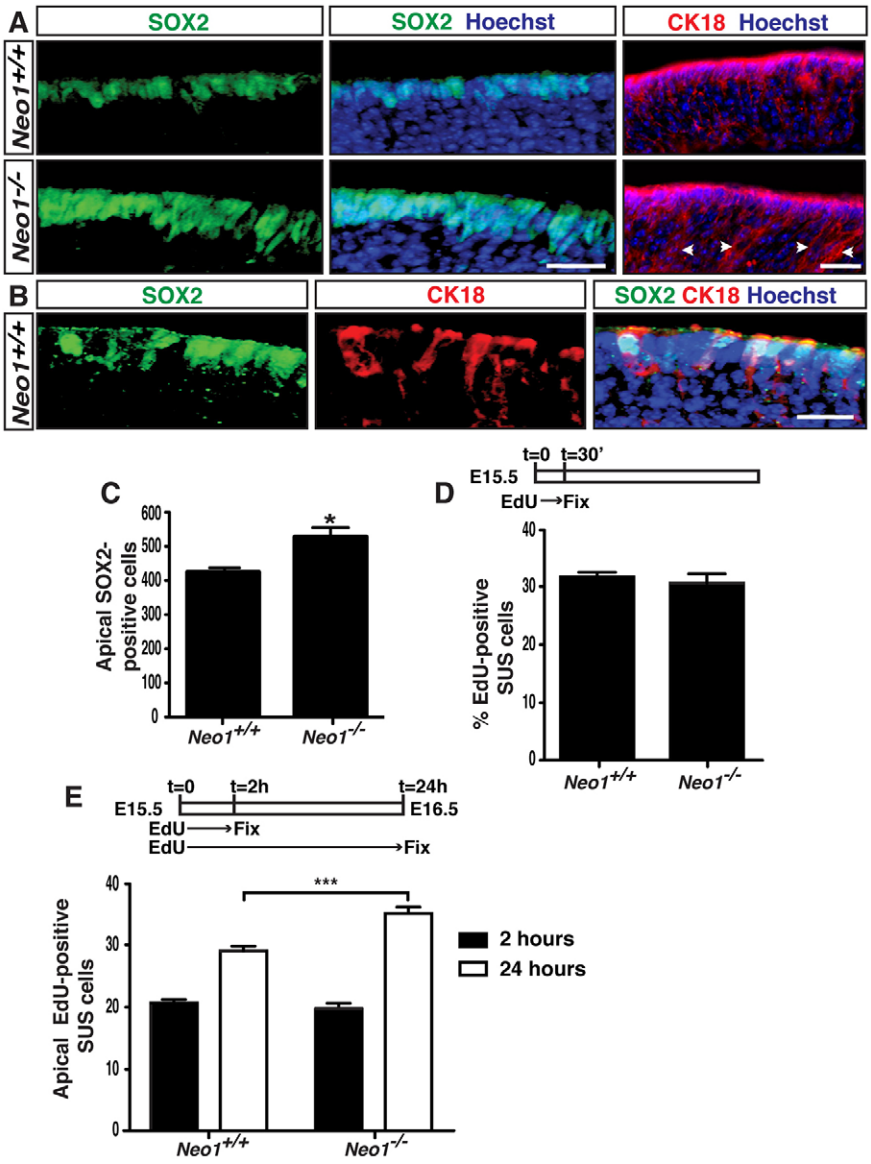


**Fig. 4. Neogenin ablation leads to overproduction of SUS cells.** (A,C) SOX2-positive and CK18-positive cells in the apical region of the OE in control and *Neo1*$^{-/-}$ embryos. An increased number of SOX2-positive cells (per 2.5 mm of OE) is observed in the apical region of the OE in *Neo1*$^{-/-}$ embryos (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4). The CK18 signal is more intense in *Neo1*$^{-/-}$ embryos, with increased labeling of SUS cell basal processes (arrowheads). (B) Immunolabeling of OE sections with SOX2 and CK18 antibodies shows that most SOX2-expressing cells in the apical region of the OE co-express the SUS cell-specific marker CK18. (D) EdU was administered to pregnant dams and the percentage of SOX2-positive SUS cells that are EdU$^{+}$ (proliferation index) was quantified at the time indicated (30 min) (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4). (E) EdU was administered to pregnant dams and double EdU$^{+}$/SOX2$^{+}$ cells in the apical compartment were quantified at the times indicated (2 and 24 h) and plotted as cells per 500 µm of OE (*Neo1*$^{+/+}$ and *Neo1*$^{-/-}$, *n*=4 for each time point). All values are mean±s.e.m. Student's unpaired *t*-test (C,D) or one-way ANOVA (E) was performed to compare values between groups, **P*<0.05, ****P*<0.001. Scale bars: 100 µm.

been implicated in the regulation of ORN development *in vitro*, but the early embryonic lethality of several BMP mouse mutants has made it difficult to assess their role in OE development *in vivo* (Bandyopadhyay et al., 2006; Winnier et al., 1995; Zhang and Bradley, 1996).

Interestingly, whereas *Rgma* was expressed throughout the OE, *Rgmb* expression appeared concentrated in the intermediate layer of the OE where immature ORNs reside (Fig. 5A). Immunostaining with an RGMB-specific antibody (characterized in Fig. S4) also revealed higher levels of expression of RGMB in the intermediate layer of the OE, where immature ORNs are located (Fig. 5B). To confirm that RGMB is preferentially expressed in immature ORNs, we examined mice carrying an *Rgmb* allele targeted with a tau-GFP cassette within the first exon. In the OE and dorsal root ganglia of these mice, GFP expression faithfully recapitulated RGMB expression (Fig. 5B-D, Fig. S4). In the OE of E16.5 *Rgmb*$^{+/-}$ embryos, GFP was not expressed in Ki-67-positive proliferating cells of the basal region, but was highly expressed in βIII-tubulin-positive immature ORNs (Fig. 5E,F). By contrast, RGMB expression appeared downregulated in mature ORNs, since most OMP-positive neurons of the apical layer of the OE did not express GFP (Fig. 5G). Thus, an intriguing possibility that arises from these expression patterns is that RGMB expressed by immature neurons in the OE could bind to neogenin on progenitor cells and mature ORNs to regulate the development of OE cell populations.

### RGMB promotes neuronal differentiation in OE explants

Since RGMB and neogenin have complementary patterns of expression in the OE, and ablation of neogenin expression leads to decreased numbers of mature ORNs and enhanced production of SUS cells, we explored the possibility that RGMB could be the ligand that mediates these effects. We first examined the effect of RGMB on the differentiation of OE progenitors using an *in vitro* olfactory explant assay (Calof and Chikaraishi, 1989; Calof et al., 1991). Explants were prepared from E14-15 embryos and plated on coverslips coated with merosin (laminin α2). Under these conditions, the majority of migrating cells observed around the explant were neuronal cells (Calof and Chikaraishi, 1989; DeHamer et al., 1994; Mumm et al., 1996). Indeed, 72% of cells surrounding the explants expressed the neuronal marker βIII-tubulin. By contrast, 84% of migrated cells expressed βIII-tubulin in explants grown in media supplemented with recombinant RGMB protein (Fig. 6A,B). Furthermore, the percentage

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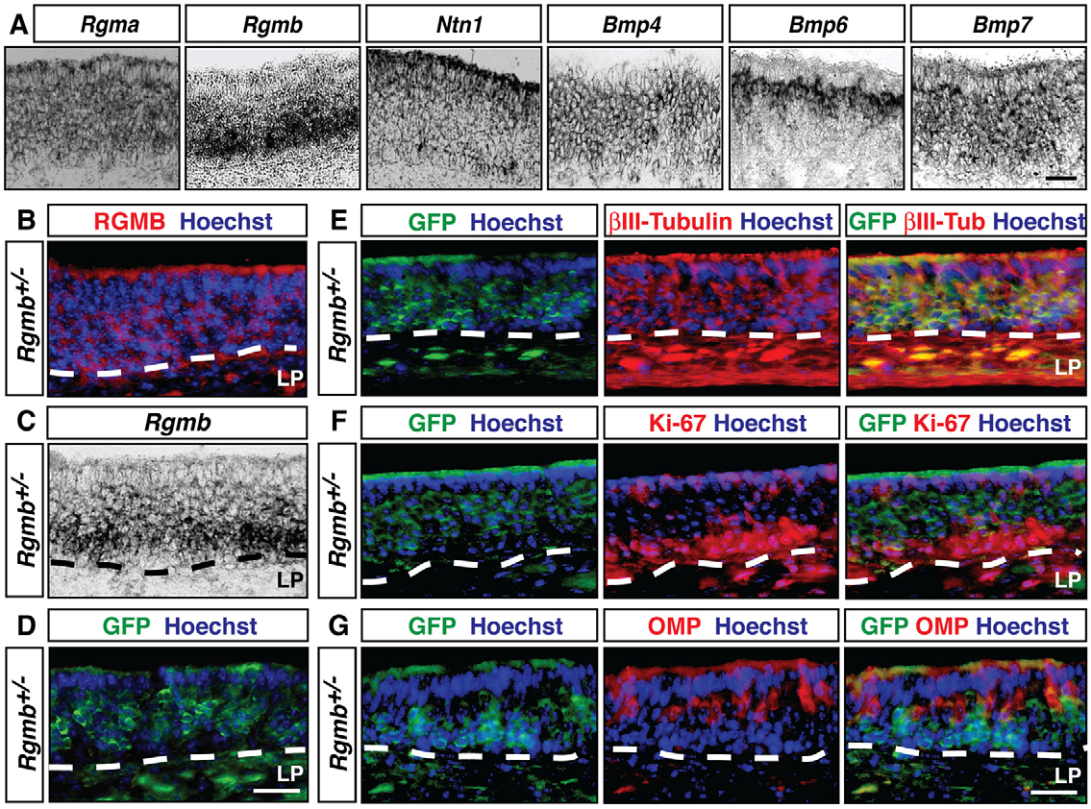


**Fig. 5. Expression of RGMB and other neogenin binding partners in the OE.** (A) ISH analysis on sections of OE from E16.5 wild-type embryos using *Rgma*, *Rgmb*, *Ntn1*, *Bmp4*, *Bmp6* and *Bmp7* cRNA probes. (B-D) Immunolabeling (B,D) and ISH (C) for RGMB (B,C) or GFP (D). (E,F) Double immunolabeling for GFP and βIII-tubulin (D), Ki-67 (F) or OMP (G). Scale bars: 100 µm.

of migrated cells that expressed the cell division marker Ki-67 decreased around RGMB-treated explants, suggesting that more progenitor cells underwent differentiation upon RGMB treatment (Fig. 6C). These results indicate that RGMB can promote neuronal differentiation of progenitor cells.

The effect of RGMB appeared to be mediated through neogenin, since the addition of antibodies against the extracellular region of neogenin to the growth medium significantly attenuated this response (Fig. 6D). Furthermore, treatment of explants from *Neo1*$^{-/-}$ embryos with RGMB did not lead to an increase in the

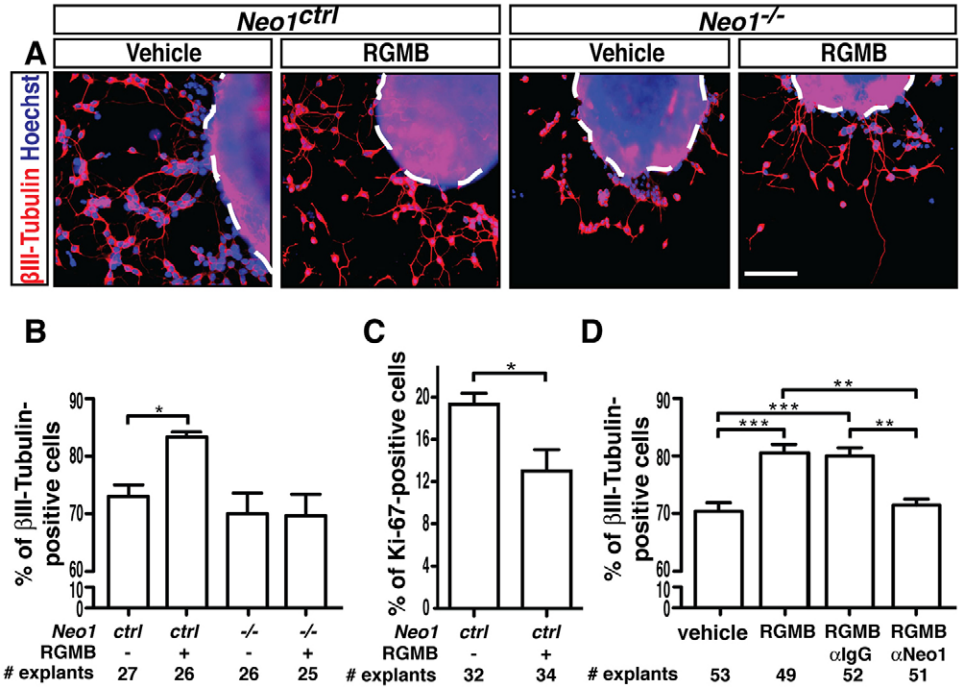


**Fig. 6. RGMB regulates OE cell differentiation *in vitro*.** (A,B) OE explants were isolated from control (*Neo1*$^{+/+}$ or *Neo1*$^{+/-}$) and *Neo1*$^{-/-}$ E14-E15 embryos and were treated with either vehicle or 1 µg/ml recombinant RGMB. Explants were stained with Hoechst and βIII-tubulin antibodies after 18 h in culture (A), and the percentage of migrated cells around the explants that were βIII-tubulin positive (B) was assessed (*n*=3 experiments). The dashed line (A) delineates the olfactory explants. (C) Percentage of cells that are Ki-67 positive around explants from control mice treated with vehicle or RGMB (*n*=4 experiments). (D) Percentage of migrated cells around OE explants isolated from wild-type embryos treated with RGMB and either a control or neogenin-specific antibody (*n*=4 experiments). Values are mean±s.e.m. One-way ANOVA, **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bar: 100 µm.

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generation of neurons from these explants, suggesting that RGMB-neogenin interactions can promote neuronal differentiation in OE explants.

### Ablation of RGMB expression leads to excess production of SUS cells in the OE

Our observation that RGMB promotes neuronal differentiation *in vitro* suggests that RGMB-neogenin signaling in progenitor cells of the OE might be crucial to regulate cell fate decisions. Since *Neo1*$^{-/-}$ embryos display an accumulation of dividing progenitor cells, increased cell death, and the overproduction of SUS cells, RGMB could be implicated in the regulation of cell fate choices in the OE *in vivo*. To study the function of RGMB *in vivo*, we generated an *Rgmb* knockout mouse in which a tau-GFP reporter cassette replaced exon 1 of the *Rgmb* locus (Fig. S4A). In these mice, *Rgmb* mRNA and protein expression were abolished, and GFP expression faithfully recapitulated RGMB expression (Fig. S4B-F). If RGMB mediates some or all of the neogenin functions in progenitor cells of the OE, we would expect that ablating its expression would phenocopy the defects observed in *Neo1*$^{-/-}$ embryos.

We performed a detailed analysis of cell populations in the OE of *Rgmb*$^{-/-}$ embryos. In these mice, the thickness of the OE, the amount of cell death and the number of mature ORNs were similar to those of wild-type mice (Fig. 7A,B, Fig. S4E). However, as we observed in *Neo1*$^{-/-}$ embryos, the number of Ki-67-positive dividing cells in the basal region of the OE was significantly increased in *Rgmb*$^{-/-}$ embryos when compared with littermate controls (Fig. 7D). Furthermore, whereas the number of SOX2-positive stem cells in the basal region of the OE was unchanged in *Rgmb*$^{-/-}$ mice (Fig. 7C), the number of RUNX1-expressing cells was increased in the basal OE, as observed in *Neo1*$^{-/-}$ embryos (Fig. 7E,F). Interestingly, unlike *Neo1*$^{-/-}$ embryos, the number of RUNX1-expressing cells observed in the intermediate layer of the OE where immature ORNs reside was unchanged in *Rgmb*$^{-/-}$ embryos, which might explain the absence of increased cell death in the OE of *Rgmb*$^{-/-}$ embryos (Fig. 7G).

Our observation that *Rgmb*$^{-/-}$ embryos contain more dividing progenitor cells, combined with the normal neuronal differentiation observed in these mice, predicts that RGMB ablation should lead to excess SUS cells. Indeed, there were 18% more SUS cells in the apical region of the OE of *Rgmb*$^{-/-}$ embryos than in controls (Fig. 7H).

Together, these results support a model whereby RGMB in immature ORNs signals to neogenin-expressing progenitor cells to control cell cycle progression and restrain the production of SUS cells. Furthermore, they demonstrate that neogenin regulates the survival of ORNs independently of RGMB, suggesting that additional ligands mediate these functions of neogenin.

## DISCUSSION

Our studies show for the first time, using an *in vivo* loss-of-function approach, that neogenin is required for multiple aspects of cell morphogenesis in the mammalian nervous system, including cell division, cell survival and cell fate choice. We also demonstrate that RGMB-neogenin signaling controls the size of the progenitor pool and the production of SUS cells in the OE, but that RGMB is not required for neogenin-dependent ORN survival (Fig. 8A).

### Neogenin in neuronal survival

Although several lines of evidence have implicated RGM-neogenin interactions in the regulation of cell death/survival in the nervous system, a consensus on the effect of their signaling on cell survival has yet to be achieved. Neogenin has been proposed to act as a dependence receptor, whereby binding of RGM to neogenin is necessary to prevent neogenin-induced cell death in the chick neural tube (Matsunaga et al., 2004). By contrast, RGMA-induced neogenin signaling promotes the survival of retinal ganglion cells in the chick retina or induces cell death in the early *Xenopus* embryo (Shin and Wilson, 2008; Koeberle et al., 2010). We found that ablation of neogenin expression induces robust cell death, which is consistent with a role for neogenin signaling in promoting survival rather than cell death in the mouse OE. Although the mechanism underlying cell death in *Neo1*$^{-/-}$ embryos remains to be fully characterized, our results suggest that dysregulated proneural gene expression may contribute to this process.

### Cell-cell interactions in OE development

In the OE, bipotent basal progenitor cells contribute to the generation of both ORN and SUS cell populations. The relative levels of expression of the transcription factors ASCL1 and SOX2 appears to underlie cell fate bias, with cells that maintain SOX2 expression committing to a glial fate (Gokoffski et al., 2011; Krolewski et al., 2012). Several secreted factors have been identified so far as regulators of cell fate choice in the OE, including activins, BMPs, GDF11 and follistatin (Gokoffski et al., 2011; Kawauchi et al., 2009; Shou et al., 2000; Wu et al., 2003). In addition to these proteins, members of the Notch family of receptors and their transmembrane ligands of the Delta family are expressed in the OE and have been proposed to regulate the development and maintenance of OE cell populations (Carson et al., 2006; Cau et al., 2000; Schwarting et al., 2007). For example, ablation of *Notch2* expression is required for the maintenance of SUS cells in adult OE (Rodriguez et al., 2008).

Our results support the idea that cell-cell interactions in the OE are important to regulate progenitor cell division and differentiation. We propose that the expression of RGMB on immature ORNs activates the neogenin receptor in progenitor cells to regulate cell cycle kinetics and exit (Fig. 8B). In their absence, dysregulated cell cycle kinetics and a reduction in cell cycle exit lead to increased numbers of progenitor cells that preferentially give rise to SUS cells in the OE. Alternatively, RGMB-neogenin signaling might actively block the differentiation of progenitor cells into SUS cells by inhibiting SOX2 expression, thereby favoring neurogenesis (Fig. 8B).

### Neogenin regulates cell cycle progression and cell fate choice in the OE

Ablation of neogenin expression leads to defects in multiple populations of OE cells, including ORN apoptosis, increased numbers of dividing progenitors, and overproduction of SUS cells. The numerous negative- and positive-feedback mechanisms that regulate both proliferation and differentiation in the OE make it challenging to distinguish between direct and indirect phenotypic effects of ablating neogenin expression on the different populations of cells. For example, the increased number of dividing cells in the OE of *Neo1*$^{-/-}$ embryos could be due to an overproliferation of progenitor cells caused by increased apoptosis of ORNs in these mice. Nonetheless, our analyses indicate that an increase in the length of S-phase and a decrease in cell cycle exit underlie the presence of supernumerary dividing progenitor cells in *Neo1*$^{-/-}$ embryos. Furthermore, ablation of RGMB led to increased numbers of dividing progenitor cells without having an effect on the survival of ORNs, supporting a direct effect for RGMB-neogenin signaling

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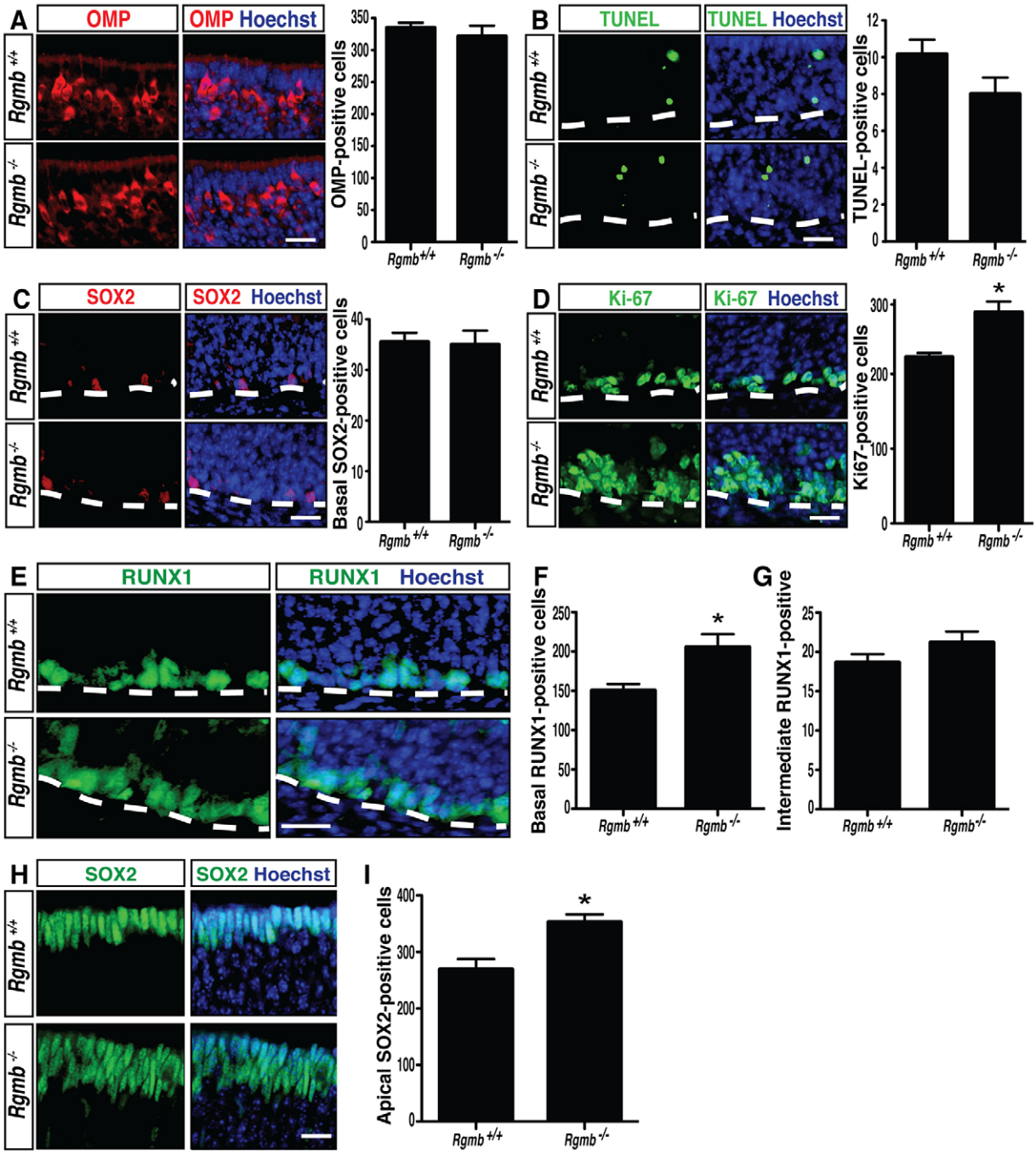


**Fig. 7. Increased number of dividing progenitor cells and SUS cells in the OE of *Rgmb*$^{-/-}$ embryos.** (A-I) Immunolabeling and cell counts in OE sections from control and *Rgmb*$^{-/-}$ E16.5 embryos for markers of mature ORNs (OMP positive; A), cells undergoing cell death (TUNEL; B), stem cells in the basal layer (SOX2 positive; C), dividing progenitor cells (Ki-67 positive; D), RUNX1-positive cells (E-G) and apical SUS cells (SOX2 positive; H,I). All counts (mean±s.e.m.) represent the number of cells per 2.5 mm of OE. Student's unpaired *t*-test, **P*<0.05. For each genotype, *n*=6 (A,D), *n*=4 (C,H,I) or *n*=5 (E-G); except (B) *Rgmb*$^{+/+}$ *n*=6, *Rgmb*$^{-/-}$ *n*=8. Scale bars: 100 µm.

in regulating cell cycle progression and exit. Interestingly, reduced neogenin expression also leads to decreased cell cycle exit in neuroblasts of the subventricular zone in adult mice (O'Leary et al., 2015).

The accumulation of dividing progenitor cells in the basal layer of the OE in *Neo1*$^{-/-}$ embryos is likely to result in the generation of supernumerary SUS cells observed in these embryos. The change in cell cycle kinetics observed in *Neo1*$^{-/-}$ embryos might affect cell fate choice in the progenitors, leading to the overproduction of SUS cells. Alternatively, RGMB-neogenin signaling might influence progenitor cell fate by regulating the balance of expression of ASCL1 and SOX2 in these cells. RGMB-neogenin signaling may restrict the expression of SOX2, thereby limiting the production of SUS cells. Ablation of either neogenin or RGMB would lead to an increase in the number of SUS cells generated, as observed in *Neo1*$^{-/-}$ and *Rgmb*$^{-/-}$ embryos. Neogenin has previously been shown to undergo cleavage of its intracellular domain, which translocates to the nucleus to regulate gene

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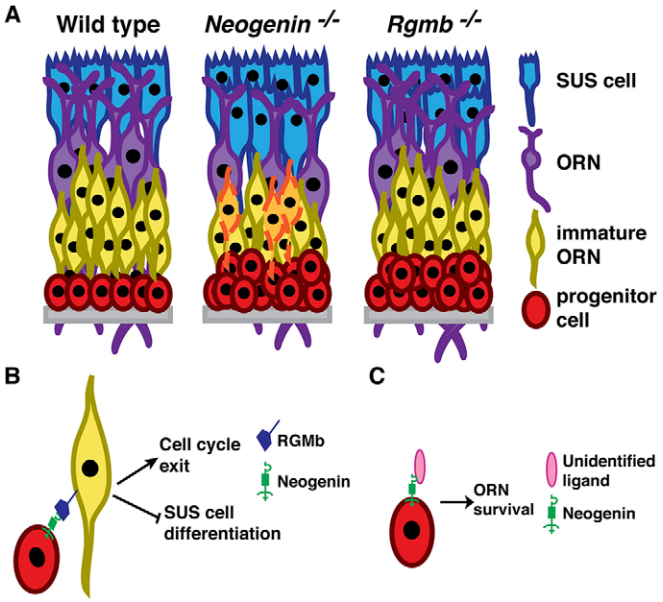


**Fig. 8. Proposed functions for RGMB and neogenin in the developing OE.** (A) Summary of cellular phenotypes observed in the OE of *Neo1*$^{-/-}$ and *Rgmb*$^{-/-}$ embryos. Progenitor cells lining the basal region of the OE produce ORNs and glial-like SUS cells. Ablation of neogenin expression leads to increased numbers of dividing progenitor cells and SUS cells, to the death of immature ORNs, and to reduced numbers of mature ORNs. Interestingly, ablation of RGMB expression also leads to increased numbers of dividing progenitor cells and SUS cells. However, it does not affect the survival of ORNs in the OE. (B) Proposed role for RGMB-neogenin-mediated cell-cell interactions in OE development. The binding of RGMB expressed on immature ORNs (yellow) to neogenin on progenitor cells (red) regulates cell cycle progression and exit. An excess of progenitors in the absence of this signal leads to the overproduction of SUS cells in the OE. Alternatively, RGMB-neogenin signaling could regulate SUS cell production by directly inhibiting SUS cell differentiation from progenitor cells. (C) In addition to regulating progenitor cell proliferation, neogenin also has RGMB-independent functions necessary for the survival of ORNs.

expression (Goldschneider et al., 2008). The cleaved intracellular domain of neogenin might therefore regulate the expression of genes that influence cell fate choice, such as *Ascl1* or *Sox2*.

Another possible explanation for the increased number of SUS cells in *Neo1*$^{-/-}$ embryos is that this effect is indirectly mediated by the changes in the number of dividing progenitor cells. If the excess dividing progenitor cells produce a factor that favors the generation of SUS cells, this could explain the increased number of SUS cells in *Neo1*$^{-/-}$ and *Rgmb*$^{-/-}$ embryos.

### RGMB regulates progenitor cell numbers and cell differentiation but not cell survival

While our results support a role for RGMB in regulating neogenin-dependent progenitor cell cycle progression and SUS cell fate choice, the ligand required for neogenin-induced cell survival remains to be identified. Indeed, although RGMB can promote the neogenin-dependent differentiation of ORNs in OE explants *in vitro*, it is not required for ORN differentiation and survival *in vivo*. It remains possible that the low levels of expression of RGMA that we observed in the OE could compensate for the loss of RGMB in this process. Alternatively, other families of neogenin ligands could regulate ORN survival (Fig. 8C).

BMP proteins have been extensively studied as regulators of cell fate choice during embryogenesis. For example, during neurulation BMPs block proneural gene expression and favor epithelial fate (Smith et al., 1993; Zimmerman et al., 1996). BMP2 has been shown to bind neogenin, which leads to inhibition of osteoblast differentiation (Hagihara et al., 2011). Furthermore, neogenin can positively regulate BMP receptor signaling by promoting its localization into lipid rafts through interactions with RGMs (Zhou et al., 2010). Several BMP family members are expressed in the OE and have dose-dependent effects on ORN production in *in vitro* OE explant assays. Treatment of OE explants with high doses of BMP2, BMP4 or BMP7 promotes ASCL1 degradation in progenitor cells and prevents ORN generation (Shou et al., 1999). By contrast, treatment with low doses of BMP4 promotes the survival of newly born ORNs in this assay (Shou et al., 2000). It therefore remains possible that neogenin-dependent BMP4 signaling could promote the survival of newly born ORNs.

We have shown that RGMB-neogenin interactions play a crucial role in regulating progenitor cell numbers and cell differentiation in the developing OE. Whether these interactions also play a role in the regeneration and maintenance of cell populations in the OE throughout adulthood will have to be addressed in the future using cell-specific temporal ablation of these two proteins in the OE. Furthermore, our results raise the interesting possibility that cell-cell interactions mediated by RGMB and neogenin might also regulate progenitor cell fate choice in other regions of the nervous system, including the ventricular zone of the brain, where neogenin is expressed by a subpopulation of progenitor cells and regulates neuroblast differentiation (Fitzgerald et al., 2006; O'Leary et al., 2015). The floxed *Neo1* allele that we have described will be an invaluable tool in the effort to uncover the *in vivo* functions of neogenin in other regions of the nervous system, as well as in other physiological systems of the developing and adult mouse.

## MATERIALS AND METHODS

### Experimental animals

Mouse embryos were obtained from timed pregnancies. Morning of the vaginal plug was considered E0.5. The *Neo1* and *Rgmb* mouse lines (see the supplementary Materials and Methods) were backcrossed for three generations in a C57BL/6J background. Primers for genotyping by PCR are described in the supplementary Materials and Methods. The gene-trap neogenin (*Neo1*$^{GT}$) (Leighton et al., 2001; Phan et al., 2011), *Ntn1*$^{GT}$ (Serafini et al., 1996) and OMP-Cre (Cho et al., 2011; Eggan et al., 2004) mouse lines have been described previously. All animal procedures have been approved by the Montreal Neurological Institute Animal Care Committee, in accordance with the guidelines of the Canadian Council of Animal Care.

### Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde (PFA) and processed for immunohistochemistry as previously described (Cho et al., 2012, 2007). For antibodies that required an antigen retrieval step, slides were placed on a hot plate for 10-30 min with 0.01 M sodium citrate before the blocking incubation. Primary antibodies and dilutions used were: rabbit anti-GFP (Invitrogen, A6455; 1:250), goat anti-OMP (Wako, 544-10001; 1:1000), mouse anti-Ki-67 (BD Pharmingen, clone B56; 1:150), rabbit anti-β-gal (MP Biomedical, 559762; 1:500), sheep anti-RGMB (R&D, AF3597; 1:500), rabbit anti-RGMB (Abcam, ab33496; 1:100), goat anti-neogenin (R&D, AF1079; 1:500), rabbit anti-neogenin (Santa Cruz Biotechnology, H-175; 1:100), rat anti-BrdU (Abcam, ab6326; 1:50), mouse anti-βIII-tubulin (Promega, G7121; 1:2000), rabbit anti-SOX2 (Millipore, AB5603; 1:250), rabbit anti-RUNX1 (Abcam, ab92336; 1:200) and mouse anti-cytokeratin 18 (Millipore, MAB3234; 1:50). Secondary antibodies used were Alexa 488, Alexa 546 and Alexa 594 labeled from Invitrogen (1:500).

### TUNEL

For terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL), tissue sections were fixed in 4% PFA for 20 min,

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washed with PBS and permeabilized with 0.1% Triton X-100/0.1% SSC for 2 min. The sections were incubated with TUNEL reaction cocktail [1% biotin-16-dUTP (Roche), TdT buffer (Promega), TdT (Promega), double-distilled water] for 1 h at 37°C and biotin-16-dUTP-positive cells were visualized with streptavidin-Alexa 488.

### S-phase duration and cell cycle exit

We estimated the length of the S-phase using dual DNA synthesis labeling as previously described (Alexiades and Cepko, 1996; Huard and Schwob, 1995). 5-ethynyl-2′-deoxyuridine (EdU) was injected (50 mg/kg), initially intraperitoneally, in E15.5 pregnant dams followed by an injection of bromodeoxyuridine (BrdU; 50 mg/kg) 2 h later. One hour later, embryos were fixed and sections were processed for EdU Click-iT reaction (Invitrogen) and anti-BrdU immunohistochemistry. S-phase length was calculated using the following formula: 2 h×($EdU^+$ cells/$EdU^+$ $BrdU^-$ cells) (Huard and Schwob, 1995).

Cell cycle exit index was measured by injecting E15.5 pregnant dams with EdU. Embryos were harvested 24 h after the injection, sectioned, and processed for EdU Click-iT reaction and Ki-67 immunohistochemistry. Under these conditions, cells that exited the cell cycle after injection of EdU should be EdU positive and Ki-67 negative, whereas cells that are still cycling should be positive for both markers. We calculated the proportion of cells that exited the cell cycle as follows: ($EdU^+$ $Ki67^-$ cells/total $EdU^+$ cells)×100.

### Image capture and analysis

Images of processed sections were obtained using a Carl Zeiss Axio Imager M1 and cell counts were performed using Eclipse (Empix Imaging) and ImageJ (NIH) software. Cells were counted over a total distance of 2.5 mm of OE (1 mm for βIII-tubulin analysis) along the septum from at least four embryos derived from a minimum of three separate litters. Statistical significance was assessed using unpaired two-tailed *t*-test and one-way ANOVA analyses performed using Prism software (GraphPad).

### *In situ* hybridization (ISH)

ISH was performed on OE cryosections from embryos as previously described (Cho et al., 2011; Prince et al., 2009).

### Nissl staining

Embryos were fixed in 4% PFA for 30 min. Sections were air dried at room temperature followed by dehydration in ethanol. The sections were then stained with cresyl violet acetate, washed in ethanol, and mounted with Permount (Fisher Scientific).

### Primary OE explant culture

OE explants were prepared from CD-1 E14/15 and *Neo1* mutant E14/15 embryos, and cultured for 18 h with or without recombinant RGMB protein (R&D Systems; 1 µg/ml) before processing for immunostaining. For the function-blocking antibody experiments, 5 µg/ml control IgG or neogenin antibodies (R&D Systems, AF1079) were added to the media. Explants were grown on merosin following previously described protocols (DeHamer et al., 1994; Gordon et al., 1995). At least 2250 migratory cells were counted, from a minimum of 25 individual explants in at least three individual experiments for each condition.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

J.W.K.K., D.M.d.S., T.E.K. and J.-F.C. designed the study. J.W.K.K., E.D., C.B., A.C.B. and D.M.d.S. performed experiments and data analysis. E.D. and M.C. generated the *Neo1* conditional and *Rgmb* null strains. J.W.K.K. and J.-F.C. wrote the manuscript, and all authors contributed to editing the manuscript before submission.

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#### Supplementary information

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## Supplementary Materials and Methods

*Mice*

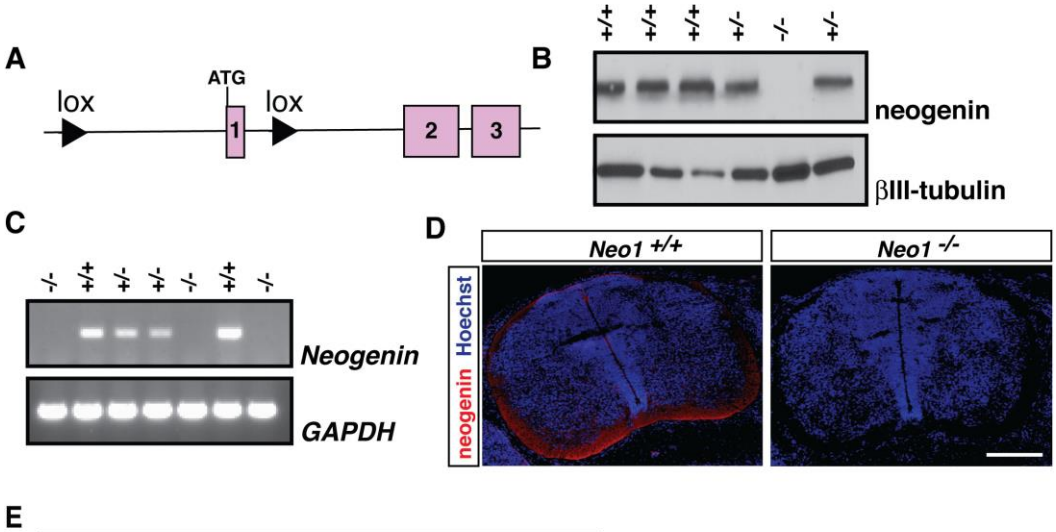
We generated *Neo1* mutant mice by standard homologous recombination methods to introduce two loxP sites flanking the first exon of the *Neo1* allele, which encodes the signal sequence. Following germline transmission, heterozygous mice (*Neo1*$^{+/lox}$) were crossed to FlpE mice to excise the Neomycin cassette used for G-418 selection of targeted ES cell clones. The *Neo1*$^{+/lox}$ mice were crossed with CMV-Cre mice to obtain germline deletion of the floxed *neogenin* allele (*Neo1*$^{+/-}$). Those heterozygous mice were bred together to obtain *neogenin* mutant mice.

We generated *Rgm-b* mutant mice by standard homologous recombination methods to introduce a tau-GFP cassette, 3' UTR and polyA signal into the first exon of the *Rgm*-b gene, in frame with the ATG start site but deleting the endogenous amino acid signal sequence of *Rgm-b*. In this way, tau-GFP is expressed under the control of endogenous *Rgm-b* regulatory sequences, but Rgm-b cannot follow the secretory pathway to reach the cell surface. Heterozygous mice (*Rgm-b*$^{+/-}$) mice were bred with FlpE mice to remove the neomycin cassette.

*Reverse transcription polymerase chain reaction (RT-PCR)*

Brains were isolated from E16.5 *Neo1*$^{+/+}$, *Neo1*$^{+/-}$, and *Neo1*$^{-/-}$ embryos. RNA was purified using the Qiazol reagent (Qiagen) followed by the RNeasy Lipid Tissue Mini Kit (Qiagen). DNase treatment was performed using DNase I (Invitrogen). cDNA was generated using the SuperScript II reverse transcriptase system (Invitrogen). PCR oligonucleotides for *Gapdh*, 5′-GCATCCTGCACCACCAACTG-3′ and 5′-CGGCCGCCTGCTTCACCACCTTCT-3′; *Neogenin Primers A, 5'-*

GAGCCCTCTCCAAACATTGA- 3, and 5'- TCAACAATGCAGCGGTAGAG- 3'; *Neogenin Primers C*, 5′- CCTCTGAAGAGCTTCGCTGT -3′ and 5′- TCCTGTACTTCAGGGGCACT -3′. PCR reactions were conducted for 30 cycles using the GoTaq system (Promega).




E

| Age(Total number of embryos) | *Neo1* $^{+/+}$ | *Neo1* $^{+/-}$ | *Neo1* $^{-/-}$ |
|---|---|---|---|
| E11 (22 from 3 litters) | 6 (27%) | 12 (55%) | 4 (18%) |
| E14 (124 from 17 litters) | 29 (24%) | 62 (50%) | 33 (26%) |
| E16 (181 from 26 litters) | 39 (22%) | 99 (55%) | 43 (23%) |
| P0 (30 from 4 litters) | 8 (27%) | 20 (67%) | 2 (7%) |

**Fig. S1: Generation and characterization of a floxed *Neogenin* mutant.**

(A) Diagram of the *Neo1* targeted allele containing two LoxP sites inserted 5' and 3' of exon 1. (B) Immunoblots on brain lysates collected from littermates of a cross between two *Neo1*$^{+/-}$ mice. Neogenin protein expression is abolished in *Neo1*$^{-/-}$ embryos. (C) RT-PCR analysis on brain mRNA samples isolated from control and *Neo1*$^{-/-}$ embryos. *Neogenin* mRNA expression is abolished in *Neo1*$^{-/-}$ embryos. (D) Immunolabeling of spinal cord sections from E16.5 embryos with a neogenin antibody. Neogenin signal is absent in *Neo1*$^{-/-}$ embryos. (E) Table indicating the number of *Neo1*$^{+/+}$, *Neo1*$^{+/-}$, and *Neo1*$^{-/-}$ embryos obtained in litters at different time points in embryonic development. None of the *neo1*$^{-/-}$ mice survive past P0. Scale bar: 200 μm.

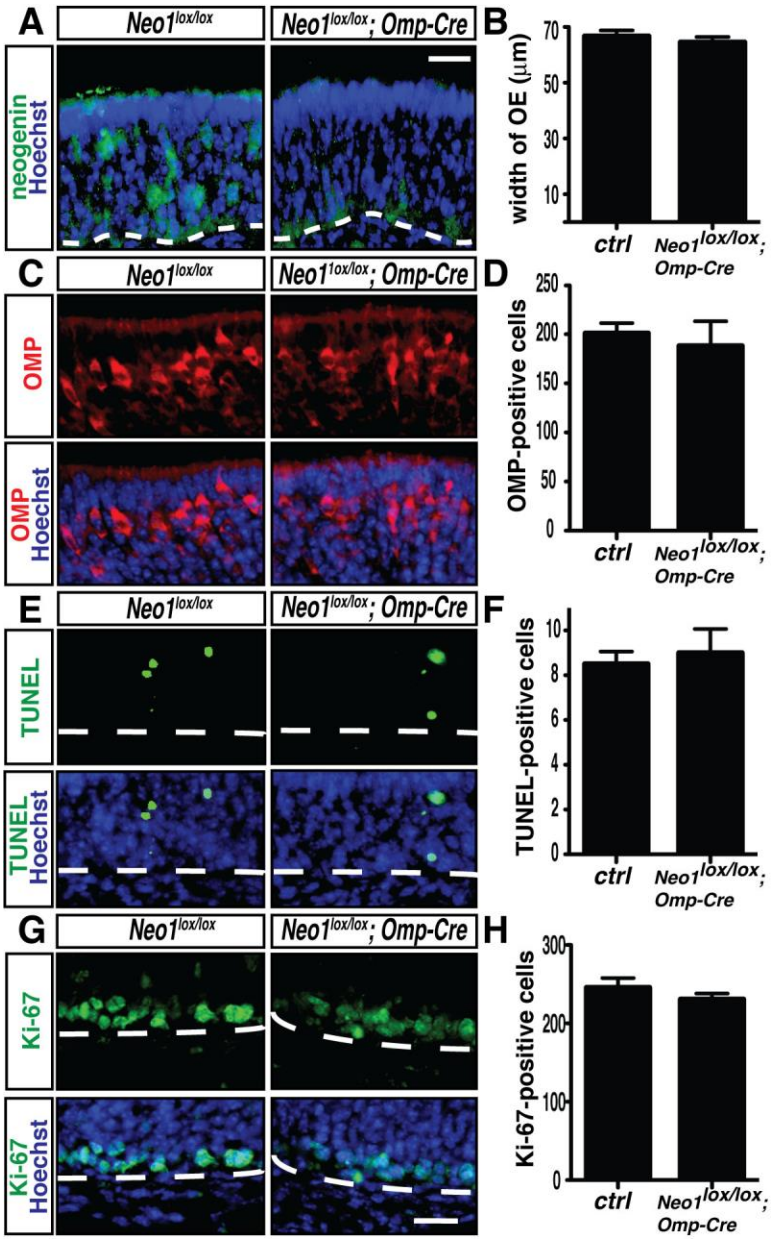



**Fig. S2: Deletion of Neogenin from OMP expressing cells does not alter ORN generation and survival.**

(A) Immunolabeling of OE sections from E16.5 embryos with a neogenin antibody. OMP-Cre mediated deletion of neogenin in the OE. The expression of neogenin is

abolished in mature ORNs but not in basal cells of the OE in E16.5 *Neo1*$^{lox/lox}$*;Omp-Cre* embyos. (B) Quantification of the width of OE shows comparable OE width between control and mutant littermates. (C-H) Immunolabeling of OE sections from E16.5 embryos reveal that the number of OMP- (C,D), TUNEL- (E,F) and Ki-67 (G,H)-positive cells is unchanged in *Neo1*$^{lox/lox}$*;Omp-Cre* embryos. All cell counts are represented as cells per 2.5 mm of OE. Students unpaired t-test was performed to compare values between groups. Scale bar: 100 μm.

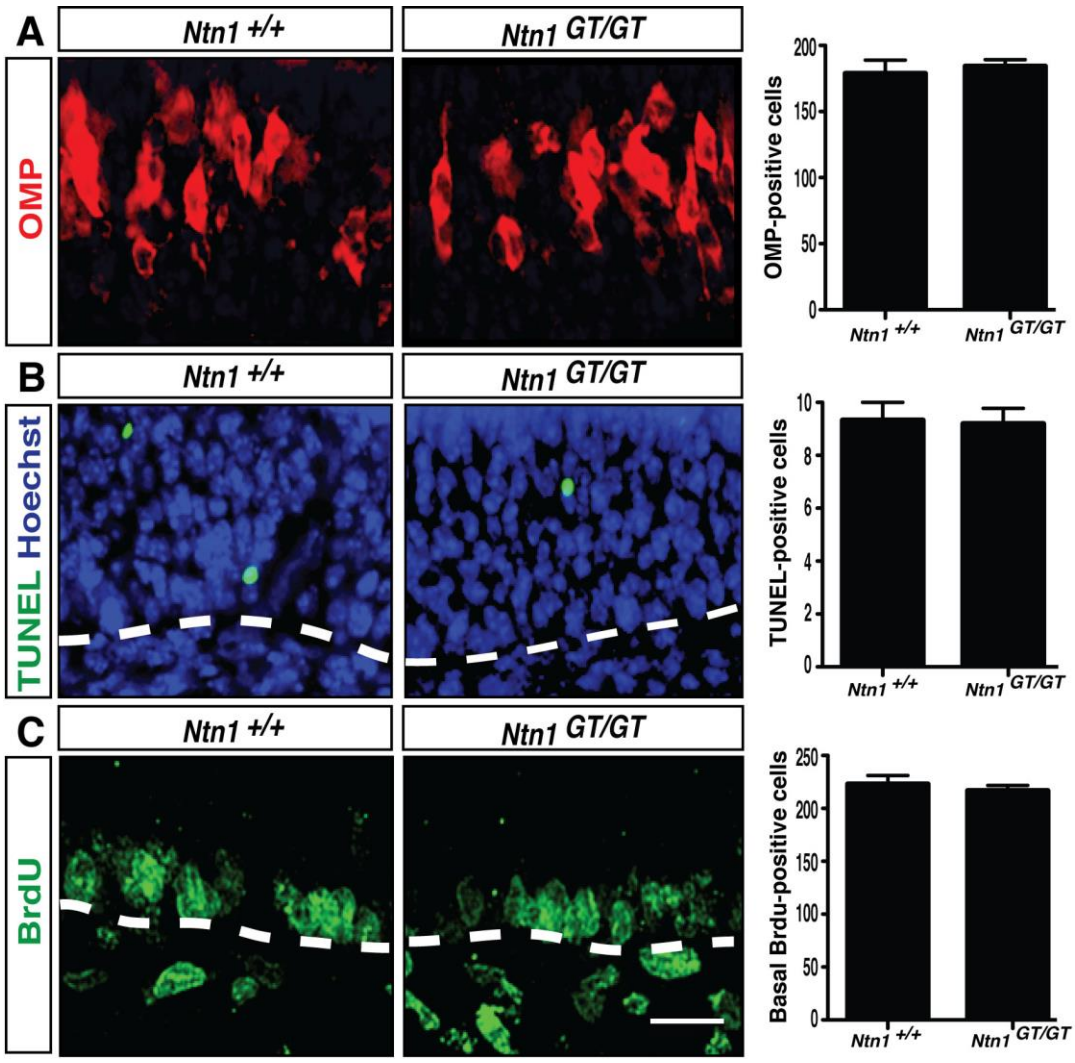



**Fig. S3: OE development is normal in *Ntn1*$^{GT/GT}$ mice.**

**(A-C)** OE sections from E16.5 embryos were processed for OMP (A), TUNEL (B), and BrdU (C) staining. The OE of *Ntn1*$^{GT/GT}$ embryos shows similar numbers of mature ORNs (A), cell death (B), and basal proliferating cells (C), as control embryos. All cell counts are represented as cells per 2.5 mm of OE. Students unpaired t-test was performed to compare values between groups. Scale bar: 100 μm.

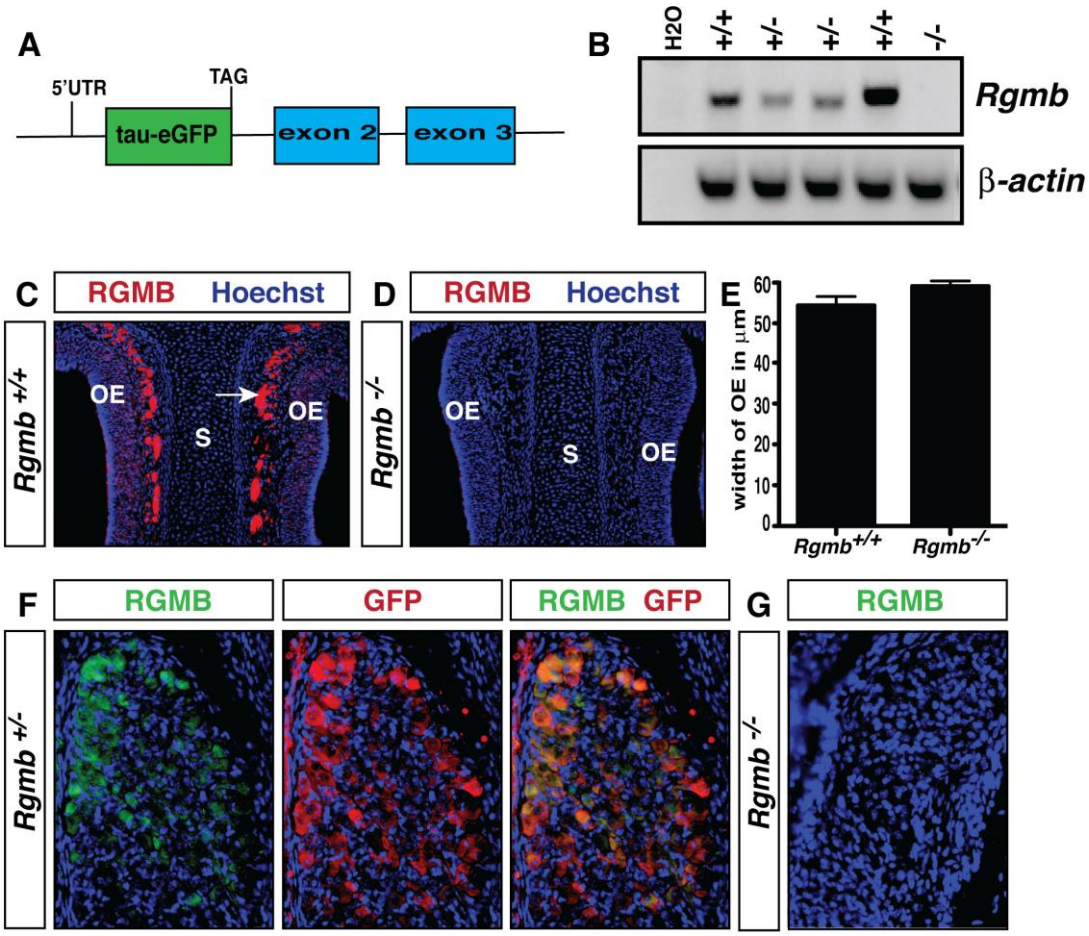



**Fig. S4: Generation and characterization of the *Rgmb* knock-in mice.**

(A) Diagram of the *Rgmb* targeted allele. An eGFP cassette was inserted into exon 1 of the *Rgmb* allele. (B) RT-PCR analysis on brain mRNA samples isolated from control and $Rgmb^{-/-}$ embryos. *Rgmb* mRNA expression is abolished in $Rgmb^{-/-}$ embryos. (C,D) RGMB immunolabeling of OE sections from E16.5 $Rgmb^{+/+}$ (C) and $Rgmb^{-/-}$ (D) embryos. RGMB protein expression is abolished in $Rgmb^{-/-}$ embryos. (E) Quantification of the width of OE (μm) in $Rgmb^{+/+}$ and $Rgmb^{-/-}$ embryos. The width of the OE is unchanged in $Rgmb^{-/-}$ embryos. (F-G) Immunolabeling of dorsal root ganglia from an

E16.5 *Rgmb*$^{+/-}$ embryo with GFP and RGMB antibodies. GFP is expressed in RGMB-positive cells and GFP expression recapitulates RGMB expression in the dorsal root ganglia (F). RGMB expression is abolished in the dorsal root ganglia of *Rgmb*$^{-/-}$ embryos (G). Students unpaired t-test was performed to compare values between groups. Scale bars: 100 μm.