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# Polycomb repressive complex 2 regulates basal cell fate during adult olfactory neurogenesis

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## SUMMARY

Adult neurogenesis occurs in the mammalian olfactory epithelium to maintain populations of neurons that are vulnerable to injury yet essential for olfaction. Multipotent olfactory basal stem cells are activated by damage, although mechanisms regulating lineage decisions are not understood. Using mouse lesion models, we focused on defining the role of Polycomb repressive complexes (PRCs) in olfactory neurogenesis. PRC2 has a well-established role in developing tissues, orchestrating transcriptional programs via chromatin modification. PRC2 proteins are expressed in olfactory globose basal cells (GBCs) and nascent neurons. Conditional PRC2 loss perturbs lesion-induced neuron production, accompanied by altered histone modifications and misexpression of lineage-specific transcription factors in GBCs. De-repression of *Sox9* in PRC2-mutant GBCs is accompanied by increased Bowman's gland production, defining an unrecognized role for PRC2 in regulating gland versus neuron cell fate. Our findings support a model for PRC2-dependent mechanisms promoting sensory neuronal differentiation in an adult neurogenic niche.

## INTRODUCTION

Adult neurogenesis, due to the presence of neural stem cells, has been identified in mammals in limited sites including the hippocampus, the subventricular zone, and the olfactory neuroepithelium (OE) (Altman and Das, 1965; Graziadei and Graziadei, 1979; Lois et al., 1996; Luskin, 1993). Understanding the regulation of adult neural stem cell renewal and differentiation is an essential step toward developing new therapies to repair nervous system disorders. In the rodent and human OE, which is the peripheral organ for olfaction, specialized olfactory sensory neurons are replaced continually by stem and progenitor cells situated in basal cell layers (Caggiano et al., 1994; Calof et al., 2002; Durante et al., 2020; Huard and Schwob, 1995; Mackay-Sim and Kittel, 1991). Following experimentally induced damage to the rodent OE, multipotent basal cells are activated to reconstitute the neurons and the non-neuronal supporting lineages, providing a system well suited to examine strategies regulating adult cell fate and neurodifferentiation (Huard et al., 1998; Leung et al., 2007).

Utilizing the adult mouse OE model, fate-mapping studies have identified heterogeneous populations of basal stem and progenitor cells and their lineage potential (Chen et al., 2014; Goldstein et al., 2015, 2016; Leung et al., 2007). Recently, single-cell transcriptional profiling approaches have helped define differentiation trajectories and key pro-neural regulatory signals (Gadye et al., 2017; Lin et al., 2017). Classic loss-of-function studies *in vivo* defined the importance of families of transcription factors, including the basic-helix-loop-helix (bHLH) transcription factors, active in progenitor basal cells (Cau et al., 1997; Guillemot et al., 1993). Many of these mechanisms are highly conserved between *Drosophila*, where they were initially discovered, and mammals. Of interest, mammalian homologs of the *Drosophila* Polycomb group (PcG) proteins, functioning as chromatin modulators to silence gene transcription, have been found to be critically important in development (Bracken and Helin, 2009; Ringrose, 2007), yet their role in the OE has not been well studied. The expression of PcG proteins has been identified in adult olfactory basal cells and immature neurons, suggesting roles for Polycomb repressive complexes (PRCs) in cell renewal or differentiation in the neuronal lineage (Goldstein et al., 2016, 2018; Lin et al., 2017).

The regulation of gene expression by epigenetic mechanisms, including PcG activity, is a fundamental strategy controlling cell fate and identity during embryonic development and neurogenesis (Hsieh and Zhao, 2016). Polycomb proteins were initially viewed as repressive elements controlling *Hox* gene expression, due to pattern malformations seen in PcG mutant flies (Lewis, 1978). However, Polycomb complexes regulate diverse processes in mammals due to differences in the composition of subunits or interactions with other co-factors (Gao et al., 2012, 2014; Morey et al., 2012). PcG proteins function by forming two types of multimeric complexes, PRC1 and PRC2. The core PRC2 complex (Figure 1A) is composed of EED,

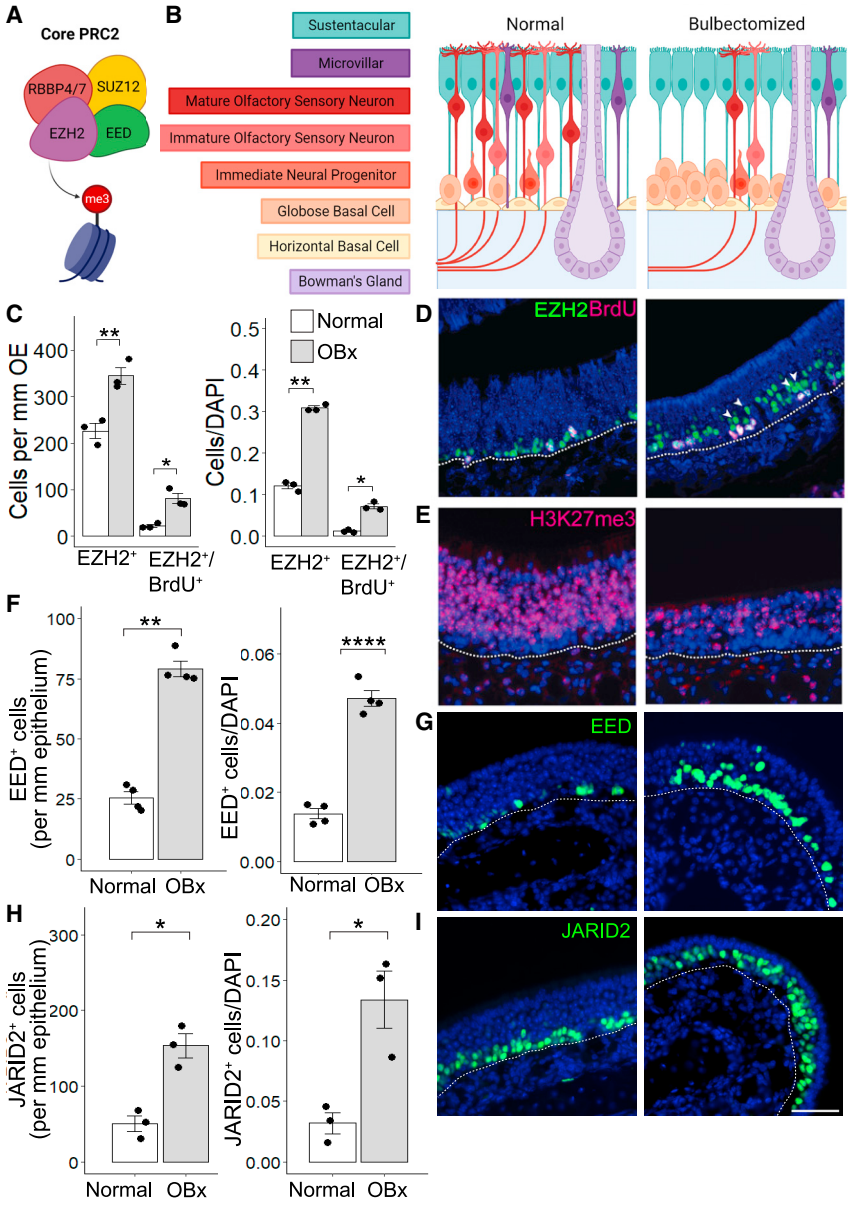



**Figure 1. PRC2 proteins are expressed in neurogenic cells in normal and regenerating mouse OE**

(A) Core PRC2 subunits.

(B) Schematic of cell types comprising the OE in normal and bulbectomized conditions; the bulbectomy model causes increased production of only neurons from basal cells, while non-neural cell lineages are unaffected.

(C–I) Representative olfactory immunohistochemistry; (D, E, G, and I) antibodies to BrdU, EZH2, H3K27me3, EED, and JARID2 in normal (left column) and bulbectomized (right column) epithelium. Bar: 50 μm. White dashed lines indicate base of the epithelium. In graphs (C), (F), and (H), points represent data from individual mice, and bars represent mean ± SEM (n = 3 per group for C and H; n = 4 per group for F) (paired t test; *p < 0.05, **p < 0.01, ****p < 0.0001).

SUZ12, RBBP4/7, and EZH1/2, the latter component being the enzymatic subunit that establishes methylation on lysine 27 of histone H3 (H3K27me1/2/3). Two main subgroups exist: PRC2.1 also contains PALI or EPOP and a PCL subunit, and PRC2.2 contains the core complex along with AEBP2 and JARID2. The unique subunits likely alter localization and function via recognition of distinct histone marks. Substantial diversity of PRC1 complex composition also provides a means for functional variation, although all PRC1s contain E3 ubiquitin ligase activity. PRC2 and canonical PRC1 classically function as transcriptional repressors, but recent evidence suggested that PRCs can also function as transcriptional activators, particularly during differentiation (Chan et al., 2018; Cohen et al., 2018; Creppe et al., 2014; Gao et al., 2014; Morey et al., 2015).

While PRC activity has been well studied in non-neuronal adult stem cell niches, including the skin and bone marrow (Cohen et al., 2021), little is known regarding PRCs and adult neurogenesis. However, recent fate-mapping and cell culture results suggest important roles for PRCs in the OE, a stem cell niche capable of remarkable adult neuroepithelial renewal (Choi et al., 2019; Goldstein et al., 2016, 2018). Here, we have focused on defining the function of PRC2 in adult olfactory neurogenesis, employing mouse olfactory lesion models,

conditional deletion, single-cell RNA sequencing (scRNA-seq) and chromatin studies.

## RESULTS

### PRC2 proteins are expressed in proliferative globose basal cells and immature neurons

Previously, we have identified the expression of PRC2 subunits SUZ12 and EZH2 in the basal portions of the adult OE (Goldstein et al., 2016, 2018). The basal layers of the OE house heterogeneous populations of reserve or active stem and progenitor cells, along with nascent and immature neurons (Figure 1B) (Schwob et al., 2017). Here, we utilized a mouse unilateral olfactory bulbectomy lesion model to further characterize PRC2 subunit-expressing cells in the OE. Bulbectomy is well studied and axotomizes mature olfactory neurons to cause neuronal degeneration, which leads to reactive globose basal cell (GBC) proliferation and increased neurogenesis (Carr and Farbman, 1993; Holcomb et al., 1995; Schwob et al., 1992; Verhaagen et al., 1990). The non-neuronal sustentacular cells remain intact in this neuron-specific lesion, providing a lesion model to focus only on increased production of neurons from their precursor basal cells but not other cell lineages. In bulbectomized, regenerating OE, we observed an increase in the $EZH2^+$ population of cells ($n = 3$, $p < 0.01$, paired t test) (Figures 1C and 1D). Bulbectomy increases basal cell mitotic activity, and co-staining demonstrated that all acutely proliferative basal cells incorporating a brief pulse of the thymidine analog bromodeoxyuridine (BrdU) co-express EZH2 (Figures 1C and 1D), which is consistent with the known role for PRC2 in the regulation of other adult stem cell niches, including the skin or hematopoietic system (Cohen et al., 2018; Di Carlo et al., 2019). PRC2 is also expressed in newly differentiating immature olfactory neurons emerging from GBCs, identifiable as $EZH2^+/BrdU^-$ cells apical to the $EZH2^+/BrdU^+$ basal layers in the bulbectomized OE (Figure 1D, arrowheads). Additionally, H3K27me3 histone marks deposited by PRC2 are present in the neuronal layers of the OE and are diminished following bulbectomy and the resulting loss of neurons (Figure 1E). Expression changes of core PRC2 subunit EED and JARID2, a PRC2.2-specific protein with potential roles in differentiation, also follow a similar pattern in normal and bulbectomized OE (Figures 1F–1I).

In a chemical olfactory lesion model caused by intraperitoneal injection of methimazole (Bergman et al., 2002; Leung et al., 2007), neurons and non-neuronal cells are killed, providing a means to study generation of all olfactory populations from spared multipotent basal cells. Therefore, the methimazole lesion model is ideal for asking whether PRC2 expression plays a role in lineage decisions. Over the course of 14 days following methimazole injury, epithelial thickness increases, along with re-establishment of the sustentacular barrier layer marked by SOX2 and progressively more neuronal cell layers marked by expression of TUJ1 (Figure S1). In this setting, horizontal basal cells are activated to reconstitute all olfactory lineages via progenitor GBCs, and we similarly identify PRC2 proteins in neurogenic lineages (Figure 2; Table S1). At 7 days post-injury, there are few mature, $OMP^+$ neurons, but by day 14, the epithelium has reconstituted, and there is an abundance of mature $OMP^+$ neurons (Figure 2B). At both 7 and 14 days post-injury, proliferative GBCs identifiable by expression of c-KIT (Fletcher et al., 2017; Goldstein et al., 2015) are present in the basal layers of the epithelium (Figure 2C), and expression of PRC2 proteins EED, EZH2, and SUZ12 are present in the GBCs and neuronal-lineage cell layers (Figures 2D–2F and 2H). H3K27me3 histone marks deposited by PRC2 are also present in the neuronal layers of the OE at 7 and 14 days post-injury (Figure 2G). Together, the pattern of PRC2 expression in the un-lesioned, bulbectomized, or methimazole-lesioned OE in proliferative GBCs and immature neurons, along with the presence of the PRC2-specific H3K27me3 mark within the neuronal layers, suggests a neuron lineage-specific role for PRC2 in adult olfactory neurogenesis.

### Loss of PRC2 subunit EED in olfactory basal cells impairs neurogenesis and increases generation of sustentacular and basal cell-derived Bowman's glands

To test for a requirement for PRC2 function in olfactory neuronal-lineage cells, we used a mouse conditional deletion approach (Figure 3A). In the OE, KRT5 is selectively expressed by reserve horizontal basal cells (HBCs). Thus, using *Krt5-CreER$^{T2}$; Eed$^{flox}$; R26R Ai9* mice, we induced recombination in HBCs to delete exon 7 of *Eed* and activate a tdTomato reporter. The methimazole lesion was subsequently induced (Fletcher et al., 2011; Leung et al., 2007) to fate-map activated HBCs, reconstituting all olfactory lineages. Quiescent HBCs do not express PRC2 proteins; EED and its binding partners are expressed in the GBCs (see Figure 1) emerging from activated HBCs. Thus, our approach permits the manipulation of PRC2 expression by conditional deletion in the reserve cell upstream of the stage at which it would normally be expressed during OE reconstitution following injury. The ability of *Eed*-deficient basal cells to repopulate the OE was then visualized by fate mapping cells following methimazole lesion, based on reporter expression.

We observed a histologic phenotype for lineage-traced cells in *Eed$^{fl/fl}$* mice (*Krt5-CreERT2; R26R Ai9; Eed$^{fl/fl}$*) based on cell morphology and expression of cell-type-specific markers at 14 days post-lesion. In other mouse models,

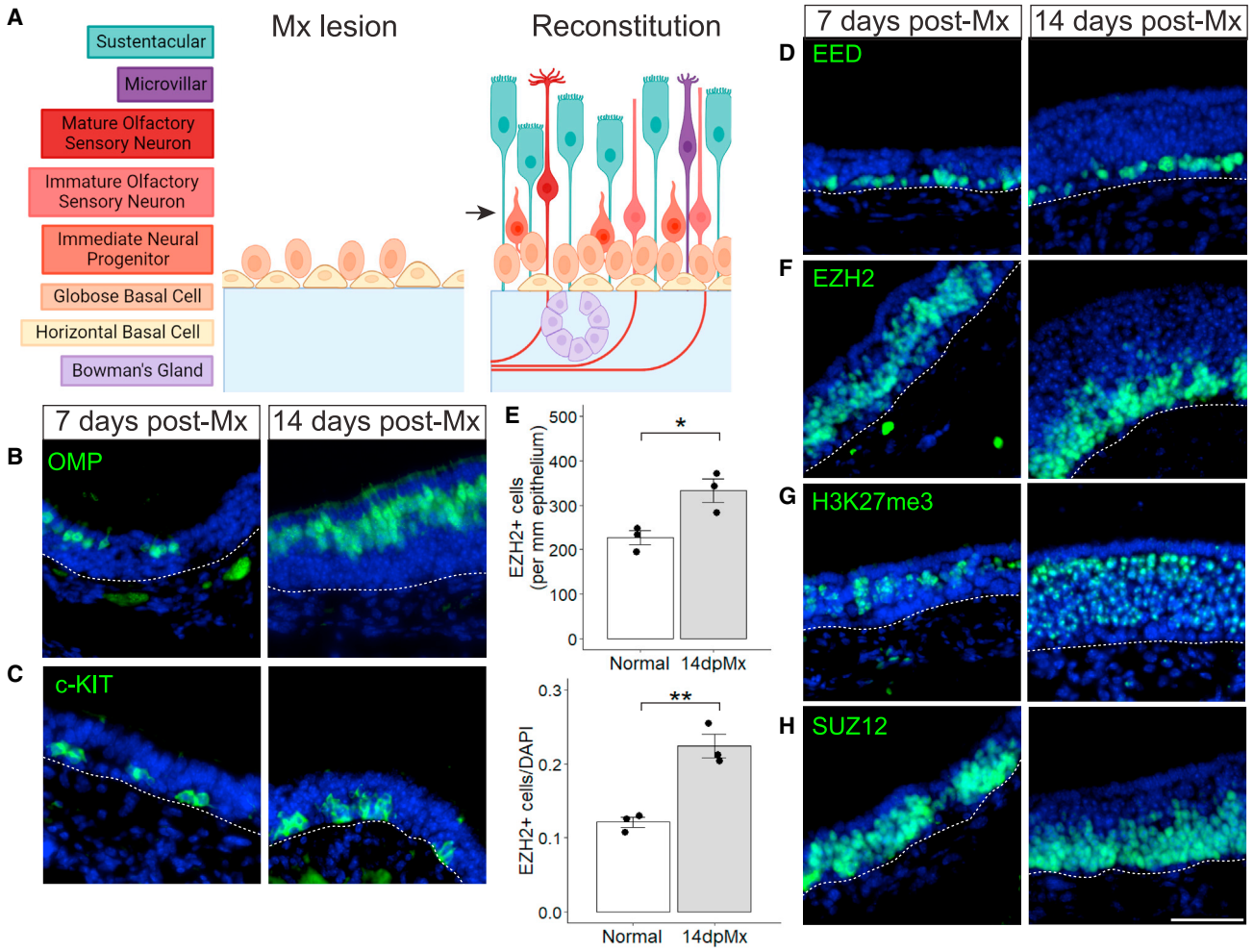



**Figure 2. PRC2 protein expression in chemically lesioned OE**

(A) Schematic of OE in normal condition, immediately following methimazole (Mx) lesion, and post-Mx reconstitution.

(B–D and F–H) Immunohistochemical staining of OE at 7 and 14 days post-Mx with antibody to (B) OMP, (C) c-KIT, (D) EED, (F) EZH2, (G) H3K27me3, or (H) SUZ12.

(E) Quantification and comparison of $EZH2^+$ cells between normal, un-lesioned OE and 14 days post-Mx OE (n = 3 per group) (t test, *p < 0.05, **p < 0.01); points represent data from individual mice, and bars represent mean ± SEM. Bar: 50 μm, dashed line indicates basal lamina.

inducible deletion of key Polycomb subunits using *Cre*ERT2 or *Cre*PRG driven by promoters of keratin genes such as *Krt5*, *Krt14*, or *Krt15*, which are expressed in many epithelia, is known to affect mouse survival, so topical applications of tamoxifen or RU-486 have been used to avoid these systemic off-target effects for conditional Polycomb deletion in skin (Flora et al., 2021b; Li et al., 2021). Since topical application of tamoxifen is not feasible in the OE, and multiple injections of tamoxifen resulted in lethality, we chose to use a single dose of tamoxifen injected intraperitoneally to avoid systemic toxicity in the *Krt5-CreERT2; Eed*$^{fl/fl}$ model. As expected, *Eed* deletion did not occur in all reporter-positive cells, and in the lineage-traced cells of *Eed*$^{fl/fl}$ mice (n = 5), there was a 52% decrease in $EED^+$/$tdTomato^+$ cells compared with control *Eed*$^{fl/WT}$ (wild-type) mice (*Krt5-CreERT2; R26R Ai9; Eed*$^{fl/WT}$) (n = 5) (Figures 3B and S2A; p < 0.01, t test). Despite incomplete recombination, this approach permits us to assess PRC2 function in lesion-induced olfactory epithelial reconstitution; we observed a significant impairment in lesion-induced olfactory neurogenesis. In the *Eed*$^{fl/fl}$ mice (n = 4), there was a decrease in lineage-traced neurons (p < 0.05, t test) and an increase in lineage-traced sustentacular cells (p < 0.05, t test) when compared with control *Eed*$^{fl/WT}$ mice (n = 5) (Figures 3C–3E). Olfactory epithelial cell-type identity was readily determined based

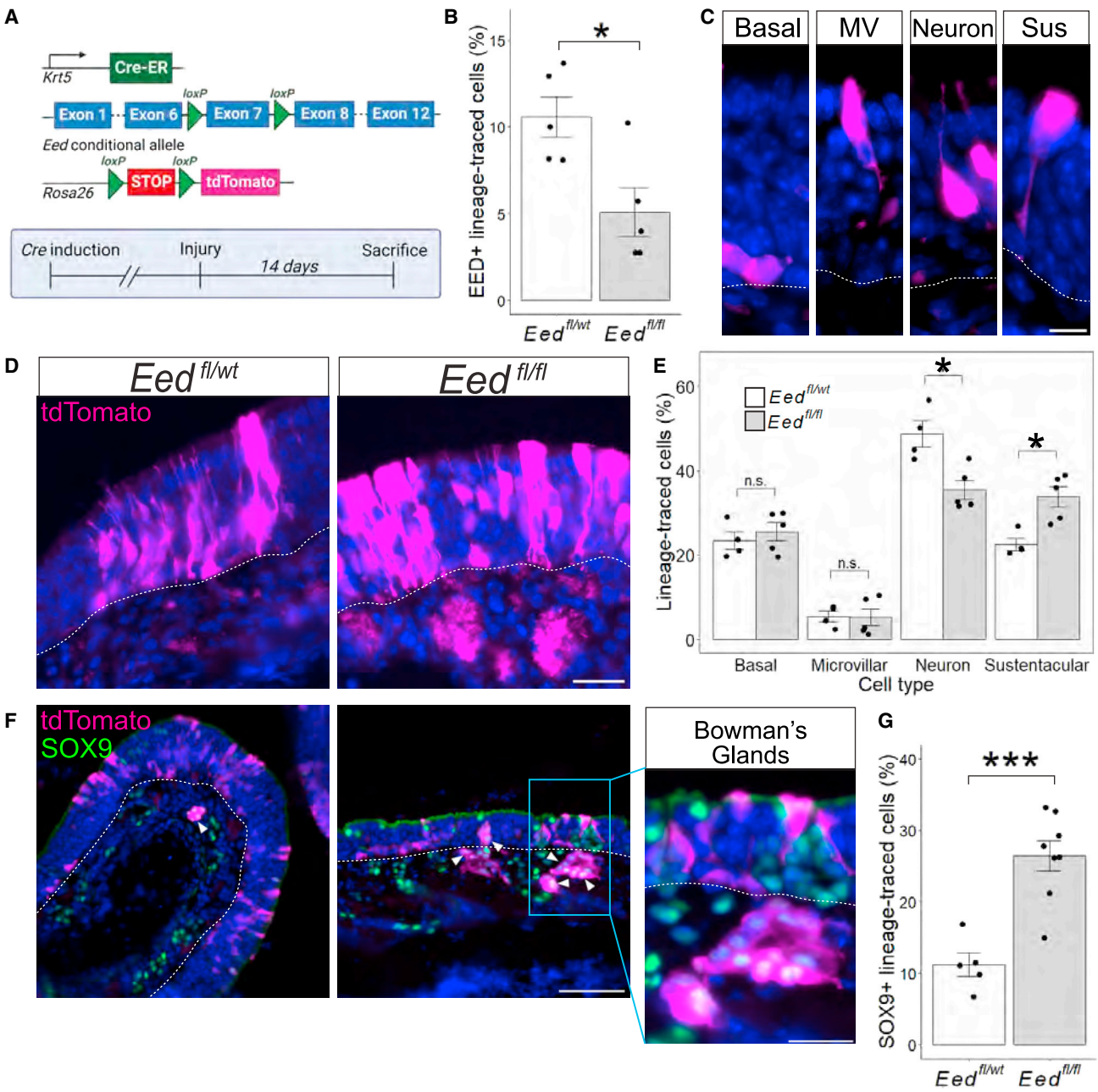



**Figure 3. Conditional deletion of *Eed* in olfactory basal cells impairs neurogenesis and increases generation of basal cell-derived Bowman's glands**

(A) Schematic of conditional deletion of *Eed* and timeline for *Cre* induction, injury, and sacrifice. *Cre* induction occurred 5–7 days prior to Mx injury. Mice were sacrificed 14 days post-Mx.

(B) Quantification of EED$^{+}$/tdTomato$^{+}$ olfactory cells as a percentage of total tdTomato$^{+}$ cells following single tamoxifen injection in *Eed*$^{fl/WT}$ and *Eed*$^{fl/fl}$ mice (n = 5 for each group) (t test, *p < 0.05).

(C) Example of cell-type-specific identifying morphology for basal cells, microvillar (MV) cells, neurons, and sustentacular (Sus) cells; bar: 10 μm.

(D) Representative images of tdTomato$^{+}$ lineage-traced cells. Bar: 25 μm.

(E) Quantification of cell types in *Eed*$^{fl/WT}$ and *Eed*$^{fl/fl}$ mice as a percentage of total tdTomato$^{+}$ cells (*Eed*$^{fl/WT}$ n = 4, *Eed*$^{fl/fl}$ n = 5) (t test, Benjamini-Hochberg correction, *p < 0.05).

(F) Representative images of immunofluorescent staining of SOX9$^{+}$/tdTomato$^{+}$ Bowman's glands cells; bar: 75 μm. Inset bar: 25 μm.

(G) Quantification of SOX9$^{+}$/tdTomato$^{+}$ cells as a percentage of total tdTomato$^{+}$ cells (*Eed*$^{fl/WT}$ n = 5, *Eed*$^{fl/fl}$ n = 8) (t test, ***p < 0.001). In graphs (B), (E), and (G), points represent data from individual mice, and bars represent mean ± SEM. Dashed lines indicate basal lamina.

on morphology and position of tdTomato$^+$ cells, verified by co-staining with appropriate cell-type-specific markers, including anti-CBX8, which specifically labels olfactory sensory neuron (OSN) nuclei (Goldstein et al., 2018; Shirazyan et al., 2023) (Figures 3C and S2I–S2K): basal cells deep in the epithelium lack cell processes with CBX8 (−) nuclei; microvillar cells have an apical cell body that narrows toward the epithelial surface with CBX8 (−) nuclei; neurons are CBX8$^+$ and have a rounded soma and bipolar neurites; and CBX8 (−) sustentacular cells have a larger apical cell body with rectangular shape and a thin basal process (see Figure S3 for quantification of non-neuronal morphologies). Other cell-type-specific markers were also used to confirm lineage-traced cell identities, such as TUJ1 and OMP for immature and mature neurons, respectively (Figures S2B and S2C).

PRC2 loss might drive the observed phenotype via several possible mechanisms: (1) due to decreased proliferation by GBC transit amplifying neural precursors; (2) due to increased cell death in maturing olfactory neurons; or (3) due to a shift in cell fate, biasing differentiation toward non-neuronal cell types. To explore these possibilities, we quantified markers of proliferation and cell death in *Eed*$^{fl/fl}$ and control mice (Figures S2E–S2H). There was no difference in expression of activated caspase-3 label in the lineage-traced cells between *Eed*$^{fl/fl}$ (n = 6) and *Eed*$^{fl/WT}$ mice (n = 4), suggesting that observed findings are not explained by neuronal death (Figures S2E and S2G). Quantification of proliferative lineage-traced GBCs by Ki67 staining was also not different in *Eed*$^{fl/fl}$ (n = 4) and *Eed*$^{fl/WT}$ (n = 5) mice (Figures S2F and S2H). These results are consistent with a mechanism involving a shift in GBC differentiation in *Eed*$^{fl/fl}$ mice driving the observed decrease in lineage-traced neurons and the increase in lineage-traced non-neural cells.

Of particular interest, we also identified a striking increase in tdTomato$^+$ Bowman's glands in *Eed*$^{fl/fl}$ mice (Figures 3F and 3G). To ensure that reporter-labeled cells in the lamina propria beneath the OE were glands, we co-stained for the gland-specific marker SOX9 and quantified SOX9$^+$/tdTomato$^+$ cells from control (n = 5) and mutant mice (n = 8) ($p < 0.001$, t test). Lineage-traced glands also expressed SOX10, another gland-specific transcription factor (Figure S2D). Although prior lineage-tracing studies have identified a lineage relationship between basal cells and Bowman's glands (Goldstein et al., 2015; Goss et al., 2016; Leung et al., 2007), mechanisms regulating the gland versus neuron cell-fate decision in multipotent GBCs have not been identified previously.

## Polycomb targets in GBCs include genes active in neurogenesis and differentiation

We next mapped genome-wide Polycomb target genes by chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq) from purified WT (n = 5) GBCs, the population of active olfactory progenitor cells. Using the well-characterized mouse methimazole lesion model and an established immunoselection protocol based on antibody to c-KIT, GBCs were purified from olfactory mucosa harvested at 7–10 days post-lesion, when this cell population is actively expanding and producing new cells to reconstitute the epithelium (Bergman et al., 2002; Goldstein et al., 2015, 2016). GBC regulation must balance self-renewal, differentiation, and cell-fate decisions, as these cells can produce neurons, microvillar cells, sustentacular cells, or glands (from "upstream" SOX2$^+$ GBCs, or activated HBCs) or can act as amplifying progenitors (Goss et al., 2016; Schwob et al., 2017). As such, chromatin modulation is likely to help organize expression patterns for key transcription factors involved in renewal or differentiation for the highly dynamic GBCs. Histone mark ChIP for the repressive H3K27me3 mark (deposited by EZH2) or the activating H3K27ac mark (Figures 4A and S4A) identified 3,099 genes marked solely by H3K27me3, 7,458 marked by solely H3K27ac, and 7,190 bivalently marked by both H3K27me3 and H3K27ac.

To confirm that conditional knockout of *Eed* in the regenerating OE disrupts PRC2-mediated histone modifications, we also performed ChIP-seq identically using GBCs purified from *Krt5-CreER$^{T2}$; Eed$^{fl/fl}$; R26R Ai9* mice (n = 10 biological replicates, n = 2 technical replicates) recovering from methimazole lesion. We observed a decrease in overall H3K27me3 density near transcriptional start sites (Figure 4B) when compared with WT GBCs. Focusing on key transcription factors marking specific non-neuronal lineages in the OE, including *Sox9* (a Bowman's gland transcription factor), *Pou2f3* (a tuft-like microvillar cell transcription factor), and *Pax6* (a sustentacular cell transcription factor), we confirmed a loss of H3K27me3 in GBCs from *Eed* conditional knockout mice (Figures 4C–4E).

Gene Ontology (GO) analysis of H3K27me3 marked genes revealed enrichment for "synaptic signaling" and "neuron fate commitment" (Figure 4F), and included 139 transcription factors (Table S2), including *Ascl1*, a pro-neural olfactory bHLH gene (Cau et al., 1997; Gordon et al., 1995). In contrast, genes marked only by H3K27ac were highly enriched for terms including "regulation of cell cycle" and "chromosome organization" (Figure S4E). These genes included stem cell potency factors such as *Klf4* and cell-cycle-associated genes such as *Pcna* (Figures S4C and S4D). The genome-wide pattern of histone marks identified in GBCs likely reflects potential for PRC2 to contribute to regulation of both neuronal-lineage identity and cellular renewal. Of particular interest, bivalently marked genes included several transcription factors restricted to specific lineages in the OE. These included *Ebf1*, a well-studied transcription factor

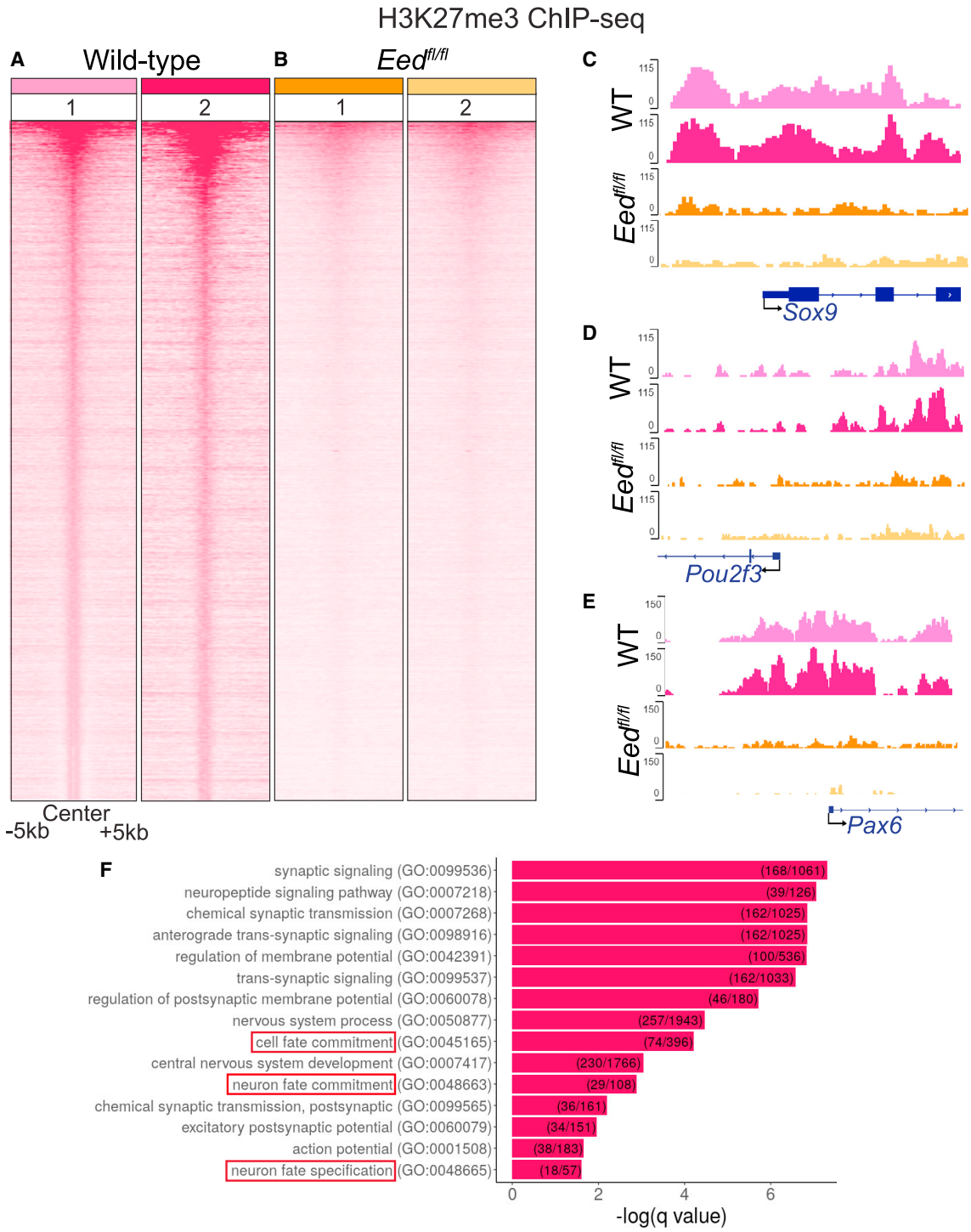


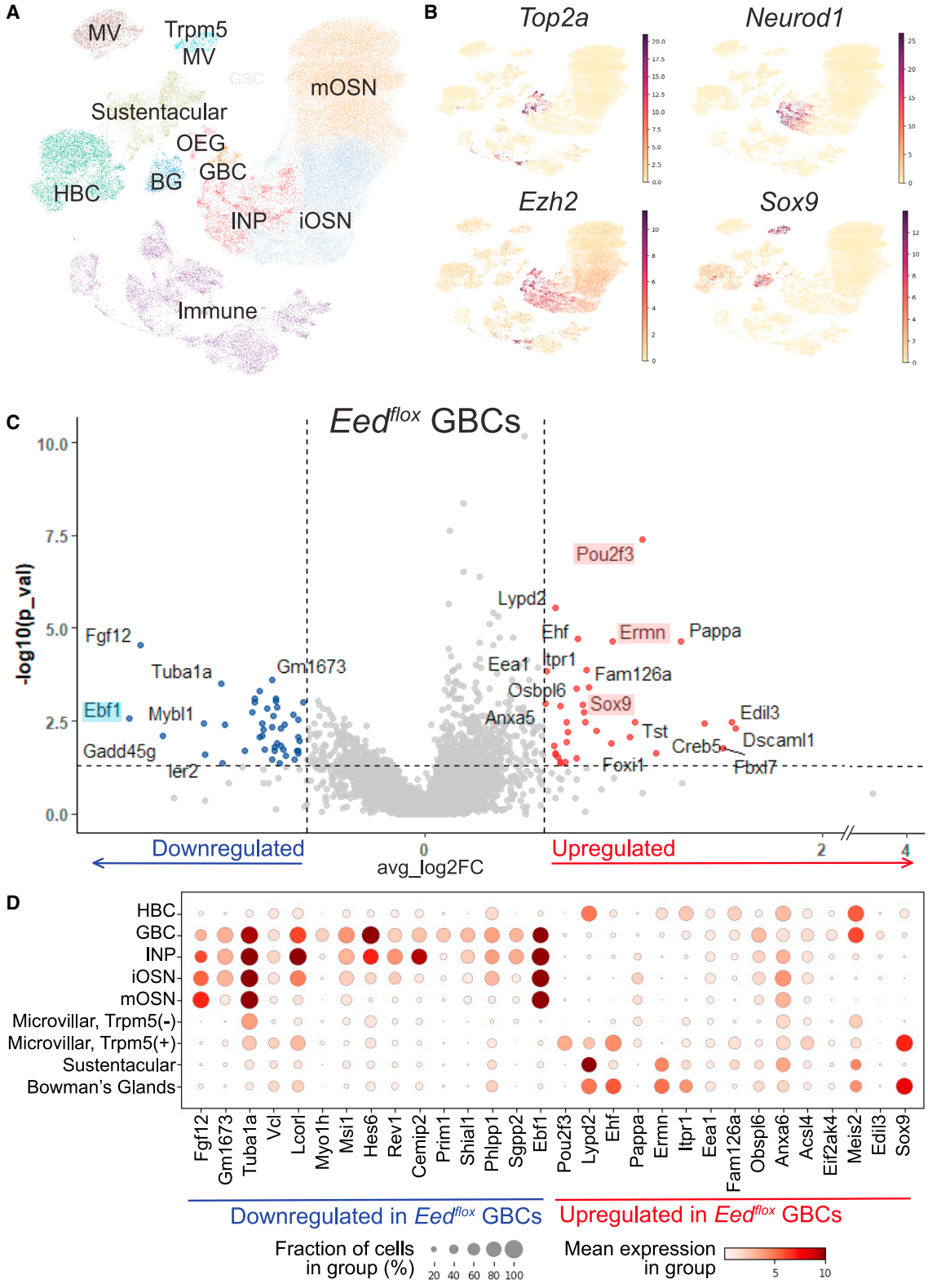

**Figure 4. Conditional deletion of *Eed* in olfactory basal cells depletes genome-wide H3K27me3 marks**

PRC2 target genes in acutely dissociated c-KIT$^{+}$ mouse GBCs were assessed using anti-H3K27me3 ChIP-seq.

(A and B) Heatmap of ChIP-seq read densities ±5 kb from peak centers (n = 2 technical replicates for each group; WT n = 5, *Eed*$^{fl/fl}$ n = 10 biological replicates).

(C–E) Genome browser views of *Sox9*, *Pou2f3*, and *Pax6*.

(F) Top significant terms from GO analysis of WT H3K27me3 ChIP-seq peaks. For *Eed*$^{fl/fl}$ mice, Mx injury occurred 5–7 days following tamoxifen induction. Globose basal cells were harvested 7–10 days post-Mx injury.

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known to activate olfactory neuron-specific genes, as well as the gland and microvillar factors *Sox9* and *Pou2f3* (Figures 4C–4E and S4) (Durante et al., 2020; Holbrook et al., 2011; Wang et al., 1997, 2004; Yamaguchi et al., 2014). Bivalent marking of key transcription factors in regenerating GBCs likely reflects functional importance, as bivalently marked genes are thought to be "poised," or capable of being rapidly expressed to direct fundamental transcriptional programs (Oguro et al., 2010).

### Loss of PRC2 subunit EED alters gene expression in olfactory basal cells, including de-repression of the Bowman's gland transcription factor *Sox9*

To further understand the observed lineage shift in the $Eed^{fl/fl}$, we performed scRNA-seq on olfactory cells from *Krt5-CreER$^{T2}$; $Eed^{flox}$; R26R Ai9* mice, either $Eed^{fl/fl}$ (n = 3) or $Eed^{fl/WT}$ (n = 3), following tamoxifen induction and methimazole lesion (Figure 5A). In scRNA-seq plots, the GBC population was identified by expression of the common GBC marker genes *NeuroD1* and *Top2a* (Figure 5B). Focusing attention to GBCs, the population first expressing PRC2 during olfactory neurogenesis, we compared mutant versus WT GBCs. We identified differentially expressed (DE) transcripts between lineage-traced (tdTomato$^+$) and control (tdTomato$^-$) GBCs from $Eed^{fl/fl}$ animals (i.e., mutant and WT cells from the same genotype group stratified by the presence or the absence of inducible Cre reporter gene expression) (n = 3) (Figure 5C). Histone marks are present on many loci across the genome (see Figure 4), representing one of many dynamic mechanisms involved in the regulation of gene expression; as such, we expect only a subset of transcripts to be significantly up- or downregulated in our analysis of PRC2-mutant GBCs. Among the most significantly upregulated transcripts in $Eed^{flox}$ GBCs is *Ermn*, a gene normally restricted to sustentacular cells in the OE (Durante et al., 2020). Among the most significantly downregulated transcripts is *Ebf1*, a neuron lineage-specific transcription factor (Davis and Reed, 1996) found to be a bivalently marked PRC2 target in our GBC ChIP-seq (Figures 4C and S4; Table S2). We verified the same pattern in DE analysis between $Eed^{fl/fl}$ (n = 3) and $Eed^{fl/WT}$ (n = 3), lineage-traced tdTomato$^+$ GBCs (Figure S5A), i.e., lineage-traced GBCs compared from mice of different genotypes, and identified GBCs with decreased EBF1 expression in $Eed^{fl/fl}$ mice using immunofluorescence (Figure S5C). Other significantly upregulated transcripts in recombined GBCs from $Eed^{fl/fl}$ mice include *Sox9* and *Pou2f3* (Figure 5C). In normal mouse or human olfactory mucosa, neither *Sox9*, *Ermn*, nor *Pou2f3* expression is observed in GBCs (Durante et al., 2020; Fletcher et al., 2017), so their identification here in $Eed^{flox}$ GBCs reflects a dysregulation of normal transcriptional programs. *Sox9* de-repression is of particular interest, as this was found to be a PRC2 target in GBCs by ChIP-seq and as conditional knockout of *Eed* decreased H3K27me3 marks at the *Sox9* promoter (Figure 4C). Furthermore, SOX9$^+$ fate-mapped Bowman's glands were increased in $Eed^{fl/fl}$ mice, verifying the de-repression of *Sox9* (Figures 3F and 3G). Additionally, transcriptional distance analysis suggests mutant basal cell shifts toward a Bowman's gland or sustentacular phenotype (Figure S5B).

We also visualized transcriptional changes by DotPlot to further define the cell populations normally expressing transcripts identified as significantly upregulated or downregulated in PRC2-mutant GBCs (Figure 5D). Downregulated transcripts normally localize to neuronal lineages, including GBCs, immediate neural precursors (INPs), immature OSNs (iOSNs), and mature OSNs (mOSNs). In contrast, upregulated transcripts localized to non-neuronal populations including sustentacular cells, microvillar cells, or Bowman's glands. Together, these findings are consistent with a model in which PRC2 regulates gene expression controlling olfactory basal cell fate.

## DISCUSSION

While the Polycomb family has been found to play fundamental roles in embryonic development, in cancer stem cells, and in certain renewing adult tissue niches such as skin and bone marrow, a role for Polycomb complexes in adult neurogenesis has not been clearly defined (Boyer et al., 2006; Bracken and Helin, 2009; Chan et al., 2018;

**Figure 5. Dysregulated gene expression in PRC2-mutant olfactory basal cells, including de-repression of the Bowman's gland transcription factor *Sox9***

(A) scRNA-seq of olfactory mucosa cells from of $Eed^{fl/WT}$ and $Eed^{fl/fl}$ 11–12 days post-lesion, visualized by uniform manifold approximation projection (UMAP) with annotation of cell clusters (n = 3 for each group). MV, *Trpm5*$^-$ microvillar cells; Trpm5 MV, *Trpm5*$^+$ microvillar cells; HBC, horizontal basal cell; BG, Bowman's gland; OEG, olfactory ensheathing glia; GBC, globose basal cell; INP, immediate neural progenitor; iOSN, immature olfactory sensory neuron; mOSN, mature OSN.

(B) Gene expression plots for GBC population markers *Top2a* and *Neurod1*, as well as PRC2 subunit *Ezh2* and Bowman's gland marker *Sox9*.

(C) Volcano plot of DE genes comparing $Eed^{fl/fl}$ tdTomato$^+$ GBCs ($Eed^{flox}$) and tdTomato$^-$ GBCs (WT). Red and blue indicate significant changes of >0.6 $\log_2$ fold change, $p < 0.05$.

(D) DotPlot showing in WT olfactory epithelial cell clusters the expression of the significant DE genes from $Eed^{flox}$ GBCs.

Flora et al., 2021a). Here, we focused on identification of PRC2 function in adult olfactory basal cells. Together, protein expression patterns in normal and regenerating OE, chromatin studies, mouse conditional deletion and fate-mapping assays, and scRNA-seq analysis suggest a model in which PRC2 contributes to adult olfactory neurodifferentiation by modulating or permitting expression of lineage-specific transcription factor networks in basal stem cells to influence cell fate.

The OE exhibits a striking capacity to reconstitute the neural population following tissue damage due to the presence of basal stem and progenitor cells within the epithelium. A central problem in self-renewing tissues involves understanding the mechanisms balancing cellular signals driving renewal and maintaining lineage differentiation. In the OE, several approaches have shown that activated reserve HBCs, or subsets of GBCs emerging from them, are multipotent cells with the potential to generate neurons, microvillar sensory cells, sustentacular cells, additional GBCs or HBCs, or Bowman's glands (Gadye et al., 2017; Goldstein et al., 2015; Goss et al., 2016; Huard et al., 1998; Leung et al., 2007). Post-injury, activated olfactory basal stem cells have been shown to divide asymmetrically, contributing progeny to clones consisting of neuronal and non-neuronal cell types (Gadye et al., 2017). Thus, mechanisms regulating cell-fate choice must be active in basal cells during lesion-induced epithelial reconstitution. Polycomb-mediated chromatin modulation provides one mechanism that can orchestrate transcriptional repression or activation to control cell fate.

Indeed, chromatin modification has been found to be a key strategy shaping gene expression and differentiation during development (Boyer et al., 2006; Cao et al., 2005) and, in the OE, appears to help regulate problems such as singular monoallelic olfactory receptor gene expression (Lyons et al., 2013; Magklara et al., 2011). Furthermore, progressive chromatin structure changes are recognized as olfactory neurons mature (Le Gros et al., 2016). The Polycomb gene family acts to modify chromatin with histone marks and to alter chromatin compaction to repress or, in some cases, activate gene expression. In the OE, immunohistochemical labeling for certain histone marks appears to be lineage specific, with the H3K27me3 mark strongly labeling neuronal-lineage cells and the activating H3K27ac mark strongly labeling proliferative basal cells. GO terms from histone mark ChIP-seq targets support this labeling pattern. The presence of H3K27ac histone marks alludes to broad transcriptional activity, with binding localizing to enhancers or super-enhancers at developmentally important genes, often in progenitor cells (Whyte et al., 2013). The neuronal-lineage specificity of the H3K27me3 mark is also of interest: we previously found that the histone methyltransferase EZH2 is expressed in GBCs and that small-molecule inhibition of EZH2 decreased renewal of neuronal-lineage GBC cultures (Goldstein et al., 2016), and subsequently, others reported that intranasal EZH2 inhibitor treatment of lesioned mice may increase sustentacular cell production; however, further mechanistic studies were not provided (Lin et al., 2017). We also find here by immunohistochemistry that the PRC2.2 component JARID2 also co-localizes with core PRC2 expression. By scRNA-seq in the human OE, we identified strong expression of PRC2.2 in INPs and immature olfactory neurons, with weak expression of PRC2.1 transcripts in upstream GBCs (Finlay et al., 2022a). In the adult epidermis, a robust self-renewing tissue, JARID2, plays complex roles in the maintenance of epithelial homeostasis (Mejetta et al., 2011).

Our analysis of a conditional PRC2 deletion targeted to mouse olfactory basal cells uncovered an unrecognized role for PRC2 in regulating gland versus neuron cell fate. Derepression of *Sox9* in PRC2-mutant GBCs is accompanied by a marked increase in lineage-traced Bowman's glands. The relationship of olfactory basal cells to the genesis of both neurons and glands, and the mechanisms regulating this process, warrants further study. Furthermore, ASCL3 is a transcription factor that has been found to have a role in OE microvillar and gland lineages (Weng et al., 2016), and a further dissection of mechanisms driving generation olfactory microvillar cell subpopulations, such as $POU2F3^{+}$ tuft/brush subsets versus $FOXI1^{+}$ ionocyte-like subsets, will be of interest. Clinically, processes such as aging, inflammation, viral-mediated olfactory damage, and other forms of wear and tear can lead to failed neuroepithelial reconstitution and a respiratory-like metaplasia with a glandular or secretory-type epithelium replacing the sensory neuroepithelium (Fitzek et al., 2022; Haglin et al., 2020; Oliva et al., 2022; Paik et al., 1992; Seiden, 2004). Whether and how basal cell exhaustion or dysregulation may contribute to this process is unclear. Furthermore, a rare but aggressive malignancy arising from olfactory mucosa, olfactory neuroblastoma, may contain cells expressing olfactory neuron lineage markers as well as SOX9, and factors driving growth of this tumor remain to be defined (Holbrook et al., 2011). In addition, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the agent causing COVID-19, directly infects olfactory sustentacular cells but has also been shown to enter ducts and acinar units of Bowman's glands (Khan et al., 2021, 2022). The maintenance of olfactory mucus function in health and disease, and whether this may be a potential therapeutic target, is a focus of ongoing study.

The lifelong presence of an active neurogenic niche in the olfactory mucosa of rodents or humans necessitates highly complex regulatory mechanisms to maintain epithelial homeostasis. The expression pattern of PRC2 subunits in olfactory basal cells, and our identification of PRC2-dependent mechanisms promoting sensory neuronal differentiation,

provides new insights into some of the mechanisms acting to regulate this highly dynamic neuroepithelium. The development of new treatments for conditions that impair the maintenance of an intact or properly functional olfactory organ, such as viral-induced damage or aging-related changes, will require an understanding of these regulatory mechanisms.

## EXPERIMENTAL PROCEDURES

### Resource availability

#### *Corresponding author*

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Bradley J. Goldstein (bjg47@duke.edu).

#### *Materials availability*

This study did not generate new unique reagents.

#### *Data and code availability*

The accession number for the scRNA-seq and ChIP-seq data reported in this paper is GEO: GSE224894. Code is available on the GitHub repository Goldstein-Lab/Ko_PRC2_olfactory_neurogenesis.

### Animals

All procedures were approved by the Duke University Institutional Animal Care and Use Committee (IACUC) and/or the University of Miami IACUC. For Cre induction, adult mice were injected with tamoxifen intraperitoneally. Methimazole lesion was induced by a single intraperitoneal injection into adult mice. Experiments included both male and female animals. For more details, see the supplemental experimental procedures.

### Cell counts from immunofluorescence

The ImageJ Cell Counts tool was used to quantify cells positive for markers of interest (Table S1) and cells exhibiting cell-type-specific morphologies. Counts were normalized to the length of olfactory epithelium and to $DAPI^+$ nuclei. Olfactory epithelial cell types were identified based on morphology, laminar position of $tdTomato^+$ reporter-labeled cells, and presence of cell-type-specific marker expression, as described in the results (see also Figures S2B, S2C, and S3). For further details, see the supplemental experimental procedures.

### ChIP-seq

ChIP was performed using the ChIP-IT High Sensitivity Kit (Active Motif) according to manufacturer's instructions on GBCs immunomagnetically purified from mouse olfactory tissue. See the supplemental experimental procedures for details.

### ChIP-seq analysis

ChIP-seq data were processed by removing low-quality bases and Illumina sequencing adapters. Reads were mapped to the GRCm38 mouse genome assembly (mm10), and amplification artifacts were removed. Regions of enrichment were called using MACS2 peak calling. See the supplemental experimental procedures for more details.

### scRNA-seq

scRNA-seq on mouse olfactory epithelium was performed using the Chromium controller (10× Genomics) for cell capture and barcoding per manufacturer recommendations (3′ Gene Expression, v.3.1). Reverse transcription, cDNA amplification, library construction, and sequencing were performed per the manufacturer's instructions. For details, see the supplemental experimental procedures.

### scRNA-seq analysis

CellRanger was used for BCL-to-FASTQ conversion and to create gene count matrices. Raw cell-by-gene count matrices were preprocessed and integrated with Scanpy and scvi-tools. The scvi-tools pipeline (Gayoso et al., 2022) was adapted from previous olfactory epithelial scRNA-seq analyses (Brann et al., 2020; Finlay et al., 2022b; Tsukahara et al., 2021). For details, see the supplemental experimental procedures.

### Statistics

All sequencing dataset analyses were conducted in Python, R, or Galaxy with the aforementioned toolkits and packages. Graphs were generated with Scanpy or ggplot2. Immunofluorescence cell count comparisons were performed with unpaired two-tailed t tests or unpaired Wilcoxon rank-sum tests, depending on the normality of the data (verified with the Shapiro-Wilks test). Blinding was performed where feasible. The Benjamini-Hochberg correction was used for multiple comparisons to control the false discovery rate, and significance was defined as $p < 0.05$. All error bars represent the standard error of the mean. GO analyses were performed using the ToppGene suite with a Bonferroni correction, and significance was defined as $p < 0.05$.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2023.09.008.


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## AUTHOR CONTRIBUTIONS

T.K., R.C., and B.J.G. designed the experiments and wrote the paper; T.K., R.G., K.I., E.L., and R.C. performed experiments; T.K., L.M., and J.B.F. analyzed and interpreted data; and T.K., R.C., L.M., and B.J.G. reviewed and edited manuscript.

## DECLARATION OF INTERESTS

B.J.G. has interest in Rhino Therapeutics.


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