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Polycomb subunits Ezh1 and Ezh2 regulate the Merkel cell differentiation program in skin stem cells

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While the Polycomb complex is known to regulate cell identity in ES cells, its role in controlling tissue-specific stem cells is not well understood. Here we show that removal of Ezh1 and Ezh2, key Polycomb subunits, from mouse skin results in a marked change in fate determination in epidermal progenitor cells, leading to an increase in the number of lineage-committed Merkel cells, a specialized subtype of skin cells involved in mechanotransduction. By dissecting the genetic mechanism, we showed that the Polycomb complex restricts differentiation of epidermal progenitor cells by repressing the transcription factor Sox2. Ablation of *Sox2* results in a dramatic loss of Merkel cells, indicating that Sox2 is a critical regulator of Merkel cell specification. We show that Sox2 directly activates *Atoh1*, the obligate regulator of Merkel cell differentiation. Concordantly, ablation of *Sox2* attenuated the *Ezh1/2*-null phenotype, confirming the importance of Polycomb-mediated repression of Sox2 in maintaining the epidermal progenitor cell state. Together, these findings define a novel regulatory network by which the Polycomb complex maintains the progenitor cell state and governs differentiation *in vivo*.

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Introduction

There is increasing evidence that chromatin structure and epigenetic regulation of gene expression play a fundamental role in the control of stem cells, development, and tissue homeostasis. One of the earliest described chromatin regulators is the Polycomb repressive complex (PRC), which establishes and maintains gene repression. PRC activity is divided between two multi-subunit complexes that work sequentially: first, PRC2 is recruited to chromatin, where it catalyses trimethylation of lysine 27 on histone H3 (H3K27me3) through the activity of Ezh1 and Ezh2 histone methyltransferases (Cao *et al*, 2002; Shen *et al*, 2008). This histone mark then allows the second complex, PRC1, to be recruited, where it assists in chromatin compaction and gene silencing (Min *et al*, 2003; Zhang *et al*, 2012). Misregulations of Polycomb function have been associated with diverse human pathologies, including cancer (Sauvageau and Sauvageau, 2010; Delgado-Olguin *et al*, 2012).

Most mammalian studies of the role of the Polycomb complex in control of stem cells and development to date have been done *in vitro* with either embryonic stem cells or progenitor cells, but the roles of Polycomb in regulating tissue-specific stem cells and governing organogenesis remain poorly understood (Caretti *et al*, 2004; Benoit *et al*, 2012; Sher *et al*, 2012). Importantly, profiling of the association of Polycomb with genomic regions in many stem cell systems identified its presence at a large set of differentiation genes (Boyer *et al*, 2006; Lee *et al*, 2006), suggesting a model wherein this complex represses differentiation. Published functional studies, however, have so far failed to support this model. Indeed, in many systems, Polycomb-null phenotypes were linked to activation of the *Ink4a/Ink4b/Arf* locus (Bracken *et al*, 2007) leading to loss of cell proliferation rather than aberrant differentiation (Molofsky *et al*, 2003; Park *et al*, 2003; Martinez and Cavalli, 2006; Chen *et al*, 2009). In skin, loss of Ezh1/2 also results in an upregulation of the *Ink4a/Ink4b/Arf* locus, leading to loss of hair follicle stem cell proliferation and ultimately degeneration of the hair follicles (Ezhkova *et al*, 2011). Thus, the importance of Polycomb-mediated repression and the gene regulatory networks involved in controlling stem cell differentiation *in vivo* need to be investigated.

Skin has proven to be an excellent model system to study the mechanisms controlling stem cell self-renewal and differentiation (Zhang *et al*, 2012). During embryonic development, a single layer of multipotent embryonic epidermal stem cells that reside in the basal layer produce multiple lineages, including the epidermis that provides barrier function, hair follicles that provide thermal protection, and Merkel cells that are involved in mechanotransduction (Blanpain and Fuchs, 2009; Mascre *et al*, 2012). While the mechanisms controlling hair follicle and epidermal development are well studied (Blanpain and Fuchs, 2009), the mechanisms controlling Merkel cell specification are largely unknown.

Merkel cells were described over a century ago (Merkel, 1875) as clusters of cells located in touch-sensitive areas of the skin, where they transduce mechanical stimuli

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via sensory neurons to aid in the perception of curvature, texture, and shape of objects (Haeberle and Lumpkin, 2008). Consistent with this function, Merkel cells express voltage-gated ion channels, neuropeptides, components of the presynaptic machinery such as Rab3c, and are innervated by sensory neurons; this is surprising, however, considering the epithelial origin of these cells (Maricich *et al*, 2009; Morrison *et al*, 2009; Van Keymeulen *et al*, 2009; Woo *et al*, 2010). The intermediate filament cytokeratins 18 and 20 (K18 and K20) are often used as a tool for the analysis and diagnosis of Merkel cell carcinoma due to their highly specific expression in Merkel cells (Houben *et al*, 2010; Donepudi *et al*, 2012). Furthermore, a variety of transcription factors involved in neuronal differentiation, such as *Sox2* and *Isl1* (Haeberle *et al*, 2004), are also found in Merkel cells, though how these factors control Merkel cell lineage specification is unknown. It has been shown that in mice, Merkel cell lineage development depends on the basic helix–loop–helix transcription factor *Atoh1* (Maricich *et al*, 2009), but despite the importance of these cells, and the previous determination of the Merkel cell signature (Haeberle *et al*, 2004), little is known about the mechanism orchestrating their development.

In this report, we provide evidence that Ezh1 and Ezh2 repress Merkel cell lineage differentiation in epidermal stem cells. We show that conditional ablation of Ezh1 and Ezh2 in mouse skin results in an increase in the number of Merkel cells due to increased differentiation of progenitor cells. We delineate the molecular pathway through which the Polycomb complex controls Merkel cell specification, and show that the PRC-dependent H3K27me3 histone mark directly targets and represses Sox2, which we posit as a novel regulator of Merkel cell lineage specification. Finally, we show that ablation of *Sox2* in Ezh1/2 2KO skin attenuates the Polycomb loss-of-function phenotype, confirming the critical role of the Ezh1/2 repression of *Sox2* in maintaining the epidermal stem cell state. Through these experiments, we have not only elucidated the molecular pathway that controls Merkel cell differentiation, but have shown that the Polycomb complex can act as a specific lineage regulator through repression of transcription factor networks in a mammalian stem cell system.

Results

Loss of Ezh1/2 leads to expansion of Merkel cells

Analysis of transcriptional profiling data of genes expressed in Merkel cells revealed that several components of the Polycomb complex (Ezh2, Eed, Pcgf2) are expressed in skin epithelium but are downregulated in Merkel cells (Haeberle *et al*, 2004). To explore the role of the Polycomb complex in controlling the Merkel cell lineage, we analysed the Merkel cell population in Ezh1/2 2KO mice (Ezhkova *et al*, 2011), where key subunits of the Polycomb complex, histone methyltransferases Ezh1 and Ezh2, were conditionally ablated in the skin epithelium. Immunofluorescent (IF) studies with antibodies against the well-characterized Merkel cell markers, cytokeratin 18 and 20 (K18 and K20), revealed an increase in the number of K18(+) and K20(+) cells in newborn (P0) and adult (P90) Ezh1/2 2KO back skin compared to WT (Figure 1A and B and Supplementary Figure 1A). Increase in the number of K18(+) or K20(+) cells was also found in the skin of the ventral, paw, and whisker interfollicular epidermis (IFE), tissues that normally contain Merkel cells (Supplementary Figure 1B–D, quantified in F). In whisker follicles, Merkel cells are about 10-fold more abundant in comparison to the other analysed zones, and interestingly, increase in Merkel cell number was not observed in Ezh1/2 2KO mice in the whiskers area (Supplementary Figure 1E and F). We speculate that the whisker follicle region has reached a maximum density in WT animals, and thus loss of Ezh1/2 has little consequence there. As expected, at P0, all Merkel cells co-express K18 and K20 (Figure 1C) and in accordance with previous reports (Maricich *et al*, 2009; Morrison *et al*, 2009; Van Keymeulen *et al*, 2009; Woo *et al*, 2010), they lose expression of epidermal progenitor marker K5 (Figure 1D). Merkel cells have been suggested to form near guard hair follicles, but histological analysis indicated no difference in the number of guard hair follicles present in Ezh1/2 2KO skin compared to WT (Supplementary Figure 1G), discounting the possibility that the observed phenotype was due to alterations in guard hair number.

To carry out their mechanosensory functions, Merkel cells are innervated by sensory neurons and express neuronal proteins. IF analysis showed the presence of Rab3c, a component of the presynaptic machinery (Fischer von Mollard *et al*, 1994; Haeberle *et al*, 2004), in Ezh1/2 2KO K18(+) cells (Figure 1E) and innervation by sensory neurons, as shown by IF staining with antibodies to the neurofilament protein NF200 (Figure 1F). Interestingly, the percentage of innervated K18(+) cells in WT and Ezh1/2 2KO skins was comparable (Figure 1F, percentages), indicating that the additional K18(+) cells formed in Ezh1/2 2KO skin become innervated successfully. Finally, transmission electron microscopy (TEM) confirmed the presence of additional Merkel cells in Ezh1/2 2KO skin, as detected by the presence of characteristic neuroendocrine granules (Tachibana *et al*, 1983); (Figure 1G) and corroborated by quantifications (Figure 1G, right). Overall, these data indicate that loss of Ezh1/2 leads to an increase in the number of fully differentiated Merkel cells.

Ezh1 and Ezh2 repress Merkel cell differentiation

We next explored the origin of Merkel cells in Ezh1/2 2KO skin. We have previously reported that in K14-Cre Ezh1/2 2KO mice, epithelial cells are *Ezh1/2* null and thus completely lack the H3K27me3 histone mark (Ezhkova *et al*, 2011), whereas cells of non-epithelial origins retain this mark. By examining the pattern of H3K27me3, we found that K18(+) cells in Ezh1/2 2KO skin also lacked H3K27me3 (Figure 2A), whereas mesenchymal dermal cells and neural crest-derived melanocytes remained H3K27me3 positive (Figure 2A and Supplementary Figure 2A). To further confirm the epithelial origin of K18(+) cells in Ezh1/2 2KO skin, we crossed WT and Ezh1/2 2KO mice with K14-GFP-H2B mice (Tumbar *et al*, 2004), where histone H2B is tagged with GFP and is expressed under the keratin 14 (K14) promoter that is active only in epidermal progenitor cells. IF analysis of K18(+) cells in WT and Ezh1/2 2KO mice showed that they were GFP positive (Figure 2B), whereas dermal or melanocyte cells were GFP negative (Figure 2B, and Supplementary Figure 2B and C). The GFP level in WT and Ezh1/2 2KO K18(+) cells was significantly lower than in the

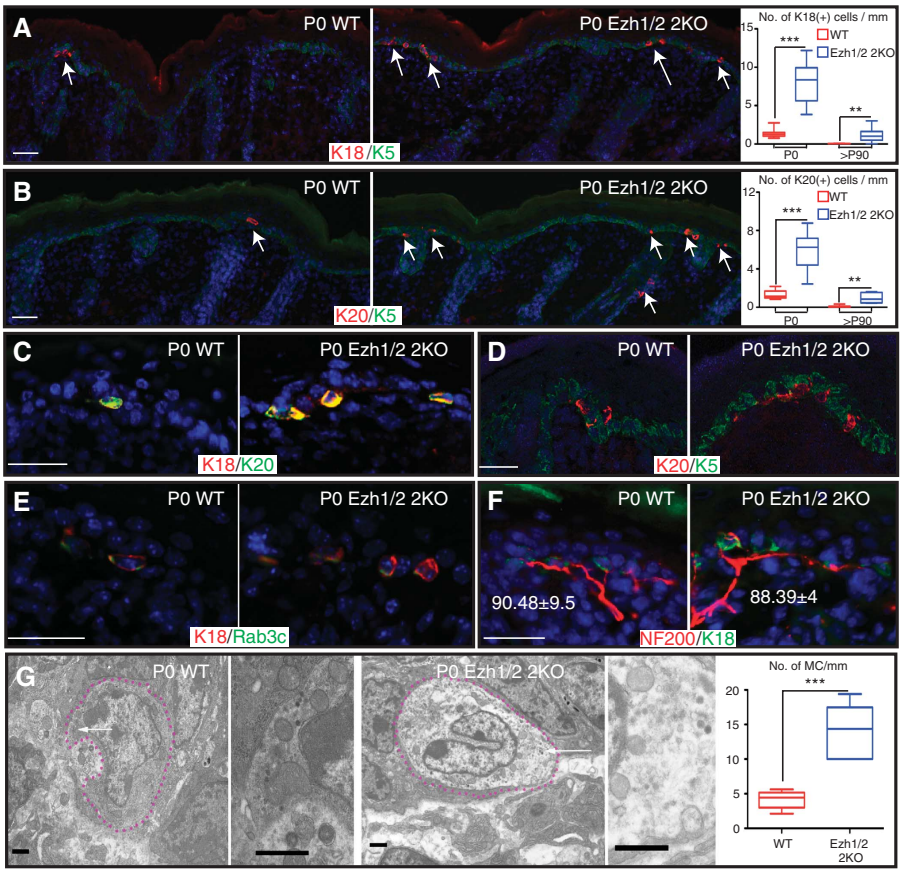

Figure 1 Number of Merkel cells is increased in Ezh1/2 2KO epidermis. (**A**, **B**) Immunofluorescent (IF) staining of WT and Ezh1/2 2KO skin with antibodies against Merkel markers K18 (**A**) or K20 (**B**) shows increase in the number of K18(+) and K20(+) cells. Arrows indicate positively stained K18(+) and K20(+) cells. Skins counterstained with basal layer marker K5. Quantifications are provided at the right and show statistically significant increase in the number of Merkel cells (P0 WT/2KO K18 $P<0.0001$, $n=127/150$ mm, 310/1285 cells; P90 WT/2KO K18 $P=0.0026$, $n=84/83$ mm, 2/95 cells; P0 WT/2KO K20 $P=0.0003$, $n=72/52$ mm, 94/298 cells; P90 WT/2KO K20 $P=0.0017$, $n=81/39$mm, 5/33 cells). (**C**) IF studies with K18 and K20 antibodies show overlap of the two markers. (**D**) Confocal analysis of K20-labeled cells showing lack of overlap with anti-K5-labeled cells. (**E**, **F**) IF studies show overlap of K18 with Merkel cell protein Rab3c (**E**), and innervation of K18(+) cells by neurons, as labeled by NF200 (**F**). Numbers in (**F**) show similar levels of innervation in WT and Ezh1/2 2KO skin (WT/2KO $P=0.8497$, $n=78/220$ cells). (**G**) TEM imaging shows fully differentiated Merkel cells in the whisker area of P0 WT and Ezh1/2 2KO mice. Dotted lines outline Merkel cells. Arrows indicate characteristic dense core neuroendocrine granules, magnified at the right. TEM quantifications are shown at the right (WT/2KO $P=0.0009$, $n=9/8.7$ mm, 37/120 cells) and confirm increase in the number of Merkel cells in Ezh1/2 2KO skin. Scale bars for (**A**–**F**) are 50 μm. Scale bars for (**G**) are 1 μm.

K14-expressing basal epidermal cells, but comparable to the level seen in K14-derived suprabasal cells (Figure 2B, right). Together, these data indicate that Ezh1/2 2KO Merkel cells are indeed K14 derived and therefore epithelial in origin, consistent with previous reports (Morrison *et al*, 2009; Van Keymeulen *et al*, 2009; Woo *et al*, 2010).

It has been shown that Merkel cells are postmitotic under normal conditions (Woo *et al*, 2010). To test whether increase in the number of *Ezh1/2*-null Merkel cells is due to their aberrant proliferation, we analysed the proliferation marker Ki67 (S + M-phase cells) and observed that, as in WT, Ezh1/2 2KO K18(+) cells were Ki67(−) both at E16 and at P0 (Figure 2C and D, respectively, and Supplementary Figure 2H). Furthermore, 4-h pulses of 5-bromo-2′-deoxyuridine (BrdU) to WT and Ezh1/2 2KO mice at E16, P0, and P14 showed that Merkel cells were BrdU negative (Supplementary Figure 2D–G), further indicating that these cells are non-proliferative. These results thus show that loss of Ezh1 and Ezh2 does not affect the proliferative status of Merkel cells and that they remain postmitotic.

Since Merkel cells showed no proliferation defects, we next analysed if differentiation of *Ezh1/2*-null progenitors to Merkel cells might be enhanced. Remarkably, K18(+) and K20(+) Merkel cells were precociously acquired in Ezh1/2 2KO skin at E16 as compared with their WT littermates, where appearance of fully differentiated Merkel cells was observed only by E17 (Figures 2E, F and 3E) in accordance with previous reports (Van Keymeulen *et al*, 2009). This suggests that the commitment of epidermal stem cells to the Merkel cell lineage is accelerated in the Ezh1/2 2KO. In hairy portions of mouse skin, Merkel cells appear in clusters referred to as touch domes (Woo *et al*, 2010), which are complexes of Merkel cells and neurons located around the guard hairs. However, in Ezh1/2 2KO skin, Merkel cell clusters form around different hair types, and can be seen in the IFE (Figure 1A and B). Our analysis of the size of these clusters, by number of Merkel cells per cluster, did not reveal significant differences between WT and Ezh1/2 2KO skin, but strikingly, we observed an increase in the number of distinct clusters in *Ezh1/2*-null skin compared to WT (Supplementary Figure 2I). Overall, these data suggest that

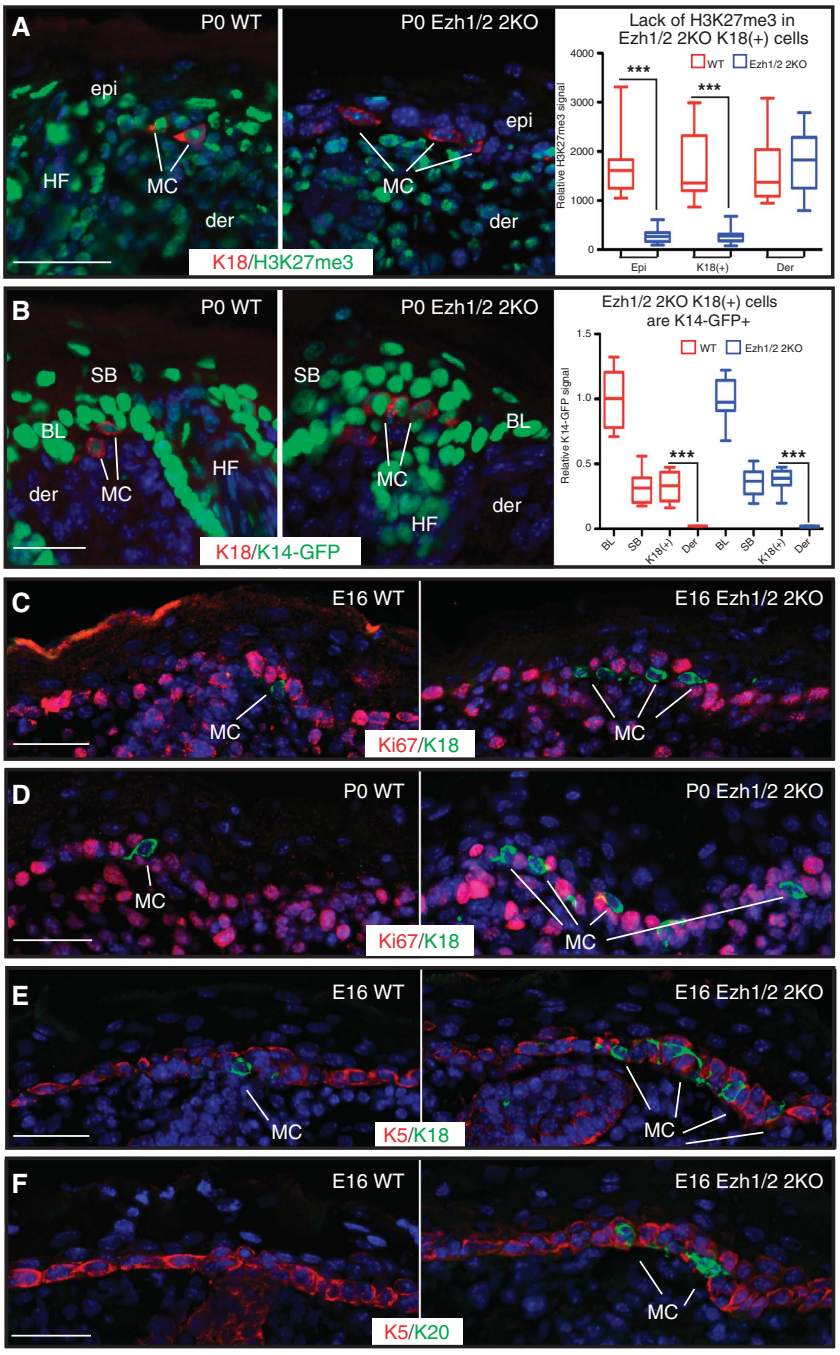

Figure 2 Loss of Ezh1/2 leads to accelerated differentiation of epidermal progenitors to Merkel cells. (**A**) IF studies of P0 WT and Ezh1/2 2KO skin using antibodies against K18 and H3K27me3 show that K18(+) Merkel cells lack H3K27me3 signal. Fluorescence intensity quantification of H3K27me3 signal (arbitrary units) is shown at the right (WT versus 2KO epi $P<0.0001$, $n=20/20$ cells; WT versus 2KO K18 $P<0.0001$, $n=13/14$ cells). (**B**) IF studies of P0 WT and Ezh1/2 2KO mice expressing K14-GFP-H2B show that K18(+) cells are GFP(+). Relative fluorescence intensity quantification of K14-GFP-H2B signal is shown at the right and normalized to the average basal layer signal (WT K18 versus der $P<0.0001$, $n=7$; 2KO K18 versus der $P<0.0001$, $n=6$). (**C**, **D**) IF analysis of E16 (**C**) or P0 (**D**) WT and Ezh1/2 2KO skin using antibodies against K18 and the proliferation marker Ki67 show that Merkel cells are postmitotic. (**E**, **F**) IF studies of E16 WT and Ezh1/2 2KO skin using antibodies against K5 and either K18 (**E**) or K20 (**F**) showing the premature formation of Merkel cells in Ezh1/2 2KO skin. In all panels, epi: epidermis; der: dermis; HF: hair follicle; BL: basal layer; MC: Merkel cell. All scale bars are 50 μm.

loss of Ezh1/2 leads to increased commitment of stem cells to the Merkel lineage, leading to formation of new Merkel cell clusters.

Polycomb represses the Merkel cell gene Sox2 in epidermal stem cells

To gain insight into how loss of Ezh1/2 might lead to the observed phenotype, we analysed genes that were (1) activated in *Ezh1/2*-null epidermal stem cells compared to WT (Ezhkova *et al*, 2011) and (2) expressed in Merkel cells (Haeberle *et al*, 2004). The analysis revealed that 78 Merkel cell signature genes (~10% of total) (Haeberle *et al*, 2004) became activated in Ezh1/2 2KO cells, while they were silenced in WT cells (Figure 3A and Supplementary Table 1). Interestingly, expression of many *bona fide* Merkel cell genes, such as keratin 20 and chromogranin B, were

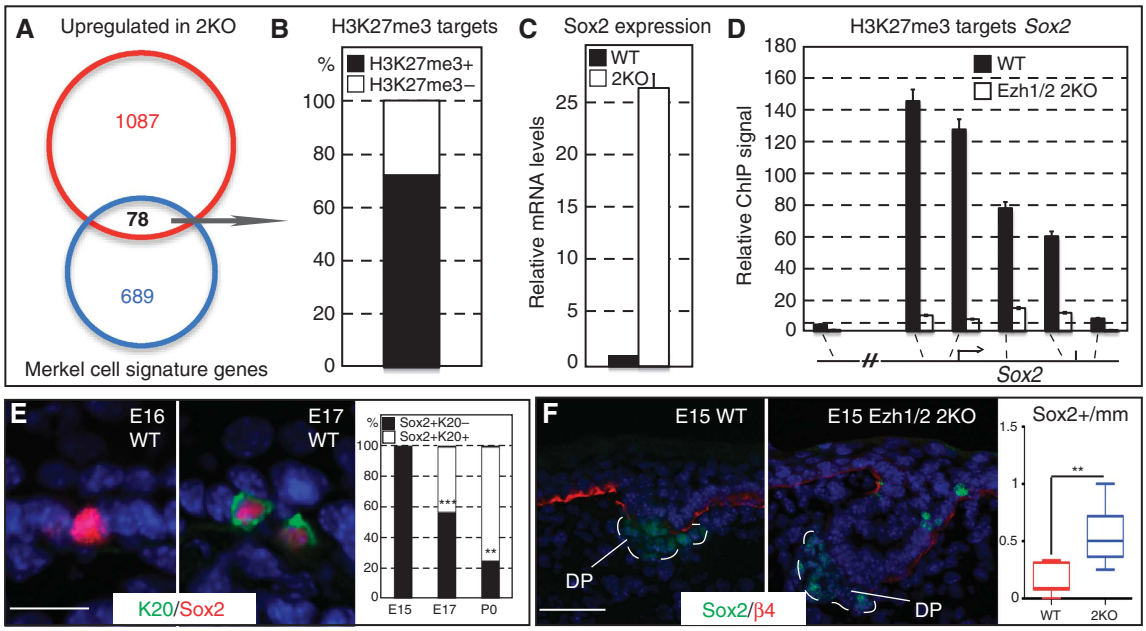

Figure 3 The Merkel signature gene *Sox2* is directly regulated by Ezh1/2-mediated repression in epidermal stem cells. (**A**) Venn diagram of Merkel cell signature genes with genes that are upregulated in Ezh1/2 2KO epidermal progenitors shows overlap of 78 genes. (**B**) Of those 78 genes, 55 (70.5%) are direct targets of H3K27me3 repression, and *Sox2* is among the most upregulated. (**C**) qPCR confirmation of microarray data shows that *Sox2* is 26-fold upregulated in Ezh1/2 2KO progenitors. (**D**) *In vivo* ChIP-qPCR confirmation of ChIP-seq data showing that Sox2 is directly targeted by the Ezh1/2-dependent H3K27me3 histone mark, and that the H3K27me3 ChIP signal is drastically decreased in total skin cells isolated from P0 Ezh1/2 2KO mice. Please note that total skin contained both epithelial and a small fraction of non-epithelial cells (melanocytes, immune cells, etc.) that were not targeted by the K14-Cre-mediated ablation of Ezh1/2. Thus, a small residual level of H3K27me3 at *Sox2* is observed in 2KO cells. (**E**) IF studies of E16 and E17 WT skin using antibodies against K20 and Sox2 reveal that Sox2 is expressed prior to other Merkel cell markers. Quantifications are provided at the right and show statistically significant decrease in the number of Sox2 + K20 − cells throughout development (E15 versus E17 $P = 0.0002$; E17 versus P0 $P = 0.0036$; E15 $n = 109$mm/0 Sox2(+)K20(+) cells/19 Sox2 + K20 − cells; E17 $n = 97/99/151$; P0 $n = 68/74/26$). (**F**) IF analysis of E15 WT and Ezh1/2 2KO skin using antibodies against Sox2 and ß4-integrin shows that Sox2 is expressed prematurely in Ezh1/2 2KO skin. Quantifications are provided at the right and show statistically significant increase in the number of Sox2(+) cells in 2KO versus WT (WT versus 2KO $P = 0.0013$, $n = 90/83$mm, 14/45 cells). All scale bars are 50 μm.

unchanged in 2KO basal cells compared to WT, indicating that only a specific subset of Merkel signature genes becomes activated in Ezh1/2 2KO epidermal stem cells (Supplementary Figure 3A). About two-thirds of these 78 genes were direct targets of the H3K27me3 repressive mark in WT epidermal stem cells (Figure 3B) (Ezhkova *et al*, 2011; Lien *et al*, 2011), indicating the direct role of the Polycomb-mediated repression in their control. Remarkably, the transcription factor Sox2 was one of the most highly upregulated genes (26-fold) of this subset, which we confirmed by semi-quantitative real-time PCR (RT-PCR) analysis (Figure 3C) and by performing IF studies where Sox2 was detectable in Ezh1/2 2KO skin in both K18(+) Merkel cells and K18(−) epidermal stem cells (Supplementary Figure 3C). We also confirmed that *Sox2* is a direct target of Polycomb repression by performing chromatin immunoprecipitation (ChIP) and showed the presence of Polycomb-dependent H3K27me3 histone marks as well as Ezh1 and Ezh2 at the *Sox2* gene (Figure 3D and Supplementary Figure 3B). Notably, IF studies indicated that Sox2 is expressed in the WT skin at an early developmental stage, just prior to expression of the first Merkel cell markers (Figure 3E), as well as precociously expressed in Ezh1/2 2KO epidermis (Figure 3F, quantified at right), suggesting that Sox2 is an early marker of Merkel cell differentiation. Importantly, the percentage of Sox2(+)K20(−) cells decreases consistently between E15 and P0, suggesting that these Sox2(+) cells eventually become fully differentiated Sox2(+)K20(+) Merkel cells (Figure 3E, graph).

Sox2 controls Merkel cell development

We next hypothesized that the loss of Polycomb repression led to activation of Sox2 expression in epidermal stem cells and resulted in increased differentiation of cells to the Merkel lineage. To test this, we first analysed the role of Sox2 in control of the Merkel cell lineage through conditional ablation of *Sox2* in skin epithelium by crossing Sox2 fl/fl mice with K14-Cre mice (Sox2cKO). IF studies confirmed that Sox2 expression was abolished in Sox2cKO epithelium (Figure 4A), but not in dermal papilla cells (Figure 4B) that are not targeted by the K14-Cre ablation strategy (Vasioukhin *et al*, 1999). A dramatic decrease in the number of fully differentiated Merkel cells was observed in Sox2cKO skin, as shown by IF analysis of the Merkel cell markers K18 and K20, as well as by EM (Figure 4C–E). This loss of Merkel cells was observed in all body regions analysed, including paws, whisker IFE, and whisker follicles (Supplementary Figure 4A–D, quantified in E). The observed phenotype persisted into adulthood (Figure 4F), indicating that the observed phenotype is not due to a delay in the development of Merkel cells. Besides the alteration in the Merkel cell number, mice deficient for Sox2 in the skin were born alive at a Mendelian ratio, bred normally, and had no gross pathological abnormalities (Supplementary Figure 4F).

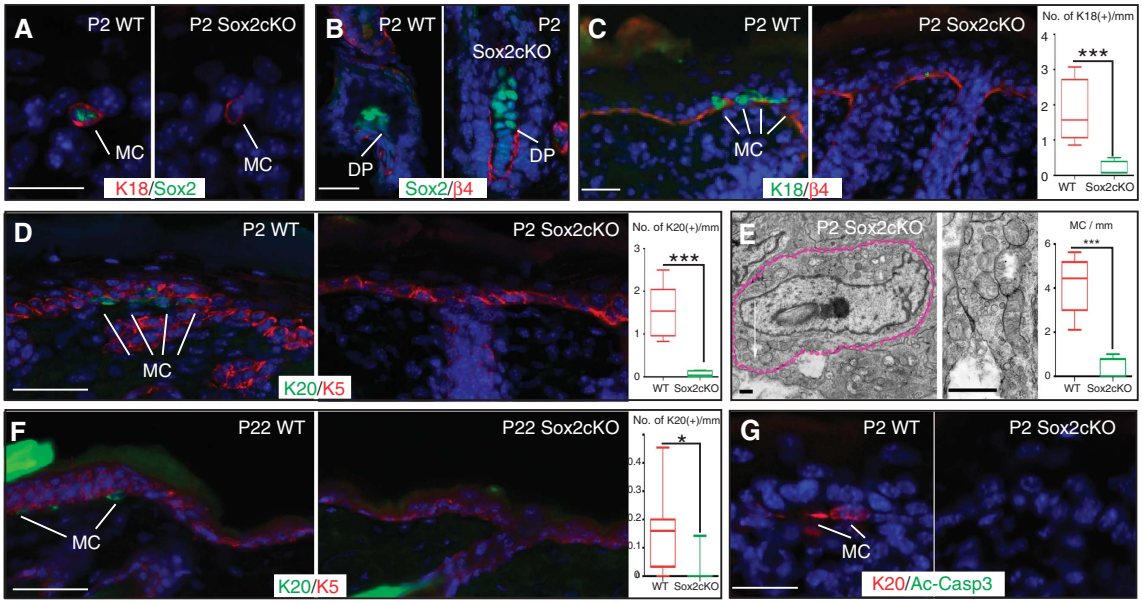

Figure 4 Sox2 is required for proper Merkel cell development. (**A**, **B**) IF analysis using antibodies against Sox2 and either K18 (**A**) or ß4-integrin (**B**) shows loss of Sox2 from Merkel cells but not from the dermal papilla. (**C**, **D**) IF using antibodies against either K18 (**C**) or K20 (**D**) shows dramatic reduction of Merkel cells from Sox2cKO skin. Quantifications are shown at right (WT versus Sox2cKO K20 $P<0.0001$, $n=82/80$mm, 156/16 cells; K18 $P=0.0002$, $n=73/80$mm, 112/5 cells). (**E**) TEM imaging (left) shows that few Merkel cells present are fully developed. Dotted lines outline Merkel cells. Arrows indicate characteristic dense core neuroendocrine granules, magnified at right. TEM quantifications are shown at right and confirm drastic decrease in the number of fully differentiated Merkel cells present in the whisker area of P0 Sox2cKO mice (WT versus Sox2cKO $P=0.0003$, $n=9/9.5$mm, 37/3 cells). (**F**) Decrease in the number of fully differentiated Merkel cells in Sox2cKO persists into adulthood as shown by IF analysis of K20. Quantifications are provided at right (WT versus Sox2cKO $P=0.0163$, $n=99/95$mm 15/2 cells). (**G**) Analysis of apoptosis using antibodies against Act-Casp3 and K20 shows no difference between WT and Sox2cKO skin. All scale bars are 50 μm.

Furthermore, histological and IF analysis of Sox2cKO epidermis showed no apparent defects in epidermal differentiation, hair formation, or skin innervation by NF200(+) neurons (Supplementary Figure 4G–J). Importantly, no increase in apoptosis of Merkel cells was observed in Sox2cKO back skin, paws, or whiskers either prior to Merkel cell specification at E16 or postnatally at P0 and P22, as shown by IF with antibodies to activated-caspase 3 (Figure 4G and Supplementary Figure 4K–S). Overall, the requirement of Sox2 for Merkel cell development positions this gene as a new critical regulator of Merkel cell lineage specification.

Sox2 regulates Atoh1 and Isl1 gene expression

To further characterize the mechanism of how Sox2 controls Merkel cells, we analysed the expression of the *Atoh1* gene, which has been shown to be an obligate transcription factor for Merkel cell differentiation (Maricich *et al*, 2009). We observed a >3-fold decrease in Atoh1 mRNA levels in Sox2cKO skin (Figure 5A). Genome scanning identified a Sox2 binding site at the enhancer region of the *Atoh1* gene, and a direct role for Sox2 in controlling the *Atoh1* gene transcription has been reported in other developmental systems (Yoon *et al*, 2011; Neves *et al*, 2012). To determine if a similar mechanism is operating in Merkel cells, we performed *in vitro* studies on cultured epidermal progenitor cells overexpressing Sox2 (Figure 5B) and observed an ~5000-fold upregulation of Atoh1 mRNA, and a small (~5-fold) increase in K20 mRNA, compared to Control (Figure 5C). To analyse if Sox2 directly binds the *Atoh1* gene, we performed ChIP assay with Sox2 antibodies. Indeed, we found that Sox2 targets the *Atoh1* enhancer region, and that the signal was significantly higher than at the 3′ end of *Atoh1*, which does not contain Sox2-binding sites (Figure 5D). We also found an association of Sox2 with its own promoter region (Figure 5D), consistent with reports in embryonic stem cells that show Sox2 autoregulation is important for stemness control (Heng *et al*, 2010). Together these data indicate that Sox2 directly activates transcription of the *Atoh1* gene.

To characterize the onset of expression of Sox2 and Atoh1, we performed a detailed temporal analysis of the appearance of Sox2(+) cells in the skin of Atoh1-GFP mice, where EGFP is fused to the 3′ end of the *Atoh1* gene. In these mice, Merkel cells are the only skin cells with GFP signal (Haeberle *et al*, 2004). In accordance with the published studies (Driskell *et al*, 2009; Clavel *et al*, 2012), Sox2 was found in the dermal papilla at E14, and those cells did not express Atoh1. However, neither Atoh1-GFP nor Sox2 is expressed in the epidermis before E15, at which point they are consistently co-expressed (Figure 5E). These Atoh1-GFP cells then begin to express K20 starting at E17 (Figure 5F). These data suggest that Sox2 and Atoh1-GFP are concomitantly expressed during Merkel cell specification and are both early markers of Merkel cell development.

To confirm the involvement of Sox2 in *Atoh1* expression and Merkel cell differentiation *in vivo*, we crossed Sox2cKO mice with Atoh1-GFP mice. In these animals, Atoh1-GFP(+) cells were found in significantly reduced numbers at P0 (Figure 5G, left and graph) and completely lacked K20 staining (Supplementary Figure 5A). Fluorescence-activated cell sorting (FACS) analysis of P0 epidermis confirmed the reduction of Atoh1 expression, as indicated by decrease in the

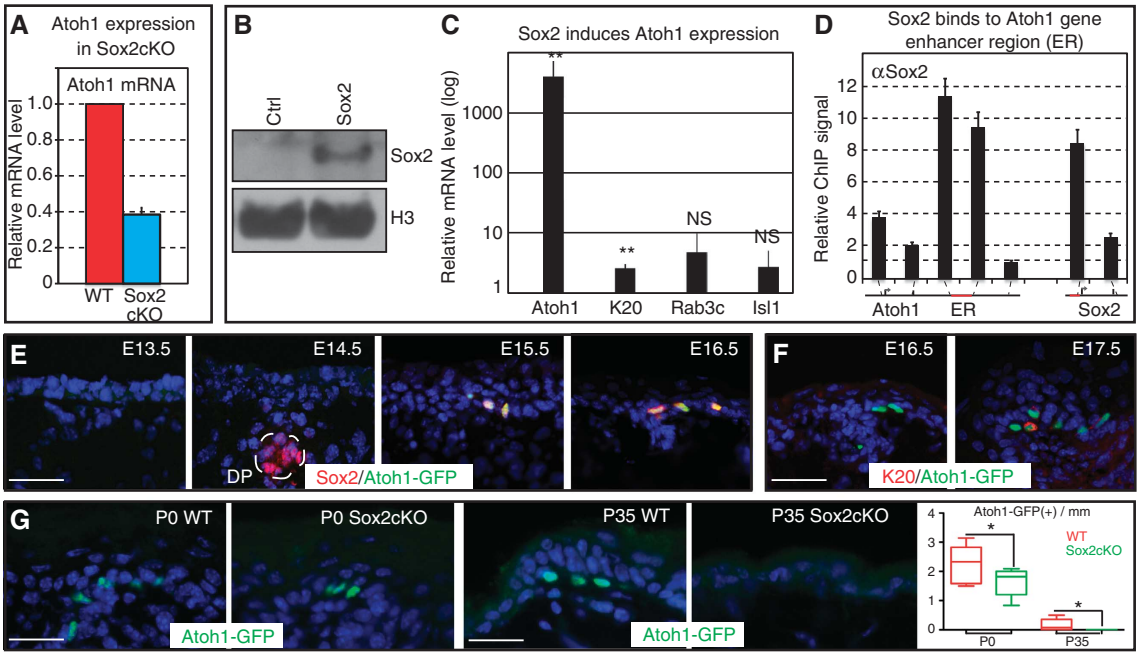

Figure 5 Sox2 regulates expression of Atoh1, a critical regulator of Merkel cell specification. (**A**) Expression of the obligate Merkel cell transcription factor Atoh1 is reduced in total skin isolated from P0 Sox2cKO mice as measured by RT-qPCR. (**B**) Overexpression of Sox2 in WT epidermal stem cells was confirmed by western blot. Histone H3 serves as a loading control. (**C**) Sox2-overexpressing cells had a dramatic increase in the level of Atoh1 mRNA and a slight upregulation of K20 mRNA, while expression of other Merkel cells markers remained unchanged. (**D**) ChIP studies on Sox2-overexpressing cells showed Sox2 binding at the *Atoh1* enhancer region and the known Sox2 autoregulatory site. ChIP-qPCR signal is normalized to experimental control cells infected with an empty vector. (**E**) Concomitant expression of Sox2 and Atoh1 is observed throughout Markel cell development, as shown by IF studies of Sox2 on Atoh1-GFP back skin samples. (**F**) As with Sox2, Atoh1 is an early marker of Merkel cell differentiation as shown by lack of co-staining between Atoh1-GFP and K20 at E16. (**G**) IF studies show that the presence of Atoh1-GFP(+) cells is dependent on Sox2, as shown by decrease in the number of Atoh1-GFP+ cells at P0 and their absence at P35. Quantifications are provided at right (P0 WT versus Sox2cKO $P = 0.0283$, $n = 103/86$mm 226/144 cells. P35 WT versus Sox2cKO $n = 103/92$mm 16/0 cells). All scale bars are 50 μm. Source data for this figure is available on the online supplementary information page.

Atoh1-GFP signal intensity (Supplementary Figure 5B, right) and showed a 3-fold decrease in the number of Atoh1-GFP(+) cells in Sox2cKO compared to WT (Supplementary Figure 5B). Importantly, by P35, no Atoh1-GFP(+) cells were observed in Sox2cKO skin, whereas they were still present in WT mice (Figure 5G, right and graph). These data indicate that Sox2 is required to sustain Atoh1 expression and to promote Merkel cell specification.

RT–PCR analysis of the Merkel cell marker Isl1 also showed a >5-fold decrease for Isl1 mRNA in Sox2cKO skin compared to WT (Supplementary Figure 5D, left) and both the number of Isl1+ cells and the IF fluorescence intensity for Isl1 in Sox2cKO K18(+) Merkel cells were reduced (Supplementary Figure 5D, right and E). Together, these data demonstrate that Sox2 is required to properly specify Merkel cells during development and suggest that Sox2 modulates transcription of *Atoh1*, as well as *Isl1*. Since Atoh1 is absolutely required for Merkel cell differentiation and Sox2 can induce *Atoh1* transcription (Maricich *et al*, 2009; Van Keymeulen *et al*, 2009), we propose that Sox2 controls Merkel cell development by activating *Atoh1*. Importantly, ChIP analysis of the *Atoh1* gene revealed the presence of the Polycomb-dependent H3K27me3 histone mark (Supplementary Figure 5C), and analysis of ChIP-sequencing data (Ezhkova *et al*, 2011; Lien *et al*, 2011) at the promoters of *Isl1* and *Atoh1* also revealed the presence of the H3K27me3 repressive mark. These data thus reveal a complex regulation of the Merkel cell lineage by Polycomb in epidermal stem cells, with tight control of both the activator Sox2 and its effector genes, *Atoh1* and *Isl1*.

Loss of Ezh1/2 promotes Merkel cell differentiation by derepression of Sox2

Finally, to determine the extent to which the increase in the number of Merkel cells in *Ezh1/2*-null skin was directly attributable to the activation of Sox2, we generated Ezh1/2 Sox2 3KO mice. IF analysis of P0 Ezh1/2 Sox2 3KO skin confirmed a complete loss of H3K27me3 and Sox2 specifically in the knockout epithelium, but not in the K14-Cre-negative dermal cells (Supplementary Figure 6A–C). No apparent defects in epidermal differentiation, skin innervation by NF200-neurons, or increase in apoptosis were observed in 3KO mice compared to WT (Supplementary Figure 6D–I). TEM analysis of the Ezh1/2 Sox2 3KO skin showed a drastic decrease of the number of fully differentiated Merkel cells when compared to Ezh1/2 2KO skin (Figure 6A) and was corroborated by IF analysis of K20, Rab3c, and K18 Merkel cell markers (Figure 6B and C, and Supplementary Figure 6J). The decrease was also observed throughout all body regions of the mice, including paws, whisker IFE, and whisker follicles (Supplementary Figure 6K–O). As shown in Supplementary Figure 6J, the number of K18(+) cells in Ezh1/2 Sox2 3KO mice was similar to that seen in WT mice. This is consistent with data showing that the *K18* gene is directly targeted by Polycomb, and leads us to speculate that loss of Polycomb

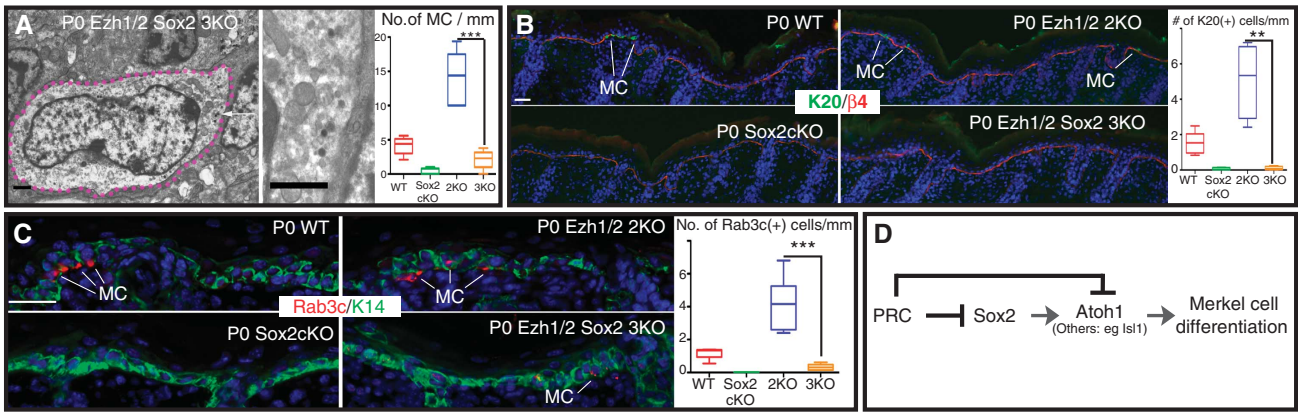

Figure 6 Loss of Sox2 attenuates the Ezh1/2 2KO Merkel cell phenotype. (**A**) TEM imaging showing fully developed Merkel cells in Ezh1/2 Sox2 3KO skin. Dotted lines outline Merkel cells. Arrow indicates dense core neuroendocrine granules shown in the magnified region at right. TEM quantifications shown at the right indicate the reduction in Merkel cells in Ezh1/2 Sox2 3KO skin relative to Ezh1/2 2KO (WT/3KO $P=0.0003$, 3KO $n=9/5.8$mm 37/13 cells). (**B**, **C**) IF analysis of Ezh1/2 Sox2 3KO skin using antibodies against either K20 (**B**) or Rab3c (**C**) confirms TEM data showing the reduction in Merkel cell number relative to Ezh1/2 2KO skin. Quantifications are shown at the right (K20 2KO versus 3KO $P=0.0033$, $n=24/53$mm 119/5 cells; Rab3c 2KO versus 3KO $P=0.0002$, $n=56/64$mm 128/10 cells). (**D**) Model. The Polycomb complex is responsible for the direct repression of Sox2 and Sox2-regulated Merkel cell genes (*Atoh1*, *Isl1*, others) to repress the Merkel cell differentiation program in epidermal stem cells. Scale bars for (**A**) are 1 μm, all others are 50 μm. WT: wild type; 2KO: Ezh1/2 2KO; 3KO: Ezh1/2 Sox2 3KO.

allows partial activation of K18 in the absence of Sox2, while addition of Sox2 greatly amplifies K18 expression. The collected evidence in this work suggests a mechanistic model for Polycomb repression of the Merkel cell lineage in epidermal stem cells (Figure 6D). We propose that Polycomb complexes are responsible for the direct repression of Sox2, a novel critical regulator of Merkel cell lineage specification, and Sox2-regulated Merkel cell genes (*Atoh1*, *Isl1*). Loss of Polycomb repression leads to activation of Sox2, which in turn promotes transcription of *Atoh1*, leading to Merkel cell differentiation.

Discussion

Mechanisms involving Polycomb proteins are attracting a lot of attention in the stem cell field due to the importance of this complex in controlling *in vitro* cultured ES cells. Here, we uncovered the relevance of the Polycomb complex in control of the stem cell state *in vivo*, and showed that Polycomb is essential to repress the lineage commitment and differentiation of epidermal progenitor cells to Merkel cells. Importantly, we dissected the molecular mechanisms of this control by showing that the downstream target of Polycomb repression, Sox2, is a critical transcriptional regulator of Merkel cell differentiation and that its repression is required to maintain the stem cell state.

The role of the Polycomb complex in controlling epidermal progenitor cells is somewhat reminiscent of its function in ES cells, where PRC has also been shown to repress key transcriptional regulators of differentiation (Boyer *et al*, 2006; Lee *et al*, 2006). Interestingly, here we show that both Sox2 and its downstream target gene *Atoh1*, which is essential for Merkel cell differentiation, are under the control of the Polycomb. We therefore propose that Polycomb, Sox2, and Atoh1 form a regulatory network to control Merkel cell lineage specification in embryonic epidermal progenitors. We speculate that the Polycomb repression is first lost from *Atoh1* and *Sox2* genes, allowing their transcription to be initiated. Sox2 activity is then required to further promote *Atoh1* expression and induce full differentiation of Merkel cells. This data thus reveal a tight regulatory network in place to maintain the epidermal stem cell state.

Despite the similarity to ES cells, our studies also uncovered clear differences between Polycomb regulation of ES and epidermal stem cells. In ES cells, the Polycomb complex has been shown to be required for proper execution of the differentiation program, and *in vitro* studies showed that Polycomb-null ES cells failed to differentiate the neuronal lineage due to failure to silence stemness genes (Pasini *et al*, 2007, 2010). Contrary to ES studies, we showed that *Ezh1/2*-null epidermal stem cells are capable of executing the terminal differentiation program and downregulating stemness genes. Taking into consideration our previous work on hair follicle stem cells (Ezhkova *et al*, 2011), we propose that in skin stem cells, the Polycomb complex is critical to maintain the stem cell state rather than to promote differentiation.

Decades of work dissecting the molecular mechanisms controlling hair follicle and epidermal suprabasal lineages identified several key regulators of these pathways (Fuchs and Raghavan, 2002; Blanpain and Fuchs, 2009). The processes regulating the Merkel cell differentiation program, however, are completely unknown despite the discovery of Merkel cells more than 100 years ago. Here we uncovered the critical role for the Sox2 transcription factor in promoting the Merkel cell lineage specification. Interestingly, in ES cells and several mammalian tissues (Masui *et al*, 2007; Arnold *et al*, 2011), Sox2 is expressed in stem cells and is required for stem cell maintenance. Our studies clearly show that in skin, *Sox2* is absent from epidermal stem cells, is expressed in early committed Merkel cells, and is required for the Merkel lineage specification.

Together, our studies clearly show the different roles for both the Polycomb complex and Sox2 in different stem cell systems. As there is an increasing interest in the role of these proteins in controlling tumorigenesis, the clear

understanding of their roles in regulation of stemness and differentiation in a specific tissue context will be essential to determine their functional involvement in cancer stem cells and, further, for the future design of proper cancer therapies. Our studies dissected that in at least one tissue, the skin, there is a clear role for the Polycomb-mediated repression of Sox2 in maintaining the stem cell state.

Materials and methods

Mice

All mice were housed and cared for according to MSSM and IACUC-approved protocols. At least two animals from independent litters were used for each analysis. Ezh1/2 2KO mice were previously reported (Ezhkova *et al*, 2011). As described, mice null for Ezh1 and Ezh2 die shortly after birth, and all analysis of these mice after P0 was performed on grafted skin obtained using the described grafting protocol (Ezhkova *et al*, 2011). K14-GFP-H2B mice were previously reported (Tumbar *et al*, 2004). K14-Cre and Atoh1-EGFP mice were obtained from The Jackson Laboratories. The *Atoh1* and *Ezh2* genes are located on chromosome 6, which prevents crossing Atoh1-GFP mice and Ezh2-flox mice to generate Ezh2 cKO Atoh1-GFP. Mice were genotyped by PCR using DNA extracted from tail skin. BrdU was administered as previously reported (Ezhkova *et al*, 2009). Briefly, BrdU was administered (50 μg BrdU per 1 g mouse weight) to mice or pregnant females 4 h before sacrificing. The back skin, whisker regions, and paws were embedded in OCT immediately following sacrifice.

Transmission electron microscopy

Tissues were fixed in glutaraldehyde-parformaldehyde in 0.1% sodium cacodylade (Karnovsky's), post fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide in distilled water and embedded in epoxy-Araldite resin. Thin sections counterstained with lead citrate were imaged with the Hitachi H7000 TEM. Electron microscopic quantification was performed on five serial sections at 30 μm intervals for each animal on 2×1 mm butvar-coated slot grids (Tachibana *et al*, 1983). Both nucleated and non-nucleated Merkel cells were counted, as the 30-μm interval between sections ensured that cells were only counted once (the diameter of Merkel cells is less than 15 μm).

Immunofluorescence

Tissues were collected from mice and embedded fresh into OCT or PFA, fixed for 2 h, and equilibrated in Sucrose before embedding, and subsequently cut into 10-μm sections using a Leica Cryostat. Slides were then fixed for 10′ in 4% PFA and blocked for 1 h in phosphate-buffered saline (PBS)-Triton with BSA/NGS/NDS. Primary antibodies were diluted in blocking solution and incubations were carried out for 1 h at room temperature, followed by incubation in secondary antibody for 1 h at room temperature. Slides were then counterstained with Hoechst and mounted using antifade mounting media.

Antibodies for immunofluorescence

Antibodies were used as follows: Keratin 5 (1:500); H3K27Me3 (Millipore, 1:300); K18 (Abcam, 1:100); Isl1 (Abcam, 1:400); Rab3C (Abcam, 1:250); NF200 (Sigma, 1:1000); Ki67 (Novacostra, 1:250); Sox2 (Stemgent, 1:150); K20 (Dako, 1:70); AcCasp3 (R&D, 1:250); CD104 (B4, 1:100); GFP (Abcam, 1:1000); CD117 (eBioscience, 1:100); BrdU (Abcam, 1:250); K10 (Covance, 1:500); and Loricrin (Covance, 1:250).

Microscopy and quantification

Slides were imaged using either Leica DM6000 or Zeiss Axioplan 2IE inverted slide microscopes and either $\times 10$ or $\times 20$ objectives. Confocal microscopy was performed using a Leica SP5 DM and either $\times 20$ or $\times 63$ objectives. Fluorescence intensity was calculated from at least three raw, single-channel grayscale images per condition. Using either Leica LAS AF or NIH ImageJ software, the mean intensity was calculated for nuclei of K14-GFP or H3K27me3 cells from the lineages specified. Fluorescence intensity was normalized to non-nuclear background for Figure 2A. For Figure 2B, intensity was normalized to the average basal cell (BL) intensity to show reduction as relative to K14-GFP-expressing basal cells.

Merkel cell quantification

Merkel cell number quantifications were calculated using a fluorescence microscope. The length of each section was measured and the number of positively stained cells was counted. Typical section lengths were between 7–14 mm. Due to the highly variable number of Merkel cells between genotypes, we have included the total length of skin quantified. For instance, when comparing WT and Sox2cKO skins, we counted a large number of MCs in the WT condition (>100) and then counted the number of Merkel cells in a similar length of skin for the Sox2cKO condition. We defined a 'MC cluster' as a group of >3 K18+ Merkel cells in which no two cells are located more than 30 μm from each other. Please note that touch domes are defined as MC clusters located in and around guard hairs. In 2KO skin, however, Merkel cell clusters appear around different hair types as well as in the interfollicular epidermis. We thus called them Merkel cell clusters and not touch domes. The total length of skin analysed has been provided in each figure legend. Complete information on the total number of Merkel cells counted for each staining is in Supplementary Table 2.

Cell culture and overexpression

Primary epidermal progenitors were collected from WT CD1 mice and cultured in calcium-restricted media. Control (empty vector) or mSox2 overexpression lentiviruses were produced from transfected 293FT cells and used to transduce keratinocytes. After 3 days, infected cells were collected and analysed by ChIP and RT–qPCR.

Chromatin immunoprecipitation

ChIP assay was performed as described (Lien *et al*, 2011). Briefly, keratinocytes were obtained from *in vitro* culture or total epidermal isolation. (See the description in FACS analysis section. Cells from total epidermal isolation do contain small contaminating populations of melanocytes and immune cells, which accounts for the low level of H3K27me3 signal in Ezh1/2 2KO cells.) Cells were crosslinked by addition of 1/10th volume of fresh 11% formaldehyde solution for 10 min at room temperature and then rinsed twice with $1\times$ PBS prior to freezing in liquid nitrogen and storing at -80C. Before ChIP, cells were resuspended, lysed, and sonicated to solubilize and shear crosslinked DNAs. For sonication, lysates were treated with Triton X-100 to 1% and then subjected to a Bioruptor Sonicator (Diagenode, UCD-200) according to a $30\times$ regimen of 30 s sonication followed by 30 s rest. The resulting whole-cell extract was incubated overnight at 4°C with 10 ml of Dynal Protein G magnetic beads (Invitrogen), which had been preincubated with ~2 ug of the Sox2 (Stemgent), H3K27me3 (Millipore), Ezh1 (Mousavi *et al*, 2012), Ezh2 (Active Motif), or pan-H3 (abcam) antibodies. After ChIP, samples were washed with low salt, high salt, LiCl, and Tris-EDTA buffers for 15 min at 4°C. Bound complexes were eluted and crosslinking was reversed by overnight (o/n) incubation at 65°C. Whole-cell extract DNA was also treated for crosslink reversal.

RNA analysis

Cells were lysed using Qiagen RLT buffer with ß-mercaptoethanol and RNA isolated using the Qiagen RNeasy Mini kit with DNaseI. Reverse transcription was performed using Invitrogen Superscript III and Oligo-dT primers. All qPCR was performed using Roche SYBR green reagents and a Lightcycler480 machine.

FACS analysis

For Atoh1-GFP analysis, whole back skin was collected from Atoh1-GFP or WT mice and placed in Dispase for 1 h at 37°C, after which the epidermis was peeled from the dermis and trypsinized to give a single-cell suspension. Cells were then stained with the viability marker DAPI and analysed using a BD LSRII and BD FACS Diva software.

FACS sorting of epidermal basal cells was performed as described in Ezhkova *et al* (2011). Briefly, cells were collected from WT and Ezh1/2 2KO mice as for Atoh1-GFP mice above. FACS gating was done on live $CD140a^-CD207^-CD117^-CD31^-Sca1^+\alpha6\text{-integrin}^{high}$ cells to collect basal epidermal cells to the exclusion of non-epithelial cell types.

Statistics

In all column bar graphs, mean value ± 1 standard deviation is presented. Box-and-whisker plots show first to third quartiles

around the median, with whiskers showing maximum and minimum values. All quantifications were performed on multiple cell populations from different animals. To determine the significance between two groups (as indicated in the figures by a bracket), comparisons were made using Student's *t*-test (GraphPad Prism 5). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance and actual *P*-values (to four decimal places) are provided in the figure legends.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: ESB, VJV, JZ, and EE designed the study and performed the experiments. SN generated the Sox2-floxed mice. SAH performed the electron microscopy analysis. JMS analysed RNA microarray data. ESB, VJV, CP, and EE wrote the manuscript with input from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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