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## Ezh2 Orchestrates Gene Expression for the Stepwise Differentiation of Tissue-Specific Stem Cells

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### SUMMARY

Although in vitro studies of embryonic stem cells have identified polycomb repressor complexes (PRCs) as key regulators of differentiation, it remains unclear as to how PRC-mediated mechanisms control fates of multipotent progenitors in developing tissues. Here, we show that an essential PRC component, Ezh2, is expressed in epidermal progenitors but diminishes concomitant with embryonic differentiation and with postnatal decline in proliferative activity. We show that Ezh2 controls proliferative potential of basal progenitors by repressing the *Ink4A-Ink4B* locus and tempers the developmental rate of differentiation by preventing premature recruitment of AP1 transcriptional activator to the structural genes that are required for epidermal differentiation. Together, our studies reveal that PRCs control epigenetic modifications temporally and spatially in tissue-restricted stem cells. They maintain their proliferative potential and globally repressing undesirable differentiation programs while selectively establishing a specific terminal differentiation program in a stepwise fashion.

### INTRODUCTION

One of the fundamental goals of modern biology is to understand the molecular mechanisms by which multipotent progenitor cells control tissue development and maintenance. Increasing evidence has pointed to a possible role for polycomb group (PcG) proteins in this process. PcG proteins form chromatin-remodeling complexes referred to as polycomb repressor complexes (PRCs) (Ringrose and Paro, 2004). Comprised of Ezh2, Eed, and Suz12, PRC2 is recruited to chromatin, where methyltransferase Ezh2 catalyzes H3 trimethylation on lysine 27 (triMeK27-H3) (Cao et al., 2002). This histone mark then provides a platform to recruit PRC1 (Cao et al., 2002; Min et al., 2003), which aids in PcG-mediated repression either by chromatin compaction

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#### ACCESSION NUMBERS

Microarray data of genes expressed in basal WT and Ezh2cKO cells at E16 have been deposited to the GEO repository under GSE14045 Series Accession Number.

or by interfering with the transcription machinery (Francis et al., 2004; Ringrose and Paro, 2004; Sarma et al., 2008). Without Ezh2 activity, PRC1 cannot be recruited to chromatin, and PcG-mediated repression is not established (Cao et al., 2002; Rastelli et al., 1993).

In vitro studies of pluripotent mouse and human embryonic stem cells (ESCs) have shown that PRC2 proteins and their triMeK27-H3 marks reside at and transcriptionally repress many regulatory genes that control specific developmental lineages (Boyer et al., 2006; Lee et al., 2006; Pietersen and van Lohuizen, 2008). Establishing functional significance, *Eed* null ESCs have elevated expression of PcG-repressed differentiation genes (Boyer et al., 2006; Chamberlain et al., 2008).

Intriguingly, the genes in ESCs that are repressed by triMeK27-H3 frequently contain the additional H3 modification, lysine 4 trimethylation (triMeK4-H3), often associated with active chromatin (Bernstein et al., 2006). This has led to speculation that, through these bivalent marks, differentiation genes controlled by PRC2 may be poised for activation upon removal of their repressive epigenetic marks (Bernstein et al., 2006; Boyer et al., 2006). That said, the role of PRC-mediated chromatin repression in regulating ESC differentiation is complex. Thus, cultured *Suz12* null ESCs treated with retinoic acid do not execute normal neuronal differentiation but, rather, fail to suppress pluripotent genes and only partially activate neuronal genes (Pasini et al., 2007). This has led to speculation that PRCs are required for both suppression and activation of differentiation programs in ESCs.

It remains an important challenge to determine whether these epigenetic mechanisms unveiled in vitro operate in vivo to govern fates of the more restricted progenitors that develop and maintain tissues (Spivakov and Fisher, 2007). Assessing the roles of PcG components in tissue organogenesis has been hampered by the early embryonic lethality caused by loss-of-function mutants of core PRC2 components. Conversely and further complicating interpretation is that conditional ablation of *Ezh2* in adult bone marrow cells does not seem to affect either hematopoietic SC survival or lineage determination, suggesting either functional redundancy and/or compensation by paralogous *PRC* genes in at least some tissues (Su et al., 2003, 2005). This also seems to be the case for *PRC1* genes such as *Bmi1*, in which mutations do not appear to affect embryogenesis (Sauvageau and Sauvageau, 2008; Valk-Lingbeek et al., 2004). Ironically, *Bmi1* mutants malfunction in maintaining hematopoietic and neuronal adult SC renewal, in part due to misregulation of the *Ink4A/Arf* locus (Bruggeman et al., 2005; Molofsky et al., 2003, 2005; Park et al., 2003). That said, triMeK27-H3 epigenetic marks are still apparent in *Bmi1* null cells (Cao et al., 2005), suggesting that the phenotype does not reflect complete abrogation of PcG-repressive functions. These findings underscore the importance of analyzing PcG functions in other in vivo biological systems in order to understand their physiological relevance in tissue development and maintenance.

Mammalian epidermis is an excellent model to address this problem. Epidermal lineages originate from a single layer of multi-potent progenitors, basal cells, that adhere to an underlying basement membrane separating epidermis from dermis (Fuchs, 2007). In mice, epidermal stratification and fate specification initiate at approximately embryonic day 14 (E14) and complete shortly before birth, when the skin surface barrier is required to keep harmful microbes out and prevent dehydration (Fuchs, 2007).

Basal cells continually fuel the production of ~10 suprabasal layers. Once cells exit the basal layer, they downregulate proliferation-associated genes and execute a terminal differentiation program that is marked by a stepwise transcriptional transition from early differentiation spinous layers to late differentiation granular layers. In the last step, all metabolic activity ceases as dead squames of the protective stratum corneum are formed and subsequently sloughed from the skin surface (Watt et al., 2006).

In this report, we use epidermis as a model to explore the functional significance and physiological relevance of PcG-mediated chromatin repression during embryonic tissue development and homeostasis. We unveil Ezh2 as a critical mediator of chromatin repression in embryonic basal cells and show that its expression diminishes with age and with commitment to terminally differentiate in vivo and in vitro. Using chromatin immunoprecipitation (ChIP) performed on purified embryonic epidermal progenitors, we show that its triMeK27-H3 mark is present both on silent epidermal genes activated late in terminal differentiation and on nonepidermal genes, e.g., neuronal and muscle transcriptional regulators. Interestingly, however, despite this broad association of triMeK27-H3 with silenced genes of different tissue lineages, loss of PcG-mediated repression does not alter the fate of epidermal cells. Rather, it reduces their proliferation through upregulation of the *Ink4A-Ink4B* locus and tempers differentiation rates and barrier function acquisition by upregulating late-stage epidermal differentiation genes, many of which are controlled by AP1 transcriptional activators. We show that the triMeK27-H3 mark prevents AP1 proteins from binding to these target genes in basal cells and that, during terminal differentiation, these late differentiation genes lose their histone mark, associate with AP1 components, and become activated suprabasally. Our findings provide new mechanistic insights into PcG-mediated repression of tissue-specific differentiation genes and suggest that the spatial balance between expression of PcG epigenetic repressors in undifferentiated cells and transcriptional activators like AP1 in differentiated cells controls proper establishment of the stepwise mechanism of epidermal differentiation during skin development.

## RESULTS

### Ezh2 Marks the Progenitor Cell Nuclei in Developing Epidermis

At E14, epidermis consists of a basal layer of progenitors and a layer of suprabasal cells that have initiated a program of terminal differentiation but are still proliferative (Figures S1A and Figure S1B available online) (Lechler and Fuchs, 2005; Watt et al., 2006). Purification of these two populations by fluorescence-activated cell sorting (FACS) and transcriptional profiling revealed that PcG components of PRC2 (Ezh2 and Eed) and PRC1 (Bmi1, Cbx2, and Pcgf2) are expressed in basal cells and downregulated suprabasally. Semiquantitative PCR (RT-PCR) and anti-Ezh2 immunofluorescence confirmed these patterns (Figures 1A and 1B and data not shown).

By E16–E18, proliferation is restricted to the basal layer, and the epidermis consists of multiple layers of differentiating suprabasal cells (Figures S1C and S1D). At these stages, Ezh2 was strong in basal but weak in spinous, granular, and stratum corneum layers (Figures 1C). After birth, Ezh2 waned, and by postnatal day 9 (P9), Ezh2 was barely detected even in basal cells (Figure 1D). The 5-Bromo-2'-deoxyuridine (BrdU) pulses administered at different developmental ages revealed that the %BrdU(+) basal cells also decreased steadily with age (Figure 1E) and that the few remaining Ezh2(+) cells were also BrdU(+) (Figure 1F).

In vitro, cultured basal epidermal cells exposed to elevated calcium stop proliferating and initiate terminal differentiation (Hennings et al., 1980). Similar to their behavior in vivo, basal mouse keratinocytes (MK) expressed high levels of PcG mRNAs and protein, which progressively declined upon calcium-induced differentiation (Figures 1G and 1H). Both in vivo and in vitro, Ezh2 in proliferating, undifferentiated MK was largely, if not solely, nuclear (Figures 1I and 1J). Therefore, we pursued the notion that, in epidermal MK, Ezh2 functions in the nucleus.

## PcG-Dependent triMeK27-H3 Globally Marks Genes of Nonepidermal Lineages and Selectively Marks Genes of Epidermal Lineages

In mouse ESCs, PRC2 marks and globally represses genes involved in the specification of lineages, including those of the epidermal lineage (Boyer et al., 2006). To evaluate the extent to which the repressed PRC2 targets of mouse ESCs are maintained in Ezh2(+) embryonic epidermis, we performed ChIP assays with FACS-purified embryonic epidermal basal cells and Abs against triMeK27-H3 and unmodified histone H3 (Figures S2A and S2B). We first focused on nonepidermal “master” transcriptional regulators of the *Hox*, neuronal (*Neurog1*, *Neurog2*, *Olig2*, and *Olig3*), and muscle (*Myod1*) families that are marked for PcG-mediated repression in ESCs (Boyer et al., 2006). Many of these genes were also transcriptionally repressed by triMeK27-H3 in basal epidermal cells (Figures 2A and 2B).

We next focused on identifying epidermal lineage genes targeted by PcG repression in Ezh2(+) embryonic basal cells. For these studies, we analyzed promoters of basal genes, which are transcriptionally active, as well as spinous and granular layer genes, which are not expressed at this stage of differentiation. Our tests included genes previously found in ESCs to be enriched for PcG-mediated repression, as well as other epidermal genes.

Interestingly, genes enriched for triMeK27-H3 in embryonic basal cells showed a good inverse correlation between Ezh2 and terminal differentiation (Figures 2C and 2D) (Fuchs, 2007). Genes coexpressed with Ezh2 in either embryonic basal or early (spinous) layers (*actinb1*, *K14*, *K1*, and *K10*) were not enriched for triMeK27-H3 in basal progenitors, whereas genes expressed in granular cells (*loricrin*, *filaggrin*, *Lce1*, *Lce1a2*, and *Crct1*) showed significant triMeK27-H3 enrichment in embryonic basal cells.

Intriguingly, the triMeK27-H3 status of genes in the embryonic basal epidermal layer in vivo differed from that in cultured ESCs, in which both early (*K1*) and late epidermal differentiation genes (*loricrin* and *Lce1a2*) scored as targets of PcG-mediated repression (Boyer et al., 2006). Thus, whereas some PcG targets established in pluripotent cells appear to be maintained in the epidermal lineage, others may lose their repressive mark during tissue development.

### Ezh2 Controls Proliferation of Basal Cells through Repression of *Ink4A/Ink4B*

To address the physiological significance of Ezh2’s interesting pattern of gene expression, we conducted conditional loss-of-function studies. Mice harboring the floxed *Ezh2* allele (Su et al., 2003) were bred to mice expressing Cre recombinase under the *keratin 14* (*K14*) promoter in embryonic epidermal progenitors (Vasioukhin et al., 1999). *Ezh2* conditional knockout (*Ezh2* cKO) mice were born alive and in the expected Mendelian ratios. Immunofluorescence and western blot analyses revealed a loss of Ezh2 and triMeK27-H3 marks in basal cells (Figure 3A–3C and Figure S3A), underscoring their reliance upon Ezh2 for establishment and maintenance of this mark. TriMeK27-H3 was still detected in suprabasal cells, most likely because the Ezh2 paralog, Ezh1, is expressed throughout embryonic epidermis and in basal cells of postnatal skin (Figure S8A). We return to this issue later.

In the absence of Ezh2, basal epidermal cells were less proliferative, exhibiting reductions in Ki67(+) cells and BrdU labeling (Figures 3D–3F). During early postnatal development when Ezh2 was still expressed in WT basal cells, the % BrdU(+) basal cells in *Ezh2* cKO skin was more than 2-fold diminished. As Ezh2 waned in postnatal WT basal cells, the % BrdU(+) basal cells within cKO and WT skins became comparable (Figure 3F).

When cultured, P0 *Ezh2* cKO and WT basal cells both formed similar-sized colonies with comparable efficiencies (Figures S3B and S3C). However, whereas WT colonies were composed of tightly packed small cells, cells within cKO colonies were often larger, flat, and loosely packed (Figure 3G). After several days in culture, the growth of *Ezh2* cKO cultures

began to slow (Figure 3H). This reduction did not appear to be attributable to increased apoptosis (Figure 3J) nor to altered growth factor-stimulated actin dynamics (Su et al., 2005) (Figure 1). Instead, cell-cycle analysis revealed an increase in G1/G0 and decrease in S phase cKO cells compared to WT (Figure 3I). Consistent with a reduction in S phase entry, the phosphorylation of retinoblastoma protein was reduced in cKO cells (Figure S3D).

The proliferation defect in *Ezh2* cKO skin appeared to be attributed, at least in part, to derepression of the *Ink4B-Arf-Ink4A* tumor suppressor locus that encodes potent cell-cycle inhibitors of the G1-to-S transition in a variety of cell types, including epidermis (Bracken et al., 2007; Bruggeman et al., 2007; Kim and Sharpless, 2006). Consistent with prior studies that established *Ink4B-Arf-Ink4A* as a PcG target, we observed an ~10-fold upregulation of *Ink4B* in FACS-purified *Ezh2* cKO cells in vivo (Figure 3K) and a similar marked upregulation of both *Ink4A* and *Ink4B* genes in *Ezh2* null MK in vitro (Figure 3L). Finally, in WT skin, *Ink4B* expression inversely correlated with *Ezh2* (Figure 3K, basal versus total).

### **Ezh2 Tempers Epidermal Development and Barrier Acquisition**

P0 *Ezh2* cKO epidermis displayed a hyperthickened stratum corneum and pronounced granular layer accompanied by the expansion of the granular markers loricrin and filaggrin (Figures 4A–4C). These defects were also apparent in E16 cKO skin concomitant with loss of *Ezh2* and basal triMeK27-H3 (Figure 4D–4F, Figure S4A, and S4B). Ultrastructurally, the immature large keratohyalin granules (KG) of WT E16 skin were already dispersed in cKO skin (Figure 4G and Figure S4C) (Fuchs, 2007; Steven et al., 1990). A well-formed stratum corneum was also evident, and with proper preservation, highly ordered lipid lamellae interconnected these cells only within the E16 *Ezh2* cKO (Figure S4D). Correspondingly, the nonepidermal periderm (P) layer that normally provides a temporary barrier during epidermal maturation was already degenerated (Figure 4D, 4G, and Figure S4C). These various signs of accelerated development (Jaubert et al., 2003; Patel et al., 2006) were manifested functionally in the precocious acquisition of the epidermal barrier capable of excluding dye (Figure 4H).

### **Ezh2 Is Required to Repress Precocious Epidermal Late Differentiation Genes in Basal Cells**

To understand how loss of *Ezh2* might result in accelerated epidermal differentiation, we conducted transcriptional profiling of FACS-purified basal cells from E16 *Ezh2* cKO and WT skins to determine the global consequences of *Ezh2* loss to epidermal gene expression. The data are compiled in Figure 5A, with upregulated genes in red and downregulated genes in green.

As expected, loss of *Ezh2* resulted mostly in gene upregulation in basal cells. Surprisingly, however, only a small fraction of the genes analyzed showed a more than 2-fold change compared to WT. Of the 94 genes (0.2%) upregulated, 75% were epidermal genes, and among the others, there did not appear to be a significant bias toward any specific developmental lineage (Table S1). Thus, despite the global association of triMeK27-H3 marks with epidermal and nonepidermal genes (Figures 2A and 2C), the loss of *Ezh2* in basal cells led primarily to selective upregulation of epidermal genes. In this regard, PRC-mediated repression in basal epidermal cells appeared to differ markedly from ESCs, in which PRC target genes encompassing multiple lineages were upregulated when PcG function was compromised (Boyer et al., 2006).

As judged by array and RT-PCR, genes associated with basal and/or early (spinous) differentiation were unaffected by loss of *Ezh2* (Figures 5B and 5C), and this was consistent with the lack of triMeK27-H3 marks at their promoters (Figure 2C). Notably, this included transcription factors, e.g., *Trp63*, *Tcf3*, *Notch*, *Hes1*, *AP2*, *cEBP*, and *Klf4*, that have been implicated in either maintenance of the undifferentiated state or induction of terminal



differentiation (Fuchs, 2007; Watt et al., 2006). This finding also contrasted with ESCs, in which many targets for global PcG repression of lineages are transcriptional regulators of fate specification and differentiation (Boyer et al., 2006).

Interestingly, 77% of genes upregulated in E16 *Ezh2* cKO basal cells were expressed more than 2-fold higher in mature WT epidermis compared to WT basal cells (Figure 5A). Moreover, genes upregulated in *Ezh2* null basal cells were primarily those expressed during the normal course of terminal differentiation. The largest differences were among genes encoding major granular proteins, e.g., filaggrin (Flg), loricrin (Lor), involucrin (Ivl), and late cornified envelope (Lce) proteins (Figures 5A and 5C and Table S1) (Marshall et al., 2001; Watt et al., 2006). Notably, these genes are within a 2Mb locus encompassing an epidermal differentiation complex (EDC) on mouse chromosome 3 (Figure 5D). These data suggest a role for *Ezh2* in ensuring that granular genes within this cluster are repressed until the appropriate stage in development and differentiation.

Studies with cultured *Ezh2* null MK provided further evidence to support this notion. Thus, under low calcium conditions that favor the basal fate, *filaggrin* and *loricrin* levels were comparable to those of WT cells exposed to high calcium, a trigger of terminal differentiation (Figure S5A). In  $\text{Ca}^{2+}$ -stimulated *Ezh2* null MK, *filaggrin* and *loricrin* were even more highly expressed than normal.

To determine the extent to which precocious induction of late-stage differentiation-specific genes in *Ezh2* null basal cells is a direct cell autonomous consequence of loss of *Ezh2*-mediated repression, we conducted ChIP assays with *Ezh2*, triMeK27-H3, and pan-H3 Abs and primers encompassing *loricrin*, *filaggrin*, and other EDC gene promoters. In WT MK, both *Ezh2* and triMeK27-H3 were enriched near transcriptional initiation sites (Figure 5E and Figure S5B). The signals for both *Ezh2* and triMeK27-H3 appeared to be specific, as judged by comparisons with *Ezh2* null cells or control ChIPs in which Abs were omitted (Figure 5E, Figure S5B–S5D).

Basal cell PcG complexes were detected over many, but not all, terminal differentiation-specific genes across the EDC locus (Figure 5E). The distal region harboring the *Lce* genes was particularly rich in triMeK27H3, whereas the small proline-rich (*Spr*) family of genes exhibited only background levels of this modification (Figure 5E). Correspondingly, expression of *Spr* genes was unchanged (Figure 5A), suggesting that *Ezh2* may differentially regulate EDC genes. Promoters of basally expressed *K14* and *actinb1* genes and nonexpressed intergenic regions did not display the *Ezh2*-mediated triMeK27-H3 mark (Figure 5E). These findings underscored the specificity of the ChIP signals and PcGs for late-stage differentiation genes and established them as direct targets for *Ezh2*-histone methyltransferase.

### More to Gene Repression Than triMeK27-H3: Analysis of Nonepidermal Genes

If expression of genes that receive triMeK27-H3 marks is governed solely by PRC-mediated chromatin modifications, then relief of these marks should result in their aberrant activation. We tested this by first examining the expression status of none-epidermal genes harboring this histone mark in WT epidermal cells and in ESCs (Figure 2A). Consistent with the global reduction of triMeK27-H3 when *Ezh2* is absent, most of the nonepidermal genes that were analyzed showed a significant reduction in triMeK27-H3 in *Ezh2* null MK (Figure S5E). Despite the loss of PcG repression, however, these genes were not activated (Figure S5F).

### More to Gene Expression Than triMeK27-H3: Analysis of Epidermal Genes

To understand why relief of PcG repression leads to selective activation of epidermal, but not nonepidermal, lineage genes in our *Ezh2* cKO mice, we began by investigating whether EDC genes in basal cells selectively contain the active epigenetic mark triMeK4-H3, known to

function antagonistically in PcG-regulated genes in ESCs (Bernstein et al., 2006). Although triMeK4-H3 was readily detected at the promoters of naturally expressed basal genes, we did not detect it in the EDC promoters that we analyzed in embryonic basal cells (Figure 6A).

We next turned to the possibility that the counteractive mark in epidermal basal cells might be a transcriptional activator involved in regulating EDC gene expression. In searching for key EDC gene regulator(s) that might be expressed basally in WT cells, we began to focus on AP1 family members, which are known to regulate *loricrin* and *filaggrin* promoter activities (DiSepio et al., 1995; Jang et al., 1996). Genome scans revealed AP1 consensus binding sites throughout the EDC cluster (Figure 6B). Importantly, Jun and Fos AP1 proteins were detected both in basal cells and total epidermis (Figure 6C, 6D, and Figure S6A), and terminal differentiation appeared to elicit an upregulation of some, but not all, AP1 proteins in vivo and in vitro (Figures 6C and 6D).

To test whether AP1 factors might account for upregulation of EDC genes upon loss of Ezh2, we used the chemical inhibitor Tanshinone IIA (TanIIA), which is thought to abrogate all AP1 binding to DNA. In the absence of TanIIA and in low calcium, only *Ezh2* null and not WT basal MK expressed the EDC genes (Figure 6E). The addition of TanIIA revealed a dose-dependent reduction in expression of EDC genes that harbored AP1 consensus sites, but not in *K14* and *Ppib* genes that lacked them (Figure 6E). The inhibitory response to TanIIA was observed even for some EDC genes that lacked obvious AP1 sequence motifs. Although consensus sequences are not always necessary for DNA binding, an alternative possibility is that close proximity of genes within this cluster contributes to their coordinate regulation through shared regulatory elements. Although pursuing this further is outside the scope of the present study, such a mechanism has been shown for other gene complexes (Dillon and Grosveld, 1993; Lomvardas et al., 2006).

To verify that EDC gene expression in *Ezh2* null basal cells is AP1 dependent, we used RNAi to attenuate expression of Jun proteins, which can homodimerize or form heterodimers with Fos. *cJun* and *JunD* shRNAs each led to specific downregulation of EDC genes without affecting *K14* or *Ppib* expression (Figure 6F and Figure S6B). Combination of both shRNAs was even more dramatic, yielding comparable EDC gene expression levels between WT and cKO basal cells (Figure 6F).

Conversely PMA, which facilitates AP1 activity (Eckert et al., 1997), had little or no effect on EDC gene expression in WT basal cells, but in *Ezh2* null cells, PMA further enhanced activation of EDC genes in low-calcium conditions (Figure 6G). By contrast, expression of PcG-repressed nonepidermal genes *Myod1*, *Olig2*, and *HoxB13* were unaffected by PMA (Figure S6C). Finally, PMA resulted only in a very modest upregulation of EDC genes when *Ezh2* cKO basal cells were exposed to *Jun* shRNAs (Figure 6H). Together, these loss- and gain-of-function studies demonstrate that, in basal epidermal cells, EDC genes are repressed by Ezh2 even though AP1 transactivating factors are present.

Consistent with our array data (Figure 5B), Ezh2 did not markedly affect AP1 mRNA or protein levels either in vivo or in vitro (Figure S7). Thus, we speculated that triMeK27-H3 marks on EDC genes in WT basal cells may interfere with AP1 recruitment, as it has been shown for Myod1 in muscle cells in vitro (Caretta et al., 2004). To test this hypothesis, we first analyzed whether coordinated relief of triMeK27-H3 and recruitment of AP1 factors control terminal differentiation and EDC gene expression under normal physiological conditions. Of the EDC genes marked by Ezh2 and triMeK27-H3, all but *Lce3b* were significantly upregulated when basal MK were induced to differentiate (Figure 7A) (Jackson et al., 2005). ChIP analysis of triMeK27-H3 at the EDC cluster detected a significant decrease in this repressive mark concomitant with EDC gene activation (Figure 7B).

Finally, ChIP analysis detected cJun at several EDC gene promoters only in cKO and not WT basal cells (Figure 7C). By contrast, cJun was not found at *Myod1* and *Olig3* promoters (Figure 7C). Moreover, upon induction of differentiation, cJun was now detected at promoters of these EDC genes in WT cells (Figure 7C). Importantly, inhibition of AP1 binding by TanIIA reduced the cJun ChIP signal at EDC genes underlying its specificity (Figure 7D).

### **Ezh1 Increases in the Epidermis as Ezh2 Wanes: Partially Overlapping Activities of Ezh2 and Ezh1**

Whereas *Ezh2* is often expressed during embryogenesis, *Ezh1* is found in adult tissues (Laible et al., 1997; Margueron et al., 2008; Su et al., 2003). This also holds true for epidermis in which postnatally, as basal *Ezh2* declined, *Ezh1* rose (Figure 1 and Figure S8A). Additionally, although triMeK27-H3 was barely detected in primary P0 *Ezh2* null MK, it increased with passage (Figure S8B).

Recently, *Ezh1* was shown to function similarly to *Ezh2* (Shen et al., 2008), which could explain the residual triMeK27-H3 modification in *Ezh2* cKO epidermis. Although an effective *Ezh1* shRNA did not appreciably affect the overall high levels of triMeK27-H3 in WT MK, it did show a marked effect on further diminishing triMeK27-H3 marks in passaged *Ezh2* null MK (Figure S8C). Together, these findings suggest that the bulk of triMeK27-H3 in embryonic and neonatal basal cells is due to *Ezh2* but that *Ezh1* can establish this histone mark in the absence of *Ezh2*.

Analysis of gene expression further suggested that certain functions of *Ezh1* and *Ezh2* in MK might be similar (Figure S8D). Thus, terminal differentiation genes were upregulated in both *Ezh2* null and *Ezh1* shRNA cells, and this effect was even stronger in passaged *Ezh2* null/*Ezh1* shRNA MK. *Ezh1* was not upregulated in *Ezh2* cKO cells either in vivo or in vitro (Figure S8E), suggesting that both *Ezh2* and *Ezh1* contribute to repression of terminal differentiation genes. This could explain why no obvious morphological defects were noted in the mature skin of our *Ezh2* cKO mice in which *Ezh1* is expressed (Figure 3B and Figure S8A). Supportive evidence for this notion stems from engraftments of primary WT and *Ezh2* cKO MK infected with control or *Ezh1* shRNAs. Cells infected selectively with *Ezh1*, but not *control*, shRNAs failed to reconstitute skin in adult mice (Figure S8F). Generation of *Ezh1* cKO and *Ezh1*/*Ezh2* cKO mice will be necessary to further dissect roles for these developmentally regulated histone methylases in skin and in other tissues.

## **DISCUSSION**

Molecular mechanisms involving PcG proteins have attracted significant attention in the stem cell field due to their critical role in controlling cultured ESCs (Boyer et al., 2006; Lee et al., 2006; Pasini et al., 2007). By studying the role of *Ezh2* in epidermal development and differentiation, we have uncovered new insights into the in vivo relevance of PRCs in the context of lineage determination and differentiation within a tissue. We have shown that *Ezh2*-dependent histone modifications act in embryonic epidermal progenitors to control both proliferative potential and differentiation. Our results are consistent with a role for PRCs in mediating repression of *Ink4A/Ink4B* in epidermal stem cells and further unveil a role for PRCs in regulating terminal differentiation of these cells by repressing genes governing epidermal barrier formation.

Our findings are intriguing in light of a recent study reporting enhanced differentiation of organotypic human epidermal cultures by overexpressing or RNAi knockdown of the histone demethylase *Jmjd3* (Agger et al., 2007; Sen et al., 2008). In contrast to *Ezh2*, *Jmjd3* levels do not appear to change in basal versus suprabasal cells of mouse epidermis. It is tempting to speculate that suprabasal *Jmjd3* might ensure proper differentiation through efficient removal



of the Ezh-mediated triMeK27-H3 mark, and in the future, it will be interesting to explore the possible interplay between histone methylases and demethylases in this process. However, from our physiological and molecular evidence, we favor the view that the primary mechanism for controlling epidermal differentiation in mouse development is by temporal expression of histone methylase Ezh2, which in basal cells, interferes with the recruitment of transcriptional activators to their differentiation-specific targets.

Our study also uncovered several notable differences in the mechanisms by which PcGs control differentiation in ESCs versus embryonic epidermal progenitors. In ESCs, triMeK27-H3 is present at and globally represses key transcriptional regulators that promote diverse cell lineages (Boyer et al., 2006; Lee et al., 2006). Such widespread repression is critical since ESCs are pluripotent. That said, ESCs must also retain the ability to respond to external cues and activate any one of the differentiation lineages repressed by PcG complexes. Once they make a commitment to differentiate, they must repress pluripotent regulators, e.g., Oct4, Nanog, and Sox2, and this process also appears to be governed either indirectly or directly by PcG-mediated events (Pasini et al., 2007). By contrast, embryonic epidermal stem cells maintain permanent repression of pluripotent regulators and selectively activate only the terminal differentiation program of the skin barrier.

Our results suggest that, like ESCs, embryonic basal progenitors use PcG-mediated repression to maintain repression of both nonepidermal and epidermal differentiation programs. However, in ESCs, bivalent active and repressive marks exist over many of the lineage-regulatory genes that are governed by PRCs, such that once repressive marks are relieved, the genes are poised to become activated (Pasini et al., 2007). By contrast, although Ezh2 loss in embryonic basal cells reduced triMeK27-H3 on many of the same genes as in ESCs, this was not sufficient for either maintaining or relieving their transcriptional repression. Rather than use a bivalent chromatin mark, embryonic basal epidermal progenitors appear to employ PRCs to prevent recruitment of AP1 and perhaps other key transcriptional activators required for terminal differentiation (Figure 7E). This additional layer of regulation ensures that, as development proceeds, most lineages remain permanently silenced while permitting the epidermal lineage to be selectively activated.

By invoking additional mechanisms to restrict activation of differentiation programs, developing tissues are poised to take advantage of relieving PRC modifications as a means to fine-tune the transcriptional program of the desired lineage. In this regard, it was notable that Ezh2 was highest in proliferative, undifferentiated basal cells. Upon induction of the basal-to-spinous switch, Ezh2 was reduced, and by the time the spinous-to-granular transcriptional switch was executed, little or no Ezh2 was detected. By employing gene-specific transcriptional activators that can operate when Ezh2 and triMeK27-H3 wane, the correct differentiation stage genes can be induced, and the undesirable nonepidermal lineages can remain silenced.

Controlling the balance between proliferation and differentiation requires different challenges in embryonic versus adult skin. In the rapidly growing embryo, basal proliferation must be high, and the program of epidermal differentiation and barrier acquisition must be orchestrated from scratch. By contrast, during homeostasis of the postnatal epidermis, proliferation rates are considerably reduced, and the program of terminal differentiation merely requires maintenance and not establishment. In this regard, it may be relevant that postnatal human basal epidermal cells exhibit low immunoreactivity to the active chromatin mark acetylated-H4 and appreciable reactivity to the repressive chromatin mark triMeK9-H3 (Frye et al., 2007). Additionally, our studies showing that Ezh2 expression wanes after birth suggest that either the Ezh2 paralog Ezh1 or alternative mechanisms come into play to control homeostasis in the mature epidermis. Although distinguishing between these possible models in vivo is predicated upon *Ezh1* and *Ezh1/Ezh2* double cKO mice, our in vitro and engraftment studies

suggest that Ezh2 may provide the bulk of triMeK27-H3 modification in basal epidermal cells, and Ezh1 may function in fine-tuning histone modifications at later stages of terminal differentiation during embryogenesis and in homeostasis of postnatal epidermis.

In closing, it is worth considering the potential clinical impact of our findings. The epidermal barrier does not form until shortly before birth. Prematurely born infants lack this essential shield, and hence, accelerating barrier acquisition becomes a critical necessity in reducing the medical risks of these infants. Our discovery that conditional loss of Ezh2 accelerates epidermal barrier formation in the embryo but does not impair postnatal development offers a hitherto unanticipated target for the development of therapies that might be useful to infant survival. More-over, Ezh2's exquisite ability to function in rapidly proliferating, tissue-restricted progenitor cells provides an intriguing explanation for why Ezh2 is overexpressed in many tumors wherein hyperproliferation and reduced differentiation are often likened to an embryonic state.

## EXPERIMENTAL PROCEDURES

### Mice, BrdU Injections, and Barrier Assays

Mice were housed in the ALAAC accredited CBC animal facility at The Rockefeller University in accordance with university and NIH guidelines. Mice conditionally ablated for *Ezh2* in skin were generated by mating *Ezh2<sup>fl/fl</sup>* mice (Su et al., 2003) with *K14-Cre* mice (Vasioukhin et al., 1999). Genotyping was confirmed by PCR of tail skin DNAs. BrdU (Sigma-Aldrich) was administered to pregnant mice or to neonatal pups by peritoneal injection (50 µg/g BrdU). Dye exclusion assays were performed as described (Hardman et al., 1998).

### Fluorescence-Activated Cell Sorting

FACS purification of epidermal cells from *K14-H2B-GFP* embryos was performed on a FACSVantage SE system equipped with FACS DiVa software (BD Biosciences). Cells were gated for single events and viability and then sorted according to α6 integrin expression and GFP. Cell-cycle/BrdU incorporation was analyzed using the BrdU-FITC Flow Kit (PharMingen) (see Blanpain et al., 2004), and cell-cycle status was analyzed using FlowJo program.

### Microarray Analysis

RNAs from FACS-purified WT and *Ezh2* cKO basal cells (see Supplemental Experimental Procedures) were provided to the Genomics Core Facility, MSKCC for quality control, quantification, reverse transcription, labeling, and hybridization to MOE430A 2.0 microarray chips (Affymetrix). Arrays were scanned per the manufacturer's specifications for the Affymetrix MOE430v2 chip. Images were background subtracted, and probeset expression estimates were generated using the MAS5 algorithm implemented in Bio-conductor. A detection p value of  $p = 0.05$  was used to threshold probesets into present ( $p \leq 0.05$ ) or absent ( $p > 0.05$ ). Probesets were identified as differentially expressed when the absolute fold change was  $> 2$ , and the probeset is present in both samples of the relatively overexpressed group. Probesets selected for visualization were log<sub>2</sub> transformed and normalized to the replicate mean (estimated from basal cells). Intensity bias between replicates necessitated this within replicate normalization, which leaves the data for each gene centered around zero in the basal cells. These normalized data were analyzed with hierarchical clustering (Pearson correlation, average linkage) and visualized with heatmaps to assist in interpretation.

## Statistics

For all graphs, mean value  $\pm$  one standard deviation was presented with the number of replicates indicated in figure legends. To determine significance between two groups indicated in figures by a bracket, comparisons were made using Student's *t* test. For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

## Cell Culture

Enzymatic separation of epidermis from skins and primary P0 MK culturing were as described (Blanpain et al., 2004). To visualize colonies, we fixed cells and stained them with 1% Rhodamine B (Sigma). To induce differentiation in culture, calcium was raised from 0.05 to 1.5 mM. To assess cell growth, we plated equal numbers of live cells on collagen-coated 6-well plates. Cell numbers were determined by hemocytometer.

To assess AP1 function, MK were treated either with Tanshinone IIA (Biomol) for 48 hr with indicated concentrations or with PMA (Sigma) 10 ng/ml for 5–10 hr. DMSO or Ethanol were used as vehicle controls, correspondingly. To attenuate cJun and JunD, cells were infected with lentiviruses that carry shRNA constructs (Sigma). Selection with puromycin (2 mg/ml) was done 2 days upon infection, and MK were analyzed a week after infection.

## Chromatin Immunoprecipitation Assays

Assays were performed with the EZ ChIP kit (Millipore). Briefly,  $2 \times 10^6$  cells were crosslinked with 1% formaldehyde for 10 min at room temperature (for ChIPs with Ezh2, H3, and triMeK27-H3 Abs) or for 15 min at 37°C (for cJun Abs). Cells were collected, lysed, and sonicated for a total of 200 s using Branson Sonifier 450 equipped with microtip (setting 3; 50% input). Chromatin lysates were used for immunoprecipitations with either Ezh2 (Lake Placid Biologicals), triMeK27-He (Upstate), H3 (Abcam), triMeK4-H3 (Abcam), cJun (Santa Cruz), or no Ab control. Immunoprecipitates were collected with Protein A/G agarose beads (Millipore), protein/DNA crosslinks were reversed by incubating at 65°C, and DNA was purified using QIAgen PCR purification kit (QIAgen). Treatment with TanIIa drug for ChIP was done for 2 hr with 100  $\mu$ M of TanIIa or DMSO (vehicle control).

ChIP DNA was analyzed by performing quantitative PCR as described above. Primers against *K5* and/or intergenic region on chromosome 5 genes were used for normalization. Sequences of primers are available upon request.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENTS

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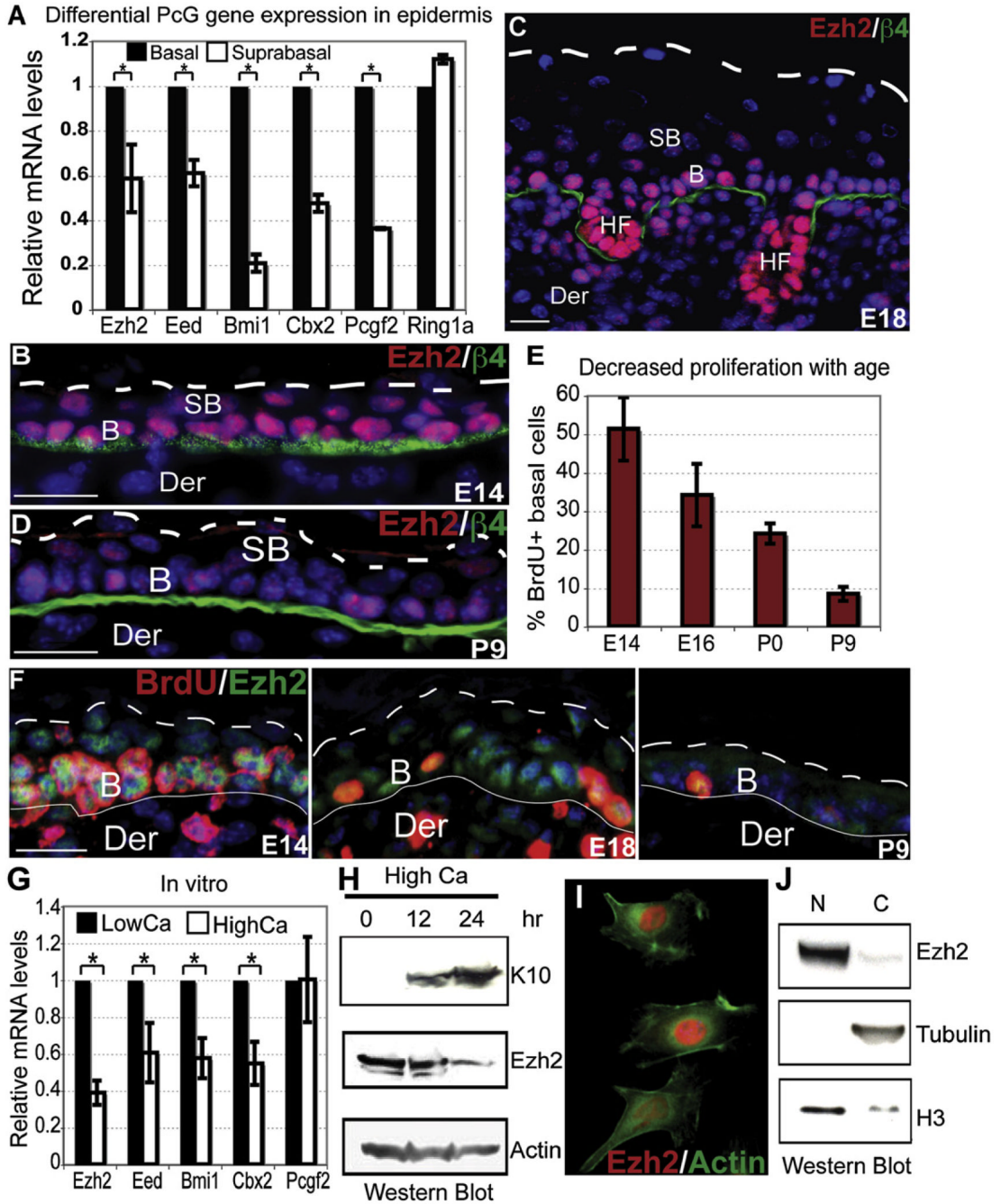
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**Figure 1. Ezh2 Marks Basal Cell Progenitors in Developing Epidermis**

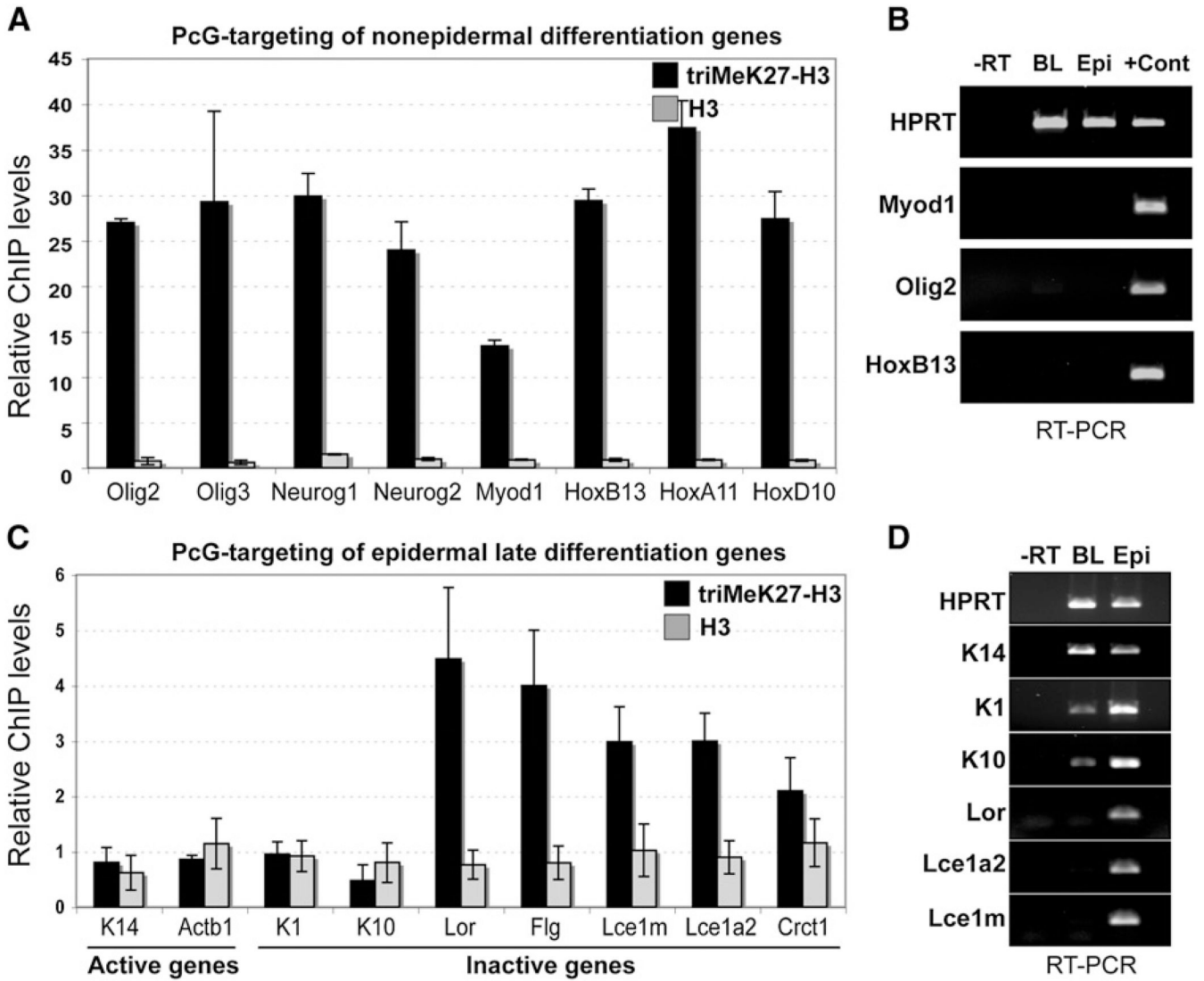
(A) Differential expression of PcG components in E14 epidermis. FACS-purified basal and suprabasal cells were analyzed by semiquantitative RT-PCR of their isolated mRNAs. Data are mean ± SD. n = 3. \*p < 0.05.

(B–F) Age-related decline in basal Ezh2 correlates with reduced proliferative potential. BrdU was administered 4 hr prior to sacrificing mice. Frozen skin sections were subjected to immunofluorescence microscopy. Primary Abs are color coded according to their secondary Abs. Nuclei are stained with Dapi (blue).

(G) Semiquantitative RT-PCR reveals decreased expression of PcG components with epidermal terminal differentiation in vitro. Primers against *HPRT* were used for normalization. Data are mean  $\pm$  SD. n = 2. \*p < 0.05.

(H) Ezh2 protein diminishes upon calcium (Ca)-induced MK differentiation in vitro. Controls are actin (unchanged) and K10 (spinous marker). (I–J) Immunofluorescence and western blot analyses show Ezh2 nuclear localization and partitioning. Note that the cytosolic fraction (C) contains minor residual nuclear material as judged by H3, whereas the nuclear fraction (N) is clean as judged by the absence of  $\beta$ -tubulin.

B, basal. SB, suprabasal. Der, dermis. HF, hair follicle.  $\beta$ 4,  $\beta$ 4 integrin. Scale bars, 30  $\mu$ m.

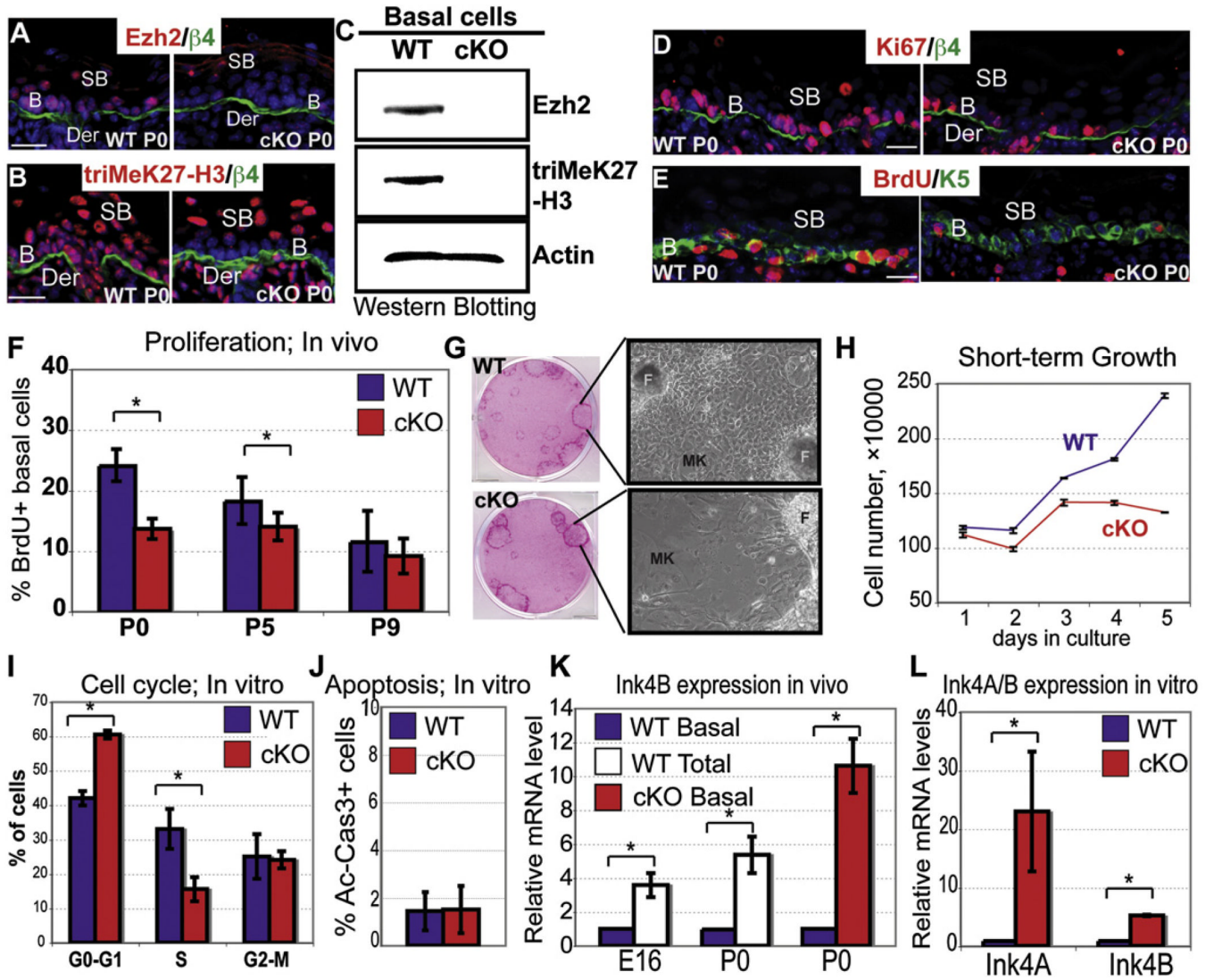


**Figure 2. In Embryonic Basal Progenitors, triMeK27-H3 Marks Silenced Differentiation-Specific Genes of Both Epidermal and Nonepidermal Lineages**

(A and C) ChIP analysis showing association of tri-MeK27-H3 and bulk H3 with promoters of nonepidermal and epidermal genes in FACS-purified E16 basal progenitors.

Semiquantitative PCR of the basal *K5* gene promoter region was used for normalization. Data are mean  $\pm$  SD. n = 2.

(B and D) RT-PCR of nonepidermal and epidermal mRNAs in E16 basal cells (BL) versus total epidermis from P0 embryos. Negative control is PCR with total RNA (-RT) as a template. RT-PCR with RNAs isolated from skeletal muscle and brain served as positive controls (+Cont) for Myod1 (muscle) and Olig2 (brain). Control is HPRT (unchanged).



**Figure 3. A Developmental Role for Ezh2 in the Proliferative Potential of Epidermal Progenitors** (A–C) Immunofluorescence and western blot reveal the absence of Ezh2 and triMeK27-H3 in P0 *Ezh2* cKO basal cells.

(D and E) Immunofluorescence shows fewer Ki67 and BrdU(+) basal cells in P0 *Ezh2* cKO epidermis.

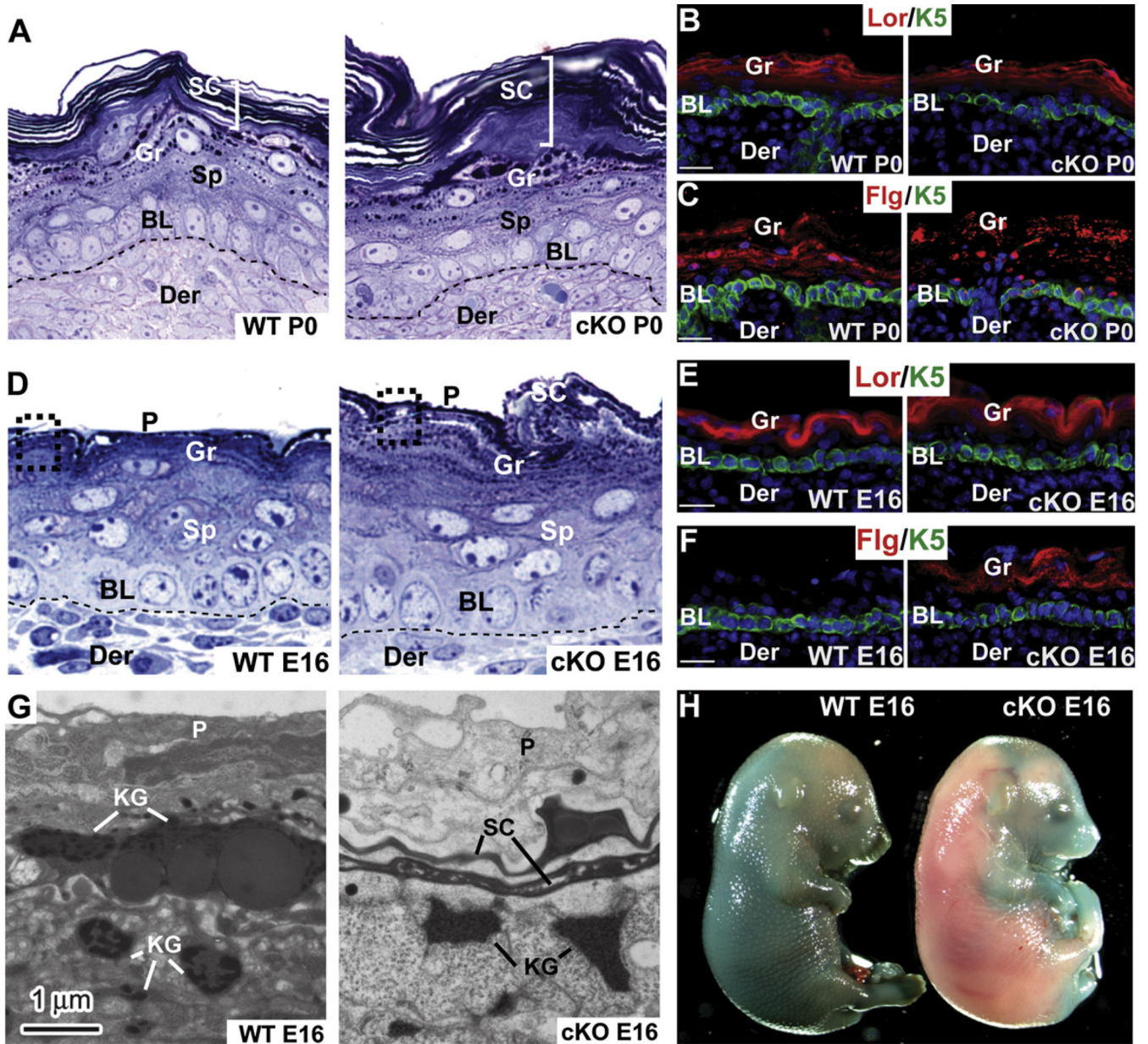
(F) Quantification of BrdU(+) basal cells from mice receiving a 4 hr BrdU pulse at indicated times. Data are mean  $\pm$  SD. WT, cKO n = 3–6. \*p < 0.05.

(G–J) Differences in morphologies, growth rates, and cell cycles, but not apoptosis (active caspase 3, Ac-Cas3), of WT versus *Ezh2* cKO epidermal cells in vitro. Cell-cycle data are mean  $\pm$  SD. WT, cKO n = 3. \*p < 0.05.

(K–L) Semiquantitative RT-PCR to measure *Ink4A* and/or *Ink4B* expression in FACS-purified basal cells in vivo (K) or cultured 1° MK in vitro (L). Data are mean  $\pm$  SD. n = 2 for in vivo and n = 3 for in vitro analysis. \*p < 0.05. For comparative purposes, WT expression in differentiating cells was determined by analyzing total epidermal mRNA. The *HPRT* gene was used for all normalizations.

MK, mouse keratinocytes. F, feeders. Scale bar, 30  $\mu$ m.





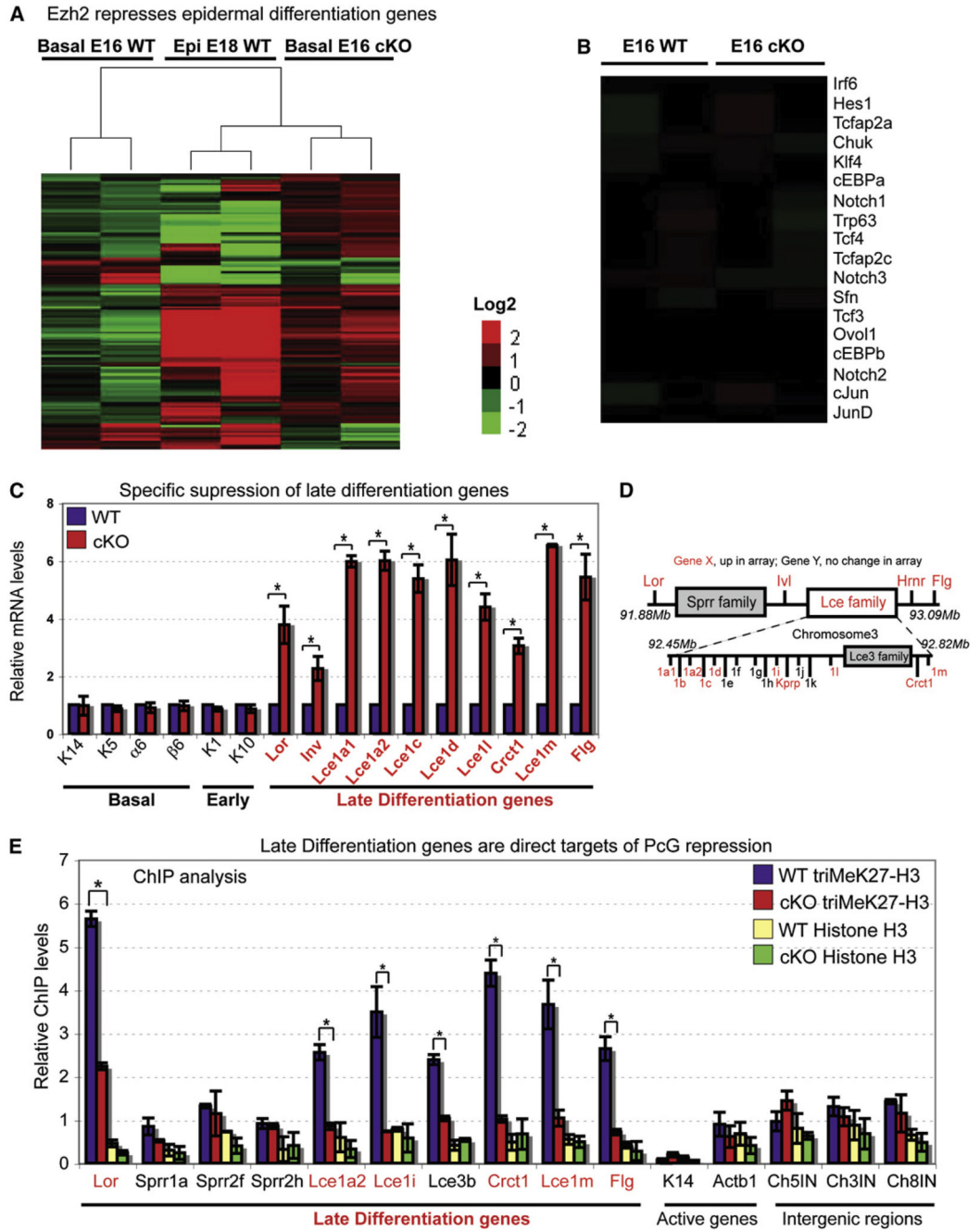
**Figure 4. A Role for *Ezh2* in Regulating Temporal Differentiation and Epidermal Barrier Acquisition during Skin Development**

(A–F) Histological and immunofluorescence microscopy reveals signs of accelerated epidermal differentiation in P0 and E16 *Ezh2* cKO epidermis. Semithin sections in (A) and (D) are toluidine blue stained. Frozen sections are labeled with Abs as indicated (color coding according to secondary Abs).

(G) Ultrastructural analyses. Note the presence of mature keratohyalin granules (KG), a thin stratum corneum layer (SC), and a partial loss of periderm (P) in *Ezh2* cKO E16 epidermis, all lacking in the WT counterpart.

(H) Blue dye exclusion assay to measure skin barrier.

Lor, Loricrin. Flg, filaggrin. BL, basal layer. Sp, spinous layer. Gr, granular layer. Der, dermis. Scale bar, 30  $\mu$ m.



**Figure 5. Transcriptional Profiling and Chromatin Analyses Reveal Late Differentiation Genes as Direct Functional Targets of Ezh2 Repression in Embryonic Basal Cells**

(A and B) Heatmap derived from cluster analysis of microarray data of E16 basal progenitors shows that genes whose expression is normally associated with late-stage terminal differentiation are elevated in the absence of Ezh2. E18 WT epidermis is used as a comparative source of genes active in late-stage terminal differentiation. Microarray hybridizations for each sample were done in duplicate and are shown as separate columns. Probesets were selected that demonstrate a 2 log change (in either direction) and present (“P”) in both instances of a condition (P in both KO or P in both WT). Red, overexpressed genes. Green, under-expressed

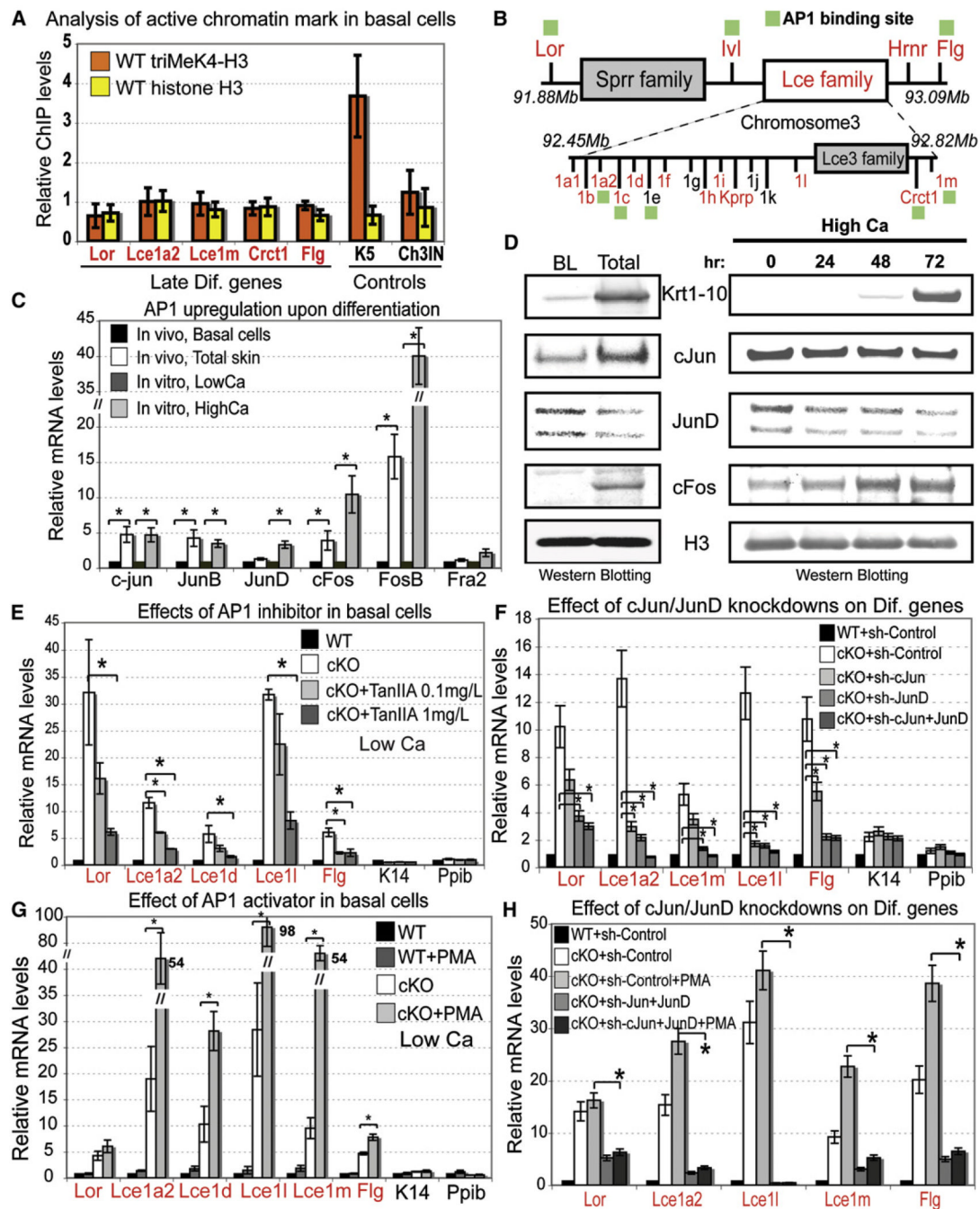
genes. No obvious changes were revealed in the basal expression of key epidermal transcriptional regulators when *Ezh2* was absent (B).

(C) Semiquantitative RT-PCR of mRNAs from FACS-purified E16 basal progenitors confirms the precocious induction of late differentiation genes when *Ezh2* is absent. Semiquantitative RT-PCR with primers against the *HPRT* gene was used for normalization. Data are mean  $\pm$  SD. n = 2. \*p < 0.05.

(D) Schematic representation of the epidermal differentiation cluster (EDC) on mouse chromosome 3. Late-stage differentiation EDC genes that were upregulated in *Ezh2*-deficient basal cells are marked in red.

(E) ChIP analyses reveal the presence of triMeK27-H3 at promoters of many genes within the EDC in WT and reduction in *Ezh2* cKO cells. Semiquantitative PCR with primers against the promoter of the active *K5* gene was used for normalization. Data are mean  $\pm$  SD. n = 2. \*p < 0.05.





**Figure 6. Transcriptional Activator AP1, Rather Than Activating Histone Modifications, Governs Upregulation of EDC Genes in Ezh2 cKO Basal Cells**

(A) ChIP analyses show no association of the active triMeK4-H3 mark with EDC genes in WT embryonic basal cells, in which the EDC is silent. Semiquantitative PCR with primers against the intergenic region on chromosome 5 was used for normalization. The basal *K5* gene was used as a positive control. Data are mean  $\pm$  SD.  $n = 2$ .

(B) AP1 consensus sites are found at promoters of EDC genes.

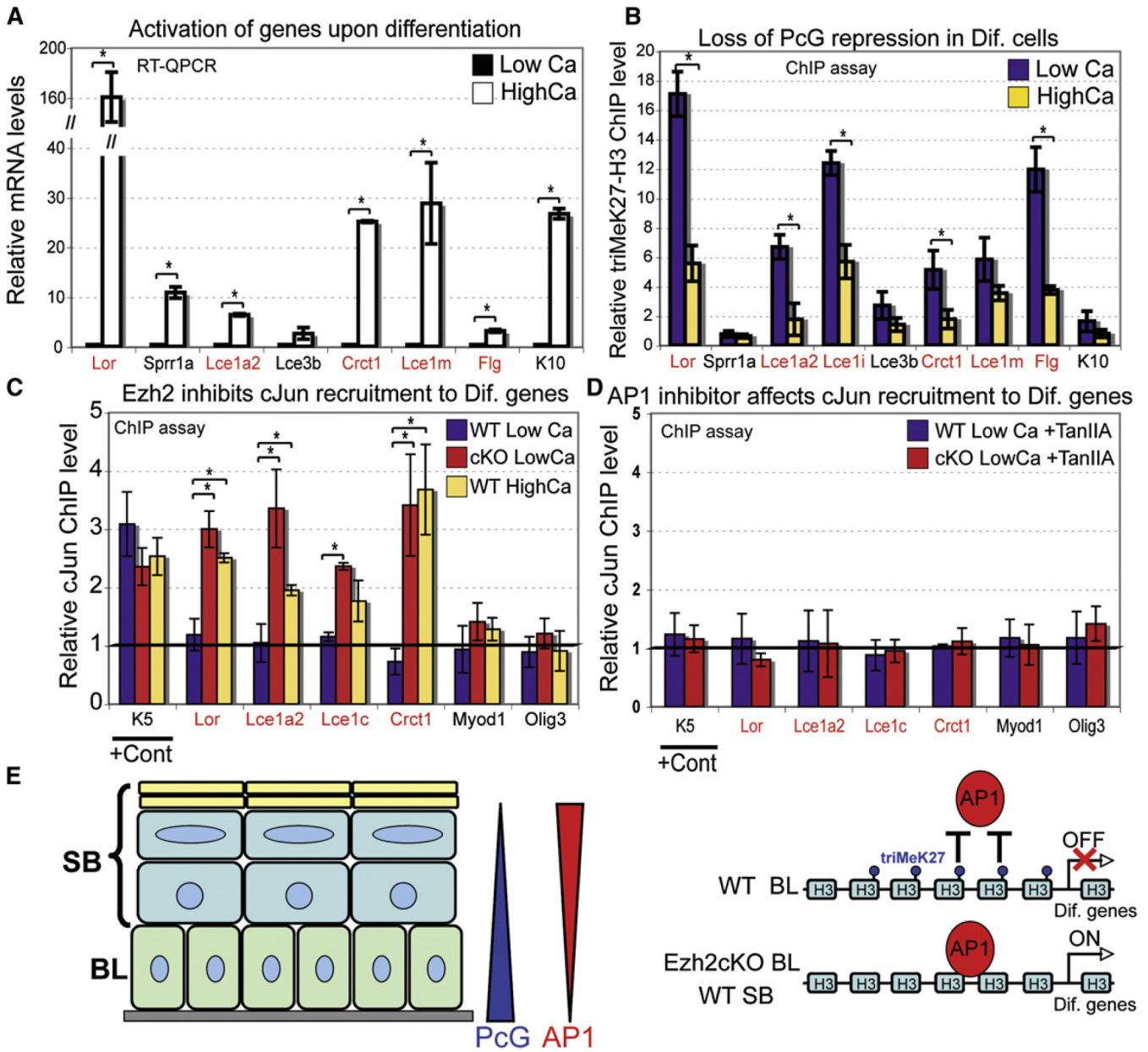
(C and D) Semiquantitative RT-PCR and western blotting detects Jun and Fos AP1 proteins in epidermis and cultured MK. Expression of some members is enhanced in terminally

differentiating cells. Positive control is HPRT (unchanged) and Flg (differentiated layers). Data are mean  $\pm$  SD. n = 2. \*p < 0.05.

(E and F) Semiquantitative RT-PCR shows that the precocious activation of EDC genes in *Ezh2* cKO basal progenitors is largely abrogated by either the AP1 inhibitor, TanIIA, or RNAi-mediated attenuation of cJun/JunD expression. Data are mean  $\pm$  SD. n = 2. \*p < 0.05.

(G and H) By contrast, PMA, which promotes AP1 activity, enhances precocious expression of EDC genes in *Ezh2* cKO, but not WT basal cells (G). EDC upregulation in PMA-treated *Ezh2* null cells is significantly lower when Jun and JunD are knocked down than in sh control (H). Data are mean  $\pm$  SD. n = 3. \*p < 0.05. Active *K14* and *Ppib* genes served as controls. BL, basal layer cells. LowCa, in vitro conditions that prevent differentiation of basal progenitors. HighCa, conditions that promote terminal differentiation.





**Figure 7. Opposing Roles for PcG Repressors and AP1 Transcriptional Activators in Epidermal Development and Differentiation**

(A and B) Semiquantitative RT-PCR shows that EDC genes are induced when WT basal cells commit to terminally differentiate (A), and ChIP analysis (B) shows that triMeK27-H3 marks associated with EDC genes in basal cells decline upon calcium-induced differentiation. Semiquantitative PCR with primers against promoter of the active *K5* gene was used for normalization (B).

(C and D) ChIP analysis shows cJun recruitment to promoters of late differentiation genes upon removal of Ezh2-dependent triMeK27-H3 mark in cKO basal cells or WT cells upon differentiation (C), but this recruitment is inhibited upon treatment of cells with AP1 inhibitor TanIIA (D). Presence of cJun at the *K5* promoter served as a positive control. No recruitment of cJun to *Myod1* and *Olig3* promoters that lack obvious AP1 sites was detected.

Semiquantitative PCR with primers against intergenic region on chromosome 5 was used for normalization. Data are mean  $\pm$  SD. n = 2. \*p < 0.05.

(E) Differential expression of PcG repressor and Ap1 activator ensures spatial and temporal program of epidermal differentiation. In WT basal cells, the presence of triM3K27-H3 mark prevents AP1 from binding and activation of late differentiation genes. In Ezh2cKO or in differentiated WT cells, loss of triMeK27-H3 mark allows Ap1 to bind and activate transcription of late differentiation genes.