Ring1b bookmarks genes in pancreatic embryonic progenitors for repression in adult β cells

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Polycomb-mediated gene repression is essential for embryonic development, yet its precise role in lineage-specific programming is poorly understood. Here we inactivated *Ring1b*, encoding a polycomb-repressive complex 1 subunit, in pancreatic multipotent progenitors (*Ring1b*^{progKO}). This caused transcriptional derepression of a subset of direct *Ring1b* target genes in differentiated pancreatic islet cells. Unexpectedly, *Ring1b* inactivation in differentiated islet β cells (*Ring1b*^{BKO}) did not cause derepression, even after multiple rounds of cell division, suggesting a role for *Ring1b* in the establishment but not the maintenance of repression. Consistent with this notion, derepression in *Ring1b*^{progKO} islets occurred preferentially in genes that were targeted de novo by *Ring1b* during pancreas development. The results support a model in which *Ring1b* bookmarks its target genes during embryonic development, and these genes are maintained in a repressed state through *Ring1b*-independent mechanisms in terminally differentiated cells. This work provides novel insights into how epigenetic mechanisms contribute to shaping the transcriptional identity of differentiated lineages.

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Throughout embryonic development, an interplay between positive and negative regulatory mechanisms promotes dynamic changes in chromatin structure that set up lineage-specific transcriptional programs. Although current knowledge supports this general model, the exact manner in which chromatin regulatory complexes contribute to differentiation programs remains to be defined.

One of the key mechanisms to create repressive chromatin states is mediated by Polycomb group (PcG) proteins (Sparmann and van Lohuizen 2006; Schwartz and Pirrotta 2007; Schuettengruber and Cavalli 2009; Vidal 2009; Margueron and Reinberg 2011). PcG proteins form two major families of complexes, named Polycomb-repressive complex 1 (PRC1) and PRC2. PRC2 comprises Eed, Suz12, and two related subunits, Ezh1 and Ezh2, that catalyze the trimethylation of histone H3K27 (H3K27me3).

PRC1 complexes include Bmi1, Cbx proteins, Mel18, and the ring finger proteins Ring1a and Ring1b (Vidal 2009). Among these, Ring1b has a primary role in catalyzing another post-translational histone modification, H2A K119 ubiquitination (de Napoles et al. 2004; Wang et al. 2004). Ring1b-mediated repression is linked to H2A ubiquitination, although Ring1b also promotes chromatin compaction independently of this histone-modifying activity (Eskeland et al. 2010). Thus, many of the molecular components and mechanisms underlying PcG-mediated repression have begun to be unraveled.

Multiple lines of evidence indicate that PcG proteins are pivotal for embryonic developmental programs. Notably, several *Drosophila* and mouse PcG mutants have shown embryonic lethality or homeotic transformations due to derepression of Hox genes (e.g., Lewis 1978; O'Carroll et al. 2001; Voncken et al. 2003). Furthermore, PcG proteins show a strong preference to bind inactive genes that encode for developmental regulators, and PcG-dependent modifications are dynamically placed and removed at key lineage regulatory genes throughout pancreatic and neural differentiation (Boyer et al. 2006;

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Bracken et al. 2006; Lee et al. 2006; Mohn et al. 2008; van Arensbergen et al. 2010). The precise role of PcG proteins in developmental programs, however, is still poorly understood. In part, this is because many germline PcG mutant models exhibit early lethality (Faust et al. 1998; O'Carroll et al. 2001; Voncken et al. 2003; Purity et al. 2005). A good example is provided by *Ring1b* mutations, which (unlike *Ring1a* deficiency) causes defective gastrulation and embryonic lethality (del Mar Lorente et al. 2000; Voncken et al. 2003). For viable genetic mutations, including *Bmi1*^{-/-} or conditionally inactive *Ezh2* mice, studies have so far largely uncovered severe self-renewal defects (e.g., Jacobs et al. 1999; Chen et al. 2009; Dhawan et al. 2009). Recent studies, however, have begun to address how PcG proteins control cellular differentiation from tissue-specific progenitors. For example, during normal differentiation of epidermal progenitors, *Ezh2* levels are down-regulated, leading to the activation of genes associated with epidermis differentiation (Ezhkova et al. 2009). Consequently, inactivation of *Ezh2* in basal skin progenitors has led to premature epidermal differentiation. In the ventral foregut endoderm, *Ezh2* has been shown to restrict the pancreatic fate choice (Xu et al. 2011). Other experiments suggest that *Ezh2* and *Ring1b* restrict neurogenesis to early developmental stages by repressing the proneural genes *Neurogenin1* and *NeuroD1* during the later astrogenic stage (Hirabayashi et al. 2009; Roman-Trufero et al. 2009). It is thus evident that PcG-dependent repression plays crucial developmental roles, although there is still a limited understanding of the detailed roles that PcG-mediated repression plays throughout different stages of lineage-specific programs.

After the specification and differentiation of cellular lineages, transcriptional states are maintained throughout multiple rounds of cell division. PcG-dependent repressive mechanisms have also been proposed to underlie long-term maintenance of cellular identity (Ringrose and Paro 2007). However, ablation of PcG genes in differentiated cells has led to proliferative defects without obvious loss of cellular identity, although genetic studies reported so far have not directly addressed effects on the transcriptional programs of differentiated lineages (e.g., Chen et al. 2009; Juan et al. 2011). Thus, further studies are required to address whether PcG proteins maintain the cellular identity of differentiated cells.

In this study, we created conditional mutations of the PRC1 subunit gene *Ring1b* to address the stage-specific functions of PcG-mediated repression during the embryonic differentiation of pancreatic β cells. Our results show that during embryonic differentiation, *Ring1b* is required to establish the transcriptional repression of target genes in the differentiated β -cell lineage, despite the fact that the maintenance of this repression in terminally differentiated β cells is independent of *Ring1b*. We created cell lines from mice with stage-specific mutations to demonstrate that the transcriptional phenotypes are mitotically stable and integrated expression and occupancy studies to show that they reflect a direct function of *Ring1b*. The results therefore reveal separate mechanisms that either establish or maintain the repression of a discrete set of

genes in a cellular lineage and provide novel insights into how PcG-mediated repression contributes to shaping the transcriptional identity of pancreatic β cells.

Results

Stage-specific inactivation of Ring1b during β -cell differentiation

To study the stage-specific functions of PRC1 during pancreatic β -cell development, we crossed mice with a conditional *Ring1b* LoxP allele and either Pdx1-Cre or Ins-Cre transgenic lines (Fig. 1A; Herrera 2000; Gu et al. 2002; Cales et al. 2008). *Ring1b* is expressed in multipotent embryonic pancreatic progenitors, the mesenchyme, and the adult islet β cells (Fig. 1B,E,G,I). The Pdx1-Cre transgene efficiently deleted *Ring1b* in embryonic pancreatic progenitors and adult β cells (*Ring1b*^{progKO}), while Ins-Cre caused efficient recombination in lineage-committed β cells (*Ring1b* ^{β KO}) (Fig. 1C,D,F,H,J).

Early but not late Ring1b inactivation leads to impaired islet endocrine function

To assess the consequences of stage-specific ablation of *Ring1b* in pancreatic cells, we first studied glucose tolerance in 4-mo-old mice. *Ring1b*^{progKO} mice displayed increased blood glucose levels during a meal test (Fig. 2A) and after intraperitoneal injection of glucose (Fig. 2B). This impairment was due to decreased blood insulin levels (Fig. 2C). In sharp contrast, *Ring1b* ^{β KO} mice showed normal glucose tolerance and insulin secretion (Fig. 2E–G). Thus, early but not late *Ring1b* inactivation caused decreased insulin output and glucose intolerance.

We next assessed whether glucose intolerance in *Ring1b*^{progKO} mice resulted from an abnormal number of β cells. Previous studies showed that deficiency of other PcG subunits induces transcripts from the *Cdkn2a* locus, leading to reduced β -cell growth (Chen et al. 2009; Dhawan et al. 2009). *Ring1b*^{progKO} and *Ring1b* ^{β KO} islets showed moderately increased *Cdkn2a* transcripts (Supplemental Fig. 1), yet this was not sufficient to cause significant changes in β -cell mass (Fig. 2D,H; Supplemental Fig. 2A). Thus, the inactivation of *Ring1b* during early pancreatic development results in a functional impairment of adult β cells, whereas *Ring1b* inactivation in lineage-committed β cells does not.

Early but not late Ring1b inactivation causes derepression of a subset of Ring1b targets

To investigate whether the impairment of islet endocrine function resulted from a transcriptional perturbation of *Ring1b* targets, we assessed transcriptional changes in *Ring1b*^{progKO} islets. Consistent with the fact that *Ring1b* is a component of a transcriptional repressive complex, we observed a marked derepression phenotype in *Ring1b*^{progKO} islets, with significantly more probes showing up-regulation ($n = 398$) than down-regulation ($n = 179$; $P < 2.2 \times 10^{-16}$) (Fig 3A; Supplemental Table 1).

To assess whether this transcriptional response reflected a derepression of direct *Ring1b* targets, we used

Figure 1. Stage-specific inactivation of *Ring1b* in β -cell development. (A) Schematic of the Cre transgenic models used for *Ring1b* inactivation. Pdx1-Cre inactivates *Ring1b* in embryonic multipotent pancreatic progenitors (*Ring1b*^{progKO}) and Ins-Cre in lineage-committed β cells (*Ring1b*^{betaKO}). (B) *Ring1b* is expressed in embryonic day 11.5 (E11.5) pancreatic progenitors ($n = 2$) and in islets from young (14 wk; $n = 4$) and 1-yr-old ($n = 2$) mice. (C,D) *Ring1b* is efficiently excised in *Ring1b*^{progKO} and *Ring1b*^{betaKO} islets ($n = 5$). Levels were normalized for *Actb* mRNA and are expressed relative to *Ring1b*^{+/+} littermate controls. Note that β cells represent $\sim 60\%$ of islet cells. Error bars indicate the SEM. (E,F) Immunofluorescence analysis of *Ring1b* (green), Pdx1 (red), and Topro (blue) shows enriched *Ring1b* expression in the multipotent progenitor trunk domain of *Ring1b*^{+/+} E13.5 embryos (E), whereas it is absent in *Ring1b*^{progKO} embryos (F). (G,H) Immunofluorescence analysis of *Ring1b* (green), insulin (red), and Topro (blue) shows enriched *Ring1b* expression in the pancreatic islets of 3-mo-old *Ring1b*^{+/+} animals (G), whereas it is absent in *Ring1b*^{progKO} islets (H). (I,J) Same as G and H, but for *Ring1b*^{betaKO} islets. The white arrows indicate "escaper" cells that have not lost *Ring1b*.

tiling arrays to profile *Ring1b* binding in islet cells (Supplemental Table 1). We identified 448 genes bound by *Ring1b* in islets and, consistent with previous studies in embryonic stem (ES) cells, found that they form a subset of the genes that are enriched in H3K27me3 in islets (Fig. 3B; Ku et al. 2008).

Of all of the *Ring1b* target genes, 5.6% were significantly up-regulated in *Ring1b*^{progKO} islets, in contrast to only 1.4% of non-*Ring1b* target genes ($P = 1.2 \times 10^{-12}$) (Fig. 3C). Gene set enrichment analysis (GSEA) consistently showed that *Ring1b*-bound genes were enriched among up-regulated genes ($P < 0.001$) (Fig. 3D). Likewise, 28% of genes that were up-regulated *Ring1b*^{progKO} islets showed H3K27me3 enrichment in wild-type islets, indicating that they were PcG targets, whereas H3K27me3 enrichment was observed in 15% of all genes ($P = 6.9 \times 10^{-11}$). Thus, genes bound by *Ring1b*—and, more generally, PcG targets—were preferentially derepressed in *Ring1b*^{progKO} islets, although only a subset of *Ring1b* targets was perturbed.

We next selected 15 genes that were bound by *Ring1b* in islets (Fig. 3E), of which 10 showed up-regulation and five showed no change in *Ring1b*^{progKO} islets in the gene chip analysis, and confirmed these findings in all cases by quantitative RT-PCR (qRT-PCR) (Fig. 3F). All 15 *Ring1b* target genes were then examined in islets from adult *Ring1b*^{betaKO} mice. In keeping with their normal glucose tolerance, the expression of *Ring1b* target genes was unperturbed (Fig. 3G). Thus, *Ring1b* deletion in pancreatic progenitors led to derepression of a subset of *Ring1b* target genes in differentiated islet cells, whereas no changes were encountered when *Ring1b* was deleted in lineage-committed β cells.

Early *Ring1b* inactivation causes islet misexpression of neural and disallowed genes

To further understand the role of *Ring1b*-dependent repression in pancreatic endocrine programming, we

Figure 2. Early but not late inactivation of *Ring1b* leads to glucose intolerance. (A,E) Post-prandial blood glucose is significantly increased in *Ring1b*^{progKO} ($n = 7$) versus *Ring1b*^{+/+} littermate controls ($n = 10$; $*P = 0.0046$), whereas no differences were observed in *Ring1b*^{βKO} ($n = 10$) versus *Ring1b*^{+/+} littermate controls ($n = 10$). (B,F) Impaired intraperitoneal glucose tolerance in *Ring1b*^{progKO} ($n = 7$) versus *Ring1b*^{+/+} littermate controls ($n = 8$) but not in *Ring1b*^{βKO} ($n = 8$) versus *Ring1b*^{+/+} littermate controls ($n = 7$; $*P < 0.05$). (C,G) Impaired insulin levels in response to an intraperitoneal glucose challenge in *Ring1b*^{progKO} ($n = 11$) versus *Ring1b*^{+/+} littermate controls ($n = 9$) but not in *Ring1b*^{βKO} mice ($*P = 0.01$). (D,H) No significant differences in β-cell mass were observed between *Ring1b*^{progKO} ($n = 4$) and *Ring1b*^{+/+} littermate controls ($n = 4$; $P = 0.54$) or *Ring1b*^{βKO} ($n = 3$) and *Ring1b*^{+/+} littermate controls ($n = 3$; $P = 0.50$). Error bars indicate the SEM.

focused on the subset of genes that were derepressed in *Ring1b*^{progKO} islets. This gene set was enriched in neural developmental regulators ($P = 5.4 \times 10^{-7}$) (Fig. 4A) and in genes that showed a tissue-specific gene expression pattern in the brain and cerebellum ($P < 0.05$) (Fig. 4B; Supplemental Fig. 3A). Thus, neural-enriched genes that are normally silent in islets, such as *Rab3c*, *Mkx*, and *Kcnj3*, were up-regulated in *Ring1b*^{progKO} islets. Of note, *Npy* is normally detected in scarce control β cells but was expressed in a sixfold higher number of *Ring1b*^{progKO} β cells (Supplemental Fig. 3B–D). Thus, *Ring1b* deletion in pancreatic progenitors causes abnormal islet expression of neural-enriched and neuroendocrine genes.

We recently showed that “β-cell disallowed genes”—a term that refers to genes that are selectively inactive in β cells and have been proposed to be deleterious for β-cell function (Pullen et al. 2010; Thorrez et al. 2011)—are selectively targeted by PcG-mediated repression during late differentiation (van Arensbergen et al. 2010). The current analysis showed that this set of genes was also enriched among genes that are up-regulated in *Ring1b*^{progKO} islets ($P < 0.001$) (Fig. 4C). Thus, during embryonic development, *Ring1b* establishes repressed states that refine the neuroendocrine phenotype of islet cells and prevent inappropriate gene activity in differentiated cells.

Early *Ring1b* inactivation prevents repression of *de novo* *Ring1b* targets

We next examined why only a subset of *Ring1b* targets was derepressed in *Ring1b*^{progKO} islets. We reasoned that if *Ring1b* is solely required to establish repressed chromatin rather than maintain it, genes that were already targeted by *Ring1b* in pluripotent cells prior to inactivation of *Ring1b* in pancreatic progenitors should not be affected in *Ring1b*^{progKO} islets. In contrast, genes that are targeted by *Ring1b* *de novo* during pancreas development should be perturbed if *Ring1b* is inactivated at this early stage.

To identify *Ring1b* targets that are established *de novo* during embryonic development, we profiled *Ring1b* in ES cells. We found that of all 448 islet *Ring1b* targets, 90 (20.1%) were not bound in ES cells (Supplemental Table 1). These were classified as *de novo* *Ring1b* targets (Fig. 5A). In keeping with our predictions, *de novo* islet *Ring1b* targets were more frequently up-regulated in *Ring1b*^{progKO} islets than targets that were already bound by *Ring1b* in ES cells (12.2 vs. 3.9%, respectively; $P = 0.005$) (Fig. 5B). Likewise, GSEA showed that *de novo* *Ring1b* targets were preferentially enriched among up-regulated genes (Fig. 5C). Thus, loss of *Ring1b* at the pancreatic progenitor stage preferentially affects genes that are targeted *de novo* by PcG proteins during embryogenesis, rather than those

Figure 3. Derepression of Ring1b targets in *Ring1b*^{progKO} islets. (A) *Ring1b*^{progKO} islets exhibit a transcriptional derepression phenotype. GeneChip expression analysis was performed with islets from adult *Ring1b*^{progKO} mice ($n = 3$) and control littermates ($n = 3$). Probes showing at least 1.5-fold up-regulation or down-regulation at $P < 0.05$ in *Ring1b*^{progKO} islets were binned in four groups according to their fold changes. Up-regulated probes were significantly more common than down-regulated probes ($P < 2.2 \times 10^{-16}$). (B) Venn diagram showing the overlap of H3K27me3 targets and Ring1b targets in islets (the overlap is 407 out of 448). (C) Ring1b targets were preferentially up-regulated in *Ring1b*^{progKO} islets. Transcript up-regulation in *Ring1b*^{progKO} islets was approximately fourfold more frequent in Ring1b-bound than nonbound genes ($[*] P = 1.2 \times 10^{-12}$), whereas transcript down-regulation did not differ between the two groups. (D) GSEA showed that genes bound by Ring1b in islets were enriched among genes that showed a high expression fold change in *Ring1b*^{progKO} islets ($P < 0.001$). An analogous enrichment analysis is shown for a random gene set of the same size. (E) H3K27me3 and Ring1b-binding patterns in mouse islets for selected Ring1b target genes that were either up-regulated or unperturbed in *Ring1b*^{progKO} islets. (F,G) qRT-PCR assessment of direct Ring1b target genes in *Ring1b*^{progKO} (F, gray bars; $n = 3$) and *Ring1b*^{betaKO} (G, gray bars; $n = 3$) islets. Levels were normalized for *Actb* mRNA and are expressed relative to *Ring1b*^{fl/fl} littermate controls (black bars; $n = 3$). This confirmed that many direct Ring1b target genes were unaffected in both Ring1b-deficient models (left panels), whereas a subset of targets were selectively up-regulated in *Ring1b*^{progKO} but not *Ring1b*^{betaKO} islets (right panels). Error bars indicate the SEM.

that were already targeted during earlier stages. Consistent with this idea, islet Ring1b targets that showed up-regulation in *Ring1b*^{progKO} islets acquired H3K27me3 de novo throughout normal pancreas development, whereas islet Ring1b targets that were unaffected in *Ring1b*^{progKO} mice already displayed H3K27me3 enrichment at the pancreatic progenitor stage (Fig. 5D,E; Supplemental Fig. 4). Thus, *Ring1b* inactivation in pancreatic progenitors preferentially alters the expression of genes that are targeted de novo by Ring1b during late pancreatic development while leaving genes that are targeted by Ring1b prior to *Ring1b* deletion in pancreatic progenitors largely unperturbed. These findings further support a role for Ring1b in establishing rather than maintaining β -cell-repressive programs.

Silencing of Ring1b targets is mitotically inherited in *Ring1b*^{betaKO} β cells

The observation that Ring1b targets are derepressed after gene ablation in progenitors but not after gene ablation in differentiated islets could theoretically reflect a defect in mitotic transmission of repressed states, given that more rounds of cell divisions occur from gene deletion to analysis in the early ablation model. To address this possibility, we generated immortalized β -cell lines from *Ring1b*^{progKO} and *Ring1b*^{betaKO} mice (Fig. 6A) using RIP-Tag transgenic mice expressing the SV40 large T-antigen in β cells (Hanahan 1985). β -Cell lines were maintained in culture for >20 estimated cell divisions. We analyzed five cell lines from separate animals for each genotype and tested six

Figure 4. Misexpression of neural and disallowed genes in *Ring1b*^{progKO} islets. (A) The top categories of gene ontology analysis of up-regulated genes in *Ring1b*^{progKO} islets. $P < 0.05$ after Benjamini correction for all uncorrected P -values shown in the table. (B) Genes that were up-regulated in *Ring1b*^{progKO} islets were frequently neural-specific ([*] $P < 0.05$ for all comparisons of cortex or cerebellum with all other tissues). Tissue-specific genes were defined as those expressed in that tissue and no more than two other tissues. (C) GSEA showed that genes that have been classified as disallowed in β cells were enriched among genes up-regulated in *Ring1b*^{progKO} islets ($P < 0.001$). An analogous enrichment analysis is shown for a random gene set of the same size ($P = 0.661$).

genes that were up-regulated in the *Ring1b*^{progKO} islets. In keeping with our observations in native islets, five out of six of these genes were consistently up-regulated in the *Ring1b*^{progKO} β -cell lines (Fig. 6B–E; Supplemental Fig. 5). Remarkably, all of these genes remained repressed in *Ring1b*^{BKO} β -cell lines, despite multiple rounds of cell divisions. Thus, *Ring1b*-deficient β cells are capable of mitotically propagating repressed states that were established in a *Ring1b*-dependent manner during pancreatic development. This finding further supports that *Ring1b* is not essential to maintain transcriptional repression of its target genes in differentiated β cells.

Ring1b is required in pancreatic progenitors for target gene DNA methylation in differentiated islet cells

We next assessed molecular mechanisms that could underlie the maintenance of repression in the *Ring1b*^{BKO}

β -cell lines in genes that showed up-regulation in the *Ring1b*^{progKO} β -cell lines. We first confirmed that *Ring1b* was bound to these genes in the cell lines (Fig. 7A). Interestingly, the related protein *Ring1a* was not strongly enriched at these same genes in control β cells, and, importantly, neither *Ring1a* nor *Bmi1* (another PRC1 subunit) showed increased binding in mutant islets (Fig. 7B; Supplemental Fig. 6A). This result argued that gene silencing in *Ring1b*^{BKO} β cells was unlikely to be maintained by PcG-mediated repressive mechanisms.

We next hypothesized that DNA methylation was responsible for maintaining repression in control and *Ring1b*^{BKO} β cells. Accordingly, methylated DNA immunoprecipitation (MeDIP) showed that *Ring1b* targets had a decreased level of DNA methylation in *Ring1b*^{progKO} cells compared with control and *Ring1b*^{BKO} β cells (Fig. 7C). Furthermore, H3K9me3, a histone mark in certain cases associated with DNA methylation (Hashimoto et al. 2010), displayed a similar trend in *Ring1b*^{progKO} β cells (Supplemental Fig. 6B). These findings thus suggest that *Ring1b*-independent repressive mechanisms—namely, DNA methylation and H3K9me3—are associated with the maintenance of a repressed state that is initiated in a *Ring1b*-dependent manner in pancreatic progenitors.

Discussion

Several distinct mechanisms are known to promote repressed chromatin states. The specific role that each mechanism plays in controlling cell fate decisions, differentiation, and long-term phenotypic maintenance, however, remains elusive. In the present study, we showed that *Ring1b* acts in pancreatic embryonic progenitors to specify the transcriptional program of differentiated β cells. We demonstrated that *Ring1b* function in pancreatic progenitors is essential to establish transcriptional repression and DNA methylation of selected target genes in differentiated β cells, whereas after cells have differentiated, *Ring1b* is no longer required to maintain the repression of these same target genes. The data therefore indicate that during lineage-specific differentiation, *Ring1b* bookmarks genes that are later locked in a stably repressed state through *Ring1b*-independent mechanisms.

Ring1b is used to refine the transcriptional program of differentiated β cells

Earlier studies showed that the differentiation of β cells entails the selective removal of PcG-mediated repression from a core set of neural regulatory genes (van Arensbergen et al. 2010). This in turn enables the activation of a transcriptional program in β cells that is remarkably similar to that of ectodermally derived neural cell types (van Arensbergen et al. 2010). The current findings suggest that during the differentiation of β cells, PcG-mediated repression is also employed to suppress the activation of selected neural genes. Plausibly, this fulfills a need to repress the indiscriminate activation of neural

Figure 5. Early loss of Ring1b leads to misexpression of de novo Ring1b targets. (A) Cluster representation of Ring1b-bound genes (blue) based on binding in ES cells and islets. The right column shows genes up-regulated in *Ring1b*^{progKO} islets in black. (B) Percentage of up-regulated genes among the three groups of Ring1b targets. De novo Ring1b targets are more frequently up-regulated than pre-existing Ring1b targets ($[*] P = 0.005$). (C) GSEA of de novo and pre-existing islet Ring1b targets among genes ranked according to their fold change in expression in *Ring1b*^{progKO} islets. Islet de novo Ring1b targets displayed strong enrichment among up-regulated genes ($P < 0.001$, maximum enrichment score = 0.52), while weaker enrichment was observed for pre-existing Ring1b targets ($P = 0.0011$, maximum enrichment score = 0.19). The two bars below the graph depict the rank position of de novo (top) and pre-existing (bottom) Ring1b targets. (D,E) H3K27me3 enrichment in wild-type pancreatic progenitors (dark red) and wild-type islets (light red) for Ring1b targets that were unperturbed ($n = 418$) (D) or up-regulated ($n = 28$) (E) in *Ring1b*^{progKO} islets. Enrichment is quantified as average posterior probability (post. prob.). Plots that show H3K27me3 as a fold enrichment value show the same pattern (Supplemental Fig. 4).

genes in cells that express many neural transcriptional activators. Existing data thus suggest that during pancreas development, PcG-dependent repression is selectively removed from neuroendocrine regulatory genes that promote islet cell differentiation, although, concomitantly, PcG-mediated repression is used to repress neural-enriched genes that do not form part of the pancreatic endocrine program.

Epigenomic profiling in pancreatic islet cells has also shown that PcG-mediated repression targets disallowed genes in β cells, defined as genes that are selectively absent and potentially deleterious for β cells (Thiel and Schuit 2008; Pullen et al. 2010; van Arensbergen et al. 2010). The present study shows that Ring1b not only binds to such genes, but is also required in pancreatic progenitors to establish their repression in β cells.

The transcriptional changes observed in *Ring1b*^{progKO} islet cells led to abnormal glucose intolerance due to defective β -cell insulin secretory function. One of the disallowed genes that we identified as up-regulated in

Ring1b-deficient islets was *Acot7*, encoding a type II acyl-CoA thioesterase. *Acot7* displays a preference for medium-chain-length acyl-CoA esters (Kirkby et al. 2010), which are implicated in the gating of ATP-sensitive K^+ channels and the control of insulin granule exocytosis (Corkey et al. 2000). The absence of *Acot7* from normal β cells may thus allow acyl-CoA to reach high levels, modulating both glycolytic flux and late events in insulin granule release. Consistent with this prediction, forced overexpression of *Acot7* in β -cell lines leads to impaired glucose and KCl-stimulated insulin secretion (GA Rutter and TJ Pullen, unpubl.). This enzyme is thus a plausible candidate contributor to the defective secretion in *Ring1b*^{progKO} mice. Another gene that is up-regulated in *Ring1b*-deficient islets is *Npy*, which is known to suppress cAMP levels and inhibit insulin secretion and can therefore also contribute to the in vivo phenotype of *Ring1b*^{progKO} mice (Wang et al. 1994; Myrsen-Axcrona et al. 1997). In addition to *Acot7* and *Npy*, we do not exclude roles of other up-regulated genes that could contribute to abnormal

Figure 6. Mitotic inheritance of repression patterns. (A) Schematic representing the generation of β-cell lines. We crossed *Ring1b^{progKO}* and *Ring1b^{betaKO}* mice with RIP-Tag transgenics to generate insulinomas. *Ring1b*-deficient β-cell lines were established from individual adult animals per genotype. (B–E) qRT-PCR analysis of *Irx2*, *Acot7*, *Slc16a1*, and *Higd1a* in cell lines established from wild-type (*n* = 5), *Ring1b^{+/+}* littermate control (*n* = 5), *Ring1b^{betaKO}* (*n* = 5), and *Ring1b^{progKO}* (*n* = 5) mice. *Ring1b^{progKO}* cell lines displayed derepression of *Ring1b* targets, whereas repression was maintained in the *Ring1b^{betaKO}* cell lines despite >20 rounds of cell division. Error bars indicate the SEM.

β-cell function due to a collective transcriptional defect of differentiated β cells.

A developmental bookmarking function of *Ring1b*

PcG complexes control numerous developmental functions, including anteroposterior patterning, gastrulation, X inactivation, stem cell differentiation, and genomic imprinting (Sparmann and van Lohuizen 2006; Margueron and Reinberg 2011). Furthermore, PcG complexes target developmental regulatory genes, consistent with a pivotal

role in development (Boyer et al. 2006; Bracken et al. 2006; Lee et al. 2006). However, the precise spatiotemporal roles of PcG proteins in lineage-specific differentiation programs are only beginning to be understood.

One limitation to understanding PcG-dependent programming has been that its deficiency often leads to premature senescence or cell death, resulting in the abrogation of the lineage of interest. Recent studies have nevertheless revealed how the PcG subunit *Ezh2* controls the differentiation of lineage-specific precursors. Thus, in skin progenitors, *Ezh2* deficiency leads to the activation

Figure 7. *Ring1b* is required in pancreatic progenitors for target gene DNA methylation in differentiated islet cells. (A,B) Chromatin immunoprecipitation (ChIP) analysis of *Ring1b* and *Ring1a* occupancy at direct *Ring1b* targets in cell lines established from *Ring1b^{+/+}* littermate controls (black bar; *n* = 3) and *Ring1b^{betaKO}* (light-gray bar; *n* = 3) and *Ring1b^{progKO}* (dark-gray bar; *n* = 3) mice. The dotted line indicates enrichment = 1. (C) As in A and B, but here, MeDIP was performed (*n* = 4). Enrichments are shown relative to controls after normalization for *Tbp*. Error bars indicate the SEM.

of genes that promote differentiation, whereas the inactivation of *Ezh2* in skeletal muscle or heart progenitors causes altered lineage differentiation (Ezhkova et al. 2009; Juan et al. 2011; Delgado-Olguin et al. 2012). Furthermore, *Ezh2* deficiency causes altered cell fate decisions of foregut endoderm multipotent progenitors (Xu et al. 2011). These results have highlighted a crucial role for *Ezh2* in establishing the transcriptional fidelity of organ-specific progenitors and their offspring.

In the present study, we exploited the fact that *Ring1b* deficiency does not disrupt pancreatic organogenesis or cell growth to understand stage-specific functions of PcG-mediated repression during lineage programming. Our results uncouple the mechanisms that establish and maintain the repression of a discrete set of genes in a cellular lineage. *Ring1b* was essential to set up repression when it first associated with specific loci, whereas repression of those loci thereafter became *Ring1b*-independent despite maintained *Ring1b* occupancy. This transient function of *Ring1b* in pancreatic progenitors was linked to the acquisition of DNA methylation at target loci in terminally differentiated cells. Based on these findings, we propose a model in which *Ring1b* marks genes for repression as lineages are formed during pancreas organogenesis, and once the terminally differentiated β -cell lineage is established, its transcriptional state is maintained through alternate mechanisms.

Ring1b is not required to maintain the differentiated β -cell phenotype

Ring1b was dispensable to maintain the transcriptional program of differentiated β cells. Interestingly, *Ezh2* deficiency in differentiated β cells only causes a cell growth phenotype that appears completely rescued by the inactivation of the cell cycle regulator *Cdkn2a* (Chen et al. 2009), consistent with other studies showing a critical role of PcG-dependent repression in controlling the growth of differentiated cells (e.g., Jacobs et al. 1999; Bracken et al. 2007). On the other hand, existing studies have shown diverse PcG-dependent functions during development to either control cell fate choices or the timing of differentiation or, as shown in the present study, refine the transcriptional program of differentiated cellular offspring. To our knowledge, however, no study has demonstrated that the inactivation of a PcG subunit in mature cells results in a global modification of its transcriptional program. This contrasts with observations that removal of DNA methylation in differentiated β cells causes transcriptional perturbations that cause cellular transdifferentiation events, suggesting that DNA methylation is a dominant mechanism for maintenance of cell identity in differentiated β cells (Dhawan et al. 2011). Taken together, existing data support a prominent role for PcG-mediated repression during developmental programming of cellular identities, whereas direct evidence for PcG-dependent maintenance of adult terminally differentiated phenotypes is still lacking.

More generally, genetic experiments have provided insight into the complexity of epigenetic regulation of

development. They illustrate how different regulatory subunits play highly stage-dependent roles in lineage differentiation. A detailed understanding of the specialized functions of epigenetic regulators, together with ongoing efforts to generate a plethora of compounds that target this class of proteins, should provide opportunities to manipulate differentiation programs for disease modeling and therapeutics.

Materials and methods

Mouse models

Mice with *Ring1b* LoxP alleles as well as RipTag, Ins-Cre, and Pdx1-Cre transgenic lines have been described (Hager and Hanahan 1999; Herrera 2000; Gu et al. 2002; Cales et al. 2008). All experiments were approved by the Institutional Animal Care Committee of the University of Barcelona.

RNA analysis

Mouse pancreatic islets were isolated from 12- to 14-wk-old male mice as described previously (Luco et al. 2008). Total RNA was extracted from pancreatic islet preparations of three individual male animals for each genotype using Trizol (Invitrogen). RNA integrity was verified with a 2100 Bioanalyzer (Agilent), and labeled cRNA was hybridized to Affymetrix Mouse Genome 430 2.0 arrays. Data normalization was performed as described (van Arensbergen et al. 2010). Expression data on other mouse tissues were described previously (van Arensbergen et al. 2010).

Chromatin immunoprecipitation (ChIP)

ChIPs were performed essentially as described (van Arensbergen et al. 2010). In short, pancreatic islets or mouse ES cells (CGR8) (Skoudy et al. 2004) were fixed in 1% formaldehyde for 10 min, after which nuclei were purified and sonicated using a Bioruptor (Diagenode) to a length of 200–1000 base pairs (bp). Samples were precleared with protein A+G-Sepharose (1:1) and immunoprecipitated with rabbit anti-*Ring1b* (Garcia et al. 1999), rabbit anti-*Ring1a* (Schoorlemmer et al. 1997), rabbit anti-H3K9me3 (Abcam, 8898), rabbit anti-H3K27me3 (Upstate Biotechnology, 07-449), and mouse anti-Bmi1 (Millipore, 05-637) overnight at 4°C. Immune complexes were collected by adsorption to protein A+G-Sepharose for 2 h at 4°C. Beads were washed, and immunocomplexes eluted prior to DNA purification with Qiaquick columns (Qiagen).

For tiling array experiments, ChIP and input DNA were amplified as described previously using the Sigma GenomePlex WGA2 kit while adding dUTPs to a final concentration of 0.4 mM during the amplification reaction to enable subsequent fragmentation (van Arensbergen et al. 2010). We fragmented 6–7.5 μ g of DNA, labeled it using the Affymetrix GeneChip WT Double-Stranded DNA Terminal Labeling kit, and hybridized it to GeneChip Mouse Promoter 1.0R arrays. Primary processing of data was performed as described (van Arensbergen et al. 2010). H3K27me3 GeneChip data sets from ES cells, purified pancreatic embryonic progenitors, and islets were described previously (van Arensbergen et al. 2010).

MeDIP

Immunoprecipitation of methylated DNA was performed as described previously with minor modifications (Weber et al.

2005). In short, we isolated the DNA from ~3 million cells using a Proteinase K digest followed by phenol-chloroform extraction. Four micrograms of DNA was sonicated in 500 μ L of TE buffer using a Bioruptor (Diagenode). DNA was then denatured in boiling water for 10 min and cooled on ice. The DNA was then diluted in 500 μ L of 2 $\times$ precipitation buffer, and 2 μ L of mouse 5-methylcytidine antibody (Eurogentec, BI-MECY-0100) was added. From this step on, samples were treated as in the normal ChIP procedure described above.

Statistical and integrated data analysis

Significant enrichment in GeneChip ChIP experiments relative to input DNA was determined using Cisgenome (Ji et al. 2008). We applied a hidden Markov model as described (Ji and Wong 2005) and used a posterior probability cutoff of 0.5 in at least five continuous probes. Using identical criteria, <0.05% of sites detected with Ring1b antibodies were observed in IgG control ChIP experiments in pancreatic acinar tissue. Enriched intervals were mapped to RefSeq genes when located within 2000 bp of transcriptional start sites.

Differential RNA expression was assessed using the ANOVA statistical analysis of the Partek software package. Genes were considered differentially expressed with a fold difference of >1.5 and a *P*-value <0.05. The presence and absence values for expression in other mouse tissues used for comparisons were determined as described previously (van Arensbergen et al. 2010).

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional annotation tool was used to study the overrepresentation of annotation terms using all RefSeq genes as the background (Huang et al. 2009). Nonredundant gene ontology categories from the top “molecular function” and “biological process” terms were selected.

GSEA was performed on preranked gene expression data sets using the default weighted enrichment (Subramanian et al. 2005). Significance of the enrichment was assessed using 1000 permutations.

Differences in glucose, insulin, β -cell mass, and gene-specific expression in isolated islets were examined with Student's two-sided *t*-test. In the β -cell line studies, a Wilcoxon test was used to account for the nonnormal distribution of the data. When a single *P*-value is given for several comparisons, it represents the *P*-value for the least significant of the comparisons.

Differences in frequency distributions were assessed in R using Pearson's χ^2 test with Yates' continuity correction, except for results in Figure 4B, where Fisher's exact test was used because several entries in the table were smaller than five.

qPCR analysis

qPCR of reverse-transcribed RNA, ChIP, or MeDIP samples was performed on a 7300 Real-Time PCR system (Applied Biosystems) using the Power SYBR Green reagent (Applied Biosystems). Quantities were determined using the $2^{-\Delta\Delta C_t}$ method. A full list of the primers used is provided in Supplemental Table 2.

Immunofluorescence

Embryos were collected at indicated times and processed for immunofluorescence analysis of paraffin-embedded pancreas as previously described (Maestro et al. 2003). The primary antibodies were guinea pig anti-insulin (1:5000; C. Van Schravendijk), rabbit anti-glucagon (1:200; Dako), rabbit anti-NPY (1:1000; Sigma, N9528), goat anti-PDX1 (1:1000; Abcam, ab47383), and rabbit anti-Ring1b (1:1000) (Garcia et al. 1999).

Physiological tests

For meal tests, male age-matched animals were fasted for 16 h overnight, and blood glucose was measured before and 1 h after refeeding. For glucose tolerance tests, fasted animals were injected intraperitoneally with 2 mg of glucose per kilogram of body weight, and glucose levels were tested from tail blood at 0, 15, 30, 75, and 120 min after injection. Glucose was measured using the Glucocard G+ meter (Menarini Diagnostics). Insulin ELISA was performed using the Insulin ELISA kit of Mercodia (catalog no. 10-1247-01) and were read using a Synergy HT Multimode microplate reader (BioTek). Control littermates were Ring1b^{fl} littermates without the Cre transgene.

β -Cell mass analysis

The pancreases of 6-mo-old mice were dissected, weighed, folded to reduce their length, fixed, and embedded in OCT. Cryosections (6 μ m) were obtained at 30- μ m intervals throughout the organs. Approximately 60–70 cryosections were obtained from each pancreas. Immunofluorescence for DAPI and insulin was performed at each fifth slice. Images were taken by automated capturing and reconstruction of ~50 frames (Leica DMI 600B). Insulin-positive area and total tissue area were determined with in-house developed ImageJ macros and are available on request. In short, after manual curation of the merged RGB images, total tissue surface was determined by binarizing a diffused image for DAPI. β -Cell surface was determined by binarizing the insulin signal based on its relative intensities compared with the other two channels. These binarized images were then again manually curated, after which the β -cell mass was obtained by multiplying the ratio of the β -cell surface to total surface with the pancreas weight.

Ring1b-deficient β -cell lines

We performed crosses to generate mice that carried a RIP-Tag transgene alone or were, in addition, homozygous for the Ring1b^{LoxP} allele and carried either Ins-Cre, Pdx1-Cre, or no Cre transgene. We dissected β -cell tumors from 10- to 14-wk-old mice when they showed a basal blood glucose level <25 mg/dL. Tumors were rinsed in PBS and then ruptured to release the inner cells. These cells were transferred to 20 separate 96-well plates, each containing ~10,000 cells. Cells were monitored for proliferation and emergence of fibroblasts, and based on this, one cell line per individual animal was used for further analysis. Cells were cultured as normal Min6 β -cell lines in DMEM supplemented with 10% FBS, 70 μ M 2-mercaptoethanol, 2 mM L-glutamine, and penicillin–streptomycin. We selected five cell lines at passages 5–8, obtained from five different mice from each genotype, and, in all cases, confirmed genotypes.

Data access

Microarray data for RNA expression and ChIP experiments are publicly available through ArrayExpress under accession numbers E-MTAB-1404 and E-MTAB-1402, respectively.

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Ring1b bookmarks genes in pancreatic embryonic progenitors for repression in adult β cells

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