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Loss of the retinoblastoma binding protein 2 (RBP2) histone demethylase suppresses tumorigenesis in mice lacking Rb1 or Men1

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Aberations in epigenetic processes, such as histone methylation, can cause cancer. Retinoblastoma binding protein 2 (RBP2; also called JARID1A or KDM5A) can demethylate tri- and dimethylated lysine 4 in histone H3, which are epigenetic marks for transcriptionally active chromatin, whereas the multiple endocrine neoplasia type 1 (MEN1) tumor suppressor promotes H3K4 methylation. Previous studies suggested that inhibition of RBP2 contributes to tumor suppression by the retinoblastoma protein (pRB). Here, we show that genetic ablation of RBP2 decreases tumor formation and prolongs survival in Rb1−/− mice and Men1-defective mice. These studies link RBP2 histone demethylase activity to tumorigenesis and nominate RBP2 as a potential target for cancer therapy.

Epigenetic alterations, like genetic alterations, can contribute to tumor initiation and progression (1, 2). Indeed, a number of genes that play roles in chromatin modifications and hence, epigenetic regulation are mutated in human cancers, including mixed-lineage leukemia (MLL1), multiple endocrine neoplasia type 1 (MEN1), and ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX) (3–6). The retinoblastoma gene (RB1) tumor suppressor gene is frequently inactivated in a wide variety of cancers (7). The retinoblastoma protein (pRB) inhibits S-phase entry by repressing E2F (7). In addition, pRB promotes senescence and differentiation (8). These latter two activities track closely with the ability of pRB to induce cell cycle arrest and senescence in the presence of DNA damage (9). In addition, RBP2 belongs to a superfamily of 2-oxoglutarate–dependent dioxygenases (18, 19), which can be inhibited with drug-like small molecules (20, 21). We, therefore, used mice carrying null or conditional Rbp2 alleles to further explore potential roles for RBP2 in pRB-defective tumorigenesis. In addition, we tested the hypothesis that loss of RBP2 H3K4 demethylase activity would inhibit tumors driven by loss of the MEN1 tumor suppressor, which is part of an H3K4 methyltransferase complex (6, 22, 23).

Results

Loss of RBP2 Inhibits Proliferation and Induces Senescence. Mouse embryonic fibroblasts (MEFs) derived from Rbp2−/− embryos on a pure genetic background proliferated more slowly than MEFs derived from WT littermate controls, especially when examined at later passages (Fig. 1 A and B). Senescence-associated β-galactosidase (SABG) staining revealed increased staining of late-passage Rbp2−/− MEFs compared with WT control MEFs (Fig. 1 C and D), suggesting that RBP2 loss promotes senescence. To study the effect of acute RBP2 inactivation, we created mice that carry a conditional (floxed or f) Rbp2 allele (11) and a transgene encoding a Cre-ER fusion protein, which can be activated by tamofoxifen (24). Treatment of Rbp2fl/fl;Cre-ER MEFs with tamofoxifen led to growth arrest, but treatment of Rbp2fl/+; Cre-ER control MEFs did not lead to growth arrest (Fig. 1 E and F). Similar results were obtained when RBP2 was acutely deleted in Rbp2fl/fl MEFs using a retroviral vector encoding Cre recombinase (Fig. S1 A and B). Collectively, these results support the earlier conclusion, obtained with siRNAs, that RBP2 loss impairs proliferation and promotes senescence.


The authors declare no conflict of interest.

Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE26446 and GSE26798).

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Inactivation of p53, using either the SV40 Large T antigen (LT) K1 mutant (25) or a dominant-negative C-terminal fragment of p53 (p53C11F) (26), immortalized Rbp2<sup>−/−</sup> MEFs, which was evidenced by their ability to be continually passaged in culture and absence of SABG staining; however, it did not correct their proliferation defect relative to similarly immortalized WT MEFs (Fig. S1C and data not shown). The availability of immortalized Rbp2<sup>−/−</sup> MEFs allowed us to ask whether the proliferation defect in Rbp2<sup>−/−</sup> cells is caused by loss of RBP2 histone demethylase activity. Reintroduction of WT RBP2, but not the histone demethylase-defective RBP2<sup>H483A</sup> mutant (11), into LT K1-immortalized Rbp2<sup>−/−</sup> MEFs using retroviral vectors rescued the proliferation defect caused by RBP2 loss (Fig. 1 G and H).

Notably, the proliferation defect of Rbp2<sup>−/−</sup> MEFs was also rescued by inactivation of pRB, achieved with either WT LT (in contrast to LT K1) (Fig. S1D) or Rb1 nullizygosity (Fig. S1 E and F). Rbp2<sup>−/−</sup>:Rb1<sup>−/−</sup> primary MEFs did, however, eventually senesce, presumably because of p53 activation. Taken together, these results suggest that the senescence defect caused by RBP2 loss is p53-dependent, whereas the proliferation defect caused by RBP2 loss is pRB-dependent. Moreover, these data, together with earlier studies (9), suggest that RBP2 acts both upstream and downstream of pRB.

Loss of RBP2 Leads to Loss of Stem Cell Markers. Many developmentally important promoters contain bivalent chromatin, which consists of H3K4me3 and H3K27me3 (27). These marks ensure that the genes are poised for activation or repression on differentiation. Because RBP2 can erase H3K4me3, we asked whether loss of RBP2 affects the maintenance of mouse ES cells. We compared the gene expression profiles of Rbp2<sup>−/−</sup> and Rbp2<sup>−/−</sup> ES cells grown either in the presence of leukemia inhibitory factor (LIF) (Fig. 2 A and B), which suppresses differentiation, or 6 d after withdrawal (Fig. 2 C and D), which promotes differentiation, using gene set enrichment analysis (GSEA) (28). GSEA was performed using two previously defined subsets of genes: an ES genes subset that included genes that are highly expressed in undifferentiated ES cells (Fig. 2 A and C) and a differentiation genes subset that included genes that are bound by H3K27me3 and repressed in undifferentiated ES cells but activated 6 d after induction of differentiation (Fig. 2 B and D) (29). These analyses showed that loss of RBP2 down-regulates many genes that are normally highly expressed in ES cells (Fig. 2 A and C) and leads to partial activation of the genes linked to differentiation (Fig. 2B), despite the presence of LIF, suggesting that RBP2 promotes or maintains a stem cell-like phenotype. Consistent with this idea, down-regulation of stem cell markers was more rapid in Rbp2<sup>−/−</sup> ES cells after LIF withdrawal compared with WT ES cells (Fig. 2C). Nonetheless, transcriptional activation of genes that are normally repressed by LIF was blunted in Rbp2<sup>−/−</sup> ES cells (Fig. 2D), suggesting that Rbp2<sup>−/−</sup> ES cells exit the stem cell compartment more rapidly than WT ES cells but are impaired in terms of fully executing a differentiation program.

To further examine this finding, we performed real-time PCR analysis of selected transcripts from the ES cells treated as above. In keeping with the GSEA, Rbp2<sup>−/−</sup> ES cells prematurely down-regulated the stem cell markers Nanog and Oct4 in response to LIF withdrawal but failed to fully up-regulate the differentiation markers Sox17 and Gata6 (Fig. 2E). Similar findings with respect to Nanog and Oct4 were also observed when WT and Rbp2<sup>−/−</sup> ES cells were induced to form embryoid bodies (EB) and then treated with retinoic acid (RA) to promote neuronal differentiation (Fig. 2F). In this model, however, Rbp2<sup>−/−</sup> ES cells displayed enhanced expression of the neuronal markers Pax3 and Msi1 (Fig. 2F). These findings suggest that Rbp2 deficiency down-regulates stem cell markers and promotes differentiation. Similar results were obtained with independently derived ES cell lines.

RBP2 Loss Mitigates Proliferation and Differentiation Abnormalities in pRB-Defective Cells. Down-regulation of RBP2 using siRNA inhibits the proliferation of pRB-defective tumor cells (9, 10) and restores the ability of Rbp2<sup>−/−</sup> MEFs to differentiate (9). The availability of Rbp2<sup>−/−</sup> mice allowed us to address the roles of RBP2 without being confounded by siRNA-mediated off-target effects. Through appropriate crosses, we generated WT, Rb1<sup>−/−</sup>, Rbp2<sup>−/−</sup>, Rbp2<sup>−/−</sup>:Rb1<sup>−/−</sup>, and Rbp2<sup>−/−</sup>:Rb1<sup>−/−</sup> embryos. Homozygous loss of Rbp2 impairs the proliferation of Rb1<sup>−/−</sup> MEFs derived from these littermate embryos (Fig. 3A).

Next, WT, Rb1<sup>−/−</sup>, Rbp2<sup>−/−</sup>, and Rbp2<sup>−/−</sup>:Rb1<sup>−/−</sup> early-passage MEFs were infected with an adenovirus-encoding MyoD and induced to differentiate in differentiation medium. RBP2 status did not influence adenoviral infection efficiency (data not shown).
Consistent with previous studies, WT MEFs, but not Rb1−/−MEFs, started to form elongated myocytes 1 d after being placed in differentiation media, and they formed multinucleated myotubes shortly thereafter, which were associated with expression of the late-differentiation marker myosin heavy chain (MYHC). Loss of Rbp2 partially rescued both MYHC expression and formation of multinucleated cells (Fig. 3 B and C). Differentiation of Rbp2−/−;Rb1−/−MEFs was also enhanced after reintroduction of WT pRB or by the pRB variant Δ663, which promotes differentiation despite an inability to bind to E2F or repress E2F-dependent promoters (8) (Fig. S2). This finding suggests that pRB has non-E2F targets in addition to RBP2 that affect differentiation.

Loss of RBP2 Suppresses Tumorigenesis Caused by Deletion of the Rb1 or Men1 Tumor Suppressor Genes. Although RBP2 regulates proliferation, senescence, and differentiation in vitro, which are processes deregulated in cancer, its potential relevance in transformation in vivo is unknown. We, therefore, asked whether Rbp2 interacts genetically with Rb1 in vivo, exploiting the fact that Rbp2−/− mice in a mixed genetic background are viable and have a normal lifespan (Fig. S3). Rb1−/−embryos die at embryonic day 14.5 (30–32), and Rb1−/−embryos supplied with Rb1+/+ extra-embryonic tissues die shortly after birth, possibly because of severe skeletal muscle defects (33, 34). No Rbp2−/−;Rb1+/− pups were born from Rbp2−/−;Rb1+/− intercrosses (Table S1), indicating that Rbp2 loss cannot rescue the embryonic developmental defects caused by Rb1 loss.

Next, we asked if loss of RBP2 would alter pRB-defective tumorigenesis. Rb1−/− mice develop pituitary and thyroid tumors that are associated with stochastic loss of the second Rb1 allele (30, 35). We, therefore, examined the Rb1+/− progeny of matings between Rbp2−/−;Rb1+/− mice. A limited number of timed necropsies were performed on 28-wk-old mice. As expected, most (3/4) Rb1+/− mice had early pituitary lesions, including small tumors, whereas no abnormalities were detected in the pituitaries of all (4/4) of the Rbp2−/−;Rb1+/− mice (Fig. 4A), suggesting that RBP2 loss suppresses tumor initiation. The remainder of the mice were monitored and killed when they became distressed or moribund because of the development of tumors.

Importantly, deletion of Rbp2 dramatically extended the life span of Rb1+/− mice (Fig. 4B). The median survival time improved from 47 wk for Rbp2−/−;Rb1+/− mice to 72 wk for Rbp2−/−;Rbp2−/− mice. Indeed, some Rbp2−/−;Rb1+/− mice lived up to 2 y, the average life span of WT mice. Importantly, loss of one Rbp2 allele also delayed tumorigenesis and partially extended the life span of Rb1−/− mice (Fig. 4B). Similar results were obtained when the analysis was restricted strictly to littermates (Fig. S4). Notably, all of the Rbp2−/−;Rb1+/− and Rbp2−/−;Rbp2−/− mice had microscopic pituitary and/or thyroid tumors at necropsy (Table S2). This finding suggests that RBP2 delays the onset of such tumors or retards their progression rather than preventing tumor initiation.

Mammals have three RBP2 paralogs called PLU-1, SMCX, and SMCY. Plu-1 mRNA levels were significantly increased in pituitary tumors arising in 12-mo-old Rbp2−/−;Rb1+/− mice compared with tumors arising in 12-mo-old Rbp2−/−;Rb1+/− mice (Fig. 4C), suggesting that compensation by Rbp2 paralogs contributes to the eventual formation of pituitary tumors in the Rbp2−/−;Rb1+/− mice.

Inactivation of the Men1 tumor suppressor gene, like inactivation of Rb1, leads to formation of neuroendocrine tumors (4, 36, 37). Menin, the MEN1 gene product, is part of a complex that promotes H3K4 methylation, and this activity is diminished by tumor-associated MEN1 mutations (6, 22, 23). We, therefore, reasoned that inactivation of the RBP2 H3K4 demethylase might partially rescue Men1 loss. To this end, we exploited the fact that Men1 inactivation in pancreatic islet cells leads to the development of insulinomas (37), which can be monitored based on changes in circulating insulin levels. Through appropriate matings, we generated Men10f/−;Rbp2−/−; Men10f/−;Rbp2−/−, and Men10f/−;Rbp2−/− mice that also expressed Cre recombinase in their pancreatic islet cells (RIP-Cre) (38). Inactivation of the floxed alleles
was confirmed by anti-menin and anti-RBP2 immunohistochemistry (Fig. S5).

Rbp2 inactivation, either systemically (Fig. 5A) or specifically in islet β-cells (Fig. 5B), substantially decreased islet cell tumor burden, which was measured by circulating insulin levels (Fig. 5C), and enhanced survival (Fig. 5A and B). The median survival for Men1+/Rbp2+/+;RIP-Cre mice was 45 wk compared with median survivals of 68 wk for Men1+/Rbp2+;RIP-Cre mice (Fig. 5A) or 69 wk for Men1+/Rbp2+/;RIP-Cre mice (Fig. 5B), respectively. Inactivation of Rbp2 in islet cells of Men1+/+ mice did not grossly affect islet histology or function, which was determined by gene expression profiling (Fig. S6), circulating insulin (Fig. 5C), and glucose levels (data not shown).

We also performed timed necropsies on a limited number of Men1+/+;Rbp2+;RIP-Cre and Men1+/Rbp2+/+;RIP-Cre mice (Fig. 6A). By 2 mo of age, 50% (5/10) of the former exhibited islet cell hyperplasia compared with zero of the latter (0/8) (Table S3). The prevalence of cellular atypia and insulinoma at 4 and 8 mo was dramatically reduced by loss of Rbp2. By 10 mo of age, all (15/15) of the Men1+/Rbp2+/+;RIP-Cre mice had insulinomas compared with 2 of 21 Men1+/Rbp2+/+;RIP-Cre mice (Table S3). These findings indicate that Rbp2 loss significantly delays the onset of hyperplasia, atypia, and insulinoma in this model.

Notably, insulinomas were observed in some (2/5) 12-mo-old Men1+/+;Rbp2+/+;RIP-Cre mice at necropsy. Comparison of insulinomas from 12-mo-old Men1+/+;Rbp2+/+;RIP-Cre mice with Men1+/+;RIP-Cre mice revealed increased expression of Plu-1 but not Smcr and Smcy after Rbp2 loss (Fig. 6B). This increase, however, was not observed in spleens from 12-mo-old Men1+/+;Rbp2+/+;RIP-Cre mice or pancreatic islets from 2-mo-old Men1+/+;Rbp2+/+;RIP-Cre mice (data not shown). These observations suggest that the eventual formation of insulinomas in Men1+/+;Rbp2+/+;RIP-Cre mice depends on increased levels of PLU-1, perhaps occurring stochastically over time.

To begin to understand the mechanisms underlying these differences, we injected 2-mo-old mice with BrdU and examined their pancreata 5 h later. As expected, BrdU incorporation was increased in the islets of Men1+/+;Rbp2+/+;RIP-Cre mice compared with WT controls (Fig. 6C and D). This increase was not observed, however, in islets that concurrently lacked Rbp2. We did not observe differences in bulk H3K4 trimethylation by immunohistochemistry (data not shown), possibly reflecting the activity of additional H3K4 methyltransferases and demethylases.

To begin to assess the molecular basis for the effect of Rbp2 loss in attenuating tumorigenesis, we performed mRNA profiling...
using DNA microarrays on pancreatic islets isolated from 2-mo-old WT, RIP-Cre, Men1	extsuperscript{ff}, Rip-Cre, Rbp2	extsuperscript{ff}, RIP-Cre, and Men1	extsuperscript{ff}; Rbp2	extsuperscript{ff}, RIP-Cre mice. The gene expression changes caused by deletion of Men1 overlap with those changes reported previously (39) (Dataset S1).

To determine the effects of Rbp2 inactivation on the gene expression changes in Men1-deficient islets, we compared the gene expression changes in Men1	extsuperscript{ff};Rbp2	extsuperscript{ff}, RIP-Cre, Men1	extsuperscript{ff};RIP-Cre, and Rbp2	extsuperscript{ff}, RIP-Cre islets with WT and RIP-Cre control islets. The effects of Men1 deletion on pancreatic islet gene expression were reversed by Rbp2 loss for a number of genes belonging to several classes, including genes involved in signaling, cell cycle, and apoptosis (Fig. 7 A and B). The reversal by Rbp2 deletion of expression changes associated with Men1 deletion in islets was confirmed by real-time RT-PCR (Fig. 7C).

**Discussion**

We confirmed that loss of RBP2 impairs proliferation, promotes senescence, and enhances differentiation in vitro. Notably, deletion of Rbp2 was insufficient to rescue the embryonic developmental defects caused by Rb1 loss but significantly suppressed pituitary and thyroid tumorigenesis in Rb1	extsuperscript{+/-} mice and islet cell tumorigenesis after inactivation of Men1 in pancreatic neuroendocrine cells.

The canonical pRB targets are members of the E2F transcription factor family, and suppression of E2F-responsive promoters contributes to cell-cycle control and tumor suppression by pRB (7). pRB also biochemically interacts with a number of chromatin modifiers, including HDACs (40–42), SWI/SNF chromatin remodeling complexes (43, 44), H3K9 methyltransferases Suv39h1 (45) and RIZ1 (46), H4K20 methyltransferase Suv4-20h (47), and DNA methyltransferase 1 (DNMT1) (48). Our findings, together with earlier biochemical and siRNA-derived data, suggest that another pRB-interacting chromatin modifier, RBP2, contributes to tumor suppression by pRB. RBP2 loss inhibits cell proliferation in a pRB-dependent manner, placing RBP2 upstream of pRB. However, RBP2 inhibits senescence and differentiation in pRB-defective tumor cells, and loss of RBP2 inhibits formation of pRB-defective endocrine tumors, suggesting that RBP2 also acts downstream of pRB. In summary, tumor suppression by pRB might involve coordinated regulation of both E2F and RBP2. Consistent with this idea, RBP2 is recruited to E2F target genes during differentiation (49).

It is increasingly clear that alterations in histone methylation play important roles in cancer in general (50, 51). For example, MLL1, a subunit of an H3K4 methyltransferase complex, is frequently translocated in leukemia (52, 53), whereas another H3K4 methyltransferase subunit gene, MEN1, is frequently mutated in endocrine tumors (4, 6, 22, 23). EZH2, the catalytic subunit of an H3K27 methyltransferase polycomb repressive complex 2, is overexpressed in aggressive prostate cancers (54). Finally, copy number changes and intragenic mutations affecting histone methyltransferases and demethylases, such as the UTX H3K27 histone demethylase, are increasingly being identified in cancers (5, 55, 56).

RBP2 is one of four proteins [together with PLU-1 (also known as KDM5B or JARID1B), SMXC (also known as KDM5D or JARID1C), and SMCY (also known as JARID1D)] capable of demethylating trimethylated H3K4 (57). This mark is usually associated with actively transcribed genes and is also found at bivalent domains in association with trimethylated H3K27, which is usually linked to transcriptional repression (27).
tumors in both species (4, 36, 37). Interestingly, inactivation of Rb1 and Men1 in neuroendocrine tumors arising in Rb1+/−; Men1 compound heterozygous mice is mutually exclusive (67, 68), suggesting that Rb1 and Men1 share a critical activity or activities relevant to neuroendocrine tumorigenesis. Our studies suggest that regulation of H3K4 methylation is one such activity.

Enzymes have historically proven to be tractable drug targets. RBP2 and its paralogs PLU-1, SMCX, and SMCY are 2-oxoglutarate–dependent dioxygenases (18, 19). These enzymes can be inhibited with drug-like small organic molecules that act competitively with respect to 2-oxoglutarate, interfere with iron use, or both (20, 21). Our findings suggest that RBP2-inhibitory drugs, should they be developed, would be anticancer activity. Furthermore, elevated expression of PLU-1 in Rb1- and Men1 knockout mice (Fig. 4C and 6B) suggests that RBP2 inhibitors that also inhibit PLU-1, if they were safe, would be more effective than inhibitors that target RBP2 alone.

Materials and Methods

Mouse Experiments. Rbp2+/− and Rbp2fl/fl mice were described previously (11) and backcrossed to C57BL/6 strain for at least five generations. Rbp2−/− mice were intercrossed to generate Rbp2−/−;Rb1−/− MEFs. Rbp2fl/fl mice were crossed with C57BL/6 chicken β-actin Cre-ER mice (24, 69) to obtain Rbp2fl/fl;Cre-ER mice. Rbp2fl/fl;Cre-ER mice were crossed with Rbp2fl/fl mice to generate Rbp2fl/fl;Cre-ER MEFs and Rbp2fl/fl;Cre-ER littermate control MEFs. Rb1fl/fl mice on a C57BL/6 background (30) were obtained from the National Cancer Institute Mouse Repository. Rb1fl/fl mice were crossed with Rbp2fl/fl mice on a mixed 129SvEv, FVB/N, and C57BL/6 background to obtain Rbp2fl/fl;Rb1fl/fl mice. These mice were then intercrossed to generate the experimental cohorts of Rb1+/+, Rbp2+/+, Rb1−/−, and Rbp2−/−. Men1 conditional KO mice were described previously (6) and maintained on a mixed 129S6, FVB/N, and C57BL/6 background. To specifically delete the Men1 gene in pancreatic islet β-cells, Men1fl/fl mice were crossed with RIP-Cre transgenic mice (38). The Men1fl/fl;RIP-Cre mice were crossed with Men1fl/fl;Rbp2−/− mice to generate Men1fl/fl;Rbp2−/−;RIP-Cre mice. Men1fl/fl;Rbp2−/− mice were intercrossed by introducing WT alleles into the Men1fl/fl;Rbp2−/−;RIP-Cre mice through appropriate matings. For in vitro proliferation and differentiation assays, Rb1+/+ mice were crossed with Rbp2+/+ mice on a pure C57BL/6 background to obtain Rbp2+/+;Rb1−/− mice, which were intercrossed to generate WT, Rbp2−/−;Rb1−/−, Rbp2−/−;Rb1−/−, and Rbp2−/−;Rb1−/− MEFs. Mice and cells carrying Men1 floxed alleles were genotyped using primers described in SI Materials and Methods, and all other mice and cells were genotyped as described (11, 24, 30, 38). All mice were maintained in the research animal facility of the Dana–Farber Cancer Institute and Yale Animal Resources Center in accordance with the National Institutes of Health guidelines. All procedures involving mice were approved by the Institutional Animal Care and Use Committees of the Dana–Farber Cancer Institute and Yale University.

ES Cell Culture and Differentiation. In Fig. 2 A–E, Rbp2fl/fl ES cells were isolated from mouse blastocysts after intercrossing Rbp2fl/fl mice on a pure C57BL/6 background and transiently transfected with pBS500/EFl-GFPCre plasmid. GFP-positive cells were isolated by FACS and plated at low density. Isolated colonies were then expanded into ES lines. Successful recombination of the Rbp2 locus was confirmed by PCR and Western blot analysis. In Fig. 2F, WT and Rbp2−/− ES cells were isolated from mouse blastocysts after intercrossing Rbp2−/− mice. WT, Rbp2fl/fl, and Rbp2−/− ES cells were maintained on mito-

SI Materials and Methods.
digestion (Qiagen). Gene expression profiling was performed using Affymetrix GeneChip mouse genome 430 2.0 arrays. Raw gene expression profiling data were analyzed using dChip (70). The two gene sets used for gene set enrichment analysis were described previously (29). The differentiation genes include all genes that are marked by both H3K27me3 and EZH2 in WT ES cells and up-regulated at least threefold 6 d after induction of differentiation by LIF withdrawal. The ES genes are genes highly expressed in pluripotent ES cells compared with differentiated cells.

Pancreatic islets were isolated as described (71). Briefly, 0.25 mg/mL Liberase solution (Roche) in serum-free M199 medium were injected into pancreata through the common bile duct of anesthetized 2 mo-old male mice. The infected pancreata were incubated at 37 °C for 20 min for digestion before filtered through mesh. Then, islets were purified through histopaque gradient purification and gravity sedimentation. Finally, islets were hand-picked from dark field dishes under a dissecting microscope for RNA isolation using the RNAqueous mini kit (Ambion). Islet RNAs were expression-profiled on Affymetrix GeneChip Mouse Gene 1.0 ST arrays. Raw gene expression profiling data were analyzed using dChip (70). Transcripts were defined to be significantly changed based on a t test P < 0.05. The expression data reported in this paper have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database under accession numbers GSE26446 and GSE26978.

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