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A systems view of epigenetic networks regulating pancreas development and β-cell function

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Abstract

The development of the pancreas and determination of endocrine cell fate are controlled by a highly complex interplay of signaling events and transcriptional networks. It is now known that an interconnected epigenetic program is also required to drive these processes. Recent studies using genome-wide approaches have implicated epigenetic regulators, such as DNA and histone-modifying enzymes and non-coding RNAs, to play critical roles in pancreas development and the maintenance of cell identity and function. Furthermore, genome-wide analyses have implicated epigenetic changes as a casual factor in the pathogenesis of diabetes. In the future, genomic approaches to further our understanding of the role of epigenetics in endocrine cell development and function will be useful for devising strategies to produce or manipulate β-cells for therapies of diabetes.

Introduction

Epigenetics refers to mechanisms that alter gene expression patterns in the absence of changes in the nucleotide sequence of the DNA. Epigenetic marks, which include DNA modifications (such as methylation) and post-translational modifications of histones (such as acetylation, phosphorylation, ubiquitination and sumoylation), are deposited on chromatin by DNA and histone-modifying enzymes. In addition, long non-coding RNAs (lncRNAs) are emerging as important epigenetic regulators by functioning as molecular scaffolds to initiate and sustain epigenetic changes [1]. Epigenetic regulators are now known to contribute to pancreas development as well as the differentiation, maintenance and function of pancreatic endocrine cells, most notably the insulin-producing β-cells. In addition, studies have shown that an altered epigenetic landscape is associated with the pathogenesis of diabetes. In this review, we highlight the growing evidence for the importance of epigenetic regulation in pancreas development, maintenance of β-cell identity and function, and the pathogenesis of diabetes.

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Epigenetic programming of pancreatic organ fate commitment

The pancreas originates from the endodermal germ layer, which also gives rise to the esophagus, stomach, intestine and organs lining the gastrointestinal tract, such as the liver, thyroid and lungs. The specification of endodermal lineage intermediates toward these different organ fates occurs stepwise and is initiated by localized signals that induce the expression of lineage-specific transcription factors (TFs). While the last two decades have provided a detailed understanding of the TFs that mediate the differentiation steps toward the different organ fates, less is known about how changes at the level of chromatin influence these developmental decisions.

The pancreas and liver arise from a common population of cells in the ventral foregut. Inductive signals for the pancreas and liver must act upon these bipotent progenitors to activate pancreas- or liver-specific genes. Work by Zaret and colleagues explored whether specific chromatin modifications are established at liver- and pancreas-specific regulatory sequences prior to gene activation and whether chromatin “pre-patterns” play a role in cell fate induction [2]. Employing analysis of select candidate genes, they found that liver- and pancreas-specific regulatory regions exhibit distinct chromatin patterns in bipotent foregut progenitors. Pancreas regulatory elements are marked by both the active H3K9K14ac and the repressive H3K27me3 marks, reflecting a “poised” state for future gene activation (Figure 1). In contrast, these marks are poorly represented at liver regulatory elements in foregut progenitors, and liver genes acquire H3K9acK14ac active marks *de novo* when cells differentiate into hepatoblasts. The authors tested whether these histone modifications direct the cell fate choice of foregut progenitors between liver and pancreas. Consistent with the observation that histones at liver-specific loci undergo *de novo* acetylation during hepatic fate induction, reduced p300 acetyltransferase activity prevents hepatic fate induction and favors the pancreatic fate [2]. Inactivation of the histone methyltransferase Ezh2, a key component of the Polycomb Gene (PcG) complex mediating H3K27me3 deposition, similarly favors the pancreatic over the hepatic fate, presumably because “poised” pancreatic genes are activated upon removal of the repressive H3K27me3 mark. These findings demonstrate distinct mechanisms for the activation of pancreas and liver genes during development and illustrate how this mechanism is predetermined by specific chromatin “pre-patterns” in developmental intermediates.

It is clear from these studies that the chromatin undergoes important alterations that are highly regulated as cells transition from an undifferentiated to a differentiated state. In pluripotent embryonic stem (ES) cells, a majority of developmental genes that contain the repressive H3K27me3 mark is also enriched for the active H3K4me3 mark, which has been coined a bivalent state [3]. Lineage-specific differentiation of ES cells is associated with a resolution of this bivalent state, with a loss of either H3K27me3 or H3K4me3 leading to the activation or stable repression of lineage-specific genes, respectively. However, bivalent domains are not only resolved but also gained during development and these dynamic changes in H3K27me3 deposition during developmental progression are thought to help facilitate rapid changes in gene expression [4]. What has been unclear is when and how the bivalent state is resolved or gained during the progression of ES cells toward a differentiated state. Early studies had been limited in that only select developmental stages were examined,

usually only comparing one undifferentiated population with differentiated cells [3, 5]. However, recent *in vitro* differentiation protocols using pluripotent stem cells (PSCs) have enabled the use of genome-wide approaches to identify changes in epigenetic modifications as cells progress through lineage intermediates toward a differentiated state [6, 7]. The development of such protocols for the pancreatic endocrine cell lineage [8, 9] has offered an opportunity to study critical epigenetic events occurring during endocrine cell development. Studies using these approaches have shown that at each step during progression toward pancreas, changes in the bivalent state of a promoter coincide with changes in the expression of the associated gene [10]. Further studies have shown that these epigenetic changes are directly relevant for the regulation of gene expression, as inactivation of H3K27me3 demethylases in ES cells prevents the induction of endodermal genes during endoderm formation [10–12] (Figure 1). The stage-specific loss of H3K27me3 repression is specifically observed at genes encoding developmental regulators, as for example *EOMES* at the endoderm stage and *PDX1* at induction of the pancreatic fate [10]. Similar observations have been reported during the development of other lineages [6, 7], suggesting that the combined analysis of gene expression and bivalent promoter states could be globally employed to identify novel developmental regulators.

It is currently unclear how these ubiquitously expressed histone-modifying enzymes, such as the PcG component EZH2, can have such specific roles in differentiation. One might expect that modulating the activity of these epigenetic regulators would widely change a cell's chromatin state, resulting in deleterious consequences to the cell. Studies so far suggest that these regulators are acting on select genes and therefore have highly specific context-dependent effects. Indeed, genome-wide studies have shown that PcG-dependent H3K27 trimethylation is not a universal repression mechanism, but actually represses a subset of genes that encode for developmental regulators [13]. In addition, the H3K27me3-mediated repressive mechanism is only employed in certain cellular contexts [14]. One hypothesis that could explain why histone-modifying enzymes act in such a specific manner is that cell type-specific TFs recruit histone modifiers to select loci during differentiation. It was recently shown that the T-box TF TBX3 directs the H3K27me3 demethylase JMJD3 to the regulatory elements of the endodermal regulator *EOMES* resulting in derepression of *EOMES* and subsequent endoderm induction [12]. By systematically mapping where epigenetic regulators are recruited and how their recruitment changes during differentiation, we may be able to define the roles for these regulators in the development of the pancreatic as well as other organ lineages.

The above studies provide initial insights at a system-wide level into how pancreatic lineage progression is regulated. There are likely additional layers of regulation, such as differential promoter and enhancer usage, DNA methylation, and non-coding RNAs that orchestrate these changes in gene expression. Genome-wide analysis of how these modifications and regulators change during endodermal differentiation will further elucidate the molecular mechanisms regulating organ specification.

Regulation of pancreatic endocrine cell development by epigenetic mechanisms

Changes in the epigenome are also important during the differentiation of the different cell types of the pancreas. Studies using inhibitors for histone deacetylases (HDACs) in embryonic pancreas explants have demonstrated that the decision of multipotent pancreatic progenitor cells whether to produce endocrine, acinar or ductal cells relies on modifications of histones (Figure 1). Maintenance of acetylation by HDAC inhibition suppresses acinar cell differentiation and promotes commitment to the ductal and endocrine fates [15]. Dynamic changes in the acetylation of histones are also important for terminal endocrine cell differentiation and the specification of the different endocrine cell types, namely alpha (α)-, beta (β)-, delta (δ), pancreatic polypeptide (PP)- and epsilon (ε)-cells. In this context, different classes of HDACs appear to have distinct effects on the development of individual endocrine cell types (Figure 2). For example, HDAC class I (HDAC-I) members specifically restrict the formation of α- and PP-producing cells, while promoting β- and δ-cell differentiation [15]. Further supporting the idea that HDACs have distinct functions during endocrine subtype specification, genetic deletion of different HDAC class IIa (HDAC-IIa) members results in either an increase of β-cells or δ-cells [16]. Thus, histone-modifying enzymes have highly specific roles during the specification of the different endocrine cell types. This knowledge could be utilized to direct the differentiation of specific endocrine cell types during *in vitro* differentiation of PSCs.

In the last decade, numerous groups have tried to generate functional β-cells from human PSCs. Early efforts in achieving proper endocrine cell differentiation *in vitro* had been met with limited success. Insulin^{+} cells produced with these early protocols expressed multiple hormones, were not glucose responsive and more closely resembled fetal than adult β-cells in regard to gene expression [10, 17, 18]. However, developmental precursors produced with these protocols develop into glucose-responsive, mature β-cells upon implantation into mice [19, 20], and based on transcriptome analysis, these *in vivo*-differentiated endocrine cells are remarkably similar to primary human endocrine cells [10]. This strategy of differentiating β-cells *in vivo* by implanting PSC-derived pancreatic precursors is currently been tested in humans in the first stem cell-based diabetes clinical trial. Furthermore, two independent studies from the Kieffer and Melton labs have recently described *in vitro* differentiation protocols that can produce functional β-like cells from human PCSs [8, 9]. Kieffer and colleagues demonstrated these cells were able to permanently reverse hyperglycemia when transplanted into diabetic mice; however the effects were not immediate, suggesting that some degree of maturation still has to occur within the host.

Earlier studies have shown that the insufficient induction of β-cell genes during terminal differentiation *in vitro* was associated with aberrant chromatin remodeling. In pancreatic progenitors, the expression of core β-cell genes is largely suppressed by H3K27me3 modifications, which become selectively removed during terminal endocrine differentiation [10, 21]. However, *in vitro* differentiated cells had inadequate changes in H3K27me3 and H3K4me3 modification patterns, especially at key β-cell gene loci [10]. Now with the Kieffer and Melton protocols in hand, it would be interesting to determine if these

in vitro-produced β-like cells have the same epigenetic makeup as primary β-cells. If there are differences, targeted manipulation of specific epigenetic regulators could help achieve complete β-cell differentiation *in vitro*. Indeed, Zaret and colleagues have shown that inhibition of EZH2 during pancreatic differentiation of human PSCs leads to an increase in endocrine progenitors and insulin-expressing cells *in vitro* [22]. However, it was not determined if the insulin$^+$ cells generated were functional β-cells.

Roles of the epigenome in cell plasticity and β-cell identity

There have been significant advances in generating insulin-producing cells from a variety of adult cell types. Several reports have achieved transdifferentiation of endoderm-derived cell types, such as liver, intestinal and gall bladder epithelium, into insulin-producing cells by ectopic expression of TFs critical for pancreas and β-cell development [23–26]. These reprogramming strategies often lack robustness and tend to be slow and inefficient, which might explain why these strategies have not found widespread applications. However, it has been noted that there is great plasticity among the different cell types of the pancreas, particularly α-cells and δ-cells, towards β-cells. In comparison to pancreatic exocrine cells, which require TF-based reprogramming with multiple endocrine TFs [27], reprogramming of α- or δ- to β-cells can be achieved by manipulating a single TF or simply by ablating the β-cell population [28–31]. Why these endocrine cell types can be more easily converted into β-cells and why similar experimental approaches are insufficient to facilitate this conversion in other cell types had been unclear. Recent studies indicate that epigenetic similarities between different but developmentally related cell types could explain some of these differences on a global scale.

Kaestner and colleagues found that the intrinsic plasticity of α-cells associates with specific histone methylation profiles [32]. They show that α-cells not only contain more bivalently marked genes than β-cells, but while most genes critical for β-cell development and function are H3K4me3 marked in β-cells, the majority of these genes is bivalently marked in α-cells (Figure 2). This bivalency may enable α-cells to be plastic upon suitable stimuli, as these β-cell-specific genes are poised for activation. This might suggest that the histone methylation profile is an indicator of a cell's potential for plasticity, with α-cells having greater plasticity toward the β-cell phenotype than, for example, exocrine cells, which carry far less of these bivalent marks and have limited potential for conversion into β-cells. Interestingly, Herrera and colleagues have found that δ-cells, like α-cells, are capable of spontaneously reprogramming into β-cells after β-cell ablation; however, the ability of δ-cells to reprogram does not extend beyond puberty [28]. Further studies on the epigenetic state of young and aged endocrine cells could reveal novel mechanisms of how the epigenome affects cellular plasticity.

Recent studies have revealed that once endocrine cells have differentiated, the epigenetic landscape is actively maintained to stabilize cellular identity. For example, to maintain β-cell identity, the α-cell fate regulator *Arx* needs to be actively repressed. In β-cells, the *Arx* promoter is highly methylated and this is facilitated by the DNA methyltransferases Dnmt3a and Dnmt1 [33, 34](Figure 2). Deletion of either of these enzymes leads to derepression of *Arx*, and subsequent transcriptional activation of the α-cell program, resulting in β- to α-cell

conversion. In β-cells, the *Arx* promoter is associated with methyl-specific binding proteins that recruit enzymatic complexes capable of locally altering histone modifications. Dnmt3, for example, facilitates transcriptional silencing of *Arx* by recruiting HDAC1 to the promoter [34]. In addition, the methyl-DNA binding protein MeCP2 recruits a complex of proteins to the *Arx* promoter, including the methyltransferase PRMT6, which can counteract activating H3K4me3 marks by mediating histone arginine methylation [33]. These studies illustrate the importance of epigenetic factors in maintaining adult β-cell identity and safeguarding β-cells from transdifferentiation.

It is possible that manipulation of specific epigenetic regulators may loosen these epigenetic constraints and increase plasticity of terminally differentiated cells. It has already been shown that treatment of islets with an inhibitor to PcG proteins results in increased expression of the β-cell TF PDX1 and insulin in α-cells [32] (Figure 2). Therefore, it will be critical in the future to identify epigenetic regulators involved in modulating cell plasticity for improved approaches to direct reprogramming toward the β-cell phenotype. Further studies are needed to systematically identify common epigenetic changes that occur during the transdifferentiation of various cell types into β-cells. Based on these marks, we may be able to predict the epigenetic regulators facilitating this process and confirm their role by genetic deletion. Employing locus-specific editing approaches [35], important epigenetic regulators could then be guided to the regulatory regions of critical lineage determinants to induce reprogramming toward the β-cell phenotype.

Epigenetic control of β-cell function and mass

Epigenetic modifiers are not only important for preventing activation of alternative lineage programs, but also for regulating cell function. For instance, epigenetic factors have been shown to dynamically regulate insulin secretion in β-cells. Mirmira and colleagues have demonstrated that the histone methyltransferase SET7/9 is required for the expression of a subset of genes involved in glucose-stimulated insulin secretion [36]. Depletion of SET7/9 results in repression of these genes through loss of activating H3K4me2 marks. SET7/9 catalyzes the transfer of methyl group(s) to histones from the diet-induced co-factor S-adenosyl-L-methionine (SAM). Indeed, the establishment of many epigenetic marks is dependent on the availability of metabolic cofactors such as SAM, nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD) and α-ketoglutarate (α-KG) [37]. Because the availability of these cofactors is regulated by nutrient status, diet could alter gene expression in β-cells through epigenetic modifiers. Recently, an impact of a methyl-deficient diet on endocrine pancreas mass and insulin secretion has been reported [38]. Prenatal methyl deficiency in rats reduces pancreatic mass and impairs glucose tolerance and insulin secretion. Since dietary methyl donors have a critical role in DNA and histone methylation, the effects mediated by this diet could be attributed to alterations in the epigenome. There is indeed evidence that poor maternal diet or an adverse intrauterine environment has a transgenerational influence on endocrine cell function through epigenetic silencing of developmental regulators, such as *Hnf4a* and *Pdx1* [39, 40]. It is likely that environmental influences early on in development has long-lasting effects on the epigenome, affecting β-cell function and the risk for diabetes later in life.

The capacity of β-cells to proliferate is critical for their ability to adapt to changing metabolic demands. An example of this is the adaptive expansion of β-cell mass in response to pregnancy, which has been linked to epigenetic changes. During pregnancy β-cell expansion is controlled by the transcriptional regulator/tumor suppressor Men1 [41]. Men1 associates with MLL1, a H3K4 methyltransferase that trimethylates H3K4 and maintains expression of the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p18^{INK4c}$ to prevent β-cell proliferation [42]. Reduction of Men1 during pregnancy facilitates β-cell expansion by reducing active marks on the loci of these cell cycle inhibitors.

The adaptive capacity of β-cells to proliferate declines with age [43] and this has been shown to be regulated by $p16^{INK4a}$, a cyclin-dependent kinase inhibitor encoded by the *Cdkn2a* gene. Increased expression of $p16^{INK4a}$ with age mediates an age-associated decline in β-cell proliferation and ability to regenerate [44]. Epigenetic modulation of the *Cdkn2a* locus plays an important role in regulating the capacity of β-cells to proliferate (Figure 2). Studies have shown that an age-dependent decrease in expression of two PcG proteins, Bmi1 and Ezh2, is associated with derepression of the *Cdkn2a* locus and increase in $p16^{INK4a}$ levels [45, 46]. In aged mice, the simultaneous knockdown of *MLL1*, which prevents deposition of activating H3K4me3 marks, and induction of Ezh2 to remove repressive H3K27me3 marks can repress $p16^{INK4a}$ expression and increase proliferation of β-cells [47]. Collectively, these studies illustrate that the epigenome is actively modified in differentiated β-cells and that it may be possible to manipulate regulators of the epigenome to reverse β-cell senescence and promote β-cell regeneration.

The epigenetic basis of diabetes

An abundance of literature has implicated epigenetic changes as a casual factor in the pathogenesis of diabetes [48]. Early studies using candidate gene approaches have shown that increased DNA methylation at promoters of genes that regulate β-cell function associates with decreased expression of the respective gene and reduced insulin secretion in islets from patients with type 2 diabetes (T2D) [49–52]. More recently, genome-wide DNA methylation profiling has led to the identification of hundreds of genes that in diabetic islets display significant changes in the methylation pattern at promoters when compared to non-diabetic donors [53, 54]. These studies indicate that altered DNA methylation at promoters of genes critical for β-cell function affects gene expression and contributes to the pathogenesis of T2D. Islet-specific TFs have been implicated in shaping the proper epigenetic landscape to maintain β-cell gene expression. For example, HNF1-α, a homeodomain-containing TF expressed in islets, interacts with co-activator proteins possessing histone acetyltransferase activity to maintain hyperacetylation of histones at promoters of β-cell-specific genes [55]. Loss of *Hnf1-α* results in hypoacetylation of HNF1-α-bound promoters, loss of H3K4me2 activating marks, and enrichment of repressive H3K27me3 marks. Interestingly, mutations in *HNF1-α* are associated with maturity onset diabetes of the young (MODY) [56], which suggests that dysregulation of epigenetic modifications caused by *HNF1-α* mutations might contribute to this subtype of diabetes.

In recent years, genome-wide association studies (GWAS) have identified multiple genetic variants that are associated with T2D [57–59]. Most disease-associated variants are non-coding

and it is unclear how these variants affect cell function. As the loci of the majority of T2D-associated variants are not in protein-coding regions, this would suggest that these loci have a regulatory function. For instance, some lncRNAs within genomic regions map very closely to small nucleotide polymorphisms associated with β-cell dysfunction and T2D, and it may be that these variants affect the expression or function of these lncRNAs [60]. Several other sequence variants associated with T2D map to open chromatin regions containing distal regulatory elements that are occupied by islet-specific TFs [61]. Studies have experimentally validated that many of these variants reside in enhancer regions and abolish enhancer activity [61–63]. For example, by analyzing the chromatin state and TF occupancy in islets, Ferrer and colleagues demonstrated that several non-coding T2D variants map to enhancers that are bound by islet-specific TFs [61]. One of the variants was found to abolish enhancer activity in β-cells, and alter sequence-specific DNA binding of the islet-enriched MODY-associated TF NEUROD1.

There are several examples of mutations in distal regulatory elements causing disease. However, it is still unclear how these distant elements affect gene transcription. Specifically, it has been difficult to define how enhancers communicate with promoters and to identify target genes for enhancers. Just recently, Hattersley, Ferrer and colleagues located an enhancer for the *PTF1* gene, encoding a TF essential for the development of the pancreas, in a distal region downstream of *PTF1* that harbors mutations associated with pancreas agenesis, a rare condition that occurs when the pancreas fails to develop before birth [64]. By using chromosome conformation capture (3C) technology, which measures the frequency of physical interaction or proximity among any pair of genomic loci, they were able to show that this region interacts with the *PTF1* promoter. Further, they demonstrated that mutations in this region prevent enhancer activity by abolishing TF binding. Recent advances combining 3C technology with deep sequencing now allows a three-dimensional (3D) view of the genome. This new technology (4C-seq) can be utilized to identify target genes of non-coding T2D genetic variants. This has been nicely demonstrated in recent work by Nobrega and colleagues [65]. Here they used 4C-seq to profile genomic interactions of obesity-associated SNPs with gene promoters. They found that sequences residing in the first intron of the *FTO* gene, which harbors a variant associated with increased risk for obesity and T2D, interact with the promoter of the homeobox gene *IRX3*, located ~500 kilobases away. Their data directly tie the obesity-associated SNPs within the *FTO* intron to reduced expression of *IRX3*. These findings were surprising, because the variant does not influence expression of the closest gene, *FTO*, but instead expression of a very distal gene.

Concluding remarks and perspectives

In this review we have highlighted recent progress showing the important roles of the epigenome in not only pancreas and endocrine cell development, but also in endocrine cell plasticity and function. With recent advances in stem cell technology and genome-wide approaches, we are now just beginning to understand how epigenetic modifications and modifiers affect these processes. In the future, systems approaches to generate high-resolution epigenome maps from isolated pancreatic cell populations, combined with genetic approaches in model organisms or cultured cells should help identify important epigenetic switches that can be manipulated to evoke changes in cell state. Furthermore, genome-wide

studies will provide a more comprehensive and systematic view of the contribution of epigenetics to diabetes by uncovering new target genes that may contribute to dysregulation of β-cell function. GWAS have identified numerous genetic variants associated with disease, yet how T2D-associated variants contribute to the pathogenesis of T2D remains largely unknown. In the future, the effects of these non-coding disease variants can be functionally validated and modeled using locus-specific gene-editing approaches in human PSC-based models. In addition, by analyzing disease variants in the context of the epigenome and creating 3D interaction maps between promoters, enhancers and their regulatory proteins, we can start to define the biological mechanisms underlying their association with T2D and potentially develop novel therapies for the treatment of diabetes.

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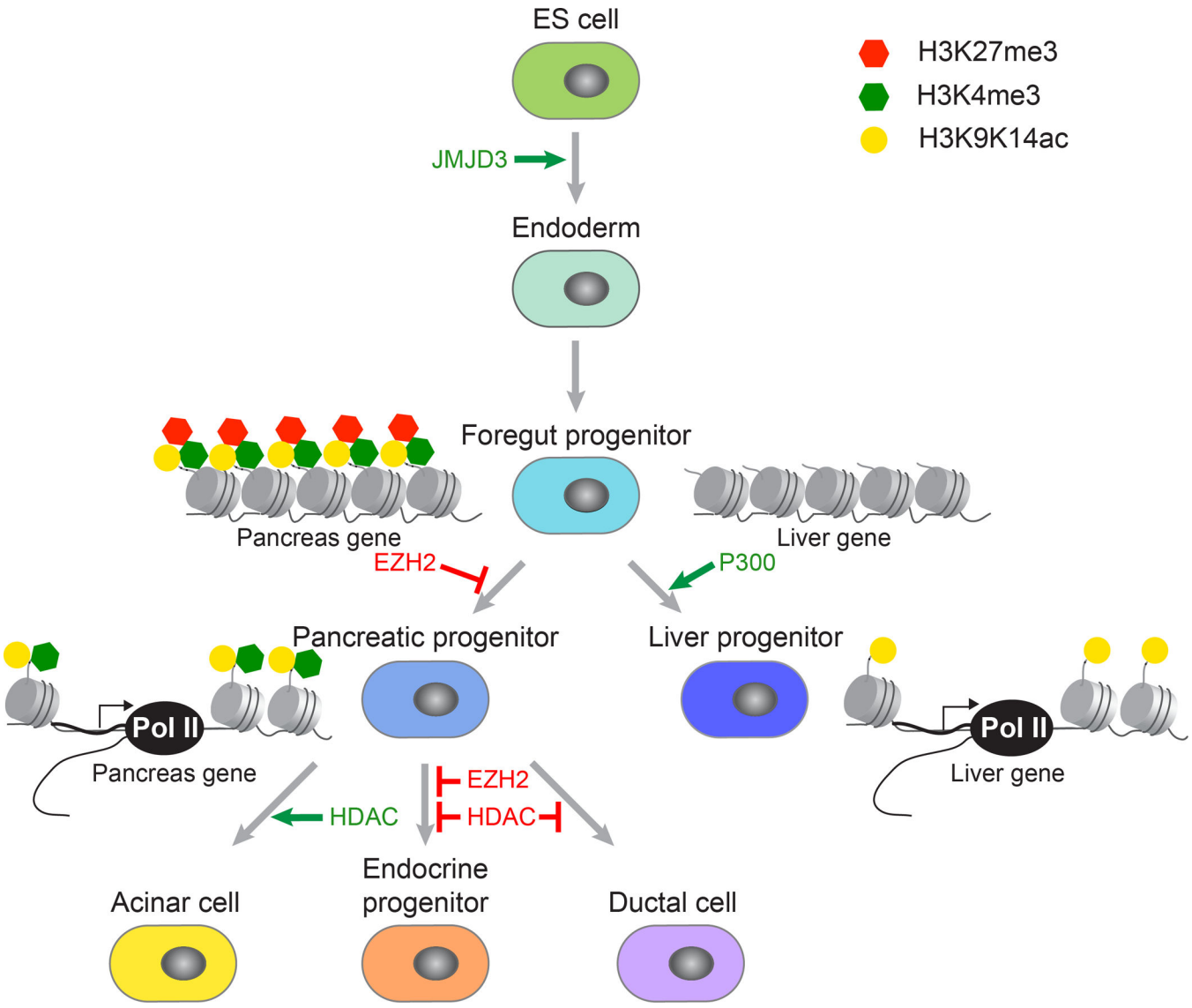

Figure 1. Epigenetic programming of pancreatic lineage specification

Overview of the key steps in pancreas development and the role of epigenetic regulators in these transitions. As cells transition from an undifferentiated to a differentiated state, the chromatin undergoes cell type-specific alterations that are highly regulated. For example, removal of the repressive mark H3K27me3 from promoters of endodermal regulators by the H3K27 demethylase JMJD3 results in endoderm induction. In foregut progenitor cells, loci for liver- and pancreas-specific genes exhibit different chromatin pre-patterns, and the histone methyltransferase EZH2 and the histone acetyltransferase P300 affect the cell fate choice between liver and pancreas. The differentiation of pancreatic progenitor cells into the different pancreatic cell types is influenced by histone deacetylases (HDACs) and EZH2.

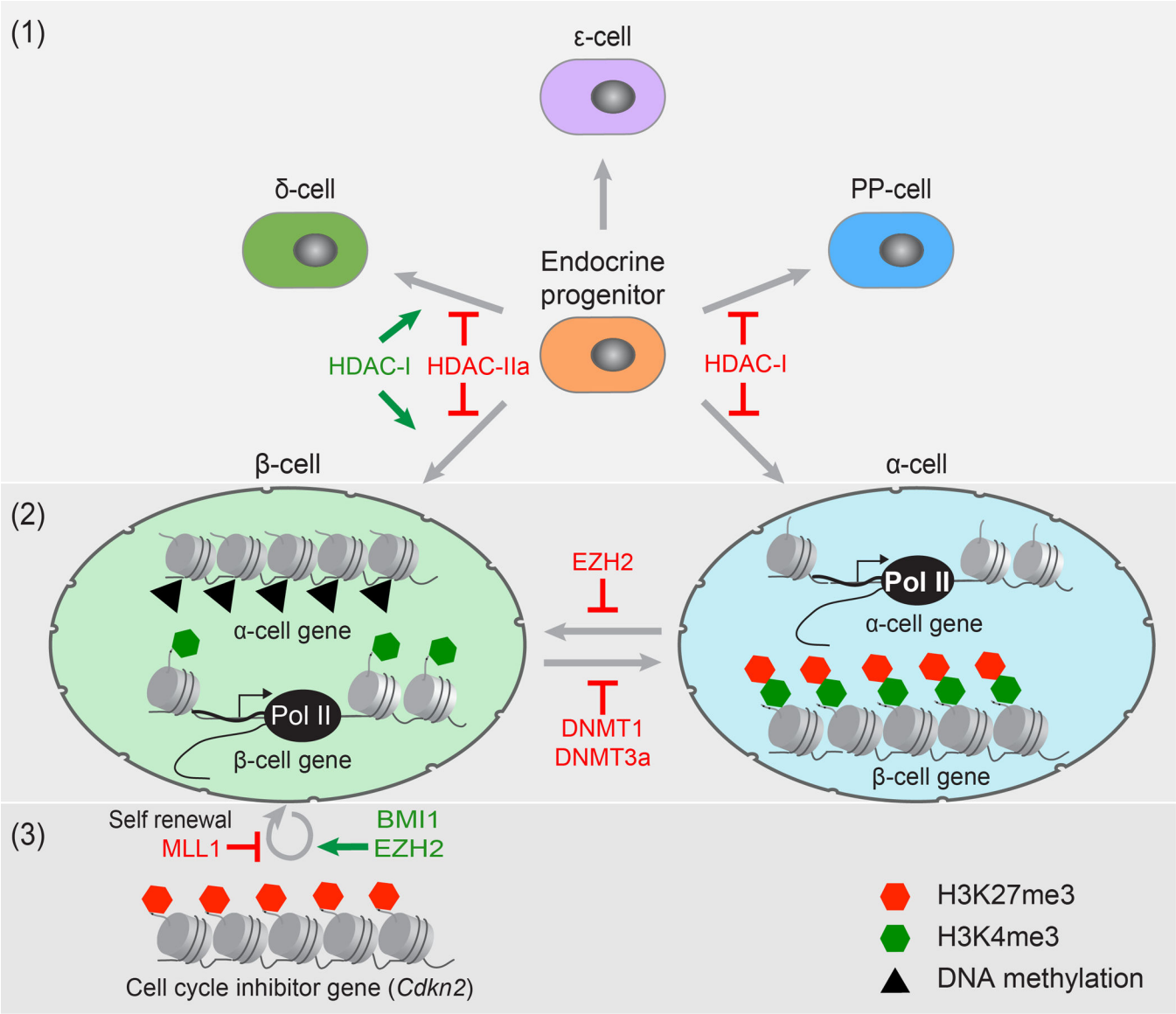

Figure 2. Epigenetic regulation in pancreatic endocrine differentiation and maintenance of β-cell function

The diagram depicts the role of epigenetic regulators as endocrine progenitor cells differentiate into the different endocrine subtypes (1), in regulating cell plasticity (2), and β-cell proliferation (3). (1) Histone deacetylase (HDAC) class I (HDAC-I) and HDAC class IIa (HDAC-IIa) family members have distinct effects on the development of individual endocrine cell types. (2) While genes critical for β-cell function are expressed and marked by H3K4me3 in β-cells, these same genes are silent and bivalently modified in α-cells. This bivalency suggests a plastic epigenetic state for key β-cell genes in α-cells. Additionally, to maintain β-cell identity, genes important for α-cell function have to be actively repressed by the DNA methytransferases DNMT1 and DNMT3a in β-cells. (3) Epigenetic modification of the *Cdkn2* locus, encoding a cell cycle inhibitor, by BMI1 and EZH2 promotes β-cell

proliferation and regeneration, whereas the methyltransferase MLL1 contributes to β-cell senescence.