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Epigenetic Mechanisms in the Development of Type 2 Diabetes

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Abstract

T2D is a disorder of complex genetics influenced by interactions between susceptible genetic loci and environmental perturbations. Intrauterine growth retardation (IUGR) is one such environmental perturbation linked to the development of T2D in adulthood. An abnormal metabolic intrauterine milieu affects fetal development by permanently modifying expression of key genes regulating β-cell development (*Pdx1*) and glucose transport (*Glut4*) in muscle. Epigenetic regulation of gene expression is one mechanism by which genetic susceptibility and environmental insults can lead to T2D. Therefore, therapeutic agents targeting epigenetic gene regulation may ultimately be used to treat T2D; still, there is much to be learned about genome-wide epigenetic programming of health and disease before these therapies can be used in patient care.

The incidence of Type 2 Diabetes (T2D) has rapidly increased over the past several decades and is now reaching epidemic proportions across the globe. In fact, incidence of T2D is estimated to reach 366 million throughout the world by 2030 [1]. Simple Mendelian inheritance patterns have failed to describe the genetics of T2D, in that studies have identified single nucleotide polymorphisms linked to the development of T2D, but no disease-causing mutations have been discovered. Recent genome wide association studies identified at least 17 genetic loci associated with T2D [2], suggesting that T2D is a complex genetic disorder influenced by interactions between multiple susceptible genetic loci and environmental perturbations.

Environmental contributions to the development of T2D potentially include exposures such as a suboptimal *in utero* environment, low birth weight, obesity, inactivity and advancing age [3]. These environmental perturbations can lead to a disease phenotype by affecting gene expression through epigenetic modifications. Epigenetic changes are defined as mitotically heritable alterations in gene expression that are not related to changes in DNA sequence. One of the key environmental perturbations associated with T2D is exposure to an adverse intrauterine milieu, such as intrauterine growth retardation (IUGR). An adverse intrauterine milieu affects fetal development by modifying gene expression of both pluripotent cells that are rapidly replicating and terminally differentiated cells that replicate poorly. Whether the effect(s) of exposure to an altered intrauterine milieu extend into adulthood depends on whether the cells are undergoing differentiation, proliferation, and/or functional maturation at the time of that exposure.

There are several examples where human exposure to an abnormal intrauterine milieu leads to abnormalities in glucose homeostasis and ultimately T2D. For example, pregnant women

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exposed to the Dutch Hunger Winter, the period in late World War II during which daily caloric intake was limited to 400–800kcal, women delivered infants with lower birth weights. By age 50, these offspring had impaired glucose tolerance compared to offspring who were *in utero* either the year before or after the famine [5]. Another epidemiological study from Hertfordshire, UK found that men who were the smallest at birth (<2.5 kg) were seven times more likely to have glucose intolerance or T2D than those who were heaviest at birth [6]. Permanent changes in the phenotype of the offspring suggest that IUGR is associated with stable changes in gene expression, potentially due to epigenetic modifications. Here we provide a general review of epigenetics and discuss the possible causal role of chromatin remodeling in the development of T2D.

Chromatin structure, DNA methylation and gene expression

Epigenetic modifications of the genome provide a mechanism that allows the stable propagation of gene expression from one generation of cells to the next [7–10]. There are at least two distinct mechanisms through which epigenetic information can be inherited: histone modifications and DNA methylation.

Histone modifications

In eukaryotes, the nucleosome consists of DNA wrapped around an octameric complex of two molecules of each of the four histones: H2A, H2B, H3, and H4. The amino termini of histones can be modified by acetylation, methylation, sumoylation, phosphorylation, glycosylation, and ADP ribosylation. The most common histone modifications involve acetylation and methylation of lysine residues in the amino termini of H3 and H4. Increased acetylation induces transcription activation, whereas decreased acetylation usually induces transcription repression. Methylation of histones, on the other hand, is associated with both transcription repression and activation. Moreover, lysine residues can be mono-, di-, or trimethylated in vivo, providing an additional level of regulation.

DNA methylation

The second class of epigenetic regulation is DNA methylation, in which a cytosine base is modified by DNA methyltransferase at the C5 position of cytosine, a reaction that is carried out by various members of a single family of enzymes. Approximately 70% of CpG dinucleotides in human DNA are constitutively methylated, whereas most of the unmethylated CpGs are located in CpG islands. CpG islands are CG-rich sequences located near coding sequences and they serve as promoters for their associated genes. Approximately half of mammalian genes have CpG islands. The methylation status of CpG islands within promoter sequences works as an essential regulatory element by modifying the binding affinity of transcription factors to DNA binding sites. In normal cells, most CpG islands remain unmethylated; however, under circumstances such as cancer [16–19] and oxidative stress, they can become methylated *de novo*. This aberrant methylation is accompanied by local changes in histone modification and chromatin structure, such that the CpG island and its embedded promoter take on a repressed conformation that is incompatible with gene transcription. It is not known why particular CpG islands are susceptible to aberrant methylation.

DNA methylation is commonly associated with gene silencing and contributes to X-chromosomal inactivation, genomic imprinting, and transcriptional regulation of tissue-specific genes during cellular differentiation [reviewed in 14,15]. While it is not known why some genes undergo aberrant DNA methylationa study by Feltus *et al* [20] suggests a “DNA sequence signature associated with aberrant methylation”. Of major significance to T2D is their finding that *Pdx*-1, a pancreatic homeobox transcription factor, was one of only 15 genes (of 1749 examined) with CpG islands within the promoter that were methylation-susceptible

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(induced by over-expression of a DNA methyltransferase). This study demonstrates that genes essential to pancreatic development, like *Pdx-1*, are susceptible to epigenetic modifications that could ultimately affect gene expression.

Histone methylation can influence DNA methylation patterns and vice versa [13]. For example, methylation of lysine 9 on histone 3 (H3) promotes DNA methylation, while CpG methylation stimulates methylation of lysine 9 on H3 [14]. Recent evidence indicates that this reciprocal relationship between histone methylation and DNA methylation might be accomplished by direct interactions between histone and DNA methyltransferases [13]. Thus, chromatin modifications induced by adverse stimuli are self-reinforcing and can propagate.

Maternal nutritional supplementation and epigenetic modifications in offspring

The role of environmental regulation of epigenetic phenomena in offspring has been established by experiments performed in agouti mice (reviewed in [21]). Wild-type expression of the Agouti protein results in a phenotypic brown coat color in the mouse. In this mouse model, an endogenous retrovirus-like transposon sequence is inserted close to the gene coding for the Agouti protein. An unmethylated retrotransposon promoter overrides the wild-type agouti promoter, resulting in ectopic agouti expression and a yellow coat color. A methylated retrotransposon is silenced and results in a wild-type agouti (brown) coat. Wolff *et al* investigated whether maternal diet alters the phenotype of the agouti mouse [22] and found that when pregnant females are fed a diet supplemented with methyl donors, a larger proportion of offspring have a wild-type Agouti coat color compared with offspring of mothers fed a standard diet. These studies indicate that the maternal methyl donor diet leads to increased methylation of the offspring's retrotransposon. Methylation silences the offspring's retrotransposon allowing the wild-type agouti promoter to be expressed, thus resulting in a mouse with a wild-type (brown) coat color. Therefore, maternal nutrition can change the stable expression of genes in offspring through epigenetic modifications occurring *in utero*.

Chromatin Remodeling and Oxidative Stress

Exposure to oxidative stress can directly mediate both DNA methylation and chromatin remodeling in multiple disease models and thus could be a mechanism by which aberrant epigenetic programming leads to T2D [16–25]. In addition to targeted DNA methylation changes in response to external stimuli, random DNA methylation changes also occur during aging in several tissue types and are associated with increased oxidative stress [17,24]. Such changes in DNA methylation patterns affect the expression of multiple genes [25]. Further, replacement of guanine with the oxygen radical adduct 8-hydroxyguanine profoundly alters methylation of adjacent cytosines [25].

Histones, because of their abundant lysine residues, are susceptible to oxidative stress [26–28]. Recent discovery of histone demethylases such as members of the jumoniji C family, which requireoxygen as a cofactor, link epigenetic processes to oxygen gradients during development (reviewed in [29]). This is particularly relevant to T2D, since there are now substantial data showing that oxidative stress plays a significant role in β-cell deterioration [30–34]. Further, intrauterine growth retardation induces mitochondrial dysfunction in the β-cell leading to increased production of ROS and oxidative stress [35].

Epigenetic regulation of gene expression in fetal growth retardation

A number of studies suggest that uteroplacental insufficiency, the most common cause of IUGR in the developed world, induces epigenetic modifications in offspring [36–40]. Fetal growth

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retardation can be induced by bilateral uterine artery ligation in the pregnant rat [41]. Following this, pups are born spontaneously with decreased levels of glucose, insulin, IGF-1 and amino acids [41]. Diabetes develops in these animals at approximately 15–26 weeks of age with underlying β-cell secretory defects and insulin resistance, the salient features of most forms of T2D in humans [41,42]. Epigenetic modifications affecting processes important to glucose regulation and insulin secretion, characteristics essential to the pathophysiology of T2D, have been described in the IUGR liver, pancreatic β-cells and muscle. The following sections describe specific epigenetic modifications induced in the IUGR model and their relationship to the development of T2D.

Chromatin Remodeling in the ƒÀ-cell of IUGR rats

Pdx-1 is a homeodomain-containing transcription factor that plays a critical role in the early development of both the endocrine and exocrine pancreas and in the later differentiation and function of the β-cell. As early as 24 hours after the onset of growth retardation, *Pdx-1* mRNA levels are reduced by more than 50% in IUGR fetal rats. Suppression of *Pdx-1* expression persists after birth and progressively declines in the IUGR animal, implicating an epigenetic mechanism.

A change in histone acetylation is the first epigenetic modification found in β-cells of IUGR animals (Figure 1). Islets isolated from IUGR fetuses show a significant decrease in H3 and H4 acetylation at the proximal promoter of *Pdx-1* [39]. These changes in H3 and H4 acetylation are associated with a loss of binding of USF-1 to the proximal promoter of *Pdx1* [39]. USF-1 is a critical activator of *Pdx-1* transcription, and its decreased binding markedly decreases *Pdx-1* transcription [43,44]. After birth, histone deacetylation progresses and is followed by a marked decrease in H3K4 trimethylation and a significant increase in dimethylation of H3K9 in IUGR islets (39). H3K4 trimethylation is usually associated with active gene transcription, while H3K9 dimethylation is usually a repressive chromatin mark. Progression of these histone modifications parallels the progressive decrease in *Pdx-1* expression that manifests as defective glucose homeostasis and increased oxidative stress in aging IUGR animals [39]. Nevertheless, at 2 weeks of age, the silencing histone modifications in the IUGR pup suppress *Pdx1* expression, since there is no appreciable methylation of CpG islands in mice at this age [39]. Reversal of histone deacetylation in IUGR islets at 2 weeks of age is sufficient to nearly normalize *Pdx-1* mRNA levels permanently, perhaps due to active β-cell replication present in the neonatal rodent [39].

In IUGR, *Pdx-1* is first silenced due to recruitment of co-repressors, including histone deacetylase 1 (HDAC1) and mSin3A [39]. Binding of these deacetylases facilitates loss of trimethylation of H3K4, further repressing *Pdx1* expression [39]. In fact, inhibiting HDAC activity by trichostatin A (TSA) normalizes H3K4me3 levels on *Pdx1* in IUGR islets [39]. These data suggest that the association of HDAC1 with *Pdx1* in IUGR islets likely serves as a platform for recruiting a demethylase to catalyze demethylation of H3K4.

The molecular mechanism responsible for DNA methylation in IUGR islets is likely dependent on the methylation status of lysine 9 on H3 (H3K9). Previous studies showed that changes in methylation of H3K9 precede changes in DNA methylation [45,46]. It has also been suggested that DNA methyltransferases may act only on chromatin that is methylated at H3K9 [47]. Another class of enzymes, the histone methyltransferases, e.g. DNA methyltransferase 3A (DNMT3A) and DNA methyltransferase 3B (DNMT3B), bind to DNA methylases to initiate DNA methylation [38].

These results demonstrate that IUGR induces a self-propagating epigenetic cycle (Figure 1) in which the mSin3A/HDAC complex is first recruited to the *Pdx1* promoter, histone tails are subjected to deacetylation and *Pdx1* transcription is repressed. At the neonatal stage, this

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epigenetic process is reversible and may define an important developmental window for therapeutic approaches. However, as dimethylated H3K9 accumulates, DNMT3A is recruited to the promoter and initiates *de novo* DNA methylation, which locks in the silenced state in the IUGR adult pancreas resulting in diabetes.

How do these epigenetic events lead to diabetes? Targeted homozygous disruption of *Pdx-1* in mice results in pancreatic agenesis, and homozygous mutations yield a similar phenotype in humans [48]. A milder reduction in *Pdx-1* protein levels, as occurs in *Pdx-1*$^{+/-}$ mice, allows for a normal β-cell mass [48], but results in the impairment of several events in glucose-stimulated insulin secretion [48]. These results indicate that *Pdx-1* plays a critical role in β-cell function [48] in addition to its role in β-cell lineage development. This may be the reason that humans with heterozygous missense mutations in *Pdx-1* exhibit early and late onset forms of T2D [48].

The discovery of a critical developmental stage during which aberrant epigenetic modifications may be reversed represents a therapeutic window for the use of novel agents that could prevent common diseases with late-onset phenotypes [39]. T2D is one such disease – where predisposed individuals could be treated with agents that normalize the epigenetic programming of key genes, thus providing protection against development of the adult diabetic phenotype.

Chromatin Remodeling in Muscle of IUGR rats

Reduced glucose transport in muscle is a trademark of insulin resistance in IUGR offspring [49,50]. Under normal physiological circumstances, glucose transport occurs by facilitated diffusion, a rate-limiting step in glucose utilization [51]. This process of glucose transport is mediated by a family of structurally related membrane-spanning glycoproteins, termed facilitative glucose transporters (GLUT; Slc2 family of transport proteins; reviewed in [52]). Of the isoforms cloned to date, GLUT4 is the major insulin-responsive isoform expressed in insulin-sensitive tissues such as skeletal muscle, adipose tissue and cardiac muscle [52]. In mice, the promoter region of *Glut4* has been well-characterized, and disruption of the myocyte enhancer factor 2 (MEF2)-binding site ablates tissue-specific *Glut4* expression in transgenic mice (52). MyoD, on the other hand, is responsible for *Glut4* expression *in vitro* during myoblast to myocyte differentiation [53]. MyoD binding to MEF2 and TR1 spans the 502- to 420-bp region of *Glut4* in skeletal muscle (Figure 2). These two proteins synergistically enhance skeletal muscle *Glut4* transcription and gene expression (53).

It has recently been shown by Raychaudhuri and colleagues [40] that IUGR is associated with an increase in MEF2D (a form of MEF2 that acts as an inhibitor) and a decrease in both MEF2A (a form of MEF2 that acts as an activator) and MyoD (a co-activator) binding to the *Glut4* promoter in skeletal muscle. Interestingly, differential methylation of these three CpG clusters in the *Glut4* promoter was not observed. This study also found that DNA methyltransferase (DNMT) binds to the *Glut4* gene at different ages – DNA methyltransferase 1 (DNMT1) binds postnatally, while DNMT3a and DNMT3b bind in adults. The increase in DNMT binding was associated with exposure to increased methyl CpG-binding protein 2 (MeCP2) concentrations. Covalent modifications of the histone code consisted of histone 3 lysine 14 (H3K14) de-acetylation mediated by recruitment of HDAC1 and enhanced association of HDAC4. This set the stage for Suv39H1 methylase-mediated di-methylation of H3K9 and increased recruitment of heterochromatin protein 1, which partially inactivates postnatal and adult IUGR *Glut4* gene transcription (Figure 2). These studies demonstrate that perinatal nutrient restriction resulting in IUGR leads to silencing histone modifications in skeletal muscle which in turn directly decrease *Glut4* gene expression, effectively creating a metabolic knockdown of this important regulator of peripheral glucose transport and insulin resistance and contributing to the adult

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T2D phenotype [40]. Hence, these studies show that histone modifications can be stably inherited in a calorie-restricted model of IUGR, mimicking the Dutch famine experience described earlier.

Histone modifications in vascular epithelium exposed to hyperglycemia

The previous examples describing the relationship between chromatin remodeling and its contribution to the development of T2D focused on the IUGR animal model. We focused on this model, since it is the only system that has so far been able to tie specific chromatin modifications to changes in gene expression relevant to alterations in glucose homeostasis *in vivo*. Brasacchio *et al* describe how transient hyperglycemia *in vitro* induces changes in histone methylation at the promoter of NFκB-p65 in vascular epithelial cells, changing NFκB-p65 expression and contributing to vascular complications similar to those seen in T2D [54].

NFκB- driven proinflammatory gene expression appears to play a major role in the pathogenesis of atherosclerosis [62,63]. *In vitro* studies were performed using bovine aortic endothelial cells and HMEC cells (a human microvascular cell line) in which cells were cultured for 16 hours under either normoglycemic or hyperglycemic conditions, all followed by normoglycemic conditions for up to one week. Initial exposure to 16 hours of hyperglycemia was associated with persistent increased expression of NFκB-p65 up to 1 week after treatment despite returning to conditions of normoglycemia after the initial 16 hour treatment. Increased NFκB-p65 gene expression was associated with persistently increased H3K4 mono-methylation at the NFκB-p65 promoter but not with H3K4 di-methylation or tri-methylation. These experiments indicate that increased NFκB-p65 gene expression is associated with persisting epigenetic marks that are maintained when the cell is removed from its hyperglycemic environment, providing evidence that epigenetic modifications contribute to altered gene expression and could form the basis for physiologic “hyperglycemic memory”.

T2D therapeutic agents targeting chromatin remodeling

Drugs that are currently used to treat patients with T2D have been shown to reverse epigenetic modifications *in vitro*. Treating INS1 (832/13) β-cells (a rat insulinoma cell line) or dispersed mouse islets with incretin hormones such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic-peptide 1 (GIP) increased global acetylation of histone H3 at lysine residues 9 and 18 and increased phosphorylation at serine 10 in a concentration-dependent manner [55]. These histone modifications at H3 increased its association with the transcription factor, phosphorylated cAMP-response element-binding protein (phospho-CREB) and with cAMP-response CREB coactivator 2. Therefore, treatment with incretin hormones (either GLP-1 or GIP) induces β-cell chromatin remodeling, which may lead to coordinated interactions between specific chromatin-modifying enzymes and transcription factors *in vitro*. However, it is to be noted that changes in histone modifications were not linked to gene expression in this study and these findings still need to be examined *in vivo.*

In addition to incretin hormones, the nuclear receptor proliferator-activated receptor γ (PPAR-γ) is an important target in diabetes therapy. Treatment of diabetic or glucose-intolerant mice with a PPAR-γ agonist improves glycemic control, increases serum insulin and enhances glucose stimulated insulin release, indicating direct effects on the islet [56–58]. Evans-Molina *et al* treated 8 week old C57BL6/J-*db/db* (*db/db*) mice or *db/db* mice fed a high fat diet (HFD) with a PPAR-γ agonist and showed that both groups of mice had significantly improved whole body glucose homeostasis and improved insulin secretion [59]. Cultured islets harvested from mice following 6 weeks of oral pioglitazone therapy showed a 15- and 7-fold increase in expression of Ins1/2 and GLUT 2, respectively. These islets also showed increased acetylated histone H3, increased dimethyl H3K4 association at the proximal promoter of Ins1/2, and increased Set7/9 (a specific dimethyl H3K4 methyltransferase) mRNA and protein levels. The

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specific chromatin remodeling mechanisms described above could be responsible for increased gene expression of both Ins 1/2 and GLUT2 in this model system, but the effects of pioglitazone treatment *in vivo* remain to be demonstrated.

Concluding Remarks

The studies described above clearly show that environmental effects can induce epigenetic alterations, ultimately affecting expression of key genes linked to the development of T2D including genes critical for pancreatic development and β-cell function, peripheral glucose uptake and insulin resistance, and atherosclerosis. Recent progress in understanding epigenetic programming of gene function has led to the development of novel therapeutic agents with epigenetic targets in diseases such as cancer. Understanding the role of developmental programming of genes crucial to the development of T2D may unveil a critical window during which epigenetic therapeutic agents could be used as a means to prevent the later development of a disease. Prior to the use of such therapeutic agents, there remains much to be learned about the programming of the epigenetic code, especially on a genome-wide scale.

Much of the recent progress in understanding epigenetic phenomena is directly attributable to technologies that allow researchers to pinpoint the genomic location of proteins that package and regulate access to the DNA. The advent of DNA microarrays and inexpensive DNA sequencing allowed many of those technologies to be applied to the whole genome. For example, it is now possible that epigenetic profiling of CpG islands in the human genome can be used as a tool to identify genomic loci that are susceptible to DNA methylation. Aberrant methylation may be then be used as a biomarker for disease [47–50].

The genome-wide mapping of histone modifications by ChIP-chip and ChIP-seq has led to important insights regarding the mechanism of transcriptional and epigenetic memory, and how different chromatin states are propagated through the genome in yeast [60] and in mammalian cells [61]. Although Chip-Seq experiments are currently being performed in human tissues, obstacles such as intrinsic human epigenetic variability (including age-related changes) and tissue-specific epigenetic variability must be characterized and mapped in the healthy, non-diseased state before this information can be applied to diseases such as T2D. Eventually genome-wide epigenetic characterization will lead to specific therapies with epigenetic targets and will also allow monitoring of genome-wide epigenetic consequences of these therapies.

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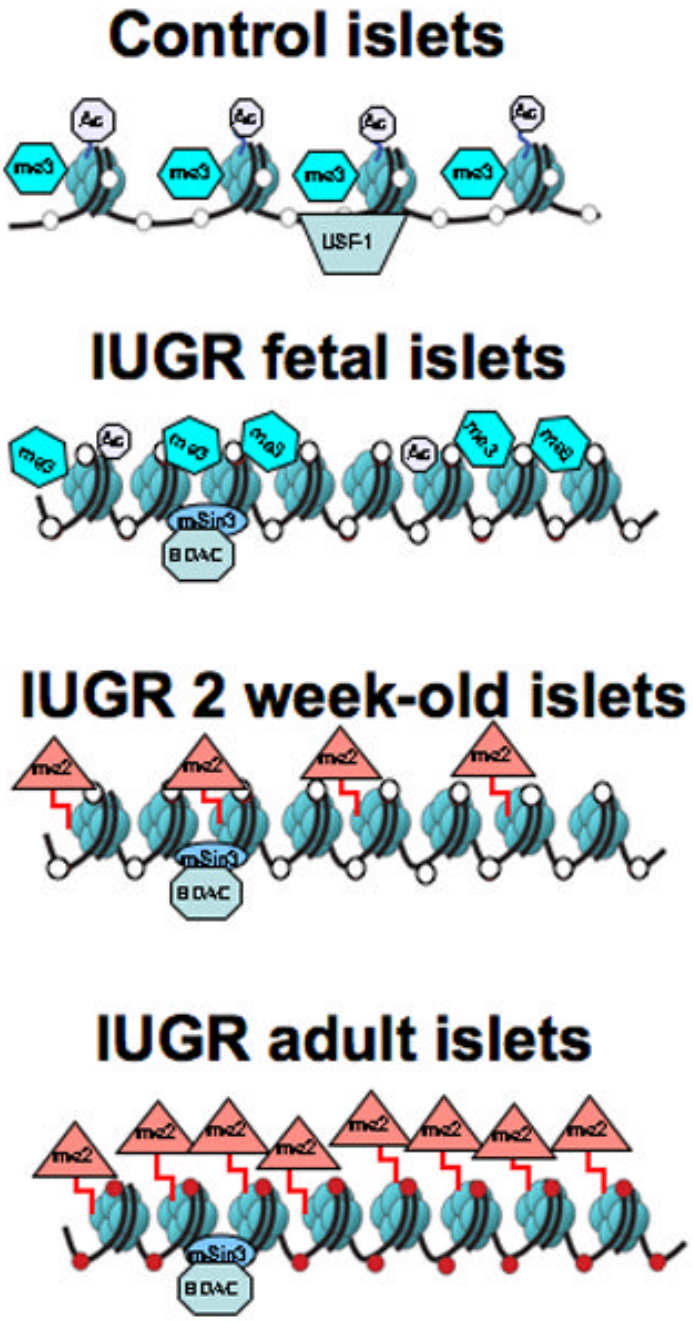

Figure 1. Epigenetic changes at *Pdx1* in IUGR rats during the development of type 2 diabetes

(a) In pancreatic β-cells, the *Pdx1* proximal promoter is normally found in an unmethylated (white circles) open chromatin state allowing access to transcription factors such as USF-1 and associated with nucleosomes characterized by acetylated (Ac, blue octagons) histones H3 and H4 and with trimethylated H3K4 (Me, green hexagons). (b) In IUGR fetal and (c) 2 week islets, histone acetylation is progressively lost through association with a mSin3A-HDAC1-DNMT1 repressor complex, with trimethylated H3K4 disappearing and dimethylated H3K9 (Me, red triangles) appearing after birth. (d) IUGR adult islets are characterized by inactive chromatin with dimethylated H3K9 and extensive DNA methylation (red circles) locking in the transcriptionally silent state of *Pdx1*.

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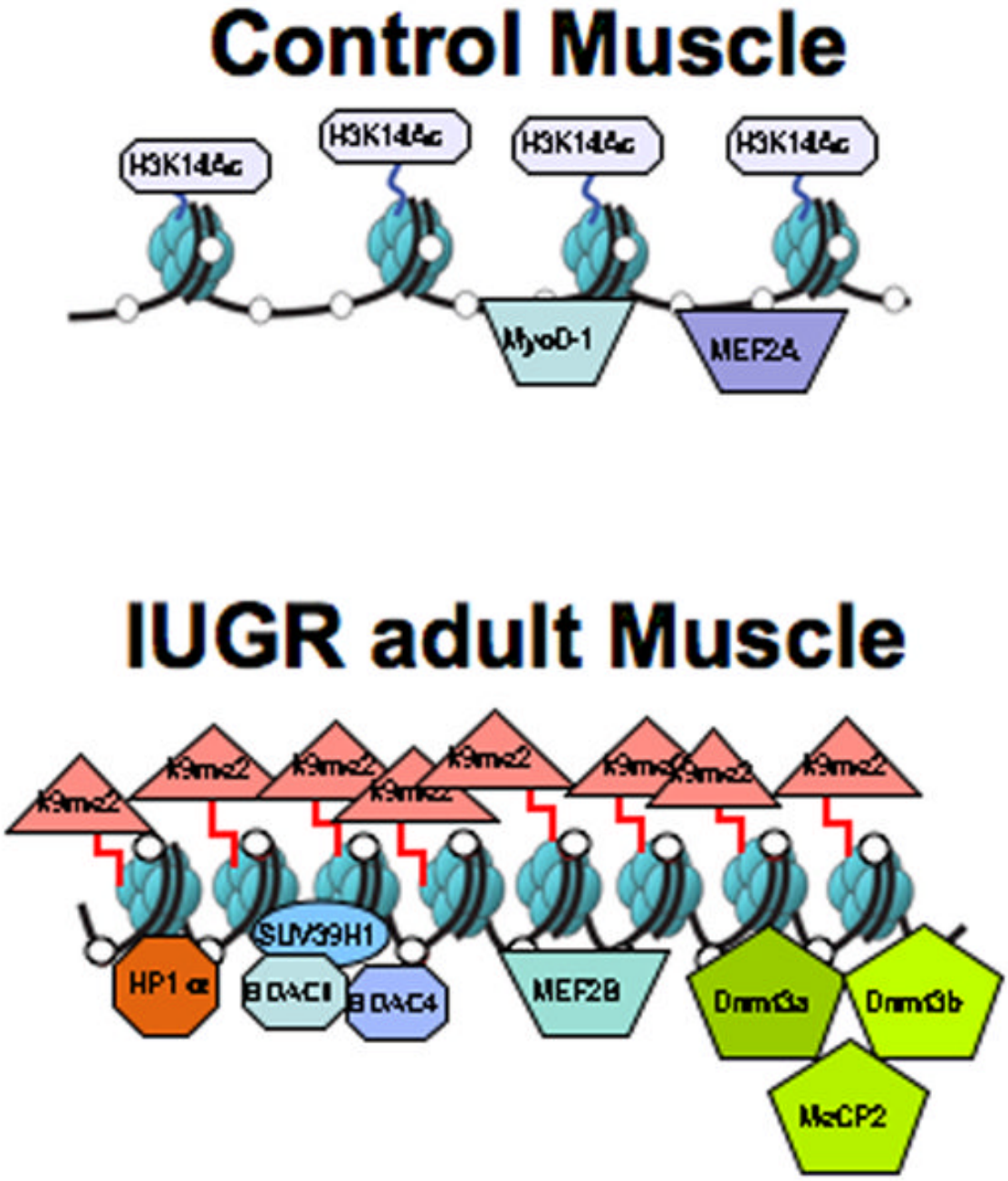

Figure 2. Epigenetic changes at *Glut4* in IUGR rats

(a) In muscle, the *Glut4* promoter is found in the unmethylated (white circles) open chromatin state allowing access to transcription factors such as MEF2A and MyoD1 and associated with nucleosomes characterized by acetylated K14Ac (H3K14Ac, blue octagons) on histones H3. (b) In IUGR adult muscle, histone acetylation is lost, mediated by the histone deacetylases HDAC1 and HDAC4. Unlike the *Pdx1* promoter in IUGR islets, no differential methylation of the CpG islands in the *Glut4* promoter occurs. In the adult IUGR animal, the CpG islands are characterized by association of the repressors DNMT3a and 3b as well as MeCP2 (green pentagons). This sets the stage for Suv39H1 methylase (blue oval)-mediated di-methylation

of H3K9 (orange triangle) and increased recruitment of heterochromatin protein 1α (HP1α-orange octagon), which partially inactivates postnatal and adult IUGR *glut4* gene transcription.

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