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Epigenetics: A New Player in the Regulation of Mammalian Puberty

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Abstract
All reproductively competent adults have gone through puberty. While key genes and signaling pathways that lead to the onset of sexual maturation are known, the molecular mechanisms that determine when an individual enters puberty are only beginning to be understood. Both genetic and environmental factors determine the timing of puberty. New advances in understanding how environmentally sensitive, yet highly heritable developmental processes are regulated have come from the field of epigenetics. Of note, studies investigating the epigenetic control of the onset of puberty suggest that epigenetic repression of key inhibitory loci may play a fundamental role in the initiation of puberty. Current technologies that not only read out the DNA sequence, but also determine how the DNA is modified in response to the environment, promise new insight into how puberty is regulated, including the identification and understanding of gene regulatory networks that control the biological pathways affecting pubertal timing. Here we review the findings to date and discuss how epigenetic investigation can further our understanding of this fundamental aspect of human development.

Introduction

When gonadotropin-releasing hormone (GnRH) secretion emerges from its childhood quiescence and the hypothalamic-pituitary-gonadal (HPG) axis is reactivated, puberty is initiated, sexual maturation ensues and boys and girls transition into adolescence. Although this is a common experience, the age at the onset of puberty varies greatly among individuals. The age of onset also varies between the sexes; females on average experience the onset of puberty at younger ages than males and are more likely to have idiopathic central precocious puberty while boys are predisposed to idiopathic delayed puberty (referred to as constitutional delay of growth and puberty) [1]. Even within the normal range of age at onset, understanding the causes of variation in the timing of puberty is important because the age of onset is associated

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with later life outcomes. For example, early age at menarche (AAM) is a risk factor for breast cancer [2–4], cardiovascular disease [5], depression [6], eating disorders [7], conduct and behavioral disorders [8], diabetes [9] and increased all-cause mortality [10]. Conversely, late AAM is associated with a decreased risk of ischemic heart disease [11] but an increased risk of osteoporotic fracture [12, 13]. Boys are also affected by alterations in pubertal timing, with delayed puberty being a cause of bullying, poor self-esteem and psychosocial distress, while early puberty is a risk factor for testicular cancer [8]. Although these associations need to be verified and their biological and/or psychosocial origins clarified, it is clear that pubertal timing impacts health during and after adolescence [14]. It is possible that the identification of pathways that regulate the timing of puberty may lead to the identification of overlapping pathways that play a role in the pathogenesis of some of the outcomes.

Why this variability exists can be explained only partially by genetic background and environmental influences. Recent genome-wide association studies (GWAS) have provided important new knowledge regarding 32 new genetic loci and pathways that regulate the timing of puberty [15–19], but the genetic variants identified, including those associated with the RNA binding protein gene *LIN28B*, explain less than 7% of the variation in the timing of menarche [17]. Importantly, for 19 of these 32 loci, the gene closest to the particular locus is a gene known to be directly involved in epigenetic regulation of the genome. Genetic investigation has also identified many other genes that lead to rare disorders of puberty, such as the GnRH gene and its receptor (*GNRH1* and *GNRHR*), the neuropeptide kisspeptin and its receptor [*KISS1* and *KISS1R* (previously referred to as *GPR54*)], and neurokinin B and its receptor (*TAC3* and *TACR3*) in hypogonadotropic hypogonadism [for reviews, see 20, 21] and malkorin RING-finger protein 3, *MKRN3*, in familial precocious puberty [22]. However, sequence variation in these and other disease-causing genes does not appear to be a substantial cause of variation in pubertal timing within the general population. Interestingly, variation in pubertal timing manifests itself even in genetically identical individuals. Although greater in dizygotic twins, variation is present in monozygotic twins [23] and even in inbred rodents sharing largely identical environmental conditions [24, 25] (fig. 1).

Environmental factors also regulate the timing of puberty. Factors such as general health, nutrition, endocrine disrupting chemicals and stress can affect pubertal timing [26–30], and timing can be influenced by environmental exposures at critical periods, including in utero [31]. It is also known that a complex gene network is involved in coordinating the regulation of pubertal onset and GnRH release, involving inhibitory, excitatory and permissive inputs that show dynamic regulation that changes over time [32]. The characteristics of transcriptional regulation and phenotypic plasticity suggest that epigenetically mediated gene-by-environment interactions regulate the timing of puberty and may underlie some of the unexplained variability in this process.

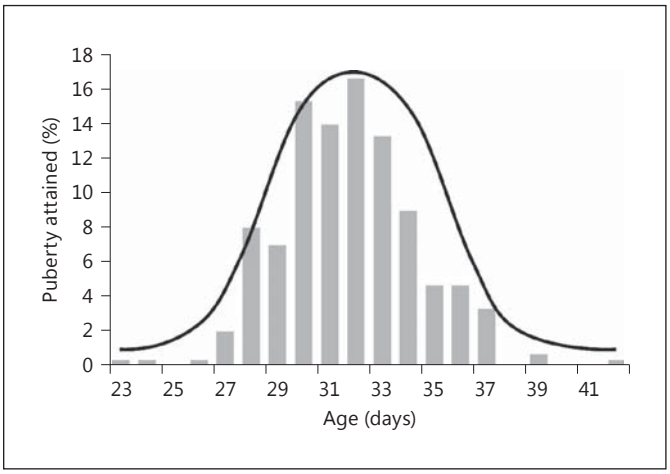


Fig. 1. The timing of puberty approximates a normal distribution. This pattern has been observed in humans, but it is also seen in model systems such as mice, as illustrated here for female mice based on data collected in our laboratory over several years (n = 298 C57BL/6J mice, mean age of vaginal opening 31.7 ± 2.6 postnatal days). Why genetically identical inbred mice display this degree of variability is unclear, but one hypothesis is that it could stem from epigenetic differences. Several macroenvironmental factors have been assessed and do not explain the variability, including litter size, male-to-female ratio in the litter, age of mother and father at mating, and mother's first or second pregnancy. Although extreme differences in litter size can affect phenotypes such as pubertal timing (likely due to the associated over- or undernutrition differences that arise) [123–125], in naturally occurring litter sizes, there is no marked effect on pubertal timing. Proposed epigenetic differences could arise from microenvironmental differences or be stochastic [126, 127].

What Is Epigenetics and How Might It Affect the Timing of Puberty?

Epigenetic control systems have emerged as strong determinants of disease and developmental phenotypes. Epigenetics refers to a mitotically heritable layer of tran-

scriptional control in the absence of changes to the DNA sequence. Common epigenetic mechanisms include differential methylation of 5-methylcytosine (5-mC) in cytosine guanine dinucleotides (CpGs) of DNA and covalent histone posttranslational modifications (HPTMs), such as acetylation and methylation [33]. These modifications affect chromatin structure and gene expression and have been shown to contribute to variation in complex phenotypes including mental disorders, stress response, appetite, metabolism and inflammation [for reviews, see 29, 34, 35].

Until recently, only a few reports speculated [20, 36, 37] or provided evidence supporting the involvement of epigenetic mechanisms in the regulation of pubertal onset [24, 38–42]. However, exciting recent developments in understanding the mechanisms underlying the reactivation of the HPG axis have pointed to the involvement of an epigenetically mediated control of neuroendocrine-related gene transcription. Foremost in this area is the recent original research paper by Lomniczi et al. [43] which ushers puberty research more forcefully into the realm of epigenetics. A major development supported by this and other studies is that the activation of neuroendocrine components involved in the initiation of puberty is, at least in part, mediated by an epigenetically mediated lifting of factors that were inhibiting HPG axis neurons during the childhood quiescence.

The search for the genes and gene regulatory networks that are under epigenetic control and involved in determining the timing of puberty is in its infancy. The future involves utilizing innovative, genome-wide scanning approaches to identify genes and pathways that are regulated by epigenetic mechanisms and that play important roles in regulating pubertal onset.

Classic Mammalian Epigenetic Control Systems

DNA Methylation

Methylation of cytosine residues (5-mC) of DNA, predominantly in the context of cytosine guanine (CpG) dinucleotides [44], is a key epigenetic mechanism for regulating gene expression. DNA methylation plays a central role in genomic imprinting where either the maternally or paternally inherited allele is methylated and silenced. This results in parent-of-origin effects on gene expression and phenotype [22, 45]. It is interesting in this regard that a recent study in children with precocious puberty identified a paternally expressed imprinted gene *(MKRN3)* which, when mutated, appears to inhibit the HPG axis less effectively, resulting in precocious puberty [22].

5-mC plays important roles outside of imprinting and is typically associated with lower levels of expression [46], with some noted exceptions [47]. 5-mC regulates gene expression through methylation at promoters but also at intragenic and intergenic sites, with DNA methylation of intragenic sites being a regulator of tissue and even cell-specific gene expression [48]. The degree of DNA methylation is controlled by the activity of DNA methyltransferases but can also be affected by other mechanisms such as transcription factor binding at or near CpG sites, which can lead to loss of methylation [49]. Transcription factor binding has been shown to be a necessary and sufficient means of generating low-methylated regions and of effecting cell-type-specific gene regulation [50].

To understand the interplay between DNA methylation and genetic variation, Gutierrez-Arcelus et al. [51] obtained gene expression and CpG methylation data from 3 cell types (fibroblasts, T cells and lymphoblastoid cells) from over 200 healthy newborns. This study showed that variability in interindividual gene expression is associated with differential methylation specifically in the genomic regions that they found to show differential methylation between the 3 cell types. This result highlights the importance of DNA methylation at specific regions during development and suggests that epigenetic changes at these select genomic regions are more likely to be functionally relevant. Importantly, this variability in gene expression was also linked to variation in DNA sequence. Overall this study gives new insight into the cross-talk between genetics and epigenetics, which is certainly reflected in recent mechanistic studies of pubertal development [43].

The pattern of 5-mC in DNA is dictated by the relative activity of enzymes acting to add, remove or maintain this epigenetic mark. The enzymes responsible for maintaining DNA methylation patterns during replication and catalyzing de novo DNA methylation are the DNA methyltransferases DNMT1 and DNMT3a and b, respectively [52]. DNA demethylation, on the other hand, can occur passively or actively. The passive loss of 5-mC occurs during DNA replication [53]. Active demethylation occurs through the oxidation of 5-mC to 5-hydroxymethyl cytosine (5-hmC) by the ten–eleven translocating enzymes, which is followed by full demethylation by thymine DNA glycosylase-mediated base excision and the regeneration of unmodified cytosines through DNA repair pathways [53]. It has recently been shown that 5-hmC is also enriched in active genes in neuronal tissues and can be

bound by the Rett syndrome protein methyl CpG binding protein 2 [54], thus providing a clear link between 5-hmC and chromatin regulation. Relevant for research on pubertal timing, a survey of 5-hmC in the mouse brain revealed high levels of 5-hmC in the hypothalamus [55].

Histone Modifications

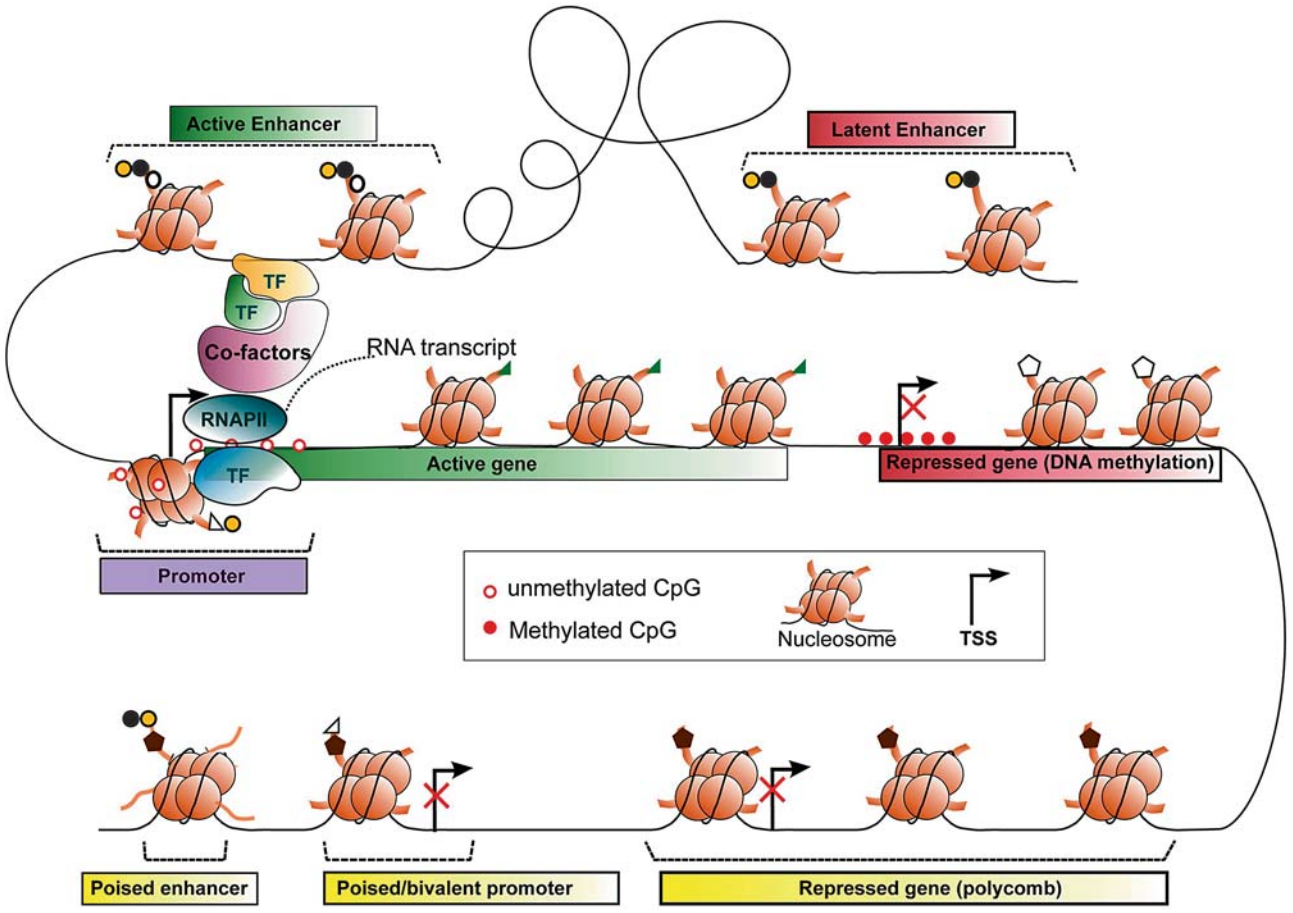
HPTMs, such as acetylation (Ac) and methylation (me), regulate gene expression during development and in response to the environment. HPTMs are a cornerstone of epigenetic regulation. Changes in HPTMs occur in response to gene regulatory events such as the binding of transcription factors to DNA and can be stably inherited through mitosis. The precise genomic location of HPTMs in a wide variety of cell lines and primary tissues from multiple species, and developmental stages are becoming increasingly available owing to the use of chromatin immunoprecipitation followed by high-throughput DNA sequencing (ChIP-Seq). In particular, the Encyclopedia of DNA Elements Consortium ENCODE [56] and the NIH Roadmap Reference Epigenome Mapping Consortium [57] have used ChIP-Seq to generate genome-wide data for several HPTMs in hundreds of different samples. Analysis of these data has shown that a subset of these HPTMs is able to annotate the entire genome into discrete chromatin states of relatively active or silenced gene expression [58, 59]. Although dozens of HPTMs have been mapped to the genome of several cell types, the HPTMs histone H3 lysine residue 4 monomethylated chromatin mark (H3K4me1), H3K4me2 and H3K27Ac (active enhancers), H3K4me3 (transcription start sites), H3K36me3 (actively transcribed gene bodies) and H3K27me3 (repressed gene regions/or poised promoters and enhancers) are a particularly informative means of annotating the genome into biologically meaningful categories (fig. 2).

HPTMs can function to physically aid or obstruct the access of transcriptional machinery to transcriptional start sites, gene regulatory regions and gene bodies [60]. Presence of histone acetylation, which is added by histone acetyltransferases [61], leads to less compact chromatin and easier access to transcription start sites. Conversely, deacetylation by histone deacetylases causes chromatin to be more tightly coiled because positively charged DNA winds around the negatively charged histones more fully in the absence of neutralizing acetyl groups [62, 63], leading to decreased gene expression.

Methylation of histones can also affect gene transcription. The inhibitory histone H3 lysine residue 27 trimethylated chromatin mark (H3K27me3) is deposited by the Polycomb group repressive complex 2 (PRC2), a dynamic complex of chromatin-associated proteins that mediate epigenetic silencing of transcription through chromatin compaction [64]. When H3K27me3 co-occurs with the activating HPTM histone H3 lysine residue 4 trimethylated chromatin mark (H3K4me3) that resides at transcription start sites, the region is often described as bivalent or developmentally poised [65] (fig. 2). RNA polymerase II is bound in this region, but its activity is paused until H3K27me3 inhibition is lifted. The mechanisms that activate these H3K27me3 silenced or poised promoters/enhancers have been implicated in puberty regulation [43] making the regulation of the PRC2 complex during HPG axis development of great interest. In related work, histone demethylation via lysine-specific demethylase (LSD1) has also been linked to alterations in pubertal timing in rodents [42].

Histone modifications can have long-term effects on gene regulation and can serve as a 'metabolic memory' that can have important consequences during disease progression. For example, vascular smooth muscle cells obtained from diabetic mice retain their diabetic phenotype after several weeks in culture [66]. This epigenetic phenomenon has been linked to a loss of the repressive histone H_3 lysine residue 9 trimethylated chromatin mark (H3K9me3) at inflammatory genes, which in turn results in hypersensitivity to inflammatory stimuli. Further supporting this idea is a recent study in macrophages that illustrates the concept of activating 'epigenetic scars' which are comprised of HPTMs (monomethylation of the lysine 4 on histone H_3; H3K4me1) that occur due to an inflammatory event and remain associated at specific DNA loci long after inflammatory stimuli have been washed away [67]. These 'epigenetic scars' are distinct from poised enhancers marked by H3K27me3/H3K4me1 and result in enhanced activation of specific genes upon restimulation. The possibility of repressive or activating 'epigenetic scars' occurring in early development that later manifest themselves in differences in pubertal timing due to additional environmental stimuli is an intriguing epigenetic mechanism that could generate variability in pubertal timing.

In summary, different activity of the various components of the epigenetic machinery leads to differing patterns of DNA methylation, histone acetylation and histone methylation. These differences provide a powerful mechanism to regulate gene expression that can vary from individual to individual, over time, and across development.



Abbreviation	Full name of HPTM	Functional associations
H3K4me1	Histone H3 lysine 4 monomethylation	Active/latent/poised enhancers
H3K4me2	Histone H3 lysine 4 dimethylation	Active/latent/poised enhancers/promoters
H3K4me3	Histone H3 lysine 4 trimethylation	Active promoters
H3K27Ac	Histone H3 lysine 27 acetylation	Actively transcribed genes
H3K36me3	Histone H3 lysine 36 trimethylation	Permanent repression at heterochromatin
H3K27me3	Histone H3 lysine 27 trimethylation	Temporary repression at latent/poised enhancers/ promoters and repressed genes
H3K9me3	Histone H3 lysine 9 trimethylation	Permanent repression at heterochromatin

Fig. 2. Overview of commonly studied epigenetic modifications to histone proteins and DNA, and their effects on gene expression. The black line represents DNA. TSS = Transcription start site; TF = transcription factor; RNAPII = RNA polymerase 2.

Evidence that DNA Methylation and Histone Modifications Regulate Pubertal Timing

Various pharmacological agents have the ability to perturb epigenetic marks on a global scale, and the prepubertal period is one during which DNA is highly susceptible to epigenetic changes [68]. These factors led three independent research groups to attempt to modify the epigenome in prepubertal rodents to determine if alterations of epigenetic profiles would lead to changes in pubertal timing. The results from all three groups were consistent with a mechanism that requires the lifting of inhibitory inputs for puberty to occur.

Pharmacological Perturbation of the Epigenome

Using 5-azacytidine (AZA), a powerful inhibitor of DNA methyltransferases (DNMTs) and hence DNA methylation, Lomniczi et al. [43] provide strong evidence that the timing of puberty is under an inhibitory control. In their work, AZA was administered to prepubertal female rats. Compared with controls, rats that received AZA showed a delay in puberty and reproductive maturation. Vaginal opening, an estrogen-dependent marker of the onset of puberty in rodents, was delayed, with control rats reaching this milestone at 31.33 ± 0.21 days and rats treated with AZA reaching vaginal opening much later at 36.67 ± 0.67 days ($p < 0.001$; fig. 3a). Ovarian morphology also showed evidence of delayed maturation with ovaries of AZA-treated rats at 28 days being smaller and having fewer mature antral follicles than ovaries of control rats (fig. 3b), and ovaries of 44-day-old AZA-treated rats lacking corpora lutea. Finally, the AZA-treated rats also showed disrupted estrous cyclicity compared to controls. Since AZA inhibits methylation, it will in general lead to increased gene expression; since this increased gene expression leads to delayed puberty, the findings argue for an effect on genes that inhibit HPG axis activation.

In parallel to the work by Lomniczi et al. [43], we asked whether administering the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) to female mice could alter pubertal timing. In unpublished experiments, Rzeczkowska et al. [24] corroborate the findings of Lomniczi et al. [43] as SAHA treatment delayed the onset of puberty. This was evidenced by significantly lighter ovarian weights than controls (1.6 ± 0.1 vs. 2.0 ± 0.1 mg, $p < 0.01$) on day 32 (fig. 3d), lighter uterine weights (11.4 ± 1.7 vs. 17.1 ± 3.0 mg, $p < 0.01$) on day 32 and a trend towards delayed age at vaginal opening (fig. 3c). As a histone deacetylase inhibitor, SAHA ultimately leads to increased histone acetylation [69–72] allowing gene transcription to proceed. Increased gene expression is enhanced further because SAHA can also cause decreased DNA methylation [73–75], like AZA. That increased gene expression leads to delayed puberty, once again implies that genes that inhibit pubertal onset were those most affected by the administration of an agent that alters the epigenome.

In complementary studies, L-methionine (L-MET) was administered to another group of prepubertal female mice. L-MET increases the supply of available methyl groups for DNMTs to use for cytosine methylation because L-MET is converted to the methyl donor S-adenosyl methionine [76]. Therefore, we hypothesized that L-MET administration would decrease gene expression and exert an opposite effect on pubertal timing compared with SAHA. Indeed, administering L-MET resulted in earlier puberty, as evidenced by vaginal opening occurring significantly earlier in treated mice than in controls (30.5 ± 2.2 vs. 31.6 ± 2.2 days, $p < 0.05$; fig. 3c) accompanied by a trend towards heavier uterine weights than controls (22.2 ± 3.6 vs. 17.1 ± 3.0 mg) [24].

Nonpharmacological Alteration of the Epigenome

Finally, a third research group determined that a rise in histone methylation early in life is associated with earlier pubertal timing [42]. Their unpublished studies show that the epigenome was altered by decreasing histone lysine demethylase levels through the use of lysine-specific demethylase heterozygous knockout mice (LSD1 +/–). The authors found increased histone methylation early in life associated with earlier age at vaginal opening. The data imply that increased expression of an LSD1 target led to the earlier puberty (since the knockout of *Lsd1* should lead to a rise in histone methylation that would be expected to lead to increased transcription). Although the downstream targets of LSD1 were not identified in this study, Lomniczi et al. [43] and others [42] have shown that *Kiss1*, the gene encoding kisspeptin, an established puberty-activating neuropeptide, is an LSD1 target. It is therefore feasible that lower levels of *Lsd1* led to increased *Kiss1* levels and earlier puberty. An alternative explanation is that the demethylase was acting to remove methylation at a mark such as H3K9, a histone methylation mark that silences transcription of its target genes. In this case, H3K9me3 could be localized to puberty repressor genes, and silencing of the repressor genes could have led to the earlier onset of puberty.

The perturbations employed in these studies, whether they were pharmacological or nonpharmacological, were administered in a systemic fashion via intraperitoneal in-

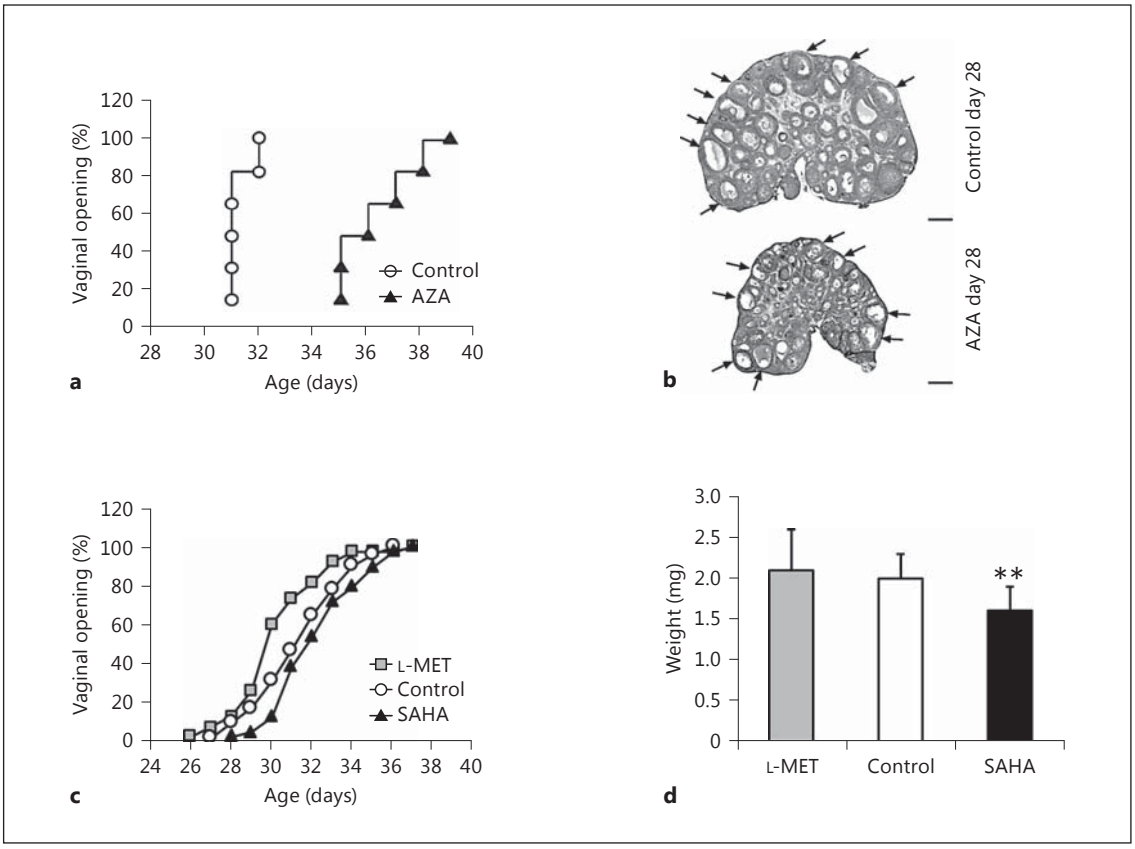


Fig. 3. In vivo perturbation of DNA methylation and histone acetylation results in changes to timing of onset of puberty in female rodents. Mean ± SEM throughout. SAHA = Suberoylanilide hydroxamic acid; L-MET = L-methionine. Panels **a** and **b** reprinted by permission from Macmillan Publishers Ltd., copyright 2013. **a**, **b** Lomniczi et al. [43] have demonstrated that rats intraperitoneally injected daily with AZA from postnatal day 22 onward have a markedly delayed vaginal opening (**a**; mean age 36.67 ± 0.67 days, n = 6) compared to controls (31.33 ± 0.21 days, n = 6; $p > 0.001$) as well as delayed ovarian maturation (**b**), indicated in day 28 ovarian micrographs from AZA-treated versus control rats by morphologically smaller ovaries and presence of fewer antral follicles (arrows), hallmarks of female rodent pubertal onset. The evidence from this group indicating that the changes in epigenetic marks can affect the timing of puberty is supported by complementary studies using L-MET and SAHA. Scale bar = 100 μm. **c** Mice treated with L-MET via drinking water from day 21 display earlier puberty (vaginal opening at 30.5 ± 2.2 days, n = 26, compared to controls 31.6 ± 2.2 days, n = 51; $p < 0.05$), and those treated with SAHA dissolved in the vehicle hydroxypropyl-β-cyclodextrin in the drinking water display a trend towards delayed puberty (32.5 ± 2.1 days, n = 37). Control mice administered water only or water and vehicle were pooled, having shown no significant differences. **d** 32-day-old mice treated with SAHA have smaller ovaries (1.6 ± 0.1 mg, n = 15, vs. 2.0 ± 0.1 mg, n = 20) than controls ($p < 0.01$), consistent with later pubertal development, whereas mice treated with L-MET have a trend toward larger ovaries at the same age (n = 22) [24].

jection [43], orally through drinking water [24] or globally as a gene knockout [42]. As such, these modes of administration do not allow for identification of the specific tissue(s) in which causative alterations occurred. However, Lomniczi et al. [43] did perform a series of experiments that allowed them to localize the effects of AZA administration on pubertal timing to the hypothalamus. In addition, these authors observed no negative effects of AZA treatment within other hormonal systems, such as prolactin and corticosterone, that might have affected the timing of puberty. Nonetheless, effects elsewhere cannot be ruled out. Further studies designed to identify responsible tissues and particular CpG and other marks that modulate the HPG axis and the timing of puberty will be an important area of future studies.

In total, the studies reported to date do provide consistent, though somewhat indirect evidence that altering the epigenome can alter pubertal timing (table 1). Although it is likely that epigenetic factors regulate nearly all developmental processes, the aforementioned studies

Table 1. Summary of effects of globally perturbing epigenome on timing of puberty in female rodents

	Epigenetic mechanisms			
Perturbation	AZA	SAHA	L-MET	+/– LSD1
Mechanism of action	↓ DNA methylation	↑ Histone acetylation	↑ DNA methylation	↑ Histone methylation
Consequence to transcription	Allow gene expression		Inhibit gene expression	
Result on pubertal timing	Delay puberty		Advance puberty	
Interpretation of mechanism	Initiation of puberty requires repression of repressors			

AZA data derive from Lomniczi et al. [43], SAHA and L-MET data derive from Rzeczkowska et al. [24], and +/– LSD1 data derive from Gill et al. [42].

together provide strong evidence that epigenetic factors are key players in the regulation of the onset of puberty. This concept is consistent with other work indicating that epigenetic mechanisms regulate the HPG axis at other stages of development [for a review, see 77]. Examples include regulation of GnRH gene expression in cultured neurons [41, 78, 79], of the estrogen-α receptor gene in the hypothalamus [80] and of *Kiss1* in the rodent brain during sexual differentiation [81] and during estrogen positive and negative feedback [82]. It is evident that further investigation of epigenetic mechanisms within the HPG axis, across tissues and developmental states, will be an important step towards further understanding the complexity of pubertal regulation and the coordinated layers of its control within gene regulatory networks [32, 83, 84].

Assay of Genome-Wide DNA Methylation

Leveraging array-based platforms, Lomniczi et al. [43] performed a genome-wide scan of DNA methylation differences in rat hypothalamic samples across the pubertal transition. Data for all RefSeq promoters and more than 15,000 annotated CpG islands were obtained, aiding in the discovery of previously unknown regulators of pubertal timing. The authors found enrichment in a cluster of chromatin/histone modification genes that are members of or interact with Polycomb group gene silencing complexes. The assay detected a rise in promoter methylation relative to CpG island methylation in many of these genes with the advent of puberty. This rise in methylation supports the probable mechanism of decreasing expression of repressive genes, facilitating the onset of puberty by lifting inhibitory signals.

Assessing the functional significance of particular genes discovered by the genome-wide assay suggests that regulation of the onset of puberty may also involve epigenetic control of epigenetic machinery. For example, a component of the PRC2, the embryonic ectoderm development gene *(Eed)*, was found to be silenced in the hypothalamus by DNA methylation at the initiation of puberty [43]. Prior to the initiation of puberty, *Eed* was bound to the *Kiss1* promoter, mediating the prepubertal repression of *Kiss1* expression. *Kiss1* repression was lifted at puberty by the epigenetic silencing of *Eed*, leading to diminished association of EED with the *Kiss1* promoter and increase in *Kiss1* mRNA expression. This was accompanied by a rise in activating histone marks H3K4me3 and H3K9,14Ac at the *Kiss1* promoter at the initiation of puberty, and a drop in the repressive histone mark H3K27me3 after the initiation of puberty, resulting in changes to chromatin structure that facilitate transcription. How this system regulates the timing of puberty in coordination with other inputs is not yet known. For example, kisspeptin is found in neurons in the arcuate nucleus of the hypothalamus that also produce neurokinin B and dynorphin, termed KNDy neurons [85]. These other peptides also regulate GnRH secretion [86], and it is feasible that the genes encoding these neuropeptides are also epigenetically regulated during pubertal onset. How epigenetic mechanisms regulate the function of the network of inputs that control GnRH secretion will likely be an important topic of research for many years [84].

Further evidence supporting a complex system derives from treatment of the rats with the DNMT inhibitor AZA. AZA decreased methylation at the *Eed* promoter and prevented the pubertal fall in its expression. Increased *Eed* expression was accompanied by continued low-level *Kiss1* expression. Conversely, when *Eed* was overexpressed, *Kiss1* expression dropped, GnRH response was blunted, and pubertal developmental was delayed. The studies by Lomniczi et al. [43] and others [24, 42, 87] need to be replicated and expanded, but they clearly represent a step forward in our understanding of the regulation of puberty and provide an important construct for future studies.

Emerging Epigenetic Mechanisms and How They Might Affect the Timing of Puberty

Epigenetic Regulation by Noncoding RNAs

Although not as well studied as DNA methylation or HPTMs, evidence for roles of noncoding RNAs, which act to reduce gene expression, in epigenetic regulation is continuing to emerge [88, 89]. Early examples of long noncoding RNAs (lncRNAs) include genes such as *Xist* and *H19,* which are involved in X inactivation and imprinting, respectively. More recently, loss of function studies in mouse embryonic stem cells show that lncRNAs can affect gene expression of distant genes and are involved in controlling pluripotency and cellular differentiation [90, 91], including the differentiation of neurons from human embryonic stem cells. Such lncRNAs may serve as scaffolds or decoy binding sites for epigenetic machinery and transcription factors [88, 89].

As noted previously, GWAS have identified 32 loci that affect the AAM in humans. The responsible gene at one of those loci, *LIN28B*, encodes a cytoplasmic RNA binding protein that acts to inhibit production of noncoding RNAs, termed microRNAs, and hence acts as a repressor of repressors. Overexpression of homologues of *LIN28B, Lin28* in *Caenorhabditis elegans* [92] and *Lin28a* in female mice [93], leads to delayed larval development and delayed puberty, respectively. Additionally, expression of various *Lin28* family members in the hypothalamus of rodents decreases prior to the onset of puberty [94, 95]. It is also relevant to note that lncRNA_N2, which is needed for neuronal specification, contains microRNA *LET7A* within its intron [90]. Together, these studies indicate that regulation by microRNAs is a key epigenetic mechanism involved in regulating pubertal timing.

Epigenetic Regulation through Transcription Factor Binding

Transcription factors can bind to DNA individually or in combination, and different combinations present in different tissues at different developmental stages are important determinants of spatial and temporal gene expression [96–98]. Transcription factor binding affects gene expression by altering chromatin structure and DNA accessibility.

Transcription factors have long been known to play important roles in the HPG axis [21, 99, 100]. For example, mutations in *PROP1, HESX1* and *LHX3* all affect pituitary development whereas mutations in *NR5A1* lead to defects in the ovaries, testes and adrenal glands. In addition, 15 of the 32 genes associated with AAM are transcription factors. Although mechanisms have not yet been defined, these GWAS data underscore the role that transcription factors play in modulating the timing of puberty. The transcription factor IRF2BPL [interferon regulatory factor 2 binding protein-like, which has been referred to as EAP1 (enhanced at puberty 1)] is another transcription factor implicated in pubertal control. Expressed in the rodent hypothalamus, EAP1 has been linked to delayed puberty in animal models [101].

Thus, it is evident that transcription factors play an important role in the epigenetic control of HPG axis development and in the control of the timing of puberty. Since many of the key transcription factors and their targets are only just beginning to be uncovered, it is likely that these transcription factors will emerge as even more important regulators of the timing of puberty in the future.

Long-Range Gene Regulation by Three-Dimensional Chromatin Configurations

The organization of chromatin can result in long-range interactions, which can bring distal tissue-specific enhancers to promoters (fig. 2) [102]. For example, mutations affecting the sex-determining *SOX9* gene occur from 50 to 1,000 kb upstream of the gene [103]. At the center of these long-range interactions are looping factors: the transcription factor CTCF and the ring-shaped cohesin complex, both of which play critical roles in controlling the epigenome [for a review, see 104]. The formation of long-range interactions directly involves a combination of several epigenetic mechanisms – transcription factor binding, histone modifications and DNA methylation – and understanding these types of complex interactions may well be part of understanding genome control during puberty more fully.

Characterizing long-range DNA interactions is a major post-GWAS problem where the fundamental challenge is often linking disease-associated loci to the responsible gene. One of the most comprehensive studies to date that mapped long-range DNA interactions across the mouse genome reported that at least 50% of genes undergoing transcription are involved in at least one long-range interaction [105]. The challenge of identifying long-range interactions presents itself in AAM GWAS results where 20 of 32 loci identified are located at least 10 kb from the nearest gene [106].

The disruption of long-range epigenetic interactions has been linked to pubertal timing. In what may be an initial example, the genetic study of a girl with precocious puberty and severe mental retardation identified an 8.9-Mb duplication whose distal breakpoint was 600 kb away

from the kisspeptin receptor *(KISS1R)* [107]. Given the prevalent role of KISS1R in puberty, a plausible explanation is that the duplication had disrupted a long distance chromatin interaction(s) that interrupted the spatial temporal expression of KISS1R during puberty.

The tissue-specific nature of 3-dimensional interactions and their regulation will likely represent a major challenge to understanding the factors that regulate the timing of puberty. However, recent developments creating and analyzing genome-wide maps of open chromatin generated by the DNA cleavage enzyme deoxyribonuclease I (DNAse I) have identified open chromatin in a wide array of tissues and cell lines and allowed predictions to be made about which of these open chromatin regions are likely to interact in 3 dimensions [108]. Relevant to puberty research, similar techniques were used to show that of all the GWAS variants reported for any disease in the NCBI database, the AAM-associated single-nucleotide polymorphisms show the highest enrichment of fetal specific regulatory elements (open chromatin) [109]. This exciting observation strongly suggests that important aspects of the epigenomic landscape of pubertal timing are established early in development.

Future Opportunities and Challenges

Importance of Constructing Gene Regulatory Networks

Linking puberty-associated genes into pathways is an important area of future research and one that has been championed by Ojeda and his group [32, 83, 84]. Meeting this need will be an important step towards further understanding the complexity of the regulation of the timing of puberty and investigating the coordinated layers of its control within gene regulatory networks. Rare human mutations, GWAS and animal models have clearly shown that puberty is driven by a complex network of genes and by hierarchical levels of stimulatory and inhibitory components [32, 83, 84].

Finding genetic interactions can give insight into how genes work within networks to carry out complex cellular processes [110]. One way to identify these interactions is to use radiation hybrid panels that measure attraction or repulsion of gene pairing higher than by chance alone. More than 7 million genetic interactions have been characterized using human, mouse, rat and dog radiation hybrid panels [111]. Applying radiation hybrid data to the genes and loci involved in regulating pubertal timing shows that many of the genes are found together in genetic networks (fig. 4). This information contrasts with the observation that the most significant GWAS identified single-nucleotide polymorphisms for AAM [106] did not occur near known hypogonadotropic hypogonadism and Kallmann syndrome genes [20] and reiterates that genetic interactions are not dependent on proximity. There are, of course, limitations to the radiation hybrid approach to finding genetic interactions in multicellular organisms that utilize hormones to signal across tissues. For example, the receptor-ligand pairs in this analysis (e.g. KISS1/KISS1R, GNRH1/GNRHR) do not show genetic interactions. Nonetheless, the novel genetic interactions identified create new testable hypotheses.

Experimental Strategies for Identifying Components of Gene Regulatory Networks

In addition to whole-genome DNA methylation assays, examining whole-genome gene expression patterns using RNA sequencing is another way to identify epigenetically controlled genes for the creation of gene regulatory networks. RNA sequencing allows for the comprehensive quantification of RNA transcripts and for the detection of alternative splicing events, both of which can be influenced by epigenetic marks. Epigenetic effects on gene expression have been discussed above; alternative splicing of pre-mRNA can result from differential DNA methylation where enrichment of methylation promotes exon recognition and produces alternatively spliced exons [112]. Histone acetylation, too, affects splicing events and can generate exon skipping [113]. Given these strengths, RNA sequencing is an ideal technique for identifying genes with expression patterns that vary across the pubertal transition.

Conducting studies in models that lead to alterations in pubertal timing might allow for identification of system components more readily. For example, pharmacological agents such as those discussed above could be used to alter epigenetic marks and alter the timing of puberty followed by the use of RNA sequencing or ChIP-Seq to identify which genes have been affected. The identified genes would be important candidate gene regulatory network members. Epigenetic marks are sensitive to other environmental perturbations, including endocrine disrupting chemicals and diet, and these perturbations could also be used to alter pubertal timing followed by assessment of how the epigenome was affected. Even without environmental manipulation, the C57BL/6 strain of female mice already displays wide variation in timing of puberty, and the epigenomes of extreme early- and late-onset mice could be compared to each other to identify key regulators (fig. 1).

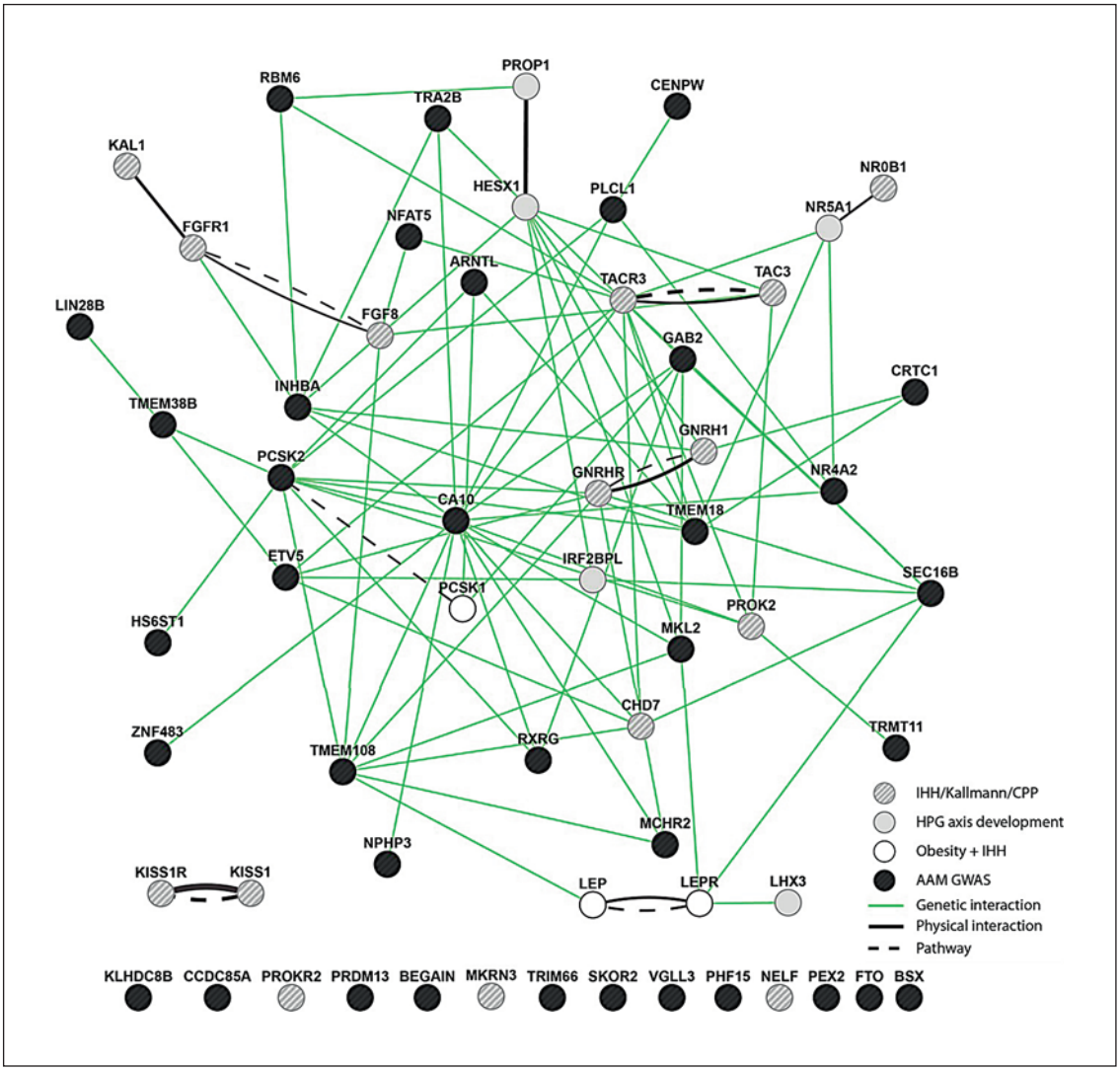


Fig. 4. Gene regulatory network illustrating putative interactions among genes known to cause hypogonadotropic hypogonadism and *Lin28B* and other AAM-associated loci. AAM genes from Elks et al. [106] are shown as black circles. Kallmann syndrome (KS; *KAL1, FGFR1, PROK2, PROKR2, FGF8, HS6ST1* and *CHD7*), isolated hypogonadotropic hypogonadism (IHH; *KAL1, GNRHR, GNRH1, GPR54/KISS1R, FGFR1, FGF8, PROK2, PROKR2, TAC3, TACR3. HS6ST1, NELF* and *CHD7*) and central precocious puberty (CPP; *MKRN3*) genes are shown as hatched gray circles. Of note, some genes have been identified as causes of Kallmann syndrome and isolated hypogonadotropic hypogonadism. Essential HPG axis development genes *(DAX1/NR0B1, NR5A1, HESX1, LHX3,* and *PROP1* and *IRF2BPL/EAP1)* are shown as gray circles. Obesity and hypogonadotropic hypogonadism genes *(LEP, LEPR* and *PCSK1)* are shown as white circles. Genetic interactions that were previously characterized using a radiation hybrid approach that utilized human, mouse, dog and rat panels are shown as solid green lines [128]. Proteins known to interact physically are connected by solid black lines, and genes known to be found in the same pathway are connected with dashed black lines. The connections were identified using GeneMania using default parameters with the 'equal weighting by network' setting [129]. Thicker lines represent the highest-confidence genetic or physical interactions.

Transgenic models could also be employed to perturb pubertal timing. For example, female mice overexpressing *Lin28a* have been shown to have delayed puberty [93], and epigenetic techniques could be used to determine which genes are modulated in transgenic versus control mice. Finally, since the timing of puberty differs between boys and girls, sex-specific differences in the epigenome could be leveraged in the discovery of gene regulatory network components.

Table 2. Summary of techniques that could be used for the identification of genes and epigenetic mechanisms involved in the regulation of pubertal timing

Technique	Purpose
DNA methylation array	Measure DNA methylation levels at CpG sites and identify areas with methylation profiles that favor more (generally less methylation) or less transcription
DNase I hypersensitive site sequencing	Genome-wide identification of nucleosome-depleted regions (open chromatin, which indicates areas with conformation that favors transcription and/or binding of regulatory elements)
ChIP-Seq	Genome-wide characterization of sites where DNA-binding proteins and particular histone protein modifications reside. Depending on mark used during immunoprecipitation, can identify areas that are more or less poised for transcription
Assay for transposase-accessible chromatin using sequencing	Genome-wide identification of both nucleosome occupancy profile and nucleosome-depleted regions. A mechanism that fragments the genome at accessible regions and simultaneously generates a sequencing library, allowing for identification of areas with open chromatin with much lower quantities of DNA than needed for DNase sequencing or ChIP-Seq, facilitating studies with a small number of cells
Next-generation sequencing of transcripts	Genome-wide sequencing of mRNA that allows for comprehensive assessment of gene expression by quantitation of transcripts and identification of alternative splicing
Transcription activator-like effector-mediated epigenetic modification	Technique that allows for targeted removal (or addition) of epigenetic marks by delivering epigenetic modifiers to specific genomic loci, allowing for assessment of the function of the particular mark. To date, this approach has been used to remove methylation at CpG sites as well as methylation at lysine 3 on histone H3

Dissecting Epigenetic Changes across Tissues and Development

Central to understanding the interplay between genes and environment is to know where (what cell type) and when (what development stage) to look. A host of genes have been documented that have defined roles in modulating pubertal timing and that show dynamic expression over the pubertal transition, including *Lin28a/b*, *Kiss1* and *Mkmr3*, among others [22, 94, 114]. Even when the genes of interest are known, it is often unclear how far in advance of the pubertal transition one should look for differential patterns. Some epigenetic marks are dynamic and lead to dynamic changes in gene expression. Other marks are persistent, placed at embryogenesis until they are functionally relevant at a certain point in time, thus representing a programmed state [115, 116]. A persistent pattern would dictate that individuals with different pubertal timing profiles (early onset versus late onset) would have different epigenetic marks from embryogenesis until (and perhaps after) the onset of puberty.

The range of tissues and developmental windows that could play a role in regulating the timing of puberty also makes it difficult to predict which transcription factors are most relevant. Thus, it is important to consider techniques that might allow for discovery even without more precise knowledge. Several methods exist to look globally for regulatory regions, including performing ChIP-Seq using antibodies to CTCF and the ring-shaped cohesin complex, both of which play critical roles in controlling the epigenome [104], or to HPTMs such as H3K27Ac or H3K4me2 (fig. 2). The gold standard for mapping open chromatin is sensitivity to the enzyme DNase I [108, 117, 118]. DNAse I hypersensitivity profiles from multiple cell types of different lineages and developmental stages have revealed that many DNAse I hypersensitivities are propagated during differentiation [117]. Translating these findings to puberty research is encouraging and suggests that many insights into precise locations of epigenetic regulation might be gained without choosing exactly the right time point to study [119].

Since tissue choice in human studies is limited to blood and peripheral tissues, or posthumous dissections, puberty research has relied heavily on animal models for advances in the field. However, even in ani-

mal models, performing analyses on pituitary samples or individual hypothalamic nuclei has been challenging due to small sample sizes. However, recent advances in obtaining signatures of open chromatin from small samples with assay for transposase-accessible chromatin using sequencing have just been reported, which may open up new opportunities for puberty research [120]. Furthermore, we may soon be able to test the importance of particular epigenetic marks in regulating the timing of puberty. Scalable genome-editing techniques such as the transcription activator-like effector nuclease have just been harnessed to make site-specific epigenetic changes. For example, CpG methylation can be erased at specific loci using transcription activator-like effector to recruit the demethylating enzyme TET1 to specific loci, allowing for erasure and then investigation of the functional significance of particular CpG methylation marks on the phenotype of interest [121]. Demethylation of enhancer-associated HPTMs has been achieved by transcription activator-like effector nuclease recruiting LSD1 to specific chromatin loci [122]. Using these and other technologies (table 2), investigators will be able to test whether altering a specific epigenetic mark alters the timing of puberty and whether that gene should be included in a puberty-related gene regulatory network.

Conclusion

Recent work by several groups has ushered the regulation of the HPG axis and of the timing of puberty into the epigenetic era. In total, the studies linking epigenetic control to pubertal timing provide consistent evidence that the initiation of puberty requires a lifting of repression, mediated by repressive epigenetic mechanisms. Global changes in specific epigenetic factors, such as the PRC2 complex [43], appear to be key players in the regulation of the onset of puberty. Further work in this area will enhance our understanding of the factors and gene regulatory networks that control the HPG axis and that underlie the wide variation in the timing of puberty observed among humans. The identification of these new gene regulatory networks is important to our understanding of puberty but may also help us understand further how the timing of puberty affects later-life health outcomes. This prediction will hold true if components of the newly identified networks overlap, at least in some cases, with genes or pathways that are involved in the pathogenesis of the medical outcomes (e.g. cardiovascular disease, diabetes, cancer) associated with age of onset of puberty.

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