ANNUAL REVIEWS

Annual Review of Biochemistry

Molecular Mechanisms of Facultative Heterochromatin Formation: An X-Chromosome Perspective

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Annu. Rev. Biochem. 2020. 89:255–82

First published as a Review in Advance on April 7, 2020

The *Annual Review of Biochemistry* is online at biochem.annualreviews.org

https://doi.org/10.1146/annurev-biochem-062917-012655

Keywords

facultative heterochromatin, epigenetics, noncoding RNA, X-chromosome inactivation, stem cells, development

Abstract

Facultative heterochromatin (fHC) concerns the developmentally regulated heterochromatinization of different regions of the genome and, in the case of the mammalian X chromosome and imprinted loci, of only one allele of a homologous pair. The formation of fHC participates in the timely repression of genes, by resisting strong *trans* activators. In this review, we discuss the molecular mechanisms underlying the establishment and maintenance of fHC in mammals using a mouse model. We focus on X-chromosome inactivation (XCI) as a paradigm for fHC but also relate it to genomic imprinting and homeobox (*Hox*) gene cluster repression. A vital role for noncoding transcription and/or transcripts emerges as the general principle of triggering XCI and canonical imprinting. However, other types of fHC are established through an unknown mechanism, independent of noncoding transcription (*Hox* clusters and noncanonical imprinting). We also extensively discuss polycomb-group repressive complexes (PRCs), which frequently play a vital role in fHC maintenance.

Contents

INTRODUCTION

Chromatin, the complex mixture of genomic DNA, RNAs, histones, and other proteins, provides the template for development. It not only contains the genetic information for all nuclear-encoded proteins, but also provides the regulatory platform for their timely expression or repression. A vital feature of chromatin is its ability to store the cellular memory of gene expression changes. Indeed, specific chemical modifications of both DNA and histones contribute to the formation of distinct chromatin compartments of transcriptionally active euchromatin and repressed heterochromatin. Initial cytological experiments have defined these genomic compartments based on their compaction and staining and thus appeared relatively static (1). However, just as gene expression patterns change rapidly during development, so does the state of chromatin itself. The precise orchestration of expression of developmentally regulated genes relies on their capacity to become activated at precise developmental stages or in a tissue-specific context. Outside these precise spatiotemporal windows, developmentally regulated genes must remain stably silenced. This can occur as a result of an absence of activatory signals or through strong repressors mediating the formation of facultative heterochromatin (fHC) (reviewed in 2). In the case of female mammals, almost all genes (developmental and otherwise) must be transcriptionally repressed on one of the two X chromosomes during development, and this is then stably propagated thanks to fHC

formation. Multiple layers of chromatin modifications and factors enable stable transcriptional silencing. Here, we review the recent advances not only in the mapping of chromatin states characterizing fHC but also in our understanding of molecular mechanisms enabling its establishment and maintenance in mice. For a recent review on fHC in other organisms, see Reference 3. We focus primarily on X-chromosome inactivation (XCI) as a paradigm but also discuss other types of fHC. We propose here that there are two main strategies of fHC formation in mice—one triggered by noncoding RNAs (ncRNAs) (or their transcription) and the other initiated independently of ncRNAs. Examples of the latter process include *Hox* clusters and noncanonical imprinting, which are characterized by a strong reliance on polycomb-group repressive complex 2 (PRC2) for their maintenance.

DEFINING FACULTATIVE HETEROCHROMATIN

fHC has been defined as developmentally regulated heterochromatinization of a region at only one allele of a homologous chromosome pair (4). This definition implies that fHC is maintained despite the presence of all *trans* activators necessary for transcription of a given region. In these cases, strong *cis*-acting repressors overriding the signals from activatory transcriptional factors maintain the silenced state. Such a narrow definition is thus mainly limited to two systems operating in mouse cells, namely, XCI and genomic imprinting. In this review, we discuss both processes with a specific focus on the former, as it serves as a useful paradigm for developmentally regulated stable gene silencing. The classical fHC definition has often been broadened to include all regions stably silenced at specific developmental stages despite the presence of all necessary *trans* activators (2). This wider definition could encompass multiple biallelically heterochromatinized loci. Here, it is difficult to prove that such regions are not silenced simply due to a lack of specific transcription factors (TFs) rather than by a specific set of overriding repressors. In some cases, however, when removing a single chromatin modifier reactivates multiple clustered genes one can assume one is dealing with a long-range silencing domain. *Hox* clusters serve as elegant examples of such fHC, and PRCs are the *trans* repressors maintaining their silencing state. These groupings of vital developmental regulators are transcriptionally silent during the first week of mouse development; however, upon the removal of the PRC2 they become reactivated (5–11). Before delving into the complex regulatory mechanisms of such regions, it is first necessary to introduce the basic chromatin regulatory pathways contributing to fHC formation.

COMMON CHROMATIN REGULATORY PATHWAYS INVOLVED IN FACULTATIVE HETEROCHROMATIN FORMATION AND MAINTENANCE

Extensive genome-wide mapping efforts in recent years have identified a set of chromatin features commonly associated with fHC. Importantly, none of these marks are exclusive for fHC, since they are frequently found in constitutive heterochromatin. What is more, the precise chromatin status of fHC differs between its various types and also between the initiation and maintenance phases of fHC formation. The initiation of fHC still remains somewhat mysterious but in some cases, such as XCI and canonical imprinting, it clearly involves the expression of ncRNAs. The act of transcription or the ncRNA itself can directly guide chromatin modifiers, which can then trigger a secondary wave of chromatin changes. This initial trigger allows specific genomic regions to embark on a sequence of chromatin alterations. Such a system has been conclusively demonstrated during XCI and relies on an ncRNA *Xist* (X inactive specific transcript) (12–14). In the case of most canonical imprints, it is transcription, which targets heterochromatinization in the germline

(15–18). However, the involvement of transcription or ncRNAs remains unlikely in the formation of other fHC regions. These include developmentally regulated *Hox* clusters, as well as recently discovered noncanonical imprinting (19–21). We discuss in detail the diverse possible mechanisms of fHC formation and maintenance for specific fHC types.

Another common feature of fHC formation is the exclusion of the majority of active histone modifications during establishment of the silent state. This usually includes extensive histone deacetylation either occurring through passive processes (during chromatin replication or histone exchange) or driven by enzymatic activity of histone deacetylases (HDACs) or sirtuins (22). Similarly, in many contexts histone H3 lysine 4 becomes demethylated thanks to the activity of specific histone lysine demethylases (KDMs) (23). Thus, fHC becomes largely depleted of activatory histone modifications; their loss might contribute to the formation of a chromatin state that is less accessible to multiple TFs (23).

In addition to the depletion of active chromatin marks, fHC also accumulates a plethora of repressive features. Most notably, fHC is often bound by PRCs. Two major groups of PRCs have been characterized, PRC1 and PRC2, first in *Drosophila* and then in many multicellular organisms including mice, where these complexes are consistently involved in embryonic development and differentiation (reviewed in 24). PRC2 consists of three core factors, enhancer of zeste 1/2 (EZH1/2), embryonic ectoderm development (EED), and suppressor of zeste 12 homolog (SUZ12) as well as some tissue- and context-specific cofactors (reviewed in 25). The catalytic activity of the PRC2 complex responsible for H3K27me3 deposition resides in EZH2 and EZH1. Although both these proteins have histone methyltransferase activity, EZH2 is the factor predominantly expressed during early mouse development (25). However, in vivo this activity entirely depends on the presence of two other core components, EED and SUZ12 (25). Apart from these factors, activity and locus specificity are also thought to be modulated by several cofactors, including Jumonji and AT-rich interaction domain containing 2 (JARID2) or metal response element binding transcription factor 2 (MTF2) (26).

The composition of PRC1 is much more diverse than that of the PRC2, and it comes in multiple subtypes (27). All of these contain either ring finger protein 1A or 1B (RING1A/B) E3-ubiquitin ligases, which are able to mediate monoubiquitinylation of histone H2A lysine 119 (H2AK119Ub). Subcomplex type is determined by which polycomb-group ring finger (PCGF) cofactor is loaded as well as on the presence or absence of chromobox proteins (CBX) (27). The canonical PRC1 is composed of PCGF2/4 and contains the CBX7 protein. However, noncanonical complexes, such as those with PCGF3/5, are bound by RING1 and YY1 binding protein (RYBP) and YY1-associated factor 2 (YAF2) cofactors rather than CBX (reviewed in 28). The complexity of PRC1 is of vital importance for fHC formation, especially in the context of XCI, and is discussed further in the section titled Molecular Mechanisms of Polycomb Recruitment During X-Chromosome Inactivation.

Classically, it was believed that H3K27me3 deposition by PRC2 led to the recruitment of PRC1 complexes via its CBX subunits recognizing H3K27me3 (29). This targeting results in the deposition of H2AK119Ub. More evidence has recently accumulated to propose that initial PRC1 recruitment could lead to subsequent binding of PRC2 thanks to one of its cofactors, JARID2, which contains an H2AK119Ub recognition motif (30). This is discussed further in the section titled Molecular Mechanisms of Polycomb Recruitment During X-Chromosome Inactivation.

Another mark associated with (but not limited to) fHC is 5-methylcytosine (5mC), or DNA methylation. Deposited de novo by DNA methyltransferase (DNMT) 3a/b/c, and maintained by DNMT1, 5mC exists mainly in the symmetrical CpG context (reviewed in 31). Importantly, high levels of 5mC at gene bodies are correlated with transcriptional elongation, whereas 5mC at 5′ ends of genes correlates with gene repression. How exactly 5mC can regulate gene expression

when present at promoters remains unclear, but classically it is thought to recruit methyl-binding domain-containing factors, which would execute its repressive function (32, 33). Alternatively, CpG methylation within binding motifs seems to change the binding affinity of multiple TFs (34, 35). DNA methylation is a vital chromatin feature of canonical imprint establishment, but we also discuss it in the context of XCI maintenance. A whole range of other chromatin marks are associated with transcriptional silencing and thus with fHC. These include not only repressive histone modifications (e.g., H4K20me1, H3K9me3) but also histone variants that replace canonical histones (e.g., MacroH2A).

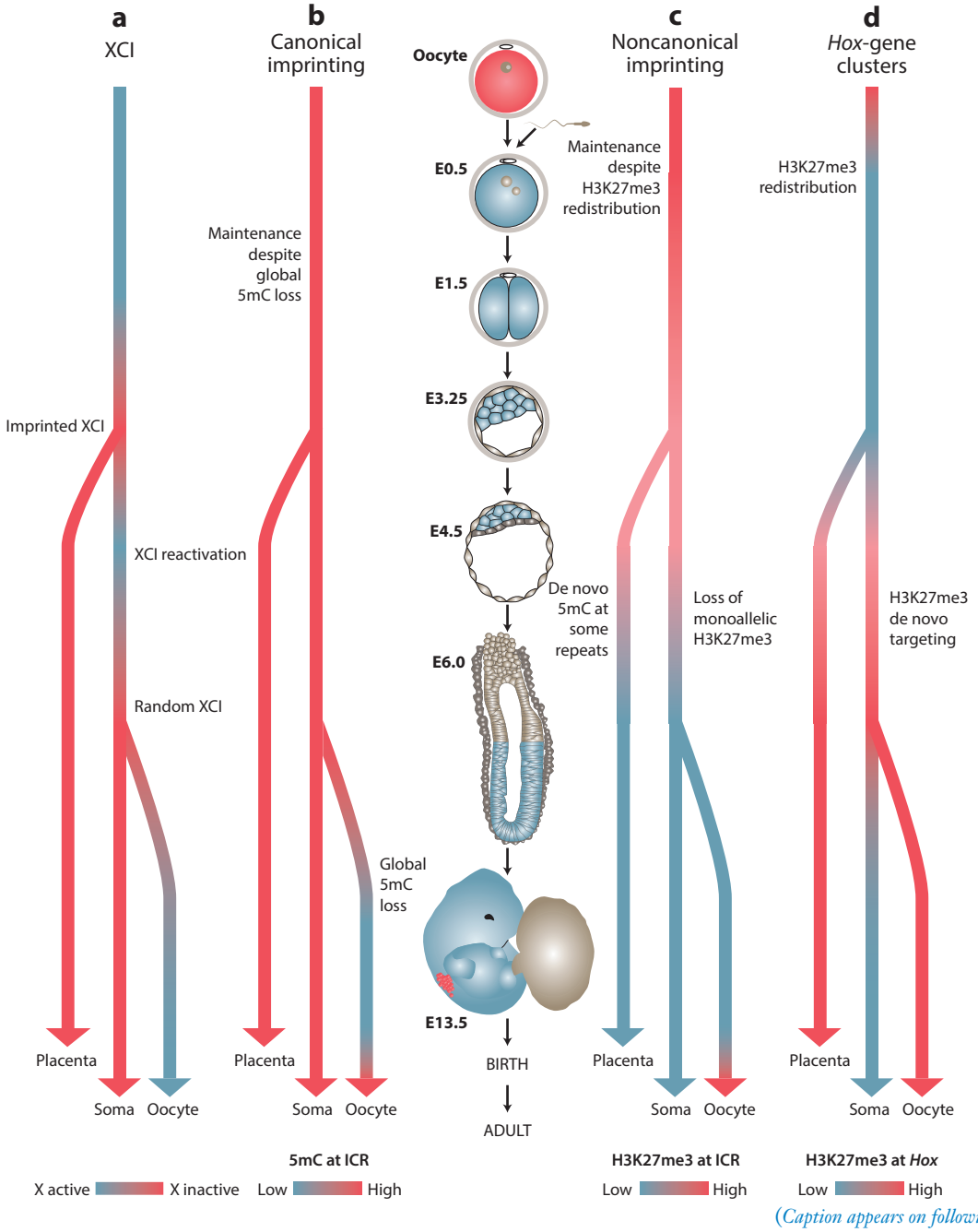
Thus, global mapping of chromatin marks has revealed a set of typical features of fHC, including the accumulation of repressive histone marks, DNA methylation, histone hypoacetylation, and H3K4 demethylation. Other features of fHC formation include a shift in DNA replication timing as well as changes in nuclear positioning (reviewed in 36). The mechanisms underlying the establishment of fHC have been more elusive, but the emerging picture is that in some (but not all) cases ncRNAs or their transcription can play a vital role in fHC formation. This is the case for XCI, which represents one of the most fascinating examples of fHC formation, as it occurs on a chromosome-wide scale, on only one of the two alleles in the same cell.

X-CHROMOSOME INACTIVATION AS A PARADIGM FOR FACULTATIVE HETEROCHROMATIN FORMATION

One of the most striking examples of developmentally regulated fHC formation is XCI. The presence of two X chromosomes in female mammalian cells results in an inherent disequilibrium of X-linked gene expression when compared to male cells. The double X-linked gene dosage in female mammals is incompatible with early development (12) and results in perturbed global gene expression (37). All placental mammals seem to use a process of heterochromatinization of one of the X chromosomes to correct for this effect (38–40). The process of transcriptional silencing of the >1,000 genes along the X chromosome is induced when one of the two alleles begins to robustly express an ncRNA, *Xist* (12–14). *Xist* RNA coats almost the entire X chromosome from which it is expressed (41, 42), inducing not only gene repression but also a cascade of chromatin alterations (43, 44). In mice, XCI occurs in two waves, first at early stages postfertilization and then at peri-implantation. Initially, female embryos inherit two active X chromosomes (Xas) from the gametes but starting from the four-cell stage the paternal allele becomes gradually inactivated (**Figure 1**) (37, 45, 46). This process of imprinted XCI (iXCI) depends on a noncanonical maternal imprinting of the *Xist* locus (21, 47), the molecular mechanism underlying this phenomenon is discussed in detail below. iXCI culminates at the early blastocyst stage when most genes along the X chromosome are partially silenced on the paternal X chromosome and expressed from the maternal allele. Next, during blastocyst expansion the paternal X chromosome becomes reactivated specifically in the epiblast lineage (48–50). This process entails rapid loss of repressive H3K27me3 and allows for a second round of XCI to occur once the embryo implants. The second XCI wave is random, with each epiblast cell independently embarking on the inactivation of the paternal or maternal allele (50). Once *Xist* is stably expressed from one allele, this decision is maintained through multiple cell divisions. Ultimately, in a postimplantation conceptus, extraembryonic lineages originating from the trophectoderm or primitive endoderm show iXCI with the paternal allele repressed, whereas cells of the embryonic lineages have undergone random XCI (rXCI) (**Figure 1**).

Due to relative inaccessibility of peri-implantation embryos, the majority of our knowledge on rXCI originates from the studies of differentiating female mouse embryonic stem cell (ESCs). ESCs are derived from the pluripotent inner cell mass (ICM), harbor two Xas, and undergo rXCI

upon differentiation. By employing immunofluorescence (IF) with RNA or DNA fluorescence in situ hybridization (FISH), early reports have uncovered the chronology of epigenetic alterations occurring at the X chromosome undergoing inactivation (Xi) (44, 51–55). This global view revealed rapid loss of active histone marks including H3K4me2/3 and H4ac and concurrent RNA



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Dynamics of fHC formation during female mouse development. Early mouse development entails rapid changes in chromatin states and gene expression patterns. Shown are the dynamics of fHC in females during XCI (*a*), canonical imprinting (*b*), and noncanonical imprinting (*c*) as well as *Hox*-gene cluster repression (*d*). fHC regions undergo waves of heterochromatin establishment, maintenance, and erasure. These processes often ensue with different dynamics between extraembryonic (*gray cells*), embryonic (*blue cells*), and germline (*red cells*) lineages. (*a*) XCI is initiated shortly after fertilization during imprinted XCI. This is reversed by E4.5 but only in the pluripotent epiblast cells. Random XCI occurs specifically in the epiblast when the embryo implants. XCI is stably maintained in the somatic and extraembryonic lineages but becomes reset during early germline development. (*b*) Maternal canonical imprinting is inherited from the oocyte as it carries DNA methylation at the ICRs. These CpG-dense regions are hypomethylated on the paternal allele. 5mC asymmetry between the alleles is maintained during the first wave of DNA demethylation at the blastocyst stage. Embryonic and extraembryonic lineages maintain asymmetric 5mC marking of ICRs, whereas in the germline this is removed during the second wave of demethylation. Maternal-specific pattern of DNA methylation is established during oogenesis only after birth. (*c*) Noncanonical imprinting relies on H3K27me3 marking inherited from the oocyte. Asymmetric H3K27me3 enrichment between maternal and paternal alleles is partially maintained during preimplantation development. Upon implantation, this becomes erased in all lineages. Only extraembryonic lineages maintain monoallelic expression of some noncanonically imprinted genes. This could be regulated by de novo monoallelic DNA methylation of specific repeat elements. The specific H3K27me3 pattern at noncanonically imprinted loci is re-established during oogenesis. (*d*) *Hox*-gene clusters are enriched with H3K27me3 in the oocyte; however, this is rapidly lost upon fertilization. H3K27me3 marking starts to be re-established only at the blastocyst stage. *Hox* genes become activated in somatic cells during gastrulation in postimplantation epiblast, and this entails H3K27me3 loss. Abbreviations: 5mC, 5-methylcytosine; fHC, facultative heterochromatin; ICR, imprint control region; XCI, X-chromosome inactivation.

polymerase II (RNAP II) exclusion from the *Xist*-coated domain in early differentiating cells (56, 57). Researchers observed subsequent accumulation of the PRC2-associated H3K27me3 (54, 55), followed by other changes, including MacroH2A and H3K9me2 enrichment (58–60). DNA hypermethylation of CpG-dense promoters is thought to be a later mark deposited with varying dynamics between genes (61, 62). All these chromatin processes result in a stable, transcriptionally silent state that can be maintained even upon the loss of *Xist* RNA coating (63, 64). Thus, two main stages of XCI can be defined: (*a*) an *Xist*-dependent phase of XCI initiation when nearly all X-linked genes become silenced, yet the process can be reversed upon *Xist* loss, and (*b*) a maintenance phase of XCI, which relies mainly on multilayered epigenetic regulatory mechanisms for gene repression and to a lesser extent on *Xist* RNA. Below, we discuss further how these epigenetic mechanisms prevent Xi from reactivation and how chromatin alterations might be involved in initiating XCI. First, we focus on the key trigger of the XCI process, *Xist* RNA itself.

Xist RNA AS AN ARCHITECT OF X-CHROMOSOME INACTIVATION

Xist RNA is the master regulator and trigger of XCI in all eutherian mammals. Murine *Xist* is a long (15,000–17,000 nt) ncRNA with relatively poor sequence conservation except for a series of unique tandem repeats named A to F (65–67; reviewed in 68). Of these, the best studied is the A-repeat region, which is required for the bulk of transcriptional silencing during XCI initiation (**Figure 2*a***) (69). It does so by recruiting protein split ends homolog (SPEN; also known as SHARP or MINT) and other repressive factors, which we discuss below (70). However, *Xist* B repeats, and to a lesser extent the C repeats, are required for polycomb-group (PcG) enrichment (71–75). *Xist* RNA also regulates appropriate Xi organization within the nucleus. Here, both the E and F repeats seem to contribute by tethering *Xist* RNA to the nuclear matrix and nuclear lamina, respectively (76–78). Finally, multiple regions mediate the capacity of *Xist* RNA to coat the chromosome in *cis* (69). Thus, *Xist* RNA is a modular ncRNA with specific regions independently permitting different functions.

XCI is not a synchronous process along the X chromosome. Different genes show different times of onset of transcriptional silencing during preimplantation embryo development (46). One explanation for this phenomenon might be the differential timing of genes becoming coated by

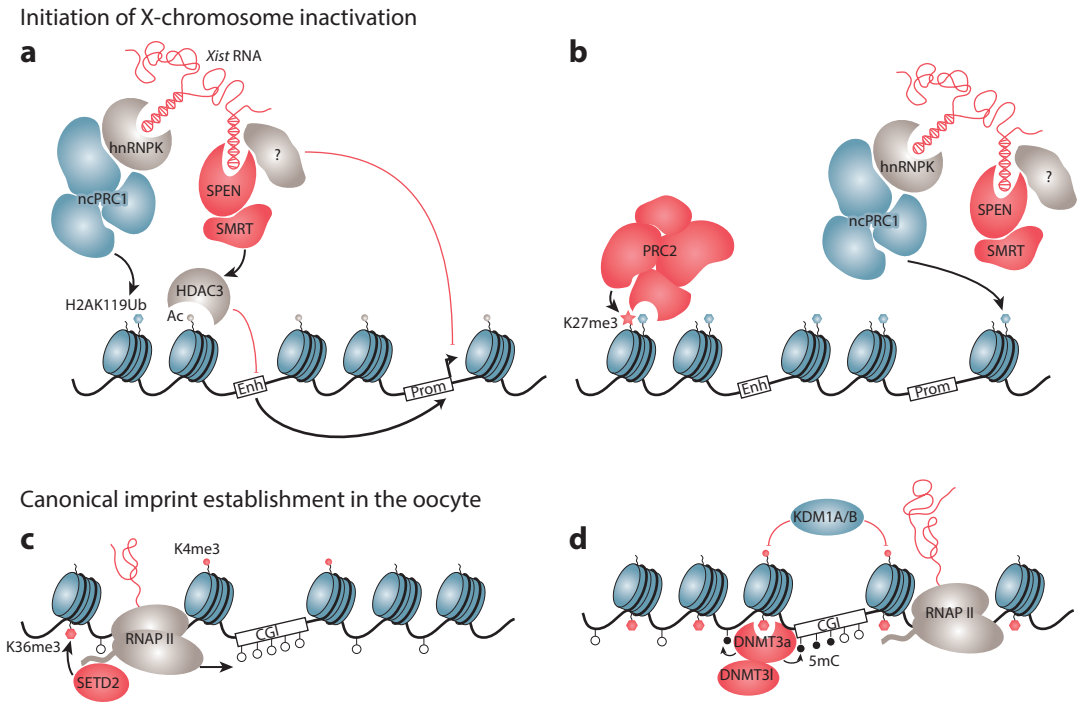


Figure 2

Putative mechanisms driving fHC formation. (*a*) Initiation of XCI is entirely dependent on *Xist* noncoding RNA. Once it coats the X chromosome in *cis*, it recruits a set of RNA binding proteins. The *Xist* RNA A-repeat region recruits SPEN and thanks to its interaction with SMRT allows for full HDAC3 activation. HDAC3 is prebound on active enhancers and after *Xist* coating promotes rapid histone deacetylation and gene repression. Although X-linked gene silencing is SPEN dependent, HDAC3-independent mechanisms must operate for some genes. These could involve m6A RNA methylation and NuRD machinery. The B- and C-repeat regions of *Xist* RNA allow for recruitment of ncPRC1 and deposition of H2AK119Ub at intergenic regions. (*b*) Once gene silencing is initiated, H2AK119Ub spreads into genic regions. PRC2 complexes are presumably recruited thanks to their ability to recognize H2AK119Ub and not due to direct interaction with *Xist* RNA. (*c*) Canonical imprinting is established during gametogenesis. In females, prenatal germ cells have extremely low levels of DNA methylation (5mC). Noncoding transcription through CGIs allows for the deposition of H3K36me3 by SETD2. (*d*) During oocyte growth, H3K4me3 at CGIs is removed by KDM1A/B, allowing for de novo DNA methylation by DNMT3A/L. Abbreviations: 5mC, 5-methylcytosine; Ac, histone acetylation; CGI, CpG island; DNMT, DNA methyltransferase; Enh, enhancer; fHC, facultative heterochromatin; H2AK119Ub, histone H2A lysine 119 monoubiquitinylation; HDAC, histone deacetylase; ICR, imprint control region; K27me3, histone H3 lysine 27 trimethylation; K36me3, histone H3 lysine 36 trimethylation; K4me3, histone H3 lysine 4 trimethylation; ncPRC1, noncanonical PRC1; Prom, promoter; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; SPEN, protein split ends homolog; XCI, X-chromosome inactivation.

Xist RNA. Two independent studies have addressed this by mapping the primary sites of *Xist* RNA enrichment along the X chromosome in ESCs (41, 42). By precipitating *Xist* RNA and sequencing the DNA it is interacting with at early stages of XCI, they mapped the primary sites of *Xist* RNA landing [so-called *Xist* entry sites (XES)]. Intriguingly, the correlation between primary XES and gene silencing seems ambiguous. Consistent with the idea that *Xist* RNA is required for gene silencing, genes escaping XCI are largely devoid of *Xist* RNA, and the silencing-deficient *Xist*:*ΔA* RNA is not able to spread into actively transcribed domains (41). However, genes within XES are silenced only marginally more rapidly than those outside, with the exception of genes lying in proximity to the *Xist* locus itself, which are typically silenced first (37, 79). This implies that, although *Xist* RNA spreading mediates gene silencing, it is not the sole factor determining the timing of gene repression.

Although XES proximity was a poor predictor for gene silencing timing, XES showed a striking correlation with the 3D folding of the X chromosome (41). Indeed, XES lie within 3D vicinity of the *Xist* locus according to HiC analysis, indicating that *Xist* RNA spreads first to regions within its immediate spatial proximity. This was further confirmed when an inducible *Xist* transgene was inserted at another X-linked locus (*Hprt*). In this case *Xist* RNA coated the X chromosome but followed the 3D proximity pattern of the *Hprt* locus rather than that of the endogenous *Xist* gene (41). Thus, *Xist* RNA first binds to DNA regions in its 3D neighborhood and only later spreads in *cis*. Importantly, XES also become strongly enriched for H3K27me3 and H2AK119Ub, indicating a correlation between *Xist* RNA binding and PcG recruitment (41, 42, 80). Recent mapping efforts have also revealed that XES are in fact already premarked by both PRC1-dependent H2AK119Ub and PRC2-dependent H3K27me3, prior to *Xist* RNA upregulation and coating (80). This prefolding of the X chromosome to bring PcG-enriched domains in 3D proximity of the *Xist* locus is somewhat reminiscent of the interactions between *Hox*-related genes in mouse ESCs (81–84). Indeed, autosomal PcG target genes preferentially interact together in a PRC1- and PRC2-dependent manner. It is thus tempting to speculate that the initial *Xist* coating pattern is instructed by the 3D conformation of the X chromosome thanks to PcG prebinding. Further experiments on how X-chromosome folding is affected by RING1A/B or EED loss are necessary. These results suggest that the prebound fraction of PcG may in fact influence the spreading of *Xist* RNA and thus the initiation of XCI. Although the initiation of *Xist* RNA spreading in a PcG mutant context has never been addressed, a recent study mapped *Xist* RNA coating in *Ring1A/B* double knockout (dKO) and *Eed* knockout (KO) mouse embryonic fibroblasts (71). By doing so, the authors revealed that PcG has a role in promoting the maintenance of *Xist* RNA coating. How this relates to the dynamics of XCI initiation remains to be determined.

CHRONOLOGY OF REPRESSIVE MARK ACCUMULATION DURING X-CHROMOSOME INACTIVATION

The coating of the X chromosome by *Xist* RNA results in a sequence of rapid chromatin alterations linked to transcriptional silencing. These include the loss of most active histone modifications and reciprocal accumulation of H3K27me3, H2AK119Ub, and H4K20me1 (53–55, 85). A delayed accumulation of H3K9me2, MacroH2A, and CpG promoter methylation follows such primary chromatin changes (60, 62, 86–89). Although initial IF/RNA FISH studies identified the key global chromatin changes induced by *Xist* RNA coating, they could not provide a precise temporal sequence of events or characterize how different regions of the X chromosome become dynamically marked.

With the advent of chromatin immunoprecipitation approaches and next-generation sequencing, it became possible to map the allele-specific chromatin state of the Xi in differentiated cells. These types of analyses rely on single-nucleotide polymorphism information to distinguish two alleles in F1 hybrid cells (derived from crosses of evolutionary divergent mouse strains). Such studies have confirmed Xi chromosome-wide accumulation of H3K27me3 and H4K20me1 (90–92). As previously mentioned, this revealed that XES are also those that accumulate H3K27me3 most efficiently (41). However, a high level of variability between single ESCs' differentiation dynamics leads to asynchronous upregulation of *Xist* RNA and thus precludes the studies on the earliest chromatin changes taking place during XCI. In a recent study, an F1 hybrid, female ESC line harboring a DOX-inducible *Xist* at one of the endogenous alleles (93) was used for rapid, synchronous, monoallelic expression of *Xist* RNA and uncoupling of XCI from ESC differentiation.

By using such *Xist*-inducible ESCs and performing chromatin immunoprecipitation sequencing (ChIP-seq) at short time intervals (on the scale of hours) the precise temporal dynamics

of chromatin marks along the X chromosome could be obtained (80). Of the repressive marks, PRC1-dependent H2AK119Ub accumulates prior to PRC2-dependent H3K27me3. The PRC1-dependent mark is deposited first intergenically, within the XES, and only later spreads into gene bodies. This process of spreading is concomitant with the initiation of gene silencing, indicating a potential functional link between transcriptional repression and PRC1 activity. Similar to H2AK119Ub, H3K27me3 accumulates first intergenically within the XES before spreading into gene bodies. However, the deposition of this mark by PRC2 occurs significantly later than gene silencing and H2AK119Ub accumulation, potentially precluding the involvement of H3K27me3 deposition in the initiation of XCI.

Important functional insights have come from studies using a silencing-deficient *XistΔA* RNA. Cells expressing this mutant RNA showed the accumulation of both H3K27me3 and H2AK119Ub at the intergenic regions but not within large gene-rich domains (80). Further analysis revealed that although H2AK119Ub spreading is initiated concomitantly with gene silencing, it is not the trigger for transcriptional repression (80). Rather, current data suggest that gene silencing needs to be initiated by other mechanisms to allow for PRC1-mark spreading (see below). This does not preclude PRC1 from playing some role in gene silencing, but it is clearly not the major molecular mechanism for transcriptional repression.

MOLECULAR MECHANISMS OF POLYCOMB RECRUITMENT DURING X-CHROMOSOME INACTIVATION

The coating of the X chromosome by *Xist* RNA results in a strong accumulation of both PRC1 and PRC2 core components. Indeed, IF/RNA FISH assays have revealed enrichment of RING1A/B as well as EED/EZH2/SUZ12 at the *Xist* cloud in differentiating cells (53–55, 94). This was further confirmed by allele-specific ChIP-seq for EZH2 in differentiated ESCs (90). However, the question of the underlying molecular mechanisms for PRC1 and PRC2 recruitment have remained controversial for a long time. Initial *Xist* mutation experiments indicated that the A-repeat region is not required for PcG recruitment (94). However, knockdown and in vitro binding assays have shown direct interaction between EZH2 and *RepA* ncRNA. *RepA* is an independent, 1.6-kb-long RNA entity that overlaps with the A-repeat region of *Xist* (95). On the basis of these findings, it was proposed that PRC2 is first recruited to the Xi by *Xist* RNA (or in fact by *RepA*), allowing for H3K27me3 deposition. This in turn would lead to the recruitment of canonical PRC1 through the H3K27me3 binding specificity of the CBX7 subunit (29). However, several recent studies do not support this model, although they do not exclude the binding of PRC2 to the *RepA* transcript. First, PRC2 has been found to bind rather promiscuously to most long, highly expressed RNAs, eroding the claims of a specific A-repeat interaction with EZH2 (96). Second, none of the core PRC2 subunits were detected in proteomic *Xist* interactome studies (70, 97, 98). Third, the core noncanonical PRC1 components, RING1B and PCGF3/5, were significantly enriched in *Xist* RNA pull-downs (independent of the *Xist* A repeat) (70, 72). Together, these studies indicate that PRC1 may be first recruited by *Xist* RNA, and PRC2 becomes enriched on the *Xist*-coated chromosome only subsequently. This secondary PRC2 recruitment could potentially occur thanks to the H2AK119Ub binding activity of the JARID2 cofactor of PRC2 (30). In line with this model, PRC1 is enriched on the Xi even in PRC2 mutant ESCs (75, 99).

Recent extensive *Xist* mutation studies have revealed that it is the B- and, to a lesser extent, C-repeat regions that recruit noncanonical PRC1 through its interaction with heterogeneous nuclear ribonucleoprotein K (hnRNPK) (**Figure 2*a*,*b***) (71–74, 100). Indeed, deletion of both the B- and C-repeat regions results in the loss of *Xist* interaction with hnRNPK and RING1B, as well as the noncanonical PRC1 subunits RYBP and PCGF3/5 (72). Importantly, ChIP-seq

analysis has revealed that during both XCI initiation and maintenance, the *Xist* B-repeat region is required for chromosome-wide deposition of H3K27me3 and H2AK119Ub (71, 72). Moreover, researchers demonstrated that in the E7.5 epiblast devoid of PCGF3/5, there is no H3K27me3 and H2AK119Ub accumulation at the Xi (100). Together, these data indicate the importance of noncanonical PRC1 in PRC2/H3K27me3 enrichment on the Xi. However, further experiments are required to show the relative dynamics of recruitment of core PRC1 and PRC2 components to the X chromosome undergoing inactivation. What is more, the importance of preloaded PcG complexes prior to *Xist* upregulation remains largely unexplored. Indeed, the striking correlations between *Xist* spreading and PcG premarking merit further investigation. Moreover, multiple transcribed genes are prebound by RING1B and premarked by H2AK119Ub, and this correlates with rapid gene silencing dynamics during XCI (79, 101). Finally, a recent finding that *XistΔA* RNA mutant cells show reduced H2AK119Ub levels at the Xi, but robust H3K27me3 enrichment, is not explained by current models (80). In this respect, questions regarding the interplay between gene silencing and PRC1 marking require further investigation.

FUNCTION OF POLYCOMB IN MEDIATING GENE SILENCING DURING X-CHROMOSOME INACTIVATION

Although there are increasing data showing the importance of PRC1 in mediating PRC2 recruitment during XCI, the precise role of these complexes in XCI remains somewhat controversial. On one hand, initiation and maintenance of random XCI appear to be independent of EED (102, 103). However, in extraembryonic lineages, loss of core PRC2 components leads to partial reactivation of the Xi, indicating an important, but context-dependent, role of PRC2 in XCI maintenance (102). Importantly in extraembryonic tissues, the Xi does not accumulate DNA methylation at CpG islands (CGIs), and the lack of this epigenetic mark could lead to a greater dependence on PRC2. The function of PRC1 in mediating gene repression is still not properly understood given only *Ring1B* KO phenotypes, and not those of *Ring1A/B* dKO, have been reported (104). On the other hand, loss of noncanonical PRC1 subunits PCGF3/5 in ESCs leads to reduced gene silencing induced by an autosomal *Xist* transgene (73, 100). This is consistent with the purification of these factors with *Xist* RNA. Nevertheless, the contributions of different PRC1 complexes to gene silencing in the context of the X chromosome remain unclear.

The studies of ESCs expressing *Xist* RNA deleted for the B and C repeats revealed initiation of chromosome-wide gene silencing but without PcG recruitment (72, 73). However, coating by *Xist:ΔB* or *Xist:ΔBC* leads to less efficient gene silencing with the strength of phenotype depending on experimental design. This deficiency becomes even more pronounced when ESCs are differentiated (71, 73). Thus, PcG recruitment by the B- and, to a lesser extent, C-repeat regions of *Xist* RNA might promote efficient gene silencing during XCI. On its own, however, PcG recruitment is not sufficient to induce gene silencing. The *Xist* A-repeat region is the main trigger for gene repression during XCI.

CHRONOLOGY OF ACTIVE HISTONE MARK DEPLETION DURING X-CHROMOSOME INACTIVATION

Upon *Xist* coating, the Xi not only accumulates an array of repressive chromatin marks but also becomes depleted for many active histone modifications. Indeed, IF/RNA FISH studies have revealed rapid loss of histone modifications associated with active promoters (H3K4me3, H4ac, H3K9ac, H3K27ac) and enhancers (H3K27ac, H3K4me1) as well as exclusion of the RNAP II (44, 56, 89, 105). This was further validated by allele-specific ChIP-seq for H3K4me3 in differentiated

cells (90). To monitor how active chromatin marks are dynamically remodeled during XCI it was necessary to use the same ESC model as for PcG marks (80). Strikingly, of all the chromatin modifications tested, it was H3K27 deacetylation that progressed with most rapid dynamics, whereas others, such as H3K4me1/3, became depleted more slowly (80). H3K27 deacetylation did not occur uniformly along the X chromosome, with some promoters being more refractory to H3K27ac loss than others. This differential promoter behavior precisely correlated with nascent transcript silencing dynamics as judged by transient transcriptome sequencing (80). Further analysis into the promoter and enhancer silencing dynamics revealed that enhancers become deacetylated slightly more rapidly than promoters. However, stable decommissioning of enhancers by the loss of H3K4me1 is a very slow process even when compared to promoter demethylation of H3K4me3. Together, these data indicate a complex sequence of events where rapid deacetylation of gene regulatory elements precedes their stable decommissioning. The tight correlation in timing of H3K27 deacetylation and gene silencing suggested a potential functional role for HDACs in XCI.

MOLECULAR MECHANISMS OF THE INITIATION OF X-CHROMOSOME INACTIVATION: SPEN, HDAC3, AND BEYOND

Recent genetic screens for XCI factors as well as the purification of *Xist* RNA's protein partners have provided exciting new insights into the mechanisms by which *Xist* induces gene silencing (70, 97, 98, 106, 107). All these studies independently identified the transcriptional repressor SPEN as a key factor directly binding to the *Xist* A repeat (**Figure 2*a***) (70, 108). Importantly, loss of SPEN results in nearly complete loss of *Xist*-dependent gene silencing along the X chromosome both in vitro and in vivo, phenocopying the *Xist* RNA A-repeat deletion (73, 109). Thus, the initiation of gene silencing is almost entirely dependent on the A-repeat region binding SPEN; however, SPEN is largely dispensable for maintaining XCI in differentiated neuronal progenitor cells (109). Another key question pertains to the mechanism underlying SPEN-mediated gene silencing. One indication of its mode of action is that efficient promoter deacetylation is dependent on the A-repeat region (80). In line with this, SPEN, through its SPOC domain, binds the corepressors silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (NCoR), potentially enabling HDAC3 activation (109–111). Indeed, HDAC3 knockdown in ESCs results in inefficient silencing of an X-linked gene, *Gpc4* (97). On the basis of this indirect evidence, it was proposed that SPEN might recruit SMRT/NCoR complexes with HDAC3 to the X chromosome, allowing for histone deacetylation and gene silencing. This model would be in line with rapid promoter deacetylation during XCI (80). However, *Hdac3* KO ESCs revealed a more nuanced phenotype, with the majority of X-linked genes showing delayed rather than abrogated transcriptional silencing following *Xist* RNA coating (80). Furthermore, a minority of genes were silenced independently of HDAC3 activity and yet are SPEN dependent (109). Thus, SPEN-mediated activation of HDAC3 contributes to XCI, but other parallel mechanisms must operate to mediate gene silencing during XCI (**Figure 2*a***). What is more, IF and allele-specific ChIP-seq assays have revealed that HDAC3 is not efficiently recruited to the Xi, contrary to the initial model, but rather it is prebound on active enhancers. It is *Xist* RNA coating, and SPEN recruitment, that most likely results in HDAC3 activation and histone deacetylation. A recent report confirmed that SPEN's mechanism of action goes beyond the activation of HDAC3 (109). Indeed, SPEN is recruited by *Xist* to the active promoters and enhancers along the Xi, where it seems to integrate transcriptional machinery with multiple repressive complexes (109). Apart from NCoR/SMRT/HDAC3, these include m6A RNA methylation machinery and nuclease remodeling deacetylase complex (NuRD). One pressing question that remains is, To what extent do

they contribute to SPEN's silencing activity? Initial experiments also reported that knockdown of SPEN and HDAC3 results in the loss of H3K27me3 enrichment at the Xi. However, allele-specific ChIP-seq analysis in *Hdac3* KO ESCs did not confirm this observation. In line with this, loss of *Xist* A, but not of the B+C repeats, is compatible with initial PcG recruitment to the Xi (72, 80).

In addition to recruiting SPEN and enabling histone deacetylation and PcG-mark deposition, *Xist* RNA also recruits several other factors (reviewed in 112, 113). These include m6A RNA methyltransferase complexes (RBM15/WTAP), as well as the lamin B receptor. Both pathways have been implicated in mediating efficient gene silencing (78, 114); however, recent ESC KO studies have revealed only minor XCI defects (73). Thus, SPEN and *Xist* A repeats integrate multiple complexes with potentially repressive roles. However, their relative importance in the process of XCI remains unclear.

MECHANISMS MAINTAINING X-CHROMOSOME INACTIVATION

Using ESCs with an inducible *Xist* gene, XCI has been shown to be fully *Xist* dependent and reversible during early differentiation. Following three days of differentiation and *Xist* expression, the inactive state becomes locked in and is largely independent of *Xist* RNA (63). Indeed, loss of *Xist* in differentiated female cells typically does not lead to global X-chromosome reactivation (64, 115). This stable epigenetic silencing occurs in spite of strong *trans* activators regulating the expression of genes on the Xa allele. How the stability of the inactive state is ensured throughout the life of an adult female remains unclear. One of the important repressive marks believed to lock in stable gene silencing is DNA methylation. Although the Xa is hypermethylated along gene bodies, the Xi shows strikingly high methylation at CGI-associated promoters (62, 86, 116). Indeed, loss of 5mC due to pharmacological inhibition of DNMTs or by genetic loss of DNMT1 leads to partial X-chromosome reactivation (117, 118). Further insight into the importance of CGI methylation in XCI was provided by the studies of structural maintenance of chromosomes flexible hinge domain-containing protein 1 (SMCHD1). SMCHD1 is necessary for efficient maintenance of gene repression of ~10% of X-linked genes, and its loss is associated with promoter hypomethylation (61, 119). However, further studies have revealed that SMCHD1 mediates its repressive role independently of DNA methylation (120). Rather, it becomes enriched at the Xi at late differentiation stages thanks to the PRC1 activity (121). This allows for SMCHD1 to weaken the 3D chromatin conformation typical of the Xa (122, 123). How precisely this structural role of SMCHD1 relates to its function in maintaining gene silencing remains unclear.

Another pathway associated with DNA methylation is di- and tri-methylation of H3K9. The H3K9me2 mark becomes enriched on the Xi in differentiating female cells (89). Researchers proposed that the majority of H3K9me2 and H3K9me3 during XCI is deposited by euchromatic histone lysine methyltransferase 2 (EHMT2) and SET domain bifurcated 1 (SETDB1), respectively (60, 124). However, the role of H3K9 methylation in XCI maintenance remains unclear. Loss of either EHMT2 or SETDB1 leads to no, or only limited, gene reactivation at the Xi (124, 125). It is likely that the lack of maintenance phenotypes is a result of redundancy between chromatin-modifying enzymes. In this respect, DNA methylation and polycomb marks might serve as an additional barrier for X-chromosome reactivation in *Ehmt2* KO and *Setdb1* KO cells.

Multiple parallel mechanisms operate together to allow for stable maintenance of silencing on the Xi; these include polycomb marks, CGI methylation, and probably H3K9 methylation. Intriguingly, stable fHC can differ between cell types, with placental tissues being more reliant on PRC repressive mechanisms and in embryonic tissues DNA methylation playing a more prominent role.

FACULTATIVE HETEROCHROMATIN AT CANONICALLY IMPRINTED LOCI

Like XCI, the process of genomic imprinting entails the establishment of a stable silent compartment at only one allele. This is linked to epigenetic asymmetry between two alleles, which originates from differential chromatin states inherited from the gametes. Imprinted genes typically form clusters of monoallelically expressed genes regulated by single imprint control regions (ICRs). In the case of canonically imprinted loci, ICRs harbor asymmetric patterns of DNA methylation originating from the oocyte or sperm. The vast majority of ICRs are maternally imprinted (i.e., containing 5mC on the maternal allele), and only three paternal ICRs have been reported thus far (126). As in the case of XCI, distinct molecular machineries are involved in the establishment of repressive chromatin patterns and in their maintenance.

The most important pathway for imprint establishment is de novo DNA methylation during oogenesis by DNMT3a and its catalytically inactive cofactor DNMT3L (127, 128). In female germline, global DNA methylation levels are relatively low, reaching ~40% of CpG methylation (129, 130). This DNA methylation positively correlates with ongoing transcription, CGIs, high H3K36me3, and low H3K4me3 (131, 132). Studies on the dynamics of ICR heterochromatinization during oogenesis have shed light on the order of chromatin events. Initially, in prenatal female germ cells, there is no transcription and the global levels of DNA methylation remain low. Already at this point the CGIs, which will undergo DNA methylation, are premarked by H3K4me2/3 (132). In postnatal growing oocytes, there is transcriptional activation of the genome and concurrent deposition of H3K36me3 at gene bodies, but this precedes de novo DNA methylation (**Figure 2*c***) (132, 133). Nevertheless, it has been demonstrated for multiple loci that the act of transcription is necessary for subsequent ICR methylation (15–18). Mechanistically, this de novo DNA methylation by DNMT3A/DNMT3L can only occur once H3K4me2/3 is removed by KDM1A and KDM1B (**Figure 2*d***) (132, 134). Despite these elegant experiments, it remains unclear what drives the recruitment of de novo DNA methylation machinery during oogenesis. Initially it was hypothesized that the proline-tryptophan-tryptophan-proline (PWWP) domain of DNMT3A allows its recruitment to H3K36me3 (135). This seems inconsistent with the phenotype of mice carrying PWWP domain point mutations abrogating such interactions (136). A potential role for PWWP interacting with dimethylated H3K36 should also be considered (137). Other compensatory mechanisms might also operate, with HDAC1/2 and DNMT1 cofactor ubiquitin-like with PHD and ring finger domains 1 (UHRF1) playing some role in de novo DNA methylation during oocyte growth (138, 139).

The mode of DNA methylation targeting at the three paternally imprinted ICRs is even more elusive than that operating in the oocyte. In fact, these loci represent an exception from the general pattern of DNA methylation in sperm. The paternal genome, unlike that of the oocyte, shows high levels of DNA methylation reaching ~90% of CpGs, but this is generally excluded from CGIs (131, 140). ICRs are a part of a small subset of mainly intergenic CGIs, which become efficiently methylated. For two of the three known paternal ICRs (*H19*, *Dlk1/Gtl2*), this largely depends on the activity of DNMT3A/DNMT3L (141, 142). However, it is still not known how this complex is recruited to chromatin, but it might rely on noncoding transcription through these ICRs in the male germline development (143). We know more about the mechanisms governing imprint establishment at the ICR of *Rasgrf1*. This paternal ICR becomes methylated in a DNMT3L-dependent, but DNMT3A-independent, manner (142). Strikingly, it is the newly discovered, rodent specific, de novo methyltransferase DNMT3C that targets this ICR (144). Establishment of the methylated state at *Rasgrf1* requires the transcription of a retrotransposon through the ICR, and this in turn produces piRNAs (145, 146). The biogenesis of these transposon-derived small

ncRNAs is dependent on PIWI proteins (for a review, see 147). Mice carrying mutations in the PIWI proteins show loss of *Rasgrf1* ICR methylation in sperm. Nevertheless, how the piRNA pathway directs the recruitment of DNMT3C to *Rasgrf1* still remains unclear.

In summary, the establishment of canonical imprinting seems to depend, all in all, on targeted de novo DNA methylation during oogenesis or spermatogenesis. The key factors involved in the epigenetic marking of most ICRs are proposed to be ongoing noncoding transcription and associated changes in histone methylation patterns. Crucially, these repressive states are established in the germline and as such do not entail epigenetic asymmetry between the alleles. Such asymmetry is only found after fertilization, when differentially methylated genomes fuse to form one nucleus. Thus, the monoallelic nature of imprints is in fact established upon fertilization, at which point specific pathways must operate to stably maintain this allelic asymmetry.

In fact, immediately following fertilization there is striking overall asymmetry in the DNA methylation patterns between the paternal and maternal genomes (148). However, only a minute subset of these gametic differentially methylated regions function as ICRs throughout embryonic development and into adult life, as the majority of this asymmetry is dynamically reprogrammed during preimplantation development. Rapid active and passive DNA demethylation ensues shortly after fertilization, leading to a globally hypomethylated state at the blastocyst stage (for a review, see 148). However, ICR elements are selectively protected from this genome-wide demethylation, and asymmetric DNA methylation between the two alleles is thus maintained. The molecular mechanisms allowing this 5mC protection relies in part on a Krüppel-associated box domain–zinc finger protein (KRAB-ZFP) called ZFP57 (35). This factor binds to a specific motif present at all ICRs, but only in the context of high DNA methylation and H3K9me3 (149). ZFP57 thus allows for the recruitment of its cofactor KRAB-associated protein 1 (KAP1, also called TRIM28) maintenance DNMT1 and histone methyltransferases specifically to the methylated alleles of ICRs (149, 150). Indeed, lack of ZFP57 at preimplantation stages leads to partial imprint erasure (151, 152). Recently, another KRAB-ZFP, ZFP445, was also found to contribute to protection from imprint erasure (153). Thus, the initial asymmetric DNA methylation at the ICRs enables the recruitment of specific ZFP proteins bringing in multiple heterochromatic proteins including the DNA methylation maintenance machinery.

Another potential alternative pathway is dependent on H3K9me2 associated with some methylated ICRs. This histone modification is postulated to bind the maternal factor developmental pluripotency-associated 3 (DPPA3; also known as STELLA), which in turn would prevent efficient 5mC to 5hmC conversion during preimplantation development (154, 155). This model, however, is somewhat confounded by a recent study showing that DPPA3 already regulates global DNA methylation levels in the oocyte (156). Further studies will be necessary to define the role of this regulatory pathway in maintaining imprinted states after fertilization.

How do ICRs ultimately result in imprinted gene expression across regions that can span several hundred kilobases? Upon fertilization, differential methylation at the ICRs can lead to transcriptional silencing of nearby genes on the imprinted allele or to the formation of fHC on the other allele. The former process typically depends on ncRNA expression from the allele with unmethylated ICR (157, 158). Indeed, this is the case for two imprinted ncRNAs: *Airn* and *Kcnq1ot1* (157). These are both maternally imprinted and as such are expressed only from the paternal allele. In the placenta, however, both of these ncRNAs are necessary and sufficient to establish a large silent PcG-enriched domain on the paternal allele (159–161). Indeed, similarly to *Xist* RNA, both of these ncRNAs interact with hnRNPK to enable PRC1 and PRC2 enrichment. On the basis of our understanding of XCI mechanisms, it seems likely that hnRNPK would first recruit PRC1 and only subsequently allow PRC2 enrichment thanks to JARID2 binding to H2AK119Ub (30). However, this model awaits experimental validation.

Thus, canonical imprinting shows some similarities with XCI. On the one hand, establishment of fHC domains seems to rely on noncoding transcription in both cases. During XCI, *Xist* RNA mediates accumulation of repressive histone marks, and some imprinted ncRNAs seem to behave in a similar manner. On the other hand, the establishment of imprinting typically depends on the act of transcription itself, allowing for efficient deposition of repressive chromatin signatures at the ICR. An important difference in both processes is the role of DNA methylation. In the case of XCI, this mark seems to be involved solely in maintaining gene silencing, whereas in genomic imprinting it plays a pivotal role in initiation. This mark also allows stable maintenance and recruitment of other chromatin-modifying activities. Another difference is the dependence of genomic imprinting on highly specific DNA binding factors. Indeed, it seems that in most cases the ZFP57 binding motif is crucial for imprint maintenance. In the case of XCI, no such factor has yet been identified and maintenance of the inactive state seems rather to be mediated by self-reinforcing multilayered chromatin marks.

NONCANONICAL H3K27me3-DEPENDENT IMPRINTING

Genomic imprinting was first formally demonstrated in 1984 by Davor Solter, Azim Surani, and colleagues (162–164). Around the same time, genetic data from Bruce Cattanach revealed differential activity of maternally and paternally derived chromosome regions in mice (165). In fact, preferential inactivation of the paternal X chromosome in extraembryonic lineages had already been discovered in the 1970s (166). Surprisingly, however, this early example of imprinting was found not to rely on a canonical DNA methylation-dependent mechanism (118). Despite significant efforts, the molecular mechanism of nonrandom *Xist* RNA expression in extraembryonic lineages remained elusive for decades. It was because of the discovery of H3K27me3-dependent, but DNA methylation-independent, imprinting that a putative mode of action could be tested (21). This novel pathway was only discovered because of the development of ultrasensitive technologies allowing for allele-specific mapping of histone modifications and open chromatin in preimplantation embryos. By using such approaches, Inoue et al. (21) identified 76 genes with maternal H3K27me3 marking and an open and transcribed state on the paternal allele. These maternal domains of H3K27me3 are inherited from the oocyte and arise during oocyte growth but are erased around the time of implantation (**Figure 1**) (167). Consistent with this, the paternal expression of noncanonically imprinted genes seems to be transient and becomes lost at the time of embryo implantation (21). A notable exception from this concerns a small set of genes that are monoallelically expressed in the placenta (21). *Xist* is one of these, and it remains repressed at the maternal allele in the extraembryonic lineages. This is due to a maternally inherited large H3K27me3 domain spanning its promoter and extending well beyond it (21). Indeed, maternal loss of EED, a core PRC2 component, leads to robust *Xist* RNA expression from the maternal allele even in male embryos where *Xist* is normally never expressed (168, 169). What is more, other noncanonically imprinted genes also become biallelically expressed upon the loss of PRC2 (169). Although the presence of H3K27me3-dependent imprinting is unquestionable, its importance for placental development beyond *Xist* regulation requires further investigation.

Furthermore, the molecular mechanism that establishes H3K27me3 domains remains unclear. The formation of fHC is typically associated with the act of transcription (canonical imprinting) and ncRNAs (e.g., *Xist*, *Airn*, *Kcnq1ot1*); however, it remains unlikely that a similar mechanism plays a role in targeting H3K27me3 in the oocyte. Consistent with the antagonism between transcription, DNA methylation, and H3K27me3 (170), it seems that de novo DNA methylation targeted by transcription in the oocyte might restrict H3K27me3 domains (167). Thus, the underlying mechanism of PcG targeting remains unknown. Another interesting aspect of this novel

pathway is how allelic asymmetry is maintained during early preimplantation development, as the H3K27me3 inherited from the sperm becomes rapidly reprogrammed, whereas the maternal pronucleus seems protected from this activity. On the basis of what is now known about XCI, we propose that there is a possible function of PRC1 allowing for a positive feedback loop in retaining H3K27me3-marked domains. What is more, unlike the canonical ICRs, those driving H3K27me3 imprinting have not yet been characterized. Even in the case of *Xist* imprinting, the H3K27me3 enrichment domain spans 400 kb; however, which part (or all) of this domain is actually necessary for *Xist*'s early monoallelic expression remains to be shown. Finally, there is the question of how noncanonical imprinting is maintained specifically in the extraembryonic lineages. Chromatin profiling of these lineages revealed that imprinted H3K27me3 domains are typically lost after implantation (171, 172). They seem to be replaced by monoallelic de novo DNA methylation at specific repeat elements. These are proposed to act as imprinted promoters (or enhancers) driving transcription from the unmethylated paternal allele (171). The functional importance of DNA methylation in this process was recently demonstrated (172). However, the validity of this model for the *Xist* locus is questionable. Indeed, the *Xist* imprint itself is removed prior to implantation (173) and monoallelic *Xist* expression is maintained in the extraembryonic lineages even in the absence of DNA methylation (118).

LONG-RANGE SILENCED DOMAINS: *Hox* CLUSTER REPRESSION

The final example of fHC that we discuss is developmentally regulated *Hox* genes. In mammals, 39 such genes are grouped within four clusters. All of these are stably repressed during pre- and peri-implantation stages (**Figure 1**). They normally become activated only during gastrulation in a spatially and temporally controlled manner (174). The reason why *Hox* clusters can be considered fHC is because once key PcG components are removed widespread, *Hox*-gene reactivation is observed both in vitro and in vivo (5–11). Indeed, in the case of both ESCs and peri-implantation embryos, *Hox* clusters are richly decorated by the PcG complexes, leading to the formation of the stably repressed domains of H3K27me3 and H2AK119Ub enrichment (10, 175). The requirement of PcG for *Hox* silencing fulfils the fHC definition, where strong repressive chromatin signals override the activatory *trans*-acting TFs.

One key question regarding *Hox*-gene regulation is how the repressive fHC state is established. ChIP-seq experiments during preimplantation development revealed that the repressive H3K27me3 state is not inherited from the gametes (167). Indeed, this mark is removed from *Hox* clusters after fertilization and only becomes strongly enriched at the peri-implantation stage (**Figure 1**). An important but yet unanswered question concerns the timing of PRC1 recruitment. Because a strong positive feedback loop exists between PRC1 and PRC2 (30, 176–178), it remains possible that one of these complexes is recruited first. A significant body of knowledge points to the initial recruitment of PRC2 and only subsequently of PRC1 (reviewed in 179). Indeed, loss of PRC2 in ESCs leads to a significant loss of PRC1 binding (6, 180). However, depletion of PRC1 does not lead to complete PRC2 loss (5, 30, 181). In ESCs it seems that the primary mode of PRC2 recruitment depends on its subunit MTF2, which selectively binds to demethylated, CpG-rich regions with a specific helical shape (182). However, single *Mtf2* KO ESCs still retain strong H3K27me3 enrichment along *Hox* clusters. An alternative recruitment mode operates through other cofactors of PRC2, such as JARID2, EPOP, and AEBP2 (26, 183, 184). Indeed, ESCs lacking all these cofactors fail to recruit PRC2 to the *Hox* clusters (183, 184). To identify the sites where MTF2/JARID2 cofactors first recruit PRC2, the Reinberg lab has utilized an EED point mutation (Y365A or F97A), which renders PRC2 unable to spread as EED loses its binding to H3K27me3 (26). Such mutant ESCs lose H3K27me3 genome-wide except at ~200 nucleation

sites. It is from these sites that wild-type PRC2 can spread in *cis* and into regions lying in their 3D proximity. Such nucleation sites have been identified in the proximity of all *Hox* clusters. Importantly, weak H3K27me3 deposition at these regions can already be demonstrated at the blastocyst stage, indicating that they might be functional not only in ESCs but also in vivo (26). Such primary PRC2 recruitment to unmethylated CGI at the blastocyst stage could in turn recruit the canonical PRC1 through its CBX7 subunit binding to H3K27me3 (29).

The reverse hypothesis has also been proposed, as PRC1 tethering in ESCs results in de novo PRC2 recruitment possibly thanks to the affinity of JARID2 to bind H2AK119Ub (30, 183). Such a model would be more reminiscent of that proposed for *Xist* RNA-mediated PcG recruitment. Unfortunately, not enough is known about the dynamics and role of PRC1 in preimplantation embryos to disentangle the primary recruitment of PRC1 from PRC2 functionally. Indeed, although studies in ESCs have enabled elegant functional analyses, in the case of the PcG complexes, ESCs seem to differ dramatically from their in vivo counterparts. In the ICM of the blastocyst, very little de novo PRC2 recruitment took place even at the *Hox* clusters (167). Why the repressive chromatin state of fHC at these regions is not yet established by the blastocyst stage remains unclear. Indeed, the ICM shows global DNA hypomethylation, and yet there is no robust PcG recruitment to the CGIs as would be predicted from the MTF2 binding specificity observed in ESCs. These and other discrepancies merit detailed in vivo investigation given that appropriate in vitro stem cell models do not yet exist.

Another frequent feature of fHC formation are ncRNAs or the act of their transcription, which operate to target repressive chromatin modifiers. In the case of *Hox* cluster silencing, this is a highly controversial topic. Initial studies described an antisense transcript to *HoxC* genes: *Hotair* (185). This ncRNA was reported to repress the *HoxD* genes in *trans* and to interact with both the PRC2 complex as well as the H3K4me3 demethylase LSD1 (185, 186). What is more, loss of *Hotair* in vivo was reported to lead to homeotic transformations reminiscent of *Hox*-gene deregulation (187). However, more rigorous phenotyping of the *Hotair* KO mouse revealed no sign of developmental defects or *HoxD* cluster derepression (20). This is in line with other reports revealing that PRC2 complex promiscuously binds long RNAs and with similar affinities to the interaction with *Hotair* (96, 188, 189). Further data arguing against the biological importance of *Hotair* RNA for *HoxD* gene repression came from *Hotair* overexpression and tethering experiments (19). Indeed, only very minor gene deregulation was observed in these experiments, and this was independent of PRC2 activity (19). Thus, it seems unlikely that *Hotair* plays a significant role in *Hox*-gene repression in vivo. This is in line with recent models proposing that PRC2 recruitment is actually impeded by ongoing transcription (179, 188, 190, 191). Such inhibition of PRC2 spreading was also visible during the early stages of XCI (80).

FACULTATIVE HETEROCHROMATIN ESTABLISHMENT CAN OCCUR INDEPENDENTLY OF NONCODING RNAs OR TRANSCRIPTION

Here, we have sought to review recent findings on the role of chromatin modifications in establishing and maintaining fHC in mouse development (**Table 1**). The latest advances in the field indicate that establishment of many types of fHC (but not all) depends on noncoding transcription. Either ncRNAs (e.g., *Xist*, *Kcnq1ot1*, *Airn*) or the act of transcription itself (canonical imprint establishment) targets chromatin-modifying activity, typically in *cis*, allowing for the initiation of heterochromatinization. In the case of XCI, *Xist* RNA recruits SPEN along with other factors, and HDAC activity is enabled, which facilitates establishment of a repressive chromatin compartment. In the case of canonical imprinting, transcription (or production of piRNAs for *Rasgrf1*) targets de novo DNA methylation machinery to establish the heterochromatin features

Table 1 Summary of mechanisms involved in fHC initiation and maintenance

	Mechanism of initiation	Mechanism of maintenance
X-chromosome inactivation	■ *Xist* RNA-dependent recruitment of SPEN, subsequent HDAC3 activation, and histone deacetylation ■ Polycomb contributes to efficient gene repression. ■ Potential function of other SPEN interactors (e.g., m6A RNA methylation or NuRD)	■ Repression is largely *Xist*-independent. ■ Promoter DNA methylation locks in silencing, especially in embryonic lineages. ■ PRC2 locks in silencing in extraembryonic lineages. ■ SMCHD1 maintains silencing of ~10% of genes.
Canonical imprinting	Oogenesis: ■ Transcription targets H3K36me3 and allows for H3K4me2/3 removal by KDM1A/B from CGIs. ■ H3K4me3 loss allows DNMT3A/L targeting to CGIs. Spermatogenesis: ■ DNMT3A/L targeting to rare CGIs through an unknown mechanism ■ DNMT3C targeting to *Rasgrf1* depends on piRNAs.	■ Asymmetric ICR methylation is maintained in part by 5mC-dependent binding of ZFP57/ZFP445. ■ Subsequent recruitment of KAP1 and DNMT1 allows 5mC maintenance.
Noncanonical imprinting	■ H3K27me3 targeting in the oocyte to broad untranscribed domains of low 5mC ■ Unclear role of PRC1 or PRC2 targeting cofactors ■ Role of noncoding transcription is unlikely	■ Presumably positive feedback between PRC2 and PRC1 allows for preimplantation maintenance. ■ Extraembryonic lineages exchange broad H3K27me3 for monoallelic 5mC at repeats. ■ Entirely unknown for *Xist* locus
Hox cluster repression	■ In ESCs, cofactors target PRC2 to unmethylated, CpG-rich, untranscribed regions. ■ Spreading of PRC2 from nucleation sites relies on H3K27me3-binding activity of EED. ■ Role of PRC1 in targeting PRC2 remains unclear as does the targeting mechanism in vivo. ■ The role of polycomb in initiating *Hox* repression is unclear.	■ A positive feedback between PRC2 and PRC1 is vital. ■ Feedback presumably depends on JARID2 recognizing H2AK119Ub and CBX7 binding H3K27me3. ■ No role for noncoding transcription

Abbreviations: 5mC, 5-methylcytosine; CBX, chromobox; CGI, CpG island; DNMT, DNA methyltransferase; EED, embryonic ectoderm development; ESC, embryonic stem cell; fHC, facultative heterochromatin; H2AK119Ub, monoubiquitinylation of histone H2A lysine 119; HDAC, histone deacetylase; ICR, imprint control region; JARID2, Jumonji and AT-rich interaction domain containing 2; KAP1, KRAB-associated protein 1; KDM, histone lysine demethylase; MTF2, metal response element binding transcription factor 2; NuRD, nuclease remodeling deacetylase complex; piRNA, Piwi-interacting RNA; PRC, polycomb-group repressive complex; SMCHD1, structural maintenance of chromosomes flexible hinge domain-containing protein 1; SPEN, protein split ends homolog; ZFP, zinc finger protein.

of ICRs. One surprising common feature of these fHC states is that their establishment does not depend on PRC2 activity. Indeed, it seems that ongoing transcription would rather hinder the rapid accumulation of H3K27me3 as is the case in XCI, where H3K27me3 accumulation can only occur when genes have been silenced (80). This, however, does not preclude PRC2 from playing an important role in maintaining gene repression within some specific developmental contexts.

However, several other fHC types seem to be entirely independent of ncRNA or transcription. In these cases, the molecular mechanisms of establishment remain unclear, although it is known that they strongly rely on PRC2 for their maintenance. This seems to be the case for both noncanonical imprinting and *Hox*-gene cluster repression. ESC experiments indicate that the establishment of such fHC relies mainly on the targeting of PRC2 by its cofactors. Recent data

point to important functions of PRC1 in both targeting and maintaining fHC. Finally, although the mechanisms that preserve asymmetric DNA methylation at imprints have been elucidated, so far the mechanisms underlying the maintenance of asymmetric H3K27me3 marking in mouse preimplantation development remain to be discovered. Mechanistic studies on developing mouse embryos will be required to elucidate this point.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

LITERATURE CITED

1. Heitz E. 1928. Das Heterochromatin der Moose. *Jahrb. Wiss. Bot.* 69:762–818
2. Trojer P, Reinberg D. 2007. Facultative heterochromatin: Is there a distinctive molecular signature? *Mol. Cell* 28:1–13
3. Allshire RC, Madhani HD. 2018. Ten principles of heterochromatin formation and function. *Nat. Rev. Mol. Cell Biol.* 19:229–44
4. Brown SW. 1966. Heterochromatin. *Science* 151:417–25
5. Endoh M, Endo TA, Endoh T, Fujimura Y, Ohara O, et al. 2008. Polycomb group proteins Ring1A/B are functionally linked to the core transcriptional regulatory circuitry to maintain ES cell identity. *Development* 135:1513–24
6. Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, et al. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 441:349–53
7. Pasini D, Bracken AP, Hansen JB, Capillo M, Helin K. 2007. The polycomb group protein Suz12 is required for embryonic stem cell differentiation. *Mol. Cell. Biol.* 27:3769–79
8. Chamberlain SJ, Yee D, Magnuson T. 2008. Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. *Stem Cells* 26:1496–505
9. Leeb M, Pasini D, Novatchkova M, Jaritz M, Helin K, Wutz A. 2010. Polycomb complexes act redundantly to repress genomic repeats and genes. *Genes Dev.* 24:265–76
10. Zylicz JJ, Dietmann S, Gunesdogan U, Hackett JA, Cougot D, et al. 2015. Chromatin dynamics and the role of G9a in gene regulation and enhancer silencing during early mouse development. *eLife* 4:e09571
11. Isono K, Fujimura Y, Shinga J, Yamaki M, O-Wang J, et al. 2005. Mammalian polyhomeotic homologues Phc2 and Phc1 act in synergy to mediate polycomb repression of *Hox* genes. *Mol. Cell. Biol.* 25:6694–706
12. Marahrens Y, Panning B, Dausman J, Strauss W, Jaenisch R. 1997. Xist-deficient mice are defective in dosage compensation but not spermatogenesis. *Genes Dev.* 11:156–66
13. Panning B, Dausman J, Jaenisch R. 1997. X chromosome inactivation is mediated by Xist RNA stabilization. *Cell* 90:907–16
14. Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N. 1996. Requirement for Xist in X chromosome inactivation. *Nature* 379:131–37
15. Chotalia M, Smallwood SA, Ruf N, Dawson C, Lucifero D, et al. 2009. Transcription is required for establishment of germline methylation marks at imprinted genes. *Genes Dev.* 23:105–17
16. Frohlich LF, Mrakovcic M, Steinborn R, Chung UI, Bastepe M, Juppner H. 2010. Targeted deletion of the Nesp55 DMR defines another Gnas imprinting control region and provides a mouse model of autosomal dominant PHP-Ib. *PNAS* 107:9275–80
17. Smith EY, Futtner CR, Chamberlain SJ, Johnstone KA, Resnick JL. 2011. Transcription is required to establish maternal imprinting at the Prader-Willi syndrome and Angelman syndrome locus. *PLOS Genet.* 7:e1002422
18. Veselovska L, Smallwood SA, Saadeh H, Stewart KR, Krueger F, et al. 2015. Deep sequencing and de novo assembly of the mouse oocyte transcriptome define the contribution of transcription to the DNA methylation landscape. *Genome Biol.* 16:209

19. Portoso M, Ragazzini R, Brencic Z, Moiani A, Michaud A, et al. 2017. PRC2 is dispensable for *HOTAIR*-mediated transcriptional repression. *EMBO J.* 36:981–94
20. Amandio AR, Necsulea A, Joye E, Mascrez B, Duboule D. 2016. *Hotair* is dispensable for mouse development. *PLOS Genet.* 12:e1006232
21. Inoue A, Jiang L, Lu F, Zhang Y. 2017. Genomic imprinting of Xist by maternal H3K27me3. *Genes Dev.* 31:1927–32
22. Seto E, Yoshida M. 2014. Erasers of histone acetylation: the histone deacetylase enzymes. *Cold Spring Harb. Perspect. Biol.* 6:a018713
23. Bannister AJ, Kouzarides T. 2011. Regulation of chromatin by histone modifications. *Cell Res.* 21:381–95
24. Schuettengruber B, Bourbon HM, Di Croce L, Cavalli G. 2017. Genome regulation by Polycomb and Trithorax: 70 years and counting. *Cell* 171:34–57
25. Margueron R, Reinberg D. 2011. The Polycomb complex PRC2 and its mark in life. *Nature* 469:343–49
26. Oksuz O, Narendra V, Lee CH, Descostes N, LeRoy G, et al. 2018. Capturing the onset of PRC2-mediated repressive domain formation. *Mol. Cell* 70:1149–62.e5
27. Gao Z, Zhang J, Bonasio R, Strino F, Sawai A, et al. 2012. PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1 family complexes. *Mol. Cell* 45:344–56
28. Brockdorff N. 2017. Polycomb complexes in X chromosome inactivation. *Philos. Trans. R. Soc. B* 372:20170021
29. Bernstein E, Duncan EM, Masui O, Gil J, Heard E, Allis CD. 2006. Mouse polycomb proteins bind differentially to methylated histone H3 and RNA and are enriched in facultative heterochromatin. *Mol. Cell. Biol.* 26:2560–69
30. Blackledge NP, Farcas AM, Kondo T, King HW, McGouran JF, et al. 2014. Variant PRC1 complex-dependent H2A ubiquitylation drives PRC2 recruitment and polycomb domain formation. *Cell* 157:1445–59
31. Zeng Y, Chen T. 2019. DNA methylation reprogramming during mammalian development. *Genes (Basel)* 10:257
32. Deaton AM, Bird A. 2011. CpG islands and the regulation of transcription. *Genes Dev.* 25:1010–22
33. Du Q, Luu PL, Stirzaker C, Clark SJ. 2015. Methyl-CpG-binding domain proteins: readers of the epigenome. *Epigenomics* 7:1051–73
34. Wang H, Maurano MT, Qu H, Varley KE, Gertz J, et al. 2012. Widespread plasticity in CTCF occupancy linked to DNA methylation. *Genome Res.* 22:1680–88
35. Quenneville S, Verde G, Corsinotti A, Kapopoulou A, Jakobsson J, et al. 2011. In embryonic stem cells, ZFP57/KAP1 recognize a methylated hexanucleotide to affect chromatin and DNA methylation of imprinting control regions. *Mol. Cell* 44:361–72
36. Marchal C, Sima J, Gilbert DM. 2019. Control of DNA replication timing in the 3D genome. *Nat. Rev. Mol. Cell. Biol.* 20:721–37
37. Borensztein M, Syx L, Ancelin K, Diabangouaya P, Picard C, et al. 2017. Xist-dependent imprinted X inactivation and the early developmental consequences of its failure. *Nat. Struct. Mol. Biol.* 24:226–33
38. Lyon MF. 1961. Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* 190:372–73
39. Sharman GB. 1971. Late DNA replication in the paternally derived X chromosome of female kangaroos. *Nature* 230:231–32
40. Escamilla-Del-Arenal M, da Rocha ST, Heard E. 2011. Evolutionary diversity and developmental regulation of X-chromosome inactivation. *Hum. Genet.* 130:307–27
41. Engreitz JM, Pandya-Jones A, McDonel P, Shishkin A, Sirokman K, et al. 2013. The Xist lncRNA exploits three-dimensional genome architecture to spread across the X chromosome. *Science* 341:1237973
42. Simon MD, Pinter SF, Fang R, Sarma K, Rutenberg-Schoenberg M, et al. 2013. High-resolution Xist binding maps reveal two-step spreading during X-chromosome inactivation. *Nature* 504:465–69
43. Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E. 2004. Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science* 303:644–49
44. Chaumeil J, Okamoto I, Guggiari M, Heard E. 2002. Integrated kinetics of X chromosome inactivation in differentiating embryonic stem cells. *Cytogenet. Genome Res.* 99:75–84

45. Okamoto I, Arnaud D, Le Baccon P, Otte AP, Disteche CM, et al. 2005. Evidence for de novo imprinted X-chromosome inactivation independent of meiotic inactivation in mice. *Nature* 438:369–73
46. Patrat C, Okamoto I, Diabangouaya P, Vialon V, Le Baccon P, et al. 2009. Dynamic changes in paternal X-chromosome activity during imprinted X-chromosome inactivation in mice. *PNAS* 106:5198–203
47. Tada T, Obata Y, Tada M, Goto Y, Nakatsuji N, et al. 2000. Imprint switching for non-random X-chromosome inactivation during mouse oocyte growth. *Development* 127:3101–5
48. Borensztein M, Okamoto I, Syx L, Guilbaud G, Picard C, et al. 2017. Contribution of epigenetic landscapes and transcription factors to X-chromosome reactivation in the inner cell mass. *Nat. Commun.* 8:1297
49. Okamoto I. 2004. Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science* 303:644–49
50. Mak W, Nesterova TB, de Napoles M, Appanah R, Yamanaka S, et al. 2004. Reactivation of the paternal X chromosome in early mouse embryos. *Science* 303:666–69
51. Boggs BA, Connors B, Sobel RE, Chinault AC, Allis CD. 1996. Reduced levels of histone H3 acetylation on the inactive X chromosome in human females. *Chromosoma* 105:303–9
52. Jeppesen P, Turner BM. 1993. The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell* 74:281–89
53. de Napoles M, Mermoud JE, Wakao R, Tang YA, Endoh M, et al. 2004. Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. *Dev. Cell* 7:663–76
54. Silva J, Mak W, Zvetkova I, Appanah R, Nesterova TB, et al. 2003. Establishment of histone H3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 Polycomb group complexes. *Dev. Cell* 4:481–95
55. Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, et al. 2003. Role of histone H3 lysine 27 methylation in X inactivation. *Science* 300:131–35
56. Chaumeil J, Le Baccon P, Wutz A, Heard E. 2006. A novel role for Xist RNA in the formation of a repressive nuclear compartment into which genes are recruited when silenced. *Genes Dev.* 20:2223–37
57. Keohane AM, O'Neill L P, Belyaev ND, Lavender JS, Turner BM. 1996. X-Inactivation and histone H4 acetylation in embryonic stem cells. *Dev. Biol.* 180:618–30
58. Mermoud JE, Popova B, Peters AH, Jenuwein T, Brockdorff N. 2002. Histone H3 lysine 9 methylation occurs rapidly at the onset of random X chromosome inactivation. *Curr. Biol.* 12:247–51
59. Rasmussen TP, Mastrangelo MA, Eden A, Pehrson JR, Jaenisch R. 2000. Dynamic relocalization of histone MacroH2A1 from centrosomes to inactive X chromosomes during X inactivation. *J. Cell Biol.* 150:1189–98
60. Escamilla-Del-Arenal M, da Rocha ST, Spruijt CG, Masui O, Renaud O, et al. 2013. Cdyl, a new partner of the inactive X chromosome and potential reader of H3K27me3 and H3K9me2. *Mol. Cell. Biol.* 33:5005–20
61. Gendrel AV, Apedaile A, Coker H, Termanis A, Zvetkova I, et al. 2012. Smchd1-dependent and -independent pathways determine developmental dynamics of CpG island methylation on the inactive X chromosome. *Dev. Cell* 23:265–79
62. Lock LF, Takagi N, Martin GR. 1987. Methylation of the Hprt gene on the inactive X occurs after chromosome inactivation. *Cell* 48:39–46
63. Wutz A, Jaenisch R. 2000. A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. *Mol. Cell* 5:695–705
64. Csankovszki G, Panning B, Bates B, Pehrson JR, Jaenisch R. 1999. Conditional deletion of Xist disrupts histone macroH2A localization but not maintenance of X inactivation. *Nat. Genet.* 22:323–24
65. Brown SD. 1991. XIST and the mapping of the X chromosome inactivation centre. *Bioessays* 13:607–12
66. Nesterova TB, Slobodyanyuk SY, Elisaphenko EA, Shevchenko AI, Johnston C, et al. 2001. Characterization of the genomic Xist locus in rodents reveals conservation of overall gene structure and tandem repeats but rapid evolution of unique sequence. *Genome Res.* 11:833–49
67. Yen ZC, Meyer IM, Karalic S, Brown CJ. 2007. A cross-species comparison of X-chromosome inactivation in Eutheria. *Genomics* 90:453–63

68. Loda A, Heard E. 2019. Xist RNA in action: past, present, and future. *PLOS Genet*. 15:e1008333
69. Wutz A, Rasmussen TP, Jaenisch R. 2002. Chromosomal silencing and localization are mediated by different domains of Xist RNA. *Nat. Genet.* 30:167–74
70. Chu C, Zhang QC, da Rocha ST, Flynn RA, Bharadwaj M, et al. 2015. Systematic discovery of Xist RNA binding proteins. *Cell* 161:404–16
71. Colognori D, Sunwoo H, Kriz AJ, Wang CY, Lee JT. 2019. *Xist* deletional analysis reveals an interdependency between Xist RNA and polycomb complexes for spreading along the inactive X. *Mol. Cell* 74:101–17.e10
72. Bousard A, Raposo AC, Żylicz JJ, Picard C, Pires VB, et al. 2019. The role of *Xist*-mediated Polycomb recruitment in the initiation of X-chromosome inactivation. *EMBO Rep*. 20:e48019
73. Nesterova TB, Wei G, Coker H, Pintacuda G, Bowness JS, et al. 2019. Systematic allelic analysis defines the interplay of key pathways in X chromosome inactivation. *Nat. Commun.* 10:3129
74. Pintacuda G, Wei G, Roustan C, Kirmizitas BA, Solcan N, et al. 2017. hnRNPK recruits PCGF3/5-PRC1 to the Xist RNA B-repeat to establish Polycomb-mediated chromosomal silencing. *Mol. Cell* 68:955–69.e10
75. da Rocha ST, Boeva V, Escamilla-Del-Arenal M, Ancelin K, Granier C, et al. 2014. Jarid2 is implicated in the initial Xist-induced targeting of PRC2 to the inactive X chromosome. *Mol. Cell* 53:301–16
76. Sunwoo H, Colognori D, Froberg JE, Jeon Y, Lee JT. 2017. Repeat E anchors Xist RNA to the inactive X chromosomal compartment through CDKN1A-interacting protein (CIZ1). *PNAS* 114:10654–59
77. Ridings-Figueroa R, Stewart ER, Nesterova TB, Coker H, Pintacuda G, et al. 2017. The nuclear matrix protein CIZ1 facilitates localization of Xist RNA to the inactive X-chromosome territory. *Genes Dev*. 31:876–88
78. Chen CK, Blanco M, Jackson C, Aznauryan E, Ollikainen N, et al. 2016. Xist recruits the X chromosome to the nuclear lamina to enable chromosome-wide silencing. *Science* 354:468–72
79. Barros de Andrade ESL, Jonkers I, Syx L, Dunkel I, Chaumeil J, et al. 2019. Kinetics of Xist-induced gene silencing can be predicted from combinations of epigenetic and genomic features. *Genome Res.* 29:1087–99
80. Zylicz JJ, Bousard A, Zumer K, Dossin F, Mohammad E, et al. 2019. The implication of early chromatin changes in X chromosome inactivation. *Cell* 176:182–97.e23
81. Denholtz M, Bonora G, Chronis C, Splinter E, de Laat W, et al. 2013. Long-range chromatin contacts in embryonic stem cells reveal a role for pluripotency factors and polycomb proteins in genome organization. *Cell Stem Cell* 13:602–16
82. Joshi O, Wang SY, Kuznetsova T, Atlasi Y, Peng T, et al. 2015. Dynamic reorganization of extremely long-range promoter-promoter interactions between two states of pluripotency. *Cell Stem Cell* 17:748–57
83. Schoenfelder S, Sugar R, Dimond A, Javierre BM, Armstrong H, et al. 2015. Polycomb repressive complex PRC1 spatially constrains the mouse embryonic stem cell genome. *Nat. Genet.* 47:1179–86
84. Kundu S, Ji F, Sunwoo H, Jain G, Lee JT, et al. 2017. Polycomb repressive complex 1 generates discrete compacted domains that change during differentiation. *Mol. Cell* 65:432–46.e5
85. Kohlmaier A, Savarese F, Lachner M, Martens J, Jenuwein T, Wutz A. 2004. A chromosomal memory triggered by Xist regulates histone methylation in X inactivation. *PLOS Biol*. 2:E171
86. Norris DP, Brockdorff N, Rastan S. 1991. Methylation status of CpG-rich islands on active and inactive mouse X chromosomes. *Mamm. Genome* 1:78–83
87. Costanzi C, Pehrson JR. 1998. Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. *Nature* 393:599–601
88. Mermoud JE, Costanzi C, Pehrson JR, Brockdorff N. 1999. Histone macroH2A1.2 relocates to the inactive X chromosome after initiation and propagation of X-inactivation. *J. Cell Biol.* 147:1399–408
89. Heard E, Rougeulle C, Arnaud D, Avner P, Allis CD, Spector DL. 2001. Methylation of histone H3 at Lys-9 is an early mark on the X chromosome during X inactivation. *Cell* 107:727–38
90. Pinter SF, Sadreyev RI, Yildirim E, Jeon Y, Ohsumi TK, et al. 2012. Spreading of X chromosome inactivation via a hierarchy of defined Polycomb stations. *Genome Res*. 22:1864–76
91. Marks H, Chow JC, Denissov S, Francoijs KJ, Brockdorff N, et al. 2009. High-resolution analysis of epigenetic changes associated with X inactivation. *Genome Res*. 19:1361–73

92. Calabrese JM, Sun W, Song L, Mugford JW, Williams L, et al. 2012. Site-specific silencing of regulatory elements as a mechanism of X inactivation. *Cell* 151:951–63
93. Schulz EG, Meisig J, Nakamura T, Okamoto I, Sieber A, et al. 2014. The two active X chromosomes in female ESCs block exit from the pluripotent state by modulating the ESC signaling network. *Cell Stem Cell* 14:203–16
94. Plath K, Talbot D, Hamer KM, Otte AP, Yang TP, et al. 2004. Developmentally regulated alterations in Polycomb repressive complex 1 proteins on the inactive X chromosome. *J. Cell Biol.* 167:1025–35
95. Zhao J, Sun BK, Erwin JA, Song J-J, Lee JT. 2008. Polycomb proteins targeted by a short repeat RNA to the mouse X-chromosome. *Science* 322:750–56
96. Davidovich C, Zheng L, Goodrich KJ, Cech TR. 2013. Promiscuous RNA binding by Polycomb repressive complex 2. *Nat. Struct. Mol. Biol.* 20:1250–57
97. McHugh CA, Chen CK, Chow A, Surka CF, Tran C, et al. 2015. The Xist lncRNA interacts directly with SHARP to silence transcription through HDAC3. *Nature* 521:232–36
98. Minajigi A, Froberg JE, Wei C, Sunwoo H, Kesner B, et al. 2015. Chromosomes. A comprehensive Xist interactome reveals cohesin repulsion and an RNA-directed chromosome conformation. *Science* 349:aab2276
99. Schoeftner S, Sengupta AK, Kubicek S, Mechtler K, Spahn L, et al. 2006. Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. *EMBO J.* 25:3110–22
100. Almeida M, Pintacuda G, Masui O, Koseki Y, Gdula M, et al. 2017. PCGF3/5-PRC1 initiates Polycomb recruitment in X chromosome inactivation. *Science* 356:1081–84
101. Loda A, Brandsma JH, Vassilev I, Servant N, Loos F, et al. 2017. Genetic and epigenetic features direct differential efficiency of Xist-mediated silencing at X-chromosomal and autosomal locations. *Nat. Commun.* 8:690
102. Kalantry S, Mills KC, Yee D, Otte AP, Panning B, Magnuson T. 2006. The Polycomb group protein Eed protects the inactive X-chromosome from differentiation-induced reactivation. *Nat. Cell Biol.* 8:195–202
103. Kalantry S, Magnuson T. 2006. The Polycomb group protein EED is dispensable for the initiation of random X-chromosome inactivation. *PLOS Genet.* 2:e66
104. Leeb M, Wutz A. 2007. *Ring1B* is crucial for the regulation of developmental control genes and PRC1 proteins but not X inactivation in embryonic cells. *J. Cell Biol.* 178:219–29
105. Terrenoire E, McRonald F, Halsall JA, Page P, Illingworth RS, et al. 2010. Immunostaining of modified histones defines high-level features of the human metaphase epigenome. *Genome Biol.* 11:R110
106. Monfort A, Di Minin G, Postlmayr A, Freimann R, Arieti F, et al. 2015. Identification of *Spen* as a crucial factor for *Xist* function through forward genetic screening in haploid embryonic stem cells. *Cell Rep.* 12:554–61
107. Moindrot B, Cerase A, Coker H, Masui O, Grijzenhout A, et al. 2015. A pooled shRNA screen identifies Rbm15, Spen, and Wtap as factors required for Xist RNA-mediated silencing. *Cell Rep.* 12:562–72
108. Lu Z, Zhang QC, Lee B, Flynn RA, Smith MA, et al. 2016. RNA duplex map in living cells reveals higher-order transcriptome structure. *Cell* 165:1267–79
109. Dossin F, Pinheiro I, Zylicz JJ, Roensch J, Collombet S, et al. 2020. SPEN integrates transcriptional and epigenetic control of X-inactivation. *Nature* 578:455–60
110. You SH, Lim HW, Sun Z, Broache M, Won KJ, Lazar MA. 2013. Nuclear receptor co-repressors are required for the histone-deacetylase activity of HDAC3 in vivo. *Nat. Struct. Mol. Biol.* 20:182–87
111. Shi Y, Downes M, Xie W, Kao HY, Ordentlich P, et al. 2001. Sharp, an inducible cofactor that integrates nuclear receptor repression and activation. *Genes Dev.* 15:1140–51
112. da Rocha ST, Heard E. 2017. Novel players in X inactivation: insights into Xist-mediated gene silencing and chromosome conformation. *Nat. Struct. Mol. Biol.* 24:197–204
113. Pinheiro I, Heard E. 2017. X chromosome inactivation: new players in the initiation of gene silencing. *F1000Research* 6:344
114. Patil DP, Chen CK, Pickering BF, Chow A, Jackson C, et al. 2016. m^{6}A RNA methylation promotes *XIST*-mediated transcriptional repression. *Nature* 537:369–73
115. Brown CJ, Willard HF. 1994. The human X-inactivation centre is not required for maintenance of X-chromosome inactivation. *Nature* 368:154–56

116. Hellman A, Chess A. 2007. Gene body-specific methylation on the active X chromosome. *Science* 315:1141–43
117. Graves JA. 1982. 5-azacytidine-induced re-expression of alleles on the inactive X chromosome in a hybrid mouse cell line. *Exp. Cell Res.* 141:99–105
118. Sado T, Fenner MH, Tan SS, Tam P, Shioda T, Li E. 2000. X inactivation in the mouse embryo deficient for *Dnmt1*: distinct effect of hypomethylation on imprinted and random X inactivation. *Dev. Biol.* 225:294–303
119. Blewitt ME, Gendrel AV, Pang Z, Sparrow DB, Whitelaw N, et al. 2008. SmcHD1, containing a structural-maintenance-of-chromosomes hinge domain, has a critical role in X inactivation. *Nat. Genet.* 40:663–69
120. Gendrel AV, Tang YA, Suzuki M, Godwin J, Nesterova TB, et al. 2013. Epigenetic functions of Smchd1 repress gene clusters on the inactive X chromosome and on autosomes. *Mol. Cell. Biol.* 33:3150–65
121. Jansz N, Nesterova T, Keniry A, Iminitoff M, Hickey PF, et al. 2018. Smchd1 targeting to the inactive X is dependent on the Xist-HnrnpK-PRC1 pathway. *Cell Rep.* 25:1912–23.e9
122. Wang CY, Jegu T, Chu HP, Oh HJ, Lee JT. 2018. SMCHD1 merges chromosome compartments and assists formation of super-structures on the inactive X. *Cell* 174:406–21.e26
123. Gdula MR, Nesterova TB, Pintacuda G, Godwin J, Zhan Y, et al. 2019. The non-canonical SMC protein SmcHD1 antagonises TAD formation and compartmentalisation on the inactive X chromosome. *Nat. Commun.* 10:30
124. Keniry A, Gearing LJ, Jansz N, Liu J, Holik AZ, et al. 2016. Setdb1-mediated H3K9 methylation is enriched on the inactive X and plays a role in its epigenetic silencing. *Epigenet. Chromatin* 9:16
125. Ohhata T, Tachibana M, Tada M, Tada T, Sasaki H, et al. 2004. X-inactivation is stably maintained in mouse embryos deficient for histone methyl transferase G9a. *Genesis* 40:151–56
126. Williamson CM, Blake A, Thomas S, Beechey CV, Hancock J, et al. 2013. *Mouse Imprinting Data and References*. MouseBook Catalog, MRC Harwell Inst., Oxfordshire, UK. **https://www.mousebook.org/imprinting-gene-list**
127. Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, et al. 2004. Essential role for *de novo* DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* 429:900–3
128. Bourc'his D, Xu GL, Lin CS, Bollman B, Bestor TH. 2001. Dnmt3L and the establishment of maternal genomic imprints. *Science* 294:2536–39
129. Shirane K, Toh H, Kobayashi H, Miura F, Chiba H, et al. 2013. Mouse oocyte methylomes at base resolution reveal genome-wide accumulation of non-CpG methylation and role of DNA methyltransferases. *PLOS Genet.* 9:e1003439
130. Wang L, Zhang J, Duan J, Gao X, Zhu W, et al. 2014. Programming and inheritance of parental DNA methylomes in mammals. *Cell* 157:979–91
131. Kobayashi H, Sakurai T, Imai M, Takahashi N, Fukuda A, et al. 2012. Contribution of intragenic DNA methylation in mouse gametic DNA methylomes to establish oocyte-specific heritable marks. *PLOS Genet.* 8:e1002440
132. Stewart KR, Veselovska L, Kim J, Huang J, Saadeh H, et al. 2015. Dynamic changes in histone modifications precede de novo DNA methylation in oocytes. *Genes Dev.* 29:2449–62
133. Gahurova L, Tomizawa SI, Smallwood SA, Stewart-Morgan KR, Saadeh H, et al. 2017. Transcription and chromatin determinants of de novo DNA methylation timing in oocytes. *Epigenet. Chromatin* 10:25
134. Ciccone DN, Su H, Hevi S, Gay F, Lei H, et al. 2009. KDM1B is a histone H3K4 demethylase required to establish maternal genomic imprints. *Nature* 461:415–18
135. Dhayalan A, Rajavelu A, Rathert P, Tamas R, Jurkowska RZ, et al. 2010. The Dnmt3a PWWP domain reads histone 3 lysine 36 trimethylation and guides DNA methylation. *J. Biol. Chem.* 285:26114–20
136. Sendzikaite G, Hanna CW, Stewart-Morgan KR, Ivanova E, Kelsey G. 2019. A DNMT3A PWWP mutation leads to methylation of bivalent chromatin and growth retardation in mice. *Nat. Commun.* 10:1884
137. Weinberg DN, Papillon-Cavanagh S, Chen H, Yue Y, Chen X, et al. 2019. The histone mark H3K36me2 recruits DNMT3A and shapes the intergenic DNA methylation landscape. *Nature* 573:281–86

138. Maenohara S, Unoki M, Toh H, Ohishi H, Sharif J, et al. 2017. Role of UHRF1 in de novo DNA methylation in oocytes and maintenance methylation in preimplantation embryos. *PLOS Genet*. 13:e1007042
139. Ma P, de Waal E, Weaver JR, Bartolomei MS, Schultz RM. 2015. A DNMT3A2-HDAC2 complex is essential for genomic imprinting and genome integrity in mouse oocytes. *Cell Rep*. 13:1552–60
140. Smallwood SA, Tomizawa S, Krueger F, Ruf N, Carli N, et al. 2011. Dynamic CpG island methylation landscape in oocytes and preimplantation embryos. *Nat. Genet.* 43:811–14
141. Bourc'his D, Bestor TH. 2004. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. *Nature* 431:96–99
142. Kato Y, Kaneda M, Hata K, Kumaki K, Hisano M, et al. 2007. Role of the Dnmt3 family in *de novo* methylation of imprinted and repetitive sequences during male germ cell development in the mouse. *Hum. Mol. Genet.* 16:2272–80
143. Henckel A, Chebli K, Kota SK, Arnaud P, Feil R. 2012. Transcription and histone methylation changes correlate with imprint acquisition in male germ cells. *EMBO J.* 31:606–15
144. Barau J, Teissandier A, Zamudio N, Roy S, Nalesso V, et al. 2016. The DNA methyltransferase DNMT3C protects male germ cells from transposon activity. *Science* 354:909–12
145. Watanabe T, Tomizawa S, Mitsuya K, Totoki Y, Yamamoto Y, et al. 2011. Role for piRNAs and noncoding RNA in de novo DNA methylation of the imprinted mouse *Rasgrf1* locus. *Science* 332:848–52
146. Watanabe T, Cui X, Yuan Z, Qi H, Lin H. 2018. MIWI2 targets RNAs transcribed from piRNA-dependent regions to drive DNA methylation in mouse prospermatogonia. *EMBO J.* 37:e95329
147. Ozata DM, Gainetdinov I, Zoch A, O'Carroll D, Zamore PD. 2019. PIWI-interacting RNAs: small RNAs with big functions. *Nat. Rev. Genet.* 20:89–108
148. Eckersley-Maslin MA, Alda-Catalinas C, Reik W. 2018. Dynamics of the epigenetic landscape during the maternal-to-zygotic transition. *Nat. Rev. Mol. Cell Biol.* 19:436–50
149. Strogantsev R, Krueger F, Yamazawa K, Shi H, Gould P, et al. 2015. Allele-specific binding of ZFP57 in the epigenetic regulation of imprinted and non-imprinted monoallelic expression. *Genome Biol.* 16:112
150. Messerschmidt DM, de Vries W, Ito M, Solter D, Ferguson-Smith A, Knowles BB. 2012. Trim28 is required for epigenetic stability during mouse oocyte to embryo transition. *Science* 335:1499–502
151. Li X, Ito M, Zhou F, Youngson N, Zuo X, et al. 2008. A maternal-zygotic effect gene, *Zfp57*, maintains both maternal and paternal imprints. *Dev. Cell* 15:547–57
152. Takahashi N, Gray D, Strogantsev R, Noon A, Delahaye C, et al. 2015. *ZFP57* and the targeted maintenance of postfertilization genomic imprints. *Cold Spring Harb. Symp. Quant. Biol.* 80:177–87
153. Takahashi N, Coluccio A, Thorball CW, Planet E, Shi H, et al. 2019. ZNF445 is a primary regulator of genomic imprinting. *Genes Dev*. 33:49–54
154. Nakamura T, Arai Y, Umehara H, Masuhara M, Kimura T, et al. 2006. PGC7/Stella protects against DNA demethylation in early embryogenesis. *Nat. Cell Biol.* 9:64–71
155. Nakamura T, Liu YJ, Nakashima H, Umehara H, Inoue K, et al. 2012. PGC7 binds histone H3K9me2 to protect against conversion of 5mC to 5hmC in early embryos. *Nature* 486:415–19
156. Han L, Ren C, Zhang J, Shu W, Wang Q. 2019. Differential roles of Stella in the modulation of DNA methylation during oocyte and zygotic development. *Cell Discov*. 5:9
157. Peters J, Robson JE. 2008. Imprinted noncoding RNAs. *Mamm. Genome* 19:493–502
158. Zhang Y, Guan DG, Yang JH, Shao P, Zhou H, Qu LH. 2010. ncRNAimprint: a comprehensive database of mammalian imprinted noncoding RNAs. *RNA* 16:1889–901
159. Schertzer MD, Braceros KCA, Starmer J, Cherney RE, Lee DM, et al. 2019. lncRNA-induced spread of Polycomb controlled by genome architecture, RNA abundance, and CpG island DNA. *Mol. Cell* 75:523–37.e10
160. Regha K, Sloane MA, Huang R, Pauler FM, Warczok KE, et al. 2007. Active and repressive chromatin are interspersed without spreading in an imprinted gene cluster in the mammalian genome. *Mol. Cell* 27:353–66
161. Pandey RR, Mondal T, Mohammad F, Enroth S, Redrup L, et al. 2008. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol. Cell* 32:232–46

162. Barton SC, Surani MA, Norris ML. 1984. Role of paternal and maternal genomes in mouse development. *Nature* 311:374–76
163. McGrath J, Solter D. 1984. Maternal Thp lethality in the mouse is a nuclear, not cytoplasmic, defect. *Nature* 308:550–51
164. Surani MA, Barton SC, Norris ML. 1984. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* 308:548–50
165. Cattanach BM, Kirk M. 1985. Differential activity of maternally and paternally derived chromosome regions in mice. *Nature* 315:496–98
166. Takagi N, Sasaki M. 1975. Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. *Nature* 256:640–42
167. Zheng H, Huang B, Zhang B, Xiang Y, Du Z, et al. 2016. Resetting epigenetic memory by reprogramming of histone modifications in mammals. *Mol. Cell* 63:1066–79
168. Harris C, Cloutier M, Trotter M, Hinten M, Gayen S, et al. 2019. Conversion of random X-inactivation to imprinted X-inactivation by maternal PRC2. *eLife* 8:e44258
169. Inoue A, Chen Z, Yin Q, Zhang Y. 2018. Maternal Eed knockout causes loss of H3K27me3 imprinting and random X inactivation in the extraembryonic cells. *Genes Dev.* 32:1525–36
170. Riising EM, Comet I, Leblanc B, Wu X, Johansen JV, Helin K. 2014. Gene silencing triggers polycomb repressive complex 2 recruitment to CpG islands genome wide. *Mol. Cell* 55:347–60
171. Hanna CW, Perez-Palacios R, Gahurova L, Schubert M, Krueger F, et al. 2019. Endogenous retroviral insertions drive non-canonical imprinting in extra-embryonic tissues. *Genome Biol.* 20:225
172. Chen Z, Yin Q, Inoue A, Zhang C, Zhang Y. 2019. Allelic H3K27me3 to allelic DNA methylation switch maintains noncanonical imprinting in extraembryonic cells. *Sci. Adv.* 5:eaay7246
173. Nesterova TB, Barton SC, Surani MA, Brockdorff N. 2001. Loss of Xist imprinting in diploid parthenogenetic preimplantation embryos. *Dev. Biol.* 235:343–50
174. Forlani S, Lawson KA, Deschamps J. 2003. Acquisition of Hox codes during gastrulation and axial elongation in the mouse embryo. *Development* 130:3807–19
175. Mallo M, Alonso CR. 2013. The regulation of Hox gene expression during animal development. *Development* 140:3951–63
176. Margueron R, Justin N, Ohno K, Sharpe ML, Son J, et al. 2009. Role of the polycomb protein EED in the propagation of repressive histone marks. *Nature* 461:762–67
177. Cooper S, Grijzenhout A, Underwood E, Ancelin K, Zhang T, et al. 2016. Jarid2 binds mono-ubiquitylated H2A lysine 119 to mediate crosstalk between Polycomb complexes PRC1 and PRC2. *Nat. Commun.* 7:13661
178. Rose NR, King HW, Blackledge NP, Fursova NA, Ember KJ, et al. 2016. RYBP stimulates PRC1 to shape chromatin-based communication between Polycomb repressive complexes. *eLife* 5:e18591
179. Laugesen A, Hojfeldt JW, Helin K. 2019. Molecular mechanisms directing PRC2 recruitment and H3K27 methylation. *Mol. Cell* 74:8–18
180. Tavares L, Dimitrova E, Oxley D, Webster J, Poot R, et al. 2012. RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target sites independently of PRC2 and H3K27me3. *Cell* 148:664–78
181. Fursova NA, Blackledge NP, Nakayama M, Ito S, Koseki Y, et al. 2019. Synergy between variant PRC1 complexes defines Polycomb-mediated gene repression. *Mol. Cell* 74:1020–36.e8
182. Perino M, van Mierlo G, Karemaker ID, van Genesen S, Vermeulen M, et al. 2018. MTF2 recruits Polycomb Repressive Complex 2 by helical-shape-selective DNA binding. *Nat. Genet.* 50:1002–10
183. Healy E, Mucha M, Glancy E, Fitzpatrick DJ, Conway E, et al. 2019. PRC2.1 and PRC2.2 synergize to coordinate H3K27 trimethylation. *Mol. Cell* 76:437–52.e6
184. Hojfeldt JW, Hedehus L, Laugesen A, Tatar T, Wiehle L, Helin K. 2019. Non-core subunits of the PRC2 complex are collectively required for its target-site specificity. *Mol. Cell* 76:423–36.e3
185. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, et al. 2007. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 129:1311–23
186. Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, et al. 2010. Long noncoding RNA as modular scaffold of histone modification complexes. *Science* 329:689–93

187. Li L, Liu B, Wapinski OL, Tsai MC, Qu K, et al. 2013. Targeted disruption of *Hotair* leads to homeotic transformation and gene derepression. *Cell Rep*. 5:3–12
188. Kaneko S, Son J, Bonasio R, Shen SS, Reinberg D. 2014. Nascent RNA interaction keeps PRC2 activity poised and in check. *Genes Dev*. 28:1983–88
189. Kaneko S, Son J, Shen SS, Reinberg D, Bonasio R. 2013. PRC2 binds active promoters and contacts nascent RNAs in embryonic stem cells. *Nat. Struct. Mol. Biol.* 20:1258–64
190. Wang X, Paucek RD, Gooding AR, Brown ZZ, Ge EJ, et al. 2017. Molecular analysis of PRC2 recruitment to DNA in chromatin and its inhibition by RNA. *Nat. Struct. Mol. Biol.* 24:1028–38
191. Beltran M, Yates CM, Skalska L, Dawson M, Reis FP, et al. 2016. The interaction of PRC2 with RNA or chromatin is mutually antagonistic. *Genome Res*. 26:896–907