Genomic Imprinting and Epigenetic Control of Development

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SUMMARY

Epigenetic mechanisms are extensively utilized during mammalian development. Specific patterns of gene expression are established during cell fate decisions, maintained as differentiation progresses, and often augmented as more specialized cell types are required. Much of what is known about these mechanisms comes from the study of two distinct epigenetic phenomena: genomic imprinting and X-chromosome inactivation. In the case of genomic imprinting, alleles are expressed in a parent-of-origin-dependent manner, whereas X-chromosome inactivation in females requires that only one X chromosome is active in each somatic nucleus. As model systems for epigenetic regulation, genomic imprinting and X-chromosome inactivation have identified and elucidated the numerous regulatory mechanisms that function throughout the genome during development.

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1 INTRODUCTION

A striking example of epigenetic mechanisms in mammalian development comes from landmark studies on the developmental potential of uniparental mouse embryos (Mann and Lovell-Badge 1984; McGrath and Solter 1984; Surani et al. 1984). Zygotes reconstituted with either two maternal or two paternal pronuclei (gynogenotes and androgenotes, respectively) fail to develop beyond midgestation, suggesting that additional layers of information beyond DNA sequence alone are transmitted to the offspring. The functional inequivalence of parental genomes can be attributed to a limited subset of genes whose allele-specific transcriptional activity is dependent on the parent of origin. These genes undergo a process termed genomic imprinting, in which differential parent-specific epigenetic modifications are established during gametogenesis, transmitted to the zygote, and stably maintained throughout somatic development and in tissues of the adult offspring. Conversely, parental genomic imprints are erased in gametes of the offspring, where new epigenetic marks are established, dependent on the sex of the offspring. As a paradigm for epigenetic gene regulation, studies of imprinted loci have been instrumental in identifying the mechanisms and gene products required for establishing, maintaining, interpreting, and erasing epigenetic information. We now know that the factors involved in the regulation of imprinted loci function throughout the genome, and have essential roles in normal development. Moreover, the misregulation of these mechanisms has been implicated in numerous human diseases, further underscoring their importance in mammalian biology.

2 PARADIGMS OF EPIGENETIC REGULATION

2.1 Characteristics of Autosomal Imprinted Genes

Nearly 100 genes displaying imprinted expression patterns in the majority of somatic tissues have been identified in the mouse, most of which also show imprinted expression in humans. In addition, many more genes are imprinted in a tissue-specific manner, for example in certain cell types of the placenta (Hudson et al. 2010, 2011) and brain (Gregg et al. 2010a,b). Studies of imprinted genes have identified several common characteristics. First, most imprinted genes are found in clusters, often spanning hundreds of kilobases. Second, almost all imprinted loci contain at least one non-coding RNA transcript. Third, the alleles of imprinted genes are differentially associated with covalent modifications to DNA and histones, and display significant differences in chromatin structure. Of these, genomic regions of differential DNA methylation, called differentially methylated domains (DMDs) or differentially methylated regions

(DMRs), have been identified at almost all imprinted gene clusters and appear to play a central role in defining parental identity. Finally, *cis*-acting sequences, termed imprinting control regions (ICRs), have been identified and are required to both establish parental identity of imprinted loci and maintain differential epigenetic patterns throughout development. Many ICRs have been experimentally defined and often overlap with DMRs. Despite these similarities, imprinted loci use a diverse number of mechanisms to establish and maintain their characteristic expression patterns.

2.2 X-Chromosome Inactivation: A Model for the Establishment of Epigenetic Information

In contrast to imprinted gene regulation, which occurs at the level of a few genes within an imprinted locus, X-chromosome inactivation (XCI) acts on an entire chromosome. Mammalian female cells contain two X chromosomes and must therefore inherently solve a problem of X-linked gene dosage. As such, XCI serves to balance sex-chromosome-linked gene dosage in female somatic cells, by rendering one of the two X chromosomes largely transcriptionally silent (Wutz and Gribnau 2007; Payer and Lee 2008).

Two different forms of XCI have been observed: imprinted XCI and random XCI. Imprinted XCI is characterized by the invariable inactivation of the paternally derived X chromosome (Xp), and occurs in the extraembryonic lineages of mice, as well as in all tissues of marsupials. In contrast, random XCI is found in the embryonic lineages of mice and all primate somatic cells (both extraembryonic and embryonic), and is characterized by the random inactivation of either the maternal X chromosome (Xm) or the Xp. Unlike imprinted XCI and autosomal imprinting, in which parent-specific epigenetic information is established in the germline, random XCI is accomplished by establishing contrasting epigenetic information on each homologous chromosome within the same somatic nucleus. Analogous to ICRs, an experimentally delimited region on the X chromosome, the X-inactivation center (XIC), is required for both imprinted and random XCI to define the epigenetic state of the X chromosome in *cis* (Brown 1991; Brown et al. 1991b). As such, XCI has also served as a vital system in understanding how epigenetic states are established during somatic development.

3 EPIGENETIC MECHANISMS OF GENE REGULATION

3.1 *H19/Igf2*: Imprinted Expression through Chromatin Structure

The best-known example of imprinted expression is the *H19/Igf2* locus. These genes are closely linked, yet oppositely

expressed: The *H19* noncoding RNA is expressed exclusively from the maternal allele, whereas insulin-like growth factor 2 (*Igf2*) is expressed from the paternal allele in most tissues (Fig. 1). Misregulation of *Igf2* has profound phenotypic consequences for development. Deletion of the paternal *Igf2* allele results in smaller mice (DeChiara et al. 1990), whereas biallelic expression results in larger animals (Leighton et al. 1995; Thorvaldsen et al. 1998; Constancia et al. 2000). The regulation of *IGF2* in humans is of clinical interest: A genetic disease characterized by somatic overgrowth, Beckwith–Weidemann syndrome, has been associated with biallelic *IGF2* expression (Weksberg et al. 2003).

In between the two genes, upstream of the *H19* promoter, lies a CpG-rich DMD. The maternal DMD allele contains very low levels of DNA methylation, whereas on the paternal allele the DMD is hypermethylated (Tremblay et al. 1995). These contrasting methylation patterns are established in the germline of the parents and maintained throughout the development of the soma in the embryo and adult. Engineered mutations have demonstrated the

DMD to be required for imprinting of both *H19* and *Igf2* (Thorvaldsen et al. 1998). Inheritance of an allele with a DMD deletion from either parent results in transcription of both *H19* and *Igf2* from the mutant allele, thus defining the DMD as the ICR for this locus.

The DMD mediates the correct imprinted expression patterns by controlling the access of the *H19* and *Igf2* promoters to enhancers located downstream of *H19* (Fig. 1). On the maternal allele, the DMD functions as an enhancer-blocking element: a sequence that can interfere with promoter–enhancer communication. This activity is attributable to the presence of binding sites within the DMD for the vertebrate insulator protein, CTCF. As inferred by chromatin conformation capture assays, the CTCF-bound maternal DMD causes the locus to adopt a three-dimensional conformation, which occludes the activation of *Igf2* by the enhancers, leading to the activation of *H19* expression (Engel et al. 2006; Kurukuti et al. 2006; Zhang et al. 2011). Importantly, association of CTCF with its binding sites is inhibited by the presence of DNA methylation. Therefore, because CTCF cannot associate with the paternally hypermethylated

Figure 1. The *H19/Igf2* imprinted locus. (A) *H19* and *Igf2* are separated by approximately 80 kb on the distal end of mouse chromosome 7. Downstream of *H19* lie shared endodermal enhancers. (B) On the paternal allele, the DMD is hypermethylated and the enhancers activate *Igf2*. (C) On the maternal allele, CTCF binds the hypomethylated sites within the DMD. The presence of CTCF forms a structure sequestering the *Igf2* promoter and allowing *H19* to be activated by the enhancers.

DMD, the enhancer-blocking structure cannot be formed, leading to *Igf2* activation by the enhancers. Collectively, these findings demonstrate that the DMD acts as a methylation-sensitive switch to guide allele-specific expression through chromatin conformation.

The *H19/Igf2* locus has been an important system for appreciating the role of chromatin structural components in gene regulation during development. Garnering the most interest is CTCF. Genome-wide analysis has revealed thousands of CTCF-binding sites in mammalian genomes (Kim et al. 2007), and other studies have implicated CTCF-driven chromatin structure in diverse developmental processes, including random XCI (Chao et al. 2002; Navarro et al. 2006; Donohoe et al. 2007) and somatic recombination in the immune system (Guo et al. 2011). Intriguingly, DNA-bound CTCF appears to be regulated through additional means. Recently, components of the cohesin complex have been found to occupy many of the same sites as CTCF during interphase in both human and mouse genomes (Parelho et al. 2008; Stedman et al. 2008; Wendt et al. 2008).

At the *H19/Igf2* locus, depletion of cohesin results in loss of insulator activity of the DMD without affecting CTCF binding. Thus, the insulator activity of CTCF is regulated not only by DNA methylation, but by assembly into larger protein complexes. Interactions between promoters and distant regulatory elements, facilitated by cohesin, have been found to occur throughout the genome in a variety of cell types (Cuylen and Haering 2010; Kagey et al. 2010). In many cases the formation of these loops is directly linked to gene expression patterns, suggesting a broad, structural mechanism for transcriptional regulation. It is therefore not surprising that mutation of CTCF and cohesin, as well as misregulation of nuclear architecture, have been observed in cancers and number of human diseases (Zaidi et al. 2007). For example, mutation of a structural component of the nuclear membrane, *LMNA*, is associated with numerous diseases including a form of progeria (premature aging). Strikingly, nuclei from these patients show drastic changes in heterochromatic histone modifications and aberrant nuclear structure (Scaffidi and Misteli 2006). Together, these findings illustrate the importance of chromatin structure in development and human disease.

3.2 Noncoding RNAs: A Common Mechanism for Imprinted Regulation and Dosage Compensation

At first believed to be a hallmark of imprinted regions, noncoding RNAs (ncRNAs) have been identified throughout mammalian genomes (Kapranov et al. 2007a,b; Guttman

et al. 2009; Khalil et al. 2009; Latos and Barlow 2009; Santoro and Barlow 2011). Conserved transcription units among disparate species such as zebrafish and humans, along with functional analysis in mice, suggest that ncRNA species may contribute significantly to gene regulation. Almost all known imprinted loci contain at least one ncRNA, and these transcripts are often closely linked with regulation of imprinted expression in *cis*. Perhaps the best-studied of such molecules are the imprinted *Airn* and *Kcnq1ot1* ncRNAs, and the *Xist* ncRNA, which is required for XCI in mammals.

Spanning ~500 kb on mouse chromosome 17, the imprinted *Igf2r* locus contains several imprinted protein-coding genes and the *Airn* ncRNA (Fig. 2). Similarly, the ~900-kb imprinted *Kcnq1* locus on mouse chromosome 7 encompasses 10 imprinted genes and the *Kcnq1ot1* ncRNA (Fig. 2). Hypomethylated DMRs on the paternal chromosomes drive monoallelic paternal expression of both *Airn* and *Kcnq1ot1*. Although the protein-coding genes within these clusters are functionally unrelated, these large imprinted domains share a number of regulatory features. At both loci the DMRs lie within introns of protein-coding genes and serve as promoters for ncRNAs that are transcribed in an antisense orientation to their protein-coding counterparts. Transcription of these ncRNAs occurs exclusively from the hypomethylated paternal DMR (Fig. 2B); ncRNA transcription is suppressed from maternally hypermethylated DMRs (Fig. 2A). Additionally, the ncRNAs are responsible for silencing an additional set of genes in the placenta (Fig. 2C).

Paternal inheritance of DMR deletions at either locus results in the absence of ncRNA expression and is accompanied by reactivation of the normally silent alleles in the region (Wutz et al. 1997; Fitzpatrick et al. 2002; Mancini-DiNardo et al. 2003). Similar effects on imprinted expression are observed when the paternal transcript of either ncRNA is terminated prematurely; however, DMRs remain hypomethylated (Sleutels and Barlow 2002; Mancini-DiNardo et al. 2006). Therefore, the DMRs function as ICRs and reflect parental identity in levels of DNA methylation, whereas the ncRNAs are required for silencing of imprinted genes in *cis*.

Perhaps the best-known model of ncRNA-mediated gene silencing is demonstrated by XCI in female mammals. In eutherians, XCI is in part regulated through a ncRNA, *Xist*, that coats the silent X chromosome (Xi) in *cis* (Fig. 3) (Borsani et al. 1991; Brockdorff et al. 1991; Brown 1991; Brown et al. 1991a; Clemson et al. 1996). The *Xist* gene is indispensable for both imprinted and random XCI: X chromosomes with deletions of the *Xist* gene are never inactivated (Kay et al. 1994; Penny et al. 1996; Marahrens et al. 1997). In addition, multicopy *Xist* transgenes located on

Figure 2. The *Igf2r* and *Kcnq1* imprinted loci. (A) On the maternal alleles, hypermethylation of the DMR silences ncRNA expression, resulting in locus-wide transcription. (B) Conversely, the repressive ncRNAs are transcribed from paternally hypomethylated DMRs. In several instances, these ncRNAs have been shown to associate with the chromatin at the promoter of the repressed alleles. (C) Both loci contain a number of genes that are only repressed by the ncRNA in a subset of extraembryonic lineages. Positions of genes, DMRs, and ncRNA transcripts are relative and not to scale.

autosomes are sufficient to locally silence adjacent genes (Lee and Jaenisch 1997). What makes XCI a particularly fascinating model for epigenetic regulation is the scope of silencing. Induction of *Xist* on the future Xi results in heterochromatinization throughout the entire chromosome.

In the mouse, inheritance of a paternally derived deletion of *Xist* leads to failed imprinted XCI in the extraembryonic tissues of female embryos (Marahrens et al. 1997). Although unknown, the imprint required for imprinted XCI is established during oocyte maturation (Tada et al. 2000). Failure of imprinted XCI leads to two active X chromosomes in the extraembryonic lineages, as the imprint on the X_m prevents it from being silenced (Tada et al. 2000). Although these embryos implant, the lack of dosage compensation is incompatible with proper placental development, leading to early embryonic lethality. Conversely, maternally inherited deletions of *Xist* are compatible with normal embryonic development, as the X_p normally undergoes imprinted XCI in female extraembryonic cells and undergoes biased random XCI in the embryonic tissues. In this latter case, the X_m is always the X_a . Female embryos

completely lacking *Xist* function are not viable and die before implantation. Males do not require *Xist* function, as dosage compensation is unnecessary.

Thus *Xist*, as well as *Airn* and *Kcnq1ot1*, has provided a robust system for identifying and understanding large-scale ncRNA regulatory mechanisms that are required for normal embryonic development.

3.3 Gene Silencing through Transcriptional Interference

Functional characterization of imprinted ncRNAs has suggested that they may involve numerous mechanisms to mediate transcriptional silencing of genes within their domains. The phenomenon of transcriptional interference occurs where sense–antisense gene pairs overlap at their transcriptional start sites. Antisense transcription of one gene by RNA polymerase II (Pol II) through the promoter of the other may displace required transcription factors at that promoter. Additionally, elongating RNA Pol II on one strand is known to promote changes in local histone

Figure 3. *Xist* induction results in chromosome-wide changes in chromatin and gene expression. After one X chromosome is chosen for inactivation in both embryos and differentiating ES cells, the *Xist* transcript can be seen coating the chromosome in *cis*. This event coincides with exclusion of RNA Pol II. Shortly thereafter, histone-modifying complexes such as polycomb repressive complex 1 and 2 (PRC1, PRC2) become enriched on the inactive X, along with the heterochromatic histone modifications they catalyze. As differentiation proceeds, the histone variant macroH2A becomes associated with the inactive X, and later CpG islands become methylated.

modifications and chromatin structure, possibly precluding antisense Pol II transcriptional initiation events (Lee and Shilatifard 2007). Both *Airn* and *Kcnq1ot1* have transcriptional start sites within introns of protein-coding genes (*Igf2r* and *Kcnq1*, respectively) and are transcribed in the antisense direction. The premature transcriptional termination of *Airn* ncRNA before the *Igf2r* promoter results in paternal *Igf2r* expression (Sleutels et al. 2002), suggesting that *Airn* functions, at least partially, through transcriptional interference (Shearwin et al. 2005). However, *Airn* and *Kcnq1ot1* transcription traverses only a subset of genes, which they repress. Therefore, transcriptional interference can only explain the repression of *Igf2r*, implying additional mechanisms of ncRNA-based transcriptional repression for neighboring genes.

3.4 ncRNA-Mediated Formation of Repressive Nuclear Compartments

ncRNA-dependent silencing at the *Igf2r* and *Kcnq1* imprinted loci extends hundreds of kilobases away from the

site of transcription. In the case of XCI, *Xist* transcripts influence hundreds of megabases, making XCI a particularly attractive model for epigenetic phenomena. Although data suggest that *Airn* and *Kcnq1ot1* may mediate the formation of a localized, transcriptionally silent nuclear compartment (Terranova et al. 2008; Redrup et al. 2009), *Xist* function during XCI has provided the best evidence of ncRNA-mediated formation of silent nuclear domains.

A critical facet of XCI is the localization of the *Xist* RNA. Using RNA fluorescence in situ hybridization (FISH), a spliced *Xist* transcript can be seen coating the entire Xi. At the onset of XCI, the future Xi up-regulates *Xist* expression, facilitating coating of the Xi by *Xist* transcripts. Once *Xist* coating is established, the chromatin of the Xi is dramatically altered (Fig. 3). The Xi becomes increasingly enriched for histone modifications similar to those found in transcriptionally silent heterochromatic regions of the genome. Like *Xist* coating, the enrichment of these modifications and other heterochromatic proteins can be readily visualized with specific antibodies. Together, these data suggest that formation of large heterochromatin domains

by ncRNAs may be a core mechanism of large-scale epigenetic silencing.

In addition to *Xist* coating, the onset of XCI is also characterized by the cytologically visible exclusion of RNA Pol II and associated general transcription factors from the Xi chromosome territory (Fig. 3) (Chaumeil et al. 2006). Following the subsequent depletion of active chromatin modifications such as di- and trimethylated lysine 4 of histone H3 (H3K4me2/3), repressive histone modifications begin to accumulate on the Xi. The first such modification enriched on the Xi is the trimethylation of lysine 27 of histone H3 (H3K27me3), catalyzed by the Ezh2 histone methyltransferase of polycomb repressive complex 2 (PRC2) (Plath et al. 2003; Okamoto et al. 2004). *Xist* is believed to directly recruit the PRC2 complex to the Xi via sequences located in the 5' end of the *Xist* RNA, termed the A repeat (Zhao et al. 2008). In both the early embryo and differentiating embryonic stem (ES) cells, chromosomes lacking *Xist* fail to accumulate H3K27me3 and are incapable of undergoing XCI. As XCI proceeds, the Xi accumulates a repertoire of histone modifications associated with silent chromatin, including H3K9me2 (dimethylated lysine 9, histone H3), H3K9me3, H4K20me1 (monomethylated lysine 4, histone H4), and H2A119Ub (ubiquitinated histone H2A) (Fig. 3).

The Xi eventually becomes enriched for the histone variant macroH2A, which is believed to be a stabilizing mechanism for the silent chromatin of the Xi (Costanzi and Pehrson 1998). At this point, *Xist* is no longer required for XCI maintenance. Finally, de novo DNA methylation of CpG islands on the Xi provide a mechanism for long-term, X-linked gene silencing. In agreement with a critical role in this late phase, mouse embryos deficient in Dnmt1 fail to maintain proper XCI patterns (Sado et al. 2000). Collectively, these observations demonstrate an orchestrated, precise chain of events that initiates with *Xist* RNA coating of the future Xi, and involves multiple epigenetic modifications and likely numerous *trans*-acting epigenetic regulators. Ultimately, these events produce a chromosome-wide heterochromatic domain refractory to efficient Pol II transcription.

4 TISSUE-SPECIFIC IMPRINTING

In addition to genes that are imprinted in all tissues, many more show tissue-specific imprinting, especially in the mouse placenta and brain (Gregg et al. 2010a,b; Hudson et al. 2010, 2011). Engineered mutations that cause both loss of activity (through targeted deletions) and overexpression (through loss of imprinting) have revealed significant effects on placental function and development, as well as on postnatal and adult behavior (Davies et al. 2007; Frost

and Moore 2010). This is particularly evident at the *Kcnq1* locus, where many of the genes within this region contribute to placental development. For example, *Ascl2* is required for the proliferation of spongiotrophoblast progenitor cells and *Cdkn1c* for the development of trophoblast giant cells (Guillemot et al. 1995; Tanaka et al. 1997; Caspary et al. 1999; Takahashi et al. 2000). Moreover, deletion of the maternal allele of *Phlda2* results in placental overgrowth (Frank et al. 2002). Conversely, reactivation of the paternal allele caused by genetic disruption of *Kcnq1ot1* expression results in the development of a smaller placenta (Salas et al. 2004).

As in the embryo, *Kcnq1ot1* is responsible for silencing of the placental genes; however, the locus has been observed to undergo a “compaction” specific to extraembryonic lineages (Terranova et al. 2008; Redrup et al. 2009). Reminiscent of the mechanism of *Xist*, the *Kcnq1ot1* ncRNA forms a repressive nuclear compartment, devoid of Pol II and other marks of active chromatin (Terranova et al. 2008). Moreover, loss-of-function studies in mice have demonstrated that the PRC1 and PRC2 histone modification complexes, as well as the G9A histone methyltransferase (responsible for catalyzing the H3K9me2 modification), have essential roles in repressing placentally imprinted genes at several imprinted loci (Mager et al. 2003; Nagano et al. 2008; Wagschal et al. 2008).

It is not yet known how genes are designated for imprinting only in a subset of tissues. In the case of the *Kcnq1* locus, where placentally and ubiquitously imprinted genes are in close proximity to one another, structural changes brought about by the *Kcnq1ot1* ncRNA may recruit the placental genes into a silent compartment. Consistent with this hypothesis, the locus has been observed to undergo a compaction specific to extraembryonic lineages (Terranova et al. 2008; Redrup et al. 2009).

However, although most imprinted loci are associated with a ncRNA, function of the ncRNA is not necessarily conserved, implying additional mechanisms to designate imprinted alleles for tissue-specific regulation. The differential use of alternative promoters appears to be a common theme. For example, the *Grb10* gene is maternally expressed in most tissues, yet predominantly paternal in the brain (Arnaud et al. 2003; Hikichi et al. 2003). This is accomplished through the use of a hypomethylated paternal DMR that is capable of serving as a promoter in this state (Arnaud et al. 2003; Hikichi et al. 2003; Yamasaki-Ishizaki et al. 2007; Sanz et al. 2008). The activity of this promoter is restricted to neuronal tissues in part through the action of PRC2 (Sanz et al. 2008). Collectively, these examples from tissue-specific imprinting illustrate a number of ways that genes may be subject to epigenetic control during development. The ordered and timely recruitment of epigenetic

modifiers to promoters and other regulatory elements is the key to producing the correct patterns of expression during the developmental program.

5 ESTABLISHING EPIGENETIC STATES AT IMPRINTED LOCI: LESSONS FROM THE GERMLINE

During development, changes in gene expression bring about the differentiation of pluripotent cells into transient progenitor stages, and ultimately into specialized, terminally differentiated cells. Accompanying these transitions, specific gene expression patterns are established and maintained, then augmented as new developmental stimuli are received. Numerous epigenetic mechanisms participate in this process, including covalent histone modifications and DNA methylation. For example, apart from regulating imprinted genes, polycomb proteins are required for managing genome-wide expression patterns. Further, loss-of-function experiments have demonstrated that *Dnmt3b*, a member of the de novo DNA methyltransferase gene family, is required for development (Okano et al. 1999). Moreover, human immunodeficiency, centromere instability, and facial anomalies (ICF) syndrome is associated with mutations in *DNMT3B* (Bestor 2000). These observations illustrate the importance of establishing epigenetic information to development and human disease. Imprinted genes have been invaluable in elucidating how these patterns are generated at specific sites.

Differential DNA methylation has been observed at nearly all known imprinted gene clusters. These patterns are established in the gametes, and inherited by the embryo. The functional relevance of DNA methylation to genomic imprinting has been demonstrated by studies from mice with null mutations in the maintenance DNA methyltransferase, *Dnmt1*. In addition to significant developmental defects, *Dnmt1*-null embryos show loss of DNA methylation patterns and imprinted expression at most imprinted loci (Li et al. 1992, 1993). Therefore, the establishment and maintenance of this modification is central to imprinted gene regulation.

Unlike the maintenance of DNA methylation that occurs in the developing soma, de novo methylation of ICRs occurs in a sex- and locus-specific manner. Before these processes begin, DNA methylation patterns are erased during the development of primordial germ cells. These cells undergo genome-wide epigenetic changes relative to their somatic counterparts, such that by the time the sexually dimorphic stages of germ cell development begin, imprinted alleles contain equivalent, low levels of DNA methylation (Davis et al. 2000; Hajkova et al. 2002). De novo methylation of paternally methylated ICRs, such as the

H19 DMD, begins during the later stages of embryogenesis in the quiescent prospermatogonia and is complete by the entry into meiosis (Davis et al. 1999, 2000; Ueda et al. 2000). In contrast, de novo methylation of ICRs in the female germline does not begin until after birth (Lucifero et al. 2002). This occurs during the period of oocyte growth, when oocytes are arrested in the first prophase of meiosis. Notably, the kinetics of de novo methylation at DMRs differs among imprinted loci, indicating that locus-specific characteristics, and perhaps even diverse mechanisms, regulate this event.

5.1 The CTCF-DMD Interaction Is Essential for the Establishment and Maintenance of the Maternal Epigenotype

The *H19/Igf2* DMD is one of the few paternally hypermethylated ICRs; it remains free of significant levels of DNA methylation in the oocyte. Apart from other ICRs, the genome is undergoing large-scale reorganization and reprogramming of chromatin during oocyte growth, including de novo methylation of repetitive and nongenic sequences. Because of the apparent accessibility of similar sequences to de novo methyltransferases, a mechanism must exist to distinguish the *H19* DMD and protect it from becoming hypermethylated.

The simplest mechanism to prevent de novo methylation is through a protective mechanism whereby the binding of a factor to DNA effectively masks the underlying CpG residues from the methyltransferase machinery. At the *H19* DMD, CTCF serves this vital role. In oocytes depleted for CTCF, the *H19* DMD acquires an abnormally high level of DNA methylation (Fedoriw et al. 2004). As the expression of the de novo methyltransferases is not limited to gametogenesis, and can occur in somatic tissues of both the embryo and the adult, the protective role of CTCF in maintaining a hypomethylated maternal *H19* DMD extends beyond the female germline. Genetically engineered mutations in the DMD that alter or completely delete CTCF-binding sites, acquire DNA methylation during development (Schoenherr et al. 2003; Pant et al. 2004; Engel et al. 2006). These results demonstrate a role for CTCF in establishing and maintaining maternal identity of the *H19* DMD. Importantly, a mutually exclusive relationship for CTCF and DNA methylation has been shown for a number of nonimprinted loci, including human *C-MYC* (Gombert and Krumm 2009) and mouse *p16* loci (Witcher and Emerson 2009). Similarly, protective roles have been described for other DNA-binding factors, including Sp1 (Brandeis et al. 1994) and YY1 (Kim et al. 2009). Therefore, association of *trans*-factors with CpG-rich DNA sequences may be a common mechanism in

5.2 Targeting of DNA Methyltransferases to DMRs during Oogenesis

discrete methylation events required for the establishment of specific methylation patterns at imprinted loci.

A

Maternal

DMR

gDMR

Nes

Gnasxl

Gnas

B

Paternal

Nespas

gDMR

gDMR 1A

Active gene

Active ncRNA gene

Silent gene

Methylated CpG

Unmethylated CpG

DMR Differentially methylated region

gDMR Germline DMR

The diagram illustrates the imprinting of the *Gnas* locus on maternal and paternal alleles. Panel A shows the maternal allele with a DMR upstream of the gDMR, and the paternal allele with a DMR upstream of the gDMR. Panel B shows the maternal allele with a DMR upstream of the gDMR, and the paternal allele with a DMR upstream of the gDMR. The diagram includes a legend for CpG methylation, DMRs, and gene activity.

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not appropriately methylated during oogenesis, demonstrating a role for transcription of the *Nesp* gene in hypermethylation of the DMR.

These results implicate an RNA as a “guide molecule” for the establishment of DNA methylation, possibly by a direct interaction of the RNA with DNMT3 proteins and/or other epigenetic regulators. Intriguingly, interactions between ncRNAs and histone methyltransferase complexes have been detected in vivo (Nagano et al. 2008; Pandey et al. 2008; Zhao et al. 2010), suggesting that ncRNAs are capable of directing epigenetic modifications in mammalian nuclei. Alternatively, the epigenetic changes brought about by transcription itself may be responsible for producing a chromatin composition conducive to the action of the de novo DNA methyltransferases. A number of histone modification enzymes and demethylases are known to associate with elongating RNA Pol II (Fuchs et al. 2011), which may produce a pattern that would serve as an optimal chromatin template for the de novo DNA methylation. In this model, transcription, rather than a transcript, plays the pivotal role in the establishment of epigenetic information.

6 ESTABLISHING DIFFERENTIAL EPIGENETIC STATES IN THE SOMA: RANDOM XCI

In contrast to the reprogramming events in the germline that erase somatic imprinting patterns to establish parental identity on both alleles, asymmetric epigenetic states are established on each X chromosome within the same nucleus during early embryonic development in female mammals. In mice, initiation of random XCI is directly linked with exit from the pluripotent state. The factors known to be required for ES cell self-renewal, Oct4, Nanog, and Sox2, all act to repress *Xist* transcription (Navarro et al. 2008); therefore, ES cells contain two active X chromosomes. Upon their differentiation in vitro, the events of random XCI can be recapitulated, comparable to what has been observed in vivo (Chow and Heard 2009). As such, ES cells have served as a surrogate to the developing mouse epiblast, resulting in the rapid identification of genomic sequences required for *Xist* regulation.

Despite exhaustive genetic manipulations of the XIC, analysis of mutations of epigenetic modifying factors, as well as observations from human pathologies, the initiation of XCI remains enigmatic. Notably, gene silencing and *Xist* coating appear uncoupled during mammalian preimplantation development and during the erasure of imprinted XCI in the developing mouse epiblast. In mice, gene silencing during imprinted XCI precedes *Xist* coating, whereas in humans and rabbits, *Xist* coating occurs in the absence of gene silencing (Kalantry et al. 2009; Okamoto et al. 2011; Williams et al. 2011). However, *Xist* is an

essential, if not central, component to the XCI process (Penny et al. 1996). Thus, although additional events may participate in XCI during the earliest developmental stages, establishing monoallelic *Xist* expression during the onset of random XCI is a critical aspect in generating a heritable, chromosome-wide, transcriptionally inert state.

Work from female mice and ES cells has identified a complex network of ncRNAs, proteins, and cytological events that may participate to establish differential *Xist* expression. Among them, an antisense RNA to *Xist*, *Tsix*, has a central role in regulating *Xist* (Fig. 5A) (Lee et al. 1999). Like imprinted sense–antisense gene pairs, the expression of *Xist* and *Tsix* from a single allele is mutually exclusive. During the onset of XCI, *Tsix* expression becomes limited to a single allele on what will become the future active X chromosome (Xa) (Fig. 5B), whereas up-regulation of *Xist* on the homologous allele leads to a chromosome-wide repressive state (Xi) (Fig. 5C) (Lee and Lu 1999; Lee et al. 1999). *Tsix* transcription traverses the *Xist* promoter and results in the accumulation of repressive histone modifications (Sado et al. 2005). As is the case with *Airn*, truncation of *Tsix* transcription before the *Xist* promoter results in a mutant allele that up-regulates *Xist* expression before and during XCI. Importantly, this mutation cannot be rescued by expression of a *Tsix* gene repositioned such that it does not overlap the *Xist* promoter (Shibata and Lee 2003, 2004; Ohhata et al. 2008). Therefore, the repression of *Xist* by *Tsix* represents another example of transcription playing a central role in establishing an epigenetic state.

Despite the known role of *Tsix* in down-regulating *Xist* transcription, it is not yet clear how this asymmetric pattern of expression is established. Several lines of evidence suggest two separate phases of the initiation process, counting and choice, which have been difficult to experimentally separate and appear very closely interconnected. Counting ensures that only one X chromosome is active per diploid set of autosomes. In mice, *Xist* itself is not required for counting, as heterozygous deletions of *Xist* preferentially inactivate the wild-type chromosome, and thus must be able to sense the existence of two X chromosomes for XCI to occur (Marahrens et al. 1998; Gribnau et al. 2005).

Counting is followed by choice, wherein a difference must be established between homologous X chromosomes, which will eventually lead to differences in transcriptional activity. For choice to occur, a difference must be recognized between two homologs, yet in mouse embryos and ES cells, the X chromosomes are often genetically identical. Not surprisingly, heterozygous deletions of *Tsix* and *Xist* favor inactivation and activation of the mutant chromosome, respectively (Marahrens et al. 1997; Lee and Lu 1999). Apart from these engineered effects, choice is skewed by *cis*-elements within the XIC. Alleles of X-controlling

Figure 5. The X-inactivation center controls chromosome-wide silencing. (A) The antagonistic relationship between *Xist* and its antisense partner, *Tsix*, controls the inactivation of the entire X chromosome. During differentiation from a pluripotent state, female cells will choose one X chromosome for silencing. This mechanism occurs through *Xist* and *Tsix* regulation. (B) *Tsix* up-regulation represses *Xist* transcription, leading to an active X chromosome. (C) When *Xist* is up-regulated, either through down-regulation of *Tsix* or its genetic ablation, XCI occurs in *cis*.

element (*Xce*) differ between mouse strains, and affect the probability of inactivation in F₁ hybrid females. However, the mechanism behind choice remains largely unknown.

Although choice is traditionally believed to take place after counting, recent data suggest an alternative possibility. Transient, stochastic differences between the alleles *before* differentiation may dictate which X chromosome is chosen for *Xist* up-regulation (Fig. 6) (Mlynarczyk-Evans et al. 2006). This effect is manifest in differences in cytological appearance of replicated alleles. During S phase, regions that have undergone DNA replication appear as two juxtaposed DNA FISH signals (a doublet), whereas regions that have not yet replicated appear as a single FISH signal (a singlet). Despite coordinated replication timing of homologous alleles among X-linked loci in undifferentiated female ES cells, one allele appears as an expected doublet, whereas the other mysteriously appears as a singlet. This phenomenon is coordinated across this chromosome, except the XIC, which shows an opposite pattern. For example, on the X chromosome on which genes appear as singlets, the XIC will appear as a doublet. These patterns

do not appear fixed, as chromosomes oscillate between the two states in an undifferentiated population. Importantly, it is predictive of which allele will be inactivated after differentiation: The singlet-to-doublet ratio is skewed between X chromosomes carrying different *Xce* alleles, reflecting the probability of silencing dictated by the *Xce* locus. Therefore, it may be plastic states in chromatin structure that serve to differentiate between identical alleles.

Apart from imprinted genes, whose identity is set forth in the germlines, random monoallelic expression is pervasive in mammalian genomes (Gimelbrant et al. 2007). The selection of a single allele is important in numerous developmental processes in mammals. For example, in olfactory neurons only one allele of hundreds of olfactory receptor genes scattered in clusters throughout the genome is chosen for activity. Similar to that of X-linked loci before XCI, olfactory receptor loci display a singlet–doublet pattern preceding differentiation (Alexander et al. 2007). Although olfactory receptor gene clusters do not possess known regulatory ncRNAs like *Tsix* or *Xist*, transient differences in chromatin structure in undifferentiated cells may be a

Figure 6. Is the future epigenetic state predetermined? Most models of counting and choice presuppose that these processes occur only after differentiation begins. However, recent data suggest that X chromosomes—and indeed other monoallelically expressed loci—fluctuate between states that will determine their activity after differentiation. (A) In undifferentiated ES cells, X chromosomes switch between a state that will predispose them to the active (green) or inactive (red) fate. The pattern observed at the XIC is opposite that of the rest of the chromosome. (B) After differentiation, the outcome of choice would reflect the state of the chromosome before XCI began.

prevalent mechanism for establishing differential epigenetic modifications at genetically identical alleles during the course of development.

7 CONCLUSIONS

Genomic imprinting is perhaps the most striking display of epigenetic phenomenon in mammalian development. Importantly, it has provided a fruitful system for understanding how epigenetic states are established, interpreted, and maintained by the transcriptional machinery to produce very different outcomes on each genetically identical allele. XCI has also become a popular model for epigenetic gene regulation, especially for the distinct regulatory events that lead to chromosome-wide gene silencing. In contrast to how genomic imprints are established during gametogenesis, differential epigenetic states on each X homolog are established within the same nucleus. Each example of monoallelic gene expression has important ramifications not only for mammalian development, but for human

disease. For example, loss of imprinted expression at numerous loci is associated with human pathologies including birth defects and cancer. Therefore, understanding the mechanisms that initiate and maintain differential epigenetic states has broad implications that extend into the clinical setting.

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