Current view of the potential roles of proteins enriched on the inactive X chromosome

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X chromosome inactivation (X-inactivation) is triggered by X-linked noncoding *Xist* RNA, which is expressed asymmetrically from one of the two X chromosomes in females and coats it in *cis* to induce chromosome-wide silencing. *Xist* RNA is thought to play a role as a platform in recruiting proteins involved in gene silencing and heterochromatinization, which mediate serial changes in epigenetic modification of the chromatin. During the last two decades, many proteins have been shown to be enriched on the inactivated X chromosome in mouse and human. Although the biological significance of most of them for X-inactivation has not been fully established, extensive studies of these proteins should provide a better understanding of the molecular basis of how X-inactivation mediated by *Xist* RNA is regulated. Here, we review the potential roles of some of these proteins in the stepwise process of *Xist* RNA-mediated chromosome silencing.

Key words: epigenetic regulation, gene silencing, heterochromatin, X chromosome, *Xist* RNA

INTRODUCTION

During early development in female mammals, one of the two X chromosomes becomes transcriptionally silenced, so that the dosage difference of X-linked genes between the sexes is compensated for (Lyon, 1961). This X-inactivation is a phenomenon which, in addition to the t-haplotype (Sugimoto, 2014), has attracted the attention of mouse geneticists for many years. The X chromosome is also intriguing in the context of hybrid male sterility (Oka and Shiroishi, 2014). In the process of X-inactivation, an X-linked noncoding RNA, *Xist*, is asymmetrically upregulated on either X chromosome as cells differentiate, and subsequently associates in *cis* with the X chromosome from which it is transcribed to induce chromosome silencing. Although the molecular mechanisms are still largely obscure, *Xist* RNA is thought to recruit proteins that are involved in gene silencing and heterochromatinization. Available evidence also suggests that the association of *Xist* RNA with the inactive X chromosome (Xi) is mediated not by hybridization with DNA to form a heteroduplex but by an interaction with chromatin proteins (Clemson et al., 1996). Given that Xi is often found at the nuclear periphery, and that *Xist* RNA remains associated with the nuclear matrix or scaffold

even after chromatin proteins and genomic DNA are removed, it is likely that *Xist* RNA serves as a bridge between chromatin and other structural components. Although many proteins such as chromatin proteins and epigenetic regulators have been shown to localize to Xi (reviewed in De La Fuente et al., 2011), it is unclear in most cases what impact *Xist* RNA has on their localization to Xi. Indeed, the biological significance of most of these proteins for X-inactivation is not fully understood. Detailed studies of these proteins, however, should help us to understand the molecular basis of how *Xist* RNA-mediated X chromosome silencing is initiated, established and maintained, and it is therefore timely to summarize our current knowledge about proteins that are enriched on Xi. Here, we provide an overview of several of these proteins (Fig. 1) in terms of their potential role in the stepwise process of *Xist* RNA-mediated gene silencing.

macroH2A AND Parp1

macroH2A was the first protein found to localize to Xi (Costanzi and Pehrson, 1998). It is a variant of histone H2A with a large C-terminal non-histone domain known as the macro domain, and is three times the size of canonical histone H2A (Pehrson and Fried, 1992). There are three isoforms of macroH2A: macroH2A1.1 and 1.2 are produced from a single gene, *H2afy*, by alternative splicing (Pehrson et al., 1997; Rasmussen et al., 1999), while

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Fig. 1. Summary of the proteins highlighted in this review among those which are enriched on the inactive X chromosome, and their potential roles in *Xist* RNA-mediated silencing.

macroH2A2 is encoded by *H2afy2*. These are enriched on Xi in a chromosome-wide fashion and form a macro chromatin body (Chadwick and Willard, 2001; Costanzi and Pehrson, 2001; Mietton et al., 2009). Their recruitment occurs at the morula stage during mouse development (Costanzi et al., 2000), and at a relatively late phase in the process of X-inactivation during embryonic stem (ES) cell differentiation (Mermoud et al., 1999; Pullirsch et al., 2010). Conditional depletion of *Xist* RNA in female mouse embryonic fibroblasts (MEFs), while not compromising the X-inactivation state, results in a loss of macroH2A1.2 from Xi (Csankovszki et al., 1999; Wutz et al., 2002). *Xist* expression from an autosomal transgene, which apparently induces silencing of neighboring genes at the integration site, is accompanied by the enrichment of macroH2A1 in differentiating ES cells (Rasmussen et al., 2001). On the other hand, mutant *Xist* RNA is still capable of recruiting macroH2A1 although it is defective in silencing because it lacks the A-repeat, one of the conserved repeats present in the 5' region of the RNA and essential for *Xist*'s function in silencing (Wutz et al., 2002). These studies demonstrate that although the deposition of macroH2A on Xi depends on the presence of *Xist* RNA, it is not sufficient for either triggering X-inactivation or maintaining the inactivated state. However, depletion of macroH2A in combination with treatment with inhibitors of DNA methylation and histone deacetylases resulted in reactivation of an X-linked GFP transgene in a portion of MEFs, suggesting that macroH2A, DNA methylation and histone hypoacetylation act synergistically in the maintenance of the X-inactivation state (Hernández-Muñoz et al., 2005).

Given the specific localization of macroH2A not only to Xi but also to the imprinting control regions of the repressed alleles, macroH2A, especially the macro

domain, is likely to be involved in gene silencing. It has been shown that the macro domain mediates an interaction between macroH2A1.1 and poly(ADP-ribose) polymerase 1 (PARP1), which also localizes to Xi (Nusinow et al., 2007; De La Fuente et al., 2011). PARP1 belongs to a group of nucleosome binding proteins that affect chromatin structure, and, when catalytically inactive, it inhibits transcription by binding to chromatin. macroH2A may therefore act as a transcriptional silencer by associating with PARP1 to repress its enzymatic activity through the macro domain (Ouararhni et al., 2006; Nusinow et al., 2007). However, targeted disruption of *H2afy* and *Parp1* in mice revealed that mutants homozygous for either gene are viable and fertile, demonstrating their nonessential roles in chromatin regulation during normal development (Wang et al., 1995; Changolkar et al., 2007). In addition, female ES cells depleted of both macroH2A1 and macroH2A2 are capable of undergoing X-inactivation upon differentiation (Tanasijevic and Rasmussen, 2011). Furthermore, although the details have not yet been provided, it has been reported that *H2afy* and *H2afy2* double knockout mice are viable and fertile (Gaspar-Maia et al., 2013). We have also found that female mice deficient for both *H2afy* and *H2afy2* are viable and fertile (unpublished observations). It is thus likely that macroH2As are dispensable for normal development as well as for the initiation and establishment of X-inactivation. A recent study, however, demonstrated that macroH2A acts as a barrier to nuclear reprogramming and that its depletion facilitates reactivation of Xi in somatic cells (Pasque et al., 2011), suggesting that macroH2As serve as an epigenetic lock against reversion of the chromatin state from the differentiated to the pluripotent cell type.

SAF-A/hnRNP U

Xist RNA exerts its effect on gene silencing by coating the chromosome from which it originates. The mechanism by which *Xist* RNA attaches to the X chromosome in *cis*, however, is still largely obscure. It has been reported that *Xist* RNA remains associated with the nuclear matrix after removal of chromatin by DNase digestion and salt extraction, suggesting that it constitutes part of a non-chromatin nuclear structure (Clemson et al., 1996). Interestingly, scaffold attachment factor A (SAF-A), also known as heterogeneous nuclear ribonucleoprotein U (hnRNP U), has been shown to localize to Xi (Helbig and Fackelmayer, 2003; Fackelmayer, 2005). SAF-A/hnRNP U is a prominent component of the nuclear matrix or scaffold and has been suggested to be involved in chromatin organization, transcriptional regulation and RNA processing. It has two functional domains, a SAF-box near the N terminus (Kipp et al., 2000) and an RGG-box near the C terminus (Kiledjian and Dreyfuss, 1992), and an Spla and Ryanodine receptor (SPRY) domain of unknown function (Ponting et al., 1997). The SAF-box and RGG-box mediate binding to AT-rich scaffold or matrix attachment regions (S/MARs) and to RNA, respectively. As is the case for *Xist* RNA, SAF-A/hnRNP U stably associates with the territory of Xi through the RGG-box even after chromatin is removed (Helbig and Fackelmayer, 2003). Given these molecular characteristics, this protein is a plausible candidate anchor point for *Xist* RNA on Xi. In fact, siRNA-mediated knockdown of SAF-A/hnRNP U results in the dissociation of *Xist* RNA from Xi (Hasegawa et al., 2010). Furthermore, the SAF-box and RGG-box are both essential for tethering *Xist* RNA to Xi. A recent computational analysis based on physicochemical properties of nucleic acid and amino acid side chains has predicted with high confidence that the SAF-box binds to the A-repeat region (Agostini et al., 2013). It has been shown, however, that a silencing-defective *Xist* RNA lacking the A-repeat region is still capable of recruiting SAF-A/hnRNP U as well as macroH2A and Polycomb group proteins (Pullirsch et al., 2010), suggesting that deposition of these proteins is insufficient for inducing X-inactivation. Furthermore, given that SAF-A/hnRNP U apparently begins to associate with Xi at the time of the transition to the maintenance phase of X-inactivation (Pullirsch et al., 2010), the recruitment of *Xist* RNA to Xi should precede that of SAF-A/hnRNP U. This appears to contradict the idea that SAF-A/hnRNP U plays a role in anchoring *Xist* RNA on the X chromosome to induce X-inactivation. Depletion of SAF-A/hnRNP U in differentiating female ES cells, however, has been shown to compromise the initiation of X-inactivation (Hasegawa et al., 2010). Although hypomorphic mutants for SAF-A/hnRNP U, which die at around embryonic day (E) 9.5, have been described

(Roshon and Ruley, 2005), it is unclear whether or not there are any sex-specific differences in the time at which developmental defects become apparent. It will be important to study the effect of functional loss of SAF-A/hnRNP U using null mutant mice.

SATB1 AND SATB2

Special AT-rich sequence binding protein 1 (SATB1) is another plausible factor that may participate in the inactive structure of Xi. SATB1 is a transcription factor that is predominantly expressed in thymocytes and binds to a core unwinding region within the S/MARs (Kohwi-Shigematsu and Kohwi, 1990). It contains a homeodomain at the C terminus and two Cut domains containing Cut-like repeats in the center as potential DNA binding domains (Nakagomi et al., 1994; Dickinson et al., 1997). SATB1 displays a cage-like distribution surrounding heterochromatin in thymocyte nuclei and this network regulates the expression of target genes through higher-order packaging of chromatin structure (Cai et al., 2003).

The ability of *Xist* RNA to induce gene silencing is likely to be restricted to a certain window during differentiation (Wutz and Jaenisch, 2000). The induction of *Xist* RNA coating can cause gene silencing in undifferentiated ES cells but not in differentiated cells such as MEFs. However, the intriguing finding that *Xist* RNA is still capable of inducing gene silencing in pre-B cells and pre-T cells (Savarese et al., 2006) suggests that not only embryonic cells but also pre-B and pre-T cells share a primary initiation factor(s) that triggers *Xist* RNA-mediated gene silencing (Agrelo et al., 2009). SATB1 and its paralog, SATB2, were found to be expressed in ES cells as well as pre-B and pre-T cells and downregulated in differentiating ES cells (Savarese et al., 2009). Furthermore, ectopic expression of either SATB1 or SATB2 in MEFs could induce gene silencing on the chromosome coated with *Xist* RNA, suggesting these proteins as the presumptive initiation factors (Agrelo et al., 2009). Notably, however, although mutants doubly deficient for SATB1 and SATB2 are embryonic lethal, MEFs that can be derived from double-mutant female fetuses contain a typical Xi, indistinguishable from that in wild-type female MEFs (Alvarez et al., 2000; Nechanitzky et al., 2012). This result suggests that SATB1 and SATB2 are dispensable for the initiation of gene silencing on Xi in mice. Although SATB1 and SATB2 are attractive factors in that they confer on differentiated cells the ability to respond to *Xist* RNA and initiate X-inactivation, further studies are required.

POLYCOMB REPRESSIVE COMPLEXES 1 AND 2

(1) PRC2 Polycomb group (PcG) proteins play an important role in developmental gene regulation and cell

fate commitment. Trimethylation at lysine 27 of histone H3 (H3K27me₃), one of the histone modifications that effects gene repression, is mediated by polycomb repressive complex 2 (PRC2), which consists of four core PcG protein components, Ezh2, Eed, Suz12 and RbAp46/48, in combination with accessory factors such as Jarid2 and PCL2 (MTF2). Although Ezh2 is a catalytic subunit of canonical PRC2, its paralog Ezh1, present in noncanonical PRC2, is also capable of catalyzing H3K27me₃ and is responsible for residual H3K27me₃ in cells lacking Ezh2 (Shen et al., 2008). Eed and Suz12 seem to be indispensable components for the activity of either canonical or noncanonical PRC2.

Enrichment of H3K27me₃ is one of the earliest epigenetic changes that happen to the X chromosome undergoing inactivation following the accumulation of *Xist* RNA on it. Eed was the first protein whose mutation was shown to cause a phenotypic difference in embryonic development between the sexes, attributable to a defect in X-inactivation (Wang et al., 2001). In the trophoblast of female embryos deficient for Eed (*Eed*^{-/-}), where X-inactivation is normally imprinted, the inactivated paternal X chromosome becomes progressively reactivated during postimplantation development due to a failure in the maintenance of the inactive state. Although the deficiency of Eed results in embryonic lethality in both sexes, it makes the females die earlier than the males. It is unclear, however, if Eed is required for the initiation of imprinted paternal X-inactivation in preimplantation embryos, because Eed protein and/or mRNA stored in the oocytes, although consumed by the late blastocyst stage (E4.5) (Kalantry and Magnuson, 2006), is initially available even in *Eed*^{-/-} embryos. In contrast to imprinted X-inactivation in the extraembryonic tissues, random X-inactivation in the embryonic tissues is properly initiated and maintained in *Eed*^{-/-} female embryos after implantation, indicating that Eed is dispensable for random X-inactivation (Kalantry and Magnuson, 2006). Suz12, like Eed, is also required for the enzymatic activity of PRC2. Functional disruption of Suz12 in the mouse resulted in embryonic lethality at the early postimplantation stages, with a global loss of H3K27me₃ (Pasini et al., 2004). Given the indispensable role of Eed and Suz12 for PRC2 activity, it is likely that H3K27me₃ is essential for the maintenance of imprinted X-inactivation in the trophoblast but for neither the initiation nor the maintenance of random X-inactivation in the embryonic lineage.

It has been reported that a short RNA transcribed from the *Xist* locus, RepA, which shares its sequence with the 5' part of *Xist* RNA, interacts with Ezh2 and recruits PRC2 to the *Xist* locus, from which H3K27me₃ propagates along the chromosome (Zhao et al., 2008). Other long noncoding RNAs (lncRNAs) such as HOTAIR have also been shown to interact with PRC2 and to facilitate H3K27me₃ at their target loci (Rinn et al., 2007; Tsai et

al., 2010). These findings have provided the basis of the concept for the role of one class of lncRNA to modulate chromatin and gene expression. Although this is an attractive and plausible model, another study based on a biochemical analysis measuring binding constants demonstrated that the binding affinities of PRC2 for lncRNAs that have been shown to interact with PRC2 and for irrelevant RNAs are comparable (Davidovich et al., 2013). Such promiscuous binding of PRC2 to various RNAs raises an issue about the specificity of the association between Ezh2 and *Xist* RNA and argues for the need for reevaluation.

Jarid2, a founder member of the Jumonji family of proteins, was recently shown to be transiently enriched on the X chromosome undergoing inactivation via an interaction with *Xist* RNA prior to the recruitment of PRC2, suggesting a role for Jarid2 in targeting PRC2 to Xi (da Rocha et al., 2014; Kaneko et al., 2014). Depletion of Jarid2, however, only delayed the recruitment of PRC2 to the X chromosome, and PRC2 was eventually loaded and catalyzed H3K27me₃ on the X chromosome (Li et al., 2010). Therefore, while Jarid2 appears to play a role in the initial efficient recruitment of PRC2 to the X chromosome, its significance in the process of X-inactivation is still obscure. It should be noted that since homozygous mutants of Jarid2, although embryonic lethal, can develop until the midgestation stage (Takeuchi et al., 1995), X-inactivation would not be significantly affected.

(2) PRC1 The inactivated X chromosome is also characterized by monoubiquitination at lysine 119 of histone H2A (H2AK119ub1), although the biological significance of this modification is not fully understood. This is catalyzed by the E3 ligases Ring1A and Ring1B (de Napoles et al., 2004), and either of them, in combination with Mel18 and Cbx proteins, among others, is incorporated into another class of PcG protein complex, PRC1. While Ring1A-deficient mice are viable and fertile, those deficient for Ring1B are developmentally arrested after implantation with gastrulation defects and die before E10.5 (Voncken et al., 2003). H2AK119ub1 on Xi is maintained in cells lacking Ring1A or Ring1B, but is lost if both are depleted (de Napoles et al., 2004). It is not clear whether depletion of H2AK119ub1 compromises X-inactivation status, as the fact that cells lacking both Ring1A and Ring1B are selected against precludes the analysis of a long-term effect of H2AK119ub1 depletion. Although the model that H3K27me₃ mediated by PRC2 forms a binding site for PRC1 to facilitate H2AK119ub1 (Cao et al., 2002; Kuzmichev et al., 2002; Fischle et al., 2003; Min et al., 2003) has been generally accepted, a subsequent study demonstrates that PRC1 on its own can target Xi in the absence of H3K27me₃, suggesting direct targeting of *Xist* RNA by PRC1 (Schoeftner et al., 2006).

Recently, a noncanonical PRC1 complex, which con-

tains RYBP in place of CBX chromodomain proteins, has been shown to become loaded at its targets independently of H3K27me3 and to mediate H2AK119ub1 as efficiently as canonical PRC1 containing CBX proteins (Tavares et al., 2012). PRC1 containing RYBP was also found to be enriched over the territory of *Xist* RNA expressed from an autosomal transgene. These findings suggest that PRC1 containing RYBP can be recruited to Xi in response to *Xist* RNA coating and can catalyze H2AK119ub1 independently of H3K27me3. The molecular basis of this noncanonical PRC1 recruitment to the *Xist* RNA territory will be an interesting subject of future studies.

SmcHD1

SmcHD1 was first identified as a protein whose functional disruption caused female-specific lethality during mouse embryonic development (Blewitt et al., 2008). It belongs to the SMC family and contains an N-terminal ATPase domain and a C-terminal SMC hinge domain. Given that the postimplantation development of female embryos is significantly compromised if X-inactivation does not occur properly, and that the gross morphology of homozygous SmcHD1 mutants observed by Blewitt et al. (2008) was indistinguishable from that of their wild-type littermates at the early postimplantation stages, it is likely that X-inactivation had been initiated and established in these mutants, at least to an extent that was sufficient to support early postimplantation development. However, derepression of X-inactivated genes became apparent in the mutants by the midgestation stage, with an occasional decrease in DNA methylation at nearby CpG islands. These initial findings suggested that SmcHD1 was involved in DNA methylation at X-linked CpG islands and contributed to the maintenance of X-inactivation. Recent transcriptome analyses, however, revealed that only about 10% of the genes on Xi in SmcHD1-deficient female MEFs were affected, and that the derepression was not necessarily associated with hypomethylation at the CpG islands (Gendrel et al., 2013; Mould et al., 2013). These findings suggest that hypomethylation at the CpG islands is not a direct cause of the derepression of the X-inactivated genes in the SmcHD1 mutants, but rather is one of the consequences. It is therefore likely that SmcHD1 exerts its effect on the maintenance of X-inactivation via different types of epigenetic regulation. It is well established that PcG proteins play important roles in maintaining the repressed chromatin state in various contexts including Xi, probably by means of PRC2-mediated H3K27me3 and PRC1-mediated H2AK119ub1. The fact that Xi still retains H3K27me3 in SmcHD1-deficient MEFs implies that SmcHD1 and PcG proteins may function in independent pathways.

HBiX1, recently identified as a protein that coimmuno-

precipitates with heterochromatin protein 1 (HP1), has been shown to interact with SMCHD1 in human cultured cells (Nozawa et al., 2013). These three proteins appear to form a complex and localize to Xi. Genome-wide analyses by ChIP-seq have revealed that SMCHD1 and HBiX1 tend to associate with H3K27me3 and H3K9me3, respectively, on Xi. Depletion of SMCHD1 or HBiX1, although affecting the distribution of neither H3K27me3 nor H3K9me3 when examined by ChIP-seq, caused an apparent decompaction of Xi, which became barely detectable in interphase nuclei by immunofluorescence using an antibody against H3K9me3. Although derepression of genes on Xi was not observed in cells upon depletion of SMCHD1 or HBiX1, these results suggest the existence of an SMCHD1-HBiX1 pathway to maintain higher-order chromatin structure. Although neither HP1 nor H3K9me3 is detected on a mouse Xi by immunofluorescence analysis in interphase nuclei, it would be of interest to study the impact on X-inactivation during development if HBiX1 were deleted in the mouse.

CONCLUDING REMARKS

Xist RNA is considered to serve as a platform for proteins that are involved in the initiation and maintenance of chromosome-wide heterochromatinization by coating the X chromosome undergoing inactivation. Although many proteins have been shown to be enriched on Xi, conclusive studies of how they are targeted to Xi and whether they interact with *Xist* RNA have not yet been performed in most cases. Although RNA immunoprecipitation and cross-linking immunoprecipitation have been powerful techniques for revealing the interaction between small RNA and proteins, they are not invariably applicable to lncRNA and proteins. It will be important, if challenging, in the future to employ more biochemical approaches to address the interactions, and their molecular basis, between *Xist* RNA and the proteins that are enriched on Xi.

REFERENCES

- Agostini, F., Cirillo, D., Bolognesi, B., and Tartaglia, G. G. (2013) X-inactivation: quantitative predictions of protein interactions in the *Xist* network. *Nucleic Acids Res.* **41**, e31.
- Agrelo, R., Souabni, A., Novatchkova, M., Haslinger, C., Leeb, M., Komnenovic, V., Kishimoto, H., Gresh, L., Kohwi-Shigematsu, T., Kenner, L., et al. (2009) SATB1 defines the developmental context for gene silencing by *Xist* in lymphoma and embryonic cells. *Dev. Cell* **16**, 507–516.
- Alvarez, J. D., Yasui, D. H., Niida, H., Joh, T., Loh, D. Y., and Kohwi-Shigematsu, T. (2000) The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes during T-cell development. *Genes Dev.* **14**, 521–535.
- Blewitt, M. E., Gendrel, A.-V., Pang, Z., Sparrow, D. B., Whitelaw,

- N., Craig, J. M., Apedaile, A., Hilton, D. J., Dunwoodie, S. L., Brockdorff, N., et al. (2008) SmcHD1, containing a structural-maintenance-of-chromosomes hinge domain, has a critical role in X inactivation. *Nat. Genet.* **40**, 663–669.
- Cai, S., Han, H.-J., and Kohwi-Shigematsu, T. (2003) Tissue-specific nuclear architecture and gene expression regulated by SATB1. *Nat. Genet.* **34**, 42–51.
- Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S., and Zhang, Y. (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* **298**, 1039–1043.
- Chadwick, B. P., and Willard, H. F. (2001) Histone H2A variants and the inactive X chromosome: identification of a second macroH2A variant. *Hum. Mol. Genet.* **10**, 1101–1113.
- Changolkar, L. N., Costanzi, C., Leu, N. A., Chen, D., McLaughlin, K. J., and Pehrson, J. R. (2007) Developmental changes in histone macroH2A1-mediated gene regulation. *Mol. Cell. Biol.* **27**, 2758–2764.
- Clemson, C. M., McNeil, J. A., Willard, H. F., and Lawrence, J. B. (1996) XIST RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. *J. Cell Biol.* **132**, 259–275.
- Costanzi, C., and Pehrson, J. R. (1998) Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. *Nature* **393**, 599–601.
- Costanzi, C., and Pehrson, J. R. (2001) MACROH2A2, a new member of the MACROH2A core histone family. *J. Biol. Chem.* **276**, 21776–21784.
- Costanzi, C., Stein, P., Worrall, D. M., Schultz, R. M., and Pehrson, J. R. (2000) Histone macroH2A1 is concentrated in the inactive X chromosome of female preimplantation mouse embryos. *Development* **127**, 2283–2289.
- Csankovszki, G., Panning, B., Bates, B., Pehrson, J. R., and Jaenisch, R. (1999) Conditional deletion of *Xist* disrupts histone macroH2A localization but not maintenance of X inactivation. *Nat. Genet.* **22**, 323–324.
- da Rocha, S. T., Boeva, V., Escamilla-Del-Arenal, M., Ancelin, K., Granier, C., Matias, N. R., Sanulli, S., Chow, J., Schulz, E., Picard, C., et al. (2014) Jarid2 is implicated in the initial Xist-induced targeting of PRC2 to the inactive X chromosome. *Mol. Cell* **53**, 301–316.
- Davidovich, C., Zheng, L., Goodrich, K. J., and Cech, T. R. (2013) Promiscuous RNA binding by Polycomb repressive complex 2. *Nat. Struct. Mol. Biol.* **20**, 1250–1257.
- De La Fuente, R., Baumann, C., and Viveiros, M. M. (2011) Role of ATRX in chromatin structure and function: implications for chromosome instability and human disease. *Reproduction* **142**, 221–234.
- de Napolles, M., Mermoud, J. E., Wakao, R., Tang, Y. A., Endoh, M., Appanah, R., Nesterova, T. B., Silva, J., Otte, A. P., Vidal, M., et al. (2004) Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. *Dev. Cell* **7**, 663–676.
- Dickinson, L. A., Dickinson, C. D., and Kohwi-Shigematsu, T. (1997) An atypical homeodomain in SATB1 promotes specific recognition of the key structural element in a matrix attachment region. *J. Biol. Chem.* **272**, 11463–11470.
- Fackelmayer, F. O. (2005) A stable proteinaceous structure in the territory of inactive X chromosomes. *J. Biol. Chem.* **280**, 1720–1723.
- Fischle, W., Wang, Y., Jacobs, S. A., Kim, Y., Allis, C. D., and Khorasanizadeh, S. (2003) Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes Dev.* **17**, 1870–1881.
- Gaspar-Maia, A., Qadeer, Z. A., Hasson, D., Ratnakumar, K., Leu, N. A., Leroy, G., Liu, S., Costanzi, C., Valle-Garcia, D., Schaniel, C., et al. (2013) MacroH2A histone variants act as a barrier upon reprogramming towards pluripotency. *Nat. Commun.* **4**, 1565.
- Gendrel, A.-V., Tang, Y. A., Suzuki, M., Godwin, J., Nesterova, T. B., Grealley, J. M., Heard, E., and Brockdorff, N. (2013) Epigenetic functions of smcHD1 repress gene clusters on the inactive X chromosome and on autosomes. *Mol. Cell. Biol.* **33**, 3150–3165.
- Hasegawa, Y., Brockdorff, N., Kawano, S., Tsutui, K., Tsutui, K., and Nakagawa, S. (2010) The matrix protein hnRNP U is required for chromosomal localization of Xist RNA. *Dev. Cell* **19**, 469–476.
- Helbig, R., and Fackelmayer, F. O. (2003) Scaffold attachment factor A (SAF-A) is concentrated in inactive X chromosome territories through its RGG domain. *Chromosoma* **112**, 173–182.
- Hernández-Muñoz, I., Lund, A. H., van der Stoep, P., Boutsma, E., Muijters, I., Verhoeven, E., Nusinow, D. A., Panning, B., Marahrens, Y., and van Lohuizen, M. (2005) Stable X chromosome inactivation involves the PRC1 Polycomb complex and requires histone MACROH2A1 and the CULLIN3/SPOP ubiquitin E3 ligase. *Proc. Natl. Acad. Sci. USA* **102**, 7635–7640.
- Kalanitry, S., and Magnuson, T. (2006) The Polycomb group protein EED is dispensable for the initiation of random X-chromosome inactivation. *PLoS Genet.* **2**, e66.
- Kaneko, S., Bonasio, R., Saldaña-Meyer, R., Yoshida, T., Son, J., Nishino, K., Umezawa, A., and Reinberg, D. (2014) Interactions between JARID2 and noncoding RNAs regulate PRC2 recruitment to chromatin. *Mol. Cell* **53**, 290–300.
- Kiledjian, M., and Dreyfuss, G. (1992) Primary structure and binding activity of the hnRNP U protein: binding RNA through RGG box. *EMBO J.* **11**, 2655–2664.
- Kipp, M., Göhring, F., Ostendorp, T., van Drunen, C. M., van Driel, R., Przybylski, M., and Fackelmayer, F. O. (2000) SAF-Box, a conserved protein domain that specifically recognizes scaffold attachment region DNA. *Mol. Cell. Biol.* **20**, 7480–7489.
- Kohwi-Shigematsu, T., and Kohwi, Y. (1990) Torsional stress stabilizes extended base unpairing in suppressor sites flanking immunoglobulin heavy chain enhancer. *Biochemistry* **29**, 9551–9560.
- Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev.* **16**, 2893–2905.
- Li, G., Margueron, R., Ku, M., Chambon, P., Bernstein, B. E., and Reinberg, D. (2010) Jarid2 and PRC2, partners in regulating gene expression. *Genes Dev.* **24**, 368–380.
- Lyon, M. (1961) Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* **190**, 372–373.
- Mermoud, J. E., Costanzi, C., Pehrson, J. R., and Brockdorff, N. (1999) Histone macroH2A1.2 relocates to the inactive X chromosome after initiation and propagation of X-inactivation. *J. Cell Biol.* **147**, 1399–1408.
- Mietton, F., Sengupta, A. K., Molla, A., Picchi, G., Barral, S., Heliot, L., Grange, T., Wutz, A., and Dimitrov, S. (2009) Weak but uniform enrichment of the histone variant macroH2A1 along the inactive X chromosome. *Mol. Cell. Biol.* **29**, 150–156.
- Min, J., Zhang, Y., and Xu, R.-M. (2003) Structural basis for specific binding of Polycomb chromodomain to histone H3

- methylylated at Lys 27. *Genes Dev.* **17**, 1823–1828.
- Mould, A. W., Pang, Z., Pakusch, M., Tonks, I. D., Stark, M., Carrie, D., Mukhopadhyay, P., Seidel, A., Ellis, J. J., Deakin, J., et al. (2013) Smchd1 regulates a subset of autosomal genes subject to monoallelic expression in addition to being critical for X inactivation. *Epigenetics Chromatin* **6**, 19.
- Nakagomi, K., Kohwi, Y., Dickinson, L. A., and Kohwi-Shigematsu, T. (1994) A novel DNA-binding motif in the nuclear matrix attachment DNA-binding protein SATB1. *Mol. Cell. Biol.* **14**, 1852–1860.
- Nechanitzky, R., Dávila, A., Savarese, F., Fietze, S., and Grosschedl, R. (2012) Satb1 and Satb2 are dispensable for X chromosome inactivation in mice. *Dev. Cell* **23**, 866–871.
- Nozawa, R.-S., Nagao, K., Igami, K.-T., Shibata, S., Shirai, N., Nozaki, N., Sado, T., Kimura, H., and Obuse, C. (2013) Human inactive X chromosome is compacted through a PRC2-independent SMCHD1-HBIX1 pathway. *Nat. Struct. Mol. Biol.* **20**, 566–573.
- Nusinow, D. A., Hernández-Muñoz, I., Fazzio, T. G., Shah, G. M., Kraus, W. L., and Panning, B. (2007) Poly(ADP-ribose) polymerase 1 is inhibited by a histone H2A variant, MacroH2A, and contributes to silencing of the inactive X chromosome. *J. Biol. Chem.* **282**, 12851–12859.
- Oka, A., and Shiroishi, T. (2014) Regulatory divergence of X-linked genes and hybrid male sterility in mice. *Genes Genet. Syst.* **89**, 99–108.
- Ouararhni, K., Hadj-Slimane, R., Ait-Si-Ali, S., Robin, P., Mietton, F., Harel-Bellan, A., Dimitrov, S., and Hamiche, A. (2006) The histone variant mH2A1.1 interferes with transcription by down-regulating PARP-1 enzymatic activity. *Genes Dev.* **20**, 3324–3336.
- Pasini, D., Bracken, A. P., Jensen, M. R., Lazzerini Denchi, E., and Helin, K. (2004) Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. *EMBO J.* **23**, 4061–4071.
- Pasque, V., Gillich, A., Garrett, N., and Gurdon, J. B. (2011) Histone variant macroH2A confers resistance to nuclear reprogramming. *EMBO J.* **30**, 2373–2387.
- Pehrson, J. R., and Fried, V. A. (1992) MacroH2A, a core histone containing a large nonhistone region. *Science* **257**, 1398–1400.
- Pehrson, J. R., Costanzi, C., and Dharia, C. (1997) Developmental and tissue expression patterns of histone macroH2A1 subtypes. *J. Cell. Biochem.* **65**, 107–113.
- Ponting, C., Schultz, J., and Bork, P. (1997) SPRY domains in ryanodine receptors (Ca²⁺)-release channels. *Trends Biochem. Sci.* **22**, 193–194.
- Pullirsch, D., Härtel, R., Kishimoto, H., Leeb, M., Steiner, G., and Wutz, A. (2010) The Trithorax group protein Ash2l and Saf-A are recruited to the inactive X chromosome at the onset of stable X inactivation. *Development* **137**, 935–943.
- Rasmussen, T. P., Huang, T., Mastrangelo, M. A., Loring, J., Panning, B., and Jaenisch, R. (1999) Messenger RNAs encoding mouse histone macroH2A1 isoforms are expressed at similar levels in male and female cells and result from alternative splicing. *Nucleic Acids Res.* **27**, 3685–3689.
- Rasmussen, T. P., Wutz, A. P., Pehrson, J. R., and Jaenisch, R. R. (2001) Expression of Xist RNA is sufficient to initiate macrochromatin body formation. *Chromosoma* **110**, 411–420.
- Rinn, J. L., Kertesz, M., Wang, J. K., Squazzo, S. L., Xu, X., Brugmann, S. A., Goodnough, L. H., Helms, J. A., Farnham, P. J., Segal, E., et al. (2007) Functional demarcation of active and silent chromatin domains in human *HOX* loci by noncoding RNAs. *Cell* **129**, 1311–1323.
- Roshon, M. J., and Ruley, H. E. (2005) Hypomorphic mutation in hnRNP U results in post-implantation lethality. *Transgenic Res.* **14**, 179–192.
- Savarese, F., Flahndorfer, K., Jaenisch, R., Busslinger, M., and Wutz, A. (2006) Hematopoietic precursor cells transiently reestablish permissiveness for X inactivation. *Mol. Cell. Biol.* **26**, 7167–7177.
- Savarese, F., Dávila, A., Nechanitzky, R., De La Rosa-Velazquez, I., Pereira, C. F., Engelke, R., Takahashi, K., Jenuwein, T., Kohwi-Shigematsu, T., Fisher, A. G., et al. (2009) Satb1 and Satb2 regulate embryonic stem cell differentiation and *Nanog* expression. *Genes Dev.* **23**, 2625–2638.
- Schoeffner, S., Sengupta, A. K., Kubicek, S., Mechtler, K., Spahn, L., Koseki, H., Jenuwein, T., and Wutz, A. (2006) Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. *EMBO J.* **25**, 3110–3122.
- Shen, X., Liu, Y., Hsu, Y.-J., Fujiwara, Y., Kim, J., Mao, X., Yuan, G.-C., and Orkin, S. H. (2008) EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. *Mol. Cell* **32**, 491–502.
- Sugimoto, M. (2014) Developmental genetics of the mouse *t*-complex. *Genes Genet. Syst.* **89**, 109–120.
- Takeuchi, T., Yamazaki, Y., Katoh-Fukui, Y., Tsuchiya, R., Kondo, S., Motoyama, J., and Higashinakagawa, T. (1995) Gene trap capture of a novel mouse gene, jumonji, required for neural tube formation. *Genes Dev.* **9**, 1211–1222.
- Tanasijevic, B., and Rasmussen, T. P. (2011) X chromosome inactivation and differentiation occur readily in ES cells doubly-deficient for macroH2A1 and macroH2A2. *PLoS One* **6**, e21512.
- Tavares, L., Dimitrova, E., Oxley, D., Webster, J., Poot, R., Demmers, J., Bezstarosti, K., Taylor, S., Ura, H., Koide, H., et al. (2012) RYBP-PRC1 complexes mediate H2A ubiquitylation at Polycomb target sites independently of PRC2 and H3K27me3. *Cell* **148**, 664–678.
- Tsai, M.-C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J. K., Lan, F., Shi, Y., Segal, E., and Chang, H. Y. (2010) Long noncoding RNA as modular scaffold of histone modification complexes. *Science* **329**, 689–693.
- Voncken, J. W., Roelen, B. A., Roefs, M., de Vries, S., Verhoeven, E., Marino, S., Deschamps, J., and van Lohuizen, M. (2003) *Rnf2 (Ring1b)* deficiency causes gastrulation arrest and cell cycle inhibition. *Proc. Natl. Acad. Sci. USA* **100**, 2468–2473.
- Wang, J., Mager, J., Chen, Y., Schneider, E., Cross, J. C., Nagy, A., and Magnuson, T. (2001) Imprinted X inactivation maintained by a mouse Polycomb group gene. *Nat. Genet.* **28**, 371–375.
- Wang, Z. Q., Auer, B., Stingl, L., Berghammer, H., Haidacher, D., Schweiger, M., and Wagner, E. F. (1995) Mice lacking ADPRT and poly(ADP-ribosylation) develop normally but are susceptible to skin disease. *Genes Dev.* **9**, 509–520.
- Wutz, A., and Jaenisch, R. (2000) A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. *Mol. Cell* **5**, 695–705.
- Wutz, A., Rasmussen, T. P., and Jaenisch, R. (2002) Chromosomal silencing and localization are mediated by different domains of *Xist* RNA. *Nat. Genet.* **30**, 167–174.
- Zhao, J., Sun, B. K., Erwin, J. A., Song, J.-J., and Lee, J. T. (2008) Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. *Science* **322**, 750–756.