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The control of polycomb repressive complexes by long noncoding RNAs

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Abstract

The polycomb repressive complexes 1 and 2 (PRCs; PRC1 and PRC2) are conserved histone-modifying enzymes that often function cooperatively to repress gene expression. The PRCs are regulated by long noncoding RNAs (lncRNAs) in complex ways. On the one hand, specific lncRNAs cause the PRCs to engage with chromatin and repress gene expression over genomic regions that can span megabases. On the other hand, the PRCs bind RNA with seemingly little sequence specificity, and at least in the case of PRC2, direct RNA-binding has the effect of inhibiting the enzyme. Thus, some RNAs appear to promote PRC activity, while others may inhibit it. The reasons behind this apparent dichotomy are unclear. The most potent PRC-activating lncRNAs associate with chromatin and are predominantly unspliced or harbor unusually long exons. Emerging data imply that these lncRNAs promote PRC activity through internal RNA sequence elements that arise and disappear rapidly in evolutionary time. These sequence elements

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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may function by interacting with common subsets of RNA-binding proteins that recruit or stabilize PRCs on chromatin.

This article is categorized under:

RNA Interactions with Proteins and Other Molecules > Protein-RNA Recognition

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1 | INTRODUCTION

The polycomb repressive complexes 1 and 2 (PRCs; PRC1 and PRC2) are conserved multisubunit enzymes that, among other actions, monoubiquitinate histone H2A on lysine119 (H2AK119ub1) and trimethylate histone H3 on lysine27 (H3K27me3), respectively. The primary function of the PRCs is to repress gene expression, often in a cooperative manner. While the enzymes can induce reversible forms of gene silencing, they are also involved in pathways that function to silence genes more permanently. Thus, the PRCs provide cells with ways to regulate gene expression, both transiently and stably, depending on the context. Accordingly, the PRCs play critical roles in embryonic development and response to stress across kingdoms of life (for recent reviews, see Almeida et al., 2020; Costa & Dean, 2019; Laugesen et al., 2019; Z. A. Lewis, 2017; Schubert, 2019; Schuettengruber et al., 2017; J. R. Yu et al., 2019).

One of the many essential functions of the PRCs is to mediate stable gene silencing during X chromosome inactivation (XCI), a conserved process that evolved to equalize X-linked gene expression between females, which have two X chromosomes, and males, which have only one. XCI occurs early during the development of all female mammals and leads to the near-complete transcriptional silencing of one of the two X chromosomes in every diploid cell. In placental mammals, XCI is orchestrated by a long noncoding RNA (lncRNA) called *Xist*. Over the last two decades, XCI has provided a paradigm to investigate the mechanisms by which a lncRNA (i.e., *Xist*) can target the PRCs to a specific region of the genome (i.e., the inactive X) to induce stable gene silencing. Investigations of XCI and other related phenomena have revealed that the relationship between the PRCs and RNA is far more complicated than initially envisioned.

Nevertheless, while central questions remain unanswered, recent progress in the field has been substantial. Evidence suggests that principles established from the study of *Xist* might be relevant to other RNAs, and also that general interactions between RNA and PRC2 (if not PRC1, as well) may simultaneously position the enzyme near its target genes while preventing it from accessing its chromatin substrates. In this review, we summarize, in turn, studies that have investigated relationships between the PRCs and RNAs throughout

the transcriptome, between the PRCs and *Xist*, and between the PRCs and other specific lncRNAs. Our primary focus is on advances made in mammalian model systems, but we also discuss an important series of advances made in plants.

2 | THE POLYCOMB REPRESSIVE COMPLEXES

PRC1 and PRC2 are multisubunit enzymes that monoubiquitinate histone H2A on lysine119 (H2AK119ub1) and trimethylate histone H3 on lysine27 (H3K27me3), respectively. The enzymes repress gene expression through complex mechanisms that include compacting chromatin, inhibiting transcriptional initiation and elongation, antagonizing co-activators, recruiting co-repressors, and mediating long-range contacts between repressed loci. The histone modification deposited by PRC1 can recruit PRC2 and vice versa; thus, at many target loci, the PRCs work together to repress gene expression (Blackledge et al., 2015; Laugesen et al., 2019; Schuettengruber et al., 2017; J. R. Yu et al., 2019). In this subsection, we summarize the protein constituents of PRC1 and PRC2 and discuss the RNA-independent mechanisms that recruit the PRCs to chromatin. This information is useful for understanding the relationship between the PRCs and *Xist* and other lncRNAs. In addition to its role in depositing H3K27me3, PRC2 also deposits H3K27me1 and H3K27me2 and methylates non-histone substrates; these latter functions are reviewed elsewhere (Laugesen et al., 2019; Schuettengruber et al., 2017; J. R. Yu et al., 2019).

In mice, core components of the PRCs appear to be most highly expressed during the early stages of embryonic development, where they play essential roles (Aloia et al., 2013; Kloet et al., 2016). As the embryo differentiates, the overall levels of the PRCs decrease, concomitant with changes in the composition of PRC accessory subunits (Aloia et al., 2013; Kloet et al., 2016; Kuzmichev et al., 2005; J. R. Yu et al., 2019). Nevertheless, the PRCs play critical roles in many lineages, including adult stem cell populations as well as in more differentiated cell types (Akerberg & Pu, 2019; Brand et al., 2019; Desai & Pethe, 2020; Di Carlo et al., 2019; P. P. Liu et al., 2018; Vidal & Starowicz, 2017; J. R. Yu et al., 2019). Accordingly, mutation or misexpression of the PRCs and their accessory factors is associated with many forms of disease, including cancer, overgrowth syndromes, malignant hematological disorders, and forms of intellectual disability (Chan & Morey, 2019; Comet et al., 2016; Cyrus et al., 2019; Deevy & Bracken, 2019; Di Carlo et al., 2019; Isshiki & Iwama, 2018; Poynter & Kadoch, 2016; Srivastava et al., 2017; Vidal & Starowicz, 2017). Moreover, both PRC1 and PRC2 have been targeted in multiple campaigns to identify chemical inhibitors, underscoring their potential as therapeutic targets (Comet et al., 2016; He et al., 2017; Lamb et al., 2019; Lingel et al., 2017; Milosevich et al., 2016; Potjewyd et al., 2020; Qi et al., 2017; Ren et al., 2015; Stuckey et al., 2016).

There is no single PRC1 or PRC2. The PRCs each exist in subcomplexes comprised of core subunits partnered with different accessory factors, described below. These subcomplexes can vary in their ability to act as enzymes and in their ability to interact with other biomolecules, including DNA, histones, other proteins, and RNA. Depending on the cell type and the genomic region, the different PRC subcomplexes play varied roles in gene silencing (Blackledge et al., 2015; Laugesen et al., 2019; Schuettengruber et al., 2017; J. R. Yu et al., 2019).

2.1 | Polycomb repressive complex 1

Polycomb repressive complex 1 (PRC1) can take the form of at least six subcomplexes (Figure 1), each of which contains a heterodimeric E3 ubiquitin ligase comprised of a RING1 protein (either RING1A or RING1B) partnered with one of six polycomb group RING finger proteins (PCGF1–6) that together form its catalytic core. PRC1 subcomplexes can be further partitioned into two groups that are collectively referred to as canonical and variant PRC1, depending on which PCGF homologue is incorporated (Blackledge et al., 2015; Gao et al., 2012; Hauri et al., 2016; Schuettengruber et al., 2017).

Canonical PRC1 (cPRC1) is comprised of RING1A/B partnered with either PCGF2 or PCGF4. cPRC1 additionally contains one of five chromobox family proteins (CBX2, CBX4, CBX6, CBX7, and CBX8) and one of three polyhomeotic-like proteins (PHC1–3; Blackledge et al., 2015; Gao et al., 2012; Hauri et al., 2016; Schuettengruber et al., 2017). A primary function of cPRC1 is to compact chromatin and restrict transcriptional activators from accessing their underlying nucleosomal substrates (J. A. Simon & Kingston, 2013). As part of cPRC1, the CBX proteins bind H3K27me3; in turn, H3K27me3-binding is one mechanism through which cPRC1 is recruited to chromatin, specifically to those regions being targeted simultaneously by PRC2 (Cao et al., 2002; Min et al., 2003; Moussa et al., 2019; L. Wang et al., 2004; Zepeda-Martinez et al., 2020). Another important targeting mechanism involves lncRNAs, which will be described in greater depth in the sections below. In parallel, the CBX proteins of cPRC1 also mediate the formation of long-range contacts between PRC-repressed loci, potentially by promoting the separation of cPRC1 and other proteins into a different chemical phase (J. Kim & Kingston, 2020; Plys et al., 2019). The same domain within CBX that promotes long-range contacts is required for compaction of nucleosomal arrays in vitro, indicating that nucleosomal compaction, the formation of three-dimensional (3D) contacts, and phase-separation are linked by the same domain (Francis et al., 2004; Grau et al., 2011). The PHC proteins within cPRC1 also potentiate gene silencing through their ability to form oligomers between cPRC1 complexes; these oligomers appear to stabilize both PRC1 and PRC2 on chromatin as well as promote the formation of long-range contacts between PRC-repressed loci (Gambetta & Muller, 2014; Isono et al., 2013; C. A. Kim et al., 2002; Kundu et al., 2018; Robinson et al., 2012). Thus, although cPRC1 appears to play a limited role in catalyzing H2AK119ub1 in vivo, its more significant functions may be to compact chromatin and orchestrate physical interactions between PRCs and their genomic targets (Blackledge et al., 2014; Boyle et al., 2020; Eskeland et al., 2010; Fursova et al., 2019; Gao et al., 2012; Isono et al., 2013; J. Kim & Kingston, 2020; King et al., 2005; Kundu et al., 2018; Lau et al., 2017; Scelfo et al., 2019). Emerging data suggest that compaction is often seeded at sites of high H3K27me3, first by the oligomerization and then by the condensation of cPRC1; subsequent compaction may be maintained not necessarily by sustained condensation of cPRC1, but by PRC1-dependent modifications to chromatin (Eeftens et al., 2020).

Variant PRC1 (vPRC1) is comprised of RING1A/B partnered with PCGF1, 3, 5, or 6. vPRC1 complexes lack the CBX and PHC proteins that define cPRC1 and instead contain RYBP or the closely related protein YAF2 (Blackledge et al., 2015; Gao et al., 2012; Hauri et al., 2016; Schuettengruber et al., 2017). In vivo, vPRC1 complexes appear to have a

higher catalytic activity than cPRC1 complexes (Blackledge et al., 2014). Presumably, this is due to the stimulatory effect that incorporation of the RYBP or YAF2 subunits imparts on the catalytic activity of vPRC1 (Gao et al., 2012; Rose et al., 2016). Unlike cPRC1, vPRC1 is recruited to the genome independently of PRC2, either by sequence-specific DNA-binding proteins such as KDM2B, E2F6-DP1, or MGA-MAX, by lncRNAs such as *Xist* (discussed more below), or by means that remain unknown (Blackledge et al., 2015; Fursova et al., 2019; Scelfo et al., 2019; Schuettengruber et al., 2017). The RYBP and YAF2 subunits also contain an H2AK119ub-binding domain (Arrigoni et al., 2006). While H2AK119ub-binding by RYBP/YAF2 does not appear to be the predominant mechanism that recruits vPRC1 to chromatin, the binding does recruit vPRC1 to a subset of its genomic targets, including the inactive X chromosome (Almeida et al., 2017; Rose et al., 2016).

In many instances, the H2AK119ub1 deposited by vPRC1 also recruits PRC2 and cPRC1 to the genome. In mouse embryonic stem cells (mESCs), the cell type in which PRC composition and targeting has been studied the most extensively, vPRC1 is required for the majority of H2AK119ub1 deposition genome-wide, as well as for transcriptional silencing at most PRC-target genes. Interestingly, however, while deletion of PCGF1, the major vPRC1 PCGF in mESCs, does cause loss of H2AK119ub1 and gene de-repression at some PRC target genes, individual deletion of any of the other PCGF proteins results in few substantive changes in H2AK119ub1or gene repression. It is only after all four vPRC1 PCGF proteins are deleted that the majority of H2AK119ub1and gene silencing is lost. Thus, at least in mESCs, vPRC1 plays a central role in establishing most PRC-repressed domains and does so in a way that depends on extensive cooperativity between vPRC1 subcomplexes (Blackledge et al., 2020; Fursova et al., 2019; Scelfo et al., 2019; Tamburri et al., 2020; Zepeda-Martinez et al., 2020). Nevertheless, a recent study showed that in the context of a heterologous reporter system, silencing induced by vPRC1 was far less stable than silencing caused by cPRC1, pointing to important roles for cPRC1 and PRC2 in the propagation of polycomb-mediated silencing through cell division (Moussa et al., 2019). The mechanistic basis for the difference in silencing stability is unclear.

2.2 | Polycomb repressive complex 2

The core components of polycomb repressive complex 2 (PRC2) are EZH1/2, EED, SUZ12, and RBBP4/7. EZH1 and EZH2 are the catalytic engines of PRC2, and in the early embryo EZH2 is the dominant one (O'Carroll et al., 2001; Son et al., 2013). EED is essential for the integrity of the PRC2 core and plays a central role in propagating PRC2-induced chromatin modifications through its ability to bind H3K27me3 and stimulate PRC2 catalytic activity. SUZ12, like EED, is essential for the integrity of the PRC2 core. It is also the protein within the core complex that interacts with the accessory factors that define the two major PRC2 subcomplexes (discussed below). The WD40 domain-containing proteins RBBP4/7 are additionally present in most if not all forms of PRC2 and are thought to help tether the complex to chromatin as well as stimulate its catalytic activity (Laugesen et al., 2019; Schuettengruber et al., 2017; J. R. Yu et al., 2019).

The two subcomplexes of PRC2, PRC2.1, and PRC2.2, are comprised of the core proteins listed above partnered with different accessory factors (Figure 2). PRC2.1 is composed of

the PRC2 core plus one PCL protein (either PHF1/PCL1, MTF2/PCL2, or PHF19/PCL3) and either the protein EPOP or PALI. PRC2.2 is comprised of the PRC2 core plus the proteins JARID2 and AEBP2. Generally speaking, these accessory factors act cooperatively to increase the catalytic activity of PRC2 at specific sites in the genome, either by directly stimulating catalysis or by increasing the residence time of PRC2 on chromatin (Laugesen et al., 2019; Schuettengruber et al., 2017; J. R. Yu et al., 2019). Underscoring their cooperativity, in mESCs, the cell type in which PRC2 has been studied the most extensively, PRC2.1 and PRC2.2 occupy many of the same genomic regions. It is only after deletion of all of PRC2's accessory factors that H3K27me3 and binding of the PRC2 core is lost at the genomic regions over which they are normally enriched (Healy et al., 2019; Hojfeldt et al., 2019; Oksuz et al., 2018). Relatedly, deleting the N-terminal region of SUZ12—the domain within the PRC2 core that interacts with all accessory factors—disrupts stable interactions between all forms of PRC2 and the genomic sites over which they are normally enriched (Hojfeldt et al., 2018; Youmans et al., 2018). Conversely, when expressed as a stand-alone protein, this same N-terminal region, which alone does not interact with any core components of PRC2, is enriched at the same genomic sites that are usually stably bound by wholly intact PRC2 (Hojfeldt et al., 2018; Youmans et al., 2018). And, in the absence of all known accessory factors, rather than being enriched at specific genomic regions, the PRC2 core interacts with chromatin in a nonspecific manner, depositing H3K27me3 over broad regions of the genome without apparent specificity or selectivity (Healy et al., 2019; Hojfeldt et al., 2019). Thus, while they are not essential for PRC2 catalytic activity, the accessory factors that define PRC2.1 and 2.2 stabilize the PRC2 core at specific genomic regions. This stabilization, coupled with the stimulation of PRC2 catalytic activity by at least a subset of the accessory factors, leads to localized genomic regions that harbor high levels of H3K27me3 (Laugesen et al., 2019; Schuettengruber et al., 2017; J. R. Yu et al., 2019).

The accessory factors of PRC2 also contribute to the cooperativity between PRC1 and PRC2. Most relevant to this review is the fact that JARID2, the defining component of PRC2.2, binds H2AK119ub1. Through this capacity, JARID2 helps recruit PRC2.2 to genomic regions that have already been targeted by PRC1, and thus is a central component in the bridge between PRC1 and PRC2 (Cooper et al., 2016; Healy et al., 2019; Kalb et al., 2014; Tamburri et al., 2020). Importantly however, in the absence of JARID2, PRC2.1 is still targeted to genomic regions bound by PRC1, where it deposits substantial levels of H3K27me3, indicating that the recruitment of PRC2.1 to chromatin does not depend exclusively on PRC2.2 (Fursova et al., 2019; Healy et al., 2019; Hojfeldt et al., 2019; Oksuz et al., 2018). Relatedly, the reduction in H3K27me3 levels upon knockout of PRC1 appears to be greater than the decrease in H3K27me3 levels upon knockout of JARID2, implying that cooperativity exists between PRC1 and PRC2.1, even though PRC2.1 may not directly bind the H2AK119ub1 modification (Blackledge et al., 2020; Fursova et al., 2019; Healy et al., 2019; Hojfeldt et al., 2019; Oksuz et al., 2018; Tamburri et al., 2020). Speculatively, this cooperativity may exist because PRC1 promotes 3D contacts and condensation between and within PRC-bound domains (Blackledge et al., 2020; Boyle et al., 2020; Eeftens et al., 2020; Isono et al., 2013; Kundu et al., 2018). In turn, PRC1-mediated contacts may increase

the concentration of PRC2.1 at subsets of target genes, perhaps in a way that depends on its accessory factors.

2.3 | CpG islands

In mammals, a class of DNA elements highly enriched in CG dinucleotides, called CpG islands, plays essential roles in recruiting the PRCs to specific regions of the genome. This recruitment is mediated in large part by sequence-specific DNA-binding proteins that themselves have a strong preference for CpG island DNA and interact robustly with the vPRC1 complexes that initiate polycomb-dependent silencing cascades (Blackledge et al., 2015; Farcas et al., 2012; Fursova et al., 2019; Scelfo et al., 2019; Schuettengruber et al., 2017). Analogous interactions with sequence-specific DNA-binding proteins may recruit PRC2 to some of its genomic targets. Still, such interactions do not appear to predominate at least in mESCs, where the majority of PRC2 depends on vPRC1 for its stable recruitment to CpG islands (Blackledge et al., 2015; Laugesen et al., 2019; Schuettengruber et al., 2017; J. R. Yu et al., 2019). PRC2 also has a strong affinity for the nucleosome-depleted DNA frequently found at CpG islands; thus, direct interactions between PRC2 and DNA may also help to stabilize PRC2 at CpG islands (Blackledge et al., 2015; Choi et al., 2017; Laugesen et al., 2019; Schuettengruber et al., 2017; X. Wang, Paucek, et al., 2017; J. R. Yu et al., 2019). In this regard, the PCL proteins of PRC2.1 were recently found to stabilize the intrinsic dimerization properties of PRC2. In turn, this stabilization may help recruit PRC2.1 to CpG islands through an avidity effect (Chen et al., 2020; Davidovich et al., 2014). Speculatively, we note that CpG islands can associate with many DNA-binding and RNA-binding proteins (Deaton & Bird, 2011; Hughes et al., 2020; Xiao et al., 2019); heterotypic interactions between these CpG-associated proteins and the PRCs may play an additional role in stabilizing PRCs at CpG islands.

3 | RNA-BINDING BY POLYCOMB REPRESSIVE COMPLEX 1 AND 2

Both PRC1 and PRC2 are recruited to the inactivating X chromosome shortly after the expression of the lncRNA *Xist* begins in the early embryo. Concurrent with this recruitment, the chromatin of the inactive X becomes highly decorated in PRC-catalyzed H2AK119ub1 and H3K27me3 (de Napoles et al., 2004; Okamoto et al., 2004; Plath et al., 2003; Silva et al., 2003). These discoveries, along with the discovery that PRC2 is essential for XCI in the extraembryonic tissues of the mouse embryo (J. Wang et al., 2001), set the stage for the investigations into lncRNAs and PRCs that form the basis for this review.

The tight temporal relationship between *Xist* expression and PRC recruitment to the inactive X suggested a parsimonious model in which high-affinity binding sites within *Xist* directly recruit the PRCs, first to *Xist*, and then to the chromatin of the inactive X. By extension, it is conceivable that other lncRNAs could operate using similar mechanisms. Indeed, results from early studies were consistent with this parsimonious model—that many lncRNAs recruit PRCs to chromatin through direct RNA–PRC interactions (Khalil et al., 2009; Rinn et al., 2007; Zhao et al., 2008, 2010).

However, work performed over the last decade has shown that the relationship between the PRCs and *Xist*—and the PRCs and RNA in general—is more complicated than initially

envisioned. Although the PRCs bind RNA directly, they harbor little apparent sequence specificity for it (references discussed below). Moreover, super-resolution microscopy indicates that a significant fraction of PRCs that surround the inactive X chromosome do not colocalize with *Xist* (Cerase et al., 2014; Sunwoo et al., 2015). Thus, how lncRNAs such as *Xist* recruit PRCs to chromatin so effectively is not yet entirely clear. Studies of the interactions between RNA and the PRCs have mostly focused on PRC2; therefore, so does our discussion in this subsection.

PRC2 binds RNA with relatively little sequence specificity. In vitro, PRC2 binds RNA with nanomolar affinity; yet, relative to length-matched, physiologically irrelevant controls such as RNA produced from the bacterial maltose-binding protein gene, PRC2 shows only a mild preference for sequences within *Xist* and other lncRNAs that are thought to bind PRC2 in vivo (Cifuentes-Rojas et al., 2014; Davidovich et al., 2013, 2015). Relatedly, in vivo crosslinking-immunoprecipitation (CLIP) experiments have shown that PRC2 directly binds—or at least forms transient contacts with—chromatin-associated RNAs produced from virtually all expressed genes, with no apparent preference for lncRNAs (Beltran et al., 2016; Kaneko et al., 2013; Kaneko, Bonasio, et al., 2014). In slight contrast, when PRC2 RNA immunoprecipitation (RIP) was performed from either formaldehyde-crosslinked or uncrosslinked cells, some sequence preference was observed. Here, PRC2 still associates robustly with many highly expressed spliced and unspliced mRNAs, again with no apparent preference for lncRNAs (Davidovich et al., 2013; Hendrickson et al., 2016; Khalil et al., 2009; Zhao et al., 2010). However, in these RIP assays, PRC2 appears to preferentially associate with regions of RNA that are rich in guanine nucleotides and are capable of forming RNA structures known G-quadruplexes (X. Wang, Goodrich, et al., 2017). PRC2's preference for G-rich sequence is also apparent in in vitro binding assays (Kaneko, Son, et al., 2014; X. Wang, Goodrich, et al., 2017). G-quadruplex motifs are degenerate and present throughout the transcriptome, consistent with the observation that PRC2 can interact with many expressed RNAs (Beltran et al., 2016; Davidovich et al., 2013; Hendrickson et al., 2016; Kaneko et al., 2013; Kaneko, Son, et al., 2014; Khalil et al., 2009; Zhao et al., 2010).

In addition to the promiscuity that underpins direct interactions between PRC2 and RNA, direct interactions with RNA appear to antagonize PRC2 function. In vitro, direct RNA-binding allosterically inhibits the PRC2 enzyme, likely by preventing PRC2 from accessing its nucleosome substrates (Beltran et al., 2016; Cifuentes-Rojas et al., 2014; Davidovich et al., 2015; Kaneko, Son, et al., 2014; X. Wang, Paucek, et al., 2017; Q. Zhang et al., 2019). Consistent with this observation, in cells, genomic regions that produce PRC2-bound RNAs are depleted in H3K27me3 (Beltran et al., 2016; Davidovich et al., 2013; Kaneko et al., 2013; Kaneko, Son, et al., 2014; Riising et al., 2014). Moreover, either the degradation of RNA or the general inhibition of transcription causes PRC2 to engage with chromatin in many of the same genomic regions that would have otherwise been transcribing PRC2-bound RNAs (Beltran et al., 2016; Davidovich et al., 2013; Kaneko et al., 2013; Kaneko, Son, et al., 2014; Riising et al., 2014). Additionally, in mESCs, the knockout of an RNA decay pathway increases the abundance of polyadenylated RNAs in the nucleus and inhibits PRC2 function dramatically, leading to a global reduction in H3K27me3, a reduced association between PRC2 and chromatin, the destabilization of PRC2 complex integrity, and the de-repression of polycomb-target genes (Garland et al., 2019). PRC2 can also be

inhibited by specific lncRNAs, including *Braveheart*, *Ppp1r1b-lncRNA*, and *linc-YY1* (Kang et al., 2020; J. Kim & Kingston, 2020; Zhou et al., 2015). Thus, in vitro and in vivo, RNA can antagonize the function of PRC2 at least locally, and global changes in the abundance of nuclear RNA can destabilize interactions between PRC2 and chromatin genome-wide. These data support a model whereby RNA can locally repel PRC2 from chromatin, and only upon displacement of the RNA from a target locus or the inhibition of transcription by polycomb-independent mechanisms is PRC2 able to engage with the underlying chromatin (Figure 3(a); Beltran et al., 2016; Davidovich et al., 2013; Kaneko, Son, et al., 2014; Kaneko et al., 2013; Riising et al., 2014).

At first glance, this model is seemingly at odds with the fact that lncRNAs such as *Xist* can cause PRC2 to engage with chromatin over large regions of the genome. However, an RNA that repels PRC2 from its own genic locus through direct interaction could still function to recruit catalytically active PRC2 to other loci that may be primed for modification by the enzyme. Furthermore, that same RNA could still recruit PRC2 indirectly, by binding proteins that themselves bind PRC2 and thereby help deposit PRC2 over broader regions.

Indeed, work from multiple labs supports the notion that both PRC1 and PRC2 can be recruited to chromatin through dedicated RNA-binding proteins that bridge interactions between the PRCs and their cognate RNAs. For example, vPRC1 can directly interact with the RNA-binding protein HNRNPK, HNRNPK directly interacts with *Xist*, and *Xist* requires HNRNPK to recruit the PRCs to the inactive X (Figure 3(b); explained in more depth below; Colognori et al., 2019; Pintacuda, Wei, et al., 2017). Similarly, RBFOX2, another dedicated RNA-binding protein, can directly interact with PRC2, and RBFOX2-binding motifs within introns of certain pre-mRNAs can recruit PRC2 to attenuate transcription of genes whose promoters are already decorated in H3K27me3 (Figure 3(c); Wei et al., 2016). Most recently, using standard chromatin immunoprecipitation and sequencing (ChIP-seq) assays, it was found in human ESCs that interactions between PRC2 and chromatin are wholly sensitive to RNase: treatment of cell lysates with RNase during the immunoprecipitation step of the ChIP results in a near-complete loss of PRC2 signal in genomic regions that are normally enriched for PRC2-binding (Long et al., 2020). Additional data from that same study suggest that the sensitivity to RNase arises from both direct and indirect interactions between PRC2 and RNA; mutation of the RNA-binding domain within EZH2 reduces but does not eliminate the interaction between PRC2 and chromatin (Long et al., 2020). Thus, even though direct interactions with RNA may inhibit the PRC2 enzyme locally, it appears that direct interactions with RNA are needed for PRC2 to accumulate at its targets genome-wide. It also remains possible that bridged interactions between PRC2, dedicated RNA-binding proteins, and lncRNAs help the enzyme accumulate at specific sites on chromatin (Figure 3(d)). Such interactions may recruit PRC2 "*in cis*" or "*in trans*":"*in cis*," meaning that the RNA recruits PRC2 to one or more regions of the same chromosome from which the RNA was expressed (but not necessarily to the same locus that produced the RNA in question); "*in trans*," meaning that an RNA expressed from one chromosome can recruit PRC2 to one or more regions of another chromosome(s).

To date, only a limited number of studies have investigated the general interactions between PRC1 and RNA. These studies suggest that, like PRC2, PRC1 also directly binds RNA with

low levels of sequence specificity and that PRC1 can associate with RNAs throughout the transcriptome, not merely lncRNAs. For example, in 2006, Bernstein et al. discovered that in vitro, the CBX proteins of cPRC1 could bind both single-stranded and double-stranded RNA; in this case, the RNA substrate used was produced from the Cyclin E gene, a protein-coding mRNA that presumably does not harbor *Xist*-like function (Bernstein et al., 2006). Still, in that same study, Bernstein et al. noted that RNase A treatment disrupted the association between PRC1 and both bulk chromatin and the inactive X chromosome, consistent with what was recently observed for PRC2 (Bernstein et al., 2006; Long et al., 2020). More recently, using a variant form of RIP performed on chromatin-enriched RNAs, it was found that the cPRC1 component BMI-1/PCGF4 associates with several lncRNAs, a subset of mRNAs, and presumably, intron-containing, chromatin-bound pre-mRNAs (M. K. Ray et al., 2016). At least one of the cPRC1-bound lncRNAs, termed *CAT7*, appeared to control PRCs in an *Xist*-like manner (M. K. Ray et al., 2016). Lastly, CLIP for the cPRC1 subunit CBX7 has been performed in both mESCs and human 293T cells (Rosenberg et al., 2017). In that study, it was found that CBX7 predominantly associated with 3′ UTRs in mRNAs through a series of divergent sequence motifs. Several of these motifs were similar to motifs bound by proteins known to be involved in splicing and RNA metabolism, suggesting that the binding of cPRC1 to mRNA 3′ UTRs may be stabilized by other proteins, perhaps analogous to the binding of vPRC1 by HNRNPK and the binding of PRC2 by RBFOX2 (Colognori et al., 2019; Pintacuda, Wei, et al., 2017; Wei et al., 2016). Surprisingly, and for reasons that are unclear, CBX7 appeared to post-transcriptionally stabilize at least a subset of its bound mRNAs (Rosenberg et al., 2017). Thus, like PRC2, PRC1 seems to bind RNA with little sequence specificity. Yet, RNA clearly plays a role in a subset of PRC1 targeting events, and specific RNAs target PRC1 to chromatin more efficiently than others.

3.1 | Xist and the polycomb repressive complexes

3.1.1 | Polycomb repressive complex 1, 2, and Xist-induced gene silencing—

The PRCs are recruited to the inactivating X within hours after the *Xist* lncRNA begins to be expressed at the onset of XCI (Okamoto et al., 2004; Zylicz et al., 2019). Accordingly, both PRC1 and PRC2 are essential for proper XCI to occur. During XCI, as in other genomic contexts, the different PRC subcomplexes act hierarchically and cooperatively. At the top of this hierarchy are specific vPRC1 complexes that contain RING1A or B, RYBP or its homologue YAF2, and PCGF3 or 5 (Almeida et al., 2017). In mESCs engineered to express a doxycycline-inducible version of *Xist*, which serve as a model for the early stages of XCI, recruitment of vPRC1 by *Xist* leads to the deposition of H2AK119ub1 that, in turn, is required for stabilization of all PRC subcomplexes over the inactive X–vPRC1, cPRC1, and PRC2.1 and PRC2.2 (Almeida et al., 2017; Nesterova et al., 2019). Accordingly, deletions of RING1A and B or PCGF3 and 5 in mESCs cause a near-complete failure of gene silencing when doxyclycline-inducible *Xist* transgenes are expressed from autosomal locations (Almeida et al., 2017; Nesterova et al., 2019). Likewise, temporally, H2AK119ub1 is deposited over the inactive X just before H3K27me3 (Zylicz et al., 2019). In contrast, deletion of EED, a core component of PRC2, causes loss of cPRC1 over the *Xist*-expressing chromosome, yet it does not affect *Xist*-induced recruitment of RING1B, nor does it obviously affect gene silencing, presumably indicating vPRC1 recruitment is unaffected

by PRC2 loss (Schoeftner et al., 2006). Moreover, despite causing a near-complete loss of H3K27me3 over the Xi, EED deletion does not affect *Xist*-mediated recruitment of JARID2, the protein within PRC2.2 that binds H2AK119ub1and whose recruitment by *Xist* is dependent on the H2AK119ub-binding domain in its N-terminus (Cooper et al., 2016; da Rocha et al., 2014). Additionally, deletion of SUZ12 in mESCs causes only modest defects in *Xist*-induced silencing (as measured by RNA-seq; Nesterova et al., 2019). Lastly, mouse models show that deletion of PRC2 from the mouse embryo has a small or no apparent effect on XCI (as measured by gene-by-gene assays; Kalantry & Magnuson, 2006; Silva et al., 2003). In contrast, deletion of *Pcgf3* and *Pcgf5* results in a precocious loss of female embryos around embryonic day (E) 9.5/10.5, implying a failure of XCI and *Xist*-induced gene silencing (Almeida et al., 2017).

Collectively, the data support a hierarchical model of PRC recruitment at the onset of XCI that parallels what is known about PRC recruitment elsewhere in the mESC genome. Specific vPRC1 complexes deposit H2AK119ub1 that is required to enrich all forms of PRC1 and PRC2 over the Xi; in turn, the collective actions of the PRCs are required for proper gene silencing during XCI (Figure 4; Almeida et al., 2017). It should be noted however, that in this model, vPRC1-deposited H2AK119ub1 does not obviate the need for *Xist*. Considering that PRC-silenced domains on the autosomes are all substantially smaller than the inactive X, it seems unlikely that PRC-induced chromatin modifications are in and of themselves sufficient to spread PRCs over the entirety of the inactive X, nor does it seem plausible that PRC recruitment to the inactive X is a default response to transcriptional silencing by another means. Rather, it is *Xist* that is the distinguishing factor. A discussion of a mechanism by which *Xist* may concentrate PRCs over the inactive X is presented in a section below.

Relatedly, because the deletion of PRC2 has a minimal effect on XCI in mESCs and in the embryo proper, a reasonable presumption is that at the onset of XCI, PRC2, and cPRC1 are of secondary importance to vPRC1. However, based on evidence demonstrating cooperativity but also redundancy between PRC2, cPRC1, and vPRC1, it seems plausible that PRC2 and cPRC1 cooperate with vPRC1 to maintain gene silencing over the inactive X—perhaps even during early stages of XCI—just as they do elsewhere in the mESC genome (Moussa et al., 2019; Zepeda-Martinez et al., 2020), and during mouse gastrulation (Grosswendt et al., 2020). Such a model would be consistent with the data that show deletion of PRC2 only modestly compromises XCI in mESCs and the embryo proper, if at all (Kalantry & Magnuson, 2006; Nesterova et al., 2019; Schoeftner et al., 2006; Silva et al., 2003), but would also predict that simultaneous deletion of both PRC2 and cPRC1 would cause a dramatic de-repression of the inactive X in mESCs and the embryo proper. This latter hypothesis remains to be tested. In the same way that simultaneous deletion of both *Pcgf3* and *Pcgf5* is needed to cause a failure of *Xist*-induced gene silencing, it may be that the simultaneous deletion of PRC2 and cPRC1 is needed to uncover the roles of the latter two complexes during the early phases of XCI. It is worth noting that upon deleting *Pcgf3* and *Pcgf5* in the embryo, while a failure of XCI was apparent from the selective loss of female embryos at E9.5/10.5, X-linked gene expression in *Pcgf3*/*Pcgf5* double knockout mice was not directly examined (Almeida et al., 2017). Because XCI initiates in the embryo proper around E5.25, the delayed embryonic lethality of female *Pcgf3*/*Pcgf5*

knockout embryos at E9.5/10.5 leaves open the possibility that PRC2 and cPRC1 cooperate with vPRC1 and partially compensate for its loss during the early stages of *Xist*-induced gene silencing. To our knowledge, no single laboratory has yet to compare the requirement of vPRC1 versus PRC2 and cPRC1 in the same experimental system using the same assay (e.g., allele-specific RNA-Seq). Thus, the extent to which PRC2 and cPRC1 may cooperate to repress genes during the early stages of *Xist*-induced silencing remains unclear.

When considering the roles of PRC2 and cPRC1 in XCI, in addition to considering that phenotypes may be masked by redundancy, it is also worth noting that different cell types utilize different PRC subcomplexes to varying extents. Most notably, unlike in tissues of the embryo proper (Kalantry & Magnuson, 2006; Silva et al., 2003), XCI depends more heavily on functional PRC2 in the extraembryonic tissues of the mouse. Deletion of EED, a core member of PRC2, results in a failure to maintain XCI in trophoblast stem and giant cells (Maclary et al., 2017; J. Wang et al., 2001). Here, the primary role for PRC2 is in the maintenance of XCI, not initiation (Kalantry et al., 2006; J. Wang et al., 2001). Work from Almaeda et al. suggests that XCI is also more sensitive to loss of vPRC1 in the trophectoderm than it is in the embryo proper (Almeida et al., 2017). We speculate that relative to the embryo proper, the heightened requirement for the PRCs in the maintenance of extraembryonic XCI reflects the latter lineage's heightened dependence on polycomb-mediated silencing pathways. Consistent with this view, silencing induced by two *Xist*-analogous lncRNAs, *Airn* and *Kcnq1ot1*, is more potent in extraembryonic tissues compared with the embryo proper and also exhibits a dependence on PRC1 and PRC2 (Andergassen et al., 2017; A. Lewis et al., 2004; Schertzer et al., 2019; Terranova et al., 2008; Umlauf et al., 2004).

Relative to more differentiated cell types, DNA methylation appears to play little role in the maintenance of *Xist*-induced gene silencing in the extraembryonic lineage (Sado et al., 2000); yet, compared with stage-matched embryonic tissue, the extraembryonic lineage harbors lower overall levels of PRCs (Silva et al., 2003). These data may explain why relative to the embryo proper, the extraembryonic lineages are more sensitive to the loss of a single arm of the PRC-silencing cascade. Relatedly, trophoblast giant cells undergo extensive endoreduplication; the polyploidy that results from this may increase dependence on the PRCs for all aspects of gene silencing (Corbel et al., 2013). Lastly, for reasons that remain unclear, deletion of EED from trophoblast stem cells leads to a loss of *Xist* expression (Maclary et al., 2017). *Xist* loss by itself would be expected to cause X-linked silencing to erode, and this erosion might be accelerated in the absence of the repressive effect of any single form of PRC1 or PRC2.

As a secondary point, the extraembryonic tissues of the mouse are subject to "imprinted XCI," in which the paternally inherited X chromosome is inactivated in all cells. In contrast, tissues of the embryo proper are subject to "random XCI," in which each cell independently selects a single X chromosome—either the maternally or paternally inherited one—for inactivation (Okamoto et al., 2004). Historically, the data that demonstrate a role for PRC2 in the maintenance of imprinted XCI in the extraembryonic tissues of the mouse may have received less attention than they warrant because of the perception that the extraembryonic tissues are subject to a "different" form of XCI than the tissues of the embryo proper. We

stress that the primary difference between imprinted and random XCI is the chromosome from which the *Xist* lncRNA is expressed: the maternally or paternally inherited X. It follows that imprinted and random XCI utilize near-identical mechanisms for gene silencing: in the context in which they have been studied, imprinted and random XCI occur in the same organism (mouse); require the same lncRNA (*Xist*; Kalantry et al., 2009; Namekawa et al., 2010; Penny et al., 1996); and result in the deposition of the same histone modifications (H3K27me3 and H2AK119ub1 among others) over the same chromosome (the inactive X; Kalantry et al., 2006; Mak et al., 2002; Okamoto et al., 2004; Plath et al., 2003; Silva et al., 2003). Moreover, *Xist* associates with the same proteins in embryonic and extraembryonic cells (Chu et al., 2015). Thus, the heightened requirement for PRC2 in the maintenance of XCI in the extraembryonic tissues seems unlikely to be due to an unusual mechanism of *Xist*-induced silencing in that lineage. Instead, it hints at a broader requirement for PRC2 in the maintenance of XCI, even within tissues of the embryo proper.

Consistent with a role for PRC2 in the maintenance of XCI, the deletion of EED in mouse embryonic fibroblasts (MEFs) has been shown to cause a dramatic reduction in the levels of both H3K27me3 and also H2AK119ub1 on the inactive X (Colognori et al., 2019). Thus, contrary to mESCs, in MEFs, a substantial, two-way cooperativity exists between PRC1 and PRC2: in order to be enriched on the inactive X, the bulk of PRC2 requires the action of PRC1, but so does the bulk of PRC1 require the action of PRC2 (Colognori et al., 2019; Nesterova et al., 2019; Schoeftner et al., 2006). Upon deletion of PRC1 or PRC2 in MEFs, gene silencing was not directly investigated, but prior works have shown that even deletion of *Xist* itself does not cause substantial reactivation of the inactive X in MEFs, owing to the multiple epigenetic pathways that function to maintain X-linked silencing in these and other differentiated cells; these pathways include the incorporation of variant histones, the methylation of histone H3 lysine9, and the methylation of DNA (Csankovszki et al., 1999; Csankovszki et al., 2001; Escamilla-Del-Arenal et al., 2013; Pasque et al., 2014). Thus, in MEFs, the substantial erosion of PRC-deposited chromatin modifications upon deletion of EED or RING1A/B would not be expected to coincide with immediate gene reactivation (Colognori et al., 2019). Notably, investigators of this same study found that deletion of EED or simultaneous deletion of RING1A and B caused a reduced ability of *Xist* to associate with chromatin of the Xi, unexpectedly suggesting that the PRCs are important not only for modifying chromatin during XCI, but also for mediating the spread of *Xist* over the inactive X (Colognori et al., 2019). Subsequent work (described below) raises the possibility that the PRCs may have a general role in mediating the spread of *Xist*-analogous lncRNAs over chromatin (Schertzer et al., 2019).

While the PRCs are important for the early maintenance of gene silencing during XCI and for establishing a silent state of the inactive X that is propagatable through cell division, they may not be as important for the initial wave of transcriptional silencing induced by *Xist*. The study that first linked PRC2 to XCI found that the timing of X-linked gene reactivation upon EED knockout was most consistent with a role for PRC2 in the maintenance of gene silencing during XCI and not the initiation (J. Wang et al., 2001). A subsequent study identified a region within *Xist* critical for the initiation of gene silencing (termed its “Repeat A” region; Wutz et al., 2002). Shortly thereafter, it was recognized that *Xist* transgenes lacking Repeat A were still capable of recruiting PRCs to the bulk of the X chromosome

even though silencing by *Xist* was severely compromised (Chaumeil et al., 2006; Kohlmaier et al., 2004; Plath et al., 2003). Carefully timed immunofluorescence assays demonstrated that hallmarks of gene silencing—visible exclusion of RNA Polymerase II, loss of Cot-1 RNA staining, and histone hypoacetylation—all occurred just before the enrichment of PRCs over the inactive X (Chaumeil et al., 2006; Okamoto et al., 2004). More recent genomic assays confirmed and extended these observations, demonstrating not only that the silencing of active transcription and histone de-acetylation precede the recruitment of PRCs to the Xi, but also that an *Xist* transgene lacking Repeat A can recruit PRCs to intergenic or already-silent regions of the Xi, yet it cannot recruit PRCs over actively transcribed loci (Barros de Andrade et al., 2019; Zylicz et al., 2019). Thus, a picture emerges in which *Xist* engages two silencing pathways at the onset of XCI: an initial pathway, which leads to the transcriptional silencing of active loci on the X and requires the Repeat A region of *Xist* and a protein called SPEN (Dossin et al., 2020), and a second pathway, which serves to lock in the silent state through subsequent cell divisions. It is this latter pathway that involves the PRCs and multiple regions within the *Xist* transcript that are described in the subsection below.

Still, it should be noted that the PRCs may do more than simply maintain gene silencing established by the Repeat A region of *Xist*. In undifferentiated mESCs, dual knockout of *Pcgf3* and *Pcgf5* causes what appears to be a complete failure of *Xist*-induced gene silencing at the earliest timepoint investigated—a mere 24 h after induction of *Xist* (Almeida et al., 2017). If the function of the PRCs were merely to maintain gene silencing induced by a Repeat A-dependent pathway, then it would appear that in the absence of *Pcgf3* and *Pcgf5*, whatever gene silencing is induced by Repeat A is rather weak—indeed, it is either undetectable or completely unstable 24 h after the induction of *Xist* (Almeida et al., 2017). In light of this result, we hesitate to categorize the PRCs as simple custodians of a Repeat A-dependent silencing pathway. Instead, it would seem that they play important roles even in the early stages of *Xist*-induced gene silencing. In support of this idea, we recently found that a transgene comprising the first 2 kb of *Xist*, which contains Repeat A but lacks all other known functional domains in *Xist*, still binds the critical silencing factor SPEN and associates robustly with chromatin, yet it is essentially incapable of inducing gene silencing, even of adjacent genes (Trotman et al., 2020). The reasons for this lack of silencing are currently unclear, but at a minimum our data indicate that Repeat A cooperates with other regions in *Xist* to induce gene silencing at the onset of XCI (Trotman et al., 2020). The findings from Almeida et al. would suggest that the PRCs play a part in filling this cooperative role (Almeida et al., 2017).

3.1.2 | Xist and the polycomb repressive complexes: Mechanisms of engagement—Since the PRCs themselves do not have strong preferences for specific RNA sequences, it remains to be determined how certain lncRNAs recruit PRCs to chromatin so effectively. In the case of *Xist*, which is overwhelmingly the best-studied lncRNA regarding its interactions with the PRCs, findings published within the last few years have brought some level of clarity to this topic.

3.1.3 | *Xist* transcript structure—*Xist*** harbors an unusual transcript structure, being one of the longest spliced RNAs in the mammalian genome. For example, in humans, the longest spliced form of *Xist* is longer than 99.9% of other spliced transcripts, and its first and last exons themselves are each longer than 99.9% of other human exons (Frankish et al., 2019; Yates et al., 2020). Interspersed between its unusually long first and last exons are four to five smaller exons that collectively make up only about 3% of the length of the longest annotated *Xist* transcript. Both the length and splicing patterns of *Xist* are some of its most conserved features, implying that they are somehow important for *Xist* function (Figure 5(a); Brockdorff et al., 1992; Brown et al., 1992; Frankish et al., 2019; Nesterova et al., 2001; Yates et al., 2020; Yen et al., 2007). However, the relevance of RNA length and splicing within *Xist* remain unclear (Yue & Ogawa, 2018).

Nevertheless, within its two longest exons, the *Xist* transcript harbors at least five domains of tandemly repeated sequence (Figures 4 and 5). Each of these domains is required for different aspects of *Xist* function, and all of them have been implicated, either directly or indirectly, in the ability of *Xist* to recruit the PRCs to the inactive X. The domains are named by the order in which they appear in the *Xist* transcript—Repeats A, B, C, D, and E. The repeats vary dramatically in both their length and sequence composition: the shortest, Repeat B, is roughly approximately 200 nucleotides in length and mostly consists of variations on a single repeated pentamer, CCCCA, whereas the longest, Repeat D, is upward of 6 kb in length and is comprised of a degenerate monomeric unit that itself is apporximately 300 nucleotides long. Repeats A, B, and E are conserved among eutherian mammals, whereas Repeat C is rodent-specific, and Repeat D or D-like sequence is present in most other non-rodent, eutherian mammals (Brockdorff et al., 1992; Brown et al., 1992; Nesterova et al., 2001; Sprague et al., 2019; Yen et al., 2007).

Over the last several years, it has become clear that each of the tandem repeats in *Xist* function as domains that directly engage with specific subsets of RNA-binding proteins (RBPs). Various forms of mass spectrometry, CLIP, and other methods to screen RNA–protein interactions have identified distinct sets of proteins that associate with each of the tandem repeats in *Xist*. Repeat A associates with the critical silencing factor SPEN along with a number of RBPs whose roles in XCI remain unknown, including many SR proteins, HNRNPC, and RALY (Chu et al., 2015; Cirillo et al., 2016; Graindorge et al., 2019; Pintacuda, Wei, et al., 2017; Trotman et al., 2020); Repeat B associates predominantly with HNRNPK (Cirillo et al., 2016; Colognori et al., 2019; Nakamoto et al., 2020; Pintacuda, Wei, et al., 2017); Repeat C also associates with HNRNPK and other RBPs, including HNRNPU (Bousard et al., 2019; Cirillo et al., 2016; Graindorge et al., 2019); Repeat D, while not studied as extensively as the other *Xist* repeats, also binds HNRNPK (Van Nostrand et al., 2016); and Repeat E associates with PTBP1, MATR3, TDP-43, CELF1, and CIZ1, among other proteins (Cirillo et al., 2016; Pandya-Jones et al., 2020; Ridings-Figueroa et al., 2017; Smola et al., 2016; Sunwoo et al., 2017; Van Nostrand et al., 2016). The underlying sequence and structural motifs that are unique to each of these repeats may underlie their ability to recruit distinct subsets of RBPs (Figures 4 and 5; Duszczyk et al., 2011; Fang et al., 2015; F. Liu et al., 2017; Z. P. Lu et al., 2016; Maenner et al., 2010; Smola et al., 2016; P. Y. Wang, Sexton, et al., 2019; Weidmann et al., 2020).

3.1.4 | *Xist* Repeats B, C, and D—In regard to controlling the PRCs, within mouse *Xist*, the most important interactions appear to be between Repeats B and C and the RBP called HNRNPK. Deletion of Repeats B and C causes a near-complete loss of the PRCs and PRC-induced chromatin modifications over the inactive X; no other region within *Xist* is known to play as dominant of a role in recruiting the PRCs (Bousard et al., 2019; Colognori et al., 2019; da Rocha et al., 2014; Nesterova et al., 2019; Pintacuda, Wei, et al., 2017; Wutz et al., 2002). Proteomic studies identified a direct interaction between HNRNPK and Repeat B (Colognori et al., 2019; Pintacuda, Wei, et al., 2017), and the significance of this interaction is supported by the fact that Repeat B itself is essentially comprised of approximately 30 tandemly arrayed copies of a consensus HNRNPK-binding motif (Backe et al., 2005; Dominguez et al., 2018; D. Ray et al., 2013; Sprague et al., 2019). Mechanistically, HNRNPK-binding by Repeats B and C is thought to directly recruit vPRC1 to *Xist*: in vitro, HNRNPK directly interacts with vPRC1 but not cPRC1 via HNRNPK's intrinsically disordered P/R/G-rich linker domain (El-Gebali et al., 2019; Pintacuda, Wei, et al., 2017); in cells, tethering HNRNPK to an *Xist* transgene that lacks Repeat B and a portion of Repeat C restores PRC recruitment to the inactive X, but tethering a mutant version of HNRNPK that lacks its vPRC1-binding domain does not (Pintacuda, Wei, et al., 2017); and RNA pulldown of native *Xist* followed by mass spectrometry reveals that while wild-type *Xist* associates robustly with HNRNPK and PCFG3/5-vPRC1, a mutant form of *Xist* lacking Repeats B and C does not (Bousard et al., 2019). HNRNPK may also interact with PRC2 (Denisenko & Bomsztyk, 1997), raising the possibility that in addition to vPRC1, HNRNPK may promote the association between PRC2 and *Xist*.

HNRNPK is one of the most abundant RBPs in the cell, is ubiquitously expressed, and has roles in virtually all aspects of RNA metabolism, including transcription, splicing, RNA stability, and translation (Bomsztyk et al., 2004; Gallardo et al., 2016; Hein et al., 2015). Thus, beyond *Xist*, HNRNPK interacts with thousands of other RNAs, many of which probably have no role in recruiting PRCs to chromatin. In this regard, it is important to consider not only that *Xist* binds HNRNPK, but also *the manner* in which *Xist* binds HNRNPK. As mentioned above, Repeat B is comprised of approximately 30 tandemly arrayed, near-consensus HNRNPK-binding sites (Dominguez et al., 2018; D. Ray et al., 2013; Sprague et al., 2019), and recent work suggests that these sites are present in a structural context optimized for HNRNPK-binding (Nakamoto et al., 2020). Thus, Repeat B engages with HNRNPK in a manner that is likely distinct from most other RNAs. Moreover, during the early stages of XCI, Repeat B alone is insufficient to recruit wild-type levels of the PRCs to the inactive X—a second domain is required—in mouse, this domain is Repeat C (Bousard et al., 2019). In terms of its sequence composition, Repeat C is far more complex than Repeat B, being comprised of 14 tandemly arrayed copies of a 120 nucleotide-long monomer (Brockdorff et al., 1992). Repeat C is also enriched in HNRNPK-binding motifs, although not to the same extent as Repeat B (Sprague et al., 2019), and CLIP data show that like Repeat B, Repeat C also associates with HNRNPK in cells (Cirillo et al., 2016). In addition to Repeat B, this requirement for Repeat C, a second adjacent domain that engages with HNRNPK, almost certainly serves to distinguish *Xist* from other chromatin-bound transcripts. Lastly, *Xist* is one of the most abundant chromatin-associated RNAs and may have one of the longest half-lives, especially when compared with pre-mRNAs, another

class of very long, chromatin-associated RNAs that bind HNRNPK (Mukherjee et al., 2017; Schertzer et al., 2019). Furthermore, the binding of HNRNPK by *Xist* may itself contribute to the abundance of *Xist* on chromatin (Beletskii et al., 2001; Colognori et al., 2019; Sarma et al., 2010; M. D. Simon et al., 2013). Thus, it seems likely that via Repeats B and C, *Xist* engages with HNRNPK using a mechanism that is distinct from most other RNAs. This distinct mechanism of engagement coupled with *Xist*'s high abundance and its stability on chromatin may be one of the major reasons why *Xist* expression recruits PRCs to chromatin so effectively.

Despite its function, Repeat C is one of the least conserved regions in *Xist*, being present only in rodents (Nesterova et al., 2001; Yen et al., 2007). However, in non-rodent eutherian mammals, there is a separate domain, Repeat D, that appears to be a functional analogue of Repeat C. Repeat D is arguably the most striking feature in non-rodent *Xist*, occupying upward of one-third or more of the transcript (Nesterova et al., 2001; Yen et al., 2007). It is also a complex and degenerate repeat. In humans, the Repeat D region contains a core of eight copies of a tandemly arrayed, approximately 290 nucleotide monomer that is surrounded by an additional approximately 18 copies of degenerate, Repeat D-like sequence (Brown et al., 1992; Nesterova et al., 2001; Yen et al., 2007). Remarkably, while Repeat D has no linear sequence similarity with Repeat C, CLIP data show that like Repeat C, Repeat D associates with HNRNPK in cells (Cirillo et al., 2016; Van Nostrand et al., 2016), and relative to other lncRNAs, both Repeats C and D are enriched in similar subsets of protein binding-motifs, including but not limited to motifs that bind HNRNPK (Sprague et al., 2019). To date, there have been only two studies of Repeat D, with one suggesting that it is essential for the function of human *Xist* (Lv et al., 2016), and another suggesting that its deletion has no effect (Lee et al., 2019). In the latter study, however, it should be noted that the targeted deletion of Repeat D included only its core and not the surrounding degenerate repeats, and that the deletion was made in human K562 cells, which for unclear reasons, do not harbor an enrichment of the PRCs over their inactive X even when expressing wild-type *Xist* (Lee et al., 2019). Therefore, we would surmise that Repeat D plays an essential role in the function of non-rodent *Xist* and that it is indeed the functional analogue of Repeat C: Repeat D-like sequence is one of the most conserved features of non-rodent *Xist* (Nesterova et al., 2001; Sprague et al., 2019; Yen et al., 2007), it shares a remarkable level of nonlinear sequence similarity with Repeat C (Sprague et al., 2019), both regions bind HNRNPK in cells (Cirillo et al., 2016; Van Nostrand et al., 2016), and defects in gene silencing have been observed upon deletion of Repeat D in human cells (Lv et al., 2016).

The expansion of Repeats C and D in rodent and non-rodent eutherian mammals, respectively, underscores just how rapidly functional domains can evolve and disappear in lncRNAs. The lack of linear sequence similarity between Repeats C and D and their sequence complexity relative to the other repeats in *Xist* implies a role for RNA structure in mediating their functions. These same features also underscore an important theme, especially in the context of this review: separate lncRNAs can and perhaps often do encode similar functions through different spatial arrangements of related sequence or structural motifs (Hezroni et al., 2015; Johnson & Guigo, 2014; Kelley et al., 2014; Kirk et al., 2018; Lubelsky & Ulitsky, 2018; Quinn et al., 2016; Shukla et al., 2018; Sprague et al., 2019; Ulitsky et al., 2011).

3.1.5 | *Xist* Repeat A—In addition to Repeats B, C, and D, Repeat A has been implicated in the recruitment of PRCs to *Xist* and the inactive X. In vitro, Repeat A directly interacts with PRC2 with measurably higher affinity than control sequences, and this interaction is sensitive to mutations in Repeat A that disrupt its ability to form secondary structures (Cifuentes-Rojas et al., 2014; Davidovich et al., 2015; Zhao et al., 2008). In vivo, deletion of Repeat A from *Xist* has been shown to reduce the accumulation of PRCs over the inactive X in some studies (Kohlmaier et al., 2004; Zhao et al., 2008), but not in another (da Rocha et al., 2014). However, the ability of *Xist* to recruit PRCs to chromatin is likely directly dependent on its abundance (Schertzer et al., 2019), and Repeat A deletions can reduce the abundance of *Xist* (Chow et al., 2007; Colognori et al., 2019; Ha et al., 2018; Hoki et al., 2009; Lee et al., 2019; Z. Lu et al., 2020; Nesterova et al., 2019; Pintacuda, Wei, et al., 2017; Royce-Tolland et al., 2010; Y. Wang, Zhong, et al., 2019). Thus, historically, it has been challenging to disentangle the effects that Repeat A deletions have on PRC recruitment from the effects that deletions have on *Xist* abundance.

Most recently, a new Repeat A deletion was made that had no discernable effect on *Xist* abundance (Colognori et al., 2020). Using this new allele, the authors discovered that while deletion of Repeat A by itself had no noticeable effect on PRC recruitment, deletion of Repeat A in concert with deletion of Repeat B caused a complete loss of PRC enrichment over the inactive X, despite the presence of a functional Repeat C (Colognori et al., 2020). Therefore, not only does *Xist* require Repeats B and C to recruit PRCs to the inactive X, but it also requires Repeat A (Colognori et al., 2020).

While it is formally possible that deletion of Repeat A affects PRC recruitment indirectly, by disrupting the RNA structure of the nearby PRC-recruitment domains Repeats B and C, we do not favor this interpretation. RNA structure probing by SHAPE has shown that in the context of native, full-length *Xist* RNA and in the context of an in vitro transcribed 5′ fragment of *Xist* (its “RepA” region), the structural properties of Repeat A are essentially identical, suggesting no significant RNA–RNA interactions occur between Repeat A and downstream regions in *Xist* (F. Liu et al., 2017; Smola et al., 2016). Analysis of RNA duplexes crosslinked by psoralen supports this view (Z. P. Lu et al., 2016).

Still, the mechanism by which Repeat A functions in PRC recruitment remains incompletely defined. Intuitively, considering that PRC2 has only a modest in vitro preference for Repeat A over other length-matched control RNAs (Davidovich et al., 2015), we would surmise that direct interactions between PRC2 and Repeat A are, in and of themselves, insufficient to explain the recruitment of PRC2 to the inactive X by *Xist*. Nevertheless, direct interactions between PRC2 and Repeat A seem likely to be important (Cifuentes-Rojas et al., 2014; Davidovich et al., 2015; Zhao et al., 2008), a notion that is reinforced by recent work demonstrating that PRC2 is evicted from chromatin upon disruption of its RNA-binding ability (Long et al., 2020). It also remains possible that in addition to direct interactions with PRC2, Repeat A could indirectly recruit PRC2 (and possibly PRC1) through bridged interactions with associated RBPs, much in the way that Repeats B and C rely on HNRNPK to recruit vPRC1 to *Xist* (Pintacuda, Wei, et al., 2017; Wei et al., 2016). Consistent with this notion are data showing that a significant fraction of the PRC2 that surrounds the inactive

X is spatially separated from *Xist* (Cerase et al., 2014; Markaki et al., 2020; Sunwoo et al., 2015).

3.1.6 | *Xist* Repeat E—Repeat E has also been implicated in the enrichment of PRCs over the inactive X. Deletion of Repeat E or several of its interacting proteins results in dispersal of *Xist* away from the inactive X and a coincident loss of H3K27me3, particularly in the latter stages of XCI (Pandya-Jones et al., 2020; Ridings-Figueroa et al., 2017; Sunwoo et al., 2017). Here, given that Repeat E deletion causes *Xist* to de-localize from the inactive X, Repeat E loss would be expected to cause a loss of PRCs over the inactive X, regardless of whether it harbored an ability to interact with the PRCs. Thus, the current consensus is that the involvement of Repeat E in PRC recruitment is indirect, being more related to Repeat E's role in *Xist* localization than being involved in PRC recruitment per se (Pandya-Jones et al., 2020; Ridings-Figueroa et al., 2017; Sunwoo et al., 2017). Nevertheless, in RIP assays performed from formaldehyde-crosslinked human cells, components of both cPRC1 and PRC2 are markedly enriched over Repeat E, raising the possibility that Repeat E also plays a more direct role in PRC recruitment (Hendrickson et al., 2016; M. K. Ray et al., 2016).

3.1.7 | Interdependency between *Xist* repeats—In considering the various roles for the *Xist* repeats in recruiting PRCs to the inactive X, it should be noted that most studies have focused their investigations on the necessity of the repeats, and not their sufficiency. For example, despite the importance of Repeats B and C in *Xist*-mediated PRC recruitment, it remains unknown to what extent, if any, the expression of a transgenic RNA comprised solely of Repeats B and C would recruit PRCs to chromatin. However, studies suggest that when expressed in isolation, the functions of the individual *Xist* repeats are not portable. In the first to identify functional domains within *Xist*, it was found that a transgenic RNA comprised of the first 3 kb of *Xist* was incapable of inducing long-distance gene silencing —this fragment contained Repeat A, the domain within *Xist* essential for long-distance gene silencing, but it lacked all other portions of the transcript, including Repeats B, C, and E (Wutz et al., 2002). Extending these findings, we similarly found that a transgenic RNA comprised of the first 2 kb of *Xist* was retained on chromatin and bound the critical *Xist* silencing factor SPEN, but was essentially incapable of inducing gene silencing even of nearby genes (Trotman et al., 2020). In another study, in the context of a 3.9-kb *Xist* transgene that contained Repeat A, Repeat B, and a portion of Repeat C, deletion of either Repeat A or the Repeat B/C portion of the transgene caused a loss of its ability to silence gene expression and recruit the PRCs (Pintacuda, Wei, et al., 2017). Thus, even though Repeats A, B, and C may all be necessary for *Xist* to silence genes and recruit PRCs to the inactive X, none of them appear to be capable of doing so on their own.

3.2 | Rsx

Although *Xist* is an essential gene in placental mammals (i.e., eutherians), it is not conserved outside of the eutherian lineage. Nevertheless, marsupial mammals (i.e., metatherians) undergo XCI, and for some time, it appeared that marsupial XCI proceeded independently from the action of any lncRNA (Al Nadaf et al., 2012; Chaumeil et al., 2011; Graves, 2016). This notion was called into question in 2012 with the discovery of a lncRNA called *Rsx*

(Grant et al., 2012). *Rsx* is located in a different region of the X chromosome and evolved independently from *Xist*. Yet, *Rsx* shares many striking similarities with *Xist*. Like *Xist*: *Rsx* is only expressed in females but is expressed across female tissues; it is spliced yet unusually long (~27 kb), with the majority of its length deriving from a single approximately 25-kb exon; the majority of its sequence can be partitioned into one of four large tandem repeats; it is exclusively nuclear, expressed only from the inactive X chromosome, and localizes in a cloud-like pattern around that chromosome; and when *Rsx* is expressed as a transgene from a mouse chromosome, it remains nuclear, localizes in a cloud-like pattern, and appears to induce modest levels of gene silencing (Grant et al., 2012). Subsequent work demonstrated that the marsupial inactive X chromosome is transcriptionally silent and decorated in H3K27me3, analogous to the eutherian inactive X (X. Wang et al., 2014). Most recently, it was shown that the expression of *Rsx* in the marsupial embryo is coincident with the initiation of gene silencing on the future inactive X (Mahadevaiah et al., 2020). Thus, while genetic dependency has yet to be demonstrated (i.e., *Rsx* has not been knocked out in a marsupial, insofar as we are aware), *Rsx* appears to be a functional analogue of *Xist* in marsupial mammals.

At first glance, given the striking similarities between *Xist* and *Rsx*, perhaps that which is most surprising is what is missing between them—linear sequence similarity. One can compare *Xist* to *Rsx* via any number of linear sequence alignment algorithms, including BLAST, nhmmer, or Stretcher, and identify essentially no regions of significant similarity between the two lncRNAs (Altschul et al., 1990; Rice et al., 2000; Sprague et al., 2019; Wheeler & Eddy, 2013). However, upon further reflection, one might consider that linear alignment algorithms were designed to detect evolutionary relationships, and that *Xist* and *Rsx* evolved independently from one another (Grant et al., 2012). Thus, simply because the two lncRNAs lack apparent linear sequence similarity does not necessarily indicate that they are completely dissimilar. Instead, it may indicate that the sequence similarity they do share is not detectable by conventional linear alignment.

With this consideration in mind, we recently compared the tandem repeats in *Xist* and *Rsx* using an approach we developed called “SEEKR,” in which sequences are compared not by their linear arrangements, but instead by their composition of short sequence substrings called *k*-mers (Kirk et al., 2018). SEEKR is analogous to what is called a “bag-of-words” model, an approach used in natural language processing in which large bodies of text are compared by breaking them up into constituent word profiles without regard to grammar or syntax (McTear et al., 2016). The idea behind SEEKR is that lncRNAs with related functions may be enriched in similar subsets of RNA “words” (i.e., *k*-mers or protein-binding motifs), even if the lncRNAs are evolutionarily unrelated and harbor no obvious linear sequence similarity (Kirk et al., 2018). By breaking up lncRNAs or their functional domains into short sequence fragments of length *k* (where *k* is typically 4, 5, or 6), then using Pearson’s correlation to compare *k*-mer profiles between RNA species, nonlinear similarity may emerge. Indeed, when comparing the repeat domains in *Xist* and *Rsx* at the level of *k*-mers, we found that they share substantial nonlinear sequence similarity (Sprague et al., 2019). This similarity was equally apparent when we examined the enrichment of protein-binding motifs. Specifically, at the level of *k*-mers and protein-binding motifs, we found that Repeats B, C, and D in *Xist* were most similar to the first repeat domain within

Rsx, whereas Repeats A and E were most similar to the final two *Rsx* repeats. These patterns of similarity were found in the sequence of both koala and opossum *Rsx*, although the trends of similarity in opossum were not as strong as they were in koala, implying that there may not be a one-to-one functional relationship between repeat domains in *Xist* and *Rsx* (Sprague et al., 2019). RIP experiments demonstrated that in a manner that is perhaps analogous to *Xist*, *Rsx* also associates with HNRNPK (Sprague et al., 2019). The data support the notion that *Xist* and *Rsx* convergently evolved to carry out similar functions, and provide indirect evidence that they do so through related mechanisms (Grant et al., 2012; Sprague et al., 2019).

In regard to recruiting the PRCs, it is important to note that on a length-normalized basis, many protein-binding motifs are more enriched in the repeats of *Xist* and *Rsx* than in essentially any other mouse lncRNA (Sprague et al., 2019). Moreover, many of the most-enriched motifs are known to recruit ubiquitously expressed RBPs, including known *Xist* co-factors HNRNPK, MATR3, and PTBP1 (Colognori et al., 2019; Pandya-Jones et al., 2020; Pintacuda, Wei, et al., 2017; Sprague et al., 2019). Thus, a hypothesis emerges in which *Xist* and *Rsx* may recruit the PRCs not necessarily by recruiting a set of dedicated RNA silencing factors, but by engaging with ubiquitously expressed RBPs in ways that are distinct from other RNAs. Specifically, when pushed to high concentrations, such as those that may be brought about by binding the repeat domains in *Xist* or *Rsx*, many RBPs form condensates that promote homotypic and heterotypic interactions with other proteins and with RNA (Roden & Gladfelter, 2020). Indeed, at least two proteins whose binding-motifs are highly enriched in *Xist* and *Rsx*, PTBP1 and MATR3, interact with *Xist* through Repeat E to promote the formation of a semi-stable condensate that helps retain *Xist* and other proteins in the vicinity of the inactive X (Pandya-Jones et al., 2020; Weidmann et al., 2020). Relatedly, HNRNPK directly interacts with vPRC1 and *Xist*, helps retain *Xist* and other RNAs on chromatin, and is soluble in condensates called stress granules (Colognori et al., 2019; Fukuda et al., 2009; Lubelsky & Ulitsky, 2018; Moujalled et al., 2015; Nakamoto et al., 2020; Pintacuda, Wei, et al., 2017). Thus, *Xist* and *Rsx* are both very long chromatin-associated RNAs that harbor domains that are enriched in similar subsets of protein-binding motifs. Some of the proteins that bind these domains form condensates that can accumulate higher concentrations of protein than could otherwise be recruited by RNA alone, in the absence of protein condensation. In turn, it is conceivable that such condensates help *Xist*, *Rsx*, and other functionally analogous lncRNAs recruit supernumerary copies of epigenetic regulators, including the PRCs. For related reviews on this subject, see (Brockdorff, 2018; Cerase et al., 2019; Cerase & Tartaglia, 2020).

4 | IMPRINTED lncRNAs

By most accounts *Xist* is an outlier among lncRNAs: it is longer, more abundant, more stable, more potent, and more conserved than most other lncRNAs in mammals (Cabili et al., 2011; Cabili et al., 2015; Clark et al., 2012; Derrien et al., 2012; Iyer et al., 2015; Mukherjee et al., 2017; Schertzer et al., 2019). Yet, it remains one of the best-studied lncRNAs and a lens through which other lncRNAs are often viewed. However, even in eutherian mammals, *Xist* is not the only lncRNA that utilizes the PRCs to repress gene expression. Studies of a regulatory phenomenon termed genomic imprinting have led to

the discovery of several lncRNAs whose expression may control the PRCs in a manner analogous to *Xist*. Genomic imprinting is itself an epigenetic process that results in genes being preferentially expressed from one parentally inherited chromosome over the other (Monk et al., 2019). In the mouse, there are at least three imprinted regions in which gene silencing appears to depend on a lncRNA and the PRCs. The lncRNAs expressed in these three regions—*Airn*, *Kcnq1ot1*, and *Meg3*—are described sequentially in the subsections below (Figure 6).

4.1 | Airn

Airn (antisense *Igf2r* RNA) is a 90 to 120 kb lncRNA that is expressed from the paternally inherited copy of chromosome 17 in mouse and transcribed in the antisense direction over the promoter of a protein-coding gene called *Igf2r* (Lyle et al., 2000). Like other RNA Polymerase II transcripts, *Airn* is capped and to a certain extent polyadenylated, but it is unstable, exclusively nuclear, and predominantly unspliced; this last characteristic especially is unusual for an RNA of *Airn*'s length (Huang et al., 2011; Schertzer et al., 2019; Seidl et al., 2006). Moreover, analysis of RNA-seq data has shown that as the distance from the *Airn* transcriptional start site increases, the level of *Airn* transcription decreases, presumably due to increased transcriptional termination (Huang et al., 2011; Koerner et al., 2012; Seidl et al., 2006). Thus, the *Airn* locus does not produce a single lncRNA product; rather, it produces a mix of lncRNAs that vary in length and 3′ end composition (Huang et al., 2011; Koerner et al., 2012; Seidl et al., 2006).

In the mouse embryo, the transcription of *Airn* results in the silencing of the single gene whose promoter it overlaps, *Igf2r* (Sleutels et al., 2002; Wutz et al., 1997). In the extraembryonic tissue of the mouse, the transcription of *Airn* represses genes to varying extents within a broad approximately 13 Mb window (Andergassen et al., 2017; Schertzer et al., 2019; Zwart et al., 2001). These functions of the *Airn* locus appear to be restricted to rodents. In humans, the locus that is syntenic to *Igf2r* does produce an antisense lncRNA, but this lncRNA does not appear to extend over the *Igf2r* promoter, and *Igf2r* and its surrounding genes do not appear to be imprinted (Killian et al., 2001; Yotova et al., 2008). Additionally, in marsupials, even though *Igf2r* is imprinted and there is evidence that an antisense lncRNA is produced from within the marsupial the *Igf2r* gene, this antisense lncRNA appears to be approximately 650 nucleotides long, nowhere near the length of *Airn*, and it does not appear to overlap the *Igf2r* promoter (Suzuki et al., 2018).

Mechanistically, the silencing of *Igf2r* by *Airn* is due to the act of *Airn* transcription over the *Igf2r* promoter and not to an intrinsic function of the *Airn* lncRNA itself (Latos et al., 2012). This observation, coupled with the atypical features of the *Airn* transcript and its lack of apparent conservation, raises the question of whether the *Airn* locus encodes a functional lncRNA, or instead, induces gene silencing by transcribing through DNA regulatory elements contained within the *Airn* gene body (Pauler et al., 2012).

While this central question remains incompletely resolved, an accumulating body of evidence supports the notion that the *Airn* locus produces a functional lncRNA that shares aspects of its mechanism with *Xist*. Silencing in the *Airn* imprinted region is partially lost upon deletion of the enzyme EHMT2/G9A, a histone H3 lysine 9 methyltransferase, which

associates with the *Airn* lncRNA in RIP assays (Nagano et al., 2008). The enrichment of EHMT2 and the chromatin modifications it catalyzes are hallmarks of the inactive X chromosome, and in mouse, EHMT2 enrichment depends on epigenetic modification by PRC2 (Escamilla-Del-Arenal et al., 2013; Rougeulle et al., 2004); thus, we would consider *Airn*'s dependence on EHMT2 as evidence in favor of *Airn* encoding a functional lncRNA that shares aspects of mechanism with *Xist*. Moreover, while gene silencing within the *Airn* imprinted domain has yet to be extensively investigated upon deletion of either PRC1 or PRC2, in cells of the extraembryonic lineage, the 13 Mb that surrounds *Airn* is heavily enriched in H2AK119ub1 and H3K27me3, and these enrichments depend on continued *Airn* expression (Andergassen et al., 2019; Schertzer et al., 2019). Extending this connection between the PRCs and the *Airn* lncRNA, RIP assays suggest that the *Airn* lncRNA associates with higher levels of HNRNPK than nearly all other chromatin-associated transcripts save *Xist*, and HNRNPK knockdown causes loss of H3K27me3 throughout the imprinted domain (Schertzer et al., 2019). Lastly, within the *Airn* locus, there are no transcriptional regulatory elements whose deletion on the maternal allele affects the maternal expression of distal *Airn*-target genes, implying that distal silencing within the *Airn* domain does not depend on transcriptional interference by the act of *Airn* transcription, and suggesting that the *Airn* lncRNA itself harbors function (Andergassen et al., 2019).

4.1.1 | Differences between the Airn and Xist lncRNAs—

While more work needs to be done, evidence suggests that the *Airn* locus produces a lncRNA whose function is analogous to *Xist*. Under this assumption, and in regard to understanding mechanism, it becomes informative to consider the differences between the *Airn* and *Xist* transcripts. Two of the clearest differences are their abundance and half-lives. In mouse trophoblast stem cells, RNA-seq analyses suggest that *Xist* is 25-times more abundant than *Airn*; the steady-state levels of the two lncRNAs being about 230 and 9 copies per cell, respectively (Schertzer et al., 2019). Relatedly, the half-life of *Xist* is about four times longer than that of *Airn*; approximately 6.2 versus 1.7 h, respectively (Schertzer et al., 2019). Thus, in this single instance, the difference in relative abundance between two lncRNAs scales proportionately with the genomic range over which expression of the lncRNAs directs PRCs to chromatin: approximately 165 Mb for *Xist* and the inactive X, and 13 Mb for *Airn* and its imprinted domain. In that same study, using CRISPR to control the expression of *Airn* from its endogenous promoter, we found that the intensity of H3K27me3 modification and gene silencing within the *Airn* imprinted domain scaled directly with *Airn* lncRNA abundance (Schertzer et al., 2019). Thus, while the act of transcription through the *Airn* gene body may yet play a role in distributing PRCs throughout the *Airn*-target domain, the data also support a direct role for the *Airn* lncRNA. Viewed in that light, at least in the case of *Xist* and *Airn*, our data suggest that lncRNA abundance may be a general feature that correlates positively with an ability to direct PRCs to chromatin.

Other notable differences between the *Xist* and *Airn* lncRNAs involve their splicing, termination, and propensity to diffuse away from their sites of synthesis. Whereas the *Xist* transcript is spliced, terminates robustly at its annotated polyadenylation signals, and can diffuse away from its site of transcription, the *Airn* transcript is predominantly unspliced, does not terminate at its annotated polyadenylation signals, and is retained near its site of

transcription. The act of splicing is now thought to promote both transcriptional termination and transcript release from chromatin (Custodio et al., 1999; Reimer et al., 2021). Thus, the splicing of *Xist*, and the lack thereof in *Airn*, may explain several differences between the two lncRNAs that, in turn, may translate into differences that affect their ability to spread PRCs over chromatin.

Lastly, at the sequence level, *Airn* harbors no obvious linear similarity to *Xist* and does not harbor large tandem repeat domains. However, almost half of *Airn* can be classified as belonging to one of several families of prevalent interspersed repeats (Braidotti et al., 2004; Lyle et al., 2000). Thus, whereas the sequence elements that give *Xist* its ability to control the PRCs are focused predominantly within discrete tandem repeats, the sequence elements that give *Airn* an ability to control the PRCs might be dispersed in broad regions over the length of the lncRNA. This notion is supported by RIP-seq data that suggest HNRNPK is broadly enriched over the 5′ half of *Airn* (Schertzer et al., 2019).

4.1.2 | Genomic features that correlate with polycomb repressive complex activity in the Airn imprinted domain—The *Airn* lncRNA is unstable, retained near its site of transcription, and in mouse trophoblast stem cells, is present at only approximately nine copies per cell. Yet, in that same cell type, *Airn* directs PRCs to a 13-Mb genomic region (Schertzer et al., 2019). In mammalian cells, RNA Polymerase II transcribes at an average rate of 1–2 kb/min (Fuchs et al., 2014; Gregersen et al., 2019; Jonkers et al., 2014; Singh & Padgett, 2009; Veloso et al., 2014; Wada et al., 2009). Thus, the time it may take to transcribe a single copy of a 90 kb *Airn* transcript—45 to 90 min—approaches the length of the lncRNA's half-life in trophoblast stem cells (Schertzer et al., 2019). These data imply that features of the genome itself facilitate the spread of PRCs within *Airn*'s imprinted domain.

There are at least two genomic features that appear to be important in mediating the extent of PRC-induced modification within the *Airn* imprinted domain: 3D genome architecture and CpG island DNA (Andergassen et al., 2019; Nagano et al., 2008; Schertzer et al., 2019). Using DNA/RNA FISH in mouse trophoblast stem cells, we found that the regions within the *Airn* domain that exhibit the highest levels of H3K27me3 on the paternal allele, which expresses the *Airn* lncRNA, are the same regions that are in closest proximity to the *Airn* locus on the maternal allele, which does not express the lncRNA (Schertzer et al., 2019). Furthermore, within the imprinted domain, the CpG islands bound by the PRCs on the maternal allele were the ones that exhibited the highest levels of H3K27me3 on the paternal allele, and deletion of one of these CpG islands on the paternal allele—the one found at the known *Airn*-silenced gene, *Slc22a3*—caused a 4.5-Mb reduction in H3K27me3 within the domain (Schertzer et al., 2019). Relatedly, a seminal study found that the *Airn* lncRNA accumulates at the *Slc22a3* CpG island in placental tissue (Nagano et al., 2008). Lastly, chromosome conformation capture in the visceral yolk sack has shown that interactions between the *Airn* locus and the *Airn*-silenced gene *Slc22a3* are enriched on the maternal allele, which does not express *Airn* (Andergassen et al., 2019). Thus, the 3D conformation of chromatin that surrounds the *Airn* locus, independent of whether *Airn* is expressed, likely plays a role in promoting the spread of PRCs to distal regions within the imprinted domain. Specific, 3D contacts between PRC-bound CpG islands and the *Airn* locus provide

a possible explanation for how a lncRNA such as *Airn*—which is low in abundance, short-lived, and retained at its site of transcription—could promote the spread of PRCs over a broad genomic window (Figure 7; Schertzer et al., 2019).

Three-dimensional contacts also appear to play an important role in spreading PRCs over the inactive X (reviewed in Pandya-Jones & Plath, 2016; see also Barros de Andrade et al., 2019; Bousard et al., 2019; Colognori et al., 2019; Cotton et al., 2014; Engreitz et al., 2013; Kelsey et al., 2015; Loda et al., 2017; Nesterova et al., 2019; Pinter et al., 2012; Schertzer et al., 2019). Here however, the stability of *Xist*, its affinity for actively transcribed loci, and its ability to diffuse away from its site of transcription may lessen its dependence on any single contact or DNA structural feature to mediate PRC spread (Barutcu et al., 2018; Froberg et al., 2018; Schertzer et al., 2019).

4.2 | Kcnq1ot1

Kcnq1ot1 is an 80–95 kb transcript located within an intron of the protein-coding gene *Kcnq1* on human chr11 and mouse chr7, and like *Airn*, is exclusively expressed from the paternally inherited allele (Fitzpatrick et al., 2002; Smilinich et al., 1999). Imprinting in the *Kcnq1ot1* domain and the *Kcnq1ot1* lncRNA itself are conserved in human and mouse and have also been documented in cow and pig (S. Li et al., 2012; Robbins et al., 2012).

Other than the difference in conservation, *Kcnq1ot1* and *Airn* are similar in many respects, implying that they share a mechanism of action. Like *Airn*, the *Kcnq1ot1* lncRNA is predominantly unspliced, unstable relative to *Xist*, associates with chromatin, and terminates precociously over the length of its gene body (Huang et al., 2011; Pandey et al., 2008; Redrup et al., 2009; Schertzer et al., 2019). Also like *Airn*, the *Kcnq1ot1* locus exhibits augmented potency in the extraembryonic tissues of the mouse; in the tissues derived from the embryo proper, the expression of *Kcnq1ot1* silences up to four genes, whereas in the extraembryonic tissues, the number of Kcnq1ot1-silenced genes increases to nine (Fitzpatrick et al., 2002; A. Lewis et al., 2004, 2006; Mancini-Dinardo et al., 2006; Schertzer et al., 2019). Moreover, several transcriptional enhancers are located throughout the length of the *Kcnq1ot1* gene body, and thus the act of *Kcnq1ot1* transcription may play a role in the silencing of the genes in the *Kcnq1ot1* imprinted domain, independent of any function of the lncRNA (Golding et al., 2011; Korostowski et al., 2012; Schultz et al., 2015).

However, in a manner analogous to *Airn*, many findings support a role for the *Kcnq1ot1* lncRNA in promoting gene silencing and PRC spread within its imprinted domain, beyond the mere act of its transcription. Firstly, imprinted silencing within the *Kcnq1ot1* domain also requires PRC1, PRC2, and EHMT2 (Mager et al., 2003; Sachani et al., 2018; Terranova et al., 2008; Wagschal et al., 2008). Moreover, the *Kcnq1ot1* lncRNA associates with PRC2, EHMT2, and the PRC1-binding and RNA-binding protein HNRNPK (Pandey et al., 2008; Schertzer et al., 2019; Zhao et al., 2010). Relatedly, the *Kcnq1ot1* imprinted domain is enriched in H2AK119ub1, H3K27me3, and H3K9me2/3 (the histone modifications deposited by EHMT2; Andergassen et al., 2019; A. Lewis et al., 2004, 2006; Pandey et al., 2008; Schertzer et al., 2019; Umlauf et al., 2004). Even more, when truncated versions of *Kncq1ot1* are expressed in heterologous contexts, local gene silencing is induced, consistent with a function for the lncRNA beyond the act of transcription over its endogenous locus

(Kanduri et al., 2006; Mancini-DiNardo et al., 2003; Thakur et al., 2003; Thakur et al., 2004).

Extending the similarity to *Airn*, 3D interactions between the *Kcnq1ot1* locus and its target genes likely play important roles in mediating gene silencing and PRC spread within its domain (Korostowski et al., 2011, 2012; Redrup et al., 2009; Schertzer et al., 2019; Schultz et al., 2015; Terranova et al., 2008). Also like *Airn*, the *Kcnq1ot1* transcript is enriched in common interspersed repeats (De Donato et al., 2017; Smit et al., 2015), and the sequences that encode its ability to engage with the PRCs may be distributed across broad regions of the transcript. This latter notion is supported by data showing that in a transgenic assay, the intensity of silencing induced by the *Kcnq1ot1* lncRNA increases with the length of the *Kcnq1ot1* fragment being expressed, and RIP-seq data which suggest that, as within *Airn*, HNRNPK-binding is broadly distributed across the 5′ half of the *Kcnq1ot1* transcript (Kanduri et al., 2006; Schertzer et al., 2019).

4.3 | Maternally expressed gene 3

Maternally expressed gene 3 (*Meg3*; also known as *Gtl2*) is an alternatively spliced, nuclear-retained lncRNA produced from a polycistronic transcript that is expressed from the maternally inherited allele located within the *Dlk1-Dio3* imprinted domain on human chromosome 14 and mouse chromosome 12. Imprinting within this region and the polycistronic transcript that contains *Meg3* are highly conserved in eutherians, being present in human, mouse, cow, and sheep (Charlier et al., 2001; Dindot et al., 2004; Edwards et al., 2008; Miyoshi et al., 2000; Paulsen et al., 2001). The polycistronic transcript that contains *Meg3* produces at least three additional lncRNAs, which are themselves precursors to scores of miRNAs and snoRNAs (Cavaille et al., 2002; Seitz et al., 2003, 2004). Accordingly, the transcriptional regulation and posttranscriptional processing of the *Meg3* lncRNA appears to be extraordinarily complex and is not discussed in this review (da Rocha et al., 2008; Girardot et al., 2012; Kota et al., 2014; Sanli et al., 2018).

Nevertheless, *Meg3* is unusually well-conserved for a lncRNA (Charlier et al., 2001; Miyoshi et al., 2000; Paulsen et al., 2001), and prior studies suggest that one of its functions may be to control the PRCs in ways that are both similar and different from *Xist*. The expression of *Meg3* is known to repress transcription (or prevent transcriptional upregulation) of its neighboring protein-coding gene, *Dlk1*, during the transition from embryonic to more differentiated cell states (Sanli et al., 2018). In a manner that is perhaps analogous to *Xist*, *Airn*, and *Kcnq1ot1*, repression of *Dlk1* by *Meg3* occurs exclusively *in cis*, on the maternally inherited allele, and this repression also requires *Ezh2* (Sanli et al., 2018). However, in differentiated mESCs, it was found that genetic ablation of *Meg3* did not appear to affect EZH2-binding or H3K27me3 levels at the *Dlk1* promoter, suggesting that the repression of *Dlk1* by *Meg3* occurs independently from the repression mediated by PRC2 (Sanli et al., 2018). In contrast, a separate study performed in mESCs found that *Meg3* associates with PRC2 in RIP assays, and that shRNA-knockdown of *Meg3* simultaneously de-represses *Dlk1* and reduces PRC2-binding at the *Dlk1* promoter, supporting the idea that one function of *Meg3* is to recruit PRC2 to *Dlk1*, *in cis* (Zhao et al., 2010). In a third study, also performed in mESCs, it was found by CLIP that *Meg3*

associated with both EZH2 and JARID2, but in this work, siRNA knockdown of *Meg3* did not decrease PRC2-binding at *Dlk1*; rather, *Meg3* knockdown appeared to decrease PRC2 occupancy at some of its other genomic targets, thereby implying that *Meg3* can regulate PRC2 function *in trans* (Kaneko, Bonasio, et al., 2014). Separately, work in human cancer cells supports a *trans* function for *Meg3* (Terashima et al., 2017). Here, it was found that *Meg3* associates with PRC2 and regulates PRC2-target genes located outside of the *Dlk1–Dio3* domain (Terashima et al., 2017). RNA FISH data in mouse and human cells support both *cis* and *trans* functions for *Meg3*, showing that the lncRNA accumulates in a large focus around its site of transcription, but also that *Meg3* molecules can be found throughout the nucleoplasm, particularly in differentiated cells (Cabili et al., 2015; Sanli et al., 2018). Lastly, we found in trophoblast stem cells that like *Airn* and *Kcnq1ot1*, the *Meg3* locus sits within a large domain enriched in H3K27me3 and that *Meg3* associates with higher levels of HNRNPK by RIP than essentially any other expressed transcript save *Xist* (Schertzer et al., 2019); these data could support both *cis* and *trans* functions of *Meg3*.

5 | NONIMPRINTED lncRNAs

Expanding upon the previous examples of imprinted lncRNAs, several nonimprinted lncRNAs have also been reported to control gene expression by modulating the PRCs. Many of these lncRNAs were first identified through differential expression analysis of cellular differentiation or disease progression. Through further characterization, it has become clear that they regulate gene expression in diverse contexts that include embryonic development, myogenesis, myeloid cell function, cardiac hypertrophy, spinal muscular atrophy, and various types of cancer. A detailed discussion of each lncRNA is beyond the scope of this review (Amandio et al., 2016; d'Ydewalle et al., 2017; Gupta et al., 2010; Holdt et al., 2013; Huarte et al., 2010; Jia et al., 2016; Jin et al., 2018; Kang et al., 2020; Klattenhoff et al., 2013; Kotake et al., 2011; Kotzin et al., 2016; L. Li et al., 2013; G. Y. Liu et al., 2016; Maamar et al., 2013; Marin-Bejar et al., 2013, 2017; Negishi et al., 2014; Pasmant et al., 2007; Portoso et al., 2017; M. K. Ray et al., 2016; Rinn et al., 2007; Schorderet & Duboule, 2011; Tsai et al., 2010; J. B. Wang, Jin, et al., 2019; Woo et al., 2017; Yap et al., 2010; Yin et al., 2015; J. Yu et al., 2020; W. Yu et al., 2008; K. J. Zhang et al., 2020; Zhou et al., 2015; Zhu & Xu, 2013). However, key aspects of each lncRNA, as well as relevant references, are summarized in Table S1.

Overall, similar experimental strategies have been used to evaluate each lncRNA's role in PRC-mediated gene repression. To varying degrees, these strategies generally include but are not limited to: (a) demonstrating correlations between lncRNA and target-gene expression in a developmental or disease-relevant context, (b) using lncRNA knockdown/knockout and/or overexpression to establish a causative role for the lncRNA in controlling target-gene expression, (c) providing evidence that PRCs regulate target-gene expression via PRC subunit knockdown and/or ChIP for PRC subunits and chromatin modifications, (d) showing that PRC-subunit and chromatin-modification ChIP signal at target genes (e.g., promoters) changes as a function of lncRNA knockdown/knockout and/or overexpression, (e) demonstrating that the lncRNA interacts (in vitro and/or in vivo) with PRC subunits, and (f) using tethering assays or methods such as ChIRP or CHART (Chu et al., 2011; M. D. Simon et al., 2011) to demonstrate that the lncRNA physically associates with the genomic

site(s) of PRC binding. If a lncRNA and target gene are on the same chromosome, ectopic overexpression of the lncRNA is often used to establish if regulation can occur *in trans*; additionally, allele-specific mutational analysis is often a strong way to establish whether regulation occurs *in cis* or *in trans*.

Much is yet to be learned about how nonimprinted lncRNAs engage with the PRCs and the extent to which their mechanisms are similar or different to each other and to the imprinted lncRNAs discussed above. While two of the nonimprinted lncRNAs—*Cat7* and *ANRIL*—have been demonstrated to control both PRC1 and PRC2, the vast majority have been studied in the context of PRC2 regulation. Thus, given the complexity surrounding the nature of PRC2–lncRNA interactions and whether such interactions generally promote or inhibit PRC2 function, it is not surprising that some lncRNAs—*Braveheart*, *Ppp1r1b–lncRNA*, and *Linc-YY1*—are reported to antagonize PRC2 to upregulate gene expression, while most others have been proposed to promote PRC2-mediated gene repression. Indeed, one study estimated that approximately 20% of intergenic lncRNAs interact with PRC2 and that knockdown of these lncRNAs mostly relieved target-gene repression, raising the possibility that the more predominant role for lncRNAs, in general, is in directing the recruitment and/or spread of PRCs at target genes (Khalil et al., 2009). The extent and range of gene regulation by each nonimprinted lncRNA varies considerably as well: some—including *Morrbid*, *Haunt*, and *ANRIL*—use PRCs to regulate the expression of local genes *in cis*, while others have been shown to have more wide-ranging effects on many genes *in trans*.

For reasons discussed in previous sections, it is not clear whether a direct lncRNA–PRC interaction detected in vitro is a biologically meaningful predictor for how the lncRNA engages PRCs inside the cell. In the example of the extensively studied *HOTAIR*, there is contradicting evidence whether the lncRNA binds PRC2 specifically (Tsai et al., 2010) or nonspecifically (Portoso et al., 2017). Indeed, the latter study tethered *HOTAIR* to a reporter locus to demonstrate, surprisingly, that *HOTAIR* represses transcription in a manner independent of PRC2 and that PRC2 is then recruited as a consequence of this silencing, consistent with the notion that PRC2 requires transcriptional silencing for recruitment to chromatin (Kaneko, Son, et al., 2014; Riising et al., 2014). *HOTAIR*'s initial, PRC2-independent silencing mechanism might involve the repressive LSD1/CoREST/REST complex, which has also been shown to interact with *HOTAIR* (Tsai et al., 2010). A similar two-step silencing mechanism has been proposed for *Xist* (Colognori et al., 2020; Zylicz et al., 2019). All of this considered, the mechanistic details and biological functions of PRC recruitment by many nonimprinted lncRNAs remain to be determined.

5.1 | Epigenetic control of the plant FLC gene by PRC2 and lncRNAs COOLAIR, COLDAIR, and COLDWRAP

PRCs regulate developmental and tissue-specific gene expression programs across kingdoms of life. Indeed, it has been proposed that the evolution of PRC-mediated epigenetic regulation was a key driving force enabling the rise of multi-cellularity (Gombar et al., 2014; Schubert, 2019; Shaver et al., 2010; Whitcomb et al., 2007). In plants, homologues of the metazoan PRCs have been well-characterized, and their function, particularly that of PRC2,

has been elucidated in part through study of the *Arabidopsis thaliana FLOWERING LOCUS C* (*FLC*) locus. The mechanisms involved in *FLC* silencing exhibit surprising parallels to those involved in XCI, and thus warrant discussion in the context of this review.

Proper seasonal control of flowering time is essential for plant reproduction, and in many plants, the transition from vegetative growth to flowering occurs in response to a prolonged exposure to cold temperatures. This response to cold, called vernalization, involves epigenetic silencing of the centrally important *FLC* gene, whose protein product is a transcription factor that represses genes essential for flowering (Michaels & Amasino, 1999; reviewed in Madrid et al., 2020). Thus, flowering is promoted by mechanisms that repress expression of *FLC*. Epigenetic silencing of *FLC* is a complex, multistep process that involves both PRC2 and several locally expressed lncRNAs (reviewed in Costa & Dean, 2019).

During exposure to cold, PRC2 induces metastable H3K27me3 modification of *FLC* in an intragenic "nucleation region" at its 5′ end (Figure 8). After a return to warmer growing conditions, PRC2 and H3K27me3 then spread over the rest of the gene to keep *FLC* in a stably silenced state that permits flowering (Angel et al., 2011; Finnegan & Dennis, 2007). Nucleation and spreading of H3K27me3 are distinct, genetically separable phases that involve dynamics of a diverse set of PRC2 core and accessory factors (De Lucia et al., 2008; Yang et al., 2017). Intriguingly, the robustness of H3K27me3 nucleation and spreading increases in proportion to the length of the cold exposure. Accordingly, PRC2-mediated silencing of *FLC* quantitatively translates an environmental stimulus into a form of epigenetic memory (Angel et al., 2011; Finnegan & Dennis, 2007).

The earliest study linking PRC2 activity to *FLC* regulation found that plants with a mutant homologue of PRC2 core component SUZ12 were defective in maintaining an epigenetic memory of cold-induced *FLC* repression (Gendall et al., 2001). Like control plants, these SUZ12 mutants rapidly lost expression of *FLC* mRNA following exposure to cold; however, while the control plants maintained *FLC* repression long after the cold exposure, the SUZ12 mutants readily recovered *FLC* expression (Gendall et al., 2001). Later work concluded that the deposition of H3K27me3 over *FLC* occurs after the *FLC* locus has been transcriptionally silenced (Buzas et al., 2011). Therefore, PRC2 serves to maintain *FLC* silencing but does not appear to cause the initial silencing on its own, resembling the model established for *Xist* and the inactive X chromosome (Chaumeil et al., 2006; Kohlmaier et al., 2004; Nesterova et al., 2019; Okamoto et al., 2004; Plath et al., 2003; Zylicz et al., 2019).

Prior to and independently of PRC2-induced epigenetic changes at the *FLC* locus, the rapid silencing of *FLC* coincides with transcriptional upregulation of a family of antisense lncRNAs, collectively referred to as *COOLAIR*, whose promoter lies near the 3′ end of *FLC* on the opposite strand (Swiezewski et al., 2009). Through unclear mechanisms, *COOLAIR* antisense transcription is thought to cause transient silencing of *FLC* sense transcription, serving as the initial trigger that enables PRC2 and other chromatin modifiers to "lock in" the silenced state (Figure 8; Costa & Dean, 2019; Csorba et al., 2014; Rosa et al., 2016; Swiezewski et al., 2009). Single-molecule RNA FISH revealed that transcription of either *FLC* or *COOLAIR* is mutually exclusive at individual loci (Rosa et al., 2016), but it remains

unknown whether *COOLAIR* silences *FLC* via transcriptional interference or if *COOLAIR* targets the *FLC* locus with repressive, perhaps chromatin-modifying activity. *FLC* silencing can be modulated by alternative splicing and 3′ end formation of *COOLAIR* (Hornyik et al., 2010; F. Liu et al., 2010; Marquardt et al., 2014). Curiously, *FLC* silencing and alternative 3′ end formation of the *COOLAIR* transcript require a homologue of the protein SPEN, the very same protein required by *Xist* to induce gene silencing at the onset of XCI (Dossin et al., 2020; Hornyik et al., 2010; F. Liu et al., 2010). Thus, it is conceivable that alternate RNA processing pathways may be relevant during the early stages of *Xist*-induced silencing in mammals.

Extending the similarity to mammalian systems, lncRNAs have also been implicated in directing PRC2 to the *FLC* locus. In addition to the *FLC* mRNA and *COOLAIR* antisense transcripts, two other lncRNAs, *COLDAIR* and *COLDWRAP*, are expressed from the *FLC* locus in response to cold (Figure 8). *COLDAIR*, which is sense to *FLC* and initiates from within the H3K27me3 nucleation region, is expressed later than *COOLAIR* but before maximum silencing of *FLC* occurs (Heo & Sung, 2011). Importantly, knocking-down *COLDAIR* via RNA interference did not impact initial *FLC* silencing during cold exposure; instead, this prevented PRC2-mediated maintenance of *FLC* silencing (Heo & Sung, 2011). *COLDWRAP*, a second, smaller lncRNA sense to and expressed upstream of the *FLC* transcription start site, reaches peak expression level later than *COLDAIR* but is also necessary for H3K27me3 enrichment over *FLC* (D. H. Kim & Sung, 2017). Both *COLDAIR* and *COLDWRAP* interact with homologues of EZH2 in vitro and in vivo (Heo & Sung, 2011; D. H. Kim & Sung, 2017; D. H. Kim et al., 2017), but given questions surrounding the specificity of EZH2-RNA interactions and allosteric inhibition of EZH2 activity by RNA, the mechanisms linking *COLDAIR* and *COLDWRAP* to PRC2-mediated silencing remain unclear. Nonetheless, *COLDAIR*, *COLDWRAP*, and PRC2 are all required for the cold-induced strengthening of a pre-existing chromatin loop at *FLC* (D. H. Kim & Sung, 2017), highlighting another potential similarity between plant and mammalian systems (Andergassen et al., 2019; Pandya-Jones & Plath, 2016; Schertzer et al., 2019). Interestingly, the vernalization response in *COLDAIR* and *COLDWRAP* mutant plants was restored upon ectopic overexpression of a wild-type form of the respective lncRNA, suggesting that each lncRNA can function *in trans* despite being transcribed from the locus it regulates, breaking the similarity to *Xist* and related mammalian lncRNAs (D. H. Kim & Sung, 2017; D. H. Kim et al., 2017). Although initial work suggested no direct role for *COOLAIR* in H3K27me3 deposition (Csorba et al., 2014; Heo & Sung, 2011), an alternative model was recently proposed wherein an RBP, FCA, bridges an interaction between *COOLAIR* and PRC2 to play an essential role in H3K27me3 deposition (Tian et al., 2019), resembling the function of HNRNPK in *Xist*-PRC1 engagement (Pintacuda, Wei, et al., 2017). Beyond lncRNA-mediated mechanisms of PRC2 recruitment, sequence-specific DNA-binding proteins such as VAL1 and VAL2 also play roles in guiding PRC2 to the *FLC* nucleation region (Questa et al., 2016; Yuan et al., 2016), which may represent yet another similarity with mammalian systems (Blackledge et al., 2015; Laugesen et al., 2019; Schuettengruber et al., 2017; J. R. Yu et al., 2019). In sum, the plant *FLC* locus exemplifies the complex interplay between lncRNAs, PRCs, and chromatin features

in epigenetic regulation, and may provide clues to understanding analogous systems in mammals.

6 | CONCLUSIONS

RNA can both antagonize and promote the association between PRCs and chromatin, likely via direct interactions as well as interactions that are bridged by RBPs. DNA-binding proteins, 3D folding of the genome, contacts between PRCs themselves, transcription, and even potentially RNA processing also influence the effects that RNAs have on PRCs. With this knowledge in hand, many key questions emerge. What are the general features of the RNAs that repel versus recruit PRCs to chromatin? Does PRC1 directly interact with RNA in a manner that resembles PRC2 (i.e., with high affinity but low specificity)? Can PRC2 be recruited to chromatin by RNA in a manner analogous to PRC1 (i.e., through a bridged interaction with a dedicated RBP)? To what extent do chromatin-associated RNAs recruit PRCs to chromatin in a manner analogous to *Xist*? We favor the hypothesis that many chromatin-associated RNAs recruit PRCs to chromatin predominantly through RBP intermediaries, but note that this hypothesis is based on relatively limited data (Colognori et al., 2019; Pintacuda, Wei, et al., 2017; Schertzer et al., 2019; Wei et al., 2016). Understanding the biophysical properties that govern the interactions between PRCs and RBPs will be key steps moving forward. Moreover, recent data implicate a direct role for RNA in essentially all PRC2 targeting events in embryonic stem cells—even to loci such as the *Hox* gene clusters that are transcriptionally repressed and ostensibly do not produce substantive amounts of RNA (Long et al., 2020). These data imply that RNAs may play major roles in loading PRCs on chromatin *in trans*. Whatever the case, the data suggest that consequential interactions between RNAs and PRCs are widespread. It is conceivable that similar principles govern other chromatin-modifying enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the public domain. Please see our list of references for specific information.

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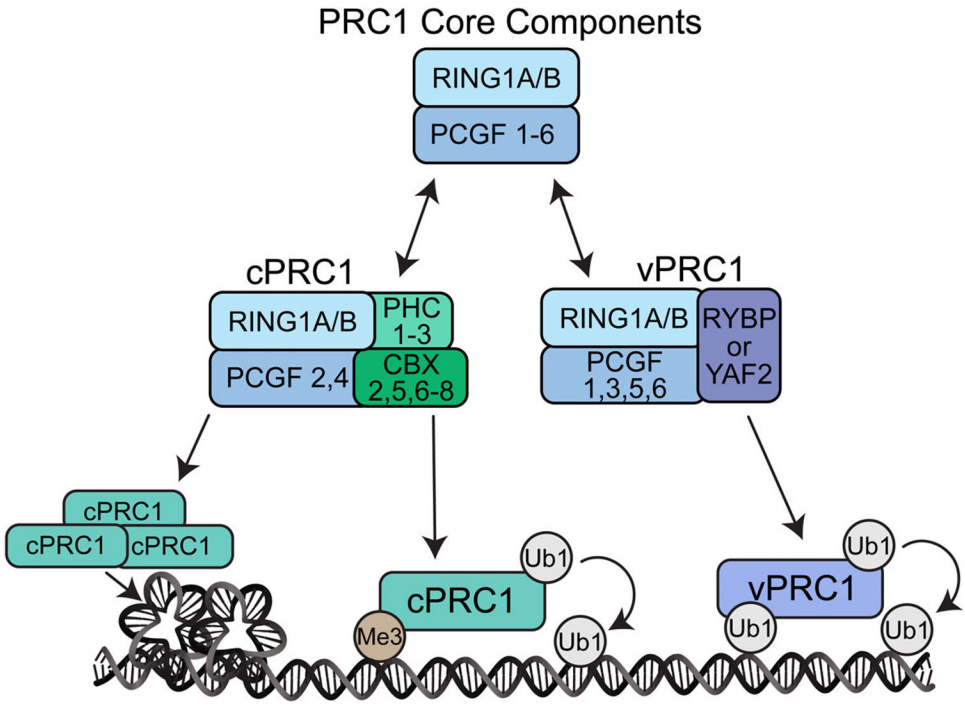

FIGURE 1.

Composition of PRC1 subcomplexes. PRC1 has two main forms, canonical PRC1 (cPRC1) and variant PRC1 (vPRC1). cPRC1 induces PRC1 oligomerization and chromatin compaction. The CBX subunit of cPRC1 recognizes and is recruited by H3K27me3. The RYBP subunit of vPRC1 stimulates vPRC1 catalytic activity and can also recognize H2AK119ub1

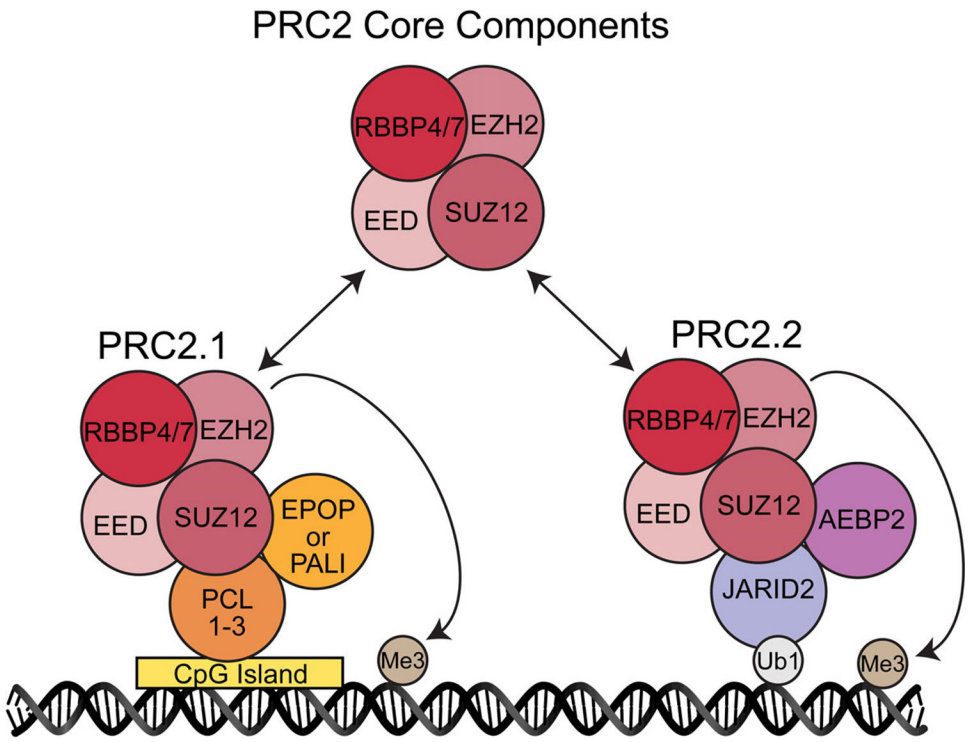

FIGURE 2.

Composition of the PRC2 subcomplexes. PRC2 has two main forms, PRC2.1 and PRC2.2. For more information, see (Laugesen et al., 2019; Schuettengruber et al., 2017; J. R. Yu et al., 2019). The PCL1–3 subunits of PRC2.1 appear to be important for recruiting PRC2.1 to CpG Islands. The JARID2 subunit of PRC2.2 recognizes and is recruited by H2AK119ub1. EPOP may function to promote a low level of transcription from within domains repressed by PRC2. PALI appears to stimulate the enzymatic activity of PRC2 in vitro *and* in vivo. Both JARID2 and AEBP2 allosterically activate PRC2. AEBP2 also binds methylated DNA and may stabilize PRC2 over a subset of genomic regions harboring methylated DNA

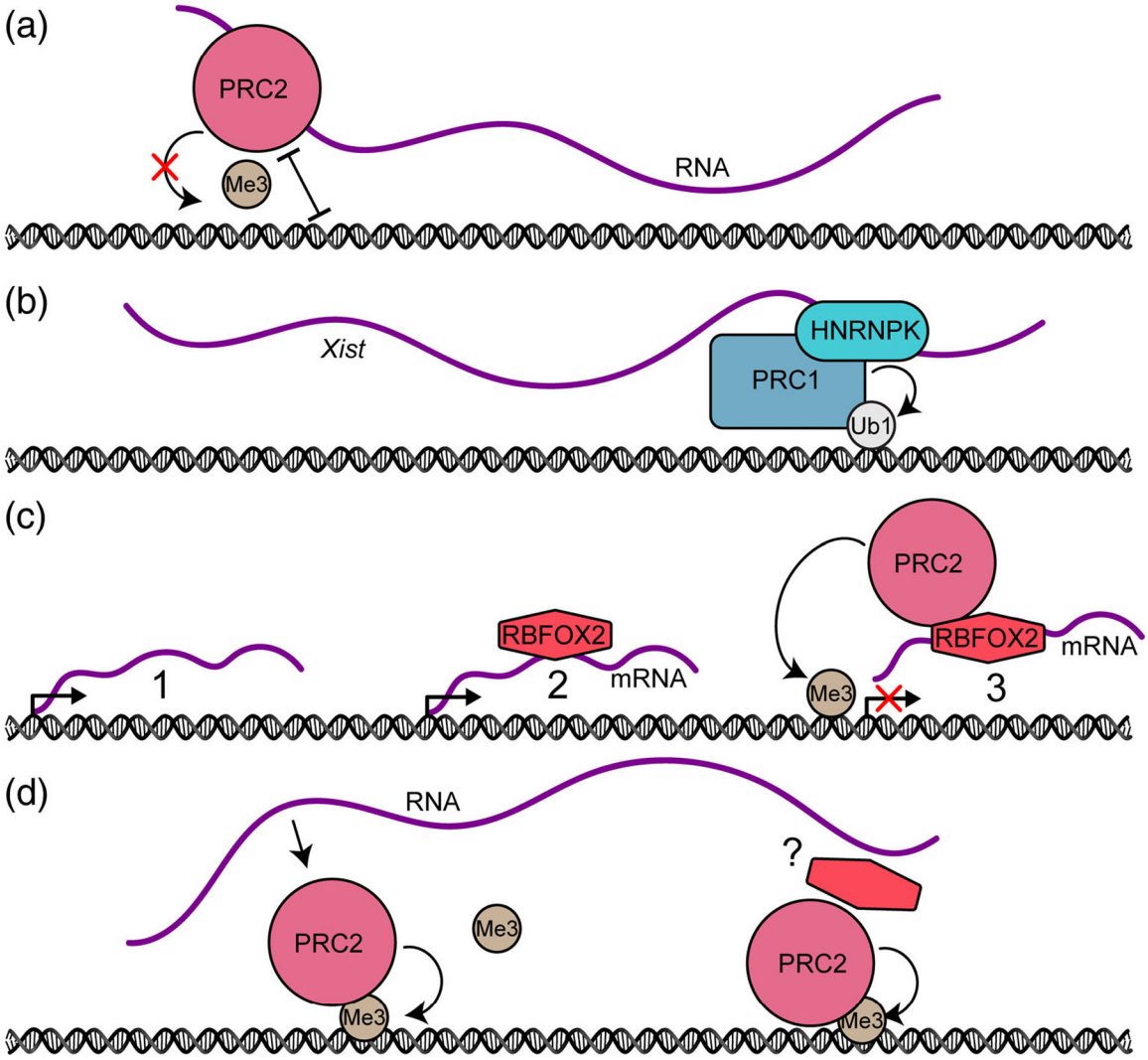

FIGURE 3.

Modes of interaction between the PRCs and RNA. (a) PRC2 binds RNA promiscuously, but this direct binding blocks the catalytic activity of PRC2, rendering it unable to place H3K27me3. (b) The RNA-binding protein HNRNPK bridges the lncRNA *Xist* and PRC1, bringing PRC1 to the X chromosome (Pintacuda, Wei, et al., 2017). (c) PRC2 can be recruited by RBFOX2 through pre-mRNA to dampen expression of genes that are already susceptible to PRC2-mediated silencing (Wei et al., 2016). (d) Either through direct or indirect interactions, RNA appears to be essential to stabilize PRC2 at specific sites throughout the genome (Long et al., 2020)

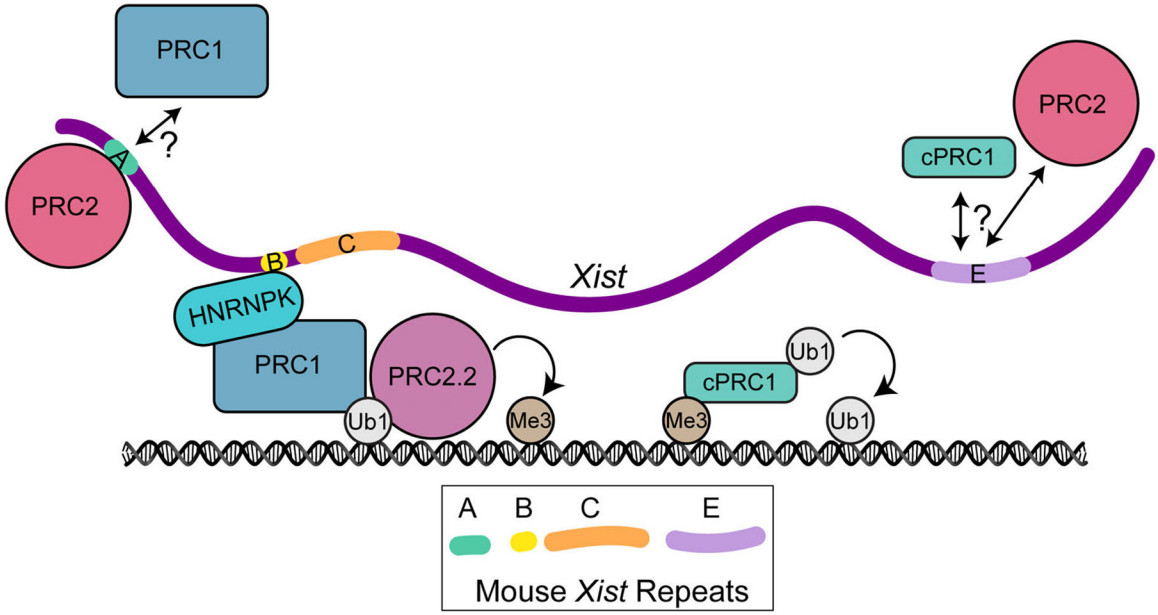

FIGURE 4.

Recruitment of PRCs by Xist during mouse XCI. vPRC1 recruitment by HNRNPK and Repeats B/C recruits PRC2 and cPRC1 to the inactive X (Almeida et al., 2017; Bousard et al., 2019; Colognori et al., 2019; Pintacuda, Wei, et al., 2017). Repeat A is also necessary to recruit the PRCs (Colognori et al., 2020). Repeat E associates with components of the PRCs and may play some role in recruitment to the inactive X (Hendrickson et al., 2016; M. K. Ray et al., 2016)

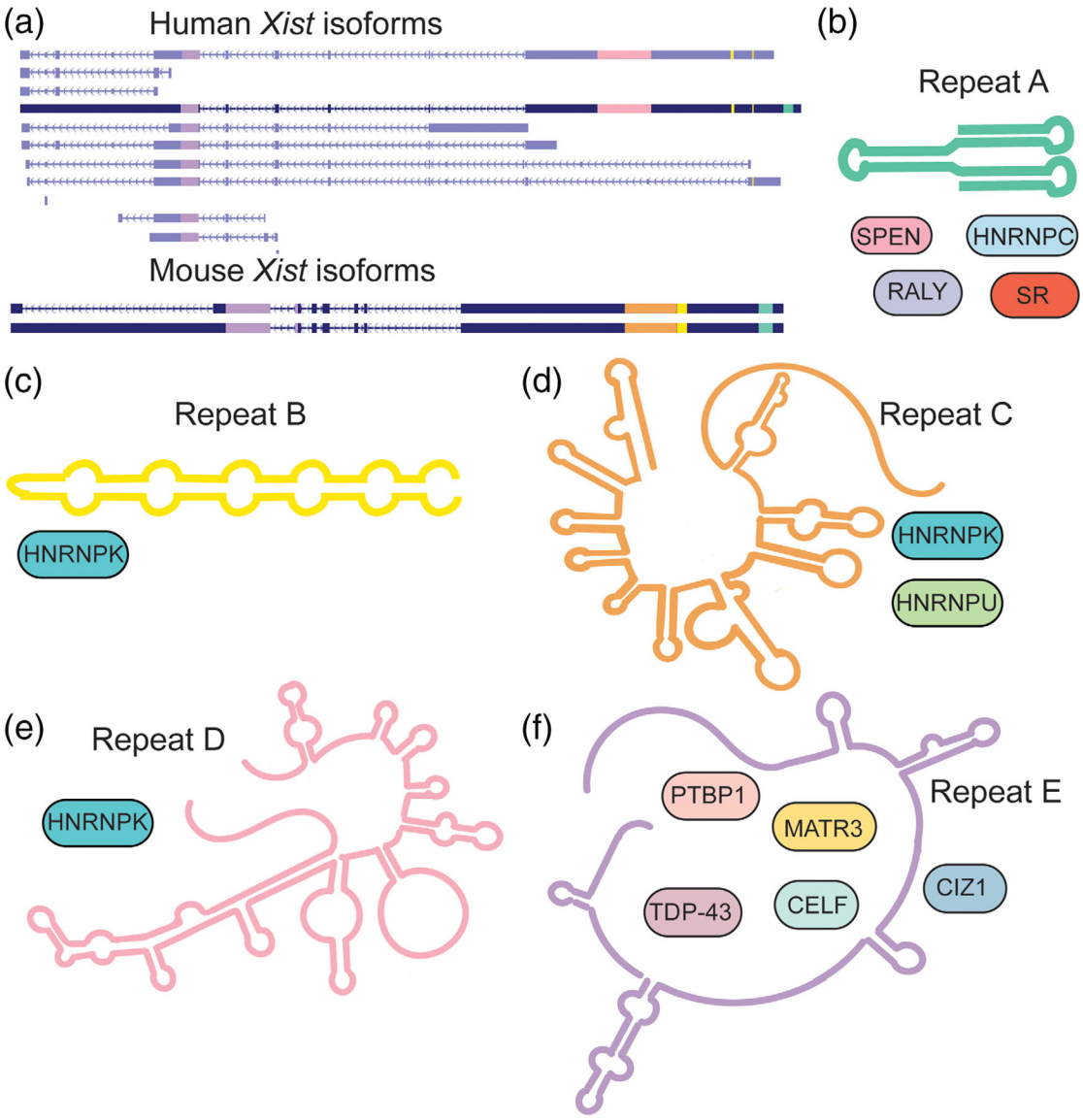

FIGURE 5.

Xist transcript structure and Repeat regions. Cartoon structures are modeled from a combination of RNA-fold predictions (Gruber et al., 2008) and the specific works referenced in each panel. (a) UCSC Genes' human and mouse *Xist* isoforms, annotated with location of *Xist* repeats (Haeussler et al., 2019). Repeat B is split by insertion in human *Xist*. (b) Repeat A is present in both human and mouse and interacts with many RBPs including SPEN, HNRNPC RALY, and several SR proteins (Chu et al., 2015; Cirillo et al., 2016; Graindorge et al., 2019; Pintacuda, Wei, et al., 2017; Trotman et al., 2020). The cartoon structure represents what is in our opinion one of the more likely conformations of Repeat A—one in which the predominant base-pairing occurs between, and not necessarily within, each repeat (Duszczyk et al., 2011; Z. P. Lu et al., 2016; Maenner et al., 2010). For more information, we direct the reader to recent reviews that summarize the structural data that have been collected for Repeat A (Jones & Sattler, 2019; Pintacuda, Young, &

Cerase, 2017). (c) Repeat B robustly binds HNRNPK and largely consists of repeating HNRNPK binding-motifs (Cirillo et al., 2016; Colognori et al., 2019; Nakamoto et al., 2020; Pintacuda, Wei, et al., 2017). Cartoon structure is modeled from (Nakamoto et al., 2020) and RNA-fold predictions. (d) Repeat C is rodent specific and binds HNRNPK and HNRNPU, among other proteins (Bousard et al., 2019; Cirillo et al., 2016; Graindorge et al., 2019). (e) Repeat D is the longest and most complex *Xist* repeat, and is present in most non-rodents (Nesterova et al., 2001; Sprague et al., 2019; Van Nostrand et al., 2016; Yen et al., 2007). (f) Repeat E is largely unstructured and is known to associate with PTBP1, MATR3, TDP-43, CELF1, and CIZ1 (Cirillo et al., 2016; Pandya-Jones et al., 2020; Ridings-Figueroa et al., 2017; Smola et al., 2016; Sunwoo et al., 2017; Van Nostrand et al., 2016). Cartoon structure is modeled from (Smola et al., 2016) and RNA-fold predictions

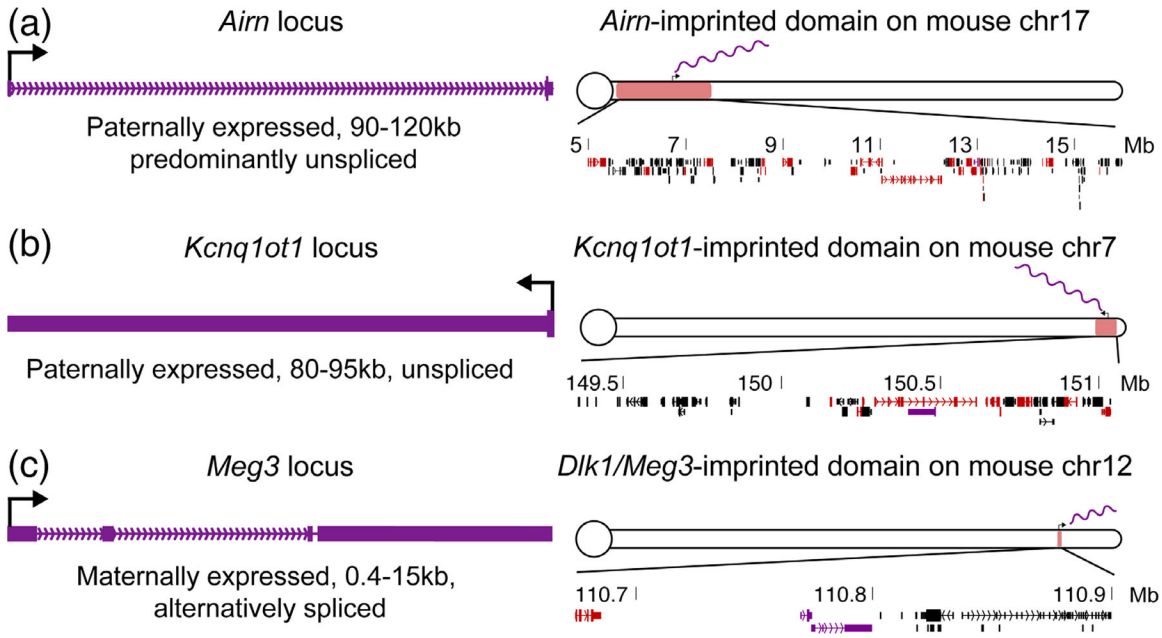

FIGURE 6.

Imprinted lncRNAs in their imprinted domains. UCSC Genes' noncoding RNA gene structures (left) and schematic of imprinted targeted domains (right) of the (a) *Airn*, (b) *Kcnq1ot1*, and (c) *Meg3* lncRNAs in mice. In the left-hand panels, filled rectangles correspond to UCSC-annotated exons, and fishbone structures correspond to UCSC-annotated introns (Haeussler et al., 2019). In the right-hand panels, the location of each imprinted domain relative to its position on its corresponding chromosome is shown. The *Airn*, *Kcnq1ot1*, and *Meg3* genes are colored purple, and the protein-coding genes that are repressed in each domain are colored red. Genes whose expression is bi-allelic within the imprinted domain are colored black. (a) The *Airn* locus is located on mouse chromosome 17. The *Airn* lncRNA is expressed predominantly as an unspliced and unstable 90–120 kb transcript from the paternally inherited allele and silences up to 13 Mb of chromatin. (b) The *Kcnq1ot1* locus is located on mouse chromosome 7. The *Kcnq1ot1* lncRNA is expressed predominantly as an unspliced and unstable 80–95 kb transcript from the paternally inherited allele and silences up to 3 Mb of chromatin. (c) The *Meg3* locus is located on mouse chromosome 12. The *Meg3* lncRNA is alternatively spliced and from the maternally inherited allele. While *Meg3* is implicated to silence neighboring genes in *cis* (shown here), it has been suggested to regulate silencing of other genes in *trans* (not shown here)

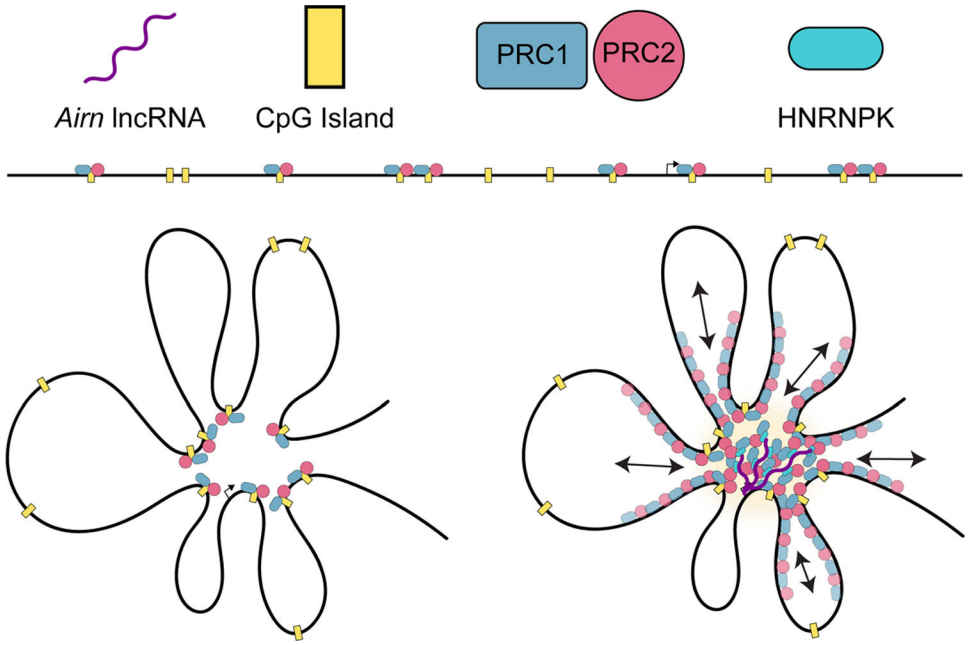

FIGURE 7.

A potential model for how the *Airn* lncRNA coordinates gene silencing and PRC deposition throughout a 13 Mb domain. (Top) Linear schematic representation of the 13-Mb *Airn* lncRNA-targeted domain on mouse chromosome 17. (Left) Predicted conformation of targeted domain on the maternal nonexpressing allele. Within the targeted domain, gene promoters that harbor CpG islands bound by the PRCs may form 3D contacts with each other and the CpG islands near *Airn*, creating a pre-existing conformational state that allows *Airn* to access distal targets despite remaining localized to its site of transcription. (Right) Predicted conformation of the targeted domain on the paternal lncRNA-expressing allele. Upon *Airn* expression, the lncRNA may preferentially contact regions of chromatin that are in close proximity to its site of transcription—in this case, we would predict that those regions of contact would harbor CpG islands that were already bound by the PRCs. HNRNPK and potentially other RBPs that interact with *Airn* help to create a high local concentration of PRCs around the lncRNA locus; these PRCs could then engage with nearby chromatin and spread over broad domains

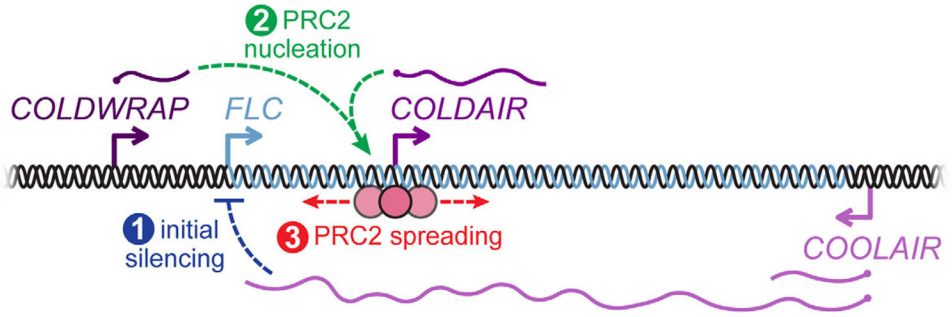

FIGURE 8.

Epigenetic control of the plant *FLC* gene by PRC2 and lncRNAs *COOLAIR*, *COLDAIR*, and *COLDWRAP*. Cold-induced repression of the *FLC* floral repressor gene occurs in three consecutive steps. First, upon exposure to cold temperatures, *FLC* transcription is transiently silenced in a PRC2-independent manner that involves expression of the antisense *COOLAIR* lncRNAs. Second, following extended time in the cold, the sense *COLDAIR* and *COLDWRAP* lncRNAs are expressed, which promote PRC2 spreading and H3K27me3 accumulation in a nucleation region at the 5′ end of FLC. Third, after a return to warmer growing temperatures, PRC2 and H3K27me3 spread outward from the nucleation region, causing *FLC* to be maintained in a silenced state that permits the transition to flowering