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Modulation of chromatin modifying complexes by noncoding RNAs in trans

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Abstract

Increasing evidence supports a central role for ncRNA in numerous aspects of chromatin function. For instance, ncRNAs can act as a scaffold for the recruitment of certain chromatin modifying complexes to specific sites within the genome. It is easily imaginable how this can occur *in cis*, but examples also exist whereby targeting of complexes by ncRNA occurs *in trans* to the site of transcription. Moreover, association of an ncRNA with a particular locus can trigger localization of the gene to a subnuclear structure harboring a specialized transcriptional environment. In this review, we discuss new insights into the mechanisms by which ncRNAs function *in trans* with respect to Polycomb Group, chromatin insulator, and dosage compensation complexes in mammals and/or *Drosophila*.

Introduction

Noncoding RNAs (ncRNAs) play key roles in chromatin function, particularly in scaffolding and recruitment of certain chromatin modifying complexes. It has long been appreciated that ncRNAs are central components of the dosage compensation machinery, and recent work has elucidated how various ncRNAs contribute to Polycomb Group (PcG) and chromatin insulator activities. In several cases, a nascent ncRNA nucleates recruitment of a chromatin complex *in cis*, serving as a simple mechanism to promote binding specificity. However, ncRNAs can also stimulate targeting of chromatin complexes *in trans*, including nucleation of complexes at distant sites or stabilization of large nuclear structures. The likely mechanisms by which these activities occur far from the site of transcription are hardly intuitive and require further study. In this review, we focus on recent studies providing key insights regarding the function of ncRNAs *in trans* with respect to PcG, chromatin insulator, and dosage compensation activities in mammals and/or *Drosophila*.

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Maintenance of cellular identity by PcG repression

The PcG proteins are required for the proper development of multicellular organisms, functioning to preserve pluripotency and/or cellular identity. Their main function is to repress the expression of genes that would otherwise promote differentiation into other cell types (Reviewed in [1]). This conserved class of proteins comprises two major subcomplexes, Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). One function of PRC2 is to methylate lysine 27 of histone H3 (H3K27) through the activity of the Ezh2 methyltransferase. Resultant H3K27me3 serves as a docking platform for the chromodomain containing Polycomb (Pc) protein of PRC1. Recruitment of PRC1 ultimately leads to stable repression through chromatin compaction and/or interference with RNA polymerase II transcription. Another property proposed to further stabilize repressive activities is the propensity of PcG complexes to concentrate at large subnuclear structures termed PcG bodies (Reviewed in [2]).

How specific recruitment of PcG complexes is achieved remains a critical outstanding question. Although a variety of DNA-binding proteins have been demonstrated to affect PcG recruitment in both *Drosophila* and mammals, recent studies have alternatively suggested that PcG recruitment may be promoted through interaction with RNA. Genome-wide purification approaches have identified a plethora of associated RNAs [3–6], likely due to the ability of PRC2 to bind RNA non-specifically [7]. An in-depth discussion regarding the role of ncRNAs in PcG recruitment *in cis* was recently presented [8]. Here we will discuss the abilities of certain ncRNAs to promote PcG activity *in trans* with respect to targeting of complexes to specific genomic locations as well as to subnuclear structures.

PRC2 recruitment to chromatin by *HOTAIR* ncRNA

The long ncRNA *HOX Antisense Intergenic* RNA (*HOTAIR*) serves as the archetypal *trans* acting PcG recruitment factor. *HOTAIR* is 2.2 kb in length and transcribed in the antisense direction with respect to the *HOXC* homeotic locus, preferentially expressed in distal and posterior cells of mammals [9]. *HOTAIR* represses the expression of the *HOXD* locus *in trans* through direct interaction and recruitment of the Suz12 and Ezh2 components of PRC2. Consistent with a key role in *HOX* gene regulation, targeted deletion of *HOTAIR* in mouse leads to skeletal malformations of the vertebrae and limbs, indicative of homeotic transformation along with extensive genome-wide loss of H3K27me3 [10].

Subsequent global analyses have determined that *HOTAIR* also regulates targets outside of the *HOXD* locus. Development of a novel method termed Chromatin Isolation by RNA Purification (ChIRP) allowed genome-wide mapping of *HOTAIR* binding sites in a breast cancer cell line [11]. These 832 binding sites overlap extensively with PRC2 occupancy in the same cell type, consistent with the ability of *HOTAIR* to serve as a recruitment factor for the complex. Whether these locations constitute natural binding sites for the ncRNA is unclear since the cell line used in this study overexpresses *HOTAIR*, which causes genome-wide mistargeting of PRC2 [12]. In the future, it will be important to improve the sensitivity of ChIRP and related techniques [13, 14] and apply them to different cell lines expressing natural levels of *HOTAIR*.

The mechanism of *HOTAIR* targeting of PcG is further complicated by its involvement in targeting a second chromatin modifying complex. *HOTAIR* harbors two independent interaction modules and can interact simultaneously with PRC2 and the H3K4 lysine demethylase LSD1 [15]. Consistent with the possibility that *HOTAIR* acts as a scaffold to coordinate both activities, *HOTAIR* knockdown leads to loss of both Suz12 and LSD1 chromatin association at shared sites. However, *HOTAIR* also affects LSD1 recruitment at non-PcG sites, suggesting that *HOTAIR* also harbors PcG-independent functions. Assuming then that *HOTAIR* alone cannot specify PcG recruitment, other factors may be required to act in concert with *HOTAIR*. This possibility is also supported by the recent finding that the affinity of PRC2 association with *HOTAIR* is no higher than its affinity for non-specific RNA [7]. In fact, *HOTAIR* binding sites are enriched for a GA-rich polypurine motif [11], which could indicate interaction with a sequence-specific DNA-binding factor to assist in targeting.

Recruitment of PcG by ncRNA to distinct nuclear structures

Two different Pc-associated ncRNAs can target genes to subnuclear compartments associated with either transcriptional activation or repression. Under serum starvation conditions, E2F1-regulated growth genes are rendered inactive and are associated with Pc2/CBX4 that is methylated on lysine 191 [16] (Figure 1). Furthermore, Pc2K191me2 interacts with the ncRNA *TUG1*, which is required for the localization of these genes to PcG bodies. Upon serum stimulation, Pc2 is demethylated and associates with the ncRNA *NEAT2/MALAT1*. These events are coincident with the relocalization of growth genes from the repressive PcG body to an interchromatin granule (ICG), which is associated with active transcription.

Of key mechanistic importance, association of Pc2 with either *TUG1* or *NEAT2* affects the specificity of Pc2 interaction with modified histones. *In vitro* binding assays showed that the Pc2 chromodomain preferentially binds H3K9me3 in the absence of RNA; however, in the presence of *TUG1*, Pc2 binding specificity is altered toward modifications associated with repression such as H3K27me2. In contrast, addition of *NEAT2* alters Pc2 specificity toward that of acetylated H2A peptides, which are associated with active transcription. Previous work showed that the chromodomain of another Pc homolog, CBX7, can bind the *ANRIL cis*-acting ncRNA simultaneously using an alternate face needed for H3K27me3 interaction [17]. However, at high concentrations the two interactions are competitive, suggesting feedback between the two domains. Since Pc2K191 methylation occurs outside of the chromodomain [16], it will be important to determine the potential structural crosstalk between these two domains. Collectively, these results provide new mechanistic insights into how PcG-associated ncRNAs can influence binding specificity as well as the spatial positioning of genes towards the appropriate transcriptional environment.

Chromatin insulators demarcate independent transcriptional domains

Chromatin insulators are multiprotein-DNA complexes capable of preventing inappropriate communication between adjacent *cis*-regulatory elements. For example, insulators can prevent the spread of PcG repression into an active region or block communication of an

enhancer and promoter when placed in between the two elements (Reviewed in [18]). Insulators minimally consist of a DNA-binding protein specificity factor, as well as a module to mediate interactions between insulator complexes in order to promote long-distance chromatin looping. The conserved zinc finger containing CCCTC-binding factor (CTCF) is the only insulator protein thus far identified in mammals. In *Drosophila*, there exist a variety of insulator complexes, such as the CTCF/CP190 and the *gypsy* insulators, defined by CTCF and Suppresor of Hairy wing (Su(Hw)) proteins respectively. Both complexes include the Centrosomal Protein 190 kDa (CP190) protein. Like PcG complexes, *Drosophila* insulator complexes coalesce within the nucleus at sites termed insulator bodies. Evidence exists suggesting that these bodies consist of DNA-bound insulator complexes that may form higher order chromatin domains. Although mammalian CTCF does not localize to an equivalent structure, it has previously been suggested that tethering of CTCF to the nucleolus promotes insulator activity [19].

The conserved p68 RNA helicase influences insulator activity in *Drosophila* and mammals

The steroid receptor RNA activator (*SRA*) ncRNA was identified as the first RNA regulator of chromatin insulator activity. *SRA* is stably associated with the p68 DEAD-box RNA helicase, and together these factors promote CTCF insulator activity, not by affecting its targeting to DNA, but by stimulating recruitment of the cofactor cohesin at a variety of sites [20]. On a genome-wide level, binding of p68 overlaps extensively with CTCF, but it is not known if *SRA* is also present at these sites. An *SRA* counterpart does not exist in *Drosophila*; however, the p68 homolog Rm62 was previously reported to act as a negative regulator of *gypsy* insulator enhancer blocking activity and insulator body localization [21]. Interestingly, mutation of Rm62 does not affect CTCF/CP190 insulator activity [22], suggesting conservation of components yet divergence of mechanisms of insulator regulation.

Jpx ncRNA as an anti-scaffold for CTCF

In contrast, ncRNA can also negatively regulate CTCF activity, by competing with its ability to bind to DNA. In mammals, inactivation of one X chromosome in females requires expression of an ncRNA termed *Xist*, which coats the entire chromosome and recruits PcG to promote silencing [8]. *Xist* transcription is positively activated by *Jpx*, an ncRNA located 10 kb upstream of *Xist* that is transcribed in antisense orientation [23]. Recent work showed that during differentiation, *Jpx* expression results in loss of CTCF binding at the *Xist* promoter, permitting activation of the gene by an unknown mechanism [24]. CTCF and *Jpx* interact directly *in vitro*, and this association can compete with CTCF binding to *Xist* promoter DNA. Therefore, it was suggested that association of *Jpx* can titrate CTCF away from its chromatin binding site. Intriguingly, *Jpx* can induce *Xist* expression when overexpressed *in trans* from an autosomal transgene at high levels [23, 24], and CTCF association is reduced at the *Xist* promoter but not several other CTCF binding sites tested. Although the specificity of this disruptive effect should be explored in more detail, this result suggests that the higher sensitivity of CTCF to titration effects at this particular site

results from some unique property of the *Xist* promoter, such as the presence of a specific factor or a specialized conformation.

Noncoding functions for mRNA in promoting *gypsy* insulator activity

Recent work in *Drosophila* revealed that instead of ncRNA, certain mRNAs are stably associated with insulator complexes. Native tandem immunoaffinity purification of *gypsy* insulator complexes followed by high-throughput sequencing identified association of mature *su(Hw)* and *Cp190* mRNAs [25]. In order to evaluate the biological importance of these associated transcripts, untranslatable versions of *su(Hw)* and *Cp190* RNAs were overexpressed *in vivo* using T7 polymerase. Ectopic expression of these transcripts resulted in an increase of the number of insulator bodies per nucleus and improvement of enhancer blocking activity. Several additional mRNA transcripts coding for chromatin-associated proteins were also identified in these purifications, suggesting the possible generality of bifunctional coding/noncoding transcripts. The majority of these transcripts do not correspond to *gypsy* insulator binding sites, suggesting that these RNAs act *in trans*, perhaps to promote scaffolding of insulator complexes (Figure 2). An obvious question raised by this finding is whether separate pools of a given mRNA are either retained in the nucleus or exported to the cytoplasm for translation, or alternately, if each mRNA can serve both functions. Recent work identified a novel RNA-binding protein in negative regulation of *gypsy* insulator activity [26]; however, it is unknown what factor(s) mediate the interaction between these mRNAs and insulator complexes.

Remodeling of ncRNAs involved in *Drosophila* dosage compensation

In *Drosophila* males, X chromosome dosage is normalized by upregulation of genes through specific recruitment of a transcription activation complex termed the dosage compensation complex (DCC). Transcribed from the X chromosome, the *roX1* and *roX2* noncoding RNAs serve redundant roles as scaffolds for DCC, despite little sequence similarity (Reviewed in [27]). In addition to the sites of *roX* transcription, DCC complex formation is nucleated at hundreds of additional chromatin entry sites along the X chromosome. A major advance in understanding DCC assembly came recently with the elucidation of secondary structures for several critical stem-loop structures in each RNA using chemical probing and nuclease sensitivity methods [28, 29]. Importantly, association of the Mle RNA helicase alters the conformation of the stem loop structure in an ATP-dependent manner, and this remodeling step stimulates the binding of the Msl2 protein, which triggers formation of the DCC (Figure 3). Incorporation of a maturation step at the site of action provides a potential mechanism by which a *trans*-acting ncRNA is prevented from acting before licensed to do so.

Conclusions

Several key concepts have emerged based on the various examples of *trans*-acting chromatin-associated ncRNAs discussed here. Due to their length, long ncRNAs can harbor multiple binding sites for different protein complexes and act as a scaffold for their interaction or coordinated recruitment to a particular site. Next, the binding of an ncRNA with a protein can alter the specificity of interaction of the protein with other factors.

Therefore, association of an ncRNA could act as a switch to trigger either assembly or disassembly of specific complexes. This capacity could also affect targeting of the complex to a specific location within the genome or nucleus. When this complex is associated with a particular locus, the ncRNA could thereby promote association of that locus to a subnuclear structure to enforce a specific transcriptional environment. Finally, to control the timing of its activity, the ncRNA itself can be regulated through a change in secondary structure by a helicase, which can affect its interaction specificity. This remodeling step would allow the ncRNA to diffuse from its site of transcription and function only at the appropriate location and context.

A major difficulty in studying long ncRNAs is that their structures are complex and difficult to predict based on sequence. As can be seen in recent DCC studies, detailed RNA analysis approaches are needed to elucidate secondary and eventually tertiary structures of these mysterious RNAs. Moreover, improvements to methods such as ChIRP will promote our understanding of ncRNA function on a genome-wide level, while application of single transcript *in situ* and single molecule tracking techniques (reviewed in [30]) should help elucidate the relationship between ncRNAs and specialized nuclear structures. Further insight into the mechanisms of ncRNA function in chromatin will certainly be obtained using a combination of these biochemistry, molecular biology, and cell biology approaches.

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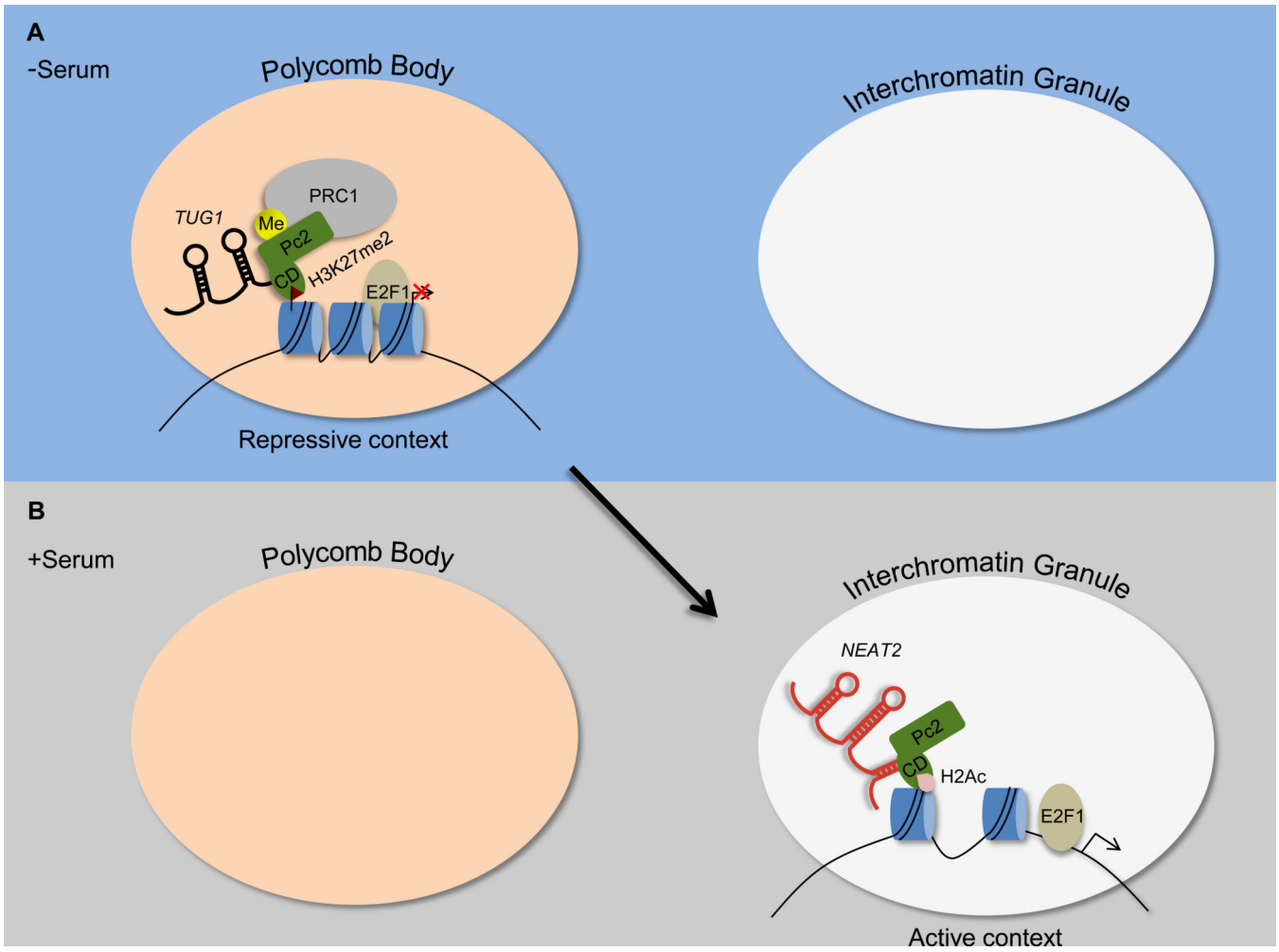

Figure 1. E2F1-responsive genes localize to a Polycomb body or interchromatin granule structure in the absence or presence of serum, respectively, dependent on the methylation status of Pc2

(A) Under serum starvation conditions, Pc2 is methylated and interacts with *TUG1* through its chromodomain (CD), which results in a higher affinity toward binding of H3K27me2. As a result, E2F1-responsive genes localize to a Polycomb body, which provides an environment to reinforce transcriptional repression.

(B) After serum stimulation, Pc2 is demethylated and interacts with *NEAT2*, which promotes association of the Pc2 CD with acetylated histone H2. As a result, E2F1-responsive genes localize to an interchromatin granule, which harbors a transcriptionally permissive environment.

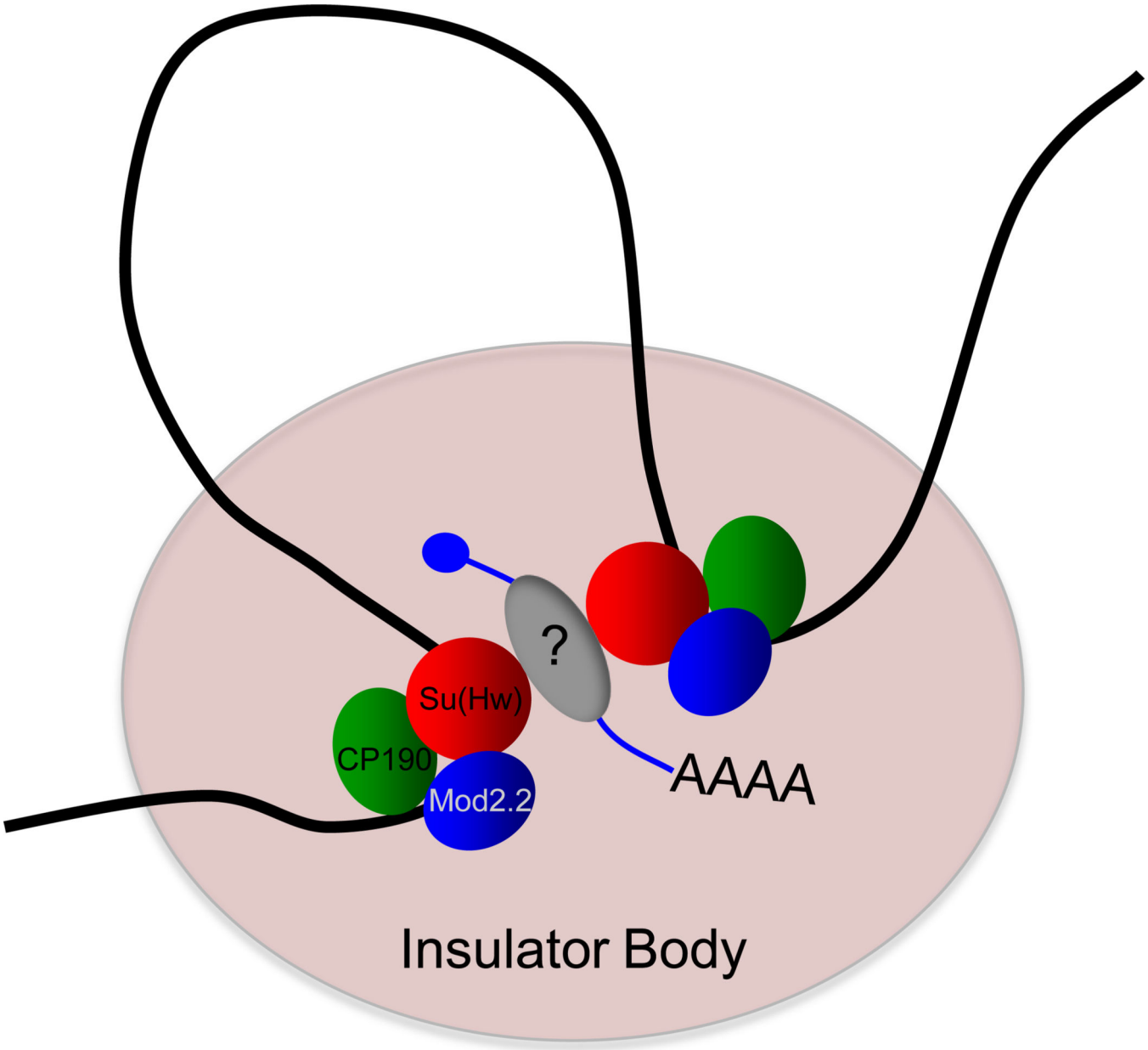

Figure 2. Model for mRNA acting as a scaffold for *gypsy* chromatin insulator complex formation

Specific mRNAs associate with the *gypsy* insulator complex, comprised of Su(Hw), CP190, and Mod(mdg4)2.2, promoting insulator-insulator interaction and loop formation, possibly in the context of an insulator body. Interaction of the mRNA and insulator complex is mediated by an unknown RNA-binding protein.

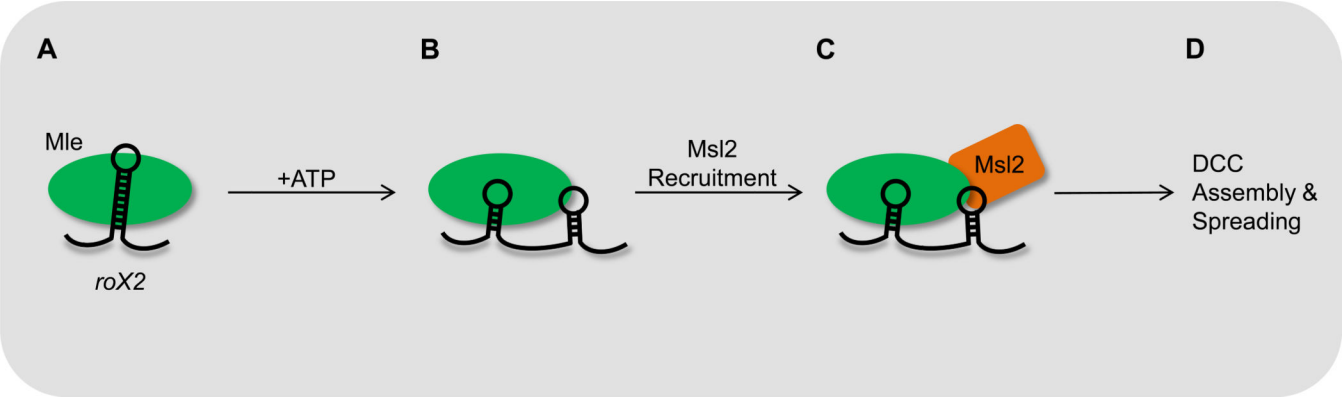

Figure 3. Remodeling of *roX2* acts a structural switch to provide a platform for Msl2 recruitment and subsequent DCC assembly

(A) The helicase Mle is recruited to the *roX2* ncRNA.

(B) Mle helicase activity remodels *roX2* conformation in an ATP-dependent manner.

(C) Remodeled *roX2* serves as a platform for Msl2 recruitment.

(D) Recruitment of Msl2 triggers DCC assembly and spreading across the X chromosome.