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Re-evaluating the foundations of lncRNA–Polycomb function

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Many lncRNAs are thought to interact with the polycomb repressive complex 2 (PRC2) in order to regulate gene expression. A central example of this lncRNA–PRC2 paradigm in gene regulation is *HOTAIR*. In this issue of *The EMBO Journal*, a study (Portoso *et al*, 2017) reports that while *HOTAIR* binds PRC2 with high affinity, the complex itself is dispensable for *HOTAIR*-mediated transcriptional silencing. This study raises important questions about the role of PRC2 interactions for lncRNA-mediated functions and argues for a re-evaluation of this lncRNA–PRC2 functional paradigm.

See also: **M Portoso *et al*** (April 2017)

The polycomb repressive complex 2 (PRC2) is a critical chromatin regulatory complex that plays an important role in transcriptional silencing in various biological contexts. Over the past decade, there has been enormous interest in the role of PRC2 and its interaction with various long non-coding RNAs (lncRNAs). This excitement derives primarily from the discovery that the Xist and *HOTAIR* lncRNAs silence transcription, physically interact with the PRC2 complex, and that perturbation of these RNAs impacts PRC2 localization at their target sites (Rinn *et al*, 2007; Zhao *et al*, 2008). Based on these observations, numerous studies have uncovered hundreds of lncRNAs that interact with PRC2 (Khalil *et al*, 2009; Zhao *et al*, 2010). Since PRC2 in mammals lacks clear DNA sequence specificity, these results led to a model whereby lncRNAs guide PRC2 to specific targets on DNA to control chromatin modification and gene expression.

However, there are several emerging challenges to this model. Recent studies have shown that PRC2 components interact "promiscuously" with virtually all RNAs, including mRNAs and RNAs derived from bacterial sequences (Davidovich *et al*, 2013; Kaneko *et al*, 2013). These results have raised important questions about the functional relevance of lncRNA–PRC2 interactions in gene regulation because it is unclear how lncRNAs can provide specificity for PRC2 localization if PRC2 binds to all RNAs across the entire genome. Indeed, it is now clear that the PRC2 complex is dispensable for Xist-mediated transcriptional silencing of the X chromosome (Kalantry & Magnuson, 2006; Schoeftner *et al*, 2006).

In this issue of *The EMBO Journal*, Portoso and colleagues sought to re-visit the remaining functional pillar of this lncRNA–PRC2 paradigm by directly testing whether PRC2 is required for *HOTAIR*-mediated transcriptional silencing. To do this, they designed a direct assay for lncRNA-dependent control of chromatin structure and gene regulation. In this assay, the *HOTAIR* lncRNA (or other control RNAs) is fused to the MS2 RNA hairpin and expressed along with the MS2 coat binding protein containing a DNA recognition domain that localizes upstream of a luciferase reporter gene integrated into the genome (Fig 1A). Consistent with the previously reported roles for *HOTAIR*, Portoso *et al* (2017) find that *HOTAIR* tethering leads to the deposition of H3K27 trimethylation marks, a hallmark of PRC2 activity, at this reporter gene locus as well as to transcriptional silencing (Fig 1B). Despite this observation, genetic deletion of PRC2 components did not impact the ability of *HOTAIR* to silence the reporter gene demonstrating that *HOTAIR*-mediated transcriptional silencing is not a consequence of PRC2 recruitment. In contrast, recruitment of various control RNAs, including several that bind PRC2 with equal affinity, had no impact on H3K27 trimethylation or transcriptional activity at this reporter gene. These results demonstrate that transcriptional silencing is dependent on the expression and localization of the *HOTAIR* lncRNA, but does not depend on its interaction with PRC2 (Fig 1C).

These results highlight the need for direct evidence when drawing conclusions about the functional importance of lncRNA–protein interactions. Beyond PRC2, many other chromatin regulatory complexes have been found to associate with lncRNAs, yet few of them have been shown to be important for lncRNA function (Guttman *et al*, 2011). Most studies have relied on indirect functional measurements such as gene expression effects seen upon perturbation of the lncRNA or the chromatin protein (Khalil *et al*, 2009; Guttman *et al*, 2011). However, a lncRNA can often impact hundreds of genes and it is unclear which of these are directly regulated by the lncRNA. Furthermore, many of these RNA-associated chromatin proteins regulate thousands of genes independently of the bound lncRNA, which may confound analysis of the *precise* role of a specific lncRNA–protein complex. The approach developed by Portoso and colleagues provides an elegant system for defining the *in vivo* function of lncRNA–protein interactions because it enables direct examination of the lncRNA function at a single target site with defined components.

These results reconcile an important disconnect between functional and

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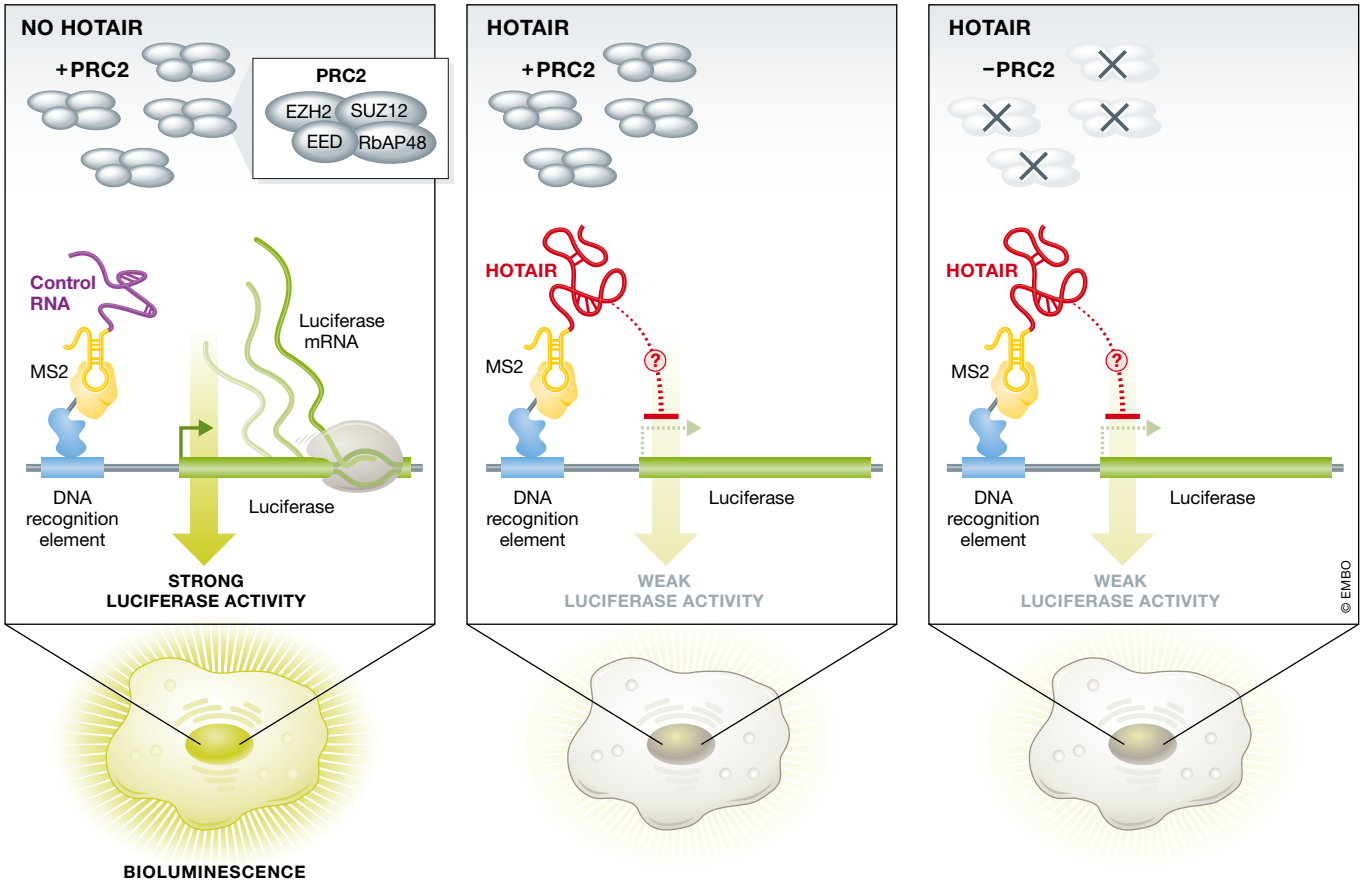

Figure 1. PRC2 is not required for *HOTAIR*-mediated silencing of gene expression.
The MS2 coat protein is fused with the Gal4-DNA binding domain to recognize an engineered UAS/Gal4-binding site (DNA recognition element) upstream of a luciferase reporter gene. In this system, a specific RNA (*HOTAIR* or control RNAs) is fused to an MS2 hairpin which binds tightly to the MS2 coat protein and is tethered upstream of the reporter gene allowing for direct measurements of their impact on gene expression through measurement of luciferase levels. Recruitment of control RNAs had no impact on luciferase expression (left panel), whereas recruitment of the *HOTAIR* lncRNA leads to silencing of the luciferase reporter (middle panel). Notably, *HOTAIR* can silence transcription of the reporter gene even in cells lacking the PRC2 components (right panel).

biochemical studies about the nature and functional consequences of lncRNA–PRC2 interactions. Rather than forming a specific interaction between *HOTAIR* and PRC2 to silence transcription, it appears that PRC2 binds to *HOTAIR* and other RNAs with comparable affinity and that these interactions are not required for regulation of gene expression. Furthermore, these results argue that *HOTAIR* recruitment of PRC2 to genomic DNA is not dependent on the RNA interaction with PRC2 since tethering of the *HOTAIR* antisense RNA, which has comparable PRC2 binding affinity, does not result in H3K27me3 deposition on the reporter locus. Instead, PRC2 recruitment and H3K27me3 modifications likely occur as a consequence *HOTAIR*-dependent gene silencing rather than through a direct RNA-mediated recruitment mechanism. This indirect PRC2 recruitment model also appears to be how the Xist lncRNA recruits PRC2—Xist directly interacts with SHARP (also called SPEN) to silence transcription and this silencing complex is essential for subsequent PRC2 recruitment on the X chromosome (McHugh *et al*, 2015). Consistent with an indirect recruitment model, it is now clear that gene silencing can lead to the recruitment of PRC2 to genomic DNA (Riising *et al*, 2014).

One important question that this study raises is how we can properly interpret PRC2 binding to RNA. This study highlights some of the difficulties with the current interpretation of *in vitro* binding data because there are RNAs that appear to have high affinity for PRC2 yet fail to recruit PRC2 when targeted *in vivo*. This may occur because affinity measurements are made in the absence of other unknown functional proteins that may bind more strongly to the RNA and without accounting for the differential localization of proteins and RNA within the nucleus. Furthermore, this problem is not restricted to *in vitro* measurements since current immunoprecipitation methods (i.e. RIP and CLIP) rely on non-denaturing conditions in order to maintain the antibody–protein interaction and therefore can have difficulty separating *bona fide in vivo* targets from those that merely occur in solution—especially with complexes that bind with high affinity *in vitro*. In support of this point, several groups performing denaturing purifications of Xist have failed to recover PRC2 despite the fact that this interaction has been identified by immunoprecipitation methods (Chu *et al*, 2015; McHugh *et al*, 2015), arguing that this interaction is

unlikely to occur at a high frequency *in vivo*. Similar to Xist, defining the mechanism of how *HOTAIR* actually silences transcription will require unbiased *in vivo* characterization of the lncRNA using denaturing purifications.

Although there are many remaining questions about what role promiscuous RNA binding of PRC2 might play, these results provide direct functional evidence that challenge the foundation supporting direct functional importance of lncRNA–PRC2 interactions for gene regulation and argues for a re-evaluation of what role (if any) PRC2 interactions play in lncRNA-mediated gene regulation.

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