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PERSPECTIVE

In preprints: revisiting RNA in PRC2

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The role of RNA in recruitment of various chromatin factors is widely acknowledged to contribute to nuclear transcriptional regulation. For instance, in PIWI–piRNA-mediated nuclear co-transcriptional silencing, piRNAs act as guides for the PIWI–piRNA complex, facilitating its localization at target nascent RNAs through sequence complementarity (Iwasaki et al., 2015). This complex recruits silencing effectors, including chromatin modifiers, thereby initiating co-transcriptional silencing. Similarly, long non-coding RNAs (lncRNAs) have diverse roles in nuclear transcriptional regulation (Yao et al., 2019). Xist serves as a prime example, orchestrating the architecture of the inactive X chromosome (Xi) during X chromosome inactivation. Xist recruits HDAC1-associated repressor proteins, SMART, HDAC3 and Polycomb repressive complex 1 (PRC1) to silence Xi (McHugh et al, 2015; Chu et al., 2015). Another lncRNA, Mhrt, prevents SWI/SNF binding to corresponding DNA loci (Han et al., 2014). Khps1 enhances RNA polymerase II (Pol II) transcription by forming an R-loop that anchors Khps1-interacting p300/CBP to the *SPHK1* promoter (Postepska-Igielska et al., 2015). Additionally, some lncRNAs, such as SLERT, promote RNA polymerase I (Pol I) transcription by binding to DDX21, altering its conformation, and releasing its inhibitory effect on Pol I (Xing et al., 2017). These examples underscore the significance of non-coding RNAs in collaborating with proteins for diverse nuclear regulatory and genome function aspects.

Polycomb repressive complex 2 (PRC2) mediates gene silencing by associating with CpG islands (CGIs) by trimethylation of histone H3 at lysine 27 (H3K27me3). Recent reports indicate that its recruitment and catalytic activity are closely linked to recognition of H2A mono-ubiquitylation given by PRC1 and unmethylated CpG dyads in CGIs (Blackledge and Klose, 2021; Kasinath et al., 2021). Nonetheless, PRC2 is also known to interact with RNA through several mechanisms to exert precise control over gene expression (Almeida et al., 2020; Davidovich and Cech, 2015). Notably, there are some models suggesting that PRC2 interacts with non-coding RNAs, which can bind to specific genes, guiding PRC2 to their genomic locations. RNA, through binding to regulatory regions of particular genes, recruits PRC2 to those specific loci, initiating H3K27 methylation. This epigenetic modification promotes gene silencing. A prominent example occurs in the Kcnq1 domain of an imprinted allele, where it has been reported that Kcnq1ot1 lncRNA interacts with PRC2 and recruits it to the imprinted allele, increasing H3K27 methylation levels and ensuring gene silencing (Pandey et al., 2008). Although it was initially postulated that RNA facilitates the recruitment of PRC2 to chromatin, another line of reports have demonstrated that RNA inhibits the interaction of PRC2 with nucleosomes (Beltran et al., 2016) and inhibits its methyltransferase activity (Kaneko et al., 2014). Therefore, proposed models of RNA-mediated PRC2 regulation remain a controversial subject. Nevertheless, the complex interaction between RNA and PRC2 contributes to precise gene regulation through H3K27 methylation and affects various biological processes, and disruptions in this regulatory process can potentially lead to diverse diseases.

Healy and colleagues (2023 preprint) and Hall Hickman and Jenner (2023 preprint) have raised concerns about a high-throughput methodology employed to investigate the requirement for RNA-PRC2 interaction in the localization of PRC2 components in their preprints. The methodology, known as RNase-ChIP (rChIP), uses RNase A treatment during chromatin immunoprecipitation to observe the reduction of PRC2 occupancy on chromatin upon RNA depletion, suggesting an ‘RNA bridge’ hypothesis between RNA molecules and chromatin modifiers (Long et al., 2020). However, a closer examination of this methodology has yielded unexpected findings. Instead of solely affecting PRC2, Healy and colleagues revealed that RNase A treatment during chromatin immunoprecipitation leads to the apparent loss of all facultative heterochromatin, including PRC2 and its associated H3K27me3 marks, across the entire genome. This phenomenon was consistent across various cell types, including mouse embryonic stem cells, human cancer cells and human induced pluripotent stem cells. The study suggests that this is linked to a global increase in pulled-down DNA, which artificially diminishes ChIP signals from facultative heterochromatin during data normalization. The second preprint from Hall Hickman and Jenner (2023 preprint) arrived at the same conclusion. In this case, the authors demonstrated that the reduction of the relative enrichment of PRC2-bound genomic regions upon RNase A treatment was caused by non-specific chromatin precipitation, which could be rescued by adding the anionic polymer poly-L-glutamic acid, which maintains chromatin solubility in RNase A-treated samples. Consequently, these two independent preprints collectively suggest that RNase A treatment may not be as reliable a method as previously thought for mapping RNA-dependent chromatin occupancy.

Importantly, a recent preprint by Long and colleagues (2023 preprint) also addresses a similar issue in their original PRC2 RNase-ChIP (Long et al., 2020), that RNase A treatment caused a substantial increase in the DNA pulled down by magnetic antibody beads. Meanwhile, the authors re-verified that chromatin binding of, for example, TBP (TATA box-binding protein) is not affected by the treatment with RNase A, suggesting that the issue may be antibody specific. They suggested that with the use of lower RNase A concentrations or the use of the RNase T1 RNA can still be effectively digested while reducing excess DNA pull-down. rChIP with lower RNase A concentration resulted in fewer genes

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significantly affected, suggesting the involvement of an 'RNA bridge' between PRC2 and chromatin, but to a lesser extent than the original high RNase A treatment protocol.

These findings challenge the established 'RNA bridge' hypothesis, emphasizing the intricate relationship between RNA and chromatin modification. Although there are lines of evidence using methods other than rChIP suggesting the transfer of PRC2 from localized RNA to DNA in cis (Cifuentes-Rojas et al., 2014; Hemphill et al., 2023; Long et al., 2020; Zhao et al., 2008), the 'RNA bridge' hypothesis may not be entirely conclusive. The extent to which PRC2 recruitment to its targets depends on RNA remains a subject of ongoing debate. In some cases, PRC2 exhibits the characteristic of being recruited to promoters even when transcription is inhibited, suggesting that its interaction may not be RNA dependent (Riising et al., 2014; Sugishita et al., 2021). Also, a cryo-electron microscopy structure-based study showed that G-quadruplex RNA association with PRC2 promotes dimerization of PRC2, leading to blockade of its interaction with nucleosome DNA (Song et al., 2023). These observations indicate that the extent to which RNA is involved in PRC2's interaction with its target region may be context dependent, relying on the loci or the RNA itself, possibly being influenced by factors such as RNA length and/or structure. Given these considerations, comprehensive, accurate and stable methods for identifying the impact on various loci have become increasingly important, enabling us to focus on discussions that consider the locus- and RNA-dependent aspects of PRC2's interactions.

In the ever-evolving field of epigenetics and chromatin biology, this research highlights the need for scientists to re-evaluate the tools and methods used to explore the intricate relationship between RNA and chromatin regulation. The studies underscore the need for a more nuanced approach when investigating RNA-mediated regulation of chromatin modifiers and suggest that previous assumptions based on rChIP experiments may need to be reconsidered. Although the lower concentration of RNase A may overcome these issues, it is essential to integrate multiple lines of evidence to analyze the extent to which RNA contributes to PRC2 regulation. Various techniques and approaches for studying these interactions, such as CLIP (cross-linking and immunoprecipitation), ChIRP-MS (comprehensive identification of RNA-binding proteins by mass spectrometry), and the use of RNA-binding mutants, are crucial for overcoming the limitations of the rChIP approach, especially regarding possible side effects resulting from the depletion of total RNA. These studies highlight the limitations of the rChIP methods employed to substantiate the existing 'RNA-bridge' hypothesis and emphasizes the crucial need for validation through multiple experimental approaches.

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