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eRNA binding produces tailored CBP activity profiles to regulate gene expression.

1,2 Daniel A Bose, 3,4 Shelley L Berger

Affiliations
1 Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield, S10 2TN, United Kingdom.
2 Sheffield Institute for Nucleic Acids, University of Sheffield, Firth Court, Western Bank, Sheffield, S10 2TN, United Kingdom.
3 Department of Cell and Developmental Biology, Genetics, Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA.
4 Epigenetics Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA.

Summary
Enhancers are cis- regulatory genetic elements crucial for controlling temporal and cell-type specific patterns of gene expression. Active enhancers generate bi-directional non-coding RNA transcripts called enhancer RNAs (eRNAs). eRNAs are important for stimulating gene expression, but precise mechanisms for this ability remain unclear. Here we highlight recent findings that demonstrate a direct interaction between RNAs and the transcriptional co-activator Creb-binding protein (CBP). Notably, RNA binding could stimulate the core histone acetyltransferase activity of the enzyme, observable in cells as a link between eRNA production, CBP-dependent histone acetylation and expression of genes regulated by specific enhancers. Although RNA binding was independent of RNA sequence, specificity arises in a locus-specific manner at transcribed sites where CBP was bound to chromatin. The results suggest a functional role for eRNAs as regulatory molecules that are able to stimulate the activity of a key epigenetic regulatory enzyme, thereby promoting gene expression. Furthermore, they suggest an intriguing role for eRNAs: by modulating the activity of chromatin modifying enzymes, they could directly impact transcription by altering the chromatin environment.
Introduction

The transcription of bi-directional, non-coding RNAs known as enhancer RNAs (eRNAs) is a key signature of active enhancer regions. It is now widely recognized that eRNA transcripts themselves play direct roles in promoting enhancer dependent gene expression; depletion of eRNAs can therefore affect transcription from enhancer-associated genes. eRNAs have been implicated in controlling looping between enhancers and promoters. In human breast cancer cells, eRNAs stabilize interactions between estrogen-receptor activated enhancers and promoters by interacting directly with cohesin. eRNAs also facilitate recruitment of Mediator to promoters and promote the release of paused RNA Polymerase II (Pol II) from the promoter into the gene body at poised genes. However, until recently eRNAs were not thought to play a direct role in directing the post-translational modification of histones to modify the chromatin environment. Indeed, enhancer specific histone methylation changes that signpost enhancer activity, such as di-methylation of histone H3 on lysine 4 (H3K4me2), arise due to transcription of the active enhancer by RNA polymerase II; these modifications are independent of eRNA transcripts. As H3K4me2 occurs at enhancers without the need for eRNAs, this raises the question of whether eRNAs are capable of sculpting the chromatin milieu.

The transcriptional co-activator CREB binding protein (CBP) contains a core enzymatic histone acetyltransferase (HAT) activity within a central catalytic domain. CBP acts as a transcriptional ‘network-hub’, interacting with hundreds of transcription factor (TF) binding partners. Enhancers are typically formed of clusters of TF binding sites; consequently, CBP is recruited to almost all enhancers through its interactions with TFs, and CBP occupancy is considered a defining characteristic of enhancer elements. Localizing CBP HAT activity to enhancers using de-activated Cas9 nuclease (dCas9) is sufficient to promote transcription of enhancer-associated genes. Moreover, CBP-dependent histone acetylation, specifically acetylation of histone H3 on lysine 27 (H3K27ac), is a characteristic epigenetic signature of enhancers across the genome.

A number of recent studies have revealed that chromatin associated proteins interact with RNA in cells at high frequency. In many cases, these interactions do not involve canonical or previously annotated RNA binding regions (RBRs) within the interacting proteins. Notably, RNA binding through non-canonical regions appears to be especially common for chromatin-modifying epigenetic enzymes. Chromatin-associated enzymes or enzyme complexes that bind to RNA, such as PRC2 and DNMT1, demonstrate a slight preference for structured RNA elements, but binding is often considered to be promiscuous as they have similar affinities for more than one RNA sequence. Moreover, a number of examples have now been described where RNA binding can directly modulate the catalytic activity of these enzymes.

We recently demonstrated an interaction between CBP and RNA, where specificity for RNA binding arises in a locus-specific manner, dictated by locations of CBP.
recruitment to chromatin. Although independent of RNA sequence, these interactions were sufficient to stimulate the catalytic HAT activity of the CBP enzyme, increasing CBP-dependent H3K27ac and thus promoting transcription \(^{29}\). Given the high frequency of interactions between RNAs and chromatin associated proteins, this raises an intriguing question: Does RNA binding convey a localized activity profile to chromatin bound epigenetic enzymes, and does this enable eRNAs to modify the local chromatin environment at a particular locus? Here, we utilize the example of CBP to explore the potential role of eRNAs as regulators of chromatin-modifying enzyme activity, and explore the implications of such a mechanism for enhancer function.

### Locus specificity for RNA binding

Our data revealed that CBP could interact with RNA in cells under native conditions, and importantly that CBP binds directly to RNA in photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) experiments \(^{29-31}\). Profiling of CBP-bound RNAs across the genome using high-throughput sequencing of PAR-CLIP libraries, revealed that bound RNAs arise from sites where CBP is recruited to chromatin. As CBP is strongly bound to enhancer regions and promoters, we observed a strong bias towards RNAs transcribed from these regions. These transcripts were especially enriched given their relatively low abundance and high turnover compared to coding transcripts from exonic regions. CBP therefore preferentially binds to RNAs transcribed from sites where it is recruited to chromatin \(^{29}\). Thus, although binding does not rely on a particular RNA sequence, RNA binding to CBP displays locus-specificity – transcripts are preferentially bound according to where CBP is bound to chromatin (Figure 1A). We note that, in common with other interactions between chromatin modifiers and RNA, a preference for specific RNA structures within eRNAs remains a possibility and requires further investigation for CBP.

One important consequence of locus-specific binding, was that RNAs interacting with CBP in vivo included a large number of eRNAs due to the high frequency of CBP recruitment at enhancers. A locus-specific binding model is logical in the context of the biological function of CBP: CBP is almost universally recruited to enhancer elements across the genome, regions that differ in their underlying DNA sequence. eRNAs arising from these enhancers will therefore also display different sequences and structural features. By retaining the ability to interact with RNAs independent of sequence, the activity of CBP could thus be modulated by RNAs wherever it binds to chromatin, i.e by eRNAs transcribed from any enhancer element where CBP is bound. Locus-specificity therefore opens the possibility that RNA binding to CBP, and potentially other epigenetic enzymes, could confer highly localized effects on specific CBP populations across the genome. Thus, RNAs, and eRNAs in particular could function as local regulators of CBP activity.
Direct binding to eRNAs stimulates CBP HAT activity

To investigate this potential regulatory function of eRNAs in more detail, we reconstituted binding to CBP in vitro. RNA pull downs and EMSAs revealed that eRNAs bind to the large, full-length CBP protein, but also to the much smaller catalytic HAT domain of CBP. We mapped RNA binding within the HAT domain to a contiguous region of 49 basic and disordered amino acids. Importantly, this RBR had been previously identified as an important regulatory motif for the HAT activity of CBP. This region, known as the activation loop, binds within the active site of CBP and blocks substrate binding until it is displaced by auto-acetylation. Consistent with the importance of the activation loop for regulating catalytic activity, we found that RNA binding to the RBR within the HAT domain caused a pronounced stimulation of the HAT activity of CBP (Figure 1B). This was evident both in radioactive filter binding HAT assays, and by western blot for specific CBP-dependent histone modifications, H3K27ac and H4K5ac, on reconstituted nucleosomes in vitro. HAT assays under steady state conditions revealed that stimulation results from displacement of the activation loop from the active site upon RNA binding (Figure 1B). Our results also revealed that eRNAs possess variable abilities to bind to CBP, and to stimulate its HAT activity. For example, eRNAs that failed to bind strongly to CBP in pull-down experiments, also failed to strongly upregulate its HAT activity, and different eRNA sequences produced different patterns of stimulation. These findings support a model that various sequences, and potentially structures of eRNAs, may have differential binding to CBP and generate alternative patterns of HAT stimulation.

Therefore, one intriguing possibility is that the activity of CBP can be modulated by eRNAs at different enhancers to generate a tailored activity profile at that enhancer (Figure 1C). Such a model could help to explain the observation that some eRNAs display strong strand-specificity in their ability to stimulate gene expression: if one strand is able to bind strongly to CBP and stimulate activity, but the other is unable to bind, then a difference in the ability of each eRNA strand to direct transcription would be observed at that enhancer (Figure 1D). We also note that while we observe a bias towards eRNA binding to CBP due to the prevalence of enhancer-bound CBP, promoter-derived transcripts could also potentially bind to and stimulate CBP activity (Figure 1C). The observation that eRNAs can control the activity of CBP in an enhancer specific manner, can also help explain the surprising observation that recruitment of CBP to enhancers and promoters is a relatively poor predictor of downstream gene activation. For example, eRNAs that strongly stimulate the activity of CBP could contribute to a stronger enhancer activity profile. However, more work is required to fully characterize the relationship between transcribed eRNAs, stimulation of CBP activity and enhancer strength.

Could eRNA have a general role as a regulatory molecule?
The regulatory activation loop within the HAT domain of CBP, which contains the RBR, binds in the active site of the enzyme and blocks substrate binding. The ability to bind within the CBP active site is derived from the positive charge of the RBR within the activation loop; this positive charge also enables the loop to bind to RNAs. Other histone acetyltransferases, such as the MYST family member Males Absent on the First (hMOF) are not regulated by an activation loop in the manner of CBP, thus their HAT activity remains unaffected by the presence of RNA.

Nevertheless, it is clear that the activity of other epigenetic-enzyme complexes, such as PRC2 and DNMT1, are sensitive to RNA binding: Binding of RNA to PRC2 decreases both its methyltransferase activity and recruitment to promoter regions and ncRNAs transcribed proximal to the C/EBPA locus interact with DNMT1 to block DNA methylation at the promoter. In both instances, RNA binding was driven more by RNA secondary structure than sequence, with more structured RNAs binding more strongly, and different RNA sequences producing different effects on enzyme activity. In cells, the ability of ncRNA to inhibit the methyltransferase activity of PRC2 is thought to arise from competition between RNA and nucleosomes for binding to PRC2. For DNMT1, RNA binding was mapped to the catalytic domain of the enzyme. This suggests that in order for a chromatin-modifying enzyme to be susceptible to direct regulation by RNA binding, there must be a link between potential RBRs within the protein and the catalytic domain. Interestingly, many epigenetic complexes - such as PRC2 - contain multiple subunits and components, often with individual abilities to bind to RNA. It remains to be determined what the combined effect of RNA binding to individual components of these complexes plays to regulate overall complex activity. Full elucidation of the mechanisms of RNA-dependent stimulation will require further biochemical and structural studies of the interactions between the enzyme and bound RNA species.

**Concluding remarks**

Recent studies have highlighted the high frequency of binding between chromatin-associated proteins or enzyme complexes and RNA in cells, and the importance of non-canonical RBRs for these interactions. The observation that locus-specific RNA binding to CBP can directly stimulate its catalytic HAT activity presents an interesting role for these interactions: RNA binding events could enable local ‘tailoring’ of enzymatic-activity profiles.

In the case of CBP, this tailoring is driven by the binding of RNAs transcribed proximal to sites where CBP is recruited to chromatin. RNAs bind to CBP through a non-canonical, positively charged RBR within the catalytic HAT domain. Because this region is crucial for the regulation of CBP activity, RNA binding can stimulate the catalytic activity of the enzyme and promote gene expression. The structure of CBP, and its method of regulation by the activation loop, renders it particularly susceptible to regulation by RNA binding to this region. Whilst it is increasingly clear that RNA binding to other chromatin-modifying enzymes is widespread, the extent to which this
binding can modulate their enzymatic activity is less well understood. A number of examples suggest that RNA does play such a regulatory role\textsuperscript{21,22,25-28}, however further biochemical work is required to fully determine the mechanisms that RNAs use to modulate the activity of these complexes.

For CBP, locus-specific RNA binding leads to preferential binding of CBP to eRNAs, as CBP is enriched at enhancers. Our results suggest a functional role for eRNAs as regulatory molecules that are able to stimulate the activity of a key epigenetic regulatory enzymes, thereby promoting gene expression. Furthermore, they suggest an intriguing, RNA sequence-independent (but potentially structure-dependent) role for eRNAs in modulating the activity of chromatin modifying enzymes, that could directly impact transcription by altering the chromatin environment. In contrast, we note that enhancer-specific methylation changes appear to be reliant on transcription at enhancers, rather than eRNA transcripts themselves\textsuperscript{10}. A key outstanding question is whether eRNA transcripts can play a role in modifying the activity of epigenetic complexes when enhancers loop to contact promoter regions.

Chromatin-modifying enzymes and enzyme complexes are crucial for directing gene expression profiles across the genome. In particular, eRNAs are attractive candidates as regulatory molecules due to their highly localized expression profiles, rapid turnover and low abundance\textsuperscript{2,3,5}. The prevalence of RNA binding to chromatin-modifying enzymes, and the potential for modulation of their catalytic activity, suggests that RNAs could modulate their activity in a similar manner to CBP to generate tailored patterns of histone modifications and gene expression profiles.

Figure Legend

Figure 1: RNA binding can generate localized CBP activity profiles and tailored patterns of histone acetylation. A) Locus specificity for RNA binding to CBP. CBP bound RNAs (dashed-line) correspond to sites of CBP recruitment to chromatin (dark-grey) and nascent RNA transcription (light-grey) at enhancers and promoters; B) RNA binding to the activation loop of CBP stimulates catalytic HAT activity by displacing the loop from the active site, and thereby promotes CBP dependent histone acetylation (H3K27ac); C) eRNA binding could generate enhancer specific patterns of CBP activity by differential regulation of CBP activity. At more active enhancers, CBP activity is stimulated more strongly by eRNA binding (arrows) than at enhancers with weak stimulation of CBP activity (crosses); D) Differences in the ability of sense and antisense eRNA transcripts to stimulate CBP activity. The eRNA strand that binds and stimulates CBP strongly (arrows) could promote associated gene expression more strongly than weaker binding eRNA strands (crosses).

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