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

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19
20
21
22 **Summary**

23
24 Enhancers are cis- regulatory genetic elements crucial for controlling temporal and
25 cell-type specific patterns of gene expression. Active enhancers generate bi-
26 directional non-coding RNA transcripts called enhancer RNAs (eRNAs). eRNAs are
27 important for stimulating gene expression, but precise mechanisms for this ability
28 remain unclear. Here we highlight recent findings that demonstrate a direct
29 interaction between RNAs and the transcriptional co-activator Creb-binding protein
30 (CBP). Notably, RNA binding could stimulate the core histone acetyltransferase
31 activity of the enzyme, observable in cells as a link between eRNA production, CBP-
32 dependent histone acetylation and expression of genes regulated by specific
33 enhancers. Although RNA binding was independent of RNA sequence, specificity
34 arises in a locus-specific manner at transcribed sites where CBP was bound to
35 chromatin. The results suggest a functional role for eRNAs as regulatory molecules
36 that are able to stimulate the activity of a key epigenetic regulatory enzyme, thereby
37 promoting gene expression. Furthermore, they suggest an intriguing role for eRNAs:
38 by modulating the activity of chromatin modifying enzymes, they could directly impact
39 transcription by altering the chromatin environment.

43 Introduction

44

45 The transcription of bi-directional, non-coding RNAs known as enhancer RNAs
46 (eRNAs) is a key signature of active enhancer regions¹. It is now widely recognized
47 that eRNA transcripts themselves play direct roles in promoting enhancer dependent
48 gene expression; depletion of eRNAs can therefore affect transcription from
49 enhancer-associated genes²⁻⁷. eRNAs have been implicated in controlling looping
50 between enhancers and promoters^{5,8}. In human breast cancer cells, eRNAs stabilize
51 interactions between estrogen-receptor activated enhancers and promoters by
52 interacting directly with cohesin⁵. eRNAs also facilitate recruitment of Mediator to
53 promoters⁴ and promote the release of paused RNA Polymerase II (Pol II) from the
54 promoter into the gene body at poised genes⁹. However, until recently eRNAs were
55 not thought to play a direct role in directing the post-translational modification of
56 histones to modify the chromatin environment. Indeed, enhancer specific histone
57 methylation changes that signpost enhancer activity, such as di-methylation of
58 histone H3 on lysine 4 (H3K4me2), arise due to transcription of the active enhancer
59 by RNA polymerase II; these modifications are independent of eRNA transcripts¹⁰.
60 As H3K4me2 occurs at enhancers without the need for eRNAs, this raises the
61 question of whether eRNAs are capable of sculpting the chromatin milieu.

62 The transcriptional co-activator Creb binding protein (CBP) contains a core
63 enzymatic histone acetyltransferase (HAT) activity within a central catalytic domain
64^{11,12}. CBP acts as a transcriptional 'network-hub', interacting with hundreds of
65 transcription factor (TF) binding partners¹³. Enhancers are typically formed of
66 clusters of TF binding sites; consequently, CBP is recruited to almost all enhancers
67 through its interactions with TFs, and CBP occupancy is considered a defining
68 characteristic of enhancer elements^{14,15}. Localizing CBP HAT activity to enhancers
69 using de-activated Cas9 nuclease (dCas9) is sufficient to promote transcription of
70 enhancer-associated genes¹⁶. Moreover, CBP-dependent histone acetylation,
71 specifically acetylation of histone H3 on lysine 27 (H3K27ac), is a characteristic
72 epigenetic signature of enhancers across the genome^{14,17}.

73

74 A number of recent studies have revealed that chromatin associated proteins interact
75 with RNA in cells at high frequency¹⁸⁻²⁰. In many cases, these interactions do not
76 involve canonical or previously annotated RNA binding regions (RBRs) within the
77 interacting proteins^{18,19}. Notably, RNA binding through non-canonical regions
78 appears to be especially common for chromatin-modifying epigenetic enzymes¹⁸.
79 Chromatin-associated enzymes or enzyme complexes that bind to RNA, such as
80 PRC2 and DNMT1, demonstrate a slight preference for structured RNA elements,
81 but binding is often considered to be promiscuous as they have similar affinities for
82 more than one RNA sequence²¹⁻²⁵. Moreover, a number of examples have now been
83 described where RNA binding can directly modulate the catalytic activity of these
84 enzymes^{21,22,25-28}.

85

86 We recently demonstrated an interaction between CBP and RNA²⁹, where specificity
87 for RNA binding arises in a locus-specific manner, dictated by locations of CBP

88 recruitment to chromatin. Although independent of RNA sequence, these interactions
89 were sufficient to stimulate the catalytic HAT activity of the CBP enzyme, increasing
90 CBP-dependent H3K27ac and thus promoting transcription²⁹. Given the high
91 frequency of interactions between RNAs and chromatin associated proteins, this
92 raises an intriguing question: Does RNA binding convey a localized activity profile to
93 chromatin bound epigenetic enzymes, and does this enable eRNAs to modify the
94 local chromatin environment at a particular locus? Here, we utilize the example of
95 CBP to explore the potential role of eRNAs as regulators of chromatin-modifying
96 enzyme activity, and explore the implications of such a mechanism for enhancer
97 function.

100 **Locus specificity for RNA binding**

101
102 Our data revealed that CBP could interact with RNA in cells under native conditions,
103 and importantly that CBP binds directly to RNA in photoactivatable ribonucleoside-
104 enhanced crosslinking and immunoprecipitation (PAR-CLIP) experiments²⁹⁻³¹.
105 Profiling of CBP-bound RNAs across the genome using high-throughput sequencing
106 of PAR-CLIP libraries, revealed that bound RNAs arise from sites where CBP is
107 recruited to chromatin. As CBP is strongly bound to enhancer regions and promoters,
108 we observed a strong bias towards RNAs transcribed from these regions. These
109 transcripts were especially enriched given their relatively low abundance and high
110 turnover compared to coding transcripts from exonic regions. CBP therefore
111 preferentially binds to RNAs transcribed from sites where it is recruited to chromatin
112²⁹. Thus, although binding does not rely on a particular RNA sequence, RNA binding
113 to CBP displays locus-specificity – transcripts are preferentially bound according to
114 where CBP is bound to chromatin (Figure 1A). We note that, in common with other
115 interactions between chromatin modifiers and RNA, a preference for specific RNA
116 structures within eRNAs remains a possibility and requires further investigation for
117 CBP.

118
119 One important consequence of locus-specific binding, was that RNAs interacting with
120 CBP in vivo included a large number of eRNAs due to the high frequency of CBP
121 recruitment at enhancers. A locus-specific binding model is logical in the context of
122 the biological function of CBP: CBP is almost universally recruited to enhancer
123 elements across the genome, regions that differ in their underlying DNA sequence.
124 eRNAs arising from these enhancers will therefore also display different sequences
125 and structural features. By retaining the ability to interact with RNAs independent of
126 sequence, the activity of CBP could thus be modulated by RNAs wherever it binds to
127 chromatin, i.e by eRNAs transcribed from any enhancer element where CBP is
128 bound. Locus-specificity therefore opens the possibility that RNA binding to CBP,
129 and potentially other epigenetic enzymes, could confer highly localized effects on
130 specific CBP populations across the genome. Thus, RNAs, and eRNAs in particular
131 could function as local regulators of CBP activity.

134 **Direct binding to eRNAs stimulates CBP HAT activity**

135
136 To investigate this potential regulatory function of eRNAs in more detail, we
137 reconstituted binding to CBP *in vitro*²⁹. RNA pull downs and EMSAs revealed that
138 eRNAs bind to the large, full-length CBP protein, but also to the much smaller
139 catalytic HAT domain of CBP. We mapped RNA binding within the HAT domain to a
140 contiguous region of 49 basic and disordered amino acids^{11,12,29}. Importantly, this
141 RBR had been previously identified as an important regulatory motif for the HAT
142 activity of CBP³². This region, known as the activation loop, binds within the active
143 site of CBP and blocks substrate binding until it is displaced by auto-acetylation³².
144 Consistent with the importance of the activation loop for regulating catalytic activity,
145 we found that RNA binding to the RBR within the HAT domain caused a pronounced
146 stimulation of the HAT activity of CBP (Figure 1B). This was evident both in
147 radioactive filter binding HAT assays, and by western blot for specific CBP-
148 dependent histone modifications, H3K27ac and H4K5ac, on reconstituted
149 nucleosomes *in vitro*. HAT assays under steady state conditions revealed that
150 stimulation results from displacement of the activation loop from the active site upon
151 RNA binding²⁹ (Figure 1B). Our results also revealed that eRNAs possess variable
152 abilities to bind to CBP, and to stimulate its HAT activity. For example, eRNAs that
153 failed to bind strongly to CBP in pull-down experiments, also failed to strongly
154 upregulate its HAT activity, and different eRNA sequences produced different
155 patterns of stimulation²⁹. These findings support a model that various sequences,
156 and potentially structures of eRNAs, may have differential binding to CBP and
157 generate alternative patterns of HAT stimulation.

158
159 Therefore, one intriguing possibility is that the activity of CBP can be modulated by
160 eRNAs at different enhancers to generate a tailored activity profile at that enhancer
161 (Figure 1C). Such a model could help to explain the observation that some eRNAs
162 display strong strand-specificity in their ability to stimulate gene expression: if one
163 strand is able to bind strongly to CBP and stimulate activity, but the other is unable to
164 bind, then a difference in the ability of each eRNA strand to direct transcription would
165 be observed at that enhancer (Figure 1D). We also note that while we observe a bias
166 towards eRNA binding to CBP due to the prevalence of enhancer-bound CBP³³,
167 promoter-derived transcripts could also potentially bind to and stimulate CBP activity
168 (Figure 1C). The observation that eRNAs can control the activity of CBP in an
169 enhancer specific manner, can also help explain the surprising observation that
170 recruitment of CBP to enhancers and promoters is a relatively poor predictor of
171 downstream gene activation³³. For example, eRNAs that strongly stimulate the
172 activity of CBP could contribute to a stronger enhancer activity profile. However,
173 more work is required to fully characterize the relationship between transcribed
174 eRNAs, stimulation of CBP activity and enhancer strength.

177 **Could eRNA have a general role as a regulatory molecule?**

179 The regulatory activation loop within the HAT domain of CBP, which contains the
180 RBR, binds in the active site of the enzyme and blocks substrate binding. The ability
181 to bind within the CBP active site is derived from the positive charge of the RBR
182 within the activation loop; this positive charge also enables the loop to bind to RNAs.
183 Other histone acetyltransferases, such as the MYST family member Males Absent on
184 the First (hMOF) are not regulated by an activation loop in the manner of CBP, thus
185 their HAT activity remains unaffected by the presence of RNA ²⁹.

186

187 Nevertheless, it is clear that the activity of other epigenetic-enzyme complexes, such
188 as PRC2 and DNMT1, are sensitive to RNA binding: Binding of RNA to PRC2
189 decreases both its methyltransferase activity and recruitment to promoter regions
190 ^{22,25,26}, and ncRNAs transcribed proximal to the C/EBPA locus interact with DNMT1 to
191 block DNA methylation at the promoter ²¹. In both instances, RNA binding was driven
192 more by RNA secondary structure than sequence, with more structured RNAs
193 binding more strongly, and different RNA sequences producing different effects on
194 enzyme activity ²¹⁻²³. In cells, the ability of ncRNA to inhibit the methyltransferase
195 activity of PRC2 is thought to arise from competition between RNA and nucleosomes
196 for binding to PRC2 ²⁵. For DNMT1, RNA binding was mapped to the catalytic
197 domain of the enzyme ²¹. This suggests that in order for a chromatin-modifying
198 enzyme to be susceptible to direct regulation by RNA binding, there must be a link
199 between potential RBRs within the protein and the catalytic domain. Interestingly,
200 many epigenetic complexes - such as PRC2 - contain multiple subunits and
201 components, often with individual abilities to bind to RNA. It remains to be
202 determined what the combined effect of RNA binding to individual components of
203 these complexes plays to regulate overall complex activity. Full elucidation of the
204 mechanisms of RNA-dependent stimulation will require further biochemical and
205 structural studies of the interactions between the enzyme and bound RNA species.

206

207

208 **Concluding remarks**

209

210 Recent studies have highlighted the high frequency of binding between chromatin-
211 associated proteins or enzyme complexes and RNA in cells, and the importance of
212 non-canonical RBRs for these interactions ^{18,19}. The observation that locus-specific
213 RNA binding to CBP can directly stimulate its catalytic HAT activity presents an
214 interesting role for these interactions: RNA binding events could enable local
215 'tailoring' of enzymatic-activity profiles.

216

217 In the case of CBP, this tailoring is driven by the binding of RNAs transcribed
218 proximal to sites where CBP is recruited to chromatin. RNAs bind to CBP through a
219 non-canonical, positively charged RBR within the catalytic HAT domain. Because this
220 region is crucial for the regulation of CBP activity, RNA binding can stimulate the
221 catalytic activity of the enzyme and promote gene expression. The structure of CBP,
222 and its method of regulation by the activation loop, renders it particularly susceptible
223 to regulation by RNA binding to this region. Whilst it is increasingly clear that RNA
224 binding to other chromatin-modifying enzymes is widespread, the extent to which this

225 binding can modulate their enzymatic activity is less well understood. A number of
226 examples suggest that RNA does play such a regulatory role^{21,22,25-28}, however
227 further biochemical work is required to fully determine the mechanisms that RNAs
228 use to modulate the activity of these complexes.
229

230 For CBP, locus-specific RNA binding leads to preferential binding of CBP to eRNAs,
231 as CBP is enriched at enhancers. Our results suggest a functional role for eRNAs as
232 regulatory molecules that are able to stimulate the activity of a key epigenetic
233 regulatory enzymes, thereby promoting gene expression. Furthermore, they suggest
234 an intriguing, RNA sequence-independent (but potentially structure-dependent) role
235 for eRNAs in modulating the activity of chromatin modifying enzymes, that could
236 directly impact transcription by altering the chromatin environment. In contrast, we
237 note that enhancer-specific methylation changes appear to be reliant on transcription
238 at enhancers, rather than eRNA transcripts themselves¹⁰. A key outstanding
239 question is whether eRNA transcripts can play a role in modifying the activity of
240 epigenetic complexes when enhancers loop to contact promoter regions.
241

242 Chromatin-modifying enzymes and enzyme complexes are crucial for directing gene
243 expression profiles across the genome. In particular, eRNAs are attractive
244 candidates as regulatory molecules due to their highly localized expression profiles,
245 rapid turnover and low abundance^{2,3,5}. The prevalence of RNA binding to chromatin-
246 modifying enzymes, and the potential for modulation of their catalytic activity,
247 suggests that RNAs could modulate their activity in a similar manner to CBP to
248 generate tailored patterns of histone modifications and gene expression profiles.
249

250

251 **Figure Legend**

252

253 **Figure 1:** RNA binding can generate localized CBP activity profiles and tailored
254 patterns of histone acetylation. A) Locus specificity for RNA binding to CBP. CBP
255 bound RNAs (dashed-line) correspond to sites of CBP recruitment to chromatin
256 (dark-grey) and nascent RNA transcription (light-grey) at enhancers and promoters;
257 B) RNA binding to the activation loop of CBP stimulates catalytic HAT activity by
258 displacing the loop from the active site, and thereby promotes CBP dependent
259 histone acetylation (H3K27ac); C) eRNA binding could generate enhancer specific
260 patterns of CBP activity by differential regulation of CBP activity. At more active
261 enhancers, CBP activity is stimulated more strongly by eRNA binding (arrows) than
262 at enhancers with weak stimulation of CBP activity (crosses); D) Differences in the
263 ability of sense and antisense eRNA transcripts to stimulate CBP activity. The eRNA
264 strand that binds and stimulates CBP strongly (arrows) could promote associated
265 gene expression more strongly than weaker binding eRNA strands (crosses).
266

267

268

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373

Figure 1

