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Boosting transcription by transcription: enhancer associated transcripts

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Abstract

Enhancers are traditionally viewed as DNA sequences located some distance from a promoter that act *in cis* and in an orientation-independent fashion to increase utilization of specific promoters and thereby regulate gene expression. Much progress has been made over the last decade toward understanding how these distant elements interact with target promoters, but how transcription is enhanced remains an object of active inquiry. Recent reports convey the prevalence and diversity of enhancer transcription and transcripts and support both as key factors with mechanistically distinct, but not mutually exclusive roles in enhancer function. Decoupling the causes and effects of transcription on the local chromatin landscape and understanding the role of enhancer transcripts in the context of long-range interactions are challenges that require additional attention. In this review we focus on the possible functions of enhancer transcription by highlighting several recent eRNA papers and, within the context of other enhancer studies, speculate on the role of enhancer transcription in regulating differential gene expression.

Keywords

Enhancer; chromatin; noncoding RNA; transcription; mediator; cohesion; histone modifications; enhancer transcripts; eRNA

Introduction

While promoters, enhancers, and insulators are all regulatory elements involved in controlling gene expression, enhancers in particular are largely responsible for rapidly modulating transcriptome profiles in response to stimuli or as cells differentiate and perform specialized functions. Enhancers contribute to dynamic gene regulation by acting as a docking site for transcription factors, which in turn recruit coactivator complexes that subsequently activate or boost transcription from distal promoters. Dynamically regulated biological processes, such as cellular differentiation or those involved in the immune response and hormone signaling, utilize enhancers as a means of coordinating developmental or tissue-specific spatial, temporal, and contextual transcription. In general, enhancer sequences are relatively small, encompassing several hundred base pairs to a few kilobases (kb), yet these sequences are able to dramatically impact distal promoters through a series of carefully coordinated processes. The particular combination and sequential binding of transcription factors (TF) regulates and primes the conditional activation of enhancers (Lupien et al. 2008; Heinz et al. 2010; Zaret and Carroll 2011). Most enhancers

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are pre-marked by DNase hypersensitivity, eviction of a central nucleosome at the TF binding site, and two well-positioned nucleosomes flanking the TF site (He et al. 2010; Wang et al. 2012), such as observed at binding sites for the glucocorticoid receptor (John et al. 2011).

Chromatin at active enhancer sequences is typically characterized by the presence of the histone variants H2A.Z and H3.3 along with a myriad of posttranslational histone modifications. Among the various histone modifications, histone H3 mono-methylated and di-methylated at lysine 4 (H3K4me1 and H3K4me2), combined with histone H3 acetylated at lysines 9 and 27 (H3K9ac and H3K27ac), are generally accepted as indicators of active enhancers (Barski et al. 2007; Consortium et al. 2007; Heintzman et al. 2007; Koch et al. 2007; Wang et al. 2008; Creyghton et al. 2010; Zentner et al. 2011; Bonn et al. 2012), and are described in detail in a recent review (Calo and Wysocka 2013). While many modifications are shared between active enhancers and promoters, a higher ratio of H3K4me1 to histone H3 trimethylated at lysine 4 (H3K4me3) at enhancers and depletion of H3K4me1 at promoters is a prominent difference between the two types of regulatory elements (Heintzman et al. 2007; Heintzman et al. 2009). In addition, the association of the histone acetyltransferases (HAT), CREB-binding protein (CBP) and p300 are commonly used to predict active enhancers (Heintzman et al. 2007; Visel et al. 2009).

Enhancer transcription

The first report of transcription at an enhancer was at the well-studied locus control region of the β-globin cluster in human erythroid cells (Tuan et al. 1992). Since that time, transcription has been detected at countless enhancers, with most reported in the last few years. The act of transcription at enhancers raises several questions including: is transcription common to all enhancers, and is it necessary for function? Advances in sequence-based technologies have made progress towards addressing these questions, as we describe below. Since the initial publications featuring widespread transcription at enhancers (De Santa et al. 2010; Kim et al. 2010), other genome-wide studies have identified enhancer transcripts linked to the immune response, hormone signaling, development, neural signaling, and cancer (Table 1).

Initial reports that focused on specific enhancers described long, often polyadenylated transcripts that were readily detectable, but such scenarios did not appear common. However, given what we now know, most RNA originating from enhancers is at low levels, with some active enhancers producing as few as 1.3 transcripts per cell (Li et al. 2013). Therefore, it is not surprising that bidirectional, short, non-polyadenylated transcripts went unnoticed for so long.

The experimental approach taken by Kim and colleagues is represented in Figure 1a. The authors set out to investigate how enhancers contribute to changes in gene expression by stimulating primary cortical neurons dissected from mouse embryos. Cells were first treated with tetrodotoxin (TTX) to reduce neural activity prior to inducing membrane depolarization with potassium chloride (KCl). These cells were then used to investigate genome-wide changes in gene expression and chromatin at enhancers. Most enhancers and promoters were pre-marked with H3K4me1 and H3K4me3 respectively, as well as certain TFs, and RNA polymerase II (Pol II) was detected at promoters. Upon stimulation with KCl, Pol II and CBP binding increased at promoters and enhancers and Neuronal PAS domain 4 (NPAS4), a TF not present before stimulation, was found almost exclusively at enhancers. Intriguingly, this coincided with an increase in the bidirectional transcripts that extend ~1–2 kb from the CBP binding site within enhancers (Fig. 1b). These RNAs were termed enhancer RNAs (eRNAs) and increases in their levels strongly correlated with expression of nearby target genes. Furthermore, significant increases in the levels of eRNA were dependent upon

interaction with an intact target promoter, as demonstrated by deleting the promoter of the Activity-Regulated Cytoskeleton-Associated gene (*Arc*) in mouse neurons. Stimulated wild-type neurons recruited Pol II to the *Arc* enhancer and generated bidirectional eRNAs, while neurons lacking the *Arc* promoter continued to recruit Pol II to the enhancer, but in the absence of CBP and eRNAs (Fig. 1c).

Further evidence supporting eRNAs in enhancer activity comes from mutating TF binding sites within enhancers, resulting in decreased luciferase expression from enhancer constructs (Ernst et al. 2011), coupled with decreased expression of an eRNA necessary for enhancer function (Melo et al. 2013). Weak or poised enhancers generally show lower eRNA levels than active, strong enhancers (Ernst et al. 2011) and therefore, a change in transcriptional activity, rather than absolute expression is a better indicator of enhancer activity. Reverse transcription of RNA coupled with the polymerase chain reaction, or massively parallel RNA sequencing (RNA-seq) measure steady-state transcripts and often fail to detect the presence of or changes to unstable or low-abundance transcripts, whereas global run-on sequencing (GRO-seq) (Core et al. 2008) detects nascent transcripts before they are released from Pol II and has been the choice for more recent eRNA studies (Table 1, and see below).

Enhancer transcripts are typically un-spliced and either short (1–3 kb), bidirectional, and non-polyadenylated, or long (>3 kb), unidirectional, and can be polyadenylated or non-polyadenylated (Table 1). The discrepancy between the types of transcripts found by different studies could be enhancer and lineage-specific, but may also reflect the different criteria used to identify enhancers. Candidate enhancers are sometimes removed from data sets because their histone modification profile is indistinguishable from that of other transcribed sequences making their assignment as true enhancers challenging (Mikkelsen et al. 2007; Guttman et al. 2009).

Basic differences do exist between transcriptionally active enhancers and those apparently not generating eRNAs. Enhancers generating polyadenylated transcripts are marked by histone H3 trimethylated at lysine 36 (H3K36me3) and have marginally higher activity than non-polyadenylated enhancers (Koch et al. 2011). Transcribed enhancers show higher occupancy of Pol II and elevated levels of H3K4me1, H3K4me3 and H3K27ac compared to those that are not transcribed (Djebali et al. 2012), and are more likely to physically interact over long distances with transcription start sites (TSS) of target genes (Hah et al. 2013).

Mediator and cohesin associate with enhancer transcripts

One of the proposed roles for eRNAs is that they act as a scaffold to assist in long-range interactions. In support of this, enhancers looped to TSS are more likely to express eRNAs and do so at higher levels than those that are not looped (Sanyal et al. 2012; Hah et al. 2013). Independent studies have confirmed that this observation is not simply a result of enhancer activity, but that eRNAs have a functional role in long-range interactions (Orom et al. 2010; Hah et al. 2013; Lam et al. 2013; Melo et al. 2013), specifically through associations with subunits of Mediator and cohesin (Lai et al. 2013; Li et al. 2013).

Orom and colleagues described a type of long noncoding RNA (lncRNA) with enhancer activity that they referred to as noncoding RNA-activating (ncRNA-a) (Orom et al. 2010). Whether these are the same as eRNA is unclear, as in general they are longer, unidirectional and polyadenylated, and share histone modification signatures typical of lncRNA. In a follow up study, they showed that enhancers generating ncRNA-a were positive for Pol II, as were the target promoters. Furthermore, chromatin immunoprecipitation (ChIP) against subunits of Mediator (MED1 and MED12) showed that both the target promoter and enhancer were positive, and chromosome conformation capture indicated that the two DNA elements were held in close proximity (Fig. 2a), an arrangement that was perturbed upon

reduction of the levels of Mediator subunits by short hairpin RNAs. Purification of Mediator revealed co-fractionation of the ncRNA-a; likewise, precipitation of the ncRNA-a pulled down Mediator, suggesting that Mediator directly interacts with the RNA (Lai et al. 2013). MED12 is mutated in patients with FG syndrome-1 (OMIM 305450). Introduction of known FG syndrome-1 mutations into a FLAG-tagged MED12 transgene substantially reduced the ability to co-purify ncRNA-a compared to wild-type sequence, supporting an important role of ncRNA-a interaction with the Mediator complex (Fig. 2b) and implicating this deficiency as a contributing factor to the disease. Intriguingly, further evidence supporting an activating role at target genes by the ncRNA-a was demonstrated *in vitro* by its stimulation of Mediator kinase activity, targeting H3 serine-10 phosphorylation (H3S10ph) at target genes (Fig. 2c). This activity was compromised when ncRNA-a levels were artificially reduced, and not stimulated by non-enhancer derived lncRNA.

Long-range looping between enhancers and promoters involves the cohesion complex in addition to Mediator (Kagey et al. 2010; Schmidt et al. 2010). Li and colleagues provide evidence to suggest that actual eRNAs rather than the act of transcription alone influences target gene expression, in part through the stabilization of enhancer-promoter looping through cohesion (Lai et al. 2013). In this study, the human breast adenocarcinoma cell line, MCF-7, was stimulated by treatment with 17β-oestradiol (E2) to induce oestrogen receptor-α (ER-α) binding. Changes to ER-α occupancy were assessed by ChIP coupled with massively paralleled sequencing (ChIP-seq), and changes in transcription assessed by GRO-seq (Fig. 3a). The majority of the up-regulated genes were less than 200 kb from ER-α binding sites that co-localized with H3K27ac and H3K4me1, consistent with enhancer sites. Approximately 90% of ER-α bound enhancers generated ~1.5-kb bidirectional transcripts similar to those found in neurons (Kim et al. 2010), while unidirectional transcripts were detected for the remaining ~10% of enhancers (Fig. 3b). However, because GRO-seq detects nascent transcripts, it is unclear if these are polyadenylated. Artificially reducing the levels of eRNA through the use of locked nucleic acids (LNA) and small interfering RNA (siRNA), did not impact ER-α binding, but did reduce target gene transcription, suggesting a functional role for the actual eRNA transcripts (Fig. 3c).

Many ER-α binding sites overlapped with cohesion sites (Schmidt et al. 2010), based on the occupancy of RAD21, which increased at putative ER-α enhancers upon E2 treatment. Reducing the levels of eRNA and cohesin subunits, RAD21 and SMC3, caused a decrease in target gene expression and physical interactions between the enhancer and promoter. Importantly, cohesin and eRNAs appear to physically interact based on pull-down experiments, and reduction of eRNA levels by LNA or siRNA treatment reduced interaction levels between enhancers and promoters (Fig. 3d), supporting a model whereby eRNA have a role in facilitating and stabilizing long-range interactions through cohesin (Li et al. 2013).

Enhancer transcription and histone modifications

The simple act of Pol II transcription is sufficient to alter the local chromatin environment (Wang et al. 2005; Ho et al. 2006; Kim et al. 2007; Petesch and Lis 2012; Kornienko et al. 2013) and has implications in enhancer activation and priming (Kaikkonen et al. 2013). Enhancer transcription correlates with the domain of association and appearance of H3K4me1 and H3K4me2 at poised and active enhancers (Kim et al. 2010; Wang et al. 2011; Kaikkonen et al. 2013). Kaikkonen and colleagues investigated chromatin and transcriptional changes at macrophage enhancers in order to understand the order of events in establishing active/poised enhancer states (Kaikkonen et al. 2013). Macrophages were treated with Kdo2-lipid A (KLA), a specific agonist of the Toll-like receptor 4 (TLR4), in order to activate an immune response. GRO-seq revealed increases and decreases in a large numbers of transcripts (Fig. 4a), with many activated genes involved in inflammation and

immunity as was expected. Putative enhancers were identified by performing ChIP-seq to H3K4me2, prior to and at various time intervals post stimulation. The vast majority of enhancers remained unchanged; however, some showed a loss of H3K4me2 signal, whereas others gained H3K4me2 (Fig. 4b), and these changes correlated directly with changes in expression of local genes. This latter class were referred to as "*de novo*", and provided a means to investigate the mechanism through which enhancer chromatin is established. *De novo* enhancer sites were enriched for motifs for various TFs, including NF-κB, interferon regulatory factors, a PU-box binding factor, and STAT (signal transducer and activator of transcription) factors. Blocking nuclear entry of NF-κB prevented H3K4me2 accumulation at some enhancers, suggesting an important role for TF binding prior to acquisition of chromatin modifications at enhancers. By monitoring chromatin and expression changes at defined time points post induction, the order of events leading to establishing the active *de novo* enhancers could be determined. As outlined in the schematic model in Figure 4c, signal-dependent TFs bind to the enhancer site and recruit co-activator complexes and HATs that coincide with gain of H3K27ac. This in turn recruits Pol II, which initiates both unidirectional and bidirectional transcription of eRNA, followed by acquisition of H3K4me1 and H3K4me2. Pausing Pol II through inhibitor treatment, did not impact TF binding or histone acetylation, but did reduce eRNA and H3K4me1/2 deposition. Interestingly, reducing eRNA levels did not block H3K4me2 deposition, suggesting that the act of transcription and not the eRNA *per se* were necessary, although the length and directionality of the eRNAs directly correlated with the peaks of H3K4me1/2. Targeted reduction of the levels of known H3K4 histone methyltransferases, implicated Mll1 and Mll3 in enhancer H3K4me1/2 deposition. MLL is recruited by CBP (Ernst et al. 2001; Goto et al. 2002), aligning with the observation that H3K9ac and H3K27ac precede H3K4me1/2 at *de novo* enhancers.

As an about-face, Lam and colleagues investigated how repressor complexes shut down gene expression through the nuclear receptors Rev-Erb-α and Rev-Erb-β (Lam et al. 2013). Rev-Erb binding sites corresponded to regions defined by a high ratio of H3K4me1 to H3K4me3, indicative of enhancers. Binding of Rev-Erb to target sites, recruited co-repressor histone deacetylase (HDAC) complexes that reduced H3K9ac, resulting in reduced eRNA levels and corresponding target gene expression (Fig. 5). Consistent with H3K4me1 providing molecular memory for sites of transcription and poised enhancers (Rada-Iglesias et al. 2011; Kaikkonen et al. 2013; Ostuni et al. 2013), this modification was not impacted by Rev-Erb binding.

Concluding remarks

Despite advances made in the past few years and the shift from the initial descriptive studies to those probing the importance of enhancer transcription, some questions still remain. Mediator-dependent interactions between enhancers and target promoters, as well as Mediator kinase activity towards H3S10, a histone modification that contributes to transcript elongation, are at least partially dependent on eRNAs and reducing these transcripts severely reduces expression of target genes. Enhancer transcripts interact with Mediator, but how and when contact is established is an important question. Some proteins are able to bind specific secondary structures in lncRNA as a means of directing non-sequence specific proteins to the appropriate genomic location (Tsai et al. 2010). Secondary structure may also contribute to the interactions between eRNA and Mediator. Several lines of evidence support complex interactions between multiple promoters and enhancers (Li et al. 2012; Sandhu et al. 2012; Sanyal et al. 2012; Thurman et al. 2012; Hwang et al. 2013) and it is conceivable that, though interactions with Mediator and cohesin (Kagey et al. 2010), eRNAs contribute to the assembly and maintenance of these topologically complex structures (Lai et al. 2013; Li et al. 2013; Melo et al. 2013).

Decoupling the causes and effects of transcription at enhancers is a difficult task. Evidence currently supports transcription as a contributor of H3K4me1 and H3K4me2 deposition, both of which are prevalent at active enhancers and may serve as indicators of recent enhancer activity or facilitate enhancer poising. Further work is necessary to elucidate the mechanisms underlying enhancer kinetics.

Taken together, transcripts and transcription are integral components of many, if not most, enhancers and given the current momentum of eRNA research, a greater mechanistic understanding of how enhancer transcription contributes to gene regulation is within reach.

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Abbreviations

AR	Androgen receptor
Arc	Activity-Regulated Cytoskeleton-Associated Protein
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
ChIP-seq	ChIP coupled with massively paralleled sequencing
E2	17β-oestradiol
ER-α	Oestrogen receptor-α
eRNA	Enhancer RNA
GRO-seq	Global run-on sequencing
H3K9ac	Histone H3 acetylated at lysine 9
H3K27ac	Histone H3 acetylated at lysine 27
H3K4me1	Histone H3 monomethylated at lysine 4
H3K4me2	Histone H3 dimethylated at lysine 4
H3K4me3	Histone H3 trimethylated at lysine 4
H3K36me3	Histone H3 trimethylated at lysine 36
H3S10ph	H3 serine-10 phosphorylation
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
kb	Kilobase
KCl	Potassium chloride
KLA	Kdo2-lipid A
LNA	Locked nucleic acids
lncRNA	Long non-coding RNA
LPS	Lipopolysaccharide
ncRNA-a	noncoding RNA-activating

NPAS4	Neuronal PAS domain 4
Pol II	RNA polymerase II
RNA-seq	Massively parallel RNA sequencing
SRF	Serum Response Factor
siRNA	Small interfering RNA
TF	Transcription factor
TLR4	Toll-like receptor 4
TSS	Transcription start sites
TTX	Tetrodotoxin
3C	Chromosome conformation capture
3D-DSL	Three-dimensional DNA selection and ligation
4C	Circularized chromosome conformation capture

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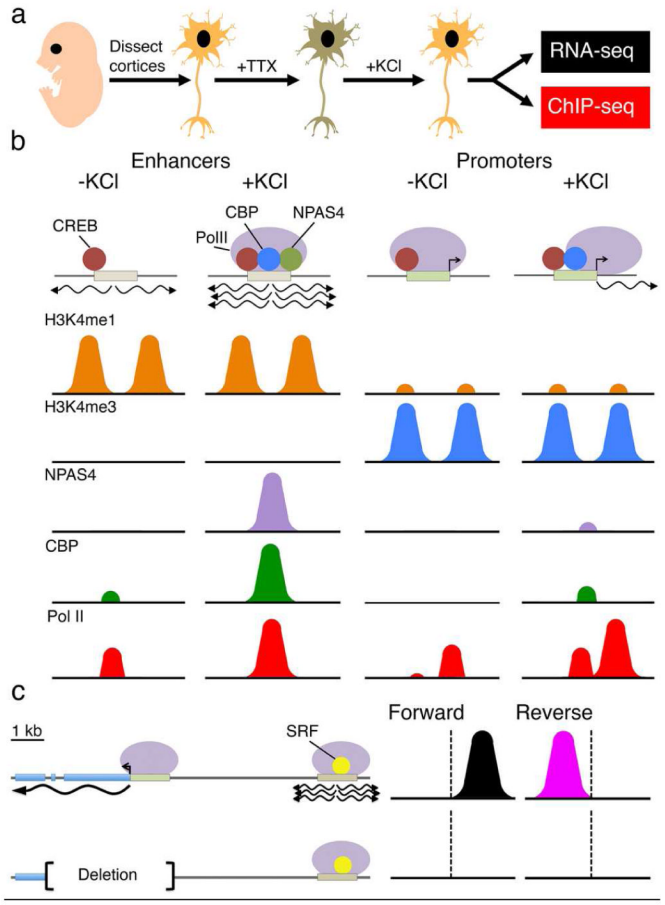

Fig. 1.

a. Schematic representation of the experimental flow used by Kim and colleagues. Briefly, cortices were dissected from E16.5 C57BL/6 mouse embryos (far left) and neurons seeded into culture dishes. Neuronal activity was dampened by treatment over night with TTX before depolarizing membranes with KCl. Changes in expression and chromatin were assessed by RNA-seq and ChIP-seq. **b**. Schematic representation of chromatin state at enhancers and promoters prior to and after KCl treatment. The enhancer and promoter are represented by a tan and green box respectively. Transcription factors and Pol II are indicated. The right facing hooked arrow at the promoter indicates the transcriptional start site. Wavy arrows represent transcripts and their direction of transcription. Below this are stylized representations of ChIP-seq peak profiles for the chromatin modifications H3K4me1 and H3K4me3, the transcription factor NPAS4, co-activator CBP and Pol II. **c**. Schematic representation showing the impact of the deletion of the *Arc* promoter on eRNA transcription. Top image shows the wild type *Arc* enhancer upstream (right side) of the *Arc* gene locus (left side). Pol II is located at both elements, whereas Serum Response Factor (SRF) is restricted to the enhancer. Bidirectional eRNA transcripts are generated at the enhancer which is represented as the RNA Seq profiles to the far right (black = forward strand; red = reverse strand). The lower image shows the loss of transcripts in the *Arc* knockout.

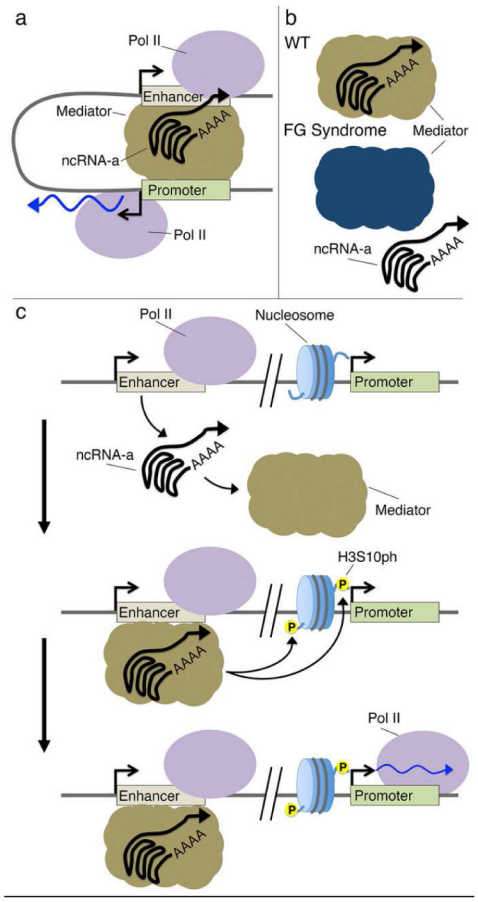

Fig. 2.

a. Stylized model showing the long-range bridging between an enhancer and promoter by the Mediator complex. The ncRNA-a that is bound to Mediator is represented by the squiggly arrow, whereas promoter generated transcripts as blue wavy arrows. **b**. Point mutations in the Mediator subunit MED12 in patients with FG syndrome-1 disrupt the ability for ncRNA-a to bind to the Mediator complex. **c**. Model depicting the stimulation of kinase activity by ncRNA-a binding to the Mediator complex, resulting in localized H3S10ph at promoters.

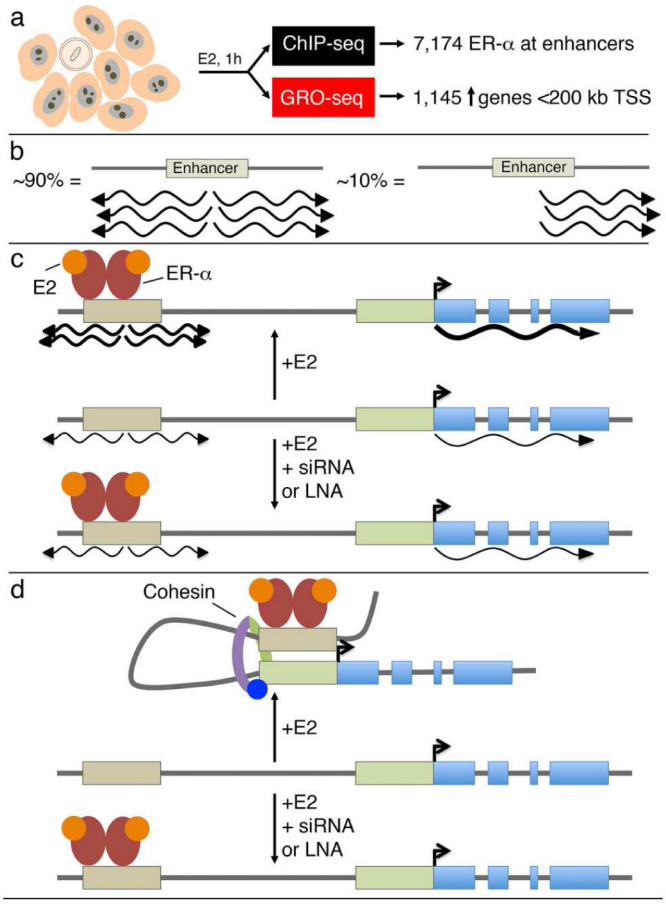

Fig. 3.

a. Experimental design performed by Li and colleagues. Briefly, MCF-7 cells were treated for one hour (1h) with E2. Changes in the levels of nascent RNA were measured by GRO-seq and changes in E2-bound ER-α chromatin occupancy were assayed by ChIP-seq. TSS = transcription start site. **b**. Approximate proportion of eRNA that were bidirectionally or unidirectionally transcribed. **c**. Model depicting the impact of E2 treatment on eRNA and promoter driven transcript levels, as compared to cells in which eRNA were reduced by siRNA or LNA treatment. The red oval with orange circle represents E2-bound ER-α. **d**. Model depicting the impact of eRNA loss on cohesion-mediated long-range interactions. E2 treatment increases the level of interaction represented by the cohesion folded image, whereas E2 treatment in cells with reduced eRNA levels reduces the overall long-distance interactions.

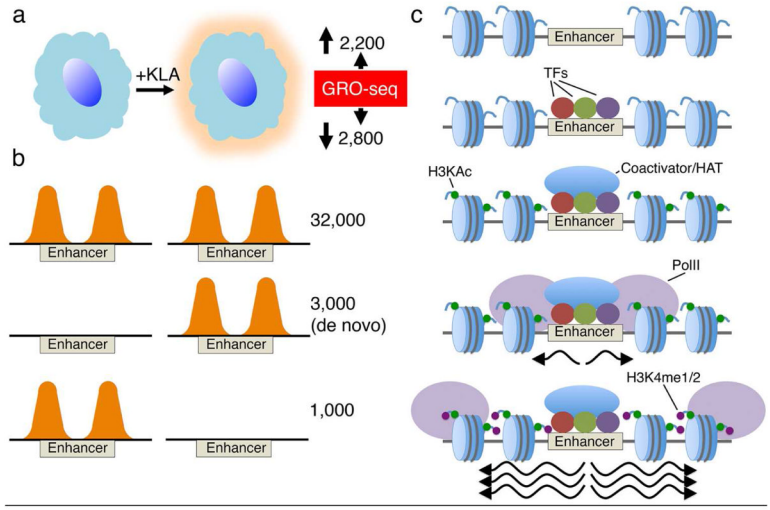

Fig. 4.

a. Experimental system used by Kaikkonen and colleagues. Macrophages were stimulated with the TLR4 agonist KLA. Up and down changes in the levels of nascent RNA were measured by GRO-seq. **b**. Schematic representation showing the gain, loss or no change in H3K4me2 ChIP-seq signals at enhancers upon KLA treatment. The number of enhancers in each category is given to the right. **c**. Model showing the sequence of events resulting in the establishment of H3K4me1/2 at enhancers. Briefly, TF binding at the enhancer sequence recruits a co-activator HAT complex that acetylates various lysine residues of core histones. Pol II is recruited and bidirectional transcription is initiated generating eRNA transcripts. The passage of Pol II, as transcripts are elongated, results in H3K4me1/2 at the enhancer nucleosomes.

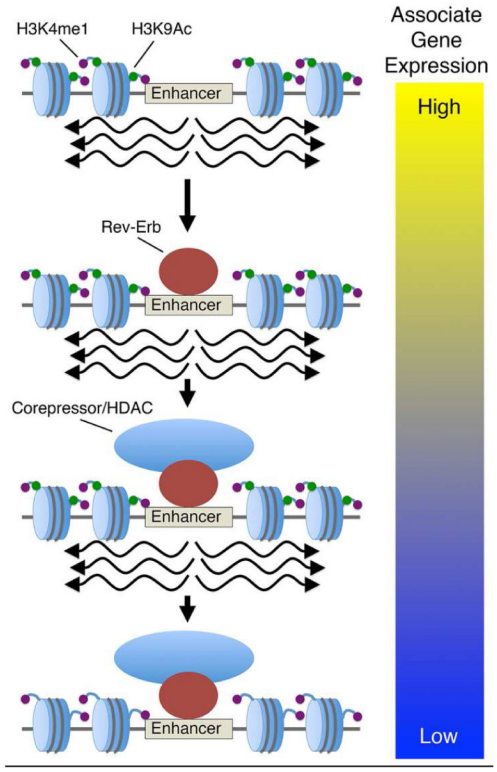

Fig. 5.

Schematic representation of Rev-Erb silencing of an active enhancer. Nucleosomes are represented at blue cylinders and covalent modifications of histone H3 N-terminal tail are represented as a purple sphere (H3K4me1) or green sphere (H3K9ac). Wavy arrows indicate eRNA transcripts and direction of transcription. Briefly, the illustration shows that binding of Rev-Erb at the enhancer results in the recruitment of a co-repressor/HDAC complex that de-acetylates K9 of H3 and shuts down eRNA transcription. To the right of the image, the impact on the expression of genes influenced by the enhancer is represented by a gradual reduction in expression from high (yellow) to low (blue).

Table 1

Recent literature focusing on enhancer transcription.

Paper	Major finding	RNA	Enhancer-promoter interactions	Org.	Cell type	Transcript directionality	Poly(A)	length (kb)
Kim et al. 2010	Widespread enhancer transcription (eRNA) in response to stimulation of neurons.	RNA-seq	NR	Mo	primary cortical neurons	bi	(−)	< 2
DeSanta et al. 2010	Pol II occupies intragenic sites and many are transcriptionally active enhancers. Enhancer activity induced by LPS.	FANTOM, RNA-seq	3C	Mo	macrophage	uni	(+)	0.1 to 7
Orom et al. 2010	Activating RNAs are intergenic transcripts capable of increasing adjacent and non-adjacent gene expression.	GENCODE, custom microarray	NR	Hu	multiple cell lines & primary cells	uni (spliced)	(+)	0.1 to 9
Wang et al. 2011	Enhancer responds to AR binding in different ways, suggesting multiple classes of transcriptionally active enhancers.	GRO-seq	3C	Hu	prostatic adenocarcinoma	bi	NR	~ 1
Koch et al. 2011	Enhancer transcripts can be poly(A)+ or poly(A)−, and poly(A)+ enhancers have H3K36me3. Enhancers are primarily bound by the initiating form of Pol II.	RNA-seq	NR	Hu	double-positive thymocytes	both*	60% (+) 40% (−)	> 4 (+) 2 to 3 (−)

Paper	Major finding	RNA	Enhancer-promoter interactions	Org.	Cell type	Transcript directionality	Poly(A)	length (kb)
Kowalczyk et al. 2012	Inter and intragenic enhancers produce transcripts that exactly mirror the gene structure with the exception of the first exon. Multi-exonic poly(A)+ RNA (meRNA)	RNA-seq	NR	Mo & Hu	multiple cell lines & primary cells	both**	uni: (+) bi: (−)	uni: length of gene bi: ~ 1
Melo et al. 2013	Enhancer-promoter interactions exist prior to activation. Tethered eRNA is able to induce transcription of reporter gene.	qRT-PCR	4C	Hu	breast adenocarcinoma & primary fibroblasts	uni	(−)	~ 0.6
Lai et al. 2013	ncRNA-a physically interact with Mediator and are required for full target gene upregulation and enhancer-promoter interactions. Transcripts stimulate H3S10ph.	qRT-PCR	3C	Hu	breast adenocarcinoma & lung carcinoma	uni (spliced)	(+)	~ 0.4
Hah et al. 2013	Inhibiting eRNA elongation does not prevent enhancer-promoter interactions.	GRO-seq	3C, ChIA-PET (Fullwood et al. 2009)	Hu	breast adenocarcinoma	both	(+)	5-Mar
Lam et al. 2013	Rev-Erb binding represses enhancer transcription and consequently enhancer activity. Transcripts are	GRO-seq, 5′-GRO-seq	NR	Mo	macrophage	bi	NR	< 2

Paper	Major finding	RNA	Enhancer-promoter interactions	Org.	Cell type	Transcript directionality	Poly(A)	length (kb)
	required for enhancer activity.							
Li et al. 2013	Enhancer transcripts are necessary for enhancer-promoter interactions and their contribution is sequence specific.	GRO-seq	3D-DSL	Hu	breast adenocarcinoma	bi	NR	NR
Kaikkonen et al. 2013	Enhancer transcription is mechanistically linked to H3K4me1/2 deposition	GRO-seq	NR	Mo	primary bone marrow	both, mostly bi	NR	uni: <1.5 bi: ~2

*The majority of both poly(A)+ and poly(A)− transcripts were unidirectional, with an increase in bidirectional transcripts in the poly(A) − fraction.

**A single enhancer can transcribed short (~1 kb), bidirectional poly(A)− transcripts in addition to long, unidirectional poly(A)+ transcripts that mirror the structure of the gene.

3D-DSL = three-dimensional DNA selection and ligation; 3C = chromosome conformation capture; 4C = circularized chromosome conformation capture; NR = not reported; LPS = lipopolysaccharide; AR = androgen receptor; Hu = human; Mo = mouse.