

Developmental Transcriptional Enhancers: A Subtle Interplay between Accessibility and Activity

Considering Quantitative Accessibility Changes between Different Regulatory States of an Enhancer Deconvolutes the Complex Relationship between Accessibility and Activity

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Measurements of open chromatin in specific cell types are widely used to infer the spatiotemporal activity of transcriptional enhancers. How reliable are these predictions? In this review, it is argued that the relationship between the accessibility and activity of an enhancer is insufficiently described by simply considering open versus closed chromatin, or active versus inactive enhancers. Instead, recent studies focusing on the quantitative nature of accessibility signal reveal subtle differences between active enhancers and their different inactive counterparts: the closed silenced state and the accessible primed and repressed states. While the open structure as such is not a specific indicator of enhancer activity, active enhancers display a higher degree of accessibility than the primed and repressed states. Molecular mechanisms that may account for these quantitative differences are discussed. A model that relates molecular events at an enhancer to changes in its activity and accessibility in a developing tissue is also proposed.

or eviction of histones is a necessary step for the exposure of recognition sites of effector DNA binding proteins such as transcription factors (TFs), polymerases, and insulator proteins.^[3,4] This level of DNA exposure in a chromatin context can be profiled genome-wide with sensitive and quantitative assays involving nuclease digestion (deoxyribonuclease I hypersensitive sites sequencing, DNase-seq^[5]), transposase fragmentation (assay for transposase-accessible chromatin using sequencing, ATAC-seq^[6]), or cross-linking and phenol-chloroform extraction to separate protein-bound and protein-free DNA fragments (formaldehyde-assisted isolation of regulatory elements sequencing, FAIRE-seq^[7]). While accessible chromatin conformation is not limited to distal *cis*-regulatory elements,^[4] it has been widely

1. Introduction

Open chromatin structure has long been associated with active functional elements of the genome.^[1,2] The destabilization


used as a marker of transcriptional enhancers (reviewed in ref. [8]). In combination with other chromatin and sequence features, accessibility has been used not only to annotate genomic positions of enhancers,^[9] but also to predict their spatiotemporal activity.^[4,10] Several recent publications, however, highlight the limitations of the predictive power of accessibility, suggesting that the relationship between accessibility and activity is more complex.

In this review, we focus on developmental enhancers and discuss how regulatory events at the enhancers impact their accessibility. We first review the current knowledge and limitations of using accessibility as a signature of enhancer activity. We then show how our picture of their relationship is reduced by commonly describing accessibility and activity only in binary terms: open versus closed, active versus inactive. We go on to present recent findings that emphasize quantitative changes in accessibility between different regulatory states of enhancers. We discuss the known and plausible mechanisms that establish the accessibility of enhancers and later quantitatively modulate it during enhancer operation. We conclude with a model of temporal control of accessibility that links molecular events at the enhancers with changes in their activity and accessibility in a developing tissue.

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2. An Imperfect Correlation between Enhancer Accessibility and Enhancer Activity

In a canonical example,^[1] enhancers are accessible exclusively in the cell type where they are active, that is where they promote transcription of their target gene. During embryonic development, the chromatin of cell-type specific enhancers (e.g., regulating axis patterning genes, growth control genes, cell differentiation genes) becomes selectively open, which correlates well with the timing of their activity *in vivo*.^[11,12] The fact that accessibility of enhancers is a strong predictor of their spatiotemporal activity has been also demonstrated with computational models.^[10,13]

Alongside reports confirming the correlation between accessibility and activity of enhancers, an increasing number of publications questions this simple one-to-one relationship.^[8,14,15] While active enhancers are indeed characterized by open chromatin, they can be also accessible in other cell types or at other developmental timepoints, as demonstrated by the following studies. Arnold et al.^[16] deployed a massively parallel reporter assay STARR-seq (self-transcribing active regulatory region sequencing) to identify cell-type specific enhancers in two *Drosophila melanogaster* cell lines, derived from ovaries and embryonic macrophages. The authors found a remarkable mismatch with DNase-seq accessibility profiles, whereby about a fifth of the enhancers active in only one cell type were accessible in both cell types. Similarly, Shashikant et al.^[17] observed that enhancers exclusively active in primary mesenchyme cells (PMCs) of the sea urchin embryo were also characterized by significant accessibility in non-PMC lineages, and were already open several hours before activation of their target genes. Although McKay and Lieb^[11] concluded a strong correlation between accessibility and temporal activity of enhancers in the course of *D. melanogaster* embryogenesis, close inspection of their data reveals that some enhancers displayed FAIRE-seq signal already 2–3 h prior to expression of the reporter gene.

These examples illustrate several potential reasons for the observed discrepancies between the spatiotemporal activity and accessibility of enhancers. First, as demonstrated by McKay and Lieb,^[11] the degree of the spatial and temporal overlap between the measured accessibility and activity depends on the resolution of experiments. While accessibility and activity might strongly correlate between broadly defined developmental stages, there might be a discrepancy between the exact timing of their establishment. Second, limited purity of samples (e.g., isolated from complex tissues or fast progressing developmental processes) can lead to contamination of accessibility profiles,^[18] in particular producing significant signal over closed and presumably inactive enhancers. Finally, comparisons between accessibility measured at endogenous loci and activity measured with transgenic enhancer reporter assays should be done with caution. Sequences tested outside of their endogenous genomic context do not necessarily provide an accurate picture of their regulatory potential. In fact, it has recently been highlighted that reporter assays produce both false positives and false negatives, and that their performance is cell-line dependent.^[19,20] The results can be confounded by the use of partial enhancer sequences that miss certain transcription factor binding sites (TFBS),^[21] but also by differences in the chromatin context for integrated transgenes^[22]

or by limited chromatinization of episomal vectors.^[23,24] For example, it has been reported that an enhancer may show activity in the episomal STARR-seq assay even if in the same cell type its endogenous counterpart does not promote transcription and is marked by repressive histone marks.^[16] Thus, STARR-seq tests the overall potential of a genomic region to function as a transcriptional enhancer (independent of the cell line), rather than characterize its cell-type specific activity.

In summary, we still lack a consensus on how well the accessibility of enhancers predicts their spatiotemporal activity. To some extent, the reported discrepancies may result from technical challenges of assessing enhancers' accessibility and activity in their endogenous context, including interpretation of enhancer reporter assays. Here, we propose that these contrasting results may also reflect biological phenomena, and could be reconciled by considering different degrees of accessibility (quantitative accessibility) and different regulatory states of enhancers. As we discuss in subsequent sections, this creates a more comprehensive framework to study the causal relationship between accessibility and activity.

3. Binary Distinction between Open and Closed Chromatin Conceals Quantitative Accessibility Differences

A vast majority of studies only consider a binary distinction between open and closed chromatin. In spite of a wide dynamic range of ATAC-seq, DNase-seq, and FAIRE-seq, most analysis is performed after peak calling, and is restricted to sets of open regions with significant signal enrichment. Thus, the measured quantitative variation in accessibility is reduced to binary terms (open versus closed intervals), raising certain caveats. First, the exact definition of open regions, and more importantly their biological relevance, depends on arbitrary significance thresholds, and varies with the different specificity and sensitivity of peak calling algorithms.^[25,26] Second, such a simplified view of chromatin organization can conceal relevant quantitative differences between accessibility profiles of enhancers in different cell types. Even if in two different samples an enhancer is identified as open by a peak caller, it might still display significantly different levels of accessibility.^[15]

In that sense, there is little focus in the literature on the quantitative aspect of accessibility, other than in the context of *de novo* enhancer discovery. In one approach, the accessibility profile of a small cell population is compared with the corresponding whole-organism sample, which represents an average of all cell types. Genomic intervals that display significantly higher accessibility, and thus interpreted as specifically open in the given cell population, have been indeed shown to drive cell-type specific transcription.^[27,28] Other studies have identified novel enhancers using quantitative accessibility differences between well-separated cell types and timepoints. Shashikant et al.^[17] (discussed above) demonstrated that PMC-specific enhancers displayed higher ATAC-seq signal in the cell type of their activity as compared to non-PMC lineages. Uyehara et al.^[29] identified a set of genomic intervals that remained accessible during an entire window of *D. melanogaster* wing development, yet displayed a quantitative increase or decrease of their FAIRE-seq

signal at different timepoints. Notably, these temporally dynamic regions drove transcription of a reporter gene at the timepoint that coincided with their highest accessibility. Importantly, both studies identified cell-type specific enhancers based on the empirical observation that activity coincides with elevated accessibility. While providing a simple rule for de novo discovery of enhancers, they did not offer an explanation for the residual accessibility in the cell type where the regulatory elements did not promote transcription.

To conclude, the binary distinction between open and closed chromatin does not exploit the full quantitative potential of the genome-wide accessibility assays. Yet, the quantitative information might be meaningful to understand the reported discrepancies between enhancer accessibility and activity. For instance, when inactive enhancers are detected as open, how often do they display lower accessibility signal than the active enhancers?

4. Inactive Enhancers Encompass Multiple Regulatory States with Different Chromatin Organizations

The binary classification that we underscored for enhancer accessibility also applies to activity, a context in which enhancers are usually classified as either active or inactive. This reflects the binary read-out of reporter assays and, more generally, stems from our interpretation of enhancers as simple on and off switches. However, enhancers exist in multiple regulatory states, and transition between them during development and in response to external stimuli (reviewed in refs. [30, 31]). While an active enhancer can be unambiguously defined as one that promotes transcription from the target promoter, an inactive enhancer can in fact correspond to several states, which differ not only in terms of their regulatory potential but also chromatin organization.

Active enhancers are bound by co-activators (e.g., p300 histone acetyltransferase and the Mediator complex) and are actively transcribed by RNA polymerase II into enhancer RNA (eRNA).^[32,33] They are characterized by accessible, nucleosome-depleted chromatin organization,^[1] and exhibit H3K4 methylation (H3K4me) together with H3K27 acetylation (H3K27ac).^[34,35] Inactive enhancers, however, can be classified into three different regulatory states: 1) silenced, 2) repressed, and 3) primed. For the sake of this review, we define silenced enhancers as sequestered in compact chromatin, depleted of active histone modifications and devoid of TF binding. Importantly, we distinguish silenced enhancers from repressed elements that are subject to direct repression by sequence-specific TFs. As regulation by TFs requires exposure of their binding sites, repressed enhancers are expected to display some degree of accessibility.^[15,36] Moreover, repressed enhancers can be also simultaneously occupied by activating TFs, as illustrated by enhancers involved in axis patterning during *D. melanogaster* embryogenesis.^[21,37] These elements are targeted both by activators and repressors, and it is the stoichiometry and affinity of TF binding that determines the net effect on transcription of the target gene. While repressed enhancers share the H3K4me signature with active elements, they are characterized by lower acetylation levels of histones, in particular by loss or reduction of H3K27ac.^[38,39] The third inactive state corresponds to enhancers primed for future activation (and

possibly repression),^[40] including a class of poised enhancers in mammalian embryonic stem cells.^[35] While primed enhancers are occupied by TFs and co-regulators, they do not receive sufficient regulatory input to promote transcription from the target promoters. Poised enhancers display similar chromatin features as repressed elements.^[41] They are characterized by accessible chromatin conformation and exhibit H3K4me in the absence or reduction of H3K27ac.^[35,40,42] Poised enhancers additionally exhibit repressive H3K27 trimethylation (H3K27me3) that is associated with Polycomb Repressive Complex 2.^[43]

Few studies provide information on how accessibility changes in quantitative terms as enhancers transition between regulatory states, and we still lack a systematic comparison between all states in different multiple organisms. Some studies report very similar levels of nucleosome depletion when active, primed, and poised enhancers are compared in the same cell type.^[35,40] However, such comparisons might be misleading since they are based on the mean signal across multiple elements. We know from examination of individual enhancers in *D. melanogaster* embryos^[15] that the range of accessibility signal between individual elements in the same regulatory state can differ even by one order of magnitude. It is more informative then to examine accessibility changes as the same set of enhancers transitions between different states. In such comparisons from mouse cells, progression from the poised or primed state to the active state is accompanied by increased accessibility.^[42,44] Several studies in *D. melanogaster* revealed that repressed enhancers are characterized by reduced accessibility in comparison to their active state, while still remaining open.^[15,36,45] In fact, compaction into inaccessible chromatin was shown to be a gradual process, which occurs with a considerable delay upon repression of enhancers.^[29] Thus, inactivation of transcription by a repressor TF and loss of enhancer accessibility are distinct regulatory events that take place on different time scales.

The notion of inactive enhancer conceals differences in the regulatory potential and chromatin organization between 1) silenced elements that are sequestered in compact chromatin, and 2) repressed or 3) primed elements that are both accessible and subject to dynamic regulation. While accessibility of repressed and primed enhancers does not correlate with the expression of their target genes, it still offers a cell-type and time-point specific indication of TF binding and regulatory decisions. Importantly, these two enhancer states can be distinguished from active elements by their reduced signal when considering quantitative accessibility. On these bases, we propose that the systematic characterization of quantitative accessibility signatures for different enhancer states might improve our ability to predict them genome-wide.

5. Resolving Enhancer Accessibility in Space and Time

Besides considering the quantitative aspect of accessibility and different regulatory states of enhancers, our view on their mutual relationship can be also improved by an increased spatiotemporal resolution of accessibility profiles in complex tissues. This has been achieved thanks to the development of ATAC-seq,^[6] which allows for a considerable reduction of the starting

material, even down to single cells.^[46] Using ATAC-seq, a handful of recent publications focusing on the early embryogenesis in *D. melanogaster* have explored in great detail how enhancer accessibility varies both along the temporal and spatial axes of development.^[15,45,47,48] Additionally, by considering quantitative accessibility differences, they provided valuable insights into chromatin organization of different enhancer states.

Early *D. melanogaster* embryogenesis offers several advantages for examining enhancer activity with high spatiotemporal resolution. In this well-studied paradigm for body patterning (reviewed in refs. [49–50]), a set of enhancers regulates the establishment of spatial coordinates immediately after zygotic genome activation. As these elements are targeted both by activators and repressors whose distribution is spatially restricted along the body axes, they exist in multiple regulatory states in the pre-gastrulation embryo.^[21,37,51] A large number of these enhancers have been validated and characterized with different experimental approaches, including identification of their input TFs and target genes.^[52] While the pre-gastrulation embryo consists of 6000 nuclei, each with a unique transcriptional profile,^[53] it has a simple morphology and can be easily subjected to genetic manipulation and mechanical dissection.

The temporal dynamics of enhancer accessibility have recently been surveyed with two different experimental approaches. First, using ATAC-seq on tightly staged individual *D. melanogaster* embryos, Blythe and Wieschaus^[47] resolved enhancer accessibility changes to 3 min intervals. The authors demonstrated that acquisition of accessibility by enhancers, and thus their priming, preceded that of their target promoters. Second, Cusanovich et al.^[48] performed single-cell ATAC-seq on broader embryonic collections and computationally ordered individual nuclei into temporal trajectories based on their accessibility profiles. As a result, the authors recreated rapid transitions of cell identities during the earliest embryogenesis and identified enhancers characterized by dynamic accessibility changes. Single-cell ATAC-seq also revealed considerable spatial heterogeneity of accessibility profiles at a single developmental timepoint.

Spatial heterogeneity was confirmed by two other studies that performed a quantitative analysis of accessibility profiles of enhancers patterning the anteroposterior (AP) axis. Haines and Eisen^[45] applied ATAC-seq to cryosliced anterior and posterior halves of the embryo, while in ref. [15] we performed genetic tagging and affinity-based isolation of nuclei from seven domains along the AP axis. Both approaches demonstrated that the enhancers, while remaining open in the entire embryo, were characterized by elevated accessibility in the embryonic domain in which they promoted transcription of their target gene. On the other hand, they displayed reduced accessibility in the domain in which they did not drive transcription. In our study,^[15] we also provided a simple framework for analyzing genomic signal from complex samples, demonstrating that the ATAC-seq signal obtained from each embryonic domain was a weighted average of accessibility signatures of active and inactive enhancer states. Importantly, as activity of the enhancers was assessed through expression patterns of enhancer-reporter constructs, neither study could discriminate between the two classes of inactive enhancers: the primed and the repressed state.

In conclusion, profiling enhancer accessibility with high spatial and temporal resolution allows for linking quantitative

changes in accessibility with different enhancer states in a complex developing tissue. The recent studies highlight the dynamic evolution of enhancer accessibility in time, but also reveal that at a single time point enhancer can display different accessibility profiles in different cell populations.

6. Enhancer Accessibility is Controlled at Different Scales, from Broad Domains to Single Enhancers

The precise and dynamic pattern of accessibility of developmental enhancers points to a tight regulation of their chromatin state. At least three non-exclusive modes of accessibility control have been examined in the literature. They may represent different ranges of regulation, as suggested by others,^[54] from 1) broad chromosome domains controlled by the Polycomb/Trithorax system to 2) the initiation of accessibility of individual enhancers by pioneer transcription factors (PTFs; this section), and to 3) the local fine-tuning of accessibility during the operation of enhancers (see next section). However, the molecular mechanisms that govern each of these modes are not resolved to the same depth. Little is known about how they are coordinated.

6.1. Accessibility of Broad Chromatin Domains and Individual Enhancers is Regulated by Different Mechanisms

The first control of chromatin accessibility is extrinsic to enhancer sequences and is mediated by the Polycomb-group proteins (PcG) and Trithorax-group proteins (TrxG) from a particular class of *cis*-regulatory elements called PREs and TREs, respectively. PREs and TREs govern chromatin compaction over broad chromosomal domains, in the order of tens or hundreds of kilobases, by recruiting chromatin modifiers and remodelers.^[55] In the plethora of studies on PcG and TrxG, the chromatin state is evaluated through different histone modifications^[56–60] and the actual chromatin accessibility is not directly examined. It is therefore difficult to reconcile the pattern of local, discrete, and dynamic peaks of accessibility seen at individual enhancers^[15,29,36] with the broad control of chromatin states from PREs and TREs. Do subtle interactions between PREs and individual enhancers modulate local accessibility within a broad Polycomb repressive domain? Exploring this question during *D. melanogaster* embryogenesis, Koenecke et al.^[41] made an interesting observation: the enrichment of the Polycomb repressive mark H3K27me3 at individual enhancers is strongly correlated with their distance to the nearby PRE, rather than with the local repression by a TF. To further understand the role of this broad control at individual enhancers, it would be necessary to directly probe their accessibility with DNase-seq or ATAC-seq upon mutation of a nearby PRE or TRE.

The second control of enhancer accessibility is built-in in the form of binding sites for PTFs. PTFs are defined as proteins that initiate accessibility, and thus prime enhancers,^[61,62] by interacting directly with nucleosomal DNA.^[63] Their occupancy at enhancers induces local nucleosome depletion that is required for the subsequent binding of the patterning activator and repressor TFs.^[14,18,64–68] Recent studies from *D. melanogaster* show that the establishment of accessibility is directly mediated through

PTF binding sites within the enhancer,^[14] and that the degree of openness positively correlates with their number.^[65,69] In a classical view, while PTFs prime enhancers, they are insufficient to activate transcription of the target gene, a job left to activator TFs.^[14,70,71] However, recent studies indicate that the distinction between PTFs as permissive factors and patterning TFs as instructive factors is somewhat blurred. For example, the activator TFs p53^[72] and Bicoid,^[73] while operating in completely different physiological contexts, appear to promote both accessibility and activity of some of their respective target enhancers. Furthermore, a classical PTF, Zelda, has been recently demonstrated not only to be required for the maintenance of enhancer accessibility during the operation of patterning TFs,^[71] but also to bear itself a transcription activating domain.^[74]

6.2. Enhancers Preserve their Local Accessibility Profiles Outside of their Endogenous Loci

Although genomic context influences their activity, enhancers are deemed to function as independent modules.^[75] How is this reflected at the level of their accessibility? We have seen above that accessibility of enhancers is determined by intrinsic information and quantitatively linked to the number of PTF binding sites,^[65,69,70] suggesting a certain degree of autonomy. How then does the broader genomic context (e.g., nearby PREs and TREs) influence the local accessibility of an enhancer?^[76] To test what aspect of enhancer accessibility is inherently determined by its sequence, in particular in ectopic reporter constructs, we reanalyzed ATAC-seq profiles from our recent study.^[15] We compared accessibility signal of selected enhancers active in *D. melanogaster* embryos under two genetic conditions (**Figure 1**): 1) embryos containing only an endogenous enhancer (blue line) and 2) transgenic embryos with an additional copy of the same enhancer in a genomic integration site (green line). Remarkably, the transgenic enhancers displayed very similar chromatin organization as their endogenous counterparts. Boundaries between the accessible portion of the enhancer and the inaccessible flanking regions were maintained at a nearly base-pair resolution. Furthermore, local modulation of accessibility, including positioning of nucleosomes, was also highly conserved in the transgenic enhancers. We concluded that the 0.7–1.7 kb of the DNA sequences that we placed in the genomic integration site were autonomous to determine and fine-tune their own accessibility with almost a base-pair resolution. It is important to note that the transgenic enhancers were placed in a permissive environment of a tested integration site.^[77] As transgenes show variable activity depending on their genomic position,^[78] it would be interesting to test how the boundaries and the overall degree of enhancer accessibility change in different genomic integrations.

6.3. Interplay of Internal and External Mechanisms of Accessibility Regulation Coordinates Activity Across Multiple Enhancers

External elements such as PREs/TREs and the internal binding sites for PTFs may represent two different, yet complemen-

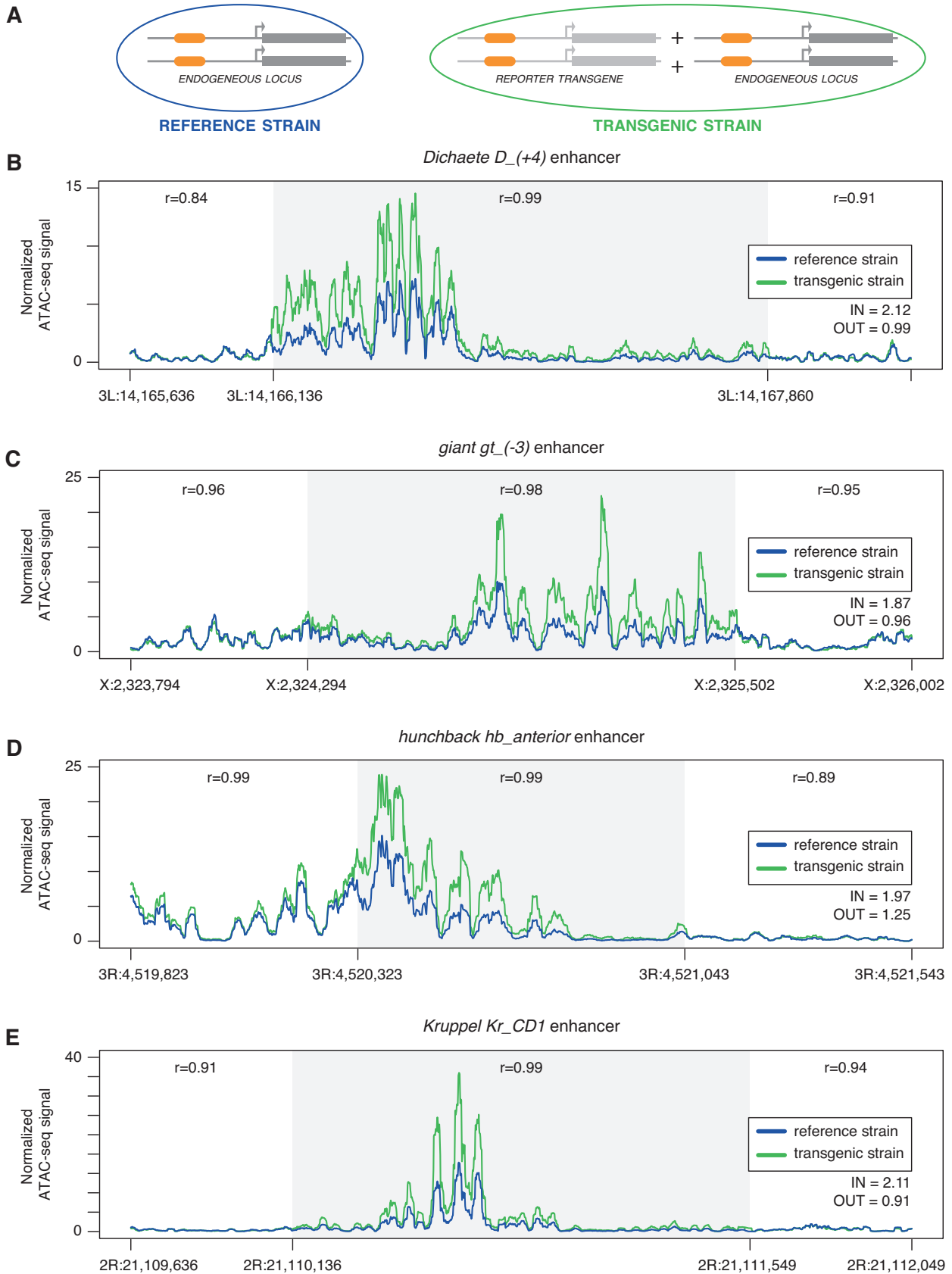
tary, regulatory mechanisms. PREs have been demonstrated to modulate higher-order chromatin structure, including looping, enhancer-promoter pairing, formation of topologically associating domains (TADs), and interactions between TADs.^[58,79] These 3D chromatin rearrangements may affect the TF target search (the ability of a TF to find its target enhancer in the nucleus),^[80] or the frequency of interactions between an enhancer and its distant target core promoter. By contrast, built-in TFBS for PTFs determine local chromatin decompaction of individual enhancers.^[14,65] As they expose TFBS to activator and repressor TFs, they play a permissive role in enhancer function. By determining boundaries of enhancer accessibility, PTFs might in fact define the exact composition of TFBS contributing to enhancer regulation.^[13] Interestingly, in the context of *D. melanogaster* embryogenesis, the same factor that primes the direct decompaction of hundreds of enhancers, Zelda, also appears to seed higher-order structures of the genome.^[81] This is perhaps where the coordination of different levels of chromatin accessibility happens. Along the same lines, another PTF, FOXA1, was showed to facilitate deposition of H3K4me by recruiting a chromatin modifier.^[82]

In conclusion, we can distinguish two scales at which accessibility of an enhancer is regulated. Locally, PTFs determine accessibility and boundaries of individual elements. On a larger scale, Polycomb and Trithorax systems determine the global permissiveness of a chromatin domain to regulatory activity, potentially coordinating the action of multiple enhancers.

7. Enhancer Activity May Directly Modulate Enhancer Accessibility

While PTFs seed and maintain enhancer accessibility,^[14,71] additional local mechanisms may subsequently modulate it during enhancer operation, resulting in the observed quantitative differences in accessibility levels between the primed and active states,^[42,44] as well as the active and repressed states.^[15,45] Accessibility signal of the ATAC-seq and DNase-seq assays represents the frequency with which naked DNA is targeted by the transposase or nuclease in the chromatin context. Therefore, the distribution and intensity of the signal are influenced both by nucleosome location along the sequence and affinity of histones to DNA.^[83] We consider here three plausible mechanisms that, by affecting nucleosome positioning and stability, quantitatively modulate accessibility of different enhancer states.

First, progression of the RNA polymerase during transcription of active enhancers^[84] results in nucleosome displacement, and transiently increases DNA exposure.^[85] Thus, the elevation in accessibility that is observed upon transition of enhancers from their primed to the active state might be a direct consequence of their transcription into eRNA. Indeed, it has been shown that signal-induced changes in transcription of genes correlate well with fold changes in eRNA levels at their corresponding enhancers.^[86] Additionally, eRNA transcription has been proposed to play an active role in chromatin remodeling, maintaining the accessibility of enhancers to transcriptional regulators.^[87] However, recent studies contest this model by demonstrating that eRNA transcription occurs also at inactive enhancers with repressive histone marks^[88] or by identifying active enhancers with undetectable eRNA transcription.^[89]



Interestingly, the rate of transcription initiation at the target promoter has been shown to correlate more strongly with the overall enhancer accessibility than the amount of transcribed eRNA.^[88]

Second, non-pioneer patterning TFs are capable of acting in a collaborative fashion to evict nucleosomes, by competing with histones for binding to their TFBS.^[90,91] If active enhancers are targeted by a larger number of regulators than their primed counterparts, we can envision that this higher TF load would result in lower nucleosome stability. Although TFs can locally protect their binding sites from cleavage by a transposase or a nuclease,^[6,92] this effect is unlikely to counterbalance the overall impact of nucleosome destabilization on enhancer accessibility.^[15] Patterning TFs bind to DNA only transiently compared to histones, their residence time being in the order of seconds,^[93,94] thus conferring greater accessibility of their TFBS. Although TFs might contribute to the overall maintenance of enhancer accessibility under a law of mass action as we have just described, it is important to note that activator and repressor TFs have been also reported to have opposing effects on the nucleosome stability. During early *D. melanogaster* embryogenesis, for instance, the activator Bicoid promotes accessibility of its target enhancers,^[73] while the repressor Knirps increases their local nucleosome occupancy.^[39] The stabilization of nucleosomes by repressors could explain the observed reduction in enhancer accessibility in the transition from an active to a repressed state. Furthermore, if a nucleosome that is stabilized by a repressor TF overlaps an activator TFBS, this might create a simple mechanism for integrating activating and repressive regulatory inputs at the enhancer.^[95] The idea that different TFs may impact local enhancer accessibility in different ways is additionally supported by studies that have examined the correlation between predicted TF motifs in regulatory elements and their overall accessibility^[4] or cell-to-cell variation in accessibility.^[46] Yet, this notion has never been thoroughly tested with experiments. Furthermore, it is still to be determined how the collaborative destabilization of nucleosomes by TFs interplays

with their stabilization by repressors, and how this impacts enhancer accessibility.

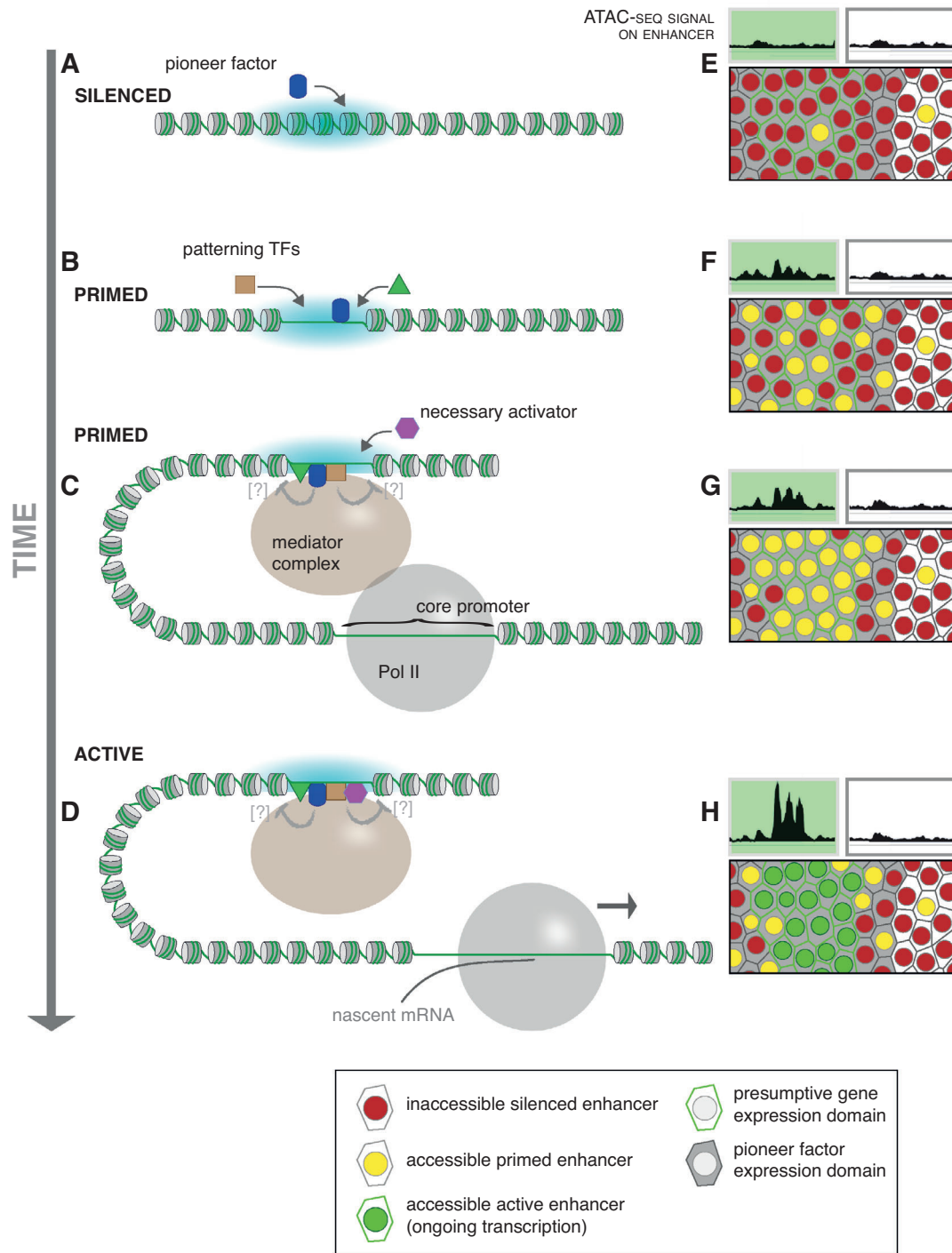
Finally, TFs may also actively shape accessibility through local modulation of epigenetic marks, by recruiting histone acetyltransferases and deacetylases via their co-activators and co-repressors, respectively.^[96] Indeed, enhancers in an active state display higher levels of H3K27ac than in a primed or repressed state.^[41] While this per se does not indicate a causal link, Li and Arnosti^[39] showed that overexpression of the repressor Knirps in *D. melanogaster* embryos led to the decrease of H4 acetylation at its target enhancers.^[97] As lysine acetylation reduces nucleosome stability and increases accessibility of linker DNA,^[98–100] differential action by co-activators and co-repressors at the enhancer could result in the observed quantitative differences between the active, primed, and repressed states.^[15]

Overall, in addition to the broad and local mechanisms seeding enhancer accessibility that were discussed in the previous section, accessibility of enhancers can be also fine-tuned during their operation, as a consequence of multiple regulatory events. Enhancer transcription as well as nucleosome stabilization and destabilization by non-pioneer patterning TFs could account for the quantitative accessibility differences that we observe between different enhancer states.

8. A Temporal Model of the Quantitative Relationship between Enhancer Accessibility and Activity

The considerations above lead us to propose a model (Figure 2) to represent the onset of enhancer activity, for instance in the context of embryonic development. We consider a hypothetical enhancer driving gene expression in a particular spatial domain of an embryo (presumptive domain highlighted with green cell outlines in Figure 2E–H) and as of a certain stage of development. Several molecular events take place around this enhancer

Figure 1. Enhancer accessibility information is contained in the enhancer sequence. A) In this experimental setup,^[15] an endogenous enhancer (homozygous) drives transcription of its target gene in the *D. melanogaster* embryo, while its transgenic copy integrated at the attP2 site^[103] (homozygous) drives transcription of a nuclear tag. When mapping ATAC-seq reads to the reference genome, we did not include a sequence of the reporter construct. As a result, reads originating from the transgenic enhancer and those originating from the endogenous enhancer mapped to the same position (endogenous locus). For each enhancer, we compare accessibility signal in the endogenous locus between the strain that carries an additional transgenic copy of the element (referred to as a transgenic strain; green line, mean over two replicates) and the three remaining strains that serve as a reference and only contain the endogenous enhancer (referred to as a reference strain; blue line, mean from three strains with two replicates each). As expected, signal intensity over the enhancer is on average twice higher in the transgenic strain than in the reference (IN ratio, grey shading). Likewise, accessibility of regions flanking each enhancer is highly comparable between the two strains (OUT ratio). Importantly, the four enhancer sequences considered here, which have been identified through genetic dissections or predicted clustering of TFBS,^[104] are not accessible along their entire sequence in the endogenous locus. All enhancer sequences contain a distinct peak of ATAC-seq signal that neighbors an inaccessible region (blue lines). Notably, the distribution of the accessibility signal originating from the transgenic strains (green lines) is very similar to the signal of reference strains with the endogenous enhancer alone (blue lines). The two strains show a strong and significant correlation between their ATAC-seq signal at individual base pairs, both within the enhancer as well as at the upstream and downstream flanking regions (r = Pearson's correlation coefficient indicated in each domain; for all coefficients: p -value < 0.001). We would not expect to observe such a conservation of the distribution of ATAC-seq signal at the base-pair resolution, or a clear difference between the accessible and inaccessible portion of the element, if local accessibility of the transgenic enhancer in the integration site differed from its accessibility profile in the endogenous locus. Tracks represent the distribution of Tn5 transposase cuts, after normalization (as in Bozek et al.^[15]) and smoothing with a moving average of 10 bp. Position of enhancers in their endogenous loci is marked with grey shading, and they are flanked by 500 bp upstream and downstream for reference: B) *Dichaete D₋(+4)*, C) *giant gt₋(-3)*, D) *hunchback hb₋anterior*, and E) *Kruppel Kr_CD1*. Upper right of each panel: ratio of the total number of transposase cuts between the transgenic and the reference strains, within the enhancer's sequence (IN) and in the two 500 bp blanking regions (OUT). Samples represent whole-embryo controls from Bozek et al.^[15] which, apart from the different transgenic enhancer, are genetically identical: D1 control, D4 control, D5 control, and D7 control. The ATAC-seq profiles were obtained at the same developmental timepoint. Genomic coordinates according to Release 5.57 of the *D. melanogaster* reference genome.^[105] Coordinates and names of the enhancers as in Segal et al.^[104] Exact sequences and details on the transgenic strains and ATAC-seq analysis in Bozek et al.^[15]



and its target promoter before transcripts start to accumulate, from a silenced inaccessible state (red nuclei in Figure 2E) to a primed and accessible state (yellow nuclei in Figure 2F,G), and to an active state (green nuclei in Figure 2H). In its original silenced state, the enhancer sequence shows a high nucleosome occupancy (Figure 2A), both in the presumptive expression domain of its target gene (Figure 2E: green cell outlines) and in the cells that will never express this gene (Figure 2E: gray and white cells). This inaccessibility of DNA prevents binding of patterning TFs that govern the spatial activity. The mean enhancer accessibility (e.g., ATAC-seq signal) both inside and outside of the presumptive expression domain of the gene is very low, at the same level as the surrounding genomic positions (green and white insets in Figure 2E). In certain nuclei, however, the enhancer may become transiently accessible as histones may stochastically dissociate from its sequence. When a PTF (Figure 2A: blue oval), expressed homogeneously or broadly in a cell population (Figure 2E-H: gray cells), binds the nucleosomal DNA, the enhancer transitions to a primed state: a first, permissive step toward activity. The PTF initiates the regulated depletion or displacement of nucleosomes (Figure 2F,G: yellow nuclei). Because this process is likely not synchronous among nuclei, the establishment of accessibility across the tissue may take some time.^[47] This will first manifest itself as a small peak of ATAC-seq signal (green and white insets in Figure 2F,G) at the enhancer locus. The peak will grow in height (but not in width) over time as the action of the PTF unfolds among nuclei and the proportion of primed states increases in the embryonic domain. Concurrently, the enhancer sequence that is now exposed becomes “open for business,” which has two consequences for the primed enhancer. The patterning TFs can bind their cognate TFBS on the enhancer (Figure 2B), and the enhancer can initiate a search of its target promoter, to finally loop onto it^[101] (Figure 2C). While the mere looping is not sufficient to activate transcription (Figure 2G: yellow nuclei), it has been shown for some developmental genes to prime the target promoter by stabilizing a paused RNA polymerase.^[101,102] A key signal is missing for the transcription to start, for instance an activator that is not expressed yet at this stage. Once this key signal comes in (Figure 2D: magenta hexagon), the enhancer switches to the active state and the transcripts start to accumulate (Figure 2D,H). Interactions of activator TFs with nucleosomes and chromatin remodelers as well as eRNA transcription may further promote nucleosome destabilization at the enhancer, resulting in a higher ATAC-seq peak in the enhancer activity domain (green inset in Figure 2H). Nevertheless, because of the transient nature of TF binding and some stochasticity in enhancer–promoter interactions, enhancers in some cells may transiently revert to a primed state (few yellow nuclei in cells with green outlines in Figure 2H), even in the presence of the full set of activators.

9. Conclusions and Prospects

Our understanding of the relationship between accessibility and activity of enhancers has recently been advanced through improved spatiotemporal resolution and a greater focus on the quantitative aspect of accessibility. Subtle differences in the degree of accessibility between primed, repressed, and active en-

hancers reconcile contradictory reports in the literature and paint a finer picture of accessibility modulation at enhancers.

The transition between different regulatory states explains why enhancers display accessibility in a broader developmental time window than their activity. The fact that at a single time point enhancers are open also outside of their activity domain indicates that in a complex tissue primed and repressed states can exist alongside the active enhancers. Therefore, the accessibility of enhancers is not a specific signature of their spatiotemporal activity, understood as promoting transcription of the target gene. Yet, accessibility is still a strong indicator of the regulatory activity at the enhancer itself. The accessible repressed and primed states, since targeted by pioneer and patterning TFs, are a site of tight regulatory control by the cell.

Nevertheless, while accessibility per se does not imply activity, active enhancers are characterized by a higher degree of accessibility than the other states. This observation comes with two important consequences. Systematic characterization of quantitative accessibility signatures of different enhancer states may aid their genome-wide identification. Second, it opens questions on the fundamental mechanisms that fine-tune chromatin organization at enhancers.

Finally, as a perspective in this context, we identify two questions that represents a frontier for the field. First, through which mechanisms is the initial accessibility promoted by PTFs maintained and modulated during activation or repression of enhancers? Second, what is the relative contribution of the overall TF occupancy, TF valence (activators versus repressors) and enhancer transcription (eRNA) to the enhancer’s quantitative accessibility, and how is their action coordinated?

Conflict of Interest

The authors declare no conflict of interest.

Keywords

accessibility, ATAC-seq, chromatin, embryonic development, enhancer, quantitative analysis, transcriptional regulation

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