Trends in Genetics

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Opinion

Non-canonical functions of enhancers: regulation of RNA polymerase III transcription, DNA replication, and V(D)J recombination

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Enhancers are the key regulators of other DNA-based processes by virtue of their unique ability to generate nucleosome-depleted regions in a highly regulated manner. Enhancers regulate cell-type-specific transcription of tRNA genes by RNA polymerase III (Pol III). They are also responsible for the binding of the origin replication complex (ORC) to DNA replication origins, thereby regulating origin utilization, replication timing, and replication-dependent chromosome breaks. Additionally, enhancers regulate V(D)J recombination by increasing access of the recombination-activating gene (RAG) recombinase to target sites and by generating non-coding enhancer RNAs and localized regions of trimethylated histone H3-K4 recognized by the RAG2 PHD domain. Thus, enhancers represent the first step in decoding the genome, and hence they regulate biological processes that, unlike RNA polymerase II (Pol II) transcription, do not have dedicated regulatory proteins.

Highlights

Enhancers generate nucleosome-depleted regions to regulate DNA-based processes that, unlike RNA polymerase II (Pol II) transcription, do not have dedicated regulatory proteins.

Increased accessibility of DNA by enhancers facilitates RNA polymerase III (Pol III) transcription, binding of the origin replication complex (ORC) to DNA replication origins, and targeting of the recombination-activating gene (RAG) recombinase for V(D)J recombination.

Enhancer-dependent transcription further regulates V(D)J recombination by generating localized regions of trimethylated histone H3-K4 that are recognized by the RAG2 PHD domain.

Enhancers contain multiple binding sites for transcriptional activator proteins

Enhancers were discovered as genetic elements that can stimulate transcription by Pol II when located at long and variable distances from the promoter [1,2]. They are responsible for transcriptional activation in response to environmental and developmental signals [3–5], and hence are the primary determinants of gene regulatory patterns [6,7]. The ability of enhancers to act at a distance makes it possible for an individual promoter, and hence a gene, to be regulated by multiple enhancers with different regulatory specificities [6,7].

Enhancers are composed of multiple binding sites for transcriptional activator proteins that are co-localized to regions of 100–300 base pairs [6,7] (Figure 1A). While a single activator binding site is sufficient for high-level transcription in yeast [8], metazoan enhancers require multiple protein-binding sites [6,7]. The requirement for multiple binding sites mechanistically imposes combinatorial activation of transcription, the fundamental principle for how multicellular organisms generate the extraordinary diversity of gene regulatory patterns. For example, if one posits that three protein-binding sites are needed for a functional enhancer, there are nearly 10 billion combinations of the ~2000 DNA-binding transcription factors in mammalian cells. Moreover, the activities of these transcription factors are regulated by physiological conditions via phosphorylation, other post-translational modifications, and interactions with small molecules or other proteins.

Enhancers activate Pol II transcription via three classes of protein–protein interactions

Transcriptional activator factors bound to enhancers directly interact with three classes of proteins to stimulate Pol II transcription. First, activator proteins recruit nucleosome remodeling

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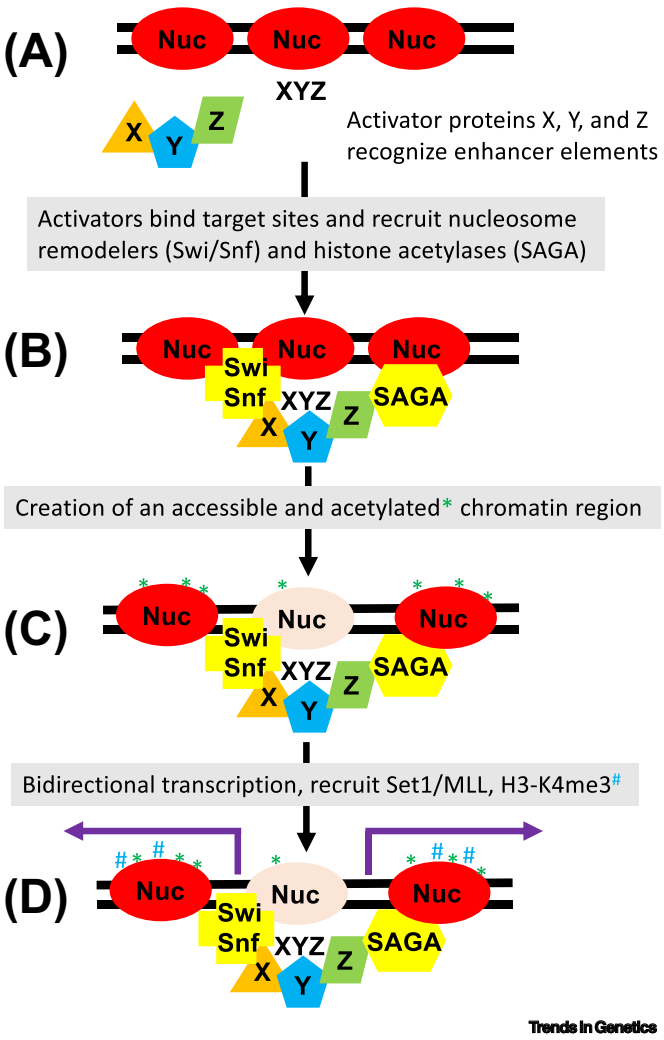

Figure 1. Mechanism by which enhancers stimulate RNA polymerase II (Pol II) transcription. (A) A segment of DNA (black bars) containing nucleosomes (Nuc, red ovals) with X, Y, Z binding sites for transcription factors (different colored shapes). (B) The combined action of the activator proteins recruits chromatin-modifying complexes (yellow shapes) that remodel nucleosomes (e.g., Swi/Snf) or acetylate histones (e.g., SAGA). (C) Structure of enhancer-modified chromatin showing depleted nucleosomes (light-red oval) acetylated histones (green asterisks). (D) Bidirectional transcription from enhancers (purple arrows) and associated recruitment of Set1/MLL histone methylases and generation of localized H3K4me3 regions of chromatin (blue number sign).

(e.g., Swi/Snf or BRG1) or histone acetylation (e.g., SAGA/Gcn5 or p300) complexes to alter chromatin structure in the vicinity of the enhancer [9–12] (Figure 1B). As such, enhancer regions are typically depleted of nucleosomes, and histones within or adjacent to the enhancers are hyperacetylated (Figure 1C). Thus, chromatin at enhancers is accessible to transcription factors, and it contains histone modifications that contribute to transcriptional activation [13].

Second, activator proteins can interact directly with components of the Pol II transcription machinery, thereby helping to recruit Pol II to promoters [14]. The major target of activator proteins is the Mediator complex, which also interacts directly with Pol II [15–20]. By directly interacting with both activators and Pol II, Mediator serves as a crucial bridge between enhancers and promoters. Some activator proteins interact with transcription factor IID (TFIID) [21–23], a component of the basal Pol II machinery consisting of the TATA-binding protein (TBP) and associated factors. The distinction between chromatin and Pol II machinery targets is not absolute. For example, the yeast SAGA complex, which acetylates and ubiquitinates histones, also has protein subunits that interact with TBP [24,25].

Third, activator proteins bound to enhancers can also interact with other proteins (often, but not necessarily, activators) bound to target sites near promoters. These promoter-proximal sites are distinct from promoter sequences (e.g., TATA and initiator elements) that are bound by the general Pol II machinery. Promoter-proximal elements and enhancers are bound by the same constellation of sequence-specific transcription factors, and both generate bidirectional 'enhancer RNAs' that initiate immediately adjacent from each side of the enhancer [26–28] (Figure 1D). As such, promoter-proximal elements are best described as proximal enhancers. Thus, protein–protein interactions between proximal and distal enhancers, which may be direct or involve an intermediary protein(s), create a physical loop/connection that is critical for enhancers to act at long distances from promoters [29–31]. By contrast, activators bound to distal enhancers do not form transcription-competent loops by interacting with Mediator (or other general transcription factors) associated with promoters [29–31], and looping is largely unaffected when Pol II transcription is eliminated [32].

Enhancers mediate the first step in decoding the genome

Genomic DNA contains information that must be decoded for functional events to happen. Unlike bacterial genomes – that are accessible to and ultimately decoded by sequence-specific DNA-binding proteins and RNA polymerase [33] – eukaryotic genomes are coated by nucleosomes that generally inhibit DNA-mediated functions. Thus, creating accessible genomic regions by removing nucleosomes is the first step in decoding the genome and functionally differentiating DNA sequences.

In yeast, poly(dA:dT) sequences are preferentially located in gene regulatory regions, intrinsically disfavor nucleosome formation [34], and are substrates for the RSC (remodels the structure of chromatin) nucleosome remodeling complex to generate nucleosome-depleted regions [35]. However, metazoan genomes lack poly(dA:dT) sequences, and nucleosome remodeling complexes generally do not recognize specific DNA sequences. While 'pioneer' transcription factors can bind DNA in the context of a nucleosome, such binding *per se* is not sufficient for nucleosome eviction [36].

Thus, in metazoans, activator-mediated recruitment of nucleosome remodeling complexes is the major, and perhaps only, mechanism by which nucleosomes can be evicted from gene regulatory regions, hence making the underlying DNA accessible. Importantly, activator-mediated eviction of nucleosomes is restricted to enhancers, and it is independent of transcriptional activity [11,37,38]. In some cases, the nucleosome-depleted region generated by enhancers can also encompass the nearby promoter sequences. As discussed in the following, direct and indirect effects on chromatin structure underlie how enhancers play key roles in transcription by Pol III, DNA replication, and V(D)J recombination.

Role of enhancers in Pol III transcription

Pol III is responsible for transcription of genes encoding tRNAs, several small nuclear RNAs (snRNAs), and a few other RNA species [39,40]. The basic Pol III machinery is simpler than the Pol II machinery, and it comprises three multiprotein complexes: the promoter recognition factor TFIIIC (a multiprotein complex that binds specific DNA sequences), the initiation factor TFIIIB (a TBP-containing complex), and Pol III itself. In metazoans, TFIIIA, a sequence-specific DNA-binding protein, is selectively required for Pol III-mediated transcription of 5S rRNA genes [41]. snRNA-activating protein complex c (SNAPc), a DNA-binding complex, is selectively required for Pol III-mediated transcription of a class of snRNA genes [42]. Aside from TFIIIA and SNAPc, DNA-binding proteins that selectively regulate expression of Pol III-transcribed genes have not been identified.

Despite the lack of gene-specific transcription factors, metazoan tRNA genes are nevertheless subject to cell-type regulation. In a given cell type, ~75% of tRNA genes are expressed, while the remaining ~25% are not [43–48]. In addition, differential expression of tRNA genes occurs during liver regeneration [49]. By contrast, all Pol III-transcribed genes are active in yeast cells [50].

Cell-type-specific regulation ultimately requires a cell-type specific entity. Components of the Pol III machinery (including TFIIIC) are present and active in all cells and hence cannot mediate cell-type-specific regulation of tRNA genes. Furthermore, even though TFIIIC can bind its target sites in the context of nucleosomal DNA [51], there is no plausible mechanism by which the Pol III machinery can distinguish between active versus inactive genes in a given cell type. The only plausible cell-type-specific entities that can distinguish between active and inactive genes in a given cell type are DNA-binding transcription factors.

The distinction between active and non-active tRNA genes is linked to differences in their chromatin structure [43–48]. Active tRNA genes are associated with nucleosome-depleted regions that are flanked by highly acetylated nucleosomes (Figure 2A), whereas chromatin of inactive tRNA genes lack these features. The chromatin structure at active tRNA genes is indistinguishable from that of enhancers, and hence can only be generated by activator-dependent recruitment of nucleosome remodeling and histone acetylase complexes. TFIIIC has intrinsic histone acetylase activity [51] and also interacts with the p300 histone acetylase [52], and these activities could contribute to chromatin structure of Pol III-transcribed genes. However, these activities are general and hence cannot mediate cell-type- and gene-specific regulation of tRNA promoters. Thus, the cell type specificity of enhancers, which ultimately reflects the cell type specificity of transcriptional activator proteins, is mechanistically responsible for the cell type specificity of tRNA expression.

The simplest mechanism to explain cell-type-specific expression of tRNA genes invokes enhancer-mediated effects on chromatin that cause differential accessibility of the Pol III machinery to Pol III promoter elements. There is no evidence that enhancer-bound proteins interact with any component of the Pol III machinery, unlike the case for the Pol II machinery. It is unknown whether such differential accessibility of the Pol III machinery due to nucleosome depletion is sufficient for cell-type-specific regulation or whether histone acetylation (or possibly methylation) of flanking nucleosomes plays a role.

As enhancers are associated with Pol II occupancy and transcription, it is also possible that enhancer effects on cell-type-specific expression of tRNA genes involve Pol II transcription. However, blocking Pol II transcription with α-amanitin or depletion of Pol II itself has limited [45,53] and in some cases repressive [54] effects on Pol III transcription. In addition, Pol III occupancy at tRNA genes correlates with the level of nearby Pol II occupancy but not with the level of Pol II-generated RNA [43]. These observations are consistent with activator-dependent and transcription-independent alteration of local chromatin structure [11,37,38]. Nevertheless, Pol II transcription can increase Pol III occupancy twofold in fission yeast [55], and Pol II occupancy peaks upstream of Pol III at enhancers [43–48], a relationship consistent with Pol II transcription *per se* affecting Pol III transcription. The relative contributions of enhancer-mediated effects on chromatin structure versus Pol II transcription remain to be dissected and are likely to be gene-specific. Most importantly, enhancers are the ultimate mediators of both chromatin changes and local Pol II transcription and hence the basis of cell-type regulation.

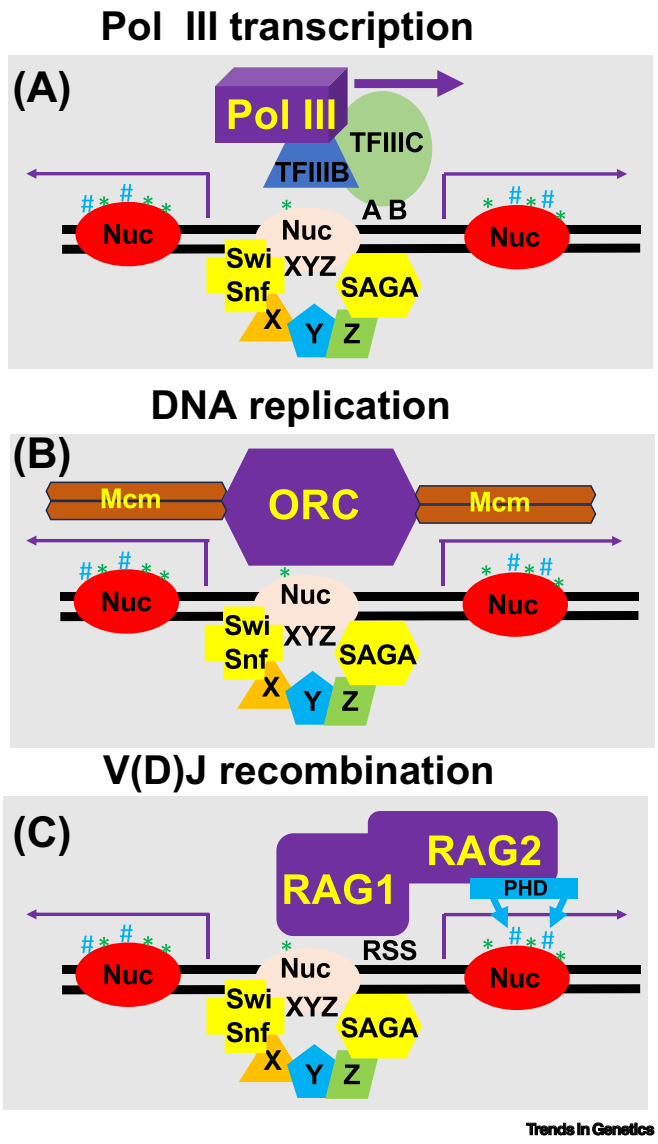

Figure 2. Enhancers, via effects on chromatin, regulate RNA polymerase III (Pol III) transcription, DNA replication, and V(D)J recombination. (A) Transcription factor IIIC (TFIIIC) binds the A and B boxes in accessible chromatin, thereby permitting recruitment of TFIIIB and Pol III resulting in transcription. Cell types where the enhancer is inactive are unable to bind TFIIIC and support Pol III transcription. (B) The origin replication complex (ORC) binds enhancer-mediated regions depleted for nucleosomes (Nuc) but having acetylated histones. ORC recruits the minichromosome maintenance (MCM) helicase to start the process of bidirectional DNA replication. (C) The RAG1/RAG2 V(D)J recombinase binds target sequences (RSS) in enhancer-mediated regions depleted for nucleosomes. Binding of RAG2 is facilitated by binding of the PHD domain of RAG2 to H3K4me3 marks (blue box, arrows pointing to methylation marks) generated by recruitment of Set1/MLL histone methylases during the generation of enhancer RNAs.

Role of enhancers in initiation of DNA replication

The initial step of DNA replication involves binding of the ORC to origins of DNA replication that are distributed throughout the genome [56]. Although yeast ORC binds to specific sequences, metazoan ORC binds most, and perhaps all, accessible genomic regions (DNase I hypersensitive and nucleosome depleted) that contain active chromatin marks such as H3 acetylation and H3 lysine 4 methylation [57,58] (Figure 2B). In accord with this observation, the ORC binding profile is broader than DNA-binding profiles of proteins that recognize short target sites, indicating that metazoan ORC is not bound to specific DNA sequences. ORC binding does not occur at accessible chromatin regions lacking acetylated histones, suggesting that ORC binding involves recognition of histones that are acetylated [58] and perhaps have other modifications such as H4K20me2/3 [59,60]. As this chromatin structure is generated exclusively by enhancers, ORC binding and subsequent initiation of DNA replication is ultimately

mediated by enhancers. As such, cell-type-specific enhancers are responsible for cell-type-specific ORC binding.

Histone acetylation also plays a separate role in replication licensing, the process that restricts genomic regions to be replicated only once per cell cycle [56]. Upon binding to replication origins, ORC recruits the licensing factor Cdt1, which in turn recruits the minichromosome maintenance (MCM) helicase necessary for DNA replication [56]. Replication licensing requires Cdt1-dependent recruitment of HBO1 histone acetylase specifically during the G1 phase of the cell cycle [61] and HBO1-mediated acetylation of histones [62]. Cdt1 function and HBO1-mediated histone acetylation is inhibited by Geminin in the context of a Cdt1–HBO1 complex associated with replication origins [62]. In addition to being a coactivator for Cdt1, HBO1 is a coactivator for AP-1 transcription factors that respond to stress-activated c-Jun N terminal kinases (JNKs). Regulated and reciprocal recruitment of HBO1 to target genes and replication origins coordinates the transcriptional and DNA replication response to cellular stress [63].

Genomic regions are replicated at distinct times in S phase [64,65], and replication licensing restricts origins to be fired only once per cell cycle. While chromatin structures at ORC sites are similar in early- and late-replicating regions, there are far more ORC sites in early-replicating regions [58]. This and other observations, as well as computational simulation, suggest that replication timing can be explained by ORC density and stochastic firing from ORC sites [58,66,67]. In this view, origin firing occurs only at a small fraction of ORC sites at any given time, so replication initiation is strongly biased to ORC-dense regions at the beginning of S phase. This bias to ORC-dense regions continues as S phase progresses, leading to early-replicating regions. Due to replication licensing, ORC sites in early replicating regions are unavailable for initiation near the end of S phase, thereby biasing ORC firing to sites in later-replicating regions, which are often heterochromatic. In addition, large genomic regions with a paucity of enhancers have few ORC binding sites, replicate poorly, and are strongly associated with common fragile sites and recurrent deletions in cancers [58]. These observations suggest that the location of replication origins, replication timing, and replication-dependent chromosome breaks are determined primarily by the genomic distribution and cell-type specificity of enhancers.

Role of enhancers in regulation of V(D)J recombination

V(D)J recombination joins the physically separate V, D, and J gene segments to generate the diverse repertoire of antibodies and T cell receptors that mediate the adaptive immune response [68]. The V(D)J recombinase, which consists of RAG1 and RAG2 proteins [69,70], recognizes and cleaves signal sequences (RSSs) flanking the V, D, and J gene segments [71,72]. Subsequently, a complex process involving multiple proteins results in ligation of the V, D, and J segments to generate intact genes that express antibodies and T cell receptors [68]. The multiple V, D, and J genes are rearranged in a combinatorial fashion to generate a wide diversity of antigen receptors that characterizes the immune response.

V(D)J recombination is tightly regulated in multiple ways [68]. In part, this regulation is mediated by restricting expression of the RAG proteins to developing B and T lymphocytes. However, this developmental restriction does not explain why antibody (B cell receptor) genes are fully rearranged only in pre-B cells, whereas T cell receptors are only fully rearranged in T cells. Furthermore, joining of D and J segments precedes joining of V segments to recombined DJ segments.

These observations and regulated changes in histone acetylation and RSS accessibility strongly suggest that differences in chromatin structure underlie the tight regulation of V(D)J

recombination [73–75]. By analogy with their role in Pol III transcription and DNA replication, developmentally regulated enhancers increase RAG protein accessibility and hence V(D)J recombination at RSS sites near the enhancers under the specific conditions where the enhancers are active [76] (Figure 2C). In addition, enhancer-mediated transcription can also increase RAG protein accessibility via eviction of H2A/H2B dimers [77].

In addition to enhancer-mediated effects on nucleosome depletion and histone acetylation, V(D)J recombination is directly affected by a specific histone modification, trimethylated lysine 4 of histone H3 (H3K4me3) [78]. RAG2 contains a PHD finger motif that specifically binds H3K4me3 [79], and mutations that block this interaction (including one that is the cause of Omenn's syndrome) are defective for V(D)J recombination. Similarly, reducing the level of H3K4me3 reduces the level of V(D)J recombination [78].

The major, and perhaps sole, mechanism for generating H3K4me3-modified nucleosomes involves targeted recruitment of the Set1 or MLL histone methylases near active Pol II promoters (including those near distal enhancers that generate non-coding RNAs) and extending a short distance downstream [80–82]. This and other observations suggest a model in which enhancers, though not directly involved in V(D)J recombination, are critical for regulating the process (Figure 2C). Specifically, proximal enhancers located near the various RSSs generate non-coding RNA transcripts (historically called 'sterile RNAs') [83–85]. Enhancers mediating the various forms of regulation are active only under the relevant developmental conditions. The enhancer-dependent RNA transcripts are not important, but their transcription by Pol II generates H3K4me3-modified nucleosomes in the vicinity of the relevant RSSs. RAG2 recognizes H3K4me3, and together with RAG1 cleaves at RSSs leading to V(D)J recombination that gives the desired product. Binding of the RAG2 PHD finger motif to H3K4me3 also induces an allosteric change in RAG1 to stimulate V(D)J recombinase activity [86,87]. Thus, developmental regulation of the enhancers, via its transcription-mediated creation of localized H3K4me3 nucleosomes, results in developmental regulation of V(D)J recombination.

Concluding remarks: non-canonical regulatory functions of enhancers are mediated through chromatin

Enhancers were discovered by their ability to activate Pol II transcription, and indeed they are primarily responsible for generating the extraordinary diversity in gene regulatory patterns. However, activator proteins bound to enhancers also recruit nucleosome remodeling and histone acetylase complexes that cause localized changes in chromatin structure. Of particular importance, enhancers generate nucleosome-depleted regions that render the underlying DNA accessible to proteins not involved in Pol II transcription. Such nucleosome-depleted regions underlie the role of enhancers in facilitating Pol III transcription and ORC binding with subsequent initiation of DNA replication. Enhancer effects on V(D)J recombination are not exclusively mediated by nucleosome depletion, but also via transcription-coupled deposition of H3K4me3 near target sites for the RAG proteins. In all these cases, the regulatory properties of the enhancers are linked, indirectly via their effects on chromatin, to regulation of DNA-mediated processes other than Pol II transcription. More generally, enhancers represent the first step in decoding the genome by virtue of their unique ability to alter local chromatin structure via specific DNA sequences and their cognate activator proteins that recruit chromatin-modifying activities. Mechanistic details between enhancer-mediated effects on chromatin and regulation of the various DNA-based processes remain to be elucidated (see Outstanding questions).

Outstanding questions

What are the functional requirements between the location and quality of the enhancer and nucleosome-depleted region to the DNA sequence elements that mediate Pol III transcription and V(D)J recombination?

Is an artificial enhancer based on activator proteins with non-native DNA binding and transcriptional activation domains sufficient (with the appropriate DNA sequence elements) to mediate and regulate these non-canonical functions?

Do enhancers regulate other DNA-based processes such as DNA repair, homologous recombination, non-homologous end joining, mutagenesis, and clustered regularly interspaced short palindromic repeats (CRISPR)-mediated activities?

What chromatin-modifying activities recruited to enhancers by activators are required to regulate DNA-based processes other than Pol II transcription?

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Declaration of interests

I declare no competing interests.

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