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Invited Review

INVITED REVIEW

Enhancers: bridging the gap between gene control and human disease

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Abstract

Enhancers are a class of regulatory elements essential for precise spatio-temporal control of gene expression during development and in terminally differentiated cells. This review highlights signature features of enhancer elements as well as new advances that provide mechanistic insights into enhancer-mediated gene control in the context of three-dimensional chromatin. We detail the various ways in which non-coding mutations can instigate aberrant gene control and cause a variety of Mendelian disorders, common diseases and cancer.

Introduction

Precise gene control is central to life. Transcriptional enhancers are *cis*-regulatory sequences that recruit transcription factors (TFs) and play a fundamental role in regulating which genes cells express, the timing of their expression and the levels of their expression. Most enhancers are located in non-coding regions of the genome, previously considered 'junk," DNA, and now widely accepted to be functional. While the number of genes is ∼20 000 in the human genome, there are millions of candidate enhancers (1,2). The enhancerome of any given human cell type occupies 5–10 times more genomic real estate than the exome. Although weakly conserved across species, enhancer elements are among the most highly constrained sequences across humans (3). They are enriched for germline variants associated with both rare and common diseases, and somatic mutations that disrupt enhancer function can cause cancer. Armed with genomic technologies that facilitate interrogation of the enhancer epigenome and 3D chromatin architecture at high resolution, new insights into enhancer–gene control are being uncovered at an unprecedented rate. This review focuses on basic principles of enhancer regulation with an emphasis on human diseases caused by germline and somatic non-coding mutations that disrupt enhancer–gene control via *trans* or *cis* mechanisms.

Features of Enhancer Elements

Enhancer elements are located in DNase I hypersensitive regions of chromatin flanked by histone H3 covalently modified with monomethylation of lysine 4 (H3K4me1) (4,5) (Fig. 1). Histone variants, H2A.Z and H3.3, are also incorporated at active enhancers (6). Covalent histone modifications in addition to H3K4me1 may also be present and the specific composition of histone marks generally correlates with the activity state of the enhancer. Enhancers can exist in decommissioned (no histone modification), poised (H3K4me1 and H3K27me3), primed (H3K4me1) or active (H3K4me1 and H3K27ac) states (7–9). During development and in terminally differentiated cells, enhancers can switch between states. Upon embryonic stem cell differentiation, for example, poised enhancers lose the repressive H3K27me3 mark and gain H3K27ac. The enhancer state change occurs concomitantly with a switch in the expression of the target gene from off to on and the transition in cell state from undifferentiated to differentiated. Enhancer activation is a step-wise process mediated by a variety of sequence-specific TFs chromatin-remodeling complexes and coactivators (10). RNA Polymerase II (Pol II) is also involved, directly binding to enhancer sequences and transcribing them into enhancer RNA (eRNA) (11). p53 may also participate in eRNA production (12).

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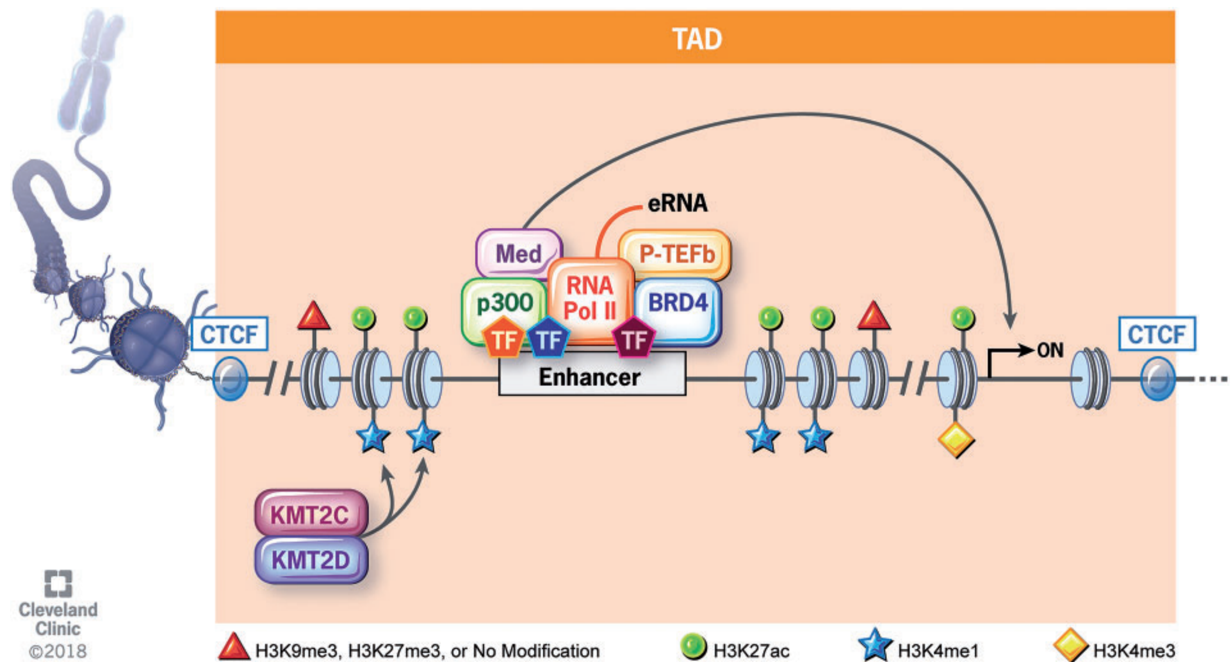

Figure 1. Features of active enhancer elements (see text). Transcription factor (TF), mediator (Med), RNA polymerase II (RNA Pol II), bromodomain-containing protein 4 (BRD4), CCCTC-binding factor (CTCF), enhancer RNA (eRNA), lysine methyltransferase 2C (KMT2C), lysine methyltransferase 2D (KMT2D).

eRNA levels correlate with enhancer activity, and eRNA can be used as a marker for genome-wide identification of active enhancer elements (13). Enhancer transcription can lead to the formation of R-loops (14), which, if left uncorrected, can induce replication stress, genomic instability and other hallmark features of cancer (15).

Enhancers communicate with genes through physical interactions with their promoters, mediated by Mediator and Cohesin complexes (16) as well as ZNF143 (17) and YY1 (18). The histone methyltransferases (MLL3/4 or KMT2C/B), and the canonical H3K4me1 enhancer mark these enzymes deposit, are also necessary for establishing enhancer–promoter interactions (19). Most enhancer–gene interactions are contained within topologically associated domains (TADs) (20), self-interacting megabase-size genomic regions bordered by the insulator protein CTCF. Within TADs, CTCF can contribute to formation of insulated neighborhoods that further seclude enhancers and their gene targets (21). Enhancers can be located close to or several hundred kilobases away from their gene targets and they do not necessarily regulate their nearest gene. Multiple enhancers often regulate a single gene, and multiple genes can be regulated by a single enhancer.

Super Enhancers

Super enhancers (SEs) and stretch enhancers are clusters of enhancers found across diverse cell types (22–24). SEs contain supranormal levels of the active enhancer histone mark H3K27ac and many other enhancer-associated proteins, like Mediator and Pol II. They are typically several kilobases in length, exquisitely cell-type specific, and regulate genes that control cell state. In embryonic stem cells for example, SEs regulate master TFs of pluripotency, Oct4, Sox2 and Nanog (23). Tumor cells often acquire SEs near oncogenes that are known tumor dependency genes, like MYC (25,26). They are also highly enriched for Genome-wide association studies (GWAS)-identified SNPs that predispose to several common diseases. The constituents of SEs physically interact with one another and their gene targets in 3D chromatin and these interactions are likely essential for maintenance of SE function (27).

Recent studies have exploited CRISPR-Cas9 editing technologies to dissect SEs and quantify the functional contribution of individual SE constituents to their target gene levels. These studies have consistently shown that SE constituents function independently and all constituents participate in the regulation of their target genes, with some enhancers contributing more than others. There appears to be no common regulatory modality; some act additively like those at the α-globin locus (28), and others work synergistically, like those at the mammary *Wap* locus (29). Common disease-associated variants in SE constituents can also influence the expression of their target genes via different modalities (30). Recently, a global analysis of SE function found that while all enhancers are necessary for maximal SE activity, there exists a set of 'hub enhancers' whose knockout results in particularly profound decrease in SE activity (31). Hub enhancers are enriched for CTCF and cohesin and are critical for maintaining physical interactions between SE constituents as well as other enhancers within the same TAD. A recently proposed 'phase separation' model of transcriptional control may explain how SEs promote consistent and high level transcription. In this model, binding of TFs and other regulators to SE constituents stimulates aggregate formation and their subsequent compartmentalization within the cell nucleus (32).

Mutations in Enhancer Regulators

Several Mendelian diseases are caused by mutations in genes encoding proteins that function to establish, maintain, or interact with enhancers (Table 1). Most are congenital, multiple malformation diseases caused by heterozygous mutations that arise *de novo*, implicating haploinsufficiency as the pathogenic mechanism. A key player in enhancer activation is the

Table 1. Diseases caused by mutations in proteins that function in establishing, maintaining, or regulating enhancers

Disease	Gene (protein) implicated	Function	Reference
Kabuki syndrome	*KMT2D* (KMT2D)	Histone methyltransferase that deposits H3K4me1 on histones adjacent to enhancers.	(39)
	KDM6A (KDM6A)	Histone demethylase that removes H3K27me3 mark.	(40)
Rubinstein-Taybi syndrome	*EP300* (P300)	Histone acetyltransferase and enhancer coactivator.	(71,72)
	CREBBP (CBP)	Histone acetyltransferase and enhancer coactivator.	(41,73)
CHARGE syndrome	*CHD7* (CHD7)	ATP-dependent chromatin remodeler that functions at enhancers.	(74,75)
Coffin-Siris syndrome	*ARID1A* (ARI1A) *ARID1B* (ARI1B) *SMARCA4* (BRG1) *SMARCB1* (SMARCB1) *SMARCE1* (SMARCE1)	Subunits of the BAF (SWI/SNF), chromatin remodeling complex that function in establishing enhancers.	(35,76)
Schwannomatosis	*SMARCB1* (SMARCB1)	See above.	(77)
Potocki-Shaffer syndrome	*PHF21A* (PF21A)	Component of BRAF-histone deacetylase complex.	(78)
Cornelia de Lange-like syndrome	*BRD4* (BRD4)	BRD4 binds to H3K27ac and mediates RNA polymerase II pause-release.	(48)

ATP-dependent BRG1/BRM associated factor complex, or BAF, which slides or evicts nucleosomes to make way for TFs and other coactivators. BAF also associates with H3K4me1 at enhancers (33). Mutations in several subunits of the BAF complex cause Coffin-Siris syndrome, a condition characterized by developmental delays and unique skeletal malformations (34,35). CHARGE syndrome (coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities, and ear abnormalities) is another disease caused by mutation of a chromatin remodeler that acts at enhancers, CHD7 (36). A related CHD family member, CHD8, is implicated in autism (37) and may also function in enhancer activation (38).

The switching of enhancers between primed and active states is mediated in part by enzymes that deposit or remove covalent histone modifications, or 'writers' and 'erasers'. An illustrative example of the crucial role of these proteins in normal development is Kabuki syndrome (KS), characterized by distinctive facies, intellectual disability, unique skeletal abnormalities and growth delay. KS is caused by mutations in *KMT2D*, a histone methyltransferase that writes the canonical H3K4me1 enhancer mark on chromatin (39). KS can also be caused by mutation of KDM6A, a histone demethylase that erases the H3K27me3 mark (40). P300 and CBP, coactivators that bind acetylated lysines at active enhancers are mutated in Rubinstein-Taybi syndrome, characterized by short stature, intellectual disability, distinctive facies and broad thumbs and toes (41). Several of the same genes with enhancer-related functions are frequently somatically mutated in cancer. Mutations in genes encoding subunits of the BAF complex occur in 20% of all human cancers (42). KMT2D, KDM6A, ARID1A, KTM2C, P300, CREBBP and KMT2A, are all constituents of machinery cells use to establish enhancers and are among the most frequently mutated genes in bladder cancer (43). Mutations in enhancer-associated histone variant H3.3 genes have been implicated in pediatric brain cancers (44), although the exact mechanism by which the oncohistone promotes tumorigenesis remains unresolved.

Although not generally considered 'enhanceropathies' like the diseases described above, Cornelia de Lange syndrome (CdLS), Roberts syndrome, and other 'cohesinopathies' are caused by mutations in various components of the cohesin complex (*SCC2/NIPBL*, *SMC1A*, *SMC3*, *RAD21*, *ESCO2* and others) that function in chromatin looping and hence play an important role in facilitating enhancer-promoter interactions (45–47). Moreover, mutations in *BRD4* were recently shown to cause a Cornelia de Lange-like syndrome (48). BRD4 binds to SEs and also engages NIPBL, which is required for loading the cohesin looping complex onto DNA (49). The proposed model is that *BRD4* mutation disrupts looping and transcription at SE-associated loci and as such, the authors noted that CdLS can be considered a "transcriptomopathy".

Mutations Disrupting Enhancer–Gene Control

There are a variety of ways in which genetic mutation of non-coding regions can disrupt enhancer–gene regulation and cause disease (Table 2). Deletions can result in either gene silencing by removing active enhancers, or gene activation by repositioning them closer to enhancers. Deletions can also remove TAD boundaries, allowing for ectopic interactions between enhancers and promoters normally insulated from one another in adjacent TADs. In a variant of bracydactyly, for example, deletions removing TAD boundaries creates ectopic interactions between the EPHA4 TAD domain and PAX3 (50). Insertions may create novel TF binding sites at enhancer sequences (51). Translocations can breakup SEs, or displace enhancers from their endogenous targets and relocate them to other chromosomes (52). Inversions can force a promoter to adopt a repositioned enhancer, resulting in increased gene transcript output (53). Alternatively, inversions can reposition an enhancer to be out of range of its promoter (54). Duplications can create ectopic enhancer sequences that can act to increase target gene transcription (55). Point mutations can modulate TF affinity at enhancer sequences (56). Virtually all of these types of non-coding mutations can arise somatically and have been implicated in oncogenesis. For example, a translocation that repositions immunoglobulin heavy chain enhancers near the *MYC* oncogene is a common driver mutation in Burkitt's lymphoma (57). In a variety of epithelial cancers, copy number gains containing SEs are known to activate oncogenes (58). In T-ALL, driver indel mutations at the *TAL1* locus can create binding sites for MYB, a pioneer transcription factor that engages the indel, instigating SE formation and *TAL1* activation (59). Microdeletions can disrupt boundaries of insulated neighborhoods, leading to oncogene activation (60).

Table 2. Various types of mutations that impact enhancer function and their associated diseases

Mutation type	Disease	Enhancer defects	References
Insertion/deletion			
	X-linked deafness type 3 (DFN3)	Multiple deletions 900 kb from *POU3F4*.	(79)
	Pierre Robin sequence	Deletions 1–1.5 Mb upstream and downstream of Sox9 on chromosome 17.	(52)
	Split-hand-split food malformation (SHFM)	7q21.3 deletion affecting enhancer sequences within *DYNC1I1*.	(80,81)
	Craniosynostosis	Multiple deletions upstream of Indian hedgehog (IHH).	(82)
	Autosomal dominant adult-onset demyelinating leukodystrophy	Deletion eliminating TAD, allowing for enhancer adoption of *LMNB1*.	(83)
	Axenfeld-Rieger syndrome	Deletion of up to 1.1 Mb upstream of *PITX2*.	(84)
	Syndromic unspecified intellectual disability	Microdeletions in *STAG1*, which makes up the core cohesin complex.	(85)
	F-syndrome	Deletions removing TAD and allowing aberrant interaction between *EPHA4* and *WNT6*.	(50)
	Brachydactyly	Deletions removing TAD and allowing aberrant interaction between *EPHA4* and *PAX3*.	(50)
	Polysyndactyly	Deletions removing TAD and allowing aberrant interaction between *EPHA4* and IHH	(50)
	Preaxial polydactyly	13 bp insertion in the zone of polarizing activity regulatory sequence (ZRS) affecting sonic hedgehog (SHH) expression.	(51)
Translocation			
	Aniridia	Involves 11p13, downstream of *PAX6*.	(86)
	Pierre Robin sequence	1 Mb away from *SOX9*. Abrogates binding of MSX1 in *in vitro* studies.	(52)
	Split-hand syndrome	t(2; 7)(p25.1; q22), separates limb enhancers in *DYNC1I1* from *DLX5/6*.	(81)
Inversion			
	Limb syndactyly	Enhancer adoption by SHH induced by a 7q inversion.	(53)
	Hand-foot-genital syndrome	Chromosome 7 inversion causing a *HOXA13* enhancer delocalization.	(54)
Duplication			
	Disorders of sex development (DSD)	16p13.3 duplication of *GNG13* and *SOX8* enhancers.	(87)
		600 kb upstream of *SOX9*.[19]	(55)
	Haas-type polysyndactyly and Laurin-Sandrow syndrome	Microduplications in SHH limb enhancer ZRS.	(88)
	Syndactyly	Multiple targets upstream of IHH.	(82)
	Keratolytic Winter Erythema	Duplication of enhancer upstream of CTSB.	(89)
	Preaxial polydactyly	Microduplications in 7q36.3 affecting ZRS.	(90)
Point mutation			
	Aniridia	*De novo* mutation 150 kb downstream of *PAX6* disrupting *PAX6* autoregulation.	(91)
	Holoproscencephaly	460 kb upstream of SHH resulting in loss of SHH brain enhancer-2 activity.	(92)
	Pancreatic agenesis	25 kb downstream of *PTF1A*.	(56)
	Van der Would syndrome	Mutation in IRF6 enhancer, abrogating p63 and E47 binding.	(93)
	Hirschsprung disease	Common non-coding variant within an enhancer-like sequence in RET intron 1.	(94)
	Isolated congenital heart defects	Mutation 90 kb dowstream of *TBX5*.	(95)
	Preaxial polydactyly	Various point mutations in the ZRS enhancer (e.g. 295 T>C)	(96,97)

GWAS SNPs and Enhancers

GWAS have successfully identified loci associated with several human traits and susceptibility to numerous common diseases, including heart disease, type 2 diabetes (T2D), multiple sclerosis and cancer. Sixty to eighty percent of the associated SNPs map to enhancers and/or SEs (61). There is also a strong correlation between associated SNPs and enhancers active specifically in tissues involved in disease pathology. SNPs associated with T2D for example, often reside within enhancers active exclusively in pancreatic islet cells. SNPs associated with risk for multiple sclerosis, generally considered to be an immune-related disease, lie in enhancers active in immune-related cell types. These findings offer mechanistic insights into the basis of

common diseases and provide a potential explanation for why some tissues are affected while others are spared. The prevailing model is that the disease-associated SNPs impact gene regulation by modulating TF binding in cell types where the enhancer is active. There is compelling support for this model. Increased risk for melanoma is conferred by an enhancer indel that increases the affinity of RECQL, leading to increased PARP1 expression and contributing to melanogenesis (62). A risk SNP for prostate cancer increases *RFX6* expression by increasing binding of HOXB13 at the *RFX6* enhancer (63). SNPs associated with fetal hemoglobin levels map to BLC11A enhancers in human erythroblasts and modulate GATA1 and TAL1 binding (64). Mechanistic studies of GWAS–enhancer SNPs have also highlighted the complexity of enhancer–gene interactions in 3D chromatin. For example, an obesity associated enhancer variant within an intron of *FTO*, presumed to influence *FTO* expression, physically interacts with and impacts the expression of an altogether different gene, IRX3, located megabases away (65). Moreover, more than one gene may be targeted. The T2D risk SNP rs7163757 resides within a B-islet cell-specific stretch enhancer and modulates expression of two inflammatory cytokines, *C2CD4B* and *C2CD4A* (66). The targets of enhancer variants may not necessarily be protein coding genes, but can also be non-coding genes including miRNAs and lincRNAs. Investigations of GWAS enhancer SNPs have also helped nominate new cell types in common disease pathophysiology. SNPs associated with Alzheimer's Disease risk correlate not only with fetal brain-specific enhancers as may have been expected, but also immune cell-specific enhancers, thereby implicating immune-related processes in AD predisposition (67).

The success of the above mentioned studies suggests that the veritable gap between SNP association and function is steadily closing. Although successful in pinpointing functional variants at GWAS loci, often these studies do not rule out other variants in the region that could also be contributing to the correlation between genotype and target gene expression. There may be more than one casual SNP, particularly at loci where multiple disease-associated SNPs lie in SE constituents that physically interact with one another and their gene targets in 3D chromatin. As recently demonstrated, the SNPs at SE-associated loci may collude to influence target gene expression and the clinical risk to disease, and the influential SNPs may not necessarily be in linkage disequilibrium with one another (30,68). Furthermore, given that enhancers respond to specific physiological, pathological or environmental conditions, the regulatory effects of enhancer SNPs may be context specific (69). While considerable progress has been made, there is a clear need for functional studies that go beyond the standard eQTL and reporter assays to nominate causal variants. Strategies in which risk SNPs are directly edited in the genome are needed.

Concluding Remarks

As highlighted above, a variety of Mendelian diseases and cancers are caused by mutations in genes encoding proteins that function in *trans* in global transcriptional control either through regulating enhancer activity or enhancer–gene interactions via chromatin looping. These have been termed enhanceropathies and cohesinopathies, but given that the true pathology lies in transcription, 'transcriptomopathies' might be more precise descriptor for these diseases. Because clinical sequencing has largely focused on the exome, the number of genetic diseases due to non-coding mutations that disrupt enhancer–gene control in *cis* is not yet known. Given the sizeable percentage of Mendelian diseases currently explained by mutations in the exome (30%), the proportion of diseases due to mutations in non-coding regions is probably much smaller, even in spite of the fact that the enhancerome is 5–10× larger than the exome. Consistent with this prediction, whole genome sequence analysis of a large cohort of patients with developmental delay found that 1–3% without a diagnostic coding mutation had *de novo* mutations in regulatory elements (compared to 42% in the exome) (70). However, with respect to risk to common diseases, the proportion of causal variants in noncoding regions likely dwarfs that of the exome. Similarly, in cancer, and in particular cancers with high mutation rates, excessive aneuploidy and other structural rearrangements, functional mutations in noncoding regions are likely to be fairly common. Whole genome sequence analysis of tumor genomes paired with epigenome analysis should help shed light on the prevalence of noncoding cancer driver mutations. These studies are essential for the full potential of targeted therapies and precision medicine to be realized.

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