Super-enhancers and cancer: how a novel regulatory element creates super-villain cells

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Abstract

Super-enhancers (SE) are regulatory regions that induce high levels of transcription due to unusually high transcription factor binding and associated chromatin modifications. Deregulation of SEs is a recently appreciated contributor to changes in cell identity and cancer development. In this review we describe mechanisms of SE-driven cancers including solid tumors and hematological malignancies. We summarize the current knowledge of the genetic and epigenetic changes in regulatory regions of the genome that allow cancer cells to acquire SEs, which ultimately alters the regulation of genes that cause cancer. Finally, we offer a perspective on the remaining barriers facing our understanding of the role of SEs in cancer and the promise of their potential as therapeutic targets.

Enhancers are essential for cell regulation

In order to survive, a cell must tightly regulate which genes are expressed, how much they are expressed, and when they are expressed. Proper gene regulation is accomplished through a variety of mechanisms including cell signaling pathways, epigenetic modifications that alter chromatin accessibility, and the coordinated interactions of transcription factors (TF) that repress or enhance gene transcription. Together, cell regulatory mechanisms ensure that the cell produces the transcripts needed for proper function. In contrast, disrupted gene regulation can have irrecoverable consequences for the cell and lead to disease. Cancer is an extreme case of transcriptional deregulation that causes excessive cell proliferation and growth. Inappropriate gene expression in cancer cells can occur through a number of mechanisms including epigenetic modifications and deviant cell signaling. Aberrant epigenetic modifications often cause regions of the DNA to become exposed and accessible to transcriptional machinery at inappropriate times. Cancer cells also often have deregulated signaling cascades that result in excessive proliferation or migration. Finally, proto-oncogenes can become oncogenic by alterations to the repressors or enhancers that regulate transcriptional machinery. These are just a few examples of how genome regulatory mechanisms must work in concert to ensure proper cell functioning.

How enhancers regulate gene expression has been the focus of research efforts for over four decades (Banerji et al., 1981). Enhancers are regions of DNA that can activate the transcription of a distal gene irrespective of orientation within the genome. TFs regulate transcription by binding the enhancer region and Mediator, a protein complex found at promoters, to affect the assembly of the other general transcription factors and RNA polymerase II (**Figure 1A**). There is no defined distance from the enhancer to the gene it regulates and enhancers do not have a consensus sequence due to the numerous TFs that bind these regions. For years, these unique characteristics have made it difficult to identify enhancer regions within the genome and determine their role in transcriptional regulation. However, advances in research tools

and technologies have allowed for robust detection of enhancers and a better understanding of how enhancers regulate transcription. Recently, a novel class of enhancers termed super-enhancers (SE) were serendipitously discovered and found to preferentially regulate genes essential for cell identity (**Figure 1B**) (Whyte et al., 2013). Since their initial discovery, SEs have been implicated in several diseases including cancer. This review will focus on the continuing debate surrounding the classification of SEs, how these regulatory elements drive cancer, and the potential of therapeutics that target cancer specific SEs.

What are Super-Enhancers?

Discovery and Traditional Classification

SEs were initially defined by Whyte *et al.* to have two characteristics: (1) clustering of individual enhancers over ~50 kb of DNA and (2) high levels of binding of the transcriptional cofactor Mediator (**Figure 2**) (Whyte et al., 2013). Mediator is a complex of proteins that acts ubiquitously as a scaffold to coordinate the recruitment of TFs and RNA polymerase II to the promoter and is therefore essential for transcription. Whyte *et al.* originally set out to determine why depletion of Mediator caused differentiation of mouse embryonic stem cells (mESC). In doing so, Whyte *et al.* inadvertently discovered SEs and demonstrated that SEs preferentially regulate genes essential for cell identity. Their study began by determining where Oct4, Sox2, and Nanog, TFs essential for maintaining a pluripotent state, bind DNA using chromosome immunoprecipitation followed by sequencing (ChIP-seq) (**Figure 2A**). ChIP-seq reveals enhancer regions through global genome profiling of protein-DNA interactions by using antibodies to immunoprecipitate any protein of interest bound to the associated DNA region (Pandey et al., 2018). Whyte *et al.* noticed that all three TFs bound to clustered enhancers that regulated genes known to be essential for mESC cell identity and that these regions had nearly 18 times more Mediator bound than typical enhancers (**Figure 2B and 2C**) (Whyte et al., 2013). These data were verified in differentiated cell types as well and suggested that SEs are a previously unidentified regulatory element characterized by high levels of transcriptional cofactor binding that regulate genes essential for cell identity.

Working toward a universal definition for super-enhancers

Since their initial discovery, SEs have been further characterized using a variety of secondary criteria. SEs are primarily identified by Mediator levels, but SEs can also be defined by secondary criteria based on the enrichment of marks indicative of transcriptionally active regions. These include epigenetic marks such as histone modifications H3K4me1 and H3K27ac, and increased occupancy of acetyltransferases p300 and CBP (Niederriter et al., 2015; Pott and Lieb, 2015; Hnisz et al., 2013). Additionally, proteins that bind to epigenetic marks are increased at SEs, including BRD4, which binds acetylated histones and directly activates RNA polymerase II (Hnisz et al., 2013; Lovén et al., 2013). Finally, SEs occupy regions of transcriptional activity marked by DNase 1 hypersensitivity (an indicator of open chromatin), production of unstable RNA products, and DNA methylation valleys, which are regions found upstream of transcriptionally active genes (Pott and Lieb, 2015). Currently, there is no unifying definition of SEs because independent research groups use different secondary criteria, the implications of which this review will discuss later. Despite this, SEs have been identified in embryonic stem cells, cancer cells, immune cells, and other differentiated cell types (Whyte et al., 2013; Hnisz et al., 2013).

Similar to typical enhancers, SEs are also characterized by their spatial interactions with promoters through bound TFs and high sensitivity to TF levels (Whyte et al., 2013; Lovén et al., 2013). In mESCs, SEs cause chromatin looping by interacting with specific TFs; these interactions change when different genes in the cell need to be expressed, for example, during differentiation (Novo et al., 2018).

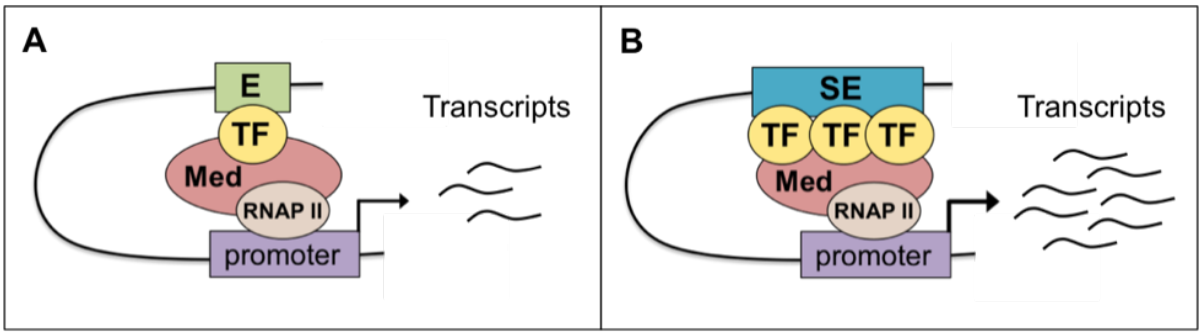

Figure 1: **Features of typical enhancers verses super-enhancers. (A)** Example of a typical enhancer. The promoter interacts with RNA polymerase II (RNAP II), Mediator (Med), transcription factors (TF), and enhancers (E) to produce a pool of transcripts. **(B)** Example of a super-enhancer (SE). SEs bind higher levels of TFs and Med (generally 18 times more binding than typical enhancers) and more strongly activate transcription to produce a larger pool of transcripts (figure adapted from (Sur and Taipale, 2016)).

Additionally, changes to TF levels at SEs associated with cell identity genes can change the cell itself. SEs drive transcription more robustly than typical enhancers, so depleting TFs at SEs has a disproportionally large effect on transcription. Sensitivity to TF levels was first described by Whyte *et al.* who found that depletion of Mediator at SE sites associated with pluripotent genes in mESCs reduced transcription of these genes and induced differentiation (Whyte et al., 2013). Loven *et al.* described a similar phenotype in cancer cells that upregulate BRD4 at SEs. When an inhibitor against BRD4 was used, they observed preferential loss of BRD4 at SEs and a greater reduction in SE-associated gene expression (Lovén et al., 2013). These independent studies suggest that SEs are highly sensitive to TF levels and that depletion of the associated TFs can induce changes in cell identity and contribute to disease.

Identification of Cancer Associated Super-Enhancers

Cancer is a heterogeneous disease characterized by the acquisition of several biological changes that drive tumor development. As reviewed by Hanahan and Weinberg, these hallmark capabilities are acquired by cancer cells in succession and contribute to uncontrolled cell proliferation, growth, and tumorigenesis (Hanahan and Weinberg, 2011). Initial research in cancer biology asked how mutations in protein-coding regions of the genome contribute to cancer, and characterized mutations in oncogenes and tumor suppressors that cause cancer. Investigating the cellular and molecular changes that occur during cancer development led to the discovery that many cancer causing mutations occur in regulatory regions of the genome and lead to transcriptional misregulation (Vogelstein et al., 2013). Recent studies have focused on deciphering how global regulatory changes and epigenetic alterations contribute to cancer. The focus of this review is centered on how these epigenetic and regulatory changes lead to the acquisition of SEs that initiate and maintain cancer.

SE involvement in cancers, including solid tumors and hematological malignancies, was identified soon after Whyte *et al.* discovered and defined SEs (Whyte et al., 2013). SEs have been detected in cancer cells using ChIP-seq and secondary criteria discussed above. Various studies demonstrate that SE regions are associated with known oncogenes in patient samples and cancer cell lines, which are absent in healthy counterpart tissue or cells (Hnisz et al., 2013; Lovén et

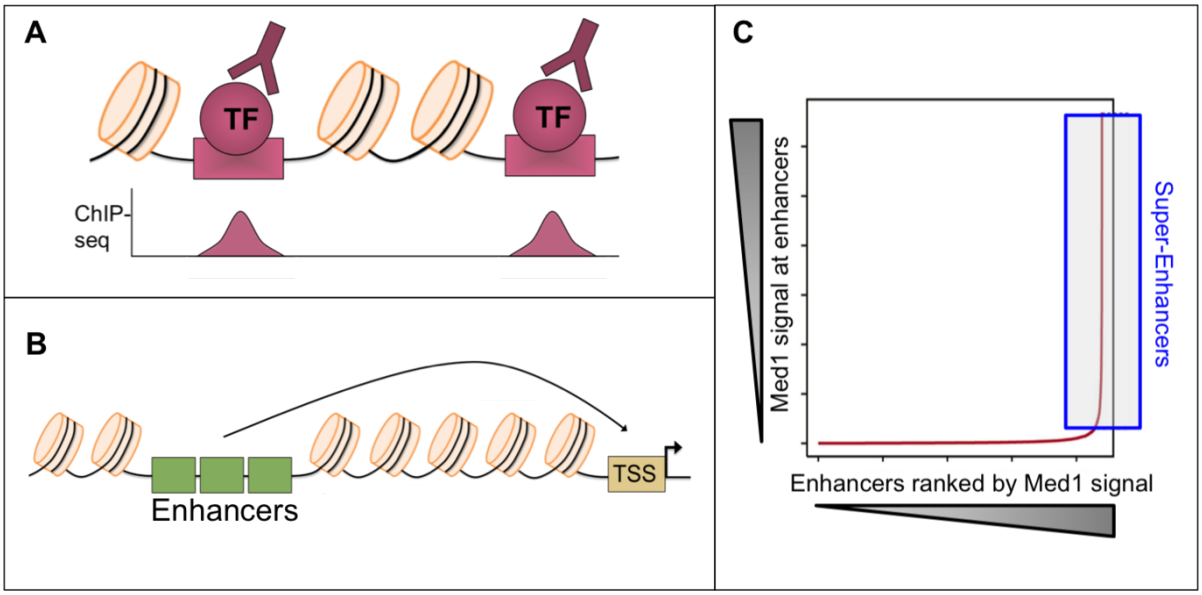

Figure 2: **Identifying super-enhancers. (A)** Identify enhancer regions using ChIP-seq against a TF or histone mark. Enrichment of DNA sequence associated with the chosen mark determines the presence of an enhancer. **(B)** Determine enhancer clustering. SE enhancer clusters are typically less than 12.5 kb apart and have a median size range of roughly 9,000 bp. **(C)** Measure Mediator binding as determined by Med1 enrichment. Regions with enrichment above the inflection point are SEs, highlighted in the grey box (figure adapted from (Pott and Lieb, 2015; Sur and Taipale, 2016)).

al., 2013). This finding led to the hypothesis that cancer cells acquire SEs by several mechanisms to provide cells with enhanced growth and proliferative abilities. For example, SEs can arise due to epigenetic alterations that make certain regions of DNA more accessible to transcriptional machinery. In fact, comparison of healthy cells and cancer cell lines has revealed distinct epigenetic characteristics of SEs associated with cancer, including aberrant DNA methylation patterns and altered activity of TFs and chromatin modifying enzymes. In addition to the finding that many SEs are associated with known oncogenes, such as MYC, SEs found in cancer cells often control transcription of other genes that promote the hallmarks of cancer and enhance oncogenic signaling (Hnisz et al., 2015; Hnisz et al., 2013). Collectively, acquisition of SEs by several mechanisms appears to drive cancer pathogenesis via transcriptional reprogramming. Understanding how cancer cells acquire SEs that drive oncogenic programs is important for understanding disease mechanisms and the development of targeted therapies.

Modes of Super-Enhancer Acquisition in Cancer

Several key genomic mechanisms contribute to the acquisition of SEs that drive cancer. The three primary means of SE acquisition in cancer include chromosomal and genomic rearrangement that place SEs near oncogenes, mutations causing novel SEs or amplification of SEs, and epigenetic modifications that affect SEs and drive an oncogenic gene program and cancer progression (**Figure 3**).

Chromosomal and genomic rearrangements affecting super-enhancer activity

The most evident examples of SE acquisition in cancer are events that position SEs in control of oncogene expression. The most well-studied

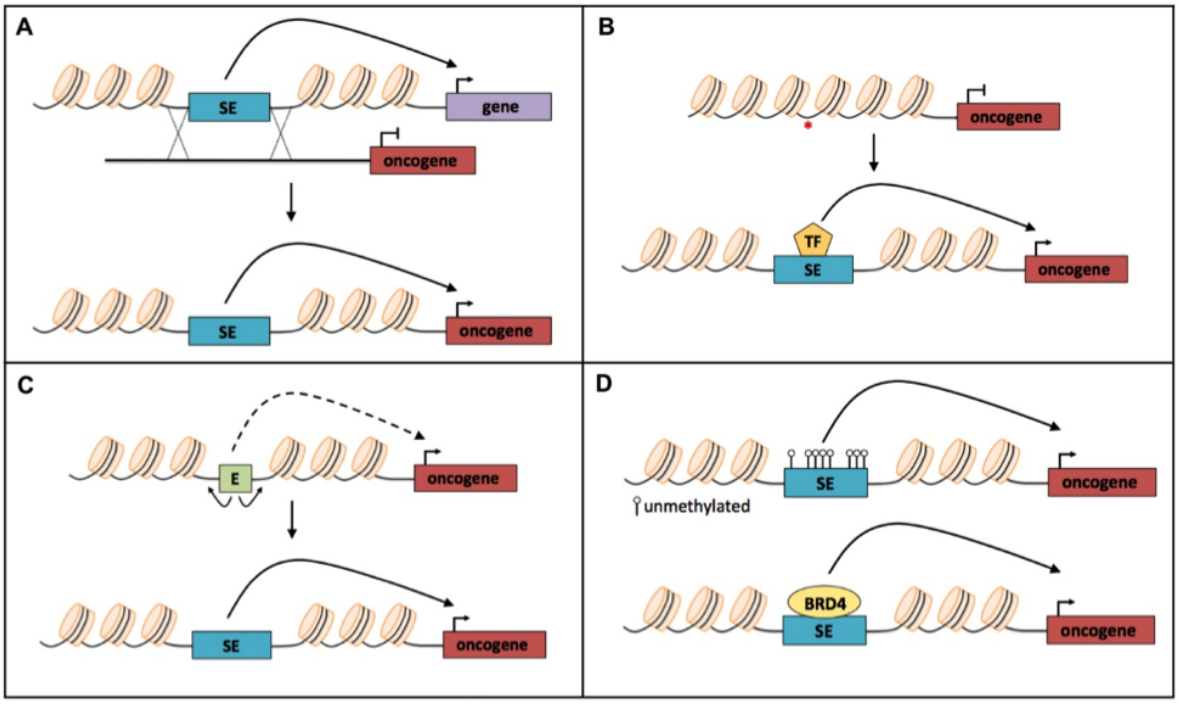

Figure 3: **Modes of super-enhancer acquisition in cancer. A.** Chromosome translocations or rearrangements that position a SE near an oncogene induces oncogene expression. **B**. Mutations causing changes in TF binding can induce SE formation and oncogene expression. **C**. focal amplifications contribute to SE acquisition in cancer. **D.** Epigenetic changes in cancer cells including hypomethylation (top) and epigenetic regulators like BRD4 (bottom) can influence oncogenic signaling at SEs in cancer cells (figure adapted from (Sur and Taipale, 2016)).

chromosomal abnormalities in cancer are rearrangements and translocations that create oncogenic fusion genes that cause aberrant gene expression (Fröhling and Döhner, 2008). Gene rearrangements can also position regulatory elements including SEs near genes that would normally be silent. In fact, the field has recently accepted the hypothesis that cancer cells are able to hijack SEs through chromosomal rearrangements to drive oncogenesis by changing the target gene of the SE (**Figure 3A**).

Consistent with this hijacking hypothesis, Affer *et al.* set out to characterize the prevalence of MYC rearrangements in multiple myeloma patient samples and found that most MYC rearrangements in cancer cells reposition MYC near a SE (Affer et al., 2014). This phenomenon has also been observed in medulloblastoma cells where Northcott *et al.* found that transcription factors GFI1 and GFI1B, usually silent in normal cells, are translocated into SE loci with actively transcribed regions containing high H3K27ac and DNA hypomethylation. Accordingly, they identified a novel mechanism where translocations proximal to SEs activate a gene to become oncogenic (Northcott et al., 2014). Another study found a chromosomal rearrangement in acute myeloid leukemia that causes the GATA2 enhancer to translocate and activate the EVI1 proto-oncogene. This translocation simultaneously creates a haploinsufficiency of GATA2, an essential hematopoietic stem cell regulator, and repositions the enhancer to activate an oncogene, creating an ideal environment for cancer progression (Gröschel et al., 2014). Thus, repositioning SEs to control gene expression that is normally silent or lowly expressed

in noncancerous cells confers a strong advantage to cancer cells that allows for progression of cancer pathogenesis.

Mutations causing formation or amplification of super-enhancers

In cancer, oncogenes often acquire SEs that are tissue and tumor type specific and drive increased levels of gene expression. In SE-driven cancers focal amplification, or increase in chromosomal copy number, of SE containing genomic regions can elevate oncogene expression. Another mechanism that induces oncogene expression by SEs are mutations or small indels that change transcription factor binding or create new regulatory binding sites (**Figures 3B and 3C**). For example, Zhang *et al.* found focal amplifications in SE containing regions of the genome in several tumor types by performing copy number analysis and epigenomic profiling of cancer databases. Two specific focally amplified SEs involved in lung adenocarcinoma and endometrial carcinoma were found to physically interact with the MYC promoter and were associated with increased MYC expression (Zhang et al., 2016). Another study found that a recurring T cell acute lymphobastic leukemia (T-ALL) mutation causes a duplication in an SE containing genomic region. They found that this SE was occupied by Notch and drove expression of MYC, a downstream target of Notch signaling, thus establishing a feed-forward loop that amplifies oncogenic signaling in T-ALL (Herranz et al., 2014). These examples demonstrate how mutations affecting copy number in SE regions can drive several cancer types. Mutations in non-coding regions not only affect existing SE activity, but can also induce the formation of SEs that drive cancer. For example, Mansour *et al.* found that mutations in noncoding regions can induce transcription factor binding sites that trigger oncogenic SEs. This group reported that in about 5% of T-ALL cases, acquired mutations in noncoding regions created new binding sites for the transcription factor MYB. MYB then recruits chromatin modifiers including H3K27 acetylase to expose regions of DNA, which facilitates SE-driven cancer progression (Mansour et al., 2014). These studies demonstrate that mutations in SE regions causing chromosomal amplification or novel transcription factor binding sites influence oncogenic activity in a variety of cancers.

Epigenetic modifications that induce Super-Enhancer activity

Epigenetic modifications to the genome affect chromatin accessibility, transcription factor binding, and, ultimately, gene expression. As such, another mechanism of SE acquisition in cancer results from changes in epigenetic modifications and altered epigenetic machinery (**Figure 3D**). A common epigenetic feature of cancer cells is aberrant DNA methylation. For example, Heyn et al. investigated global DNA methylation of cancer cells and found enrichment of unmethylated DNA sequences within SE regions associated with expression of cancer-driving genes (Heyn et al., 2016). Another broad class of epigenetic change discussed by Sur *et al.* postulates that mutations altering the activity of histone modifying enzymes like histone deacetylases increase chromatin accessibility for tumor-specific transcription factor binding and upregulation of gene expression, a mechanism they hypothesize is facilitated at SEs (Sur and Taipale, 2016). Likewise, mutations in cohesin, a protein complex required for chromosome architecture and thought to be involved in enhancer function, can contribute to transcriptional deregulation in cancer (Kagey et al., 2010; Solomon et al., 2014). A number of recent studies have also identified that BRD4, a bromodomain protein and transcriptional activator that ubiquitously associates with acetylated chromatin, is associated with SE-driven cancers. BRD4 was found to be preferentially increased at SEs near known oncogenes in multiple myeloma and non-Hodgkin's lymphoma, and found to have similar binding patterns as Mediator (Lovén et al., 2013; Chapuy et al., 2013). Thus, BRD4 occupancy at SEs likely represents the need for chromatin modifiers to create accessible chromatin to influence an oncogenic gene program.

Super-Enhancers as a Therapeutic Target

Given the commonality that several oncogenes are driven by SEs in diverse cancers, SEs may serve as a reliable biomarker for cancer diagnosis. Further, due to the recent appreciation of SEs as cancer drivers, efforts have been made to exploit these regulatory regions as therapeutic targets. Thus far, indirect approaches using small molecule inhibitors to target SE-associated TFs and transcription elements have been promising. Most notably, BET bromodomain inhibitors and inhibitors of cyclin dependent kinases that regulate RNA polymerase II have been explored as interventions for SE-driven cancers.

Several groups have independently observed BRD4 upregulation and preferential occupancy of SEs in cancer (Lovén et al., 2013; Chapuy et al., 2013). Delmore *et al.* first observed that MYC expression is regulated by enhancers containing BET bromodomains bound by BRD4 in multiple myeloma (MM). They tested JQ1, a BET inhibitor that binds competitively to BRD4 bromodomains and prevents their interaction with enhancers, and that found this inhibitor depleted BRD4 bound to enhancers and decreased MYC transcription (Delmore et al., 2011; Filippakopoulos et al., 2010). Building on this work, Loven *et al.* sought to understand how inhibition of BRD4 lead to the selective inhibition of MYC in MM. They revealed that SEs are associated with increased levels of BRD4 and Mediator, so they are more sensitive to depletion, which suppresses the high level of oncogenic transcription associated with SEs (Lovén et al., 2013). This work was confirmed by another group that targeted SEs in non-Hodgkin's lymphoma cells using JQ1, which leads to preferential BRD4 depletion at SEs and downregulation of the associated target genes including MYC (Chapuy et al., 2013). Another strategy to decrease SE driven oncogenic transcription is aimed at targeting cells that are actively transcribing growth and cell cycle associated genes. Recently, these strategies have focused on targeting cyclin dependent kinases involved in RNA polymerase initiation and elongation. Chipomuro *et al.* used THZ1, a covalently linked CDK7 inhibitor that prevents RNA polymerase II activation, in neuroblastoma cells and found decreased transcription from SE associated genes (Chipumuro et al., 2014). Another recent study found that using THZ1 against SE-mediated and MYC driven metastatic osteosarcoma was more effective than JQ1 treatment, though both drugs lead to decreased proliferation, migration, and invasion of cancer cells (Chen et al., 2018). As demonstrated in these studies, targeted treatment using JQ1 and THZ1 reveal that many cancers are dependent on SE mediated aberrant gene expression. Future efforts will likely focus on developing effective and specific interventions that target SE dependencies in cancer and spare healthy cells.

Remaining Issues with Super-Enhancers

Continued issues with classifying super-enhancers

How to distinguish SEs from typical enhancers remains a contentious topic in the literature. As previously discussed, inconsistencies arise when independent research groups use different secondary criteria to distinguish SEs from typical enhancers. Additionally, many cell types do not have complete data sets for defined histone marks or protein occupancies, thus hindering consistent classification of SEs across cell types. Furthermore, SEs have primarily been identified through correlative data and many SEs have not been functionally validated. Functional testing is necessary to distinguish SEs as a novel class of regulatory elements and to determine how their component enhancers interact to form a SE. A number of studies have used luciferase reporter assays to demonstrate that SEs produce more transcripts than typical enhancers, however this is a low throughput and burdensome method to demonstrate functional relevance of SEs for all cell types (Whyte et al., 2013; Lovén et al., 2013). CRISPR/Cas9 site directed mutagenesis represents an alternative approach for future studies to determine the components of SEs that are necessary and sufficient for their function.

Another consequence of inconsistent classification of SEs is how to differentiate them from previously identified regulatory regions that are distinct from typical promoters or enhancers (Hnisz et al., 2013; Xie et al., 2013; Li et al., 2002; Parker et al., 2013; Song et al., 2011). These regions include stretch enhancers (regions of DNA with enhancers distributed over large distances), locus control regions (LCR; genomic regions that regulate the expression of one or multiple genes), and DNA methylation valleys (regions with low methylation proximal to promoters). Future work with reporter assays and gene editing techniques will be necessary to determine if these regulatory mechanisms serve independent or redundant functions.

Continued issues with using super-enhancers as therapeutic target

Targeting SEs using small molecules has shown promise as a cancer therapy, but issues with specificity need to be resolved before clinical applications are feasible. To date, most SE therapies target transcriptional elements. While the components that drive transcription can differ between healthy and cancer cells, such as elevated levels of the TF *MYC* in cancer cells, the transcriptional machinery is largely the same. Therefore, any therapy that targets a factor found in both healthy and diseased cells, such as Mediator or BRD4, could have unintended consequences *in vivo*. Furthermore, therapeutics that change the regulation of SEs is problematic because researchers cannot yet predict how SEs influence the onset of cancer. As previously described, many factors, including mutations that alter TF binding or chromosome accessibility and structure, can alter how SEs regulate their associated genes. Future work characterizing whether specific cancers uniformly adopt or alter SEs through the same mechanism will enhance our ability to target aberrant SE function.

Conclusion

SEs are a newly discovered regulatory mechanism that influence cell identity via transcriptional regulation in both healthy and diseased cells. In cancer, aberrant transcription is associated with the deregulation or acquisition of SEs, making these regions attractive targets for therapeutics. Since SE regulation is highly sensitive to the levels of TF and cofactor binding, small molecule inhibitors for these factors may preferentially target SE associated oncogenes. Additionally, SEs could be targeted with CRISPR/Cas9 technologies that modify sequences specifically within deleterious SEs. Finally, further characterization and validation of SEs is necessary to determine the components that are necessary and sufficient for their function. Together, these efforts will determine how SEs function in healthy and diseased cells and how SEs can be harnessed as novel cancer therapeutic.

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