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Spatial promoter–enhancer hubs in cancer: organization, regulation, and function

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Abstract

Transcriptional dysregulation is a hallmark of cancer and can be driven by altered enhancer landscapes. Recent studies in genome organization have revealed that multiple enhancers and promoters can spatially coalesce to form dynamic topological assemblies, known as promoter–enhancer hubs, that strongly correlate with elevated gene expression. In this review, we discuss the structure and complexity of promoter–enhancer hubs recently identified in multiple cancer types. We will further discuss underlying mechanisms driving dysregulation of promoter–enhancer hubs and speculate on their functional role in pathogenesis. Understanding the role of promoter–enhancer hubs in transcriptional dysregulation can provide insight into new therapeutic approaches to target these complex features of genome organization.

Keywords

promoter–enhancer hub; multiway interaction; cancer

Multiple enhancer and promoter elements can spatially coalesce

Spatial organization of mammalian genomes contributes to gene expression control and cell identity. The genome's spatial organization is set at various length scales, ranging from **chromosome territories** (see Glossary) to megabase-scale transcriptionally active and inactive **compartments, topologically associating domains (TADs)**, and fine-scale chromatin loops [1-4]. Compartments, TADs, and chromatin loops contribute to positioning of regulatory elements, including enhancers and promoters, influencing their activity and specificity (reviewed in [3]). Enhancers are non-protein coding, cis-regulatory sequences

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containing numerous binding sites for **transcription factors (TFs)**. Upon binding to an enhancer, TFs facilitate increased chromatin accessibility and recruit coactivators and histone modifying enzymes [5,6]. Because TF binding depends on contextual information specified by cell lineage and/or signaling pathways, enhancers can integrate environmental and developmental cues to fine-tune gene expression [7].

Despite several proposed models (reviewed in [8]), it is not yet fully understood how distal enhancers exert their regulatory function across great genomic distances. Most models agree that proximity between the enhancer and promoter, even transiently, is essential for enhancer function [8,9]. Promoter–enhancer interactions could be mediated and/or stabilized by cohesin-mediated loop extrusion [10], transcriptional machinery and coactivator bridging [11], and TF/cofactor-mediated phase separation and oligomerization [12,13]. Adding to the complexity is the fact that the number of active genes in a cell is 2-3 times less than that of active enhancers [14]. Thus, it is often possible that the expression of a single gene is controlled by multiple enhancers, giving rise to complex gene expression control circuits [15,16]. In some cases, multiple enhancers in close genomic proximity cluster together, forming super-enhancers [17]. There are also cases where multiple gene promoters are controlled by the same set of enhancers, potentially allowing regulatory information to be relayed across multiple genes [18,19].

Technical advancements in **chromatin conformation capture** technologies have pointed to the existence of **promoter–enhancer hubs** or cliques, which we define here as dynamic topological assemblies with one or more promoters that interact, even transiently, amongst themselves and with two or more enhancers that may or may not be connected (Figure 1). Although most studies were limited to examining pairwise interactions between enhancers and promoters, recent work started to reveal that multiway interactions are more prevalent and complex than previously appreciated [20-24]. The frequency of multiway contacts is relatively low, suggesting that promoter–enhancer hubs are dynamic topological assemblies rather than rigid physical structures (Figure 2). Although the organizational principles, regulators, and functions of promoter–enhancer hubs remain largely unclear, detailed examination of multiple loci shows that spatial interactivity of enhancers with each other and cognate promoters strongly correlates with transcriptional activation and often regulate genes critical for cell identity [18,25-29]. Several reviews discussed the role of promoter–enhancer hubs as well as proposed mechanisms of their formation in normal differentiation and cell function [30-33] and hence will not be our focus here.

Recent evidence suggests that promoter–enhancer hubs can also play a role in regulating disease susceptibility and pathogenesis, including cancer. A key hallmark of cancer is aberrant gene expression control, which can be influenced by both genetic and epigenetic events that disrupt enhancer activity and nuclear positioning [34-41]. But how do cancer-specific promoter–enhancer hubs contribute to transcriptional dysregulation? This review will focus on recent evidence from diverse cancer types suggesting that coalescence or separation of promoter–enhancer hubs contribute to transcriptional regulation in cancer (Table 1, Key table). We will further speculate on their mechanisms of assembly and functional implication in pathogenesis based on available evidence from literature. We will

start by describing new technological advancements that enable better detection of these dynamic topological assemblies.

New technologies to study promoter–enhancer hubs

Proximity ligation-based chromatin conformation capture technologies such as Hi-C have enabled the genome-wide study of long-range enhancer–promoter interactions (Box 1). Yet, these assays can only measure pairwise interactions with high confidence. Further, results from chromatin conformation capture assays cannot distinguish between co-occurring interactions in the same cells and mutually exclusive interactions occurring in different cells (Figure 3). To overcome these limitations, variants of chromatin conformation capture protocols based on targeted (Tri-C, TM3C, COLA), long-read (MC-4C), and molecular barcoding (C-walks) technologies were developed [42-46]. Except for TM3C, these assays are not genome-wide. Further, the targeted-based methods cannot resolve beyond three-way interactions. Pore-C, a more recent approach, relies on long-read sequencing like MC-4C, but in addition enables genome-wide study of multiway contacts [47].

Approaches without proximity ligation have also been developed to study interactions among regulatory elements, including GAM and SPRITE [20,48,49]. SPRITE uniquely identifies fragment ends and overcomes resolution limitations of chromatin conformation capture-based techniques in identifying multiway interactions by repetitive pooling, splitting, and barcoding DNA fragments [20,48].

In addition to sequencing technologies, microscopy techniques have enabled the single-cell resolution study of the physical location and proximity of promoters and enhancers. For the past two decades, 3D DNA fluorescence in situ hybridization (FISH) has identified many features of chromatin architecture [50], but these studies were limited to probing a few genomic elements at a time (Figure 3). Advancements in **oligopaint**-based FISH probes combined with high-throughput imaging led to the development of chromosome-tracing technologies, including ORCA, Hi-M, MINA, DNA-MERFISH, and DNA-seqFISH+ [51-55]. The common principle of all these techniques is sequential cycling through adding and imaging a fluorophore that uniquely binds to a barcoded primary oligo corresponding to a distinct genomic locus, followed by removing and replacing it with another fluorophore that recognizes the adjacent barcoded primary oligo. Together, these chromosome-tracing technologies enabled the reconstruction of DNA polymer folding in individual cells. Furthermore, the single-cell resolution of these studies revealed that enhancer–promoter interactions are heterogeneous and occur in domain-like structures consisting of contacts among multiple regulatory elements [53-55]. More recently, these advanced imaging-based technologies also provided evidence of multiway contacts between multiple enhancers, promoters, and **insulators** [21,22,56]. Despite these intriguing findings, current chromatin tracing studies are limited to a few genomic loci. Future work using higher-throughput microscopy-based technologies can further elucidate the extent of enhancer cooperativity or competitiveness and the involvement of RNA and protein molecules in hub formation at single-cell resolution.

Promoter–enhancer hubs in health and disease

Recent investigations into cellular differentiation suggest that the dynamic rewiring of promoter–enhancer hubs may play a key role in cell-fate decision. In pluripotent stem cells, immune cells, and neurons, promoters exhibiting higher levels of chromatin interactivity are enriched for lineage-specific genes, enabling fine-tuning of transcription during differentiation [18,23,29,57]. For example, Enhancer Capture Hi-C conducted during differentiation of human mesenchymal stem cells into adipocytes and osteoblasts revealed that transcription of lineage-specific genes is regulated by the coordinated activation and convergence of multiple enhancers to promoters, forming promoter–enhancer hubs, amplifying TF occupancy, and increasing cofactor recruitment [58].

In addition to integrating spatiotemporal signals, enhancers within a promoter-enhancer hub may exhibit context–specific behavior to fine-tune transcription [29]. Prior studies of multi-enhancer gene regulation without long-range interaction measurement revealed cooperative, redundant, or repressive functions of enhancers [15,59-62]. Although it remains unclear whether enhancers and promoters examined in these studies participate in spatial hubs, recent work revealed that enhancers in spatial hub can behave additively, synergistically, or redundantly [24,63-65].

Given the role of promoter–enhancer hubs in controlling gene expression during normal development, it is unsurprising that the re-wiring of these topological assemblies contributes to aberrant transcription in various diseases. Disease-associated sequence variations have been linked to mis-regulation of promoter–enhancer hubs, increasing the risk of developing metabolic, immune, and brain disorders [19,23,66-68]. Additionally, accumulating evidence suggests that promoter–enhancer hub dysregulation coincides with aberrant oncogene and tumor suppressor expression in cancer. Next, we will describe the complexity, regulation, and function of promoter–enhancer hubs gleaned from recent studies on cancer genome organization. As this is a nascent topic in cancer biology, in some cases, we interpret and describe the data from the perspective of promoter–enhancer hubs even when the original study did not specifically examine these topological assemblies.

Promoter–enhancer hubs in cancer

Promoter–enhancer hubs in cancer could be influenced by a plethora of genetic and epigenetic mechanisms (Figure 4). As described in the following sections, structural variants and deregulation of TFs and epigenetic regulators can alter enhancer function across different cancer types. The extent to which these events can mis-regulate gene expression often depends on a combination of enhancer activity and positioning relative to the target promoter [41,69]. Recent studies in various cancers uncovered cases where aberrant promoter–enhancer hubs dysregulate expression of oncogenes and tumor suppressors, contributing to malignant phenotypes.

Prostate Cancer

Prostate cancer is characterized by widespread transcriptional dysregulation [70]. To investigate the underlying mechanisms driving aberrant transcription in prostate cancer,

several groups recently mapped the chromatin conformation in normal and malignant cells [71,72]. These studies identified major chromatin reorganization, including at the *FOXO1* [71] and androgen receptor (*AR*) loci [72]. *FOXO1*, a tumor suppressor, is frequently downregulated in prostate cancer in the absence of genetic alterations [73]. Concomitant with *FOXO1* downregulation, the promoter loses interactions with active compartments and connects to heterochromatic regions [71,74]. Although links between promoter–enhancer hubs and compartments remain unclear, this data shows the repression of multiple enhancers located at this locus, which can be interpreted as potential loss of the *FOXO1* promoter–enhancer hub during tumorigenesis. In contrast, *AR*, whose upregulation is essential during tumorigenesis [75], acquires and interacts with oncogenic enhancers independent of CTCF binding changes [72]. Although the authors did not specifically define the *AR* locus as a hub, their observations suggest that *AR* participates in a cancer-restricted promoter–enhancer hub. Both studies showed that transcriptional changes correlate with alterations in enhancer activity and positioning in prostate cancer [71,72]. However, the chain of causality of these events in driving prostate cancer pathogenesis remains to be understood.

Squamous Cancers

Squamous cancer cells exhibit similar transcriptional programs and binding profiles of KLF5, a pro-proliferation TF, regardless of tissue of origin [76,77]. It was shown that KLF5 partners with lineage-specific TFs in squamous cancer cells to activate enhancers through CBP/EP300 methyltransferase complex and chromatin reader BRD4 recruitment [78]. KLF5-mediated histone acetylation promotes long-range interactions at loci with multiple enhancers, potentially forming promoter–enhancer hubs. This hypothesis is supported by the observation that several oncogenic enhancers synergize to promote Pol II elongation and activate transcription of oncogenes including *FOXE1* and *TP63*. Similar to other TFs, direct inhibition of KLF5 is challenging. Liu et al. showed that degrading BRD4 can be an alternative strategy to selectively target KLF5-dependent cancer cells [78].

NUT carcinoma, a rare and aggressive squamous cell carcinoma subtype [79], is characterized by fusion between *NUTM1* and a number of genes, mainly *BRD4* (BRD4-NUT) [80,81]. BRD4-NUT drives the formation of mega-domains, or massive active genomic regions that contiguously bind BRD4-NUT as well as wild-type BRD4 and p300 [82]. Mega-domains are even larger than super-enhancers and coincide with key oncogenes, including *MYC, TP63*, and *SOX2* (reviewed in [83]). The mega-domains form topological assemblies termed sub-compartment M that are characterized by high intradomain interactions across extremely large linear genomic distances. Sub-compartment M may potentiate an auto-regulatory feed-forward network to coregulate the expression of constituent genes [83,84]. Nevertheless, additional research is needed to investigate whether these topological assemblies can be selectively targeted in cancer.

Sarcoma

Ewing’s sarcoma is characterized by low mutation burden and the fusion between *EWSR1* and ETS TFs, most commonly *FLI1* [85]. The main oncogenic driver in this malignancy is EWS-FLI1, an aberrant fusion TF that drives epigenetic remodeling in permissive cells, resulting in their transformation [86]. Sanalkumar et al. demonstrated

that EWS-FLI1 converts repetitive GGAA genomic sequences into highly inter-connected topological assemblies, connecting megabase-sized loci containing numerous enhancer–enhancer and enhancer–promoter interactions and promoting oncogenic expression [87]. Depletion of EWS-FLI1 induces chromatin reorganization in tumor cells and restores latent differentiation programs characteristic of the tumor precursor cells. Similar to NUT carcinomas, this study in Ewing's sarcoma supports the idea that a single fusion protein can promote genome-wide nuclear reorganization and formation of oncogenic promoter–enhancer hubs.

Breast Cancer

Triple negative breast cancer (TNBC) accounts for more than 15% of breast cancer cases and exhibits an aggressive phenotype and higher relapse rate [88]. A recent study mapped promoter–enhancer interactions genome-wide in TNBC and found 140 hubs or cliques of enhancers and promoters with more than 100 interactions [24]. The spatial positioning of regulatory elements within cliques are preferentially regulated by oncogenic Notch, a TF that is crucial for TNBC tumorigenesis and its hyperactivation correlates with poor prognosis [88]. The authors found that many promoter–enhancer hubs form at key oncogenes, including *MYC, CCND1, TMPRSS2*, and *RIPK4*. The *MYC* promoter resides within a highly connected promoter–enhancer hub containing five super-enhancers [24]. The activity of the most distal and proximal super-enhancers in the *MYC* hub are the most Notch-dependent and behave synergistically. In contrast, the Notch-dependent enhancers within the *CCND1* promoter–enhancer hub behave redundantly [24]. Notch can also coregulate expression of multiple genes by promoting looping within spatial hubs containing multiple promoters. For example, the *TMPRSS2 / RIPK4* promoter–enhancer hub consists of highly interacting enhancers within a single super-enhancer. Comparison of the *MYC* and *TMPRSS2 / RIPK4* loci suggests that some promoter–enhancer hubs closely align with aggregation of enhancer elements within a super-enhancer, while others involve multiple distally located super-enhancers. Overall, these results suggest that oncogenic TFs can markedly increase expression of key oncogenes by selectively targeting and positioning enhancers within highly connected promoter–enhancer hubs.

Gastrointestinal (GI) Cancers

Structural variations including tandem duplications can cause amplification of enhancer regions and activate proto-oncogenes [89]. ZFP36L2, an RNA-binding protein, behaves as an oncogene in pancreatic [90] and gastric cancers [91]. Xing et al. recently identified a novel tandem duplication hotspot in a 500 Kb region downstream of *ZFP36L2*, which is present in 10% of gastric cancers and associates with elevated *ZFP36L2* [91]. The duplicated region contains up to eleven distal enhancer elements highly interacting with the *ZFP36L2* promoter. The enhancers are organized hierarchically as deletion of one of the enhancers, but not the others, almost completely abrogates *ZFP36L2* expression [91]. Although this study only reported enhancer–promoter but not enhancer–enhancer interactions, the data suggests the hypothesis that *ZFP36L2* and its interacting enhancers form a promoter–enhancer hub. By contrast, *ZFP36L2* is lowly expressed and functions as a tumor suppressor in esophageal squamous cell carcinoma, where unlike gastric cancer, the

ZFP36L2 downstream region is hypermethylated and inactive [92]. These results illustrate that ZFP36L2 can exert opposite functions in different cancers.

Changes in TF specificity and DNA methylation are proposed epigenetic mechanisms altering promoter-enhancer hubs [78,93,94]. In colorectal cancer, KLF5, an essential TF, exhibits distinct binding patterns compared to squamous cell cancers [78]. Altered KLF5 binding promotes spatial hub formation at *CEACAM5* and *ETV4* loci in colorectal cancer, consistent with their aberrant upregulation [78]. CEACAM5 is overexpressed in 90% of colorectal cancer cases and correlates with poor prognosis [95], while ETV4 promotes proliferation and migration in this cancer type [96].

Succinate dehydrogenase (SDH) and isocitrate dehydrogenase (IDH) mutations are common initiating events in many tumor types [93]. In SDH and IDH-deficient tumors, the accumulation of succinate or 2-hydroxyglutarate, respectively, inhibits demethylases and causes DNA hypermethylation [97]. Increase in methylation disrupts CTCF binding [94], resulting in tumors with widespread insulator inactivation, local genome folding reorganization, and aberrant gene activation [93,98]. For example, in SDH-deficient GI stromal tumors, hypermethylation-mediated disruption of an insulator 3’ of oncogenes *FGF3* and *FGF4* leads to their upregulation and ectopic interactions between their promoters and multiple interconnected enhancers within a super-enhancer, potentially forming a hub consisting of two promoters and multiple enhancers [93]. This alteration is allele-specific and represents a stable epigenetic event, indicative of propagation through a malignant clone. SDH-deficiency similarly leads to the loss of an insulator 5’ of tyrosine kinase *KIT* in primary tumors, increasing looping between the *KIT* promoter and its upstream enhancers. Although *KIT* is already highly expressed in GI stromal tumors, loss of this insulator further upregulates *KIT*. Simultaneous FGF and KIT hyperactivation in these tumors increases crosstalk between the two signaling pathways, conferring resistance to KIT inhibitors [99].

Proto-oncogene *EPHA2* encodes another receptor tyrosine kinase with an altered enhancer landscape in GI cancers. In colorectal tumors, one of the three enhancers in *EPHA2*’s promoter–enhancer hub gains activity and interactions with the promoter [100]. In the same samples, tumor suppressor *PDCD4* is downregulated and less frequently interacts with one of its two distal enhancers located within a spatial hub [100]. Although changes in enhancer connectivity are observed across multiple colon tumor samples, the underlying mechanisms of these epigenetic changes remain unknown.

B Cell Malignancies

Cancer-associated mutations in non-coding regions are prevalent and growing evidence suggests that these mutations can impact chromatin organization. Inherited genetic variations are important determinants of susceptibility to acute lymphoblastic leukemia (ALL), but how these variants in non-coding sequences increase leukemia risk is less understood. Yang et al. showed that the rs3824662 variant at the *GATA3* locus associates with susceptibility to pediatric Philadelphia chromosome-like (Ph)-like ALL [101]. This variant creates a *de novo* enhancer that loops to *GATA3* and other putative *GATA3* enhancers, forming a hub consisting of a single promoter and multiple enhancers. Alteration of the *GATA3* promoter–enhancer hub directly increases *GATA3* expression in lymphoblasts and is associated with

higher *GATA3* expression in multiple ALL subtypes [101]. Further, GATA3 upregulation associates with widespread genome reorganization in Ph-like ALL patients with rs3824662 variant. For example, increased GATA3 binding is associated with concomitant *CRLF2* upregulation, formation of a new enhancer–promoter loop, and increased interactions with other enhancers within the *CRLF2* promoter–enhancer hub [101]. CRLF2 mis-regulation is a hallmark of Ph-like ALL, mediates constitutive activation of the JAK-STAT pathway, and promotes leukemogenesis [102]. This study exemplifies how a single germline variation can increase cancer risk by altering TF binding and inducing genome reorganization, setting a key oncogenic transcriptional program.

Somatic 13q12.2 microdeletions at the *FLT3* locus is one of the most common drivers of ALL [103]. In B-cell precursor ALL, the deletion disrupts insulation at the *FLT3* locus, enabling the gene promoter to hijack and interact with an additional enhancer distal from the deletion breakpoints, leading to allele-specific *FLT3* mis-regulation [103]. Although this study only showed enhancer–promoter and not enhancer–enhancer interactions, we hypothesized that changes in chromatin looping leads to gain of a promoter–enhancer hub comprised of *FLT3* and its two interacting enhancers.

H1 proteins are linker histones that limit chromatin accessibility and act as transcriptional repressors [104]. Recurrent H1 mutations occur in approximately 30-40% of diffuse large B-cell lymphomas (DLBCL) [105]. Loss of H1 in germinal center (GC) B cells, the DLBCL cell-of-origin, decompacts and upregulates stem cell genes, including *Klf5* [105]. *Klf5* gains interactions with two distal enhancers that become accessible in H1-mutant GC B cells, consistent with *Klf5* upregulation in H1-deficient lymphomas [105]. Enhanced self-renewal properties in lymphoma cells due to H1 loss may partly explain why H1-mutant DLBCL is highly aggressive. Further, the newly *Klf5*-interacting enhancers are enriched for OCT2 binding. Interestingly, another study on DLBCL found the enrichment of OCT2, OCA-B, and MEF2B ternary complexes as a key regulator of *BCL6*'s promoter–enhancer hub [106]. Sustained *BCL6* expression is essential and regulated by a locus control region (LCR) containing 10 active enhancers highly interacting with the promoter in GC B and DLBCL cells [106-108]. Loss of one LCR enhancer reduces interaction frequency between the *BCL6* promoter and non-targeted LCR enhancers, suggesting the LCR forms a cooperative promoter–enhancer hub with *BCL6*. A CRISPRi screen of the LCR revealed hierarchical organization of enhancers within the hub. Their individual essentiality in driving *BCL6* expression and promoting cell survival is regulated by enrichment of the OCT2-MEF2B-OCA-B ternary complex and not enhancer activity [106]. Further studies in DLBCL are required to determine whether OCT2 and its partners can access closed chromatin to activate enhancers or if additional events like H1 loss are necessary to enhance chromatin accessibility.

Mutations affecting TFs can lead to their signal-independent activation and aberrant chromatin binding. For instance, activating *NOTCH1* mutations can lead to ligand-independent release of NOTCH1 into the nucleus, recruiting transcriptional cofactors to chromatin [109]. In mantle cell lymphoma (MCL), aberrant Notch signaling upregulates crucial genes for cancer survival, including *MYC, LYN*, and *SH2B2* [24]. In addition to TNBC, Notch signaling is also implicated in multi-gene coregulation in MCL by

promoting hub formation at loci with multiple promoters, including the *SH2B2* / *ORAI2* and *LDLRAD4 / FAM210A* loci. Oncogenic NOTCH1 transcriptionally controls these genes by positioning their respective enhancers within promoter–enhancer hubs, even when the enhancer activity is NOTCH-independent. However, the factors determining why certain NOTCH-dependent promoter–enhancer hubs exhibit NOTCH-independent enhancer activity remain unclear.

Promoter–enhancer hubs can also be cancer-protective. For instance, EBF1 is a TF that is expressed and regulated by highly interconnected enhancers in naïve and mature B cells [110], which are thought to be the cells of origin for chronic lymphocytic leukemia (CLL) [111]. In CLL, most of the looping interactions and enhancer activity at the *EBF1* locus are lost [110]. Loss of the promoter–enhancer hub results in *EBF1* silencing, reducing levels of multiple B cell signaling factors and leading to anergy and low susceptibility to recognition by the host's immune system, characteristics that are often observed in CLL patients [110].

T Cell Acute Lymphoblastic Leukemia (T-ALL)

T-ALL is an aggressive hematological malignancy resulting from transformed T cell progenitors [112]. Several groups mapped differential genome folding in primary T-ALL blasts and peripheral T cells to characterize chromatin reorganization during malignant transformation [113,114]. Widespread differences were observed in leukemic cells, including over 6000 differential loops [114].

NOTCH1 is mutated in more than 50% of T-ALL cases and contributes to leukemogenesis [112]. Like TNBC and MCL, *MYC* is a key Notch target in T-ALL [24,113-116]. Kloetgen et al. showed that loss of a *MYC* insulator element increases *MYC* expression and interactions with a NOTCH1-bound and a NOTCH1-unbound super-enhancer [113]. Although this study did not show interactions among the two *MYC*-interacting super-enhancers, another study found that the *MYC* promoter and its two super-enhancers are highly interconnected [115]. Zhou et al. further used Oligpaint DNA FISH and confirmed the formation of the *MYC* promoter–enhancer hub in individual T-ALL cells. Notch inhibition reduces *MYC* expression and NOTCH1-bound super-enhancer activity [113,115,116]. However, unlike TNBC and MCL [24], loss of Notch signaling has no impact on *MYC* promoter–enhancer looping in T-ALL. In contrast to Notch inhibition, CDK7 inhibition significantly reduces *MYC* promoter–enhancer interactions, suggesting that additional factors including CDK7 maintain enhancer positioning in a subset of T-ALL enhancers [113]. Further understanding of the factors maintaining or establishing genome folding could lead to new targets for T-ALL therapy.

Proto-oncogene *SOX4* is another T-ALL associated TF. Yang et al. showed that *SOX4* is derepressed in T-ALL and gains new loops to three distal enhancers, forming a T-ALL-restricted promoter–enhancer hub [114]. Differential looping is associated with changes in CTCF binding and H3K27ac loading, but not copy number alterations. In addition, *HOXA* genes are also commonly derepressed and correlates with poor prognosis [117]. Fusion proteins including SET-NUP214 can transactivate *HOXA* genes, increasing *HOXA* enhancer activity and promoter-interactions and credentialing the hypothesis that a spatial hub containing multiple promoters and enhancers forms at this locus. In line with sarcoma and

NUT carcinomas, these observations in T-ALL provide another example where oncogenic fusion proteins can commission promoter–enhancer hubs.

Although Kloetgen et al. and Yang et al. reported widespread differential genome folding in T-ALL compared to healthy peripheral T cells [113,114], precaution must be taken when interpreting these results as many differences might be due to genome reorganization during normal T cell differentiation and not malignant transformation. Further studies using the corresponding cell-of-origin are needed to elucidate T-ALL-specific changes in genome organization.

Acute Myeloid Leukemia (AML)

To study genome organization changes in AML blasts compared to normal hematopoietic progenitors, Xu et al. integrated chromatin conformation, accessibility, enhancer activity, gene expression, and whole-genome sequencing data [118]. The authors identified several structural variations associated with enhancer hijacking events creating AML-specific enhancer–promoter loops, an observation also made in other cancer types [89,91,103]. For instance, a fusion between chromosomes 11 and 7 connects several enhancers to *CDK5* and inv(16) creates new loops linking *HSF4* to multiple enhancers [118]. Additionally, various translocation events in different patients can connect *MYC* and *CBL* to disjoint sets of enhancers [118]. Elevated *CDK5, MYC* and *CBL* are implicated in AML progression [119], but the clinical relevance of HSF4 hyperactivation is currently unclear. From this data, one could postulate that structural variants contribute to promoter–enhancer hub formation involving key oncogenes, a hypothesis that warrants further investigation.

Therapeutic opportunities

The role of promoter-enhancer hubs in regulating various oncogenes makes them an attractive target for treatment. One way to target hubs is by inhibiting their regulators. Because TF occupancy at one enhancer stabilizes the occupancy of TFs and cofactors at interacting enhancers [58], inhibiting TFs can specifically disrupt oncogenic promoter–enhancer hubs. For instance, Notch inhibition in multiple cancers is promising due to its strong anti-tumor effects and ability to disrupt Notch-dependent long-range interactions within promoter–enhancer hubs [24,115]. However, treatment resistance is still a challenge and could potentially be overcome with combination therapy [39,115,120,121]. Kloetgen et al. demonstrated that simultaneous inhibition of Notch and CDK7 disrupts Notch-insensitive enhancer–promoter loops [113]. Additionally, Notch-inhibitor-resistant T-ALL cells are more responsive to BRD4 inhibition [116], whose therapeutic value has been explored in other cancers [122].

Loss of insulation at oncogenic loci is prevalent [98,123-125]. Here, we provide specific examples from SDH-deficient GI stromal tumors, B-cell precursor ALL, and AML where insulator disruption could be interpreted as gain of enhancer–promoter hubs involving proto-oncogenes [93,103,118]. For instance, as we discussed, DNA hypermethylation coincide with altered enhancer–promoter interactions of key oncogenes. DNA methyltransferase inhibitors show promising anti-tumor effects and could disrupt AML-specific enhancer–promoter interactions [118], rationalizing for their use as potential therapeutic options.

Yet, to fully leverage the potential of agents targeting promoter–enhancer hubs as part of combination therapies, several challenges need to be overcome, including toxicity and off-target effects as well as treatment resistance.

Concluding remarks

Our knowledge of enhancer biology and its contribution to cancer has drastically improved over the last few decades. Technological advancements continue to broaden our understanding of enhancer positioning and its functional importance in transcriptional regulation in cancer. Genome-wide and/or locus-focused examination of cancer genome folding show that promoter–enhancer hubs contribute to pathogenesis by preferentially promoting oncogenic expression. Some studies focused on loci with one promoter and multiple enhancers (Figure 1, top), while others examined loci with multiple promoters and enhancers (Figure 1, bottom).

Yet, a full understanding of enhancer-mediated gene expression control that can explain varying configurations of enhancers within the nuclear space remains enigmatic. Several long-standing questions remain (see Outstanding Questions). For instance, most studies discussed in this review identified promoter-enhancer hubs based on chromatin conformation capture data generated from populations of cells (Figure 3). But how many individual cancer cells do exhibit multiway interactions among enhancers and promoters? What is the functional importance of their formation? Do multiway contacts promote stronger gene expression than pairwise contacts? Further, while an association between changes in gene expression and alterations in promoter–enhancer hubs were observed, only a few studies dissected the details of these potential regulatory interactions. Hence, it remains unclear which multiway interactions are structurally competitive, cooperative, or independent, and what factors determine this context dependency. Moreover, despite recent evidence suggesting that dynamic rewiring of promoter–enhancer hubs may play a role in cell-fate decision, further systematic studies are needed to fully credential the hypothesis that these topological assemblies are lineage-restricted and contribute to cancer transformation and progression. To address these questions and develop better models of promoter–enhancer hub regulation and function, there is a need for systematic perturbations of their constituent components and testing their effects on gene expression.

In addition, the precise identification of genome folding changes that happen during cancer transformation require comparison with the respective cells of origin, which is not known for many cancer types, or are not amenable to chromatin conformation capture assays [113,114]. Future studies using improved experimental setups and assays with lower input requirements can elucidate the underlying mechanisms driving chromatin architectural changes in cancer.

Further developments in chromatin-tracing methods and single-cell chromatin conformation capture assays allow the examination of chromatin folding heterogeneity at individual loci. Multi-modal extensions of these single-cell resolution methods, incorporating RNA and protein colocalization, will enable in-depth exploration of promoter–enhancer hub regulation and function. These techniques could also investigate links between promoter–enhancer

hubs and nuclear bodies, including phase-separated condensates and PML nuclear bodies, which have been linked to super-enhancers [126-128]. On the other hand, super-resolution live-cell imaging help to elucidate the dynamics of promoter–enhancer hubs. However, genome-wide characterization of these topological assemblies in individual cells are still lacking and requires further technological advancements [129]. Overall, addressing these questions is an exciting avenue of research in genome architecture and may pave the way for reprogramming of promoter–enhancer hubs and exploiting the therapeutic potential of their disruption and/or formation in cancer treatment.

Glossary:

Chromatin conformation capture
a class of experimental technique used to measure pairwise contact frequencies between two DNA sequences based on proximity ligation.

Chromosome territory
the specific region of the nucleus occupied by different chromosomes.

Compartments
domains found in Hi-C data formed by differential chromatin activity, and not CTCF binding.

DNA binding transcription factors (TFs)
proteins with DNA binding domains that regulate transcription by binding to specific DNA sequences in regulatory elements.

Insulator
a class of protein-binding DNA regulatory elements that protect genes from inappropriate signals from their surrounding environment.

Oligopaint
a FISH method that labels DNA using short fluorescently labeled oligonucleotides for high-resolution imaging of chromatin.

Promoter–enhancer hub
dynamic topological assemblies with one or more promoters that interact, even transiently, amongst themselves and with two or more enhancers that may or may not be connected.

Topologically associating domains (TADs)
spatially self-associating intra-chromosomal regions.

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Box 1.

Hypothesizing promoter–enhancer hubs' existence from Hi-C-based assays.

Proximity ligation-based chromatin conformation capture technologies such as Hi-C enables the genome-wide study of genome folding, painting a more comprehensive picture of the organizational principles and function of DNA looping in health and disease [2,130,131]. Despite limited sequencing depth and resolution, earlier Hi-C-based studies confirmed the presence of lineage-restricted long-range pairwise enhancer–promoter, enhancer–enhancer, and promoter–promoter interactions [2,132]. Furthermore, these studies led to the hypothesis that promoter–enhancer hubs exist within mammalian genomes, formed as a result of long-range contacts among gene promoters and a number of distal enhancer elements [2,132,133].

To improve on the ability of Hi-C in detecting enhancer–promoter interactions, antibody or oligo-based enrichment steps can be added to the protocol. Antibody enrichment of chromatin factors including RNA Pol II, cohesin complex components, and active enhancer marks using HiChIP [134], ChIA-PET [135], or PLAC-seq [136] drastically improved the discovery rate of enhancer–promoter contacts by selectively enriching for the factors that are associated with these genomic elements. On the other hand, Promoter [137] and Enhancer [58] Capture Hi-C leverage oligo-based enrichment to enable higher resolution detection of either distal promoter-interacting or enhancer-interacting regions, respectively.

Outstanding questions:

- Do promoter–enhancer hubs form in individual cancer cells, and if they do, what is the extent of their formation and function?
- What are the regulators of promoter–enhancer hubs?
- Why does a subset of genes require promoter–enhancer hubs for their regulation?
- What chromatin features distinguish the different types of multiway interactions within enhancer clusters?
- To what extent do multiway enhancer interactions predict gene expression?

Highlights:

- Genetic and epigenetic events can influence promoter–enhancer hubs in cancer.
- Deregulation of promoter–enhancer hubs contributes to cancer pathogenesis through misregulation of oncogenes and tumor suppressors.
- Promoter–enhancer hubs can also be cancer-protective when a tumor suppressor is activated.
- Recent technological advancements have paved the way for in-depth study of the cause-and-effect relationships between transcriptional regulators, promoter–enhancer hubs, and gene expression control in cancer.

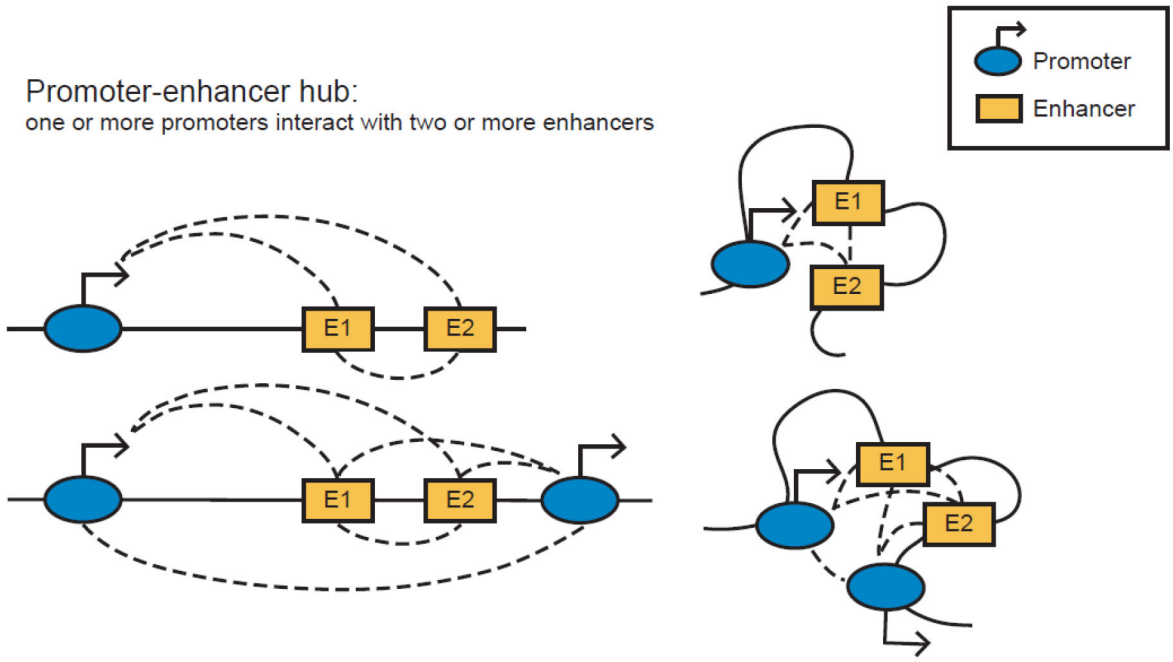

Figure 1: Graphical representation of promoter–enhancer hubs linearly and spatially.
Top: one promoter regulated by two enhancers. Bottom: two promoters that are regulated by two enhancers.

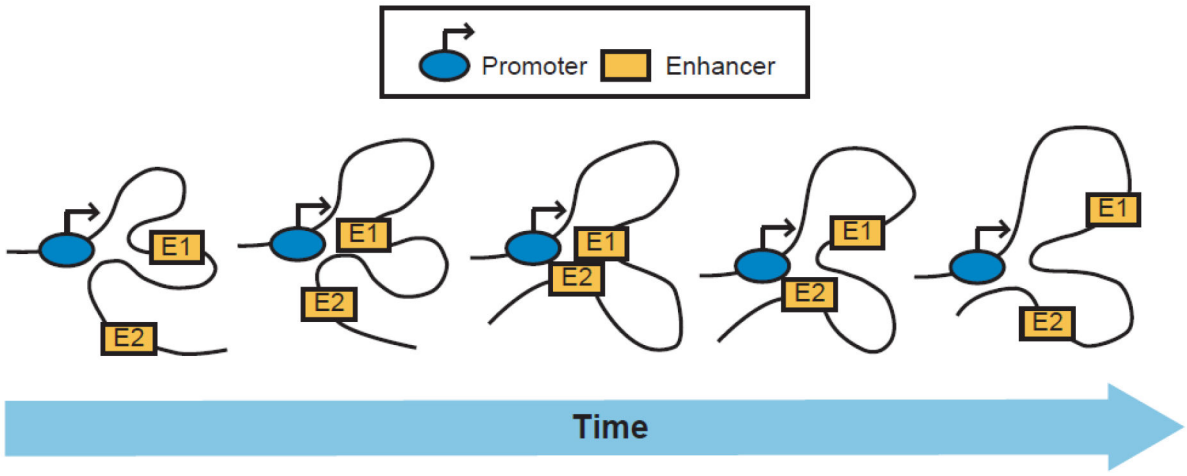

Figure 2: Evidence suggests that multiway interactions among enhancers and promoters are dynamic.

Hence, a promoter may be interacting with one, both, or none of its enhancers at a given time.

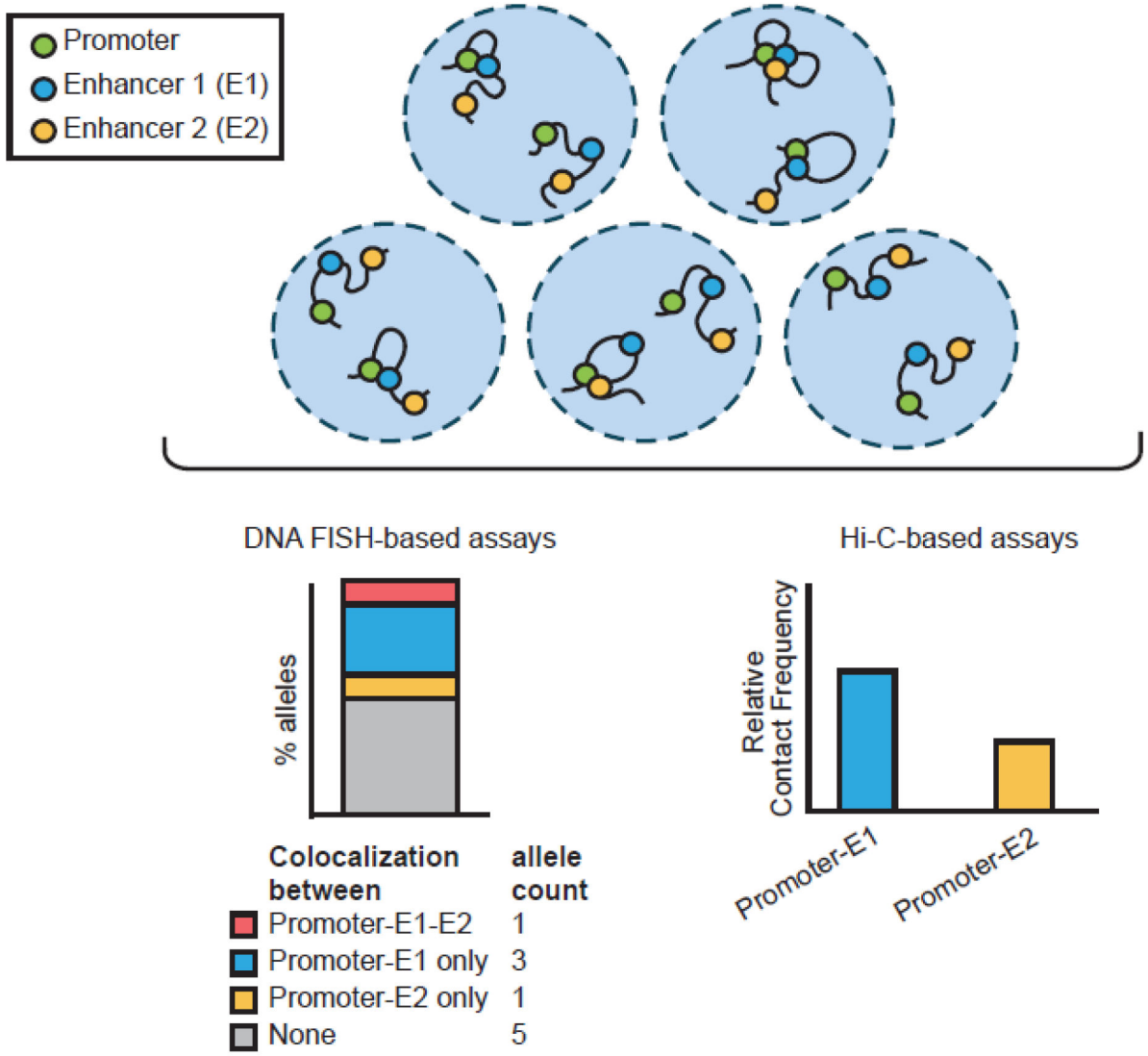

Figure 3: Various assays offer different perspectives of enhancer–promoter interactions.

Top: five nuclei depicting two alleles that contain a promoter (green), and two enhancers (blue and yellow). Bottom-left: DNA-FISH-based assays can be used to measure pairwise and multiway interactions at individual alleles of a given locus. Bottom-right: Hi-C-based assays cannot measure multiway interactions but can be used to find highly connected promoter–enhancer pairs genome-wide.

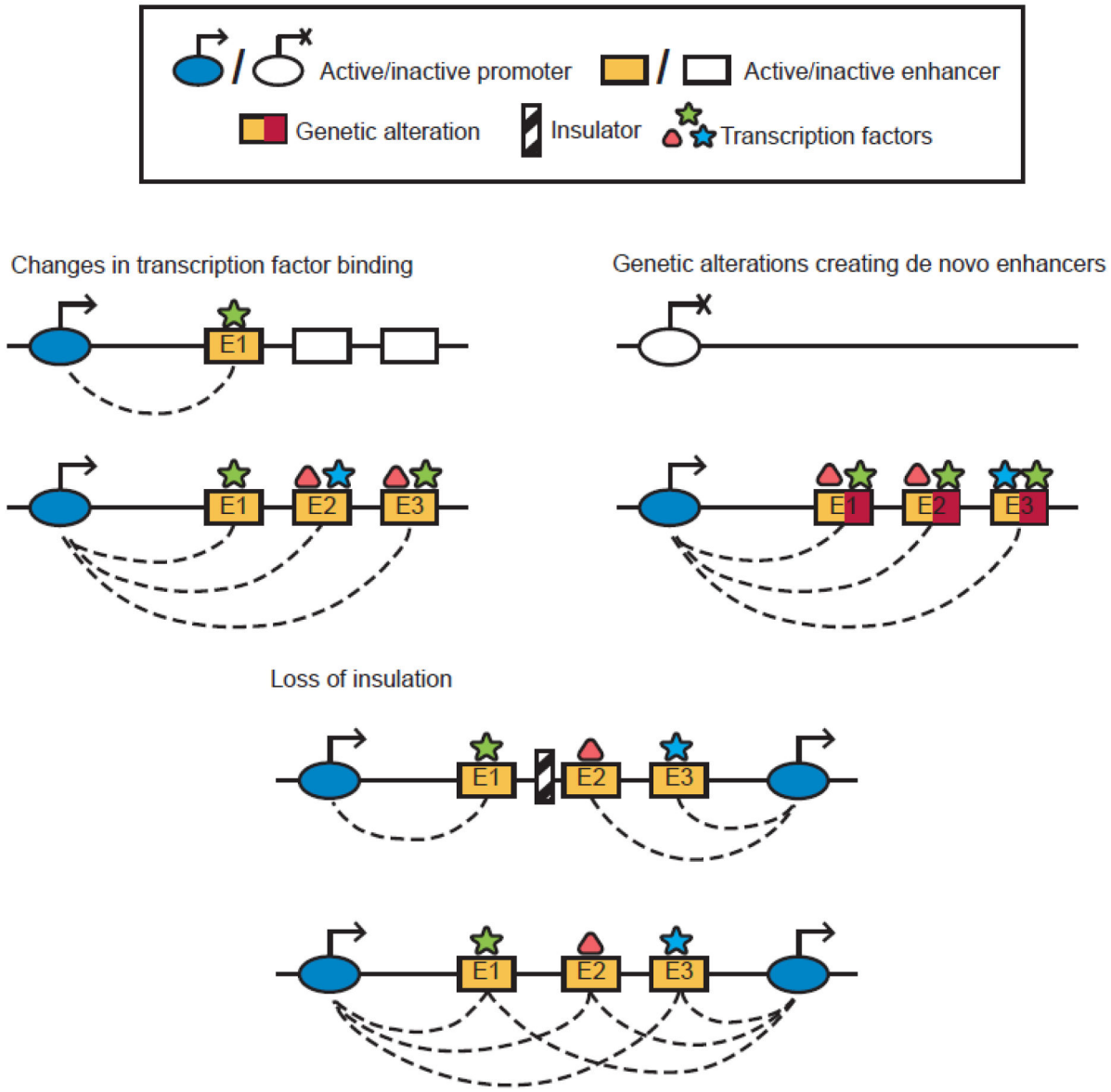

Figure 4: Example of events influencing oncogenic promoter–enhancer hubs.

Top-left: changes in binding of one TF can activate previously inactive enhancers and recruit other partner TFs. Top-right: Genetic alterations can create novel binding sites for TFs and alter enhancer activity, inducing gene expression. Bottom: loss of insulation can lead to *de novo* contacts to enhancers.

Table 1. Key table.

Summary of loci/genes discussed in this review that potentially form a promoter–enhancer hub in different cancer types.

Cancer type	Gene(s) affected	Potential mechanism	Gene function	Refs
Prostate Cancer	*FOXO1*	Unknown	tumor suppressor	[71]
	AR	Unknown; CTCF independent	oncogene	[72]
Squamous cell lung carcinoma	*FOXE1*	KLF5 binding	oncogene	[78]
	TP63	KLF5 binding	oncogene	[78]
NUT carcinoma	*MYC*	BRD4-NUT fusion	oncogene	[83]
	TP63	BRD4-NUT fusion	oncogene	[83]
	SOX2	BRD4-NUT fusion	oncogene	[83]
Triple negative breast cancer	*MYC*	Notch Transcription Complex binding	oncogene	[24]
	CCND1	Notch Transcription Complex binding	oncogene	[24]
	TMPRSS2	Notch Transcription Complex binding	oncogene	[24]
	RIPK4	Notch Transcription Complex binding	oncogene	[24]
Gastric cancer	*ZFP36L2*	Tandem duplication	oncogene	[91]
Gastrointestinal stromal tumor	*FGF3*	Disruption of insulation	oncogene	[93]
	FGF4	Disruption of insulation	oncogene	[93]
	KIT	Disruption of insulation	oncogene	[93]
Colorectal cancer	*CEACAM5*	KLF5 binding	oncogene	[78]
	ETV4	KLF5 binding	oncogene	[78]
	EPHA2	Unknown	oncogene	[100]
	PDCD4	Unknown	tumor suppressor	[100]
Ph-like acute lymphoblastic leukemia	*GATA3*	Single-nucleotide substitution rs3824662	oncogene	[101]
	CRLF2	GATA3 binding	oncogene	[101]
B-cell precursor acute lymphoblastic leukemia	*FLT3*	Somatic 13q12.2 microdeletions	oncogene	[103]
Diffuse large B-cell lymphomas	*Klf5*	H1 loss	oncogene	[105]
	BCL6	OCA-B-OCT2-MEF2B complex binding	oncogene	[106]
Mantle cell lymphoma	*MYC*	Notch Transcription Complex binding	oncogene	[24]
	LYN	Notch Transcription Complex binding	oncogene	[24]
	SH2B2	Notch Transcription Complex binding	oncogene	[24]
	ORAI2	Notch Transcription Complex binding	unknown	[24]
	LDLRAD4	Notch Transcription Complex binding	unknown	[24]
	FAM210A	Notch Transcription Complex binding	unknown	[24]
Chronic lymphocytic leukemia	*EBF1*	Unknown	tumor suppressor	[110]
T-cell acute lymphoblastic leukemia	*MYC*	Disruption of insulation, Notch binding	oncogene	[113]
	SOX4	Unknown	oncogene	[114]
	HOXA genes	Fusion proteins such as SET-NUP214	oncogene	[114]
Acute Myeloid Leukemia	*CDK5*	Chromosome 11 and 7 fusion	oncogene	[118]

Cancer type	Gene(s) affected	Potential mechanism	Gene function	Refs
	HSF4	inv(16)	unknown	[118]
	MYC	translocations	Oncogene	[118]
	CBL	translocations	Oncogene	[118]