1 *Review*

2 # Perturbing Enhancer Activity in Cancer Therapy

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8   **Abstract:** Tight regulation of gene transcription is essential for normal development, tissue
9   homeostasis and disease-free survival. Enhancers are distal regulatory elements in the genome that
10 provide specificity to gene expression programs and are frequently misregulated in cancer. Recent
11 studies examined various enhancer-driven malignant dependencies and identified different
12 approaches to specifically target these programs. In this review, we describe numerous features
13 that make enhancers good transcriptional targets in cancer therapy and discuss different
14 approaches to overcome enhancer perturbation. Interestingly, a number of approved therapeutic
15 agents such as cyclosporine, steroid hormones, and thiazolidinediones actually function by
16 affecting enhancer landscapes by directly targeting very specific transcription factor programs.
17 More recently, a broader approach to targeting deregulated enhancer programs has been achieved
18 via Bromodomain and Extraterminal (BET) inhibition or perturbation of transcription-related
19 cyclin-dependent kinases (CDK). One challenge to enhancer-targeted therapy is proper patient
20 stratification. We suggest that monitoring of enhancer RNA (eRNA) expression may serve as a
21 unique biomarker of enhancer activity that can help to predict and monitor responsiveness to
22 enhancer-targeted therapies. A more thorough investigation of cancer-specific enhancers and the
23 underlying mechanisms of deregulation will pave the road for an effective utilization of enhancer
24 modulators in a precision oncology approach to cancer treatment.

25 **Keywords:** Enhancers; BET inhibitors; CDK7 inhibitors; HDAC inhibitors; Transcription factors;
26 eRNAs; cancer
27
28

29 **1. Introduction**

30   Cancer is a disease of aberrant transcription which is addicted to mechanisms enabling
31 deregulated gene expression [1-3]. Enhancers are short genomic elements which are bound by
32 specific tissue- or cell type-specific transcription factors (TFs) that activate target gene transcription
33 in a distal and autonomous manner [4]. Soon after their discovery, enhancers were reported to drive
34 differential transcriptional regulation in a more diverse and versatile manner compared to
35 transcriptional regulation occurring (primarily) at proximal promoter regions [5]. Accordingly, it is
36 not surprising that misregulation of these transcriptional hubs was linked to various diseases
37 including cancer [6-8]. For example, a chromosomal rearrangement in acute myeloid leukemia
38 (AML) was found to bring an enhancer into the proximity to the oncogenic *MDS1 and EVI1 complex*
39 locus (*MECOM*), precipitating the malignancy [9]. Amplification of enhancers has also been found to
40 play a role in the pathophysiology of prostate cancer and neuroblastoma [10,11]. Furthermore,
41 hijacked enhancers led to the activation of the oncogenic *Growth Factor Independent 1* family in
42 medulloblastoma [12]. Additionally, reprogramming of the enhancer landscape in pancreatic cancer
43 was reported to play a significant role in promoting a more aggressive phenotype [13-15]. Moreover,
44 enhancers were implicated in therapy resistance in leukemia [16]. Accordingly, enhancer biology has
45 become a focal point of interest when investigating novel therapeutic targets in cancer. In this report,

46 we review recent studies supporting the rationale of targeting enhancers in cancer. Additionally, we
47 summarize the reported use of enhancer modulators in different cancer types. Finally, we discuss
48 the challenges facing the use of enhancer modulators in the clinical setting.
49
50 **2. Targeting transcription factor-related programs in cancer**

51 Sequence-specific binding of transcription factors (TF) underlies the selective activation of
52 enhancers in different systems [17]. TFs provide a high degree of specificity in gene regulation by
53 binding to their cognate DNA sequences across the genome to activate (or repress) transcription via
54 recruitment of various co-activators such as chromatin remodeling proteins and histone modifying
55 enzymes [18,19]. Certain TFs are reported to be lineage-specific and drive the differentiation of
56 certain cellular states through the activation of different enhancer repertoires [18,20]. Moreover, it
57 was reported that certain TFs, including the majority of tissue-specific TFs, display a larger number
58 of binding sites at enhancers compared to promoters [21]. Accordingly, agents specifically targeting
59 the function of such transcription factors will, in turn, perturb the activity of the select set of
60 enhancers controlled by the given TF. A primary example of such targeting is the perturbation or
61 activation of steroid hormone receptors in various cancers such as breast cancer [22-24], prostate
62 cancer [25-27], and lymphomas [28,29]. For example, 70% of breast cancers are estrogen
63 receptor-positive (ER+) and are, at least initially, highly responsive to endocrine therapy [30].
64 Estrogen receptor-alpha (ERα) is a master transcription factor in breast cancer which can be
65 activated by estradiol. Estrogen binding to the ligand binding domain of ERα leads to
66 conformational changes which promote dimerization, subsequent binding to specific targets in the
67 genome called estrogen response elements (EREs) and recruitment of co-activator proteins [31].
68 When ERα localization was investigated throughout the genome, it was quickly recognized that it
69 rarely binds to promoter regions, but rather shows a tendency to localize to enhancer regions [32].
70 Interestingly, ERα was recently reported to nucleate phase separated condensates at highly active
71 enhancers [33], thereby promoting transcription at these extremely active hubs [34]. Thus, while
72 endocrine therapy has been a central approach for treating a large number of breast cancer patients
73 for over four decades, it has only recently been appreciated that the main molecular mechanisms by
74 which tamoxifen and similar steroid hormone receptor antagonists exert their effects is by the
75 modulation of enhancer activity. This principle is also applicable to other steroid hormone receptors
76 where (positively or negatively) targeting the enhancer function of the androgen receptor (AR)
77 [35-37] or glucocorticoid receptor [38] has been shown to be very effective in other malignancies.

78 Other therapeutic agents that are used for other indications, such as the insulin sensitizing
79 thiazolidinediones, also modulate the enhancer landscape by acting as agonists for the nuclear
80 receptor Peroxisome Proliferator-Activated Receptor-γ (PPARG) [39]. In this case, treatment with
81 Rosiglitazone leads to the selective activation of PPARG-occupied enhancers. While
82 thiazolidinediones were reported to have an inhibitory proliferative effect in hepatocellular and
83 esophageal cancers, enhancer modulation by glitazones in cancer is still not very well studied
84 [40-42]. Interestingly, a retrospective study observed a significant negative correlation of
85 administration of thiazolidinediones and colorectal cancer, suggesting these agonists may have a
86 protective role in preventing cancer [43].

87 While targeting nuclear receptors is one of the best examples of how directly perturbing
88 transcription factor activity can be achieved, other frequently utilized therapeutic agents have a
89 similar mechanism of action. For example, calcineurin inhibitors, which attenuate the
90 calcium-dependent translocation of the Nuclear Factor of Activated T cells (NFAT), were shown to
91 have inhibitory growth effects in various types of cancer such as hepatocellular carcinoma,
92 melanoma, and retinoblastoma [44-46]. NFAT was shown to elicit its effects at enhancer regions in
93 blood vessel maturation [47] and function together with STAT3 at enhancers downstream of KRAS
94 signaling in pancreatic cancer [48]. Thus, the use of calcineurin inhibitors will directly impact the
95 activity of NFAT-driven enhancer programs and can be a promising approach in cancer therapy,
96 especially in cases such as breast and pancreatic cancer where the NFAT pathway has been shown to

be activated [39,49]. Another approach to modulate the effects of a transcription factor can be by targeting its stability. For example, the Hypoxia Inducible Factor Alpha subunit (HIF1A) is a transcription factor that is known for its role in mediating the hypoxic response and it was shown to correlate with poorer prognosis in various types of cancer [50,51]. Enhancers are highly implicated with HIF1A, affecting the expression of its target genes [15,52-54]. Interestingly, topoisomerase I inhibitors were observed to inhibit the translation of HIF1A and may therefore function in part by this mechanism in the case of cancers where this factor plays a significant role [55].

As these drugs target particular transcriptional programs driven by the activity of a highly specific group of enhancers, their effectiveness has shed light on the benefits of targeting deregulated enhancer programs in specific disease contexts. Accordingly, targeting deregulated enhancer activity is, in fact, already an established paradigm and mainstay in the clinical treatment of cancer.

3. Perturbing enhancer activity by therapeutic agents and inhibitors

Expansion of our understanding of complex processes controlling gene regulation uncovered numerous novel targets that can (potentially) be therapeutically modulated in cancer. However, such exponential growth in knowledge rendered the task to identify and invest in a select few effective and relatively safe transcriptional targets immensely challenging. A hypothetical "ideal" transcriptional target in cancer therapy would necessarily exhibit certain attributes which can lead to a perceptible change in the quality of life, prognosis and the therapeutic management of patients. To do so, a target should be firstly easily modulated, preferably by small molecule inhibitors that have good bioavailability at the target site with an acceptable half-life (i.e., good pharmacokinetic and pharmacodynamics properties). Also important is that such a target must have a specificity that spares non-transformed cells, thereby avoiding or minimizing any potential unwanted side effects caused by perturbations of normal cellular processes in healthy tissue effects (adverse or severe adverse events). Additionally, this target should be indispensable to cancer cells, rendering them highly dependent on such a target. Ideally, this dependence should be shared by all or a high percentage of the malignant cell population. As enhancers exhibit all of these characteristics, they are generally considered good transcriptional targets (Figure 1).

3.1 Enhancers are context-specific and indispensable to cancer cells

One of the major characteristics of a subgroup of enhancers is context-specificity. In general, enhancers direct lineage-specific transcriptional programs in a more predominant manner compared to promoters [56]. Around half of the enhancers identified in different tissues including brain, heart, ovaries, and placenta were tissue-specific [57]. Consistently, enhancers were the most distinctive identifying feature of tissue of origin upon analysis of hundreds of patient samples and human cell lines [58]. Interestingly, not only is there a distinct pattern of enhancer activation through tissues, but there is also a distinctive pattern of association [59]. Notably, interactions between enhancers and their target genes are variable in different systems and show more tissue-specificity than differential activation of enhancers themselves, thus providing an additional layer of complexity.

In addition to having tissue-specificity, in order to be safely targetable, enhancers must exhibit specific activation in malignant cells compared to the tissue of origin. In this case, this activation should represent a new cancer cell-specific dependence that is not shared by healthy cells from the same tissue. Consistently, aberrant hypermethylation of enhancers in renal cancer cells led them to be more sensitive to the DNA methyltransferse (DNMT) inhibitor, Decitabine, compared to healthy renal tissue [60]. More specifically, loss of *Lysine Demethylase 6A* (KDM6A) resulted in aberrant activation of a set of enhancers leading to an aggressive phenotype of pancreatic cancer [61]. Importantly, inactivation of enhancers through BET inhibitor treatment was effective in targeting this specific subtype of pancreatic cancer compared to other subtypes. This shows that enhancer specificity can extend to certain subtypes of cancer, adding a further layer of specificity and potentially increasing safety in targeting those elements.
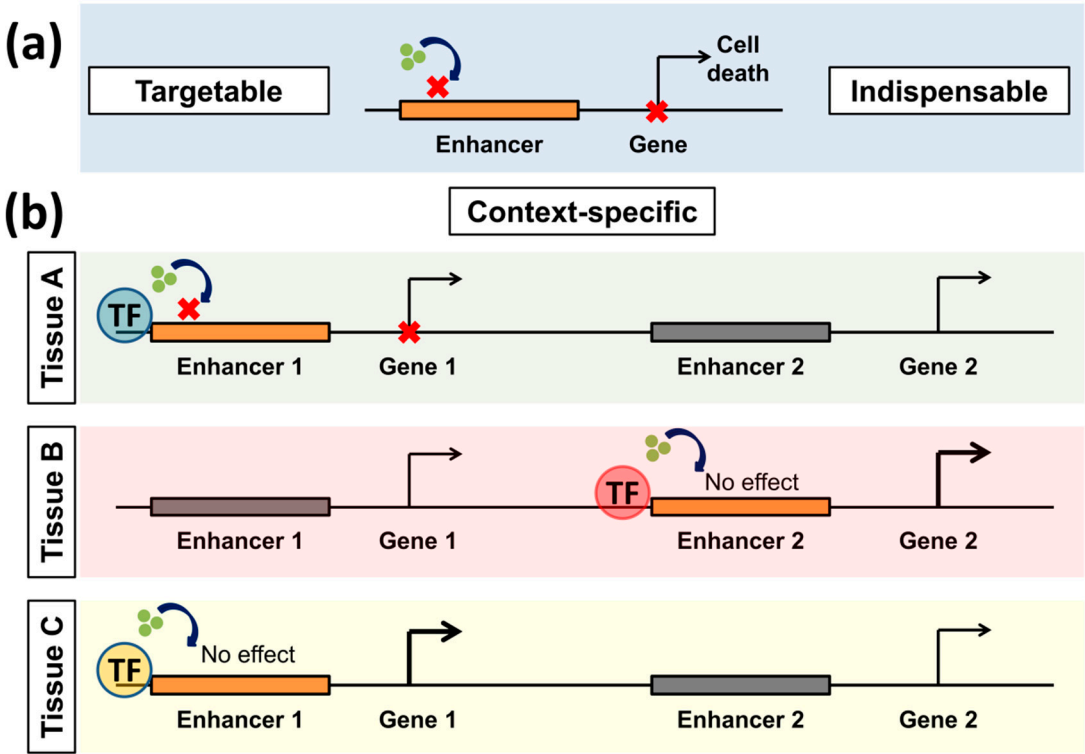
Figure 1. Positive features rendering enhancers good transcriptional targets. (a) Enhancers can be pharmacologically manipulated using different small molecule inhibitors (indicated by green dots). They are also indispensable for cancer cells as they activate important oncogenes. (b) Enhancers are also context-specific. In this example, enhancers are activated by different transcription factors in various tissues (A,B, and C). The same inhibitor affects only a specific enhancer in a tissue if it is activated by a certain TF. Thus the illustrated inhibitor only affects Enhancer 1 in tissue A but not C. It also has no effect on the other active enhancers in tissue B. Gray enhancers are inactive while orange ones are active. Bold arrows represent active transcription.

146   *3.2 Activity of enhancers can be pharmacologically perturbed*

147   Enhancers were shown to be specifically targetable by various small molecule inhibitors.
148   Preferential dependence of enhancers on Bromodomain and Extraterminal (BET) proteins has been
149   consistently reported in various cancer types such as lymphoma [62], ovarian cancer [63], breast
150   cancer [64,65], pancreatic cancer [61,66], leukemia [67], multiple myeloma and glioblastoma [67,68].
151   Other modulators with reported efficacy on enhancers include inhibitors of the transcriptional
152   Cyclin Dependent Kinases-7 (CDK7) and -9 (CDK9).

153   3.2.1 Epigenetic modulators

154   Epigenetic regulation enables cells to control gene transcription in a manner complementary to
155   sequence-dependent transcription factor-based mechanisms. Such regulatory mechanisms include
156   post-translational modification of histones, DNA methylation, nucleosome remodeling, and
157   non-coding RNAs (ncRNAs) [69]. Histone marks do not act independently from each other but
158   rather co-exist and cooperate to control gene transcription in what is referred to as "histone
159   crosstalk" [70]. Eminent factors in the epigenetic machinery are so-called epigenetic "readers",
160   which recognize specific histone marks and recruit additional effectors [71]. An extensively studied
161   example is the BET family of proteins which each contain two bromodomains that can interact with
162   acetylated lysine residues on target proteins via a hydrophobic pocket, thereby endowing BET
163   proteins with the ability to recognize acetyl marks on chromatin [72]. JQ1 is a thienodiazepine that
164   displaces the BET family member Bromodomain containing 4 (BRD4) from acetylated lysines by
165   forming hydrogen bonds with a conserved asparagine residue that is situated in the hydrophobic

166 pocket of BRD4 [73]. Many other BET inhibitors have also been developed such as I-BET151,
167 I-BET762, and OTX-015 [73-75]. In Diffuse Large B-Cell Lymphoma, BET inhibitors showed a
168 marked effect on a subset of enhancers, termed super enhancers, that are highly enriched with BRD4
169 [62]. Super enhancers (SEs) were first identified as major drivers of gene expression that are highly
170 enriched with transcription factor binding sites and include clusters of highly active distal
171 regulatory elements [68,76]. SEs were observed to drive lineage-specific programs in various
172 systems such as epithelial differentiation, mesenchymal multipotency, and estrogen-dependent
173 mammary gland malignancy and showed sensitivity to BET inhibition [68,77-79]. Consistently,
174 treating ovarian cancer cells with BET inhibitors diminished the activity of a super enhancer
175 activating the chemoresistance-related aldehyde dehydrogenase and led to increased sensitivity to
176 cisplatin treatment [63]. Additionally, treating various sensitive colorectal cancer cells with BET
177 inhibitors attenuated the activity of enhancers gained in cancer compared to normal crypts [80].
178 While different active super enhancer programs were identified in various subtypes of
179 ependymomas, a general sensitivity to BET inhibition was reported in ependymoma cells [81]. The
180 same pattern of activation of distinct BET-dependent super enhancers was also reported in chronic
181 lymphocytic leukemia [82]. Enhancers driving the transcription of receptor tyrosine kinases that
182 play a fundamental role in gastrointestinal stromal tumor have also shown dependence on BET
183 family members [83].

184 Other important members of the epigenetic machinery include writers which act by selectively
185 adding chemical moieties to a specific histone residue. Histone acetyltransferases (HATs), such as
186 p300 and CREB-binding protein (CBP), transfer an acetyl group from acetyl-CoA to histone tails [84].
187 Inhibiting HATs in pancreatic cancer affected the activation of a certain subset of enhancers that are
188 enriched by the Wnt-signaling transcription factor, Transcription Factor 7 Like 2 (TCF7L2) [79].
189 Furthermore, the *Polycomb* Repressive Complex-1 (PRC1) and -2 (PRC2) are extensively studied
190 complexes which mediate monoubiquitination of H2A at lysine 119 (H2Aub1) and tri-methylation of
191 histone 3 lysine 27 (H3K27me3), respectively [85]. As H2K27me3 is a histone mark which is highly
192 associated with gene inactivation [86,87], targeting constituents of the PRC complex led to specific
193 de-depression of enhancers in leukemia [88]. Consistently, targeting a subunit of the PRC complex,
194 *Enhancer of Zeste* Homolog 2 (EZH2), led to de-depression of enhancers controlling the pro-apoptotic
195 B cell lymphoma-2 like 11 (*BIM*), thereby mediating apoptosis in breast cancer cells [89].

196 Other classes of important epigenetic factors include "erasers", enzymes that remove histone marks
197 [90]. This includes histone deacetylases (HDACs), which mediate the removal of lysine acetylation
198 and consist of multiple classes that can also mediate de-acetylation of non-histone proteins [91].
199 HDAC inhibitors were found to affect the enhancer landscape in colorectal, pancreatic and breast
200 cancer [89,92,93]. While methylation was previously considered to be an irreversible modification,
201 Lysine-Specific histone Demethylase 1A (LSD1, also called KDM1A) was identified in 2002 as a
202 selective mediator of the de-methylation of histone 3 lysine 4 [94,95]. Mono-methylation and
203 tri-methylation of histone 3 lysine 4 (H3K4me1 and H3K4me3) are known marks for gene activation
204 [69,96]. LSD1 inhibitors affect a specific subset of enhancers controlling differentiation in acute
205 myeloid leukemia by disrupting their interaction with the SNAG-domain transcription repressor
206 (GFI1) [97]. LSD1 has also been shown to influence enhancer activity in a number of other systems
207 including embryonic stem cell differentiation [98], androgen receptor function in prostate cancer
208 [99,100], and ERα activity in breast cancer [101]. Altogether, epigenetic modulators provide us with a
209 plethora of targets which can be manipulated to modulate the cancer-specific enhancer landscape
210 and affect transcriptional programs. While current research is largely focused on BET inhibitors and
211 their role in affecting enhancers, many other epigenetic inhibitors may potentially also be used in the
212 context of enhancer activity manipulation as further mechanisms and contexts are better defined.

3.2.2 Cyclin-dependent kinase inhibitors

The recruitment of RNA Polymerase II (RNA Pol II) to the proximal promoter enables the initiation of transcription, which is signified by the phosphorylation of serine 5 within the heptapeptide repeats of the carboxy-terminal domain (CTD) and the subsequent capping of nascent RNA [102,103]. Pol II is frequently temporarily paused by the negative elongation factor (NELF) and DRB-sensitivity inducing factor (DSIF) within the first 100 nucleotides after the transcription start site (TSS) [102,104,105]. Thereby, promoter proximal pausing has recently been regarded as a crucial rate-limiting step for gene transcription in metazoans [106]. To proceed to productive elongation, the Positive Transcription Elongation Factor-b (P-TEFb) phosphorylates Pol II at serine 2 of the CTD as well as components of both the NELF and DSIF complexes [107,108] via its catalytic subunit Cyclin-Dependent Kinase-9 (CDK9) and the cognate cyclin T1 [109]. These phosphorylation events release Pol II from promoter proximal pausing and allow transcription elongation until polyadenylation sequences are transcribed, which leads to the cleavage and subsequent polyadenylation of mRNAs [110]. Notably, inhibition of CDK9 not only attenuates transcription elongation of the pre-mRNA, but also decreases eRNA production at enhancer regions [111]. Consistent with complementary functions in controlling enhancer function, the combination of CDK9 inhibition along with BET inhibitors has shown enhanced effects in both AML [112] and malignant rhabdoid tumor cells [113]. As a monotherapy, CDK9 inhibitors were observed to highly inhibit the expression of genes associated with super enhancers, such as *MYC* [114]. In chordoma, a highly aggressive tumor of the bone, inhibition of CDK9 and CDK7 has been reported to be highly effective [115].

As part of the TFIIH complex, CDK7 plays an important role in gene transcription via phosphorylation of the Pol II CTD at Ser5 [116,117]. It was reported that phosphorylation of the CTD by CDK7 leads to the dissociation of the CTD with DNA and the initiation of transcription [118]. CDK7 also plays a dual role in controlling cell cycle progression by phosphorylating and activating CDK1 and CDK2 [119]. Specific inhibition of CDK7 by the covalent inhibitor, THZ1, was found to be highly toxic to cancer cells, presumably by specific inactivation of super enhancers [120]. Indeed, super enhancers controlling the *MYCN* proto-oncogene were selectively inactivated by THZ1 in neuroblastoma [121]. Interestingly, several reports followed observing a selective perturbation of super enhancer programs by inhibition of CDK7 in small cell lung cancer [122], triple-negative breast cancer [123], ovarian cancer [124], esophageal carcinoma [125], melanoma [126], gliobastoma multiforme [127], and pancreatic cancer [128]. However, inhibition of CDK7 was reported to increase characteristics associated with metastasis in colorectal cancer cells [129]. THZ1 was found to attenuate the normal transition of the various stages of transcription starting from initiation into elongation [130]. Conversely, while THZ1 appears to preferentially affect super enhancer-associated genes, this effect does not appear to be due to altered RNA Pol II activity directly at the enhancers themselves [131]. Moreover, a more recent report suggests that the effects of THZ1 on super enhancer-associated genes may, in fact, be due to the off target inhibition of CDK12 and CDK13, rather than CDK7 [132]. Further studies to understand the exact mechanism of selective attenuation of super enhancer activation are necessary before the use of CDK7 (or CDK12/13) inhibitors can be precisely tested in the clinical setting on a mechanistic basis. Currently, there are two early phase clinical studies ongoing investigating the use of CDK7 inhibitors in patients with advanced solid malignancies (NCT03363893, NCT03134638).

In addition to the previously mentioned regulators, the Mediator complex plays a crucial role in transcriptional regulation [133,134]. Mediator is a large multi-subunit complex that plays a crucial role in the assembly and activation of the pre-initiation complex (PIC) by forming a bridge between various sequence-specific transcription factors and components of the PIC [135]. In addition to its important role at gene promoters, Mediator is reported to connect initiating promoters with active distal enhancers through chromatin loop formation [136]. The first evidence of chromatin loop formation where a distal region affected the transcription of a target gene promoter was first

263 reported in 1984 by Dunn et al. [137] in bacteria. Approximately 20 years later, cohesin, which also
264 plays a central role in sister-chromatid adhesion, was revealed to orchestrate the formation of DNA
265 loops with the help of the insulator, CCTC-Binding Factor (CTCF), and the cohesin loader,
266 Nipped-B-Like (NIPBL) [138-140]. Mediator was found to bind cohesin and NIPBL to bring active
267 enhancers and promoters into close proximity [136]. As Mediator is composed of approximately 30
268 subunits, it can have different conformations [141]. One conformation includes the kinase module
269 containing Cyclin-Dependent Kinase 8 (CDK8), which does not appear to directly phosphorylate the
270 RNA Pol II CTD, but also shown to have more preference toward affecting active enhancers [142]. In
271 contrast to BET and CDK7 inhibition, CDK8 inhibitors have been reported to have an activating
272 effect on super enhancers in leukemic cells [143]. Given the proposed dose-dependence of these
273 enhancers, interestingly, leukemic cells were still impaired in their growth following treatment with
274 a CDK8 inhibitor. Additionally, CDK8 inhibitors were reported to be crucial in mediating the
275 transcriptional effects of the aforementioned transcription factor HIF1A [144] as well as beta-catenin
276 in colorectal cancer [145]. Accordingly, CDK8 inhibition can lead to remodeling of the enhancer
277 landscape by various mechanisms. General targets in modulating enhancer activity are illustrated
278 and summarized in Figure 2.
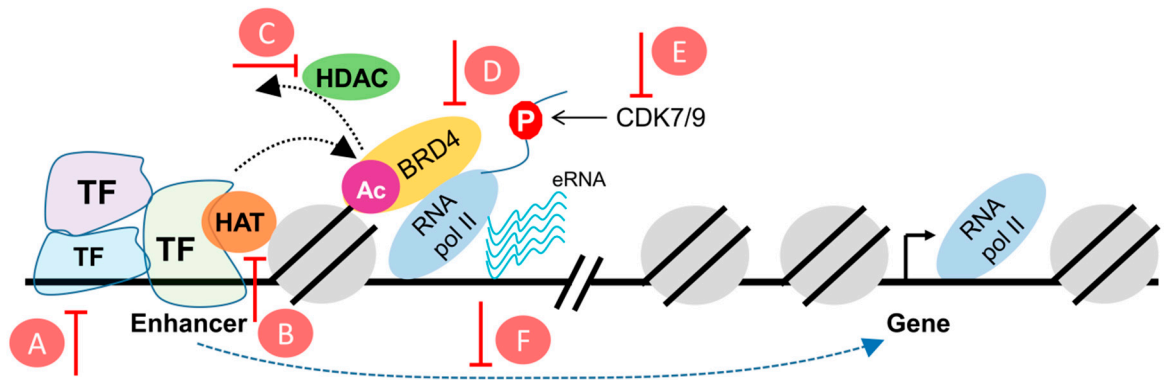
Figure 2. Schematic representation of putative targets to reprogram the enhancer landscape in cancer. (a) modulators of transcription factors, (b) HAT inhibitors, (c) HDAC inhibitors, (d) BET inhibitors, (e) CDK7/9 inhibitors, (f) CDK inhibitors.

279 **4. Challenges facing the utility of enhancer modulators in the clinical setting**

280 While targeting transcriptional enhancers is still under investigation, compensatory resistance
281 mechanisms upon inhibition of active enhancers have already been described. For example, the BET
282 inhibitor, JQ1, was reported to induce resistance mediated by transcriptional activation in
283 bromodomain-independent pathways in castration-resistant prostate cancer [146]. Interestingly, this
284 resistance uncovered an alternative dependency on CDK9-mediated activation of androgen receptor
285 signaling. In pancreatic cancer, upregulation of the *GLI Family Zinc Finger 2* (*GLI2*) was found to
286 enable resistance to BET inhibition [147]. In leukemia, resistance to BET inhibition was partly caused
287 by an in increase in the Wnt signaling pathway [148,149]. Interestingly, while MAPK/ERK kinase
288 inhibition (MEKi) sensitized colorectal cancer cells to BET inhibition, BET inhibitors sensitized
289 MEKi-resistant cells in breast cancer [64,150]. Similarly, inhibitors of the Nuclear Factor
290 Kappa-light-chain-enhancer of activated B cells (NFKB) pathway led to sensitization to BET
291 inhibitors in uveal melanoma [151]. Consequently, it is not unlikely that using enhancer modulators
292 will reveal various challenges for their use in the clinical setting. These will include the development
293 of resistance in addition to the difficulty in predicting responsiveness.

294 Although enhancer sequences do not contain genes, they were found to be transcribed, in
295 contradiction to the general trends of energy conservation inside the cell [152]. The functions and
296 mechanisms of the resulting products, named enhancer ribonucleic acids (eRNAs), are still not fully

297 elucidated [153]. Functionally, eRNAs were reported to augment gene transcription as their
298 knockdown led to decreased target gene transcription [154,155]. Furthermore, chromatin loop
299 formation and eRNA production were reported to precede transcription of the mRNA [156] and
300 more recently eRNAs were reported to promote the formation of phase-separated nuclear
301 interchromatin granules associated with actively transcribed genes [33]. Irrespective of their
302 function, we suggest that these products may provide particularly useful clinical markers for
303 predicting and monitoring therapeutic responsiveness and resistance (Figure 3).
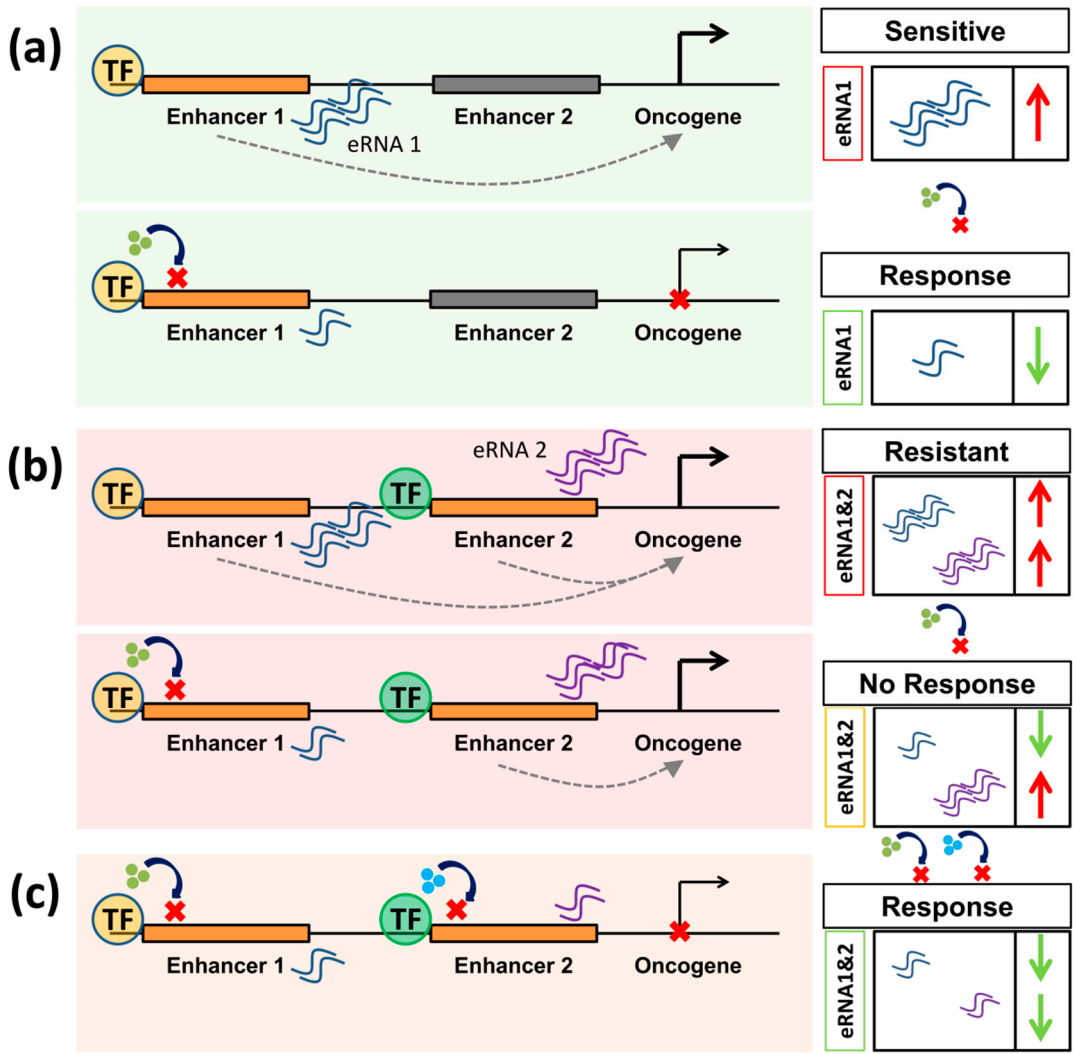
Figure 3. eRNAs are putative biomarkers for responsiveness and resistance in perturbation of enhancer activity. (a) In a responsive context, inhibiting an enhancer leads to a decrease in the activity of oncogenic target genes. In this case, high levels of eRNA can predict responsiveness to a specific inhibitor by providing a direct readout of enhancer activity. (b) In case of resistance, compensating mechanisms such as the activation of a different enhancer program can occur. Thereby, high levels of different eRNAs can predict resistance to a certain therapy. (c) To re-sensitize cells, compensatory mechanisms should also be targeted to ensure therapeutic success.

304 This is of particular interest as eRNAs were reported to be highly enriched at tissue-specific
305 enhancers [157]. Indeed, the eRNA *CCAT1* was proposed as a therapeutic biomarker that can predict
306 responsiveness to BET inhibition [158]. Interestingly, the largely known marker of prostatic cancer,
307 Prostate-Specific Antigen (PSA), was identified to be associated with an enhancer, with the resulting
308 eRNA having a central role in gene transcriptional regulation in prostate cancer cells [159].
309 Additionally, Kaczkowski et al. [160] identified 90 eRNAs that are generally upregulated in cancer

cells upon screening over 200 cell lines and approximately 300 primary samples. Identification of eRNAs has been made feasible due to the development of techniques such as global run-on sequencing (GRO-seq) [161], transient transcriptome sequencing (TT-seq) [162], precision nuclear run-on sequencing (PRO-seq) [163], and more recently chromatin run-on and sequencing (ChRO-seq) [164]. Notably, length-extension ChRO-seq enables the detection of nascent RNA from tissue samples that were stored for longer periods up to 30 years. Thus, current technologies allow us to more easily identify eRNAs from patient samples irrespective of sample quality, further enabling a potential utilization of eRNAs as enhancer biomarkers.

Notably, other technical chellenges face the elucidation of the mechanisms of enhancer functions and their targeting. Enhancers are usually identified using highly complex bioinformatic analyses that are not always accessible to clinicians and scientists alike. Identification of important enhancers has also been accompanied by the emergence of different subclasses of enhancers. Since the recent identification of super enhancers in 2013 [6,68], approximately 300 scientific papers discussing this subclass have been published. Another class of enhancers called "stretch enhancers" are sometimes used interchangeably with super enhancers [165,166]. This comparison is, however, somewhat inaccurate as studies indicate that stretch enhancers meet only the requirement of spanning long stretches of DNA but, unlike super enhancers, are not necessarily rich with transcription factors or cell-specific [167]. An additonal subclass includes "shadow enhancers", which are a group of "secondary" enhancers that are superfluous and redundant to an active enhancer, thereby ensuring the precision of gene transcriptional regulation [168]. Such a concept, which was first identified in *Drosphila*, has also been reported in mammals [169]. This led to the sometimes imprecise use of the term "shadow enhancers" to describe typical enhancers, which are not necessarily supportive of other enhancers and may play decisive roles in tissue- and cancer-specific gene regulation in their own right. A clearer definition of these new classifications will significantly help in a better and more precise understanding of enhancer activity and its modulation.

Another crucial hurdle facing the investigation of the role of enhancers in transcriptional activation is the complexity of defining the target genes of each enhancer. In the cell, targets of enhancers are not necessarily in close linear (genomic) proximity and can be separated by many unaffected genes [170]. As previously reported, interactions between enhancers and their target genes are variable in different systems and can show more tissue specificity than differential activation of enhancers themselves [59]. Chromatin conformation capture assays to detect interactions between cis-regulatory elements were first established in 2002 and have been followed by many techniques that extended our knowledge about the interactions between enhancers and their target promoters [171-174]. As these techniques are diffciult to perform in patient samples and are generally not very cost-effective, identification of target genes in a concise manner in patient samples remains challenging.

5. Conclusion

Aberrant gene transcriptional regulation is one of the characteristics of malignancy which can be most efficiently and specifically manipulated through enhancer elements. A greater breadth of knowledge about activated enhancers or super enhancers, interconnected with dependencies and biomarkers, may play a significant role in the optimization of therapy for patients suffering from cancer and other diseases. In conclusion, enhancers exhibit many attributes of an ideal transcriptional target and are highly promising to be leveraged in cancer therapy and management. This is due to the fact that they are targetable, specific, and indispensable. They also frequently produce products that may potentially be utilized as biomarkers (eRNA) and the targeting of compensatory mechanisms in response to their modulation should be considered as well. Given the clear significance of targeting enhancers in cancer, more studies are needed to further expand the currently available agents modulating the activity of these extremely important transcription targets.

359   **Author Contributions:** FHH and SAJ conceived the ideas for this work and wrote this manuscript.

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