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1 **Current challenges in understanding the role of enhancers in disease**

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37

38 **Preface**

39 Enhancers play a central role in the spatiotemporal control of gene expression and tend to
40 work in a cell type-specific manner. In addition, they are suggested to be major contributors
41 to phenotypic variation, evolution and disease. There is growing evidence that enhancer
42 dysfunction due to genetic, structural, or epigenetic mechanisms contributes to a broad range
43 of human diseases referred to as enhanceropathies. Such mechanisms often underlie the
44 susceptibility to common diseases, but can also play a direct causal role in cancer or
45 Mendelian diseases. Despite the recent gain of insights into enhancer biology and function,
46 we still have a limited ability to predict how enhancer dysfunction impacts gene expression.
47 Here, we discuss major challenges that need to be overcome when studying the role of
48 enhancers in disease etiology and highlight opportunities and directions for future studies,
49 aiming to disentangle the molecular basis of enhanceropathies.

50

51 **Main text**

52 Regulation of gene expression is accomplished through the integration of events at regulatory
53 elements that are proximal (promoters) and distal (enhancers) to gene transcription start
54 sites (TSSs). Forty years after their discovery¹, enhancers are recognized as playing a central
55 role in the spatiotemporal control of gene expression underlying human development and
56 homeostasis². Enhancers are short stretches of DNA that act as positive regulators of
57 transcription via their ability to bind key proteins – transcription factors (TFs) – and complexes
58 that control gene expression. Enhancer regulation of genes involves the three-dimensional
59 topology of chromatin, affecting the frequency by which enhancers and gene promoters come
60 into close proximity. Through this topology, several configurations can arise beyond single
61 enhancer-gene pairs, including one-to-many and many-to-many enhancer-gene wirings,
62 which may affect the robustness, strength or specificity of gene expression (**Fig. 1A**).

63 Enhancer dysfunction has emerged as a central mechanism in the pathogenesis of certain
64 diseases^{3,4} (**Table 1**). In particular, the dysfunction of enhancers by either point mutations or
65 structural variants is a significant mechanism underlying aberrant gene regulation in cancer⁵ and
66 Mendelian diseases⁶. Moreover, genetic variants associated with common diseases are
67 frequently found in *cis*-regulatory elements including enhancers^{7–12}. Depending on the nature
68 of the genetic alteration, enhancer dysfunction can be classified into two main types⁴. The
69 first type involves small single nucleotide variants (SNVs) and indels in the enhancer sequence
70 that lead to changes in enhancer activity (**Fig. 1B**). Such variations can for instance alter the
71 affinity of bound TFs or create new binding sites. The second type involves structural variants
72 that lead to deletion, duplication or relocation of the entire enhancer, which impacts
73 chromatin topology and enhancer function (**Fig. 1C**). Chromosomal rearrangements can lead
74 to rewiring of enhancer-gene connections, which may involve both enhancer
75 adoption/hijacking (gain-of-function; e.g., ref. ^{13,14}), and enhancer disconnection (loss-of-
76 function; e.g., ref. ¹⁵). Depending on the genomic alteration, enhancer dysfunction may result
77 in either gain or loss of gene expression in a given tissue as well as more complex alterations
78 of expression patterns (**Table 1**).

79 Elucidating the molecular basis of enhancer function in normal and pathological conditions
80 has far-reaching translational implications. Here, we discuss important challenges that need

81 to be considered in the study of enhancer dysfunction in disease and highlight critical areas
82 of research to address these challenges in the near future (**Fig. 2**).

83

84 **Current challenges in characterizing enhancer dysfunction in disease**

85

86 ***1) Identifying and validating functional enhancers***

87 A persisting challenge when studying transcriptional regulation in health and disease is to
88 systematically identify functionally meaningful enhancers in a given cell type. Partially, this is
89 because enhancers encompass a diverse group of regulatory sequences, which may utilize
90 various mechanisms to control gene expression^{2,16}. Conceptually, a nucleotide sequence can
91 be assigned as a biologically meaningful enhancer once it is experimentally demonstrated that
92 it modulates the transcription of a gene in *cis* in its native context. Unfortunately, there is no
93 high-throughput assay that is able to do this globally outside of cell lines. As a result,
94 enhancers are often defined in an indirect, operational, manner². For example, a sequence
95 may be functionally qualified as an enhancer if it increases the activity of a (minimal) promoter
96 in a plasmid reporter assay, or it may be qualified as an enhancer by association, when linked
97 with chromatin accessibility, transcription of enhancer RNAs (eRNAs), or marked by
98 epigenomic features that have been linked to enhancer activity (e.g. p300, H3K4me1,
99 H3K27ac). Functionally cataloging sequences as candidate enhancers has been boosted by
100 the development of Massively Parallel Reporter Assays (MPRAs) that allow systematic large-
101 scale testing of enhancer activities of any sequence in episomal contexts², while genome-wide
102 approaches to find putative enhancers by association were employed by several international
103 consortia (ENCODE¹⁷, ROADMAP¹⁸, FANTOM⁷ and BLUEPRINT¹⁹). While these approaches
104 have greatly expanded our ability to map enhancers, they suffer from some limitations. One
105 is that not all sequences that are predicted to act as enhancers based on MPRAs or association
106 strategies necessarily function to increase gene transcription in their endogenous
107 chromosomal contexts. Furthermore, MPRAs do not account for the effects that linear
108 distance and chromatin environment might have on enhancer activity. In principle, CRISPR-
109 based enhancer screens are able to overcome these limitations and can be used to assess the
110 importance of enhancers in their endogenous context²⁰⁻²². However, these assays may suffer

111 from the intrinsic redundancy or additive effects of enhancers (see Challenge 3), and high
112 false-negative rates². To further complicate things, some proven biologically meaningful
113 enhancers may lack the expected biochemical marks while others may not show enhancer
114 potential in reporter assays^{23,24}, perhaps because these assays are based on plasmids and may
115 not reproduce the function of chromatin-dependent enhancers^{24,25}. Therefore, it is not
116 surprising that there is no strict overlap¹⁶ between the hundreds of thousands of putative
117 enhancers in the human genome predicted based on indirect assays and the number of
118 biologically confirmed enhancers. Future comparisons between predicted and functionally
119 validated enhancers, including CRISPR screens with high-sensitivity and low false-negative
120 rates, should help improve our definition of the fundamental features of functionally
121 operational enhancers.

122

123 ***2) Defining the spatiotemporal window in which a regulatory variant affects enhancer*** 124 ***activity***

125 Enhancer activity is highly specific during development, across cell types, cell states, and
126 stimulatory conditions (e.g., inflammatory response, diet, drug treatment). As a consequence,
127 the effect of enhancer dysfunction (**Fig. 1B**), may only be revealed in specific contexts or
128 tissues. This leads to the challenge of defining the spatiotemporal window in which an
129 enhancer dysfunction has a measurable and meaningful impact. This is particularly important
130 for interpreting disease-associated genetic variants in non-coding regions since the cell type
131 in which an enhancer is active can be informative about the disease mechanism. For instance,
132 the finding that genetic variants associated with Alzheimer's disease, a neurodegenerative
133 condition, overlap enhancers in myeloid cells, rather than neurons²⁶, has led to a shift in the
134 research focus of the pathology²⁷. Similarly, functional assessment of obesity-associated
135 variants has identified putatively causal variants with regulatory properties in both adipose
136 and neuronal cell lines²⁸.

137 To capture inducible and context-dependent enhancers, as well as those restricted to rare cell
138 types or developmental stages, efforts in enhancer mapping need to focus on different
139 stimulatory conditions, environmental contexts, developmental stages and rare cell types^{29–}
140 ³¹. Single-cell technologies are particularly well-suited for studying rare cell types. Specifically,

141 single-cell chromatin accessibility assays (scATAC-seq, Assay for Transposase-Accessible
142 Chromatin and sequencing) can serve to operationally predict enhancer activity and
143 have facilitated the functional interpretation of disease-associated non-coding variants in
144 adult and fetal tissues³²⁻³⁴. scATAC-seq will be key for expanding putative enhancer maps in
145 diverse (rare) cell types. Furthermore, since chromatin accessibility does not necessarily
146 reflect enhancer activity⁷, further development of single-cell technologies that employ
147 orthogonal measures of enhancer activity, e.g., large-scale perturbation assays^{35,36}, will be
148 crucial to get more confidence in assessing enhancer function for rare cell types.

149 In line with the importance of cataloging enhancers and their restricted activities, there is an
150 urgent need to assess the functional impact of regulatory variants during development and
151 differentiation. To this end, recent years have seen a promising development of biological
152 models such as transgenic mice and zebrafish³⁷⁻³⁹, genetically manipulated human-induced
153 Pluripotent Cells (hiPSC) or immortalized precursor cells^{15,27,40,41}, human-mouse chimeras^{42,43}
154 and organoids⁴⁴. These *in vivo* and *ex vivo* models, in combination with assays to assess
155 developmental and differentiation potential, will facilitate the study of genetic variants and
156 determine their impact in contexts closer to human diseases.

157

158 **3) Understanding the interplay between cis-regulatory elements**

159 Enhancers are not only highly context-dependent, but they also often work together in
160 regulatory domains to achieve the correct gene expression output. Thus, a major challenge is
161 to understand the interplay between enhancers and other regulatory elements, including
162 promoters, and how the joint activity of a domain is influenced by disruptions of individual
163 enhancers. Multiple enhancers for the same gene may allow distinct enhancers to either be
164 activated under different conditions or to cooperate, both of which can lead to robustness in
165 gene activity^{24,37,45-47}. For instance, many developmental genes are associated with “shadow”
166 enhancers with similar transcription factor (TF) binding to ensure robust expression under
167 suboptimal conditions^{45,48,49}, an observation that has been confirmed by 3D topology-based
168 methods that revealed a complex landscape of multiple enhancer interactions per gene⁵⁰⁻⁵³.
169 In fact, highly coordinated enhancer activity has been linked to the regulation of cell identity
170 genes⁵⁴, signal integration and compartmentalization of the genome⁵⁵. As a consequence of

171 such regulatory complexity, many enhancers might not, individually, reveal a strong
172 phenotype when disrupted in their endogenous context^{23,56}, while still possessing
173 endogenous enhancer activity. Thus, the presence of multiple enhancers⁵⁷ *per* gene may
174 either additively or synergistically achieve a higher transcriptional output of a gene or provide
175 redundancy and mutational robustness to its expression. Systematic testing of enhancer-
176 promoter compatibilities will help to better understand the still unclear connectivity rules^{58,59}
177 that control gene transcription in the human genome.

178 Elucidating the mechanisms and contexts, including the cell type-specific 3D topology, by
179 which regulatory domains and TFs establish robustness or synergism will therefore be crucial
180 to further our understanding of enhanceropathies. Combinatorial interference or
181 perturbation of multiple enhancers within a regulatory domain will be necessary to
182 understand the principles by which enhancers act together and their effects on gene
183 regulation.

184

185 ***4) Identifying the target genes of enhancers***

186 Enhancers ultimately need to be defined by their role in enhancing endogenous gene
187 expression, which leads to the next challenge: the identification of their target genes. This is
188 particularly challenging for enhancers that are located distally to any gene promoter. It is
189 assumed that distal enhancers have to come into physical proximity to their target gene in
190 order to function, as first demonstrated by chromosome conformation capture (3C) methods
191 in the beta-globin locus⁶⁰. Thus, for the operational mapping of target genes, chromatin-
192 topology assays are key to determine the physical proximity between enhancers and their
193 putative genes. These technologies can map direct contacts (chromatin loops) and at the
194 same time identify larger domains, so-called topologically associating domains (TADs), which
195 have a high density of physical chromatin interactions⁶¹. The main caveats of using direct
196 contacts for mapping enhancer-gene pairs are that the 3C-technologies typically require large
197 cell numbers (with some exceptions^{50,52} and may thus miss enhancer-gene pairs that are
198 looped only in a subset of cells or contacts that are highly transient. TAD-based analyses suffer
199 from low resolution since they typically comprise multiple genes and enhancers and can, on
200 their own at best, restrict the search space for putative target genes⁶². There are a

201 complementary set of approaches to map enhancer-gene pairs such as targeted Hi-C, where
202 chromatin interactions of regions of interest such as promoters and/or enhancers are captured to
203 increase resolution^{55,63}, or expression quantitative trait loci (eQTL) mapping, where enhancer
204 genetic variants are associated with mRNA expression changes across individuals⁶⁴. Other
205 approaches use covariation between molecular phenotypes (e.g., histone marks, chromatin
206 accessibility, expression) of enhancers and genes across individuals or cell types^{9,65–67} or
207 combine chromatin states and long-range interactions⁶⁸, to construct genome-wide maps of
208 enhancer–gene connections in a given cell type. The advantage of these methods is that
209 relying on enhancer-gene co-variation does not assume a specific mechanism of how
210 enhancers regulate gene expression, and can therefore also capture transient enhancer-gene
211 contacts⁶⁹. Here, the caveats are that these methods require molecular data across a large
212 number of individuals or cell types, and they may miss constitutively active enhancers that do
213 not vary much across samples. Given their descriptive (for the 3C technologies) and
214 correlative (for the co-variation methods) nature, all of these approaches provide an
215 operational prediction of putative enhancer-gene pairs. For a functional mapping of target
216 genes, CRISPR-mediated enhancer deletion or inactivation, followed by gene expression
217 analysis^{29,68}, is the most direct way to search for target genes. However, such CRISPR-based
218 approaches may miss links due to low effect sizes and are often limited to cultured cells. In
219 conclusion, current approaches still have difficulties identifying with high confidence the
220 target genes of enhancers, and likely, the combination of different strategies might improve
221 the efficiency of identifying disease-targeted genes⁷⁰. Recent advances in applying machine
222 learning to predict cell-type specific expression based on DNA sequence⁷¹ show great promise
223 to generate defined and experimentally testable hypotheses. These models were enabled by
224 the vast resources of transcriptomics and genomics data that have been assembled by the
225 community, and additional data, particularly from less accessible cellular states and
226 developmental stages, will further improve the power of these methods.

227

228 **5) Understanding the grammar of enhancer activity**

229 Gene regulatory elements, including enhancers^{7–9}, are regulated by TFs, or TF-recruited co-
230 activators, which bind to the enhancer element at any given time and cellular state. It is thus
231 not surprising that genetic variants that disrupt a TF binding motif are enriched among

232 variants associated with molecular phenotypes, such as histone marks⁸ or tissue-specific
233 expression levels⁷², and can be disease-causative⁷³. For example, a mutation in a *SOX9*
234 enhancer, associated with Pierre Robin Syndrome, disrupts the binding of the TF *MSX1*⁷⁴ (see
235 other examples in **Table 1**). However, the majority of molecular trait-associated Single
236 Nucleotide Polymorphisms (SNPs) do not disrupt known TF binding sites⁸, leading to the next
237 open challenge in understanding enhancer dysfunction: to identify the rules by which
238 enhancer sequence determines its activity. Concepts, such as Variable Chromatin Modules
239 (VCMs), where the effects of a lead SNP affecting a local chromatin domain (e.g. through TF
240 binding site disruption) spread into the local vicinity, can explain the missing mechanism to
241 some extent^{8,10,75}. Recent studies revealed that flanking regions of TF binding sites are highly
242 informative for some TFs to bind⁷⁶ and they impact the enhancer potential of the
243 encompassing regulatory element⁷⁷, suggesting we are still missing part of the grammar for
244 TF binding. In line with this, up to 30% of human TFs have no characterized binding motif⁷⁸.
245 Consequently, interpreting regulatory variant-to-phenotype associations requires
246 fundamental insights into the sequence determinants of TF binding and enhancer activity.
247 Here, sequence-based machine learning to model TF binding^{76,79,80}, enhancer activity^{25,77,81}
248 and topologies⁷¹ show promise. However, major challenges remain, including the difficulty to
249 accurately interpret such models, the lack of sufficient training or validation data, and the
250 need to improve accuracy and generalization across cell types/contexts. In parallel,
251 experimental approaches that measure the functional impact of genetic variants on
252 regulatory activity and TF binding in a large-scale, such as MPRA-based approaches^{82–86} and
253 SNP-SELEX⁸⁷, can provide comprehensive experimental fine-mapping of likely causal variants.
254 Overall, these insights will be crucial for the interpretation of the potential effect and severity
255 of enhancer dysfunction, and thus the potentially implicated genetic variants, within complex
256 regulatory domains.

257

258 **6) Understanding how TF cooperation defines enhancer activity and specificity**

259 Enhancers integrate non-mutually exclusive layers of molecular information: their function
260 can be impacted by genetic variants/mutations, by epigenetic chromatin remodeling that is
261 typically set up by lineage-specific TFs, or by signaling cascades regulated by stimulus-
262 responsive TFs⁸⁸ (**Fig. 1**). Here, we focus on the challenge of understanding the role of TFs and

263 epigenetics on enhancer dysfunction. Lineage- and developmental-stage specific enhancers,
264 typically regulated by lineage-specific TFs, may define the gene expression potential of a cell,
265 and whether or not it will be able to mount a specific response to a given stimulus⁸⁹. In
266 particular, during development or differentiation, enhancers and whole chromatin domains
267 can be primed in progenitor cells towards certain lineages before gene expression changes
268 are obvious, e.g., during adipogenesis⁵⁵. In contrast, enhancers that are under the control of
269 stimulus-responsive TFs essentially act as signaling response elements and connect cell-
270 extrinsic signals to gene expression programs. Conceptually, lineage-specific TFs and the
271 chromatin accessibility landscape they set up determine the scope of stimulus-regulated TFs.
272 This way, stimulus-responsive TFs can access enhancers that are pre-marked and kept
273 accessible by lineage-specific TFs, thus integrating the two layers of regulation⁹⁰. As a
274 consequence, some response-TFs, such as NF- κ B, bind completely different enhancers
275 depending on the cell type in which they are activated⁹¹. This is consistent with observations
276 that a TF can regulate completely different sets of genes depending on the cell type⁹², which
277 is partially explained by the cooperative interaction of TFs⁹³. Yet, apart from a couple of well-
278 studied examples, very little is known about the contribution of TF cooperativity, enhancer
279 priming (that can also be TF mediated) and permissive chromatin, which in turn may define
280 the TF regulon (i.e., the set of target genes regulated by a given TF). To fully understand
281 enhancer dysfunction, it is important to study the cell type- or condition-specific TF regulons,
282 and how they are defined by the combinatorial or cooperative binding grammar of enhancer
283 sequences in normal and pathological conditions. Diverse TF-centric studies are even more
284 important given the current literature bias with many studies focusing on a small set of TFs
285 while the majority of TFs are vastly understudied⁹⁴.

286

287 ***7) Deciphering the impact and interactions of regulatory mutations in disease***

288 The challenges above culminate in the ultimate challenge of identifying and understanding
289 pathogenic enhancer dysfunction and eventually using this knowledge in clinically relevant
290 studies (**Fig. 2**). The specific challenges that need to be solved for understanding a certain
291 disease depend on the type of enhancer dysfunction and the nature of the genetic alteration
292 (rare vs. common). For rare diseases, few examples of causal enhancer mutations have been
293 established as compared to mutations in the coding genome. It is currently unclear whether

294 this limited number of reported enhancer mutations in rare disorders is because they do not
295 exist or because we have not been able to find them due to the lack of data and statistical
296 power. Either way, the additional challenge for identifying causal mutations in enhancers vs.
297 coding regions is that each genome carries around 2,000 structural and 8,500 private non-
298 coding variants⁹⁵, which are often not even captured since exome-sequencing is still the
299 standard for diagnosing rare diseases. On the other hand, GWA studies have revealed
300 hundreds of non-coding variants of significance for common disease risk, suggesting that the
301 aggregated effect of variants in multiple enhancers modulate common disease risk. Fine-
302 mapping studies aimed at identifying the causal variant(s) among those linked in a haplotype
303 block typically integrate significantly associated variants with experimentally determined
304 enhancer characteristics, as discussed above. While successful for the identification of some
305 causal variants (e.g. ^{96,97}), this is often difficult because the relevant cell type and trans-acting
306 nuclear environment are not known (challenge 2), the role of the encompassing regulatory
307 domain is not well understood (challenge 3) and the target gene of the affected enhancer is
308 not identified (challenge 4). Fine-mapping of causal signals and effect size predictions can be
309 improved by expanding the battery of GWA studies with cohorts of diverse ancestries^{98,99}, and
310 computational tools ranking genes based on their dosage-dependent pathogenicity. This
311 allows hypothesis-driven studies where candidate target genes and enhancers are tested
312 simultaneously to measure their combined effects on inferred functions¹⁰⁰. Furthermore, for
313 common diseases, both genetic and environmental factors contribute to the disease etiology.
314 Therefore, the effects of certain non-coding genetic variants might only or preferentially be
315 manifested under certain environmental conditions. Together with a significant shift for using
316 whole-genome instead of whole-exome sequences as a diagnostic utility, and consequently,
317 an increasing amount of whole-genome data accumulating thanks to biobanks and cohort
318 studies¹⁰¹, these tools will likely provide much better constraints on assessing disease
319 causality and could pave the way towards systematic prediction of pathogenicity of regulatory
320 variants and mutations for both rare and common variants¹⁰².

321

322 **Future directions**

323 As disease-associated regulatory mutations at enhancers are increasingly identified, there is
324 an urgent need to fully characterize enhancer mutations to enable their use in functional and

325 clinical genomic studies (**Box 1**). Besides the complexity of studying enhancer function in
326 normal contexts, the characterization of noncoding variants affecting enhancer activity in
327 disease adds additional challenges ranging from the identification of a credible set of
328 regulatory variants to the identification of tissues and developmental contexts in which
329 variants have an effect. Despite the wealth of data on enhancer activity across multiple cell-
330 and tissue-types, it is challenging to fully utilize the vast potential of such datasets,
331 highlighting the importance of good data-sharing practices. In addition, the majority of
332 available data informing on enhancer activities are derived from populations of cells,
333 disregarding the stochasticity and plasticity of regulatory events across individual cells.
334 However, due to the complexity of the regulatory landscape, we propose that the field should
335 move beyond the generation of enhancer catalogs and invest more in experimental and
336 computational efforts to identify their target genes, in particular for the prioritization of
337 disease-relevant genes susceptible to dysfunction upon misregulation. This can only be
338 uncovered using Systems Biology, computational modeling approaches, and targeted
339 experimental systems. Focused efforts and datasets will enable hypothesis-driven
340 investigations of a set of variants or genes for a given disease phenotype and further inform
341 the modeling of enhancer function from catalog data. Ultimately, the acquired knowledge
342 should allow the implementation of novel strategies to genetically or epigenetically modify
343 enhancer function to treat the associated diseases.

344

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350 consortium in the continuum of basic, translational and clinical research on enhancers and
351 associated diseases.

352

353 **Competing interests**

354 The authors declare no competing interest.

Table 1. Representative examples of enhancer dysfunction driving disease

Type of disease	Disease	Affected gene(s)	Enhancer*	Type of disruption	Effect on gene exp.	ref
Monogenic (Mendelian)	□-Thalassaemia	□-globin genes	LCR	Enhancer deletions	LOE	103,104
	□-Thalassaemia	□-globin genes	□-globin enhancers	Deletion or insertion of promoters alter enhancer-gene connectivity	LOE & GOE	105,106
	PDD2	<i>SHH</i>	ZRS	Rare variant introducing a TFBS	GOE	107
	HPE	<i>SHH</i>	SBE2	Rare variant disrupting a TFBS	LOE	108
	Limb malformations	<i>PAX3, IHH, WNT6</i>	EPH4 enhancers	Deletions, duplications and inversions disrupt the boundaries of a TAD containing the <i>EPH4</i> enhancer and rewire the connectivity with different genes	GOE	13
	5q14.3 microdeletion syndrome	<i>MEF2C</i>	MEF2C enhancers	TAD disruption disconnects MEF2C from associated enhancer	LOE	109
	Pierre Robin syndrome	<i>SOX9</i>	SOX9 enhancer	A point mutation in a conserved enhancer disrupts the binding of MSX1	LOE	74
	Cooks syndrome	<i>SOX9, KCNJ2</i>	SOX9 enhancers	Duplication of a TAD boundary at the <i>SOX9</i> locus causes neo-TAD formation and <i>KCNJ2</i> misexpression	GOE	110
	Isolated atrial defect	<i>TBX5</i>	90 kb downstream	Rare variant abrogates heart-specific enhancer activity	LOE	111
	Isolated pancreatic agenesis	<i>PTF1A</i>	25 kb downstream	Rare variants abolish enhancer activity and disrupt the binding of FOXA2 and PDX1	LOE	41
Common (multifactorial)	Obesity	<i>IRX3, IRX5</i>	FTO intronic	Multiple variants on a common haplotype increase the activity of several enhancers	GOE	97
	Type 2 diabetes	<i>ZFAND3</i>	Upstream	SNP disrupts the binding of NeuroD1 and decreases enhancer activity	LOE	112
	Vascular diseases	<i>EDN1</i>	<i>PHACTR1</i> intronic	SNP located in a distal region interacting with <i>EDN1</i> enhancer	LOE	96
	HBF level	<i>BCL11A</i>	Downstream	SNP disrupts TF binding and diminishes expression in erythroid cells	LOE	113
	Cardiac disorders	<i>SNC5A</i>	<i>SNC10A</i> intronic	SNP in <i>SNC10A</i> modulates <i>SNC5A</i> expression in the heart	LOE	114
	Hirschsprung disease	<i>RET</i>	Several enhancers	Several SNPs located in <i>RET</i> enhancers act synergistically to reduce gene expression	LOE	115
	Parkinson	<i>SNCA</i>	Intronic	SNP alters the binding of EMX2 and NKX6-1	LOE	40
Cancer	Burkitt lymphoma	<i>MYC</i>	IgH enhancer	Somatic translocation (enhancer hijacking)	GOE	116,117
	Lung Adenocarcinoma	<i>MYC</i>	450 kb downstream	Somatic duplication of the enhancer	GOE	118
	T-ALL	<i>TAL1</i>	7 kb upstream	Somatic insertions introduce a MYB binding site and induce the formation of a Neo-enhancer	GOE	119,120
	Ph-like ALL	<i>GATA3</i>	Intronic	A rare variant increases enhancer activity	GOE	121
	CLL	<i>AXIN2</i>	Upstream	Common variation in the <i>AXIN2</i> enhancer modulates CLL susceptibility via differential MEF2 binding	GOE	122
	AML	<i>GATA2, EVI1</i>	<i>GATA2</i> enhancer	Large somatic inversion relocated <i>GATA2</i> enhancer in the vicinity of <i>EVI1</i>	LOE & GOE	14
	Prostate cancer	<i>PCAT19, CEACAM21</i>	<i>PCAT19</i> Epromoter	Common variant changes the affinity of TFs and switch promoter and enhancer activities	GOE	123,124

356 to Table 1: *Enhancer location relates to the regulated gene unless otherwise stated. Abbreviations: LOE: Loss-of-expression; GOE: gain-
357 expression; LCR: Locus control Region; PDD2, preaxial polydactyly type II; HPE: holoprosencephaly; HBF: Fetal hemoglobin; T-ALL, T
358 Acute Lymphoblastic Leukemia; CLL: Chronic Lymphoblastic Leukemia; MLL: Myeloid Lymphoblastic Leukemia; Ph-like ALL:
359 Philadelphia chromosome-like Acute Lymphoblastic Leukemia; TF, transcription factor; IgH, immunoglobulin heavy chain
360

361

362

363 **Figure legends**

364 **Figure 1: Different mechanisms of enhancer function and dysfunction. A)** Variations in the
365 interplay between enhancers and target genes. Multiple enhancers can cooperate in a tissue
366 to increase the transcription of a target gene or be active in different tissues to control a
367 complex developmental gene expression pattern. Enhancers can further control multiple
368 genes in a mutually exclusive or shared way. Color code indicates the enhancer activity and
369 gene expression in different tissues or developmental contexts. **(B-C)** Erroneous regulatory
370 wiring between enhancers and genes, by either enhancer disruption **(B)** or altered enhancer-
371 gene connectivity **(C)**, can result in dysregulation of gene expression and ultimately cause
372 disease. Enhancer dysfunction can originate from deletions, duplications and mutations,
373 which can result in either loss or gain of gene expression. Altered enhancer-gene connectivity
374 can be caused by chromosomal translocations or large structural variations that can distort
375 or merge Topologically Associating Domains (TAD). As a consequence, enhancer-gene
376 connectivity can be lost or gained resulting in dysregulated gene expression. Changes in gene
377 expression are indicated by the number of arrows.

378

379 **Figure 2: Challenges to unravel enhancer-associated diseases.** Elucidating the molecular
380 basis of enhancer dysfunction in disease requires critical areas of research to be addressed,
381 each corresponding to one of the challenges described in the main text. Resolving challenges
382 I to VI should lead to the ultimate challenge (VII) of identifying the causal variants, the
383 impacted molecular mechanisms as well as the affected genes of a disease. TF: transcription
384 factor. MPRA: Massively Parallel Reporter Assay.

385

386

Box 1. Critical areas of research to further our understanding of enhancer dysregulation in disease

We emphasize the following critical areas of research to advance our understanding of enhancer dysregulation in disease and for better translation of enhancer research into clinical practice:

Area 1

The ongoing community-driven comparison and assessment of experimental approaches for the discovery of enhancer activity should be strengthened. This will help improve our definition of the fundamental features of functionally operational enhancers as well as determine the most appropriate assay given a biological or disease context. We particularly

see the benefits of further developments of CRISPR screens that improve sensitivity and allow measurements in non-cultured cells.

Area 2

We foresee major benefits in further efforts towards developing assays that will allow accurate assessment of enhancer activity in single cells. scATAC-seq is key for expanding the enhancer map repertoire, particularly in rare cell types. In addition, further development of single-cell technologies that employ large-scale perturbations of enhancers or TFs will be key to assessing enhancer function for such cell types. The output of such studies will also help building models of enhancer regulation informed by the dynamics and stochasticity of regulatory events as well as discover mechanisms by which their perturbation contributes to pathology.

Area 3

To better understand the rules by which enhancers work together in regulatory domains to achieve robustness, specificity, or synergism, further efforts are needed to derive assays and strategies that allow combinatorial interference or perturbation of multiple enhancers. It is further imperative to develop *in vivo* (i.e. *in situ*) assays that allow the study of the activity of an enhancer in isolation or synergy with other enhancers. The outcomes of such studies would enable us to identify the biological mechanisms by which regulatory domains are formed and the rules by which TFs and the interplay between multiple regulatory elements yield robustness or additive effects. These insights will aid the interpretation of the potential effect and severity of regulatory genetic variants and enhancer dysfunction within complex regulatory domains.

Area 4

Experimental disease systems, such as humanized animal models, organoids, and engineered tissues, are becoming increasingly available for genetic engineering and *in situ* or *ex vivo* functional experiments. It will be key to fully employ these advanced disease models for assessing the functional and pathological consequences of non-coding regulatory variants (genetic and structural). Such experimental systems will allow

interrogation of enhancer activity under a relevant internal or external stimulus for their dynamic and contextual assessment.

Area 5

The research community should increase the already promising work towards developing interpretable and generalizable computational models that can accurately predict TF binding, the activity of enhancers and their target genes, using molecular measurements in any given cell type and condition. From these, the main efforts should ideally be focused on deriving the underlying regulatory DNA code, allowing for direct interpretation of the effects of genetic variants across cell types. Relatedly, we foresee great benefits in putting effort into developing approaches to computationally predict dosage-sensitive and responsive genes, as they are more likely to be adversely affected by cis-acting mutations.

Area 6

To fully understand the molecular basis of enhancer dysfunction, we foresee the need to further develop and apply large-scale TF perturbation assays coupled with GRN analysis to study cell type- or condition-specific TF regulons, and how they are defined by the combinatorial or cooperative binding grammar of enhancer sequences in normal and pathological conditions.

Area 7

Last but not least, we foresee great potential for implementing tools (e.g. CRISPR-based) to genetically or epigenetically modify the functions or chromatin contexts of enhancers to treat enhanceropathies. By targeting enhancers, one can avoid the potential pleiotropic effects associated with drugs/tools directed toward proteins or gene promoters.

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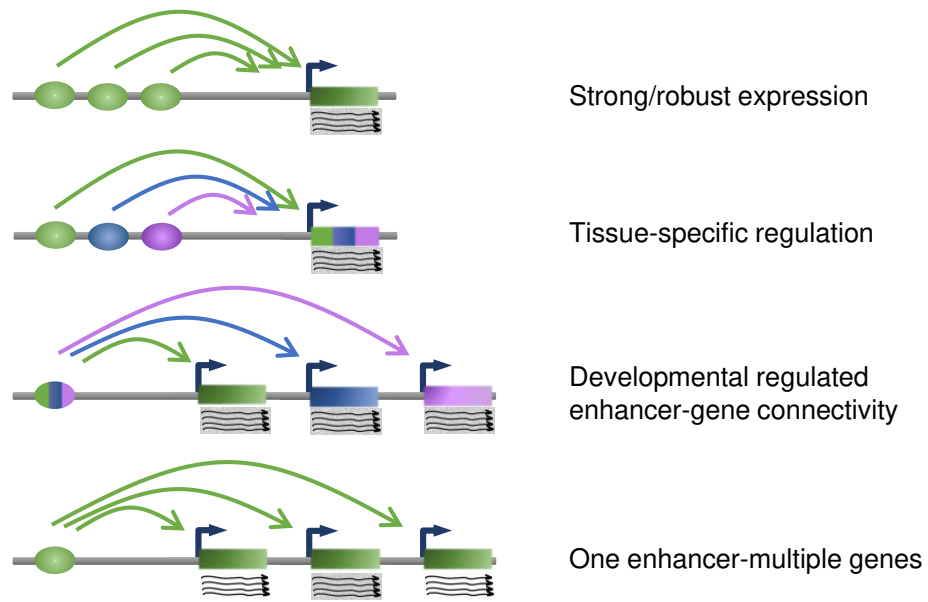
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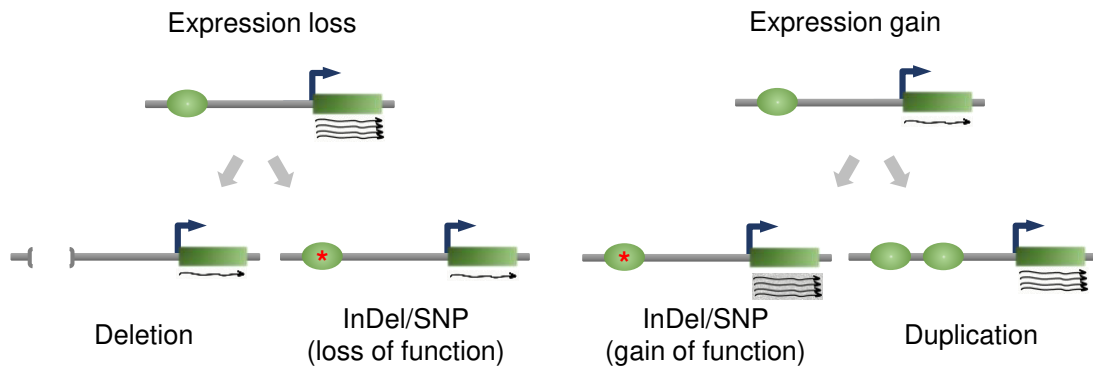
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A

Functional interplay between enhancers and target genes

**B**

Enhancer disruption

**C**

Altered enhancer-gene connectivity

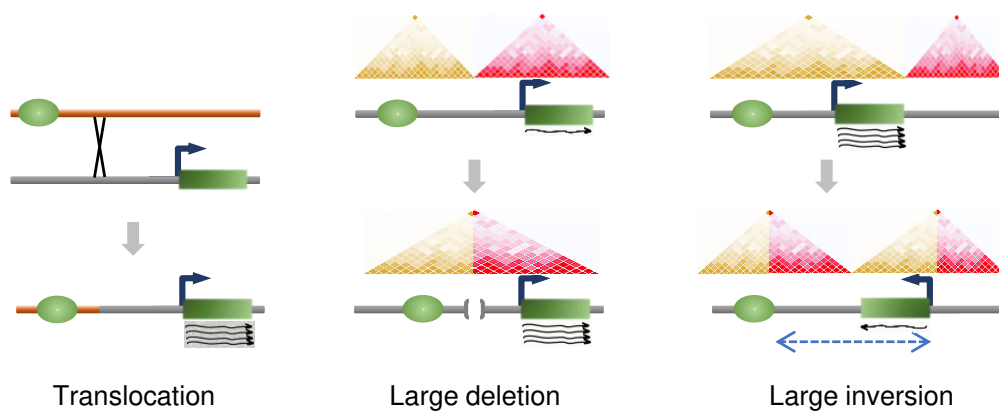


Figure 1 (REVISED)

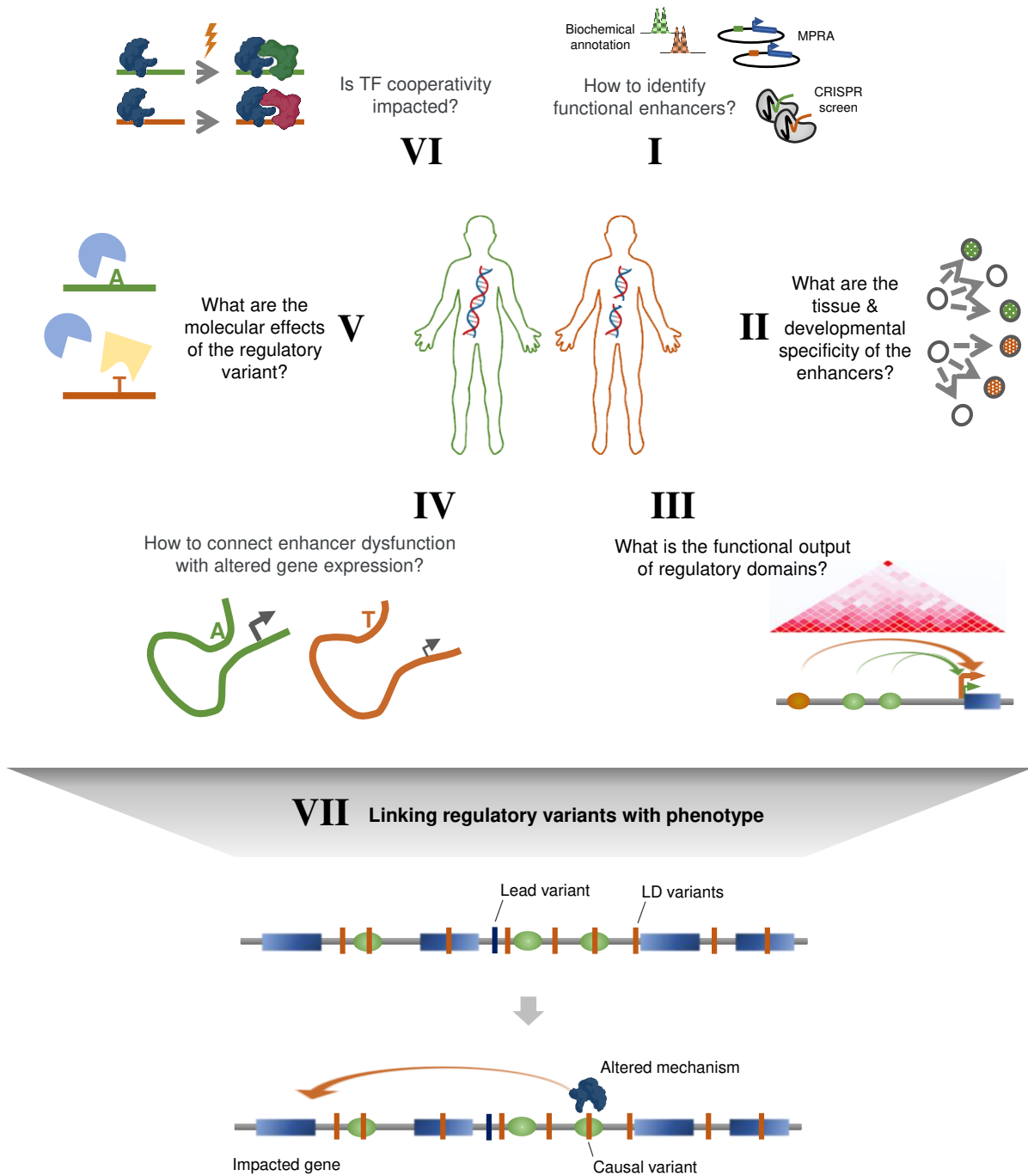


Figure 2