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Review

REVIEW

From enhanceropathies to the epigenetic manifold underlying human cognition

Alessandro Vitriolo[1,†], Michele Gabriele[2] and Giuseppe Testa[1,2,*]

[1]Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy and [2]European Institute of Oncology IRCCS, Milan, Italy

*To whom correspondence should be addressed at: European Institute of Oncology, Via Adamello 16, Milan 20138, Italy. Tel: +39 0294375105; Fax: +39 0294375990; Email: giuseppe.testa@ieo.it

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Abstract

A vast portion of intellectual disability and autism spectrum disorders is genetically caused by mutations in chromatin modulators. These proteins play key roles in development and are also highly expressed in the adult brain. Specifically, the pivotal role of chromatin regulation in transcription has placed enhancers at the core of neurodevelopmental disorders (NDDs) studies, ushering in the coining of the term enhanceropathies. The convergence of these disorders is multilayered, spanning from molecular causes to pathophysiological traits, including extensive overlaps between enhanceropathies and neurocristopathies. The reconstruction of epigenetic circuitries wiring development and underlying cognitive functions has gone hand in hand with the development of tools that increase the sensitivity of identifying regulatory regions and linking enhancers to their target genes. The available models, including loop extrusion and phase separation, have been bringing into relief complementary aspects to interpret gene regulation datasets, reinforcing the idea that enhancers are not all the same and that regulatory regions possess shades of enhancer-ness and promoter-ness. The current limits in enhancer definition, within the emerging broader understanding of chromatin dynamics in time and space, are now on the verge of being transformed by the possibility to interrogate developmentally relevant three-dimensional cellular models at single-cell resolution. Here we discuss the contours of how these technological advances, as well as the epistemic limitations they are set to overcome, may well usher in a change of paradigm for NDDs, moving the quest for convergence from enhancers to the four-dimensional (4D) genome.

Introduction

Convergence and specificity in intellectual disability and autism spectrum disorders

Neurodevelopmental disorders (NDDs) encompass a diverse range of conditions that affect the lives of many individuals and their families. Understanding their unfolding in molecular detail holds key to reduce such burden by enabling drug discovery and/or repurposing approaches anchored both to a mechanistic understanding and an elucidation of the most relevant levels of phenotypic convergence. Specifically, intellectual disability (ID) prevalence has recently been estimated between 0.05% and 1.55% (1) and is associated to 3963 genes in the OMIM database. The Simons Foundation Autism Research Initiative (SFARI) lists 1089 genes and 2291 copy number variation (CNV) loci associated to autism spectrum disorder (ASD) within a range of scores reflecting the strength of the causative evidence. These numbers alone outline the complexity underlying the regulation of cognition, sociality and their alterations. Such complexity in the genetic architecture of ID and ASD is compounded by the fact that the acquisition of such mutations is increasingly recognized to occur *de novo* (mostly germline, with mosaicism detected thus

[†]Alessandro Vitriolo, http://orcid.org/0000-0002-1462-8064
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far in a small percentage of individuals) (2). Moreover, while mutations of each gene in the SFARI list are fully penetrant and deemed causative mostly via haploinsufficiency, variations of other genes are considered risk factors, and their combination can lead to polygenic forms of such disorders (3) where each gene contributes, thanks to its non-deterministic and rather incomplete penetrance, toward ID and/or autistic spectrum outcomes.

Despite the striking diversity of genetic causes identified to date and the significant extent of phenotypic variation notwithstanding, the major phenotypic and molecular commonalities shared across NDDs clearly warrant a systematic effort in studying them jointly, starting from the most homogenous subgroups, in order to elucidate the atlas of molecular convergences onto which specificities will hopefully acquire actionable relief (4). In fact, several shared, opposite or unique phenotypes and endophenotypes, ranging from craniofacial features to cardiovascular abnormalities, have been identified across neurodevelopmental syndromes, including some in the autism spectrum.

Among the most common genetic causes of ID and ASD are mutations in chromatin regulators and transcription factors: 152 in the SFARI list, of which 97 enrich the gene ontology category "positive regulation of gene expression" and 124 enrich "DNA-templated regulation of transcription" (both with false discovery rate $< 2e-12$ using Enrichr (5)). Mutations in genes that regulate transcription, both affecting specific gene regulatory networks and in a broader setting - such as the bulk deposition of a histone mark, or chromatin organization - are likely to have an immediate transcriptional impact, which reverberates through effects that can dramatically impinge upon proper cell fate acquisition or cell physiology. The majority of epigenetic modulators causing NDDs are highly expressed during a "window of vulnerability" along the early phases of neuronal development. Their expression in the fully developed central nervous systems is mostly in the cortex, which contains neuronal circuits involved in higher cognitive functions such as language processing, social awareness and visuospatial construction (6). Still, the centrality of other regions of the brain in their manifestation, such as the hippocampus and cerebellum, cannot be neglected (7–9). Furthermore, the concomitant presence in NDDs of recurrent anomalies in neural crest-derived tissues, mainly including peripheral nervous system, craniofacial, skeletal and cardiovascular defects, has been revealing an increasing spectrum of comorbidity between the cognitive/behavioral features that are hallmarks of NDDs and the neurocristopathies (10,11).

The enhancer-centric view of NDDs

Thus far, the most successful attempts at grounding convergence are arguably reflected in the two categories of synapthopathies and enhanceropathies. While the former refers to NDDs sharing phenotypes that can be traced to synaptic dysfunction, the latter has gained increasing traction in recent years, encompassing disorders in which either enhancer elements *per se* or, more usually, the genes involved in the control of enhancer elements are mutated (12,13). The physiopathological relevance of mutations in enhancer elements for developmental abnormalities is already well established (13), with prototypical examples found in the mutations at the sonic hedgehog enhancer locus, which are responsible for polydactyly, or in the long-range mutations that affect globin expression in hemoglobinopathies (14,15). More recently, brain-relevant enhancer activity in *in vivo* murine reporter assays has been associated to the 5p14.1 region, which is a significant locus of genome-wide association study hits of ASD-associated variants, a claim that may well represent the tip of the iceberg for analogous NDD-relevant findings across neural specific enhancers. The pathogenic role of mutations in regulators of enhancer function is instead already more amply documented (6), including the example that we recently characterized of Gabriele-de Vries syndrome (16) which is caused by haploinsufficiency of the YY1 protein that mediates looping between enhancers and promoters (17,18).

Notwithstanding these advances, the fundamental challenge for the notion of "enhanceropathies" in terms of illuminating NDD convergence appears now to be twofold. Specifically, it involves the very challenge of enhancer identification as well as our evolving definition of their mode of actions, both aspects that intersect our still emergent understanding of the three-dimensional (3D) chromatin architecture, its dynamics, cell specificity and pathogenic potential. Thus, it has been proposed that the combination of the H3K4me1 and H3K27Ac histone modifications suffices to identify active enhancers (19), while H3K27Ac alone does not always suffice to call all active enhancers (20). While H4K16ac is enriched together with H3K27ac, globular modifications of H3, such as H3K64ac and H3K122ac, have been proposed to more robustly identify active enhancers, in comparison to H3K27ac (21,22). The dynamicity of transcriptional enhancers remains however, in this context, a salient and well recognized problem (22), which eludes also the power of recent techniques derived from chromatin conformation capture (3C), such as Hi-C, that instead permit identification of topologically associated domains (TADs) mediated by CCCTC-binding factor (CTCF) and cohesin (23), largely conserved across cell types and even across species (24). Indeed, the observation that the removal of CTCF and cohesin, albeit impacting TAD structures, does not dramatically alter global transcription has yielded a model in which TADs are not responsible for general transcriptional regulation but play a role in inducible gene expression driven by environmental stimuli and/or developmental cues. This was confirmed through the ablation of cohesin in macrophages and hematopoietic stem and progenitor cells (HSPCs), which impaired their capacity to activate inflammatory gene expression and HSPCs differentiation following lipopolysaccharide treatment (25).

Such chromatin domains are mainly formed through two main proposed mechanisms: i) the loop extrusion model, in which DNA is actively extruded through a cohesin ring, until two CTCF molecules respectively bound to CTCF convergent binding sites are found (26,27), and ii) a phase separation model in which BRD4 and proteins with intrinsically disordered regions interact with each other inducing a phase separated region, in which the local concentration of transcription factors ends up being higher than that for the freely diffusing ones (28). The loop extrusion model is mainly responsible for TAD formation and boundaries between physically interacting regions found within chromosome compartments. Moreover, CTCF is also found inside TADs, together with YY1, mediator, and cohesin, where it may contribute to cell-specific enhancer–promoter interactions (29–31). Indeed, mutations of mediator and cohesin proteins themselves can lead to NDDs (32,33).

Several models of how enhancers exert their regulatory function have been proposed (24): the active ones are known to be transcribed, independent of their distance from their target genes. Among them, the closest ones (within the kb range) typically loop with target promoters, while a consensus on the mode

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of activity of the farther ones is not known. In fact, they might act solely by virtue of being transcribed into RNA molecules which, in specific cases, have been observed to bind chromatin regulatory complexes and mediate chromosomic structure rearrangements along distant interactions. Another mode of action of distal enhancers is suggested by the fact that small RNAs, upon binding, can modulate crucial chromatin regulators such as the Creb-binding protein and polycomb repressive complex 2 (34,35). Still concerning distant enhancers, the possibility that they might also act by attracting proteins responsible for introducing rigidities and steric hindrances impacting on the chromosomal organization cannot be *a priori* excluded. Moreover, when looping is schematized and graphically represented, the double strand nature of DNA is often not considered, and the effective binding mode of transcription factors and RNAPII is often forgotten. To this end, computational work by Hegyi posits the hypothesis that transient 4G quadruplets, formed through an equal contribution of two 2G sequences by a couple of promoter and enhancer binding as single strands, might play an important role to transcriptional regulation (36).

Disease modeling, human-derived 3D cultures and single-cell omics

In the last decade, the power of patient-derived induced pluripotent stem cells (iPSCs), coupled with an expanding range of differentiation paradigms, has enabled the attainment of increasingly sophisticated *in vitro* recapitulations of pathologically affected tissues (37). In the context of neurodegenerative and neuro-developmental disorders studies, this fast-evolving field has moved from a more heterogeneous dual SMAD inhibition protocol to the ability of quickly producing homogeneous cultures of adult cortical neurons (38,39). A crucial further advancement has been posed by stem-cell derived 3D human brain organoids (40). These models have the ability to robustly recapitulate, in terms of cellular composition and connectivity, the districts of the brain that are mostly affected in each condition, both separately and assembled (40).

Most cases of ID and ASD appear in a syndromic form, and given their secondary traits, such as craniofacial features, peripheral nervous system impairment and cardiovascular defects, many of them can also be included under the rubric of neurocristopathies. In fact, neural crest stem cells migrate in the very early stages of development to populate and constitute several districts of the body after several specific differentiation stages (41–44). The advent of somatic cell reprogramming (45,46) allows us now to ask fundamental questions regarding shared and specific vulnerabilities of the neural crest during development (47–50), with models of different types of neural crest cells have recently become available (51).

Mutations of epigenetic factors that cause NDDs generally come in the form of haploinsufficiencies, and knock-out of the same genes in cell lines often results in lethality (6). These disorders affect only certain organs and tissues, in a dynamic and time-specific fashion. Single-cell RNA-seq has further highlighted how certain mechanisms and conditions can be highly cell-type specific (52,53). Moreover, individual genetic backgrounds are strongly associated with enhancer and chromosomal organization, as recently demonstrated by a thorough characterization of human single nucleotide polymorphisms (54). This further supports the previous observation that, to precisely identify and describe relevant transcriptional differences in the human context, a substantial number of biological replicates is required (55).

The genome-wide focus at the level of individual cells has gained momentum and is catalyzing a technological revolution that is rapidly extending such gaze beyond the transcriptome to encompass several other omic layers (56). Such technologies become fundamental in order to reconstruct the epigenetic circuitries defining the convergences of NDDs. Even recent techniques like HiC have advanced to the single-cell domain (single-cell HiC), and their application has revealed a foundational heterogeneity in chromosomal contacts defining TADs (31,57–59) and highlighted how TAD structures can be largely conserved across species and along evolution, above the megabase scale, while while typically less conserved at smaller levels. Nevertheless, HiC is a technique that does not allow for a nucleosomic resolution, which has been made possible only by micro-HiC, which employs micrococcal nuclease instead of restriction enzymes, and it has been proposed to obtain a resolution up to ∼100/200 nt (60).

NDDs converge beyond types of genetic lesions and affected chromatin remodelers

By analyzing in a concerted way, along *in vitro* differentiation, patient-derived iPSCs, carrying a diverse set of mutations that cause developmental disorders with shared clinical manifestations, one could aim at identifying critical shared and exclusive nodes of transcriptional (and later on functional) dysregulation caused by mutations in chromatin modulators.

Among NDDs caused by mutations or CNVs affecting chromatin regulators, we selected some for their partially overlapping clinical manifestations as a template example (Table 1). Activity Dependent Neuroprotector Homeobox (ADNP) is mutated in Helsmoortel van der AA syndrome (HVDAS) (ADNP-ASD or HVDAS), with a 0.17% estimated prevalence of pathogenic variants in individuals diagnosed with ASD, thus defining it as one of the most commonly mutated single genes causing autism (61). Patients with mutations in this gene also share characteristic faces with a high hairline, prominent forehead, eversion or notch of the eyelid, broad nasal bridge, and thin upper lip. BAZ1B and GTF2I are both located in the Williams Beuren syndrome chromosome region (WBSCR), spanning around 27 genes on chromosome 7. CNVs of this region, which is flanked by highly repeated sequences, lead, during chromosome crossing-over at meiosis, to a partial or complete hemizygous deletion or duplication of the WBSCR, causing Williams Beuren syndrome (WBS) (62–64) or 7q11.23 micro-duplication disorder (7DupASD), respectively (65,66). WBS features broad forehead, bitemporal narrowing, wide mouth, full lips, small jaw, and prominent earlobes. Usually, WBS individuals also show small spaced teeth and Iris stellate, which is a very common characteristic. In contrast to WBS, 7DupASD can be subtler in cranio-facial phenotypic terms and is supposed to have been underdiagnosed (63,64,67). Nevertheless, when spotted, 7DupASD patients' facial features are usually opposite to those of WBS patients. Intellectual abilities are only partially impaired in both disorders in a symmetric way, with WBS individuals showing hypersociability and good grammar skills compared to individual with comparable overall ID, and 7DupASD showing social withdrawal and severe language impairments. Additionally, EED, EZH2, and SUZ12 mutations have been found to be causative of Weaver syndrome (WS), while KMT2D and KDM6A mutations cause Kabuki syndrome (KS). It

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Table 1. Neurodevelopmental disorders show shared and unique clinical features

Clinical	Williams Beuren Syndrome	7q11.23 μDup	Cornelia De Lange Syndrome	Kabuki Syndrome	Weaver Syndrome	GADEVS	ADNP ASD	Summary
Intellectual disability	+	+	+	+	+	+	+	*******
Visuospatial impairment	+	+	+	optical	-	-	+	*****
ASD features	rare	+	rare	rare	-	-	+	****
Hypersociability	+	-	-	-	-	-	-	**
Craniofacial dysmor-phism	+	+	+	+	+	+	+	******
Cardiovascular defects	+	+	+	+	+	rare	+	******

* = number of NDDs featuring the given clinical trait

is noteworthy that these two syndromes are respectively caused by mutations in genes either implicated with transcription silencing (Polycomb Repressive Complex 2) or enhancement (COMPASS complex) and show several opposite traits, such as tall vis a vis short stature and distinctive cranio-facial features (68–73). WS features macrocephaly, tall stature and micrognathia, with mild ID, while its facial features can include a broad forehead, hypertelorism, large low-set ears and dimpled chin. KS features long and narrow lid fissures, and the lateral third of the lower lids are often everted. Eyebrows are highly arched and broad with some sparsity, especially in the lateral portion and thick eyelashes. Ptosis and strabismus are both quite recurrent. Cleft lip and palate are seen in about a third of patients, and the palate is highly arched in most of them. Similarly to WBS, teeth are usually small and widely spaced, but they can also be malformed. Cornelia de Lange syndrome (CdL) is caused by mutations in cohesin subunits and partners such as NIPBL, SMC1A, HDAC8, RAD21, and SMC3. Characteristics of these disorders largely overlap with those of KS including small stature, microcephaly, and small and widely spaced teeth (74).

Finally, YY1, as previously mentioned, is mutated in the recently characterized Gabriele-De Vries syndrome (GADEVS) (17), which features low-set ears and general fullness to the periocular area. Alike WBS and KS patients, slant downward lid fissures and strabismus are often present.

Among these disorders, CdL and GADEVS can serve as exemplary cases in which NDDs unmask a higher level of convergence than "enhancer" regulation. In fact, these two disorders are caused by mutations of genes involved in the regulation of mechanisms that have been used in a complementary manner but also alternatively to explain transcriptional regulation through enhancers. Namely, by affecting cohesin subunits and its loading effector, CdL is characterized by phenotypic manifestations that are comparable to GADEVS ones, which instead is caused by mutations of YY1. Cohesin is fundamental for loop extrusion models, working in concert with CTCF, while YY1 is at the base of enhancer–promoter association and has been involved with super-enhancer states, which rely on mechanisms of phase separation (28).

Discussion

The ontological rationale for grouping several NDDs under the rubric of enhanceropathies has contributed to our understanding of their pathogenic mechanism and has enabled relevant first strides toward the elucidation of their convergence. The unifying traction of this notion is coming, however, under increasing tension due to our rapidly evolving understanding of transcriptional regulation and the multiple scales that such understanding must confront.

First, the relevance of chromosomes' 3D structure for the regulation of gene expression is being dissected at accelerated pace and ever-increasing resolution, triggering a fundamental reassessment of the hierarchical relationship between this level of organization and previously defined enhancer/promoter-centered models. In this respect, cohesins have been shown to be necessary for the formation of TADs, and they have been identified as the main, albeit not the only, building blocks of genome 3D organization. In fact, accumulating evidence shows that the formation of chromosome territories — regions of the nucleus where chromatin partitions are active and closely regulated (75) — is independent of TADs. In turn, among chromatin architects, CTCF and YY1 have been shown to define, respectively: i) insulation and TADs boundaries, and ii) looping of enhancers and promoters (Fig. 1A). The relationship between these architectural features and the actual process of transcription is itself however highly dynamic and appears to be increasingly mutually constitutive. Thus, 1) chromatin looping was found to be stabilized by active transcription (76), 2) promoter–promoter interactions can also serve the purpose of enhancing transcription, and 3) enhancers are themselves transcribed, ushering in a rich tapestry of imbricated regulatory controls recently condensed in the notion that genomic locations can possess shades of "promoter-ness" and "enhancer-ness" (77).

A case in point, in this ongoing reassessment of regulatory hierarchies with the attending disentanglement of architectural structures from functional readouts, is the current debate on the overlap between the notion of "transcription factories" and the more recent phase-separation-based models of transcriptional control. Indeed, the concept of "transcription factories" (78) largely predates the phase-separation model that is currently on the rise and may serve to sharpen some elements of the enhancer discourse. The two notions are in fact clearly distinguishable, at least in their origins. "Transcription factories" describe the basic mechanism of aggregation of the transcriptional machinery around active genes, hence the formation of nuclear compartments that include regulatory elements, RNAPII, and eventually other complementary proteins

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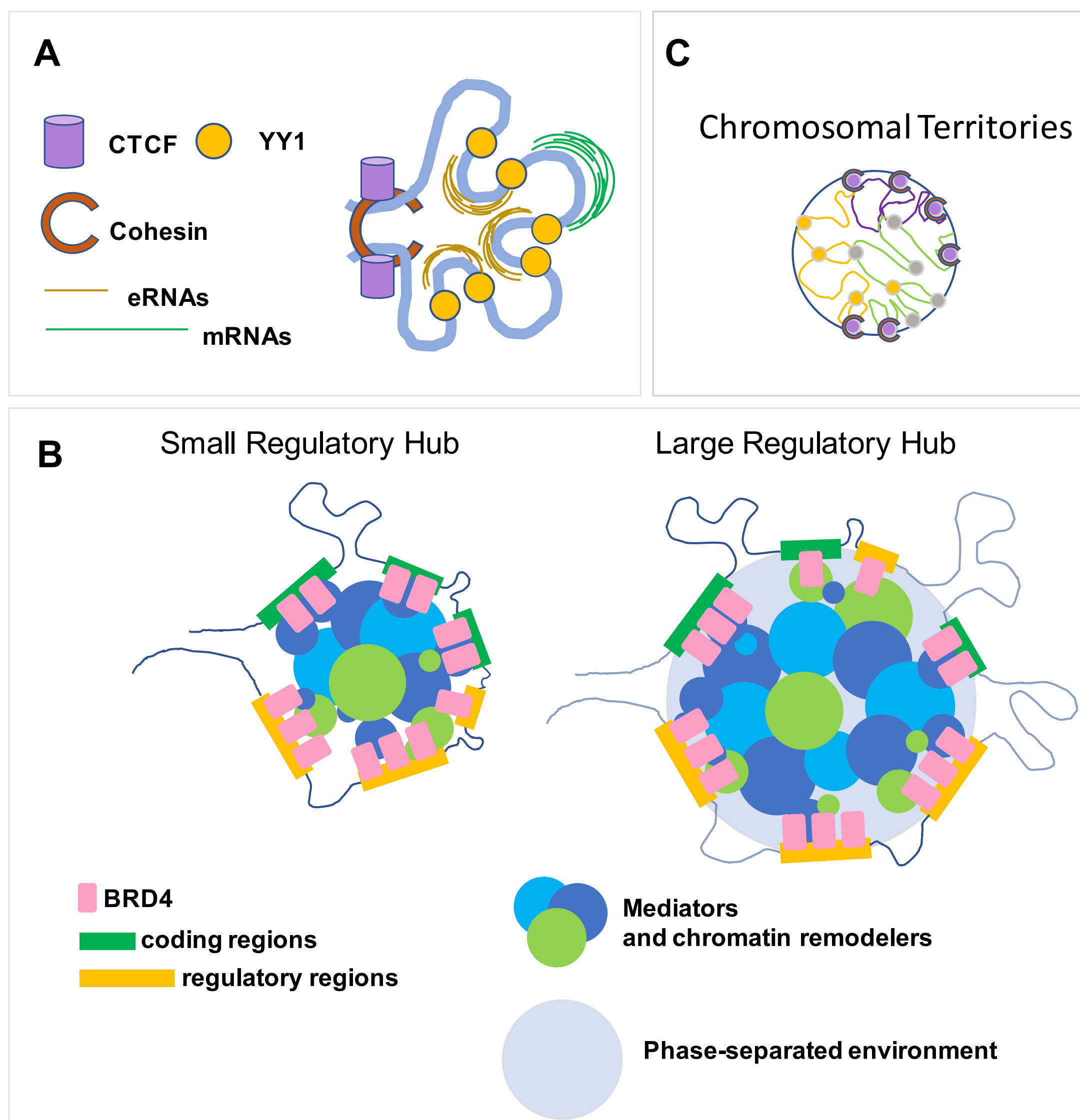

Figure 1. Schematic representation of the main models of chromatin architecture. (**A**) The loop extrusion model requires two CTCF binding chromatin in opposite orientation, blocking cohesin and fostering the formation of loops by extrusion through cohesin ring. YY1 is depicted binding enhancer–promoter connections. eRNAs and mRNAs produced by the transcription of enhancers and gene body are depicted. (**B**) Regulatory hubs [drawing inspired by Sabari *et al.* (28)]. Small ones may or not form condensates, while large have been observed. (**C**) Cohesin (orange) and CTCF (in purple) favor the formation of TADs and chromosomal territories. Inside these macro-partitions, YY1 (in yellow) and other factors (in grey) determine active and inactive subcompartmentalization.

defining independent subnuclear compartments. The phase-separation model identifies instead the specific situation in which large and abundant regulatory regions are combined with chromatin modulators possessing intrinsically disordered tails (IDTs), favoring dynamic and highly specific protein complexes formation. In order to form, such condensates need thresholds of physicochemical requirements, such as the concentration of certain histone marks and histone-binding proteins themselves. We argue however that, although the two concepts are originally distinct, in terms of both their historical and epistemic roots, the basis of the process giving rise to either the historically defined transcriptional factories or the currently investigated phase-separated compartments, is the same mechanism, namely, the capacity of bromodomain proteins (BRD4 *in primis*) to recognize active enhancer marks, such as H3K27ac. Once multiple BRD4 proteins (or the likes) bind H3K27ac tails, they start to build multimeric clusters (with the help of their Asn- and Pro-rich amino acidic residues composition), further attracting IDT-featuring partners. Such condensate would acquire physicochemical and biochemical characteristics capable of enhancing the gathering of specific chromatin and transcriptional modulators. This mechanism might occur in small hubs of regulatory and coding regions, without giving rise to detectable condensates, as well as in large ones with full-fledged outcome (Fig. 1B). Such principles could easily be applied also to poised and inactive regulatory regions, where alternative histone modifications and chromatin binding proteins could respectively favor the rapid access to “poised” or “repressive” transcriptional machineries. In the latter case, the sudden removal of active marks could ablate the presence of active transcribers and their substitution by Polycomb repressive complexes.

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A second challenge for the heuristics of the notion of "enhanceropathies" comes from the challenge of identifying enhancers by intersecting multiple analytical techniques, only some of which currently permit to tackle the structural-functional cross-talk we outlined above at relevant resolution. Thus, on the one hand, the identification of enhancers still requires the combination and validation through orthogonal techniques such as classical genetics, biochemistry, super-resolution imaging and genomics (24,79). However, precisely because chromatin conformation capture and ChIP techniques rely on cell collection and destruction, live imaging such as super-resolution imaging and single-molecule tracking is needed to study chromatin loops and enhancer–promoter dynamics during development (76,80–82). On the other hand, the more we deepen our gaze into the molecular details of the hierarchies of transcriptional control, the more the problem of enhancer identification evolves into a definition issue. In fact, we are witnessing the historical moment of convergence of three crucial developments: the understanding of what enhancers really are, the increasingly resolved molecular characterization of transcriptional regulation, and finally the ongoing appreciation of the multi-dimensional nature of the genome. Thus, while the search for distal elements positively and temporally affecting transcription (i.e. enhancers) has transformed the field of developmental biology, the availability of single-cell resolved tools and the realization that several NDDs converge, in terms of their causative mutations, at a genome-architecture level beyond the limits of enhancer definition, call for a paradigm shift. Specifically, we propose that a contemporary roadmap for understanding NDDs in a patient-tailored and actionable manner should entail the following: i) increasing the resolution of our molecular enquiries via single-cell and single-molecule methods in patient-specific, physiopathologically relevant 3D models of brain development; ii) changing our focus, from "identified enhancers" along with the specific mutated chromatin modulators underlying each condition, to the derangements in chromatin architecture that accrues in those models at the meaningful developmental stages, thereby shifting up one layer our quest for convergence; and iii) changing our theoretical framework accordingly, not to replace the established working merits of the notions of enhancer and enhanceropathies, but to embrace the need of defining types of enhancers, and eventually new types of regulatory regions, to realize a less enhancerocentric and more structure-aware vision of genome regulation. We would thus like to propose, taking cues from the knowledge available thus far on NDDs, that the regulation underlying human cognition at the level of chromatin and transcriptional regulation is productively captured by the notion of the manifold, a multi-dimensional object whose constituents can be projected in an Euclidean space. The identification of local interactions between genomic regions and proteins binding to them, along with the reconstruction of their supramolecular structures, can thus help define 3D epigenetic neighborhoods that build, along development, our 4D genome. In this view, what enables the deployment of our cognitive functions is thus the constant and temporally fine-tuned cross-talk within and between chromosomal territories and their subcompartments (Fig. 1C), unfolding through neural development at the level of individual cells in their mutual set of proximal and distal connections.

Conflict of Interests statement.

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