

Balanced chromosomal rearrangements offer insights into coding and noncoding genomic features associated with developmental disorders

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ABSTRACT

Balanced chromosomal rearrangements (BCRs), including inversions, translocations, and insertions, reorganize large sections of the genome and contribute substantial risk for developmental disorders (DDs). However, the rarity and lack of systematic screening for BCRs in the population has precluded unbiased analyses of the genomic features and mechanisms associated with risk for DDs versus normal developmental outcomes. Here, we sequenced and analyzed 1,420 BCR breakpoints across 710 individuals, including 406 DD cases and the first large-scale collection of 304 control BCR carriers. We found that BCRs were not more likely to disrupt genes in DD cases than controls, but were seven-fold more likely to disrupt genes associated with dominant DDs (21.3% of cases vs. 3.4% of controls; $P = 1.60 \times 10^{-12}$). Moreover, BCRs that did not disrupt a known DD gene were significantly enriched for breakpoints that altered topologically associated domains (TADs) containing dominant DD genes in cases compared to controls (odds ratio [OR] = 1.43, $P = 0.036$). We discovered six TADs enriched for noncoding BCRs (false discovery rate < 0.1) that contained known DD genes (*MEF2C*, *FOXG1*, *SOX9*, *BCL11A*, *BCL11B*, and *SATB2*) and represent candidate pathogenic long-range positional effect (LRPE) loci. These six TADs were collectively disrupted in 7.4% of the DD cohort. Phased Hi-C analyses of five cases with noncoding BCR breakpoints localized to one of these putative LRPEs, the 5q14.3 TAD encompassing *MEF2C*, confirmed extensive disruption to local 3D chromatin structures and reduced frequency of contact between the *MEF2C* promoter and annotated enhancers. We further identified six genomic features enriched in TADs preferentially disrupted by noncoding BCRs in DD cases versus controls and used these features to build a model to predict TADs at risk for LRPEs across the genome. These results emphasize the potential impact of noncoding structural variants to cause LRPEs in unsolved DD cases, as well as the complex interaction of features associated with predicting three-dimensional chromatin structures intolerant to disruption.

INTRODUCTION

Balanced chromosomal rearrangements (BCRs), including translocations, insertions, and inversions, are a unique class of rare genomic variation that occur roughly five-fold more frequently in individuals with developmental disorders (DDs) than in the general population.¹⁻⁶ Delineation of BCR breakpoints has long represented an approach to discover novel disease genes,^{7,8} and has been accelerated by innovative methods using whole genome sequencing (WGS) with long-inserts (liWGS) to capture BCR breakpoints.⁹⁻¹⁶ Our previous WGS analyses suggested that

26.6% of cytogenetically visible BCRs contribute to risk for DDs due to direct gene disruption.¹¹ The observation, while substantial, also implies that alternative mechanisms of disease are likely to be mediating additional genetic risk for DDs due to BCRs. However, the lack of sufficient sample sizes, and the virtual absence of large cohorts of unaffected control BCR carriers with sequence-resolved breakpoints, have precluded a systematic evaluation of the full spectrum of pathogenic mechanisms associated with BCRs to date.

Beyond the direct disruption of disease-associated protein-coding genes, emerging evidence has begun to implicate a small number of noncoding elements in the etiology of DDs. Recent analyses have emphasized the challenges with the statistically rigorous genome-wide discovery of rare and *de novo* noncoding regulatory risk variants, including their small average effect sizes, the lack of a cipher equivalent to trinucleotide codons for variant interpretation, and the large number of noncoding functional categories that could be tested.^{17–19} Nonetheless, there are examples of noncoding variants with strong regulatory consequences and considerable influence on risk for DDs,^{19–21} including long-range positional effects (LRPEs) that result from disruption of or topological associating domains (TADs)^{20,24–26} or long intergenic noncoding RNAs (lincRNAs).^{22,23} The disruption of TADs, which are megabase-sized regulatory domains of folded chromatin that contain most *cis*-regulatory interactions,^{27–30} can lead to loss of physical connections between enhancers and their target genes^{24,25} and/or the generation of ectopic enhancer-promoter contacts through a process known as “enhancer adoption”.^{31,32} While relatively few studies have systematically evaluated the contribution of rare noncoding variants to risk for disease, BCRs represent a unique class of highly penetrant genomic variation from which we might begin to understand the mechanisms of pathogenic noncoding variants in DDs given the outsized impact of BCRs on genome structure and function.^{11,32,33}

In this study, we analyzed 1,420 BCR breakpoints from 710 unrelated individuals, including 406 DD cases as well as the first large-scale sequence-resolved cohort of 304 unaffected BCR carriers (*i.e.*, controls), and evaluated a range of mechanisms by which BCRs may increase risk for DDs. Our analyses revealed a series of significant chromosomal, genic, and noncoding loci associated with DDs, as well as features that distinguished BCRs occurring in DD cases versus unaffected controls. In addition to refining DD risk estimates for BCRs directly disrupting dominant DD genes, we identified six TADs significantly associated with recurrent disruption by noncoding BCRs in DD cases, representing strong-effect LRPEs in DDs. Collectively, 7.4% of our DD cohort harbored a noncoding BCR breakpoint that disrupted one of these six TADs. We also defined a subset of core genomic features that, when considered together, can aid in the interpretation of DD-associated LRPEs throughout the genome.

RESULTS

International aggregation, sequencing, and genome-wide analyses of BCRs in 406 DD cases and 304 controls

We have previously shown that chromosomal rearrangements that appear balanced at cytogenetic resolution can involve extensive complexity ranging from multiple cryptic breakpoints to balanced chromosomal shattering, or chromothripsis, at sequence resolution.^{11,15,34} Here, we focused analyses on the most interpretable classes of BCRs by aggregating a cohort of 710 unrelated individuals harboring a “simple” BCR (*i.e.*, breakpoints at two genomic positions without significant imbalance or additional complexity) initially identified by cytogenetic methods and subsequently resolved using either short-insert or long-insert WGS (Fig. 1A; Supplementary Fig. 1; Supplementary Methods). This cohort included 406 cases diagnosed with a DD or congenital anomaly in which a BCR was confirmed to have arisen *de novo* or segregate with phenotype and 304 unaffected control adults with no early-onset pediatric phenotype (see Fig. 1B and Supplementary Table 1 for complete descriptions).

Over 55% of the BCR breakpoints have not been previously published. As a comparison for these 710 empirically-identified BCRs ($n = 1,420$ breakpoints; Supplementary Table 2), we also generated a set of 30,400 simulated BCRs under the null hypothesis that breakpoints should be randomly distributed throughout the genome. These simulated BCRs were randomly sampled *in silico* from the genome while matching properties of the 304 BCRs empirically identified in controls, including structural variant (SV) type, inversion size, as well as excluding N-masked regions known to be inaccessible to short read alignments (Supplementary Methods).^{35,36}

We first sought to understand the global patterns of BCRs throughout the genome by comparing the rates of breakpoints per chromosome between cases, controls, and simulations (Supplementary Fig. 2). We found that BCR frequency was approximately proportional to chromosome length with two exceptions: translocations were enriched on chromosome 14 in DD cases ($P = 5.8 \times 10^{-6}$ for cases vs. random simulations) and were depleted on chromosome X in controls ($P = 1.4 \times 10^{-6}$ for controls vs. random simulations) (Fig. 1C and Supplementary Fig. 2). The distribution of breakpoints across chromosome 14 did not appear to cluster in any particular location (Supplementary Fig. 3) and we did not find any features (*i.e.*, compartment state, replication timing, recombination frequency, or gene disruption) that could account for the enrichment. In contrast, when we subset chromosome X, we observed that both cases and controls were 2.7-fold to 5.3-fold depleted for breakpoints on the q-arm (Supplementary Fig. 2 and 3). The Xq depletion in controls may be partly explained by the exclusion of males with oligo/azoospermia and females with premature ovarian failure given their known association with X-autosome translocations.^{37,38} However, most of our DD cases were too young to be assessed for infertility, thus the Xq depletion in cases is unlikely to be related to a similar ascertainment bias. Overall, we identified that most (65%; 20/31) translocation breakpoints on chromosome X localized to the p-arm (length=58.6 Mb), which exhibited a 6.9-fold enrichment of case vs. control breakpoints ($P = 0.002$). Interestingly, 90.0% (18/20) of the Xp translocations identified in DD cases were found in females. Finally, controls were depleted for translocations involving either sex chromosome: just 2.1% of control translocations involved either chromosome X or Y, which was significantly less than translocations in DD cases (8.9%; $P = 9.2 \times 10^{-4}$) or randomly simulated BCR carriers (12.1%; $P = 5.86 \times 10^{-7}$) (Fig. 1D).

Our assessments of BCR breakpoint distributions per chromosome led to two additional discoveries. First, a single cytoband on chromosome 17 was significantly enriched for BCRs in cases ($P = 1.2 \times 10^{-5}$ vs. random simulations) and surpassed a genome-wide significance threshold adjusted for all 862 cytobands tested across all chromosomes (Fig. 1E; Supplementary Fig. 4).³⁹ This cytoband, 17q24.3, matches the location of a well-described pathogenic LRPE in DDs and congenital anomalies caused by SVs altering the local TAD organization and dysregulating SOX9 and KCNJ2.^{24,25} Second, after transforming the position of each breakpoint into a percentile relative to the length of its corresponding chromosome arm (*i.e.*, meta-chromosome), we found that translocation breakpoints in controls were biased towards the most distal ends of chromosomes (Kolmogorov-Smirnov test; $P = 0.002$ for control vs. simulation and $P = 0.021$ for control vs. cases; Fig. 1F; Supplementary Fig. 5). For example,

control BCR breakpoints were roughly three-fold enriched within the terminal 2% of each chromosome arm: 2.7-fold vs. DD cases and 3.3-fold vs. random simulations. This might suggest that translocations occurring near telomeres—which do not rearrange most of the affected chromosome—are more likely to be tolerated in the general population without leading to severe disease.

We also sought to identify genomic features that predispose to BCR formation by annotating all BCR breakpoints with features relating to chromosome maintenance (e.g., recombination rate, replication timing), chromatin accessibility, sequence context (e.g., repetitive elements, sequence homology), and three-dimensional (3D) nuclear organization (e.g., Hi-C contact frequency, nuclear compartment state).⁴⁰ Most features showed no significant differences from expectations after correcting for multiple testing. One feature of note was that our empirically-observed translocations (*i.e.*, those sequenced in DD cases and controls) were slightly more likely to form between pairs

of chromosomes in close proximity to each other in 3D within the nucleus than predicted from simulated breakpoints that did not account for this biological organization (1.08-fold increase; $P = 0.002$; **Fig. 1G**). This result was true in a fetal lung fibroblast cell line (IMR90) and replicated in a second dataset derived from embryonic stem cells.⁴⁰ These findings might suggest a weak influence on the formation of BCRs between chromosomal regions that co-localize within the nucleus, as suggested by analyses of tumor genomes and cytogenetic data from germline BCR carriers.⁴¹

BCRs in DD cases are strongly enriched for direct disruption of established disease genes

Previous studies have demonstrated that BCRs confer substantial risk for DDs through direct disruption of haploinsufficient, developmentally critical genes.^{8,11,15} However, the absence of matched cohorts of unaffected control BCR carriers has historically hindered the quantification of disease risk contributed

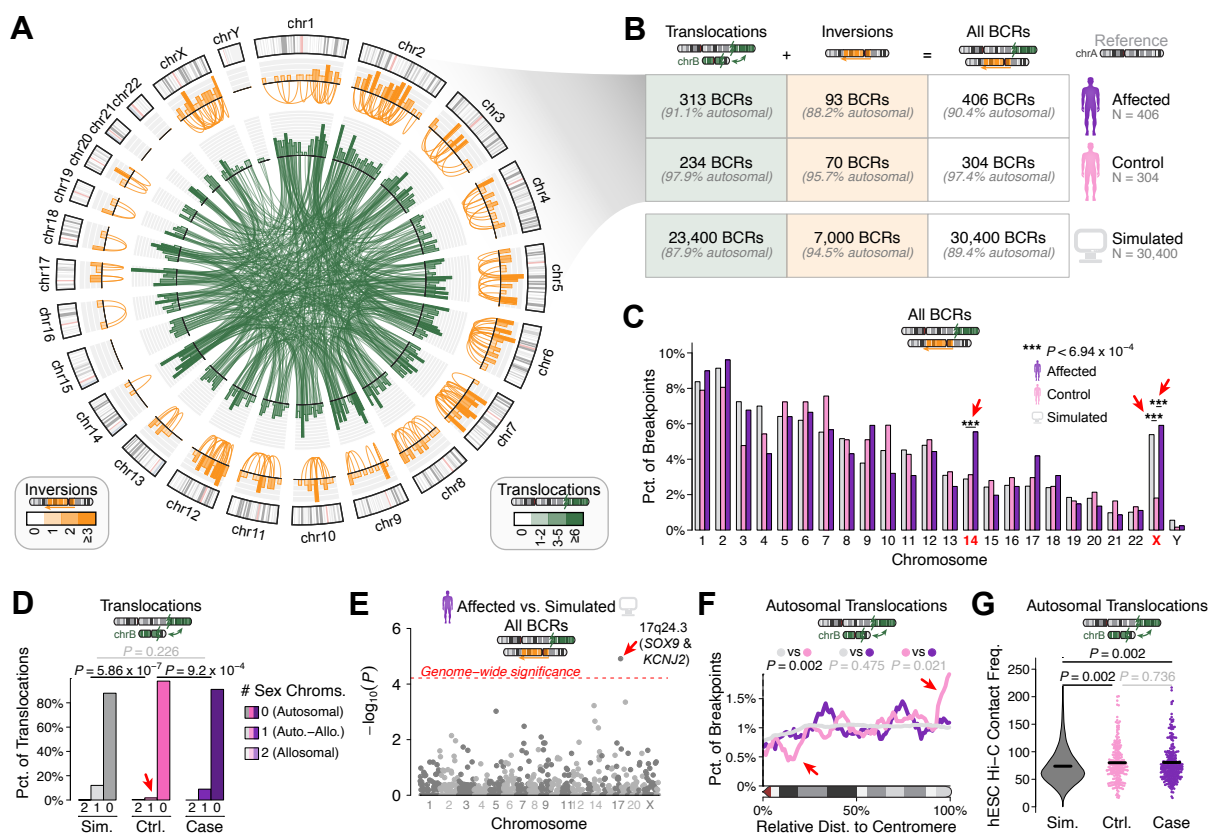


Fig. 1 | The properties of BCRs in the healthy human germline and DD cases

(A) We mapped the breakpoints of 710 simple (*i.e.*, two-breakpoint) BCRs that were originally detected with cytogenetic methods. Here, we provide the genome-wide BCR breakpoint density in 10Mb windows per chromosome. (B) These 710 BCRs were identified in the genomes of 406 individuals affected by DDs and 304 unaffected controls. For purposes of comparison, we also generated 30,400 synthetic BCRs *in silico* by resampling the distribution of control BCRs 100 times randomly from the genome. (C) BCR breakpoints were distributed across the chromosomes as expected between affected, control, and simulated subsets except for chromosome 14, which exhibited a significant enrichment of breakpoints in affected samples, and chromosome X, which was depleted of breakpoints in controls. All comparisons were Bonferroni-adjusted for 72 total tests. (D) Balanced translocations involving at least one sex chromosome were significantly depleted in control samples compared to either affected samples or simulated null expectations. (E) We conducted association tests per cytoband for BCR breakpoints in affected samples vs. simulated null expectations. After correcting for 862 independent tests, just one cytoband was significant at the Bonferroni-adjusted threshold of $P \leq 5.8 \times 10^{-5}$: 17q24.3, which corresponds to a locus with well-described LRPEs in DDs involving *SOX9* and *KCNJ2*.^{24,25} (F) The distribution of autosomal BCR breakpoints in controls across a “meta-chromosome” arm (*i.e.*, chromosome size-normalized position) was significantly different from those of affected samples or simulated null expectations, with controls exhibiting depletions near centromeres and enrichments very close to telomeres. (G) Loci corresponding to empirically identified autosome-autosome translocation breakpoints (*i.e.*, those sequenced in affected or unaffected genomes) contacted each other in 3D within the nucleus of human embryonic stem cells 1.08-fold more frequently than expected vs. simulated null expectations.

by gene-disruptive BCRs. Here, we annotated all BCR breakpoints for direct gene disruptions using Gencode v19 and compared the frequency of gene-disrupting autosomal BCRs between DD cases, controls, and random simulations.⁴² Most BCRs disrupted at least one protein-coding gene and there was no difference between cases and controls (68.1% of DD cases and 67.6% of controls) or expectations from random simulations (69.0% expected; **Fig. 2**). We further subdivided protein-coding genes into four tiers based on the evidence for their association with disease (**Supplementary Table 3**). Briefly, these included genes associated with dominant DDs (*i.e.*, Tier 1, $n = 812$), genes associated with all other diseases (Tier 2, $n = 3,129$), mutationally constrained genes with no prior disease association (Tier 3; $n = 1,257$), and all remaining protein-coding genes (Tier 4; $n = 15,188$). We found a strong enrichment of cases with BCR breakpoints directly disrupting Tier 1 genes compared to controls (21.3% of cases versus 3.4% of controls; odds ratio [OR] = 7.45; 95% confidence interval [CI] = 3.74-16.50; Fisher's exact test; $P = 1.60 \times 10^{-12}$) and compared to simulations (21.3% of cases vs. 6.6% of simulations; OR = 3.67; 95% CI = 2.80-4.76; $P = 2.06 \times 10^{-18}$), but not for Tiers 2-4.

Motivated by the strong association between disruption of dominant DD genes and BCRs in cases, we systematically searched for genes that were recurrently disrupted by BCRs in cases beyond expectations by conducting association tests for each autosomal gene (**Supplementary Table 4**). These analyses identified four protein-coding genes disrupted in at least three independent DD cases and none in controls (**Table 1**). Among these, just one gene, *TCF4*,^{11,15} surpassed a strict exome-wide significance threshold (disrupted in 1.6% of DD cases vs. 0.01% of simulated BCRs; $P = 4.3 \times 10^{-10}$; OR = 149.5). Haploinsufficiency of *TCF4* is the dominant genetic cause of Pitt-Hopkins Syndrome and has been associated with autism spectrum disorder (ASD) and broadly defined neurodevelopmental disorders (NDDs).^{43,44} The three remaining protein-coding genes did not reach exome-wide significance but had suggestive ($P \leq 0.005$) evidence of association with DDs in our analyses. These included two established DD genes (*AUTS2*, *MBD5*)^{45,46} and one candidate DD gene, *CDK6*, which was disrupted in three cases that presented with developmental delay ($n = 3$), speech delay ($n = 2$), microcephaly ($n = 2$), and cardiac defects ($n = 1$). *CDK6* is highly constrained against damaging point mutations,⁴⁷ is ubiquitously expressed across tissues,^{48,49} and encodes a cyclin-dependent kinase with major roles in skin, blood, and breast cancers.⁵⁰ *CDK6* has also been associated with a recessive form of primary microcephaly,⁵¹ but has not been previously associated with dominant germline disease.

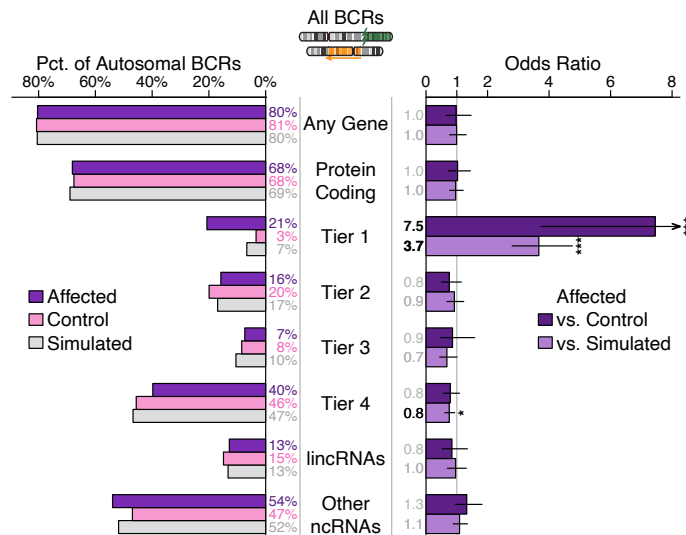


Fig. 2 | Risk conferred for DDs by BCRs disrupting genes and other transcribed loci

We annotated all BCR breakpoints for predicted overlap with protein-coding and noncoding genes present in Gencode v19.¹⁴ Here, we further subset these BCRs based on the properties of the gene(s) disrupted at either breakpoint, including: any gene present in Gencode; protein-coding genes; "Tier 1" genes including those known to be associated with dominant DDs; "Tier 2" genes including all remaining disease-associated genes; "Tier 3" genes including all genes in the top decile of loss-of-function constraint⁴⁷ but with no existing disease association; "Tier 4" genes including all remaining genes not captured in the preceding tiers; lincRNAs; all noncoding RNAs other than lincRNAs present in Gencode. For each subset of genes, we computed the rate of BCRs disrupting at least one qualifying gene between cases, controls, and simulated BCRs, and further computed the odds ratios of cases vs. controls and cases vs. simulated null expectations. Only BCRs disrupting Tier 1 genes were significantly enriched in cases vs. controls and cases vs. simulated BCRs after correcting for multiple comparisons.

Gene Info		gnomAD constraint		Case BCRs		Control BCRs		Simulated BCRs		Cases vs. Simulated	
Symbol	Biotype	LOEUF	Mis. Z	Count	Pct.	Count	Pct.	Count	Pct.	OR	P-value
<i>TCF4</i>	Protein-coding	0.22	4.10	6	1.63%	0	0.00%	3	0.01%	149.64	4.37E-10
<i>AUTS2</i>	Protein-coding	0.23	2.22	4	1.01%	0	0.00%	21	0.07%	14.24	3.14E-04
<i>RP11-562L8.1</i>	lincRNA	NA	NA	3	0.82%	0	0.00%	2	0.01%	111.74	2.30E-05
<i>CDK6</i>	Protein-coding	0.28	3.06	3	0.82%	0	0.00%	6	0.02%	37.28	1.85E-04
<i>MBD5</i>	Protein-coding	0.14	0.86	3	0.82%	0	0.00%	14	0.05%	15.98	1.39E-03
<i>MEF2C-AS1</i>	Antisense transcript	NA	NA	3	0.82%	0	0.00%	14	0.05%	15.98	1.39E-03
<i>RP11-444A22.1</i>	lincRNA	NA	NA	3	0.82%	0	0.00%	24	0.08%	9.32	5.41E-03

Table 1 | Genes recurrently disrupted by BCRs in DD cases

A list of seven genes that are disrupted by BCRs from ≥ 3 DD cases and zero controls. The type ("biotype"), constraint information,⁴⁷ P value (for case versus control and simulated breakpoint comparisons, respectively), and odds ratio (OR) for each gene are also shown. Only one gene, *TCF4*, met a strict exome-wide significance threshold. LOEUF, loss-of-function observed/expected upper bound fraction; Mis., missense; Pct, percent; BCR, balanced chromosomal rearrangement.

Pathogenic positional effects from disruption of three-dimensional chromatin structures

Our analyses identified an unambiguous, strong association between DDs and BCRs disrupting dominant DD genes; however, the majority of autosomal BCRs in DD cases did not disrupt a known disease gene ($n = 289$; 78.7%), and one-third ($n = 113$; 30.8%) did not disrupt any annotated protein-coding gene. We therefore considered three other models by which BCRs might confer DD risk through noncoding mechanisms based on: (i) their disruption of noncoding genes (e.g., lincRNAs), (ii) their linear distance from known disease genes, and (iii) the disruption of TADs containing known disease genes. We excluded all 78 (21.3%) cases and 10 (3.4%) controls with autosomal BCRs that directly disrupted a Tier 1 gene, which we reasoned would largely exclude the confounding influence of BCRs associated with pathogenic effects via direct gene disruption. We first tested whether direct disruption of noncoding genes could be responsible for pathogenic effects in DD cases and observed no difference in the fraction of cases versus controls that disrupted any subgroup of noncoding genes (Fig. 2). We next assessed whether pathogenic LRPEs could be predicted based on the absolute distance between disease genes and BCR breakpoints and observed no difference between DD cases and controls for proximity to any tier of genes (e.g., Tier 1 genes; Kolmogorov-Smirnov test; $P = 0.159$; Fig. 3A-B). These analyses confirm that linear distance to disease genes alone is insufficient to predict pathogenic LRPEs. However, when we tested the third model by comparing the fraction of cases to controls with a BCR breakpoint disrupting a TAD containing genes from each tier, we observed a significant effect for TADs containing Tier 1 genes (OR = 1.43;

95% CI = 1.68-3.18; Fisher's exact test $P = 0.033$; Fig. 3C-D), supporting the role of 3D chromatin topology disruption in pathogenic LRPEs. Given these results, we next performed genome-wide analyses to define the TADs most strongly associated with DD phenotypes.

We searched for specific TADs associated with risk for DDs by evaluating each autosomal TAD identified from a fetal lung fibroblast (IMR90) cell line⁴⁰ for an enrichment of case BCRs against a Poisson null model fit to the distribution of control breakpoints. Overall, we identified 26 recurrently disrupted TADs with suggestive evidence for association with DDs based on a Benjamini-Hochberg false discovery rate (FDR) $q \leq 0.1$ (Fig. 4 and Supplementary Table 5). Five of these TADs surpassed a Bonferroni-adjusted genome-wide significance threshold of 2.2×10^{-5} , including three known LRPE loci at *MEF2C*, *FOXG1*, and *SOX9* (Fig. 5).^{11,14,52-54} These five TADs also remained significant when we compared BCRs from DD cases against simulated breakpoints, suggesting that our models were not simply capturing rearrangement hotspots. Consistent with the finding that most TAD boundaries are tissue-invariant,^{28,29,40} tissue source had no impact on the genome-wide significant TADs (Supplementary Fig. 6). Given that we only removed cases and controls with a direct disruption of a Tier 1 gene, our TAD results represented a combination of true LRPEs, genic effects not previously associated with DDs, and other unknown mechanisms of disease etiology. For example, one of the genome-wide significant TADs was altered by three cases that all directly disrupted *CDK6*, a novel candidate DD gene identified from our exome-wide gene association analysis, suggesting that it likely represents a genic effect and not a LRPE.

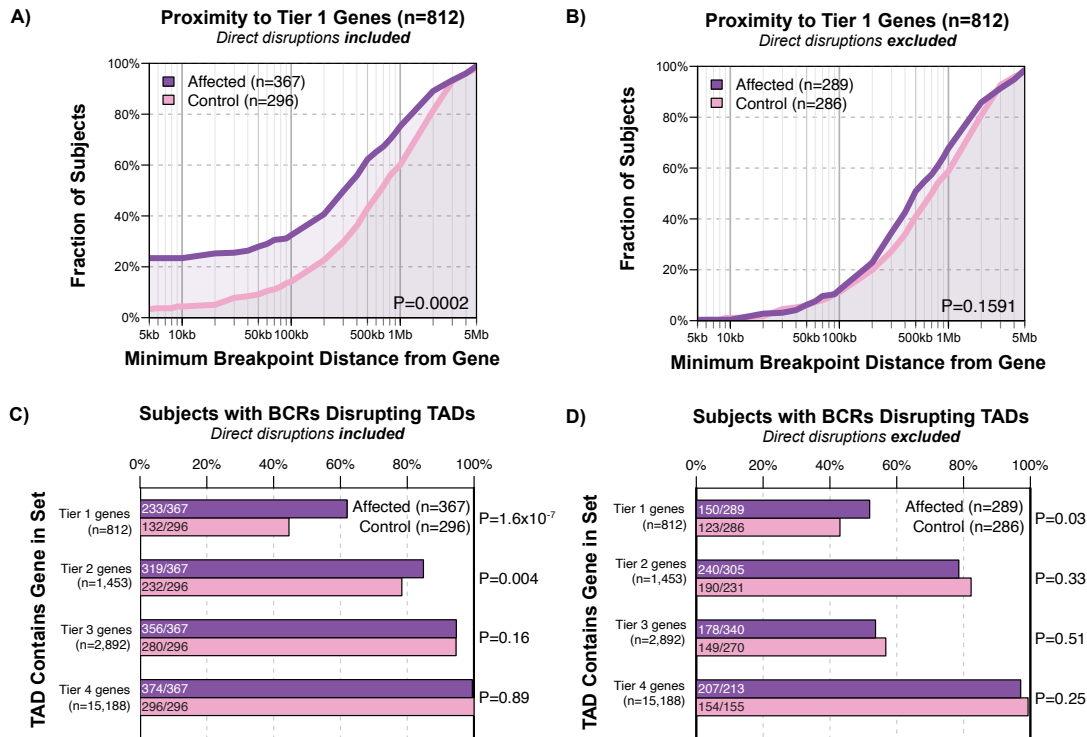


Fig. 3 | Disruption of TADs, and not proximity to known dominant DD genes, is predictive of LRPEs

(A-B) Fraction of cases and controls with an autosomal BCR breakpoint in proximity to a Tier 1 dominant DD gene (Supplementary Table 3) when direct disruption of Tier 1 genes are included (A) and excluded (B). P value corresponds to a two-sample Kolmogorov-Smirnov test. (C-D) Fraction of cases compared to controls with a BCR breakpoint directly disrupting TADs⁴⁰ containing each of the four gene tiers when direct disruption of genes within that gene tier are included (C) and excluded (D).

To identify candidate pathogenic LRPE loci among the 26 TADs with $q \leq 0.1$, we required that each TAD: (i) be disrupted by a noncoding BCR breakpoint (e.g., does not disrupt a protein-coding gene) in at least 50% of cases contributing to the signal, (ii) contain a Tier 1 gene representing a plausible target gene, and (iii) have multiple cases disrupting the same TAD that present with phenotypes that are frequently observed in cases with direct disruption of the candidate target gene (**Supplementary Table 6**). This resulted in six candidate pathogenic TADs containing the known DD genes *SATB2*, *MEF2C*, *FOXP1*, *SOX9*, *BCL11B*, and *BCL11A* (**Fig. 4**). Supporting our statistical enrichments, five of the six significant TADs have been previously associated with pathogenic LRPE loci (*SATB2*, *MEF2C*, *FOXP1*, *BCL11B* and *SOX9*) based on individual case reports,^{11,14,52,54-56} suggesting that we are accessing *bona fide* LRPE signals. The novel candidate LRPE at 2p16.1 contains *BCL11A*, which encodes a zinc finger protein involved in the BAF SWI/SNF chromatin remodeling complex, and has been previously associated with an intellectual disability syndrome.⁵⁷ All four of the cases with noncoding BCRs disrupting the TAD containing *BCL11A* presented with DD or ASD.⁵⁷ Overall, these six TADs were disrupted by 30 cases (7.4%) and one control (0.32%; Fisher's exact test; OR = 21.2; 95% CI 4.5-500.1; $P = 7.12 \times 10^{-7}$), suggesting that they represent highly-penetrant LRPE loci.

Hi-C analyses of individuals with noncoding BCRs disrupting the TAD containing *MEF2C*

We previously implicated the 5q14.3 locus as a putative pathogenic LRPE with *MEF2C* as the target gene based on a statistically significant enrichment of noncoding BCR breakpoints that all disrupted the same TAD containing *MEF2C* and observed down-regulation of this gene in multiple cases harboring the noncoding BCRs.¹¹ Based on these data, we hypothesized that the disruption of 3D topological organization could represent the underlying mechanism for this LRPE. To functionally validate this hypothesis, we generated high-throughput chromatin conformation capture (Hi-C) data from lymphoblastoid cell lines from five cases harboring BCRs disrupting the TAD containing *MEF2C* and developed a 3D resequencing workflow (see **Supplementary Methods and Fig. 5A**) to facilitate analysis of the resulting data. The goal of this workflow was to use Hi-C datasets to: (i) identify single nucleotide polymorphisms (SNPs), small insertions and deletions (indels), and SVs, (ii) phase these variants onto chromosome-length haploblocks, thereby reconstructing the end-to-end sequences of each molecule, and (iii) use the resulting diploid assembly to generate homolog-specific 3D contact maps.

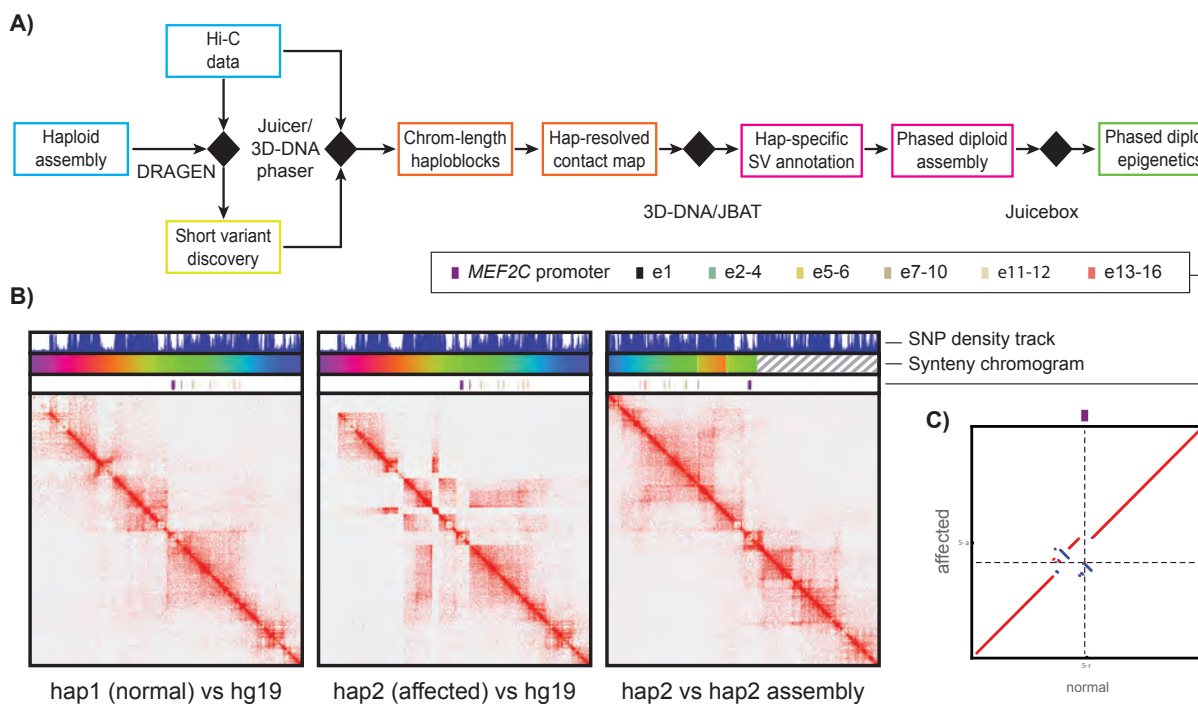


Fig. 5 | Hi-C analysis of DD case with complex BCR disrupting the TAD containing *MEF2C*

(A) The 3D resequencing pipeline starts by using Hi-C data to call short variants (SNPs and indels) against a haploid reference. In this paper we used the DRAGEN software,⁷⁹ but similar results can be achieved with other publicly available variant callers such as GATK.⁸³ We then use the Hi-C alignment data as generated by Juicer⁸⁴ in conjunction with the 3D-DNA phaser to phase the variants and produce chromosome-length haploblocks. The phased variants enable the generation of molecule-specific contact maps, which in turn allow for molecule-specific annotation of SVs. Using the assembly tools from the 3D-DNA/Juicebox Assembly Tools ecosystem we then create assisted assemblies congruent with the annotated SVs and remap the contact data against the new reference to allow for phased diploid epigenetic analyses. (B) Molecule-specific Hi-C contact maps showing DNA-DNA interactions in the vicinity of the *MEF2C* promoter in LCLs derived from patient DGAP101: “normal” haplotype Hi-C data mapped to hg19 reference genome (left); haplotype with a chromothriptic chromosome 5 (middle, notice the numerous signal depletions along the diagonal corresponding to breaks and off-diagonal enrichments in the signal corresponding to fusion points); chromothriptic haplotype remapped against a reference that accounts for the chromothriptic rearrangements (right). The 1D tracks show the phased SNP density, highlight the syntenic regions between the three maps (rainbow colors are reserved for sequences in the vicinity of *MEF2C* in the “normal” reference, while hatching corresponds to sequences juxtaposed into the genomic segment of interest from elsewhere on chromosome 5 in the affected haplotype), as well as show the position of the promoter and 16 known enhancers⁶¹ in the ‘standard’ human reference as well as in the SV-corrected reference. (C) A dotplot of the whole chromosome 5 showing the correspondence between the affected and the normal molecules (100Kb syntenic blocks are used, with direct syntenic blocks colored red, and inverted blocks colored blue). The position of the *MEF2C* promoter is highlighted with dashed lines.

We applied this workflow to two simple (one inversion and one translocation) and three additional complex BCRs from prior studies with noncoding breakpoints disrupting the TAD containing *MEF2C*.^{11,14} Each rearrangement was genotypically distinct with different resultant derivative chromosomes. While complex BCRs were excluded from other aspects of this study, we included three in our Hi-C analysis because their impact on 3D genome organization has not been previously examined in a homolog-specific manner.^{59,60} Comparing the results of rearrangement detection using Hi-C to those using liWGS, we found that the 3D resequencing pipeline detected 92.7% ($n = 51/55$) of the breakpoints found by the combination of both methods. Three of the four breakpoints missed by Hi-C were short segments (<10kb) of DNA that had been rearranged and inserted into a new position. The missed breakpoints were visible in the Hi-C map but had not been identified by the computational analysis. Conversely, liWGS detected 96.4% ($n = 53/55$) of the Hi-C identified breakpoints, failing to detect a breakpoint associated with a short interval, as well as a 78kb deletion. Crucially, Hi-C was able to reliably order and orient the rearranged sequences on each homolog, even when a breakpoint was missed (**Supplementary Table 7, Supplementary Figs. 7A-E**). By contrast, it is challenging to reliably order and orient the rearranged sequencing using liWGS data alone if breakpoints are missed. Taken together, these results demonstrate that Hi-C can be used to robustly generate both homolog-specific sequences and architectural maps.

In all five BCR cases we examined, 3D resequencing via Hi-C also revealed significantly altered 3D organization of the rearranged homolog (**Supplementary Figs. 7A-E**). Moreover, we observed reduced frequency of contact between the *MEF2C* promoter and 16 distal enhancers⁶⁰ (**Supplementary Table 8**), consistent with the dysregulation of *MEF2C* expression observed in the same cases. In the majority of the cases, the reduction in contact frequency appeared to result from the BCR greatly increasing the distance between the promoter and its enhancers (**Supplementary Fig. 7**). However, in one case with chromothripsis (DGAP101), the BCR breakpoints only had a modest effect on the linear distance between the *MEF2C* promoter and its enhancers. Instead, the promoter and enhancers were separated into distinct architectural domains through the creation of a new boundary, which likely prevented physical contact between the promoter and enhancers (**Fig. 5B-C**). The observed 3D remodeling suggests a reduced frequency of contact between sequences that influence *MEF2C* expression, providing a plausible explanation for how a noncoding BCR breakpoint can result in a DD phenotype through disruption of 3D genome architecture.

Genomic features predict TADs associated with pathogenic LRPEs

Motivated by our discovery of multiple genome-wide significant LRPE loci in DDs, coupled with our validation of functional changes in *MEF2C* and alterations to the 3D organization associated with the putative 5q14.3 LRPE, we exploited this unique BCR dataset to identify additional features contributing to pathogenic TAD disruptions that could be used for future LRPE predictions. As described above, we demonstrated that the genic content of TADs alone is insufficient to unequivocally predict the pathogenicity of an individual BCR, as 43.0% of BCRs in controls disrupted a TAD containing a Tier 1 gene. We also found that 9.4% of all TAD boundaries encompassing a Tier 1 gene were overlapped by at least one

large polymorphic deletion in the genome aggregation database (gnomAD),⁶¹ which excludes adults with a history of early onset developmental conditions. Thus, additional genomic features beyond the presence of disease-associated genes are required to predict TADs preferentially disrupted by BCRs in DD cases.

To identify genomic features that characterize TADs intolerant to disruption, we annotated all autosomal TADs⁴⁰ in the genome with 54 features that can be broadly grouped into five categories: genes, *cis*-regulatory elements, primary sequence conservation, repetitive elements, and ‘other’ (**Figs. 6A** and **Supplementary Methods**). We defined 45 “positive” training TADs (disrupted by ≥ 2 BCR cases and zero BCR controls) and 261 “negative” TADs (disrupted by ≥ 1 BCR control and no BCR cases) and performed a univariate logistic regression for each of the 54 features, which identified 26 features at a FDR < 0.05 (**Supplementary Table 9**). Next, given that many genomic features are highly correlated (**Supplementary Fig. 8**), we trained an elastic net regression on the positive and negative training TADs that included all 26 features from the univariate analysis and identified six features that were individually associated with case status after controlling for the effects of all other features: VISTA enhancers,⁶² ultraconserved elements (UCEs),⁶³ transposon-free elements,⁶⁴ TAD size,⁴⁰ the presence of at least one Tier 1 gene, and primary sequence conservation^{65,66} (**Fig. 6B**).

We tested this model’s predictive accuracy on an independent set of 372 TADs (see **Supplementary Methods** for selection criteria) that were not used in our training data and determined that these six features alone were moderately predictive of LRPE pathogenicity (area under the receiver operating characteristics curve = 0.633; **Fig. 6C**). We next defined a “LRPE pathogenicity” cutoff score of ≥ 0.43 (TADs ranked in the top 10th percentile from our model) based on the point in which case-control and case-simulation enrichments surpassed OR > 1.5 (**Fig. 6D**). We demonstrated that 75.0% of previously established LRPE loci (**Supplementary Table 10**) in the human genome that were not represented in our training dataset surpassed this score, a highly significant enrichment when compared to all other TADs in the genome (Fisher’s exact test; $P = 2.55 \times 10^{-5}$). These orthogonal approaches collectively confirmed that this relatively simple six-feature regression model was able to prioritize TADs likely intolerant to disruption. While much larger cohorts will be required to power more sophisticated predictive models, these analyses demonstrate the potential of this approach to improve noncoding variant interpretation and shed greater light on the genomic features associated with noncoding mechanisms of disease.

DISCUSSION

It has been well-established that *de novo* BCRs are associated with increased risk of congenital anomalies and a broad range of DDs,^{2,4,6,10} yet little is known about the pathogenic mechanisms through which this risk occurs outside of direct gene disruption. Using a large, aggregated cohort of cytogenetically-defined simple BCRs from which we derived sequence-resolved breakpoints, including several hundred BCRs from unaffected controls, our analyses reveal new insights into the mechanisms through which BCRs confer risk for DDs. These data identified an enrichment of BCRs impacting chromosomes 14 and Xp in DD cases, the latter of which was predominantly driven by X-autosome translocations in females. A previous cytogenetic study observed an association between telomeric breakpoints on

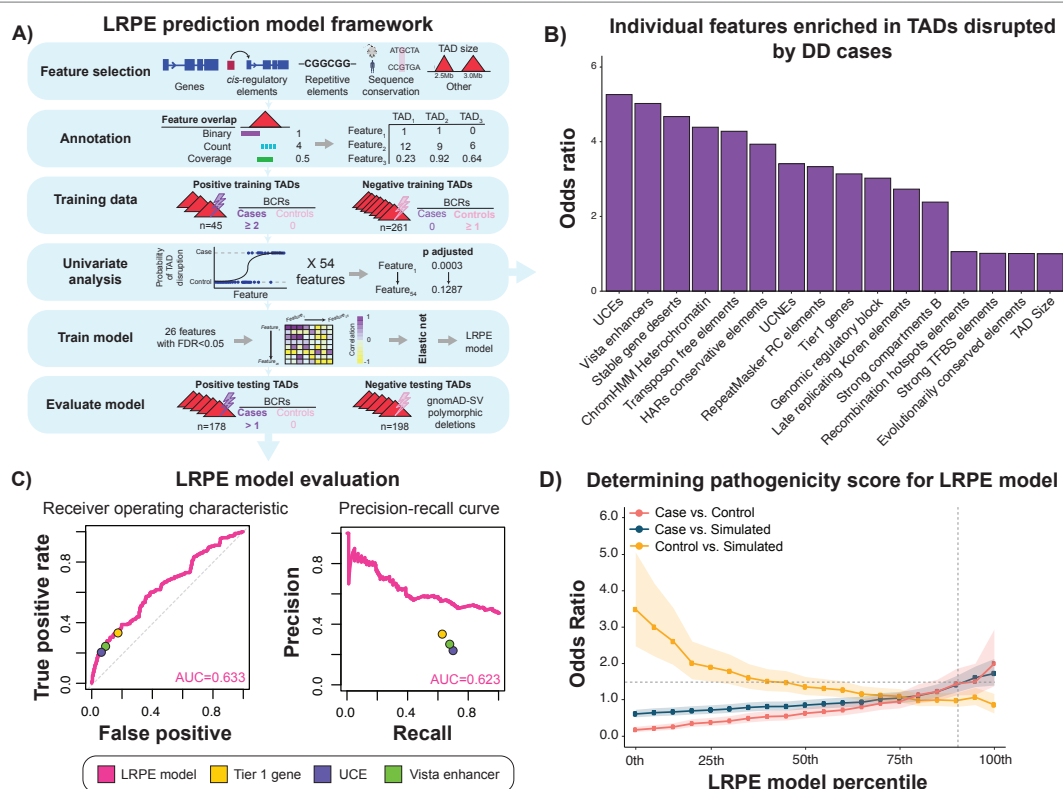


Fig. 6 | Identification of genomic features associated with pathogenic LRPEs

(A) An overview of the framework used to generate a model to predict pathogenic LRPEs across the genome based on disruption of 3D genome architecture. (B) Features identified to be potentially associated with TADs preferentially disrupted by BCRs from DD cases based on a BH-FDR < 0.05 from a univariate logistic regression performed for each feature, ordered by effect size. (C) Evaluation of the LRPE model using an independent set of 372 TADs. Performance of TADs overlapping other common functional annotations are shown as a comparison. (D) To determine a cut-off score that could be used to identify TADs that are especially intolerant to disruption, we compared the fraction of cases to controls and simulated breakpoints that disrupt a TAD in each percentile and identified the inflection point at which a case enrichment (OR > 1.5) begins to emerge.

the X chromosome, particularly at Xp22 and Xq28, and DDs in females with X-autosome translocations.⁶⁷ Our analyses confirm and broaden these results by suggesting that this enrichment extends beyond Xp22 and likely encompasses the entire p arm. We further demonstrated that there is a seven-fold enrichment of BCR breakpoints that directly disrupt known DD genes in affected cases compared to controls, but that roughly 79% of DD BCR carriers cannot be explained by the direct disruption of currently recognized disease genes. We discovered TADs containing known DD genes that were recurrently disrupted by noncoding BCRs in DD cases far more frequently than expected by chance, suggesting that additional risk for DDs from BCRs are mediated through noncoding mechanisms, which represents an enticing area for future investigations.

These data implicate disruption of 3D chromatin domains as a mechanism likely mediating some of these noncoding effects. Our analyses identified six TADs enriched for BCR breakpoints in DD cases beyond what would be expected by chance. Notably, all three of the most significant TAD associations that exceeded genome-wide significant thresholds and matched previously established pathogenic LRPEs (*MEF2C*, *FOXG1*, and *SOX9*).^{11,52,54} The three additional LRPE loci are particularly compelling candidates given that they harbor well-known DD genes, *BCL11A*, *SATB2*, and *BCL11B*,^{56,57,68-70} and are disrupted by cases with phenotypes that match those seen in individuals with direct disruption of these DD genes. We note that, collectively, 7.4% of DD cases in this cohort harbor noncoding BCRs that disrupt these six LRPEs.

This result is also certainly an underestimate of the pathogenic impact of disruptions of 3D topology as it is restricted to only the six most prominent LRPE loci identified in our dataset. Many decades of disease gene discovery have biased our findings considerably towards identifying and prioritizing pathogenic gene disruptions, whereas much less is known about the molecular mechanisms, pathogenic processes, and genomic features of pathogenic noncoding variation. We anticipate that the fraction of BCR carriers associated with identifiable pathogenic LRPEs will continue to increase as future studies increase sample size and further clarify the essential features necessary for cis-regulatory disruption of disease-associated genes.

To investigate the potential underlying mechanism of LRPEs due to 3D topology disruption, we performed Hi-C analyses on five cases with noncoding BCRs that disrupted our top putative LRPE locus, the TAD containing *MEF2C*. While Hi-C has been performed on human cells from a small number of DD cases harboring SVs,^{58,59} none of these studies have explicitly isolated the impact of heterozygous SVs on 3D topology. This is largely due the fact that existing computational workflows do not effectively combine the phased *de novo* assembly of genomes with the discovery of SVs. Our 3D resequencing pipeline merged these tasks into a single workflow that was able to recapitulate the structure of each BCR detected by liWGS. In addition, the Hi-C analyses provided data on the 3D architecture at the 5q14.3 locus, revealing extensive disruption to the normal TAD structure of the region. The Hi-C analyses demonstrated that the 5q14.3 BCRs disrupted the

physical contact between the *MEF2C* promoter and enhancers via two mechanisms, either by increasing the linear distance between them, or creating a new boundary that prevented the promoter from interacting with the enhancers. The creation of a novel boundary is consistent with a recent study that functionally dissected the TAD containing *SOX9*, one of our genome-wide significant TADs, and demonstrated that repositioning of the TAD boundary itself via inversion or insertion of novel CTCF sites created a new boundary that decoupled the *SOX9* promoter from its enhancers, resulting in downregulation of *SOX9* and abnormal phenotypic outcomes in mice.²⁴ Additional Hi-C analyses of different SV classes at various loci will be critical for determining the full range of potential LRPE mechanisms, the relative prevalence of each, and what features predispose certain genomic regions to their pathogenic effects.

Our study further illustrates the complexity of interpretation of TAD disruption in human disease. We find that the disruption of TADs containing DD genes by control BCRs usually does not result in an appreciable disease phenotype, nor does the deletion of boundaries from these TADs as identified in population controls.⁶¹ This result refutes the utility of interpretation approaches that simply seek to match TAD disruption with the presence of a DD gene within the domain, particularly when assessing DD phenotypes that can be plausibly linked to hundreds of dominant disease genes throughout the genome. To systematically identify additional genomic features that were predictive of TAD intolerance, we leveraged our BCR cohort to build a model that prioritized six genomic features independently associated with TADs preferentially disrupted by DD cases. In addition to DD-associated (Tier 1) genes we also identified TAD size as being positively correlated with risk for DDs, which is consistent with developmentally regulated genes having complex *cis*-regulatory landscapes that likely occupy greater genomic space.⁷¹ We discovered an enrichment of primary sequence conservation and UCEs in TADs disrupted by BCRs from DD cases, which aligns with the report of an enrichment of UCEs in the vicinity of SVs associated with NDDs.⁶³ Despite this initial progress towards identifying features associated with TADs intolerant to disruption, this model lacks sufficient predictive power to discriminate individual BCRs identified in cases from controls. However, we anticipate that larger sample sizes and improved noncoding functional predictions will eventually power increasingly sophisticated statistical models to aid in the clinical interpretation of noncoding BCRs. These data, together with our Hi-C analyses from the 5q14.3 locus, suggest that LRPEs are likely to be modified by the type of structural rearrangement as well as the complex interplay of genomic features within the TAD.

In conclusion, these data demonstrate that BCRs exert highly penetrant effects in DDs through both coding and noncoding mechanisms and that the disruption of 3D chromatin structures is associated with pathogenic LRPEs. We provide statistical evidence to support highly penetrant LRPEs at previously known and novel loci. Our feature selection analysis demonstrates that additional features within the TAD structure will also be critical for identifying novel LRPEs as well as provide insights into underlying molecular mechanisms through which this risk for disease occurs. The ongoing aggregation of population-scale datasets through international biobanks promises to further define the features associated with LRPEs and three-dimensional structures that are intolerant to disruption by SVs in the human genome.

METHODS & SUPPLEMENTARY INFO

Detailed methods and supplementary information for this manuscript have been provided in a separate document, which will be linked directly from *medRxiv*.

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AUTHOR CONTRIBUTIONS

Study design: C.L., M.M.M, R.L.C., M.C.B, O.D., H.B., C.C.M., E.L., J.F.G., P.J., E.L.A., I.B., M.E.T., and N.T. **Sample recruitment and data generation:** M.M.M, Z.D., M.B.R., L.N.P., A.S.F, Y.M., A.L.T., J.M.M.M., X.C., A.C., C.H., M.B., R.V.B., T.V., T.L., J.E., A.M.V.M., A.C.S.F., J.F.M., K.T.A., J.R.V., K.V.D.B., C.S., C.A., P.E., A.A.S., D.S., C.S.B., V.M.K., M.W., H.G.K., K.O., L.R., S.M., M.B., A.L.T., J.E., J.O., D.N., M.P., E.B., E.R.S., F.S., F.J.S., A.N., E.F., M.F., P.S., N.K.V., C.D.H., D.M.F., D.H.S., M.A.R.A., M.M.I., L.A., P.M.K., G.D.R., T.A.P.V., S.L., W.H., J.D., M.H., M.H., Z.S., I.V., T.D.H., R.S.M., C.F., L.B.O., B.S.G., M.L., J.P., C.P.R., S.J., N.E., E.S., C.M., K.G., A.D., U.R.D., R.S., F.L., O.Z., G.H., D.M., A.M., N.W.K., I.M.C., J.B.M., L.R.M., M.G., L.L., J.G., K.B.M.H.,

C.D.A.E., Y.A., J.R.B., X.L., J.L., A.S., C.H.H., L.N.K., J.T., K.L., R.H., R.G.S., C.A.S., J.C.J., B.L., A.T., B.N., E.M., O.A.C., E.S.W., T.K., R.E.P.A., S.G.T., A.K., A.R.H., S.B., S.L.P.S., N.Y., S.G., W.K.C., S.R., I.M., N.O., N.K.G., K.R.F., B.N.H., C.C.M., K.W.C., P.J., I.B., and N.T. **Individual case breakpoint identification:** M.M.M., M.C.B., C.L., M.B.R., H.B., Z.D., R.L.C., A.M.V.M., H.D.W., C.A., E.B., F.S., N.K.V., G.D.R., Z.T., L.B.O., C.A.S., J.C.J., K.N., S.G.T., S.O.S., B.T., K.W.C., P.J., I.B., S.C., S.C.T., A.Z., M.I.M., A.U., B.A. and N.T. **Aggregation data analysis and interpretation:** C.L., R.L.C., H.B., M.M.M., M.C.B., P.J., M.E.T., and N.T. **HiC data generation and/or analysis:** O.D., H.G., D.W., C.L., K.M.S., C.S.B., M.E.T., and E.L.A. **Generation of figures and writing of the manuscript:** C.L., R.L.C., M.M.M., M.C.B., O.D., H.B., H.G., K.M., E.L.A., M.E.T., and N.T. All authors revised the manuscript.

COMPETING INTERESTS

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