Transcript expression-aware annotation improves rare variant discovery and interpretation

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Abstract

The acceleration of DNA sequencing in patients and population samples has resulted in unprecedented catalogues of human genetic variation, but the interpretation of rare genetic variants discovered using such technologies remains extremely challenging. A striking example of this challenge is the existence of disruptive variants in dosage-sensitive disease genes, even in apparently healthy individuals. Through manual curation of putative loss of function (pLoF) variants in haploinsufficient disease genes in the Genome Aggregation Database (gnomAD)[1], we show that one explanation for this paradox involves alternative mRNA splicing, which allows exons of a gene to be expressed at varying levels across cell types. Currently, no existing annotation tool systematically incorporates this exon expression information into variant interpretation. Here, we develop a transcript-level annotation metric, the proportion expressed across transcripts (pext), which summarizes isoform quantifications for variants. We calculate this metric using 11,706 tissue samples from the Genotype Tissue Expression project[2] (GTEx) and show that it clearly differentiates between weakly and highly evolutionarily conserved exons, a proxy for functional importance. We demonstrate that expression-based annotation selectively filters 22.4% of falsely annotated pLoF variants found in haploinsufficient disease genes in gnomAD, while removing less than 4% of high-confidence pathogenic variants in the same genes. Finally, we apply our expression filter to the analysis of *de novo* variants in patients with autism spectrum disorder (ASD) and developmental disorders and intellectual disability (DD/ID) to show that pLoF variants in weakly expressed regions have effect sizes similar to those of synonymous variants, while pLoF variants in highly expressed exons are most strongly enriched among cases versus controls. Our annotation is fast, flexible, and generalizable, making it possible for any variant file to be annotated with any isoform expression dataset, and will be valuable for rare disease diagnosis, rare variant burden analyses in complex disorders, and curation and prioritization of variants in recall-by-genotype studies.

A primary challenge in the use of genome and exome sequencing to predict human phenotypes is that our capacity to identify genetic variation exceeds our ability to interpret their functional impact[3,4]. One underappreciated source of variability for variant interpretation involves differences in alternative mRNA splicing, which enables exons to be expressed at different levels across tissues. These expression differences mean that variants in different regions of a gene can have different phenotypic outcomes depending on the isoforms they affect. For example, variants occurring in an exon differentially included in two isoforms of *CACNA1C* with diverse tissue expression patterns result in distinct types of Timothy syndrome[5]. Pathogenic variants in the isoform that exhibits multi-tissue expression result in a multi-system disorder[5-7], whereas those on the isoform predominantly expressed in heart result in more severe and specific cardiac defects[8]. In addition, Mendelian variants have been found on tissue-specific isoforms[9,10] and isoform expression levels in *TTN* have been used to show that pLoF variants found in healthy controls occur in exons that are absent from dominantly expressed isoforms, whereas those in dilated cardiomyopathy patients occur on constitutive exons[11], emphasizing the utility of exon expression information for variant interpretation.

We find that isoform diversity is also a contributor to the paradoxical finding of disruptive variants in dosage-sensitive disease genes in ostensibly healthy individuals. In the gnomAD database, we identify 401 high-quality pLoF variants that pass both sequencing and annotation quality filters, in 61 haploinsufficient disease genes where heterozygous pLoF variants are established to cause severe developmental delay phenotypes with high penetrance (Methods). Given the severity of these phenotypes and their extremely low worldwide prevalence, ranging from 1 in 10,000 to less than 1 in a million, very few, if any true pLoF variants would be expected to be found in the gnomAD population. As such, most or all of these observed pLoF variants are likely to be sequencing or annotation errors[12]. Manual curation of these variants reveal common error modes that result in likely misannotation of pLoFs, with diversity of transcript structure, mediated by alternative mRNA splicing, emerging as a major consideration (Figure 1, Supplementary Figure 1, Supplementary Tables 1-3). However, no existing tools systematically incorporate information on transcript expression into variant interpretation.

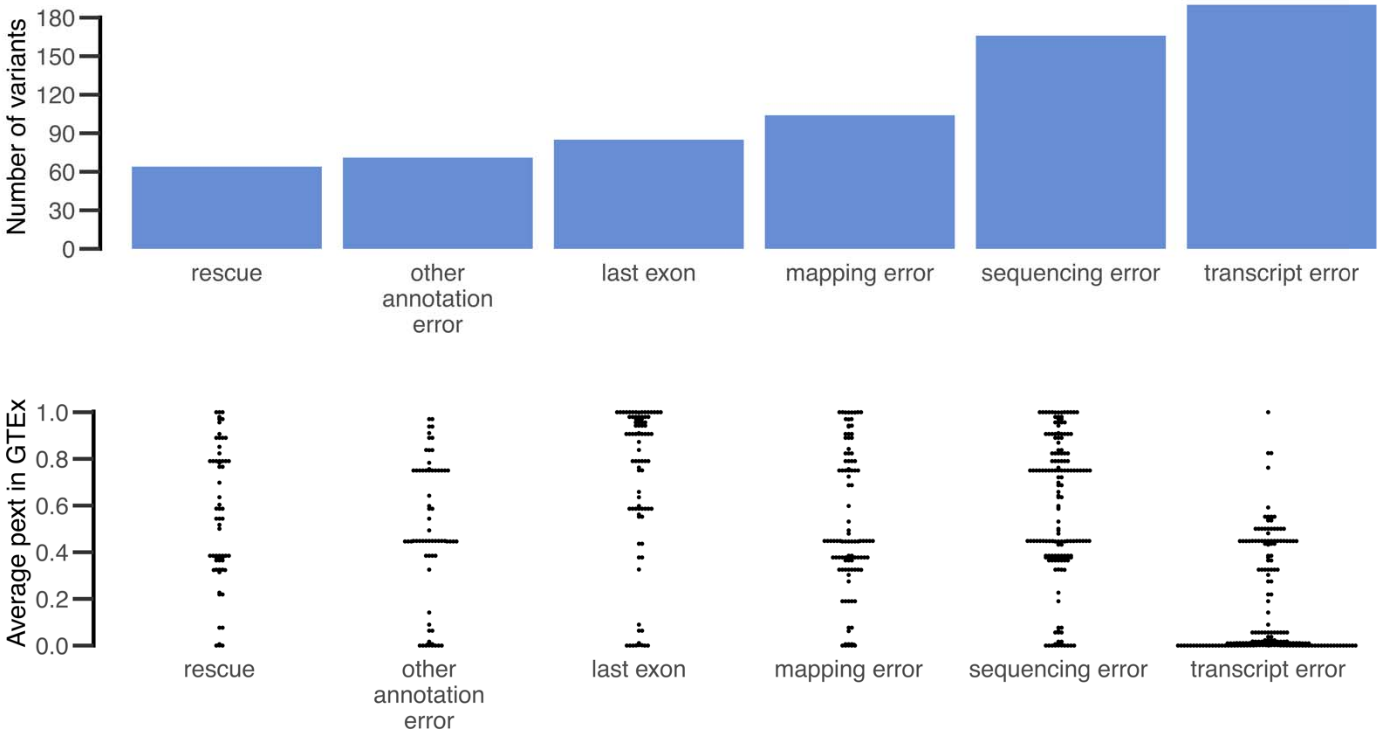
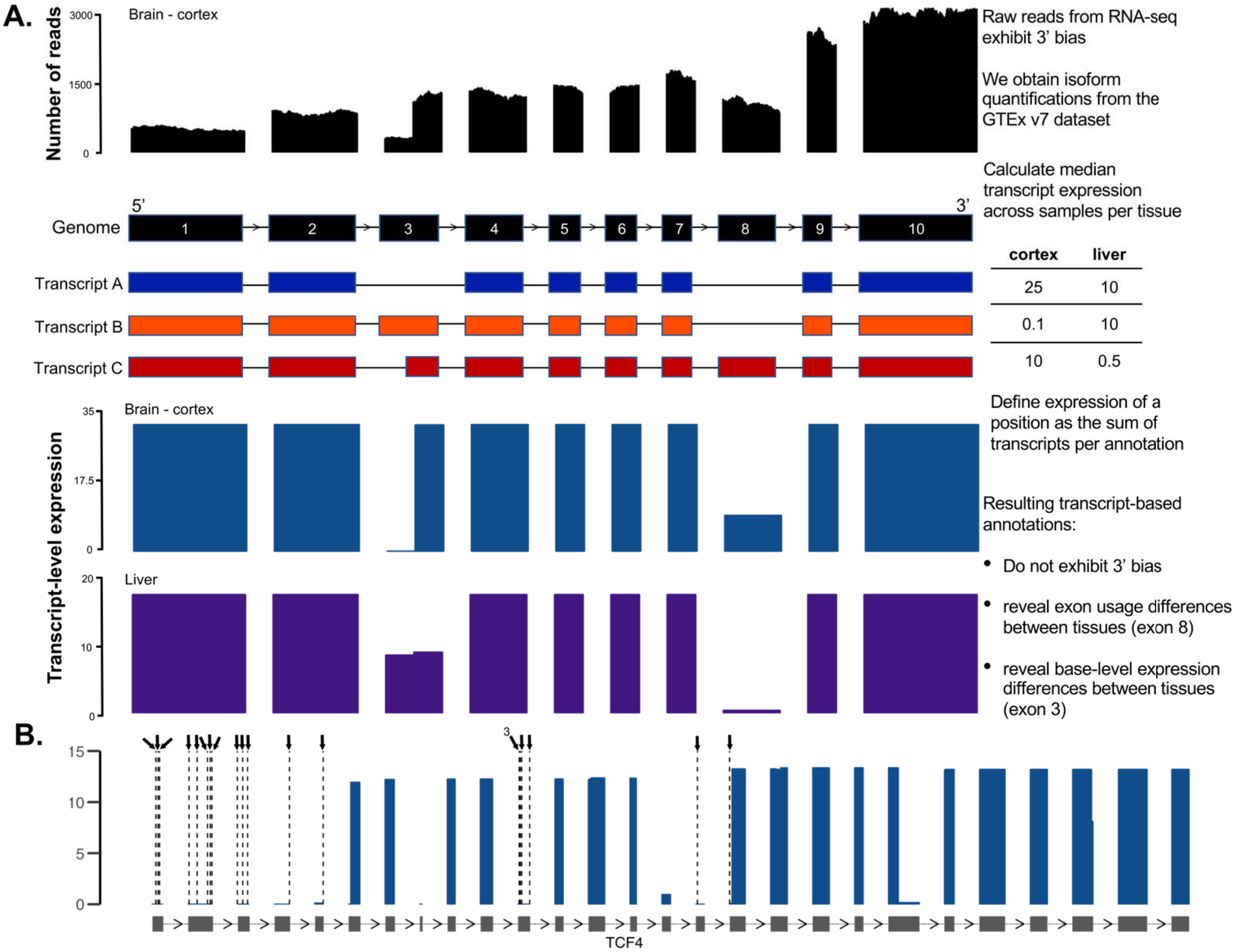


Figure 1: Curation of pLoF variants in haploinsufficient disease genes found in gnomAD reveals transcript errors as a major confounding error mode in variant annotation. We identified and manually curated 401 pLoF variants in the gnomAD dataset in 61 haploinsufficient severe developmental delay genes and flagged any reason the pLoF may not be a true LoF variant. Top plot shows the frequency of each error mode present in the 306 variants classified as unlikely to be a true LoF. Transcript errors emerge as a major putative error mode in the annotation of these pLoF variants. Beeswarm plot on bottom shows the average pext score across GTEx tissues presented in the manuscript for each variant in the error categories. This shows that pext values are discriminately lower for variants that are annotated as possible transcript errors.

The advent of large-scale transcriptome sequencing datasets, such as GTEx[2], provides an opportunity to incorporate cross-tissue exon expression into variant interpretation. However, the current formats of these databases do not readily allow for unbiased estimation of exon expression. The GTEx web browser offers information on exon-level read pileup across tissues, but this approach is confounded by technical artifacts such as 3' bias[13] (preferential coverage of bases close to the 3' end of a transcript; Supplementary Figure 2A). Such systematic biases mean that simple exon-level coverage in a transcriptome dataset cannot be used as a reliable proxy for exon expression, especially in longer genes (Figure 2A, Supplementary Figure 2B).

Isoform quantification tools provide estimates of isoform expression levels that correct, albeit imperfectly[13,14], for confounding by 3' bias as well as other technical artifacts such as isoform length, isoform GC content, and transcript sequence complexity[15-17]. Here, we utilize isoform-level quantifications from 11,706 tissue samples from the GTEx v7 dataset to derive an annotation-specific expression metric. For each tissue, we annotate each variant with the expression of every possible consequence across all transcripts, which can be used to summarize expression in any combination of tissues of interest. We first compute the median expression of a



Raw reads from RNA-seq exhibit 3' bias

We obtain isoform quantifications from the GTEx v7 dataset

Calculate median transcript expression across samples per tissue

cortex	liver
25	10
0.1	10
10	0.5

Define expression of a position as the sum of transcripts per annotation

Resulting transcript-based annotations:

- Do not exhibit 3' bias
- reveal exon usage differences between tissues (exon 8)
- reveal base-level expression differences between tissues (exon 3)

Figure 2: Summary of transcript-expression based annotation method A. Overview of transcript aware annotation. Most genes have many annotated isoforms, which can have varying expression patterns across tissues. Utilizing number of reads aligning to exonic regions in transcriptome datasets as a proxy for exon expression (top panel black) has confounding effects, due to 3' bias. In this example, while exon 3 and 8 have markedly different expression levels in Brain – Cortex, the number of reads aligning to the two exons are similar, masking exon usage differences. Transcript-aware annotation defines the expression of every variant as the sum of transcripts that have the same annotation. The resulting transcript-level expression plots do not exhibit 3' bias, and reveal exon usage differences, such as those in exons 3 and 8, across tissues. **B.** Example of utility of transcript-expression based annotation. There are 20 high quality pLoF variants in the haploinsufficient developmental delay gene *TCF4* in gnomAD, annotated as dashed lines and arrows. All 20 variants have no evidence of expression in the GTEx dataset, suggesting functional TCF4 protein can be made in the presence of these variants.

transcript across tissue samples, and define the expression of a given variant as the sum of the expression of all transcripts for which the variant has the same annotation (Figure 2A, Supplementary Figure 3A). By normalizing the expression of the annotation to the total gene expression, we define a metric (proportion expression across transcripts, or pext), which can be interpreted as a measure of the proportion of the total transcriptional output from a gene that would be affected by the variant annotation in question (Supplementary Figure 3B).

The pext metric allows for quick visualization of the expression of exons across a gene. Figure 2B shows *TCF4,* a haploinsufficient gene in which heterozygous variants result in Pitt-Hopkins syndrome[18], a highly penetrant disorder associated with severe developmental delay. This gene harbors 20 unique high quality pLoF mutations across 56 individuals in the gnomAD database. All 20 variants lie on exons with no evidence of expression across the GTEx dataset (Figure 2B, Supplementary Figure 4) indicating that functional TCF4 protein can be made in the presence of these variants. This visualization is now available for all genes in the gnomAD browser (gnomad.broadinstitute.org), and can aid in rapid identification of variants occurring on exons with little to no evidence of expression in GTEx.

To explore whether expression-based annotation marks functionally important regions, we compared the distribution of the pext metric in evolutionarily conserved and unconserved regions using phyloCSF[19]. Exons with patterns of multi-species conservation consistent with coding regions have higher phyloCSF scores, and should exhibit detectable expression patterns, whereas regions with lower scores will be enriched for incorrect exon annotations, which are expected to have little evidence of expression in a population transcriptome dataset. As expected, we observe significantly lower expression for unconserved regions, and near-constitutive expression in highly conserved regions (Figure 3A, Supplementary Figure 5A). This difference remains statistically significant after correcting for exon length (logistic regression $p < 1.0 \times 10^{-100}$), which can influence both phyloCSF scores and isoform quantifications, indicating that transcript expression-aware annotation marks functionally relevant exonic regions.

While the metrics are associated, we find that pext provides orthogonal information to conservation for variant interpretation. For example, regions with low evidence of conservation but high expression (in Figure 3A) are enriched for genes in immune-related pathways (Methods), which are selected for diversity but represent true coding regions. In addition, the pext value is higher for pLoF variants annotated as high confidence (HC) by the Loss of Function Transcript Effect Estimator[1] (LOFTEE) with no additional flags than those flagged as having found on unlikely open reading frames or weakly conserved regions (Figure 3B, Supplementary Figure 5B). However, LOFTEE-HC variants with no flags can also have low pext values, suggesting transcript-expression aware annotation adds additional information to the currently available interpretation toolkit.

We undertook manual evaluation of 128 regions marked as unexpressed (pext < 0.1 in all tissues and in GTEx brain) in 61 haploinsufficient genes following the GENCODE manual annotation workflow[20] to evaluate the annotation quality in these coding sequence (CDS) regions. A third of flagged regions were associated with low quality models that have been removed or switched to non-coding biotypes in subsequent GENCODE releases (Supplementary Figure 6) while 70% of the remaining regions correspond to models that satisfy only minimum criteria for inclusion in the gene set, corresponding to ‘putative’ annotations that lack markers for CDS functionality (Supplementary Table 4). Nonetheless, we find support for some highly conserved CDS’, several of which show evidence of transcription in fetal tissues, underlining the importance of incorporating multiple isoform expression datasets for interpretation (Supplementary Figure 6D).

Nonsynonymous variants found on constitutively expressed regions would be expected to be more deleterious than those on regions with no evidence of expression. To test this, we defined expression bins based on the average pext value across GTEx tissues where an average pext value less than 0.1 was defined as low (or unexpressed), above 0.9 as high (or near-constitutive) and intermediate values as medium expression. We compared the mutability-adjusted proportion singleton (MAPS), a measure of negative selection on variant classes[21], partitioned on the LoF Observed Upper-bound Fraction (LOEUF) decile, a measure of constraint against pLoF variants in the gnomAD dataset[1] in each of these expression bins. MAPS scores differed substantially between pLoF variants found on low-expressed and high-expressed regions in genes intolerant to pLoF variation (Figure 3C, Supplementary Figure 5C, D). This skew of nonsynonymous variation in high-expressed regions suggests that variation arising in such exons tends be more deleterious, whereas nonsynonymous variants on regions with low expression are similar to missense variants in their inferred deleteriousness.

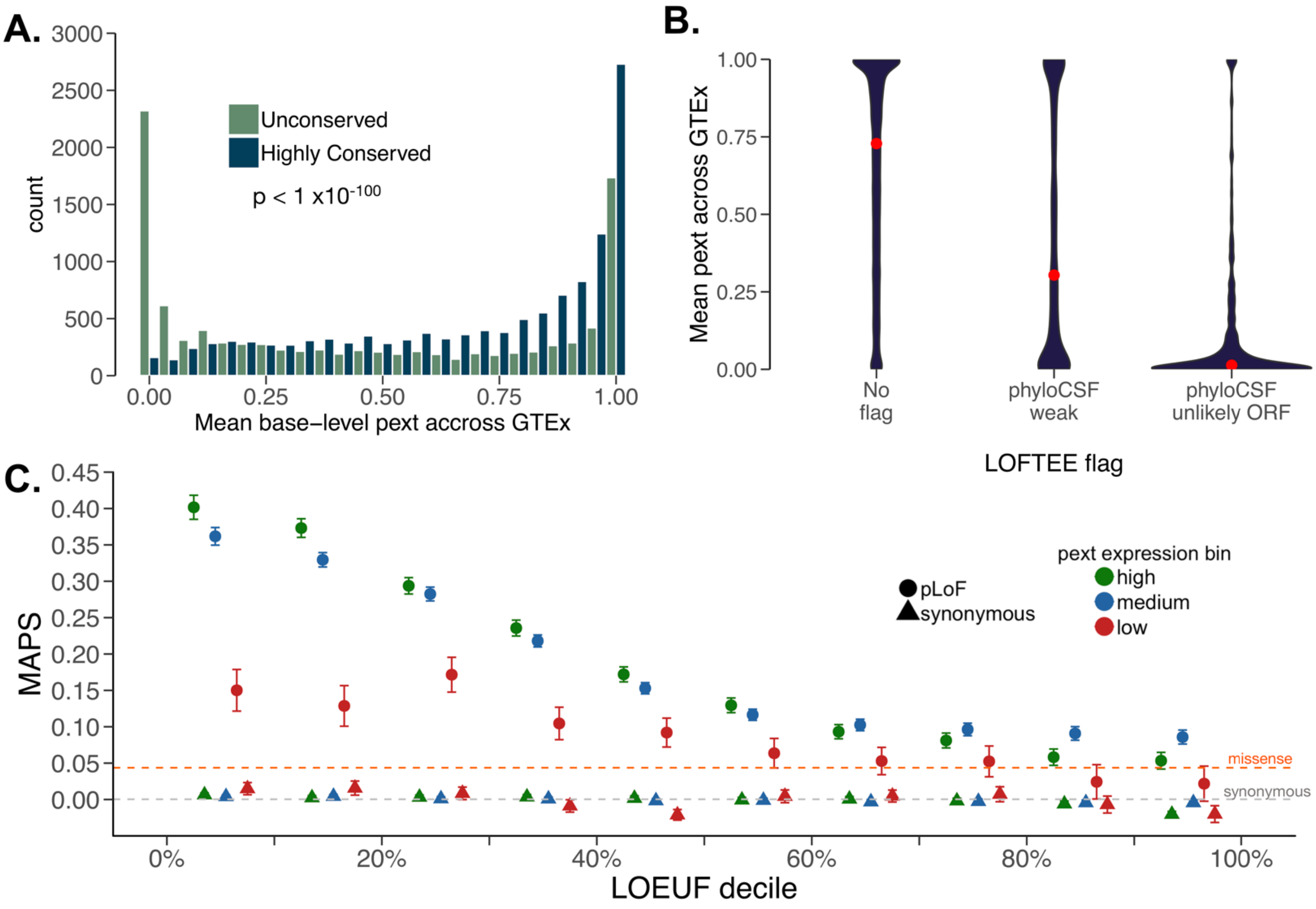


Figure 3: Functional validation of transcript-expression based annotation A. We define highly conserved and unconserved regions and compared the expression status of these regions across GTEx. Highly conserved regions are enriched for having near-constitutive expression whereas unconserved regions are enriched for having little to no usage across GTEx. This difference is significant after correcting for gene length (logistic regression p value $< 1 \times 10^{-100}$). We note that unconserved regions with high levels of expression (pext > 0.9) are enriched for immune-related genes, which are selected for diversity and thus have low conservation, but represent true coding regions. **B.** Transcript-expression based annotation recapitulates, and adds information to, existing interpretation tools. LOFTEE-HC pLoF variants in gnomAD with no flags are enriched for higher pext values, whereas HC pLoF variants falling on low phyloCSF or unlikely ORF regions are enriched for low expression. However HC-pLoF variants can also be filtered based on a low pext score. Red dots represent median pext value across GTEx **C.** Nonsynonymous variants found on near-constitutive regions tend to be more deleterious. We compared the mutability adjusted proportion singleton (MAPS) score for variants with low (<0.1), medium (0.1 ≤ pext ≤ 0.9) and high (pext > 0.9) expression. Variants with near-constitutive expression have a higher MAPS score, indicating higher deleteriousness than those with little to no evidence of expression. Dashed grey and orange line represent MAPS values for all gnomAD missense and all synonymous variants, respectively.

To evaluate the utility of transcript expression-based annotation in Mendelian variant interpretation, we assessed the number of variants that would be filtered based on a pext cutoff of <0.1 (low expression) across GTEx tissues for three gene sets. Firstly, we evaluated high-quality pLoF variants in the 61 manually curated haploinsufficient genes in gnomAD and ClinVar[22]. The low pext expression bin resulted in filtering of 22.4% of pLoF variants in haploinsufficient developmental delay genes in gnomAD, but only 3.8% of high quality pathogenic variants in ClinVar (Figure 4A; $p = 5.2 \times 10^{-34}$; Methods). We next compared pLoF variants in autosomal recessive disease genes found in a homozygous state in at least one individual in gnomAD and any pLoF variant in these genes in ClinVar and observed similar results: expression-based annotation filters 35% of variants in gnomAD while only filtering 3.8% of variants in ClinVar (Figure 4B; $p = 2.3 \times 10^{-81}$).

Finally, we evaluated gnomAD pLoF variants in genes that are constrained against pLoF variation[1] (LOEUF score < 0.35). Given that these genes are depleted for loss-of-function variation in the general population, we expect the observed pLoF variants in these genes to be enriched for annotation errors. We compared the proportion filtered to synonymous variants in the same genes, which we expect to be neutrally distributed. Our metric removes 17.2% of pLoF variants in high pLI genes, but only 5.6% of synonymous variants (Figure 4C; $p < 1.0 \times 10^{-100}$).

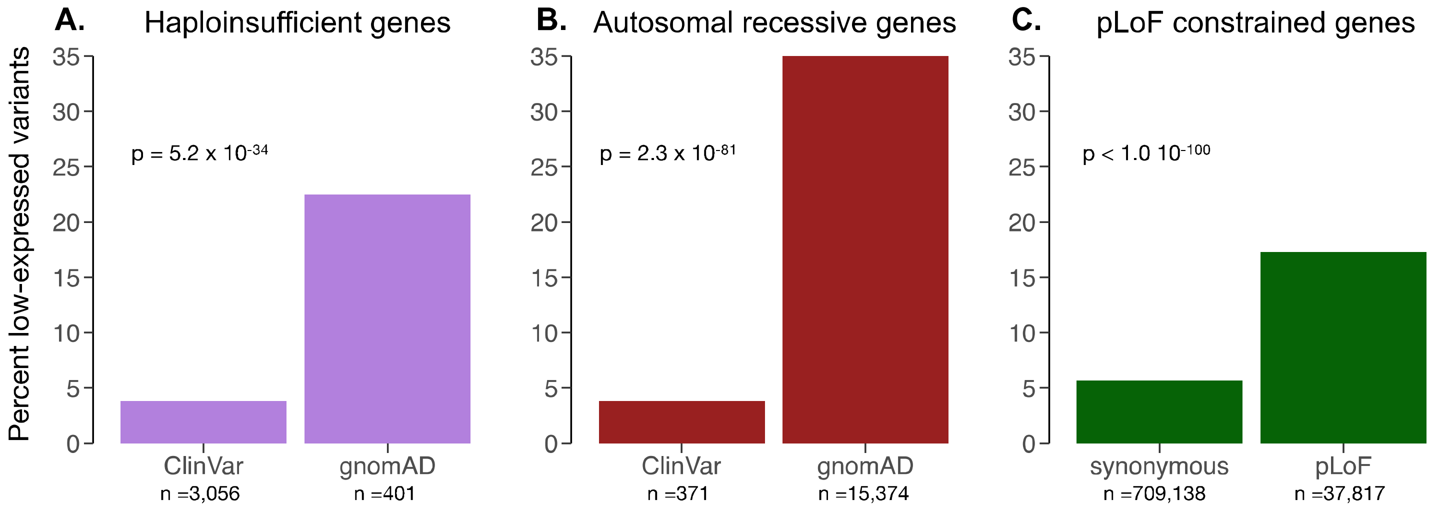


Figure 4: Transcript-expression based annotation aids Mendelian variant interpretation. A. Comparison of the proportion of high quality pLoF variants filtered in a curated list of 61 haploinsufficient developmental delays genes in gnomAD vs ClinVar with a cutoff of average pext across GTEx ≤ 0.1 (low expression). Expression-based filtering results in removal of 22.4% of gnomAD pLoFs and less than 3.8% of a curated high-confidence set of pLoFs in ClinVar. **B**. Expression-based annotation filters 35% of pLoF variants found in gnomAD in a homozygous state in at least one individual, and 3.8% of any pLoF variants found in the same genes in ClinVar. **C**. We extended this filtering approach to pLoF and synonymous variants in gnomAD pLoF-intolerant genes (defined by LOEF < 0.35). This filters 17.2% LoF and 5.6% of synonymous variants. Numbers below bar plots indicate the total number of high quality variants considered in each group. For pLoFs only LOFTEE-HC variants were considered, p-values calculated from fisher's exact test for counts.

To explore the benefits of this approach for rare variant analyses, we applied pext binning to burden testing of *de novo* variants in patients with developmental delay / intellectual disability or autism spectrum disorder using a set of 23,970 *de novo* variants collated from several studies including the Deciphering Developmental Disorders (DDD) project and the Autism Sequencing Consortium (ASC)[23-28]. We find that *de novo* pLoF variants in DD/ID patients in low-expressed regions have effect sizes similar to those of synonymous variants (rate ratio, denoted as RR, of low-expressed pLoFs = 0.66, p = 0.054) whereas pLoF variants in highly expressed regions have much larger effect sizes (RR = 2.98, p = 2.45 x 10^{-25}; Figure 5A). This observation is consistent for *de novo* variants in autism (RR for low-expressed pLoFs = 0.87, p = 0.48; RR for high-expressed pLoFs = 1.89, p = 2.87 x 10^{-8}; Figure 5B) and congenital heart disease with co-morbid neurodevelopmental delay (Supplementary Figure 7A) as well as rare variants (AC ≤ 10) identified in highly constrained genes in patients with autism spectrum disorder and attention-deficit/hyperactivity disorder (Supplementary Figure 7B). Overall, we consistently observe low-expressed pLoFs exhibiting effect sizes similar to those of synonymous variants, and pLoF variants in constitutive regions having larger effect sizes, suggesting that incorporating transcript expression-aware annotation in rare variant studies can boost power for gene discovery.

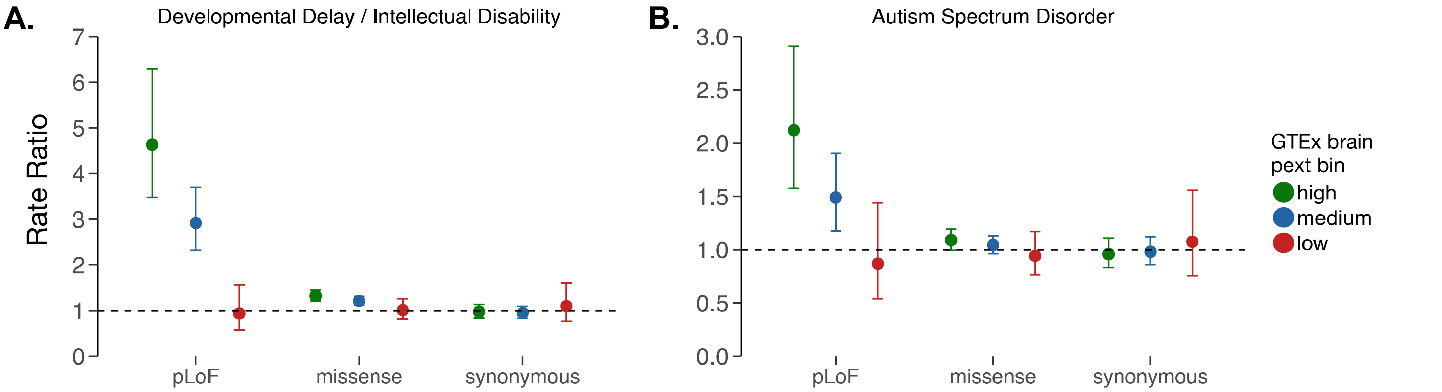


Figure 5: Application of transcript-expression based annotation to *de novo* variant analyses in **A.** developmental delay and/or intellectual disability (DD/ID) and **B.** autism spectrum disorder (ASD). We find that de novo pLoF variants found on constitutively expressed regions in GTEx brain tissues have larger effect sizes than *de novo* LoF variants in weakly expressed regions in both disorders. Strikingly, *de novo* pLoF variants found on regions with little evidence for expression are equally distributed in cases vs controls as *de novo* synonymous variants, suggesting such variants can be removed from gene burden testing analyses to boost discovery power. The high pext expression bin contains 45.6% and 40.7%, and the low expression bin contains 4.5% and 8.2% of pLoF variants found in DD/ID and ASD patients, respectively. Rate ratio represents estimate from the Poisson exact test.

We have described the development and validation of a transcript expression-based annotation framework to integrate results from transcriptome sequencing experiments into clinical variant interpretation. While our initial analysis utilizes GTEx, our method can be used with any isoform expression dataset to annotate any variant file rapidly in the scalable software framework Hail (https://hail.is). For example, annotation of >120,000 gnomAD individuals with GTEx takes under an hour using 60 cores, at a cost of about $1 on public cloud compute, which can be further scaled to larger datasets. In addition, the annotations we provide are flexible: while we have described the use of average transcript-level expression across many tissues, alternative approaches such as using minimum expression across any tissue, may prove useful depending on variant interpretation goals.

We note that while this metric successfully discriminates between near-constitutive and low expression levels, which are useful for prioritizing and filtering variants respectively, regions with intermediate expression levels are more challenging to interpret. Regions tagged as low expression are often corroborated by expert opinion of CDS curation, but domain knowledge of a gene will outperform this summary metric.

An important caveat in our approach is the imprecision of isoform quantification methods using short-read transcriptome data. However we note that repeating key analyses in the manuscript with a different isoform quantification tool showed highly consistent results (Methods, Supplementary Figure 8), suggesting robustness to the precise pipeline used. The utility of this framework will increase as our ability to quantify isoform expression across tissues improves, including refinement of methods and gene models, as well as availability of long-read RNA-seq data from human tissues. In addition, improvement of single-cell RNA-seq technologies and generation of data across human tissues will provide insight into cell type-specific exon usage for incorporation into variant interpretation[27].

The code used to generate pext is available as open source software (https://github.com/macarthur-lab/tx_annotation). In addition, we provide a precomputed file of the transcript-expression value for every possible single nucleotide variant in the human genome. This metric has already proven useful in variant curation for drug target identification[29] and for filtering variants for identification of human knockouts[1]. Overall, our metric can be incorporated into variant interpretation in Mendelian disease pipelines, rare variant burden analyses, and the prioritization of variants for recall-by-genotype studies.

Methods

Datasets

We utilized the gnomAD v.2.1.1 sites Hail 0.2 (https://hail.is) table which is accessible publicly at gs://gnomad-public/release/2.1.1 and at https://gnomad.broadinstitute.org. The GTEx v7 gene and isoform expression data were downloaded from dbGaP (http://www.ncbi.nlm.nih.gov/gap) under accession phs000424.v6.p1. The GTEx pipeline for isoform quantification is available publicly (https://github.com/broadinstitute/gtex-pipeline/) and briefly involves 2-pass alignment with STAR v2.4.2a[30], gene expression quantification with RNA-SeQC v1.1.8[31], and isoform quantification with RSEM v1.2.22. The LOEUF constraint file was downloaded from gs://gnomad-resources/lof_paper/. Variants used in all gnomAD analyses in the manuscript passed random forest filtering, and all pLoF variants were annotated as high confidence (HC) by LOFTEE v.1.0, which is described in an accompanying manuscript[1]. All files used in the analyses in the manuscript are available in gs://gnomad-public/papers/2019-tx-annotation/data and the scripts to generate files for the analyses are available at https://github.com/macarthur-lab/tx_annotation.

Curation of pLoF variants in haploinsufficient developmental disease genes

For identification of haploinsufficient developmental delay genes, we selected genes curated by the ClinGen Dosage Sensitivity Working Group[32]; 58 of the 61 genes had a score of 3 with sufficient evidence for pathogenicity, while two genes (*CHAMP1*, *CTCF*) had a score of 2 (some evidence) and one gene (*RERE*) was not yet scored. The penetrance of pathogenic variants in each gene was reviewed in the literature, and only genes with >75% reported penetrance were included. These conditions consistent of those too severe to expect to see an individual in gnomAD (likely unable to consent for a study without guardianship). The 61 gene list includes 50 autosomal genes of high severity and high penetrance and 11 genes on chromosome X where the phenotype is expected to be severe or lethal in males and moderate to severe in females. The resulting gene list is available at gs://gnomad-public/papers/2019-tx-annotation/data/HI_genes_100417.tsv.

We extracted pLoF variants, defined as essential splice acceptor, essential splice donor, stop gained, and frameshift variants, identified in the 61 haploinsufficient disease genes from the gnomAD v2.1.1 exome and genome sites tables, and considered only those pLoF variants that passed random forest filtering in the gnomAD dataset, and were annotated as high confidence (HC) by LOFTEE v1.0. We performed manual curation of 401 pLoF variants using a web-based curation portal to identify any reason a pLoF may have been a variant calling or annotation error, and categorized the likelihood of each variant being a true pLoF.

Evidence to refute a true LoF variant was categorized into the following groups: mapping error, strand bias, reference error, genotyping error, homopolymer sequence, in phase multi-nucleotide variant or frame-restoring indel, essential splice site rescue, minority of transcripts, weak exon conservation, last exon, and other annotation error. All possible reasons to reject a LoF consequence were flagged, even when a single criterion would categorize the variant as not LoF. Variants were then categorized as LoF, likely LoF, likely not LoF, and not LoF based on criteria outlined in Supplementary Table 2. Supplementary Figure 1A shows the distribution of the LoF verdicts for the 401 pLoF variants.

Technical errors comprised genotyping errors, strand biases, reference errors, and repetitive regions that could be detected by visual inspection of reads in the Integrative Genomics Viewer[33] (IGV) and from the UCSC genome browser[34]. Genotyping errors comprised skewed allele balances (conservative cutoff of ≤35%), low complexity sequences, GC rich regions, homopolymer tracts (≥ 6 base pairs or ≥ 6 trinucleotide repeats) and low quality metrics (GQ < 20). Strand bias was flagged when a variant was skewed preferentially on the forward or reverse strand, or when the majority (>90%) of a given strand covered a region; this was often observed around intron/exon boundaries. Strand biases despite balanced coverage of the forward and reverse strands were weighted towards likely not LoF, whereas a strand bias due to skewed strand coverage were weighted alongside other genotyping errors. Reference errors were uncommon, but identified by a small deletion in a given exon, posing as a <5 base pair intron. Most genotyping errors and strand biases in isolation were not deemed critical in deciding whether a variant was likely not LoF or not LoF, with the exception of allele balance ≤25%. Mapping errors were often identified by an enrichment of complex variation surrounding a variant of interest. Furthermore, the UCSC browser was used to highlight mapping discrepancies, such as self-chain alignments, segmental duplications, simple tandem repeats, and microsatellite regions.

In-frame multi-nucleotide variants (MNVs), essential splice site rescue, and frame-restoring insertion-deletions are rescue events that are predicted to restore gene function. MNVs were visualized in IGV and cross checked with codons from the UCSC browser; in frame MNVs that rescued stop codons were scored as not LoF. Essential splice site rescue occurs when an in frame alternative donor or acceptor site is present, which likely has a minimal effect on the transcript. Thirty-six base pairs upstream and downstream of the splice variant were assessed for splice site rescue. Cryptic splice sites within 6 base pairs of the splice variant were considered a complete rescue, rendering the variant “not LoF”. Rescue sites > 6 base pairs away but within +/- 20 base pairs were weighted with less confidence, scoring as likely not LoF. All potential splice site rescues were validated using Alamut v.2.11 (https://www.interactive-biosoftware.com/alamut-visual/). Frame-restoring indels were identified by scanning approximately +/- 80 base pairs from the annotated indel and counting any insertions/deletions to assess if the frame would be restored.

Transcript errors encompass issues surrounding alternative transcripts, variants within a terminal coding exon, poorly conserved exons, and re-initiation events. Coding variants that occupied the minority (<50%) of NCBI coding RefSeq transcripts for a given gene were considered not LoF. These variants often affected poorly conserved exons, as determined by PhyloP[35], PhyloCSF[19], and visualization in the UCSC browser[34]. The only exception to the minority of transcript criteria were cases where the exon was well conserved, which relegated the categorization to likely not LoF. Variants within the last coding exon, or within 50 base pairs of the penultimate coding exon were also considered not LoF, unless 25% < x <50% of the coding sequence was affected, in which case the variant was deemed likely not LoF. If >50% of the coding sequence was disrupted by a variant in the last exon, this was deemed likely LoF. Other transcript errors included: re-initiation errors; upstream stop codons of a given LoF variant; variants that fell on exactly 50% of coding RefSeq transcripts; and/or partial exon conservation. Re-initiation events were flagged when a methionine downstream of the variant in the first coding exon was predicted to restart transcription, and were predicted to be likely not LoF. Variants occurring after a stop codon in the last coding exon were considered not LoF, particularly across the region of the exon or transcript in question. Error categories were grouped for Figure 1 as follows: Minority of transcripts and weak exon conservation were grouped as transcript errors, genotyping errors and homopolymers as sequencing errors, essential splice rescue and MNV grouped as rescue and strand bias was included in other annotation errors.

The criteria above were strictly adhered throughout and manual curation was performed by two independent reviewers to ensure maximum consistency and minimize human error. Any discordance in curation was re-curated by both curators together and resolved. Full results of manual curation are available in Supplementary Table 3.

Calculation of transcript-expression aware annotation

We first imported the GTEx v7 isoform quantifications into Hail and calculated the median expression of every transcript per tissue. This precomputed summary isoform expression matrix is available for GTEx v7 in the tx_annotation github repository. We also import and annotate a variant file with the Variant Effect Predictor (VEP) version 85[36] against Gencode v19[20], implemented in Hail with the LOFTEE v1.0 plugin.

We use the transcript consequences VEP field to calculate the sum of the isoform expression for variant annotations, i.e. the annotation-level expression across transcripts (ext). For variants that have multiple consequences for one transcript (for example, a SNV that is both a missense and a splice region variant) we provide an expression value per annotation, which can be extracted based on study-specific transcript choice. In this manuscript, we used the worst consequence, ordered by VEP (in this example, missense takes precedence over splice region). We filter the consequences to those only occurring on protein coding transcripts. Full ordering of the VEP consequences is available at: https://useast.ensembl.org/info/genome/variation/prediction/predicted_data.html).

We sum the expression of every transcript per variant, for every combination of consequence, LOFTEE filter, and LOFTEE flag for every tissue (Supplementary Figure 3A). For example, if a SNV is synonymous on ENST1, a LOFTEE HC stop-gained on ENST3 and ENST4, and LOFTEE low-confidence (LC) stop gained variant on ENST 5 and ENST6, the ext values will be synonymous: ENST1, stop-gained HC: ENST 3 + ENST4, and stop-gained LC: ENST5 + ENST6. This can be computed with the tx_annotate() function by setting the tx_annotation_type to “expression”. We foresee the non-normalized ext values to be useful when only considering one tissue of interest.

To allow for taking average expression values across tissues of interest, we normalize the expression value for a given value to the total expression of the gene on which the variant is found. This is carried out by dividing the ext value with the median gene expression value per tissue in transcripts-per-million (TPM) from RNASEQC v1.1.8[31] (Supplementary Figure 3B), which is publically available via gtexportal.org. The resulting pext (proportion expression across transcript) value can be interpreted as the proportion of the total transcriptional output from a gene that would be affected by the variant annotation in question. If the gene expression value (and thus the denominator) in a given tissue is 0, the pext value will not be available for that tissue. When taking averages across tissues, such unavailable pext values are not considered. This value can be computed with the tx_annotate() function by setting the tx_annotation_type to “proportion”. For the analyses in this manuscript, we remove reproduction-associated GTEx tissues (endocervix, ectocervix, fallopian tube, prostate, uterus, ovary, testes, vagina), cell lines (transformed fibroblasts, transformed lymphocytes) and any tissue with less than one hundred samples (bladder, brain Cervicalc-1 spinal cord, brain substantia nigra, kidney cortex, minor salivary gland) resulting in the use of 38 GTEx tissues.

The full transcript-expression aware annotation pipeline, implemented in Hail 0.2, is fully available at https://github.com/macarthur-lab/tx_annotation with commands laid out for analyses in the manuscript. Passing a Hail table through the tx_annotate() function returns the same table with a new field entitled “tx_annotation” which provides either the ext or pext value per variant-annotation pair, depending on parameter choice. We provide a helper function to extract the worst consequence and the associated expression values for the new annotation. All analyses in the manuscript are based on the worst consequence of variant, ordered by VEP[36].

Functional validation of transcript-expression aware annotation

Conservation analysis was performed using phyloCSF scores using the same file utilized for the LOFTEE plugin, available publically in gs://gnomad-public/papers/2019-tx-annotation/data. We denoted exons with a phyloCSF max open reading frame (ORF) score > 1000 as highly conserved and those with phyloCSF max ORF score < -100 as lowly conserved (Supplementary Figure 5A) and evaluated their average usage in GTEx.

Using the precomputed baselevel pext values that are used in the gnomAD browser, we filtered to intervals with high or low conservation, and calculated the average pext value in the interval. To evaluate regions with low conservation but high expression, we identified genes harboring unconserved regions with the pext value > 0.9 for pathway enrichment analysis and used the web browser for FUMA GENE2FUNC feature[37], which incorporates Reactome[38], KEGG[39], Gene Ontology[40] (GO) as well as other ontologies. Results from FUMA pathway analysis are available in Supplementary Figure 9, and the gene list used in the FUMA analysis, and full results are available in Supplementary Table 5.

Analysis of pext values for LOFTEE flags and the MAPS calculation were performed utilizing the gnomAD v2.1.1 exome dataset. Calculation of MAPS scores was previously described in Lek et al. 2016[21] and is implemented as a Hail module, as described in Karczewski et al. 2019[1]. MAPS is a relative metric, and so cannot be compared across datasets, but is a useful summary metric for the frequency spectrum, indicating deleteriousness as inferred from rarity of variation (high values of MAPS correspond to lower frequency, suggesting the action of negative selection at more deleterious sites). The MAPS scores were calculated on the gnomAD v.2.1.1 dataset partitioning upon the LOEUF score and expression bin. The script for generating MAPs scores is available at
https://github.com/macarthur-lab/tx_annotation/blob/master/analyses/maps_submit_per_class.py

Manual evaluation of unexpressed regions in haploinsufficient developmental delay genes using the GENCODE workflow

As an orthogonal evaluation of regions flagged as unexpressed with the pext metric, we identified any region in 61 haploinsufficient disease genes with a pext value < 0.1 in all GTEx tissues and in GTEx brain samples, due to the relevance of brain tissues for these disorders, regardless of mutational burden in gnomAD. The resulting list of 128 regions was evaluated by the HAVANA manual annotation group of the GENCODE project[20].

The manual evaluation first established whether the transcript model corresponding to the region in question was correct in terms of structure, comparing exon / intron combinations, and the accuracy of splice sites against the RNA evidence supporting the model. Second, the functional biotype of each model was reassessed; in particular, whether the decision to annotate the model as protein-coding in GENCODE v19 was still considered appropriate. Note that GENCODE models that incorporate alternative exons or exon combinations in comparison to the 'canonical' isoform are likely to be annotated as coding if they contain a prospective CDS that is considered biologically plausible, based on a mechanistic view of translation. These re-annotations are summarized in Supplementary Table 4.

We binned cases into three main categories, according to confidence in both the accuracy and potential functional relevance of the overlapping models: (1) 'error', where the model was seen to have an incorrect transcript structure and/or a CDS that conflicted with updated GENCODE annotation criteria (these annotations had been or will be changed in future GENCODE releases based on this evaluation); (2) 'putative', where the model structure and CDS satisfied our current annotation criteria, although we judged the potential of the transcript represented to encode a protein with a functional role in cellular physiology to be nonetheless speculative (these have been maintained as putative protein-coding transcripts in GENCODE); (3) 'validated', where we believe it is highly probable that the model represents a true protein-coding isoform. High confidence in the validity of the CDS was based on comparative annotation, i.e. the observation of CDS conservation and also the existence of equivalent transcript models in other species. GENCODE also annotates transcript models as 'nonsense-mediated decay (NMD) and 'non-STOP decay' (NSD), where a translation is found that is predicted to direct the RNA molecule into one of the two cellular degradation programs. While it has been established that such 'non-productive' transcription events can play a role in gene regulation and thus disease, the interpretation of variants within NMD and NSD CDS remains challenging[41]. These models were therefore classed in a separate category.

Gene list comparisons

To evaluate the filtering power of the pext metric for Mendelian variants, we evaluated the number of variants that would be filtered with an average GTEx pext cutoff of 0.1 (low expression) in the ClinVar and gnomAD datasets. We downloaded the ClinVar VCF from the ClinVar FTP (version dated 10/28/2018), imported it into Hail, annotated it with VEP v85 against Gencode v19, and added pext annotations with the tx_annotate() function. All evaluated variants were annotated as HC by LOFTEE v1.0, and ClinVar variants were filtered to those marked as pathogenic, with no conflicts, and reviewed with at least one star status.

For variants in 61 haploinsufficient genes, we identified any variant identified in at least one individual with any zygosity in both datasets. For variants identified in autosomal recessive disease genes, we used a list of 1,183 OMIM disease genes deemed to follow a recessive inheritance pattern by Blekhman et al.[42] and Berg et al.[43] (available as https://github.com/macarthur-lab/gene_lists/blob/master/lists/all_ar.tsv). We compared the pext value for all pLoF variants identified in ClinVar versus any variant in a homozygous state in at least one individual in the gnomAD exome or genome datasets. Finally, we used a LOEUF cutoff of 0.35 to denote constrained genes, and compared any synonymous or pLoF variant in these genes in the gnomAD exome or genome datasets.

De novo and rare variant analysis

De novo variants were collated from previously published studies. We collected de novo identified in 5,305 probands from trio studies of intellectual disability/developmental disorders (Hamdam et al[26]: n = 41, de Ligt et al[27]: N = 100, Rauch et al[28]: N = 51, DDD[23]: n = 4,293, Lelieveld et al[25]: n = 820), 1,073 probands with congenital heart disease with co-morbid developmental delay (Sifrim et al[39]: n = 512, Chih Jin et al[44] : 561), 6,430 ASD probands, and 2,179 unaffected controls from the Autism Sequencing Consortium[24]. We also utilized a previously published dataset of variants in 8,437 cases with ASD and/or attention-deficit/hyperactivity disorder and 5,214 controls from the Danish Neonatal Screening Biobank[45]. In this analysis, we analyzed pLoF variants identified in highly constrained genes (first LOEUF decile) with a combined total allele count of ≤10 in cases and controls.

We annotated both de novo and rare variants with VEP v85 against Gencode v19 and added pext annotations with the tx_annotate() function. We then calculated the average pext metric across 11 GTEx brain samples and binned them as low (pext < 0.1), medium (0.1 ≤ pext ≤ 0.9) or high (pext > 0.9) expression. We then calculated the number of pLoF, missense, and synonymous variants per pext expression bin. To obtain case-control rate ratios and the 95% confidence intervals for *de novo* variant analyses, we used a two-sided Poisson exact test on counts[46]. To obtain the odds ratio for the rare variant analysis in ASD/ADHD, we used the Fisher's exact test for count data.

Isoform quantifications via salmon

To evaluate whether use of a different isoform quantification tool would affect results, we compared results of *TCF4* baselevel expression (shown in Figure 2B), MAPS (Figure 2C) and comparison of the number of variants filtered in HI developmental disease genes in ClinVar vs gnomAD (Figure 4A) using RSEM quantifications used in this study with quantifications using salmon v.0.12[47]. Due to the intractability of re-quantifying the entire GTEx dataset, we downloaded 151 GTEx brain – cortex CRAM files from the V7 dataset. We first converted CRAMs to fastq files using Picard 2.18.20 and ran salmon with the "salmon quant –i index -fastq1 – fastq2 –minAssignedFrag1 –validateMappings" command. The index was created with the "salmon index –t transcript.fa –type quasi –k 31" command using the GENCODE v19 FASTA files of protein-coding and lncRNA transcripts. The existing GTEx RSEM isoform quantifications were filtered to the same GTEx brain – cortex samples. The WDL script for the quantification pipeline is available at : gs://gnomad-public/papers/2019-tx-annotation/results/salmon_rsem/salmon.wdl and the commands to obtain results for each individual analysis at https://github.com/macarthur-lab/tx_annotation/blob/master/analyses/rsem_vs_salmon.py.

Transcript expression aware annotation with fetal isoform expression dataset

While our analyses were based on transcript expression aware annotation from the GTEx v7 dataset, we provide necessary files for pext annotation with the Human Brain Development Resource (HBDR) fetal brain dataset[48] in gs://gnomad-public/papers/2019-tx-annotation/data/. HBDR includes 558 samples from varying brain subregions across developmental timepoints. We downloaded HDBR sample fastq files from European Nucleotide Archive (study accession PRJEB14594/) and obtained RSEM isoform quantification on HBDR fastqs using the GTEx v7 quantification pipeline, publicly available at https://github.com/broadinstitute/gtex-pipeline/) which briefly involves 2-pass alignment with STAR v2.4.2a[30] and and isoform quantification with RSEM v1.2.22. The dataset was used for the analysis of baselevel expression values in *SCN2A* shown in Supplementary Figure 6D. The commands to obtain the results is available in https://github.com/macarthur-lab/tx_annotation/blob/master/analyses/get_scn2a_baselevel_fetal.py

Supplementary Materials and Methods

Fig S1: Details of manual curation of 401 pLoF variants in 61 HI developmental disease genes
Fig S2: Technical artifacts in transcriptome sequencing experiments prevent the use of read pileup at exons as an unbiased proxy for expression
Fig S3: Details of calculating transcript-expression annotation
Fig S4: Baselevel *TCF4* expression per GTEx tissue
Fig S5: Functional validation of pext
Fig S6: Results of GENCODE curation of 128 unexpressed regions in HI developmental disease genes
Fig S7: Application of pext binning to *de novo* and rare variant analysis in additional datasets
Fig S8: Comparison of key results using Salmon vs RSEM.
Fig S9: Results from FUMA GENE2FUNC analysis in unconserved regions with high expression values

Table S1 : Summary of manual curation flags for 401 pLoFs in 61 HI developmental disease genes identified in gnomAD
Table S2: Summary of criteria for LoF verdicts of 401 pLoF in 61 HI developmental disease genes identified in gnomAD
Table S3: Manual curation results of 401 pLoFs in 61 HI developmental disease genes identified in gnomAD
Table S4: GENCODE curation results of 128 regions flagged as unexpressed by pext
Table S5: FUMA GENE2FUNC analysis results and run information

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We have complied with all relevant ethical regulations. This study was overseen by the Broad Institute's Office of Research Subject Protection and the Partners Human Research Committee, and was given a determination of Not Human Subjects Research. Informed consent was obtained from all participants.

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