Large-scale discovery of novel genetic causes of developmental disorders

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Introduction

Despite three decades of successful, predominantly ***phenotype-driven***, discovery of the genetic causes of monogenic disorders 1, up to half of children with severe developmental disorders (DDs) of likely genetic origin remain without a genetic diagnosis. Especially challenging are those disorders rare enough to have eluded recognition as a discrete clinical entity, those whose clinical manifestations are highly variable, and those that are difficult to distinguish from other, very similar, disorders. Here we demonstrate the power of embracing an unbiased ***genotype-driven*** approach 2 to identify subsets of patients with similar disorders. By studying 1,133 children with severe, undiagnosed DDs, and their parents, using a combination of exome sequencing 3–11 and array-based detection of chromosomal rearrangements, we discovered 12 novel genes causing DDs. These newly implicated genes increase by 10% (from 28% to 31%) the proportion of children that could be diagnosed. Clustering of missense mutations in six of these newly implicated genes suggest that normal development is being perturbed by an activating or dominant negative mechanism. Our findings demonstrate the value of adopting a comprehensive strategy, both genomewide and nationwide, to elucidating the underlying causes of rare genetic disorders.

We established a network to recruit 1,133 children (median age 5.5, Extended Data Fig. 1A) with diverse, severe undiagnosed DDs, through all 24 regional genetics services of the UK National Health Service and Republic of Ireland. Among the most commonly observed phenotypes (Extended Data Fig. 1B, Supplementary Table 1) were intellectual disability or developmental delay (87% of children), abnormalities revealed by cranial MRI (30%), seizures (24%), and congenital heart defects (11%). These children are predominantly (~90%) of Northwest European ancestry (Extended Data Fig. 1C), with 47 pairs of parents (4.1%) exhibiting kinship equivalent to, or in excess of second cousins (Extended Data Fig. 1D, Supplementary Information). In most families (849/1,101), the child was the only affected family member, but 111 children had one or more parents with a similar DD, and 124 had a similarly affected sibling (Supplementary Information). Prior clinical genetic testing would have already diagnosed many children with easily recognized syndromes, or

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large pathogenic deletions and duplications, enriching this research cohort for less distinct syndromes, and novel genetic disorders.

We exome sequenced 1,133 affected children and their parents, from 1,101 families, representing 1,071 unrelated children and 30 sibships. We also performed exome-focused array comparative genomic hybridization (exome-aCGH) on the children (N=1,009) and UK controls (N=1,013) and genome-wide genotyping on the trios (N=1,006) to identify deletions, duplications, uniparental disomy (UPD) and mosaic large chromosome rearrangements. From our exome sequencing and exome-aCGH data, we detected an average of 19,811 coding or splicing single nucleotide variants (SNVs), 491 coding or splicing indels and 148 Copy Number Variants (CNVs) per child (Supplementary Information). From analyses of the genotyping array data 12 we identified 6 children with UPD and 5 children with mosaic large chromosomal rearrangements (Supplementary Information). The SNVs, indels and CNVs were analysed jointly in the following analyses, allowing, for example, the identification of compound heterozygous CNVs and SNVs affecting the same gene.

We discovered 1,618 *de novo* variants (1,417 SNVs, 114 indels and 87 CNVs) in coding and non-coding regions (Supplementary Tables 2 and 3), of which 1,596 (98.6%) were validated using a second, independent assay, and the remainder were validated clinically. This represents an average of 1.12 *de novo* SNVs and 0.09 *de novo* indels in coding or splicing regions per child, which is within the range of similar studies 3–11. The distribution of *de novo* SNVs and indels per child closely approximated the Poisson distribution expected for random mutational events (Extended Data Fig. 2).

We classified 28% (N=317) of children with likely pathogenic variants (Supplementary Table 4 and 13) in 1,129 robustly implicated DD genes (published before Nov 2013), or with pathogenic deletions or duplications. The majority of these diagnoses involved *de novo* SNVs, indels or CNVs (Table 1). Females had a significantly higher diagnostic yield of autosomal *de novo* mutations than males (p=0.01, Fisher exact test). Among the single gene diagnoses, most DD genes (95/148) were only observed once, although eight (*ARID1B*, *SATB2*, *SYNGAP1*, *ANKRD11*, *SCN1A*, *DYRK1A*, *STXBP1, MED13L*) each accounted for 0.5-1% of children in our cohort (Extended Data Figure 3). For 17 of these children we identified two different genes with pathogenic variants, resulting in a composite clinical phenotype.

Analyses that assess the enrichment in patients of a particular class of variation, so-called 'burden analyses', both highlight classes of variants for detailed analysis, and enable estimation of the proportion of a particular class of variant that is likely to be pathogenic. We observed a significant (p=0.0004) burden of 87 *de novo* CNVs in the 1,133 DD children compared to 12 in 416 controls (Scottish Family Health Study14) despite most children (77%) having previously had clinical microarray testing (Extended Data Figure 4).

We used gene-specific mutation rates that account for gene length and sequence context 15 to assess the burden of different classes of *de novo* SNVs and indels (Supplementary Information). We observed no significant excess of any functional class of *de novo* SNVs or

indels in autosomal recessive DD genes (Extended Data Figure 5), suggesting that few of these mutations are causally implicated. By contrast, we observed a highly significant excess of all 'functional' classes (coding and splice site variants excepting synonymous changes) of *de novo* SNVs and indels in the dominant and X-linked DD genes (Extended Data Figure 5) within which *de novo* mutations can be sufficient to cause disease. Not all protein-altering mutations in known dominant and X-linked DD genes will be pathogenic, and these burden analyses inform estimates of positive predictive values for different classes of mutations. The remaining, non-DD, genes in the genome also exhibit a more modest, but significant, excess of functional, but not silent, *de novo* SNVs and indels (Extended Data Figure 5).

We observed 96 genes with recurrent, functional mutations (Figure 1A), a highly significant excess compared to the expected number derived from simulations (median=55, Supplementary Information). This enrichment is even more pronounced (observed:29, expected:3) for recurrent LoF mutations (Figure 1B). Among undiagnosed children, we observed an excess of 22 genes (observed: 45, expected: 23) with recurrent functional mutations (Figure 1A), and an excess of 8 genes (observed:9, expected:1) with recurrent LoF mutations (Figure 1B), implying that an appreciable fraction of these recurrently mutated genes are novel DD genes.

To identify individual genes enriched for damaging *de novo* mutations (Supplementary Information), we tested for a gene-specific overabundance of either *de novo* LoF mutations or clustered functional *de novo* mutations in 1,130 children (excluding one twin from each of 3 identical twin-pairs). To increase power to detect DD genes, we also meta-analysed our data with published *de novo* mutations from 2,347 DD trios with intellectual disability 4,9, epileptic encephalopathy 3, autism 6–8,10, schizophrenia 5, or congenital heart defects 11 (the 'meta-DD' dataset). These analyses (Figure 2) successfully re-discovered 20 known DD genes at genome-wide significance ($p < 1.31 \times 10^{-6}$, a Bonferroni p value of 0.05 corrected for 38,504 tests [Supplementary Information]). Thus, despite the broad phenotypic ascertainment in these datasets, we can robustly detect DD genes solely on statistical grounds.

To increase our power to detect novel DD genes, we repeated the gene-specific analysis described above excluding the 317 individuals with a known cause of their DD. In this analysis the statistical genetics evidence was integrated with phenotypic similarity of patients, available data on model organisms and functional plausibility. We identified 12 novel disease genes with compelling evidence for pathogenicity (Table 2), nine of which exceeded the genome-wide significance threshold of 1.36×10^{-6} (Supplementary Information), with the remaining three genes (*PCGF2*, *DNM1* and *TRIO*) just below this significance threshold. The two children with identical Pro65Leu mutations in *PCGF2*, which encodes a component of a Polycomb transcriptional repressor complex, share a strikingly similar facial appearance representing a novel and distinct dysmorphic syndrome. *DNM1* was previously identified as a candidate gene for epileptic encephalopathy (EE) 3. Two of the three children we identified with *DNM1* mutations also had seizures, and a heterozygous mouse mutant manifests seizures 16. In addition to two *de novo* missense SNVs in *TRIO*, we identified an intragenic *de novo* 82kb deletion of 16 exons. For several of these novel DD genes, the meta-DD analysis increased the significance of enrichment.

For example, a total of five *de novo* LoF variants in *POGZ* were identified, two from our cohort, two from recent autism studies and one from a recent schizophrenia study. We also identified six genes with suggestive statistical evidence of being novel DD genes, defined as being a p value for mutation enrichment less than 1×10^{-4} and being plausible from a functional perspective (Extended Data Table 1). We anticipate that the majority of these genes will eventually accrue sufficient evidence to meet the stringent criteria we defined above for declaring a novel DD gene.

Strikingly, we observed identical missense mutations in unrelated, phenotypically similar, patients for four of these novel DD genes (*PCGF2*, *COL4A3BP*, *PPP2R1A* and *PPP2R5D*), and for a fifth gene, *BCL11A*, we identified highly significant clustering of non-identical missense mutations (Figure 3). We hypothesise that the mutations in some of these genes may be operating by either dominant negative or activating mechanisms. This hypothesis is supported by prior functional evidence for several of the mutated amino acids. The three identical Ser132Leu mutations in *COL4A3BP*, which encodes an intracellular transporter of ceramide, remove a serine that when phosphorylated down-regulates transporter activity from the ER to the golgi 17, presumably resulting in intra-cellular imbalances in ceramide and its downstream metabolic pathways. The two mutated amino acids (Arg182Trp and Pro179Leu) in *PPP2R1A*, which encodes the scaffolding A subunit of the Protein Phosphatase 2 complex, have been previously identified as sites of driver mutations in endometrial and ovarian cancer 18. It has previously been shown that mutating either of these two residues results in impaired binding of B subunits of the complex 18. Intriguingly, *PPP2R5D* encodes one of the possible B subunits of the same Protein Phosphatase 2 complex, suggesting that the clustered missense mutations (Pro201Arg and Glu198Lys) in this gene may similarly perturb interactions between subunits of this complex. Further functional studies will be required to confirm this hypothesis.

We assessed transmission biases of potentially pathogenic inherited SNVs in our probands (Supplementary Information) and observed a genome-wide trend (p=0.015) towards over-transmission to probands of very rare (MAF < 0.0005%) LoF variants, but not damaging missense variants. We also observed a 1.8-fold enrichment (p=0.04) of rare (MAF<5%) biallelic LoF variants (Supplementary Table 5) among probands without a likely dominant cause of their disorder, compared to those with either a diagnostic *de novo* mutation or an affected parent. Again we saw no enrichment in biallelic damaging missense variants (Extended Data Table 2), consistent with a similar observation in children with autism 19. These observations imply that although inherited LoF variants (both monoallelic and biallelic) are likely contributing to DD in our patients, much larger sample sizes will be required to pinpoint specific DD genes in this way.

To direct future, detailed functional experiments on the developmental role of a subset of candidate genes from this study we used two approaches. First, knockdown-induced phenotypes were recorded in early zebrafish development. Second we performed a systematic review of perturbed gene function in human, mouse, xenopus, zebrafish and drosophila. In both approaches the animal phenotypes were compared to those seen in individuals in our cohort

We undertook an antisense-based loss of function screen in zebrafish to assess 32 candidate DD genes with *de novo* LoF, *de novo* missense or biallelic LoF variants from exome sequencing (Supplementary Information and Supplementary Table 6). These candidate genes corresponded to 39 zebrafish orthologues. Knockdowns of these zebrafish genes were repeated at least twice and all morpholinos were co-injected with *tp53* morpholino to eliminate off-target toxicity. Successful knockdown of the targeted mRNA could be confirmed using RT-PCR for 82.4% of genes (28/34) and 9/11 (82%) of genes that were tested gave an equivalent phenotype when knocked down by a second, independent morpholino. Knock-down of at least one or a pair of zebrafish orthologues of 65.6% of candidate DD genes (21 out of 32) resulted in perturbed embryonic and larval development (Figure 4, Extended Data Table 3, Supplementary Data and Supplementary Table 7). Large-scale mutagenesis 20 and morpholino 21 studies suggest knockout or knockdown of 6-12% genes give developmental phenotypes, suggesting at least a five-fold enrichment of developmentally non-redundant genes among the 32 selected for modelling. We then compared the phenotypes of the zebrafish morphants to those of the DDD individuals with *de novo* mutations or biallelic LoF variants in the orthologous genes (Extended Data Table 3). 11/21 (52.4%) of the genes were categorised as *strong* candidates based on phenotypic similarity (Figure 4A). 7/11 were potential microcephaly genes whose gene knockdown in zebrafish gives significant reductions in both head measurements, and neural tissue (Figure 4B, Supplementary Information). 6/21 (28.6%) genes resulted in severe morphant phenotypes which could not be meaningfully linked to patient phenotypes. As many of our candidate DD genes carried heterozygous LoF variants (*de novo* mutations), it is to be expected that the severity of LoF phenotypes in zebrafish may exceed that observed in our patient cohort. The genes with proven non-redundant developmental roles can reasonably be assigned higher priority for downstream functional investigations and genetic analyses.

Our systematic review of gene perturbation in multiple species sought both confirmatory and contradictory (e.g. healthy homozygous knock-out) evidence from other animal models for these 21 apparently developmentally important genes. We identified 16 genes with solely confirmatory data, often from multiple different organisms, none with solely contradictory data, two with both confirmatory and contradictory evidence and three with no evidence either way (Supplementary Table 8).

In summary, our analyses validate a large-scale, genotype-driven strategy for novel DD gene discovery that is complementary to the traditional phenotype-driven strategy of studying patients with very similar presentations, and is particularly effective for discovering novel DDs with highly variable or indistinct clinical presentations. Our meta-analysis with previously published DD studies increased power to detect novel DD genes and highlights the shared genetic etiologies between diverse neurodevelopmental disorders such as intellectual disability, epilepsy, autism and schizophrenia 22. We identified significantly more pathogenic autosomal *de novo* mutations in females compared to males. An increased burden of monogenic disease among females with neurodevelopmental disorders has become more apparent 23,24, and our observations strengthen this proposition. Further investigations are required to assess whether males might be enriched for poly/oligogenic causation.

The 35 patients with pathogenic mutations in the 12 novel DD genes we discovered increased our diagnostic yield from 28% to 31%. What, then, are the causes of the DDs in the other 69% of patients? The undiagnosed patients are not obviously less severely affected than the diagnosed patients (e.g. fewer phenotype terms, older age of recruitment). We anticipate that there are many more pathogenic, monogenic, coding mutations in these undiagnosed patients that we have detected, but for which compelling evidence is currently lacking. This hypothesis is supported by four strands of evidence: (i) modeling statistical power suggests that studying ~1,000 trios has only 5-10% power to detect an averagely mutable haploinsufficient DD gene (Extended Data Figure 6A, Supplementary Information), (ii) the expectation that our power to detect novel DD genes that operate recessively or by gain-of-function mechanisms will be lower than for haplosufficient genes, (iii) the significant enrichment in undiagnosed patients of functional mutations in genes predicted to exhibit haploinsufficiency (Extended Data Figure 6B), and (iv) the strong enrichment for developmental phenotypes in the zebrafish knock-down screen.

Given our limited power to detect pathogenic mutations that act through dominant negative or activating mechanisms, it was notable that in four of our novel genes (*COL4A3BP*, *PPP2R1A*, *PPP2R5D* and *PCGF2*) we observed identical *de novo* mutations in unrelated trios. Two hypotheses might explain this observation: first, that there is a vast number of different gain-of-function mutations, of which we are just scratching the surface in this study, or second, that these particular variants are enriched in our cohort due to these mutations conferring a positive selective advantage in the germline 25. Analysis of larger datasets will be required to assess these hypotheses, although they are not necessarily mutually exclusive.

These considerations of the limited power of even nationwide studies such as ours motivate the international sharing of minimal genotypic and phenotypic data, for example through the DECIPHER web portal (http://decipher.sanger.ac.uk), to provide diagnoses for patients who would otherwise remain undiagnosed. Plausibly pathogenic variants observed in undiagnosed patients in our study (*de novo* SNVs, indels and CNVs, and biallelic LoF in genes not yet associated with disease) are shared through DECIPHER, and we encourage other, comparable studies to adopt a similar approach.

Extended Data

EDT1

Novel genes with suggestive evidence for a role in DD

Six genes with suggestive evidence to be novel DD genes. The number of unrelated patients with independent functional or LoF mutations in the DDD cohort or the wider meta-analysis dataset including DDD patients is listed. The p value reported is the minimum p value from the testing of the DDD dataset and the meta-analysis dataset. The dataset that gave this minimal p value is also reported. Mutations are considered to be clustered if the p value of clustering of functional SNVs is less than 0.01. Predicted haploinsufficiency is reported as a percentile of all genes in the genome, with ~0% being highly likely to be haploinsufficient and 100% very unlikely to be haploinsufficient, based on the prediction score described in Huang et al 26 updated to enable predictions for a higher fraction of genes in the genome. *NAA10* is already known to cause an X-linked recessive DD in males, but here we identified missense mutations in females, suggesting a different, X-linked dominant, disorder.

Evidence	Gene	*de novos* DDD (Missense, LoF)	*de novos* Meta (Missense, LoF)	P Value	Test	Mutation Clustering	Predicted Haploinsufficiency
De novo enrichment + additional evidence	NAA15	1 (0,1)	3 (0,3)	1.64E-06	Meta	No	7.5%
	ZBTB20	3 (1,2)	3 (1,2)	4.84E-06	DDD	No	0.2%
	NAA10	2 (2,0)	3 (3,0)	8.28E-06	Meta	No	34.1%
	TRIP12	3 (1,2)	4(2,2)	2.13E-05	Meta	No	3.8%
	USP9X	3 (1,2)	3 (1,2)	5.14E-05	DDD	No	3.8%
	KAT6A	2 (0,2)	2 (0,2)	7.91E-05	DDD	No	19.0%

EDT2
Biallelic Loss of function and damaging functional variants

Rare (MAF < 5%) biallelic loss-of-function and damaging functional variants in uninherited diplotypes and probands. 'Likely dominant probands' refers to probands with a reported *de novo* mutation or affected parents, and 'other probands' to all remaining probands. 'DDG2P Biallelic' refers to confirmed and probable DDG2P genes with a biallelic mode of inheritance. See Supplemental methods for details of variant processing.

Biallelic Variant Types	Untransmitted Diplotypes (n=1080)	Likely Dominant Probands (n=270)	Other Probands (n=810)
LoF/LoF (Genome-wide)	110	17	86
LoF/Dam (Genome-wide)	87	21	71
Dam/Dam (Genome-wide)	312	90	264
LoF/LoF (DDG2P Biallelic)	1	1	3
LoF/Dam (DDG2P Biallelic)	2	0	6
Dam/Dam (DDG2P Biallelic)	26	7	25

EDT3

Zebrafish modeling identifies 21 developmentally important candidate genes

This table summarises the 21 genes whose knockdown results in developmental phenotypes in zebrafish. "# patients" column indicates how many patients were identified as carrying variants in these genes. Split numbers indicate the breakdown of variant types (eg. for *BTBD9*, 2/1 is two biallelic LoF and one *de novo* missense carrying patients). A summary of the patient phenotypes is listed, as well as the relevant phenotypes observed in zebrafish knockdown experiments. Phenotypic concordance categories indicate the degree of overlap between the zebrafish phenotyping and the patient phenotypes. Weak concordance typically is the result of severe, multisystem phenotypes in zebrafish. See Supplemental Materials for more detailed phenotype information.

| Gene	# patients	Variant	Patient phenotypes	Phenotypic concordance	Relevant knockdown phenotypes
BTBD9	2/1	Biallelic LoF/*De novo* Missense	Seizures, microcephaly, hypertonia	Strong	Reduced head size, brain volume
CHD3	1/2	*De novo* LoF/Missense	CNS and craniofacial defects	Strong	Abnormal head shape
DDX3X	1/3	*De novo* LoF/Missense	Moderately short stature, microcephaly, CNS defects	Strong	Reduced head size, brain volume
ETF1	1	*De novo* LoF	CNS and craniofacial defects, seizures, microcephaly, hypertelorism	Strong	Reduced head size, brain volume
FRYL	1	*De novo* LoF	Short stature, craniofacial and cardiac defects	Strong	Cardiac defects, reduced axis length
PKN2	1	*De novo* Missense	CNS, cardiac, ear, and craniofacial defects, growth retardation	Strong	Cardiac, craniofacial cartilage, and growth defects
PSMD3	1	*De novo* Missense	Microcephaly, muscular hypotonia, seizures, growth abnormality	Strong	Reduced head size and neural defects
SCGN	1	Biallelic LoF	Seizures, microcephaly, CNS defects	Strong	Reduced head size, brain volume
SETD5	1	*De novo* LoF	Seizures, CNS and cardiac defects, poor motor coordination	Strong	Reduced head size, cardiac defects, abnormal locomotion
THNSL2	2	Biallelic LoF	Microcephaly, CNS and ear defects	Strong	Reduced head size, brain volume, neural defects
ZRANB1	2	*De novo* Missense	Microcephaly, muscle defects, seizures	Strong	Reduced heea size and neural defects
DPEP2	1	Biallelic LoF	CNS defects, growth retardation	Moderate	Growth reduction
PSD2	1	*De novo* LoF	CNS defects, hypertonia, seizures	Moderate	Abnormal musculature, CNS and locomotion
SAP130	1	*De novo* LoF	Short stature, hypotonia, hypotelorism	Moderate	Abnormal locomotion
CN0T1	1/1	*De novo* LoF/Missense	Short stature, cardiac, CNS, ear and craniofacial defects	Weak	Multisystem
DTWD2	1	*De novo* LoF	CNS defects, seizures	Weak	Multisystem
ILVBL	1	*De novo* LoF	CNS and craniofacial defects	Weak	Multisystem
NONO	1	*De novo* LoF	CNS and ear defects, hypotonia, growth retardation	Weak	Multisystem, with otic and growth defects
POGZ	2	*De novo* LoF	CNS and ear defects, hypotonia, seizures, coloboma	Weak	Multisystem
SMARCD1	1/1	*De novo* LoF/Missense	CNS defects, hypotonia	Weak	Multisystem

| Gene	# patients	Variant	Patient phenotypes	Phenotypic concordance	Relevant knockdown phenotypes
WWC1	1	*De novo* Missense	CNS defects, hypertelorism	None	None
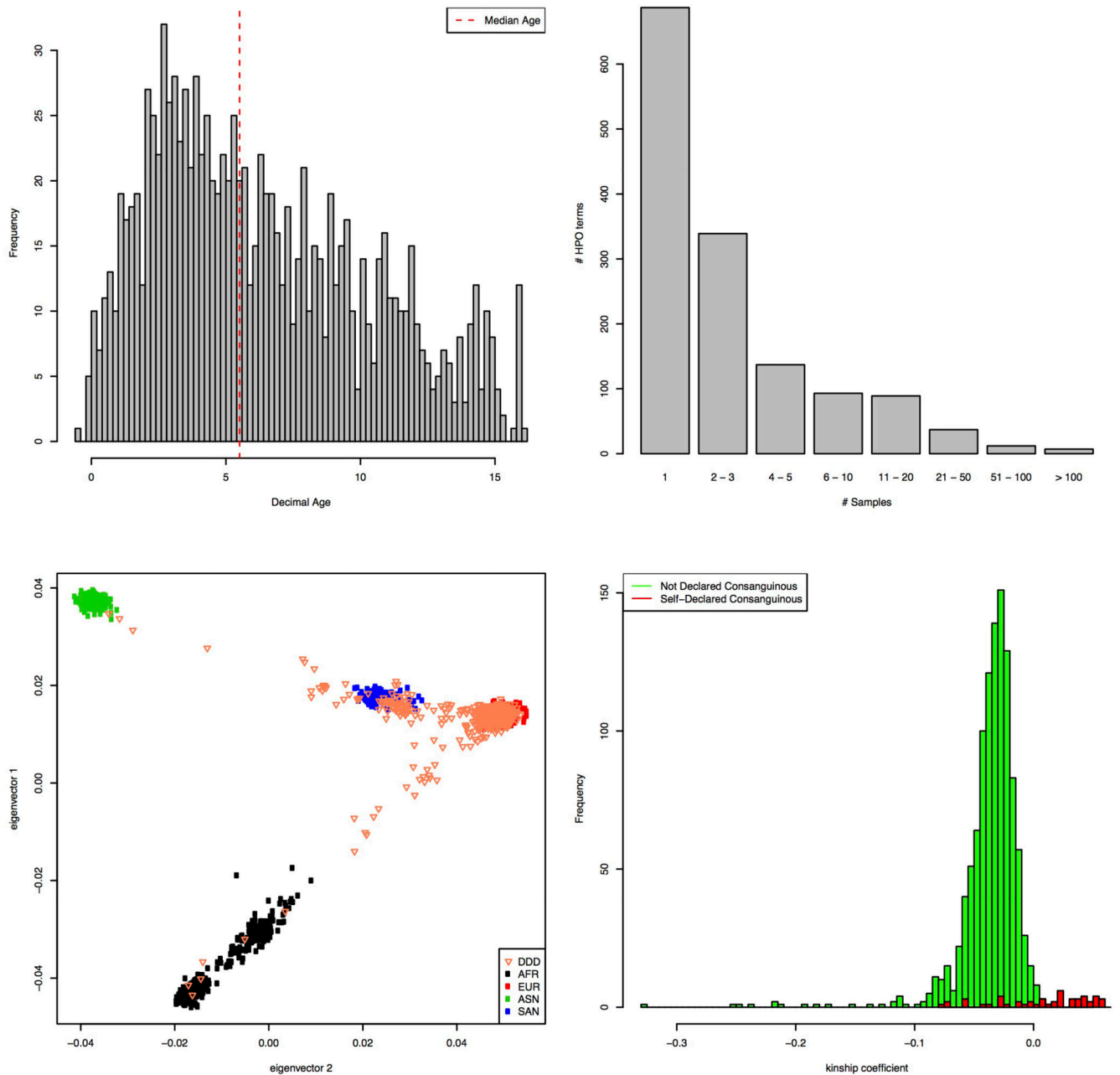
EDF1. Characteristics of the families

A. Gestation Adjusted Decimal Age at Last Clinical Assessment. Histogram showing the distribution of the gestation adjusted decimal age at last clinical assessment across the 1133 probands. The dashed red line shows the median age. **B. Frequency of HPO Term Usage**. Bar plot showing, for each used HPO term, the number of times it was observed across the 1133 proband patient records. **C. Projection PCA plot of the 1133 probands**. PCA plot of 1133 DDD probands projected onto a PCA analysis using 4 different HapMap populations from the 1000 genomes project. Black: African, Red: European, Green: East Asian, Blue: South Asian and the 1133 DDD probands are represented by orange triangles. **D. Self**

Declared and Genetically Defined Consanguinity. Overlaid histogram showing the distribution of kinship coefficients from KING comparing parental samples for each trio. Green: Trios where consanguinity was not entered in the patient record on DECIPHER. Red: Trios consanguinity was declared in the patient record on DECIPHER.
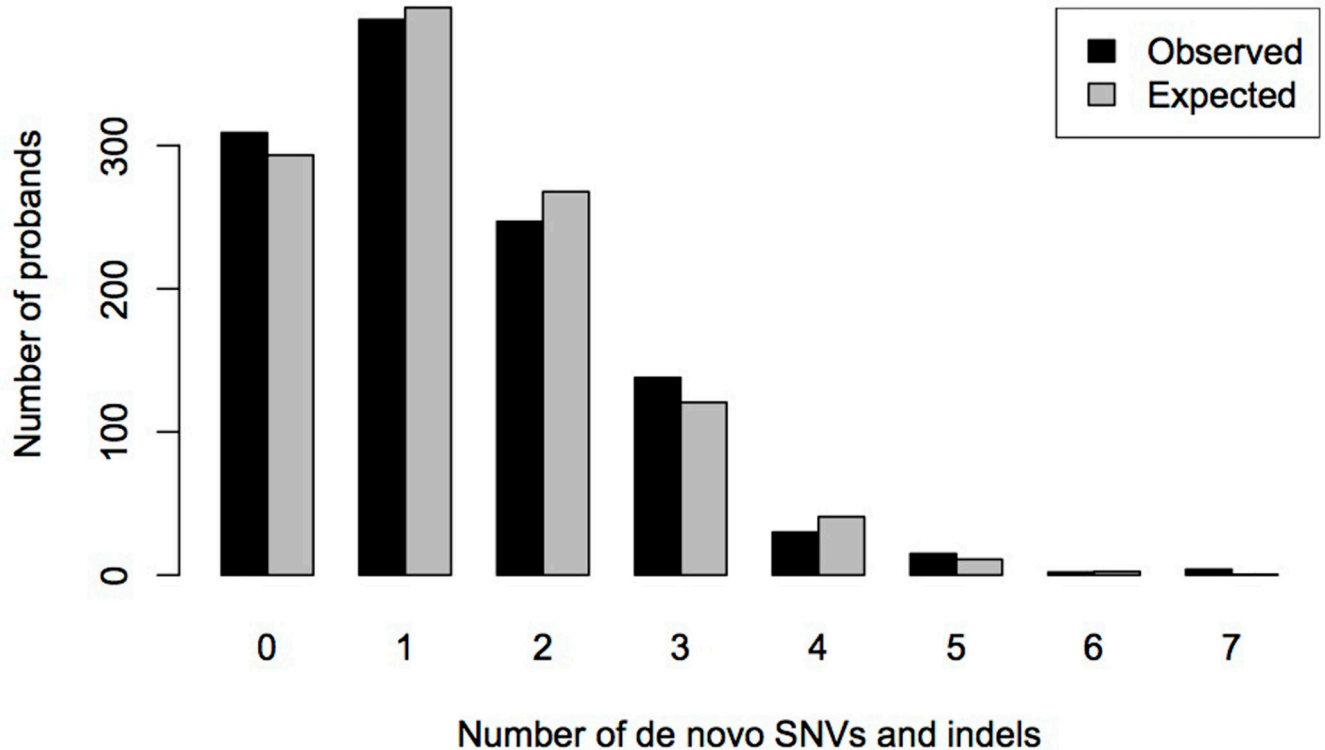
EDF2. Number of Validated *de novo* SNVs and indels per Proband
Bar plot showing the distribution of the observed number of validated SNVs and indels per proband sample, and the expected distribution assuming a Poisson distribution with the same mean as the observed distribution.
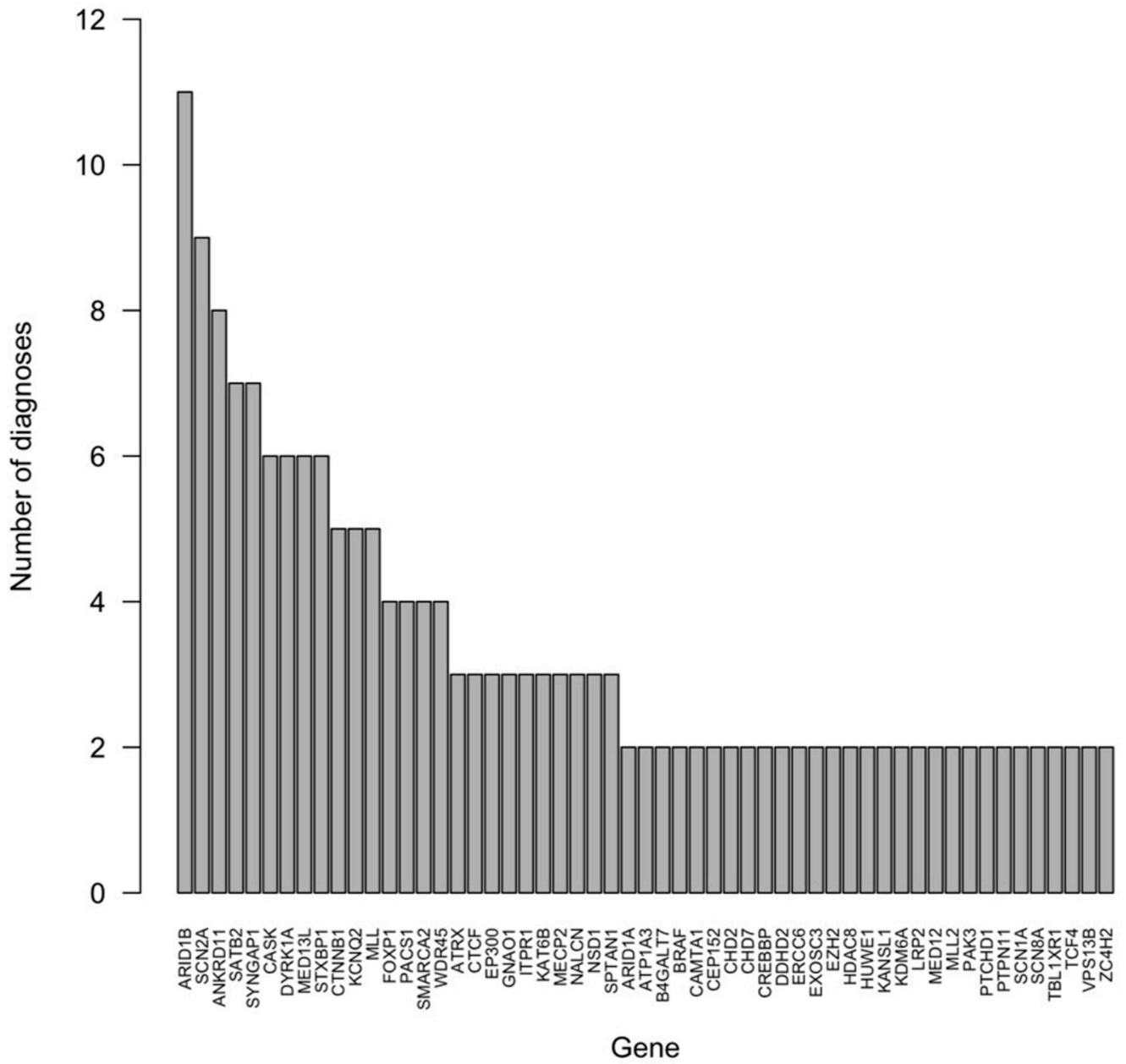
EDF3. Number of Diagnoses per Gene

Histogram showing the number of diagnoses per gene for genes with at least two diagnoses from different proband samples.
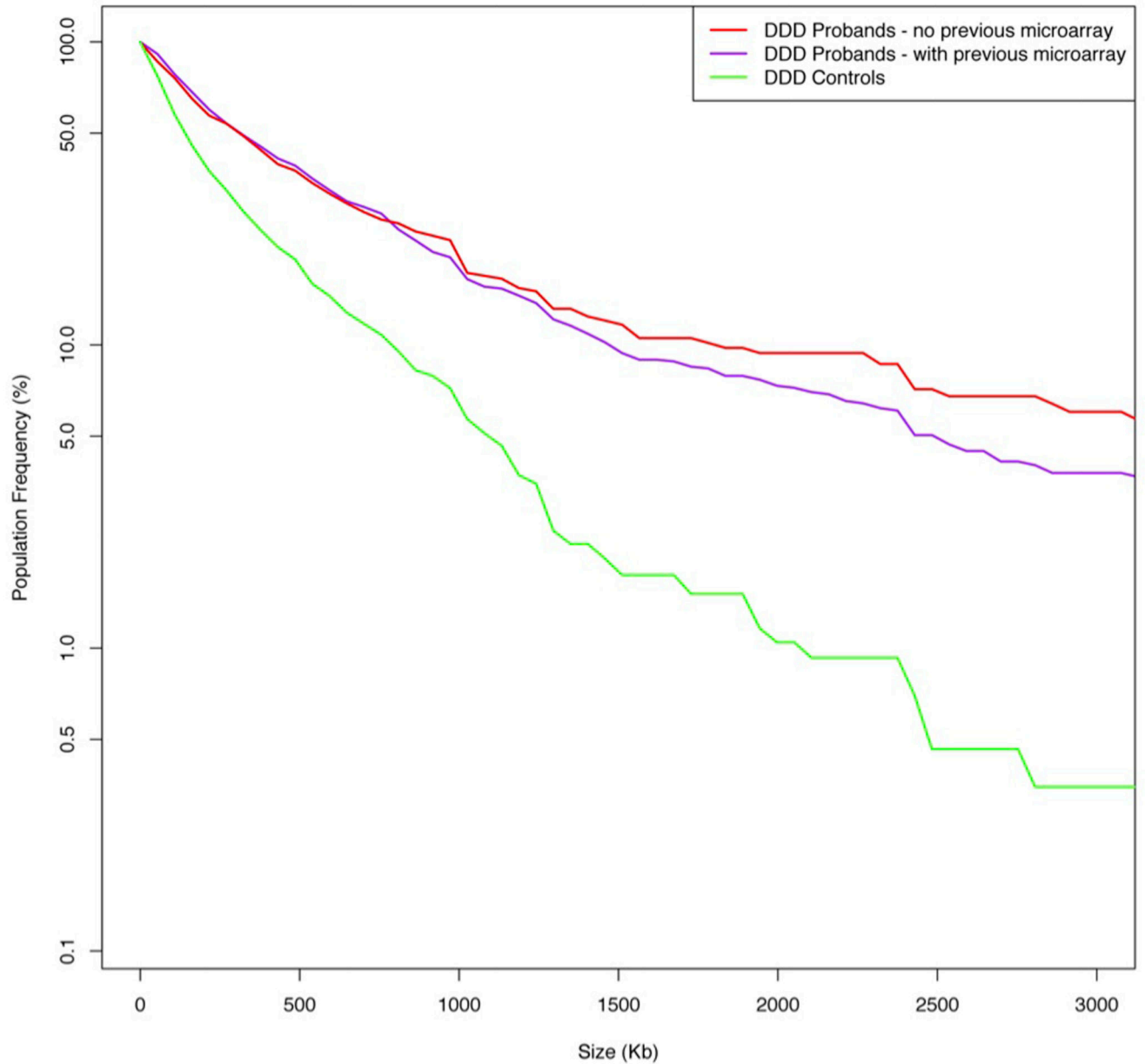
EDF4. Burden of Large CNVs in 1133 DDD Proband Samples

Plot comparing the frequency of rare CNVs in three sample groups against CNV size. Y-axis is the on a log scale. Red: DDD probands who have not had previous microarray based genetic testing, Purple: DDD probands who have had negative previous microarray based genetic testing Green: DDD controls.
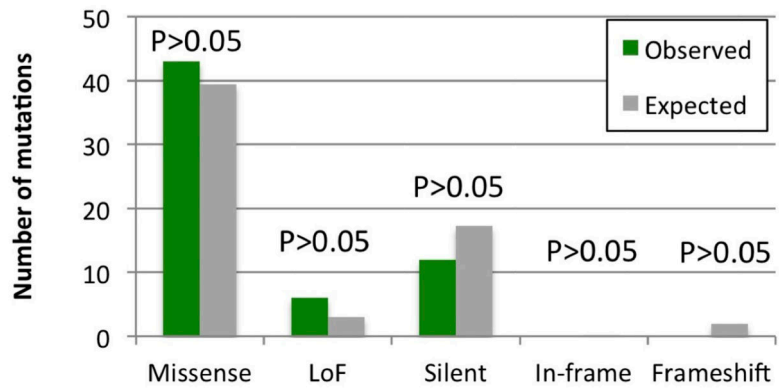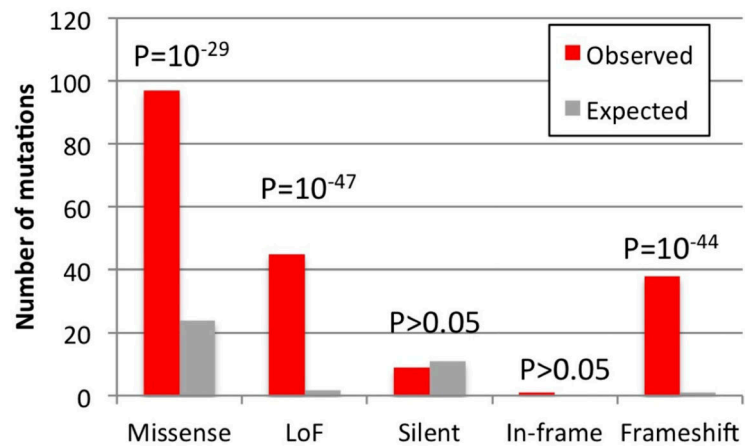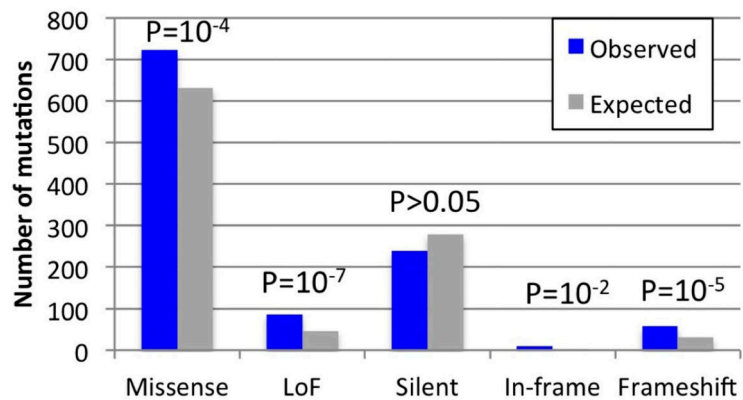
EDF5. Expected and observed numbers of *de novo* mutations

The expected and observed numbers of mutations of different functional consequences in three mutually exclusive sets of genes are shown, along with the p value from an assessment of a statistical excess of observed mutations. The three classes of genes are described in the main text.

A
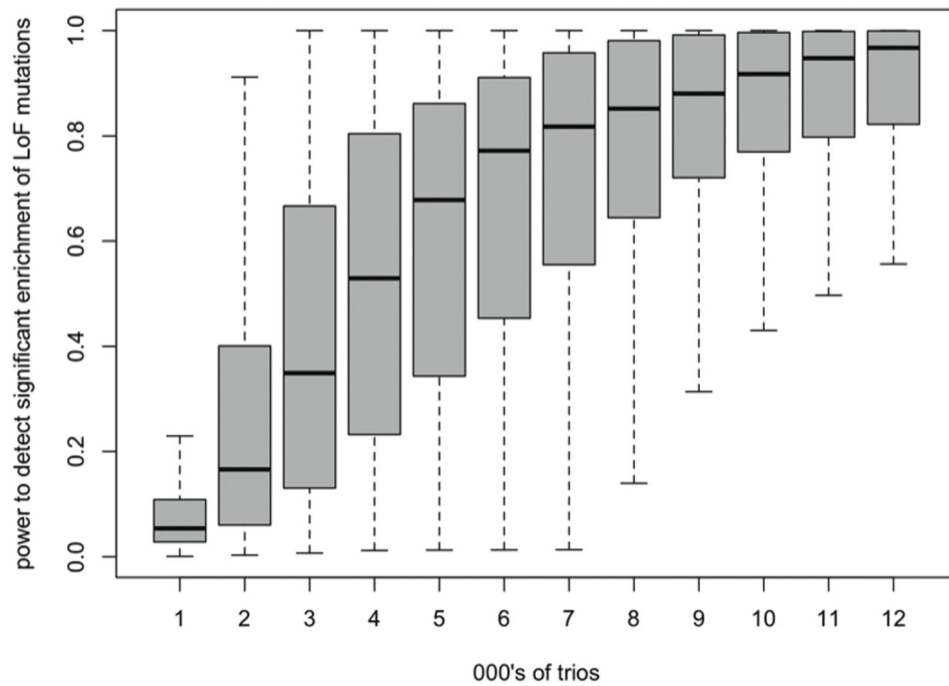
B
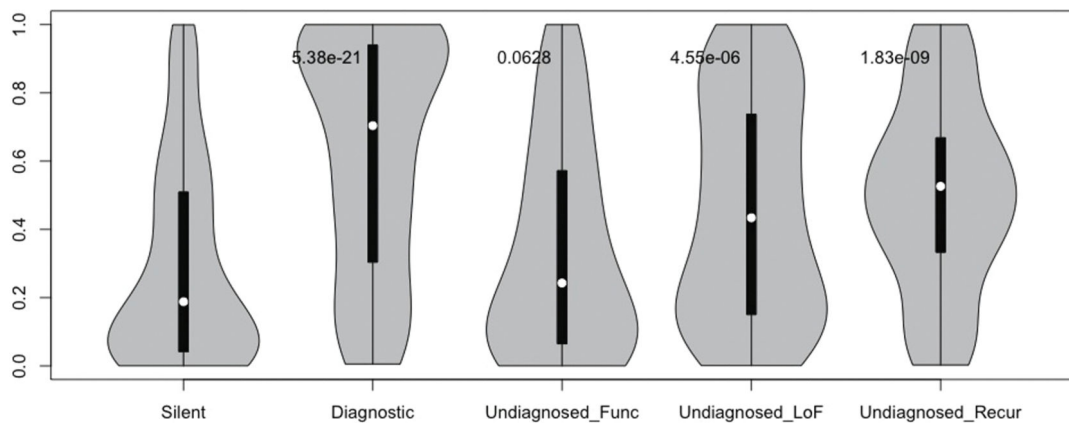
EDF6. Haploinsufficiency analyses

A. Saturation analysis for detecting haploinsufficient DD genes. A boxplot showing the distribution of statistical power to detect a significant enrichment of LoF mutations across 18,272 genes in the genome, for different numbers of trios studied, from 1,000 trios to 12,000 trios. **B. Distribution of haplinsufficiency scores in selected sets of *de novo* mutations**. Violin plot of haploinsufficiency scores in five sets of *de novo* mutations: Silent - all synonymous mutations, Diagnostic - mutations in known DD genes in diagnosed individuals, Undiagnosed_Func - all functional mutations in undiagnosed individuals,

Undiagnosed_LoF - All LoF mutations in undiagnosed individuals, Undiagnosed_recur - mutations in genes with recurrent functional mutations in undiagnosed individuals. P values for a Mann-Whitney test comparing each of the latter four distributions to that observed for the silent (synonymous) variants are plotted at the top of each violin.

Supplementary Information

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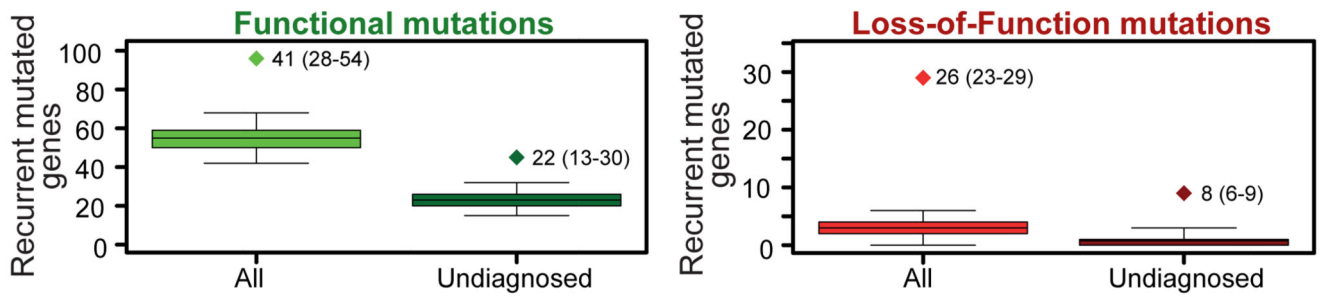
Figure 1. Excess of recurrently mutated genes

Each panel shows the observed number of recurrently mutated genes (diamond) and the distribution of the number of recurrently mutated genes in 10,000 simulations (box indicates interquartile range, whiskers indicates 95% confidence interval) under a model of no gene-specific enrichment of mutations: **a**. all protein-altering mutations in all DDD children and undiagnosed DDD children, **b**. all LoF mutations in all DDD children and undiagnosed DDD children. Each diamond is annotated with the median excess of recurrently mutated genes, with 95% confidence intervals in brackets. P value of observed excess is <0.0001 for all four tests.
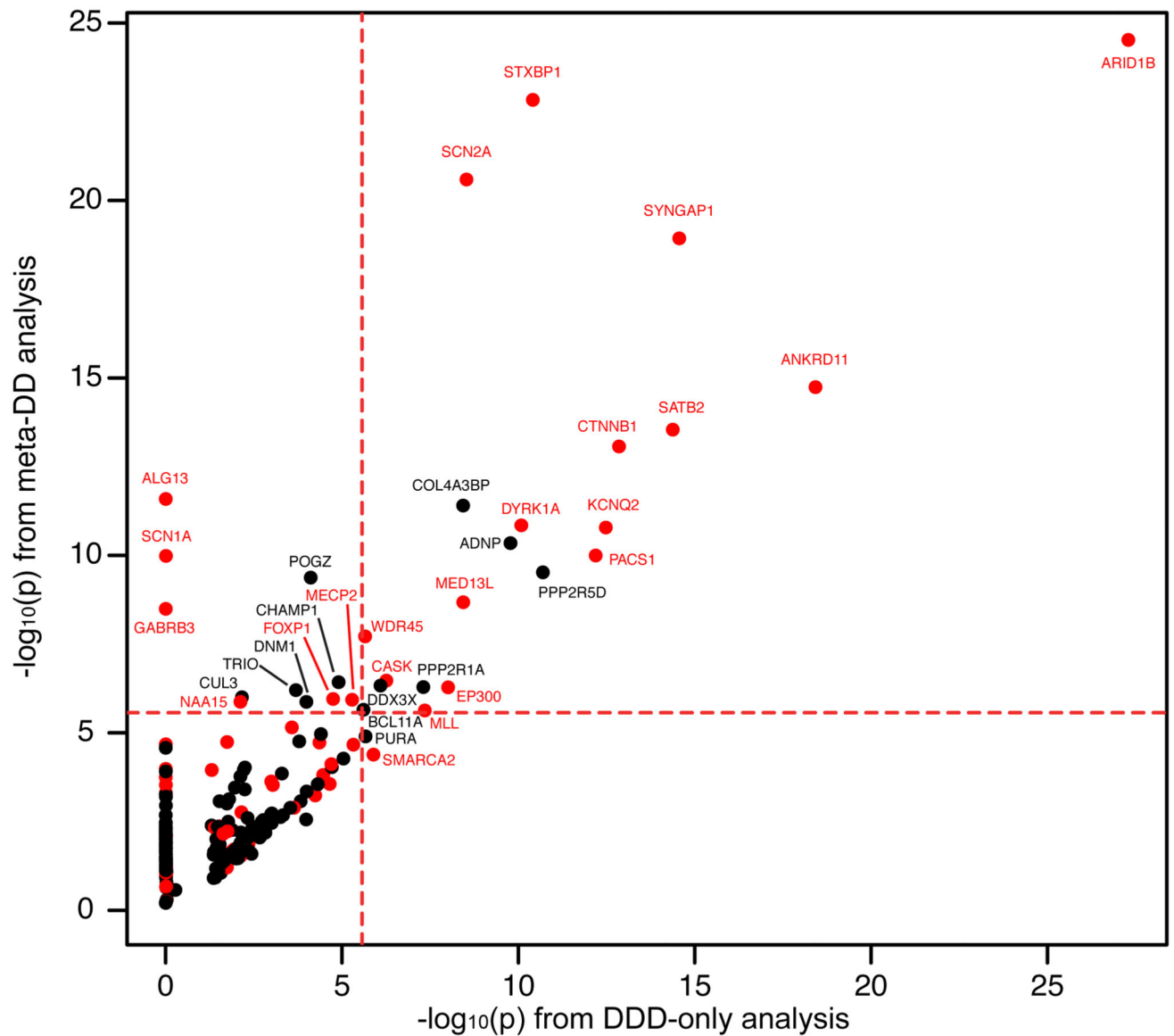
Figure 2. Gene-specific significance of enrichment for DNMs

The –log₁₀(p) value of testing for mutation enrichment is plotted only for each gene with at least one mutation in DDD children. On the X-axis is the p value of the most significant test in the DDD dataset, and on the Y-axis is the minimal p value from the significance testing in the meta-analysis dataset. Red indicates genes already known to be associated with DDs (in DDG2P). Only genes with a p value of less than 0.05/18,272 (red lines) are labeled.
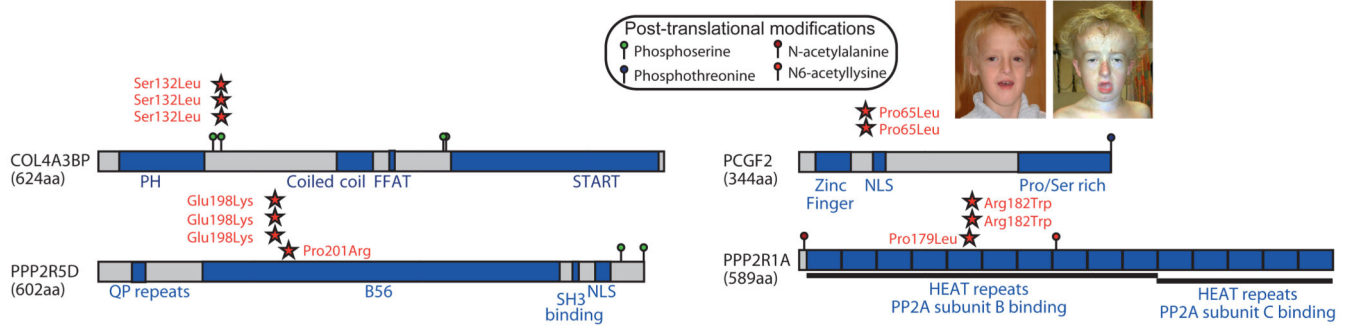
Figure 3. Five novel genes with clustered mutations

The domains (blue), post-translational modifications, and mutation locations (red stars) are shown for five proteins with highly clustered *de novo* mutations in unrelated children with severe, undiagnosed DDs. For two proteins (COL4A3BP and PCGF2) where all observed mutations are identical, photos are shown to highlight the facial similarities of patients carrying the same mutation.
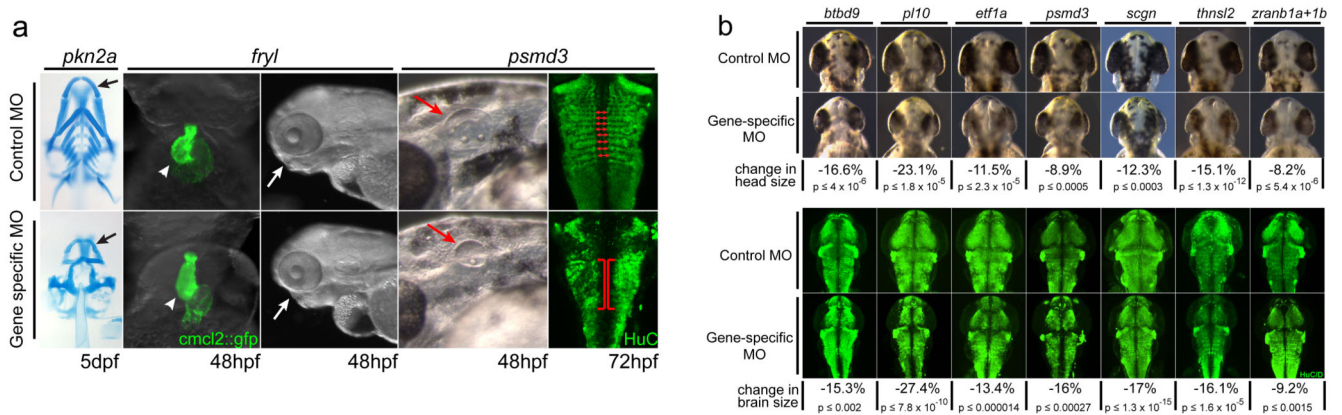
Figure 4. Candidate gene Loss of Function modeling in zebrafish reveals enrichment for developmentally important proteins

a, Examples of developmental phenotypes: Knockdown of *pkn2a* results in reduced cartilaginous jaw structures (black arrows), knockdown of *fryl* results in cardiac and craniofacial defects (white arrowheads and arrows, respectively), while knockdown of *psmd3* results in smaller ear primordia (red arrows), and mis-patterned CNS neurons (compare red double arrows and brackets). **b**, Knockdown outcomes of 7 genes with variants present in microcephaly patients: Interocular measurements of brightfield images from control and LoF embryos reveal significant decreases in head size. A neuronal antibody stain (anti-HuC/D, green channel) labels the brains of control and morphant zebrafish. Measurements taken across the widest extent of the midbrain identify significant reductions in brain size, likely underlying the concomitant head size reductions seen in brightfield. In **b**, tables show average percentage reduction in head and brain width, and p-values of a *t*-test.

Table 1

Breakdown of diagnoses by mode and by sex

	Female (%)	Male (%)	Total (%)
Undiagnosed	**383 (69.6%)**	**433 (74.3%)**	**816 (72.0%)**
Diagnosed	**167 (30.4%)**	**150 (25.7%)**	**317 (28.0%)**
De novo mutation	124 (22.5%)	80 (13.7%)	204 (18.0%)
chrX	*24 (4.4%)*	*5 (0.9%)*	*28 (2.6%)*
autosomal	*100 (18.2%)*	*75 (12.9%)*	*176 (15.5%)*
Autosomal Dominant [*]	9 (1.6%)	11 (1.9%)	20 (1.8%)
Autosomal Recessive	20 (3.6%)	26 (4.5%)	46 (4.1%)
X-linked Inherited	1 (0.2%)	19 (3.3%)	20 (1.8%)
UPD/Mosaicism	4 (0.7%)	6 (1.0%)	10 (0.9%)
Composite	9 (1.6%)	8 (1.4%)	17 (1.5%)
Total	**550**	**583**	**1133**

[*] Inherited from an affected parent

Table 2

Novel genes with compelling evidence for a role in DD

Evidence	Gene	de novos DDD (Missense, LoF)	de novos Meta (Missense, LoF)	P Value	Test	Mutation Clustering	Predicted Haploinsufficiency
De novo enrichment	*COL4A3BP*	3 (3,0)	5 (5,0)	4.10E-12	Meta	Yes	14.7%
	PPP2R5D	4 (4,0)	5 (5,0)	6.01E-12	DDD	Yes	19.7%
	ADNP	4 (0,4)	5 (0,5)	4.59E-11	Meta	No	9.8%
	POGZ	2 (0,2)	5 (0,5)	4.31E-10	Meta	No	30.0%
	PPP2R1A	3 (3,0)	3 (3,0)	2.03E-08	DDD	Yes	23.5%
	DDX3X	4 (3,1)	5 (3,2)	2.26E-07	DDD	No	12.7%
	CHAMP1	2 (0,2)	3 (0,3)	4.58E-07	Meta	No	52.9%
	BCL11A	3 (3,0)	4 (3,1)	1.03E-06	DDD	Yes	0.6%
	PURA	3 (1,2)	3 (1,2)	1.14E-06	DDD	No	9.4%
De novo enrichment + additional evidence	*DNM1*	3 (3,0)	5 (5,0)	1.43E-06	Meta	No	13.5%
	TRIO	2 (2,0)	7 (7,0)	5.16E-06	Meta	Yes	25.7%
	PCGF2	2 (2,0)	2 (2,0)	1.08E-05	DDD	Yes	37.7%

The table summarises the 12 genes with compelling evidence to be novel DD genes. The number of unrelated patients with independent functional or LoF mutations in the DDD cohort or the wider meta-analysis dataset including DDD patients is listed. The p value reported is the minimum p value from the testing of the DDD dataset and the meta-analysis dataset. The dataset that gave this minimal p value is also reported. Mutations are considered to be clustered if the p value of clustering of functional SNVs is less than 0.01. Predicted haploinsufficiency is reported as a percentile of all genes in the genome, with ~0% being highlight likely to be haploinsufficient and 100% very unlikely to be haploinsufficient, based on the prediction score described in Huang et al 26 updated to enable predictions for a higher fraction of genes in the genome. During submission, a paper was published online describing a novel DD caused by mutations in *ADNP27*.