SUPPLEMENTAL MATERIAL

Clinical genetic risk variants inform a functional protein interaction network for tetralogy of Fallot

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Supplemental Methods

Exome sequencing data

We studied the exome sequencing data of a multi-centre cohort of probands with TOF, accessed through the European Genome-phenome Archive (EGA [46]; https://www.ebi.ac.uk/ega), study accession EGAS00001003302. Exome sequencing had been performed at the McGill University/Genome Quebec Innovation Centre (MUGQIC; Montreal, Canada), and the methodology is described in detail in the supplementary information of Page et al. Briefly, exome capture was performed using Agilent SureSelectXT Human All Exon 50 Mb version 4 kit (Agilent). Trimming of paired-end sequencing reads (100 bp), using Trimmomatic, and read mapping with removal of duplicate reads (BWA-MEM algorithm[47]) were done at the McGill Genome Centre. Participants had given written informed consent for participation in the study. The cohort was reported to be of Northern European ancestry, not to exhibit features of recognized malformation or developmental syndromes[48], and to have previously excluded 22q11.2 microdeletions by multiplex ligation-dependent probe amplification. We downloaded 829 bam files from EGA, but subsequently excluded 18 data files that were: corrupted (n = 2), aligned to a different reference genome: bwa VN:0.7.6a-r433, b37 (n = 9), or found to be identical to other samples in the cohort (n = 7). We studied the resulting n = 811 unique exome sequencing datasets, aligned to the bwa VN:0.7.4-r385, hg19 reference genome.

For the analyses presented here, we considered n = 811 unique exome sequencing datasets (Supplemental Table I), aligned to GRCh37 (hg19). The data were analyzed at The Centre for Applied Genomics (TCAG, The Hospital for Sick Children, Toronto, Canada) under a research protocol of the Hospital for Sick Children (REB# 0019980189).

Variant calling and annotation

The genome analysis tool kit (GATK v3.7; GATK Best Practices recommendations[49, 50]) was used for base quality score recalibration and indel realignment prior to variant calling using the HaplotypeCaller. Hard-filters were applied to the small nucleotide variants (SNVs) (QD < 2.0 || FS > 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum< -8.0 || SOR > 3.0) and insertions/deletions (indels) (QD < 2.0 || FS > 200.0 || ReadPosRankSum< -20.0 || SOR > 10.0). Variant calls were annotated using a custom pipeline developed at TCAG based on ANNOVAR[51].

Variant assessments

We prioritized the dataset for rare (minor allele frequencies <0.1%) variants (substitutions and small insertions/deletions) affecting genes associated with congenital heart defects (https://omim.org/, http://chdgene.victorchang.edu.au/, https://pubmed.ncbi.nlm.nih.gov/; as of September 2020), and other cardiac conditions putatively relevant to outcome (Supplementary information). Variants covered by less than 10x were omitted, and all reported variants were visualized using IGV (http://software.broadinstitute.org/software/igv/).

(i) Allele frequency and control databases

Overall and population-specific allele frequencies for SNVs and small indels were derived from 1000 Genomes (African, American, East Asian, European, South Asian; http://www.internationalgenome.org/), ExAC (African, American, East Asian, Finnish, Non-Finnish Europeans, South Asians, Others; http://exac.broadinstitute.org/)[52], and gnomAD (African, American, Ashkenazi Jewish, East Asian, Finnish, Non-Finnish Europeans, South Asians, Others; http://gnomad.broadinstitute.org/).

(ii) Predicted loss-of-function alleles

We predicted the following as high-confidence loss-of-function (LOF) or null alleles: frameshift insertions, deletions or substitutions; substitutions creating a premature stop codon; and alterations of the intronic dinucleotide adjacent to a coding-exonic splice junction. Variants that were predicted to result in a premature stop codon in the last exon (or in the last 50 nuleotides of the penultimate exon) were not considered high-confidence loss-of-function alleles.

(iii) Disease gene and variant databases

Online Mendelian Inheritance in Man (OMIM; https://www.omim.org/) and CHDgene (http://chdgene.victorchang.edu.au/) were used as disease gene databases. The Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk/ac/index.php) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) were used as disease variant databases.

Pathogenic/likely pathogenic variants and variants of uncertain significance

For the yield of pathogenic/likely pathogenic variants, we pursued a conservative interpretation strategy as per American College of Medical Genetics (ACMG) consensus guidelines (Richards et al.). Primary analysis was performed by M.S.R.; results were reviewed by a team of clinical and molecular geneticists (consensus was required to report variants as pathogenic/likely pathogenic). Details on the evidence supporting each variant are provided in Supplemental Tables II, IV, VII, and VIII). Parental samples or inheritance information (other than previously published findings) were not available.

Emerging candidate genes

We also analyzed for other genes with emerging evidence that they are associated with TOF or other congenital heart defects (https://pubmed.ncbi.nlm.nih.gov/). We assessed the available evidence in the literature, in combination with statistical support from the cohort reported here.

For candidate genes *KDR* and *IQGAP1*, we compared the loss-of-function variant counts to those identified by genome sequencing of three control cohorts: (i) participants in the 1000 genomes project (n = 2,504 genomes, all ancestries; https://www.internationalgenome.org/), (ii) parents of European ancestry from the Autism Speaks MSSNG database (n = 3,697 genomes in DB6; https://www.mss.ng/), and (iii) gnomAD v.3.1.1 (n = 76,156 genomes; https://gnomad.broadinstitute.org/). The prevalence of the *GDF1* stopgain variant p.Cys227* was only compared to the n = 3,697 European controls from MSSNG and 33,079 European controls from gnomAD, as this variant is known to be more common among Europeans.

Validation of variants

We only report on variants that were covered by read depths of ≥10x. Read alignments for all putative disease-associated variants were manually inspected using IGV (http://software.broadinstitute.org/software/igv/).

Pathway enrichment analyses

Functional protein interaction analyses were performed with Cytoscape [53] software v.3.8.2, using the STRING app [54]. Genes annotated to ontology terms[55] were extracted using the Bioconductor R package GO.db v 3.5, while the pathways were built starting from their respective websites (for Reactome[56] and KEGG[57]), and Broad Institute website (for Biocarta) to create a custom gene-set collection. Gene-sets were filtered to retain gene ontology (GO) terms with a number of annotated genes between 15 and 1000, while Reactome, KEGG and Biocarta were filtered to retain only pathways with a number of

annotated genes between 5 and 500. One-tailed Fisher exact test (Ha > H0) was used to calculate the enrichment p values, using all genes in the filtered collection as the universe. P values and odd ratios are reported in Supplemental Table V (provided as a separate file); the Benjamini-Hochberg procedure was used for the multiple test comparison correction.

Supplemental Tables

Table I. Exome sequencing coverage statistics.

Included datasets (n = 811)	Mean target coverage per exome	Proportion with coverage ≥ 10x
Average	107.9x	98.4%
Median	111.0x	98.6%
Range	33.4x - 158.6x	90.8% - 99.6%

Table II. Pathogenic/likely pathogenic risk variants for congenital heart defects (CHD), identified in a cohort of 811 probands with tetralogy of Fallot (n = 39 loss-of-function, n = 10 missense in 23 genes; results summarized in Figure 1).

Sample	Gene	Variant	Variant type	MAF	Disease mechanism/evidence	OMIM-P #	OMIM disease (inheritance) or supporting literature
B009FTV[a]	*FLT4* (NM_182925.4)	c.2758C>T, p.(Gln920*)	LOF	4.18E-06	FLT4 haploinsufficiency (PVS1, PM2)	618780	Congenital heart defects, multiple types (AD)
B009FV1_R[a]	*FLT4* (NM_182925.4)	c.2300-1G>C, p.?	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	618780	Congenital heart defects, multiple types (AD)
B00B0EY_R[a]	*FLT4* (NM_182925.4)	c.1267dupC, p.(Gln423Profs*4)	LOF	4.11E-06	FLT4 haploinsufficiency (PVS1, PM2)	618780	Congenital heart defects, multiple types (AD)
B00B0HD[a]	*FLT4* (NM_182925.4)	c.2849_2850+18del CGCAGGCCGCCCGCTCACCG, p.?	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	618780	Congenital heart defects, multiple types (AD)
B00B0KK[a]	*FLT4* (NM_182925.4)	c.1083C>A, p.(Tyr361*)	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	618780	Congenital heart defects, multiple types (AD)
B00B0O8[a]	*FLT4* (NM_182925.4)	c.2686G>T, p.(Glu896*)	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	616589	Congenital heart defects, multiple types (AD)
B00B0PK[a]	*FLT4* (NM_182925.4)	c.2714delA, p.(Asn905Thrfs*21)	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	616589	Congenital heart defects, multiple types (AD
B00B0QE_R[a]	*FLT4* (NM_182925.4)	c.2559dupC, p.(Gly854Argfs*21)	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	616589	Congenital heart defects, multiple types (AD)
B00B16G[a]	*FLT4* (NM_182925.4)	c.3002-1G>A, p.?	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	616589	Congenital heart defects, multiple types (AD)
B00BDV3_R[a]	*FLT4* (NM_182925.4)	c.3376C>T, p.(Gln1126*)	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	616589	Congenital heart defects, multiple types (AD)
B00BDV4[a]	*FLT4* (NM_182925.4)	c.3002-2A>G, p.?	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	616589	Congenital heart defects, multiple types (AD)
B00BET4[a]	*FLT4* (NM_182925.4)	c.1107C>G, p.(Tyr369*)	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	616589	Congenital heart defects, multiple types (AD)
B00BETB[a]	*FLT4* (NM_182925.4)	c.1902_1906dupCACGC, p.(Leu636Profs*5)	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	616589	Congenital heart defects, multiple types (AD)
B00DKZL_R[a]	*FLT4* (NM_182925.4)	c.3091C>T, p.(Arg1031*)	LOF	0	FLT4 haploinsufficiency (PVS1, PM2)	616589	Congenital heart defects, multiple types (AD)
B00B0O5[a]	*NOTCH1* (NM_017617.3)	c.5385-2delA, p.?	LOF	0	NOTCH1 haploinsufficiency (PVS1, PM2)	616028, 109730	Adams-Oliver syndrome (AD)[b], Aortic valve disease (AD)
B00B0SE[a,d]	*NOTCH1* (NM_017617.3)	c.1342C>T, p.(Arg448*)	LOF	0	NOTCH1 haploinsufficiency (PVS1, PM2)	616028, 109730	Adams-Oliver syndrome (AD)[b], Aortic valve disease (AD)
B00B0SS[a]	*NOTCH1* (NM_017617.3)	c.344delG, p.(Gly115Alafs*8)	LOF	0	NOTCH1 haploinsufficiency (PVS1, PM2)	616028, 109730	Adams-Oliver syndrome (AD)[b], Aortic valve disease (AD)

B00BDT8[a]	*NOTCH1* (NM_017617.3)	c.3966delC, p.(Cys1322Trpfs*123)	LOF	0	NOTCH1 haploinsufficiency (PVS1, PM2)	616028, 109730	Adams-Oliver syndrome (AD)[b], Aortic valve disease (AD)
B00BEAA[a]	*NOTCH1* (NM_017617.3)	c.5197C>T, p.(Gln1733*)	LOF	0	NOTCH1 haploinsufficiency (PVS1, PM2)	616028, 109730	Adams-Oliver syndrome (AD)[b], Aortic valve disease (AD)
B00DLD1[a]	*NOTCH1* (NM_017617.3)	c.440delA, p.(Asn147Thrfs*130)	LOF	0	NOTCH1 haploinsufficiency (PVS1, PM2)	616028, 109730	Adams-Oliver syndrome (AD)[b], Aortic valve disease (AD)
B009FQG_R[a]	*NOTCH1* (NM_017617.3)	c.599G>T, p.(Gly200Val)	Missense	0	De novo in Page et al., found in two unrelated individuals (PS4-M, PM2, PM6, PP3)	616028, 109730	Adams-Oliver syndrome (AD)[b], Aortic valve disease (AD)
B00B0KM[a]	*NOTCH1* (NM_017617.3)	c.599G>T, p.(Gly200Val)	Missense	0	De novo in Page et al., found in two unrelated individuals (PS4-M, PM2, PM6, PP3)	616028, 109730	Adams-Oliver syndrome (AD)[b], Aortic valve disease (AD)
B009G03_R[a]	*NOTCH1* (NM_017617.3)	c.5624A>G, p.(Asn1875Ser)	Missense	0	De novo, reduced Notch signaling in Page et al. (PS3, PM2, PM6, PP3)	616028, 109730	Adams-Oliver syndrome (AD)[b], Aortic valve disease (AD)
B009G7N[a]	*NOTCH1* (NM_017617.3)	c.1820G>A, p.(Cys607Tyr)	Missense	0	Reduced Notch signaling in Page et al., cysteine substitution in EGF domain (PS3, PM1, PM2, PP2, PP3)	616028, 109730	Adams-Oliver syndrome (AD)[b], Aortic valve disease (AD)
B009FUJ_R	*JAG1* (NM_000214.2)	c.1984delG, p.(Ala662Profs*81)	LOF	0	JAG1 haploinsufficiency (PVS1, PM2)	187500, 118450	Tetralogy of Fallot (AD), Alagille syndrome (AD)[b]
B00B0GY	*JAG1* (NM_000214.2)	c.2639_2640delGT, p.(Cys880*)	LOF	0	JAG1 haploinsufficiency (PVS1, PM2)	187500, 118450	Tetralogy of Fallot (AD), Alagille syndrome (AD)[b]
B00B0IL	*JAG1* (NM_000214.2)	c.2372+2T>G, p.?	LOF	0	JAG1 haploinsufficiency (PVS1, PM2)	187500, 118450	Tetralogy of Fallot (AD), Alagille syndrome (AD)[b]
B00B0KQ	*TBX1* (NM_080646.1)	c.549delG, p.(Lys184Argfs*24)	LOF	0	TBX1 haploinsufficiency (PVS1, PM2)	187500	Tetralogy of Fallot (AD)
B00DL8V	*TBX1* (NM_080646.1)	c.908+1G>A, p.?	LOF	0	TBX1 haploinsufficiency (PVS1, PM2)	187500	Tetralogy of Fallot (AD)
B00B0RO	*GATA6* (NM_005257.6)	c.1502C>A, p.(Ser501*)	LOF	0	GATA6 haploinsufficiency (PVS1, PM2)	187500, 600001	Tetralogy of Fallot (AD), Pancreatic agenesis and congenital heart defects (AD)[b]
B00BEBP	*GATA6* (NM_005257.6)	c.1367G>A, p.(Arg456His)	Missense	0	De novo in Allen et al.[58] (PS4-M, PM2, PM6, PP3)	187500, 600001	Tetralogy of Fallot (AD), Pancreatic agenesis and congenital heart defects (AD)[b]
B009FO4_R	*KAT6A* (NM_006766.3)	c.1506delT, p.(Asp503Ilefs*42)	LOF	0	KAT6A haploinsufficiency (PVS1, PM2)	616268	Arboleda-Tham syndrome (AD)[b]
B009FO7[c]	*PSMD12* (NM_002816.5)	c.430C>T, p.(Arg144*)	LOF	0	PSMD12 haploinsufficiency (PVS1, PM2)	617516	Stankiewicz-Isidor syndrome (AD)[b]
B009G6B	*CSNK2A1* (NM_177559.2)	c.131_132delAA, p.(Lys44Ilefs*5)	LOF	0	CSNK2A1 haploinsufficiency (PVS1, PM2)	617062	Okur-Chung neurodevelopmental syndrome (AD)[b]

B00B0KC	*ATRX* (NM_000489.5)	c.3736+1G>T, p.?	LOF	0	ATRX deficiency (PVS1, PM2)	301040	Alpha-thalassemia/mental retardation syndrome (XLD)[b]
B00BEAD_R	*NF1* (NM_000267.3)	c.5206-1G>C, p.?	LOF	0	NF1 haploinsufficiency (PVS1, PM2)	162200	Neurofibromatosis, type 1 (AD)[b]
B00DLD6	*CHD7* (NM_017780.4)	c.7160C>G, p.(Ser2387*)	LOF	0	CHD7 haploinsufficiency (PVS1, PM2)	214800	CHARGE syndrome (AD)[b]
B009FO7[c]	*ASXL1* (NM_015338.6)	c.2728C>T, p.(Gln910*)	LOF	0	ASXL1 haploinsufficiency (PVS1, PM2)	605039	Bohring-Opitz syndrome (AD)[b]
B009G7S	*GATAD2B* (NM_020699.4)	c.520C>T, p.(Arg174*)	LOF	0	GATAD2B haploinsufficiency[59] (PVS1, PM2)	615074	GAND syndrome (AD)[b]
B009G79_R	*PIK3CA* (NM_006218.4)	c.2809_2810del, p.F937fs	LOF	0	PIK3CA haploinsufficiency[60] (PVS1, PM2)	602501	Megalencephaly-capillary malformation-polymicrogyria syndrome (postzygotic variants)[b]
B009G7Z	*RASA1* (NM_002890.2)	c.2150_2151delTC, p.(Ile717Asnfs*8)	LOF	0	RASA1 haploinsufficiency[61] (PVS1, PM2)	608354	Capillary malformation-arteriovenous malformation (AD)[b]
B00B0IX	*NODAL* (NM_018055.5)	c.692G>A, p.(Trp231*)	LOF	4.06E-06	NODAL haploinsufficiency[62, 63] (PVS1, PM2)	270100	Heterotaxy (AD)[b]
B00DKWE	*ARHGAP31* (NM_020754.4)	c.2047C>T, p.(Gln683*)	LOF	0	ARHGAP31 haploinsufficiency (PVS1, PM2)	100300	Adams-Oliver syndrome (AD)[b]
B00DKW1	*SMAD2* (NM_005901.6)	c.998-2A>C, p.?	LOF	0	SMAD2 haploinsufficiency (PVS1, PM2)	NA	[64, 65, b]
B00B0S0	*DLL4* (NM_019074.4)	c.949A>C, p.(Thr317Pro)	Missense	0	De novo in Meester et al.[66] (PS4-M, PM2, PM6, PP3)	616589	Adams-Oliver syndrome (AD)[b]
B009FV4	*RAF1* (NM_001354689.3)	c.1532C>T, p.(Thr511Ile)	Missense	0	RAF1 gain-of-function, curated by ClinVar expert panel (PS3, PM1, PM2, PP2, PP3, PP5)	611553	Noonan syndrome (AD)[b]
B009FVM_R	*CACNA1C* (NM_001167625.1)	c.1216G>A, p.(Gly406Arg)	Missense	0	De novo in Napolitano et al. (PS3, PS4, PM2, PM6, PP3, PP5)	601005	Timothy syndrome (AD)[b]
B00B0LX	*LZTR1* (NM_006767.4)	c.742G>A, p.(Gly248Arg)	Missense	0	De novo or segregating in Chinton et al.[67] (PS4, PM2, PM6, PP3)	616564	Noonan syndrome (AD)[b]
B00BDTA	*EP300* (NM_001429.4)	c.4783T>G, p.(Phe1595Val)	Missense	0	De novo in Retterer et al.[68] (PS4-M, PM2, PM6, PP3)	613684	Rubinstein-Taybi syndrome (AD)[b]

All variants were heterozygous. Variant counts as displayed in Figure 1 (confirmed CHD genes). Minor allele frequencies (MAF) were derived from gnomAD_exomes_All.

[a] Variant also reported in Page et al. Note: Reporting of *NOTCH1*, *FLT4* and other variants may differ on the basis of differences in design, approach, and methodology of the current study to that of Page et al., e.g., nomenclature, frequency cut-offs, in silico prediction methods, exclusion of samples (see discussion in manuscript text).

[b] Multi-systemic, or potentially multi-systemic condition.

[c] Individual B009FO7 had two likely pathogenic variants, one in *PSMD12* and one in *ASXL1*, both associated with an increased risk for CHD.

[d] Individual B00B0SE had two likely pathogenic/pathogenic variants, one in *NOTCH1* and one in *MYBPC3* with potential implications for cardiovascular outcome (Supplemental Table VIII).

AD, autosomal dominant; LOF, loss-of-function; NA, Not available; XLD, X-linked dominant.

Table III. Loss-of-function variants in three emerging TOF/CHD candidate genes, identified in three or more individuals in the cohort studied (and with statistical support, as presented in Figure 1 and Results).

Sample	Gene	Variant	Variant type	MAF	Disease mechanism/evidence	OMIM-P #	OMIM disease (inheritance) or supporting literature
B009FP4	*KDR* (NM_002253.2)	c.3580_3581delCT, p.(Leu1194Alafs*15)	LOF	0	KDR haploinsufficiency	NA	Reuter et al., Morton et al.
B00B0PI_R	*KDR* (NM_002253.2)	c.2605delA, p.(Met869Cysfs*2)	LOF	0	KDR haploinsufficiency	NA	Reuter et al., Morton et al.
B00BQTD	*KDR* (NM_002253.2)	c.3170delC, p.(Pro1057Glnfs*13)	LOF	0	KDR haploinsufficiency	NA	Reuter et al., Morton et al.
B00DKWY	*KDR* (NM_002253.2)	c.2373+1G>A, p.?	LOF	0	KDR haploinsufficiency	NA	Reuter et al., Morton et al.
B009FQA_R	*IQGAP1* (NM_003870.3)	c.650-2A>T, p.?	LOF	1.33E-05	IQGAP1 haploinsufficiency	NA	Reuter et al., Sevim Bayrak et al., Petrovski et al.
B00B1D6	*IQGAP1* (NM_003870.3)	c.1393dupA, p.(Arg465Lysfs*26)	LOF	0	IQGAP1 haploinsufficiency	NA	Reuter et al., Sevim Bayrak et al., Petrovski et al.
B009FOS_R	*IQGAP1* (NM_003870.3)	c.1150C>T, p.(Gln384*)	LOF	4.15E-06	IQGAP1 haploinsufficiency	NA	Reuter et al., Sevim Bayrak et al., Petrovski et al.
B009FNV	*GDF1* (NM_001492.4)	c.681C>A, p.(Cys227*)	LOF	9.00E-04[a]	GDF1 haploinsufficiency	613854	Congenital heart defects, multiple types (AD)
B009FTS_R	*GDF1* (NM_001492.4)	c.681C>A, p.(Cys227*)	LOF	9.00E-04[a]	GDF1 haploinsufficiency	613854	Congenital heart defects, multiple types (AD)
B00B0H7	*GDF1* (NM_001492.4)	c.681C>A, p.(Cys227*)	LOF	9.00E-04[a]	GDF1 haploinsufficiency	613854	Congenital heart defects, multiple types (AD)
B00B0JI	*GDF1* (NM_001492.4)	c.681C>A, p.(Cys227*)	LOF	9.00E-04[a]	GDF1 haploinsufficiency	613854	Congenital heart defects, multiple types (AD)
B00BDT4	*GDF1* (NM_001492.4)	c.681C>A, p.(Cys227*)	LOF	9.00E-04[a]	GDF1 haploinsufficiency	613854	Congenital heart defects, multiple types (AD)
B00BDTU	*GDF1* (NM_001492.4)	c.681C>A, p.(Cys227*)	LOF	9.00E-04[a]	GDF1 haploinsufficiency	613854	Congenital heart defects, multiple types (AD)
B00DL0L	*GDF1* (NM_001492.4)	c.681C>A, p.(Cys227*)	LOF	9.00E-04[a]	GDF1 haploinsufficiency	613854	Congenital heart defects, multiple types (AD)
B009G0C	*GDF1* (NM_001492.4)	c.1047_1050delCTTT, p.(Phe349Leufs*35)	LOF	1.00E-04[a]	GDF1 haploinsufficiency	613854	Congenital heart defects, multiple types (AD)

All variants were heterozygous. Variant counts as displayed in Figure 1 (candidate genes).

[a] Minor allele frequencies (MAF) for *GDF1* were derived from gnomAD_genomes_Non-Finnish Europeans. A comprehensive assessment of the *GDF1* locus could not be performed due to insufficient coverage (defined as read depths <10x; supplementary methods). Other MAF were derived from gnomAD_exomes_All.

LOF, loss-of-function; NA, Not available.

Table IV. Pathogenic/likely pathogenic and candidate loss of function variants (n = 17) in 8 genes observed in a published independent cohort of 424 probands with tetralogy of Fallot (Jin et al.).

Sample	Gene	Variant	MAF	Disease mechanism/evidence	OMIM-P #	OMIM disease (inheritance) or supporting literature
1-00645	*FLT4* (NM_182925.4)	c.2206C>T, p.(Gln736*)	0	FLT4 haploinsufficiency (PVS1, PM2)	618780	Congenital heart defects, multiple types (AD)
1-01795	*FLT4* (NM_182925.4)	c.1088dupC, p.(Pro364Alafs*63)	0	FLT4 haploinsufficiency (PVS1, PM2)	618780	Congenital heart defects, multiple types (AD)
1-00788	*FLT4* (NM_182925.4)	c.503_506delCGCT, p.(Thr168Serfs*76)	0	FLT4 haploinsufficiency (PVS1, PM2)	618780	Congenital heart defects, multiple types (AD)
1-05967	*FLT4* (NM_182925.4)	c.89delC, p.(Pro30Argfs*3)	4.05E-05	FLT4 haploinsufficiency (PVS1, PM2)	618780	Congenital heart defects, multiple types (AD)
1-04970	*FLT4* (NM_182925.4)	c.1083C>G, p.(Tyr361*)	0	FLT4 haploinsufficiency (PVS1, PM2)	618780	Congenital heart defects, multiple types (AD)
1-06642	*FLT4* (NM_182925.4)	c.2804delT, p.(Leu935Profs*72)	0	FLT4 haploinsufficiency (PVS1, PM2)	618780	Congenital heart defects, multiple types (AD)
1-03410	*FLT4* (NM_182925.4)	c.89delC, p.(Pro30Argfs*3)	4.05E-05	FLT4 haploinsufficiency, de novo (PVS1, PS2, PM2)	618780	Congenital heart defects, multiple types (AD)
1-03825	*FLT4* (NM_182925.4)	c.2844_2845delCT, p.(Cys949Argfs*53)	0	FLT4 haploinsufficiency, de novo (PVS1, PS2, PM2)	618780	Congenital heart defects, multiple types (AD)
1-00305	*NOTCH1* (NM_017617.3)	c.273_277delGGGCT, p.(Gly92Leufs*49)	0	NOTCH1 haploinsufficiency (PVS1, PM2)	616028, 109730	Adams-Oliver syndrome (AD)[d], Aortic valve disease (AD)
1-00692	*NOTCH1* (NM_017617.3)	c.1800_1801dupCG, p.(Glu601Alafs*31)	0	NOTCH1 haploinsufficiency (PVS1, PM2)	616028, 109730	Adams-Oliver syndrome (AD)[d], Aortic valve disease (AD)
1-02338	*NOTCH2* (NM_024408.3)	c.4299delA, p.(Ala1434Leufs*119)	0	NOTCH2 haploinsufficiency (PVS1, PM2)	610205	Alagille syndrome (AD)
1-00534	*CHD7* (NM_017780.3)	c.4795C>T, p.(Gln1599*)	0	CHD7 haploinsufficiency, de novo (PVS1, PS2, PM2)	214800	CHARGE syndrome (AD)
1-08360	*CHD7* (NM_017780.3)	c.4393C>T, p.(Arg1465*)	0	CHD7 haploinsufficiency, de novo (PVS1, PS2, PM2)	214800	CHARGE syndrome (AD)
1-04135	*MEIS2* (NM_170675.4)	c.383delA, p.(Lys128Serfs*19)	0	MEIS2 haploinsufficiency, de novo (PVS1, PS2, PM2)	600987	Cleft palate, cardiac defects, and mental retardation (AD)
1-00141	*NAA15* (NM_057175.3)	c.2282C>A, p.(Ser761*)	0	NAA15 haploinsufficiency, de novo (PVS1, PS2, PM2)	617787	Mental retardation (AD)
1-08084	*RPL5* (NM_000969.3)	c.67C>T, p.(Arg23*)	0	RPL5 haploinsufficiency, de novo (PVS1, PS2, PM2)	612561	Diamond-Blackfan anemia (AD)
1-07375	*KDR* (NM_002253.2)	c.1585A>T, p.(Lys529*)	0	KDR haploinsufficiency	NA	Reuter et al., Sevim Bayrak et al., Petrovski et al.

Variants were extracted from variant lists in Jin et al., Tables S7 and S9, and interpreted according to consensus guidelines. This approach resulted in a lower yield, compared to the re-analysis of raw exome files from Page et al. (Supplemental Tables II and III). Minor allele frequencies (MAF) were derived from gnomAD_exomes_All.

NA, not available.

Table V. Pathway enrichment analyses (provided as a separate file).

Table VI. Additional rare variants, meeting criteria for predicted deleterious variants of uncertain significance (n = 24 loss-of-function, n = 47 missense, n = 9 other) in CHD-relevant genes (n = 19) or candidate genes (n = 8).

Sample	Gene	Variant	Variant type	MAF	Annotation	OMIM-P #	OMIM disease (inheritance) or supporting literature
VUS in CHD-relevant genes (OMIM, http://chdgene.victorchang.edu.au/)							
B00DLES[a]	*FLT4* (NM_182925.4)	c.4011delT, p.(Tyr1337*)	Other VUS (premature stopcodon in the last exon)	0	NA	618780	Congenital heart defects, multiple types (AD)
B00B0KK[a,b]	*FLT4* (NM_182925.4)	c.781G>T, p.(Gly261Cys)	Missense VUS	0	Predicted damaging (CADD 24.3)	618780	Congenital heart defects, multiple types (AD)
B00B0LF[a]	*FLT4* (NM_182925.4)	c.153C>G, p.(Cys51Trp)	Missense VUS	0	Predicted damaging (CADD 34)	618780	Congenital heart defects, multiple types (AD)
B00BDS0	*FLT4* (NM_182925.4)	c.513G>A, p.(Ser171Ser)	Other VUS (synonymous)	0	Predicted splice effect (CADD 20.5)	618780	Congenital heart defects, multiple types (AD)
B00BDS9[a]	*FLT4* (NM_182925.4)	c.89C>T, p.(Pro30Leu)	Missense VUS	0	Predicted damaging (CADD 25.6)	618780	Congenital heart defects, multiple types (AD)
B00BDSV[a]	*FLT4* (NM_182925.4)	c.89C>G, p.(Pro30Arg)	Missense VUS	0	Predicted damaging (CADD 24.9)	618780	Congenital heart defects, multiple types (AD)
B00BECK[a]	*FLT4* (NM_182925.4)	c.482C>T, p.(Ser161Phe)	Missense VUS	0	Predicted damaging (CADD 25.4)	618780	Congenital heart defects, multiple types (AD)
B00DL8L[a]	*FLT4* (NM_182925.4)	c.2284A>G, p.(Ser762Gly)	Missense VUS	0	Predicted damaging (CADD 24.1)	618780	Congenital heart defects, multiple types (AD)
B009FTP[a]	*NOTCH1* (NM_017617.3)	c.4646G>A, p.(Cys1549Tyr)	Missense VUS	0	De novo in Page et al.	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B009FNG_R[a]	*NOTCH1* (NM_017617.3)	c.428C>T, p.(Pro143Leu)	Missense VUS	0	Predicted damaging (CADD 24.7), in three probands	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00BDSY_R[a]	*NOTCH1* (NM_017617.3)	c.428C>T, p.(Pro143Leu)	Missense VUS	0	Predicted damaging (CADD 24.7), in three probands	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00BDTA[a,b]	*NOTCH1* (NM_017617.3)	c.428C>T, p.(Pro143Leu)	Missense VUS	0	Predicted damaging (CADD 24.7), in three probands	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B009G8F[a]	*NOTCH1* (NM_017617.3)	c.1057C>T, p.(Arg353Cys)	Missense VUS	0	Predicted damaging (CADD 33), in two probands	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)

B00B16Z[a]	*NOTCH1* (NM_017617.3)	c.1057C>T, p.(Arg353Cys)	Missense VUS	0	Predicted damaging (CADD 33), in two probands	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00B0D6[a]	*NOTCH1* (NM_017617.3)	c.5497G>C, p.(Asp1833His)	Missense VUS	0	Predicted damaging (CADD 24.3)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00B0IB	*NOTCH1* (NM_017617.3)	c.1687T>C, p.(Cys563Arg)	Missense VUS	0	Predicted damaging (CADD 25.3)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00B0IH[a]	*NOTCH1* (NM_017617.3)	c.875G>A, p.(Cys292Tyr)	Missense VUS	0	Predicted damaging (CADD 25.9)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00B0IW[a]	*NOTCH1* (NM_017617.3)	c.6011G>T, p.(Arg2004Leu)	Missense VUS	0	Predicted damaging (CADD 33)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00B0KE[a]	*NOTCH1* (NM_017617.3)	c.545G>A, p.(Cys182Tyr)	Missense VUS	0	Predicted damaging (CADD 27.6)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00B0KI[a]	*NOTCH1* (NM_017617.3)	c.5017G>C, p.(Gly1673Arg)	Missense VUS	0	Predicted damaging (CADD 28.2)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00B0KR[a]	*NOTCH1* (NM_017617.3)	c.4428_4430delCGG, p.(Gly1477del)	Other VUS (in-frame deletion)	0	NA	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00B0M0[a]	*NOTCH1* (NM_017617.3)	c.4483C>A, p.(Gln1495Lys)	Missense VUS	0	Predicted damaging (CADD 23.2)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00B0SN[a]	*NOTCH1* (NM_017617.3)	c.1412T>A, p.(Ile471Asn)	Missense VUS	0	Predicted damaging (CADD 25.4)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00B16Y[a]	*NOTCH1* (NM_017617.3)	c.1934G>A, p.(Cys645Tyr)	Missense VUS	0	Predicted damaging (CADD 27.6)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00B187[a]	*NOTCH1* (NM_017617.3)	c.3974C>T, p.(Ala1325Val)	Missense VUS	0	Predicted damaging (CADD 25.2)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00BEAA[a,b]	*NOTCH1* (NM_017617.3)	c.3880G>A, p.(Glu1294Lys)	Missense VUS	0	Predicted damaging (CADD 25.2)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00BEAD_R[a,b]	*NOTCH1* (NM_017617.3)	c.598G>C, p.(Gly200Arg)	Missense VUS	0	Predicted damaging (CADD 28.6)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00DKZ8[a]	*NOTCH1* (NM_017617.3)	c.490T>G, p.(Cys164Gly)	Missense VUS	0	Predicted damaging (CADD 26.8)	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00DKZI_R[a]	*NOTCH1* (NM_017617.3)	c.436_450dup TCCAACCCCTGCGCC, p.(Ser146_Ala150dup)	Other VUS (in-frame duplication)	0	NA	616028, 109730	Adams-Oliver syndrome (AD), Aortic valve disease (AD)
B00BDSL	*JAG1* (NM_000214.2)	c.185G>A, p.(Gly62Glu)	Missense VUS	0	Predicted damaging (CADD 22.5)	187500, 118450	Tetralogy of Fallot (AD), Alagille syndrome (AD)
B00BESY	*JAG1* (NM_000214.2)	c.376T>C, p.(Phe126Leu)	Missense VUS	0	Predicted damaging (CADD 33)	187500, 118450	Tetralogy of Fallot (AD), Alagille syndrome (AD)
B00BETA	*JAG1* (NM_000214.2)	c.2365C>T, p.(His789Tyr)	Missense VUS	0	Predicted damaging (CADD 23.3)	187500, 118450	Tetralogy of Fallot (AD), Alagille syndrome (AD)

B00B0SJ	*TBX1* (NM_080646.1)	c.1095_1101delAGGTGGC, p.(Gly366Valfs*2)	Other VUS (premature stopcodon in the last exon)	0	Does not affect all transcripts	187500	Tetralogy of Fallot (AD)
B00B0RM	*TBX1* (NM_080646.1)	c.607G>C, p.(Gly203Arg)	Missense VUS	0	Predicted damaging (CADD 31)	187500	Tetralogy of Fallot (AD)
B00BEST	*TBX1* (NM_080646.1)	c.840+5G>A, p.?	Other VUS (intronic)	0	Predicted splice effect (CADD 22.8)	187500	Tetralogy of Fallot (AD)
B00DKWP	*TBX1* (NM_080646.1)	c.956C>G, p.(Ala319Gly)	Missense VUS	0	Predicted damaging (CADD 28)	187500	Tetralogy of Fallot (AD)
B009FZL	*DLL4* (NM_019074.4)	c.1285C>T, p.(Arg429Cys)	Missense VUS	0	Predicted damaging (CADD 33), in three probands	616589	Adams-Oliver syndrome (AD)
B00BDSW	*DLL4* (NM_019074.4)	c.1285C>T, p.(Arg429Cys)	Missense VUS	0	Predicted damaging (CADD 33), in three probands	616589	Adams-Oliver syndrome (AD)
B00BDUG	*DLL4* (NM_019074.4)	c.1285C>T, p.(Arg429Cys)	Missense VUS	0	Predicted damaging (CADD 33), in three probands	616589	Adams-Oliver syndrome (AD)
B009FWD_R	*DLL4* (NM_019074.4)	c.1240G>A, p.(Gly414Arg)	Missense VUS	0	Predicted damaging (CADD 34)	616589	Adams-Oliver syndrome (AD)
B00BDUC	*CHD4* (NM_001273.2)	c.4515+1G>T, p.?	LOF VUS	0	Uncertain haplosensitivity of CHD4[69]	617159	Sifrim-Hitz-Weiss syndrome (AD)
B00B0H9	*CHD4* (NM_001273.2)	c.2084G>A, p.(Arg695Gln)	Missense VUS	0	Predicted damaging (CADD 22.6)	617159	Sifrim-Hitz-Weiss syndrome (AD)
B00BDU2	*CHD4* (NM_001273.2)	c.4513_4515delAAG, p.(Lys1505del)	Other VUS (in-frame deletion)	0	NA	617159	Sifrim-Hitz-Weiss syndrome (AD)
B00B0J2	*ELN* (NM_000501.4)	c.1150+1G>A, p.?	Splice-site VUS	5.69E-05	ELN haploinsufficiency in aortic stenosis and other CHD[70]	185500	Supravalvar aortic stenosis (AD)
B009FVY_R	*ECE1* (NM_001397.3)	c.1021-2A>G, p.?	LOF VUS	0	Uncertain haplosensitivity of ECE1	613870	Hirschsprung disease, cardiac defects, and autonomic dysfunction (AD)
B009G7T	*CACNA1C* (NM_001167625.1)	c.6169G>T, p.(Glu2057*)	LOF VUS	0	Uncertain haplosensitivity of CACNA1C	601005	Timothy syndrome (AD)
B009G10_R	*TLL1* (NM_001204760.1)	c.1159-2A>C, p.?	LOF VUS	2.00E-04	Uncertain haplosensitivity of TLL1	613087	Atrial septal defect (AD)
B009FT1	*TLL1* (NM_001204760.1)	c.713T>C, p.(Val238Ala)	Missense VUS	2.00E-04	Predicted damaging (CADD 22.7)[71], in two probands	613087	Atrial septal defect (AD)

B00B160_R	*TLL1* (NM_001204760.1)	c.713T>C, p.(Val238Ala)	Missense VUS	2.00E-04	Predicted damaging (CADD 22.7)[71], in two probands	613087	Atrial septal defect (AD)
B009G8X	*CRELD1* (NM_001077415.2)	c.959delA, p.(Gln320Argfs*25)	LOF VUS	3.00E-04	Uncertain haplosensitivity of CRELD1	606217	Atrioventricular septal defect, partial, with heterotaxy syndrome (AD)
B00B0KJ[b]	*CRELD1* (NM_001077415.2)	c.977_984dupGCGGTTAT, p.(Arg329Alafs*19)	LOF VUS	5.28E-05	Uncertain haplosensitivity of CRELD1	606217	Atrioventricular septal defect, partial, with heterotaxy syndrome (AD)
B00BDUD	*CRELD1* (NM_001077415.2)	c.484C>G, p.(Pro162Ala)	Missense VUS	0	Predicted damaging[72]	606217	Atrioventricular septal defect, partial, with heterotaxy syndrome (AD)
B00B0T7	*SMAD6* (NM_005585.5)	c.223C>T, p.(Arg75*)	LOF VUS	0	Uncertain haplosensitivity of SMAD6	179300, 614823, 617439	Radioulnar synostosis, nonsyndromic (AD), Aortic valve disease (AD), Craniosynostosis (AD)
B009FTM	*PRKD1* (NM_002742.2)	c.2241delC, p.(Ala748Leufs*8)	LOF VUS	0	Uncertain haplosensitivity of PRKD1	617364	Congenital heart defects and ectodermal dysplasia (AD)
B009G10_R	*PRKD1* (NM_002742.2)	c.802A>T, p.(Lys268*)	LOF VUS	2.44E-05	Uncertain haplosensitivity of PRKD1	617364	Congenital heart defects and ectodermal dysplasia (AD)
B00BET4	*GLI3* (NM_000168.6)	c.2119C>T, p.(Pro707Ser)	Missense VUS	3.00E-04	Predicted damaging[73]	146510	Pallister-Hall syndrome (AD)
B00DKW1	*KANSL1* (NM_001193466.2)	c.190C>T, p.(Arg64*)	Other VUS (premature stopcodon in the last exon)	4.06E-06	NA	610443	Koolen-De Vries syndrome (AD)
B009FR7_R	*MYH6* (NM_002471.3)	c.642+1G>T, p.?	LOF VUS	0	Uncertain haplosensitivity of MYH6	614089	Atrial septal defect
B009FNJ_R	*NKX2-6* (NM_001136271.2)	c.797delG, p.(Gly266Valfs*?)	LOF VUS	0	Uncertain haplosensitivity of NKX2-6	217095	Conotruncal heart malformations, Persistent truncus arteriosus
B009G3M_R	*NKX2-6* (NM_001136271.2)	c.274+1G>A, p.?	LOF VUS	0	Uncertain haplosensitivity of NKX2-6	217095	Conotruncal heart malformations, Persistent truncus arteriosus
B009G8Y	*NKX2-6* (NM_001136271.2)	c.455dupA, p.(Gln153Alafs*?)	LOF VUS	3.28E-05	Uncertain haplosensitivity of NKX2-6	217095	Conotruncal heart malformations, Persistent truncus arteriosus

B00B0LO	*NKX2-6* (NM_001136271.2)	c.455dupA, p.(Gln153Alafs*?)	LOF VUS	3.28E-05	Uncertain haplosensitivity of NKX2-6	217095	Conotruncal heart malformations, Persistent truncus arteriosus
B00DL8V	*TAB2* (NM_001292034.3)	c.688C>A, p.(Gln230Lys)	Missense VUS	1.63E-05	Predicted damaging[74]	614980	Congenital heart defects, nonsyndromic (AD)
B00B0SL	*TBX20* (NM_001077653.2)	c.456C>G, p.(Ile152Met)	Missense VUS	2.03E-05	Predicted damaging[75]	611363	Atrial septal defect
VUS in CHD candidate genes							
B00B0KS	*KDR* (NM_002253.2)	c.3817G>T, p.(Glu1273*)	Other VUS (premature stopcodon in the penultimate exon)	4.07E-06	NA	NA	Reuter et al., Morton et al.
B00B0RT_R	*KDR* (NM_002253.2)	c.2248G>C, p.(Ala750Pro)	Missense VUS	0	Predicted damaging (CADD 21.9)	NA	Reuter et al., Morton et al.
B00BEB6	*KDR* (NM_002253.2)	c.2234G>C, p.(Cys745Ser)	Missense VUS	0	Predicted damaging (CADD 25.4)	NA	Reuter et al., Morton et al.
B00DL8L	*KDR* (NM_002253.2)	c.358G>C, p.(Asp120His)	Missense VUS	0	Predicted damaging (CADD 34)	NA	Reuter et al., Morton et al.
B00B16V	*IQGAP1* (NM_003870.3)	c.641A>G, p.(Glu214Gly)	Missense VUS	0	Predicted damaging (CADD 28.8)	NA	Reuter et al., Sevim Bayrak et al., Petrovski et al.
B00BDSJ_R	*IQGAP1* (NM_003870.3)	c.3887A>G, p.(Tyr1296Cys)	Missense VUS	0	Predicted damaging (CADD 32)	NA	Reuter et al., Sevim Bayrak et al., Petrovski et al.
B00DLDA	*IQGAP1* (NM_003870.3)	c.353A>C, p.(Gln118Pro)	Missense VUS	0	Predicted damaging (CADD 24.6)	NA	Reuter et al., Sevim Bayrak et al., Petrovski et al.
B00BET7	*VEGFA* (NM_001171623.1)	c.28delT, p.(Trp10Glyfs*32)	LOF VUS	0	VEGFA dysregulation in mouse models with TOF, candidate gene in humans with TOF	NA	Reuter et al.
B009FQG_R	*BCAR1* (NM_001170715.1)	c.1957C>T, p.(Arg653*)	LOF VUS	0	Candidate gene for TOF	NA	Reuter et al.
B009FWJ_R	*KIRREL3* (NM_032531.3)	c.634delC, p.(Leu212Serfs*105)	LOF VUS	0	In Jacobsen syndrome critical region	NA	NA
B009FUS_R	*LTBP3* (NM_001130144.2)	c.3376_3377delAG, p.(Ser1126Profs*85)	LOF VUS	0	Uncertain haplosensitivity of LTBP3, associated with cardiac defects[76]	617809	Geleophysic dysplasia (AD)
B00B0DN_R	*LTBP3* (NM_001130144.2)	c.1075delT, p.(Cys359Alafs*29)	LOF VUS	4.06E-06	Uncertain haplosensitivity of LTBP3, associated with cardiac defects[76]	617809	Geleophysic dysplasia (AD)
B00B0E9	*ZFPM1* (NM_153813.2)	c.886delC, p.(Gln296Serfs*28)	LOF VUS	0	Candidate gene for TOF	NA	[77]

B00B0KF	*ZFPM1* (NM_153813.2)	c.1614dupC, p.(Ala539Argfs*133)	LOF VUS	2.55E-05	Candidate gene TOF	NA	[77]
B00B0NS_R	*MESP1* (NM_018670.3)	c.310G>T, p.(Glu104*)	LOF VUS	6.28E-05	Candidate gene for TOF	NA	[78, 79]
B00B16X	*MESP1* (NM_018670.3)	c.370G>T, p.(Glu124*)	LOF VUS	4.99E-05	Candidate gene for TOF	NA	[78, 79]

All variants were heterozygous. Minor allele frequencies (MAF) were derived from gnomAD_exomes_All.

[a] Variant also reported in Page et al. Reporting of *NOTCH1*, *FLT4* and other variants may differ on the basis of differences in methodology of the current study to that of Page et al., e.g., nomenclature, frequency cut-offs, in silico prediction methods, exclusion of samples (see Discussion in manuscript text).

[b] Individuals with other likely pathogenic variants (Supplemental Table II, III).

LOF, loss-of-function; NA, Not available; VUS, variant of uncertain significance; CADD, Combined Annotation Dependent Depletion (https://cadd.gs.washington.edu/).

Table VII. Other very rare, pathogenic/likely pathogenic variants in 7 genes for childhood-onset disorders (n = 8 loss-of-function, n = 1 missense), but with uncertain relevance currently to TOF.

Sample	Gene	Variant	Variant type	MAF	Disease mechanism/evidence	OMIM-P #	OMIM disease (inheritance) or supporting literature
B00DKWN	*TCF12*[b] (NM_207037.1)	c.556delG, p.(Val186Cysfs*59)	LOF	0	TCF12 haploinsufficiency (PVS1, PM2)	615314	Craniosynostosis (AD), Morton et al.
B00B0KJ	*TCF12*[b] (NM_207037.1)	c.1640_1641delAA, p.(Lys547Argfs*4)	LOF	0	TCF12 haploinsufficiency (PVS1, PM2)	615314	Craniosynostosis (AD), Morton et al.
B00B1CK	*POLR1A*[b] (NM_015425.6)	c.5062+1G>C, p.?	LOF	0	POLR1A haploinsufficiency (PVS1, PM2)	616462	Acrofacial dysostosis, Cincinnati type (AD), [80]
B00BEAR	*POLR1A*[b] (NM_015425.6)	c.3394C>T, p.(Arg1132*)	LOF	0	POLR1A haploinsufficiency (PVS1, PM2)	616462	Acrofacial dysostosis, Cincinnati type (AD), [80]
B00BDSE	*GLI2*[b] (NM_005270.5)	c.2293+1G>C, p.?	LOF	0	GLI2 haploinsufficiency (PVS1, PM2)	615849, 610829	Culler-Jones syndrome (AD), Holoprosencephaly (AD)
B00DL0I	*APC*[b] (NM_000038.6)	c.1213C>T, p.(Arg405*)	LOF	0	APC haploinsufficiency (PVS1, PM2)	175100	Adenomatous polyposis coli (AD)
B00B15G	*EDA* (NM_001399.5)	c.466C>T, p.(Arg156Cys)	Missense	0	De novo in Monreal et al.[81] (PS4-M, PM2, PM6, PP3)	313500	Tooth agenesis, selective, X-linked 1 (XLD)
B00B0K7	*ZMYND11* (NM_006624.5)	c.1203_1206delTCAA, p.(Asn401Lysfs*17)	LOF	0	ZMYND11 haploinsufficiency (PVS1, PM2)	616083	Mental retardation (AD)
B00BEAF[a]	*TRIO* (NM_007118.2)	c.3217dupG, p.(Ala1073Glyfs*34)	LOF	0	TRIO haploinsufficiency (PVS1, PM2)	617061	Intellectual developmental disorder, with microcephaly (AD)

All variants were heterozygous. Minor allele frequencies (MAF) were derived from gnomAD_exomes_All.

[a] Individual with another likely pathogenic variant in *SCN5A* with potential implications for cardiovascular outcome (Supplemental Table VIII).

[b] Despite their uncertain relevance to TOF, the encoded proteins connect to the network map of TOF predisposing genes/proteins (Figure 2): GLI2 (NOTCH1, SMAD2, JAG1, PSMD12), APC (NOTCH1, IQGAP1, PSMD12, CSNK2A1), TCF12 (NOTCH1, SMAD2, EP300), POLR1A (EP300).

AD, autosomal dominant; LOF, loss-of-function; XLD, X-linked dominant.

Table VIII. Pathogenic/likely pathogenic variants (n = 16) with potential implications to cardiovascular outcome and management.

Sample	Gene	Variant	Variant interpretation/evidence	MAF	Disease	Cardiovascular risk	
						CHD	Other cardiovascular outcomes
Variants considered causative for the CHD (as presented in Table S2)							
B00B0LX	*LZTR1* (NM_006767.4)	c.742G>A, p.(Gly248Arg)	Pathogenic (PS4, PM2, PM6, PP3)	0	Noonan syndrome	TOF, pulmonary valve stenosis, septal defects, aortic coarctation, and other CHD[82]	Cardiac hypertrophy
B009FV4	*RAF1* (NM_001354689.3)	c.1532C>T, p.(Thr511Ile)	Pathogenic (PS3, PM1, PM2, PP2, PP3, PP5)	0			
B009FVM_R	*CACNA1C* (NM_001167625.1)	c.1216G>A, p.(Gly406Arg)	Pathogenic (PS3, PS4, PM2, PM6, PP3, PP5)	0	Timothy syndrome	TOF, patent ductus arteriosus, patent foramen ovale, septal defects	Arrhythmias, cardiac hypertrophy/dysfunction, sudden cardiac death
B00BEAD_R	*NF1* (NM_000267.3)	c.5206-1G>C, p.?	Likely pathogenic (PVS1, PM2)	0	Neurofibromatosis	TOF and other CHD	Cardiac hypertrophy, pulmonary hypertension, intracardiac neurofibroma, arterial hypertension, strokes
B009G7Z	*RASA1* (NM_002890.3)	c.2150_2151delTC, p.(Ile717Asnfs*8)	Likely pathogenic (PVS1, PM2)	0	Capillary malformation-arteriovenous malformation syndrome	TOF and other CHD[61]	Cardiac overload/heart failure due to arteriovenous malformations/fistulas
Other cardiac risk variants							
B00BDVA	*MYBPC3* (NM_000256.3)	c.1483C>G, p.(Arg495Gly)	Likely pathogenic (PS4, PM5, PP1, PP3, PP5)	4.06E-06	Hypertrophic cardiomyopathy	Unknown	Cardiac hypertrophy, arrhythmias, heart failure, sudden cardiac death
B00B0SE[a]	*MYBPC3* (NM_000256.3)	c.1504C>T, p.(Arg502Trp)	Likely pathogenic (PS4-M, PM5, PP3, PP5)	4.87E-05			
B00DLDV	*MYBPC3* (NM_000256.3)	c.3288delG, p.(Glu1096Aspfs*93)	Pathogenic (PVS1, PM2)	0			
B00BET1	*MYH7* (NM_000257.4)	c.2594A>G, p.(Lys865Arg)	Likely pathogenic (PS4-M, PM5, PP3, PP5)	4.06E-06			
B00B0HU	*MYL2* (NM_000432.4)	c. 64G>A, p.(Glu22Lys)	Likely pathogenic (PS3, PS4-M, PP1, PP3, PP5)	2.03E-05			
B00BDUN	*TNNI3* (NM_000363.5)	c.484C>T, p.(Arg162Trp)	Likely pathogenic (PS4-M, PS3-S, PP1, PP3, PP5)	4.07E-05			
B00B0JX	*DSC2* (NM_024422.6)	c.501_502delTA, p.(Thr168Hisfs*11)	Likely pathogenic (PVS1, PM2)	0		Unknown	Arrhythmias, heart failure, sudden cardiac death

B00B178	*DSP* (NM_004415.4)	c.7756C>T, p.(Arg2586*)	Likely pathogenic (PVS1, PM2)	0	Arrhythmogenic right ventricular cardiomyopathy		
B00BDS5	*DSP* (NM_004415.4)	c.8077_8080delAAAG, p.(Lys2693Profs*3)	Likely pathogenic (PVS1, PM2)	0			
B00B0D3	*DMD* (NM_000109.4)	c.1789-1G>C, p.?	Likely pathogenic (PVS1, PM2)	0	Dilated cardiomyopathy	Unknown	Cardiac dilation, arrhythmias
B00BEAF[a]	*SCN5A* (NM_198056.2)	c.2692G>T, p.(Glu898*)	Likely pathogenic (PVS1, PM2)	0	Brugada syndrome	Unknown	Arrhythmias, sudden cardiac death

All variants were heterozygous. Minor allele frequencies (MAF) were derived from gnomAD_exomes_All.

[a] Individuals with additional likely pathogenic variants (Supplemental Tables II, VII).

Supplemental Figures

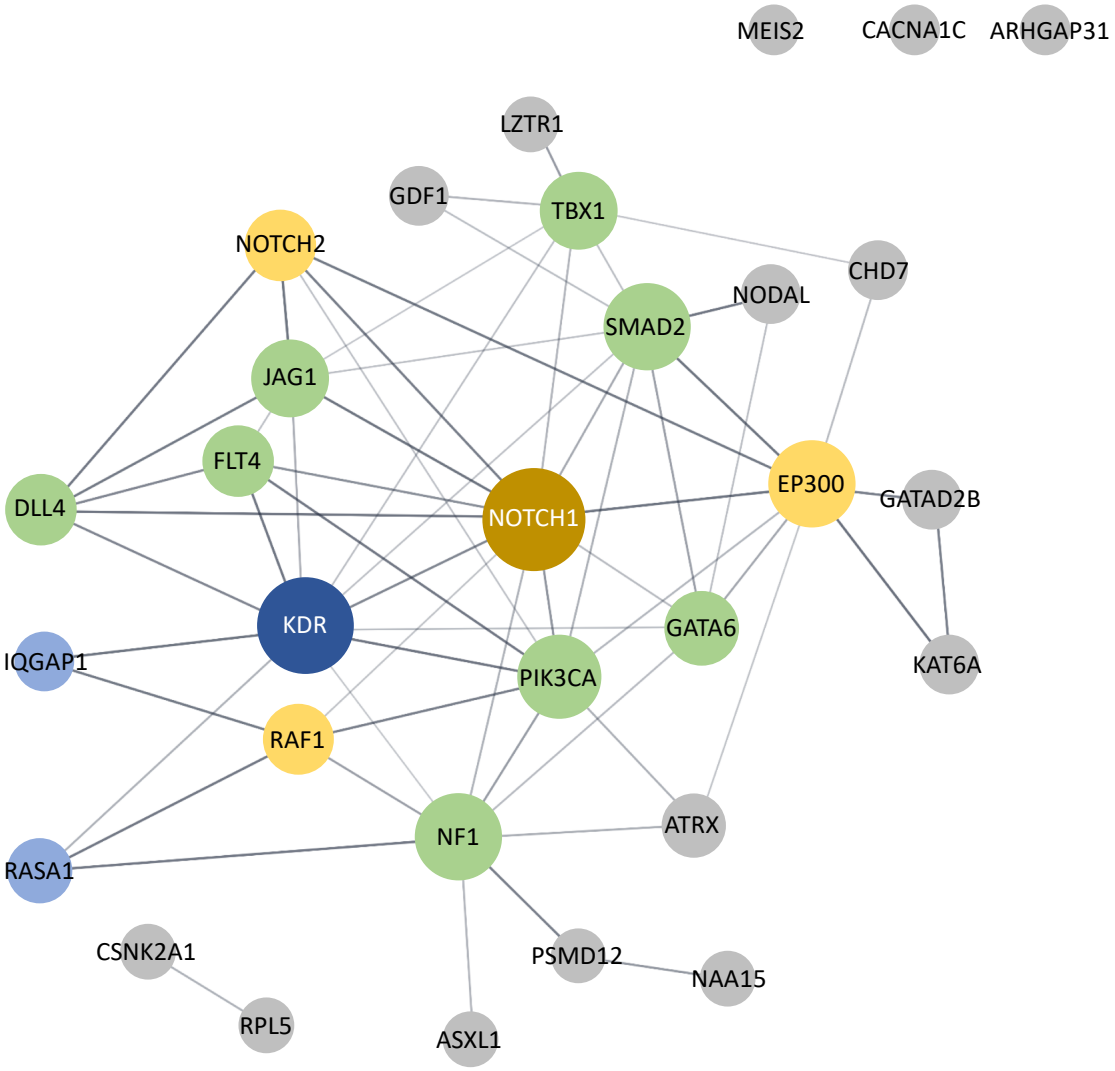

Figure I. Confirmed and candidate genes, identified for tetralogy of Fallot (from two independent cohorts), encode functionally interacting proteins – extended network. In addition to using rare high-impact variant data from the n = 811 TOF exomes from EGAS00001003302 (Supplemental Tables II, III, Supplemental Figure II), this network also considers likely pathogenic loss-of-function variants available from an independent sample of n = 424 TOF exomes (Jin et al. , Supplemental Table IV). Network analysis was performed using Cytoscape, STRING and the total 30 CHD genes with clinically relevant variants identified in the two samples (Supplemental Tables II, III, IV; STRING interaction enrichment *p* value = 1.0E-15). Node sizes (circles) represent the connectivity (number of edges to other proteins). Node colors represent the interaction with VEGFR2/KDR (blue), NOTCH1 (yellow), or both (green). Edge widths represent the confidence (strength of data support for functional and physical protein associations, including textmining, experiments, databases, and co-expression). VEGFR2/KDR and NOTCH1 form central nodes within the network, each connecting directly with 11 or 12 other proteins, respectively. NOTCH2 adds 5 interactions to the extended network, compared to the one presented in Figure 3, for the original n = 811 probands with TOF.

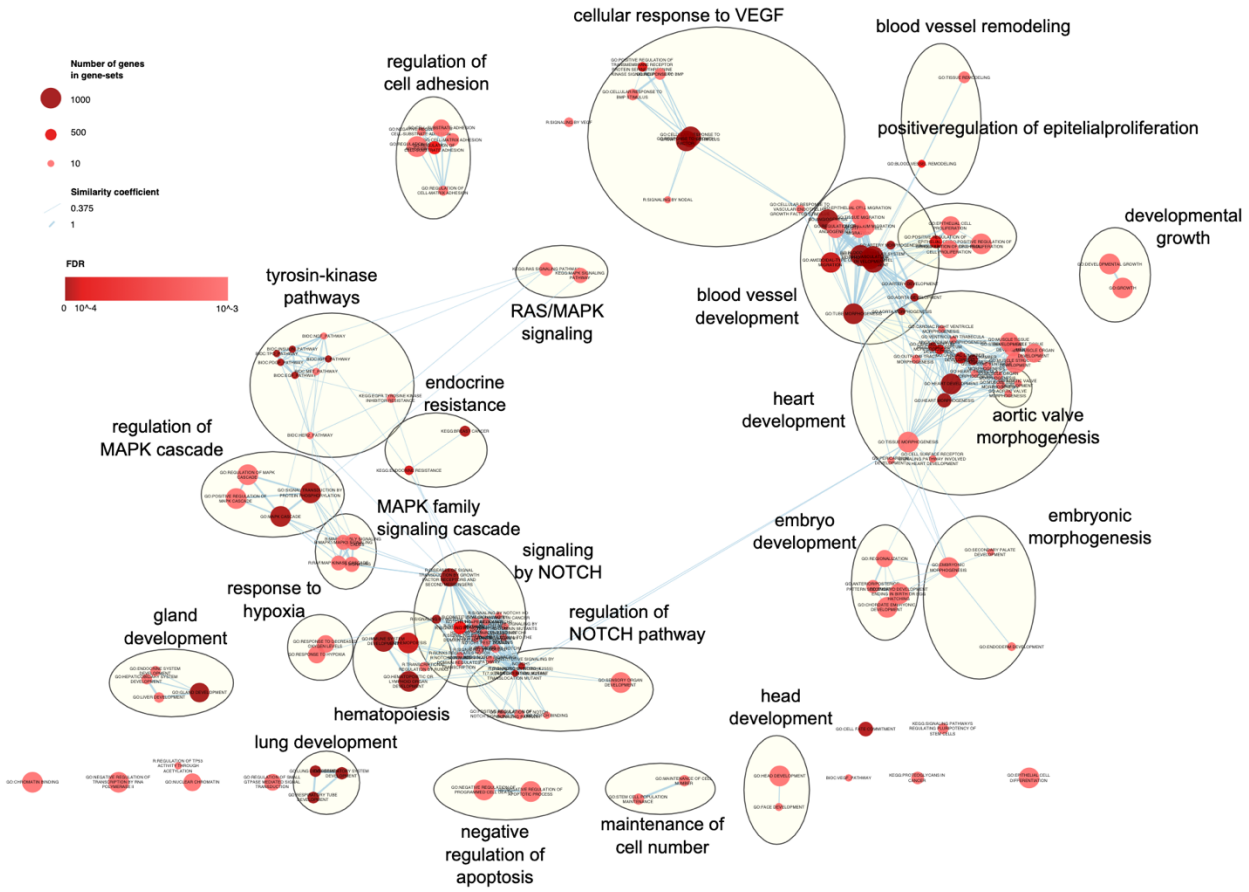

Figure II. Pathway enrichment map of confirmed genes (n = 23) and emerging candidate genes (n = 3) for CHD/TOF identified in n = 811 individuals with TOF. P values and odd ratios are reported in Supplemental Table V (provided as a separate file).