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Whole exome sequencing reveals the major genetic contributors to non-syndromic Tetralogy of Fallot

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Short title: Major genetic contributors to Tetralogy of Fallot

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Abstract

Rationale: Familial recurrence studies provide strong evidence for a genetic component to the predisposition to sporadic, non-syndromic Tetralogy of Fallot (TOF), the most common cyanotic congenital heart disease (CHD) phenotype. Rare genetic variants have been identified as important contributors to the risk of CHD, but relatively small numbers of TOF cases have been studied to date.

Objective: We used whole exome sequencing (WES) to assess the prevalence of unique, deleterious variants in the largest cohort of non-syndromic TOF patients reported to date.

Methods and Results: 829 TOF patients underwent WES. The presence of unique, deleterious variants was determined; defined by their absence in the Genome Aggregation Database (gnomAD) and a scaled combined annotation-dependent depletion (CADD) score of ≥20. The clustering of variants in two genes, *NOTCH1* and *FLT4*, surpassed thresholds for genome-wide significance (assigned as $P<5x10^{-8}$) after correction for multiple comparisons. *NOTCH1* was most frequently found to harbour unique, deleterious variants. 31 changes were observed in 37 probands (4.5%; 95% confidence interval [CI]:3.2-6.1%) and included seven loss-of-function variants 22 missense variants and two in-frame indels. Sanger-sequencing of the unaffected parents of seven cases identified five de novo variants. Three *NOTCH1* variants (p.G200R, p.C607Y and p.N1875S) were subjected to functional evaluation and two showed a reduction in Jagged1-induced NOTCH signalling. *FLT4* variants were found in 2.4% (95% CI:1.6-3.8%) of TOF patients, with 21 patients harbouring 22 unique, deleterious variants. The variants identified were distinct to those that cause the congenital lymphoedema syndrome Milroy Disease. In addition to *NOTCH1*, *FLT4* and the well-established TOF gene, *TBX1*, we identified potential association with variants in several other candidates including *RYR1*, *ZFPM1*, *CAMTA2*, *DLX6* and *PCM1*.

Conclusions: The *NOTCH1* locus is the most frequent site of genetic variants predisposing to non-syndromic TOF, followed by *FLT4*. Together, variants in these genes are found in almost 7% of TOF patients.

Keywords: Congenital heart disease; Tetralogy of Fallot; whole exome sequencing

Subject Codes: Congenital Heart Disease; Genetic; Association Studies

Abbreviations: ASD, atrial septal defect; CADD, combined annotation-dependent depletion; CHD, congenital heart disease; CI, confidence interval; CNV, copy number variant; ExAC, Exome Aggregation Consortium; gnomAD, Genome Aggregation Database; GWAS, genome wide association study; HD, heterodimerisation domain; Ig, immunoglobulin; JAG1, Jagged1; LOF, loss-of-function; MAF, minor allele frequency; NICD, NOTCH intracellular domain; SNP, single nucleotide polymorphism; TOF, Tetralogy of Fallot; VEGFR3, vascular endothelial growth factor receptor 3; VSD, ventricular septal defect; WES, whole exome sequencing.

Introduction

Congenital heart disease (CHD) is the most common type of birth defect, affecting 8/1000 live births (1). CHD covers a large spectrum of heterogeneous cardiovascular phenotypes that range from single, localised defects to more complex structural abnormalities. Tetralogy of Fallot (TOF) is the most common complex, cyanotic CHD with a prevalence of 1/3000 births (1,2). TOF is considered a malformation of the cardiac outflow tract which comprises four specific structural characteristics postnatally; a ventricular septal defect (VSD), anterocephalad deviation of the outflow septum with resultant overriding of the aorta, variable obstruction of the right ventricular outflow tract (pulmonary stenosis) and consequent hypertrophy of the right ventricle (2,3). Surgical interventions during infancy mean that 85-90% of TOF patients now survive until at least 30 years of age (1,4). However, this is not without consequence; event-free survival is just 25% after 40 years of age (5), since resultant scar tissue from surgery and pulmonary regurgitation cause significant morbidity in adulthood (6,7).

The cause of TOF is elusive and no single candidate gene can be held accountable for the disease phenotype. However, the genetic status of syndromic TOF sufferers has provided valuable insights into causative genes in some patients. Approximately 20% of cases are associated with a recognised syndrome or chromosomal anomaly (2). Most significantly, approximately 15% of TOF patients have 22q11.2 deletion syndrome, wherein the major causal gene is *TBX1* (8,9). Approximately 80% of TOF cases are non-syndromic and there is generally no identifiable cause, largely due to their non-Mendelian patterns of inheritance (10-13). Accordingly, a polygenic genetic architecture has been hypothesised and genome-wide approaches have been undertaken to provide insights into the complex genetic alterations responsible for TOF and other CHDs (11,13-18).

Whole exome sequencing (WES) has been used successfully to identify new CHD candidate genes (14,17,19,20). Many lines of evidence indicate a degree of phenotypic specificity of variants in particular genes. For example, the spectrum of phenotypes caused by 22q11.2 deletion or mutations in *TBX1* typically involves the outflow tract and great vessels (9,21,22), while Down syndrome or mutations in *NKX2-5* typically cause septal defects (23,24). To date, no WES study of CHD has included substantial numbers of any homogeneous phenotype, which should *a priori* have the highest power to identify causal variants.

Here, we present findings from WES of the largest cohort of non-syndromic TOF patients reported to date. We performed WES in 829 TOF probands and identified the rarest and most deleterious protein-coding variants genome-wide. We sought evidence of pathological relevance for a subset of variants in the most significantly over-represented genes, based on the variants' *de novo* occurrence and functional consequences in cellular models.

Methods Summary

829 TOF probands were subjected to WES and unique (absent in the Genome Aggregation Database [gnomAD]), deleterious (combined annotation-dependent depletion [CADD] score of ≥ 20) variants were identified. Any variants observed in 1252 reference exome samples, that were analysed using the same approach as our case data, were eliminated from further consideration. Clustering analysis within the cases was then used to identify genes in which significantly more variants were observed than expected given background levels of variation across all genes. *De novo* variants were identified by Sanger sequencing of proband and parent samples where possible. Immunoblotting and luciferase assays were used to assess the expression and signalling activity of selected variants in the most strongly supported candidate gene. Detailed methods can be found in the **Supplementary Materials.**

Results

Exome-wide analysis of unique, deleterious variants identifies the highest risk loci for non-syndromic TOF

We assessed the incidence of unique, deleterious variants for 829 non-syndromic TOF cases. Any variants observed in 1252 reference exomes were removed from consideration as potential TOF susceptibility variants. The statistical significance of these findings was assessed for each gene using clustering analysis, which corrected for gene size (supplementary table 1). Two genes, *NOTCH1* and *FLT4*, surpassed the threshold for genome-wide significance (assessed as $P<5 \times 10^{-8}$) (figure 1) and the unique variants identified in these genes are likely to be contributors to the pathogenesis of TOF. Combined, variants in *NOTCH1* and *FLT4* account for 6.9% of our TOF cohort, with no overlap between probands with variants in these genes. Additionally, several other genes that harbour an excess of variant clustering are also of interest; including *RYR1* and *TBX1*, which have previously been implicated in CHD (25,26). In particularly, *TBX1* is a well-established TOF risk gene which is principally responsible for the cardiac manifestations of 22q11 deletion; additionally, deleterious single nucleotide variants and small functionally significant intragenic deletions in *TBX1* have been demonstrated in TOF patients (9,21). A further two genes, *ZFPM1/FOG1* and *CAMTA2*, have roles in heart development and growth, respectively (27). *DLX6* is negatively regulated by HAND2, a crucial transcription factor for heart morphogenesis (28) and *PCM1* is a regulator of ciliogenesis, a process strongly linked to CHD (29). In addition, we specifically looked at the number of unique, deleterious variants in key cardiac transcription factors including *NKX2.5* (30), *GATA4* (31), *HAND2* (12) and *GATA6* (32), since pathogenic variants have previously been identified in TOF cases, typically by targeted candidate gene sequencing. Variants in these genes account for just 1.2% of cases in our cohort. When considering the top nine genes (or a P value cut-off of <0.01), 129 TOF cases had a unique, deleterious variant in one or more genes, accounting for over 16% of our patient cohort (table 1). Just eight samples had variants in more than one of the top nine genes, highlighting the minimal overlap between probands with variants in these genes. Overall, *NOTCH1* and *FLT4* were found to be by far the most significant contributors to TOF; we therefore explored the variants in these two genes in greater detail.

Variants in *NOTCH1* are most commonly present in non-syndromic TOF

The *NOTCH1* locus was most frequently found to harbour a unique, deleterious variant among TOF patients ($P<2.22 \times 10^{-16}$), with 37 probands harbouring 31 *NOTCH1* variants (supplementary table 2), accounting for 4.5% of our TOF patient cohort (95% CI: 3.2% - 6.1%). Seven of the variants identified were loss-of-function (LOF), including three premature stop codons (p.R448X, p.W1638X and p.Q1733X), three single base pair deletions resulting in frameshifts and eventual premature truncation (p.G115fsX6, p.N147fsX128 and p.C1322fsX121) and a single base pair deletion in a splice site consensus sequence (c.5385-1delC). Of the remaining 24 variants, two were in-frame indels and 22 were missense variants. *NOTCH1* is highly intolerant to LOF and missense variation, having a pLI of 1 and a missense z score of 4.48 on the Exome Aggregation Consortium (ExAC). We mapped the distribution of the 31 variants to the various domains of NOTCH1 (figure 2) and found the variants to be located throughout the protein with no significant clusters. The three frameshift mutations were located in the EGF-like repeats in addition to one truncating mutant, p.R448X, whereas the remaining two truncating variants were located in the heterodimerisation domain. Of particular interest, one variant located in EGF-like repeat 5, p.G193A (figure 2, bold), was identified in five unrelated patients and p.P143L (figure 2, bold) located in EGF-like repeat 4 was identified in three unrelated patients. Together, these two variants account for almost 1% of our TOF patient cohort. Interestingly, a further six *NOTCH1* variants that map to the EGF-like repeats alter evolutionary conserved cysteine residues that contribute to disulphide bonds essential for maintaining the EGF structure (33). Of the four intracellular domain mutants, a missense variant in the Ankyrin repeats region, p.R2004L is particularly notable (figure 2, bold). R2004 is a surface exposed residue in Ankyrin domain 4 which is located in an interface region with the CSL transcription factor complex (34) and also located at an interface that binds the positive Notch regulator, Deltex (35).

Deleterious mutations in other NOTCH pathway genes have been identified in patients with TOF including *HEY2* (36) and *JAG1* (37,38). For this reason, we compiled a list of NOTCH pathway genes using the MGI Gene Ontology Project and assessed the clustering of variants in these genes. Of 166 genes tested, only *NOTCH1* was found to have an excess of unique, deleterious variants (supplementary table 3). Hence, variants in other NOTCH pathway genes are not a major cause of TOF in our cohort.

Evidence of pathological consequences for *NOTCH1* variants

We investigated the occurrence rate of *de novo* variants in probands with *NOTCH1* variants. Of the 31 probands in our TOF patient cohort that harboured unique, deleterious variants in *NOTCH1*, samples from both parents were available for seven probands and analysed for variant inheritance. Following Sanger sequencing, five of the seven *NOTCH1* variants tested were identified as *de novo*; two of these were truncating variants, whereas the remaining three *de novo* variants were missense (table 2). These findings are in keeping with the results of previous WES experiments in CHD, where rare transmitted variants with strong bioinformatic support for functional impact, which are of presumed incomplete penetrance, have been uniformly encountered (14,17,20).

The *NOTCH1* gene encodes an evolutionarily conserved transmembrane receptor that mediates cell-cell communication to govern cell fate decisions during development (39). S1 cleavage is an important step in the maturation of the NOTCH1 receptor. During this process, the 300 kDa translation product of NOTCH1 undergoes cleavage in the Golgi by furin-like convertase to generate two polypeptides of 180 and 120 kDa (40). To determine whether *NOTCH1* variants affect S1 cleavage, we assessed the expression of three NOTCH1 variants in comparison to wild type (WT) NOTCH1 by immunoblotting. The variants assessed were p.G200R, p.C607Y and p.N1875S (see figure 2); p.G200R is located in a conserved residue located within a β-hairpin turn within EGF5, and p.C607Y, located in EGF16, removes a conserved disulphide bond that normally would be expected to stabilise the EGF-domain conformation. p.N1875S is located in a residue that lies in a linker region between the RAM and Ankyrin repeat regions of the Notch intracellular domain. As expected, we observed two bands at 300 kDa (P300) and 120 kDa (P120), representing full length and cleaved NOTCH1 protein (40); the remaining 180 kDa product was not detectable due to the positioning of our FLAG-tag at the C-terminus (figure 3a). For WT NOTCH1, p.G200R and p.N1875S variants, we observe similar levels of both P300 and P120 (figure 3a). However, the p.C607Y variant exhibited perturbed S1 cleavage. Indeed, quantification confirmed that 5%±0.37% of NOTCH1 p.C607Y underwent cleavage in comparison to 57%±3.96% of WT NOTCH1 (P=0.0002; figure 3b). Hence, the p.C607Y variant affects S1 cleavage of NOTCH1, whereas the receptor is processed normally in the p.G200R and p.N1875S NOTCH1 variants.

Heterodimeric NOTCH1 is membrane tethered and undergoes further cleavage by γ-secretase which releases the NOTCH intracellular domain (NICD). NICD subsequently translocates to the nucleus where it interacts with transcription factor RBPJ to activate NOTCH target genes (39). To determine whether p.G200R, p.C607Y and p.N1875S variants affect NOTCH1 canonical signalling function, we assessed NOTCH signalling through the RBPJ transcription factor-dependent pathway following stimulation with immobilised Jagged1 (JAG1) ligand. The variants were overexpressed in HeLa cells and NOTCH1 signalling was assessed by RBPJ luciferase activity. Two of the three variants demonstrated reduced NOTCH signalling via RBPJ (figure 3c). The p.C607Y variant, that exhibited perturbed cleavage, significantly reduced NOTCH signalling by 47%±0.12% (P=0.008) compared to WT NOTCH1. Similarly, *de novo* variant p.N1875S reduced NOTCH signalling by 38%±0.13% (P=0.02). The p.G200R variant exhibited similar canonical NOTCH signalling to WT NOTCH1 (P=0.67) (figure 3c), yet mapping of this variant to the three-dimensional NOTCH1 protein suggests structural implications (supplementary figure 2). Furthermore, p.G200R has also been reported in an independent study to segregate with CHD, supporting its pathogenicity (41). No significant differences were observed between WT NOTCH1, p.G200R, p.C607Y and p.N1875S variants in the absence of JAG1 ligand. In each transfection experiment, mRNA expression of WT

NOTCH1 and the three *NOTCH1* variants was equal (supplementary figure 3), thus the differences in NOTCH1 signalling observed were not due to reduced mRNA expression of the variants. Hence, two variants identified in patients that were subjected to functional testing were shown to affect canonical NOTCH1 signalling.

FLT4 variants found in TOF are distinct from those that cause Milroy Disease

The second most frequent locus of variant clustering in our TOF cohort was *FLT4* ($P=4.44 \times 10^{-16}$). *FLT4* encodes a receptor tyrosine kinase known as vascular endothelial growth factor 3 (VEGFR3). VEGFR3 is indispensable for lymphatic development and *FLT4* mutations are a known cause of the hereditary lymphoedema, Milroy disease. Strikingly, all mutations reported for Milroy disease are missense variants or in-frame indels located in the VEGFR3 protein kinase domain (figure 4). In our TOF cohort of 829 probands, we report 22 unique, deleterious *FLT4* variants in 21 TOF probands, accounting for 2.4% of cases (supplementary table 4). 16 of the *FLT4* variants were LOF, including six premature stop codons (p.Y361X, p.Y369X, p.E896X, p.Q920X, p.R1031X and p.Q1126X) six indels resulting in frameshifts and premature truncation (p.P363fsX25, p.Q423fsX3, p.L636fsX3, p.Y853fsX20, p.N905fsX20 and p.Y1337fsX19) and four splice variants (c.3002-1C>T, c.3002-2T>C, c.2300C>G and c.2849del21). One premature stop codon, p.Y361X, was reported previously in a TOF proband and affected mother (25). The remaining six variants were missense, all of which were located in the immunoglobulin (Ig) domains of VEGFR3. *FLT4* is extremely intolerant to both LOF and missense variation, as demonstrated by a pLI of 1 and missense z score of 3.73 on ExAC, respectively. In our 1252 reference exomes, no novel, LOF *FLT4* variants were identified. Parent DNA was available for four probands. Three of the variants (p.Q920X, p.Y853fsX20 and c.2300C>G) were inherited from unaffected parents indicating incomplete penetrance, and one missense variant, p.C51W, was *de novo* (supplementary table 5). Frameshift variant Y853fsX20 was identified in two siblings with TOF and was inherited from the mother who was unaffected. Crucially, no missense or in-frame variants were found in the kinase domain, a feature unique to Milroy disease (figure 4). Our findings are in line with a recent publication by Jin *et al* (2017) that reports LOF variants in *FLT4* in 2.3% of 426 TOF probands. Hence, we confirm this finding in the largest TOF cohort reported to date, approximately twice the size of previous studies, endorsing the importance of *FLT4* as a major contributor to the incidence of TOF.

Discussion

Despite TOF being the most common, severe cyanotic CHD, variants that could account for the high degree of genetic susceptibility, inferred from familial recurrence risk studies (42), are as yet unidentified. This study represents the largest WES investigation of sporadic, non-syndromic TOF performed to date. Using variant clustering analysis and stringent filtering, we identify two genes that reach genome-wide significance: *NOTCH1* and *FLT4*. As an additional safeguard against false positive results due to systematic methodological differences between our cohort and the studies which contributed to the gnomAD database, we studied a set of over 1000 reference exomes in patients free from CHD; analysed in the same fashion as the case exomes, stringently removing any variant that appeared even once in the reference exome set from consideration as a potential TOF susceptibility variant.

We identify *NOTCH1* as the major TOF susceptibility gene; 4.5% of patients carry heterozygous variants in *NOTCH1,* which based on gnomAD allele frequency, bioinformatic *in silico* prediction, and functional characterisation, we judged to be likely susceptibility alleles. With the exception of the 22q11 deletion, no single gene locus has been found to account for more TOF cases than *NOTCH1*. Seven of the variants were LOF, including truncating, frame shift and splice variants, whereas the remaining 24 variants were missense or in-frame indels and anticipated to be pathogenic. Five out of seven variants tested were *de novo,* adding to the evidence for pathogenicity; the remaining variants were transmitted from unaffected parents indicating incomplete penetrance. Previous sequencing studies of CHD have identified an association of *NOTCH1* variants in cardiac

malformations including bicuspid aortic valve, aortic valve stenosis, coarctation of the aorta and hypoplastic left heart syndrome and TOF (43-47). However, the extent of *NOTCH1* variant contribution to TOF has not been recognised until now. There are no clear distinctions between the type and location of *NOTCH1* variants identified in TOF compared to those reported in other isolated cardiovascular abnormalities. We therefore propose that genetic background and/or environmental influences may specify phenotypic expressivity.

A possible role for *NOTCH1* in non-syndromic TOF has previously been suggested by copy number variant (CNV) analysis. A study of 34 infants with non-syndromic TOF revealed two patients with CNVs encompassing the *NOTCH1* gene (48). Additionally, a microdeletion including the *NOTCH1* locus in a patient with TOF was identified in a study of CNVs in 114 TOF patients (49). A recent study that focused primarily on families with left-sided CHD also identified family members with TOF harbouring pathogenic mutations in *NOTCH1* (44). Further indirect evidence for *NOTCH1* contribution to TOF came from a study that analysed the gene expression patterns in TOF patient right ventricles and found many genes from the *NOTCH* and *WNT* signalling pathways were significantly reduced. Interestingly, down-regulation of *NOTCH* signalling components was also observed in TOF patients with a 22q11.2 deletion (50), highlighting a common transcriptional signature between both syndromic and non-syndromic TOF, initiated by different genetic events. More recently, exome sequencing of 426 TOF patients that focused solely on LOF heterozygous variants did not identify an enrichment of NOTCH1 mutations in TOF patients (25). However, the present study involves, by a substantial margin, the largest TOF cohort studied by WES to date, including both LOF and damaging missense variants, hence providing the most accurate quantification thus far of the contribution of *NOTCH1* variants to TOF risk.

Autosomal dominant germ-line mutations in the *NOTCH1* gene are also one of the causes of Adams-Oliver syndrome (AOS) which is chiefly characterised by *aplasia cutis congenita* and terminal transverse limb defects. In addition to these features, around half of patients have congenital cardiac anomalies, including atrial septal defect (ASD), VSD, aortic valve stenosis, pulmonary valve stenosis and TOF (51,52). AOS is an extremely rare syndrome, with a prevalence of approximately 1 in 225,000 (52). No patient in our cohort had diagnostic features of AOS. As with other CHDs associated with *NOTCH1* variants, there are no clear distinctions between the *NOTCH1* variants we have identified in TOF versus those that cause AOS, though no previously described AOS variant was present in our cases (51,52). Interestingly, the extra-cardiac features of AOS have been suggested to occur due to early embryonic vascular abnormalities (53), raising the possibility that AOS, TOF and other cardiac anomalies that occur due to mutations in *NOTCH1* may be a spectrum of disorders. Other examples of syndromic genes that can cause isolated CHD, including TOF, are *PTPN11* (Noonan syndrome), (13,54), *TBX5* (Holt-Oram syndrome) (55) and *JAG1* (Alagille syndrome) (38). Determining the role of genetic background, environmental context and the specific *NOTCH1* variants in determining the severity of the cardiac phenotype and the occurrence of extra-cardiac malformations requires further research.

The association of *NOTCH1* with a range of cardiac defects is consistent with the reported roles of NOTCH1 during heart development. Active NOTCH1 is observed in the trabecular endocardium and both global and endothelial-specific knockout of *Notch1* in mice results in abnormal ventricular trabeculae and abnormal cardiomyocyte patterning (56). Relevant to TOF, Notch1 plays a role in the organisation of the outflow tract, which requires the specification of cells from both the neural crest and secondary heart field (57). Furthermore, Notch1 is important for endocardial epithelial-to-mesenchymal transition, a process that is essential for cardiac valve formation (46,58). It should however be noted that all *NOTCH1* variants we report are heterozygous. There are numerous reports of global and tissue specific *Notch1* heterozygous mutant mice that appear phenotypically normal, with no obvious cardiovascular pathologies (59,60), although mice lacking endothelial/endocardial *Notch1* in various backgrounds do present with TOF-like characteristics including septal defects and abnormal heart valves (61,62). This suggests endothelial NOTCH1 may be partly responsible for the cardiac malformations associated with TOF, and again, emphasising the importance of genetic

background. In further support of this, *Notch1*$^{+/-}$ in a predominantly 129S6 background developed aortic root dilation whereas *Notch1*$^{+/-}$ in a mixed background did not (63). Altogether, these reports highlight the importance of genetic background in disease expressivity and are consistent with the incomplete penetrance observed.

De novo mutations are a significant cause of early-onset genetic disorders, including CHD. Of the *NOTCH1* variants identified in this study where parents were available, five of seven variants were found to be *de novo*. Similarly, we also found *de novo* variation in *FLT4*. For both of our genome-wide significant TOF genes, variants were also found to be inherited from unaffected parents, confirming the role of incompletely penetrant variants observed for other CHD genes and phenotypes (17,20). The incomplete penetrance is in keeping with the complex genetic aetiology of non-syndromic TOF, in which families segregating the condition in a Mendelian fashion are rarely encountered and genetic background, in addition to *in utero* environmental factors, can be inferred to play significant roles.

For a subset of *NOTCH1* variants, we provide evidence of functional impact by assessing canonical NOTCH1 signalling. The p.C607Y missense variant perturbed NOTCH1 receptor S1 cleavage by the calcium-dependent enzyme, furin-like convertase. The S1 cleavage site is located at amino acids 1651 - 1654, some distance away from the variant. A similar observation has been reported by McBride *et al* (2008) where *NOTCH1* variant p.A683T, identified in two patients with left ventricular outflow tract malformations, also perturbed S1 cleavage by similar levels. In both cases, this led to a 50% reduction in RBPJ luciferase activity (64). The mechanism by which such variants alter S1 cleavage to such an extent and reduce signalling by just 50% is unclear and requires further research. Furthermore, *de novo* variant p.N1875S was shown to have significantly reduced JAG1-induced NOTCH signalling relative to WT NOTCH1, providing further support as to the pathogenicity of *de novo* variants. p.G200R exhibited signalling levels similar to WT. However, in support of this variants pathogenicity, Blue *et al* (2014) identified the same *NOTCH1* variant in an independent study; p.G200R segregated with disease in two cousins with right-sided CHD, including persistent truncus arteriosus, VSD, pulmonary atresia, and major aorto-pulmonary collateral arteries. Furthermore, a case of TOF was also reported in the preceding generation, although sequencing analysis was not carried out on this relative.

FLT4 was first associated with isolated TOF in a CNV analysis that identified a *de novo* duplication including *FLT4*, and a deletion of unknown inheritance upstream of *FLT4* (18). Recent WES studies have also identified *FLT4* to be a significant contributor to the incidence of TOF. Jin *et al* (2017) found 2.3% of TOF patients to have LOF *FLT4* mutations. Furthermore, Szot *et al* (2018) also identified a *FLT4* variant in a family with TOF (65). Using our larger cohort, we confirm *FLT4* variants to be a significant contributor to the incidence of TOF, with 2.4% of our cohort exhibiting deleterious *FLT4* variants. In addition to LOF variants, we also identify a small number of pathogenic missense variants, including one variant that is *de novo*. The encoded product of *FLT4*, VEGFR3, has a well-established role in lymphatic development and in the adult, VEGFR3 expression is almost entirely restricted to lymphatic vessels (66,67). During embryonic development, VEGFR3 is also expressed in vascular endothelial cells and is crucial for blood vessel development. Loss of VEGFR3 in mice leads to lethality at E9.5 due to defects in blood vessel formation and cardiovascular failure (68-70). This is prior to the emergence of lymphatics, suggesting VEGFR3 plays a unique role in cardiovascular development, independent of lymphangiogenesis. Importantly, patients with VEGFR3 variants causing Milroy disease are not reported to have congenital heart malformations. The distinction between the locations of the mutations in *FLT4* that cause Milroy disease in comparison to TOF may shed light on the evidently differing roles of the receptor in lymphatic versus heart development.

In addition to *NOTCH1* and *FLT4*, we also report an excess of clustering in several other genes of interest including *RYR1, ZFPM1/FOG1, CAMTA2, DLX6, PCM1* and known TOF gene, *TBX1*. A summary of *in vivo* and *in vitro* functional data currently available for these genes can be found in

supplementary table 7. Biallelic heterozygous mutations in *RYR1* have previously been linked to CHD, including TOF, in a small number of cases (25,26). In addition, a mouse homozygous for the missense mutation I4895T, displayed notable delays in cardiogenesis including abnormal orientation, improper formation of the outflow tract and an ASD (71), suggesting a role in early heart development. *ZFPM1/*FOG1 encodes a GATA cofactor previously implicated in heart development. *Fog1* null and endothelial lineage knockout mice develop heart malformations including a double outlet right ventricle and abnormal valve formation (27). Morpholino knockdown of *fog1* also results in defective cardiac looping in zebrafish (72). While *in vivo* models suggest a role for *FOG1* in heart development, we report a suggestive association of human *FOG1* mutations with CHD for the first time. *CAMTA2* interacts with *NKX2-5*, one of the core transcription factors controlling heart development. Together, *Camta2* and *Nkx2-5* promote cardiac hypertrophy in mice (73). CAMTA2 was also identified as the likely candidate gene from a *de novo* CNV deletion at 17p13.2 in a patient with congenital pulmonary atresia (74). *DLX6* encodes a homeobox protein involved with known role in cranial-facial morphogenesis. Interestingly in mice, Dlx6 is negatively regulated by Hand2 (28), a transcription factor crucial for cardiac morphogenesis. The significance of the relationship between HAND2 and DLX6 in the developing heart is not clear, although the formation of the great vessels and coronary arteries is reported to be independent of *Dlx6* in mice (75). *PCM1* encodes Pericentriolar Material 1, which is essential for centrosomal proteins and microtubule organisation. PCM1 also positively regulates ciliogenesis (76), a process which has been strongly linked to the development of CHDs (29). Following validation in an independently ascertained cohort, investigations of the role these genes during heart development may be of interest. It should be mentioned that *ZNF717* also appears amongst our top TOF-associated genes. ZNF717 is a relatively small gene (less than 4kb) yet of all genes, exhibits the highest frequency of non-synonymous mutations per base pair in our reference exomes. For this reason, we do not consider *ZNF717* to be a TOF candidate gene.

In summary, our findings which, in addition to *NOTCH1* and *FLT4*, identified a number of potential novel TOF gene candidates, concur with previous studies regarding the marked locus heterogeneity of the condition. Among the genes that have been implicated in TOF thus far, our large study indicates that *NOTCH1* is the most commonly involved. The two most commonly involved genes (*NOTCH1* and *FLT4)* are also both crucial to angiogenesis, suggesting further investigation of common pathways between heart development and angiogenesis may be fruitful. In our top gene candidates, some mutations were *de novo,* but others were present in apparently asymptomatic individuals, indicating incomplete penetrance. Such incomplete penetrance has been frequently observed, for example, in Mendelian aortopathies, emphasising the importance of genetic background in structural cardiac and vascular diseases. Detailed phenotypic studies of mutation carriers who do not have overt CHD using advanced imaging may be of interest to delineate quantitative phenotypes potentially relevant to CHD.

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Disclosures

None

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Tables

Table 1: The top gene candidates, ordered by levels of significance, following the clustering analysis of unique, deleterious variants

Gene	Variants	P value	Samples	Cumulative sample count
NOTCH1	31	$<2.22 \times 10^{-16}$	37	37
FLT4	22	4.44×10^{-16}	21	57
RYR1	21	1.43×10^{-06}	22	78
TBX1	8	6.50×10^{-05}	8	86
ZFPM1	11	0.000266817	12	98
ZNF717	9	0.001125519	10	106
DLX6	7	0.002583786	8	114
PCM1	11	0.003208801	11	123
CAMTA2	9	0.007243157	9	129

Table 2: Sequencing of parent samples to determine *NOTCH1* variant inheritance

Amino acid change	Ref	Alt	LOF	Impact	Inheritance status
p.G200V	C	A	NO	Missense variant	DE NOVO
p.C292Y	C	T	NO	Missense variant	FROM UNAFFECTED MOTHER
p.R448X	G	A	YES	Stop gained	DE NOVO
p.Q1495K	G	T	NO	Missense variant	FROM UNAFFECTED FATHER
p.C1549Y	C	T	NO	Missense variant	DE NOVO
p.W1638X	C	T	YES	Stop gained	DE NOVO
p.N1875S	T	C	NO	Missense variant	DE NOVO

Ref, reference allele; Alt, alternate allele; LOF, loss of function.

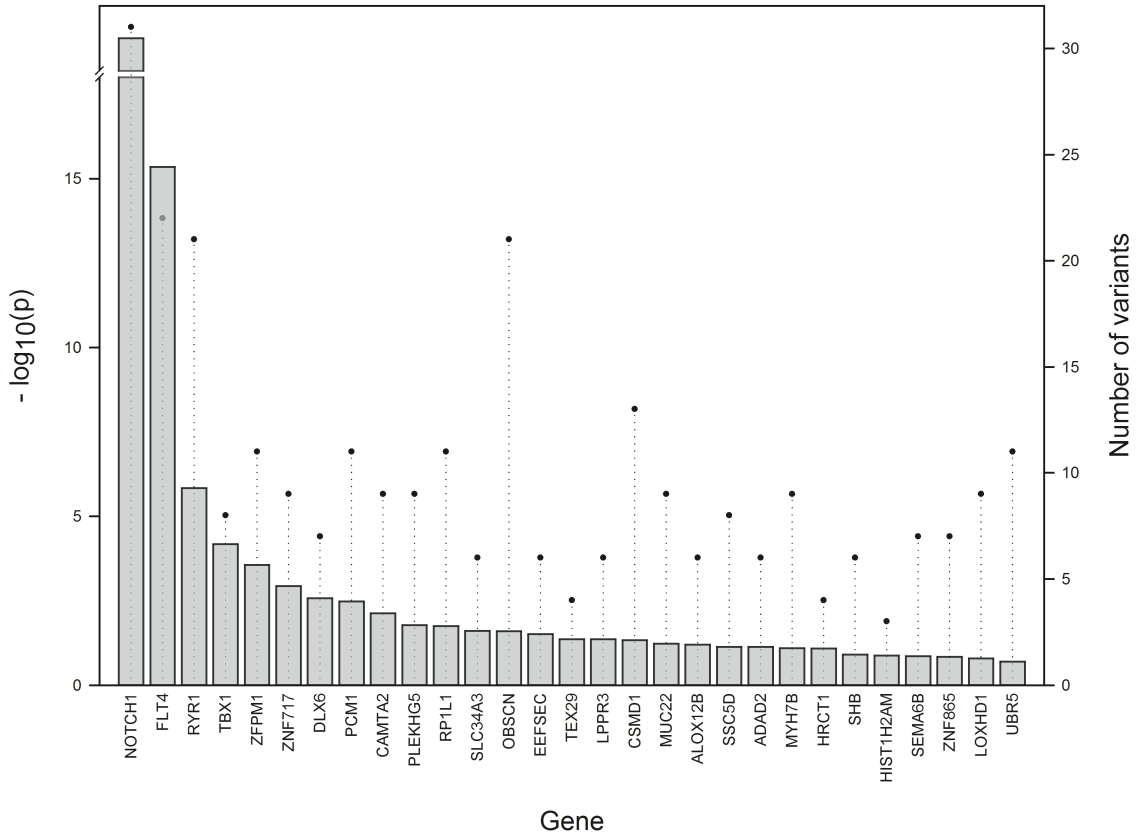
Figures with Figure Legends

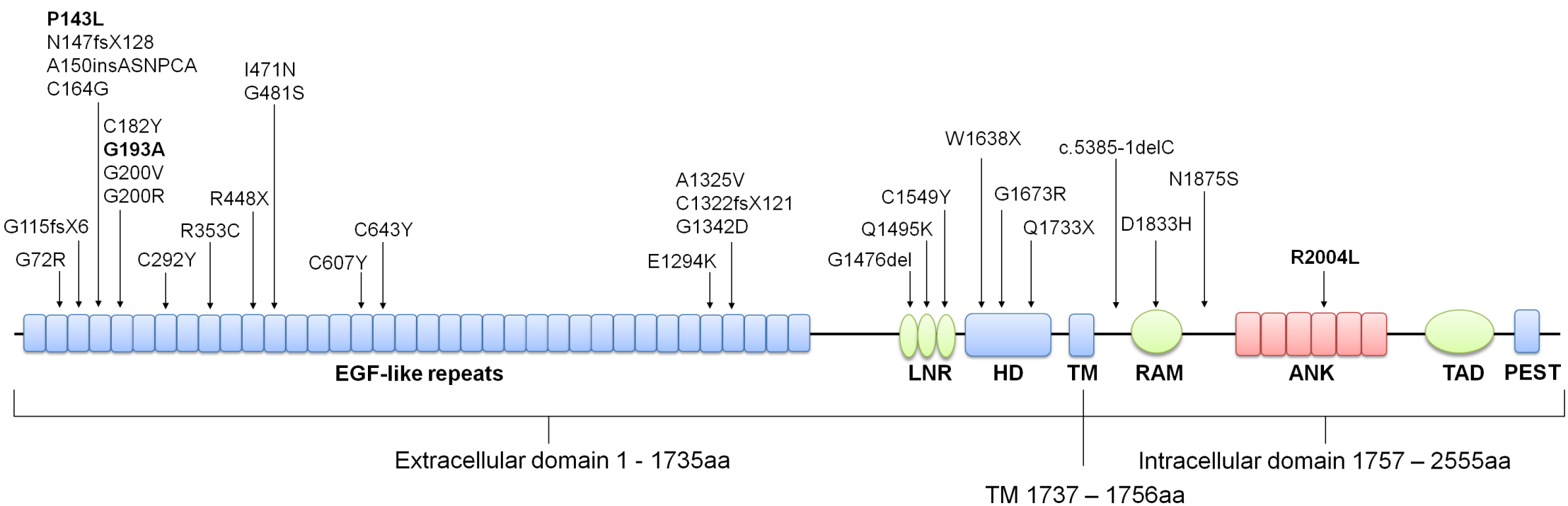
Figure 1: The top genes, in order of significance, in which non-syndromic TOF patients carry unique, deleterious variants. Bars indicate the respective significance levels of variant clustering for each gene, represented as –log P values. Circles represent the number of variants. The -log10(p) column for NOTCH1 ($P<2.22 \times 10^{-16}$) goes towards infinity and is
shown as arbitrarily high.

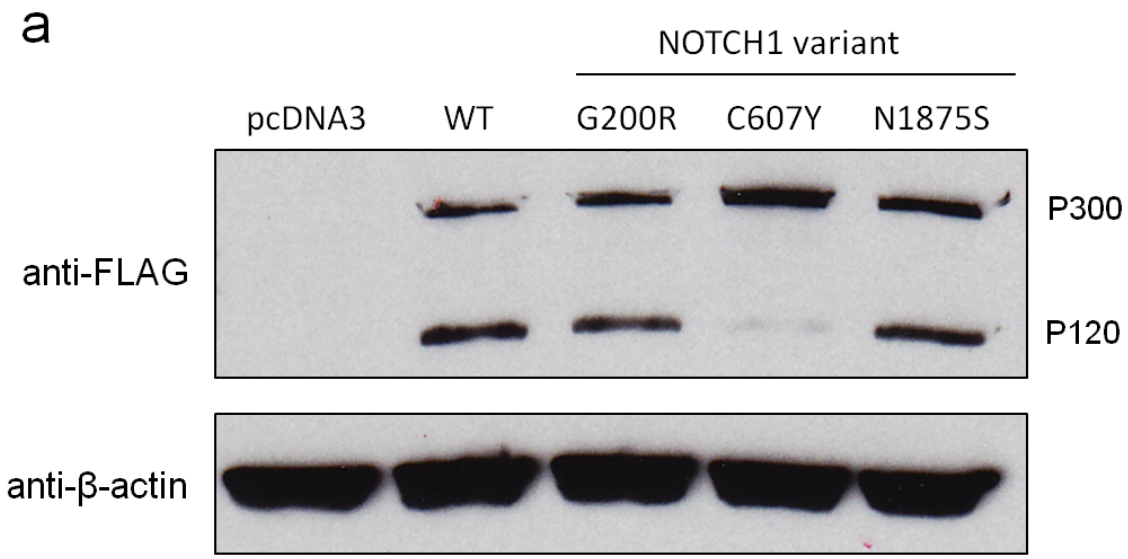
Figure 2: Unique, deleterious NOTCH1 variants in TOF patients. Diagrammatic representation of the NOTCH1 protein with known protein domains indicated. The location of *NOTCH1* variants identified in our TOF cohort is shown. p.P143L, p.G193A and p.R2004L discussed in the main text are indicated (bold). ANK, ankyrin repeats; EGF, epidermal growth factor; HD, heterodimerisation domain; LBR, ligand binding region; LNR, Lin/Notch repeats; PEST, PEST domain; RAM, RBPJ-associated molecule domain; TAD, transactivation domain; TM, transmembrane domain.

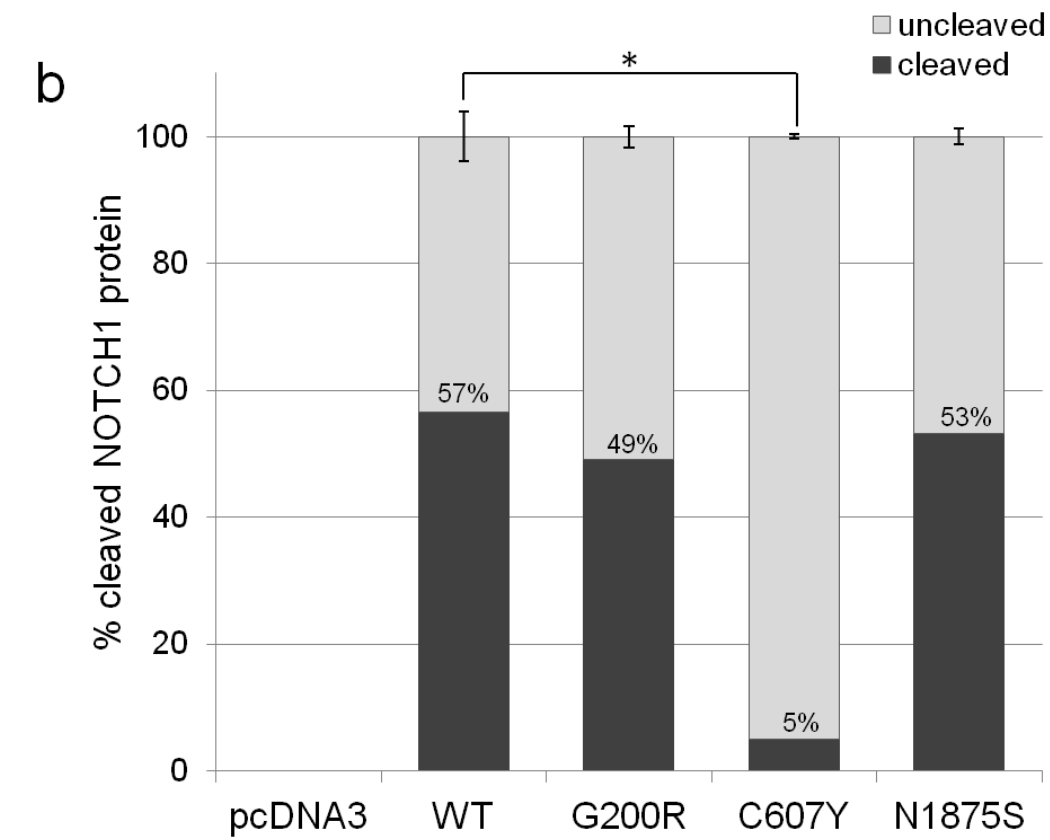
Figure 3: (a) Immunoblot for FLAG to determine the expression and S1 cleavage of NOTCH1 variants p.G200R, p.C607Y and p.N1875S in comparison to WT NOTCH1 following overexpression in HeLa cells. The two bands at 300 kDa (P300) and 120 kDa (P120) represent the full length and the S1-cleaved NOTCH1 protein. β-actin was used as a loading control. (b) Quantification of the percentage of S1 cleaved versus uncleaved NOTCH1 protein for WT NOTCH1 and NOTCH variants p.G200R, p.C607Y and p.N1875S. Error bars: mean ±SEM from three biological replicates and statistical significance was determined using two-tailed paired *t*-tests. (c) The effect of rare, deleterious *NOTCH1* variants on Jagged-induced NOTCH signalling levels. NOTCH signalling activity was measured using a luciferase-based reporter system (RBPJ). HeLa cells were cultured with or without immobilised JAG1 ligand and co-transfected with RBPJ reporter constructs and WT NOTCH1, p.G200R, p.C607Y or p.N1875S. Firefly luciferase readings were normalised to Renilla luciferase readings to control for transfection efficiency and cell number. RBPJ activity was expressed relative to WT NOTCH1 for comparison. Error bars: mean ±SEM from four biological replicates, each with three technical replicates. Statistical significance was assessed using two-tailed paired *t*-tests and the Hochberg step-up procedure to control for family-wise error rate.

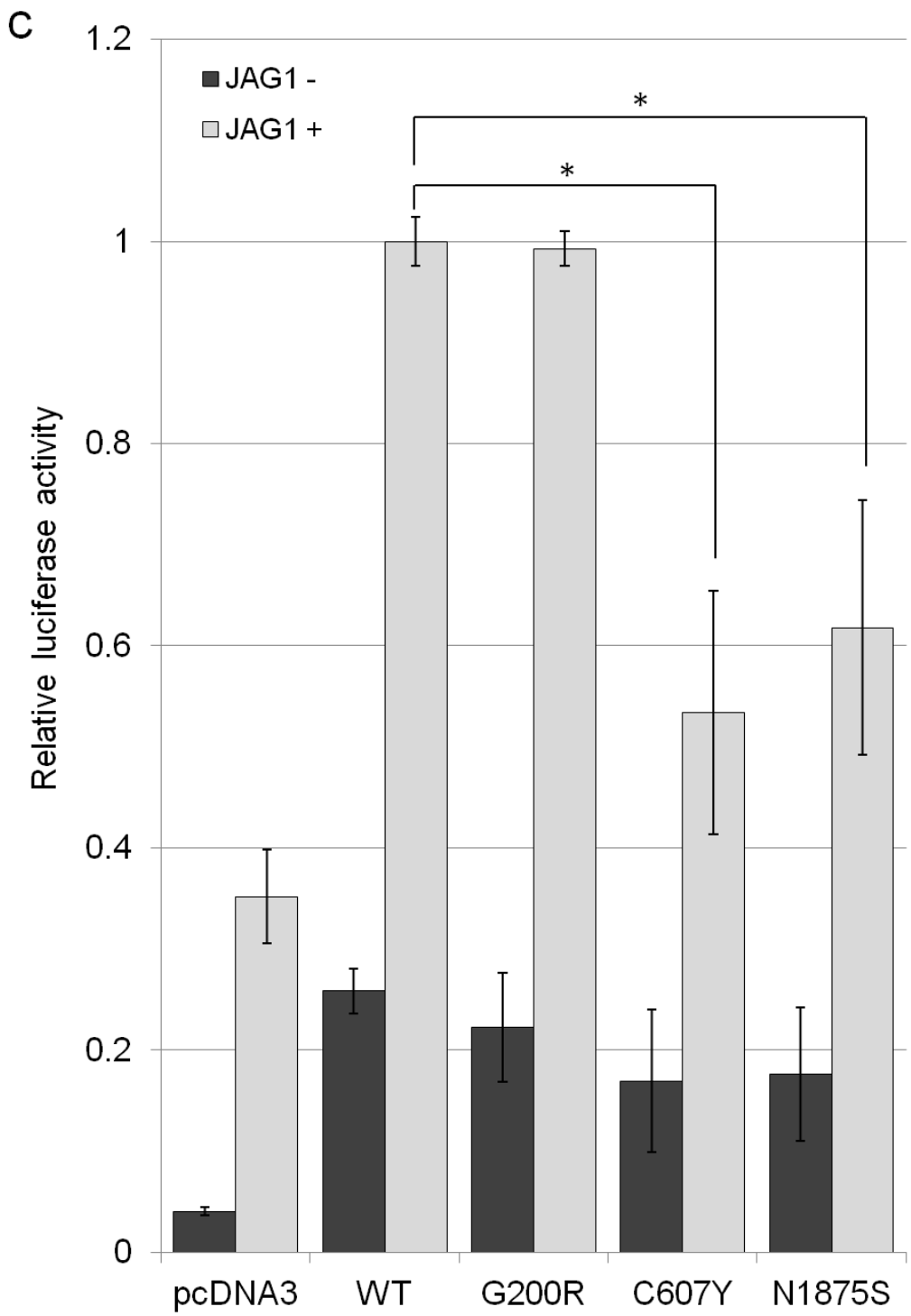
Figure 4: Unique, deleterious *FLT4* variants in TOF patients. Schematic representation of FLT4 structure with immunoglobulin (Ig) domains and protein kinase domain, indicated. Top: *FLT4* variants identified in our TOF cohort (black) and those previously reported (grey). Bottom: *FLT4* missense or in-frame mutations reported in Milroy disease, all located in the protein kinase domain.

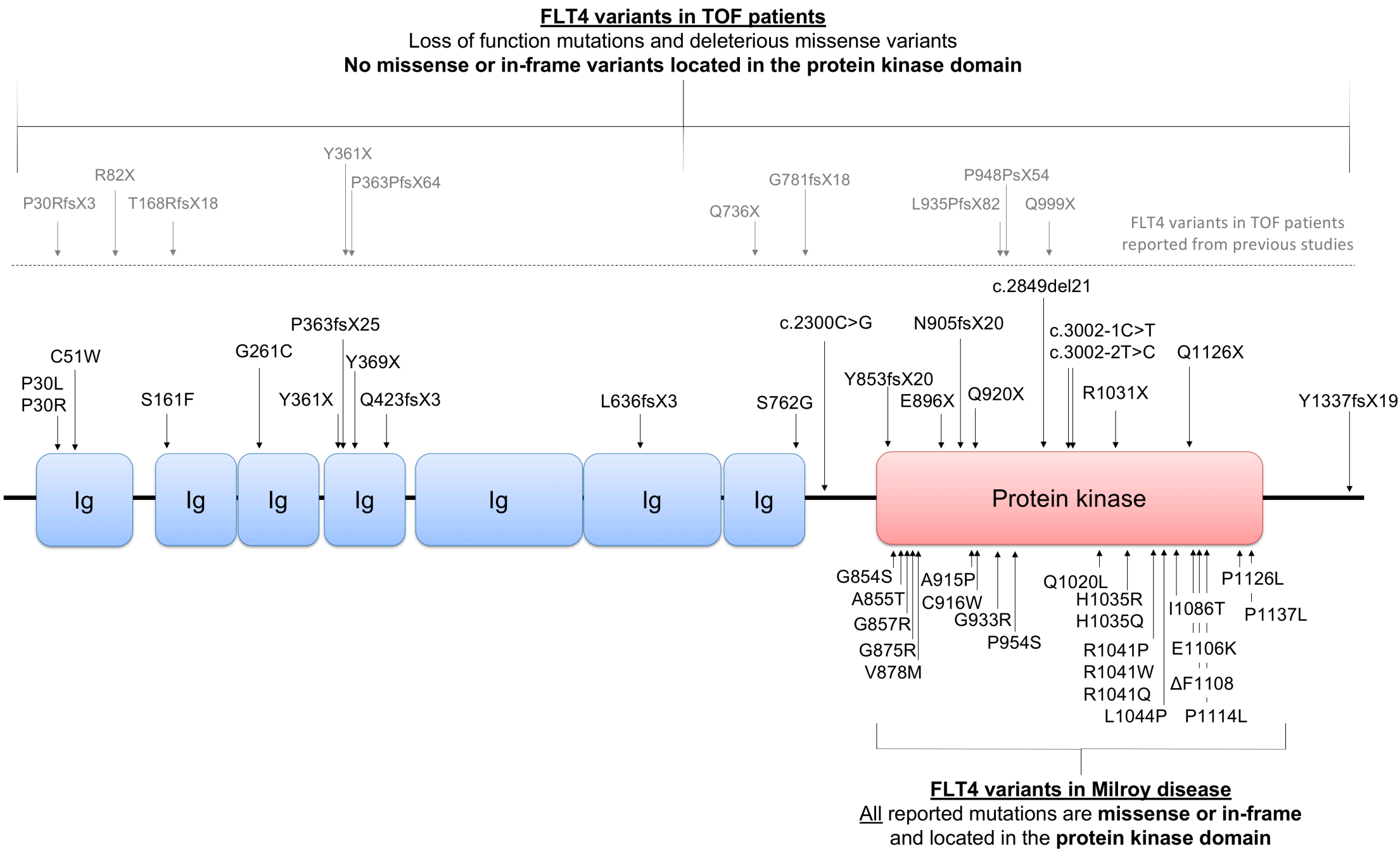

- log10(p)
Number of variants
Gene
0
5
10
15
0
5
10
15
20
25
30
NOTCH1
FLT4
RYR1
TBX1
ZFPM1
ZNF717
DLX6
PCM1
CAMTA2
PLEKHG5
RP1L1
SLC34A3
OBSCN
EEFSEC
TEX29
LPPR3
CSMD1
MUC22
ALOX12B
SSC5D
ADAD2
MYH7B
HRCT1
SHB
HIST1H2AM
SEMA6B
ZNF865
LOXHD1
UBR5


P143L
N147fsX128
A150insASNPCA
C164G
C182Y
G193A
G200V
G200R
I471N
G481S
G115fsX6
G72R
C292Y
R353C
R448X
C607Y
C643Y
A1325V
C1322fsX121
G1342D
E1294K
C1549Y
Q1495K
G1476del
W1638X
G1673R
Q1733X
c.5385-1delC
D1833H
N1875S
R2004L
EGF-like repeats
LNR
HD
TM
RAM
ANK
TAD
PEST
Extracellular domain 1 - 1735aa
TM 1737 – 1756aa
Intracellular domain 1757 – 2555aa


a
NOTCH1 variant
pcDNA3
WT
G200R
C607Y
N1875S
anti-FLAG
P300
P120
anti-β-actin


b
uncleaved
cleaved
*
% cleaved NOTCH1 protein
100
80
60
40
20
0
57%
49%
5%
53%
pcDNA3
WT
G200R
C607Y
N1875S


c
JAG1 -
JAG1 +
*
*
Relative luciferase activity
1.2
1
0.8
0.6
0.4
0.2
0
pcDNA3
WT
G200R
C607Y
N1875S


FLT4 variants in TOF patients
Loss of function mutations and deleterious missense variants
No missense or in-frame variants located in the protein kinase domain
Y361X
R82X
P363PfsX64
P30RfsX3
T168RfsX18
G781fsX18
P948PsX54
Q736X
L935PfsX82
Q999X
FLT4 variants in TOF patients reported from previous studies
c.2849del21
P363fsX25
c.2300C>G
N905fsX20
c.3002-1C>T
c.3002-2T>C
Q1126X
C51W
G261C
Y369X
P30L
P30R
S161F
Y361X
Q423fsX3
L636fsX3
S762G
Y853fsX20
E896X
Q920X
R1031X
Y1337fsX19
Ig
Ig
Ig
Ig
Ig
Ig
Ig
Protein kinase
G854S
A855T
G857R
G875R
V878M
A915P
C916W
G933R
P954S
Q1020L
H1035R
H1035Q
R1041P
R1041W
R1041Q
L1044P
I1086T
E1106K
ΔF1108
P1114L
P1126L
P1137L
FLT4 variants in Milroy disease
All reported mutations are missense or in-frame
and located in the protein kinase domain