REPORT

Mutations in *DDX3X* are a common cause of unexplained intellectual disability with gender-specific effects on Wnt signaling

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ABSTRACT

Intellectual disability (ID) affects approximately 1% of humans with a gender bias towards males. Previous studies have identified mutations in over 100 genes on the X chromosome in males with ID, but there is less evidence for *de novo* mutations on the X chromosome causing ID in females. In this study we present 34 unique deleterious *de novo* mutations in *DDX3X* identified by whole exome sequencing in 37 females with ID and various other features including hypotonia, movement disorders, behavior problems, corpus callosum hypoplasia and epilepsy. Based on our findings, mutations in *DDX3X* are one of the more common causes of ID accounting for 1-2% of unexplained ID in females.

Although no *de novo DDX3X* mutations were identified in males, we present three families with segregating missense mutations in *DDX3X,* suggestive of an X-linked recessive inheritance pattern. In these families males with the *DDX3X* variant all had ID, while carrier females were unaffected. To explore the pathogenic mechanisms accounting for the differences in disease transmission and phenotype between affected females and affected males with *DDX3X* missense variants, we used canonical Wnt defects in zebrafish as a surrogate measure of DDX3X function *in vivo*. We demonstrate a consistent loss of function effect of all tested *de novo* mutations on the Wnt-pathway, and we further show a differential effect by gender. The differential activity possibly indicates a dose dependent effect of *DDX3X* expression in the context of functional mosaic females versus one-copy males, which reflects the complex biological nature of *DDX3X* mutations.

MAIN TEXT

Intellectual disability (ID) affects approximately 1% of humans with a gender bias towards males.[1] Though mutations causing monogenic recessive X-linked intellectual disability (XLID) have been reported in over 100 genes[2; 3], the identification of conditions caused by *de novo* mutations on the X chromosome affecting females only, is limited. [4; 5]

By undertaking a systematic analysis of whole exome sequencing (WES) data on 820 individuals (461 males and 359 females) with unexplained ID (from the Department of Genetics Nijmegen, the Netherlands), seven *de novo* variants in *DDX3X* were identified in females. Given the prominent role of *de novo* mutations in ID[6], exome sequencing was performed in the probands and their unaffected parents, to identify *de novo* mutations.[7] Exome sequencing and data analysis were performed essentially as previously described.[8] To replicate these findings, we examined a second cohort of 957 individuals (543 males, 414 females) from GeneDx, United States (sequencing methods as previously published[9]) and a third cohort of 4295 individuals (2409 males, 1886 females) from the DDD study[10], United Kingdom. We identified 12 *de novo* alleles in the second cohort and 20 *de novo* alleles in the DDD cohort. In total, 39 *de novo* variants were identified in 2659 females, indicating that mutations at this locus contribute as much as 1-2% to unexplained ID in females. No *de novo* variants in *DDX3X* were identified in males in these three cohorts (Fisher's Exact test: $p = 4{,}815e^{-9}$). Additional females with *de novo DDX3X* variants were collected from other clinical and diagnostic centers in the Netherlands, Belgium, Germany, Italy and Canada. In this study, we present the clinical and molecular details of in total 37 females from the entire collected cohort, who had 34 distinct *de novo* variants in *DDX3X.* Of these 34 alleles, 19 alleles are predicted to be loss of function alleles (9 frameshift mutations leading to premature stop codon, 6 nonsense mutations, and 4 splice site mutations producing exon skipping; Table 1), suggesting

haploinsufficiency as the most likely pathological mechanism. All 14 *de novo* missense variants are located in the Helicase ATP-binding domain or Helicase C-terminal domain (Figure 1).

Analysis of the clinical data suggested a syndromic disorder with variable clinical presentation. All females (age range 1 - 33 years) showed mild to severe ID or developmental delay with associated neurological abnormalities, including hypotonia (28/37, 76%), movement disorders comprising dyskinesia, spasticity and a stiff-legged or wide based gait (17/37, 46%), behavior problems including autism spectrum disorder (ASD), hyperactivity and aggression (20/37, 54%), and epilepsy (6/37, 16%). Several recurrent additional features were noted, including joint hyperlaxity, skin abnormalities (mosaic-like pigmentary changes in some females), cleft lip and/or palate, hearing and visual impairment and precocious puberty. A summary of the clinical data is presented in Table 2 and Supplemental Table 1, and facial profiles of 30 of the 37 females are shown in Figure 2. There is no evidence for a diffence in clinical presentation between the frameshift/nonsense mutations and the missense mutations in terms of severity of ID.

DDX3X is known as one of the genes that are able to escape X-inactivation[11; 12.]. X-linked dominant conditions often show a remarkable variability among affected females which in particular holds true for genes that escape X inactivation[5; 13]. It is known that most of the transcripts escaping X-inactivation are not fully expressed from the inactivated X-chromosome, which means that the escape is often partial and incomplete[5]. Based on this, phenotypic severity may be influenced by the amount of gene expression of *DDX3X* in females, which could be affected by possibly skewed X-inactivation or incompleteness of the escape. Different expression of DDX3X in different tissues could also be a contributing factor. To further explore this, we determined inactivation using the androgen receptor gene (AR) methylation assay[14] on DNA from lymphocytes in 15 females and found an almost completely skewing (>95%) of X-inactivation in seven individuals and random skewing in the remainder, with no

evidence of correlation with disease severity. As expected, X-inactivation in females with *DDX3X* variants seems uninformative for the disease phenotype.

Given the high frequency (1-2%) of *DDX3X* mutations in females with unexplained ID, we sought to determine whether males carry deleterious alleles. We identified no *de novo* variants in males in any of our cohorts. However, upon sequencing of the X-chromosome (X-exome) of 405 ID families with apparent X-linked inheritance pattern[3], we identified two families with segregating missense variants in *DDX3X.* Moreover, one additional family was identified by diagnostic whole exome sequencing in Antwerp, Belgium. In these three families males with the *DDX3X* variant have borderline to severe ID, while carrier females are unaffected. Pedigrees of these three families are shown in Supp Figure 1, and a summary of the clinical features of the affected males is presented in Supp Table 2. All three missense mutations were predicted to be pathogenic by prediction programs PolyPhen 2 and SIFT (Table 1) and map within the Helicase ATP-binding domain (Figure 2). With three dimensional protein analysis we could not discern any clear difference between the missense mutations found in affected males and the *de novo* mutations found in females that could possibly explain the gender specific pathogenicity (Supp Figure 2, Supp Table 3). While in the first two families with affected males the phenotype consisted mainly of intellectual disability, family 3 was more complex. The male proband had severe ID and various other features such as a dysplastic pulmonary valve, hypertonia and strabismus. His mother had recurrent miscarriages of unknown gender. A second initially viable pregnancy was terminated because of ultrasound anomalies that had also been noted in the proband, including a thickened nuchal fold and absent nasal bone. In retrospect, the male fetus was tested and found to have the same missense mutation in *DDX3X* as his brother. Sequencing of other family members demonstrated the mutation arose *de novo* in the proband's mother. X-inactivation studies in this mother demonstrated a random X-inactivation pattern (68/32). X-inactivation studies in female carriers in the other families showed that in

Family 1 the obligate carrier female (II-2) had highly skewed X-inactivation (>95%), while X-inactivation studies in Family 2 were not informative.

Neither of the three *DDX3X* variants found in these families with affected males were reported in the ExAC Database or in the Exome Variant Server (ESP), nor was one of the *de novo* variants found in females reported in these databases. We downloaded all variants from the ExAC database, containing exome data of 60,706 individuals, and calculated per gene the number of missense and synonymous variants. These numbers were then normalized by dividing through the total number of possible missense and synonymous variants per gene. The ratio of corrected missense over synonymous variants was then used as a measure for tolerance of the gene to normal variation, similar as was done previously.[15] When genes were ranked according to their tolerance score, *DDX3X* was among the most intolerant genes (1.09% of genes, rank 194 out of 17,856), showing that normal variation in this gene is extremely rare.

DDX3X encodes a conserved DEAD-box RNA helicase important in a variety of fundamental cellular processes that include transcription, splicing, RNA transport and translation.[16; 17] DDX3X has been associated with many cellular processes, such as cell cycle control, apoptosis and tumorigenesis. [18; 19] It is also thought to be an essential factor in the RNAi pathway [20] and it is a key regulator of the Wnt/β-catenin pathway, acting as a regulatory subunit of CSNK1E and stimulating its kinase activity.[21]

Notably, of the 34 different *de novo* variants, 19 alleles are predicted to give a loss of function effect. Antisense-based knockdown of the *DDX3X*-orthologue PL10 in zebrafish is already described and shows a reduced brain size and head size in zebrafish embryo's at two days post fertilization.[10] As missense changes in affected females were located in the same protein domain as missense changes in affected males, we explored the pathogenic mechanisms accounting for the differences in disease transmission and phenotype between affected females and affected males with *DDX3X* missense

variants. We therefore employed a combination of *in vitro* and *in vivo* assays based on the known role of DDX3X in the regulation of Wnt/β-catenin signaling.[21] Wnt signaling is a critical developmental pathway; the zebrafish is a tractable model in which to study Wnt output [22-27] and to interrogate alleles relevant to neurocognitive traits.[28] We tested several missense variants, including the female-specific *de novo* variants p.Ile214Thr, p.Arg326His, p.Arg376Cys, p.Ile507Thr, and p.Arg534His as well as the inherited variants p.Val300Phe, p.Arg351Gln, and p.Arg362Cys identified in males. A variant from the Exome Variant Server (EVS, http://evs.gs.washington.edu/EVS/) encoding p.E196K (rs375996245; MAF <0.002) was chosen as a negative control.

We first tested whether the missense variants identified in our ID cohort resulted in dominant effects by overexpressing either WT or mutant *DDX3X* human transcript in zebrafish embryos and examining the phenotype. Zebrafish (Danio rerio) were raised and mated as described[29]. Embryos from ZDR strain fish were injected into the yolk with 1 nL of solution containing mRNA at the 1-2 cell stage using a Picospritzer III microinjector (Parker). Phenotyping was carried out at 2 days post-fertilization (dpf). The wild-type (WT) human DDX3X open reading frame (ORF) construct was obtained from the Ultimate ORF Collection (LifeTechnologies; clone ID: IOH13891), sequenced fully and sub-cloned into the pCS2+ vector using Gateway LR clonase II- mediated cloning (LifeTechnologies). Capped mRNA was generated using linearized constructs as a template with the mMessage mMachine SP6 transcription kit (LifeTechnologies). Injection of 100 pg of WT or mutant *DDX3X* transcripts did not produce a discernible phenotype at 36 hours post fertilization (hpf) (n=50-75 embryos/injection; repeated twice with masked scoring; Supp Figure 3A) or at 72 hpf (not shown). To corroborate these data in a different system, we used a mammalian cell-based assay of canonical Wnt signaling (TOPFlash).[30] Briefly, HEK293T cells and mouse L-cells (both control and Wnt3a expressing) were grown in 10% FBS/DMEM. Wnt3a containing media was prepared by incubating confluent L-cells with serum-free media for 24 hours, removing and filtering the media. This was subsequently used to stimulate transfected HEK293T cells. HEK293T cells at

a density of $1x10^4$ cells/well of 24-well plate were transfected with 1.025 □g total of DNA containing 0.5 μg of pGL4.18 TOPFLASH vector, 25 ng of renilla luciferase plasmid (pRL-SV40), and 0.5 μg of pCS2+ with or without DDX3X using XtremeGene9 (Roche). After 24 hours the media was removed and replaced with serum-free media collected as above from L-cells with and without *WNT3A*. After 24 hours cells were harvested and luciferase activity measured using the Dual-Luciferase Reporter Assay System (Promega). Results were normalized internally for each well to renilla luciferase activity and then to the unstimulated wells (conditioned with control L-cell media). Consistent with the in vivo result, transfection of expression constructs harboring DDX3X variants did not differ from WT in the modulation of WNT3A-β-catenin mediated luciferase activity (Supp Figure 3B; triplicate wells with 3-5 biological replicates). Taken together, our results suggest that neither the female nor the male DDX3X variants produce detectable dominant changes in the context of Wnt-signaling. As a consequence, we then pursued a loss of function paradigm.

Previous studies have shown that co-expression of a canonical Wnt ligand and *DDX3X* is necessary to produce a phenotype *in vivo.*[21] Expression of either of the canonical Wnt ligands *WNT3A* or *WNT8A* results in ventralization of zebrafish embryos that ranges from mild (hypoplasia of the eyes, which we term Class I embryos) to moderate (absence of one or both eyes; Class II), severe (loss of all anterior neural structures; Class III), and radialized phenotypes at 2 days post fertilization (dpf) (Class IV).[24] We recapitulated these defects by injecting increasing doses of human *WNT3A* or *WNT8A* mRNA into embryos (Figure 3A, 3B). Full-length human *WNT3A* and *WNT8A* ORFs were cloned into pCS2+ from the Ultimate ORF Collection (Clone ID *WNT8A*: IOH35591; Clone ID *WNT3A*: IOH80731). The *WNT3A* clone required site-directed mutagenesis to introduce a stop codon using primers (ctgcaaggccgccaggcacTAGGGTGGGCGCGCCGA and its reverse complement). We selected a dose of WNT3A mRNA (200 fg), which produced a modest effect (15-20% Class I+II embryos, no Class III or IV) and we co-injected it with progressively increasing concentrations of human *DDX3X* mRNA. At 2 dpf, we

observed a dose-response curve concomitant with increasing doses of *DDX3X* (Figure 3D). At low doses of mutant *DDX3X* (15-20 pg/embryo), the ventralization phenotype produced by *WNT3A* is exacerbated, producing 40-50% Class I+II embryos, with a greater percentage in class II (10-15%), indicative of augmented severity (Figure 3D).

Because of the significant ($p<0.0001$) augmentation of *WNT3A*-mediated ventralization at 15 pg of *DDX3X*, we next used this combination of doses to test the effect of the missense alleles on Wnt signaling. *WNT3A* mRNA (200 fg) was co-injected with 15 pg of WT or variant *DDX3X* mRNA, and we scored embryos for ventralization at 2 dpf. We found differential effects of the variants on ventralization (Figure 3E; triplicate experiments; pooled for the final result). All *de novo* variants tested were significantly different from WT ($p<0.001$) and indistinguishable from *WNT3A* alone, suggesting that they all confer complete loss of function to *DDX3X*. By contrast, the control variant p.E196K, resulted in phenotypes similar to WT. Notably, all three inherited variants found in the male patients were also indistinguishable from WT-injected, and each was statistically different from *WNT3A* alone ($p<0.0001$). Given this apparent dichotomization of effect between female *de novo* and male inherited alleles, we tested whether pooling of results from the female versus male variants would exhibit a "class effect" separation between the two groups. We found this to be true; there was significant differential effect of the gender-specific sets of variants on Wnt signaling (Figure 3F).

Taken together, our *in vivo* testing of the nonsynonymous *DDX3X* mutations demonstrated marked alteration in Wnt-mediated ventralization for changes arising *de novo* in affected females. Our data also reinforce the notion that disruption of β-catenin signaling during neurodevelopment has profound consequences. Loss of Wnt-signaling inhibits neuroblast migration, neural differentiation, and suppression of the development of the forebrain.[31-34] Moreover, we previously reported that mutations in β-catenin contribute to ID in humans[35; 36], while mutations in δ-catenin, some of which abrogate its

biochemical interaction with β-catenin, can contribute to severe autism in females[28], strengthening further the link between WNT-signaling and human neurodevelopmental disorders.

DDX3X has recently been proposed as a candidate ID gene.[10; 37] Our data substantiate this hypothesis and suggest that mutated *DDX3X* is amongst the most common causes of sporadic intellectual disability in females. Our data also suggest that the genetic architecture of DDX3X-mediated pathology in males is different. All detected male alleles were inherited from unaffected mothers, suggesting either a gender specific buffering effect or a milder effect of those inherited alleles on protein function. Both our *in vivo* and our *in vitro* studies indicate that none of the tested alleles (male or female) confer dominant effects and that the male alleles are indistinguishable from WT in our system. Given that human genetics and computational predictions support the causality of these alleles in the families studied, we speculate that the effect of these alleles is beyond the dynamic range of our assays and could thus not be detected; a substantially larger assay of embryos might detect a signal, although the transient nature of our system will always be limited at detecting mild allele effects. Alternatively, the mechanism of the male-derived alleles might be qualitatively different from the *de novo* female variants and reflective of the complex biology of *DDX3X*. Of note, we found that increased amounts of wild type *DDX3X* ameliorated the Wnt3a-induced ventralization phenotype (Supp Figure 4B), an observation that we reproduced with Wnt8a, another β-catenin-dependent ligand (Supp Figure 4A) and our TOPFlash reporter assay (Supp Figure 3B). This bimodal activity of *DDX3X* has been intimated previously by Cruciat et.al., in which knockdown of *DDX3X* caused loss of *WNT3A* signaling which was restored by transfection with WT construct while overexpression alone resulted in decreased WNT3A signaling.[21] We reproduced this latter finding when solely overexpressing *DDX3X* in HEK293 cells (Supp Figure 3B).

Summarized, these data suggest that *DDX3X* is dosage-sensitive and may have a differential activity in females versus males. Contribution of the *DDX3X* homologue at the Y-chromosome, *DDX3Y*, to

phenotypic variability is unlikely.[38] *DDX3X* and *DDX3Y* have 92% amino acid sequence in common[11] but DDX3Y is translated only in spermatocytes and is essential for spermatogenesis.[39; 40] Men with a deletion of *DDX3Y* have severe testicular pathology but no cognitive dysfunction or other reported abnormalities.[40]

In conclusion, *de novo* variants in *DDX3X* are a frequent cause of intellectual disability, affecting 1-2% of all so far unexplained ID in females. While no *de novo* mutations were identified in males, we found missense variants in DDX3X in males from three families with ID suggestive of X-linked recessive inheritance. Our phenotypic read out in zebrafish shows a gender difference in identified variants with a loss of function effect of all tested *de novo* mutations on the Wnt-pathway. The differential activity possibly indicates a dose dependent effect of *DDX3X* expression in the context of functional mosaic females versus one-copy males which reflects the complex biological nature of pathogenic *DDX3X* variants.

DESCRIPTION OF SUPPLEMENTAL DATA

Supplementary tables:

- Supplementary Table 1: Clinical details of affected females with *DDX3X de novo* variants

- Supplementary table 2: Clinical details of affected males with *DDX3X* variants

- Supplementary table 3: Predicted consequences of several missense variants on protein function and structure

Supplementary figures:

- Supplementary figure 1: Families with affected males and *DDX3X* mutations

- Supplementary figure 2: Location of several amino acid substitutions in three dimensional model of DDX3X

- Supplementary Figure 3: Overexpression of *DDX3X* variants does not produce alterations in Wnt signaling

- Supplementary Figure 4: Co-injection of *DDX3X* and *WNT8A* produces the same dose-dependent changes in ventralization as *WNT3A*.

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WEB RESOURCES

PolyPhen 2 (version 2.2.2r398): http://genetics.bwh.harvard.edu/pph2/)

SIFT (version1.03, SIFT/PROVEAN Human SNPs): http://sift.jcvi.org/

ExAC: http://exac.broadinstitute.org/

Exome Variant Server (NHLBI Exome Sequencing Project): http://evs.gs.washington.edu/EVS/

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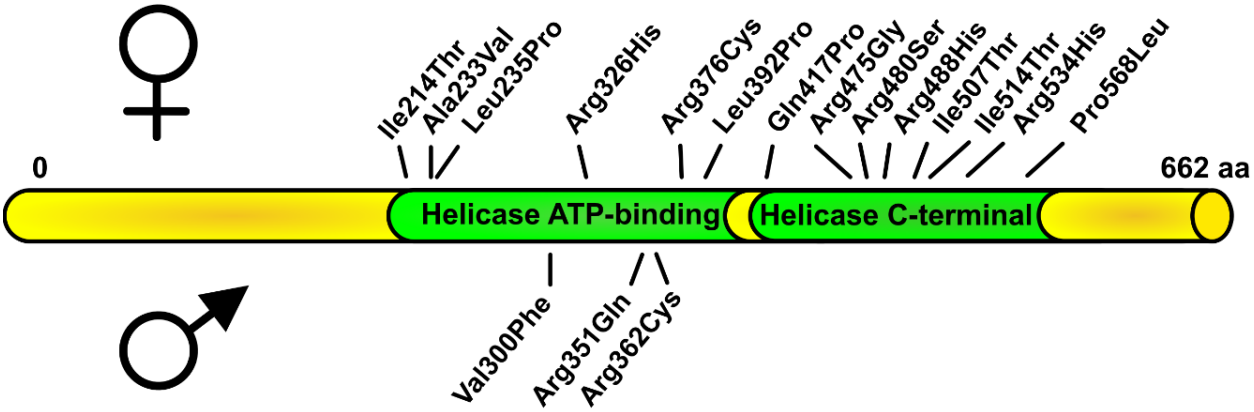
Table 1: Mutation characteristics

		Nucleotide change	Amino acid change	SIFT	PolyPhen2
Females	Individual 1	c.1126C>T	Arg376Cys	0.00	1.000
	Individual 2	c.233C>G	Ser78*	NA	NA
	Individual 3	c.1126C>T	Arg376Cys	0.00	1.000
	Individual 4	c.136C>T	Arg46*	NA	NA
	Individual 5	c.1601G>A	Arg534His	0.00	1.000
	Individual 6	c.641T>C	Ile214Thr	0.01	0.998
	Individual 7	c.1520T>C	Ile507Thr	0.00	0.995
	Individual 8	c.977G>A	Arg326His	0.00	1.000
	Individual 9	c.868del	Ser290Hisfs*31	NA	NA
	Individual 10	c.1229_1230dup	Thr411Leufs*10	NA	NA
	Individual 11	c.1105dup	Thr369Asnfs*14	NA	NA
	Individual 12	c.865-2A>G	p. (?)	NA	NA
	Individual 13	c.1600dup	Arg534Profs*13	NA	NA
	Individual 14	c.269dup	Ser90Argfs*8	NA	NA
	Individual 15	c.1440A>T	Arg480Ser	0.00	0.991
	Individual 16	c.873C>A	Tyr291*	NA	NA
	Individual 17	c.1693C>T	Gln565*	NA	NA
	Individual 18	c.1535_1536del	His512Argfs*5	NA	NA
	Individual 19	c.766-1G>C	p.(?)	NA	NA
	Individual 20	c.599dup	Tyr200*	NA	NA
	Individual 21	c.1321del	Asp441Ilefs*3	NA	NA
	Individual 22	c.1383dup	Tyr462Ilefs*3	NA	NA
	Individual 23	c.1384_1385dup	His463Thrfs*34	NA	NA
	Individual 24	c.1535_1536del	His512Argfs*5	NA	NA
	Individual 25	c.1541 T>C	Ile514Thr	0.00	1.000
	Individual 26	c.704 T>C	Leu235Pro	0.00	1.000
	Individual 27	c.1175 T>C	Leu392Pro	0.00	1.000
	Individual 28	c.1463 G>A	Arg488His	0.00	0.844
	Individual 29	c.1126 C>T	Arg376Cys	0.00	1.000
	Individual 30	c.1250 A>C	Gln417Pro	0.00	1.000
	Individual 31	c.698C>T	Ala233Val	0.02	1.000
	Individual 32	c.931C>T	Arg311*	NA	NA
	Individual 33	c.46-2A>C	p.(?)	NA	NA
	Individual 34	c.1678_1680del	Leu560del	NA	NA
	Individual 35	c.1423C>G	Arg475Gly	0.04	0.998
	Individual 36	c.46-2A>G	p.(?)	NA	NA
	Individual 37	c.1703C>T	Pro568Leu	0.00	1.000
Males	Family 1	c.1084C>T	Arg362Cys	0.00	1.000
	Family 2	c.1052G>A	Arg351Gln	0.00	0.865
	Family 3	c.898G>T	Val300Phe	0.02	1.000

Table 2: Clinical features of females with *de novo DDX3X* mutations

Development	Percentage	Number
Intellectual disability or developmental delay	100%	37/37
Mild or Mild-Moderate	27%	10/37
Moderate or Moderate-Severe	27%	10/37
Severe	38%	14/37
Developmental delay	8%	3/37
Growth		
Low weight	32%	12/37
Microcephaly	30%	11/37
Neurology		
Hypotonia	76%	28/37
Epilepsy	16%	6/37
Movement disorder	46%	17/37
Behavior problems	54%	20/37
Brain MRI		
Corpus callosum hypoplasia	36%	13/36
Cortical malformation	11%	4/36
Ventricular enlargement	36%	13/36
Other		
Skin abnormalities	38%	14/37
Hyperlaxity	35%	13/37
Visual problems	35%	13/37
Hearing loss	8%	3/37
Cleft lip or palate	8%	3/37
Precocious puberty	14%	5/37
Scoliosis	11%	4/37

Figure 1: Location of amino acid substitutions in DDX3X protein

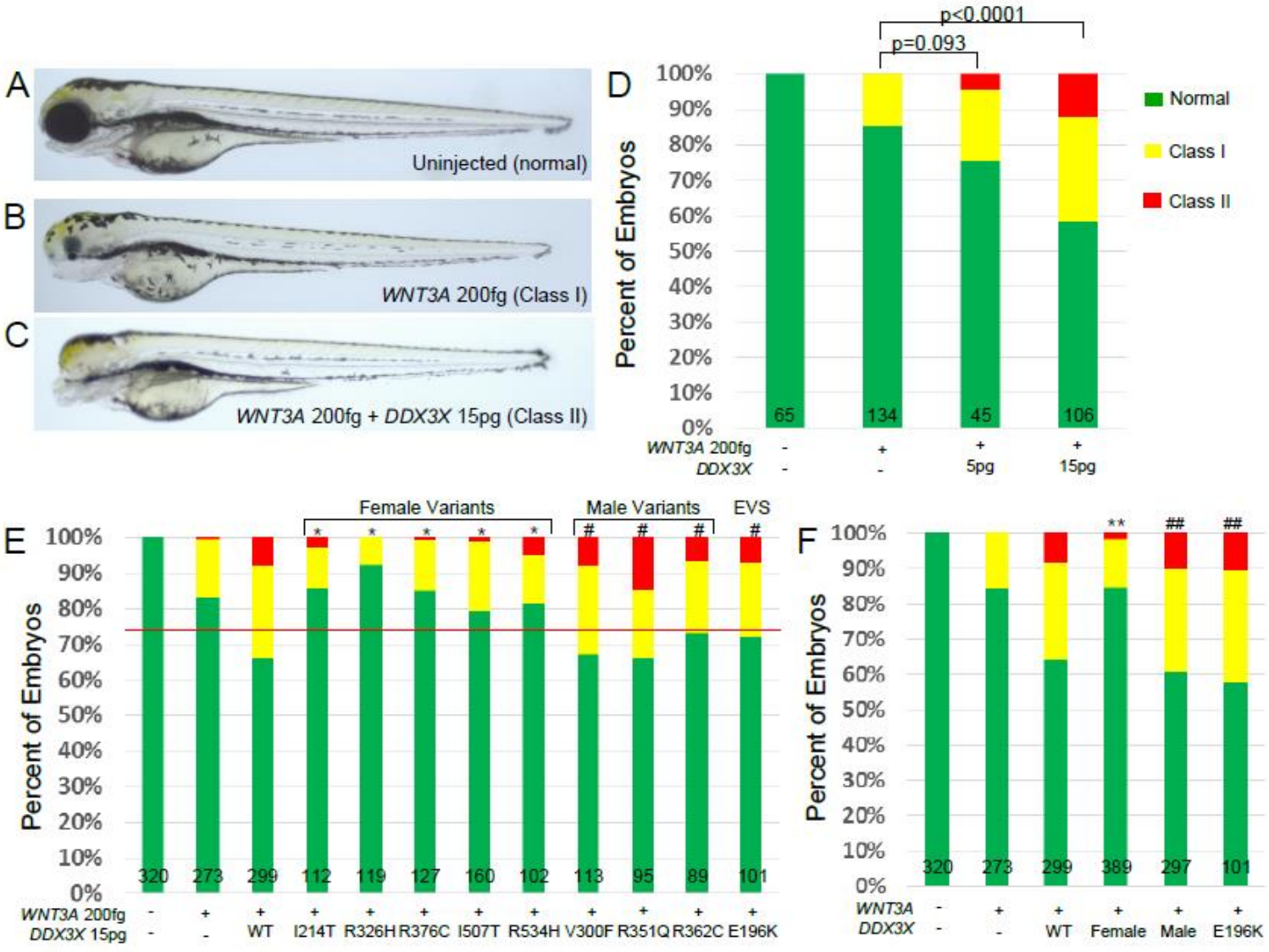


Location of amino acid substitutions in DDX3X protein sequence. The DDX3X protein consists of two subdomains: a N-terminal domain 1 and a C-terminal domain 2. All amino acid variants found in affected females are located within these two protein domains. The three amino acid substitutions found in affected males are all located within the Helicase ATP-binding domain.

Figure 2: Facial profiles of females with *de novo DDX3X* mutations

Facial features of 30 of 37 females with a *de novo* variant in *DDX3X.* Common facial features include a long and sometimes hypotonic face (e.g. individuals 2, 4, 5, 12, 22, 32), a high and/or broad forehead (e.g. individuals 1, 7, 9, 23 , 24, 26), a wide nasal bridge and/or bulbous nasal tip (e.g. individuals 11, 13, 15, 16), narrow alae nasi and/or anteverted nostrils (e.g. individuals 2, 8, 9, 12, 14, 18, 24, 27, 32, 35) and hypertelorism (e.g. individuals 5, 7, 8, 20, 27). Informed consent was obtained for all 30 individuals shown. The individual numbers correspond to the numbers mentioned in Table 1 and Supp Table 1.

Figure 3 The effect of wildtype and variant *DDX3X* mRNA on WNT3A-mediated ventralization



Zebrafish embryos at two days post fertilization (dpf) either uninjected (A) or injected with human *WNT3A* without (B) or with (C) human *DDX3X* show a range of ventralized phenotypes. These were scored according to Kelly et. al. [24] as normal, Class I, or Class II ventralization. No injection condition resulted in severe ventralization (Class III or IV).

(D) Co-injection of *WNT3A* and *DDX3X* shows an augmentation of *WNT3A* mediated ventralization with increasing dose up to 15pg. (E) Individual variants of *DDX3X* were tested for their effect on increasing *WNT3A* mediated ventralization using 200fg of *WNT3A* mRNA and 15pg of *DDX3X* mRNA per embryo (dose of maximal response from (D)). Scoring is the same as in (D). The female *de novo* variants and the male familial variants are shown together. The mutation E196K is a rare allele from the Exome Variant Server (EVS). The red horizontal line delineates a division between those variants which behave as "wild-type" (e.g. the male variants and E196K) and those that do not. All female *de novo* variants are significantly different from wild-type. *p < 0.001 compared with *WNT3A* + wild-type *DDX3X* co-injection.

#p < 0.0001 vs. *WNT3A* alone. (F) Graph illustrating the effect of combining the results from the female *de novo* variants and comparing to the male familial variants and to the E196K variant from the EVS. There is clear segregation of effect based on the source of the genetic variant. **p < 0.0001 vs. *WNT3A* + wild-type *DDX3X*. ##p< 0.0001 vs. *WNT3A* alone. For each graph total (n) is shown at the bottom of each bar.