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1 Genetic evidence of the association of DEAH-box helicase 37 defects as a novel
2 cause of 46,XY gonadal dysgenesis spectrum
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38 Short title: *DHX37* defects in 46,XY Gonadal Dysgenesis

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59

ABSTRACT

Context: 46,XY gonadal dysgenesis (GD) is a heterogeneous group of disorders with a wide phenotypic spectrum, including embryonic testicular regression syndrome (ETRS). Most patients with GD remain without a molecular diagnosis. **Objective:** To report a novel gene for 46,XY GD etiology, especially for ETRS. **Design:** Screening of familial cases of 46,XY GD using whole exome sequencing and sporadic cases by target gene panel sequencing. **Setting:** Tertiary referral center for Differences/Disorders of sex Development (DSD). **Patients and methods:** We selected 87 patients with 46,XY DSD (17 familial cases from eight unrelated families and 70 sporadic cases); 55 patients had GD (among them, ten patients from five families and eight sporadic cases had ETRS) and 32 patients had 46,XY DSD of unknown etiology. **Results:** We identified four heterozygous missense rare variants classified as pathogenic or likely pathogenic in *DEAH-box helicase 37* (*DHX37*) gene in five families (n=11 patients) and in six sporadic cases. Two variants were recurrent: the p.Arg308Gln (in two families and in three sporadic cases) and the p.Arg674Trp (in two families and in two sporadic cases).

The variants were specifically associated with ETRS (7/14 index cases; 50%). The frequency of rare, predicted to be deleterious DHX37 variants in this cohort (0.14) is significantly higher than that observed in gnomAD population database (0.004; $p<0.001$). Immunohistochemistry analysis in human testis showed that DHX37 is mainly expressed in germ cells, at different stages of testis maturation, in Leydig cells and rarely in Sertoli cells. Conclusion: This strong genetic evidence identifies DHX37 as a new player in the complex cascade of male gonadal differentiation and maintenance.

85 **Introduction**

86 46,XY gonadal dysgenesis (GD) represents a heterogeneous group of
87 disorders/differences of sex development (DSD) characterized by abnormal gonadal
88 development leading to a wide phenotypic spectrum. Variable degrees of external
89 genitalia undervirilisation are observed, ranging from micropenis to female-like genitalia
90 and partially- or fully-developed Mullerian derivatives.The gonads from these patients
91 display a wide spectrum of histological abnormalities, ranging from ovarian-like stroma
92 with disorganized seminiferous tubules to complete absence of gonadal tissue (1).
93 Embryonic testicular regression syndrome (ETRS) is considered a part of the clinical
94 spectrum of 46,XY gonadal dysgenesis (2). Most individuals with ETRS present with
95 micropenis or atypical genitalia and lack of gonadal tissue on one or both sides (2).
96 Partial or complete Mullerian duct regression associated with micropenis suggests an
97 intrinsically functional testis in the first months of fetal life subsequent loss of testicular
98 function before the last trimester of gestation, when the increase in penile length occurs.
99 Numerous genes are known to be involved in the process of gonadal determination (3).
100 However, a genetic diagnosis is identified in less than 40% of the patients with 46,XY
101 GD (4). Moreover, few patients with ETRS were included in large cohorts of 46,XY DSD
102 previously studied (4). However, the fact that some familial cases of ETRS were reported
103 indicates a genetic etiology (5,6).
104 In the present work, high throughput parallel sequencing methods, including whole-
105 exome sequencing (WES) and targeted DSD-gene panels, were used to investigate the
106 underlying genetic etiology in a large cohort of 46,XY patients with GD and 46,XY DSD
107 patients with unknown etiological cause.

108 We identified recurrent rare variants in DEAH (Asp-Glu-Ala-His) box polypeptide 37
109 (*DHX37*) in several affected individuals from distinct families, establishing a novel
110 genetic cause for 46,XY gonadal dysgenesis spectrum, including ETRS.

111 **Ethics**

112 This study was approved by the Ethics Committee of the Hospital das Clínicas da
113 Faculdade de Medicina da Universidade de São Paulo, the Institutional Review Board of
114 the University of Michigan Medical School, the Hospital de Garrahan Escuela de
115 Medicina, Pontificia Universidad Católica de Chile, and the Hospital Nacional Prof. Dr.
116 A. Posadas, Buenos Aires, Argentina. Written informed consent was obtained from all
117 patients, their parents or legal guardians.

118 **Subjects and Methods**

119 We studied eighty-seven 46,XY DSD patients without previous molecular diagnosis,
120 including 17 familial cases of 46,XY GD from 8 non-consanguineous families and 70
121 sporadic cases (38 with GD and 32 with 46,XY DSD of unknown etiology). Out of the
122 55 patients with GD, 10 patients from 5 families and 8 sporadic cases had an ETRS
123 phenotype. The patients had different nationalities: Brazilian (81 patients), Argentinian
124 (three siblings), Chilean (two siblings) and Chinese-American (one patient).

125 All patients have a normal GTG-banded metaphases 46,XY karyotype.

126 The 46,XY DSD patients were classified as having complete GD (CGD) if they had
127 female external genitalia, Mullerian derivatives and streak gonads; as partial GD (PGD)
128 if they had atypical external genitalia, Mullerian derivatives and at least one gonad with
129 histopathological features of dysgenetic testis; as ETRS if they had micropenis, partially
130 developed Mullerian derivatives and no gonadal tissue or small area of gonadal stroma;
131 and as 46,XY DSD of unknown etiology if hormonal profile was not conclusive or not
132 available due to previous gonadectomy. In this latter group, molecular defects of LHCG

133 and androgen receptors, *CYP17A1, HSD17B3*, *HSD3B2*, and *5ARD2* genes were ruled
134 out by DNA sequencing.

135 **Genomic DNA**

136 For molecular diagnosis, genomic DNA was extracted from peripheral blood leukocytes
137 by the proteinase K-SDS salting-out method (7).

138 **Genetic study**

139 Whole exome sequencing (WES) was performed in 14 familial cases from 7 families. In
140 all but one family, the probands and their first-degree relatives and other affected family
141 members were studied.

142 Sixty-eight sporadic cases were studied by targeted massively parallel sequencing.
143 *DHX37* was studied by Sanger sequencing in two sporadic cases and in three patients
144 from Family 2 (Figure 1). (Supplementary information, in DOI:
145 10.13140/RG.2.2.35903.76968).

146 Enrichment for massively parallel sequencing was performed with Nextera Exome
147 Enrichment Kit (Illumina, San Diego, CA), followed by paired-end sequencing on the
148 Illumina HiSeq 2500 System (Illumina, San Diego, CA)

149 For target sequencing, we designed an amplicon-based capture panel against exonic
150 regions of 63 genes, including 43 genes known to be associated with human DSDs and
151 20 candidate genes, including *DHX37* (3) (see Table S1, Supplementary information
152 in DOI: 10.13140/RG.2.2.35903.76968). Target sequences were captured using a custom
153 Sure Select Target Enrichment System Kit (Agilent Technologies, Santa Clara, CA,
154 USA) and sequencing was performed on the Illumina MiSeq platform (San Diego, CA,
155 USA).

156 Sanger sequencing was used to confirm the potentially pathogenic variants identified by
157 massively parallel sequencing and for segregation analysis. Sequencing was performed

158 on the ABI 3730XL DNA Analyzer (Applied Biosystems) using the BigDye (Applied
159 Biosystems), followed by data analysis using a Genetic Analyzer (ThermoFisher
160 Scientific).
161 The identified variants were classified according to American College of Medical
162 Genetics (ACMG) criteria (8).
163 **Data analysis**
164 The exome and the targeted panel sequencing data were screened for rare variants (minor
165 allele frequency < 0.1% in the public databases: Genome Aggregation Database
166 (gnomAD) (9), 1000 Genomes (10), and in the Brazilian population database (ABraOM)
167 (11), located in exonic and consensus splice site regions. Subsequently, the filtration
168 pipeline prioritized potentially pathogenic candidate variants (loss of function variants
169 and variants classified as pathogenic by multiple *in silico* programs). For variants
170 identified by WES, we selected variants that fitted an autosomal-dominant model.
171 The sequencing reads carrying candidate variants were visually confirmed using the
172 Integrative Genomics Viewer (Broad Institute, Cambridge, MA). Candidate variants were
173 segregated in the family members by Sanger method.
174 The filtering of the variants is provided in Supplementary data (Figure S1) DOI:
175 10.13140/RG.2.2.35903.76968)
176 **Histological analysis**
177 **Immunohistochemical staining**
178 Eight formalin-fixed paraffin-embedded testicular autopsy samples from 46,XY
179 individuals with different chronological ages (27 and 33 weeks gestational age, 1, 53, and
180 180 days of age, 13, 23 and 53 years of age) were collected and used for DHX37
181 expression analysis by immunohistochemistry. All samples were sliced into 3-μm-thick
182 sections using an automatic Leica RM2255 microtome (Leica Biosystems, Nussloch,

183 Germany). The sections were briefly stretched in xylol at 600°C for 20 min, cooled in
184 xylol, and dried in an incubator (Fanem Orion 515, São Paulo, Brazil) at 600ºC. Sections
185 were subjected to hematoxylin-eosin (HE) staining for histological analysis. For
186 immunohistochemical study, slides were deparaffinized with xylene, hydrated in ethanol,
187 washed in phosphate-buffered saline (0.01 M/pH 7.4), and blocked using methanol and
188 hydrogen peroxide. Epitope exposure was carried out by placing the slide in boiling 10
189 mM citric acid (pH 6) or 100 mM EDTA (pH 9), followed by blocking non-specific
190 protein. Rabbit polyclonal anti-DHX37 antibody (NB110-40581; Novus Biologicals,
191 USA) was added at a dilution factor of 1:50. Dilution was standardized after testing on
192 ovarian and skin tissues where protein expression was identified in cytoplasm of oocytes
193 and nuclear membranes of ovarian stromal and squamous cells. The samples were
194 incubated with universal secondary antibodies using the Novo Link Detection Systems
195 kit (Leica Biosystems, USA) according to the manufacturer's instruction.

196 **Statistical analysis**

197 To test the genetic evidence for the association between *DHX37* and GD phenotype, we
198 performed aggregate variant analyses comparing allele frequencies among our 46,XY
199 DSD cohort and public databases [gnomAD and ABraOM].

200 Variants with similar characteristics of the *DHX37* variants observed in our cohort (rare
201 nonsynonymous variants with a minor allele frequency of 0.01 and located in the two
202 highly conserved protein (ATP-binding and Helicase C-terminal domains) that are
203 predicted to be pathogenic by at least four *in silic*o tools (Mutation Taster, SIFT,
204 PolyPhen-2, Mutation Assessor and PROVEAN) were selected. Allele frequency
205 differences between groups were analyzed by X^2 test, and statistical significance was set
206 at $p<0.05$. Statistical analyses were performed using SIGMAstat statistical software
207 package (Windows version 3.5; SPSS Inc., San Rafael, CA).

208
209 **Results**
210
211 **Patient phenotype and *DHX37* variants**

212 Firstly, WES identified the same *DHX37* variant p.Arg308Gln (c.923G>A) (GenBank:
213 NM_032656.3) in heterozygous state in two unrelated Brazilian families with ETRS
214 (Families 1 and 2). All the affected individuals have the same phenotype (micropenis and
215 absence or bilateral rudimentary gonadal tissue) (Figure 1, Table 1). A founder effect for
216 p.Arg308Gln variant was ruled out in Families 1 and 2.

217 The p.Arg308Gln variant was also identified by WES in a Chinese-American sporadic
218 case of ETRS from Michigan University performed in Eric Vilain's laboratory (sporadic
219 case F6:II-1, Figure 1, Table 2).

220 As a novel candidate gene for 46,XY DSD, *DHX37* was included in our target DSD-
221 panel. The same p.Arg308Gln variant was identified in another two sporadic cases: one
222 had ETRS (sporadic case F7:II-1) and the other had PGD (sporadic case F8:II-1) (Figure
223 1; Table 2).

224 A further three different heterozygous *DHX37* missense variants (the p.Arg674Trp,
225 p.Ser595Phe and p.Thr304Met) were identified in seven affected members from three
226 families and in three sporadic cases (Figure 2).

227 All of these four variants are predicted to be pathogenic by at least four *in-silico*
228 prediction tools (Table 3) and are absent in genomic population databases, except for the
229 p.Arg308Gln, which has a very low allele frequency (0.00003) in the gnomAD database
230 (Tables 4-5).

231 The p.Arg674Trp (c.2020C>T) variant was identified in the two Chilean brothers, both
232 with ETRS (cases F3:II-1 and F3:II-2, Family 3), and also in the three Argentinian
233 affected members (two brothers with ETRS and their uncle with PGD; cases F4:III-1,
234 F4:III-2 and F4:II-4, respectively, Family 4) (Figure 1, Table 1). In addition, the

235 p.Arg674Trp variant was also identified in another two Brazilian sporadic cases, one
236 patient with ETRS (sporadic case F10:II-1) and the other with PGD (sporadic case F11:II-
237 1) (Figure 1, Table 2)
238 The p.Ser595Phe (c.1784C>T) variant was identified in two affected individuals from the
239 same Brazilian family (Family 5). The proband had PGD and her nephew had ETRS
240 (F5:II-6 and F5:III-1, respectively) (Figure 1,Table 1).
241 The p.Thr304Met (c.911C>T) was identified in a Brazilian female (sporadic case F9: II-
242 5), who had previously undergone bilateral gonadectomy and genitoplasty (Figure
243 1,Table 2).
244 The p.Arg308Gln variant is classified as pathogenic and the other three variants,
245 p.Arg674Trp, p.Ser595Phe and p.Thr304Met, are classified as likely pathogenic
246 accordingly the ACMG criteria (Tables 4-5).

247 **Segregation analysis of *DHX37* variants**

248 Segregation analysis of the *DHX37* variants in eight families displayed a sex-limited
249 autosomal dominant pattern, maternally inherited in five families (F2, F3, F4, F5, F11).
250 In the Family 1, the presence of the p.Arg308Gln variant in the asymptomatic father
251 suggests an autosomal dominant pattern of inheritance with incomplete penetrance
252 (Figure 1). In two sporadic cases (F6. II-1 and F8.II-1), the confirmed paternity displayed
253 a *de novo* status of the p.Arg308Gln *DHX37* variant.

254 ***DHX37* gene and its protein structure**

255 *DHX37* is located in the 12q24.31 region. It is a member of the large DEAH family of
256 proteins and encodes an RNA helicase (12). The DHX37 protein (NP_116045) comprises
257 1157 amino acids and four main domains. The conserved motifs of the helicase core
258 region contain the Helicase ATP-binding domain (position 262-429) and the Helicase
259 superfamily c-terminal domain (position 585-674); the two other domains are the helicase

260 associated domain (position 768-859) and the oligonucleotide/oligosaccharide-binding-
261 fold domain (position 894-1011) (Figure 2). All the identified variants are located in the
262 helicase core region (Figure 2).

263 **DHX37 protein was identified in different testicular cells**

264 DHX37 expression was characterized in testes from newborns, children and adults using
265 immunohistochemistry. DHX37 was expressed in fibroblasts, endothelial cells and
266 epithelial cells of epididymis. These cells were used as internal positive controls for
267 immunohistochemistry. We found DHX37 expression in Leydig cell cytoplasm and in
268 germ cells at different stages of maturation. Our analysis indicates that DHX37
269 expression in spermatogonia is characterized by a regular perinuclear halo pattern in both
270 newborns (five samples) and adults (three samples). This pattern of staining differs from
271 that seen in Leydig cells (granular cytoplasmatic) and during other stages of maturation
272 of germ cells. A progressive condensation of protein around the nucleus was observed as
273 cells differentiate from spermatocytes to spermatids, generating a localized paranuclear
274 dot-like pattern. There was no staining in spermatozoa. Rare Sertoli cells displayed a
275 weak and focal cytoplasmatic stain (Figure 3).

276 **Frequency of the *DHX37* variants in our 46,XY DSD cohort**

277 The allele frequency of rare and predicted to be deleterious *DHX37* variants identified in
278 our cohort of 46,XY DSD patients [11/78 index cases (0.14)] was markedly higher than
279 that observed in individuals from gnomAD [568 /141456 individuals (0.004; $p<0.001$)]
280 and from a Brazilian cohort [1/609 individuals (0.002); $p<0.001$)].

281

282 **Discussion**

283 The present study analyzed a large cohort of 46,XY DSD patients without a molecular
284 diagnosis, most of whom had a GD phenotype, including a large number of familial and

285 sporadic cases with ETRS.

286 Pathogenic or likely pathogenic allelic variants in the *DHX37* were identified in 11
287 familial cases from 5 unrelated families and in six sporadic cases. Deleterious variants
288 are recurrent in familial and sporadic cases of 46,XY GD in patients of different
289 nationalities.

290 The *DHX37* gene has never been directly associated with gonadal development, but
291 deletions or rearrangements of the 12q24 chromosomal region, which contains *DHX37*
292 gene, have been associated with atypical genital development (13). Four syndromic
293 patients with micropenis or perineal hypospadias, and/or hypergonadotropic
294 hypogonadism are reported to have deletions or rearrangements involving the 12q24
295 region (13-15).

296 The *DHX37* gene encodes a RNA helicase protein which is involved in RNA-related
297 processes, including transcription, splicing, ribosome biogenesis (16), translation and
298 degradation (12,17). *DHX37* is required for maturation of the small ribosomal subunit in
299 human cells, through its catalytic activity, required for dissociation of the U3 snoRNA
300 from pre-ribosomal complexes (18). Disturbance of human ribosome production is
301 associated with cancer and genetic diseases known as ribosomopathies (19).

302 Disease-causing variants in the DExH-box helicase 30 (DHX30), were previously
303 described in syndromic patients with global developmental delay, intellectual disability,
304 severe speech impairment and gait abnormalities. Functional studies of allelic variants in
305 *DHX30* demonstrated that they affect protein folding or stability interfering with the RNA
306 binding (mutations located in Motif Ia) or with ATPase activity (mutations located in
307 Motif II and VI) (17,18). Two DHX37 allelic variants found in the present study are
308 located in the same motifs.

309 Despite lack of experimental evidence to formally demonstrate the deleterious effects of

310 the four variants identified in the present study, they are located in the highly conserved
311 helicase core region of the DHX37 protein.
312 The spontaneous p.Leu489Pro Dhx37 pathogenic variant was identified in Zebrafish in
313 association with behavior scape defects (20). This study demonstrated that Dhx37 is
314 involved in pre-mRNA splicing reinforcing the role of Dhx37 in RNA-related processes.
315 Although there is no direct evidence of *DHX37* being involved in mRNA processing
316 during gonadal development, DExD/H-box RNA helicase genes are differentially
317 expressed between males and females during the critical period of male sex differentiation
318 in channel catfish (21).
319 Further, we show population evidence that the *DHX3*7 variants are enriched among the
320 46,XY GD patients in comparison with the population database. The statistical analysis
321 confirmed that the predicted deleterious *DHX37* variants located in the helicase core
322 region are more frequently identified in our 46,XY DSD cohort than in the public
323 databases, emphasizing that this finding was not by chance ($p<0.01$).
324 Therefore*, in vitro* and *in vivo* studies on DHX37 mechanism have demonstrated a role
325 of DHX37 in ribosome biogenesis (23). Based on this new knowledge, 46,XY gonadal
326 dysgenesis could be classified as a ribosomopathy, expanding the etiological mechanisms
327 of dysgenetic 46,XY DSD spectrum.
328 Since the discovery of the sex-determining region Y (*SRY*) variants in patients with GD
329 in 1990 (22), several genes have been associated with the molecular etiology of this
330 disorder. The nuclear receptor subfamily 5 group A member 1 (*NR5A1*) and Mitogen-
331 Activated Kinase Kinase Kinase 1 (*MAP3K1*) variants are the most frequent causes of
332 46,XY gonadal dysgenesis identified to date (23-26).
333 In this study we found pathogenic/ likely pathogenic variants in *DHX37* in patients with
334 46,XY GD at a frequency of 14%, which is slightly higher than the frequency of *NR5A1*

335 defects (11%) in our whole cohort (24,27). Considering only the ETRS phenotype
336 (micropenis and absence of uni or bilateral testicular tissue) this frequency increases to
337 50% (7/14 families).

338 In the literature, different inheritance patterns have already been described in 46,XY
339 gonadal dysgenesis kindreds (28), including the description of asymptomatic male
340 carriers of proven pathogenic variants of genes involved in testicular determination, such
341 as *SRY* and *NR5A1* genes (29,30). Uncertain mechanisms might prevent the appearance
342 of the phenotype in asymptomatic 46,XY carriers.

343 Maternal inheritance was observed in all familial cases with pathogenic/ likely pathogenic
344 variants in *DHX37* with the exception of family 1, where the variant was inherited from
345 a seemingly unaffected father carrier.

346 In adult humans, the DHX37 protein is expressed in the ovarian stroma and in the cells
347 within seminiferous tubules (Human Protein Atlas database) (31-33). In our study, the
348 immunohistochemistry analysis of normal testicular tissue from newborn, pubertal and
349 adult males revealed that DHX37 is expressed during specific stages of germ cell
350 maturation, in Leydig cells and rarely in Sertoli cells.

351 An elaborate paracrine cell-cell network transporting signaling molecules between germ
352 cells and Sertoli cells has been described (34). Indeed, *in vitro* studies have shown that
353 there is a bidirectional trafficking between Sertoli and germ cells, and that each cell type
354 regulates the function of the other (35-38). In addition, RNA expression profiles of
355 DHX37 in human testicular cancer cells are higher than in other tissues (The Human
356 Protein Atlas – Pathology), suggesting that DHX37 may be involved in the regulatory
357 process of the cell proliferation in the testis (31-33).

358 The present study provides several lines of genetic evidence to indicate that defects in
359 *DHX37* are associated with 46,XY GD spectrum, mainly with ETRS. First, we observed

360 that the variants segregate with the DSD phenotype in a dominant inheritance pattern in
361 most of the families and that two *de novo* variants were identified. Second, we provide
362 statistical evidence that rare *DHX37* variants are enriched in the analyzed 46,XY DSD
363 cohort in comparison with public databases involving a large number of individuals not
364 selected by this phenotype.

365 In conclusion, our findings indicate that *DHX37* is a new player in the complex cascade
366 of male gonadal differentiation and maintenance, thus establishing a novel and frequent
367 molecular etiology for 46,XY gonadal dysgenesis spectrum, which includes a high
368 proportion of individuals with embryonic testicular regression syndrome.

369 **Supplementary information:** displayed in DOI: 10.13140/RG.2.2.35903.76968).

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552

553 **Table 1.** Phenotype of 46,XY DSD patients with familial embryonic testicular regression syndrome with rare and predicted pathogenic or likely pathogenic *DHX37*
554 variants

Nationality / **Variables**	**Brazilian**		**Brazilian**		**Chilean**		**Argentinian**			**Brazilian**	
Patient	F1:III-2	F1:III-3	F2:II-4	F2:III-1	F3:II-1	F3:II-2	F4:III-1	F4:III-2	F4:II-4	F5:II-6	F5:III-1
Sex of rearing	Male	Male	Male	Female	Male	Male	Male	Male	Male	Female	Male
Age at presentation (yrs)	2.2	1.8	14.0	1.8	0.6	24 days	Newborn	4	2	18	10
Diagnosis	ETRS	ETRS	ETRS	ETRS	ETRS	ETRS	ETRS	ETRS	PGD	PGD	ETRS
External genitalia	Micropenis	Micropenis	Micropenis	Micropenis	Micropenis	Micropenis	Micropenis	Micropenis	Micropenis	Atypical	Micropenis
Gonads	Non-palpable	Non-palpable	Non-palpable	Non-palpable	Non-palpable	Non-palpable	Non-palpable	Non-palpable	Left testis - scrotum	Previous gonadectomy	Non-palpable
Histologic analysis	Small bilateral dysgenetic gonads	Left gonad not found. Right dysgenetic gonad	No gonadal tissue found	Left gonad not found. Small right dysgenetic gonad.	Small bilateral dysgenetic gonads	Small bilateral dysgenetic gonads	No gonadal Tissue	No gonadal tissue	Right gonad not found Left dysgenetic testis with GCNIS*	Bilateral dysgenetic gonads/	No gonadal tissue
Wolffian derivatives	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present
Mullerian Derivatives – Tubes	Present¥	Absent	Present¥	Present¥	Absent	Present	Present¥	Present¥	Absent	Absent	Absent
Mullerian Derivatives – Uterus	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
LH (IU/L)	14.5	12	3.5	1.9	<0.5	<0.5	<0.5	<0.5	26	NA	19
FSH (IU/L)	117	133	87	56	10.9	9.5	NA	9	112	NA	43
Basal Testosterone (ng/dL)	<10	<10	<10	NA	16	<10	38	27	16	NA	21
Testosterone after hCG test (ng/dL)	<10	NA	29	26	18	<10	40	29	NA	NA	NA
Allelic variant	p.Arg308Gln	p.Arg308Gln	p.Arg308Gln	p.Arg308Gln	p.Arg674Trp	p.Arg674Trp	p.Arg674Trp	p.Arg674Trp	p.Arg674Trp	p.Ser595Phe	p.Ser595Phe
Variant state	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous

555 NA- not available; PGD- partial gonadal dysgenesis; GCNIS - germ cell neoplasia in-situ; * testicular biopsy; ¥ - Rudimentary Fallopian tubes;
556 Conversion factors to SI units: T, ng/dL to nmol/L, multiply by 0.0347.
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560 **Table 2**. Phenotype of 46,XY DSD patients with sporadic gonadal dysgenesis spectrum and heterozygous rare pathogenic or likely pathogenic *DHX37* variants

<table>
<tr><th colspan="2">Nationality / Variables</th><th>Chinese-American</th><th colspan="5">Brazilian</th></tr>
<tr><td colspan="2">Patient</td><td>F6:II-1</td><td>F7:II-1</td><td>F8:II-1</td><td>F9:II-5</td><td>F10:II-1</td><td>F11:II-1</td></tr>
<tr><td colspan="2">Social sex</td><td>Male</td><td>Male to Female</td><td>Female</td><td>Female</td><td>Male to female</td><td>Female</td></tr>
<tr><td colspan="2">Age at presentation (yrs)</td><td>0.18</td><td>30</td><td>7.7</td><td>35</td><td>19</td><td>3.7</td></tr>
<tr><td colspan="2">Diagnosis</td><td>ETRS</td><td>ETRS</td><td>PGD</td><td>Previous gonadectomy</td><td>ETRS</td><td>PGD</td></tr>
<tr><td colspan="2">External genitalia</td><td>Micropenis</td><td>Micropenis</td><td>Female</td><td>Previous genitoplasty</td><td>Micropenis</td><td>Atypical</td></tr>
<tr><td colspan="2">Gonads</td><td>Non-palpable</td><td>Non-palpable</td><td>Non-palpable</td><td>NA</td><td>Non-palpable</td><td>Non-palpable</td></tr>
<tr><td colspan="2">Histological analysis</td><td>No gonadal tissue</td><td>No gonadal tissue</td><td>Bilateral dysgenetic gonads</td><td>NA</td><td>No gonadal tissue</td><td>Bilateral dysgenetic gonads</td></tr>
<tr><td colspan="2">Wolff derivatives</td><td>Present</td><td>Present</td><td>Present</td><td>NA</td><td>NA</td><td>Present</td></tr>
<tr><td rowspan="2">Mullerian derivatives</td><td>Tubes</td><td>Absent</td><td>Present</td><td>Absent</td><td>NA</td><td>Absent</td><td>Present</td></tr>
<tr><td>Uterus</td><td>Absent</td><td>Absent</td><td>Absent</td><td>Absent</td><td>Absent</td><td>Absent</td></tr>
<tr><td colspan="2">LH (IU/L)</td><td>0.1</td><td>10</td><td>0.1</td><td>NA</td><td>23</td><td>NA</td></tr>
<tr><td colspan="2">FSH (IU/L)</td><td>0.4</td><td>40</td><td>4.9</td><td>NA</td><td>62</td><td>NA</td></tr>
<tr><td colspan="2">Basal Testosterone (ng/dL)</td><td><10</td><td>NA</td><td><10</td><td>NA</td><td>21</td><td>25</td></tr>
<tr><td colspan="2">Testosterone after hCG test (ng/dL)</td><td><10</td><td><10</td><td>NA</td><td>NA</td><td>NA</td><td>33</td></tr>
<tr><td colspan="2">Allelic variant</td><td>p.Arg308Gln</td><td>p.Arg308Gln</td><td>p.Arg308Gln</td><td>p.Thr304Met</td><td>p.Arg674Trp</td><td>p.Arg674Trp</td></tr>
<tr><td colspan="2">Variant state</td><td>Heterozygous</td><td>Heterozygous</td><td>Heterozygous</td><td>Heterozygous</td><td>Heterozygous</td><td>Heterozygous</td></tr>
</table>

561 NA: not available; PGD- Partial gonadal dysgenesis; Conversion factors to SI units: T, ng/dL to nmol/L, multiply by 0.0347.

562

563 **Table 3.** *In silico* prediction analysis of *DHX37* allelic variants identified in 46,XY DSD patients

Families	Nucleotide changed	AA changed	Functional domain	*In silico* prediction tools						
				Mutation Taster	**Mutation Assessor**	**SIFT**	**Polyphen-2**	**PROVEAN**	**CADD**	**GERP**
F1, F2, F6, F7, F8	c.923G>A	p.Arg308Gln	Helicase ATP-binding	Disease Cause (score: 0.999)	High functional impact (score: 4.38)	Protein function affected (score 0.001)	Probably damaging (score 1.000)	Deleterious (score -3.93)	35	5.3
F3,F4,F10, F11	c.2020C>T	p.Arg674Trp	Helicase superfamily C-terminal domain	Disease Cause (score 1.000)	Middle functional impact (score: 4.83)	Protein function affected (score 0.001)	Probably damaging (score 1.000)	Deleterious (score -7.42)	33	4.2
F5	c.1784C>T	p.Ser595Phe	Helicase superfamily C-terminal domain	Disease Cause (score: 1.000)	High functional impact (score: 4.26)	Protein function affected (score 0.001)	Benign (score 0.24)	Deleterious (score -5.57)	24.4	4.13
F9	c.911C>T	p.Thr304Met	Helicase ATP-binding	Disease Cause (score 1.000)	High functional impact (score: 4.45)	Protein function affected (score 0.001)	Probably damaging (score 1.000)	Deleterious (score -5.89)	29.8	5.3

564

565 **Table 4.** *DHX37* missense allelic variants identified in 46,XY DSD patients and their frequency in population databases

Families	cDNA position	AA change	Phylogenetic Conservation	State	dbSNP	MAFs in population databases				
						1000 Genomes	ExAC	gnomAD	ABraOm	ESP6500
F1, F2, F6, F7, F8	c.923 G>A	p.Arg308Gln	Highly conserved	Heterozygous	Not available	Absent	Absent	0.00006677 Non-Finnish European	Absent	Absent
F3, F4, F10, F11	c.2020C>T	p.Arg674Trp	Highly conserved	Heterozygous	Not available	Absent	Absent	Absent	Absent	Absent
F5	c.1784C>T	p.Ser595Phe	Highly conserved	Heterozygous	Not available	Absent South Asian	Absent	Absent	Absent	Absent
F9	c.911C>T	p.Thr304Met	Highly conserved	Heterozygous	Not available	Absent	Absent	Absent	Absent	Absent

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568 **Table 5**. Pathogenicity classification of DHX37 variants according to the American College of Medical Genetics and Genomics guidelines

Families	Nucleotide changed	AA changed	Population Data	Computational and prediction data	De novo data	Other data	Classification
F1, F2, F6, F7, F8	c.923G>A	p.Arg308Gln	PM2 [a]	PP2 [b] PP3 [c]	PS2 [d]	PM1[e]	Pathogenic
F3, F4, F10,F11	c.2020C>T	p.Arg674Trp	PM2 [a]	PP2 [b] PP3 [c]		PM1[e]	Likely Pathogenic
F5	c.1784C>T	p.Ser595Phe	PM2 [a]	PP2 [b] PP3 [c]		PM1[e]	Likely Pathogenic
F9	c.911C>T	p.Thr304Met	PM2 [a]	PP2 [b] PP3 [c]		PM1[e]	Likely Pathogenic

PM2: moderate piece of evidence for pathogenicity; PP3: supporting evidence for pathogenicity by computational (*in silico*) data;

PS2: strong support for pathogenicity when the variants are *de novo*; PP4: supporting evidence using phenotype; PM1: pathogenic moderate;

VUS: Variant of Uncertain Significance.

a Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium.

b Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease

c Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)

d De novo (both maternity and paternity confirmed) in a patient with the disease and no family history.

e Located in a mutational hot spot and/or critical and well-established functional domain without benign variation.

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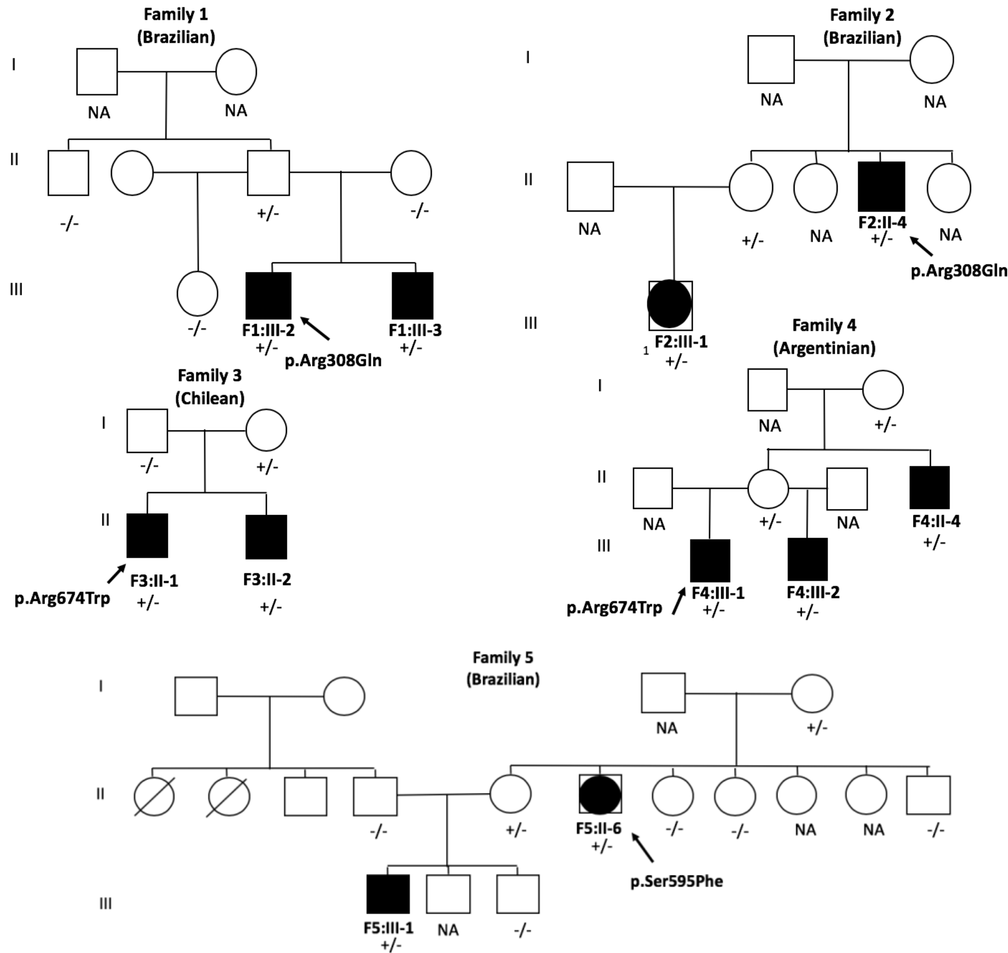
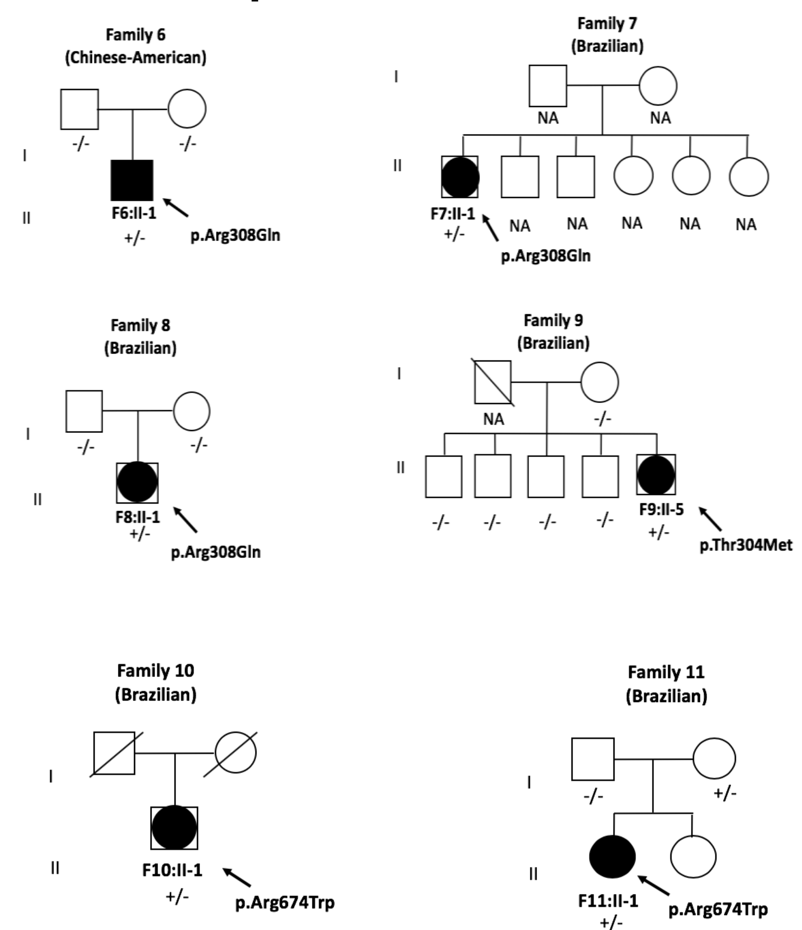
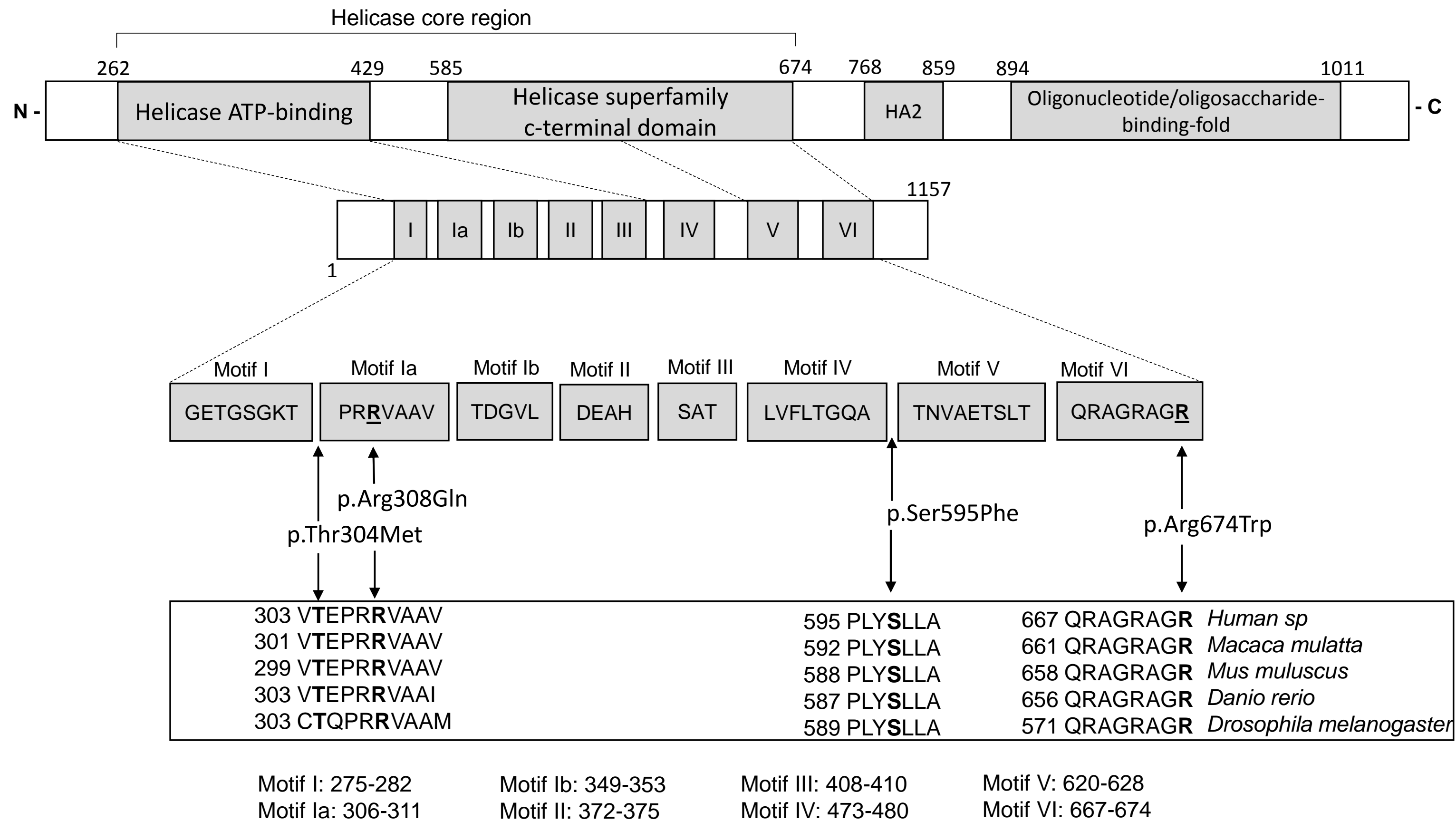




571
572 **Figure 1.** Pedigrees of the eleven families with potential disease-causing *DHX37*variants. Filled symbols represent affected individuals. The affected males (46,XY males) are
573 indicated by filled squares and the affected individuals raised as females (46,XY females) are shown by large dark dots within the squares. Symbols with a diagonal line
574 represent deceased individuals. The *DHX37* genotype is shown for the individuals whose DNA sample was available; +/- indicates a heterozygous state and -/- indicates a
575 homozygous state for wild-type allele. NA- DNA not available. Paternity and maternity was confirmed in families 6 and 8.

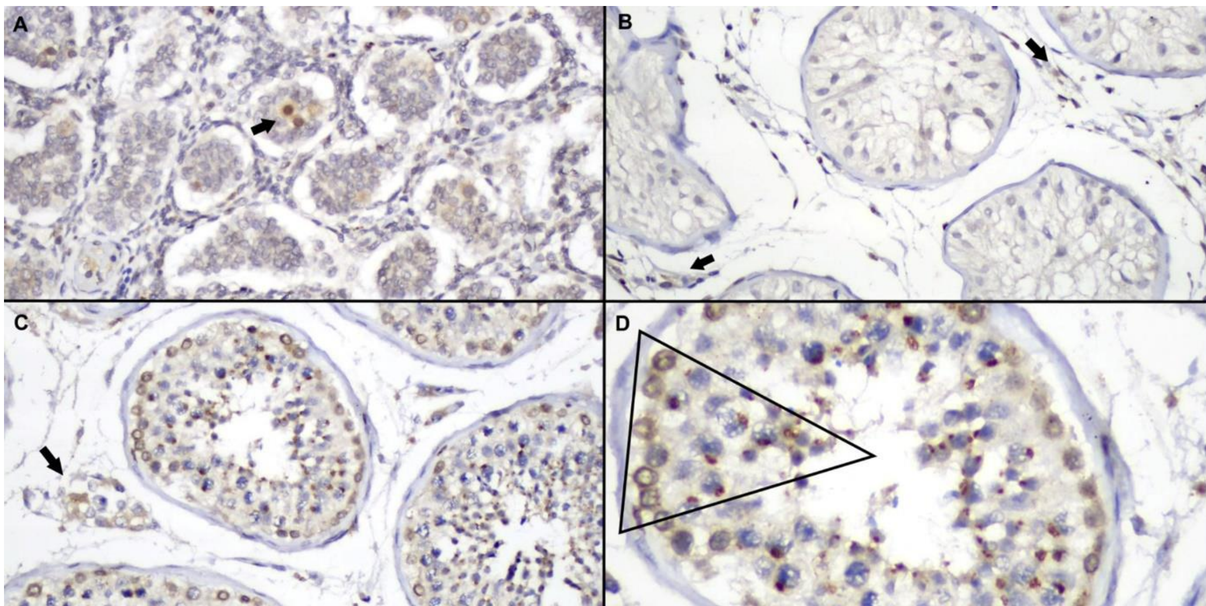
576



577

578 **Figure 2.** The identified variants are localized within conserved helicase domains of *DHX37*. Top: Schematic protein structure of DHX37 showing conserved
579 motifs of the helicase core region, the helicase associated domain (HA2) and the oligonucleotide/oligosaccharide-binding-fold. Middle: Nucleotide-interacting
580 motifs (I, II, and VI), nucleic acid-binding motifs (Ia, Ib, and IV), motif V, which binds nucleic acid and interacts with nucleotides, and motif III, which couples
581 ATP hydrolysis to RNA unwinding (N- N terminus; C- C terminus). Bottom: Amino acids within conserved motifs of the helicase core region. The position of
582 the first and last amino acid within each motif is denoted below left and right, respectively. The position of the allelic variants identified in this study are
583 indicated with vertical arrows and shown in bold in the different species sequence.

584



585

586 **Figure 3**. Immunoexpression patterns of DHX37 in testis tissues. **A-** Newborn testis showing
587 strongly positive staining in occasional spermatogonia (arrow) among numerous Sertoli cells,
588 some of which show weak cytoplasmic staining (original magnification 100X). **B-**
589 Seminiferous tubules of a 13 year old boy demonstrating tubules with predominance of Sertoli
590 cells, all of them negative for DHX37. Note some positive stromal cells (arrow) (original
591 magnification 100X). **C-** Adult testis of a 54 year old man showing positive Leydig (arrow)
592 and germ cell staining. **D-** Detail of (**C**) showing the different pattern of stain in different stages
593 of germ cells. Note strong perinuclear halo in spermatogonia, progressive paranuclear
594 condensation in spermatocytes and spermatids, and absence of DHX37 expression in
595 spermatozoa (original magnification ×20X.

596