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DHX37 and 46,XY DSD: A New Ribosomopathy?

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Abstract
Recently, a series of recurrent missense variants in the RNA-helicase *DHX37* have been reported associated with either 46,XY gonadal dysgenesis, 46,XY testicular regression syndrome (TRS), or anorchia. All affected children have non-syndromic forms of disorders/differences of sex development (DSD). These variants, which involve highly conserved amino acids within known functional domains of the protein, are predicted by in silico tools to have a deleterious effect on helicase function. DHX37 is required for ribosome biogenesis in eukaryotes, and how these variants cause DSD is unclear. The relationship between DHX37 and human congenital disorders is complex as compound heterozygous as well as de novo heterozygous missense variants in *DHX37* are also associated with a complex congenital developmental syndrome (NEDBAVC, neurodevelopmental disorder with brain anomalies and with or without vertebral or cardiac anomalies; OMIM 618731), consisting of microcephaly, global developmental delay, seizures, facial dysmorphia, and kidney and cardiac anomalies. Here, we will give a brief overview of ribosome biogenesis and the role of DHX37 in this process. We will discuss variants in *DHX37*, their contribution to human disease in the general context of human ribosomopathies, and the possible disease mechanisms that may be involved.

Ribosome Biogenesis

Ribosome biogenesis has been studied extensively in the eukaryotic model organism *Saccharomyces cerevisiae*, although more studies are being conducted in human cells reflecting the increased recognition of the contribution of ribosome biogenesis factors to human disease. For a detailed overview on eukaryote ribosome biogenesis, the reader is invited to see the review by Baßler and Hurt [2019]. Although the process and general mechanism are conserved in yeast and human, there are key differences. An extensive comparison of yeast and human ribosome formation and functions has recently been described by Bohnsack and Bohnsack [2019].

The ribosome performs the essential function of translating mRNA into proteins. Eukaryotic ribosome assembly is characterized by the sequential modular assembly

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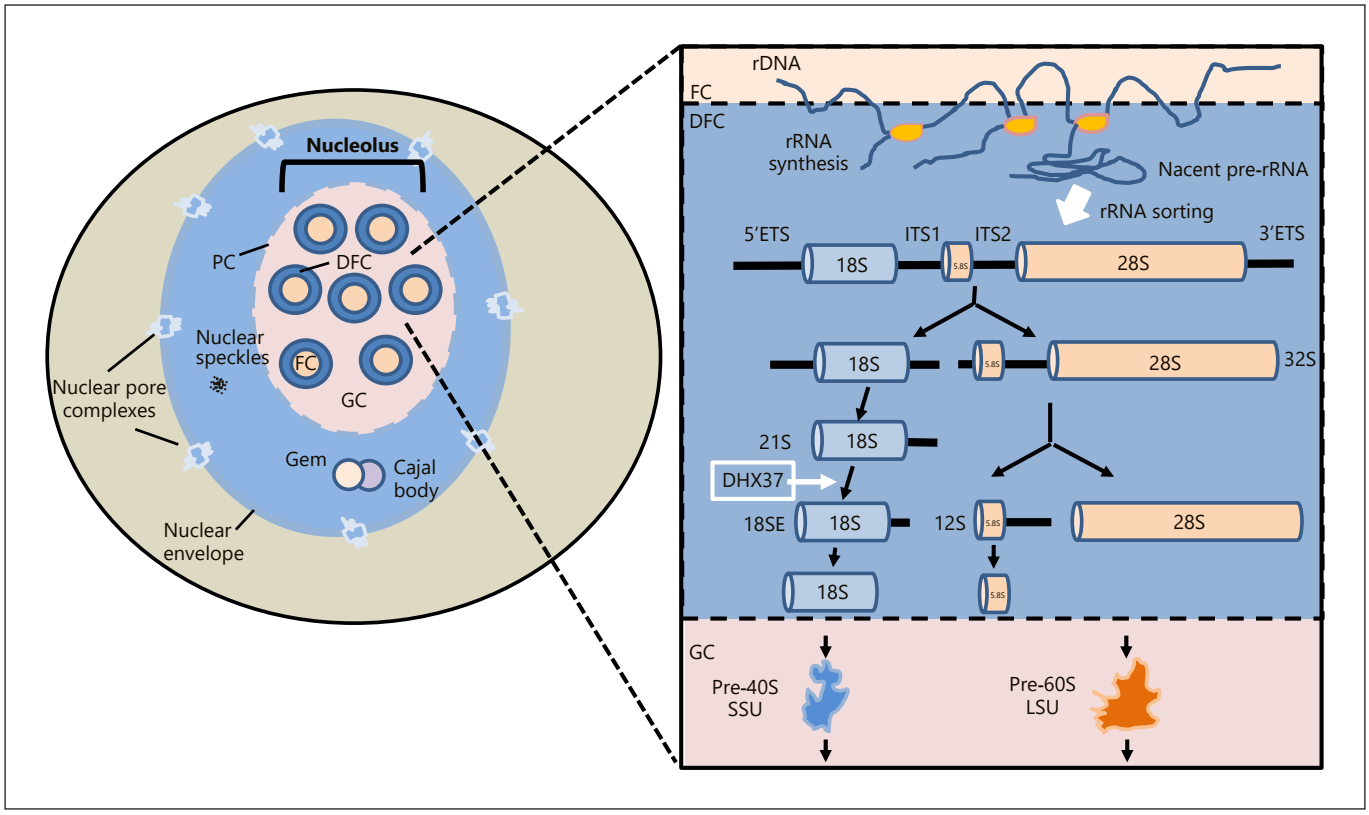

Fig. 1. Schematic representation of human ribosome biogenesis. Left: ribosome biogenesis occurs in the nucleolus, a nuclear compartment that contains the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). Right: Three of the 4 ribosomal RNAs arise from a long primary transcript (47S pre-rRNA) synthesized by RNA polymerase I from loci containing highly-repeated ribosomal DNA (rDNA) genes. Transcription of 47S pre-rRNA by RNA polymerase I occurs at the boarders of the FC and DFC (right). Processing and cleavage of the pre-rRNA occurs in the DFC. The sequences of 18S, 5.8S, and 28S rRNAs are flanked by external transcribed spacers (ETS) and internal transcribed spacers (ITS). During the maturation process, these spacer elements are progressively removed by ribosomal endo- and exonucleases, which are synthesized in the cytoplasm, enter the nucleolus, and assemble with the rRNAs. As ribosome subunit assembly and maturation continues, they enter the GC which contains GTPases that function in subunit maturation and nucleolar release. Human DHX37 is specifically required in the maturation process of the small subunit to displace the U3 snoRNA from the 5′-terminal part of the pre-18S rRNA (arrow). SSU, small subunit; LSU, large subunit.

of pre-ribosomal complexes (Fig. 1). The human ribosome consists of ribonucleoprotein complexes with a small 40S subunit (SSU), containing the 18S rRNA chain and 33 proteins (RPS), and a large 60S subunit (LSU), which has the 28S, 5S and 5.8S rRNA chains, and 47 proteins (RPL). Ribosome biogenesis is an intricate, complex, and coordinated process that takes place initially in the nucleolus and later in the cytoplasm [Henras et al., 2015; Baßler and Hurt, 2019]. This complex process requires at least 200 transacting factors in yeast and several hundred transacting factors in human that act sequentially during maturation of the ribosome subunits [Tafforeau et al., 2013; Thomas et al., 2013; Badertscher et al., 2015; Farley-Barnes et al., 2018; Klinge and Woolford, 2019; Frazier et al., 2020]. In the nucleolus, RNA polymerase I in a complex with multiple transcription initiation factors synthetizes a large polycistronic transcript (47S) from the rDNA genes that are present in hundreds of copies within each cell. This 47S pre-ribosomal RNA (pre-rRNA) transcript contains the future 18S, 5.8S, and 28S rRNAs separated by 2 internal non-coding transcribed spacers (ITS1 and ITS2) and flanked by 2 external transcribed spacers (5′-ETS and 3′-ETS; Fig. 1). These spacer elements are important for ribosome biogenesis as they contain multiple target sites for nucleases that are essential for the correct maturation of each of the rRNAs. Spacer elements differ in size in eukaryotes and are up to 5 times longer in human than in yeast. The pre-rRNA

transcript undergoes extensive processing, modification, and folding that is associated with ribosomal protein recruitment. These proteins include exo- and endonucleases, ATPases, GTPases, and DExH/D-box RNA helicases, as well as at least 70 small nucleolar RNAs (snoRNAs) [Thomas et al., 2013; Klinge and Woolford, 2019; Frazier et al., 2020]. The snoRNAs guide rRNA folding and are required for site-specific modifications. An early cleavage event within ITS1 separates the 18S rRNA and 5.8S-28S rRNA fragments, and this generates independent assembly pathways for the 40S and 60S subunits, which are subsequently exported to the cytoplasm where the final stages of maturation and quality control steps take place. A simplified representation of ribosome biogenesis is shown in Figure 1.

The Role of DHX37 in Ribosome Biogenesis

RNA helicases, including DHX37, play prominent roles in ribosome biogenesis by recruitment or dissociation of ribosomal proteins or binding factors. Their role in ribosome biogenesis has largely been defined in yeast, and it is assumed that the pathway is essentially the same in eukaryotes since highly conserved orthologues of yeast helicases have been described in mammals including human.

The majority of RNA helicases belong to the DEAD and DExH box families within the superfamily 2 (SF2) of helicases. RNA helicases bind and remodel RNA and RNP complexes in an ATP- (or NTP-) dependent manner. In contrast to DEAD helicases, which unwind RNA secondary structures by locally destabilizing and distorting RNA duplexes in an ATP-dependent manner, the DExH helicases generally unwind RNA duplexes or remodel RNPs by an ATP-driven translocation along the RNA [Büttner et al., 2007; Pyle, 2008]. Members of both DEAD and DExH families share a similar core structure that consists of 2 flexibly linked RecA domains within, which are conserved sequence elements and structural motifs. The 2 RecA-like domains of DHX37 use the conserved residues and motifs to both bind ATP (motifs I, II, III, Va, and VI) as well as target RNA sequences (motifs Ia, Ib, Ic, IV, IVa, and V) [Jankowsky and Fairman 2007; Sloan and Bohnsack, 2018]. It is important to note that these RNA helicases by themselves do not possess intrinsic substrate specificity, and they must work in conjunction with a large number of cofactors to recruit specific RNA molecules [Sloan and Bohnsack, 2018]. The main role of these RNA helicases appears to involve 2 related processes: (i) removal of specific snoRNAs from pre-ribosomal complexes and (ii) initiating structural changes in the ribosomal subunits for further processing during ribosome formation [Martin et al., 2014].

The role of DHX37 in ribosome biogenesis was first elucidated in the yeast orthologue Dhr1, and recent data have indicated a similar role for DHX37 in human cells [Sardana et al., 2015; Boneberg et al., 2019; Choudhury et al., 2019]. The recruitment of DHX37 to the pre-ribosomal complex is facilitated in yeast and human by direct physical interaction with UTP14A [Zhu et al., 2016; Boneberg et al., 2019]. An early stable intermediate of 40S assembly is the pre-ribosomal complex termed the SSU processome. It is generated by a subset of RPSs and RNA binding factors (RBFs) that are recruited to the nascent pre-rRNA and consists of several sub-complexes that mediate important steps in SSU biogenesis. A core component of the SSU processome is the U3 snoRNA that creates key interactions within early pre-ribosomes. Hybridization occurs between the U3 snoRNA 3′ hinge region and the 5′ ETS as well as the pre-18S rRNA sequence that will eventually form the central pseudoknot, a universally conserved structural element of the small ribosomal subunit. These molecular interactions mould the SSU processome to a specific conformation that allows essential pre-rRNA processing events to occur, including the nucleolytic cleavage within the 5′ ETS and ITS1. Both human DHX37 and Dhr1 are required to displace the U3 snoRNA from the 5′-terminal part of the pre-18S rRNA [Sardana et al., 2015; Choudhury et al., 2019], which promotes the formation of the central pseudoknot. Consistent with this role, a lack of DHX37 in HeLa cells was found to impair maturation of the 18S rRNA leading to reduced levels of both the mature 18S rRNA and 40S subunits (Fig. 1) [Choudhury et al., 2019]. The lack of DHX37 in these cells also triggers a surveillance pathway that leads to degradation of pre-ribosomal particles indicating the importance of DHX37 in human ribosome biogenesis [Choudhury et al., 2019].

DHX37 Variants and Human Disease

In recent years, high-throughput sequencing of DNA from individuals with DSD have resulted in identification and characterization of a number of new genes and regulatory motifs which are required for testis determination and differentiation [Bashamboo et al., 2017; Audi et al., 2018; Croft et al., 2018; Rey et al., 2000]. This has both aided the diagnostic yield in DSD and revealed insights

Table 1. Summary of DSD phenotypes associated with published DHX37 pathogenic variants

DHX37 variant (patients, *n*)	XY DSD phenotypes
p.T304 (3)	Female, GD + WD
p.R308 (15)	Female, GD; female, CGD + WD; female, PGD; female 46,XY DSD, virilized external genitalia; male, TRS, micropenis, hypospadias + bilateral cryptorchidism; male, TRS, micropenis, bilateral cryptorchidism
p.R334 (2)	Female, GD + WD; male, TRS, micropenis, bilateral cryptorchidism
p.R390 (1)	Female, GD
p.T477 (2)	Female, GD; male, TRS micropenis, bilateral cryptorchidism[a]; male, unilateral anorchia
p.S595 (2)	Sibs – female, GD + WD; male, TRS, micropenis
p.S626 (1)	Male, TRS, micropenis, bilateral cryptorchidism
p.R674 (9)	Female, CDG; female, GD + WD; male, PGD + left testis; male, TRS, micropenis
p.G1030 (1)	Male, TRS, micropenis

[a] Homozygous variant. The father, who presented with unilateral anorchia, is considered an obligate carrier although he has not been tested for the variant. WD, wolffian derivatives; GD, gonadal dysgenesis; CGD, complete gonadal dysgenesis; PGD, partial gonadal dysgenesis; TRS, testicular regression syndrome.

into the fundamental biology of early human gonad formation. One of the most unexpected findings from exome sequencing studies was the observation of recurrent missense variants in the RNA helicase *DHX37* (Table 1). Four studies have been published demonstrating a strong genetic association between missense variants of highly conserved residues located within functional domains of the DHX37 protein and 2 forms of human DSD – nonsyndromic 46,XY gonadal dysgenesis and 46,XY testicular regression syndrome (TRS) [da Silva et al., 2019; Buonocore et al., 2019; McElreavey et al., 2020; Zidoune et al., 2021].

In human, 46,XY gonadal dysgenesis comprises of 46,XY complete (CGD) or partial gonadal dysgenesis (PGD) [Berkovitz, 1992]. CGD comprises of a lack of testis determination and is characterized by female external genitalia, well-developed müllerian structures, and gonads of fibrous ovarian-like stroma with no evidence of testicular differentiation. PGD, on the other hand, has limited testis formation and is characterized by partially developed internal ducts usually consisting of a mixture of wolffian (epididymis, vas deferens, and seminal vesicle) and müllerian ducts (fallopian tube, uterus, and upper third of the vagina). The external genitalia show varying degrees of virilization depending on the amount of testicular tissue present. 46,XY TRS is defined by a 46,XY chromosome complement, ambiguous or atypical external genitalia, anomalies of sexual duct formation, and absence of gonadal tissue on one or both sides [Edman et al., 1977; Josso and Briard, 1980; Naffah, 1989; Pirgon and Dündar, 2012]. Testicular determination is considered to have occurred in boys with TRS, but the tissue disappeared before the 16th week gestation when testis formation is complete. Some boys with TRS are born with normal external genitalia but present with cryptorchidism and may even have one or both palpable testes that subsequently involute. TRS is a rare disorder and has been estimated to affect approximately 1:2,000 boys. Anorchia is defined by the absence of testicular tissue in a 46,XY male but contrasts with TRS by the presence of male-typical differentiation of the genital tract and the development of typical male external genitalia. Since ductal identity and formation of external genitalia is dependent on the production of anti-müllerian hormone (AMH) and androgens, in individuals with anorchia the testis must have been present at least up to the 16th week of gestation [Brauner et al., 2011]. It is important to note that anorchia may also occur secondary to a vascular event or testicular torsion. There is no data available to determine what proportion of cases are due to errors in testicular differentiation as opposed to those due to vascular events or testicular torsion. Both bilateral and unilateral congenital anorchia are rare disorders with a prevalence of approximately 1:20,000 males and 1:6,500 males, respectively [Smith et al., 1991; Behre et al., 2000]. There are reports of both TRS and 46,XY CGD or PGD within the same family leading to the proposal that both 46,XY gonadal dysgenesis and TRS can be regarded as a continuum of phenotypes due to errors in testis determination and maintenance and might have a common genetic aetiology [Josso and Briard, 1980; Naffah, 1989; Fechner et al., 1993; Marcantonio et al., 1994].

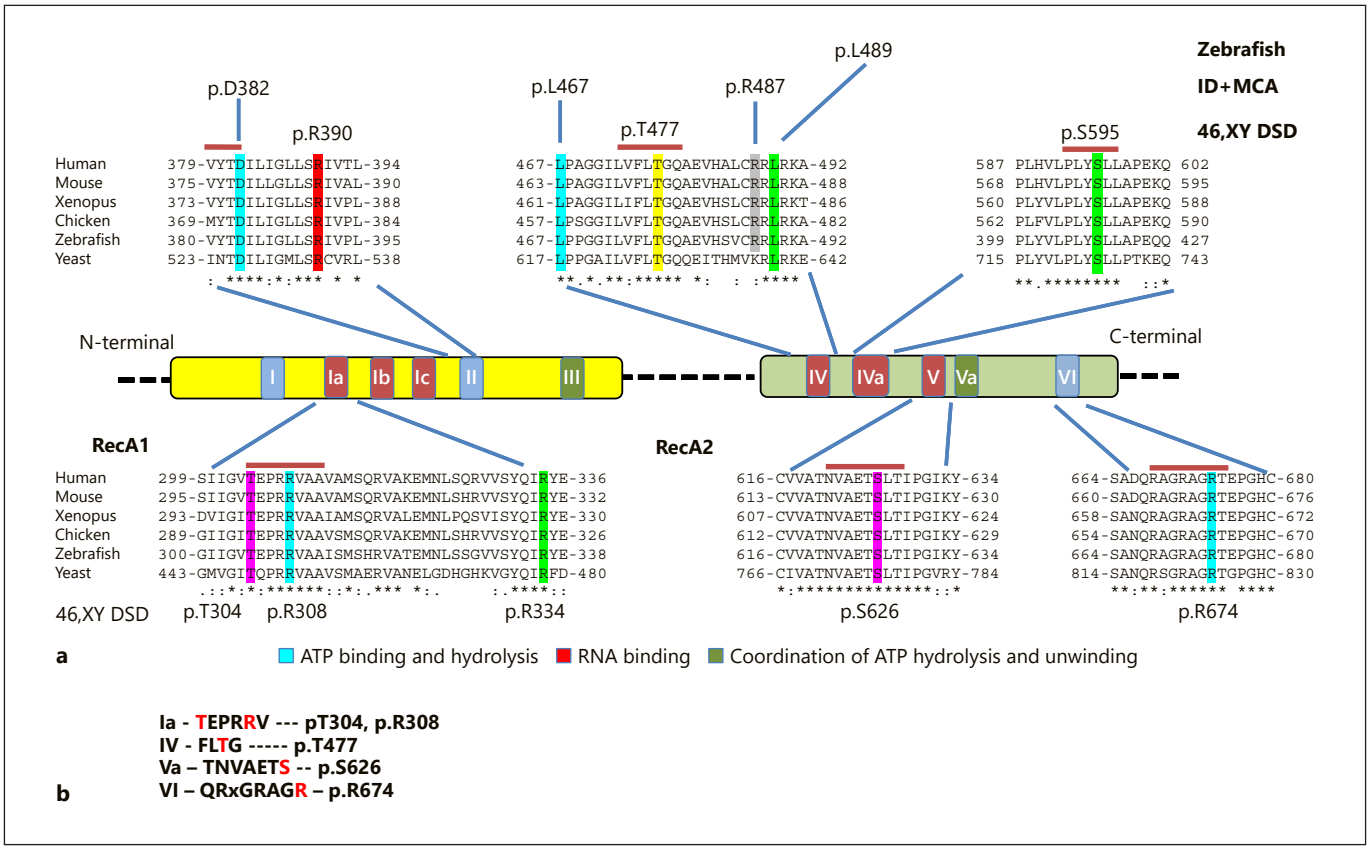

Fig. 2. Variants in DHX37 cause specific developmental anomalies. **a** Pathogenic variants of DHX37 associated with DSD, NEDBAVC, and zebrafish developmental anomalies. Schematic diagram of the RecA-like domains in DEAH-box RNA helicases. Colors represent main helicase functions. Clustal Omega sequence alignment highlighting the position and evolutionary conservation of recurrent variants causing either 46,XY DSD, NEDBAVC, or zebrafish developmental anomalies. Only pathogenic variants in the region of the RecA domains are shown. **b** Pathogenic variants in core RecA motifs which are conserved in all human DEAD/DExH helicases. Mutated variants causing XY DSD are indicated in red. NEDBAVC, neurodevelopmental disorder with brain anomalies and with or without vertebral or cardiac anomalies.

To date a total of 36 individuals are reported to have 46,XY DSD associated with novel or very rare missense variants in *DHX37*. All the reported pathogenic variants of *DHX37* causing 46,XY DSD and their associated phenotypes are described in Table 1 and highlighted in Figure 2a. For the most part these variants are located within or immediately adjacent to highly conserved motifs within the RecA1 and RecA2 domains (Fig. 2a). Pathogenic variants in *DHX37* have been observed in approximately 10–15% of all cases of non-syndromic 46,XY CGD [Buonocore et al., 2019; da Silva et al., 2019; McElreavey et al., 2020; Zidoune et al., 2021]. This is similar to the reported prevalence of pathogenic variants in the *SRY*, *MAP3K1*, and *NR5A1* genes, which, with the exception of *NR5A1*, are all associated with non-syndromic 46,XY gonadal dysgenesis [Berta et al., 1990; Pearlman et al., 2010; Suntharalingham et al., 2015]. *DHX37* variants also account for approximately 20% of all cases of TRS [Buonocore et al., 2019; da Silva et al., 2019; McElreavey et al., 2020; Zidoune et al., 2021]. No other gene variants are known to cause TRS. Pathogenic variants often involve the same amino acid and yet there is no obvious genotype-phenotype correlation. In several cases the exact clinical diagnosis of the child was difficult to define since it was often challenging to find remnants of testicular tissue for histopathological analysis in the cases presenting with 46,XY gonadal dysgenesis. This difficulty in finding gonadal tissues in children with *DHX37* variants sets them apart from other cases of CGD where a streak gonad is present. The clinical data clearly shows that there is a continuum of 46,XY DSD phenotypes associated with DHX37 (Table 1).

The most commonly observed variant is the p.R308Q amino acid change [Buonocore et al., 2019; da Silva et al., 2019; McElreavey et al., 2020; Zidoune et al., 2021]. In the DEAH-box family of proteins, p.R308 is a highly conserved residue within sugar-phosphate binding site Ia, located at the DNA/RNA contact interface which provide an electrostatic environment to accommodate nucleic acids. The recurrent variant p.R308Q removes the positive charge and thereby weakens this interaction. Fifteen individuals carrying this variant in association with 46,XY DSD have been described to date [Buonocore et al., 2019; da Silva et al., 2019; McElreavey et al., 2020; Zidoune et al., 2021], including 2 families with 2 affected individuals in each. The gonadal phenotypes of individuals carrying the p.R308Q variant range from 46,XY PGD and CGD raised as female (6 individuals), 46,XY DSD with atypical external genitalia raised as female (2 individuals), to 46,XY TRS with severe micropenis (with or without cryptorchidism and hypospadias) raised as male (9 individuals). Where the transmission of the variant p.R308Q can be established, 5 are de novo, 2 are maternally, and 1 is paternally inherited. This is consistent with a sex-limited autosomal dominant mode of inheritance.

Although the genetic association between heterozygous *DHX37* variants and 46,XY DSD has been established, there have been other reports of *DHX37* variants associated with complex syndromic forms of intellectual deficiency with an apparent absence of DSD [Karaca et al., 2015; Paine et al., 2019]. Compound heterozygous as well as de novo heterozygous missense variants in *DHX37* are associated with a complex congenital developmental syndrome consisting of microcephaly, global developmental delay, seizures, facial dysmorphia, kidney and cardiac anomalies, as well as cortical atrophy. This syndrome has been termed NEDBAVC (neurodevelopmental disorder with brain anomalies and with or without vertebral or cardiac anomalies; OMIM 618731) In 2 unrelated families each with 1 affected proband, Karaca et al. [2015], found 2 different homozygous variants (p.R487H and p.N419K) in the *DHX37* gene (Fig. 2a). The p.R487 variant is immediately adjacent both to the RecA2 IV domain and the p.K489 residue that is mutated in a zebrafish model of *Dhx37* function in the central nervous system [Hirata et al., 2013]. The p.N419 variant is located adjacent to domain III, which consists of a characteristic serine-alanine-threonine (SAT) amino acid motif that interacts with the g-phosphate of ATP and mediates communication between the ATP- and RNA-binding sites. The same group identified 2 other individuals with NEDBAVC carrying potentially pathogenic biallelic variants in *DHX37* [Paine et al., 2019]. An adult female, who presented with developmental delay, non-progressive impairment of eye, eyelid, and facial movements, asymmetric cerebellar hypoplasia, seizures, and scoliosis carried compound heterozygous variants (p.V731M and p.L467V), which were confirmed to each be inherited from different unaffected parents [Paine et al., 2019]. The p.L467 variant is highly conserved and is located adjacent to domain IV (Fig. 2a), required for substrate binding through interactions with RNA. Similarly, a boy identified with developmental delay, hypotonia, vertebral and cardiac anomalies, and dysmorphic features carried compound heterozygous DHX37 variants (p.R93Q and p.E167A), each inherited from different unaffected parents. Paine et al. [2019] also identified potentially pathogenic de novo heterozygous *DHX37* variants. A boy, presenting with developmental delay, cardiac anomalies, hypotonia, and dysmorphic features carried a heterozygous p.T1094M variant. This variant was not carried by the healthy mother and the father was not available for testing. A second child, a girl with intellectual disability, brain anomalies, chorioretinal lacunae, seizures, scoliosis, and dysmorphic features carried a de novo p.D382G variant. This highly conserved variant is located within the RecA1 domain II, which consists of a Walker B motif containing the aspartate-glutamate-alanine-histidine (DEAH) sequence motif and participates in ATP binding and hydrolysis. DSD was not reported in any of these 6 cases.

These results highlight an unusual feature of missense mutations involving *DHX37*. Pathogenic variants in the gene, even within the same functional domain, generate 2 very distinct, non-overlapping phenotypes. The first is limited to the formation and maintenance of Sertoli cells in the embryonic gonad of 46,XY individuals with no other reported developmental anomalies, and the second is a complex syndromic form of developmental delay and/or intellectual disability that may be associated with vertebral, cardiac, or kidney anomalies, as well as dysmorphic features but no gonadal anomalies. Both phenotypes are due to missense variants, the majority of which involve highly conserved amino acids that are located in or immediately adjacent to functional domains of the helicase. Indeed, several of the amino acid residues that when mutated cause 46,XY DSD are conserved across all human DEAD/DExH helicases (Fig. 2b). To date, no common variant has been reported between XY DSD and NEDBAVC. The most simple hypothesis to explain the different phenotypes is that DHX37 variants associated with NEDBAVC may be loss-of-function (LOF) variants, al-

though no obvious LOF variant (such as a nonsense variant) has yet been reported, whereas the highly specific DHX37 variants associated with DSD may be gain-of-function. Alternatively, the variants associated with NEDBAVC may also be gain-of-function that impact differentially on ribosome function (see section below) compared to the DSD variants. Variants may also impact differently on aspects of DHX37 biology other than ribosome biogenesis that are currently unknown. Functional studies on these variants, such as measurement of ATPase activity, are not yet available for any mutant protein, nor is it known if ribosome biogenesis is affected. In silico modelling and structural analysis of the mutant proteins predict that all variants will disrupt biological function. It cannot be excluded that some of the variants that have been observed only once (e.g., p.R390H or p.G1030E) may be chance findings that are unrelated to the phenotype. Whereas, other variants such as p.R308Q and p.R674Q are recurrent, often de novo, and are strongly associated with the phenotype.

Evidence in support of specific biological roles for DHX37, independent of its role in ribosome biogenesis, comes from studies of zebrafish mutants and in genome-wide screens to identify factors that modulate human T cell function. Zebrafish, carrying a homozygous missense variant p.K489P in DHX37 (Fig. 2a) [Hirata et al., 2013], exhibit changes in behavior of typical tactile-evoked escape swimming response. Wild-type fish turn and then swim away whereas mutant fish show an atypical dorsal bend, followed by swimming. Aspects of the mutant behavior strongly resemble zebrafish embryos treated with strychnine, which blocks glycine receptors. The glycine receptor is a pentameric receptor composed of alpha and beta subunits that mediates postsynaptic inhibition in the spinal cord and other regions of the central nervous system. This suggested that the abnormal motor response in mutants may be attributable to a deficit in glycinergic synaptic transmission [Hirata et al., 2013]. This was confirmed by both decreased expression levels of GlyR α1, α3, α4a, and βa subunits and gephyrin b transcripts in mutants, as well as an increase in unspliced transcripts for the GlyR α4a subunit. RNA immunoprecipitation assay demonstrated that zebrafish Dhx37 physically interacts with GlyR α1, α3, and α4a subunit transcripts. Remarkably, the amounts of 28S and 18S rRNA were comparable between wild-type and mutant fish, indicating no apparent effects on rRNA biogenesis. This data suggest that DHX37 has specific biological functions that are independent of ribosome biogenesis. All of the individuals with variants in *DHX37* described by Karaca et al. [2015] and Paine et al. [2019] have CNS anomalies with, in some cases, seizures and dysmorphic features. These features are consistent with perturbations of GlyR function but the phenotype is in stark contrast to that associated with DSD where there are no reports of somatic anomalies.

CD8 T cells play essential roles in anti-tumor immune responses. Recently, genome-wide CRISPR screens using CD8 T cells in a cancer immunotherapy setting have identified *DHX37* as an important regulator of anti-tumor effects [Dong et al., 2019]. Tumor infiltrating lymphocytes lacking *DHX37* had upregulated expression of genes in multiple immune response pathways, including lymphocyte activation, positive regulation of cytokine production, regulation of cell-cell adhesion, regulation of immune effector process, and positive regulation of interferon-gamma production. This ability of Dhx37 to suppress CD8 T cell activity in response to antigenic stimulation is associated with physical interaction with components of NF-κB pathway including NF-κB p65 and PDCD11. The association with PDCD11 is particularly interesting as it has been previously shown to be involved in ribosome biogenesis [Sweet et al., 2008]. This suggests a link between ribosome biogenesis and tissue-specific gene expression profiles.

DHX37 and Gonad Development

When a new candidate gene is proposed that may be involved in gonad differentiation, one of the first questions to address is when and where is the gene expressed. Considering the phenotypes associated with *DHX37* variants, the gene should be expressed in the early differentiating testes. In mice, data from transcriptomic analyses indicate an expression of *Dhx37* in all gonadal cell types from E11.5 to E13.5, but with the highest expression in the germinal male and female compartment at all stages, especially for the XY testes [Jameson et al., 2012b; McElreavey et al., 2020]. In order to determine the conservation of *DHX37* expression profiles in mammals, we detected the number of reads for this gene in an RNA-sequencing experiment carried on goat gonads. In goats, the polled intersex syndrome (PIS) mutation associates polledness and intersexuality. The sex reversal affects exclusively the XX individuals in a recessive manner, whereas the absence of horns is dominant in both sexes. PIS is due to a deletion at the *Foxl2* locus [Pailhoux et al., 2001]. We analysed samples from different PIS genotypes, XY males, XX females, and XX PIS$^{-/-}$ males, at 36 days of gestation, an early stage of gonadal differentiation in goats

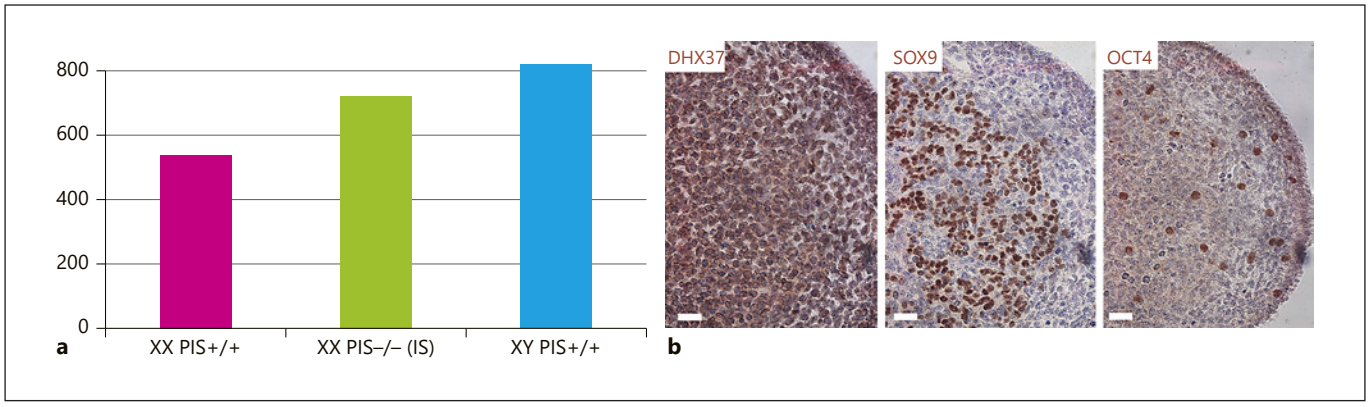

Fig. 3. DHX37 mRNA and protein expression in early differentiating goat gonads. Details of the biological materials used for this figure and of ethical agreements have been described in a previous study [Elzaiat et al., 2014]. **a** RNA-seq analysis on XX female, XX PIS$^{-/-}$ male, and XY male goat gonads at 36 days of gestation. DHX37 is higher expressed in early developing testes than ovaries, and interestingly, its expression increases in XX sex-reversed gonads. **b** Immuno-detection of DHX37 (antibody from Sigma, HPA047607, used at 1:50), SOX9 (antibody from F. Poulat, used at 1:200) [Rahmoun et al., 2017], and OCT4 (antibody from Santa Cruz, Oct3/4 (N-19) sc-8628, used at 1:500) in XY goat testis at 36 days of gestation. Immunodetection was done by using the ampli kit Vectastain from ABC Vector with DAB staining. DHX37 is found more expressed in the cells of the coelomic epithelium, some few cells between the epithelium and the medulla, and a majority of cells of the medulla. This profile is compatible with an expression of DHX37 in the pre-supporting then supporting testicular Sertoli cells.

(Fig. 3a) [Elzaiat et al., 2014]. Interestingly, *DHX37* shows a higher expression in male gonads as compared to female (816 vs. 541 reads, respectively). Moreover, similar to *DMRT1,* the expression of *DHX37* increases in XX PIS$^{-/-}$ gonads (724 reads) in pre-Sertoli-like cells immediately prior to *SOX9* expression [Elzaiat et al., 2014]. These RNA-seq results indicate a sexually dimorphic expression pattern for *DHX37* in male supporting cells that is the first population affected by the PIS mutation at 36 dpc. To define the cellular localization of DHX37 in a subpopulation of developing testis we performed immunohistochemistry on sections of goat testis in comparison with OCT4 (germ cell marker) and SOX9 (Sertoli cell marker). DHX37 showed a strong expression in cells of the coelomic epithelium in XY goat testis and relatively lower expression in few cells between the epithelium and the medulla and a population of cells of the medulla corresponding to SOX9-positive Sertoli cells (Fig. 3b). Therefore, the pre-Sertoli then Sertoli cell expression of DHX37 is in agreement with the human gonadal phenotypes observed with DHX37 protein variants. Furthermore, DHX37 seems to accumulate in the peri-nuclear region similar to the expression described for the goat SRY protein [Montazer-Torbati et al., 2010]. Does a common sub-cellular localization indicate a collaborative role for DHX37 and SRY? This poses an interesting question that needs to be addressed in the future.

The Ribosomopathies

Errors in the process of ribosome production, including defects in ribosomal proteins, rRNA processing, or ribosome assembly factors, lead to the development of a highly specific group of pathologies affecting selective organs or cell types that are collectively termed ribosomopathies [Kampen et al., 2020]. The most studied ribosomopathies include Diamond-Blackfan anemia (DBA), Shwachman-Diamond syndrome (SDS), and Treacher Collins syndrome (TCS). DBA is an autosomal dominant disorder which usually presents in early childhood as bone marrow failure [Da Costa et al., 2018; Venturi and Montanaro, 2020]. Patients may also display a series of distinct congenital birth defects, including skeletal abnormalities and cardiac and genitourinary malformations, together with an increased cancer susceptibility. DBA-associated mutations have been identified in at least 15 different genes encoding ribosome proteins (RPs) of both the small and large subunit, with LOF variants in *RPS19*, *RPL5*, *RPL11,* and *RPS10* being the most common [Venturi and Montanaro, 2020]. In approximately 30–35% of all cases, a genetic diagnosis cannot be obtained, suggesting that there are other ribosomal genes involved [Mirabello et al., 2017; da Costa et al., 2018]. Mutated ribosomal proteins could impair ribosome biogenesis at different levels during ribosome assembly, and in general, this

leads to haploinsufficiency for ribosomal protein function, affecting the maturation of the ribosomal subunit containing the protein and ultimately reducing the amount of available functional 80S ribosomes.

SDS is a multisystem autosomal recessive disorder characterized by exocrine pancreatic dysfunction, bony metaphyseal dysostosis, cognitive impairment, and bone marrow failure with cytopenia [Venturi and Montanaro, 2020]. Myelodysplastic syndrome and acute myeloid leukemia occur in up to one third of patients. Approximately 90% of SDS cases are caused by mutations in the Shwachman-Bodian-Diamond Syndrome (*SBDS*) gene [Warren, 2018]. This factor is required for the cytoplasmic maturation of 60S subunits by promoting the release of eukaryotic initiation factor 6 (EIF6) from pre-60S subunits. EIF6 keeps the nascent 60S subunit in a functionally inactive state during cytoplasmic 60S assembly but needs to be released for final 60S maturation and association with the 40S subunit. A GTPase termed ELF1 acts in concert with SBDS in the cytoplasmic maturation of the 60S ribosomal subunit by catalyzing GTP-dependent removal of EIF6. Recently, biallelic pathogenic variants in *ELF1* have been identified as causing Shwachman-Diamond syndrome-2 (SDS2), which is phenotypically similar to the SBDS (exocrine pancreatic dysfunction, hematopoietic abnormalities, short stature, and metaphyseal dysplasia) [Stepensky et al., 2017].

Pathogenic variants in SBDS are predicted to cause reduced ribosome assembly and, like DBA, this could affect the overall global translation which would impact on highly proliferative tissues such as haematopoiesis or skeletal development that require high protein synthesis.

Anomalies of skeletal development also characterize TCS, which is a rare autosomal dominant congenital disorder presenting with severe craniofacial defects, including hypoplasia of the jaw and cheek bones, downward slant of palpebral fissures, and cleft palate. More than 150 heterozygous variants in the gene *TCOF1*, which encodes the protein Treacle, have been reported to cause TCS. This is the most common genetic cause of TCS. Despite the fact that most variants are de novo frameshift deletions or duplications that are predicted to result in premature termination codons, there is no obvious genotype-phenotype correlation. Incomplete penetrance and high intra- and interfamilial clinical heterogeneity are common in TCS due to *TCOF1* variants. TCOF1 participates in rRNA synthesis via direct binding of upstream binding factor and RNA polymerase I and interacts with components of the pre-rRNA processing complex in the nucleolus [Valdez et al., 2004; Gonzales et al., 2005]. Rare pathogenic variants in the gene RNA polymerase I and III subunit D (*POLR1D*), the RNA polymerase I and III subunit C (*POLR1C*), and the RNA polymerase subunit B (*POLR1B*) cause TCS, with *POLR1D* and *POLR1B* pathogenic variants inherited in an autosomal dominant and *POLR1C* pathogenic variants in an autosomal recessive fashion [Dauwerse et al., 2011; Sanche et al., 2020]. Deficient ribosome biogenesis in TCS causes a reduced proliferation of the progenitors of the craniofacial skeleton cells, the neural crest cells.

What is remarkable about this group of diseases is the degree of tissue-specificity associated with pathogenic variants that impact on different components of ribosome biogenesis and ribosome function. Understanding how defects in a fundamental and ubiquitous cellular process such as ribosome synthesis can cause tissue specific human pathologies remains a major challenge. Although it has been proposed that these pathologies reflect tissue/organ specific needs for optimum protein production during development, this does not adequately explain the striking differences in the phenotypic presentation of these diseases. One possibility is that differing phenotypes reflect ribosome heterogeneity and functional specialization.

Ribosomes were assumed to be a uniform macromolecular complex, identical in structure and function in every cell. The observation of different human disease phenotypes associated with variants in different ribosome proteins raises the controversial possibility that there may be different groups of ribosomes that during development have a functional specialization and translate specific groups of mRNAs. Ribosome heterogeneity has been recognized in the past few years. The heterogeneity can be due to a wide range of sources including changes in individual protein components [Shi et al., 2017], posttranslational modifications [Simsek and Barna, 2017] and differential stoichiometry [Shi et al., 2017]. Ribosome heterogeneity can also occur at the level of rRNA. In human there is extensive variation in rDNA copy number varies with considerable intra- and interindividual nucleotide variation in the 5S, 5.8S, 18S, and 28S rRNAs [Parks et al., 2018]. rRNA alleles show tissue-specific expression in mice suggesting some degree of developmental regulation [Parks et al., 2018]. Changes in RP transcript levels have also been observed between different cell and tissue types which is suggestive of altered ribosome composition [Kondrashov et al., 2011; Guimaraes and Zavolan, 2016]. Regulation of ribosome composition likely occurs at least in part during ribosome biogenesis and is possibly done by ribosome biogenesis

factors. Although differences in ribosomal composition have been established and continue to be fully characterized, there is still a lack of data indicating the extent to which ribosomes are heterogeneous and how dynamic heterogeneity may be during development and within cells themselves. Strong data to equate heterogeneity with a specialized developmental function have until recently been lacking. Shi et al. [2017] found that heterogeneity in RP composition in mouse embryonic stem cells resulted in specific ribosomes that displayed a differential selectivity for translating sub-pools of transcripts, including those controlling metabolism, cell cycle, and development in embryonic stem cells. For example, ribosomes containing RPS25 or RPL10 but not RPL22 were found to preferentially translate distinct sets of several hundred mRNAs from related functional groups. Data also suggested that IRES elements present within mRNA 5′UTRs appear to guide translational control by specific types of ribosomes. This leaves open the possibility that specific mutations involving distinct components of the ribosome assembly process could impact tissue-specific or cell-specific function instead of being detrimental for the entire organism.

Ribosomopathies and Cancer Risk

Overall, ribosomopathy patients have a 2.5–8.5-fold higher risk to develop cancer throughout their life, and for particular cancer types these risks can be up to 200-fold higher [Sulima et al., 2019a]. Independently of ribosomopathies, somatic mutations in ribosomal proteins have been discovered in a variety of hematopoietic and solid tumors [Sulima et al., 2017]. An increased risk of lung, cervical colon cancer, and osteogenic sarcoma is associated with Diamond-Blackfan anemia [Alter et al., 2018; Vlachos et al., 2018]. How defects in ribosome biogenesis or function can lead to cancer is currently unknown although 3 non-exclusive hypothesis have been proposed. The first is altered protein translation due to misassembled and/or structurally unique ribosomes that could lead to the production of growth-promoting and oncogenic proteins. The second is that some ribosomal proteins may have secondary functions, independent of their ribosome function, in the regulation of oncogenes. Thirdly, ribosome defects may result in cellular stress including high cellular oxidative stress due to increased levels of reactive oxygen species (ROS). This could indirectly lead to cancer formation since elevated ROS levels are linked to increased DNA damage and genomic instability [Sulima et al., 2019b]. The cancer risk in individuals carrying pathogenic DHX37 variants is unknown. This is due to recent identification of DHX37 as a cause of DSD and cancer risk has not yet been evaluated.

Conclusions

There is currently no evidence available to indicate a possible direct functional role for DHX37 in human testis determination, but recently an intriguing link between nucleolar stress and the activation of WNT signaling has been described. In mammals, the formation of the ovary requires activation of the canonical WNT/β-catenin pathway [Capel, 2017]. Binding of extracellular WNT ligands to their respective receptors activates the WNT/β-catenin pathway and triggers the stabilization of β-catenin, in the cytosol [Nusse and Clevers, 2017]. β-Catenin then translocates to the nucleus and interacts with transcription factors to regulate gene expression. In the absence of WNT ligands, a destruction complex is formed that mediates the phosphorylation of β-catenin. Subsequent poly-ubiquitination results in the proteasomal degradation of β-catenin in the cytosol. In gonad development, the ectopic stabilization of β-catenin in transgenic XY mice disrupts testis development [Maatouk et al., 2008] and XY mice lacking *Znrf3*, a WNT signaling antagonist, exhibit gonadal sex reversal [Harris et al., 2018]. Human ZNRF3 variants have been described that disrupt ZNRF3's anti-WNT activity in a cell line and zebrafish embryos [Harris et al., 2018]. These variants lead to errors in testis determination causing a spectrum of phenotypes from 46,XY CGD to a 46,XY undervirilized boy. The inhibition of WNT β-catenin signaling is therefore required for correct testis formation in XY individuals [Jameson et al., 2012a].

The 46,XY individuals with DSD due to recurrent variants in DHX37 form a distinct group that are characterized by the absence of gonadal tissue. In 46,XY CGD, the gonad usually consists of a streak of undifferentiated fibrous tissue, however, the patients with gonadal dysgenesis due to DHX37 variants often have little gonadal tissue present [McElreavey et al., 2020 and unpubl. data]. This group of DSD is therefore characterized by both a disruption of testis formation and also apoptosis. The development and function of the ovary is apparently normal in mothers carrying pathogenic DHX37 variants. The 2 observations – disruption of testis formation and apoptosis leading to the absence of

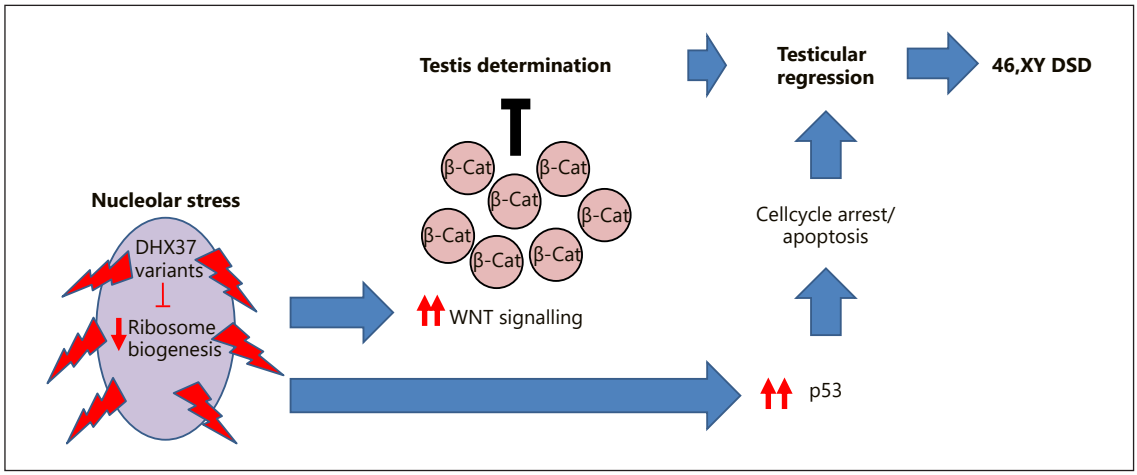

Fig. 4. Model of DHX37-associated 46,XY gonadal dysgenesis and testicular regression syndrome (TRS). A disruption of ribosome biogenesis due to DHX37 variants causes nucleolar stress. Cells such as pre-Sertoli cells, which experience nucleolar stress, accumulate cytosolic pro-ovary β-catenin that in turn disrupts testis determination. Nucleolar stress response also results in cell cycle arrest and apoptosis through the activation of p53 stabilization which contributes to the TRS.

gonadal tissue – can be reconciled by recent observations on nucleolar stress responses. Classically, impaired ribosome biogenesis and/or the loss of nucleolar integrity trigger nucleolar stress responses through stabilization of the tumour suppressor p53 (and other stress signaling pathways) resulting in cell cycle arrest and apoptosis [Fig. 4) [Bursac et al., 2012; Yang et al., 2018]. Recently, nucleolar stress was also found to result in the activation of WNT/β-catenin signaling [Dannheisig et al., 2021]. Depletion of the *SBDS* gene, the key factor involved in the ribosomopathy SBDS, was shown to activate canonical WNT signaling in human cancer cell lines. Human cells exposed to compounds that induce nucleolar stress repeatedly show early activation of the WNT/β-catenin pathway, perhaps as a compensation mechanism to induce cell proliferation and maintain ribosome biogenesis in response to the nucleolar stress. Activation of the WNT/β-catenin pathway is then followed by the classical activation of p53-dependent apoptotic pathways. This pattern of increased pro-proliferative WNT signaling followed by a switch to a p53-dependent pro-apoptotic response is consistent with the phenotypes observed in 46,XY DSD caused by DHX37 variants. DHX37 is expressed in somatic cell lineages of the mouse and human gonad during testis determination, including cells which express the essential testis-determining gene *SOX9* [McElreavey et al., 2020]. In this model (Fig. 4), nucleolar stress due to DHX37 variants results in a rapid and transient rise in WNT signaling leading to β-catenin stabilization. Human testis-determination is highly sensitive to gene dosage [Bashamboo and McElreavey, 2016] and inappropriate pro-ovary WNT-signaling in the somatic XY gonadal progenitor cells may be sufficient to disrupt testis determination but not sufficient enough to cause other complex somatic phenotypes reported elsewhere [Karaca et al., 2015; Paine et al., 2019]. The WNT signaling in the XY somatic cells of the developing gonad would thus be followed by the p53-dependent pro-apoptotic response resulting in the absence of gonadal tissue. The results of this 2-step process in the somatic XY gonadal cells would be 46,XY gonadal dysgenesis and 46,XY TRS.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

All authors contributed to the writing of this text.

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