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GENETICS

Heterozygous *TP63* pathogenic variants in isolated primary ovarian insufficiency

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Abstract

Purpose Our study aimed to identify the genetic causes of non-syndromic primary ovarian insufficiency (POI) in female patients.

Methods We performed whole exome sequencing in females suffering from isolated POI and in their available family members. Copy number variations were validated by long-range PCR and Sanger sequencing, and conservation analysis was used to evaluate the impact of sequence variants on protein composition.

Results We detected two pathogenic *TP63* heterozygous deleterious single nucleotide variants and a novel *TP63* intragenic copy number alteration in three unrelated women with isolated POI. Two of these genetic variants are predicted to result in loss of transactivation inhibition of p63, whereas the third one affects the first exon of the ΔNp63 isoforms.

Conclusion Our results broaden the spectrum of *TP63*-related disorders, which now includes sporadic and familial, isolated, and syndromic POI. Genomic variants that impair the transactivation inhibitory domain of the TAp63α isoform are the cause of non-syndromic POI. Additionally, variants affecting only the ΔNp63 isoforms may result in isolated POI. In patients with isolated POI, careful evaluation of genomic variants in pleiotropic genes such as *TP63* will be essential to establish a full clinical spectrum and atypical presentation of a disorder.

Keywords TP63 · Primary ovarian insufficiency · Isolated POI · Pleiotropy · DNA repair

Introduction

Primary ovarian insufficiency (POI) is defined as primary or secondary amenorrhea with hypergonadotropic hypogonadism and hypoestrogenism prior to age 40, resulting from follicle dysfunction or follicle depletion. It can be observed either as an isolated or a syndromic condition arising from the dysregulation of various discrete molecular pathways [1]. A factor that strongly influences the maintenance of a viable ovarian reserve is the ability of germ cells to protect genomic integrity [2]. Genes involved in DNA repair have been associated with the regulation of both the establishment of the ovarian reserve and its depletion [3].

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One of these genes is *TP63*, which encodes the p63 transcription factor that is activated in response to DNA double-strand breaks and is required for p53-dependent apoptosis. p63 is implicated in several biological processes ranging from ectodermal development to limb formation, maintenance of genomic stability, and female fertility. These multiple functions are governed by controlled tissue-specific expression and alternative splicing of distinct *TP63* isoforms [4]. All isoforms contain a DNA-binding domain (DBD) and an oligomerization domain (OD), but they differ in their N- and C-termini. The longer isoforms, such as TAp63α, have a transactivation domain (TAD) in their N-terminus, while expression from an alternative promoter results in shorter ΔN isoforms that lack the TAD. Similarly, the C-terminus of the alpha isoforms contains a second transactivation domain (TAD2), a sterile alpha motif (SAM) domain, and a transactivation inhibitory domain (TID), while shorter isoforms have only some or none of these domains [5]. The TAD and TID are key to control the activity of p63 as they interact with each other to form inactive homodimers that have reduced affinity for DNA due to conformational inaccessibility of the DBD [6, 7]. Isoforms that do not have either a TAD or a TID, such as the ΔNTp63 isoforms, are always present in a constitutively active tetrameric form [5].

The two most well characterized p63 isoforms are the ΔNp63α isoform, expressed in ectodermal and endodermal-derived tissues [8, 9], and the TAp63α isoform, predominantly found in female germ cells [10]. TAp63α plays a crucial role in the genetic quality control of oocytes. It is expressed in a dimeric inactive conformation by primary oocytes during the dictyate arrest of meiotic prophase I [10]. In response to DNA damage, inactive TAp63α homodimers become phosphorylated and adopt an active tetramer conformation that exposes the DBD and allows for transcription of pro-apoptotic target genes [11–13]. After phosphorylation, TAp63α is quickly degraded [7]. In mice, replacement of TAp63α by TAp63β, which lacks the TID, leads to the formation of constitutively active tetramers that results in an almost complete absence of follicles, and consequent POI, through uncontrolled oocyte apoptosis [14]. Thus, proper interaction between the TAD and TID in the TAp63α isoform appears to be the key factor enabling a measured response to DNA damage, essential for the maintenance of the integrity of the female germline.

Heterozygous pathogenic *TP63* variants tend to alter multiple isoforms and are linked to syndromic phenotypes such as ectodermal defects, cleft lip or palate, and limb malformations, which are collectively known as *TP63*-related disorders [15]. Of note, some patients affected by these allelic disorders also present with POI or infertility [16–19]. Several cases of isolated POI in association with *TP63* pathogenic variants have also been reported. A paternally inherited intragenic duplication involving exons 2–9 was detected in two sisters diagnosed with primary amenorrhea [20]. Two nonsense variants resulting in truncation of p63 at the N-terminus and loss of the TID were identified in two unrelated patients [21]. More recently, three additional individuals with isolated POI were described with *TP63* heterozygous missense variants [22]. Two of these mutations were shown to result in defective homodimerization and subsequent constitutive, open tetramer conformation. While the third one, located within the unique TA* domain of the TA*p63 isoform, had an uncertain deleterious effect on protein conformation or activity [22]. Mechanistic studies have shown that impaired interaction between the TAD and the TID results in an open active tetrameric form of TAp63α that likely compromises the ovarian reserve [22].

In this study, we show that autosomal dominant pathogenic single nucleotide variants (SNVs) and intragenic copy number variations (CNVs) in *TP63* can cause isolated POI. We identified a novel CNV associated with isolated POI, expanded the phenotypic spectrum linked to a known SNV now also linked to non-syndromic POI, and found that variants that alter only the ΔNp63 isoforms can cause isolated POI. In accordance with prior studies, we hypothesize that loss of transactivation inhibition leads to the inability of TAp63α to form a stable inactive conformation, causing unregulated oocyte apoptosis and subsequent depletion of the ovarian reserve.

Materials and methods

Human subjects

The study was approved by the University of Pittsburgh IRB STUDY19060330. Patients with a clinical diagnosis of non-syndromic POI, negative for premutation in the *FMR1* gene, and their family members (when available) were recruited as part of a multi-institutional study over a 15-year period. Informed written consents were obtained from participating subjects and their family members (if enrolled). DNA was isolated from peripheral blood samples using a Puregene Blood Kit B (QIAGEN, Hilden, Germany).

Exome sequencing and variant analysis

Exome sequencing of SPOF-36 and IPOF-44 samples was performed on a HiSeq X Ten platform (Illumina, San Diego, USA) and analyzed as previously described [23]. The whole exome solution (Sophia Genetics, Boston, MA) was used for sequencing and CNV detection in PPOF-178 and her parents. An average depth coverage for target regions of 100× was obtained. Variants were annotated with ANNOVAR software [24]. The results were

interpreted using the SOPHiA DDM platform (Sophia Genetics, SA). Protein-altering SNVs with a minor allele frequency (MAF) ≤ 0.1% (GnomADv2) were retained for analysis. To identify potential CNVs, depth coverage information of reads in target regions in a given sample was normalized and compared with average depth in the same region of normal samples. CNV plots with affected regions were produced for each sample. Simultaneous SNV and CNV analysis was performed using a virtual gene panel containing a list of POI relevant genes assembled upon the scientific literature search [1]. Sanger dideoxy sequencing was used to confirm plausible variants.

Long-range PCR and deletion analysis

Long-range PCR (Takara Bio) was performed on PPOF-178 and the patient's family members in order to confirm the deletion of exons 11–13. Two primers, pF (5′-CTTTCA AATT GGCTGAGTTTATTCAGCCTTGCTTC-3′) and pR (5′-CTACTTATGCTTGAATTTACCTGGCTAGTC ACTAG-3′), mapping to introns 10 and 13, respectively, were designed to amplify the deletion breakpoints junction and the wild-type fragment. DNA sequencing of a deletion-specific PCR product was completed by conventional Sanger dideoxy sequencing. The chromatograms were analyzed using the Sequencher 4.2 (Gene Codes) software.

Genomic and protein conservation analysis

The human *TP63* genomic sequence was inspected in the USCS Genome Browser (hg19) and conservation scores among 7 different species were extracted. Protein sequences of human TP63 and its homologs in *Pan troglodytes*, *Mus musculus*, *Bos taurus*, *Canis lupus*, *Gallus gallus*, *Xenopus tropicalis*, and *Danio reiro* were aligned using Clustal Omega [25].

Results

SPOF-36

SPOF-36 was a Caucasian woman diagnosed with POI at 15 years of age. She presented with primary amenorrhea, normal stature, low bone density, low estradiol, and anti-Müllerian hormone (AMH) levels, and high follicle–stimulating hormone (FSH) and luteinizing hormone (LH) measurements. Pelvic ultrasound revealed no visible ovaries (Table 1). The family history was unremarkable. The patient had a normal 46,XX karyotype and tested negative for *FMR1* premutation. Exome sequencing detected a heterozygous c.1794G>A (p.W598*) (rs1560311010) nonsense variant in exon 14 of *TP63* (Fig. 1). This variant has not been observed in heterozygous state in the 1000 Genomes, dbSNP, or gnomADv2.1 databases, but has been reported

Table 1 Clinical laboratory measurements in affected individuals

Findings	Normal range	SPOF-36	IPOF-44	PPOF-178
Ethnicity	-	Caucasian	Caucasian	Caucasian
Age at diagnosis (y)	-	15	23	16
ES findings		*TP63*; NM_003722: c.1794G>A (p.W598*)	*TP63;* NM_001114981: c.16A>C (p.N6H); *SIRT6;* NM_016539: c.362 G>A (p.R121H)	*TP63;* NM_003722:g. (?_ 189604142)_ (189608684_?)del
Diagnosis (PA/SA)	-	PA	SA	PA
Age at menarche (y)	-	-	13	-
Age at menopause (y)	-	-	23	-
Familial case	-	No	No	Paternally inherited
Height (cm)	-	172	172	158
Ovarian volume (cm^3)	6.6	Not visualized	Not measured	Not visualized
Autoimmune disease	-	Anti-thyreoglobulin antibodies	-	-
BMD results	-	Low bone density	-	Low bone density
FSH (mIU/mL)	1.8–22.5	128	73.6	145.5
LH (mIU/mL)	1.37 to 9	48	28.1	34.4
Estradiol (pg/mL)	30 to 300	12	-	11
Prolactin (ng/mL)	3.3–26.7	21	-	14.5
TSH (mIU/L)	0.5–5.0	3	-	2.16

PA primary amenorrhea, *SA* secondary amenorrhea, *BMD* bone mineral density, *FSH* follicle-stimulating hormone, *LH* luteinizing hormone, *TSH* thyroid-stimulating hormone

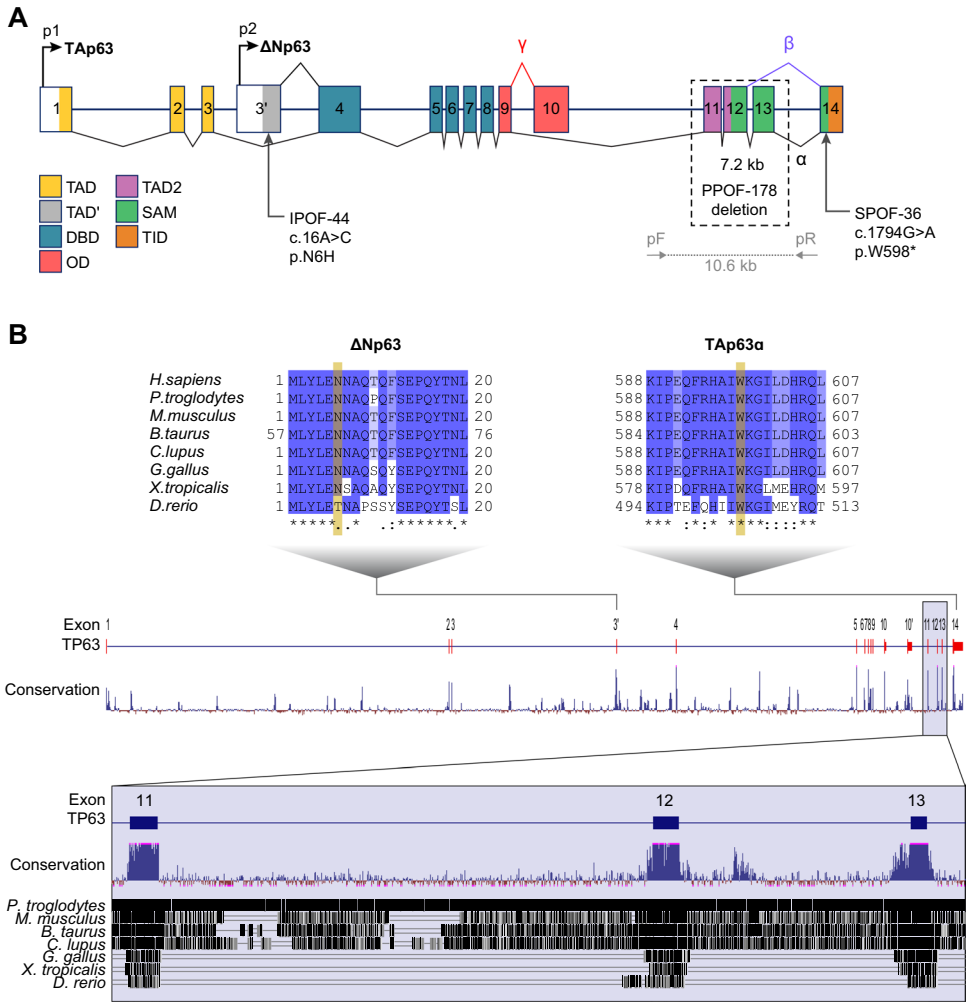

Fig. 1 Identification of *TP63* mutations in three unrelated patients with POI. **A** Schematic representation of the genomic structure of the human *TP63*. Alternative splicing (*α*, *β*, and *γ*) and alternative promoters (p1 and p2) are indicated. Vertical arrows designate the location of single nucleotide variants identified in patients IPOF-44 and SPOF-36. Dashed rectangle indicates the deletion of exons 11–13 and the surrounding intronic sequences in PPOF-178. Exons are color-filled in correspondence to the protein domains encoded. DBD, DNA-binding domain; OD, oligomerization domain; SAM, sterile alpha motif; TAD, transactivation domain; TAD2, second transactivation domain; TID, transactivation inhibitory domain. **B** (Top) Protein sequence alignment of human p63 and its homologs in *Pan troglodytes*, *Mus musculus*, *Bos taurus*, *Canis lupus*, *Gallus gallus*, *Xenopus tropicalis*, and *Danio reiro*. Residues are shaded based on similarity. Symbols denote: (*) conserved residue; (;) conservation between groups of strongly similar properties (>0.5 in the Gonnet PAM 250 matrix); (.) conservation between groups of weakly similar properties (≤0.5 in the Gonnet PAM 250 matrix). (Bottom) Conservation profile of the *TP63* gene visualized through the UCSC Genome Browser. Conservation track of a 100 vertebrates base-wise conservation displaying phyloP scores in blue and red across the *TP63* gene. In purple, inset showing the *TP63* genomic region spanning exons 11–13. In addition to the conservation scores in this region, a multi-sequence alignment of 7 different species where each genome is aligned to the reference human sequence (darker shading indicates higher BLASTZ scores) is also shown

previously in a patient with isolated POI [21]. This SNV is classified as pathogenic in ClinVar according to the ACMG guidelines and is predicted to lead to the formation of truncated p63α isoforms missing the TID.

IPOF-44

Patient IPOF-44 was a 23-year-old Caucasian woman who underwent menarche at 13 years of age and presented with secondary amenorrhea. She had elevated FSH and LH levels, and normal height and weight. Pelvic ultrasound revealed a small uterus and small ovaries (Table 1). Family history was unremarkable, and the patient tested negative for *FMR1* premutation. Exome sequencing detected two SNVs. A heterozygous c.362G>A (p.R121H) missense variant in the *SIRT6* gene (rs771714154) that is described as likely pathogenic in ClinVar and and a heterozygous missense variant c.16A>C (p.N6H) (rs113993963) located in the first exon of the ΔNTp63

isoforms (exon 3′) (Fig. 1A). This *TP63* variant is absent in the gnomADv2.1 database and affects a residue that is highly conserved among multiple species (Fig. 1B). It is listed as pathogenic in ClinVar and has been reported in a patient with acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome [16].

PPOF-178

Patient PPOF-178 was a 16-year-old Caucasian female evaluated for primary amenorrhea. She presented with normal height and weight, low bone density for her age (Z score ≤ -2.0), low estradiol, and high-FSH and LH measurements. Pelvic ultrasound revealed a small uterus, measuring approximately 1.9 × 0.6 × 1.0 cm, and small, hypoechoic structures presumed to be ovaries (Table 1). No parental consanguinity was reported, and family history was unremarkable except for the presence of isolated cleft lip/palate in the father (Fig. 2A). The patient had a normal 46,XX karyotype, normal chromosomal microarray analysis, and was negative for *FMR1* premutation.

Exome sequencing detected a novel heterozygous intragenic deletion of exons 11–13 of the *TP63* gene encompassing at least a 4.5 kb segment (chr3:189604142_189608684; hg19) (Fig. 1). The same *TP63* deletion was identified in the patient's father (Fig. 2B). Long-range PCR confirmed the deletion of exons 11–13 and further characterized the breakpoints. Two PCR products were observed in the patient and her father, corresponding to one normal allele and one *TP63* allele with a 7195 bp deletion (Fig. 2C). Subsequent testing of paternal grandparents using deletion-specific long-range PCR showed no evidence of a deletion in either grandparent. Therefore, this deletion most likely represents a de novo event in the patient's father. The deletion (chr3:189,602,917_189,610,111; hg19) breakpoints were located within unique sequences in introns 10 and 13, respectively (Fig. 2D) and no repetitive elements or microhomology were identified at the breakpoints in the reference human genome (hg19). This CNV is predicted to cause an out-of-frame deletion resulting in a frameshift in the TAp63α protein sequence (p.T451Ifs*5), thereby generating a truncated product that lacks the TID encoded in exon 14 (Fig. 2E). Deletion of this region is not reported as a benign genomic CNV in ClinVar, the Database of Genomic Variants (DGV), or the DECIPHER database.

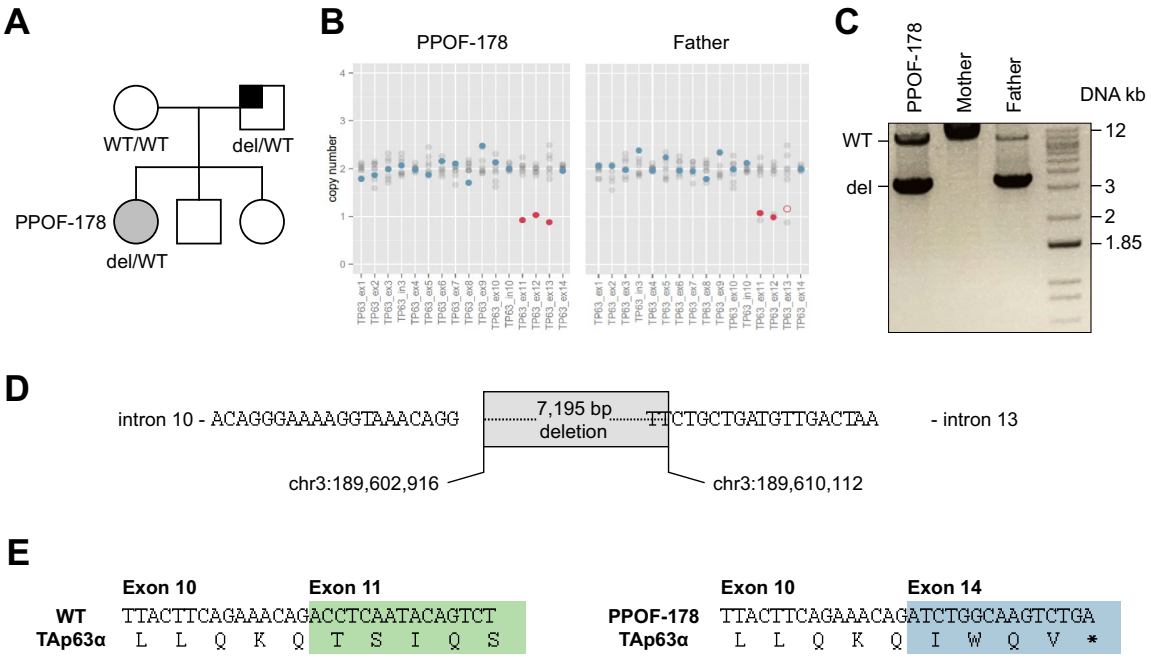

Fig. 2 Novel *TP63* deletion detected in PPOF-178. **A** PPOF-178 family pedigree. Proband affected by POI is designated by a grey filled circle. Black square specifies individuals affected by an isolated cleft lip/palate. The *TP63* genotype is given for tested individuals. WT indicates a wild-type allele. Allele with deletion of exons 11–13 denoted as "del." **B** Copy number plots produced after exome sequencing. Each plot displays the normalized coverage in each sample (blue and red dots) compared to the reference coverage levels (gray dots). Blue dots correspond to regions with normal copy number. Red dots indicate regions affected by deletions. Solid dots denote high-confidence calls. Empty dots designate medium-confidence predictions. **C** Long-range PCR products visualized by electrophoresis in a 1.2% agarose gel with ethidium bromide. PCR yielded a single wild-type (WT) ~10.6 kb band in the patients' mother (expected normal allele). In the patient (PPOF-178) and her father, two products were observed: an expected normal ~10.6 kb fragment and a ~3.4 kb fragment, corresponding to an allele with a ~7.2 kb deletion (del). **D** Sequence analysis of the deletion junction in PPOF-178. **E** Predicted nucleotide and amino acid sequence resulting in a deletion of exons 11–13 and a subsequent frameshift at the beginning of exon 14

Discussion

Pathogenic variants of *TP63* have been implicated in 7 distinct syndromes that have substantial phenotypic overlap: ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome (Hay-Wells syndrome, OMIM #106260), ADULT syndrome (OMIM #103285), ectrodactyly, ectodermal dysplasia, cleft lip/palate (EEC) syndrome (OMIM #604292), Rapp-Hodgkin syndrome (RHS) (OMIM #129400), limb-mammary syndrome (LMS) (OMIM #603543), split-hand/foot malformation (OMIM #605289), and isolated orofacial cleft (OMIM #618149).

Isolated primary ovarian insufficiency described in female patients carrying *TP63* variants (OMIM #620311) can generally be attributed to two types of genomic changes in an oocyte-specific isoform: those that promote p63 protein aggregation and those that alter protein domains directly involved in the regulation of p63 transcriptional activity [14, 22]. Within the first group are variants linked to AEC and RHS, which are primarily C-terminus missense and frameshift pathogenic mutations with aggregation-associated phenotypes. Mouse models of these *TP63* variants form either aggregated TAp63α dimers with low or no activity that cannot appropriately respond to DNA damage or aggregated active tetramers with reduced but constitutive activity that leads to a slow depletion of oocytes and infertility [14, 26]. This is consistent with a report of early onset menopause secondary to oocyte depletion in a patient with RHS [17]. The second group comprises frameshift and missense variants that cause loss of the TID as well as missense mutations that impair homodimerization. In both cases, the result is the presence of constitutively active tetramers that are associated with uncontrolled oocyte apoptosis [14, 22]. *TP63* variants that generate truncated p63 isoforms lacking the TID have been reported in patients presenting with LIM symptoms and POI [18, 19] and in patients diagnosed with isolated POI [21]. A more recent study of two patients with isolated POI detected two variants located in the TAD and the TID, respectively, that disrupt dimerization [22].

In the present study, we identified two *TP63* mutations in patients who presented with primary amenorrhea and non-syndromic POI. We found the previously reported p.W598* variant [21] that causes protein truncation and loss of the TID in patient SPOF-36. Subject PPOF-178 had a novel paternally inherited deletion of exons 11–13 that is predicted to generate p63 proteins truncated after the oligomerization domain, therefore lacking the SAM and TID. In these patients, the postulated mechanism of POI is through loss of the TID, uncontrolled transcription of pro-apoptotic genes, and excessive oocyte apoptosis.

The third patient, IPOF-44, presented with secondary amenorrhea and isolated POI, and carried a p.N6H missense variant in exon 3′ (the first exon encoding ΔNTp63) previously described in a female individual with ADULT syndrome [16]. The mechanism of ovarian dysfunction for this variant remains unclear. Although ΔNp63 lacks the TAD, it has been reported to exhibit some transactivation activity [27]. For example, deletion of the ΔN-specific first 14 amino acids was found to block ΔNp63α-dependent ATM promoter stimulation [28]. However, the p.N6H variant had no effect on the ATM transcriptional activity of ΔNp63α [28], suggesting that the clinical phenotypes associated with this mutation are not linked to a loss of ATM function. ΔNp63 can also repress the transactivation activity of TAp63 as well as p53 by competing for common DNA-binding sites or by forming inactive heterocomplexes with TAp63 and TAp73 [29–31]. It is therefore possible that impaired function of ΔNp63 due to the p.N6H variant leads to defective TAp63 negative regulation. Additionally, the variant in the *SIRT6* gene detected in IPOF-44 may contribute to the manifestation of POI. Considering that *SIRT6* is also involved in the genetic quality control of oocytes through recognition of DNA double-strand breaks [32], it is possible that variants in both genes act synergistically resulting in POI in this patient.

Heterozygous variants of *TP63* have been linked to several autosomal dominantly inherited conditions. The variants described in this study show the same inheritance pattern. Interestingly, the CNV found in patient PPOF-178 was also detected in her father, who presented with isolated cleft lip/palate. The p63 p.N6H variant was previously reported in a female patient with ADULT syndrome who inherited it from her father, in whom cleft lip/palate was the only feature of ADULT [16]. Similarly, POI was the only manifestation of ADULT syndrome in patient IPOF-44. Our study supports the notion that ADULT syndrome is associated with incomplete penetrance and variable expressivity, which has also been shown for other *TP63*-related conditions [16, 33–35], even in monozygotic twins [36]. Similar to patient PPOF-178, two cases of *TP63*-associated POI with primary amenorrhea have been described in two sisters carrying a paternally inherited *TP63* variant (p.R643*) [19]. Together with the fact that no male individuals carrying *TP63* variants present with impaired fertility, this suggests that *TP63* variants linked to POI with primary amenorrhea may be exclusively paternally transmitted.

While the role of TP63 in oocyte genetic quality control has been relatively well studied, its function in the male germline remains poorly understood. As mentioned above, clinical data show that males carrying *TP63* variants can reproduce. Similarly, while deletion of the TID and SAM domain in one of the TAp63α alleles in mice leads to primary ovarian insufficiency due to constitutive tetramerization and activation of p63, and subsequent uncontrolled oocyte apoptosis in females, no morphological differences or functional defects are observed in the testis [14]. Additionally, as opposed to female mice, TAp63 deficiency does not prevent spermatocyte

apoptosis in a mouse defective for DSB repair [37], indicating that the mechanisms of quality control differ in male and female germ cells. Indeed, it is possible that male and female germ cells require distinct types of quality control due to their different developmental programs. Spermatocytes are steadily mass produced, divide in a symmetrical manner, and do not spend more than a few hours in prophase. While oocytes are limited in number, divide asymmetrically, and remain arrested in a tetraploid state in prophase I for extended time (up to more than 1 year in mice and decades in humans). The length of this phase may have, as a consequence, an increased state of vulnerability of these immature oocytes, therefore demanding effective mechanisms to maintain genomic integrity for the preservation of the species. Although expression of TAp63 has been reported in male germ cells [37], the effect of *TP63* variants in spermatogenesis remains unclear.

In women undergoing cancer treatments, TAp63 expression can be activated in oocytes by cytotoxic drugs, leading to depletion of ovarian reserve [38, 39]. However, in females with *TP63* heterozygous variants, germ cell death and POI occurs in the absence of DNA damaging factors. TAp63 expression may also be triggered in response to oxidative stress, environmental exposures, or otherwise. The list of the *TP63*-related disorders should be expanded to recognize isolated POI as an additional OMIM entity caused by defects in the *TP63* gene. Future research studies may elucidate the molecular mechanisms of TAp63 activation and help in developing effective approaches for fertility assessment and preservation. Whole genome sequencing can overcome limitation of exome sequencing in identifications of changes in non-coding and regulatory regions as well as variants in mitochondrial genome, and facilitate the characterization of genetic variants that could provoke oocyte apoptosis or predispose women to the accelerated depletion of germ cells.

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Author contribution SHC, SAY, and AR collected the clinical information; SAY and AR conceived the experiments; RKV, MRE, SAY, and AR analyzed experimental data; MRE and AJB performed conservation analysis and in silico modeling. The manuscript was written by RKV, MRE, and SAY; reviewed by AJB, SHC, and AR. All authors read and approved the final manuscript.

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Data availability Some or all datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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