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RESEARCH ARTICLE

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Dominant TP63 missense variants lead to constitutive activation and premature ovarian insufficiency

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Abstract

Premature ovarian insufficiency (POI) is a leading form of female infertility, characterised by menstrual disturbance and elevated follicle-stimulating hormone before age 40. It is highly heterogeneous with variants in over 80 genes potentially causative, but the majority of cases having no known cause. One gene implicated in POI pathology is *TP63*. *TP63* encodes multiple p63 isoforms, one of which has been shown to have a role in the surveillance of genetic quality in oocytes. *TP63* C-terminal truncation variants and N-terminal duplication have been described in association with POI, however, functional validation has been lacking. Here we identify three novel *TP63* missense variants in women with nonsyndromic POI, including one in the N-terminal activation domain, one in the C-terminal inhibition domain, and one affecting a unique and poorly understood p63 isoform, TA*p63. Via

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blue-native page and luciferase reporter assays we demonstrate that two of these variants disrupt p63 dimerization, leading to constitutively active p63 tetramer that significantly increases the transcription of downstream targets. This is the first evidence that *TP63* missense variants can cause isolated POI and provides mechanistic insight that *TP63* variants cause POI due to constitutive p63 activation and accelerated oocyte loss in the absence of DNA damage.

KEYWORDS
genomic diagnosis, infertility, p63 isoforms, premature ovarian insufficiency, TAp63α, TP63

1 | INTRODUCTION

The *TP63* gene encodes the transcription factor p63, which has varied roles from ectodermal development to the maintenance of genomic integrity and female fertility. The varied roles are achieved via regulated and cell-specific expression of alternative isoforms. The p63 protein can have one of four different N-termini, denoted TA, TA*, GTA, and ΔN and one of five different C-termini denoted α, β, γ, δ, and ε (Figure 1). The longer isoforms, such as TAp63α or TA*p63α, have a transactivation domain (TAD) in their N-terminus and a transactivation inhibition domain (TID) in their C-terminus. These domains are pivotal for the controlled activation of the corresponding p63 isoforms (Krauskopf et al., 2018; Pitzius et al., 2019). The TAD and TID interact with each other to generate an inactive dimer conformation that has reduced affinity for DNA, as well as coactivators such as p300 (Deutsch et al., 2011; Krauskopf et al., 2018). In certain physiological

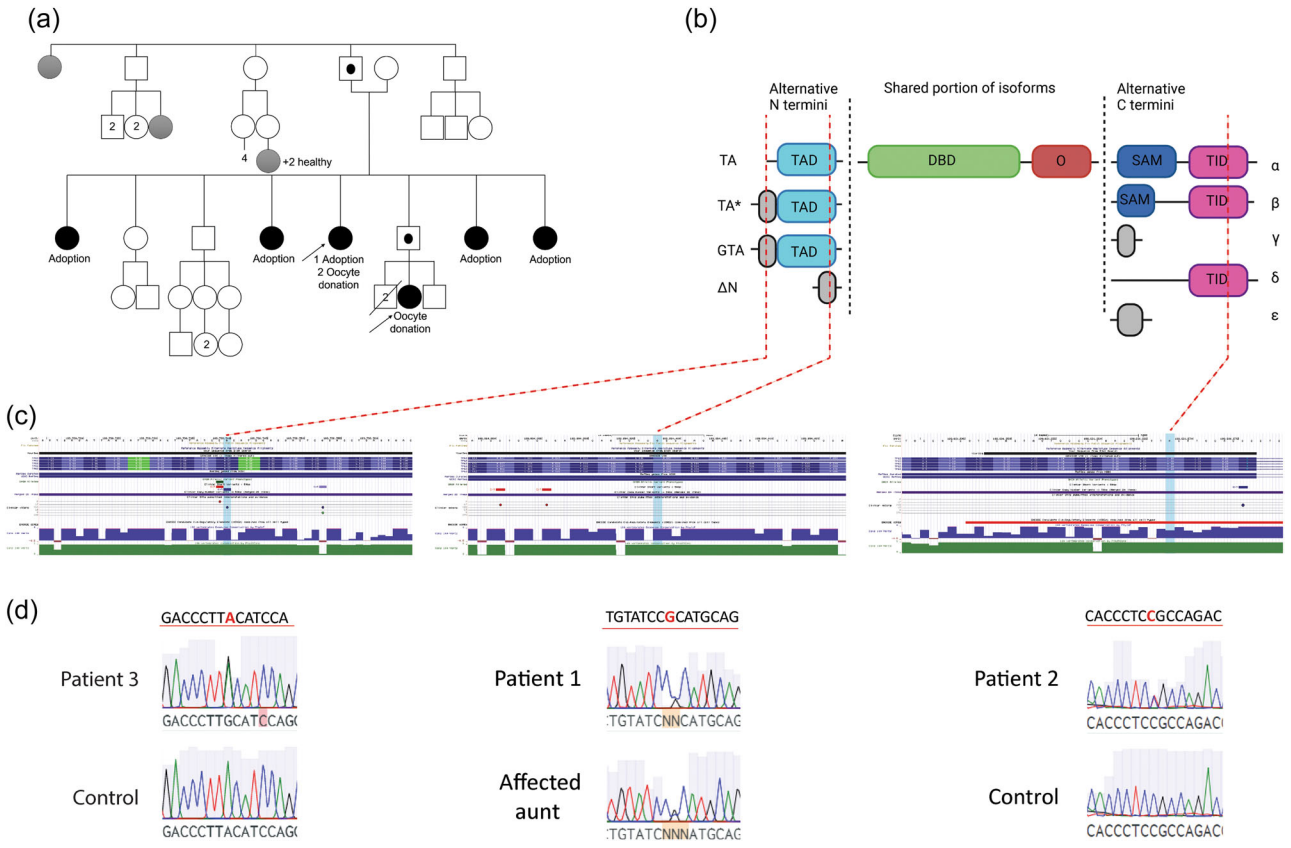

FIGURE 1 Three novel heterozygous *TP63* missense variants are identified in women with premature ovarian insufficiency. (a) Family pedigree of Patient 1. Black, atrophic ovaries; Gray, infertility with no available information on ovarian form. Arrows indicate the proband and affected aunt who underwent genomic sequencing, identifying a *TP63* missense variant. (b) Schematic diagram demonstrates p63 isoforms with four alternative N termini and five alternative C termini. The location of the three missense variants identified in POI women is indicated by red dashed lines. (c) UCSC Genome Browser https://genome.ucsc.edu/ view showing high conservation (blue bar) of the residue altered by patient missense variants. (d) Sanger validation of patient variants. POI, premature ovarian insufficiency.

conditions, a series of phosphorylation events can break this interaction, forcing p63 into an active tetramer conformation that can then activate downstream gene transcription.

Although the shorter ΔN isoforms have been shown to have a role in the development of ectodermal structures, such as the skin, hair, teeth, and nails (Koster et al., 2009; Medawar et al., 2008), the TAp63α isoform is best known and characterised for its role in the genomic integrity of oocytes and female reproduction (Suh et al., 2006). This isoform is almost exclusively expressed in female germ cells with a tightly regulated temporal expression that reflects its role in the management of DNA damage (Suh et al., 2006). It is not expressed until after homologous recombination, which involves orchestrated and permissible DNA double-strand breaks. Expression of TAp63α remains high throughout the entire dictyate arrest and only ceases when oocytes resume meiosis (Suh et al., 2006). In resting oocytes TAp63α adopts the inactive dimeric form (Coutandin et al., 2016; Deutsch et al., 2011). DNA damage leads to the phosphorylation of TAp63α by the priming kinase CHK2 followed by phosphorylation by the executioner kinase CK1 (Tuppi et al., 2018). These phosphorylation events alter the conformation of TAp63α into an active tetrameric form that induces expression of NOXA and PUMA culminating in the apoptosis of oocytes that have experienced DNA damage (Kerr et al., 2012). This maintains genomic integrity, thereby protecting female fertility (Suh et al., 2006).

In contrast to TAp63α, the alternative isoform GTAp63α is highly and exclusively expressed in spermatogenic precursors of human testes (Beyer et al., 2011). Here, its role is analogous to TAp63α in human ovaries, protecting the genomic integrity of male germ cells. Upon DNA damage it is activated by caspase cleavage of its C terminus, resulting in the induction of an apoptotic program that eliminates damaged germ cells (Beyer et al., 2011).

The role of the TA* isoform remains elusive. It has been shown to have greater activation and greater repression due to its extended N terminus (Pitzius et al., 2019) but its relevance to human biology is yet to be established. Recently a *TP63* variant affecting only this isoform of p63 was found to cause furrowed tongue, implicating a role of TA*p63 in development (Schmidt et al., 2021), but whether it also has a role in oocyte integrity remains to be established.

Not surprisingly, given its integral role in development, variants in *TP63* have been shown to cause syndromes with variable features including, but not limited to, ectodermal dysplasia, split-hand/foot malformation/syndactyly, lacrimal duct obstruction, hypoplastic breasts and/or nipples, ankyloblepharon filiforme adnatum, and cleft lip/palate (Sutton & van Bokhoven, 1993). Causative variants tend to cluster in the DNA binding domain and the downstream SAM domain (Figure 2). There is some genotype:phenotype correlation with missense variants in the DNA binding domain tending to cause Ectrodactyly-Ectodermal Dysplasia-Clefting (EEC) Syndrome characterised by missing or irregular fingers and/or toes (ectrodactyly or split hand/foot malformation) as well as cleft lip/palate (Celli et al., 1999; Russo et al., 2018) (Figure 2). In contrast, mutations in the SAM domain and frameshift variants in the C-terminal tend to cause the more severe phenotype of Ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome characterised by fusion of eyelids (ankyloblepharon), mild to severe skin erosions, abnormal hair and nails, and cleft lip/palate (Figure 2) (Russo et al., 2018).

Specific *TP63* variants have recently been found to cause premature ovarian insufficiency (POI) without syndromic features (Bestetti et al., 2019; Tucker et al., 2019). POI is defined as loss of ovarian activity associated with elevated follicle-stimulating hormone (FSH) before the age of 40. Previously reported POI variants are either truncating variants in the terminal exon of *TP63* predicted to generate a protein lacking the TID, or an intragenic duplication that causes an extra TAD. Both variants are likely to promote p63 activation, leading to accelerated oocyte death. However, this theory has not been tested via functional studies.

Despite *TP63* C-terminal nonsense and N-terminal duplication variants being a known cause of POI, the genetic basis of most POI

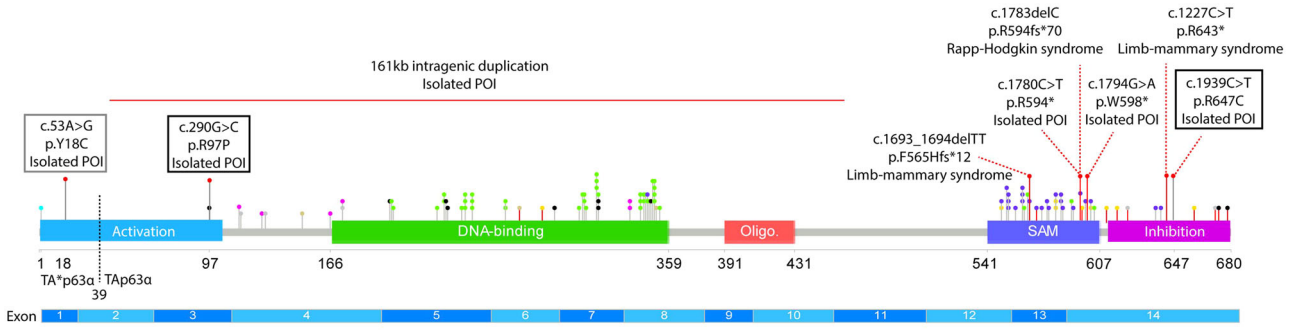

FIGURE 2 Lollipop diagram depicts pathogenic/likely pathogenic variants described in the literature, ClinVar and/or HGMD with respect to variants identified in Patients 1–3. Colour indicates associated phenotype. Boxed variants are novel missense variants associated with isolated POI and functionally analysed in this study. Black box indicates functional support for causation. Gray box indicates no functional support for causation. Lollipop colour indicates phenotype as follows: Black, split hand foot malformation; Blue, AEC syndrome; Gray, limb mammary syndrome; Green, EEC syndrome; Light blue, furrowed tongue; Magenta, ADULT syndrome; Red, POI or POI-related syndrome; Tan, Clef palate; Yellow, Rapp Hodgkin syndrome. Stem colour indicates variant type: Gray stem, missense variant; Red stem, LOF variant.

patients remains elusive. There are over 80 causative genes and these affect a variety of processes (Tucker et al., 2016). Understanding the genetic basis of POI is important because it helps to minimise the risks of comorbidities and enables cascade screening for potentially affected family members who can benefit from early intervention.

2 | MATERIALS AND METHODS

2.1 | Ethics

Written informed consent was obtained from all participants. All procedures were approved by the Ethics Committee of Rennes University Hospital and the French law (CCTIRS Comité Consultatif sur le Traitement de l'Information en matière de Recherche dans le domaine de la Santé) or the Human Research Ethics Committee of the Royal Children's Hospital, Melbourne (HREC/22073).

2.2 | Participants

Patients were recruited after clinical consultation as part of our ongoing research program investigating the genetics of POI. Family and personal medical history was collated and is included in Table 1. All patients had POI, defined by menstrual disturbance and elevated FSH (>20 mIU/ml) measured twice at least 1 month apart as per the European Society of Human Reproduction (ESHR) guidelines (https://www.eshre.eu/Guidelines-and-Legal/Guidelines/Management-of-premature-ovarian-insufficiency.aspx) (European Society for Human Reproduction and Embryology (ESHR) et al., 2016). There was no history of ovarian surgery, infection, or gonadotoxic therapy that could explain their condition. Karyotyping or microarray was performed to confirm normal 46,XX chromosomal complement, and to exclude patients with causal chromosomal rearrangements. All included cases were negative for *FMR1* premutation and negative for ovarian auto-antibodies.

2.3 | General molecular techniques

Genomic DNA was extracted from EDTA-blood manually with the NucleoSpin® Blood XL kit (Macherey-Nagel) or with an automated system, Hamilton Microlab STAR and Nucleospin® Blood L kit (Macherey-Nagel), and were assessed by NanoDrop™ 1000 spectrophotometer and Qubit dsDNA BR Assay (Thermo Fisher Scientific). Selected variants were validated by Sanger sequencing using BigDye v3.1 Terminators (Applied Biosystems) and ABI 3130X. Primer sequences are available on request.

2.4 | Whole-exome sequencing (WES)

DNA underwent WES at the Victorian Clinical Genetics Service (VCGS) or CHU-Rennes with exome capture using SureSelect QXT Clinical Research Exome V1 (Agilent) or SureSelect Human All Exon V7 (Agilent) respectively and sequencing performed on the HiSeq 4000 (Illumina) or NextSeq 500/550 (Illumina). WES data were processed using Cpipe (Sadedin et al., 2015) or C-GeVarA pipeline (Constitutional Genetc Variant Analysis) and deposited into SeqR for analysis (https://seqr.broadinstitute.org/).

We performed two phases of analysis as previously described (Tucker et al., 2016)—the first was gene-centric using a candidate POI gene list and the second was variant-centric. Variant-centric analysis focused on high-priority variants (frameshift, nonsense, or splice site variants) in any gene and with any inheritance, or potentially bi-allelic moderate-high priority variants (missense, in-frame indels, frameshift, nonsense, or splice spite). Only variants with MAF <0.005 in 1000 genomes and gnomAD (https://gnomad.broadinstitute.org/) and with high-quality scores (Q>50 and allele balance >25) were considered. Variant pathogenicity was predicted in silico using Mutation Taster (http://www.mutationtaster.org/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT/Provean (http://provean.jcvi.org/), and CADD (Combined Annotation-Dependent Depletion) (https://cadd.gs.washington.edu/snv). The conservation of affected residues was assessed by Multiz Alignments of 100 vertebrates (UCSC Genome Browser https://genome.ucsc.edu/).

2.5 | Cell culture, BlueNative-PAGE, and transactivation assays

The non-small cancer cell line H1299 (p53 null) was cultured in RPMI medium 1640 (Gibco) with 10% fetal bovine serum (Capricorn Scientific) and 1% Penicilin/Streptomycin (Gibco) at 37°C and 5% CO_2. BlueNative-PAGE and Transactivation (TA) assays were performed as described before (Pitzius et al., 2019).

For TA assays, 0.8×10^5 cells were seeded in a 12-well plate 24 h before transfection. Afterwards, equal amounts (267 ng) of p63 pCDNA3.myc, pBDS2-Luc, and pRL-CMV plasmids were transfected using Lipofectamine®2000 and grown for another 24 h. Quantification of relative luciferase activities was performed using the Promega Dual-Glo® luciferase reporter assay kit according to the manufacturer's instructions. Empty pCDNA3.myc vector served as control. Significance was calculated with one-way analysis of variance using GraphPad Prism.

For oligomeric state analysis of p63 isoforms and mutants via BlueNative (BN)-PAGE, 0.8×10^5 H1299 cells were transfected in a 12-well plate with 800 ng pCDNA3.myc p63 constructs and harvested in lysis buffer (20 mM Tris, 150 mM NaCl, 2 mM $MgCl_2$, 20 mM CHAPS, 1 mM DTT, 1× PhosSTOP [Roche], 1× cOmplete [Roche]). Cell lysis was supported by multiple freeze-thaw cycles. Subsequently, 1 µl Benzonase (Merck) was added to each sample and further incubated for 1 h on ice. The supernatant was separated by BN-PAGE (3%–12%; Thermo Fisher) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (4%–12%, Mini-PROTEAN TGX; Bio-Rad) before blotting on polyvinylidene fluoride membranes.

Membranes were blocked with 5% skim milk in TBS-T for 1 h at room temperature and probed with anti-myc primary antibody (4A6; Merck) overnight at 4°C. Signal detection was performed using goat anti-mouse HRP (A9917; Sigma Aldrich).

For stability determination of N-Terminal TA*p63α mutants via urea assay, H1299 cells were transfected in 10 cm dishes with 12 µg pCDNA3.myc p63 constructs, grown for 24 h and harvested in lysis buffer without CHAPS. The supernatant was split and incubated with different urea concentrations (0–4 M) for 1 h on ice. Equal volumes of lysis buffer containing 40 mM CHAPS was added to the samples and incubated for another 10 min on ice. BN-PAGE and immunoblotting was performed as described. Signal intensities were quantified with ImageJ.

3 | RESULTS

3.1 | Clinical history of three patients presenting with premature ovarian insufficiency

The first patient experienced menarche at 13 years of age, with one period at age 13 and one period at age 15, before commencing oral contraceptive pill (OCP). At 24 years of age, she experienced secondary amenorrhea when ceasing OCP. At this time, her FSH was elevated at 78 IU/L and estradiol was low at 20 pg/ml. Repeat hormonal assessment at 29 years of age showed high FSH (69.9 IU/L) and LH (30.5 IU/L), low estradiol (<25 pg/ml), and AMH (<0.10 ng/ml). Ultrasound examination showed atrophic ovaries and a small uterus. She was overweight, and had mild hirsutism including facial hairiness. She obtained one pregnancy through oocyte donation. She had a similarly affected paternal aunt who experienced menarche at 13 years of age with only one period then secondary amenorrhea and had a unique right atrophic ovary and small uterus on ultrasound. Exploratory endoscopy did not visualize any ovary. She also experienced facial hirsutism. She obtained two pregnancies through oocyte donation and adopted one child (Table 1). Familial history found other members in the paternal family branch with infertility, including four additional paternal aunties with atrophic ovaries and other members with infertility but without information on ovarian status (Figure 1a). None of the men of the family are reported to have infertility.

The second patient experienced menarche at 13 yo with regular menstruation from 15 to 17 years of age, and OCP from 17 to 27 years with secondary amenorrhea when ceased. Hormonal assessment at this time showed high FSH (82.67 IU/L) and LH (100.23 IU/L) and low estradiol (<5 pg/ml). Ultrasound examination showed atrophic ovaries devoid of follicles and a small uterus (Table 1). Personal history includes allergy and eczema, but no familial history of infertility.

The third patient experienced delayed puberty and primary amenorrhea (Table 1). Her FSH was elevated at 84 IU/L and AMH was low at 0.4 ng/ml. No ovaries could be observed on ultrasound and she had an atrophic uterus and short vagina. Clitoral hypertrophy was also noted.

TABLE 1 Clinical details of Patients 1–3

Patient	Age at diagnosis	Karyotype	Amenorrhea	Secondary sex characteristic	US	FSH (IU/l)	LH (IU/l)	Estradiol (pg/ml)	AMH (ng/ml)	Other
1 PROBAND	24	46,XX	Early secondary	Menarche at 13	Atrophic ovaries streak gonads	78	30.5	20	<0.10	5 Affected aunts
						69.9		<25		Hirsutism
1 AUNT	Early adulthood	46,XX	Early secondary	Menarche at 13-14	Atrophic unique ovary	-	-	-	-	Aunt of Patient 1
										4 Affected sisters
										Hirsutism
2	27	46,XX	Early secondary	Menarche at 13	Atrophic ovaries without follicles	82.67	100.23	<5	-	-
3	21	46,XX	Primary	Delayed puberty	No detectable ovaries	83	20	5	0.4	Mali descent

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Given all three patients presented only with ovarian dysfunction and related hormonal abnormalities/symptoms without other (syndromic) comorbidity, they received a diagnosis of isolated POI.

3.2 | WES identified heterozygous *TP63* missense variants

Given the number of genes potentially causing POI, the optimal strategy for genetic diagnosis is WES. As part of our larger study using WES to investigate the genetic basis of POI, we identified three rare heterozygous missense variants in *TP63* in three unrelated POI patients (Figure 1).

Patient 1 and her affected aunt shared a novel heterozygous c.290G>C, p.Arg97Pro variant (Figure 1). This variant is absent from gnomAD and alters a highly conserved residue within the N terminal TAD of TAp63α (Figure 1 and Supporting Information: Figure S1) and is predicted pathogenic based on most online algorithms (Table 2). Consistent with this site having a critical role for *TP63*, an alternative missense mutation at this site (p.Arg97Cys) is associated with isolated ectrodactyly (Zenteno et al., 2005).

Patient 2 harbored a paternally inherited novel heterozygous c.1939C>T, p.Arg647Cys variant (Figure 1), also absent from gnomAD. This variant altered a highly conserved residue within the C terminal TID and was consistently predicted pathogenic using online algorithms (Table 2 and Supporting Information: Figure S2).

In Patient 3, WES identified a novel heterozygous c.53A>G, p.Tyr18Cys variant (Figure 1), absent from gnomAD. This variant affected a residue within the unique TA* isoform of *TP63*. The residue is moderately conserved (Supporting Information: Figure S3), and pathogenicity predictions using online algorithms were conflicting (Table 2).

3.3 | *TP63* missense variants can alter protein oligomerisation

To investigate the potential causation of these three rare novel *TP63* variants, we analysed their influence on protein structure and function. We first assessed complex conformation using BN-PAGE. Using this method, dimeric and tetrameric p63 proteins can be distinguished. As a positive control, we used p63 isoforms with mutation of three residues (Phe605Ala/Thr606Ala/Leu607Ala, FTL>AAA) that disrupt dimerization and lead to constitutive open conformation, as previously described (Straub et al., 2010). We found that wild-type (WT) p63 isoforms exist mostly in the dimeric state (Figure 3).

Two of the patient variants disrupted p63 complex conformation. The p.Arg58Pro/Arg97Pro/Arg95Pro variant found in Patient 1 causes an open tetramer conformation of the TAp63α, TA*p63a, and GTAp63α isoforms, respectively (Figure 3). In contrast, changing this site to a cysteine to model the previously reported pathogenic variant at this site (Zenteno et al., 2005) or alanine, leads to

TABLE 2 Molecular detail of candidate variants in Patients 1–3

Patient	gDNA variant (GrCH38)	cDNA variant	Protein variant	gnomAD	Polyphen	Mutation Taster	CADD	SIFT	Provean	Functional support
1	chr3:189738740G>C	c.290G>C	p.Arg97Pro	0	Possibly damaging (score 0.922)	Disease-causing (score 0.997)	23.5	Damaging (score 0.007)	Neutral (score -1.12)	Yes
2	chr3: 189894398C>T	c.1939C>T	p.Arg647Cys	0	Probably damaging (score 1.000)	Disease-causing (score 1.000)	32	Damaging (score 0.000)	Deleterious (score 3.33)	Yes
3	chr3:189631568A>G	c.53A>G	p.Tyr18Cys	0	Benign (score 0.000)	Disease-causing (score 0.741)	22.3	Damaging (score 0.004)	Neutral (score -0.24)	No

Note: Variants described according to NM_003722.5 and NP_003713.3.
Abbreviations: cDNA, complementary DNA; gDNA, genomic DNA.

significant disruption of only the TAp63α isoform. Similarly, the p.Arg608Cys/Arg647Cys/Arg644Cys variant found in Patient 2 causes open active tetramer conformation of TAp63α and to a lesser extent of TA*p63a and GTAp63α. An alternative residue at this site, alanine, similarly induced the active conformation, as did alteration of a nearby arginine, p.Arg604/Arg643/Arg640 (Figure 3).

The third variant of interest, p.Tyr18Cys, alters a residue previously predicted to be important for the potential helical

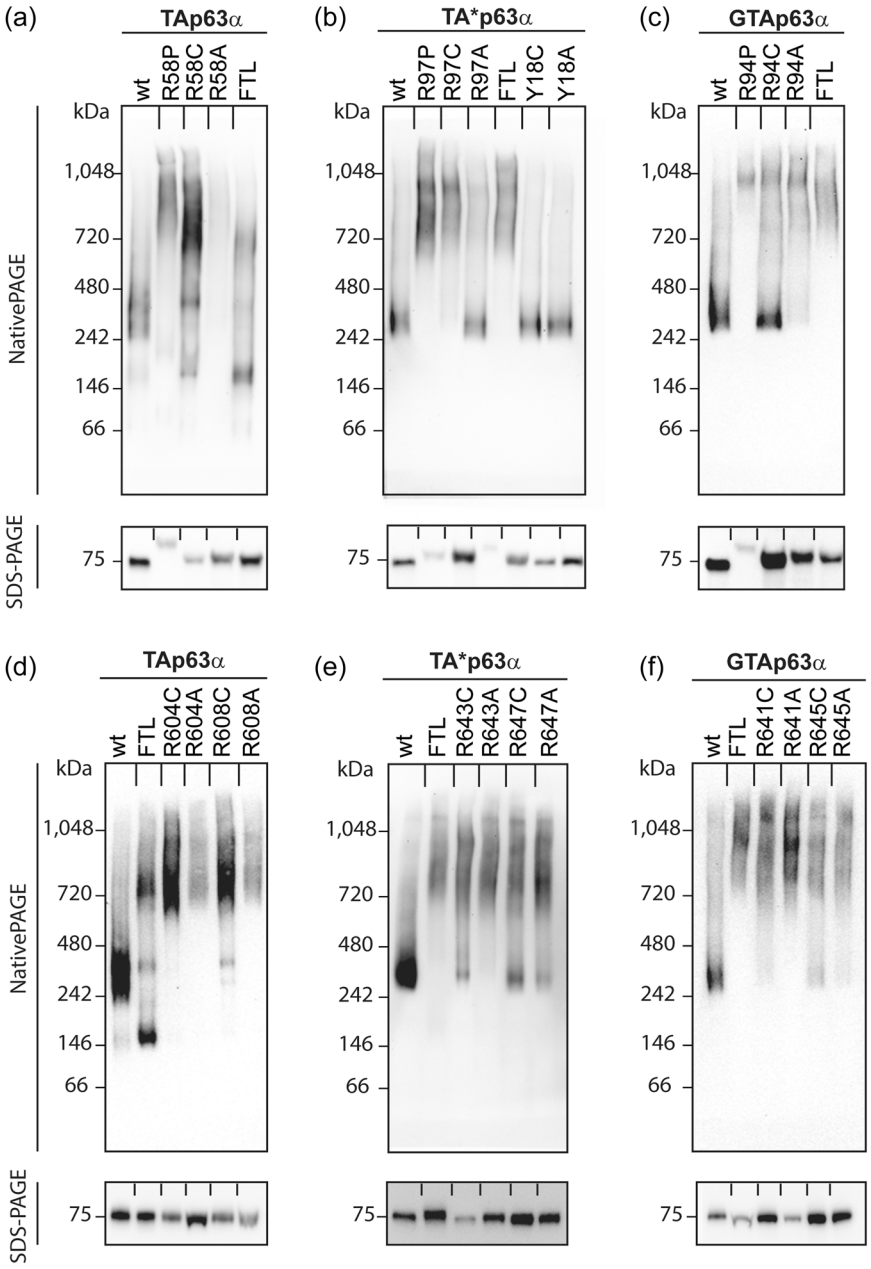

FIGURE 3 Conformation analysis of TAp63α, TA*p63α, GTAp63α mutants. Myc-tagged p63 constructs were transfected in H1299 cells and harvested after 24 h. Cell lysates were separated on BlueNative-PAGE to probe p63's oligomeric state and SDS-PAGE to probe the protein level followed by immunoblotting with an anti-myc antibody. The FTL p63 protein has mutation of three residues within the TID, known to be critical for TP63 dimerisation and acts as a tetrameric control (Coutandin et al., 2016; Straub et al., 2010). The variant of Patient 1 (NM_003722.5: c.290G>C) leads to p.Arg58Pro (TAp63α), Arg97Pro (TA*p63α), and Arg95Pro (GTAp63α), shown in panels (a–c), respectively. The variant in Patient 2 (NM_003722.5: c.1939C>T) leads to p.Arg608Cys (TAp63α), Arg647Cys (TA*p63α), and Arg644Cys (GTAp63α) shown in panels (d–f), respectively. The variant in Patient 3 (NM_003722.5: c.53A>G) causes p.Tyr18Cys in TA*p63α (b) but does not affect the other isoforms. In addition to the variants reported in this manuscript, mutation of the same residues to alternative amino acids was also investigated (a–f) as was the effect of mutation of a nearby Arg residue known to be critical (Coutandin et al., 2016) (d–f). Molecular weight markers are indicated on the left. Closed dimers are located between the 242 and 480 kDa markers. Open tetrameric conformations migrate above 720 kDa and show higher molecular species due to aggregation through the TID that is accessible in the open tetrameric state. Open tetramers can also be disrupted into monomers in the electric field showing bands above the 146 kDa mark. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TID, transcription inhibition domain; WT, wild-type.

conformation of the N-terminal extension of TA*p63 (Pitzius et al., 2019). In contrast to the other variants investigated, the p.Tyr18Cys variant and an alternative variant affecting the same residue (p.Tyr18Ala) had no detectable impact on the conformation of TA*p63α, which is the only isoform in which the residue is present (Figure 3). To determine whether the p.Tyr18Cys variant might cause a more subtle defect in protein dimerization, we performed a urea titration followed by analysis on BN-PAGE. We have previously shown that low amounts of urea can convert TA*p63α into the open tetrameric state (Pitzius et al., 2019). In this experiment, we found no difference in the concentration of urea required to disrupt the WT or variant TA*p63α dimers (Supporting Information: Figure S4).

3.4 | *TP63* missense variants can alter transcriptional activity

To confirm that the tetrameric mutant protein complexes containing the p.Arg58Pro/Arg97Pro/Arg95Pro (Patient 1) or p.Arg608Cys/Arg647Cys/Arg644Cys (Patient 2) variants have enhanced transcription of target genes, we performed luciferase reporter assays. These assays demonstrated that the p.Arg58Pro/Arg97Pro/Arg95Pro variant and the p.Arg608Cys/Arg647Cys/Arg644Cys variant cause a significant increase in transcriptional activity on a promoter with an optimized p63 binding site compared to the corresponding WT p63 isoforms (Figure 4). In contrast, and in keeping with the retained dimeric state of TA*p63 harboring the p.Tyr18Cys variant,

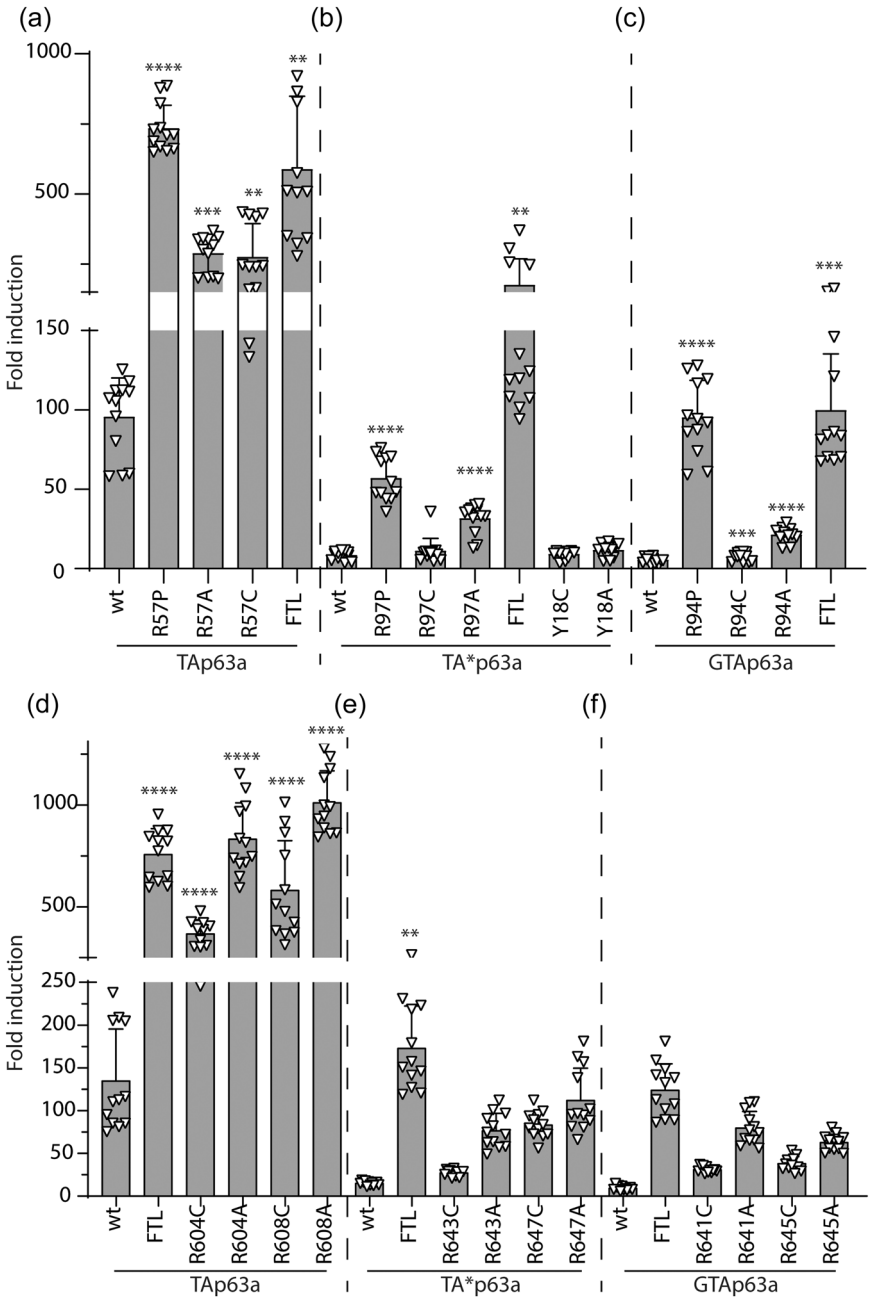

FIGURE 4 Transactivation analysis of TAp63α, TA*p63α, GTAp63α mutants. H1299 cells were cotransfected with p63 pCDNA3.myc, pBDS2-Luc, and pRL-CMV p63 constructs and relative luciferase activity after 24 h was assayed. The variant of Patient 1 (NM_003722.5: c.290G>C) leads to p.Arg58Pro (TAp63α), Arg97Pro (TA*p63α), and Arg95Pro (GTAp63α), analysed in panels (a–c), respectively. The variant in Patient 2 (NM_003722.5: c.1939C>T) leads to p.Arg608Cys (TAp63α), Arg647Cys (TA*p63α), and Arg644Cys (GTAp63α) analysed in panels (d–f), respectively. The variant in Patient 3 (NM_003722.5: c.53A>G) causes p.Tyr18Cys in TA*p63α (b) but does not affect the other isoforms. In addition to the variants reported in this manuscript, mutation of the same residues to alternative amino acids was also investigated (a–f) as was the effect of mutation of a nearby critical Arg residue (d–f). The fold induction relative to the empty vector control is shown. Experiments were performed in triplicate with technical quadruplicates. One-way ANOVA (Prism) was used for *p*-value analysis of p63 isoform mutants in comparison to the respective wildtype protein. $^{**}p < 0.005$, $^{****}p < 0.0001$. ANOVA, analysis of variance; n.s., not significant; WT, wild-type.

this variant caused no significant change in transcriptional activity (Figure 4b).

4 | DISCUSSION

4.1 | *TP63* missense variants are a novel cause of POI

Studies in mice have demonstrated the requirement of the TAp63α p63 isoform for maintenance of oocyte integrity (Suh et al., 2006). More recently, the identification of heterozygous *TP63* C-terminal truncation variants in human POI patients, demonstrated the likely requirement of functional TP63 for human ovarian function (Tucker et al., 2019). The proposed mechanism was that truncation variants remove the C-terminal repression domain leading to constitutive activation and excessive oocyte death. A N-terminal duplication has also been reported in a POI patient (Bestetti et al., 2019). Although not demonstrated, it can be hypothesized that duplication of the N terminal activation domain could similarly lead to constitutive activation and subsequent oocyte depletion. The finding of rare heterozygous missense variants in three independent POI patients, raised the possibility of missense variants also being causative. We used functional validation to prove the deleterious impact of two of these novel variants and to provide conclusive evidence that *TP63* missense variants can cause POI in humans.

4.2 | POI-associated *TP63* variants disrupt oligomerisation and transcriptional activity

To investigate the potential causation of the three rare novel *TP63* variants, we analysed their influence on protein structure and function. Via blue-native page, we demonstrated that the missense variant within the N-terminal activation domain and the missense variant within the C-terminal repression domain both disrupted TP63 dimerisation leading to an active tetrameric confirmation. We coupled this finding with luciferase reporter assays that concordantly demonstrated a corresponding increase in transcriptional activity of the mutant p63 proteins. These experiments demonstrated a clear deleterious impact on TP63 structure and function, and supports our hypothesis that POI-related variants lead to constitutive TAp63α activation, increasing expression of downstream targets that can initiate the apoptotic pathway in oocytes (Figure 5).

4.3 | The role of TA*p63 remains unknown

Although prior studies have demonstrated the critical role of the TAp63α isoform for ovarian function (Suh et al., 2006), less is known about the role of the TA*p63α and GTAp63α isoforms. These isoforms have been shown to have tighter transcriptional regulation than TAp63α due to their extended N termini (Pitzius et al., 2019). It has been proposed that GTAp63α may be the TAp63α equivalent in testes,

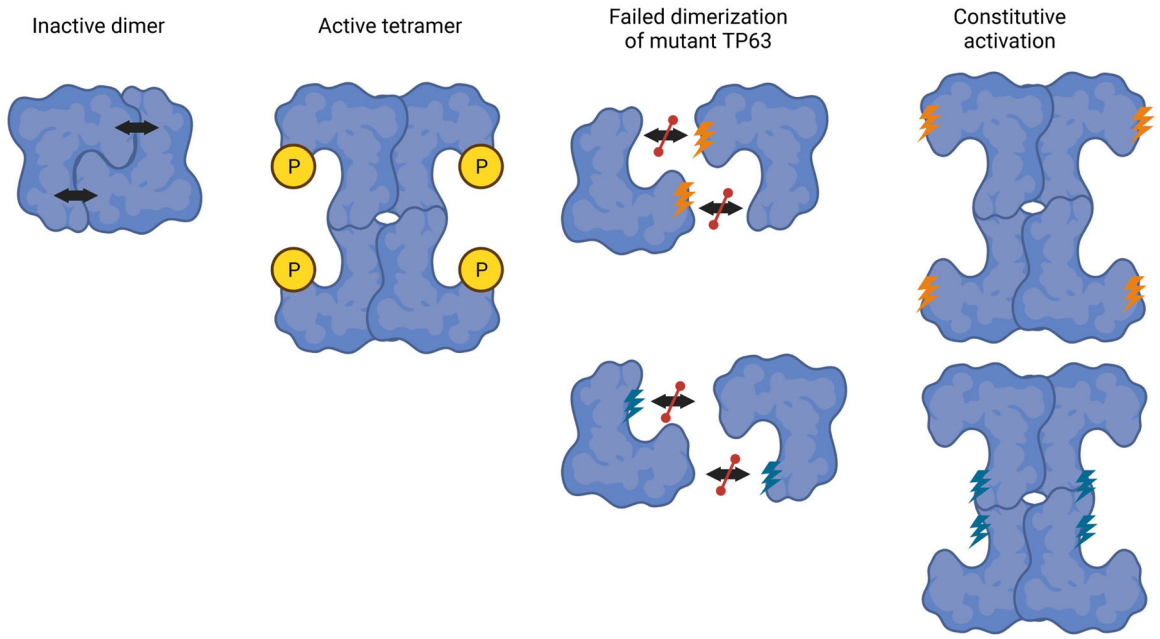

FIGURE 5 Disease model. In the absence of DNA damage TAP63α forms a closed and inactive dimer via interaction of the N terminal transcription activation domain (TAD) with the C terminal transcription inhibition domain (TID). In the presence of DNA damage, phosphorylation events occur that prevent interaction between TAD and TID leading to an open and active conformation that activates an apoptotic program leading to death of poor-quality oocytes. Pathogenic missense variants (depicted by lightning bolts) prevent interaction between TAD and TID forcing a constitutively active confirmation that leads to excessive oocyte loss.

with a role in the maintenance of germ cell integrity (Beyer et al., 2011). Whether the TA*p63α isoform has an important role in development remains to be elucidated, however, the recent identification of a variant affecting the unique N-terminus of this isoform that associated with familial cleft tongue indicates a biological role (Schmidt et al., 2021). Although we identified a rare heterozygous missense variant affecting this isoform alone in a POI patient, our functional studies were not able to demonstrate any deleterious impact on TA*p63α conformation or activity. Our study, therefore, has failed to provide further insight into the role of this isoform and further work is required to establish its biological function.

4.4 | Functional experiments allow accurate variant curation

WES is increasingly being used for the management of patients with a wide range of genetic conditions, such as POI. Following WES variants have to be carefully filtered and appropriately curated to determine those that are clinically relevant. After WES, we applied American College of Medical Genetics (ACMG) criteria to the three heterozygous *TP63* missense variants identified in the patients of this study, each of which was curated as a variant of uncertain significance (Supporting Information: Tables S1-3). With the inclusion of functional validation, however, two of the variants could be upgraded to "likely pathogenic." This demonstrates the importance of combining experimental data with genomic data for accurate diagnoses and optimal clinical outcomes.

5 | CONCLUSION

In conclusion, we have identified three novel *TP63* variants in individuals with isolated POI. One variant was in the N-terminal TAD, one was in the C-terminal TID and one was present only in the N-terminal region unique to the TA*p63 isoform. Using functional analyses, we provide evidence that two of the three variants, promote an active tetrameric conformation and an increase of transcriptional activity. We conclude that the c.290G>C, p.Arg97-Pro and c.1939C>T, p.Arg647Cys novel variants are likely pathogenic, disrupting the interaction of the activation domain with the inhibition domain and rendering the protein constitutively active, signalling for excessive oocyte death in the absence of DNA damage (Figure 5). This is the first evidence that human heterozygous missense variants can disrupt dimeric p63 conformation and cause isolated POI. The third variant remains a variant of uncertain significance. It is possible that our assays are not sensitive enough to detect the impact of the variant, or that the variant is benign, and another cause of POI exists in the patient. Whether the TA*p63 isoform has a role in ovarian development or oocyte maintenance remains elusive. This study provides better understanding of POI pathogenesis as well as the structure and function of the transcription factor, p63.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

WEB RESOURCES

https://www.eshre.eu/Guidelines-and-Legal/Guidelines/Management-of-premature-ovarian-insufficiency.aspx
https://gnomad.broadinstitute.org/
http://www.mutationtaster.org/
http://genetics.bwh.harvard.edu/pph2/
http://provean.jcvi.org/
https://cadd.gs.washington.edu/snv
https://genome.ucsc.edu/
https://varsome.com/

DATA AVAILABILITY STATEMENT

Described variants are submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/, SUB11348440). Further data generated during and/or analysed as part of the current study are available from the corresponding author on reasonable request.

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