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BRIEF REPORT

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TP63-truncating variants cause isolated premature ovarian insufficiency

Elena J. Tucker[1] | Sylvie Jaillard[1,4,5] | Sonia R. Grover[1,3,6] | Jocelyn vanden Bergen[1] | Gorjana Robevska[1] | Katrina M Bell[2] | Simon Sadedin[2] | Chloe Hanna[1,6] | Jérôme Dulon[7] | Philippe Touraine[7] | Andrew H. Sinclair[1]

[1]Reproductive Development, Murdoch Children's Research Institute, Melbourne, Victoria, Australia

[2]Bioinformatics, Murdoch Children's Research Institute, Melbourne, Victoria, Australia

[3]Department of Paediatrics, University of Melbourne, Melbourne, Victoria, Australia

[4]CHU Rennes, Service de Cytogénétique et Biologie Cellulaire, F-35033, Rennes, France

[5]INSERM U1085-IRSET, Université de Rennes1, Frances Pathologies Gynécologiques Rares, F-35042, Rennes, Paris, France

[6]Department of Paediatric and Adolescent Gynaecology, Royal Children's Hospital, Melbourne, Victoria, Australia

[7]Department of Endocrinology and Reproductive Medicine, AP-HP, Sorbonne University Medicine, Centre de Référence desMaladies Endocriniennes Rares de laCroissance et du Développement, Centre des Pathologies Gynécologiques Rares, Paris, France

Correspondence
Andrew H. Sinclair and Elena J. Tucker, Murdoch Children's Research Institute, Flemington Road, Parkville, Melbourne, VIC 3052, Australia.
Email: andrew.sinclair@mcri.edu.au (A.H.S.); elena.tucker@mcri.edu.au (E.J.T.)

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Abstract

Premature ovarian insufficiency involves amenorrhea and elevated follicle-stimulating hormone before age 40, and its genetic basis is poorly understood. Here, we study 13 premature ovarian insufficiency (POI) patients using whole-exome sequencing. We identify *PREPL* and *TP63* causative variants, and variants in other potentially novel POI genes. PREPL deficiency is a known cause of syndromic POI, matching the patients' phenotype. A role for *TP63* in ovarian biology has previously been proposed but variants have been described in multiorgan syndromes, and not isolated POI. One patient with isolated POI harbored a de novo nonsense *TP63* variant in the terminal exon and an unrelated patient had a different nonsense variant in the same exon. These variants interfere with the repression domain while leaving the activation domain intact. We expand the phenotypic spectrum of TP63-related disorders, provide a new genotype:phenotype correlation for *TP63* and identify a new genetic cause of isolated POI.

KEYWORDS
POI cohort, premature ovarian insufficiency, PREPL, TP63, whole-exome sequencing, ovarian dysgenesis

Premature ovarian insufficiency (POI) occurs when a woman experiences amenorrhea associated with elevated follicle-stimulating hormone (FSH) before the age of 40. POI can be secondary to chemotoxic/cancer therapy, physical trauma, ovarian surgery, infection, or autoimmunity, however, many cases are sporadic and unexplained. There is a clear genetic component to POI, however, it is highly heterogeneous (Tucker, Grover, Bachelot, Touraine, & Sinclair, 2016). POI-related genes have diverse roles including metabolism, folliculogenesis, gonadogenesis, oogenesis, DNA damage repair, apoptosis, hormone signaling, autoimmunity, and more. The known genes explain only a minority of cases and our understanding of POI genetics is far from complete. In addition to the genes known to be involved in human POI, there are hundreds of

candidate genes identified by studies in model organisms, by genome-wide association studies of age at natural menopause or POI, implicated by the identification of copy number variants or single-nucleotide variants (SNVs) in POI patients, by in vitro studies or a relationship to known POI genes.

To provide molecular diagnoses to patients and to identify new genes underpinning this incompletely understood condition, we recruited a diverse cohort of 13 patients through the pediatric gynecology department at the Royal Children's Hospital, Melbourne. Clinical information is included in Table S1. The diversity of our cohort reflects clinical reality, with variability in age of onset and associated features. 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 (European Society of Human Reproduction et al., 2016). Karyotyping was performed to confirm the 46,XX chromosomal complement and to exclude patients with causal chromosomal rearrangements. All included cases were negative for *FMR1* premutation and negative for ovarian autoantibodies. Written informed consent was obtained from all participants and procedures were in accordance with the ethical standards of the Human Research Ethics Committee of the Royal Children's Hospital, Melbourne. The familial DNA was obtained from eight patients, with pedigrees depicted in Figure S1. The DNA underwent high-density microarray analysis and whole-exome sequencing (WES), followed by the gene- and variant-centric data analyses. Detailed methods are provided in the Supporting Information materials and the genes prioritized for gene-centric analysis are listed in File S1. Sequencing statistics including the mean depth of coverage are provided in File S2. Reported SNVs are submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).

We were able to identify the genetic cause of POI in Patient 8, who presented with primary amenorrhea and had a diagnosis of isolated POI. WES of the patient and both her parents revealed 572 high-quality coding variants in the patient. Ten of these were within candidate POI genes, although based on their segregation, pathogenicity predictions/conservation scores, minor allele frequency, and/or the lack of a second variant in the same gene, these did not represent strong causal candidates.

Variant-centric analysis revealed 29 high-priority variants, none of which showed recessive-type inheritance, and 10 of which had a potential dominant inheritance, being absent in the unaffected mother (presence in the father was permissible given POI is female-specific). The strongest candidate variant was a de novo nonsense variant in *TP63* (MIM #603273). The NM_003722.4 (TP63):c.1780C>T p.(Arg594Ter) variant is within the terminal exon of *TP63* and is predicted to introduce a premature stop codon. Its location in the final exon means the resulting transcript likely escapes nonsense-mediated decay, and a truncated p63 protein is predicted. TP63 has at least six different isoforms (Figure 1a). Variants have been described in congenital multiorgan syndromes (Figure 1b). The c.1780C>T p.(Arg594Ter) variant falls within the sterile α motif domain and truncates TP63 before the transactivation inhibitory domain. Using the American College of Medical Genetics and Genomics (ACMG) guidelines (American College of Medical Genetics and Genomes, 2015), this variant was curated as pathogenic (Table S2).

To investigate the contribution of *TP63* to POI, we screened the terminal exon of *TP63* for variants by Sanger sequencing in an additional 107 POI patients. This revealed a different *TP63* C-terminal-truncating variant in an unrelated patient (Figure 1c), who also presented with primary amenorrhea and had a diagnosis of isolated POI. She carried a heterozygous NM_003722.4 (TP63):c.1794G>A p.(Trp598Ter) variant, also considered pathogenic by ACMG guidelines given it was a null-type variant in a known disease gene, was absent from controls and was associated with the same phenotype as the original patient who carried a pathogenic variant in this gene (Table S2). We performed WES on this patient and verified that no other likely causative variant was present.

Other *TP63* variants detected by Sanger sequencing or WES were not considered likely pathogenic. One patient carried an intronic NM_003722.4 (TP63):c.1747−34T>C variant, which was not detected in the public databases and fell within a predicted branch-site region for RNA splicing, however, was predicted to enhance its strength as a branch site (http://www.umd.be/HSF3/). Two patients carried a common SNP in the 3′-untranslated region, NM_003722.4 (TP63):c.2043+45C>T. No other *TP63* variants were detected in the remaining 103 patients.

We failed to detect the *TP63* causative variant in Patient 8 using our gene-centric protocol. Although this gene had not previously been recognized as a POI candidate by us, literature search confirmed a clear potential for involvement. The longest TP63 isoform, referred to as TAp63α, is almost exclusively expressed in female germ cells and is considered a "supervisor and executioner" that ensures germ-cell fidelity (Crum & McKeon, 2010). The timing of TAp63α expression is consistent with a role in the management of DNA damage of oocytes. It is not expressed during the nonreductive DNA replication, during which homologous recombination occurs and DNA double-strand breaks are permissible. As oocytes enter dictyate arrest, TAp63α expression is initiated and maintained until oocytes are recruited for ovulation (Suh et al., 2006). During this time, TAp63α induces apoptosis in oocytes that have undergone DNA damage (Gonfloni, 2010; Suh et al., 2006). Despite this important role in the protection of oocytes, *TAp63* null mice have no ovarian phenotype in normal conditions (Suh et al., 2006; Yang et al., 1999). The role of TAp63, however, becomes evident under genotoxic stress. Irradiated wild-type mice experience a severe and specific loss of small primordial follicles. In contrast, the same radiation dose does not lead to loss of oocytes in TAp63 null mice, confirming that TAp63 is essential for oocyte death in response to DNA damage (Suh et al., 2006).

Pathogenic variation in *TP63* is usually associated with autosomal dominant congenital malformation of multiple organs, particularly those of ectodermal origins, such as hair, teeth, and skin. These include ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome (MIM #604292), acro-dermato-ungual-lacrimal-tooth syndrome (MIM #103285), ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome (MIM #106260), Hays-Wells syndrome (MIM #106260), Rapp-Hodgkin syndrome (MIM #129400), and limb-mammary syndrome (MIM #603543; Rinne, Hamel, van Bokhoven, & Brunner, 2006). Two TP63-related cases are described in the literature with POI as a component of their syndromic phenotype (Guazzarotti et al., 2008; Holder-Espinasse, Martin-Coignard, Escande, & Manouvrier-Hanu,

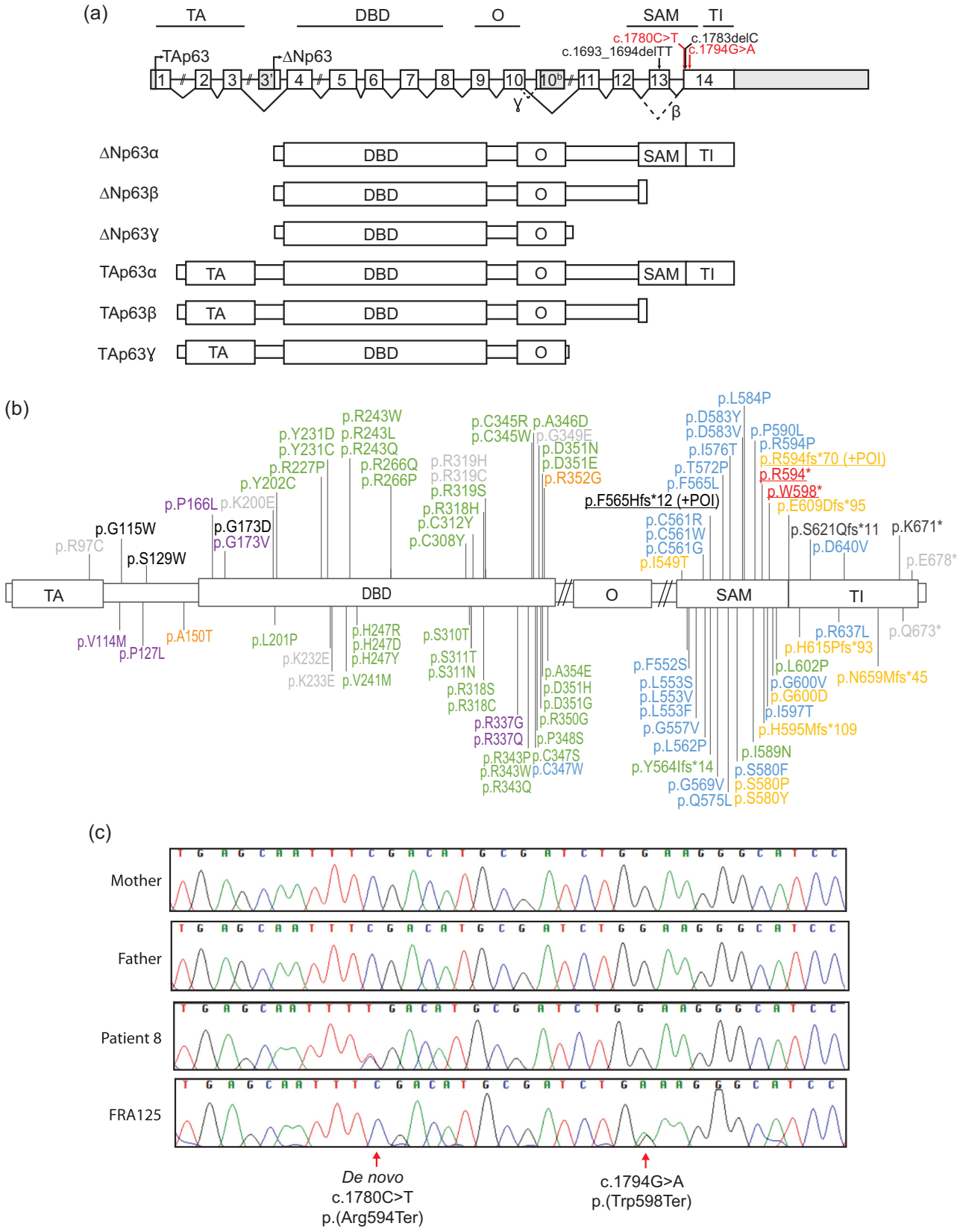

FIGURE 1 Continued.

2007). No syndromic malformations were noted in the two patients with *TP63* nonsense variants detected by our study. All variants causing TP63-related POI affect the C-terminal transactivation inhibition domain (Figure 1b), but the syndromic cases are associated with frameshift variants in contrast to the isolated POI cases described here with nonsense variants. Phenotypic and genetic comparison of the patients with syndromic POI described in the literature and the two patients identified by this study are provided in Table S3.

Taken together, these cases clearly demonstrate the importance of the C-terminal transactivation inhibition domain of TP63 to oocyte function. In nonstressed oocytes, TAp63α exists as an inactive dimer (Deutsch et al., 2011). This conformation relies on the interaction between the N-terminal transactivation domain and the C-terminal transactivation inhibition domain (Deutsch et al., 2011; Serber et al., 2002; Straub et al., 2010). DNA damage and oocyte stress lead to the phosphorylation of TP63, disrupting these inhibitory interactions, allowing TP63 to tetramize. This active tetramer is then able to bind DNA, activate transcription, and promote oocyte destruction (Deutsch et al., 2011). The TP63 truncation variants detected in our patients likely disrupt TP63 inhibition, leading to uncontrolled oocyte death and subsequent POI. The genotype:phenotype correlation for *TP63* has been investigated previously, establishing that variants causing the life-threatening AEC syndrome tend to be C-terminal frameshift variants that promote protein aggregation (Russo et al., 2018). In contrast, variants causing the milder EEC syndrome tend to be missense variants within the DNA-binding domain and are not associated with protein aggregation. Frameshift variants that introduce aggregation signals in the C terminus of TP63, not only promote self-aggregation but also cause aggregation with the related TP73 (Russo et al., 2018), thereby impacting multiple developmental processes and having a broader phenotypic consequence. It is possible that the frameshift variants in the cases of TP63-related syndromic POI described in the literature introduce such aggregation signals, but the nonsense variants in the two patients described here, do not, leading to their milder phenotype of isolated POI.

A second genetic diagnosis was achieved in our POI cohort, for two sisters (Patient 3 and 3S) diagnosed postnatally with an undefined neuromuscular condition. They both experienced hypotonia and delayed mobility in infancy, and primary amenorrhea in adolescence. Slight dysmorphism was noted (mainly high-arched palate), as well as short stature, nasal speech, mild learning difficulties, and intermittent ptosis. A gene-centric analysis focusing on the candidate genes did not identify any likely causative variants, but variant-centric analysis revealed an apparently homozygous NM_006036.4 (PREPL): c.881_955delinsTTTTA p.(Lys294IlefsTer8) variant within the *PREPL* gene (MIM #609557) shared by the sisters. The patients' mother was heterozygous for the same variant, whereas no likely pathogenic *PREPL* variant was detected in the patients' father (Figure S2A). The lack of paternal variant indicated a potential deletion had been missed by WES. We performed high-density microarrays which showed that, as suspected, both sisters carried a heterozygous deletion, arr[GRCh37] 2p21(44505149_44579929)x1. By polymerase chain reaction and sequencing, we determined the base pair resolution of the deletion to be seq[GRCh37] del(2)(2p21) chr2:g.44504991_44580502del, and confirmed that the 75.5-kb deletion was paternally inherited (Figure S2B). The heterozygous deletion spans the last nine exons of the *SLC3A1* gene and the last 13 exons of the *PREPL* gene (Figure S2C). Homozygous deletion of this region, which encompasses *SLC3A1* as well as *PREPL* is a well-established cause of hypotonia-cystinuria (MIM #606407), a condition with clinical overlap with the sisters' phenotype. The combination of the large deletion and the novel indel affecting only *PREPL*, led to the diagnosis of isolated PREPL deficiency. At the time of genetic diagnosis, isolated PREPL deficiency (MIM #616224) had only been described once in the literature with no long-term follow-up of the affected patient (Regal et al., 2014). Recently, endocrine involvement, including hypergonadotropic hypogonadism (POI), has been described in PREPL deficient patients (Regal et al., 2018). Scoring the *PREPL* variants using ACMG guidelines (American College of Medical Genetics and Genomes, 2015), reveals these null-type compound heterozygous variants that segregate with disease in the family, are pathogenic (Table S2). This enabled clarification of the patients' undefined neuromuscular condition thereby improving patient management and confirms the involvement of endocrine dysfunction and POI in this recently characterized condition.

We were unable to provide conclusive diagnoses to the remaining 11 patients in our cohort, however, data analysis identified intriguing candidate variants, as summarized in Table 1 and File S3. A detailed discussion of candidate variants is provided in the Supporting Information materials. These candidate variants are found in DNA damage repair genes (*RAD50*, *MRE11A*, *RAD51C*, *NEK8*, *MCM9*, and *RAD51C*), genes involved in transcriptional function, and downstream genome stability (*ERCC6L2* and*CCNT2)*, genes involved in DNA replication (*CDC7* and *DDI1*), chromosomal alignment (*CENPI* and *REC8*), chromosomal segregation (*CAMSAP2*, *SSH1*, and *CROCC1*), and genes involved in folliculogenesis (*HERC1*, *GREM1*, *ESRRG*, and *ESRRB*).

FIGURE 1 Pathogenic *TP63* variants in Patient 8 and an unrelated POI patient. (a) Schematic diagram depicts the *TP63* exonic structure (top) and protein isoforms (bottom). Transactivation (TA), DNA-binding (DBD), oligomerization (O), sterile α motif (SAM), and transactivation inhibition (TI) domains are indicated. The location of the two pathogenic variants found in this study is indicated by red arrows. The location of the pathogenic variants associated with syndromic POI described in the literature is indicated by black arrows. (b) Pathogenic *TP63* variants are indicated with respect to their location within the isoform relevant to oocyte function, TAp63α. Color depicts the phenotype of described patients. Gray: split hand/foot malformation, purple: acro-dermato-ungual-lacrimal-tooth syndrome, black: limb-mammary syndrome, orange: cleft palate, hypohidrosis, and oligodontia, green: ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome, yellow: Rapp-Hodgkin syndrome, blue: ankyloblepharon-ectodermal defects-cleft lip/palate syndrome, red: isolated POI. Underlined variants have been described with POI as a feature. (c) Sequence chromatogram demonstrates the de novo c.1780C>T p.(Arg594Ter) variant in *TP63* (NM_003722.4) in Patient 8, and the heterozygous c.1794G>A p.(Trp598Ter) variant in an unrelated patient, FRA125. POI: premature ovarian insufficiency

TABLE 1 Variants of interest

ID	Phenotype	Gene	RefSeq	Variant
Patient 1	POI, facial palsy, ear deformity, and deafness	*ERCC6L2*	NM_020207.4	c.2189delG p.(Gly730AspfsTer50)
		ESRRG	NM_001243518.1	c.1103A>G p.(Tyr368Cys)
		ESRRB	NM_004452.3	c.3G>A p.(Met1?)
Patient 2	POI, Wilm's tumor, and multiple sclerosis	*CDC7*	NM_001134419.1	c.683_689delCCCACAT p.(Ser228Ter)
		DDI1	NM_001001711.2	c.692_714dup p.(Gly239Ter)
Patient 3[a]	POI, and undefined neuromuscular condition	*PREPL*	NM_006036.4	c.881_955delinsTTTTA (p.K294Ifs8[a]), seq[hg19] del (2)(2p21) chr2:g.44504991_44580502del
Patient 4	POI	*SOX3*	NM_005634.2	c.1297G>T p.(Ala433Ser)
		USP53	NM_019050.2	c.173G>A p.(Arg58Gln), c.1253G>A p.(Arg418Gln)
		UBR4	NM_020765.2	c.2012G>A p.(Arg671Gln)
Patient 5	POI	*DNAH1*	NM_015512.4	c.2717A>G p.(Asp906Gly), c.4994T>C p.(Met1665Thr)
		USP53	NM_019050.2	c.173G>A p.(Arg58Gln), c.2975T>G p.(Phe992Cys)
		ESRRG	NM_001243518.1	c.55_56dupGT p.(Pro20PhefsTer36)
Patient 6	POI	*MRE11A*	NM_005591.3	c.305G>T p.(Gly102Val)
		NUP107	NM_020401.3	c.61C>T p.(Arg21Trp)
		CAMSAP2	NM_203459.2	c.1868T>C p.(Met623Thr), c.2510T>G p.(Ile837Arg)
		STAG1	NM_005862.2	c.634G>A p.(Asp212Asn)
Patient 7	POI	*MCM9*	NM_017696.2	c.911A>G p.(Asn304Ser)
		BMPR1B	NM_001203.2	c.605A>G p.(Lys202Arg)
		SSH1	NM_018984.3	c.151_175dup p.(Gln59LeufsTer23)
		NEK8	NM_178170.3	c.1795C>T p.(Arg599Ter), c.1100G>C p.(Ser367Thr)
Patient 8[a]	POI	*TP63*	NM_003722.4	c.1780C>T p.(Arg594Ter)
FRA125[a,b]	POI	*TP63*	NM_003722.4	c.1794G>A p.(Trp598Ter)
Patient 9	POI	*CENPI*	NM_006733.3	c.497G>A p.(Arg166His)
Patient 10	POI (familial)	*NTRK3*	NM_002530.3	c.1495G>A p.(Asp499Asn)
Patient 11	POI	*REC8*	NM_005132.2	c.872C>T p.(Pro291Leu)
		AMHR2	NM_020547.3	c.1280T>G p.(Leu427Trp)
		HERC1	NM_003922.3	c.8236A>G p.(Thr2746Ala), c.4931T>C p.(Ile1644Thr)
		RAD51C	NM_058216.2	c.431T>C p.(Ile144Thr)
Patient 12	POI, and polyglandular disease, Type II	*NUP155*	NM_004298.3	Chr5:37370756_37370766del (5′-UTR splice site)
		CCNT2	NM_058241.2	c.430 + 1G>C Essential splice site
		FOXK2	NM_004514.3	c.1436C>T p.(Ala479Val), c.154C>T p.(Arg52Cys)
Patient 13	POI	*GREM1*	NM_013372.6	c.466C>T p.(Leu156Phe)
		RAD50	NM_005732.3	c.2177G>A p.(Arg726His), c.3907A>G p.(Ser1303Gly)

Note. Further information is provided in File S3 and Supporting Information materials.
5′-UTR: 5′-untranslated region; POI: premature ovarian insufficiency.
[a]Pathogenic variants according to the American College of Medical Genetics and Genomics guidelines, 2015 (American College of Medical Genetics and Genomics, 2015). Refer to Table S2.
[b]From a different POI cohort.

Interestingly, the two genetic diagnoses achieved in our small cohort did not harbor causative variants in known and well-established POI genes. They were therefore filtered out in the first phase of analysis, which was "gene-centric" and focused on variants within genes of a predefined list (File S1). Indeed, several lists of POI-related genes have been published and/or used in next-generation sequencing studies (Arboleda et al., 2013; Bramble et al., 2016; Patino et al., 2017; Qin, Jiao, Simpson, & Chen, 2015; Tucker et al., 2016). A list of suspected POI genes is difficult to compile, and there is a need to compromise between the number of candidate genes included and the ease of downstream analysis. Including only genes with evidence linking them directly to human POI may significantly reduce the number of variants that need to be assessed for their potential involvement, but may cause many variants to be overlooked in genes that are not so well-linked to POI. In contrast, a comprehensive candidate list that includes any gene with a direct or indirect link to ovarian function does not so effectively reduce the number of variants of interest and the effort of downstream analysis.

In keeping with this, all reported lists have a varied capture of POI genes, and there has been only a modest success using panels for the genetic analysis of patients with POI. Recognizing the shortcomings of stringent criteria for analysis, WES data from published POI cohorts have been reanalyzed to identify previously missed diagnoses in *KHDRBS1*, *ATG7*, and *ATG9A* (Delcour et al., 2018; Wang et al., 2017). Our gene list contained 482 genes which had a predicted role in POI based on known variants in human cases, relevant phenotypes in mice with gene disruption, genetic associations in POI cohorts, and/or in vitro studies (File S1). We, too, acknowledge the shortcomings of our gene list and modified our analysis protocol to investigate three types of variants, (a) any variant in a candidate POI gene, (b) high-priority variants in any gene, and (c) recessive-type variants in any gene. This enabled the genetic diagnosis of patients with causative *TP63* and *PREPL* variants, neither of which was in our candidate gene list (nor those published by others) despite a potential role in human ovarian biology.

Despite the successful genetic diagnosis in this small POI cohort (two cases of 13 analyzed), the majority of cases remain unexplained and warrant further investigation. As presented in Table 1 and Supporting Information File S3 and discussed in Supporting Information materials, most patients harbor multiple variants of interest. It is possible that multiple variants cause POI in a multigenic manner. An example of potential oligogenic inheritance is demonstrated in Patient 7 who carries a likely damaging variant in *MCM9* as well as *BMPR1B*. The particular *MCM9* variant has previously been reported in at least three other POI patients, one of whom also harbors a likely damaging *BMPR1B* variant (Desai et al., 2017; Patino et al., 2017). The repeated detection of this *MCM9* variant in POI patients could be due to chance or could reflect a genetic predisposition to POI, with POI only manifesting if another gene, such as *BMPR1B*, is also compromised.

The selection of variants for functional validation requires careful consideration. It is important to sequence familial DNA, when available, as the inheritance pattern can inform the likelihood of causation. The sequencing of larger cohorts enabling the recognition of multiple patients and families affected by variants in the same gene will further aid prioritization of variants for functional validation. The experiments required for functional validation are gene-dependent and could involve DNA damage repair assays, reporter gene assays, gene knockdown in cell lines or animal models, and more. The variants detected and discussed in our patient cohort will provide insight for future studies into the genetic basis of POI.

In summary, we have performed WES on a small cohort of 13 POI patients providing molecular diagnoses to two families, one with pathogenic *PREPL* variants and one with a pathogenic *TP63* variant. We have established that C-terminal truncation variants of *TP63* can cause isolated POI, expanding the phenotypic spectrum of the TP63-associated syndromes, providing a new genotype:phenotype correlation for *TP63* variants, and expanding the knowledge of the genetic cause of isolated POI. The cause of POI in the remaining patients remains elusive but many interesting candidates were identified in genes involved in DNA damage repair, transcription, DNA replication, chromosomal alignment, and segregation, as well as folliculogenesis. These variants will be the focus of future studies, as will be the sequencing of larger POI cohorts to identify additional affected families.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

E. J. T., S. J., S. G., P. T., and A. H. S. conceived and designed the study. E. J. T., S. J., S. G., C. H., J. B., and G. R. acquired the data. E. J. T., S. J., K. B., S. G., P. T., and A. H. S. analyzed and interpreted the data. E. J. T., S. J., S. G., C. H., J. B., G. R., K. B., J. B., P. T., and A. H. S. wrote and/or revised the manuscript.

ORCID

Elena J. Tucker http://orcid.org/0000-0002-6380-5100

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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