



# Whole-exome sequencing reveals *SALL4* variants in premature ovarian insufficiency: an update on genotype–phenotype correlations

Qiqi Wang<sup>1,2,3</sup> · Da Li<sup>4</sup> · Baozhu Cai<sup>1</sup> · Qing Chen<sup>1,2,3</sup> · Caihua Li<sup>5</sup> · Yanhua Wu<sup>1</sup> · Li Jin<sup>1</sup> · Xiuxia Wang<sup>4</sup> · Xiaojin Zhang<sup>1,3</sup> · Feng Zhang<sup>1,2,3</sup>

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## Abstract

Premature ovarian insufficiency (POI) is a severe female disorder characterized by primary or secondary amenorrhea before 40 years of age. Genetic factors have been implicated in the pathogenesis of POI, but known POI-associated genes account for only a small fraction of heritability. Here, we performed whole-exome sequencing (WES) to explore pathogenic genes in Han Chinese subjects with POI. Intriguingly, we identified novel or rare heterozygous missense variants of *SALL4* (*spalt-like transcription factor 4*) in 3 (6%) of 50 POI subjects. The *SALL4* c.541G>A and c.2279C>T variants were paternally inherited, while c.1790A>G was inherited from an affected mother with early menopause. *SALL4* encodes a transcription factor that is highly expressed in oocytes and early embryos. Our in vitro functional assays suggested that all of these *SALL4* missense variants had significantly increased *SALL4* protein expression with enhanced regulatory activity in regard to its downstream target *POU5F1* compared to that of wild-type *SALL4*. Notably, previous studies demonstrated the genetic involvement of *SALL4* loss-of-function variants in Okihiro syndrome and related syndromic developmental disorders. Through our analysis of genotype–phenotype correlations, we suggest that different variation types of *SALL4* might have different effects on *SALL4* activity, resulting in phenotypic variability. Our findings highlight the genetic contribution of *SALL4* missense variants with enhanced regulatory activities to POI and underscore the importance of variant classification and evaluation for molecular diagnosis and genetic counseling.

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✉ Xiaojin Zhang  
zxjgcp@sina.com

✉ Feng Zhang  
zhangfeng@fudan.edu.cn

- <sup>1</sup> Obstetrics and Gynecology Hospital, NHC Key Laboratory of Reproduction Regulation (Shanghai Institute of Planned Parenthood Research), School of Life Sciences, Fudan University, Shanghai 200011, China
- <sup>2</sup> Center for Global Health, School of Public Health, Nanjing Medical University, Nanjing 211116, China
- <sup>3</sup> Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai 200011, China
- <sup>4</sup> Department of Obstetrics and Gynecology, Shengjing Hospital of China Medical University, Shenyang 110004, China
- <sup>5</sup> Genesky Biotechnologies Inc., Shanghai 201315, China

## Introduction

Premature ovarian insufficiency (POI) is defined as the cessation or disturbance of the menses for four or more months in women under 40 years old that is accompanied by two measurements of elevated serum follicle-stimulating hormone (FSH) levels (> 25 IU/l) at least 4 weeks apart (The ESHRE Guideline Group on POI et al. 2016). POI affects approximately 1–5% of women worldwide (De Vos et al. 2010). This disorder occurs through two major pathogenic mechanisms: follicle dysfunction or follicle depletion (Nelson 2009). Though the etiology of POI remains unclear, genetic aberrations, autoimmune disorders, and iatrogenic and environmental factors have been confirmed to be involved in the pathogenesis of POI (Goswami and Conway 2005). Among them, genetic factors have been regarded as the primary causes of POI. Notably, 14–31% of subjects with POI have at least one affected relative with POI or early menopause, supporting a strong genetic predisposition (Tucker et al. 2016).

Recently, whole-exome sequencing (WES) studies have provided solid evidence for genetic contributions to POI pathogenesis (Qin et al. 2015). For example, *NOBOX* (Li et al. 2017b) and *SOHLH1* (Bayram et al. 2015), two genes encoding transcription factors that regulate oocyte-specific gene expression during the early stages of folliculogenesis, have been reported to cause POI in humans. However, known POI-associated genes account for only a small fraction of POI pathogenesis (Qin et al. 2015). Other causative or susceptible genes for POI have yet to be identified.

In this study, we performed a genetic analysis using WES of 50 Han Chinese subjects with POI and identified novel or rare missense variants in *SALL4* (*spalt-like transcription factor 4*) from three unrelated families. Further in vitro functional assays suggested that these *SALL4* missense variants with enhanced regulatory activities contribute to the etiology of POI.

## Materials and methods

### Study subjects

A total of 50 Han Chinese women with POI (48 sporadic cases and 2 familial cases) were enrolled at the Obstetrics and Gynecology Hospital of Fudan University and at Shengjing Hospital of China Medical University between September 2014 and February 2017. The inclusion criteria consisted of primary or secondary amenorrhea for at least 4 months before 40 years of age, along with two measurements of abnormal serum FSH levels ( $> 25$  IU/l). All the subjects with POI in this study had a normal 46, XX karyotype. *FMRI* premutations were excluded using a FragileE-ase™ PCR Reagent Kit (Chen et al. 2017). Women with ovarian surgery or radiotherapeutic or chemotherapeutic interventions were excluded. This study was approved by the institutional review boards at Fudan University and at Shengjing Hospital of China Medical University. Written informed consent was obtained from all participants.

### Whole-exome sequencing and variant calling

Genomic DNA was extracted from peripheral blood samples using a Puregene Blood Core Kit B (QIAGEN, Hilden, Germany) according to standard procedures. WES was performed for the 50 POI subjects using a SureSelectXT Human All Exon V6 kit (Agilent Technologies, Santa Clara, USA). For each subject, 1.5 µg of genomic DNA was used to prepare a captured library that was then sequenced on a HiSeq X Ten platform (Illumina, San Diego, USA), generating 150 bp paired-end reads. Raw data of approximately 10 GB per exome were mapped to a human reference genome sequence (GRCh37/hg19) using the Burrows–Wheeler

Alignment (BWA) tool (Li and Durbin 2010). Variant calling was performed using the Genome Analysis Toolkit (GATK) (McKenna et al. 2010). All variants were further annotated with ANNOVAR software (Wang et al. 2010).

### Gene filtration and bioinformatic analysis

Rare and novel protein-altering variants (nonsense, frameshift, essential splicing-site and missense) were preferred in this study. Variant frequency was analyzed based on different ethnic subgroups from the Exome Aggregation Consortium (ExAC) and the Genome Aggregation Database (gnomAD) (Lek et al. 2016) as well as the 1000 Genomes Project (The 1000 Genomes Project Consortium et al. 2012). Single-nucleotide variants (SNVs) were analyzed with the SIFT (Kumar et al. 2009), PolyPhen-2 (Adzhubei et al. 2010), MutationTaster (Schwarz et al. 2014), CADD (Kircher et al. 2014) and DANN tools (Quang et al. 2015) for functional prediction. Relevance to phenotype was considered on the basis of OMIM, gene ontology terms (Ashburner et al. 2000), KEGG pathway analysis (Kanehisa et al. 2017) and mouse model studies.

### Variation validation

After filtering against multiple databases, Sanger sequencing was used to validate the novel and rare *SALL4* gene variants. PCR products were sequenced on a 3730XL genetic analyzer (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. The designed primers are shown in Table S1.

### Array-based comparative genomic hybridization analysis

For three subjects with *SALL4* missense variants, we further conducted a copy number variation (CNV) analysis using an Agilent SurePrint G3 Human 1 × 1M comparative genomic hybridization (CGH) microarray. Some of the experimental details were described previously (Boone et al. 2010). DNA processing, microarray handling and data analysis were conducted according to the Agilent oligonucleotide CGH protocol (version 6.3). CNV calling was performed using Agilent Genomic Workbench software.

### Plasmid construction and mutagenesis

A luciferase reporter plasmid containing the *POU5F1* promoter was constructed as described previously (Chew et al. 2005). A 3-kb fragment of the human *POU5F1* promoter was cloned into the pGL3-Basic vector (Promega, Madison, USA). The human full-length *SALL4* expression plasmid (RC213089) and pCMV6-Entry vector (PS100001) were

obtained from OriGene Technologies (Rockville, USA). Site-directed mutagenesis was performed to generate three missense variants (c.541G>A, c.1790A>G and c.2279C>T) and a null variant (c.2279delC) of *SALL4* according to the instructions of a KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan). The relevant primers are shown in Table S2. The constructs were verified by direct Sanger sequencing prior to the functional studies.

### Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, USA) and 1% Plasmocin (InvivoGen, San Diego, USA) at 37 °C with 5% CO<sub>2</sub>. To evaluate the effect of the variants on *SALL4*, HeLa cells were transfected with the wild-type or mutated *SALL4* constructs using Lipofectamine 3000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

### Western blotting

Whole-cell lysates were separated by SDS-PAGE and transferred onto PVDF membranes. After being blocked with non-fat milk, each membrane was incubated with specific antibodies against different proteins at 4 °C overnight, followed by incubation with an HRP-conjugated secondary antibody. Antibodies against the MYC Tag (Abmart, Arlington, USA) were used at a 1:2000 dilution, and an anti-GAPDH antibody (1:5000 dilution, Sigma, St. Louis, USA) was used as an internal control. The secondary antibody was a goat anti-mouse IgG (1:10,000 dilution, Abmart, Arlington, USA).

### Luciferase reporter assay

HeLa cells that were replated in 24-well plates were cotransfected with 200 ng of the *POU5F1* promoter luciferase reporter and 200 ng of wild-type or mutated *SALL4* plasmid per well. The pRL-TK plasmid (10 ng/well, Promega, Madison, USA) was used as an internal control. After 24 h of transfection, luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega, Madison, USA) according to the manufacturer's protocol. Assays were performed for three independent experiments, and each experiment was assayed in quadruplicate.

## Results

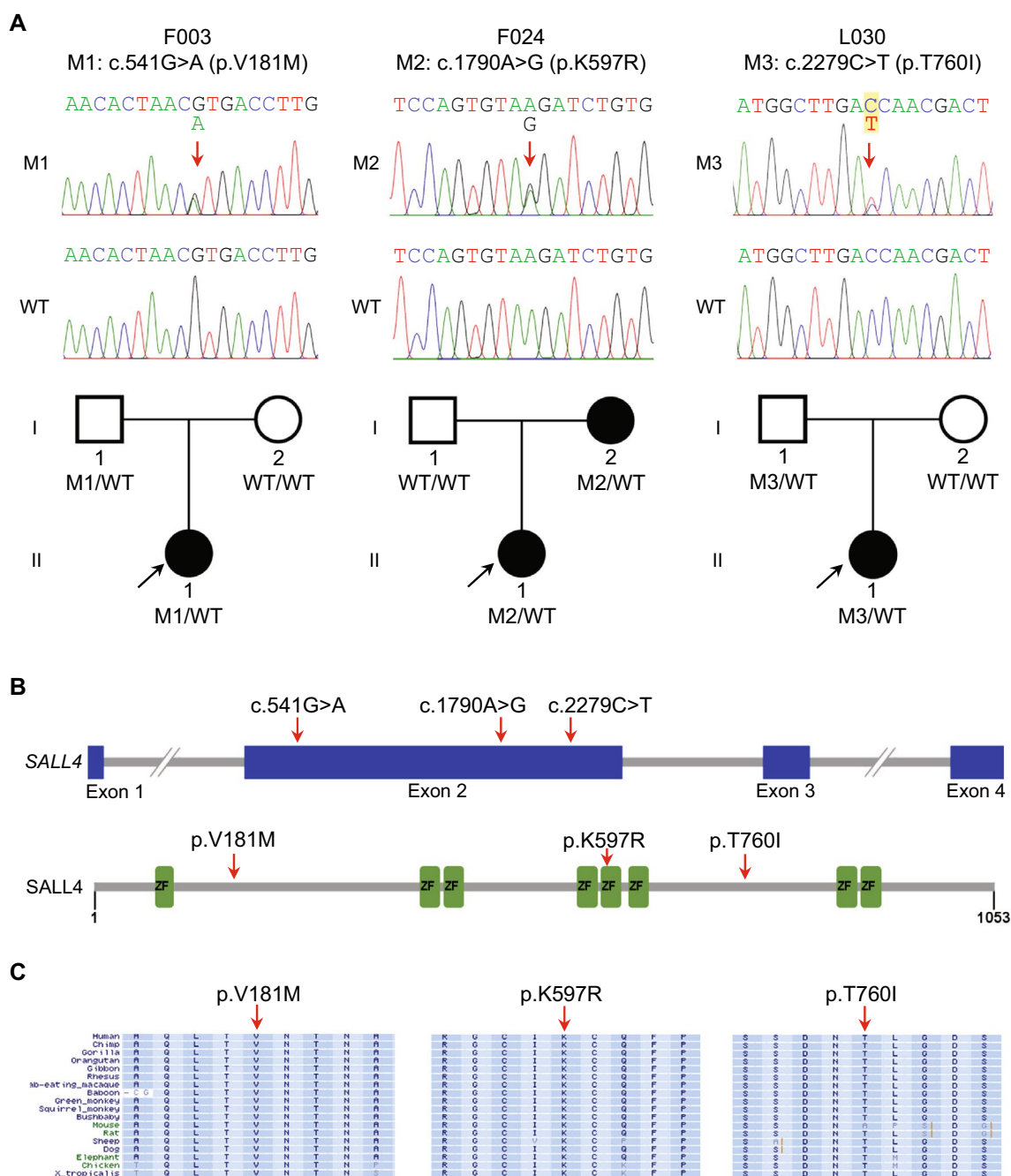
### Identification of novel and rare *SALL4* variants by WES

The mean coverage depth of the target regions in our exome sequencing data was 106×, with more than 93% of the target bases covered at more than 20×. First, we investigated genetic variants with minor allele frequencies (MAF) ≤ 0.1% according to the public population genomic databases (1000 Genomes Project, ExAC and gnomAD). Furthermore, deleterious variants predicted using no fewer than four in silico tools were chosen for further analysis. We then investigated recurrently mutated genes among the study subjects. Intriguingly, we identified three heterozygous missense variants in exon 2 of *SALL4* in three unrelated subjects with POI (Fig. 1). In these subjects with *SALL4* variants, no rare variants were identified in known POI-associated genes, according to the OMIM database (Tables S3–S5). Furthermore, no potential causative CNVs were identified in these cases (Tables S6–S8).

The *SALL4* variants were all confirmed by Sanger sequencing (Fig. 1a). Among them, the *SALL4* c.541G>A (p.V181M) and c.2279C>T (p.T760I) variants were paternally inherited (Fig. 1a). Both variants have extremely low frequencies in human populations (Table 1). The *SALL4* c.1790A>G (p.K597R) variant was inherited from the affected mother of the subject and is a novel missense variant (Fig. 1a; Table 1). We also found that the coding positions of these three *SALL4* variants were evolutionarily conserved among species (Fig. 1c). For example, the novel *SALL4* variant c.1790A>G (p.K597R) is located in a highly conserved C2H2 zinc finger motif. We conducted functional prediction analyses for the novel and rare *SALL4* variants using the SIFT (Kumar et al. 2009), PolyPhen-2 (Adzhubei et al. 2010), MutationTaster (Schwarz et al. 2014), CADD (Kircher et al. 2014) and DANN tools (Quang et al. 2015). Notably, the vast majority of these bioinformatic tools predicted that all three *SALL4* variants were deleterious (Table 1). According to the American College of Medical Genetics (ACMG) guidelines, the c.541G>A and c.2279C>T variants are considered to be variants of uncertain significance (VUS), while the c.1790A>G variant is classified as likely pathogenic (Richards et al. 2015). In view of these heterogeneous results by in silico prediction, we performed further functional studies to assess the biological impact of the novel and rare *SALL4* missense variants.

### Clinical characteristics of the affected individuals with *SALL4* variants

Clinical information of the POI subjects affected by *SALL4* variants is summarized in Table 2. Subject F003 had normal puberty and secondary amenorrhea. Her menarche



**Fig. 1** Identification of *SALL4* variants in POI. **a** Novel/rare *SALL4* missense variants (M1, M2 and M3) were identified in three families with POI. Sanger sequencing confirmed that the *SALL4* c.541G>A (M1) and c.2279C>T (M3) variants were both paternally inherited, while the c.1790A>G variant (M2) was inherited from the affected mother with early menopause. The red arrows indicate the positions of the heterozygous variants. Reference DNA sequences in the wild-

type (WT) individuals are also shown. The black arrows in the pedigree plots indicate the probands with POI. **b** Schematic representation of the *SALL4* gene and protein. The red arrows indicate the variant positions. ZF, zinc finger motif. **c** All three novel/rare *SALL4* missense variants identified in this study were located at amino acid residues conserved among different species

occurred at 13 years of age and was followed by regular cycles until 22 years of age. Pelvic ultrasonography revealed a normal uterus but small ovaries with few antral follicles. Further consecutive hormonal measurements

revealed elevated FSH levels (75.8 IU/l and 110.4 IU/l) with decreased estradiol concentrations (5 pg/ml and 4 pg/ml). Table 2 shows data from a later measurement for subject F003.

**Table 1** Overview of the *SALL4* variants observed in the POI subjects

Subject	SNP ID	cDNA change <sup>a</sup>	Protein change	Minor allele frequency <sup>b</sup>			Functional prediction <sup>c</sup>				
				IKG	ExAC	gnomAD	SIFT	PolyPhen-2	MutationTaster	CADD	DANN
F003	rs139382539 <sup>d</sup>	c.541G>A	p.V181M	0	0.0004	0.0004	Damaging	Probably damaging	Disease causing	5.5441	0.999
F024	NA	c.1790A>G	p.K597R	0	0	0	Damaging	Probably damaging	Disease causing	2.9234	0.999
L030	rs755503899	c.2279C>T	p.T760I	0	$8.3 \times 10^{-6}$	$4.1 \times 10^{-6}$	Damaging	Probably damaging	Disease causing	3.6902	0.997

NA not available

<sup>a</sup>The GenBank accession number of *SALL4* is NM\_020436.3<sup>b</sup>Allele frequencies were estimated according to the 1000 Genomes (IKG) Project, ExAC and gnomAD databases<sup>c</sup>Mutation assessment using the SIFT, PolyPhen-2, MutationTaster, CADD and DANN tools. High CADD and DANN scores suggest that a variant is likely to have deleterious effects. The CADD cutoff is usually set at 4, while 0.93 is used for the DANN cutoff<sup>d</sup>Information from ClinVar: uncertain significance or likely benign

Subject F024 was 29 years old at examination due to primary infertility. Her menses became irregular and completely stopped at 18 years of age. She was diagnosed with POI and was placed on sex steroid replacement therapy. Physical examination showed a normal body mass index and breast development. However, pelvic ultrasound imaging revealed small ovaries with no visible antral follicles. Her mother presented with early menopause at an age of 44 without known cause. Family history has been suggested to be considered 'positive' when a first- or second-degree relative had either POI or early menopause (Jiao et al. 2017). Therefore, the mother was considered to be an affected individual related to subject F024. No other known chronic diseases, endocrinopathies or autoimmune disorders were observed for subject F024 or her affected mother. After oestrogen treatment, subject F024 had visible follicles.

Subject L030 had normal puberty and established regular menses, with her menarche at 14 years old. She was diagnosed with POI at the age of 28 years when she developed amenorrhea. Subject L030 exhibited normal growth and development, and no other significant clinical or dysmorphic features were identified. Her basal serum gonadotropin levels were elevated (FSH = 52.0 IU/l and LH = 41.1 IU/l), whereas her serum estradiol was low (< 20 pg/ml).

### Genotype–phenotype correlations for the *SALL4* variants

To date, 24 pathogenic variants of *SALL4* have been identified in subjects with typical Okhiro syndrome (also known as Duane-radial ray syndrome, DRRS) and Holt–Oram syndrome (HOS) (Al-Baradie et al. 2002; Alves et al. 2016; Borozdin et al. 2004; Brassington et al. 2003; Chacon-Camacho et al. 2016; Kohlhase et al. 2002b, 2003, 2005; Terhal et al. 2006). We summarized these previously reported syndrome-associated *SALL4* variants and conducted a comparative analysis to the POI-associated *SALL4* variants identified in this study (Table S9). Furthermore, we briefly predicted the possible effects of the *SALL4* variants using multiple bioinformatic tools (Table S9). Notably, all heterozygous *SALL4* null variants resulted in syndromic developmental disorders (DRRS and HOS), whereas the subjects with pathogenic missense variants of *SALL4* herein presented with POI (Table S9). Based on the differential distribution of *SALL4* variants between POI and developmental syndromes, a statistically significant correlation was observed using the two-tailed Fisher's exact test ( $P = 3 \times 10^{-4}$ ) (Table S10).

### In vitro functional characteristics of the *SALL4* variants

The putative impact of the p.V181M, p.K597R and p.T760I variants on *SALL4* function was investigated using

**Table 2** Clinical characteristics of the POI subjects affected by *SALL4* variants

Characteristic	F003	F024	L030
First menses (years old)	13	13	14
Age of POI (years old)	22	18	28
Weight (kg)	54	49	66
Height (cm)	164	159	160
FSH (IU/l)	110.4	38.2	52.0
LH (IU/l)	43.5	14.7	41.1
PRL (ng/ml)	9.6	7.6	8.6
E2 (pg/ml)	4	10	<20
T (ng/ml)	0.5	0.1	0.2
Ultrasound imaging	Small ovaries	Small ovaries	Small ovaries
Ovary size (right/left) (mm)	17×15/17×17	12×8/11×10	19×12/18×8
Follicles per ovary (right/left) ( <i>n</i> )	4/4	0/0	NA
<i>FMRI</i> CGG repeats ( <i>n</i> )	29/29	29/29	29/29
Familial case	No	Yes	No

*FSH* follicle-stimulating hormone, *LH* luteinizing hormone, *PRL* prolactin, *E2* estradiol, *T* testosterone, *NA* not available

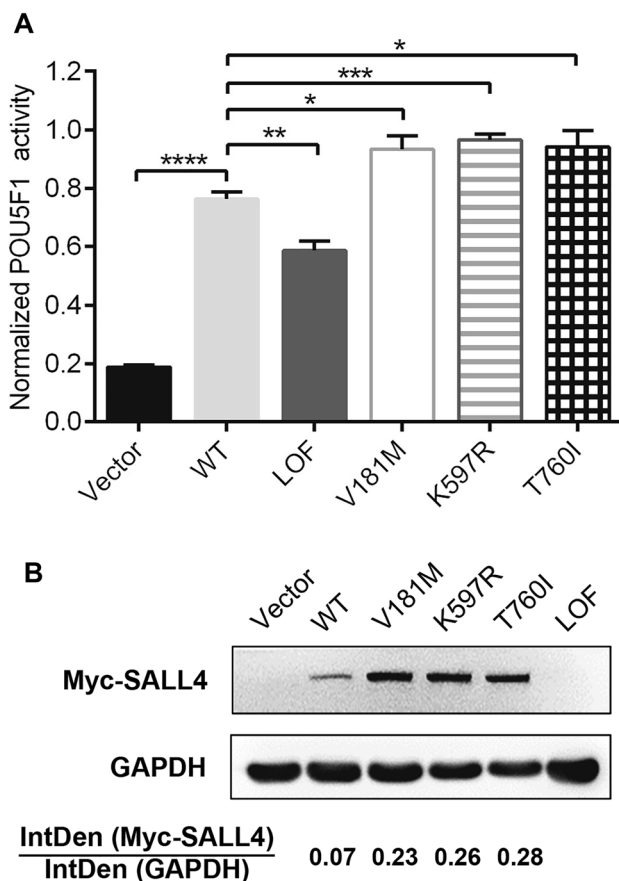
luciferase reporter assays (Fig. 2a) and western blotting (Fig. 2b). Previous experimental evidence suggested that *Sall4* is a transcriptional activator of *Pou5f1* (Zhang et al. 2006). Here, we showed that the wild-type *SALL4* protein had obvious regulatory activity and activated the *POU5F1* promoter compared with the empty vector (Fig. 2a). The *SALL4* loss-of-function (LOF) variant p.N761Tfs\*6 had remarkably suppressed regulatory activity, with substantially less activation of its downstream target *POU5F1*. Intriguingly, the *SALL4* variants identified in our POI subjects (p.V181M, p.K597R and p.T760I) exhibited significantly enhanced regulatory activities compared with that of the wild-type *SALL4*. Consistently, western blotting analysis further revealed that all three variants were expressed at higher levels than wild-type *SALL4* (Fig. 2b), which might at least partially explain their enhanced regulatory activity observed in the luciferase reporter assays (Fig. 2a). Given these findings, we presumed that the POI *SALL4* variants identified in this study might be gain-of-function variants. Our experimental observations illustrate the differential biological consequences of the *SALL4* LOF variants and the three *SALL4* missense variants identified in our subjects with POI.

## Discussion

The *SALL4* gene (NM\_020436.3) is located on human chromosome 20q13.2 and contains four exons. The *SALL4* protein (NP\_065169.1) belongs to the spalt transcription factor family, which plays an important role in regulating the embryonic development of many organisms (Al-Baradie et al. 2002; Sweetman and Munsterberg 2006). *Spalt* was

initially identified in *Drosophila* as a homeotic gene required for the early development of the posterior head and anterior tail regions (Kuhnlein et al. 1994). Murine *Sall4* is ubiquitously expressed in embryos, especially in the primitive inner cell mass. In mouse embryonic stem cells, *Sall4* binds to the highly conserved regulatory region of the pluripotent master gene *Pou5f1* and activates its expression, suggesting that *Sall4* is essential for early embryonic cell-fate decisions (Zhang et al. 2006). Mice with *Sall4* heterozygous LOF variants display increased postnatal lethality, as well as anencephaly, deafness, renal agenesis, anorectal malformations, and skeletal defects (Warren et al. 2007). In humans, heterozygous LOF variants of *SALL4* have been previously reported to cause Okhiro syndrome/Duane-radial ray syndrome, which is characterized by Duane anomaly and radial ray defects involving the thumbs and other parts of limbs, anorectal and renal malformations, and deafness (Kohlhase et al. 2002b). *SALL4* is also involved in Holt–Oram syndrome, which is characterized by radial forelimb malformations and congenital heart abnormalities and associated features (Brassington et al. 2003). No reproductive phenotypes have been reported for these syndromic cases with *SALL4* LOF variants.

*SALL4* is pleotropic, and it plays important roles in oogenesis. During germ cell development, murine *Sall4* is involved in ensuring the correct specification and migration of primordial germ cells (PGCs) (Yamaguchi et al. 2015). In adult mice, *Sall4* expression is restricted to the germline (Kohlhase et al. 2002a). The expression patterns of *SALL4* in human embryos and adult tissues are basically consistent with those that have been described in mice (Miettinen et al. 2014). Human *SALL4* is highly expressed in mitotic PGCs and fetal germ cells (Guo et al. 2015; Li et al. 2017a).



**Fig. 2** Functional evaluation of the *SALL4* variants. **a** Luciferase reporter assays with the *POU5F1* promoter in HeLa cells. The cells were transfected with equal amounts of different *SALL4* expression constructs, individually: empty vector (pCMV6-Entry), WT (wild-type), LOF variant (p.N761Tfs\*6) or rare missense variants (p.V181M, p.K597R, or p.T760I). The relative luciferase activity was normalized to Renilla activity. Error bars indicate the SEM in quadruplicate. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . **b** Western blotting analysis of the *SALL4* protein expression levels in HeLa cells transfected with equal amounts of different *SALL4* expression constructs, individually: empty vector, WT, missense variants or LOF variant. GAPDH was used as a loading control. Band density was quantified with ImageJ software. Representative images of three independent experiments are shown. IntDen, integrated density

Moreover, *Sall4* is highly expressed in oocytes at different developmental stages and could regulate oocyte meiosis resumption in female mice, indicating that *Sall4* is indispensable for follicular development and the completion of oogenesis (Xu et al. 2017). According to the Human Protein Atlas (Ponten et al. 2008), immunohistochemical analysis of human ovaries showed significant *SALL4* protein expression in follicle cells.

In this study, we recruited 50 Han Chinese subjects clinically diagnosed with POI and performed WES to identify pathogenic variants. After stringent filtration and validation, our WES analysis revealed novel and rare

missense variants of *SALL4* in three (6%) unrelated subjects. All three probands presented with normal puberty, secondary amenorrhea, and elevated FSH levels. According to the guidelines of ACMG, the *SALL4* c.541G>A and c.2279C>T variants are considered to be VUS, and the c.1790A>G variant is likely pathogenic (Richards et al. 2015). Therefore, we conducted *in vitro* functional assays on these three *SALL4* missense variants for further functional characterization. Our experimental observations suggested that compared to wild-type *SALL4*, all three *SALL4* missense variants identified in our POI subjects significantly increased the *SALL4* protein expression levels, leading to enhanced *SALL4* regulatory activity. Since no significant differences were found in the *SALL4* mRNA levels, as investigated by quantitative RT-PCR (Fig. S1), we presumed that these missense variants contribute to protein stability regulation, with posttranslational regulation at the protein level possibly being the key to the molecular mechanism underlying POI. Our findings suggest the important genetic involvement of *SALL4* in the etiology of POI.

Considering the pleiotropism of *SALL4* variants in both developmental syndromes and POI (McKusick 2007), we further investigated genotype–phenotype correlations for the *SALL4* variants identified in the POI subjects in this study as well as in other disorders. All 24 previously reported *SALL4* variants in typical Okhiro syndrome and Holt–Oram syndrome were frameshift or nonsense variants (Table S9). Variants of unclear pathogenicity or variants in atypical Okhiro and related syndromic developmental disorders were excluded. The heterozygous null alleles of *SALL4* are thought to cause these syndromic phenotypes via a haploinsufficiency mechanism (Kohlhase et al. 2005).

In contrast, only heterozygous missense variants of *SALL4* were identified in the nonsyndromic POI subjects in this study (Table S9). Interestingly, the *SALL4* c.541G>A variant has been recurrently reported in POI (Wang et al. 2009). Although it was reported as a VUS in 2017 in ClinVar, our *in vitro* functional evidence supports the pathogenicity of the c.541G>A variant. Furthermore, all three *SALL4* missense variants identified in our POI subjects showed enhanced *SALL4* regulatory activity. Therefore, we speculate that *SALL4*-associated POI does not share the same molecular mechanism as that of *SALL4* haploinsufficiency in Okhiro syndrome and Holt–Oram syndrome. Future efforts may elucidate the detailed mechanism underlying *SALL4*-associated POI.

In summary, our genetic analysis using WES of Chinese POI subjects illustrated the important genetic contribution of *SALL4* to POI and broadened the phenotypic range of heterozygous *SALL4* variants. We also described for the first time the genotype–phenotype correlations for *SALL4* variants in POI and DRRS/HOS based on *in vitro* functional

assays. These updated genotype–phenotype correlations enhance the current knowledge of *SALL4*-related disorders.

## Web resources

The URLs for the data presented herein are as follows:

1000 Genomes Project, <http://browser.1000genomes.org>.  
 CADD, <http://cadd.gs.washington.edu>.  
 DANN, [http://cbcl.ics.uci.edu/public\\_data/DANN](http://cbcl.ics.uci.edu/public_data/DANN).  
 ExAC Browser, <http://exac.broadinstitute.org>.  
 GnomAD browser, <http://gnomad.broadinstitute.org>.  
 Human Protein Atlas, <http://www.proteinatlas.org>.  
 MutationTaster, <http://www.mutationtaster.org>.  
 OMIM, <http://www.omim.org>.  
 PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>.  
 SIFT, <http://sift.bii.a-star.edu.sg>.  
 UCSC Genome Browser, <https://genome.ucsc.edu>.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no conflicts of interest.

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