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Title: *FANCL* gene mutations in premature ovarian insufficiency

Running title： *FANCL* gene mutations in POI

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Abstract

The Fanconi anemia (FA) pathway is mainly involved in DNA interstrand crosslinks (ICLs) repair in the genome. Several FA genes, including *FANCD1/BRCA2*, *FANCM*, and *FANCU/XRCC2*, have been identified as causative genes for premature ovary insufficiency (POI). Fanconi anemia group L protein (FANCL) cooperates with FANCT/UBE2T to ubiquitinate the FANCI-D2 dimer, which is a crucial event in the process of ICLs repair. *Fancl*-knockout mice phenocopy human POI, but the role of *FANCL* mutations in POI pathogenesis has not been confirmed. In the present work, potentially pathogenic mutations in the *FANCL* gene were screened in 200 Chinese patients with idiopathic POI and in 200 matched controls. Two novel heterozygous frameshift mutations, c.1048_1051delGTCT (p.Gln350Valfs*18) and c.739dupA (p.Met247Asnfs*4), were identified in the *FANCL* gene in POI patients but not in controls. Wild-type FANCL protein was predominantly localized in the nuclei, while both mutant FANCL proteins were retained in the cytoplasm. In addition, the FANCL variants exhibited impaired ubiquitin-ligase activity and compromised DNA repair ability after mitomycin C treatment. Furthermore, the FANCL variants were deleterious and might be associated with haploinsufficiency. Our results show that *FANCL* mutations are potentially causative for POI by disrupting DNA damage repair processes.

Key words: Premature ovarian insufficiency, mutation, FANCL, DNA repair

Introduction

Premature ovarian insufficiency (POI), also referred to as premature ovarian failure, is characterized by cessation of ovarian function before the age of 40 years, and it manifests as menstrual disturbances with increasing gonadotrophins and low estradiol (Webber et al.， 2016). The prevalence of POI is approximately 1% (Coulam et al.， 1986), and POI can be either isolated or syndromic. Ovarian insufficiency is one of the manifestations in syndromic POI patients, such as those with blepharophimosis-ptosis-epicanthus inversus syndrome, Perrault syndrome, and Cockayne syndrome. The etiology of POI is highly heterogeneous and consists of genetic, autoimmune, infectious, and iatrogenic factors (Vujovic， 2009). Genetic causes explain the pathogenesis in about 20–25% of POI patients (Qin et al.， 2015), and numerous genes have recently been confirmed as pathogenic genes for POI, including *MCM8*, *MCM9*, *HFM1*, *SYCE1*, *CSB-PGBD3*, and *MSH5*, which are involved in the processes of meiosis and/or DNA repair (Jiao et al.， 2018). Not unexpectedly, other DNA repair-related genes are receiving increasing attention in the exploration of POI etiology.

Interstrand crosslinks (ICLs) formed by the covalent linking of two bases on complementary DNA strands are among the most detrimental DNA lesions. The Fanconi anemia (FA) pathway maintains genome integrity by orchestrating the repair of ICLs during DNA replication (Räschle et al.， 2008). When ICLs occur, the FA core complex (FANCA, B, C, E, F, G, L, and M) is recruited to the stalled replication

forks that occur at ICLs. Subsequently, FANCL, the E3 ubiquitin-ligase subunit of the core complex, promotes the ubiquitination of the FANCI-D2 heterodimer in cooperation with FANCT/UBE2T. The ubiquitylated FANCI-D2 complex allows the recruitment of nucleases (FAN1, FANCP, FANCQ, SLX1, MUS81, etc.) that remove one of the linked nucleotides thus removing the ICL. Lesion bypass is achieved through translesion synthesis, and double strand breaks (DSBs) are repaired by homologous recombination proteins (FANCS/BRCA1, FANCN/PALB2, FANCD1/BRCA2, FANCR/RAD51, etc.) (Ceccaldi et al., 2016; Wang and Smogorzewska, 2015). To date, germline mutations in 22 genes (*FANCA–W*) involved in this pathway have been shown to result in FA (Niraj et al.，2019).

Recently, mutations in three FA genes, including *FANCD1/BRCA2*, *FANCM*, and *FANCU/XRCC2* (Howlett et al., 2002; Singh et al., 2009; Shamseldin et al., 2012), have been identified in patients with POI (Fouquet et al.，2017; Qin et al.，2019; Weinberg-Shukron et al.，2018; Zhang et al.，2019), thus suggesting the importance of the FA pathway in ovarian development and function. Ubiquitination of the FANCI-D2 complex is a key event in this pathway, and thus failure of ubiquitination due to impaired function of FANCL will prevent the activation of the FA pathway (Wang and Smogorzewska，2015). Interestingly, deletion of the mouse *Fancl* gene does not phenocopy FA, but exclusively results in a POI-like phenotype in adult female mice that is attributed to reduced proliferation of primordial germ cells (PGCs) during embryonic development (Agoulnik et al.，2002; Pellas et al.，1991). Nevertheless, it remains unknown whether *FANCL* mutations contribute to the pathogenesis of human POI.

In this study, we identified two novel frameshift mutations of the *FANCL* gene in patients with POI that compromised the ubiquitin-ligase activity and DNA repair capacity, suggesting that *FANCL* mutations are causative for human POI.

Materials and methods

Subjects

A total of 200 Han Chinese women with idiopathic POI were enrolled from the Center for Reproductive Medicine, Shandong University, from 2013 to 2016. The inclusion criteria were having primary amenorrhea or secondary amenorrhea before 40 years of age and at least two independent serum FSH (follicle stimulating hormone) measurements exceeding 40 IU/L. Exclusion criteria included syndromic POI, karyotype abnormalities, a history of ovarian surgery, chemotherapy, radiotherapy, and autoimmune disorders. The control group consisted of 200 age-matched women with regular menstrual cycles and normal basal serum FSH levels (<10 IU/L). The clinical characteristics of cases and controls are presented in Table 1.

Ethical approval

This study was approved by the Institutional Review Board of Reproductive Medicine of Shandong University. Written informed consent was obtained from each participant.

Variant sequencing

Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA Blood Kit (QIAGEN) according the manufacturer's protocol. All exons and exon-

intron boundaries of the human *FANCL* gene were amplified by polymerase chain reaction (PCR) using the 13 pairs of primers listed in Supp. Table S1. The PCR products were purified by polyethylene glycol precipitation followed by labeling with BigDye (ABI-Prism BigDye Terminator Ready Reaction Cycle Sequencing, Applied Biosystems) and sequencing on an ABI 3730XL DNA analyzer (Applied Biosystems). The sequencing data were compared with the human *FANCL* gene sequence using Sequencer software version 4.9. All of the variants were validated by three independent PCR reactions and bidirectional sequencing.

Generation of the *FANCL*-knockout cell line

FANCL-knockout human embryonic kidney 293 (HEK293) cells were generated using the CRISPR/Cas9 system. In brief, the *FANCL* targeting plasmid was constructed by inserting *FANCL*-sgRNA into the PsPca9(BB)-2A-Puro(PX459) plasmid. HEK293 cells were transfected with the plasmid, and the cells were selected with puromycin for 4 days after 48 h of transfection. Single clones of HEK293 cells were selected and cultured. The genotypes of single clones of HEK293 cells were determined by genomic DNA Sanger sequencing, and a $FANCL^{-/-}$ clone with c.590_660del71bp mutation was used for the following experiments.

RNA interference

The *FANCL*-shRNA lentivirus was purchased from Genechem (Shanghai, China), and the shRNA sequence was CTTATTGAAGAGATAGGAA. HEK293 cells were harvested at 72 h after infection with the lentivirus according to the manufacturers'

instructions. The knockdown efficiency of *FANCL* was determined by real-time RT-PCR.

Plasmid construction

The wild-type vector was generated by directly fusing the human *FANCL* cDNA fragment into a modified pXF plasmid with two N-terminal FLAG tags (In-Fusion HD Cloning Kits, Takara). The mutants were generated with the Quikchange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). All vectors were confirmed by Sanger sequencing.

Cell culture and plasmid transfection

HEK293 cells were cultured in Dulbecco Modified Eagle Medium (Gibco) with 10% fetal bovine serum (Biological Industries) and 1% penicillin-streptomycin in a 37°C incubator with 5% CO_2. For functional analysis, the cells were transiently transfected with wild-type or mutant plasmids with the X-treme GENE HP reagent (Roche) and harvested at the desired time points.

Immunofluorescence microscopy

HEK293 cells were grown on coverslips in 12-well plates and then transfected with wild-type or mutant plasmids. Thirty-six hours after transfection, cells were fixed in 4% paraformaldehyde for 20 min at room temperature. The cells were permeabilized and non-specific binding was blocked by incubating for 1 h with 0.3% Triton X-100 and 10% bovine serum albumin. The cells were then incubated with FLAG antibody (1:2,000 dilution, Sigma) at 4°C overnight and subsequently incubated with goat anti-

mouse secondary antibody conjugated with Alexa Flour 488 (1:1,000 dilution, Invitrogen) for 1 h at room temperature. The coverslips were mounted with antifade reagent with DAPI and observed under a confocal microscope (ANDOR Technology). Immunofluorescence images were obtained through a z-axis scan of the cells with an interval of 5 μm.

DNA damage assay

HEK293 cells infected with *FANCL*-shRNA lentivirus or *FANCL*$^{-/-}$ HEK293 cells transfected with the desired *FANCL* plasmids were continuously cultured for 24–48 h. The cells were exposed to 60 ng/mL mitomycin C (MMC, Melonepharma) for 24 h to induce DNA damage. Cells were harvested and boiled at 100°C for 10 min in lysis buffer (0.05 M Tris-HCl (pH 6.8), 6% β-mercaptoethanol, and 2% SDS). The proteins were resolved on 15% SDS-PAGE gels or NuPAGE 3%–8% Tris-acetate gels (Invitrogen) and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat milk/TBST and incubated with primary antibodies (FLAG, 1:5,000 dilution, Sigma; γH2AX, 1:1,000 dilution, Cell Signaling Technology; FANCD2, 1:5,000 dilution, Novus Biologicals; and β-Actin, 1:3,000 dilution, Proteintech) at 4°C overnight. Horseradish peroxidase-conjugated anti-rabbit or mouse IgG (1:5,000 dilution, Proteintech) was used as the secondary antibody. Images were acquired using the Chemidoc MP System (Bio-Rad).

Statistical analysis

The continuous variables were expressed as mean ± SD. Comparisons of genotype distributions and allele frequencies between groups were performed using the chi-

square test or Fisher's exact test when appropriate, and $P < 0.05$ was considered statistically significant.

Results

Identification of two novel heterozygous frameshift mutations in the *FANCL* gene

As shown in Table 2, 11 variants in the *FANCL* gene were identified in 200 Chinese idiopathic POI patients. Two novel heterozygous frameshift mutations, NM_018062.3: c.1048_1051delGTCT (p.Gln350Valfs*18) and c.739dupA (p.Met247Asnfs*4), were found in exon 13 and exon 9, respectively, and neither of them was present in any of the 200 controls. The p.Gln350Valfs*18 mutation was located in the ring domain and led to premature translation termination, and the p.Met247Asnfs*4 mutation was located in the DRWD domain and generated a truncated protein containing 250 amino acids and abolished the entire ring domain, which is the putative E3 ubiquitin-ligase catalytic region of the FANCL protein (Figure 1). The carrier of p.Gln350Valfs*18 presented with primary amenorrhea, and the carrier of p.Met247Asnfs*4 experienced menarche at 13 years of age but suffered from oligomenorrhea at that time and amenorrhea occurred at the age of 16 years. Furthermore, whole exome sequencing was performed on the two patients carrying the *FANCL* mutations, and no other likely causative variants were identified. The remaining nine variants were previously identified as single-nucleotide polymorphisms (SNPs). Among them, one was synonymous and localized in exon 12 (rs848291), five were in introns (rs17049422, rs149883795, rs10445896, rs3732136 and rs3732137), and three were in UTRs (Untranslated Regions) (rs41281511,

rs3732137 and rs192754138). None of the SNPs showed significant differences between POI patients and the control population in terms of genotype or allelic frequencies (Table 2).

Mislocalization of the mutant FANCL proteins

The overexpression of the FANCL protein in HEK293 cells transfected with wild-type or mutant *FANCL* was confirmed by Western blotting using FLAG antibody. The complete form of FLAG-FANCL at about 47 kDa was detected in cells overexpressing wild-type *FANCL*, but the p.Met247Asnfs*4 mutation resulted in a truncated protein weighing about 35 kDa, while the p.Gln350Valfs*18 mutation had no noticeable effect on the molecular weight of FANCL (Figure 2A). In contrast to the nuclear localization of wild-type FANCL, the two mutant proteins were retained in the cytoplasm and were absent in the nuclei (Figure 2B).

Impaired ubiquitin-ligase activity and DNA repair capacity of the mutant FANCL proteins

Monoubiquitination on the lysine 561 residue of FANCD2 is catalyzed by the E3-ubiquitin conjugating enzyme FANCL in concert with the E2 ligase UBE2T (Alpi et al.， 2008), and the detection of the ubiquitinated form of FANCD2 after MMC treatment can be used to evaluate the function of FANCL. As shown in Figure 3, an increase in the ubiquitinated form of FANCD2 was observed in *FANCL*$^{-/-}$ HEK293 cells complemented with wild-type FANCL, but no ubiquitinated FANCD2 was seen in the cells complemented with either the p.Gln350Valfs*18 or p.Met247Asnfs*4

mutants, implying that the two mutations abolished the ubiquitin-ligase activity of the FANCL protein (Figure 3A).

To further elaborate the impact of the mutations on FANCL protein, the DNA damage repair capacity of the cells was measured. The phosphorylation of serine 139 of the histone variant H2AX (indicated as γH2AX) is an early marker of DSBs, and detection of γH2AX is highly sensitive and specific for monitoring the initiation and resolution of DNA lesions (Mah et al.， 2010). In contrast to cells complemented with wild-type FANCL, more γH2AX was observed in *FANCL*$^{-/-}$ cells overexpressing the p.Gln350Valfs*18 or p.Met247Asnfs*4 mutation after MMC treatment, suggesting that mutant *FANCL* did not repair DNA damage to the same level as wild-type (Figure 3A and B). This indicated that the two FANCL mutants displayed compromised DNA repair capacity.

FANCL mutants exert no dominant-negative effect

We also examined whether the mutant FANCL proteins had a dominant-negative effect on the wild-type protein. The HEK293 cells were transiently transfected with wild-type or mutant *FANCL* plasmids, and the level of ubiquitinated FANCD2 was increased in cells overexpressing wild-type FANCL, but the abundance of ubiquitinated FANCD2 in the cells overexpressing either the p.Gln350Valfs*18 or p.Met247Asnfs*4 mutation was comparable to that of the control group (Figure 4A and B). In addition, the γH2AX in HEK293 cells transfected with wild-type *FANCL* plasmid dropped to nearly basal levels after an 8 h recovery period, indicating that the MMC-induced DNA damage was rapidly resolved. Meanwhile, the γH2AX expression in cells transfected with control or mutant plasmids was maintained at

indistinguishably high levels, suggesting that the two FANCL mutants had no dominant-negative effects (Figure 4A and B).

Haploinsufficiency of FANCL protein compromised ubiquitin-ligase activity and DNA repair ability

FANCL mRNA in HEK293 cells infected with *FANCL*-shRNA lentivirus decreased to 40% of the control group (Figure 5A). Also, compared with the control group, less ubiquitinated FANCD2, but more γH2AX was induced in the FANCL-knockdown group after MMC treatment (Figure 5B and C), suggesting that functional haploinsufficiency of FANCL attenuated the ubiquitin-ligase activity and DNA repair capacity.

Discussion

In the present study, we identified two novel heterozygous frameshift mutations of the *FANCL* gene in POI patients, p.Gln350Valfs*18 and p.Met247Asnfs*4, that resulted in the loss of ubiquitin-ligase activity and impaired DNA damage repair capacity. It is well documented that the maintenance of genome stability and integrity is a vital determinant of cell viability, and the germ cell genome is especially vulnerable to ubiquitous DNA insults (Winship et al.，2018). Moreover, increasing evidence suggests that mutations of the genes involved in DNA repair underlie the pathogenesis of POI (Huhtaniemi et al.，2018; Jiao et al.，2018). ICLs, which are among the most harmful DNA lesions and impede both transcription and replication, can be induced by endogenous metabolites (e.g., alcohol and acetaldehyde) and exogenous inducers (e.g., MMC and platinum compounds) (Clauson et al.，2013). Therefore, as an

obstacle to basic cellular processes ICLs are particularly harmful to rapidly dividing cells. It has been reported that as few as 20–40 ICLs would be lethal to mammalian cells without efficient repair (Clauson et al.，2013; Phillips，1996).

The FA pathway is responsible for the repair of ICLs, and the repair process encompasses ICL recognition, DNA incision, lesion bypass, and DSB repair (Ceccaldi et al.，2016). Cells with a deficient FA pathway are highly susceptible to DNA cross-linking agents and display increased chromosome breaks, decreased cell viability, and arrested cell cycle (Auerbach，2009; Ceccaldi et al.，2012; Kim et al.，2011; Knies et al.，2017; Luebben et al.，2014). The monoubiquitination of FANCI-D2 complex is the key node for the recruitment of downstream proteins involved in DNA incision and lesion bypass. FANCL, the E3 ubiquitin-ligase subunit of the FA core complex, specifically interacts with UBE2T/FANCT and leads to the monoubiquitination of the FANCI-D2 complex (Wang and Smogorzewska, 2015). FANCL contains three domains, including an E2-like fold domain at the N-terminus that is associated with FA complex assembly, a DRWD domain in the central part that is required for substrate binding and a ring domain at the C-terminus that has E3 ubiquitin ligase activity (Cole et al.，2010). Biallelic mutations of the *FANCL* gene result in a typical FA phenotype, including developmental abnormalities, progressive bone marrow failure, predisposition to tumors, and fertility defects (Ali et al.，2009; Auerbach，2009; Meetei et al.，2003). However, *Fancl*$^{-/-}$ mice only manifested reproductive defects, and other somatic systems were apparently unaffected. The infertility of *Fancl*$^{-/-}$ mice was attributed to reduced proliferation of PGCs between embryonic day 9.5 and 10.5 when they were in the mitotic phase (Agoulnik et al.，

2002; Pellas et al.， 1991). In addition, mutation in the zebrafish *fancl* gene causes Tp53-dependent germ cell apoptosis, which leads to female-to-male sex reversal (Rodríguez-Marí et al.， 2010). It is speculated that the reduced number of germ cells might be attributed to decreased proliferation and/or increased apoptosis due to the failure of ICL repair during the period of rapid division. These observations in animal models imply that FANCL is essential for germ cell development, and thus its role in human fertility is anticipated.

In this study, two novel heterozygous frameshift mutations, p.Gln350Valfs*18 and p.Met247Asnfs*4, were identified in patients with POI. Both mutations disrupted the ring domain and led to abnormal subcellular localization, suggesting that the nuclear localization signal may resides in the ring domain of the C-terminus. Additionally, both mutations impaired the ubiquitin-ligase activity of FANCL, and this along with γH2AX levels in *FANCL*$^{-/-}$ cells transfected with *FANCL* plasmids indicated that both mutants also compromised DNA repair capacity (Figure 3). Moreover, MMC treatment induced ubiquitination of FANCD2 in wild-type HEK293 cells, indicating that FANCL protein, which is the E3 ubiquitin-ligase for FANCD2, was present and that the FA pathway was activated. However, the abundance of ubiquitinated FANCD2 and γH2AX in the cells overexpressing either p.Gln350Valfs*18 or p.Met247Asnfs*4 mutants was comparable to that of the control group (Figure 4), suggesting that two FANCL mutations had no dominant-negative effect. Further investigation by knockdown of FANCL showed haploinsufficiency and resulted in functional defects of FANCL (Figure 5). Taken together, our results suggest that the heterozygous p.Gln350Valfs*18 and p.Met247Asnfs*4 mutations led to functional

haploinsufficiency and resulted in compromised DNA repair, which might decrease the proliferation of PGCs and/or lead to premature depletion of oocytes in the ovary, ultimately causing POI.

Fertility defects are a common feature in FA gene knockout female mice, as well as in FA patients (Tsui and Crismani，2019), which highlights the vital role of FA genes in germ cell development. Up to now, besides *FANCD1/BRCA2*, *FAMCM* and *FANCU/XRCC2* (Fouquet et al.，2017; Qin et al.，2019; Weinberg-Shukron et al.，2018; Zhang et al.，2019), novel potential causative mutations in the *FANCL* gene were also found in the present study. Considering that individuals with deficiency in FA genes are highly susceptible to multiple malignant tumors, such as breast and ovarian carcinoma, head and neck squamous cell carcinoma, and leukemia (Ceccaldi et al.，2016; Chandrasekharappa et al.，2017; Nalepa and Clapp，2018), the long-term surveillance of tumorigenesis is strongly suggested in patients with POI carrying FA gene mutations.

In conclusion, two novel heterozygous frameshift mutations p.Gln350Valfs*18 and p.Met247Asnfs*4 in the *FANCL* gene were identified in POI patients, and the impaired function of the resulting proteins in DNA repair might be causative for POI.

Authors' roles

Yingying Qin and Shidou Zhao concepted and designed the study, and critical revisions and suggestions were given by them. Yajuan Yang performed the experiments and wrote the manuscript. Ting Guo and Hanni Ke collected the subjects.

Weiwei Xu and Ran Liu helped analysis the data. All authors participated in the discussion of the results and agreed the final version to be published.

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Conflict of interest

The authors declare that there is no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in the Global Variome shared LOVD http://www.lovd.nl, reference number 0000624757 and 0000624758.

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Figure legends

Figure 1. Two novel frameshift mutations identified in the *FANCL* gene.

The schematic structure of the FANCL protein. FANCL contains three functional domains, including an N-terminal E2-like domain (first box), a DRWD domain (second box), and a C-terminal ring domain (third box). Sanger sequencing shows the mutations and the reference chromatograms. The red arrows indicate the locations of the two heterozygous frameshift mutations.

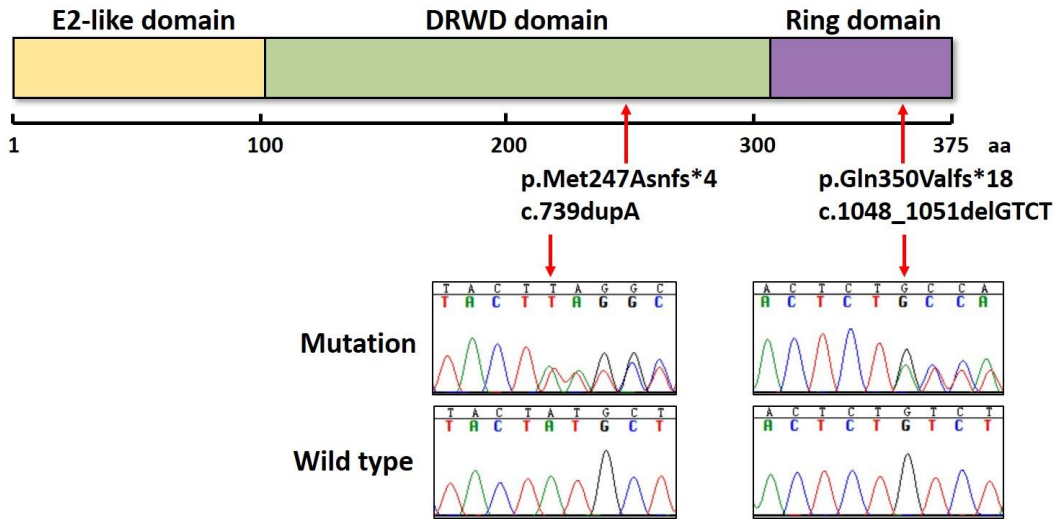

Figure 2. Mislocalization of the FANCL protein caused by the mutations.

A: The overexpression of FANCL in HEK293 cells was confirmed by Western blotting using FLAG antibody. The complete form of FLAG-FANCL was detected at about 47 kDa, and the p.Gln350Valfs*18 (Del) mutant showed no noticeable change in the molecular weight of FANCL, while the p.Met247Asnfs*4 mutation (Dup) generated a truncated protein weighing about 35 kDa.

B: Wild-type FANCL was mainly located in the nuclei, but the mutant forms of FANCL were absent in the nuclei. Cell nuclei were counterstained with DAPI. Scale bar = 20 μm. WT, wild type; Del, c.1048_1051delGTCT (p.Gln350Valfs*18); Dup, c.739dupA (p.Met247Asnfs*4).

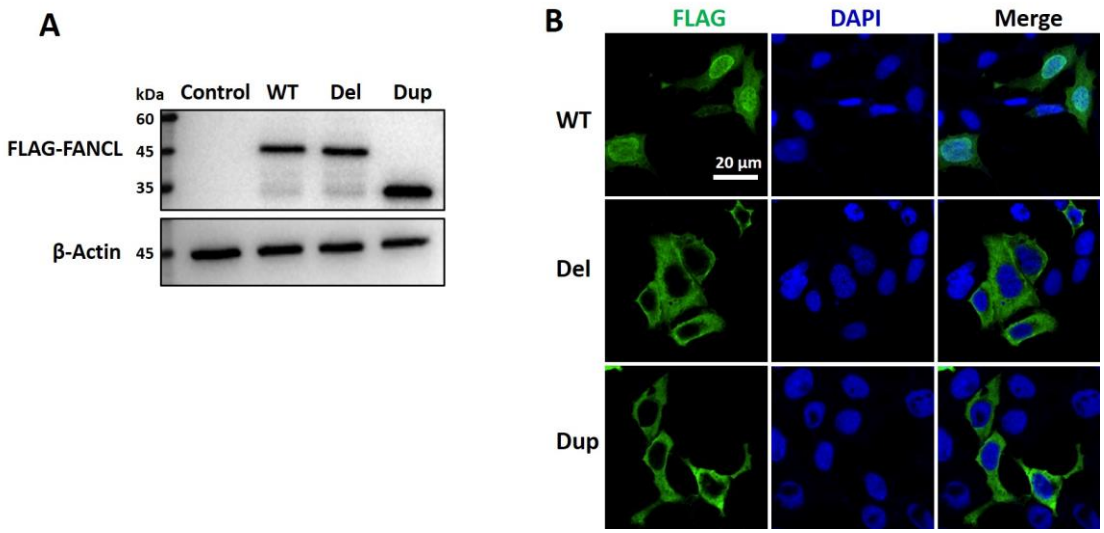

Figure 3. Impaired function of the mutant FANCL.

A: Ubiquitination of FANCD2 was induced by MMC treatment (60 ng/ml for 24 h). The ubiquitinated form of FANCD2 was significantly increased in *FANCL*$^{-/-}$ cells supplemented with wild-type FANCL, but ubiquitination was not observed in the *FANCL*$^{-/-}$ cells transfected with either the p.Gln350Valfs*18 (Del) or p.Met247Asnfs*4 (Dup) mutant. Less γH2AX was seen in the wild-type supplementation group, but complementation of the p.Gln350Valfs*18 or p.Met247Asnfs*4 mutant did not reduce the γH2AX levels in *FANCL*$^{-/-}$ cells after MMC treatment.

B: Quantification of γH2AX in *FANCL*$^{-/-}$ HEK293 cells complemented with wild-type or mutant FANCL after MMC treatment. β-Actin was used as the loading control. Error bars denote SD. *, $P < 0.05$. KO, *FANCL*$^{-/-}$ HEK293; Con, control; WT, wild type; Del, c.1048_1051delGTCT (p.Gln350Valfs*18); Dup, c.739dupA (p.Met247Asnfs*4).

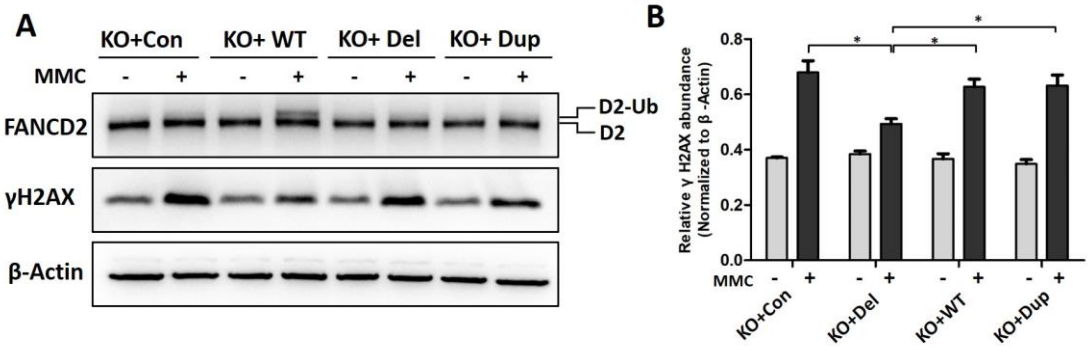

Figure 4. No dominant-negative effect displayed by the mutant FANCL.

A: Increased levels of the ubiquitinated form of FANCD2 were observed in cells overexpressing wild-type FANCL (with 0 h recovery time), but the level of ubiquitinated FANCD2 in the cells overexpressing either the p.Gln350Valfs*18 (Del) or p.Met247Asnfs*4 (Dup) mutant was comparable to that of the control group (with 0, 4, or 8 h recovery time). The γH2AX levels in cells transfected with the wild-type FANCL plasmid nearly recovered to the basal level after 8 h of recovery time. However, in contrast with the wild-type group, increased γH2AX was observed after MMC treatment in HEK293 cells overexpressing the control construct as well as in those expressing either the p.Q350Rfs*18 (Del) or p.M247Nfs*5 (Dup) mutant. No marked difference was observed in γH2AX levels in cells transfected with the control and mutant FANCL plasmid.

B: Quantification of ubiquitinated form of FANCD2 (FANCD2-Ub) and γH2AX in HEK293 cells after MMC inducement. β-Actin was used as the loading control. Error bars denote SD. Significant differences were observed between wild-type group and the mutant groups in the level of FANCD2-Ub with 0 h of recovery and in the level of γH2AX after 8 h of recovery time; but no significant differences were found for the abundance of FANCD2 and γH2AX between the control group and mutant group. *, $P < 0.05$. Con, control; WT, wild type; Del, c.1048_1051delGTCT (p.Gln350Valfs*18); Dup, c.739dupA (p.Met247Asnfs*4).

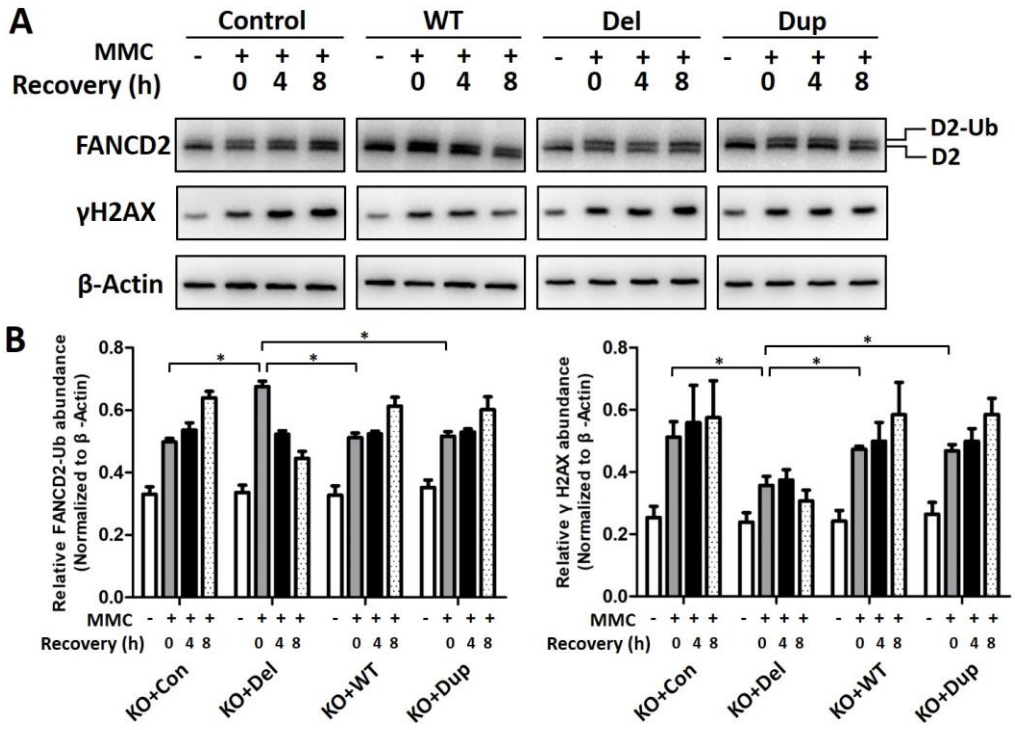

Figure 5. Haploinsufficiency of the FANCL protein decreased ubiquitin-ligase activity and DNA repair function.

A: Compared with the control group, mRNA expression of *FANCL* was reduced to 40% in the siFANCL group.

B: Knockdown of FANCL resulted in a significantly lower abundance of the ubiquitinated form of FANCD2 and an increased abundance of γH2AX.

C: Quantification of ubiquitinated form of FANCD2 (FANCD2-Ub) and γH2AX in HEK293 cells after FANCL knockdown and MMC treatment. β-Actin was used as a loading control. Error bars denote SD. Significant differences were observed in the level of FANCD2-Ub and γH2AX between the control group and the knockdown group. *, $P < 0.05$.

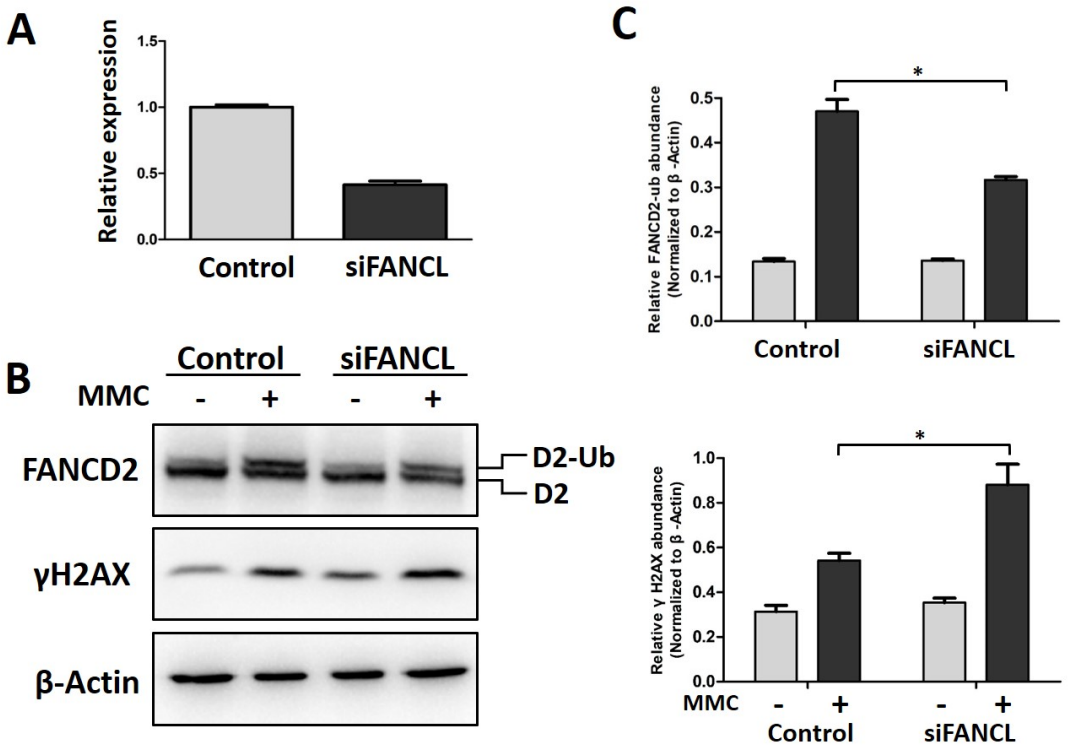

Table 1. Clinical characteristics of 200 patients with POI and 200 controls

Characteristics	POI patients		Control group
	Primary amenorrhoea	Secondary amenorrhoea	
Number	65	135	200
Age (years)	26.22±6.77	26.63± 3.86	26.10 ± 3.91
Age at menarche (years)	NA	15.28. ± 2.63	14.59 ± 2.52
Age at amenorrhea (years)	NA	19.86±3.02	NA
Serum FSH (IU/l)	69.95±27.19	78.62± 26.37	6.71 ± 3.22
Estrogen (pg/ml)	14.72±12.97	18.99 ± 14.64	46.67 ± 12.89

POI, premature ovarian insufficiency； FSH, follicle stimulating hormone. NA, not applicable.

Table 2. Variants of the *FANCL* gene identified in Chinese women with premature ovarian insufficiency

Location	**dbSNP ID**	**variation**	**Allele**	**Allele frequency**		**Genotype**	**Genotype frequency**	
				POI, %(n)	**Control, %(n)**		**POI, %(n)**	**Control, %(n)**

Exon9	Novel mutation	c.739dupA p.M247Nfs*4	- A	99.7(399) 0.3(1)	100(400) 0	- - - dup dup dup	99.5(199) 0.5(1) 0	100(200) 0 0
Exon13	Novel mutation	c.1048_1051delGTCT p.Q350Vfs*18	AGAC del	99.7(399) 0.3(1)	100(400) 0	AGAC AGAC AGAC del del del	99.5(199) 0.05(1) 0	100(200) 0 0
5’UTR	rs41281511	c.-39T>G	T G C	99.8(399) 0.2(1) (0)	100(114) 0 0	TT TG GG	99.5(199) 0.5(1) 0	100(57) 0 0
Intron3	rs17049422	c. 217-141C>T	C T	92.8(371) 7.2(29)	90.4(103) 9.6(11)	CC CT TT	86.5(173) 12.5(25) 1.3(2)	80.7(46) 19.3(11) 0

Intron5	rs149883795	c.375 -117_375 -116insAATT	-	93.3(373)	90.4(103)	--	86.5(173)	80.7(46)
			AATT	6.7(27)	9.6(11)	-	12.5(27)	19.3(11)
						insAATT	0	0
						insAATT		
						insAATT		
intron9	rs10445896	c.775+22G>A	G	99(396)	97.4(111)	GG	98(196)	94.7(54)
			A	1(4)	2.6(3)	GA	2(4)	5.3(3)
						AA	0	0
Exon12	rs848291	c.981G>A Synonymous	G	54(216)	59.7(68)	GG	33(66)	38.6(22)
			A	46(184)	40.3(46)	GA	42(84)	42.1(24)
						AA	25(50)	19.3(11)
Intron13	rs3732136	c.1093-65C>T	CC	72.2(289)	67.5(77)	CC	54(105)	50.9(29)
			T	27.8(111)	32.5(37)	CT	37.3(79)	32.8(19)
						TT	8.7(16)	15.8(9)
Intron13	rs3732137	c.1093-74G>C	G	99(396)	100(114)	GG	98(198)	100(57)
			C	1(4)	0	GC	2(4)	0

						CC	0	0
3’UTR	rs192754138	c.*127G>A	G	98.7(395)	100(114)	GG	97.5(195)	100(57)
			A	2.3(5)	0	GA	2.5(5)	0
						AA	0	0
3’UTR	rs147811379	c. *333T>C	T	99.3(397)	100(114)	TT	98.5(197)	100(57)
			C	0.7(3)	0	TC	1.5(3)	0
						CC	0	0

Accession number of the relevant reference sequence of FANCL were ENSG00000115392 and NM_018062.3. When comparing the genotype and allele frequencies of SNPs, 57 Chinese Han Beijing individuals were used as the control group, and the data was obtained from Ensembl database (http://asia.ensembl.org/index.html). All SNPs displayed no significant differences between POI patients and controls in both genotype and allele frequencies. SNPs, Single-nucleotide polymorphisms; POI, premature ovarian insufficiency.