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## Mutant Cohesin in Premature Ovarian Failure

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### Summary

Premature ovarian failure is a major cause of female infertility. The genetic causes of this disorder remain unknown in most patients. Using whole-exome sequence analysis of a large consanguineous family with inherited premature ovarian failure, we identified a homozygous 1-bp deletion inducing a frameshift mutation in *STAG3* on chromosome 7. *STAG3* encodes a meiosis-specific subunit of the cohesin ring, which ensures correct sister chromatid cohesion. Female mice devoid of *Stag3* are sterile, and their fetal oocytes are arrested at early prophase I, leading to oocyte depletion at 1 week of age.

Premature ovarian failure, the end point of primary ovarian insufficiency, affects approximately 1% of women worldwide. Patients with premature ovarian failure present with at least a 6-month history of amenorrhea and elevated plasma levels of follicle-stimulating hormone (>40 mIU per milliliter). The disorder can result from premature depletion of the follicle pool, follicular atresia, follicle growth arrest, or ovarian dysgenesis. Although a majority of cases are idiopathic, premature ovarian failure can be caused by infectious agents, chemotherapy, pelvic surgery, autoimmune disease, environmental factors, or genetic conditions.<sup>1</sup> The disorder is observed in syndromic diseases — for

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example, Turner's syndrome and BPES (blepharophimosis, ptosis, and epicanthus inversus syndrome) — or in isolated disease.

In at least 10 to 15% of patients with premature ovarian failure, a genetic cause has been determined.<sup>2,3</sup> To date, genetic alterations including chromosomal deletions, rearrangements, and autosomal and X-linked mutations have been identified in patients with this disorder.<sup>2,4,5</sup> Genomewide association studies have also provided insight into novel genomic regions that are important in ovarian insufficiency and failure.<sup>6,7</sup> However, in most cases of premature ovarian failure, no genetic cause has been identified.<sup>2,3,4,6</sup>

In analyses of samples obtained from a consanguineous Palestinian family with premature ovarian failure, we previously identified a 10-Mb region on 7q21.3–22.2 and a 3-Mb region on 7p21.1–15.3 that had significant linkage with premature ovarian failure (maximum logarithm of the odds [LOD] score, 3.26), which was consistent with homozygosity by descent.<sup>8</sup> By combining linkage data and exome sequencing in this family, we have identified a homozygous 1-bp deletion in the gene encoding stromal antigen 3 (*STAG3*), a finding that is further supported by the phenotype of female mice with a homozygous disruption in *Stag3*.

## Case Report

The Middle Eastern Family 1 (MO1DA), whose members have been described in detail previously,<sup>8</sup> presented at the Department of Genetics at Hadassah Hospital in Jerusalem for treatment of premature ovarian failure, the extreme end of the spectrum of primary ovarian insufficiency (Fig. 1A). The proband (Family Member IV-1) was the eldest of six sisters. The family history showed that in this consanguineous Middle Eastern family of Palestinian origin, five women presented with premature ovarian failure. Of the six sisters in generation IV, four were affected. The mother (Family Member III-1) and father (Family Member III-2) were first cousins and were linked by at least three other previous consanguineous marriages. In addition to the four sisters in generation IV, premature ovarian failure had also been diagnosed in a maternal aunt. The nonaffected sisters of the proband (Family Members IV-3 and IV-4) had menarche at ages within the normal range and were fertile; they naturally conceived two and three children, respectively. The two brothers (Family Members IV-2 and IV-5) were also fertile, with three children each.

All four affected sisters had received the diagnosis of premature ovarian failure between the ages of 17 and 20 years and initially presented with primary amenorrhea. On physical examination, they all had small and undeveloped breasts; the height was within the normal range. Ultrasonographic examination showed bilateral streak gonads (i.e., composed mainly of fibrous tissue). Hormonal studies that were performed at the time of diagnosis showed the expected high gonadotropin levels and low estradiol levels, with follicle-stimulating hormone levels of more than 45 mIU per milliliter (normal range, 3 to 21 mIU per milliliter), luteinizing hormone levels of more than 18 mIU per milli-liter (normal range, 1 to 18 mIU per milliliter), and estradiol levels of less than 22 pg per milli-liter (81 pmol per liter; normal range, 30 to 190 pg per milliliter [110 to 697 pmol per liter]). We ruled out the presence of autoantibodies in all four sisters.

We performed genetic testing on samples obtained from the four sisters with the disorder to rule out known genetic causes of premature ovarian failure. The results of this testing showed a normal 46,XX karyotype and no premutations in the gene encoding fragile X mental retardation 1 (*FMRI*). At the age of 19, the youngest sister with premature ovarian failure (Family Member IV-10) was found to have simultaneous bilateral ovarian tumors consisting of a gonadoblastoma on the right ovary and a complex tumor consisting of a dysgerminoma, embryonal carcinoma, and choriocarcinoma in the left ovary. No other relevant medical finding was identified in the other family members with premature ovarian failure.

## Methods

### Participants and Genetic Studies

We obtained written informed consent from all participants in the study. After whole-exome capture on Agilent SureSelectXT 50 Mb Exon Capture, we performed high-throughput sequencing of the DNA obtained from one participant with premature ovarian failure and from one of her unaffected sisters. We performed Sanger sequencing to confirm the segregation of the identified variants in the unaffected parents, affected sisters, and unaffected siblings (Table S2 in the Supplementary Appendix, available with the full text of this article at NEJM.org).

### Mouse Model

The OVE2312C mouse line, obtained from the Jackson Laboratory (Sacramento, CA), was generated with the use of a lentiposon insertional mutagenesis strategy and carries an insertion in *Stag3* that leads to a null allele (Fig. S1, S3, and S5 in the Supplementary Appendix). All experiments involving animals were performed in accordance with procedures approved by the institutional animal ethics committee at the Cancer Research Center in Salamanca.

## Results

### Homozygous Mutation in STAG3

We performed whole-exome sequencing of DNA obtained from two of the sisters, one who was affected with premature ovarian failure (Family Member IV-1) and the other who was unaffected (Family Member IV-3) (Fig. 1A). Exome sequencing generated 3.3 billion reads per sample and covered 92.6% of the targeted sequence at 10× or greater (Table S3 in the Supplementary Appendix). We filtered the single-nucleotide variants and insertions or deletions using the following criteria: location in the linkage peaks, homozygosity in the affected sibling, heterozygosity in the unaffected sibling, and absence from the Single-Nucleotide Polymorphism Database (dbSNP), build 132, and the Human Gene Mutation Database. Our data confirmed the large homozygous regions on chromosome 7 in Family Member IV-1 but not in Family Member IV-3. The filtering algorithm identified eight variants in six genes, all located within the 7q21.3–22.2 linkage peak. Of these six genes, five were ruled out as unlikely to harbor a causal mutation for premature ovarian failure on

the basis of known function or domain of expression (Tables S4 and S5 in the Supplementary Appendix).

The remaining gene, *STAG3*, carried a 1-bp deletion, c.968delC. *STAG3* encodes a subunit of cohesin, a large protein complex that is essential for proper pairing and segregation of chromosomes during meiosis.<sup>9-11</sup> The deletion results in a deleterious frameshift p.F187fs\*7 mutation and a premature stop codon, which predicts omission of exons 8 to 34 from translated *STAG3* messenger RNA. The resulting transcript would either undergo nonsense-mediated decay<sup>12</sup> or result in a severely truncated protein. In the mutant protein, the STAG domain would be compromised by truncation (Fig. 1B) and the armadillo (ARM)-type domain (which is predicted to interact with a nucleic acid or another protein) would be absent. Human *STAG3* expression is restricted to early meiosis in fetal ovaries in females and from the onset of spermatogenesis in males.<sup>13-15</sup>

The *STAG3* mutation was confirmed on Sanger sequencing, and the mutation cosegregated with the phenotype for premature ovarian failure (Fig. S2 in the Supplementary Appendix). The four sisters with premature ovarian failure were homozygous for the *STAG3* mutation, whereas the unaffected family members who were analyzed were either heterozygous or homozygous for the nonmutant allele (Fig. 1A).

### Inactivation of *Stag3* in Murine Model

To confirm that the loss of function of *STAG3* is associated with premature ovarian failure, we characterized the female phenotype of mice carrying an insertional null mutation in *Stag3*. Hemizygous mice transmitted the mutation to their offspring at mendelian frequencies (1:2:1). Homozygous mutant mice were identified by means of the Southern blot technique (Fig. S3 in the Supplementary Appendix). The absence of both transcript and protein was shown on Northern blot analysis and immunofluorescence, indicating that the insertional mutation is a null allele (Fig. S4 and S5 in the Supplementary Appendix). *STAG3* expression is restricted to the germline in mice,<sup>16</sup> and *Stag3*<sup>-/-</sup> mice showed no overt phenotype apart from sterility.

In mouse oocytes, the depletion of genes that encode subunits of the meiotic cohesin complex induces phenotypes ranging from sterility associated with premature arrest of meiotic prophase I at a zygotene-like stage (in *Rec8*)<sup>17</sup> to subfertility caused by mild defects in the homologous chromosome synapsis at the pachytene stage (in *Rad21*).<sup>18</sup> Histologic analysis of whole ovaries of *Stag3*<sup>-/-</sup> female mice at 1, 2, and 6 weeks of age showed a distinctive lack of oocytes and ovarian follicles and a dense stroma, indicating a severe and very early ovarian dysgenesis (Fig. 2A). Since female germ cells enter meiosis during embryogenesis,<sup>19</sup> we analyzed meiotic defects in *Stag3*<sup>-/-</sup> fetal oocytes. We analyzed oocytes from 15.5 to 19.5 gestational days (corresponding to the leptotene to diplotene stages of prophase I), using immunofluorescence staining of the SYCP3 component of the synaptonemal complex on chromosome spreads. Pachytene nuclei, characterized by 20 pairs of synapsed homologous chromosomes in control oocytes, were not observed in mutant *Stag3* oocytes (Fig. 2B). The axial elements, the first parts of the synaptonemal complex to be assembled, did not progress beyond the leptotene stage in the mutant oocytes. Instead, they were very short and did not elongate to form the thin SYCP3 threads observed in late

leptotene and zygotene wild-type oocytes (Fig. 2B, and Fig. S5 in the Supplementary Appendix). *Stag3*<sup>-/-</sup> oocytes did not show positive staining for SYCP1, a component of the transverse filament of the synaptonemal complex, which assembles after axial elements (Fig. 2B). We concluded that synapsis between homologues was disrupted at an early leptotene-like stage and that this premature arrest did not impair the formation of programmed double-strand breaks or the recruitment of the RAD51 recombinase on recombination foci, because these processes appeared to be similar in normal and mutant oocytes (Fig. S6 in the Supplementary Appendix).

Because the lack of the two meiosis-specific cohesin subunits RAD21L and REC8 leads to defective loading of other cohesin subunits and defective assembly of axial elements,<sup>11</sup> we asked whether the loss of *STAG3* also compromised the loading of other cohesins in fetal oocytes. Immunolabeling of the various meiotic cohesin subunits and SYCP3 in wild-type and *Stag3*<sup>-/-</sup> fetal ovaries showed absent or very limited colocalization of other cohesins with SYCP3 in the *Stag3*<sup>-/-</sup> mice (Fig. S7 in the Supplementary Appendix). We would therefore suggest that STAG3 forms complexes in vivo with REC8, SMC3, SMC1 $\beta$ , and RAD21L, independent of the somatic or meiotic kleisin (RAD21), and that STAG3 is essential for the formation of a functional cohesin ring and synaptonemal complex in mice and humans.

In addition to its role in the formation of a synaptonemal complex,<sup>11,18</sup> the cohesin complex holds sister chromatids together.<sup>10,20</sup> Therefore, we analyzed centromeric sister chromatid cohesion by means of immunolocalization of centromeres in fetal oocytes. Mutant oocytes showed an obvious loss of centromeric sister chromatid cohesion (Fig. S8 in the Supplementary Appendix). These results suggest that STAG3-containing cohesin complexes also play a central role in the maintenance of sister chromatid cohesion in mammalian oocytes (Fig. S9 in the Supplementary Appendix).

## Discussion

Using a combination of linkage data and exome sequencing, we identified a frameshift mutation in *STAG3* in a large consanguineous family with premature ovarian failure. All affected family members who provided samples for analysis were homozygous for the mutation. Our results are supported by the sterile phenotype and the very early ovarian defects observed in *Stag3*<sup>-/-</sup> female mice. The early meiotic arrest and the centromeric chromosomal cohesion defects observed in *Stag3*<sup>-/-</sup> fetal oocytes provide further evidence that *Stag3* is essential for the assembly of the meiotic cohesin ring and the synaptonemal complex. Given the essential role of *STAG3* in meiosis, we expect that a homozygous defect would lead to infertility in both sexes. However, since the brothers of the proband are heterozygous, we cannot draw conclusions from this family with regard to male infertility. Nevertheless, we found that male *Stag3*<sup>-/-</sup> mice were infertile (Fig. S10 in the Supplementary Appendix).

Mutations in mice that affect other meiotic cohesin subunits also result in abnormal oocyte development. Specifically, oocytes show meiotic defects because of abnormal formation of axial elements (in mutant REC8),<sup>17</sup> impaired sister chromatid cohesion leading to massive

aneuploidy during the second meiotic division (in mutant *SMC1β*),<sup>21</sup> or mild defects in synapsis resulting in premature depletion of the oocyte pool (in mutant *RAD21L*).<sup>18</sup> Furthermore, the gradual loss of meiotic cohesins or their protector, shugoshin-like 2, is an important mechanism of meiotic nondisjunction and age-related aneuploidy and infertility, as shown in mice and postulated in humans.<sup>22-25</sup>

Although some human cases of premature ovarian failure have been attributed to defective chromosomal cohesion at later stages of meiosis because of supernumerary chromosomes,<sup>26</sup> our results show that this disorder can also result from a defect in sister chromatid cohesion much earlier, in prophase. The very few mutations that have been identified in meiotic genes in association with human infertility result in azoospermia or recurrent pregnancy loss or alter processes other than the formation of the synaptonemal complex.<sup>27-30</sup> Genetic analyses of samples obtained from four other families with recessive inheritance of infertility ruled out linkage with the *STAG3* locus, highlighting the genetic heterogeneity of this disease and suggesting that its recessive forms are infrequent (see Section I in the Supplementary Appendix).

Cohesins are implicated in the repair, replication, and recombination of DNA, as well as in chromosomal stability, transcription regulation, stem-cell pluripotency, and cell differentiation.<sup>10,20,31</sup> Our study expands the spectrum of cohesinopathies, which include severe constitutional disorders such as the Cornelia de Lange syndrome and the Roberts syndrome, to one with isolated infertility.

In addition to having roles in crucial developmental processes, genes such as *SMC1*, *SMC3*, *STAG3*, *STAG2*, and *RAD21* are mutated in a variety of cancers<sup>32,33</sup> (see also the Catalogue of Somatic Mutations in Cancer [COSMIC] database). Indeed, somatic mutations in *RAD21*, *SMC1A*, *SMC3*, and *STAG2* (a paralogue of *STAG3*) are considered to be driver mutations in acute myeloid leukemias and several solid tumors,<sup>34-37</sup> and loss of heterozygosity in *STAG3* occurs in epithelial ovarian carcinomas.<sup>38</sup> It is worth noting that at the age of 19, the youngest sister with premature ovarian failure (Family Member IV-10) presented with bilateral ovarian cancer. The germinal origin of these tumors suggests a possible role for the germline *STAG3* truncating mutation in genomic instability in the few oocytes that survived ovarian degeneration. Accordingly, it would be interesting to investigate whether women carrying *STAG3* mutations resulting in premature ovarian failure have an increased risk of germ-cell tumors.

Our results implicate *STAG3* mutation in premature ovarian failure and the meiosis-specific cohesin complexes in enabling fertility. Further studies are required to understand the association between genetic variants in *STAG3*, human infertility, and ovarian tumorigenesis. We propose that mutations affecting other meiosis-specific subunits of the cohesin complex could lead to similar functional defects and explain ovarian failure in women with premature ovarian failure.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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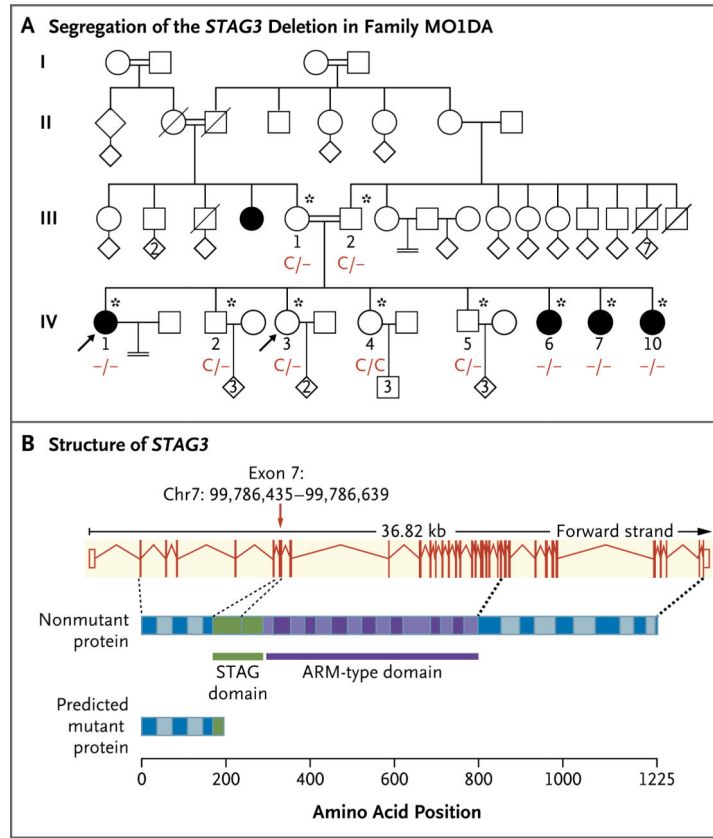
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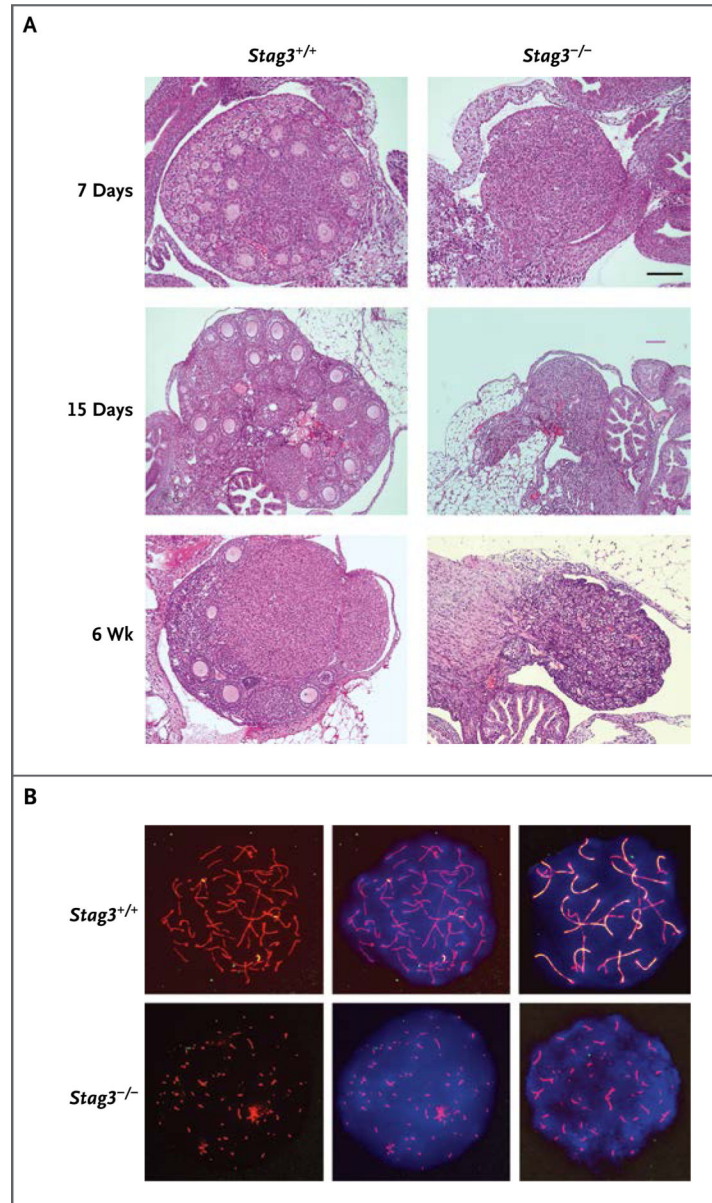


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**Figure 1. Identification of a Mutation in the Coding Sequence of *STAG3* in a Consanguineous Family with Premature Ovarian Failure**

Panel A shows the family pedigree, with double horizontal lines indicating consanguineous unions. Squares denote male family members, circles female family members, diamonds offspring for whom information on sex was not available (with the number of children indicated inside the symbols if it was known), solid symbols affected family members, open symbols unaffected family members, and slashes deceased family members. Arrows point to the two sisters who provided samples for whole-exome sequencing. The genotypes for the mutation in *STAG3* are indicated below each family member whose DNA was available for Sanger sequencing, as shown with an asterisk. Panel B shows the organization of *STAG3* (from Ensembl, reference transcript ENST00000426455). Exons are depicted as red vertical bars and introns as dashed lines. The solid bars indicate coding exons, and the open bars at each end indicate noncoding exons. The structure of the nonmutant protein is shown below the exons for the longest isoform of 1225 amino acids, with coding exons shown as bars with alternating colors. The mutation lies in exon 7 (indicated by the arrow), which encodes the first part of the STAG domain (in green). The predicted length of the mutant protein (if translated) is 194 amino acids, as induced by the frameshift mutation and premature stop codon. In the mutant protein, the armadillo (ARM)-type domain, which is predicted to interact with a nucleic acid or another protein, would be absent.



### Figure 2. Premature Ovarian Failure in *Stag3*<sup>-/-</sup> Mice

Panel A shows a comparative histologic analysis of ovarian sections from wild-type and *Stag3*<sup>-/-</sup> mice at 7 days, 15 days, and 6 weeks of age. Growing oocytes and follicle development are evident in wild-type animals at each age. Ovaries with no follicles or oocytes were observed in their *Stag3*-deficient littermates. At 6 weeks, *Stag3*-deficient mice showed atrophic and fibrotic ovaries. The scale bar represents 100  $\mu$ m in 7-day sections and 200  $\mu$ m in 15-day and 6-week sections. Panel B shows immunostaining assays for SYCP3 (detected in red) and SYCP1 (detected in green) in murine fetal oocytes. The yellow lines indicate the formation of synapses. SYCP1, a marker for transverse filaments of the synaptonemal complex, is located along the synapsed lateral elements in the wild-type oocyte from the early-zygotene stage (top left and middle panels) until the late-zygotene stage (top right panel), when almost all the lateral elements are synapsed. However, the

*Stag3*<sup>-/-</sup> oocytes that are arrested at the leptotene-like stage (bottom panels) do not show SYCP1 staining or synapsis; the top and bottom middle and right panels also show DAPI staining of DNA in blue.