Major spliceosome defects cause male infertility and are associated with nonobstructive azoospermia in humans

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Processing of pre-mRNA into mRNA is an important regulatory mechanism in eukaryotes that is mediated by the spliceosome, a huge and dynamic ribonucleoprotein complex. Splicing defects are implicated in a spectrum of human disease, but the underlying mechanistic links remain largely unresolved. Using a genome-wide association approach, we have recently identified single nucleotide polymorphisms in humans that associate with nonobstructive azoospermia (NOA), a common cause of male infertility. Here, using genetic manipulation of corresponding candidate loci in *Drosophila*, we show that the spliceosome component SNRPA1/U2A is essential for male fertility. Loss of U2A in germ cells of the *Drosophila* testis does not affect germline stem cells, but does result in the accumulation of mitotic spermatogonia that fail to differentiate into spermatocytes and mature sperm. Lack of U2A causes insufficient splicing of mRNAs required for the transition of germ cells from proliferation to differentiation. We show that germ cell-specific disruption of other components of the major spliceosome manifests with the same phenotype, demonstrating that mRNA processing is required for the differentiation of spermatogonia. This requirement is conserved, and expression of human SNRPA1 fully restores spermatogenesis in U2A mutant flies. We further report that several missense mutations in human SNRPA1 that inhibit the assembly of the major spliceosome dominantly disrupt spermatogonial differentiation in *Drosophila*. Collectively, our findings uncover a conserved and specific requirement for the major spliceosome during the transition from spermatogonial proliferation to differentiation in the male testis, suggesting that spliceosome defects affecting the differentiation of human spermatogonia contribute to NOA.

GWAS | NOA | spliceosome | spermatogonia | spermatogenesis

Approximately 30–55% of cases of infertility in humans are related to male factors, and of these, 10–15% have been attributed to azoospermia, defined as the complete absence of sperm in semen (1). Nonobstructive azoospermia (NOA), in which the testicles fail to produce mature sperm in the ejaculate because of abnormal spermatogenesis, accounts for approximately one-half of azoospermia cases (2, 3). NOA is a heterogeneous disorder caused by complex genetic and environmental factors. Hereditary changes, including chromosome abnormalities and gene mutations, have been implicated in NOA (4–6); however, the etiology of this disease remains largely unclear.

Spermatogenesis is one of the most conserved biological processes from *Drosophila* to humans (7, 8). For example, mutations in *boule* cause sterility in *Drosophila*, and mutants of the human homologous gene, *DAZ*, are associated with azoospermia. Moreover, *DAZ* can restore the meiosis of the *boule* mutant in

Drosophila (9). Therefore, *Drosophila* provides a simple system for investigating the complex genetic basis and related molecular mechanisms of human male fertility. We recently successfully used *Drosophila* to test the reproductive function of conserved genes identified by a genome-wide association study (GWAS), leading to the identification of seven previously unidentified factors essential for male fertility (10).

The vast majority of genes in higher eukaryotic genomes are interrupted by introns, which are removed from pre-mRNAs by the spliceosome. Most pre-mRNAs are processed through the major spliceosome pathway, which relies on the major spliceosome, a conserved, very large complex consisting of five small nuclear ribonucleoprotein (snRNP) complexes (U1, U2, U4, U5, and U6) and approximately 150 proteins (11). Compared with the large number of *cis*-acting splicing mutations discovered in human disease, only a few mutations in spliceosome-associated genes have been shown to cause diseases, including retinal degeneration and Taybi–Linder syndrome (12). Whether the spliceosome core components are involved in human reproductive disease remains unknown.

In the present study, using *Drosophila* as a model, we evaluated the function of an SNP locus strongly associated with NOA

Significance

The major spliceosome is required for mRNA processing and is believed to be essential for cell survival. Here we report that mutations affecting the major spliceosome are associated with nonobstructive azoospermia (NOA), a common but poorly understood cause of male infertility in humans. Specifically, we report that spliceosome dysregulation impairs the differentiation of spermatogonia, abolishing the maturation of germ cells into sperm. The requirement for the major spliceosome during spermatogonial differentiation is highly conserved among species. Our study has uncovered genetic causes and molecular mechanisms underlying NOA; these results will likely provide direction for the genetic testing and treatment of patients with NOA.

Fig. 1. The locus 15q26.3 (rs7166401) is susceptible to male infertility. (A) Regional association plot of SNP rs7166401. SNP rs716640 was identified by genome-wide association study (GWAS) in Han Chinese men. SNPs are shown in purple, and r^2 values for other SNPs are indicated in color. Genes within the region of interest (1 Mb) are annotated with direction of transcription marked by arrows. (B) Schematic diagram of spermatogenesis in *Drosophila*. At the blind apical end of the testis, germline stem cells that surround stromal hub cells divide asymmetrically to self-renew and to produce goniablasts. Goniablasts undergo four rounds of transient amplifying divisions to form 16 spermatogonia; these enter meiosis and undergo differentiation into spermatocytes, spermatids, and mature sperm.

(13, 14). We report that U2A, the *Drosophila* homolog of human SNRPA1, is essential for male fertility. Specifically, we demonstrate that the transition of spermatogonia from proliferation to differentiation depends on major spliceosome function and is abolished by missense mutations in *SNRPA1*, implicating dysregulation of the spliceosome as a cause of NOA in humans.

Results

Locus 15q26.3 (rs7166401) Is Susceptible to Male Infertility. Our recent data from a GWAS comprising 981 NOA cases and 1,657 controls identified several SNPs that associated with NOA ($P < 10^{-7}$), including rs7166401 at 15q26.3 ($P = 6.68 \times 10^{-13}$) (Fig. 1*A* and *SI Appendix*, Fig. S1 *A* and *B*). Rs7166401 maps to a large intron of the *CHSY1* gene, and six surrounding genes, including *ALDH1A3, LRRK, CHSY, SNRPA1, SELS,* and *PCSK6* are localized within 800 kb. Four of these six genes are evolutionarily conserved between humans and *Drosophila*. We used *Drosophila*

spermatogenesis (Fig. 1*B*) as a system to test the reproductive function of these genes by performing ubiquitous knockdown of each of the four candidate genes in adult flies using RNA interference (RNAi) (15).

To bypass the potential lethality of these knockdowns, we used a temperature-sensitive conditional approach incorporating the GAL4 upstream activation sequence (UAS) bipartite expression system with a temperature-sensitive mutant of GAL80 (Gal80[ts]) that reversibly suppresses GAL4 activity at 18 °C but allows GAL4 binding to UAS at 29 °C. In flies transgenic for the three components of this system (UAS-RNAi; GAL-4 driver, here a ubiquitous *tub-gal4*; and *tub-Gal80[ts]*), expression of RNAi and resulting knockdown of the target gene were suppressed at permissive temperature, such that *tub-gal80[ts]; tub-gal4 > UAS-RNAi* males raised at 18 °C were fertile (*SI Appendix*, Fig. S1*C*). Knockdown of each of the four target genes on exposure to the restrictive temperature (29 °C) resulted in reduced male fertility (*SI Appendix*, Fig. S1*D*). In particular, knockdown of U2A, the homologous gene of *hSNRPA1* that is in close proximity to rs7166401, caused complete male sterility (Table 1 and *SI Appendix*, Fig. S1*E*). These results suggest the locus 15q26.3 (rs7166401) and *hSNRPA1* as a susceptibility locus and a candidate gene, respectively, for male infertility.

**Drosophila Spermatogenesis Requires Availability of U2A in Germ Cells.** To validate the outcomes with RNAi-mediated knockdown of U2A, we generated a U2A null mutant fly line using CRISPR/Cas9-mediated genome editing. The resulting null allele contained a 7-bp deletion in exon 1 (bases 253–259 following the U2A start codon), which caused a frame shift and loss of the conserved leucine-rich repeat C-terminal (LRRCT) and U2A domains (*SI Appendix*, Fig. S2*A*). Flies homozygous for the *U2A¹* allele were not viable. To rescue these flies, we introduced a compensatory transgene (*hs-U2A*) expressing U2A cDNA under the control of a heat-shock promoter. Continuous heat-shock treatment resulting in transgene expression (*hs-U2A^ON*) permitted the survival of *U2A¹; hs-U2A* flies to adulthood, and on removal of heat-shock treatment (*hs-U2A^OFF*), these flies were converted to U2A mutants. Western blot analysis (Fig. 2*A*) and whole-mount immunostaining of testes (*SI Appendix*, Fig. S2*B*) confirmed the absence of U2A protein in testes from adult *U2A¹; hs-U2A^OFF* flies, but not from adult *U2A¹; hs-U2A^ON* flies. After withdrawal of heat-shock treatment at adulthood, male WT flies (*w¹¹¹⁸* strain) remained fertile, whereas *U2A¹; hs-U2A^OFF* flies were completely sterile and lacked mature sperm (100%; n = 37) (Fig. 2*B* and *SI Appendix*, Table S1). These results strongly support that idea that U2A is required for *Drosophila* spermatogenesis.

To determine the developmental role of U2A in the adult testis, we used cell type-specific GAL4 drivers to eliminate U2A in germ cells, spermatogonia, and surrounding somatic cells (Fig. 1*B* and *SI Appendix*, Fig. S2 *C–E*). Knockdown of *U2A* with the germ cell-specific driver *nos-gal4* lowered the fertility rate of male flies to 70% (n = 58), whereas somatic cell-specific drivers, including *c587* and *upd*, had little or no effect on fertility (*SI Appendix*, Table S1 and Fig. S2*F*). Strikingly, male flies in which U2A was specifically knocked down in spermatogonia and early spermatocytes using a *bam-gal4* driver were completely sterile (100%; n = 42) (*SI Appendix*, Table S1), suggesting a specific role of U2A in these cell types. Although U2A is expressed in the

Table 1. Fertility screen for essential genes underlying the GWAS signal rs7155401 in *Drosophila*

Gene (human)	Homologous gene (fly)	CG no.	Chromosome (fly)	Identity, %	Male sterility rate, % (tub-gal80[ts];tub-gal4 > RNAi; n = 40)
ALDH1A3	*CG3752*	CG3752	2L	61	12.5
LRRK	*lrrk*	CG5483	3R	14	5
CHSY1	*CG9220*	CG9220	X	41	15
SNRPA1	*U2A*	CG1406	2R	53	100

tub-gal80[ts];tub-gal4 flies (n = 54) served as a positive control; the sterility rate was 3.7%.

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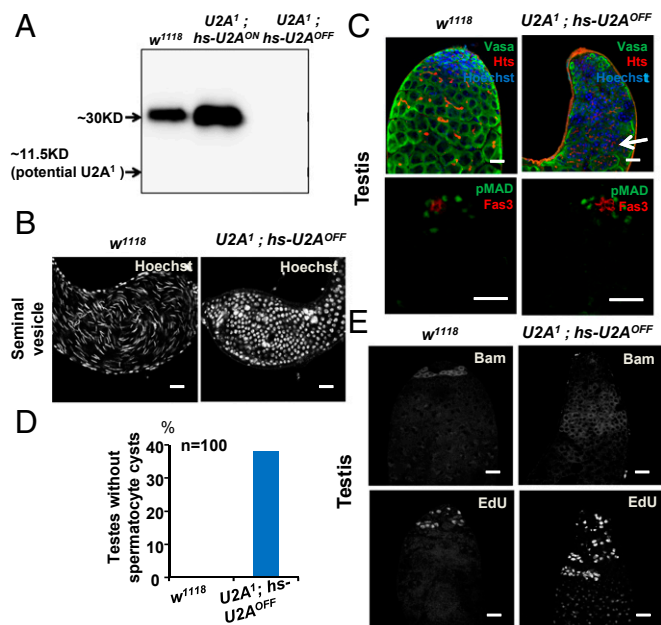
Fig. 2. U2A is required for spermatogonial differentiation in the *Drosophila* testis. (*A*) Western blot of testis lysates from control and *U2A* mutant flies confirms the absence of U2A protein (~30 kDa). Exogenous expression of U2A in *U2A¹; hs-U2A* flies was induced by heat-shock treatment (*U2A¹; hs-U2A^ON*) until adulthood. In the absence of heat-shock treatment (*U2A¹; hs-U2A^OFF*), adults were converted to null mutants with complete absence of U2A protein in the testis. (*B*) In contrast to WT (*w¹¹¹⁸*) controls, seminal vesicles from mutant *U2A¹* (*U2A¹; hs-U2A^OFF*) flies did not contain mature sperm. (Scale bar: 20 μm.) (*C*) Distribution of germ cells in the apex of the testis of control (*w¹¹¹⁸*) and mutant *U2A¹* (*U2A¹; hs-U2A^OFF*) flies. Immunostaining using anti-Vasa (green), anti-Hts (red), and Hoechst 33342 (blue) (*Upper*; scale bar: 20 μm), and anti-pMAD and anti-Fas3 (*Lower*; scale bar: 20 μm). The arrow indicates a cyst with >16 cells. (*D*) Absence of spermatocyte cysts in the apical third of the testis of *U2A* mutant flies (*U2A¹; hs-U2A^OFF*). Shown is the percentage of testes not containing spermatocyte cysts in the apical third; 100 testes were assessed per genotype. Spermatocyte cysts were identified by Hoechst staining. (*E*) Distribution of spermatogonia (Bam⁺) and cells in S phase (EdU) in the apical tip of testes from *w¹¹¹⁸* and *U2A* mutant flies (*U2A¹; hs-U2A^OFF*). (Scale bar: 20 μm.)

gonads of both sexes (*SI Appendix*, Fig. S2 *B* and *G*), germ cell-specific knockdown did not affect female fertility (*SI Appendix*, Table S1). These results suggest that U2A may play a specific autonomous role in directing male germ cell development.

Lack of U2A Blocks the Transition Between Mitotic and Meiotic Programs During Male Germ Cell Development. The apical region of *U2A* (*U2A¹; hs-U2A^OFF*) testes contained many germ cells resembling early germ cells (Fig. 2*C* and *SI Appendix*, Fig. S2*H*), but frequently (38%) lacked differentiating germ cell cysts (Fig. 2*D*). To characterize the small germ cells seen in *U2A¹* testes, we immunostained for p-Mad and for Bag of marbles (Bam), which mark spermatogonial stem cells (GSCs) and spermatogonia, respectively (16). Similar to wild type (WT) testes, in which between five and nine GSCs surround the hub, *U2A¹* testes contained a distinct small population of GSCs at the hub, suggesting that lack of U2A did not affect GSC self-renewal and differentiation (Fig. 2*C*). Mutant *U2A¹* testes exhibited an abnormally widespread Bam expression pattern throughout the entire apical third, however. Normally, Bam expression is restricted to a narrow domain representing 2-cell to early 16-cell germline cysts (Fig. 2*E*). Therefore, the small early germ cells filling the mutant testes could represent spermatogonial cells.

Notably, the apical region of *U2A* mutant (*U2A¹; hs-U2A^OFF*) testes contained many cysts with more than 16 spermatogonia (Fig. 2*C*, arrow). In WT testes, 96% of spermatocyte cysts located

in the apical third of the testis contained 16 spermatocytes per cyst (*SI Appendix*, Fig. S2*I*). In a comparable region of U2A mutant testes, 64% of cysts contained many small germ cells (≥32 small cells), and only 25% of cysts progressed to the spermatocyte state. Of these latter cysts, approximately two-thirds (16% of total cysts) had ≥32 spermatocytes per cyst (*SI Appendix*, Fig. S2*I*). In contrast to WT testes in which mitotic cells were restricted to the Bam-expressing domain, germ cell cysts throughout the apical third of U2A mutant testes were largely EdU positive and phospho-histoneH3 positive (Fig. 2*E* and *SI Appendix*, Fig. S2*J*). Therefore, the accumulating early-stage cysts in the mutant were undergoing active mitotic division and represent transit amplifying cells. Collectively, these results suggest that a lack of U2A causes excessive proliferation of spermatogonia that fail to enter the developmental programs of meiosis and differentiation.

Meiotic P26 Pre-mRNA Is One Target of U2A During Spermatogonial Differentiation. Meiotic P26 (Mei-p26) facilitates the transition from transient amplifying to differentiating spermatogonia, and its expression is regulated by splicing (*SI Appendix*, Fig. S3*A*). In normal testes, Mei-p26 is highly expressed in GSCs, gonialblasts, early spermatogonial cells, and spermatocytes. U2A mutant testes (*U2A¹; hs-U2A^OFF*) were almost devoid of Mei-p26 protein, suggesting that *mei-p26* transcript may be a downstream target of U2A (Fig. 3*A*). We evaluated the relative amount of *mei-p26* mRNA in U2A mutant testes, controlling for the lower number of spermatocytes by also evaluating transcript levels in flies with germ cell-specific *bam* knockdown, which similarly results in blockage of the spermatogonia-to-spermatocyte transition (*SI Appendix*, Fig. S3*B*). Mutant *U2A¹* (*U2A¹; hs-U2A^OFF*) testes contained less *mei-p26* mRNA than WT and *bam* knockdown controls (Fig. 3*B* and *SI Appendix*, Fig. S3*C*), whereas mRNA levels of otefin (*ote*), an intronless gene encoding a widely expressed nuclear membrane protein, did not differ (Fig. 3*B* and *SI Appendix*, Fig. S3*D*). Real-time quantitative PCR (qPCR) analyses confirmed that *U2A¹* (*U2A¹; hs-U2A^OFF*), but not WT, testes contained detectable amounts of incompletely spliced *mei-p26* mRNA (Fig. 3 *C* and *D*).

To further confirm the role of U2A in *mei-p26* pre-mRNA processing in vivo, we generated a fly line transgenic for an expression construct containing the full genomic sequence of *mei-p26* [*mei-p26(gs)*] under control of the UAS promoter.
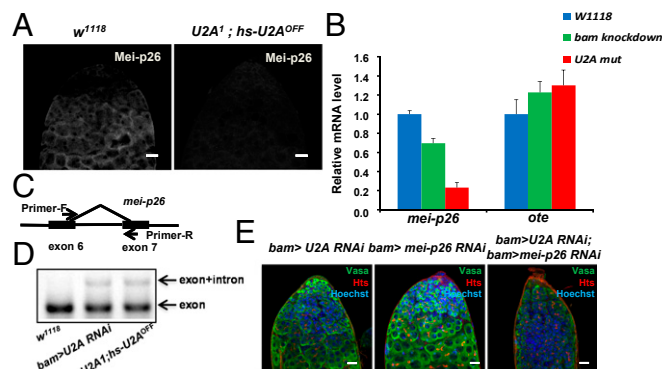
Fig. 3. U2A is required for *mei-p26* pre-mRNA processing during spermatogonial differentiation. (*A*) Immunostaining of WT (*w¹¹¹⁸*) and U2A mutant (*U2A¹; hs-U2A^OFF*) testis with anti–Mei-p26 reveals reduced levels in the mutant. (Scale bar: 20 μm.) (*B*) Real-time qPCR analysis of *mei-p26* and *ote* mRNA levels in testes. Values were normalized to WT. *w¹¹¹⁸* (blue); *bam* knockdown is shown in green; *U2A¹/hs-U2A^OFF*, in red. (*C*) Diagram of *mei-p26* pre-mRNA fragments detected by PCR. Amplicons may include exon 6, exon 7, and intron 6–7 sequences. (Exon numbering refers to transcript RE; *SI Appendix*, Fig. S3*A*.) (*D*) Partially processed *mei-p26* transcripts containing intron 6–7 sequences in U2A mutant and *bam > U2A* RNAi testis. (*E*) Simultaneous knockdown of *U2A* and *mei-p26* causes a more severe phenotype compared with individual RNAi-mediated knockdown. Anti-Vasa is shown in green; anti-Hts, in red; and Hoechst 33342, in blue. (Scale bar: 20 μm.)
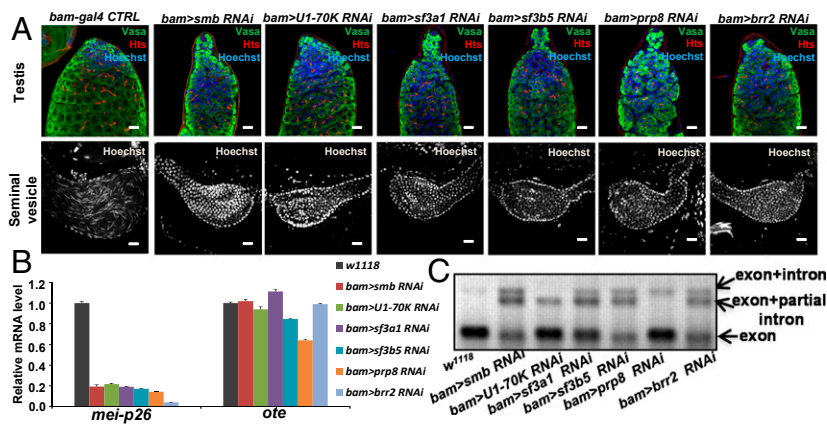
Fig. 4. The major spliceosome plays a role during the transition from spermatogonial proliferation to differentiation in *Drosophila*. (*A*) Germ cell-specific RNAi-mediated knockdown of *smb*, *U1-70*, *sf3a1*, *sf3b5*, *prp8*, and *brr2* in adult flies phenocopies the *U2A* mutant. (*Upper*) Immunostaining of testes with anti-Vasa (green) and anti-Hts (red). (*Lower*) Counterstaining of nuclei in testes and seminal vesicles using Hoechst 33342. (Scale bar: 20 μm.) (*B*) Reduced level of processed *mei-p26* mRNA in the testes of adult flies with knockdown of snRNP factors. The intronless *ote* transcript served as a control. (*C*) Partially processed *mei-p26* transcripts containing intron 6–7 sequences were detected in testes of flies with knockdown of snRNP factors.

Overexpression of *mei-p26(gs)* in germ cells using the *nos-gal4* driver caused a complete absence of germ cells (*SI Appendix*, Fig. S3E). Simultaneous expression of *U2A* RNAi dramatically suppressed this germ cell loss, and testes of adult *UASp-mei-p26(gs)/UASp-U2A RNAi; nos-gal4* flies appeared normal, containing both early-stage and cyst-stage germ cells (*SI Appendix*, Fig. S3E). Furthermore, knockdown of both *mei-p26* and *U2A* in spermatogonia and early spermatocytes (*bam-gal4* driver) produced a more severe phenotype than seen with the knockdown of *mei-p26* or *U2A* alone, with increased accumulation of spermatogonial cysts and fewer spermatocytes in the apical region (Fig. 3E). Collectively, these findings indicate that the presence of U2A is required for *mei-p26* pre-mRNA splicing, and that this processing step is essential for the differentiation of spermatogonia.

We next assessed the effect of U2A deficiency on the splicing of individual introns of *mei-p26* and on the processing of other transcripts. qPCR with six different primer pairs located in flanking exons of *mei-p26* transcripts (*SI Appendix*, Fig. S3A) detected only partially processed transcripts containing intron 6–7 (numbering based on transcript RE; *SI Appendix*, Fig. S3F), revealing that splicing defects were specific to intron 6–7. Loss of U2A was associated with reduced mRNA levels of *how* and *topi*, whereas *βtub60D* and *aly* mRNA levels were unaffected (*SI Appendix*, Fig. S3G). Moreover, qPCR products of incompletely processed *how* and *topi*, but not *βtub60D* and *aly*, transcripts were detected in mutant testes (*SI Appendix*, Fig. S3H). Thus, it appears that U2A deficiency affects the processing only of certain transcripts and specific exons.

The Major Spliceosome Plays a Role in Regulating Spermatogonial Differentiation in *Drosophila*. U2A/SNRPA1 is associated with snRNP U2 and participates in assembly of the major spliceosome. To evaluate the requirement of the major spliceosome for spermatogonial differentiation, we performed RNAi-mediated knockdown of the *Drosophila* homologs of SNRPB, SNRNP70, SF3A1, SF3B5, PRPF8, and SNRNP200 in adult flies. These proteins belong to different snRNP complexes, including U1, U2, U4, and U5 (*SI Appendix*, Table S2). Germ cell-specific (i.e., *bam-gal4* driver) knockdown of each of these factors caused male sterility (*SI Appendix*, Table S2), with defects in spermatogonial differentiation and absence of mature sperm (Fig. 4A). Consistent with defects in splicing, *mei-p26* mRNA transcript levels were reduced in all knockdown lines, whereas levels of the intronless *ote* transcript remained unaffected (Fig. 4B). Incompletely spliced transcripts were detectable in all mutants (Fig. 4C). Taken together, these results suggest that the major spliceosome is required for spermatogonial differentiation in *Drosophila* spermatogenesis.

Human SNRPA1 Restores Spermatogonia Development in *Drosophila* U2A Mutant or Knockdown Flies. Human SNRPA1 has a 47% sequence difference from *Drosophila* U2A. To assess whether human SNRPA1 (hSNRPA1) protein could functionally replace

U2A, we first examined whether hSNRPA1 binds *Drosophila* spliceosome components and *mei-p26* mRNA. To do so, we performed RNA immunoprecipitation of recombinant proteins expressed in *Drosophila* S2 cells, and found that, similar to *Drosophila* U2A, hSNRPA1 interacted with *mei-p26* mRNA, whereas GFP protein did not (Fig. 5A and *SI Appendix*, S4A). We next expressed *hSNRPA1* cDNA from the transgene *P{uasp-hSNRPA1}* in flies with *bam-gal4* driver-induced knockdown. In *bam > U2A* RNAi; *bam > hSNRPA1* flies, spermatogenesis and fertility were fully restored (*SI Appendix*, Table S3), and spermatogonial differentiation proceeded normally, resulting in the production of mature sperm (Fig. 5B). *Mei-p26* mRNA was processed
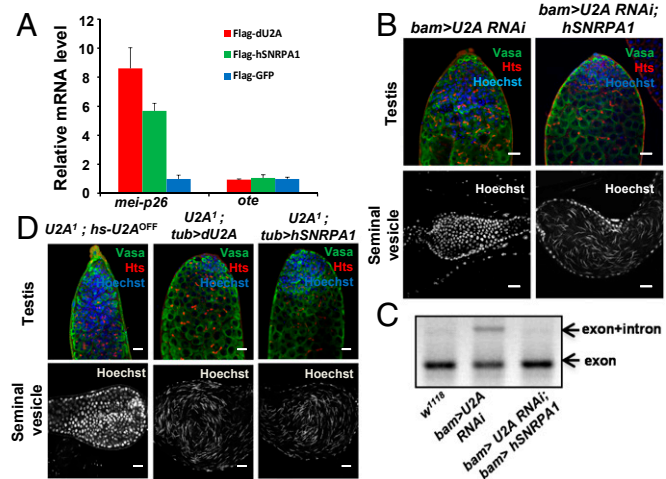
Fig. 5. Human SNRPA1 restores spermatogonial development in *Drosophila* U2A mutant and knockdown flies. (*A*) Human SNRPA1 interacts with *mei-p26* RNA in *Drosophila* S2 cells. Shown in qRT-PCR analysis of RNA isolated by immunoprecipitation of Flag-U2A (red), Flag-SNRPA1 (green), and Flag-GFP (blue). (*B*) Expression of *hSNRPA1* rescues germ cell development in *U2A* knockdown flies. Shown is normal germ cell development in the testis apex of *bam > U2A* RNAi; *bam > hSNRPA1* flies (*Upper*; anti-Vasa in green, anti-Hts in red, and Hoechst 33342 in blue) and the presence of mature sperm in the seminal vesicles (*Lower*; Hoechst 33342 staining). (Scale bar: 20 μm.) (*C*) Expression of hSNRPA1 restores *mei-p26* mRNA processing in U2A knockdown flies. Incompletely spliced *mei-p26* transcripts were present in *bam > U2A* RNAi flies, but not in *bam > U2A* RNAi; *bam > hSNRPA1* flies. (*D*) Expression of *hSNRPA1* restores spermatogenesis in *U2A*[1] null mutant flies. Shown is immunostaining of testis with anti-Vasa (green) and anti-Hts (red); nuclei were counterstained with Hoechst 33342. In *U2A* mutant flies (*U2A*[1]; *hs-U2A*[OFF]), the testes are filled with small spermatogonial cysts (*Upper*), and seminal vesicles lack mature sperm (*Lower*). In the presence of U2A or hSNRPA1, mutant flies undergo normal germ cell maturation (*Upper*) and produce mature sperm (*Lower*). (Scale bar: 20 μm.)

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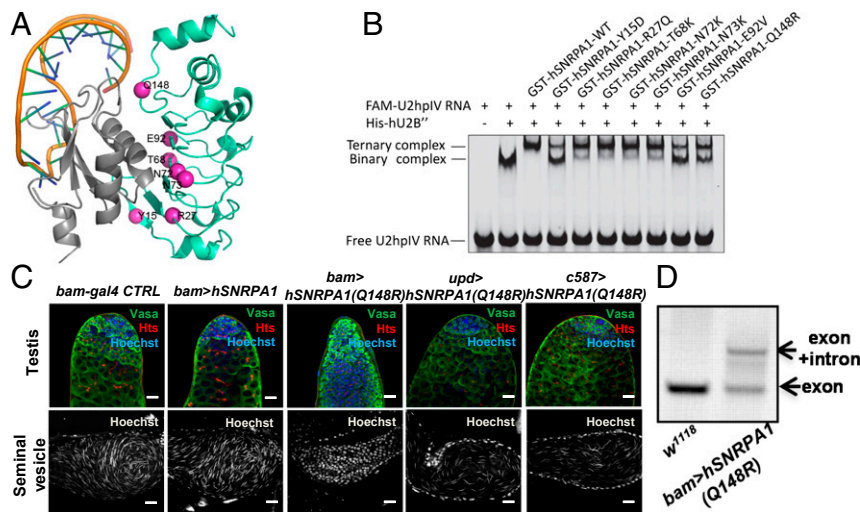
Fig. 6. A human SNRPA1 missense mutation deregulates major spliceosome assembly and disrupts spermatogenesis dominantly. (*A*) Location of the mutated amino acids (shown in magenta) in the crystal structure of the spliceosomal U2B-hSNRPA1-hairpin IV of the U2 snRNA complex (Protein Data Bank ID code 1A9N). (*B*) Gel-retardation analysis of hU2B binding to 6-carboxyfluorescein (FAM)-labeled U2hpIV RNA in the presence of hSNRPA1 or hSNRPA1 missense mutants. (*C*) Overexpression of hSNRPA1 (Q148R) in germ cells, but not in somatic cells, phenocopies the *U2A* mutant. Staining was done with anti-Vasa (green), anti-Hts (red), and Hoechst 33342. (Scale bar: 20 μm.) (*D*) Incomplete processing of *mei-p26* pre-mRNA in flies expressing hSNRPA1 (Q148R). Shown is qRT-PCR amplification of fragments containing intron 6–7 sequences (Fig. 3*C*).

normally in U2A knockdown flies expressing hSNRPA1, incompletely spliced transcripts were not detectable, and relative mRNA levels were similar to those in controls (Fig. 5*C* and *SI Appendix*, Fig. S4*B*). Furthermore, ubiquitous expression of hSNRPA1 induced by the *tub-gal4* driver rescued the lethal phenotype of *U2A* null mutant (*U2A¹*) flies. *U2A¹; tub-gal4>hSNRPA1* flies were viable and fully fertile (*n* = 21; 86%) (*SI Appendix*, Table S3) and had normal testis morphology and spermatogonial development resembling that of WT and *U2A¹; tub-gal4>U2A* flies (Fig. 5*D*). These results strongly suggest that hSNRPA1, in its function as part of the splicing mechanism, may play a role during the development of human spermatogonia.

Missense Mutations of Human SNRPA1 Deregulate Major Spliceosome Assembly. Guided by the crystal structure of the ternary complex between the spliceosomal hSNRPA1-U2B protein complex and the hairpin loop IV of U2 small nuclear RNA (U2hplV) (Protein Data Bank ID code 1A9N), we designed missense mutants of human hSNRPA1, specifically Y15D, R27Q, T68K, N72K, N73K, E92V, and Q148R (Fig. 6*A*), affecting residues located at the interface between hSNRPA1 and U2B, such that resulting structural alterations may affect the assembly of the U2 SNP complex in the major spliceosome. In vitro pull-down assays using histidine (his) tag-purified proteins expressed in *Escherichia coli* (*SI Appendix*, Fig. S5*A*) did not reveal detectable changes in the binding of hSNRPA1 mutants to U2B (*SI Appendix*, Fig. S5*B*). Presumably, replacement of one residue at the binding interface was insufficient to disrupt the stable hSNRPA1-U2B complex in vitro.

Electrophoretic mobility shift assay (EMSA) analyses showed that, as expected, U2B and U2hplV RNA formed the binary complex, which was entirely converted to the ternary complex on the addition of WT hSNRPA1 (Fig. 6*B*); however, in reactions involving three different missense hSNRPA1 mutants (Y15D, E92V, and Q148R), only very low levels of ternary complex were formed, indicating that the binding of these mutants to the binary complex was largely prohibited. The remaining four mutants formed ternary complexes similar to WT protein (Fig. 6*B*). EMSA with different protein concentrations ranging from 0.2 to 1.0 μM also revealed that WT hSNRPA1 forms the ternary complex more effectively than these three hSNRPA1 mutants (*SI Appendix*, Fig. S5*C*). Thus, missense mutations in hSNRPA1 may deregulate assembly of the major spliceosome.

Human SNRPA1 (Q148R) Disrupts Spermatogenesis in a Dominant Negative Fashion. To further evaluate the biological function of missense mutations in hSNRPA1 affecting spliceosome assembly, we focused on hSNRPA1 (Q148R), which affects a highly conserved residue within the LRRCT domain (*SI Appendix*, Fig. S5*D*). Germ cell-specific (i.e., *bam-gal4* driver) expression of this mutant [*P{uasp-hSNRPA1 (Q148R)}*] in *Drosophila* caused male sterility (*n* = 32; sterility rate, 62.5%), whereas germ cell-specific expression of the WT protein or somatic cell-specific expression of hSNRPA1 (Q148R) did not affect male fertility (*n* = 48; sterility rate, 0%) (*SI Appendix*, Fig. S5 *E* and *F*). Testes from hSNRPA1 (Q148R) transgenic flies lacked mature sperm and exhibited extensive defects in spermatogonial development resembling the *U2A* mutant (Fig. 6*C* and *SI Appendix*, Fig. S2*H*). Overexpression of hSNRPA1 (Q148R) did not restore fertility and spermatogonial maturation in *U2A* knockdown flies (*SI Appendix*, Table S3 and Fig. S5*G*), and hSNRPA1 (Q148R) did not interact with *mei-p26* RNA (*SI Appendix*, Fig. S5 *H* and *I*). Testes expressing hSNRPA1 (Q148R) contained unprocessed *mei-p26* transcript (Fig. 6*D*) and reduced levels of *mei-p26* mRNA (*SI Appendix*, Fig. S5*J*). Combined with our findings from the in vitro U2 SNP assembly assay, these results suggest that hSNRPA1 (Q148R) may play a dominant negative role in assembly of the major spliceosome, leading to disruption of spermatogenesis in vivo.

Discussion

NOA is a common cause of male infertility, but its molecular nature remains to be elucidated. Here, using *Drosophila* as a model, we found that the spliceosome component *U2A*, the homolog of *hSNRPA1*, is required for spermatogonial differentiation and associated with NOA. Specifically, spliceosome dysregulation impairs the differentiation of spermatogonia, abolishing the maturation of germ cells into sperm.

U2A/SNRPA1 belongs to the U2 snRNP A family. Although in vitro studies have indicated that U2A/SNRPA1 is associated with snRNP U2 and participates in the assembly of the major spliceosome, the roles of this gene and related pathways in specific developmental processes remain largely unknown (17, 18). So far, evidence of the relevance of spliceosome components for male gametogenesis has been provided only by a cell culture study, which suggested that changes in phosphorylation of the spliceosome component SAP155 may suppress proliferation of

mouse spermatogonia (19). In the present study, we have identified a specific role of the major spliceosome during germ cell development: control of spermatogonial differentiation.

The major spliceosome is a highly dynamic protein complex. Its U2A component appears to neither play an essential role during earlier stages of germ cell development, including asymmetric divisions of germ cell stem cells, nor have a relevant function in the formation of cyst cells. We cannot exclude a requirement for U2A as part of the major spliceosome in later stages of germ cell development, which would be masked by the early phenotype of the U2A mutants. The intriguing sensitivity of spermatogonia to spliceosome activity can be explained in part by the fact that essential factors expressed at this stage are extensively regulated by splicing, including *mei-p26* gene products (*SI Appendix*, Fig. S6).

The *mei-p26* gene has a very large gene body size of nearly 30 kb, and five different transcripts have been identified (20, 21). Mei-p26 is involved in germ cell development in *Drosophila* (22–24). Previous reports have shown that translation of *mei-P26* mRNA is controlled by Bam, Bgcn, CCR4, Vasa, and Tut (22, 25–27). Therefore, Mei-p26 may represent a key node in the mRNA metabolism network that controls distinct developmental programs during germ cell formation and maturation. The homologous human gene of *mei-p26* is *TRIM3*, which is highly expressed in testis (www.proteinatlas.org); thus, it may be interesting to investigate the male reproductive function of TRIM3 in mammals.

Different transcripts and exons interact specifically with components of the spliceosome machinery and thus likely differ in their sensitivity to a lack of these factors (28, 29). Defects in spliceosome components can cause specific human diseases, including retinal degeneration and Taybi–Linder syndrome (12). Here we show that defects in the major spliceosome cause failure of spermatogonial differentiation and are associated with NOA

in human. Thus, given its complexity (~150 proteins) and necessity during spermatogonial maturation, a large number spliceosome component-encoding factors may represent susceptibility factors for NOA in human.

In summary, our biological and clinical evidence indicates that the conserved major spliceosome specifically controls spermatogonial differentiation, and that dysfunction of this complex may cause NOA. Our findings suggest U2A/SNRPA1 as a potential candidate for characterizing and treating patients with NOA. The methodology applied here also may serve to analyze other risk-associated SNPs in GWAS.

Materials and Methods

Fly stocks used in this study were maintained under standard culture conditions. Unless specified otherwise, all primary stocks were obtained from Tsinghua Fly Center. Strains P{nos-gal4:vp16} (30), P{bam-gal4:vp16} (16), P{c587-Gal4} (31), P{upd-gal4} (32), P{uasp-shbam} (33), and P{tub-gal4} (34) have been maintained in our laboratory. Strains P{UASp-shmei-p26}, P{UASp-U2A}, P{UASp-SNRPA1}, P{hs-U2A}, and U2A¹ were generated for this study. Additional information on the fly stocks used in this study is provided in *SI Appendix*, Table S5. The following primary antibodies were used: mouse anti-Hts (Developmental Studies Hybridoma Bank), rabbit anti-GFP (Invitrogen), rabbit anti-pMad (pSmad1/5; Cell Signaling), rabbit anti-Histone H3 (phospho S10; Abcam), rabbit anti-U2A (generated by our group), and rabbit anti-BamC and rabbit anti-Vasa antibodies (gifts from Dr. Dahua Chen, Institute of Zoology, Chinese Academy of Sciences, Beijing) (35, 36). Additional information is provided in *SI Appendix*, *Materials and Methods*.

1. Lee JY, Dada R, Sabanegh E, Carpi A, Agarwal A (2011) Role of genetics in azoospermia. *Urology* 77(3):598–601.
2. Hamada AJ, Esteves SC, Agarwal A (2013) A comprehensive review of genetics and genetic testing in azoospermia. *Clinics (Sao Paulo)* 68(Suppl 1):39–60.
3. Ferlin A, Arredi B, Foresta C (2006) Genetic causes of male infertility. *Reprod Toxicol* 22(2):133–141.
4. Dam AH, et al. (2007) Homozygous mutation in SPATA16 is associated with male infertility in human globozoospermia. *Am J Hum Genet* 81(4):813–820.
5. Dieterich K, et al. (2007) Homozygous mutation of AURKC yields large-headed polyploid spermatozoa and causes male infertility. *Nat Genet* 39(5):661–665.
6. Wang XN, et al. (2013) The Wilms tumor gene, Wt1, is critical for mouse spermatogenesis via regulation of Sertoli cell polarity and is associated with non-obstructive azoospermia in humans. *PLoS Genet* 9(8):e1003645.
7. de Cuevas M, Matunis EL (2011) The stem cell niche: Lessons from the *Drosophila* testis. *Development* 138(14):2861–2869.
8. Spradling A, Fuller MT, Braun RE, Yoshida S (2011) Germline stem cells. *Cold Spring Harb Perspect Biol* 3(11):a002642.
9. Xu EY, et al. (2003) Human BOULE gene rescues meiotic defects in infertile flies. *Hum Mol Genet* 12(2):169–175.
10. Yu J, et al. (2015) Identification of seven genes essential for male fertility through a genome-wide association study of non-obstructive azoospermia and RNA interference-mediated large-scale functional screening in *Drosophila*. *Hum Mol Genet* 24(5):1493–1503.
11. Wahl MC, Will CL, Lührmann R (2009) The spliceosome: design principles of a dynamic RNP machine. *Cell* 136(4):701–718.
12. Singh RK, Cooper TA (2012) Pre-mRNA splicing in disease and therapeutics. *Trends Mol Med* 18(8):472–482.
13. Hu Z, et al. (2014) Association analysis identifies new risk loci for non-obstructive azoospermia in Chinese men. *Nat Commun* 5:3857.
14. Hu Z, et al. (2012) A genome-wide association study in Chinese men identifies three risk loci for non-obstructive azoospermia. *Nat Genet* 44(2):183–186.
15. Ni JQ, et al. (2011) A genome-scale shRNA resource for transgenic RNAi in Drosophila. *Nat Methods* 8(5):405–407.
16. Chen D, McKearin DM (2003) A discrete transcriptional silencer in the bam gene determines asymmetric division of the *Drosophila* germline stem cell. *Development* 130(6):1159–1170.
17. Blencowe BJ, Issner R, Nickerson JA, Sharp PA (1998) A coactivator of pre-mRNA splicing. *Genes Dev* 12(7):996–1009.
18. Elliott DJ, Grellscheid SN (2006) Alternative RNA splicing regulation in the testis. *Reproduction* 132(6):811–819.
19. Eto K, Sonoda Y, Abe S (2011) The kinase DYRKIA regulates pre-mRNA splicing in spermatogonia and proliferation of spermatogonia and Sertoli cells by phosphorylating

20. a spliceosomal component, SAP155, in postnatal murine testes. *Mol Cell Biochem* 355(1-2):217–222.
21. Page SL, McKim KS, Deneen B, Van Hook TL, Hawley RS (2000) Genetic studies of mei-P26 reveal a link between the processes that control germ cell proliferation in both sexes and those that control meiotic exchange in *Drosophila*. *Genetics* 155(4):1757–1772.
22. Glasscock E, Singhania A, Tanouye MA (2005) The mei-P26 gene encodes a RING finger B-box coiled-coil NHL protein that regulates seizure susceptibility in *Drosophilia*. *Genetics* 170(4):1677–1689.
23. Insco ML, et al. (2012) A self-limiting switch based on translational control regulates the transition from proliferation to differentiation in an adult stem cell lineage. *Cell Stem Cell* 11(5):689–700.
24. Neumüller RA, et al. (2008) Mei-26 regulates microRNAs and cell growth in the *Drosophila* ovarian stem cell lineage. *Nature* 454(7201):241–245.
25. Li Y, Maines JZ, Tastan OY, McKearin DM, Buszczak M (2012) Mei-P26 regulates the maintenance of ovarian germline stem cells by promoting BMP signaling. *Development* 139(9):1547–1556.
26. Joly W, Chartier A, Rojas-Rios P, Busseau I, Simonelig M (2013) The CCR4 deadenylase acts with Nanos and Pumilio in the fine-tuning of Mei-P26 expression to promote germline stem cell self-renewal. *Stem Cell Rep* 1(5):411–424.
27. Chen D, et al. (2014) Three RNA binding proteins form a complex to promote differentiation of germline stem cell lineage in *Drosophila*. *PLoS Genet* 10(11):e1004797.
28. Liu N, Han H, Lasko P (2009) Vasa promotes *Drosophila* germline stem cell differentiation by activating mei-p26 translation by directly interacting with a (U)-rich motif in its 3′ UTR. *Genes Dev* 23(23):2742–2752.
29. Hoskins AA, Moore MJ (2012) The spliceosome: A flexible, reversible macromolecular machine. *Trends Biochem Sci* 37(5):179–188.
30. Faustino NA, Cooper TA (2003) Pre-mRNA splicing and human disease. *Genes Dev* 17(4):419–437.
31. Van Doren M, Williamson AL, Lehmann R (1998) Regulation of zygotic gene expression in *Drosophila* primordial germ cells. *Curr Biol* 8(4):243–246.
32. Kai T, Spradling A (2003) An empty *Drosophila* stem cell niche reactivates the proliferation of ectopic cells. *Proc Natl Acad Sci USA* 100(8):4633–4638.
33. Halder G, Callaerts P, Gehring WJ (1995) Induction of ectopic eyes by targeted expression of the eyeless gene in *Drosophila*. *Science* 267(5205):1788–1792.
34. Wang H, Mu Y, Chen D (2011) Effective gene silencing in *Drosophila* ovarian germline by artificial microRNAs. *Cell Res* 21(4):700–703.
35. Lee T, Luo L (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* 22(3):451–461.
36. Xia L, et al. (2010) The Fused/Smurf complex controls the fate of *Drosophila* germline stem cells by generating a gradient BMP response. *Cell* 143(6):978–990.
37. Yang L, et al. (2007) Argonaute 1 regulates the fate of germline stem cells in *Drosophila*. *Development* 134(23):4265–4272.

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