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Mutation of *Eif4g3*, encoding a eukaryotic translation initiation factor, causes male infertility and meiotic arrest of mouse spermatocytes

Fengyun Sun, Kristina Palmer and Mary Ann Handel*

SUMMARY

The ENU-induced *repro8* mutation was identified in a screen to uncover genes that control mouse gametogenesis. *repro8* causes male-limited infertility, with failure of spermatocytes to exit meiotic prophase via the G2/MI transition. The *repro8* mutation is in the *Eif4g3* gene, encoding eukaryotic translation initiation factor 4, gamma 3. Mutant germ cells appear to execute events of meiotic prophase normally, and many proteins characteristic of the prophase-to-metaphase transition are not obviously depleted. However, activity of CDC2A (CDK1) kinase is dramatically reduced in mutant spermatocytes. Strikingly, HSPA2, a chaperone protein for CDC2A kinase, is absent in mutant spermatocytes in spite of the presence of *Hspa2* transcript, consistent with the observation that the *repro8* phenotype is markedly similar to the phenotype of the *Hspa2* knockout. Thus, EIF4G3 is required for HSPA2 translation in spermatocytes, a finding that provides the first genetic evidence for selective translational control of meiotic exit in mammalian spermatocytes.

KEY WORDS: Meiosis, Spermatogenesis, Translation initiation, Mouse

INTRODUCTION

Spermatogenesis requires coordination of gene and protein expression during mitotic proliferation, meiosis and postmeiotic spermiogenic differentiation; failure to ensure timely completion of these events leads to infertility. About half of human couple infertility is related to failure in male reproductive function and much of this is idiopathic in nature (Matzuk and Lamb, 2002; Matzuk and Lamb, 2008). Such infertility could be better understood if there was more knowledge about genes expressed during spermatogenesis and required for the process. Genes controlling meiosis, a defining event of both male and female gametogenesis, are of particular interest. In spite of evolutionary conservation of the process, many of the genes and proteins that control mammalian meiosis are still unknown (Handel and Schimenti, 2010).

One of the least understood aspects of meiosis is control over exit from meiotic prophase. Neither the 'clock' that controls this, nor the requisite checkpoints and participating players, are well defined. Moreover, both exit from prophase and onset of the division phase are sexually dimorphic aspects of meiosis (Handel and Eppig, 1998; Handel and Schimenti, 2010). Oocytes experience arrest (diakinesis) after desynapsis of homologous chromosomes, and meiotic maturation is initiated by factors extrinsic to the oocyte. Conversely, in the male, after desynapsis, there is rapid progress through the diplotene stage to the two meiotic divisions. Experimental separation of spermatocytes from the surrounding somatic Sertoli cells suggests that processes intrinsic to the germ cell control meiotic exit (Handel and Eppig, 1998). Exit from meiotic prophase, or the prophase I to metaphase I transition (G2/MI), is initiated by disassembly of the central element of the synaptonemal complex (SC), followed by disassembly of lateral elements of the SC, chromatin condensation and final compaction of distinct MI bivalent chromosomes. The universal cell-cycle regulator MPF (metaphase-promoting factor), comprising CDC2A (CDK1 – Mouse Genome Informatics) kinase and a cyclin B regulatory subunit, is involved in this process (Cobb et al., 1999a; Sun and Handel, 2008). Both formation of condensed MI bivalents and disassembly of the SC are regulated by cyclin-dependent kinases (CDKs) and aurora kinases (AURKs), and AURKs also mediate phosphorylation of histone H3 (Sun and Handel, 2008). However, neither CDKs nor AURKs initiate disassembly of the central element of the SC (Sun and Handel, 2008), the first step of the G2/MI transition.

Genetic analyses have revealed that cyclin A1 is required for MPF activation and the G2/MI transition (Liu et al., 2000; Liu et al., 1998). The heat shock chaperone protein HSPA2 (previously known as HSP70-2) is also required for CDC2A kinase activity, as well as for desynapsis (Zhu et al., 1997). Similar to the mouse, components of MPF are required for exit from meiotic prophase by *Drosophila* spermatocytes (Maines and Wasserman, 1998). Moreover, in *Drosophila* and *C. elegans*, there is evidence for translational control of the meiotic G2/MI transition. Translation initiation complex proteins coordinate spermatogenesis in *Drosophila* (Baker and Fuller, 2007; Franklin-Dumont et al., 2007); mutation of the gene encoding the translation initiation factor eIF4G2 causes germ cells to skip the meiotic division phase and affects translation of transcripts for cyclin B and twine, a CDC25 phosphatase. In *C. elegans*, another translation initiation factor, eIF4E (IFE-1), is required for the meiotic division (Amiri et al., 2001). During mammalian spermatogenesis, translational control is exerted by differential polyadenylation, as well as by various RNA binding proteins (Hecht, 1998), but until now there has been no evidence that exit from meiosis is regulated at the level of translation.

Here, we used an unbiased forward genetics strategy (Handel et al., 2006; Schimenti and Bucan, 1998; Ward et al., 2003) to find phenotypes causing meiotic arrest and aberrant exit from meiotic prophase in mouse spermatocytes. A mutation, *repro8*, was identified in the *Eif4g3* gene, encoding a eukaryotic translation

The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609, USA.

*Author for correspondence (maryann.handel@jax.org)

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initiation complex protein, EIF4G3. The mutation leads to failure in translation of the transcript for HSPA2, a chaperone of CDC2A kinase. The meiosis and spermatogenesis arrest phenotypes caused by the mutant *Eif4g3*^{repro8} allele provide the first evidence that some events of meiotic exit during mammalian spermatogenesis are selectively regulated at the level of protein translation.

MATERIALS AND METHODS

Animals

Mice were maintained following protocols approved by the Jackson Laboratory (JAX) Institutional Animal Care and Use Committee (IACUC). The *repro8* mutation was induced in a C57BL/6J (B6) background and subsequently outcrossed to C3HeB/FeJ (C3H). After defining the candidate region for the *repro8* mutation, a congenic line on C3H was created; all *Eif4g3*^{repro8} mice and control wild-type littermates used for biological analyses were from the congenic line.

Mutagenesis, genetic fine mapping and sequencing

Mutagenesis of adult male B6 mice with ethylnitrosourea (ENU) and identification of the infertile *repro8* phenotype in a three-generation breeding scheme were conducted as previously described (Handel et al., 2006).

Genotyping was performed by PCR amplification of tail DNA. For initial regional mapping of the *repro8* mutation to distal chromosome 4, a genome scan was carried out by Genome Sciences Service at JAX with microsatellite markers polymorphic between B6 and C3H. For the genetic fine mapping, mutant females were crossed to CAST/EiJ (CAST) males and F2 individuals were genotyped with additional polymorphic markers.

For sequencing, DNA was extracted from tails of *repro8*/*repro8*, *repro8*/+ and B6 mice. After PCR amplification, products were electrophoresed on 2% SeaKemLE Agarose gels (Lonza, Rockland, ME, USA). Suitable bands were cut, DNA extracted using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and DNA sequencing performed by the JAX Genome Sciences Service using standard methods. Exons and intron-exon boundaries of all candidate genes were sequenced.

Creation of gene-trap mice and complementation analysis

An ES cell line, XC431, was obtained from Bay Genomics (International Gene Trap Consortium). The position of the XC431 gene trap construct is near the 3′ end (between exon 34-35 of transcript ENSMUST00000105831) and was confirmed by sequencing ES cell cDNA. Chimeras were generated by microinjection of ES cells into B6 blastocysts by the Reproductive Sciences Service at JAX. Chimeric males with germ cell transmission were crossed to *repro8*/*repro8* females and testes were removed from male offspring for histology and western blot analyses.

Histology and immunohistochemistry

Testes were fixed in Bouin's solution or 4% paraformaldehyde in PBS overnight and paraffin-embedded. Sections (5 μm) were stained with Periodic Acid Schiff (PAS) by standard procedures. For immunohistochemistry, sections were blocked with 5% goat serum in PBS at room temperature for 30 minutes. Primary antibodies (Table 1) were applied and slides incubated at 37°C for 1 hour. Antigens were localized using SuperPicTure Polymer Detection Kit (Zymed/Invitrogen, South San Francisco, CA, USA) and sections were counterstained with Hematoxylin (Sigma Aldrich, St Louis, MO, USA), then mounted in Clearmount (Zymed/Invitrogen). The TUNEL assay (Roche, Indianapolis, IN, USA) was performed according to the manufacturer's instructions. Images were acquired with a Leica DMRXE microscope equipped with a CCD camera (Leica Microsystems DI, Cambridge, UK).

Okadaic acid-induced meiotic prophase-to-metaphase (G2/MI) transition

Enriched germ cells were prepared as previously described (Bellve, 1993; La Salle et al., 2009). After incubation in culture medium at 32°C for 4 hours (Handel et al., 1995; La Salle et al., 2009), cells were induced to undergo the G2/MI transition by addition of okadaic acid (CalBiochem, EMD Chemicals, La Jolla, CA, USA) dissolved at 300 μM in ethanol and used at 4 μM in the culture; control cells were treated with the same volume of ethanol. After 4 hours of culture, the cells were collected for surface-spread chromatin and air-dried chromosome preparations.

Surface-spread chromatin and air-dried chromosome preparations

Spermatocytes were collected by centrifugation, surface-spread in wells of multi-spot slides (Shandon, Pittsburgh, PA, USA) and fixed as previously described (Cobb et al., 1999b; Cobb et al., 1997; Sun and Handel, 2008). Primary antibodies (Table 1) were used as previously described (Sun and Handel, 2008). Secondary antibodies conjugated with Alexa 594 or 488 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) were used at 1:500 dilution. Images were acquired with a Leica DMRXE epifluorescence microscope equipped with a 100× plan-neofluar oil-immersion objective lens and a triple filter (set no. 61000V2 BS&M, Chroma Technology, Rockingham, VT, USA). For image capture, a Micromax cooled CCD camera (Princeton Instruments, Trenton, NJ, USA) equipped with a high-speed shutter (Sutter Instrument Company, Novato, CA, USA) was used and Metamorph software (Molecular Devices, Sunnyvale, CA, USA) was used for image analysis.

Chromosome condensation was evaluated from air-dried chromosome preparations (Evans et al., 1964; Sun and Handel, 2008) stained with Gurr Giemsa (Invitrogen). Germ-cell stages were scored using an Olympus microscope with a 40× plan objective and 10× ocular lenses, and images captured with a Hamamatsu C5810 color-chilled camera (Photonic System, Bridgewater, NJ, USA).

Table 1. Primary antibodies used in this study

Antibody	Host	Producer	Cat. number	Dilution	
				IF	WB
GATA-1	Rabbit	Sigma	G4671	1:50	n/a
GATA-4	Rabbit	Santa Cruz	SC-9053	1:50	n/a
CDC25A	Rabbit	Cell Signaling	07-459	1:100	1:250
CDC25B	Rabbit	Upstate	9525	1:100	1:1000
CDC25C	Rabbit	Handel Laboratory	Inselman et al., 2004	1:200	1:1000
CCNB1	Mouse	Abcam	Ab72	1:400	1:2000
CCNB2	Rabbit	Santa Cruz	Sc-22776	1:50	1:100
CCBN3	Rabbit	Aviva Systems	ARP39739	1:100	1:500
HSPA2	Rabbit	Protein-Tech	12797-1-AP	1:100	1:1000
BAT3	Chicken	GenWay	15-288-22823	1:200	1:500
SYCP1	Rabbit	Novus	NB 300-229	1:100	n/a
SYCP3	Rabbit	Novus	NB100-2065	1:100	n/a
SYCP3	Rat	Handel Laboratory	Eaker et al., 2001	1:1000	n/a
H1t	G. Pig	Handel Laboratory	Cobb et al., 1999b	1:500	n/a
RAD51	Rabbit	Calbiochem	PC130	1: 200	n/a
γH2AX	Rabbit	Upstate	07-146	1:200	n/a
MLH1	Mouse	BD Pharmingen	51-1327GR	1:50	n/a
Phospho-H3	Rabbit	Upstate	29480	1:200	n/a

Immunoprecipitation and in vitro CDC2A kinase assay

Testes were collected from mutant and wild-type littermates at postnatal day 14 (P14) and total testis lysates were prepared by homogenization in fresh ice-cold protein lysis buffer [50 mM Tris pH7.5, 150 mM NaCl, 0.1% NP-40, 50 mM NaF, 1 mM DTT and protease inhibitor cocktail (Roche Diagnostics)]. The extracts were cleared by centrifugation at 20,000 ***g*** for 10 minutes at 4°C and protein concentrations were determined by the BCA method (Pierce Biotechnology, Rockford, IL, USA). Samples were used immediately or stored at –80°C.

For immunoprecipitation, 500 μg of total protein was clarified by incubation for 30 minutes at 4°C with rocking with 2 μg mouse IgG (Santa Cruz, sc-2025, Santa Cruz, CA, USA) and 20 μl of resuspended Protein G PLUS-Agarose beads (Santa Cruz, sc-2002) in protein lysis buffer containing 1 mM EDTA. Beads were pelleted by centrifugation at 1000 ***g*** for 5 minutes at 4°C and the supernatant transferred to another tube. Immunoprecipitation was performed by incubating aliquots of the precleared lysate with 10 μl of pre-immune serum (control) or CDC2 antibody (Santa Cruz, sc-54) for 4 hours at 4°C with rocking. Thirty microliters of Protein G PLUS-Agarose beads were added and incubated overnight at 4°C with shaking. The pellets were collected by centrifugation at 1000 ***g*** for 5 minutes at 4°C, washed 3 times with 50 μl kinase assay buffer (Promega, Madison, WI, USA), and resuspended in 15 μl CDC2A kinase assay buffer.

The in vitro kinase assay was performed with SignaTECT CDC2 Protein Kinase Assay System (Promega, TB227). Histone H1 kinase activity was determined in the kinase assay buffer with 50 μM ATP, 25 μM CDC2 protein kinase biotinylated peptide substrate (derived from histone H1 and highly selective for CDC2A) and 40 μCi/ml [γ-^{32}P] ATP with or without CDC2A kinase inhibitor olomoucine, based on the manufacturer's instructions. The total reaction volume was 25 μl. The reaction was incubated at 30°C for 30 minutes, terminated by addition of termination buffer, and after addition of 2× sample buffer (Santa Cruz) and boiling for 5 minutes, 20 μl samples from each group were separated on an 8% SDS-polyacrylamide gel, dried and scanned with Fujifilm FLA-5100 (Life Science, Tokyo, Japan).

RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and 10 ng RNA was used in a one-step RT-PCR reaction (Qiagen), according to the manufacturer's instructions, with the following modifications: denaturation for 45 seconds, annealing at 60°C for 45 seconds and extension for 1 minute for a total of 36 cycles. Table 2 presents the sequences of the PCR primers.

Table 2. Primers used in this study

Name	Sequence (5′-3′)
To detect alternative *Eif4g3* transcripts	
Eif4g3-t forward	CTTCCCACCTACGGTCTCTG
Eif4g3-t reverse	GATTTGAGGCCTCTGGAAAA
To define stage of meiotic arrest	
Dmc1 forward	CAGTATGACACTATCCAAAAATGGTATG
Dmc1 reverse	CAATCACACACACTCAAAAACAATGTTC
Clgn forward	GAGAGAGAGATCGGGCATCTAGAGT
Clgn reverse	TTCAAAGACCAGTTAACTTCAAAGTTAC
Prm2l forward	AGCCCCAGTGAGGGTCCGCACCA
Prm2l reverse	GTGATGGTGCCTCCTACATTTCCT
To amplify region of *Eif4g3* containing the mutation	
Eif4g3-m forward	ACAAATGGGAGAGCAGCAAG
Eif4g3-m reverse	ATTGGCGAAGACGTGAAACT
To detect transcription of *Hspa2*	
Hspa2 forward	CACCTTCGATGTGTCCATCC
Hspa2 reverse	CGAGTAACAGCAGATCCTGC
Actin control	
Actb forward	TAAAGACCTCTATGCCAACACAGT
Actb reverse	CACGATGGAGGGGCCGGACTCATC

Western blot analysis

Total protein was extracted from testes of P14 or adult mice using RIPA buffer (Santa Cruz) containing protease inhibitor cocktail (Santa Cruz). Protein concentration was measured by the BCA method (Pierce Biotechnology). Ten micrograms of protein from each group was boiled for 3 minutes and proteins were separated by electrophoresis using 8 or 10% SDS-PAGE. Proteins were transferred onto PVDF membrane (Millipore, Temecula, CA, USA). The membranes were blocked overnight at 4°C with 5% dried milk in Tris-buffered saline with 0.1% Tween-20 and then probed with antibodies at specified concentrations (Table 1). The blots were incubated for 2 hours at room temperature and then incubated with horseradish peroxidase-conjugated secondary antibodies made from mouse (Invitrogen), rabbit (Invitrogen) or chicken (ProSci Inc., Poway, CA, USA) for 1 hour at room temperature. Proteins were detected using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Pittsburgh, PA, USA).

RESULTS

The *repro8* phenotype: male infertility with meiotic arrest before the division phase

The *repro8* mutant line was identified by the ReproGenomics program at JAX (Handel et al., 2006). The mutation was produced on a B6 background and carriers were subsequently mated to C3H individuals in a standard three-generation breeding scheme to identify autosomal recessive phenotypes causing infertility. The homozygous *repro8*/*repro8* males (hereafter referred to as *repro8* mutant individuals) identified in this screen were overtly normal except that the testis weight of the mutant adults was about one third of the wild-type littermates (26.02 mg versus 83.67 mg; see Table S1 in the supplementary material). Intercrosses of heterozygous animals from a C3H congenic line generated homozygous offspring at a frequency of 23.8% (96/404), slightly, but not significantly, less than 25%, providing evidence that the *repro8* mutation segregates as a simple recessive Mendelian trait. Although no epididymal sperm were recovered from adult mutants, *repro8* mutant males produced plugs in female mice, suggesting normal mating behavior.

Arrest of spermatogenesis in *repro8* mutant males occurs in meiotic prophase, before the division phase. All stages of spermatogenesis were observed in seminiferous tubules of adult wild-type mice (Fig. 1A), whereas in the mutant, only spermatogonia and spermatocytes (Fig. 1B) were observed. The absence of postmeiotic germ cells was also supported by RT-PCR assays for transcripts that are developmentally expressed during spermatogenesis; markers of meiotic prophase (*Dmc1* and *Clgn*, calmegin), but not one for spermiogenesis (*Prm2*, protamine 2), were expressed in *repro8* mutant testes (see Fig. S1 in the supplementary material). Analysis of the first wave of spermatogenesis more precisely confirmed meiotic prophase arrest. At postnatal day 8 (P8), P12 (data not shown) and P14 (Fig. 1C,D), there were no obvious differences in either morphology or histological relationships of various cell stages and germ cell number between mutants and wild-type littermates. By P17 and P21, seminiferous tubules of mutant testes contained spermatocytes with highly condensed chromatin (Fig. 1E-H). At P21, MI spermatocytes were not observed in mutant testes, although present in wild-type testes (Fig. 1G,H). To determine whether spermatogenesis became arrested in *repro8* mutants before or after the mid-pachytene stage of meiotic prophase, we assessed the presence of histone H1t, a mid-pachynema marker (Cobb et al., 1999a; Drabent et al., 1993; Inselman et al., 2003). Histone H1t was detected in nuclei of *repro8* mutant spermatocytes (see Fig. S2 in the supplementary material), indicating that germ cell development reaches at least the mid-pachytene stage before arrest. By TUNEL cell-death detection, we found a slight increase in the frequency of apoptotic germ cells at

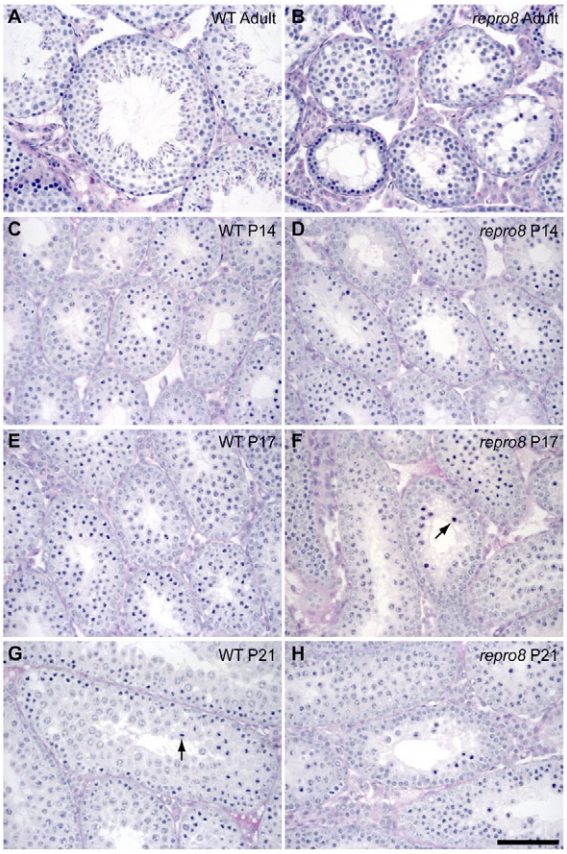

Fig. 1. PAS-stained testis sections at different developmental stages reveal meiotic arrest in *repro8* mutants. (**A**,**B**) In adult males, spermatogonia, spermatocytes and spermatids are present in wild-type testes (A), whereas in mutant testes (B), only spermatogonia and spermatocytes were found. (**C**,**D**) At P14, no major differences were observed in spermatogenic cell content between wild-type (C) and *repro8* mutant (D) testes. (**E**,**F**) By P17, *repro8* mutant testes (F) exhibited more spermatocytes with highly condensed chromatin (arrow) compared with wild-type testes (E). (**G**,**H**) At P21, metaphase spermatocytes were present (G, arrow) in wild-type testes; however, no metaphase I spermatocytes were found in *repro8* mutant testes (H) and many spermatocytes exhibited highly condensed chromatin. Scale bar: 100 μm.

P14 in *repro8* mutant testes and, by P17, a significantly greater number of dead cells per tubule and increased frequency of tubules with dead cells (see Fig. S3 and Table S2 in the supplementary material).

Because failure of *repro8* mutant spermatocytes to exit meiotic prophase could be due to abnormal or absent recombination, we assessed markers of these events. As shown in Fig. 2A,B, the labeling pattern of SYCP1 and SYCP3, components of the SC, in the mutant spermatocytes was indistinguishable from that in the wild-type littermates. A marker of DNA double-strand breaks, phosphorylated histone H2AFX (commonly known as γH2AX) exhibited the same localization pattern in wild-type and *repro8* mutant spermatocytes from the leptonema to pachynema (Fig. 2C-F). As did wild-type spermatocytes, mutant *repro8* leptotene spermatocytes exhibited accumulation of the early recombination repair marker RAD51, followed by a decline in the number of RAD51 foci as cells progressed to the pachytene stage (Fig. 2G-J). Detection of MLH1, a marker of crossovers, revealed no differences in number and location of MLH1 foci between *repro8* mutant and wild-type spermatocytes (Fig. 2K,L). Likewise, condensation of the X and Y chromosomes to form the sex body appeared to be normal in mutant spermatocytes, as revealed by the accumulation of γH2AX in the sex body (Fig. 2E,F). Taken together, these observations suggest that recombination and meiotic chromosome synapsis are cytologically normal in mutant *repro8* germ cells, and that failure of these spermatocytes to exit meiotic prophase is probably due to other causes.

The *repro8* genotype: a mis-sense mutation in *Eif4g3*

Further elucidation of cause of the *repro8* meiotic arrest came from identification of the gene mutation. A genome-wide scan of polymorphic markers on each autosomal chromosome in DNA from affected (infertile) and non-affected mice mapped the *repro8* mutation to chromosome 4 between *D4mit203* and *D4mit42*, a region of about 21.69 Mb. For fine mapping within this region, homozygous *repro8* females were crossed to CAST/EiJ males and the F1 offspring were intercrossed to produce F2 individuals that were subjected to phenotype analysis and typed for additional polymorphic markers within the region. Also, recombinants obtained from the C3H-*repro8* maintenance line were used for narrowing the crucial region. Analysis of 4954 meioses narrowed the candidate region for the *repro8* mutation to an interval of about 0.74 Mb between *D4mit67* and *D4mit170*, containing 13 genes.

Of these 13 genes, we selected four candidate genes based on testis expression inferred from our unpublished microarray data, public gene expression databases and the literature (Zhang et al., 2008): a mitochondrial ubiquitin ligase activator of NFKB1 (*Mul1*), von Willebrand factor A domain containing 5B1 (*Vwa5b1*), kinesin family member 17 (*Kif17*) and eukaryotic translation initiation factor 4 gamma 3 (*Eif4g3*). Exonic regions from these genes were sequenced and only one mutation was identified, a G-to-C transversion in *Eif4g3* (Fig. 3A). EIF4G (eukaryotic translation initiation factor 4 gamma) is an integral scaffold protein of the translation initiation machinery. There are three EIF4G isoforms in mammals (EIF4G1, EIF4G2 and EIF4G3), each encoded on a different chromosome in the mouse. EIF4G3 has three conserved domains: an MIF4G domain, an MA3 domain and a W2 domain with unknown function (Fig. 3B). The identified *repro8* mutation is a mis-sense mutation in the W2 domain, producing an amino acid change from alanine to proline. The alanine side-chain is non-reactive and thus it is not often directly involved in protein function. By contrast, prolines can play important roles in molecular recognition and can be disruptive for protein secondary and tertiary structure.

To provide genetic proof that the *repro8* phenotype is caused by the identified *Eif4g3* mutation, we conducted a complementation analysis using a known *Eif4g3* gene-trap mutation. Transcript ENSMUST00000105831 has 36 exons, and the mutation of *repro8* was identified in exon 36 (Fig. 3C). We selected the XC431 cell line from the International Gene Trap Consortium for complementation analysis because the gene-trap insertion is close to the *repro8* mutation, between exons 34 and 35 (Fig. 3C). The consequence of the insertion of gene trap would be to produce a truncated EIF4G3 protein, with loss of part of the W2 domain where the *repro8* mutation was found. The XC431 ES cells harboring this gene trap mutation of the *Eif4g3* gene (*Eif4g3*$^{Gt(XC431)Byg}$, herein referred to as *Eif4g3*^{Gt}) were injected into B6 blastocysts; chimeric males with germ-line transmission of the gene trap allele were identified and mated to homozygous *repro8* females. Offspring were genotyped for both sequencing the B6 *repro8* region and flanking markers of the

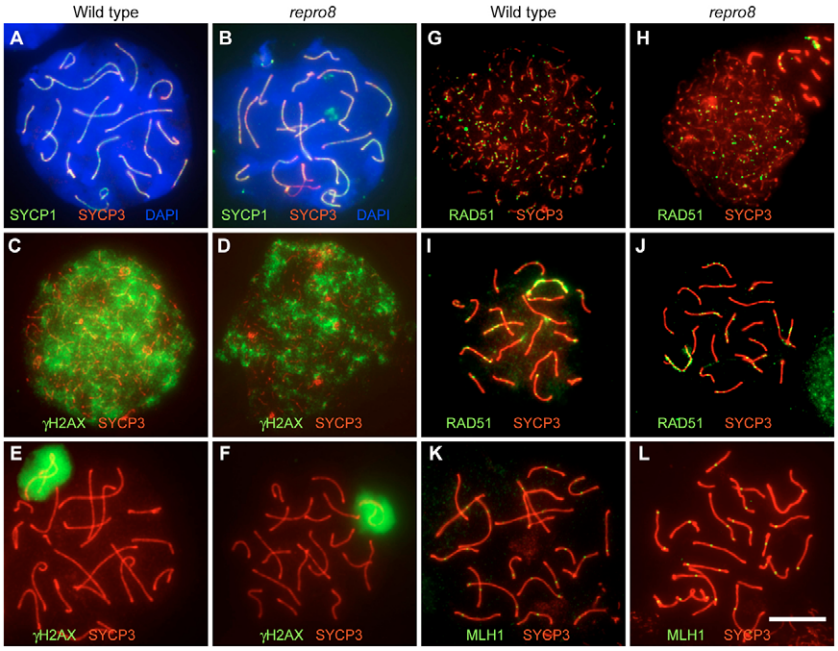

Fig. 2. Essential events in the prophase of meiosis I appear normal in *repro8* mutants. (**A**,**B**) The extent of synapsis, reflected by the pattern of antibody labeling of SYCP1 (green), a central element protein of the synaptonemal complex (SC), and SYCP3 (red), a protein of the lateral element of the SC, was indistinguishable between wild-type (A) and *repro8* mutant (B) spermatocytes. (**C-F**) The pattern of phosphorylated histone H2AFX (γH2AX) labeling, a marker of DNA double-strand breaks and repair, was identical between wild-type (C,E) and *repro8* mutant (D,F) spermatocytes early in prophase (C,D) and at the mid-to-late pachytene stage (E,F). (**G-L**) Progress of recombination events was similar between wild-type and *repro8* mutant spermatocytes. Labeling for RAD51, which accumulates at sites of double-strand breaks, was intense in early prophase (G,H), largely restricted to the X and Y chromosomes by the mid-pachytene stage (I,J), and similar in wild-type (G,I) and *repro8* mutant (H,J) spermatocytes. MLH1 foci, marking sites of reciprocal recombination, were observed in similar numbers in both wild-type (K) and *repro8* mutant (L) spermatocytes. Scale bar: 10 μm.

Eif4g3^{Gt} allele. Four adult *repro8*/*Eif4g3*^{Gt} males were analyzed; they were overtly normal, but their testes were small, similar to those of *repro8*/*repro8* males (30.45 mg versus 89.55 mg for heterozygous *repro8*/+ testes; see Table S1 in the supplementary material). No sperm were recovered from epididymis of the compound heterozygous *repro8*/*Eif4g3*^{Gt} mouse. The cellular composition of *repro8*/*Eif4g3*^{Gt} seminiferous tubules (Fig. 3D) was similar to that of homozygous *repro8* mutants (Fig. 1) and distinctly different from the normal appearance of *repro8*/+ testes, with no postmeiotic spermatids found in the compound heterozygous *repro8*/*Eif4g3*^{Gt} testes. This complementation test provided genetic evidence that *repro8* and *Eif4g3*^{Gt} are alleles of the same gene and, henceforth, we refer to *repro8* as *Eif4g3*^{repro8}.

Because the *repro8* mutation is located in an exon contributing to the W2 domain of EIF4G3, we performed RT-PCR and determined that the W2 domain-encoding region was amplified from testis RNA of both *Eif4g3*^{repro8} mutant and wild-type mice (Fig. 4A). Seven variant combinations of exons 3-8 of *Eif4g3* are annotated in the Ensembl database, version 56 (diagrammed in Fig. 4B). Because the *Eif4g3*^{repro8} mutation does not cause any apparent deleterious effects in other tissues or embryo lethality, we hypothesized that there could be a spermatocyte-specific or testis-specific variant of this ubiquitously expressed gene. We used sequence information from Ensembl to design primers flanking the polymorphic region of the seven transcripts (shown in Fig. 4B). Results of one-step RT-PCR with RNA isolated either from a whole testis or other tissues are shown in Fig. 4C. We observed and sequenced four PCR products; a, b, c and d were present in the testis, whereas only two (b and d) were observed in other tissues. The b product contains a 129 bp exon that was not present in the d product. More importantly, products a and c, which are testis-specific, each contained a 33 bp exon that was not present in testis products b and d. Sequencing revealed that products c and d contain combinations of exons 3-8 that are not among the predicted variants (Fig. 4B). To determine whether testis-specific products a and c represent germ-cell-specific transcripts, we performed RT-PCR analysis with total RNA isolated from testis of *Kit*^{w}/*Kit*^{Wv} mice, which are deficient in germ cells. Sequencing showed that the transcripts amplified from the testis RNA of *Kit*^{w}/*Kit*^{Wv} mice were identical to those amplified from the testis RNA of wild-type mice (see Fig. S4 in the supplementary material), indicating that transcripts a and c are testis-specific, but not necessarily germ-cell-specific.

Mutation of the *Eif4g3* gene affects regulation of CDC2A kinase activity, but not translation of its components and activators

Our findings led to the hypothesis that the cell cycle machinery required for prophase exit might not be present or functional in *Eif4g3*^{repro8} mutant spermatocytes. The CDC2A kinase, encoded in the mouse by the *Cdc2a* gene, is a universal regulator of the transition from prophase to metaphase in both mitosis and meiosis (Handel et al., 1999; Ohi and Gould, 1999; Sun and Handel, 2008). As shown in Fig. 5A, CDC2A kinase activity at P14 in *Eif4g3*^{repro8} mutant spermatocytes was dramatically reduced compared with wild-type spermatocytes. Furthermore, unlike wild-type P18 spermatocytes, P18 spermatocytes from *Eif4g3*^{repro8} mutants did not form fully condensed metaphase I chromosomes after treatment in vitro with the phosphatase inhibitor okadaic acid, an experimental test of the competence of the intracellular machinery for mediating the G2/MI transition (Cobb et al., 1999a) (Fig. 5B). These data indicate that arrest of spermatogenesis at prophase I in *Eif4g3*^{repro8} mutants involves failure to activate CDC2A kinase.

We next investigated whether components and regulators of CDC2A kinase were present in *Eif4g3*^{repro8} mutant spermatocytes. MPF is composed of a catalytic subunit, CDC2A, and a regulatory subunit, cyclin B (CCNB). Three isoforms of CCNB (CCNB1, CCNB2 and CCNB3) have been identified in mammals (Nguyen et al., 2002). Each of the three can form a complex with CDC2A, and all play roles in the prophase-to-metaphase transition during cell division. Moreover, CCNB3 has been reported to be the predominant pre-pachytene cyclin in mammals (Nguyen et al., 2002). We performed immunohistochemistry for these proteins on testis histological sections. As shown in Fig. 6A,B,D,E and Fig. S5 in the supplementary material, these proteins were present in *Eif4g3*^{repro8} spermatocytes, also confirmed by western blotting, where band intensities for mutants were not markedly reduced from those of wild-type controls (Fig. 6C,F; see Fig. S5 in the supplementary material).

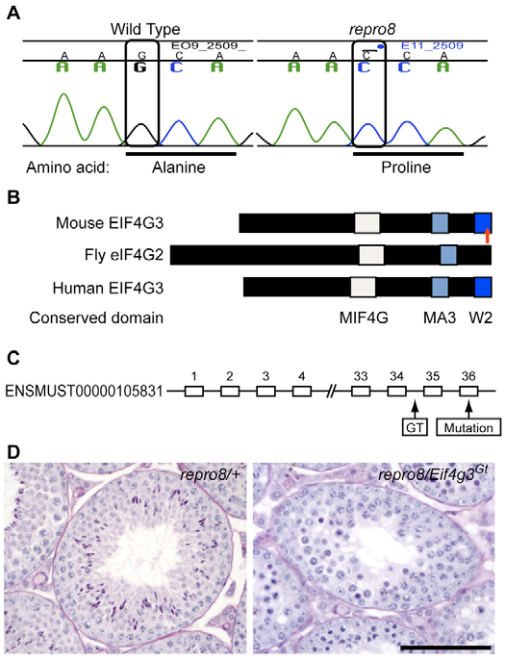

Fig. 3. A mutation in *Eif4g3* causes the *repro8* phenotype. (**A**) The G-to-C mutation is marked by an open box. This mutation results in an amino acid change from alanine to proline. The sequencing result was confirmed by sequencing DNA from each of three *repro8* mutant, *repro8*/+ heterozygous and B6 mice. (**B**) Alignment of conserved protein sequence domains of mouse and human EIF4G3 and fly eIF4G2; note that the fly eIF4G2 does not contain the W2 domain. The red arrow shows the position of the *repro8* mutation. (**C**) A diagram of exons 1-4 and 33-36 of *Eif4g3* transcript ENSMUST00000105831 showing the approximate locations of the gene trap (GT) construct in XC431 and the *repro8* mutation (exons and introns in this panel not drawn to scale). (**D**) Representative testis cross-sections from *repro8*/+ and compound heterozygous *repro8*/*Eif4g3*^{Gt} males, revealing meiotic arrest in the latter, and thus demonstrating allelism of *repro8* and *Eif4g3*. Scale bar: 100 μm.

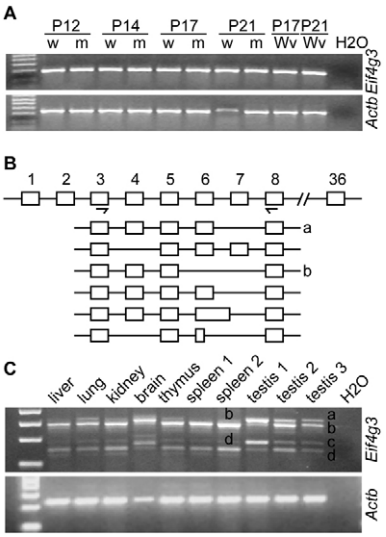

Fig. 4. RT-PCR reveals testis *Eif4g3* transcripts. (**A**) RNA from the W2 domain affected by the *repro8* mutation was amplified by RT-PCR at different developmental stages. m, *Eif4g3*^{repro8} mutant; P, postnatal day; w, wild type; Wv, germ-cell-free *Kit*^{w}/*Kit*^{Wv} testis. Size of the RT-PCR product is 192 bp. The internal control was *Actb* (actin B), with a PCR product of 240 bp. (**B**) Diagram of variant *Eif4g3* transcripts annotated by Ensembl showing the position of primers for the RT-PCR presented in C, and identifying transcripts a and b displayed in C. (**C**) Evidence for testis-specific *Eif4g3* transcripts; testis 1, adult testis; testis 2, P14 testis (B6); testis 3, P14 testis (C3H). Sizes of RT-PCR products: a, 313 bp; b, 280 bp; c, 184 bp; d, 151 bp.

Activation of CDC2A kinase can be achieved through removal of inhibitory phospho-modifications on tyrosine and threonine residues of the CDC2A by members of the CDC25 phosphatase family – CDC25A, CDC25B and CDC25C. We found that all three CDC25 phosphatases were expressed in *Eif4g3*^{repro8} mutant spermatocytes, as demonstrated by both western blotting analysis and immunohistochemistry (Fig. 6G-I; see Fig. S6 in the supplementary material). Therefore, we conclude that the loss of CDC2A kinase activity in *Eif4g3*^{repro8} spermatocytes is not due to lack of CDC2A, CCNBs or CDC25 phosphatases, but might be attributed to failure of interaction among any or all of these regulators.

The *Eif4g3*^{repro8} mutation affects translation of HSPA2, a chaperone for CDC2A kinase

Activation of the CDC2A kinase in mouse spermatocytes requires the HSPA2 chaperone protein (Zhu et al., 1997). Heat-shock protein HSPA2 is expressed highly only in spermatogenic cells (Allen et al., 1988; Eddy, 1998), and mutational analysis demonstrated that it is required for CDC2A-cyclin B1 complex formation (Dix et al., 1996a; Dix et al., 1997; Zhu et al., 1997). Like *Eif4g3*^{repro8} spermatocytes, *Hspa2*-null spermatocytes exhibit arrest during the first meiotic prophase and failure in desynapsis. Analysis of histological sections by immunohistochemistry revealed that HSPA2 was barely detectable in *Eif4g3*^{repro8} mutant (Fig. 7A,B) and *repro8*/*Eif4g3*^{Gt} heterozygous spermatocytes (data not shown); also, western blotting revealed a dramatic reduction of HSPA2 in *Eif4g3*^{repro8} mutant and *repro8*/*Eif4g3*^{Gt} heterozygous spermatocytes (Fig. 7C,E). However, as shown in Fig. 7G, *Hspa2* transcript was present in *Eif4g3*^{repro8} mutant testes, suggesting that EIF4G3 could be required for translation of the *Hspa2* transcript or, indirectly, for stability of the HSPA2 protein. BAT3 deficiency accelerates the degradation of HSPA2 during mouse spermatogenesis via the ubiquitin proteosome pathway (Sasaki et al., 2008), but expression of BAT3 protein and its localization in *Eif4g3*^{repro8} mutant testes were similar to wild-type testes (see Fig. S7 in the supplementary material). Therefore, lack of HSPA2 protein in *Eif4g3*^{repro8} mutant spermatocytes is probably not due to a deficiency in BAT3 expression.

Taken together, these observations suggest that EIF4G3 is required specifically for the translation of HSPA2 (and perhaps of other proteins as well), and that the *repro8* mutation in the *Eif4g3* gene interferes with this function.

DISCUSSION

The discovery that mutation of the *Eif4g3* gene causes the *repro8* meiotic prophase arrest phenotype provides the first evidence that exit from meiotic prophase can be controlled, at least in part, at the level of specific or selective protein translation in mammalian spermatocytes. Spermatocytes of *Eif4g3*^{repro8} mutants appear to execute key events of meiotic prophase normally; DNA double-strand breaks that initiate meiotic recombination occur and DNA repair and recombination proteins, including MLH1, localize normally in mutant spermatocytes, suggesting that reciprocal recombination and crossing over occur in mutant spermatocytes. However, CDC2A, a key protein

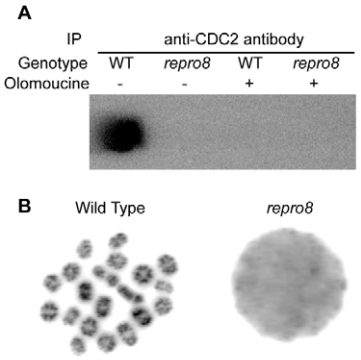

Fig. 5. CDC2A kinase activity is absent and *Eif4g3*^{repro8} mutant spermatocytes are unable to undergo meiotic prophase I to metaphase I transition. Lysate for immunoprecipitation with anti-CDC2 was prepared from whole testes at P14, before obvious germ-cell loss in *Eif4g3*^{repro8} mutant testes. (**A**) Results of the immunoprecipitation and kinase activity assay, with and without olumoucine, a selective inhibitor of CDC2A kinase. CDC2A kinase activity was present in the wild-type immunoprecipitate but absent in the *Eif4g3*^{repro8} mutant immunoprecipitate and both olomoucine-treated controls. IP, immunoprecipitation; *repro8*, *Eif4g3*^{repro8} mutant testes; WT, wild type. (**B**) After okadaic acid (OA) treatment to induce the prophase I to metaphase I transition, pachytene spermatocytes from wild-type spermatocytes form highly condensed and individualized metaphase I chromosomes, whereas *Eif4g3*^{repro8} mutants (*repro8*) do not.

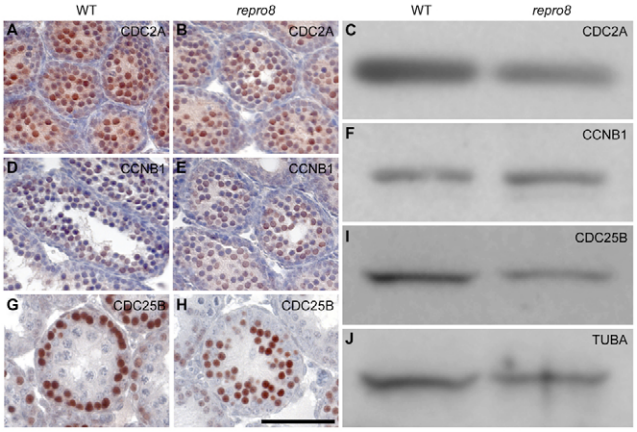

Fig. 6. Protein components and activators of MPF are present in *Eif4g3*^{repro8} mutant spermatocytes. (**A-J**) Immunohistochemistry (A,B,D,E,G,H) and western blotting (C,F,I,J) revealed that CDC2A (A-C), CCNB1 (D-F) and the MPF activator CDC25B (G-I) were present in both wild-type (A,D,G) and *Eif4g3*^{repro8} mutant (B,E,H) spermatocytes. TUBA (tubulin alpha) was detected as a loading control for western blots (J). Scale bar: 100 μm.

kinase that governs onset of the division phase, is present but not activated in mutant spermatocytes. Arguably, the most interesting aspect of the *repro8* phenotype is the presence of *Hspa2* transcript but virtual absence of the HSPA2 protein. This finding could explain the *repro8* meiotic prophase arrest phenotype. HSPA2 is required for association of CDC2A with its regulatory cyclin subunit and subsequent kinase activation. Therefore, absence of HSPA2 protein in *Eif4g3*^{repro8} mutant spermatocytes could explain both the failure to activate CDC2A kinase and the meiotic prophase arrest of mutant spermatocytes, and, indeed, the phenotype of *Hspa2* knockout mutants (Dix et al., 1996a; Dix et al., 1997) is strikingly similar to the *Eif4g3*^{repro8} mutant phenotype.

Translational control of spermatogenesis

The translation of eukaryotic mRNAs occurs via three basic steps: initiation, elongation and termination. Translation initiation requires binding of the translation initiation complex proteins to the 5′ cap of the mRNA, tethering of the 3′ end of the mRNA and subsequent recruitment of ribosome subunits. The eukaryotic translation initiation factor EIF4G is a multipurpose adapter between mRNA and ribosomes, acting as a scaffold protein for other factors in the initiation complex (Hentze, 1997; Hinton et al., 2007). EIF4G1 interacts with poly(A) binding protein [PABP (PABPC1 – Mouse Genome Informatics)] and EIF3 mRNA 5′ cap-binding complex (Haghighat and Sonnenberg, 1997; Hinton et al., 2007; von Der Haar et al., 2000), thus anchoring both ends of the mRNA in the translation initiation complex. In the mouse, there are three EIF4G isoforms encoded by three distinct genes, *Eif4g1*, *Eif4g2* and *Eif4g3*. The EIF4G2 protein, a repressor of translation, has only 16% sequence conservation with EIF4G1, the more abundant isoform (Prevot et al., 2003). Although EIF4G3 and EIF4G1 contain homologous protein binding domains and could interact with the same factors, there is only 46% identity in their primary amino acid sequence (Gradi et al., 1998), suggesting different biological function of the two isoforms, and thus it is reasonable that there might be special functions for EIF4G3.

Considerable evidence supports translational regulation as an important control mechanism during mammalian spermatogenesis (Braun, 2000; Mazeyrat et al., 2001; Hecht, 1998). Furthermore, genetic analyses link selective translational control to coordination of meiotic progress and onset of spermiogenic differentiation in *Drosophila*, where a novel EIF4G2 homolog is required for translation of proteins that govern exit from meiotic prophase (Baker and Fuller, 2007; Franklin-Dumont et al., 2007). Flies with mutated *eIF4G2* are male-sterile. Mutant spermatocytes do not reach normal size and skip the meiotic division and, furthermore, in the absence of functional eIF4G2, specific proteins required for transition from meiosis to spermiogenesis are not accumulated normally. In *C. elegans*, the EIF5A homolog IFF-1 is required for proliferation of germ cells and exit from meiosis (Hanazawa et al., 2004), and depletion of an isoform of EIF4E causes failure of spermatogenesis (Amiri et al., 2001). These and other lines of evidence demonstrate that selective translational regulation is a crucial mechanism controlling spermatogenesis and meiosis in diverse species. Similar RNA-binding and translational regulatory proteins also play crucial roles in meiotic maturation of oocytes and early embryo development (Brook et al., 2009; Mendez and Richter, 2001; Vasudevan et al., 2006).

Role of EIF4G3 in mammalian male meiosis

The *repro8* phenotype of meiotic prophase arrest is caused by a mutation in the *Eif4g3* gene encoding a translation initiation factor that is ubiquitously expressed. Yet, *Eif4g3*^{repro8} mutants are viable, and the only apparent phenotype is male infertility and azoospermia, with arrest at meiotic prophase I. A specific role for EIF4G3 in regulation of spermatogenesis might be explained by testis-specific variants. RT-PCR analysis with primers flanking the testis variable regions resulted

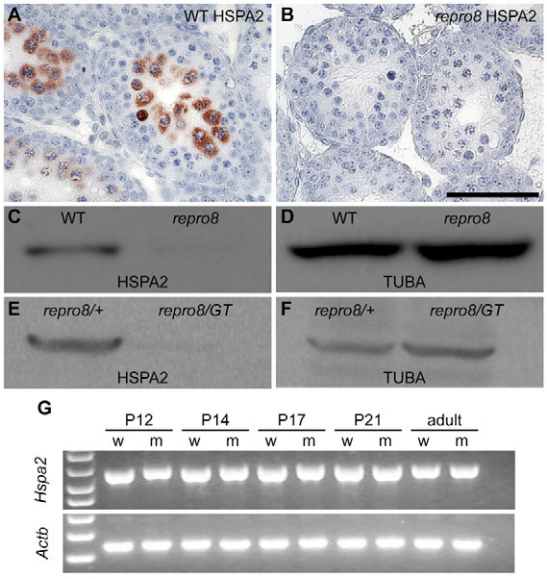

Fig. 7. HSPA2 is absent, but *Hspa2* transcript present, in *Eif4g3*^{repro8} mutant spermatocytes. (**A-F**) Both immunohistochemistry (A, wild type; B, *Eif4g3*^{repro8} mutant) and western blotting (C, wild type and *Eif4g3*^{repro8} mutant; E, *repro8*/+ heterozygote and *repro8*/*Eif4g3*^{Gt} heterozygote) revealed the presence of HSPA2 in wild-type spermatocytes, but its absence in *Eif4g3*^{repro8} mutant and *repro8*/*Eif4g3*^{Gt} heterozygous spermatocytes. TUBA was used as a loading control (D,F). (**G**) *Hspa2* transcript was detected by RT-PCR at different developmental stages. P, postnatal day; m, *Eif4g3*^{repro8} mutant; w, wild type). The size of the *Hspa2* PCR product is 575 bp. The internal control was *Actb* (actin B), with a PCR product of 240 bp. Scale bar: 100 μm

in amplification of two testis-specific transcripts of *Eif4g3*. Moreover, the gene region containing the mutation and encoding the W2 domain with unknown function was transcribed in both wild-type and mutant testes. However, we were unable to amplify the long fragment between the variable region and the W2 domain from either wild-type or mutant transcript, so it is not yet known if the W2 domain carrying the mutation is part of a testis-specific transcript.

The mouse EIF4G3 is not homologous to the *Drosophila eIF4G2*; there is only 12% amino acid sequence identity and, importantly, the fly eIF4G2 does not contain the W2 domain that is mutated in *Eif4g3*^{repro8} mice (NCBI, MGI, Ensembl and UCSC databases). Also, in contrast to the phenotype observed in *eIF4G2* mutant male flies (Baker and Fuller, 2007; Franklin-Dumont et al., 2007), the *repro8* mutation of *Eif4g3* does not affect the translation of cell cycle regulators including CDC25 phosphatases, cyclin Bs or CDC2A. Instead, the failure in meiotic exit and arrest at prophase in *Eif4g3*^{repro8} mutant spermatocytes is due, at least in part, to the fact that CDC2A kinase is not activated, apparently caused in turn by the failure of HSPA2 translation.

How might EIF4G3 specifically control levels of the HSPA2 protein? We considered three possibilities. First, EIF4G3 might regulate translation of a protein involved in HSPA2 degradation. To our knowledge, the only protein that is known to be involved in the degradation of HSPA2 is BAT3, which can stabilize HSPA2 by inhibiting polyubiquitination (Sasaki et al., 2008). However, BAT3 was present in *Eif4g3*^{repro8} mutant spermatocytes. Second, EIF4G3 might indirectly affect HSPA2 expression through regulation of primary transcription regulators of *Hspa2*, such as heat-shock transcription factors (HSF) (Nakai et al., 2000; Widlak et al., 2007). However, we consider this possibility unlikely because *Hspa2* was transcribed, as revealed by RT-PCR analysis. Third, and most probable, EIF4G3 might be specifically and directly required for regulating initiation of the synthesis of HSPA2 protein. This hypothesis is supported by presence of *Hspa2* transcript, by markedly reduced levels of HSPA2 protein and by presence of the BAT3 regulator of HSPA2 protein degradation.

Translation initiation in eukaryotic cells occurs by cap-dependent and cap-independent pathways. The cap-dependent pathway is canonical and involves the assembly of the pre-initiation complex at the 5′ cap structure in the mRNA. The cap-independent pathway involves recruitment of the 40s ribosome directly to the mRNA, provided that an IRES (internal ribosome entry site) exists. In each pathway, connecting mRNAs and 40s ribosomal subunits is thought to involve EIF4G (Hentze, 1997). A number of IRES-containing mRNAs are translated by a cap-independent mechanism. This might be the case for human *HSPA* mRNA, which is reported to contain an IRES element at its 5′ untranslated region (Rubtosova et al., 2003) and is dependent on the integrity of EIF4G3 for in vitro translation (Castello et al., 2006). The mouse *Hspa2* also has a long 5′ untranslated leader sequence, although no common DNA sequence in the 5′ untranslated region is shared between mouse *Hspa2* and other *Hspa* genes (Dix et al., 1996b). Human *HSPA2* and rat *Hspa2*, homologs of mouse *Hspa2*, share high nucleotide sequence similarity within the coding region and upstream of the ATG codon (Bonnycastle et al., 1994; Scieglinska et al., 2001). Taken together, unique features of EIF4G3 and the 5′ leader sequence of *Hspa2* might be required for selective regulation of translation of *Hspa2* transcript in spermatocytes.

Identification of the causative mutation for the *repro8* meiotic arrest provides novel genetic evidence that meiotic exit requires selective protein translation in mouse spermatocytes and that EIF4G3 is specifically required for translation of *Hspa2* transcript. However, many interesting questions remain. We do not know whether absence of HSPA2 is the sole factor determining the *Eif4g3*^{repro8} mutant meiotic arrest phenotype or how EIF4G3 activity is regulated. Future studies will clarify other downstream effects of *Eif4g3* mutation as well as structure and function of the EIF4G3 isoforms in germ cells, including whether there is selective regulation of translation of other proteins. Finally, the etiology of most male infertility with non-obstructive azoospermia has been elusive (Matzuk and Lamb, 2002; Matzuk and Lamb, 2008). Alignment of human EIF4G3 with mouse EIF4G3 revealed over 85% identity in amino acid sequence (Ensembl) and conservation of the W2 domain containing the mutation (Fig. 3B). Decreased HSPA2 protein has been noted in infertile males with spermatogenesis arrest at prophase I of meiosis (Feng et al., 2001; Son et al., 2000); it will be interesting to test whether these patients have a mutation in *EIF4G3*, which could provide insight into the etiology of their azoospermia.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material

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