***SUPPLEMENTal***

***MATERIALS & METHODS***

***Mouse colony maintenance and genotyping***

The *hmmrm/m*colony was maintained by breeding heterozygous animals. Backcrossing to the parental C57BL/6J strain using F1 hybrids, routinely every 10th generation, was employed to avoid production of inbred lines. Animals were provided with standard laboratory chow and tap water ad libitum and kept in accordance with local regulations (TLLV Thüringen, Erfurt, Germany) at constant temperature (22°C) and light cycle (12hr-light, 12hr-dark). Animals were sacrificed by CO2 inhalation. Genomic DNA was obtained from tail biopsies according to standard protocols. Genotyping was performed by PCR, as previously described (1), to discriminate between *hmmr+/+* and *hmmrm/m*alleles. The genotyping conditions for line *Trp53-/-* (primers: ex6.5frw: 5’-ACAGCGTGGTGGTACCTTAT-3’, ex7rev: 5’-TATACTCAGAGCCGGCCT-3’, neo-rev: 5’-CATTCAGGACATAGCGTTGG-3’) with an annealing temperature of 65°C, were used to discriminate between wild type and mutated *Trp53-/-* alleles with expected sizes of 400 bp and 600 bp respectively (2).

***Sperm motility analysis***

Sperm cells were obtained from adult male mice (n=3 per group) by exerting pressure on the cauda epididymis and were collected in HTF medium. The sperm cells were capacitated for 30min at 37°C in 5% CO2. Sperm samples were transferred onto pre-warmed glass slides, covered with a coverslip and assayed for motility at 37°C using a computer-assisted sperm analysis (CASA) system to measure the following sperm motility parameters: velocity curve line (VCL), velocity straight line (VSL), amplitude of lateral head displacement (ALH), velocity average path (VAP), beat-cross frequency (BCF).

***Human testis biopsies***

All human testis biopsies were obtained as formaldehyde-fixed and paraffin-embedded (FFPE) samples, from the Biobank Graz of the Medical University of Graz under the conditions described in the main *Materials & Methods* section. Analysis of the biopsies (histopathological evaluation, immunohistochemical labeling, targeted genomic sequencing, quantification of mRNA expression of selected genes by real-time PCR) was performed on the FFPE samples, as described below.

***Targeted sequencing of human testis biopsies***

Target enrichment was performed as described previously (3). Genomic DNA was extracted from 8 FFPE sections of each sample with the QIAamp DNA FFPE tissue kit (QIAGEN). To make the DNA accessible to Illumina (4) sequencing target libraries of the human RHAMM locus (chr5: 162885000-162921000 bp) were prepared using the SureSelect Target Enrichment kit (Agilent) according to the manufacturer’s instructions. 3μg of genomic DNA was sheared to 150~200bp fragments by a Focus-Ultrasonicator (Covaris) with the following parameters: 10% duty factor, 175 of peak incident power, 200 cycles per burst, 300 seconds treatment time, 4°C. The sheared DNA was purified with Agencourt AMPure XP beads (Beckman Coulter) and eluted with 50μL nuclease-free water. The eluted DNA was end-repaired, ligated with indexing-specific adapter and amplified. The amplified pre-capture library was purified by Agencourt AMPure XP beads and eluted with 30μL nuclease-free water. The quality of the pre-enrichment library was assessed and quantified with 2100 Bioanalyzer (Agilent). For enrichment, 500ng of pre-enrichment library was individually hybridized at 65°C for 16~24h with a custom SureSelect Capture library covering 80.1% sequence of RHAMM loci within 5q34. After hybridization, the hybridized fragments were captured with Dynabeads MyOne Streptavidin T1 beads, followed by on-bead amplification and index tagging. Post-capture libraries were pooled (HiSeq2500: pool1: 12 samples, pool2: 9 samples; MiSeq: pool3: 14 samples) and sequenced on the Illumina HiSeq2500 (rapid mode, paired end, 2x100 bp, one pool per lane) and MiSeq (paired end, 2x250 bp, one run of pool3) platforms, respectively. The sequencing approach resulted for MiSeq in around 1.4mio and for HiSeq2500 pool1 12.8mio and pool2 16.5mio read pairs per sample. Sequence information was extracted in FastQ format using bcl2fastq v1.8.4 (Illumina). Reads were mapped to the human genome reference sequence (GRCh37, hg19 feb 2009) using the CLC workbench (<http://www.clcbio.com/products/clc-genomics-workbench/>). Single nucleotide variants, identified by CLC, were annotated by the Seattle Sequence Annotation program (http://snp.gs.washington.edu/SeattleSeqAnnotation137/). Varscan2 was used to detect variants with low allele frequency (less than 20%) (5). The impact of missence variants on protein structure was evaluated using PolyPhen (6).

***Real time PCR***

Total RNA was extracted either from testis biopsy FFPE blocks (using the RNeasy FFPE kit, QIAGEN) or 1-3 x106 HeLa cells (using the NucleoSpin RNA isolation kit, Macherey-Nagel).

1μg total human testis RNA was reversely transcribed to cDNA (using the QuantiTech Reverse Transcription kit, QIAGEN). 100ng cDNA were used per reaction. The reactions were set-up using the Rotor-Gene SYBR Green PCR kit (QIAGEN) with the primer sets indicated in the following table. Reactions were run on Rotor-Gene Q cycler (QIAGEN) under the following program: initial template denaturation and polymerase (HotStartTaq) activation at 95oC for 5min, denaturation at 95oC for 5sec, combined annealing/extension at 60oC for 10sec; the last two steps were repeated for 35 cycles. Serial dilutions of total cDNA from HeLa cells were used for calibration in order to plot the standard curve. The amount of RHAMM or cyclin B1 cDNA in each sample was calculated according to the standard curve and normalized, for variation in the amount and quality of RNA/cDNA among samples, by division with the amount of GAPDH cDNA. These normalized values were used to calculate the ratio of RHAMM/cyclin B1.

Alternatively, 1μg total HeLa RNA was reversely transcribed to cDNA (using the Transcriptor cDNA Synthesis kit, Roche). 100ng cDNA were used per reaction. The reactions were set-up using the iQ SYBR Green Supermix (Bio-Rad) with the primer sets indicated in the following table. Reactions were run on Bio-Rad CFX384 Touch real time PCR system (Bio-Rad) under the following program: initial template denaturation and polymerase (HotStartTaq) activation at 95oC for 3min, denaturation at 95oC for 30sec, annealing at 60oC for 40sec, extension at 72oC for 30sec; the denaturation, annealing and extension steps were repeated for 35 cycles. Serial dilutions (0.1, 1, 10, 100, 1000pg) of plasmid DNA, of the gene whose expression was quantified in each sample, were used to plot the standard curve. The amount of cDNA in each sample was calculated according to the standard curve and normalized to GAPDH cDNA.

*Primer pairs used in cDNA amplification of the indicated human genes:*

| ***gene name*** | ***forward primer (5’->3’)*** | ***reverse primer (5’->3’)*** |
| --- | --- | --- |
| ATF7IP | GAAATGCAGGCACAGTGAGA | CAACTGCTGGAGGAGTGACA |
| BAK1 | TGGTCACCTTACCTCTGCAAC | ATGTCGTCCCCGATGATG |
| CFIm25 | GCTACCCCATGTGTTACTGCT | GGGTTAAGTTCACCACCAGGTA |
| CyclinB1 | ACATGGTGCACTTTCCTCCT | AGGTAATGTTGTAGAGTTGGTGTCC |
| DAZL | GGGGAGCAAAGGAGCTATGT | TGAACATTCATTTGGCACAAC |
| DMRT1 | GTGCAAGAAGTGCAACCTGA | TGGCTGATACCCAATTCCTC |
| GAPDH | AGCCACATCGCTCAGACAC | GCCCAATACGACCAAATCC |
| HMMR | GCTGAAGAATTAAAACTCCTAGAAGAA | GCAGCACTACTTTTCTCCAGTTC |
| HPGDS | GGGGGAGAGAATGGCTTATT | GGTCAGGCTTAAAGACCAAAAG |
| KITLG | GCGCTGCCTTTCCTTATG | CCTTCAGTTTTGACGAGAGGA |
| MAD1L1 | GGACCTACGTCGGGCTTC | CACAAGGTGAGGAACCCAGG |
| PRDM14 | CGTTCTGTACGGGGTCACTC | TTGAGGAAGAGAATCAGATCCAG |
| RFWD3 | CCTTCTCCTCAGGCCTCTTT | CCATGCATCGGAATGTACTG |
| SPRY4 | CCACGTACCCTGTATTCCCC | GCCCCTGCATTGTCTGTTTG |
| TERT | AGCCACGTCTCTACCTTGACA | CAGGTGAGCCACGAACTGT |
| UCK2 | TCCAGATCCCCGTGTATGAC | ACGTCTGCGGGATAGACAGT |

***DNA cloning & mutagenesis***

For localization and overexpression studies, the open reading frame (ORF) of the genes listed in the following table were PCR-amplified from cDNA clones (obtained from Dhamarcon-GE Healthcare) and attB sites were attached. PCR was performed using the PCR Extender System (5 PRIME) in two steps. Primers without attB sites targeting the full length ORF were used in the first step under the conditions: activation at 94°C for 2min, denaturation at 94°C for 20sec, annealing at 55°C for 20sec, extension at 72°C for 1 to 4 min; the denaturation, annealing and extension steps were repeated for 35 cycles. The corresponding PCR product was purified from 1.5% agarose gel with PureLink Quick Gel Extraction kit (Life Technologies) and used for the second step PCR, in which primers with attB sites were employed.

*Accession and ID numbers of cDNA clones encoding the ORF of the indicated human genes:*

| ***gene name*** | ***clone ID number*** | ***accession number*** |
| --- | --- | --- |
| ATF7IP | 6181437 | BC063855 |
| BAK1 | 2819507 | BC004431 |
| CFIm25 | 3139379 | BC001403 |
| DAZL | 4829381 | BC027595 |
| DMRT1 | 5168563 | BC040847 |
| HMMR | 40007903 | BC108904 |
| HPGDS | 4731411 | BC020734 |
| KITLG | 30915176 | BC074725 |
| MAD1L1 | 4299982 | BC009964 |
| PRDM14 | 6671984 | BC052311 |
| SPRY4 | 40118267 | BC125095 |
| UCK2 | 3940564 | BC002906 |
| RFWD3 | 5266451 | BC059371 |

Alternatively, the truncated RHAMM-∆(302-726) ORF was generated in a single step PCR, using primers with attB sites (underlined) which are listed in the following table.

The attB-flanked PCR products were recombined with the Gateway donor vector pDONR201 (Invitrogen) and subsequently shuttled into the EYFP expression vector pcDNA/FRT/TO\_YFP\_hygR (7).

The point-mutated RHAMM ORFs were generated by site-directed mutagenesis of RHAMM in the expression vector, using Pfu High Fidelity DNA polymerase (Agilent Technologies) and the primers listed in the following table, under the following conditions: activation at 95°C for 30sec, denaturation at 95°C for 30sec, annealing at 55°C for 1min, extension at 68°C for 5 min; the denaturation, annealing and extension steps were repeated for 16 cycles. The PCR reaction mix was incubated with Dpn1 enzyme at 37°C for 1h, followed by transformation into DH5a *E.coli*cells.

*Primers used in the indicated truncation or point mutation of the RHAMM ORF:*

| ***RHAMM truncation/mutation*** | ***forward primer (5’->3’)*** | ***reverse primer (5’->3’)*** |
| --- | --- | --- |
| ∆(302-726) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTCCTTTCCTAAGGCGCCC | GGGGACCACTTTGTACAGAAAGCTGGGTTCACTGCATCTCAGCAC |
| c277t | GAAGATATTAGAGAAAGAGATTTGTGTTCTTCTACAGGAACGTG | CACGTTCCTGTAGAAGAACACAAATCTCTTTCTCTAATATCTTC |
| c386g | TAAATGCTGCACTAAGGGAAAAAACATGTCTCTCTGCAAATAATG | CATTATTTGCAGAGAGACATGTTTTTTCCCTTAGTGCAGCATTTA |
| g998a | GAAGTTTATTCTTGAACAACAGGAACATGAAAAGCTTCAACAAAAAGAATTAC | GTAATTCTTTTTGTTGAAGCTTTTCATGTTCCTGTTGTTCAAGAATAAACTTC |
| c1786a | AACTATATAATAAAACAAAACCTTTTCAGATACAACTAGATGCTTTTGAAGTAGAAAAA | TTTTTCTACTTCAAAAGCATCTAGTTGTATCTGAAAAGGTTTTGTTTTATTATATAGTT |

All cloned ORFs were verified by sequencing.

***Preparation & analysis of cell lines expressing RHAMM variants***

To generate human HEK293 cell lines inducibly expressing the proteins of interest, Flp-InTM-T-RExTM-293 cells (Life Technologies) were co-transfected with pcDNA/FRT/TO\_YFP\_hygR (encoding the gene of interest) (7) and pOG44 (encoding the Flp recombinase). The cells were cultured in DMEM (Sigma) supplemented with 10% tetracycline-free FBS (Clontech) and 2mM glutamine (Biowest) at 37oC in 5% CO2. Positive selection of cells carrying the ORF was performed by the addition of hygromycin (200µg/ml) into the culture media at 48hr post transfection and for one week. Expression of the ORF was induced by treatment with doxycycline (1µg/ml) for 24hr, unless otherwise indicated.

For analysis of spindle localization of the RHAMM variants, the resulting cell lines (carrying mutated RHAMM ORFs) were treated with siRNA targeting endogenous RHAMM (see *RNA Interference*). 72hr post siRNA transfection, doxycycline was added into the growth media (0.5μg/ml) to induce expression of the mutated RHAMM ORF. The cells were incubated at 37oC for 3hr and subsequently processed for immunofluorescence microscopy (see *Immunolabeling*).

***RNA Interference***

The nucleotide sequences of the siRNAs (Dharmacon-GE Healthcare) used are listed in the following table. Pools of siRNAs duplexes were employed, with each pool containing 4 independent oligonucleotides targeting the same gene transcript. A pool of non-targeting siRNAs was used as negative control.

*Nucleotide sequences of the siRNAs used to silence the transcript of the indicated genes:*

|  |  |
| --- | --- |
| ***gene name*** | ***sense sequence of siRNA duplexes*** |
| ATF7IP | UAUAGCCUCUAGUGAAAUA |
| GGAGGUGGAUCGAAGCUGU |
| GGUGAUCUAUCCUCUAGUG |
| GAGUGGAGCUUCACAAGAC |
| BAK1 | CAGAGAAUGCCUAUGAGUA |
| CAACCGACGCUAUGACUCA |
| CGACAUCAACCGACGCUAU |
| GCUUCGUGGUCGACUUCAU |
| BRCA1 | CAGCUACCCUUCCAUCAUA |
| GGGAUACCAUGCAACAUAA |
| GAAGGAGCUUUCAUCAUUC |
| CUAGAAAUCUGUUGCUAUG |
| CFIm25 | GCGCAUGAGGGAAGAAUUU |
| CUAAGGAACAUAAGAAGUU |
| UGACAAUGCACCAGGAUAU |
| ACGCUUAAUGACAGAGAUA |
| DAZL | GGGCAAUGCUUAAAUCUGU |
| GAGCAAAGGAGCUAUGUUG |
| GAAGCUUCUUUGCUAGAUA |
| CGAUGAAUCCUAUAACUCA |
| DMRT1 | GAACACACCUGACCUGGUU |
| GCAAUGAGCUCCCAGUACA |
| CAAAGGCAGUGCUUGAAUG |
| GAGGAGGAAUUGGGUAUCA |
| HMMR | GAGCUCAAAUCAAGAAUAU |
| GAAUAAAGUUCUAGGUAUC |
| GUUAACAGCCAGUGAGAUA |
| GGACUAAUGAACUACUAAA |
| HMMR\_UTR | GAAAUAAGGACAAGCCUAAUU |
| GCAAAUACCUCCUCCCUAAUU |
| UGGCUUUCCAAUUGGCUAAUU |
| GGGAGAUUUGAAUUUGAAAUU |
| HPGDS | CAAGCUGACUGGCCUGAAA |
| ACACAGAUUUGGCUGGAAA |
| UAAUGCGCCUCAUCUUAUG |
| UGCCGUCGCUAACUGGAUA |
| KITLG | GGAAUCGUGUGACUAAUAA |
| AUAAGUAUGUUGCAAGAGA |
| UAAGCGAGAUGGUAGUACA |
| CGUCAAAACUGAAGGGAUC |
| MAD1L1 | GCUCUGGACUGGAUAUUUC |
| AGAGGGAGCUUGCCUUGAA |
| GAGCAGAUCCGUUCGAAGU |
| GCGGACACGCUCAGGUUGA |
| Non-Targeting control | UAAGGCUAUGAAGAGAUAC |
| AUGUAUUGGCCUGUAUUAG |
| AUGAACGUGAAUUGCUCAA |
| UGGUUUACAUGUCGACUAA |
| PRDM14 | GAGAUAAGCACCUCAAGUA |
| GUUCACAGCCUCCAGCAUA |
| GAAGACCUACGGAGACAAU |
| CAGUGUGUGUAUUGUACUA |
| SPRY4 | GCACGAAUGAGGACGAUGA |
| UGUGGAGAAUGACUACAUA |
| CAACGGCUCUUAGACCACA |
| UGUGGGAAGUGUAAAUGCA |
| TERT | GGUAUGCCGUGGUCCAGAA |
| CCACGUCUCUACCUUGACA |
| UCACGGAGACCACGUUUCA |
| GAACGGGCCUGGAACCAUA |
| UCK2 | GAAUUCUGCUUGCCAACAA |
| UACGAGACCUGUUCCAGAU |
| AGAAUGAGGUGGACUAUCG |
| CGGCAAGUCUUCCGUGUGU |
| RFWD3 | GGACCUACUUGCAAACUAU |
| GGAAACAGGCCGAGUUAGA |
| GUUAAGAUGUUGAGUACUG |
| GCAGUCAUGUGCAGGAGUU |

Flp-InTM-T-RExTM-293 cells were reverse transfected with 25nM of an siRNA pool targeting the UTR (untranslated region) of RHAMM mRNA, in order to silence endogenous RHAMM. 72hr post transfection, the cells were further processed as described under *Preparation & analysis of cell lines expressing RHAMM variants*

* HeLa cells (ATCC CCL-2), cultured at 37oC and 5% CO2 in modified Eagle's medium (MEM) supplemented with 10% FCS and 2mM L-glutamine. (all from Biowest), were reverse transfected with 25nM siRNA pool targeting the indicated gene transcript.

For both cell lines, transfection complexes were formed in a microfuge tube by incubating 200μl siRNA duplexes (from a 500nM siRNA pool) with 200μl of 0.75% INTERFERin (Polyplus Transfection) in OptiMEM (Invitrogen), for 15min at room temperature, in 3 replicates per experiment. The transfection complexes were transferred into a 6cmØ cell culture dish, to which 1x105 cells in a total volume of 4ml growth medium were added. The cells were incubated for 72hr at 37oC, 5% CO2, and subsequently processed for immunofluorescence microscopy or immunoblotting - unless otherwise indicated.

The RNAi screening experiment was performed in HeLa cells, according to (8) with the following modifications: In-well reverse transfection was performed in 384-well microscopy plates (BD Falcon). All pipeting steps were performed in a liquid handling workstation (Freedom EVO, TECAN) unless otherwise indicated. Transfection complexes were formed in these plates by incubating 10µl siRNA duplexes from a 500nM siRNA pool) with 10µl of 0.038% DharmafectI in OptiMEM (Invitrogen) for 15min at room temperature, in triplicate wells. 1200 cells per well, suspended in 50µl MEM, were added onto the transfection complexes using an automatic dispenser (Biotek) and incubated for 72hr at 37°C and 5% CO2. The cells were processed for immunofluorescence microscopy (see *Immunolabeling*) using anti-RHAMM and -pH3 antibodies and DAPI (final concentration of 5mg/ml, added to the solution of goat a-mouse Alexa488- plus a-rabbit Alexa594-conjugated secondary antibodies, and incubated for 60min). Following cell labeling, 70µl of PBS containing 0.01% (w/v) NaN3 was added to each well.

Phenotypic analysis was carried out on the images acquired with an ArrayScan VTI microscope (Cellomics) at 200x magnification, using algorithms designed with the associated vHCS software, according to (9) with the following modifications: Automated identification of single cells and segmentation of cell clumps was based on nuclear labeling. For each metaphase cell, total nuclear intensity (DAPI signal), mitotic chromatin intensity (pH3) and (spindle-associated) RHAMM intensity were determined. The cell cycle stage of individual cells was determined based on the intensity of DAPI and pH3 intensity in the nuclear area (Fig.S5A). In order to define the cell cycle stages, the DAPI signal was normalized against a standard histogram. The first peak of the histogram of the control wells was set to “2n” (G1) the second to “4n” (G2). The averaged pH3 signal for each well was normalized against the center of the histogram of the control wells for the whole plate. Both plate-wise normalized values were used in the cell cycle analysis. A threshold which separated G1 from G2 population in the normalized DAPI-channel (“3n”) was selected and another threshold to distinguish between low (G1,G2) and high pH3-signal (M,Ana-/Telophase). The RHAMM intensity of metaphase cells (high DAPI “4n” and high pH3) was quantified and the fold-change of RHAMM intensity compared to control cells (non-targeting siRNA-treated) was calculated. For each condition, the RHAMM signal of the triplicates was averaged. The values in Fig.S5B represent the average distances in fold standard deviation of the controls. An average of n=500 mitotic cells per knock-down and experiment were analyzed. Phenotypes were considered to be statistically significant when (ii) they had a p-value of the student’s t-test of less than 5%, (iii) they exhibit at least a change of RHAMM-signal of 2-fold downregulation or 1.5-fold upregulation.

***Antibodies***

The following primary antibodies were used in immunofluorescence (IF), immunohistochemistry (IHC) and western blotting (WB) experiments, at the indicated dilutions.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **antibody against**  (clone name) | **source** | **catalogue #** | **species** | **dilution IF** | **dilution IHC** | **dilution WB** |
| CFIm25 | Proteintech | 10322-1-AP | rabbit |  | 1:100 | 1:1000 |
| CHICA | Abcam | Ab112899 | rabbit |  |  | 1:100 |
| DYNLL1 (EP1660Y) | Abcam | Ab51603 | rabbit |  |  | 1:500 |
| GFP (D5.1) | Cell Signaling | 2956 | rabbit |  |  | 1:1000 |
| NPT | Millipore | 06-747 | rabbit |  |  | 1:500 |
| p53 | Cell Signaling | 2524 | mouse |  |  | 1:1000 |
| p53 (CM5) | Vector Lab | NCL-p53-CM5p | rabbit |  | 1/100 |  |
| pericentrin | Balco | PRB-432C | rabbit | 1:200 |  |  |
| phospho-histone H3 (pH3) | Abcam | ab14955 | mouse | 1:500 |  |  |
| PLZF | Millipore | OP128 | mouse | 1:100 |  |  |
| RHAMM | Li *et al.*2015 |  | rabbit | 1:100 |  | 1:100 |
| SCP3 | Abcam | ab97672 | mouse | 1:100 |  |  |
| α-tubulin (DM1α) | Sigma | T9026 | mouse | 1:200 |  | 1:1000 |
| β-actin (AC-74) | Sigma | A-5316 | mouse |  |  | 1:1000 |
| β-catenin | Sigma | C2206 | rabbit | 1:100 |  |  |
| γ-tubulin | Sigma | T3559 | rabbit | 1:2000 |  |  |
| γ-tubulin (GTU-88) | Sigma | T6557 | mouse | 1:200 |  |  |

Immunoaffinity-purified Alexa-conjugated goat or HRP-conjugated donkey secondary antibodies were used in the following dilutions: Alexa-fluor-488-conjugated (1/300) or -594-conjugated (1/400) (Molecular Probes, Invitrogen); HRP-conjugated (1/5000) (Jackson Immunoresearch Laboratories); HRP-conjugated (1/100) (Vector Lab).

***Immunoprecipitation***

Following induction of RHAMM variant expression, Flp-InTM-T-RExTM-293 cells (see *Preparation & analysis of cell lines expressing RHAMM variants*) were harvested by "scraping" into ice-cold PBS (containing 1mM Na3VO4, 50mM NaF, 50mM β- glycerophosphate, 1mM PMSF, 10μg/ml antipain, 10μg/ml chymostatin, 100μg/ml pepstatin A, 2μg/ml leupeptin, 200μg/ml AEBSF-HCl, 2μg/ml aprotinin) and centrifugation (100 x *g*, 15min, 4°C). One pellet volume of 2x extraction buffer (20mM Tris-HCl, 150mM NaCl, 1% NP40, 1mM EGTA, protease and phosphatase inhibitors as above) was added and cells were extracted for 1hr, at 4°C with constant agitation. Protein extracts were cleared by ultracentrifugation (100,000xg, 15min, 4°C).

Anti-GFP antibody was conjugated to protein G *Dynabeads* (Invitrogen) at a concentration of 0.15μg/μl of beads, for 1hr, at 4°C. Antibody-coupled beads were washed extensively in 0.01% Tween-20 in PBS, followed by several washes in 1x extraction buffer, and incubated with protein extract (1.5μl beads per μl extract), for 4hr, at 4°C with constant agitation. Beads were washed five times with 1x extraction buffer and once with PBS. Antibody-bound proteins were eluted by addition of 20μl 2x Laemmli sample buffer (10% w/v SDS, 50% glycerol, 0.5% w/v bromophenol blue, 390 mM β-mercaptoethanol, 100 mM Tris-HCl pH 6.8) to the beads and incubation for 30min at room temperature with gentle agitation. The resulting protein suspension was denatured for 10min at 100°C and stored at -80°C. Analysis by *SDS-PAGE & western blotting* were performed according to Li *et al.* 2015.

***Immunolabeling***

MEF or HeLa (ATCC CCL-2) cells, grown attached on glass coverslips, were fixed in 3% paraformaldehyde, 0.2% glutaraldehyde, 0.2% triton-X100 in BRB80 (80mM PIPES pH6.8, 1mM MgCl2, 1mM EGTA), treated (10min × 3changes) with NaBH4 (1mg/ml in PBS), washed in PBS and incubated with IF blocking buffer (1% BSA, 2% FCS in PBS) for 20min. Incubation with the primary antibody/ies for 1hr was followed by washing in 3 changes of PBS over 20min, incubation with the respective secondary antibody/ies and washing as before. Nuclei were labeled with 1mg/ml DAPI in ddH2O for ~30sec, the cells were washed as above. Coverslips were rinsed once by immersion in ddH2O, excess H2O was drained-off and they were mounted on glass slides in Mowiol mounting medium containing 1 mg/ml p-phenylenediamine (Sigma).

Immunofluorescence labeling of FFPE testis sections was performed according to (1).

De-paraffinized and rehydrated FFPE sections were immunohistochemically labeled as follows: Heat-mediated antigen retrieval was performed by immersion of the sections in citrate buffer (10mM, pH 6.0) and heating in a pressure cooker at 100°C for 10min. The sections were allowed to cool-down for 30min, washed in PBS, incubated in 3% H2O2 in PBS for 10min, washed in PBS and incubated with IHC blocking buffer (5% BSA, 5% goat serum, 0.1% Triton X-100 in PBS) for 1hr, followed by primary antibody incubation at 4°C overnight. The primary antibody was washed in 3 changes PBS over 30min. The sections were incubated in biotin-conjugated secondary antibody for 1hr, followed by incubation in HRP (horseradish peroxidase)-conjugated streptavidin for 30min. (Two drops of Avidin were mixed in 10ml biotinylated HRP to prepare the biotinylated HRP-Avidin complex; Vectastain ABC kit, Vector Lab). To visualize the antibody labeling, sections were incubated with 0.05% DAB (3,3'-diaminobenzidine) for 5min; the reaction was stopped by rinsing the sections in double-distilled H20. The cells were counter-stained with hematoxylin for 1min, followed by dehydration through 6 changes of ethanol (50%, 70%, 95%, 95%, 100% and 100%) and 2 changes of xylene, 1min each. Finally the sections were mounted using Vision Mount media (Thermo). All steps were performed at room temperature, unless otherwise indicated.

Images were acquired on an Axiovert200 microscope equipped with a 12-bit grayscale cooled CCD AxioCamMRm camera (Zeiss) or on a VS100 Virtual Slide Microscopy (Olympus) microscope. Representative images were brought to a resolution of 300ppi without re-sampling using Adobe Photoshop (Adobe) and the area of interest was cropped.

***Spindle assembly***

Cultures of MEF cells, grown attached on coverslips, were fixed and processed for immunofluorescence microscopy (see *Immunolabelling*) with anti-α- and -γ-tubulin anti antibodies plus DAPI. Phenotypic scoring was carried out by visual inspection of the samples at an Axiovert200 microscope (Zeiss). A minimum of 100 mitotic cells were counted, in each of three independent experiments**.**

***Orientation of germ cell division***

Mouse testes were dissected, fixed and processed according to (1). For analysis of the orientation of spermatogonial cell division testis sections were labeled with anti-pH3 and -pericentrin antibodies (see *Immunolabelling*), to visualize mitotic chromosomes and centrosomes respectively. Mitotic spermatogonia were identified as pH3-positive cells adjacent to the ST basal membrane. Images were acquired at an Axiovert 200 microscope (Zeiss) and imported in ImageJ. From the images, first the cell division axis of spermatogonia at metaphase or anaphase was determined: The long spindle axis, of these cells, defined as a line across the two centrosomes, was used to indicate the cell division plane (Fig.4E,I, orange line). Second, the plane of the ST basal membrane, adjacent to the mitotic spermatogonium, was defined by a line parallel to the membrane passing by the membrane/germ cell contact point (Fig.4E,I, white line). For each spermatogonium, the angle θ between the long spindle axis and the ST basal membrane plane was measured (Fig.4E,I) in ImageJ.

For undifferentiated spermatogonia, the difference of the two populations in θ was 24.9° with a 95% confidence interval of 12.9° to 36.8° (Fig.4F,G,H). Testes of 3 *hmmr+/+* mice (n=41 mitotic undifferentiated spermatogonia) and 3 *hmmrm/m* mutants (n=45) were used in the quantification. For differentiating spermatogonia, the difference of the two populations in θ was 20.0° with a 95% confidence interval of 8.3° to 33.9° (Fig.4J,K,L). Testes of 2 *hmmr+/+* mice (n=58 mitotic differentiating spermatogonia) and 3 *hmmrm/m* mutants (n=51) were used in the quantification.

The hypothesis that germ cell spindle orientation in spermatogonia is random was tested by the Kolmogorov-Smirnov test. The test was applied on the distribution of spindle angles

(i) in undifferentiated spermatogonia against a random distribution, normalizing the angle θ between 0 and 1 (D=0.1789, p=0.09891 for *hmmrm/m*; D=0.6935, p<2.2x10ˆ-16 for *hmmr+/+*);

(ii) in differentiating spermatogonia against a random distribution, normalizing the angle θ between 0 and 1 (D=0.1914, p=0.04772 for *hmmrm/m*; D=0.2541, p=0.001118 for *hmmr+/+*).

***TUNEL assay***

FFPE testis sections were de-parafinized, rehydrated and incubated with proteinase K at 37oC for 30min. After washing with PBS, sections were incubated with enzyme solution and TUNEL reaction mixture (In Situ Cell Death Detection kit, Roche) at 37oC for 1hr. The sections were counterstained with DAPI (see *Immunolabelling*). Phenotypic scoring of TUNEL-positive cells (n=3 animals per group, one section per animal) was carried out by visual inspection of the samples at an Axiovert200 microscope (Zeiss). A minimum of 8 tubules with TUNEL positive cells per animal were used in the quantification; the number of TUNEL-positive cells within these tubules was counted.

***Statistical analysis***

Germ cell spindle orientation was analyzed by the Mann-Whitney test, atrophy and atypia by the Fischer's exact test and all other assays by the two tail Student’s t-test. Results are presented as mean ± standard deviation, with error bars denoting the standard deviation. The hypothesis that germ cell spindle orientation in spermatogonia is random was tested by the Kolmogorov-Smirnov test. See also *Orientation of germ cell division & statistical analysis.*

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***SUPPLEMENTAL MATERIALS & METHODS***

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