Genetic Deficiency of Mtdh Gene in Mice Causes Male Infertility via Impaired Spermatogenesis and Alterations in the Expression of Small Non-coding RNAs*

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Background: MTDH is associated with poor prognosis in cancer, yet its biologic function is unclear.

Results: Mtdh-null male mice are infertile, with a lack of mature sperm in testes and altered expression of small non-coding RNAs.

Conclusion: Mtdh deficiency results in male infertility due to impaired spermatogenesis.

Significance: The oncogene MTDH plays an indispensable role in male fertility.

Increased expression of metadherin (MTDH, also known as AEG-1 and 3D3/LYRIC) has been associated with drug resistance, metastasis, and angiogenesis in a variety of cancers. However, the specific mechanisms through which MTDH is involved in these processes remain unclear. To uncover these mechanisms, we generated Mtdh knockout mice via a targeted disruption of exon 3. Homozygous Mtdh knockout mice are viable, but males are infertile. The homozygous male mice present with massive loss of spermatozoa as a consequence of meiotic failure. Accumulation of γ-H2AX in spermatocytes of homozygous Mtdh knockout mice confirms an increase in unrepaired DNA breaks. We also examined expression of the DNA repair protein Rad18, which is regulated by MTDH at the post-transcriptional level. In testes from Mtdh exon 3-deficient mice, Rad18 foci were increased in the lumina of the seminiferous tubules. The Piwi-interacting RNA (piRNA)-interacting protein Mili was expressed at high levels in testes from Mtdh knockout mice. Accordingly, genome-wide small RNA deep sequencing demonstrated altered expression of piRNAs in the testes of Mtdh knockout mice as compared with wild type mice. In addition, we observed significantly reduced expression of microRNAs (miRNAs) including miR-16 and miR-19b, which are known to be significantly reduced in the semen of infertile men. In sum, our observations indicate a crucial role for MTDH in male fertility and the DNA repair mechanisms required for normal spermatogenesis.

Metadherin (MTDH, also known as AEG-1 and 3D3/LYRIC) has been implicated in many cancer-related processes, including cellular proliferation, survival, invasion, chemoresistance, and metastasis (1–3). MTDH is located on chromosome 8q22, a region that is frequently amplified in cancers and is correlated with poor survival in patients with breast cancer and hepatocellular carcinoma (4). MTDH is overexpressed in a wide variety of cancers and in 90% of cases of hepatocellular carcinoma (5). Other mechanisms that are known to cause the up-regulation of MTDH include reduced post-transcriptional regulation by miR-375, increased stabilization by monoubiquitination, and interaction with cytoplasmic polyadenylation element-binding protein 1 (CEBP1) (6, 7).

MTDH is a poorly understood protein with few conserved domains that indicate its biological function. There are, however, three lysine-rich nuclear localization signals and several regions required to interact with other proteins such as PLZF (promyelocytic leukemia zinc finger) (8), BCCIPα (BRCA2 and CDKN1A-interacting protein α isoform) (9), SN1D1 (staphylococcal nuclease and Tudor domain-containing 1) (10, 11), and NFXb subunit p65 (12). Thus, although the underlying biological functions of MTDH have not been completely elucidated, others have suggested that it acts in the nucleus as a co-factor to promote the transcription of downstream genes involved in metastasis and drug resistance (1, 2, 12). Alternatively, several studies have identified roles for MTDH in regulation of protein translation. The interaction between MTDH and SN1D1 facilitates RNAi-mediated gene silencing in the cytoplasm. MTDH also increases the translation of multidrug resistance protein 1 (MDR1) and coagulation factor XII (FXXII) proteins by promoting loading of their respective mRNAs onto the polysome (13–15). We recently reported that MTDH contains several putative RNA binding sites and that it interacts with a large number of mRNAs and ribosomal proteins in the cytoplasm (16).

As the vast majority of studies have been performed in malignant cells, the primary function(s) of MTDH in non-cancerous cells remains to be determined. MTDH was originally reported...
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to be induced by HIV infection in astrocytes (accounting for its original name, AEG-1, astrocyte elevated gene 1) and as a lysine-rich CEACAM1 co-isolated (LYRIC) protein associated with tight junctions in prostate epithelial cells (17). Unlike cancer cells where the predominant location of MTDH is in the cytoplasm, MTDH is also present in the nucleus and nucleolus of normal cells (15). Although MTDH has been implicated in diverse physiological and pathological processes (18, 19), the precise mechanisms remain undefined. Using Mtdh exon 3-deficient mice, we herein report a novel functional role for MTDH in male fertility through the regulation of small non-coding RNAs and spermatogenesis.

EXPERIMENTAL PROCEDURES

Generation of Mtdh Knock-out Mice—Chimeric mice were generated by injecting the mouse embryonic stem cell (ESC) line generated by the International Knock-out Mouse Consortium (IKMC) (Mtdh, project CSD 48311) into the blastocysts of mouse embryos. High-contribution chimeras were mated with C57BL/6 females to confirm germline transmission of the ESCs. Mtdh heterozygous mice were bred with FLPe (The Jackson Laboratory, Stock number: 003800) and Cre (The Jackson Laboratory, Stock number: 003724) mice to delete the reporter genes and exon 3 of Mtdh, respectively (see Fig. 1A). Mtdh heterozygous knock-out mice were genotyped by PCR using Mtdh-specific genotype primers (5'-TGGAAAATGATGTGGTGGATGTTTG-3'; 5'-CACGGTTTACGCTGTTGTCGT-3') and were interbred to obtain Mtdh homozygous knock-out mice. The Mtdh heterozygous knock-out mice were maintained by intercrosses on the C57BL/6 background. Animal protocol 4071085 has been approved by Institutional Animal Care and Use Committee Office of Animal Resources (through the Animal Care and Use Review Form (ACURF)).

Western Blotting—The following antibodies were used: anti-MTDH (40–6500, Invitrogen); anti-β-actin (A1978, Sigma); anti-Rad51 (PC130, EMD Millipore); and anti-Mili and anti-Miwi (5940 and 6915, Cell Signaling Technology). Whole-cell protein lysates were prepared and analyzed by Western blotting as described previously (16).

Histology—Formalin-fixed, paraffin-embedded (FFPE) testes sections from WT or homozygous Mtdh exon 3 knock-out mice were dehydrated, deparaffinized, and stained with specific primary antibodies Mili, Miwi, SYCP3 (ab97672, Abcam), SYCP1 (ab15090, Abcam), Rad18 (ab188235, Abcam), and γ-H2AX (2577, Cell Signaling Technology), followed by staining with Alexa Fluor 546-conjugated anti-rabbit or Alexa Fluor 488-conjugated anti-mouse secondary antibodies and visualization by confocal microscopy. Nuclei were counterstained with DAPI (Vector Laboratories) or TO-PRO 3 iodide (Invitrogen).

miRNA Sequencing and Data Analysis by Illumina HiSeq 2000—Total RNA from individual tests of two WT mice and three homozygous Mtdh exon 3-depleted mice was used to prepare the miRNA sequencing library by the following steps: 1) 3'-adapter ligation with T4 RNA ligase 2 (truncated); 2) 5'-adapter ligation with T4 RNA ligase; 3) cDNA synthesis with RT primer; 4) PCR amplification; and 5) separation by PAGE, extraction, and purification of ~135–155-bp PCR-amplified fragments (corresponding to ~15–35 nt small RNAs). After the completed libraries were quantified with an Agilent 2100 bioanalyzer, the DNA fragments in the libraries were denatured with 0.1 M NaOH to generate single-stranded DNA molecules, captured on Illumina flow cells, amplified in situ, and sequenced for 36 cycles on an Illumina HiSeq 2000 according to the manufacturer’s instructions. Image analysis and base calling were performed using Off-Line Basecaller software (OLB V1.8.0). Subsequently, 3'-adapter sequences were trimmed from clean reads (reads that passed a Solexa CHASTITY quality filter), and any reads shorter than 15 nt were discarded. Next the 3'-adapter-trimmed-reads (≥15 nt) were aligned to the latest known human reference miRNA precursor set (Sanger miRBase 19) using NovoAlign (v2.07.11). Reads (counts < 2) were discarded when calculating miRNA expression. To characterize the miRNA isoform variability, any sequences that matched the miRNA precursors in the mature miRNA region ± 4 nt (no more than one mismatch) were accepted as mature miRNA isoforms, which were grouped according to the 5-prime (5p) or 3-prime (3p) arm of the precursor hairpin. The full dataset has been deposited at GEO (accession number GSE62330).

piRNA Sequencing and Data Analysis by Illumina HiSeq 2000—Total testicular RNA from two WT mice and three homozygous Mtdh exon 3-deficient mice was sequenced individually by the same procedures used for miRNAs. Specifically, the 3'-adapter-trimmed reads (length ≥15 nt) were aligned to the latest piRNA set in piRNABank using NovoAlign software (v2.07.11). Expression of each piRNA was defined as the mapped tag counts. The full dataset has been deposited at GEO (accession number GSE62330).

mRNA Sequencing and Data Analysis by Illumina HiSeq 2000—Total RNA from individual tests of two WT mice and three homozygous Mtdh exon 3-depleted mice was used to prepare the mRNA sequencing library by the following steps: 1) 3'-adapter ligation with T4 RNA ligase 2 (truncated); 2) 5'-adapter ligation with T4 RNA ligase; 3) cDNA synthesis with RT primer; 4) PCR amplification; and 5) separation by PAGE, extraction, and purification of ~135–155-bp PCR-amplified fragments (corresponding to ~15–35 nt small RNAs). After the completed libraries were quantified with an Agilent 2100 bioanalyzer, the DNA fragments in the libraries were denatured with 0.1 M NaOH to generate single-stranded DNA molecules, captured on Illumina flow cells, amplified in situ, and sequenced for 36 cycles on an Illumina HiSeq 2000 according to the manufacturer’s instructions. Image analysis and base calling were performed using Off-Line Basecaller software (OLB V1.8.0). Subsequently, 3'-adapter sequences were trimmed from clean reads (reads that passed a Solexa CHASTITY quality filter), and any reads shorter than 15 nt were discarded. Next the 3'-adapter-trimmed-reads (≥15 nt) were aligned to the latest known human reference miRNA precursor set (Sanger miRBase 19) using NovoAlign (v2.07.11). Reads (counts < 2) were discarded when calculating miRNA expression. To characterize the miRNA isoform variability, any sequences that matched the miRNA precursors in the mature miRNA region ± 4 nt (no more than one mismatch) were accepted as mature miRNA isoforms, which were grouped according to the 5-prime (5p) or 3-prime (3p) arm of the precursor hairpin. The full dataset has been deposited at GEO (accession number GSE62330).
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**RESULTS**

*Mtdh Exon 3-deficient Male Mice Are Sterile*—To generate *Mtdh*-knock-out mice, we used the mouse ESC line generated by IKMC (*Mtdh*, project CSD 48311), which contains loxP sites flanking exon 3 of *Mtdh* (Fig. 1A). Injection of CSD 48311 ES cells into blastocysts generated chimeric mice, and germine transmission was confirmed (Fig. 1A). *Mtdh* heterozygous knock-out (*Mtdh*+/−) mice were bred with FLP-e mice to delete the reporter genes and with Cre mice to delete exon 3 of *Mtdh* (Fig. 1B). *Mtdh*+/− mice were interbred to obtain *Mtdh* homozygous knock-out (*Mtdh*−/−) mice. In *Mtdh*+/− adult mice, Mtdh protein was detected in a variety of tissues, including liver, brain, and spleen, whereas it was undetectable in *Mtdh*−/− tissues (Fig. 1C). These data indicate that this deletion strategy results in a truncated Mtdh protein that either is not expressed or is unstable.

Unexpectedly, breeding *Mtdh*+/− male and female mice produced *Mtdh*−/− offspring at Mendelian ratios significantly below the expected ratio (*Mtdh*+/+, 52.7%; *Mtdh*+/−, 41.3%; *Mtdh*−/−, 6%; supplemental Table S1A). The surviving male *Mtdh*−/− mice were found to be infertile (supplemental Table S1B) and significantly smaller as compared with their WT or heterozygous littersmates (data not shown). In addition, *Mtdh*−/− males had smaller testes than WT and heterozygotes (Fig. 1D). Female *Mtdh*−/− mice were also smaller in weight than other littersmates (data not shown). Crossing *Mtdh*−/− female mice with *Mtdh*+/− male mice resulted in only 9.5% *Mtdh*−/− offspring (supplemental Table S1C), indicating that the female *Mtdh*−/− mice are subfertile.

Data available through the Human Protein Atlas demonstrate expression of MTDH in seminiferous ducts, Leydig cells, follicle cells, and ovarian stroma cells of human origin. We confirmed that testes from WT mice retain Mtdh expression by immunohistochemistry, whereas testes from *Mtdh*−/− male mice are devoid of Mtdh (Fig. 1E). Similar results were observed with ovaries from WT and *Mtdh*−/− female mice (supplemental Fig. S1).

**Morphological Alterations of the Testes from Mtdh Exon 3-deficient Mice**—To determine the mechanism of infertility in *Mtdh*−/− male mice, H&E staining of testes from WT, *Mtdh*+/+, and *Mtdh*−/− mice was performed. No significant morphological differences were observed between WT and heterozygous *Mtdh* exon 3-deficient mice. However, all four of the homozygous *Mtdh* exon 3-deficient mice had moderate to severe multifocal to coalescing testicular degeneration characterized by the accumulation of multinucleated giant cells within the seminiferous tubules and a lack of spermatozoa within the lumina of the seminiferous tubules (Fig. 2, A and B). The epithelial lining of the seminiferous tubules in *Mtdh*−/− testes was intact and composed of the appropriate germ cell types along with scattered Sertoli cells. By contrast, there was a complete lack of spermatozoa and rare spermatids within the majority of *Mtdh*−/− seminiferous tubules and epididymis (Fig. 2, B and C).

To further examine morphological changes in testes associated with *Mtdh* deficiency, we performed electron microscopy. Both chromatoid bodies (Fig. 3A) and inter-mitochondrial cement (also called inter-mitochondrial material/bar/cloud, Fig. 3B) were observed in WT and homozygous *Mtdh* exon 3-deficient testicles. However, multinuclear cells were only observed in homozygous *Mtdh* exon 3-deficient testicles (Fig. 3C). Prominent clusters of mitochondria were also observed in the multinuclear giant cells in homozygous *Mtdh* exon 3-deficient testicles as opposed to the random distribution of mitochondria in WT testes (Fig. 3D). Vacuolated vesicles were observed in homozygous *Mtdh* exon 3-deficient testes but not in WT testes (Fig. 3, A and D).

**Synopsis Is Impaired in Homozygous Mtdh Exon 3-deficient Spermatocytes—SYCP3 is a synaptonemal complex marker,** highly expressed in zygotene spermatocytes and confined to the axes of paired chromosomes during pachytene (21). To determine whether *Mtdh*−/− spermatocytes have impaired progression through prophase I during meiosis, the expression and localization patterns of SYCP3 were determined. SYCP3 was mainly present in the epithelial lining of the seminiferous tubules in WT mice (Fig. 4A). However, SYCP3 expression was dramatically higher both in the epithelial lining and near the lumina of the seminiferous tubules of *Mtdh* exon 3-deficient mice, indicating that many spermatocytes accumulated at pachytene of meiosis prophase I (Fig. 4B). We also analyzed expression of SYCP1, another component of the synaptonemal complex, in *Mtdh* exon 3-deficient testes. As shown in Fig. 4C, the staining pattern of SYCP1 is similar to that observed with SYCP3. These data support that deletion of *Mtdh* results in accumulation of spermatocytes in pachytene.

**piRNA-interacting Protein Mili Is Overexpressed in the Testes of Mtdh Exon 3-deficient Mice**—Previous studies have demonstrated that MTDH associates with SND1 to regulate the activity of the RNA-induced silencing complex (RISC) in the cytoplasm (11). The MTDH/SND1 interaction promotes oncogenic miRNA-mediated degradation of some tumor suppressor mRNAs during carcinogenesis (11). SND1 belongs to the Tudor family and shares a common Tudor domain. Multiple members of the Tudor family, including Tdrd1, Tdrd5, and Tdrd9, are required for spermatocyte development (22). Similar to MTDH and SND1, the Piwi family of genes plays important roles in RNA silencing and translational regulation during the meiotic differentiation of spermatocytes (23). Mili is one of three mouse homologs of Piwi (23). Previous observations from Mili-null mice indicate that Mili plays an essential role in meiotic differentiation of spermatocytes (24). Others have confirmed that spermatogenesis is blocked completely at zygote of meiosis I in Mili-null mice, causing male infertility (24–26). Because *Mtdh* exon 3-deficient mice also display defective spermatogenesis, we examined Mili expression in WT and *Mtdh* exon 3-deficient testes. Interestingly, Mili expression was dramatically increased in *Mtdh* exon 3-deficient testes (Fig. 5A) as compared with WT and heterozygotes, indicating a correlation with accumulation of spermatocytes at pachytene when
Mili is normally expressed. Similar to SYCP3 staining, increased expression of Mili was observed in multiple layers both in the epithelial lining and near the lumina of the seminiferous tubules in homozygous Mtdh exon 3-deficient mice as compared with WT (Fig. 5B). In contrast, no significant alteration of Miwi expression was observed in homozygous Mtdh exon 3-deficient testes as compared with WT (Fig. 5C).
Expression of miRNAs and piRNAs Is Altered in the Testes of Mtdh Exon 3-deficient Mice—Both MTDH and Mili play critical roles in the function of non-coding small RNAs (23). To identify the effect of Mtdh exon 3 depletion on the expression levels of miRNAs and piRNAs, these were assayed from homozygous Mtdh exon 3-deficient testicles and WT adult testicles. Reduced expression of miRNAs was detected in homozygous Mtdh exon 3-deficient testes (Table 1). By contrast, miRNA levels were not reduced in the liver from Mtdh exon 3-deficient as compared with WT mice (supplemental Table S2), indicating that the observed miRNA changes in testes are germ cell-specific. As shown in Table 2, piRNA expression levels were also dysregulated, with some being reduced and others increased in homozygous Mtdh exon 3-deficient testes as compared with WT mice.

\[ \gamma-H2AX \] is Increased in Homozygous Mtdh Exon 3-deficient Spermatocytes—Abnormally high \( \gamma-H2AX \) levels indicate DNA damage in cells. Expression normally increases during leptotene, but then diminishes as DNA damage is repaired during zygotene, eventually becoming confined mainly to the XY body, which is prominent during pachytene (27). The XY body forms as a consequence of chromosomal alignment, particularly the more difficult pairing of the X and Y chromosomes. As a result, the X and Y chromosomes only partially synapse through their pseudo-autosomal regions during normal pachytene (27, 28). The co-localization of \( \gamma-H2AX \) with a Y chromosome FISH probe confirms the accumulation of \( \gamma-H2AX \) at the XY body in testis from a WT mouse (Fig. 6A). In Mtdh-/- testes, abnormally high expression of \( \gamma-H2AX \) was observed in the epithelial lining and near the lumina of the seminiferous tubules as compared with WT testes (Fig. 6B). This is an indicator of ongoing and persistent DNA damage in Mtdh-/- testes.

Deficiency of Mtdh Modifies DNA Damage Repair Protein Rad18 in Testes—We previously reported that MTDH associates with multiple mRNAs, including the DNA damage repair protein RAD18 (16). Because RAD18 has been implicated in spermatogenesis (29), we examined a possible mechanistic link between MTDH and RAD18 in infertility in Mtdh exon 3-deficient male mice. We found that mRNA and protein levels of Rad18 were reduced in homozygous Mtdh exon 3-deficient testes (Fig. 6, C and D). However, testes from Mtdh exon 3-depleted mice had a pronounced increase in Rad18 foci, specifically near the lumina of the seminiferous tubules. In addition, the majority of the Rad18 foci did not co-localize with Y chromosome probe (Fig. 6E), suggestive of a role for MTDH in DNA damage repair in somatic chromosomes as well as in sex chromosomes.

DISCUSSION

To date, extensive studies of MTDH have identified it as a key driver of cellular behavior, including promoting metastasis and chemoresistance in cancer. However, the function of MTDH in
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normal physiology is still unknown. Others have reported that in WT mice, Mtdh co-localizes with Ki-67, a biomarker of cell proliferation (19). Mtdh is expressed in mid-to-hindbrain, fronto-nasal processes, limbs, and pharyngeal arches in the early developmental period from E8.5 to E9.5. At stages E12.5–E18.5, Mtdh is localized in the brain, liver, olfactory, and skeletal systems as well as in skin, including hair follicles (19). Herein we identify a role for Mtdh in the regulation of sper-
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matogenesis, male fertility, and the expression of small non-coding RNAs during spermatogenesis. Specifically, we found that Mtdh deletion mediated by exon 3 disruption results in the altered expression profile of small non-coding RNAs, increased expression of Mili, and accumulation of spermatocytes in pachytene uniquely in male mice. As a result, spermatogenesis is disrupted, and depleted mice demonstrate significantly abnormal testicular histology with accumulation of multinucleated giant cells demonstrating abnormal mitochondrial distribution. In addition, Mtdh-null mice largely fail to develop spermatozoa and are therefore infertile. There also appears to be an enhanced loss of Mtdh\textsuperscript{−/−} fetuses in utero as birth Mendelian ratios are skewed, suggesting that Mtdh deficiency is sublethal.

Interestingly, our model differs from the Mtdh knock-out mice generated by Wan et al. (30), who recently reported normal Mendelian ratios and no significant phenotypic alterations.

FIGURE 5. Expression of piRNA-associated protein Mili is increased in testes of Mtdh\textsuperscript{−/−} mice. A, expression of Miwi, Mili, and Rad51 in testes lysates from WT (+/+), heterozygous (+/-), and homozygous Mtdh knock-out mice (-/-) by Western blotting. B, Mili expression in testes from WT and Mtdh\textsuperscript{−/−} mice by immunofluorescent staining. Green, Mili; blue, nuclei (DAPI). C, Miwi expression in testes from WT and Mtdh\textsuperscript{−/−} mice by immunofluorescent staining. Arrows indicate nuclei of sperm in WT testes, which are devoid of Miwi staining. Green, Miwi; blue, nuclei (DAPI), blue.
To create the knock-out animals, these investigators used the ESC line XB780 from the Bay Genomics gene trap database, which contains an insertion in the second intron of Mtdh resulting in premature termination of transcription at exon 2. Only a transient delay in ductal outgrowth of mammary glands from 3- and 5-week-old knock-out mice and no significant differences in branching morphogenesis at later time points or during pregnancy and lactation were observed as compared to wild-type mice.

FIGURE 6. Alterations in the DNA damage marker γ-H2AX and DNA repair protein Rad18 in testes of Mtdh−/− mice. A, co-localization of γ-H2AX with Y chromosome in seminiferous tubule WT mice. Green, γ-H2AX; red, Y chromosome-specific FISH probe; blue, nuclei (DAPI). B, expression of γ-H2AX is increased in the seminiferous tubule of Mtdh exon 3-deficient mice as compared with WT mice. Green, γ-H2AX; blue, nuclei (DAPI). C, effect of Mtdh deficiency on Rad18 protein levels as detected by immunofluorescent staining in seminiferous tubules. Green, Rad18. D, effect of Mtdh deficiency on Rad18 mRNA levels as detected by RT-qPCR in testes or liver. Data were normalized to β-actin and reported as the fold change relative to WT. *p < 0.01 versus WT. E, co-localization of Rad18 with Y chromosome in seminiferous tubule from Mtdh exon 3-deficient mice. Green, Rad18; red, Y chromosome-specific FISH probe; blue, nuclei (DAPI). Inset is an enlarged image to depict punctate staining of Rad18 that does not co-localize with the Y chromosome in Mtdh−/− seminiferous tubules.
with WT littersmates (30). The mice were reported to be fertile. In contrast, our Mtdh knock-out mice, generated through a different targeting strategy, clearly show abnormal Mendelian ratios and have a pronounced sterile phenotype in males. Robertson et al. (5) generated another Mtdh (also called AEG-1) knock-out mouse model with deletion of the promoter region, exon 1, and part of intron 1 of the Mtdh gene using a Cre-loxP system. These mice were viable; however, the litter sizes generated from homozygous Mtdh knock-out mice were very small (1–2 pups per litter). Litters generated by crossing heterozygous Mtdh+/− breeding pairs were also reported to be very small (2–3 pups per litter), indicating that there may be some reproductive and/or sublethal consequences of Mtdh depletion in this model as well.

There are several possible explanations for the differences between the three models. First, our model utilizes the ESC line of EPD02917_A08 from IKMC Project 48311 with the deletion of exon 3, which results in an unstable peptide fragment that is degraded. We were unable to detect any Mtdh species (even truncated molecules) in homozygous Mtdh knock-out mice reported herein. The Robertson et al. model (5) targeted the N terminus, and again, no expression was found. In addition, the Wan et al. model (30) utilized an insertion in the first intron to cause premature termination at exon 2, but theoretically, these animals may retain expression of exon 1. If this region is present in the Wan et al. model (30), there are functional implications that could contribute to the differing impact of the deletion strategies. For example, the 162 amino acid residues corresponding to exons 1 and 2, if expressed, contain the transmembrane domain and the first nuclear localization signal motif. This region is also required for the protein-protein inter-

### TABLE 1

| miRNA | Average copy number | Mtdh−/− vs. WT Δ-fold change | p value |
|-------|---------------------|-----------------------------|---------|
| mmu-tno-101b-3p | 309 485 | 0.64 | 0.012 |
| mmu-tno-106b-3p | 148 257 | 0.57 | 0.034 |
| mmu-tno-162-2-3p | 11 20 | 0.55 | 0.006 |
| mmu-tno-182-5p | 637 1022 | 0.62 | 0.006 |
| mmu-tno-19b-3p* | 473 1250 | 0.38 | 0.016 |
| mmu-tno-28a-3p | 35 60 | 0.59 | 0.008 |
| mmu-tno-293-5p | 12 24 | 0.49 | 0.0105 |
| mmu-tno-340-5p | 905 1561 | 0.58 | 0.0009 |
* miRNAs that are also down-regulated in infertile men (34).

### TABLE 2

| piRNA ID | piRNA Sequence | Average copy number | Mtdh−/− vs. WT Δ-fold change | p value |
|---------|----------------|---------------------|-----------------------------|---------|
| mmu-piR_000219 | ACCTCTCCATGACCAAAGAATTTGTCATGACAAC | 118.63 | 24 | 4.94 | 0.026 |
| mmu-piR_006207 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 224.57 | 80.2 | 2.80 | 0.042 |
| mmu-piR_033479 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 68.57 | 27.05 | 2.53 | 0.028 |
| mmu-piR_031363 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 558.43 | 230.2 | 2.43 | 0.011 |
| mmu-piR_030012 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 1584.7 | 655.85 | 2.42 | 0.030 |
| mmu-piR_038202 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 368.57 | 152.45 | 2.42 | 0.007 |
| mmu-piR_022877 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 188.2 | 78.9 | 2.38 | 0.024 |
| mmu-piR_002024 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 68.1 | 28.7 | 2.37 | 0.034 |
| mmu-piR_019994 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 370.8 | 157.25 | 2.36 | 0.011 |
| mmu-piR_009031 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 55.23 | 23.85 | 2.32 | 0.033 |
| mmu-piR_021054 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 35.67 | 15.7 | 2.27 | 0.021 |
| mmu-piR_038322 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 35.57 | 15.75 | 2.26 | 0.009 |
| mmu-piR_022855 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 61.87 | 28.95 | 2.13 | 0.019 |
| mmu-piR_008329 | TAAAGAGTTTAAAAAGGTCTTATGAGG | 39.03 | 18.65 | 2.09 | 0.039 |

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action between MTDH and BCCIP, PLZF, CBP (CREB-binding protein), and YY1 and is involved in the association of MTDH with mRNA targets (1−3). Recently, the N-terminal region encoded by exon 1 was shown to interact with retinoid X receptor (RXR) and profoundly inhibit retinoid X receptor/retinoic acid receptor (RARR)-mediated transcriptional activation (31). In our model, where no fragments of Mtdh were detected by Western blotting, all of the functionality as described above is lost. Therefore, we believe that our findings, unlike those of Wan et al. (30), clearly point to Mtdh as an important factor in spermatogenesis. When Mtdh is lost, normal DNA processes required for meiosis and male fertility are greatly impacted. We hypothesize that the stable expression of the putative Mtdh N terminus peptide encoded by exons 1 and 2 may be adequate to compensate for the loss of full-length Mtdh in reproduction. Another possible explanation for the difference in fertility among models is the contribution of different backgrounds. Our model and the Robertson et al. model (5) are on the C57BL/6 background, whereas the mice generated by Wan et al. (30) were backcrossed to FVB background.

Our observations of increased expression of Mili and altered expression of piRNAs in the testes of Mtdh exon 3-deficient mice identify an essential role for MTDH in the regulation of small non-coding RNAs in spermatogenesis. Previous experiments have demonstrated that MTDH associates with SND1 and is involved in functional regulation of the RNA-induced silencing complex to inhibit tumor suppressor genes in cancer cells (10, 11). SND1 belongs to the Tudor domain-containing protein family. Our data support the hypothesis that MTDH is involved in the functional regulation of piRNAs via a mechanism similar to the Tudor family proteins. Argonaute proteins are crucial components in diverse gene-silencing processes. The Piwi family is a subgroup of Argonaute proteins that are required for germ and stem cell development. Mili and Miwi are Piwi family homologs essential for spermatogenesis in the mouse, and Mili expression is highest during the early stages of spermatogenesis (25). We observed Mili overexpression in our mouse model, which could simply be the result of severely impaired spermatogenesis at an early stage as Mili is expressed uniquely early in spermatogenesis (23, 25).

There are two populations of piRNAs that are expressed at the pre-pachytene and pachytene stages of meiosis in the male germ cells of mammals (32). Pre-pachytene piRNAs are mostly encoded by retrotransposons and silence the respective retrotransposons. Pachytene piRNAs are derived from ~3,000 genomic clusters and function in maintaining post-meiotic genome integrity by regulating other key aspects of translational control during spermatogenesis. Accordingly, we found that Mtdh exon 3 deletion alters the expression profile of piRNAs in male testes. Although the role of piRNAs in spermatogenesis is well defined, few studies have addressed the role of miRNAs in this process (33). We found a significant decrease in nine miRNAs, including miR-16, miR-19b, miR-28a, miR-101b, miR-106b, miR-144, miR-182, miR-293, and miR-340, in testes but not liver in response to loss of Mtdh expression. Importantly, low miR-19b and miR-16 levels are also present in the semen of infertile men (34).

What is the role of Mtdh in pachytene of male mice? The pachytene checkpoint is necessary to effect alignment of the X and Y chromosomes and to exchange genetic information during meiosis in male mice (35). The XY body, which is prominently seen during this period, is required to recruit DNA repair proteins to silence the sex chromosomes and to accomplish the unique sex chromosome partial synopsis at meiotic prophase in males. Accumulation of high levels of γ-H2AX during pachytene indicates significant DNA damage as a result of Mtdh loss. Our studies also suggest that deficiency of Mtdh induces the pachytene checkpoint because the majority of spermatocytes fail to progress further in meiosis.

A key step in meiosis is the generation and repair of DSBs. Because we observed a pronounced accumulation of cells in pachytene, along with increased expression of γ-H2AX, we speculate that MTDH is required for DSB repair, perhaps specifically in male germ cells. Furthermore, several lines of additional evidence support the role of MTDH in DNA repair in general. First, MTDH has been reported to interact with BCCIP, a BRCA2- and CDKN1-interacting protein involved in DNA damage repair and cell cycle regulation (36). Second, as with previously reported knock-out mouse models targeting other classic, general DNA repair molecules, Mtdh deficiency using our strategy results in male sterility. For example, deficiency of Atm, Brca2, and Rad18 similarly results in defects in spermatogenesis (29, 37−39). ATM, the gene mutated in the human autosomal recessive disorder ataxia telangiectasia, plays a crucial role in the detection of DSBs and is a signal transduction protein in the DNA damage surveillance network. Male Atm-null mice are sterile, and spermatogenesis is arrested at mid-pachytene, consistent with our observations in our Mtdh knock-out model (37). BRCA2 and BRCA1 play critical roles in homologous recombination repair to maintain genomic stability. Brca2-deficient spermatocytes can undergo the early steps of recombination (DNA DSB formation), but fail to complete this process and to progress beyond prophase I of meiosis (38, 39). In contrast to spermatocytes, some Brca2-deficient oocytes can progress through meiotic prophase I and can be fertilized to produce embryos. Deficiency of Rad18 is also associated with impaired spermatogenesis (29). Further confirming a role for MTDH in DNA damage repair, we observed increased Rad18 foci in the lumina of the seminiferous tubules in testes from Mtdh exon 3-depleted mice, and some punctate staining that did not co-localize with the Y chromosome. In addition, the decrease in Rad18 expression under Mtdh deficiency is consistent with our previous report that MTDH associates with multiple miRNAs, including Rad18, to regulate their translation (16). These data suggest a novel mechanism whereby MTDH regulates DNA damage repair of both somatic and sex chromosomes in spermatogenesis.

In conclusion, we report that complete deletion of Mtdh in mice, based upon the targeting strategy described herein, results in male infertility characterized by accumulation of spermatocytes in pachytene and the absence of spermatozoa. Mtdh deficiency has significant effects on the expression of multiple non-coding RNAs critical for spermatogenesis and repair of DSBs. These findings further define the normal function of MTDH, which was heretofore best known as an ampli-
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