ZMYND15 ENCODES A HISTONE DEACETYLASE-DEPENDENT TRANSCRIPTIONAL REPRESSOR ESSENTIAL FOR SPERMIogenesis AND Male Fertility*

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Spermatogenesis is a complex process through which male germline stem cells undergo a multi-step differentiation program and sequentially become spermatogonia, spermatocytes, spermatids and eventually spermatozoa. In this process, transcription factors act as switches that precisely regulate the expression of genes which in turn control the developmental program of male germ cells. Transcription factors identified to be essential for normal haploid gene expression all display transcription-activating effects and thus serve the “on” switch for the haploid gene expression. Here, we report that ZMYND15 acts as a histone deacetylase-dependent transcriptional repressor and controls normal temporal expression of haploid cell genes during spermiogenesis. Inactivation of Zmynd15 results in early activation of transcription of numerous important haploid genes including Prml1, Tnp1, Spem1 and Catpser3, depletion of late spermatids and male infertility. ZMYND15 represents the first transcriptional repressor identified to be essential for sperm production and male fertility.

Spermatogenesis is a process of cellular multiplication and differentiation through which male germ cells develop from spermatogonial stem cells to spermatozoa with highly specified functions (1,2). Spermatogenesis can be divided into three phases that include the mitotic phase (proliferation and differentiation of spermatogonia), the meiotic phase (differentiation of spermatocytes), and spermiogenesis (differentiation of haploid germ cells from round spermatids to elongated spermatids and spermatozoa) (1,2). Within the seminiferous tubules, the developing male germ cells including spermatogonia, spermatocytes and spermatids are all in close contact with the cytoplasm of supporting Sertoli cells, and these spermatogenic cells form specific cellular associations, which have been termed stages (3). For example, at stage VII, type A1 spermatogonia, preleptotene spermatocytes, step 7 round spermatids and step 16 elongated spermatids are associated with each other, whereas Type A3 spermatogonia, zygotene spermatocytes, meiotically dividing spermatocytes, and elongating step 12 spermatids are grouped together. Twelve (I-XII) stages can be identified in the mouse seminiferous tubules (3). Meiosis is unique to germ cells and spermiogenesis is unique to male germ cell development. Unique processes often require unique genes and gene products to execute the functions. This may explain why ~10% of the entire protein-encoding genes are dedicated to the regulation of spermatogenesis, and spermiogenesis alone involves at least 500 testis-specific genes (4-6).
Another unique feature in the control of gene expression during spermiogenesis is the uncoupling of transcription and translation (7-12), which results from the fact that genes required for late spermiogenesis (steps 9–16 spermatids in mice) have to be transcribed in round spermatids (steps 1–8) because transcription ceases when nuclear elongation and condensation start at step 9 of spermatid development in mice. Therefore, the most active transcription occurs at a time window between the late pachytene spermatocyte and round spermatid stages during spermatogenesis. The transcripts are then stabilized and stored until they are translated in later steps where their encoded proteins are needed (8,9,12).

Transcription factors are critical regulators of gene expression and serve as switches that control the normal spatiotemporal expression of gene sets during specific cellular processes (12). Given that so many testis-specific genes are involved in sperm production, the regulation of gene expression in spermatogenesis by testis-specific transcription factors is a foreseeable mechanism. Efforts in gene discovery in conjunction with gene knockout technology over the past 15 years have identified a number of transcription factors that are preferentially or exclusively expressed in the testis and are required for different phases of male germ cell development (13-15). ZBTB16 (16,17), SOX3 (18,19) and SOHLH (20) are essential for the mitotic phase of spermatogenesis and a lack of these transcription factors causes depletion of spermatogonia and eventually “Sertoli-only” testes. CREM-tau is essential for post-meiotic germ cell development, and a lack of this transcription factor leads to the arrest in the first step of spermiogenesis (21). CREM-tau acts as a main switch for genes required for haploid germ cell development, including Prm1, Prm2, Tnp1 and Tnp2 (21,22). TBPL1 (TATA box binding protein-like 1) is also required for spermiogenesis and inactivation of Tbp11 results in a complete arrest of spermiogenesis at step 7 (23). Many genes that are under the control of Tbp11 fail to be expressed in the Tbp11-null mice.

In the present study, we report that Zmynd15 encodes a MYND-containing zinc binding protein that is exclusively expressed in haploid germ cells during spermatogenesis. ZMYND15 acts as a transcription repressor through the recruitment of histone deacetylase enzymes (HDACs). Inactivation of Zmynd15 in mice results in late spermiogenic disruption, causing azoospermia and complete male infertility.

**Experimental Procedures**

**Zmynd15** mice- We previously generated Cxcl16 mice in which the entire coding region and part of the 5' untranslated region (UTR) of Cxcl16 were deleted (24). We later discovered that Zmynd15 and Cxcl16 genes overlap in their 5'UTRs (Fig. 1A), and the deletion of the Cxcl16 5'UTR in fact also inactivated Zmynd15 (Fig.1B). The Cxcl16-null mice are actually Cxcl16-Zmynd15 double knockout mice (see results below). Because the testicular phenotype is caused by Zmynd15 deficiency (see results and discussion below), we henceforth call these mice Zmynd15 mice.

**Testes weight and sperm count**- Whole testes were dissected and weighed. Spermatozoa were collected from caudal epididymides and incubated in 2 ml of PBS pre-warmed to 37°C. Sperm counts were performed using a hemocytometer (Hausser Scientific, Horsham, PA).

**Generation of ZMYND15 antibody**- Keyhole limpet hemocyanin-conjugated peptide corresponding to amino acids 176–190 of murine ZMYND15 (PREDERAPEKRKGQKN) was synthesized and used for immunization of rabbits (Invitrogen Life Technologies, Carlsbad, CA). ELISA was used to quantify anti-ZMYND15 titers in serum, and the IgG fraction was then purified by chromatography on protein A columns (Pierce, Rockford, IL), following the manufacturer’s protocol.
**Histology and immunohistochemistry**—Wild-type and Zmynd15−/− testis were collected, fixed in 4% paraformaldehyde in PBS, and processed for sectioning. For histology the sections were deparaffinized in xylene, rehydrated by standard procedures, and stained with the Periodic acid-Schiff solution followed by hematoxylin counterstaining (25). Immunohistochemistry was performed as described (26) and anti-ZMYND15 antibodies were diluted at 1:600.

**Quantitative and semi-quantitative PCR**—Whole testes were homogenized in Trizol (Invitrogen) and total RNA was isolated following manufacturer’s instructions. RNA samples were further purified using RNeasy mini columns (Qiagen, Los Angeles, CA) and then incubated with DNase (DNAfree, Ambion, Houston, TX) to eliminate potential genomic DNA contamination. For qPCR analyses, 1μg of total RNA was reverse transcribed in a 20μl reaction with SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen). The cDNA samples were diluted 10-fold and 5 μl was added to a 50-μl PCR reaction. To quantify Zmynd15 expression, 2.5 μl of 20X predetermined Taqman primer/probe sets from Applied Biosystems were used. Samples underwent the following protocol: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; and stage 3, 95°C for 15 s, and followed by 60°C for 1 min. Stage 3 was repeated 40 times. Relative expression levels were calculated by the comparative Ct method as outlined in the manufacturer’s technical bulletin. Measured RNA levels were normalized to GAPDH. Semi-qPCR analyses were performed as described (27). The cycle numbers were tested empirically to assure the PCRs were within the exponential range. The primers and PCR conditions used in this study are listed in the Supplementary Table 1.

**In situ hybridization**—In situ hybridization was performed as described (28,29). Briefly, Bouin-fixed, paraffin-embedded adult mouse testes were cut into 5μm sections, dewaxed, fixed, hybridized, and washed. A PCR-generated cDNA fragment (505bp) corresponding to n.t. 1-505 of the full-length Zmynd15 cDNA was subcloned into the pGEM-T vector (Promega, Madison, WI). Sense and antisense riboprobes were generated and labeled with [α-35S] UTP with the Riboprobe Labeling System (Promega, Madison, WI). Hybridization signals were detected by autoradiography with an NTB-2 emulsion (Eastman Kodak, Rochester, NY). After development and fixation, the slides were counterstained with hematoxylin and mounted for photography.

**TUNEL assay**—We performed TUNEL assays on paraformaldehyde-fixed paraffin sections using an ApoTag Plus peroxidase kit (Intergen, Purchase, NY) according to the manufacturer’s instructions.

**Transcription repression assay**—10t1/2 cells were grown to 75–80% confluency in three 6-well plates. All cells were transfected with 1mg of total DNA and Fugene6 (Roche) and with 200ng of SV40-luc and 200ng of CMV-lacZ. One 6-well plate was transfected with 600 ng of Gal4-DBD, another plate with 300 ng of Gal4-DBD and 300 ng Gal4-Zmynd15, and a final plate with 600 ng of Gal4-Zmynd15. After 24 h, half of each plate was treated with the HDAC inhibitor trichostatin A (TSA) (Calbiochem, Gibbstown, NJ) and the other half of each plate with dimethyl sulfoxide (DMSO). After another 24h, cells were washed with 1x PBS, and cell lysates were harvested with 250 ml of Reporter Lysis buffer (Promega). Luciferase activity was measured in 50 ml of sample lysates with the Luciferase Assay System (Promega). Transfection efficiency was normalized to β-galactosidase, which was measured in 50 ml of sample lysate by a β-galactosidase Enzyme Assay System (Promega). Experiments were performed in three replicates and statistic significance was evaluated using the student t test (Sigma Stat).

**Cell-based pull-down assays**—Constructs for expressing FLAG-tagged histone deacetylases
1, 3, 5, 6, 7, and SIRT1 were prepared as described previously (30-33). 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-products, Woodland, CA), 1% penicillin-streptomycin, and 2 mM L-glutamine (Hyclone Invitrogen Corporation). 1x10⁶ 293T cells were plated per 100 mM dish, 24 hrs before calcium-phosphate transfection. A total of 20 µg of DNA (10µg pCDNA HDAC C-term Flag tag + 10µg ZMYND15 HA-tag) was brought up to 876 µL in H₂O. 124 µl of 2M CaCl₂ was added to the DNA, 1ml of 2x HBS pH 7.05 was bubbled in for 10sec, and then the mixture was applied to the cells. 48 hours after transfection the cells were washed with 1X PBS and lysed directly in the 100 mm plate in 333 µl of cold lysis buffer (50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 500 mM NaCl, 0.5% NP-40, and 1x complete protease inhibitors (Roche, Penzberg, Germany) for 1hr, and then diluted with 666 µl lysis buffer with no NaCl to get a final concentration of 150 mM NaCl. The cell lysates were cleared by centrifuged for 15 min at 14,000 x g, and 0.01% of the lysate was analyzed to check for expression of transfected proteins. 40 µl of pre-washed M2 agarose beads (Cat#. A2220, Sigma-Aldrich, St. Louis, MO) were added to the lysates to immunoprecipitate the flag-tagged HDACs and the lysates were rotated at 4°C for 2-16 hrs. The beads were collected by centrifugation for 30 sec at 5,000 x g, and the supernatants were removed by aspiration. The pellets were washed five times with 0.5 ml of lysis buffer. 75 µl of 2x sample buffer was added to the M2 agarose beads, the samples were boiled for 3 min, and the immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotted using anti-ZYMND15 antibody and a rabbit anti-FLAG antibody (Cat#. F7425, Sigma).

Microarray analyses- Total RNAs were isolated from Zmynd15⁻/⁻ and wild-type testes at P20. Duplicates for each genotype were subjected to microarray analyses. Affymetrix mouse genome 430A 2.0 microarrays (Affymetrix Inc., Santa Clara, CA) were hybridized at the Nevada Genomic Center. In brief, 5 µg of total RNA was converted to first-strand cDNA by using Superscript II reverse transcriptase primed by a poly(T) oligomer that incorporated the T7 promoter. Second-strand cDNA synthesis was followed by in vitro transcription for linear amplification of each transcript and incorporation of biotinylated CTP and UTP. The cRNA products were fragmented to 200 nt or less, heated at 99°C for 5 min, and hybridized for 16 h at 45°C to MOE430A microarrays. The microarrays were then washed at low [6× standard saline phosphate/EDTA (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA] and high (100 mM Mes/0.1M NaCl) stringency and stained with streptavidin–phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin–phycoerythrin stain. A confocal scanner was used to collect fluorescence signal at 3-µm resolution after excitation at 570 nm. Affymetrix gcos 1.2 software was used to analyze and quantify the hybridized arrays. Affymetrix’s mas5 algorithm (with default settings, as encoded in gcos 1.2) was used to generate signal values and to determine present/absent/marginal flags for each probe set on each array. Probe sets flagged by mas5 as present are described as detected.

Analysis of microarray data- Analysis was performed using Bioconductor, a publicly available group of software packages as described (34). Briefly, the packages used included Simpleaffy, Limma, Annaffy, and GOstats. Simpleaffy was used to preprocess individual probe intensities from CEL files into expression values from which fold changes were derived using Limma. Robust multiple-array analysis (RMA), which uses quantile normalization for cross-chip normalization, was used to preprocess the data. The main advantage of quantile normalization is that it
controls outliers while not significantly reducing sensitivity. Significance of differential expression was determined using an empirical Bayes approach (Limma) for controlling the standard error of intensity of each probe set based on the standard errors of the intensities of all other probe sets in the comparison. After P values were obtained for each gene, they were adjusted using the Benjamini-Hochberg method (34). This method converts P values, which are measures of the false positive rate, into Q values, which are measures of false discovery rate. The Benjamini-Hochberg method allows a more direct control of false results while not reducing sensitivity as much as other methods for P value adjustment.

Annaffy was used to annotate each gene probe set and to tabulate information such as GenBank accession numbers, chromosome locations, and gene ontology terms.

RESULTS

Deletion of the 5' UTR of Cxcl16 resulted in inactivation of Zmynd15. In our earlier studies designed to investigate the role of scavenger receptors in cardiovascular diseases, we generated Cxcl16 knockout mice by deleting the 5' UTR and the entire coding region of Cxcl16 using homologous recombination in murine ES cells (24). Surprisingly, we found that all Cxcl16-/- males were infertile. We expected no fertility problems in Cxcl16-null male mice because levels of Cxcl16 mRNA in the testis were very low compared to other organs (e.g. heart and kidney), and mRNA for CXCR6, the only known receptor for CXCL16, was not detected in the testis. Furthermore, male and female Cxcr6-/- mice are completely fertile (35). By re-examining the targeting site flanking Cxcl16, we found that in fact Cxcl16 and Zmynd15 are adjacent to each other on chromosome 11 and possess overlapping 5' UTRs (Fig. 1A). Our targeting vector deleted all Cxcl16 coding exons and most of the 5' UTR, which is shared by Zmynd15. It was thus highly likely that the Cxcl16 targeting vector also inadvertently disrupted the Zmynd15 promoter. Our qPCR analyses on levels of Cxcl16 and Zmynd15 mRNAs in wild-type and Cxcl16+/ mice confirmed that in our previously-thought Cxcl16-null testes, both Cxcl16 and Zmynd15 were totally inactivated (Fig. 1B). In addition, in wild-type (WT) testes levels of Zmynd15 mRNAs were ~10 times greater than those of Cxcl16.

Zmynd15 is a testis-specific gene highly expressed in spermiogenesis. We examined the expression of Zmynd15 in 12 adult mouse organs and also in developing testes using RT-PCR (Fig.2). Unlike Cxcl16 which was expressed in a ubiquitous manner, Zmynd15 was exclusively detected in the testis (Fig. 2A). We also searched the Genehub-GEPIS database (36,37) (http://www.cgl.ucsf.edu/Research/genentech/genehub-gepis/genehub-gepis-search.html) for Zmynd15 and found that ESTs for human ZMYND15 were exclusively derived from the testis. In developing testes, Cxcl16 was detected in all ages with comparable levels (Fig. 2B). In contrast, Zmynd15 was first detected in postnatal day 17 (P17) and its levels increase at P21 and thereafter (Fig. 2B), suggesting that Zmynd15 mRNA started to be expressed in late pachytene spermatocytes and later in spermatids. Moreover, our data are consistent with previous microarray data on Zmynd15 expression in purified spermatogenic cells and developing testes in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=geo), showing that Zmynd15 is expressed in pachytene spermatocytes and round spermatids (GEO profile GDS2390), the onset of Zmynd15 expression is at P18 and its levels increase thereafter and peak in adult testes (GEO profile GDS606).

Zmynd15 encodes a protein of 703 a.a. in mice, which contains a zinc-finger MYND motif and a nuclear localization sequence (Fig. 3). Alignment analyses revealed that ZMYND15 is highly conserved among mice, rats and humans (Supplemental Fig. 1), as
evidenced by >85% homology in their amino acid sequences.

To further determine the cellular origin of Zmynd15 expression, we performed in situ hybridization and immunohistochemistry to localize Zymnd15 mRNA and protein in the testis (Fig. 3). Hybridization signals (represented by silver grains in dark-field images) were detected in pachytene spermatocytes and all developing spermatids (steps 1–16) (Fig. 3A–D). No signals were observed in either Sertoli cells or Leydig cells. The hybridization signals were first detected in early pachytene spermatocytes and the intensity of the hybridization signals were higher in steps 1–13 spermatids than in pachytene spermatocytes and late spermatids at steps 14–16. Zmynd15-null testis sections were used as a negative control and no hybridization signals were detected (data not shown). Our in situ hybridization data are consistent with the RT-PCR data on developing testes (Fig. 2B), demonstrating that Zmynd15 mRNA is expressed predominantly in pachytene spermatocytes and spermatids.

To determine Zmynd15 protein localization, we next performed immunohistochemistry on wild-type adult mouse testis sections using a polyclonal anti-ZMYND15 antibody. Immunoreactivity was first detected in step 2 round spermatids (Fig. 3E–F). The intensity of the staining continuously increased thereafter and peaked at steps 7–9 spermatids. ZMYND15 levels started to decrease after step 9, and interestingly, in steps 9–11 elongating spermatids, ZMYND15 immunoreactivity appeared to shift from the nucleus to the cytoplasm. No specific staining was detected when Zmynd15−/− testis sections were used (data not shown). Semi-quantitative PCR analyses using cDNAs prepared from purified testicular cell populations further confirmed that unlike Cxcl16 which was ubiquitously expressed in virtually all cell types within the testis, Zmynd15 mRNA was mainly expressed in pachytene spermatocytes and spermatids (Fig. 3G). Taken together, these data demonstrate that Zmynd15 is exclusively expressed in the testis and is localized to the developing haploid germ cells in the testis. A schematic summary of the localization patterns of Zmynd15 mRNA and protein during spermatogenesis is presented in Supplemental Fig. 2.

Inactivation of Zmynd15 causes spermiogenic disruption, azoospermia and male infertility in mice. Despite the fact that the Cxcl16 knockout mice are in fact Cxcl16-Zmynd15 double knockouts, their spermatogenic defects are likely caused by the inactivation of Zmynd15 based upon the abundant testis-specific expression and consistency between the late spermiogenic defects and the expression sites and function of ZMYND15 (see below).

We initially observed that when male Zmynd15−/− male mice were mated with wild-type female mice for >6 months, no litters were born. Further timed mating experiments revealed that 10 out of 10 wild-type female mice that were mated with Zmynd15+/− male mice (as judged by the presence of vaginal plugs next morning after adding the females to the male cages) became pregnant and later produced 10 litters with normal litter size (~6-8 pups in the C57Bl/6:129Sv/Ev hybrid background), whereas none of the 10 wild-type females that were mated with Zmynd15−/− males were pregnant and produced any litters. Zmynd15−/− females, on the other hand, displayed normal fertility. Zmynd15-null testes showed ~25% reduction in weight (Fig. 4A). No spermatozoa were found in the caudal epididymis (Fig. 4B) of Zmynd15-null mice. Reduced testicular weight and the lack of spermatozoa in the epididymis suggest a spermatogenic disruption in the Zmynd15-null testes. Histological examination of the Zmynd15-null testes revealed a severe depletion of haploid spermatids characterized by spermatids detaching from the seminiferous epithelium and sloughing into the lumen of the seminiferous tubules (Fig. 5A–D). Most of the seminiferous tubules in the Zmynd15-null testes lacked or showed reduced number of late spermatids.
haploid cells (elongating and elongated spermatids). It was difficult to stage each cross section of the Zmynd15-null seminiferous tubules due to the severe spermatid depletion. But the tubules displayed less severely disrupted histology, and were mainly stage IX-XII. We thus estimated that those tubules with severe spermatid depletion were mainly stage I-VIII tubules (For criteria of the mouse seminiferous epithelium staging see Ref. (3) and Supplemental Fig. 2). Round spermatids were still present in the majority of the stages I-VIII tubules, and thus it appeared that the depleted haploid cells were mainly steps 13-16 elongated spermatids. Closer examination of the seminiferous tubules indicated that few, if any, steps 13-16 spermatids were present, and in stages I-VIII tubules where elongated spermatids were actively depleted, round spermatids appeared to be being depleted as well although they were still present (Supplementary Fig. 3). The depletion of spermatids appeared to be achieved via detaching from the epithelium followed by sloughing into the lumen. The depleted haploid cells mostly existed as clusters of multiple cells probably due to the fact that they were all interconnected by intercellular bridges (Fig. 5A-D). TUNEL assays demonstrated that spermatids that appeared to be depleted were TUNEL-negative, suggesting that apoptosis is not the mechanism underlying the depletion of defective late spermatids (Fig. 5E-F). A lack of apoptosis in late spermatids has been well documented in numerous previous studies (38). However, it is noteworthy that the number of TUNEL-positive germ cells resembling mainly spermatocytes was increased in the tubules with severe spermatid depletion (Fig. 5E-F). Given that the majority of spermatocytes were present and appeared normal, it is likely that this enhanced spermatocyte apoptosis was secondary to the primary spermatid defects which disrupted the microenvironment of the seminiferous tubules or communications between spermatocytes and spermatids. Normal size of the accessory sex organs including the seminal vesicle, and normal histology of the Leydig cells in the testis suggest that the testosterone levels were normal in the Zmynd15-null males. Indeed, we measured both FSH and LH levels and found no significant differences between wild-type and Zmynd15-null males (data not shown). Taken together, these data demonstrate that inactivation of Zmynd15 gene causes severe depletion of late spermatids (steps 13-16), characterized by detaching from the seminiferous epithelium followed by sloughing into the lumen of the seminiferous tubules. Zmynd15-null males thus display azoospermia and complete male infertility.

**Function of ZMYND15 as a transcriptional repressor through interaction with histone deacetylases (HDACs).** ZMYND15 belongs to a family of MYND domain-containing zinc finger proteins (Fig. 3), which are commonly involved in transcriptional repression through interacting with histone deacetylases in chromatin remodeling (39,40). We, therefore, performed a transcriptional repression assay to determine if ZMYND15 also acted as such a repressor. ZMYND15 fused to Gal-4 inhibited SV40-driven luciferase expression in a dose-dependent manner in 10t1/2 cells (Fig. 6A). The HDAC inhibitor trichostatin A (TSA) (41,42) was then used to determine whether the ZMYND15-mediated repression of transcription was HDAC-dependent. An addition of TSA (150 μM) significantly inhibited the GAL4-ZMYND15 repression (Fig. 6A). Similar results were obtained when COS-1 cells were used (data not shown). These results suggest that effects of ZMYND15 on the luciferase expression are mediated, at least in part, through recruitment of HDACs.

We then performed pull-down (co-transfection followed by immunoprecipitation) assays to test the potential direct physical interaction
interactions between ZMYND15 and HDACs (Fig. 6B). We first verified the expression of all transfected expression vectors in 293T cells (Fig. 6B, upper two panels). We then used Anti-FLAG to pull down different HDAC complexes and detected the presence or absence of ZMYND15 using anti-HA antibody (Fig. 6B). ZMYND15 was co-immunoprecipitated with HDAC1, HDAC3, HDAC6, and to a lesser extent with HDAC7, indicating that it indeed binds both Class I and Class II HDACs (Fig. 6B). Little or no binding to HDAC5 or the Class III HDAC SIRT1 was detected.

Deregulation of transcription of haploid genes. Given that ZMYND15 interacted with HDACs and acted as a transcription repressor, we further examined the expression of 5 well-characterized haploid genes known to be essential for normal spermiogenesis and male fertility, including Prm1 (43), Tnp1 (44), Klhl10 (45), Catsper3 (46,47), and Spem1 (26) during testicular development in wild-type, heterozygous and Zmynd15-null male mice (Fig. 7). Round spermatids first appear in the seminiferous epithelium at ~P20, and at P27 spermatids have developed to step 13 or 15 (48). Previous studies have shown that these 5 genes begin to be transcribed in round spermatids between steps 1-6, and that levels increase gradually thereafter and peak in steps 8-12 spermatids (26,27,43,45,46,49). Consistent with the transcription onset in round spermatids, in wild-type P20 testes, levels of mRNAs for Prm1, Tnp1, Catsper3 and Spem1 were barely detectable, whereas in Zmynd15-null testes, levels of the four mRNAs were drastically increased. At P27, levels of the four haploid mRNAs increased in the wild-type testes, but decreased in the Zmynd15-null testes due to the onset of spermatid depletion (Fig. 7). Klhl10 is another haploid gene and its levels remained unchanged at P20 and down-regulated at P27 due to spermatid depletion in the Zmynd15+/- testes compared to in wild-type testes. This suggests that Zmynd15 selectively represses transcription of a subset of haploid genes. Pgk2 is mainly expressed in pachytene spermatocytes (50,51) and its mRNA levels remained unchanged, suggesting that the defects were mainly confined to early haploid phase (round spermatids). The depletion of steps 13-16 spermatids observed is thus a delayed reflection of the primary defects in haploid gene expression that occur in early round spermatids.

To further determine global changes in testicular mRNA transcriptome in the absence of Zmynd15, we conducted gene chip analyses using the Affymetrix mouse genome 430A 2.0 microarrays. P20 was chosen as the time point for transcriptome comparison because no morphologically discernable disruptions have occurred at this point and thus the cellular composition between the Zmynd15-null and WT testes remains the same. The microarray data showed that among ~20,000 unique probe sets analyzed, 341 genes displayed more than 2-fold increases in mRNA levels in the Zmynd15-null testes at P20, whereas only 18 genes showed more than 2 fold of decreases in their mRNA levels (Supplementary Table 2). The remaining testicular mRNAs showed no or less than 2 fold changes in their levels. The microarray data are consistent with our qPCR data on 5 spermiogenesis-specific genes, showing significantly increased levels of Prm1, Tnp1, Catsper3 and Spem1 mRNAs and similar levels of Klhl10 and Pgk2 mRNAs in Zmynd15-null testes compared to WT testes at P20 (Highlighted in yellow in Supplementary Table 2). Absence of both Cxcl16 and Zmynd15 in the gene chip analyses of Zmynd15-null testis samples further demonstrates the validity of the microarray data. We then compared the 341 upregulated genes in the P20 Zmynd15-null testes (Supplementary Table 2) with a list of 348 testis-specific genes that are mainly expressed in haploid male germ cells (spermatids) (Our unpublished data) and found that 53 out of the 341 up-regulated genes are spermatid-specific ones (highlighted in green in Supplementary Table 2). The lack of a repressor does not
necessarily lead to accumulation of mRNAs. Nevertheless, these data do further support a role of ZMYND15 acting as a transcription repressor to regulate spatiotemporal expression of a large subset of haploid genes.

**DISCUSSION**

Although both Cxcl16 and Zmynd15 are inactivated in the mouse line analyzed in this study, it is highly likely that the spermiogenic disruption is caused by Zmynd15 inactivation. Several lines of evidence support this claim. First, the only known receptor for CXCL16 is CXCR6, which is not expressed in the testes (35). Moreover, both male and female Cxcr6−/− mice are fertile (35). Second, Cxcl16 is expressed in essentially all testicular cell types including Sertoli cells, Leydig cells, spermatogonia, spermatocytes and spermatids at low levels (Fig. 3G). In contrast, ZMYND15 is exclusively expressed in spermatids, implying a specific role in spermatids. The primary defects in these male mice are confined to spermatids, which coincides with the expression site of ZMYND15. Third, abnormal spatiotemporal transcription of numerous haploid genes is consistent with a role in transcriptional regulation of ZMYND15 because it functions as a repressor of transcription by interacting with HDACs. Therefore, we believe that it is the Zmynd15 deficiency that causes the disruption in haploid gene expression and consequently the late spermatid depletion and male infertility.

Transcription is very active in the late meiotic and early haploid phases because many genes required for late spermiogenesis have to be transcribed before transcription ceases due to nuclear condensation and elongation from step 9 onward (7-12). CREM-tau has been shown to act as a main transcription activator for the expression of many haploid genes including Prm1, Prm2, Tnp1 and Tnp2 (21,22). ZMYND15 is, to our knowledge, the first spermatid-specific transcription repressor identified to date. This notion is supported by the transcription repression assays in vitro, and the enhanced transcription of numerous haploid genes in the absence of ZMYND15. This finding is of significance because it indicates that the haploid gene transcription not only requires transcriptional activators, but also needs transcription repressors, and the balance between the transcriptional activation and repression is essential for normal spatiotemporal haploid gene expression. Zmynd15 thus represents the first transcription repressor identified to be essential for normal spatiotemporal expression of haploid genes.

Human ZMYND15 shares a high degree of sequence homology with mouse ZMYND15 (~85%) and is also exclusively expressed in the testis, suggesting that they have similar physiological roles. The necessity of ZMYND15 in the regulation of normal spatiotemporal expression of critical haploid genes in mice implies that similar azoospermia phenotype would occur in humans if the human ZMYND15 gene is disrupted by mutations. Therefore, it would be informative to screen the non-obstructive azoospermia patients for potential ZMYND15 mutations.

Like many of the MYND domain-containing zinc finger proteins, ZMYND15 selectively binds Class I and Class II HDACs and can suppress transcription in a HDAC-dependent manner in our in vitro assays. Future direct immunoprecipitation assays using testis protein lysates and reporter assays using promoters of ZMYND15 target genes will further corroborate these findings under more physiological conditions. In addition, the downstream events after its binding to different members of HDACs need to be determined in future studies. Microarray analyses identified numerous haploid transcripts that are upregulated in the absence of ZMYND15. Moreover, the number of upregulated transcripts is much greater than that of downregulated transcripts (241 vs. 18). These data are supportive of a transcriptional suppressive role of ZMYND15. However, these upregulated transcripts may not be necessarily the target genes of ZMYND15.
because many of these upregulated transcripts may reflect secondary effects due to the absence of ZMYND15. Therefore, future transcriptional suppression assays using promoters of these upregulated transcripts will enable us to determine the true target genes of ZMYND15.

In summary, we have discovered that ZMYND15, a previously uncharacterized MYND domain-containing zinc finger protein, interacts with histone deacetylases and acts as a testis-specific transcriptional repressor that plays an essential role in the regulation of spatiotemporal expression of many haploid genes. Since transcription factors are key regulators of gene expression, identification of novel transcription factors essential for spermatogenesis enables us to not only reveal the gene network utilized in male germ cell development, but also provide mechanistic insights for the identification of novel causative genes of male infertility and future development of male non-hormonal contraceptives.

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FOOTNOTES

*This work was supported by NIH Grants HL52773 and HL 63894 to I.F.C., HD 048855 and HD 05028 to W.Y.. The Nevada Genomic Center is supported by a NIH grant (P20-RR-016464).

FIGURE LEGENDS

Fig. 1. Inadvertent co-inactivation of Cxcl16 and Zmynd15 in mice. A. Genomic structural relation between Cxcl16 and Zmynd15. Cxcl16 and Zmynd15 are in the opposite orientation with overlapping 5’ untranslated regions (UTRs) and our targeting strategy deleted the 5’UTRs for both genes. B. qPCR analyses of levels of Cxcl16 and Zmynd15 mRNAs in wild-type and Cxcl16-null testes.

Fig. 2. Expression profiles of Cxcl16 and Zmynd15 mRNAs in mice. A. RT-PCR analyses of levels of Cxcl16 and Zmynd15 mRNAs in 12 organs of adult mice. M, 100 bp molecular marker. NTC, non-template control. Gapdh was used as a loading control. B. RT-PCR analyses of Cxcl16 and Zmynd15 mRNA expression in developing testes. M, 100bp molecular marker, C, non-template control. Gapdh was used as a loading control.

Fig. 3. Localization of Zmynd15 mRNA and protein in the mouse testis. A–D. In situ hybridization analyses of Zmynd15 mRNA localization in the testis of adult mice. Bright- (A and C) and corresponding dark- (B and D) field images are shown. Lower magnification images (A and B) show that the hybridization signals (black dots in bright-field images and silver grains in dark-field images) are confined to the luminal and adluminal compartments, and higher power images (C and D) reveal that the hybridization signals are over pachytene spermatocytes and spermatids. Arabic numbers stand for steps of spermatid development, and Roman numerals indicate stages of the seminiferous epithelial cycles. Sg, spermatogonia; Sp, spermatocytes; Sd, spermatids; L, Leydig cells. Scale bars =20µm. 

E–F. ZMYND15 immunoreactivity is detected mainly in the nuclei of steps 2–8 round spermatids (E and F). In steps 9–11 spermatids, ZMYND15 immunoreactivity appears to shift to cytoplasm (E). Arabic numbers stand for steps of spermatid development, and Roman numerals indicate stages of the seminiferous epithelial cycles. P, pachytene spermatocytes; P1, preleptotene spermatocytes; Sd, spermatids. Scale bars=20µm. G. RT-PCR analyses of expression of Cxcl16 and Zmynd15 mRNAs in purified testicular cell types. M, 100bp molecular marker. NTC, non-template control.
**Fig. 4.** Zmynd15-null male mice display reduced testis weight and azoospermia. A. Zmynd15-null testes display ~25% reduction in weight. B. Epididymal sperm counts in wild-type and Zmynd15-null male mice. C. Numerous spermatozoa were collected from the cauda epididymis of wild-type mice (upper panel), whereas no spermatozoa were found in the cauda epididymis of Zmynd15-null mice (lower panel).

**Fig. 5.** Spermatogenic disruption in the testes of Zmynd15−/− mice. A-D. Wild-type testes display robust spermatogenesis (A), whereas Zmynd15−/− testes showed severe spermatid depletion characterized by spermatid clusters that are detaching from the seminiferous epithelium followed by sloughing into the lumen of the seminiferous tubules (arrows in B-D). Roman numerals indicate stages of the seminiferous epithelial cycles. Panels A-D are in the same magnification. Scale bar =20μm. E-F. TUNEL analyses of cell apoptosis showed very few apoptotic germ cells in wild-type testes (E), whereas in Zmynd15-null testes many TUNEL-positive germ cells resembling mainly round spermatids and spermatocytes are present (F). Panels E and F are in the same magnification. Scale bar =20μm.

**Fig. 6.** ZMYND15 acts as a histone deacetylase (HDAC)-dependent transcription repressor. A. 10T1/2 cells were transfected with GAL4-DBD or GAL4-DBD/ZMYND15 along with 5XGAL4-SV40-luc as indicated. The HDAC inhibitor trichostatin A (TSA, 150mM) was added 24 hours after transfection. Transfection efficiency was normalized to β-galactosidase. Results shown represent the average of three replicates (Means ± SEM). The response for GAL4-DBD alone is set at 100%. Bars labeled with different letters are significantly different (n=3, P<0.01). B. ZMYND15 co-immunoprecipitates with class I and IIb HDACs. Protein extracts from 293T cells co-transfected with C-terminal FLAG-tagged HDACs [empty vector (lane1), HDAC1 (lane2), HDAC3 (lane3 and lane 4) HDAC5 (lane5), HDAC6 (lane6), HDAC7 (lane7), or SIRT1 (lane8)] and HA-tagged ZMYND15 (lanes1–3 and lanes5–8) were analyzed by Western blots using anti-FLAG or anti-ZYMND15 antisera to demonstrate protein expression (upper two panels of B). Lower two panels show the co-immunoprecipitation of FLAG-tagged HDACs with HA-tagged ZMYND15.

**Fig. 7.** Selective deregulation of haploid gene expression. Semi-qPCR analyses were used to determine mRNA levels of 5 spermatid-specific genes (Prm1, Tnp1, Klhl10, Catsper3 and Spem1) in the wild-type and Zmynd15−/− testes. mRNAs for the 5 haploid genes are barely detectable in the wild-type testes at postnatal day 20 (P20) because the onset of their transcription is in early round spermatids (steps 1-5). Four (Prm1, Tnp1, Catsper3 and Spem1) out of the 5 spermatid-specific genes examined are drastically elevated in the Zmynd15−/− testes at P20. At P27, mRNA levels of the 5 haploid genes are much higher than those in Zmynd15−/− testes most likely due to the ongoing depletion of spermatids in the absence of ZMYND15. Images represent one of the three independent experiments using three sets of mice with different genotypes. M, 100bp molecular marker, Gapdh was used as a loading control.
Figure 1

A

Genomic structural relation between Cxcl16 and Zmynd15

Deleted Region

Overlapping 5' UTRs

B

Relative Abundance

Zmynd15  CXCL16

Wild-Type  Cxcl16^-/-
Figure 2

A

|       | M | Brain | Heart | Liver | Spleen | Lung | Kidney | Stomach | Intestine | Colon | Ovary | Uterus | Testis | NTC |
|-------|---|-------|-------|-------|--------|------|--------|---------|-----------|-------|-------|-------|--------|-----|
| Cxcl16|   |       |       |       |        |      |        |         |           |       |       |       |        |     |
| Zymnd15|   |       |       |       |        |      |        |         |           |       |       |       |        |     |
| Gapdh |   |       |       |       |        |      |        |         |           |       |       |       |        |     |

B

|       | M | 0.5 | 7 | 10 | 14 | 17 | 21 | 28 | 35 | 84 | C |
|-------|---|-----|---|----|----|----|----|----|----|----|---|
| Cxcl16|   |     |   |    |    |    |    |    |    |    |   |
| Zymnd15|   |     |   |    |    |    |    |    |    |    |   |
| Gapdh |   |     |   |    |    |    |    |    |    |    |   |
Figure 4

A

Average Testes Weight (g)

Wild-type
Zymnd15-null

* $p = <0.0001$

B

Sperm/ml ($x 10^7$)

Wild-type
Zymnd15-null

C

Wild-type
Zymnd15-null
Figure 6

A

Luciferase Expression (%)  

- TSA  + TSA

B

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Empty vector | + | - | - | - | - | - | - | - |
| FLAG-tagged HDAC1 | + | + | - | - | - | - | - | - |
| FLAG-tagged HDAC3 | - | + | + | - | + | - | - | - |
| FLAG-tagged HDAC5 | - | - | - | - | + | - | - | - |
| FLAG-tagged HDAC6 | - | - | - | - | - | + | - | - |
| FLAG-tagged HDAC7 | - | - | - | - | - | - | + | - |
| FLAG-tagged SIRT1 | - | + | + | + | + | + | + | + |
| HA-tagged ZMYND15 | - | + | - | + | - | + | - | + |

Anti-FLAG

Anti-ZMYND15

Total protein lysate

Anti-FLAG

Anti-ZMYND15

IP: Anti-FLAG
Figure 7

| M | P20 (+/+ +/− −/−) | P27 (+/+ +/− −/−) |
|---|-------------------|-------------------|
|   | Zmynd15           |                   |
|   | Cxcl16            |                   |
|   | Prm1              |                   |
|   | Tnp1              |                   |
|   | Klhl10            |                   |
|   | Catsper3          |                   |
|   | Spem1             |                   |
|   | Pgk2              |                   |
|   | Gapdh             |                   |
ZMYND15 encodes a histone deacetylase-dependent transcriptional repressor essential for spermiogenesis and male fertility
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J. Biol. Chem. published online July 30, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.116418

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