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Gtsf1/Cue110, a gene encoding a protein with two copies of a CHHC Zn-finger motif, is involved in spermatogenesis and retrotransposon suppression in murine testes

Takuji Yoshimura [a,b], Shuichi Toyoda [b], Satomi Kuramochi-Miyagawa [c], Tatsushi Miyazaki [b], Satsuki Miyazaki [b], Fumi Tashiro [b], Eiji Yamato [b], Toru Nakano [c], Jun-ichi Miyazaki [b,*]

[a] *Supply Center of Inbred Animals of Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
[b] *Division of Stem Cell Regulation Research, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
[c] *Department of Pathology, Graduate School of Medicine and Frontier Biosciences, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*

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ABSTRACT

We recently reported that the *Gtsf1/Cue110* gene, a member of the evolutionarily conserved *UPF0224* family, is expressed predominantly in male germ cells, and that the GTSF1/CUE110 protein is localized to the cytoplasm of these cells in the adult testis. Here, to analyze the roles of the *Gtsf1/Cue110* gene in spermatogenesis, we produced *Gtsf1/Cue110*-null mice by gene targeting. The *Gtsf1/Cue110*-null mice grew normally and appeared healthy; however, the males were sterile due to massive apoptotic death of their germ cells after postnatal day 14. In contrast, the null females were fertile. Detailed analyses revealed that the *Gtsf1/Cue110*-null male meiocytes ceased meiotic progression before the zygotene stage. Thus, the *Gtsf1/Cue110* gene is essential for spermatogenesis beyond the early meiotic phase. Furthermore, the loss of the *Gtsf1/Cue110* gene caused increased transcription of the long interspersed nucleotide element (Line-1) and the intracisternal A-particle (IAP) retrotransposons, accompanied by demethylation of their promoter regions. These observations indicate that *Gtsf1/Cue110* is required for spermatogenesis and involved in retrotransposon suppression in male germ cells.

Introduction

Spermatogenesis is a dynamic and complicated process that consists of mitosis, meiosis, and spermiogenesis. In the meiotic phase, one spermatocyte eventually divides into four round spermatids, each containing a haploid genome. Meiotic prophase I, during which the cells prepare for metaphase I, which follows, is divided into five stages: leptonema, zygonema, pachynema, diplonema, and diakinesis. Mice deficient in genes involved in the process of meiotic prophase I show an arrest of spermatogenesis at various points in prophase I, followed by the apoptosis of meiocytes. Our understanding of the molecular mechanisms underlying the process of meiotic prophase I, and of spermatogenesis as a whole, should improve with the comprehensive identification of the genes involved.

Some of the genes specifically expressed in germ cells are essential for germ-cell maintenance and differentiation, including the progression of meiotic prophase I (Tanaka et al., 2000; Crackower et al., 2003; Kuramochi-Miyagawa et al., 2004; Ballow et al., 2006; Chuma et al., 2006; Toyoda et al., 2009). Hence, we used an *in silico* subtraction method to identify novel genes specifically expressed in germ cells, and succeeded in identifying a novel transcript expressed in the unfertilized eggs, ovaries, and testes of the mouse (Yoshimura et al., 2007). The open reading frame (ORF) encodes a 167-amino-acid protein belonging to the *UPF0224* (unknown protein family 0224) family. This protein contains two tandem repeats of a CHHC Zn-finger domain, which is predicted to function as an RNA recognition and binding module (Andreeva and Tidow, 2008). We designated the gene as *Cue110*, which is herein referred to as *Gtsf1*, according to the Mouse Genome Informatics nomenclature. The *Gtsf1* transcript is expressed in both male and female gonads from embryonic day (E) 13.5 to adulthood, and GTSF1 protein resides predominantly in the cytoplasm of spermatocytes and round spermatids. These results suggested that the *Gtsf1* gene might play important roles in the meiotic and/or post-meiotic phases during spermatogenesis.

Gene products in various subcellular locations cooperate to promote meiotic progression. For example, many intranuclear proteins are implicated in the cell cycle, DNA replication, DNA recombination, or synaptonemal complex formation during meiosis (Cohen and Pollard, 2001; Cohen et al., 2006). On the other hand, some cytoplasmic proteins, such as MAEL, which is encoded by a murine homolog of the *Drosophila* gene *maelstrom*, MVH, which is a

* Corresponding author. Fax: +81 6 6879 3829.
E-mail address: jimiyaza@nutri.med.osaka-u.ac.jp (J. Miyazaki).

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DEAD box-containing ATP-dependent RNA helicase encoded by a murine homolog of the *Drosophila* gene *Vasa*, and TDRD1, which is encoded by a *tudor*-related gene, localize to an intracellular compartment called the nuage (Toyooka et al., 2000; Chuma et al., 2006; Soper et al., 2008), which is implicated in mitochondrial function, RNA regulation, and transposon control during spermatogenesis (Chuma et al., 2009).

Retrotransposons comprise roughly 40% of the mammalian genome, and have propelled genome evolution (Kazazian, 2004). Although most retrotransposons are inactive, some are capable of transposing to other genomic regions when derepressed (Ostertag and Kazazian, 2001). They are mainly classified into long terminal repeat (LTR) and non-LTR types. Line-1, a non-LTR retrotransposon, is expressed in spermatocytes during meiotic prophase I in the adult mouse testis (Branciforte and Martin, 1994). IAP is an LTR-type retrotransposon, probably derived from a retrovirus; it is expressed only in gonocytes and premeiotic undifferentiated spermatogonia (Dupressoir and Heidmann, 1996). Recently, a transcriptional silencing mechanism involving DNA methylation was found to regulate the expression of Line-1 and IAP in the male gonad (Aravin and Bourc'his, 2008). This mechanism requires two *piwi* family genes, which encode the MILI and MIWI2 proteins. These proteins play a central role in the generation of *piwi*-interacting RNAs (piRNAs), which are thought to be guiding molecules that recognize specific methylation targets, such as those involved in the silencing of transposons. The integrity of this mechanism is proposed to be important for restraining the unregulated expansion of retrotransposons in the germline, which would be deleterious for subsequent generations (Aravin et al., 2007a).

In the current study, to investigate the role of the *Gtsf1* gene, we generated a *Gtsf1* knockout mouse. The *Gtsf1*-null mice showed male-specific sterility owing to a disturbance of spermatogenesis in the early meiotic phase. Furthermore, we demonstrated that the loss of the *Gtsf1* gene reduced the methylation of the regulatory regions of retrotransposons and increased their transcription in testes, similar to observations in *Mael*-, *Mili*-, or *Miwi2*-null mice (Soper et al., 2008; Aravin et al., 2007b; Carmell et al., 2007).

Materials and methods

Generation of Gtsf1$^{-/-}$ mice

The *Gtsf1* targeting vector was designed to replace the DNA sequences from exons 2 to 4 of the mouse Gtsf1 gene locus (Yoshimura et al., 2007) with an IRES (internal ribosomal entry site)-*βgeo* cassette (Mountford et al., 1994) and a PGK-puro cassette. A 6-kb fragment containing exon 1 and exon 1.1 was PCR-amplified using LA Taq (Takara, Shiga, Japan) with primers 5′-CACAAG-CATCCTGTCTCATGTG-3′ and 5′-CTACACTTCTGGTCTGGGATTAC-3′. A 4-kb fragment containing intron 3 was PCR-amplified using LA Taq with primers 5′-CTGTTGTTTCAGTCTCCAGAGA-3′ and 5′-GGCAGGG-TATCATCTTTCTATTC-3′. The 6-kb and 4-kb fragments were inserted upstream and downstream of the IRES-*βgeo*-PGK-puro cassette, respectively (Fig. 1A). The resulting targeting vector was linearized by *Swa*I digestion and introduced into EB3 embryonic stem (ES) cells by electroporation (Robertson, 1987; Ramirez-Solis et al., 1993). EB3 is a germ line-competent subclone derived from E14tg2a ES cells (Miyazaki et al., 2004, 2005). Genomic DNAs from puromycin-resistant colonies were screened for homologous recombination by long genomic PCR using the P2–P3 primers shown in the Supplementary Table. Two targeted clones were identified and injected into blastocysts collected from superovulated C57BL/6 female mice on E3.5. The treated blastocysts were then transferred into the uterus of pseudopregnant MCH-ICR female mice (CLEA Japan Ltd., Tokyo, Japan) to obtain chimeric mice. Male chimeras were mated with female C57BL/6 mice, resulting in germ-line transmission of the mutant allele. Genomic DNAs from the offspring obtained by intercrossing *Gtsf1*$^{+/-}$ mice were analyzed by PCR for the presence of the mutant and wild-type alleles, using the P4–P6 and P5–P6 primers (Supplementary Table).

The mutant mice used in this study were of a mixed genetic background (129/Ola×C57BL/6). We confirmed that the phenotype of the *Gtsf1*$^{-/-}$ testis was the same as that of the mutant mice after backcrosses with C57BL/6 mice to the fourth generation (data not shown). All the animal experiments were performed according to protocols approved by the Animal Care and Use Committee of Osaka University Graduate School of Medicine.

RT-PCR and quantitative RT-PCR

Total RNA was extracted from the testes of mice at different ages by the acid guanidine–phenol–chloroform (AGPC) method. RNA was reverse-transcribed using an oligo (dT) primer and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). Reverse transcription was performed for 1 h at 42 °C in a total volume of 40 μl with 1 μg RNA pretreated with RNase-free DNase per sample, according to the manufacturer's instructions.

One microliter of the cDNA products was amplified by PCR using gene-specific primers. The PCR conditions were 98 °C for 15 s, 60±5 °C for 30 s, and 72 °C for 60 s for 20–30 cycles, followed by an extension for 10 min at 72 °C. The amplified products were separated by electrophoresis on a 1.5% agarose gel and detected by ethidium bromide staining. As an internal control, GAPDH cDNA was amplified as a 450-bp product. The primers used for RT-PCR are shown in the Supplementary Table.

Quantitative RT-PCR was performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster, CA). *Actb* mRNA was used as a standard control (primer sequences are shown in the Supplementary Table). The amplification conditions consisted of an initial denaturation at 90 °C for 10 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 30 s. The primer pairs for the amplification of Line-1 and IAP, respectively, L1ORF2-F and -R and IAP3LTR-F and -R, were used as described previously (Carmell et al., 2007).

Histology and immunostaining

For histological analysis, tissues were fixed overnight in 10% formalin, embedded in paraffin wax, sectioned, mounted, and stained with hematoxylin and eosin. E17.5 testis sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, followed by three 10-min washes with PBS. For the staining of meiocyte spreads or frozen sections, the cells or tissue sections were blocked with blocking buffer containing 3–10% goat serum in PBS for 1 h at room temperature or overnight at 4 °C, and incubated with primary antibody in blocking buffer for 1 h at room temperature or overnight at 4 °C. The primary antibodies were anti-GTSF1 (1:1000; Yoshimura et al., 2007), anti-RAD51 (1:400; Ab-1; Oncogene, San Diego, CA), anti-SYCP1 (1:50; NB300-229; Novus Biologicals, Littleton, CO), anti-SYCP3 (1:100; ab12452; Abcam, Cambridge, UK), and anti-γ-H2AX (1:333; #07-164; Upstate, Lake Placid, NY). After three 15-min washes with PBS, the cells or tissue sections were incubated with the secondary antibody (1:250–500; Alexa 488- or 568-conjugated goat anti-rabbit or mouse immunoglobulin; Molecular Probes, Eugene, OR) in blocking buffer for 60 min at room temperature. DAPI (300 nM; Sigma-Aldrich, St. Louis) was used for nuclear counterstaining of the tissue sections. The cells or tissue sections were washed three times with PBS for 15 min each, and the signals were examined by fluorescence microscopy (Olympus, Tokyo, Japan).