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Ccdd33, a Predominantly Testis-Expressed Gene, Encodes a Putative Peroxisomal Protein

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Coiled-coil domain · Peroxisomal proteins · Peroxisomal targeting signal · Spermatogenesis

Abstract
Many genes crucial for male fertility are often predominantly or exclusively expressed in male germ cells. The analysis of mouse models has demonstrated the functional importance of peroxisomes in spermatogenesis. The CCDC33 protein has been reported to be a cancer/testis (CT) antigen. We found that mouse Ccdc33 is predominantly expressed in the testis and undergoes alternative splicing to produce at least 4 different transcripts. The protein encoded by Ccdc33 contains 3 coiled-coil domains, a C2-domain, 2 ER membrane retention signal-like motifs and 2 putative peroxisomal targeting signals type 2 (PTS2). We could demonstrate that the second PTS2 sequence is functional and responsible for the targeting of CCDC33 to peroxisomes. Moreover, in HeLa cells Ccdc33-dsRED fusion protein co-localized with a known peroxisomal protein, namely PXT1, and showed punctuate intracellular distribution. Taken together, the mouse Ccdc33 encodes a putative peroxisomal protein and is predominantly expressed in male germ cells. The expression starts at the primary spermatocyte stage, suggesting an important role of this protein during spermatogenesis.

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For a long time it was believed that the existence of peroxisomes within the testis is restricted to somatic Leydig cells [Fawcett and Burgos, 1960; Reddy and Svoboda, 1972; Zini and Schlegel, 1996; Baumgart et al., 1997] or Sertoli cells [Lester et al., 1996]. The presence of peroxisomes in male germ cells was first reported in the GC-1 spg cell line [Luers et al., 2003] and later, using mRNA in situ hybridization, immunohistochemistry, Western blotting and electron microscopy, in spermatogonia of mouse testis [Huyghe et al., 2006; Luers et al., 2006]. Recently, in 2007 Nenicu et al. demonstrated different peroxisomal markers in most male germ cell stages, except in mature spermatozoa. Although peroxisomes seem to be indispensable for spermatogenesis, their function in the testis is poorly understood. Recent evidence indicates that peroxisomes are involved in acrosome formation [Moreno and Alvarado, 2006]. Catalase, an enzyme normally present in peroxisomes in somatic cells, is located in the acrosome region of sperm [Figuerola et al., 2000].

Peroxisomes are involved in different cellular functions such as hydrogen peroxide degradation, β-oxidation of fatty acids [van den Bosch et al., 1992], glyoxylate metabolism, synthesis of plasmalogens and gluconeogenesis [Flatmark et al., 1988; Masters, 1996]. Two peroxisomal targeting signals (PTS) are known to be involved in trafficking of proteins to peroxisomes: PTS1 and PTS2 [reviewed in Holroyd and Erdmann, 2001]. The consen-
sus sequence of the PTS2 motif, which is located in the N-terminal or internal region of the peroxisomal proteins [Gould et al., 1989; Swinkels et al., 1991], is defined as: (R/K)(L/V/I/Q)XX(L/V/I/H/Q)(L/S/G/A/K)X(H/Q) (L/A/F) [Petriv et al., 2004]. However, it should be noted that some peroxisomal matrix proteins contain neither PTS1 nor PTS2 signals, but rather a poorly defined sequence sometimes termed PTS3 [Kragler et al., 1993; Elgersma et al., 1995].

Human Ccdc33 was reported to be a cancer/testis (CT) protein [Chen et al., 2005]. In the current work, we characterized the mouse Ccdc33 gene, which is predominantly expressed in the testis. During mouse spermatogenesis its expression starts at the primary spermatocytes stage. Ccdc33 gene transcripts undergo alternative splicing and at least 4 different splice products were identified. Moreover, we could demonstrate that the Ccdc33 protein contains a functional PTS2 motif responsible for its targeting to the peroxisomes.

Materials and Methods

Data Base Searches and Computational Analysis

Nucleotide sequences and deduced protein sequences of mouse Ccdc33 (Gene ID: 382077) were subjected to homology searches in the NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html) databases. The sequence similarity was analyzed using the BLAST [Altschul et al., 1990] and GC-4 [spermatocytes; Tascou et al., 2000]. After reverse transcription, using the Superscript II reverse transcriptase system and the oligo (dT)12-18 primer (Invitrogen, Karlsruhe, Germany), an aliquot of cDNA (1 μl) was subjected to 35 rounds of PCR amplification with Taq DNA polymerase (Immola; Bölinge, Luckenwalde, Germany). For the expression analysis of mouse Ccdc33 gene primers Ccdc2RTFP (5’-GAGGACACCTG-TACGGCATGC-3’) and Ccdc2RTRP (5’-GAATGGGACGGTTC-CTTC-3’) were used, amplifying a PCR product of 409 bp in size. The amplification conditions were 4 min at 94°C followed by 35 cycles of 30 s at 94°C, 45 s at 68°C, and 1 min at 72°C and finally 5 min at 72°C. To test the cDNA quality mHPRT-For-Q: 5’-ACGG CACCA-3’ and mHPRT-Rev-Q: 5’-GGATATGAAGTGGATGCGAGACA-3’ primers amplifying a 222-bp fragment of mouse Hprt1 gene (GeneID: 15452) were used. The alternative splicing of Ccdc33 transcripts was analyzed by primer sets located in different exons of the Ccdc33 gene (Supplementary table 1; for all supplementary material, see www.karger.com/doi/10.1159/000251961) using RT-PCR conditions as described above.

Preparation of Constructs for Expression of Fusion Proteins

For subcellular localization analysis of Ccdc33 a Ccdc33-dsRED fusion protein was generated. A C-terminal part of the coding region of Ccdc33-containing PTS2 motifs was amplified by RT-PCR using mouse Ccdc33-specific primers Ccdc33Red1N1 HindIIIIFP (5’-ATGGTAAAGCTTGCCACCATGGCGAACCA-GTCTTTCTTCTT-3’) and Ccdc33Red1N1KpnIRP (5’-ATGGTACGGTGTTCAACTGCTGAGGTTC-3’) primers amplifying a 222-bp fragment of mouse Hprt1 gene (GeneID: 15452) were used. The alternative splicing of Ccdc33 transcripts was analyzed by primer sets located in different exons of the Ccdc33 gene (Supplementary table 1; for all supplementary material, see www.karger.com/doi/10.1159/000251961) using RT-PCR conditions as described above.

Reverse Transcription PCR Analysis

For reverse transcription (RT)-PCR, DNase-I- (Sigma, Deisenhofen, Germany) treated total RNA isolated from different murine tissues was used. In addition, RNAs were extracted from testes of male mice at different postnatal stages namely: at 5, 10, 15 and 20 days post partum (dpp) and from testes of mouse mutants with disruption of spermatogenesis at different germ cell stages: W/W mutant mice with no germ cells, Ins3-/- mutants with germ cells until the primary spermatocyte stage, oilt/oilt mice with germ cells until the round spermatid stage and ak/qk mutant mice with germ cells until the elongated spermatid stage [Lyon and Searle, 1989; Zimmermann et al., 1999]. Furthermore, RNA was extracted from different immortalized cell lines: MA10 [Leydig cells kindly provided by M. Ascoli, University of Iowa, USA; Ascoli, 1981], 15P-1 [Sertoli cells; Rassoulzadegan et al., 1993] and GC-4 [spermatocytes; Tascou et al., 2000]. After reverse transcription, using the Superscript II reverse transcriptase system and the oligo (dT)12-18 primer (Invitrogen, Karlsruhe, Germany), an aliquot of cDNA (1 μl) was subjected to 35 rounds of PCR amplification with Taq DNA polymerase (Immola; Bioline, Luckenwalde, Germany). For the expression analysis of mouse Ccdc33 gene primers Ccdc2RTFP (5’-GAGGACACCTG-TACGGCATGC-3’) and Ccdc2RTRP (5’-GAATGGGACGGTTC-CTTC-3’) were used, amplifying a PCR product of 409 bp in size. The amplification conditions were 4 min at 94°C followed by 35 cycles of 30 s at 94°C, 45 s at 68°C, and 1 min at 72°C and finally 5 min at 72°C. To test the cDNA quality mHPRT-For-Q: 5’-ACGG CACCA-3’ and mHPRT-Rev-Q: 5’-GGATATGAAGTGGATGCGAGACA-3’ primers amplifying a 222-bp fragment of mouse Hprt1 gene (GeneID: 15452) were used. The alternative splicing of Ccdc33 transcripts was analyzed by primer sets located in different exons of the Ccdc33 gene (Supplementary table 1; for all supplementary material, see www.karger.com/doi/10.1159/000251961) using RT-PCR conditions as described above.

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mutation in the first PTS2 was used as template for the next round of site-directed mutagenesis to introduce the mutation into the second PTS2 sequence. All constructs were verified by sequencing. The cloning of Pxt1 ORF into the pEGFP.C1 vector (Clontech, Heidelberg, Germany) and generation of PXT1-EGFP fusion protein was described previously [Grzmil et al., 2007].

Cell Culture and Cell Transfection

Human cervical adenocarcinoma (HeLa) cells were obtained from the American Type Culture Collection (ATCC) (Rockville, Md., USA) and maintained as described previously [Zimmermann et al., 1998]. HeLa cells (ca. 5 × 10⁵) were plated onto the chamber slides 24 h before transfection. Then, 1 μg (for single transfection) or 2 × 0.5 μg (for co-transfection) of construct(s) DNA was introduced into the cells using the Lipofectamine 2000 transfection reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. Twenty-four hours later, transiently transfected cells were fixed with 4% paraformaldehyde, 0.1% Tween 20 for 15 min at RT, mounted with Vectashield mounting medium with DAPI (Vector, Burlingame, Calif., USA) and observed under a fluorescence microscope (BX-60, Olympus).

Results

Ccdc33 Is Conserved during Evolution and Encodes a Putative Peroxisomal Protein

Mouse Ccdc33 gene (geneID: 382077) is located on chromosome 9, contains 25 exons and spans a genomic segment of 74 kb. Database search revealed that this gene is conserved in human (geneID: 80125, with 78% similarity to the mouse gene), rat (geneID: 315712, 91% similarity to the mouse gene), macaque (geneID: 707960, 72%) and dog (geneID: 478364, 77%). The ClustalW multiple sequence alignment is given in supplementary figure 1.

The computational analysis revealed the presence of a Ca²⁺-dependent membrane-targeting module (C2 domain), 2 ER membrane retention signal-like motifs: NNQK at the C-terminus and GRQK at the N-terminus and 3 coiled-coil domains in mouse CCDC33 (NP_083488). Moreover, 2 peroxisomal targeting signals at positions 484 and 645 in the CCDC33 amino acid sequence were identified. These 2 PTS2 signals suggest that CCDC33 is a peroxisomal protein. Using the protein calculator program the theoretical molecular weight (MW = 82793.0859) and the isoelectric point (pI = 8.23) of mouse CCDC33 were calculated.

Expression of Mouse Ccdc33 Gene

To analyse the expression profile of mouse Ccdc33 gene, total RNA was isolated from different adult mouse tissues and subjected to RT-PCR with Ccdc2RTFP and Ccdc2RTRP primers. The strongest expression was detected in the testis, but weaker expression was observed in brain, ovary and mammary gland (fig. 1A). During postnatal mouse testis development the expression of Ccdc33 was detected first at 15 dpp. No expression could be detected in RNA derived from the testis of W/W* mutants, whereas the expected 409-bp PCR product was observed in Insl3−/−, olt/olt and qk/qk mutants’ testes. Mouse Ccdc33 gene is expressed in germ cell line (GC-4) but not in Leydig (MA-10) or Sertoli (15P-1) cell lines (B). The cDNA quality was proved with housekeeping Hprt1 gene-specific primers (A and B, lower panel).
Ccdc33 transcript was not detectable in testis of a W/W<sup>v</sup> mutant mouse (without germ cells, fig. 1B). To confirm that Ccdc33 is specifically expressed in germ cells, RNA isolated from different immortalized cell lines was used. No expression of Ccdc33 could be observed in Leydig (MA-10) or Sertoli (15P-1) cell lines, whereas in the spermatocyte cell line (GC-4) the expected 409-bp product was amplified (fig. 1B). All RT-PCR products were sequenced to verify the specificity of the reaction. The RNA quality was checked with housekeeping gene-specific primers (Hprt1, fig. 1A, B). Our findings clearly demonstrate that in the testis the Ccdc33 gene is expressed exclusively in germ cells and the expression starts in primary spermatocytes.

Fig. 2. Genomic organization and alternative splicing of mouse Ccdc33 gene. The mouse Ccdc33 gene consists of 25 exons. In addition, exon 8 can be divided into 2 alternative forms (not interrupted by intron sequence) (A). Four alternative splice variants were identified in RT-PCR reaction in different tissues. Two long variants, Ccdc33a and Ccdc33c, each encoded a protein containing all characteristic domains. A shorter variant Ccdc33b was found only in the testis and encoded a C2 domain-only protein. The second short variant Ccdc33d was isolated from mammary gland (B). Northern blot analysis with the cDNA probe containing exons 6 and 7 demonstrated the presence of splice variants Ccdc33a and Ccdc33b only in the testis (C). The RNA quality was confirmed by rehybridization of the membrane with Actb probe. ATG – translation start codon, STOP – translation termination codon. Grey, numbered boxes represent exons and characteristic protein domains encoded by particular transcripts are marked. The probe used for NB is given.
Ccdd33 Undergoes Alternative Splicing

During RT-PCR amplification a shorter fragment of Ccdd33 transcript was also amplified (data not shown), which led us to analyse alternative splicing forms of this gene. The genomic organization of mouse Ccdd33 was obtained from the Ensembl database (www.ensembl.org) and is schematically represented in figure 2A. A series of primers were designed to cover the whole mRNA sequence of this gene (supplementary table 1). Alternative splicing was analyzed using RNA isolated from testis, ovary, brain and mammary gland. Our study revealed that at least 4 different splice products are generated from the mouse Ccdd33 gene (fig. 2B). The Ccdd33a splice variant, containing exons 6 to 21 and 23 to 25 with ATG located in exon 6 and the STOP codon located in exon 25, was amplified in all analyzed tissues. This splice product contains an open reading frame (ORF) encoding all 3 coiled-coil domains, both PTS2 signals, C2 domain and a C-terminal ER membrane retention signal-like motif (C-ER). In a shorter Ccdd33b variant exons 6–8 and an exon 8A (an alternative, longer variant of exon 8 containing a STOP codon) are included. Its ORF contains neither coiled-coil domains nor peroxisomal targeting signal, but only the C2 domain. This variant was amplified only in the testis. The Ccdd33c splice product was detected in brain, ovary and mammary gland, but not in the testis. This variant contains all 3 coiled-coil domains, both PTS2 signals, C2 domain and 2 ER membrane retention signal-like motifs (N-terminal and C-terminal). An additional short splice product, namely Ccdd33d, containing exons 1 to 5 was obtained from a mammary gland cDNA sample. This variant encodes a protein with the N-terminal membrane retention signal. It should be noted that during this analysis we could amplify some additional PCR products representing further putative splice variants of this gene, but these products were very weak and could not be cloned and sequenced. Therefore, we cannot exclude that Ccdd33 produces additional splice products. To confirm obtained results, Northern blot analysis with RNA isolated from testis, ovary, brain and mammary gland was performed. Because the probe used for this study was located in exons 6 and 7 (fig. 2B), the ubiquitously expressed Ccdd33a and the shorter testis-specific Ccdd33b transcripts should be detected. Two bands, 3.0 and 1.0 kb in size, could be observed only in the testis (fig. 2C). The 3.0-kb band represents the Ccdd33a and the 1.0-kb band the Ccdd33b transcript. This result corresponds to our RT-PCR findings and confirmed that Ccdd33 is predominantly expressed in testis. The expression in ovary, brain and mammary gland was very weak and not detectable in Northern blot analysis. To check the RNA quality, the membrane was re-hybridized with beta-actin-specific probe (fig. 2C).

Co-Localization of the CCDC33-dsRED Fusion Protein with a Known Peroxisomal Protein

The PSORTII analysis revealed that mouse CCDC33 protein contains 2 peroxisomal targeting signals type 2 (PTS2), which indicate that CCDC33 can be imported into peroxisomes. To determine the peroxisomal targeting of CCDC33, a co-localization experiment with the CCDC33-dsRED fusion protein and the known peroxisomal protein PXT1 [Grzmil et al., 2007] was performed. The part of Ccdd33 cDNA containing exons 13 to 25 was amplified by PCR and subcloned in pDsRed-Mono-N1 vector. In this fragment both PTS2 signals are present. Finally, CCDC33-dsRED was produced with dsRED fluorescent protein fused to the C-terminus of CCDC33. HeLa cells were co-transfected with the CCDC33-dsRED and PXT1-EGFP fusion vectors and cultured for 24 h. Red signal from CCDC33-dsRED fusion protein was present in the cytoplasm of HeLa cells (fig. 3A), green fluorescence was observed for PXT1-EGFP (fig. 3B), and cell nuclei were counterstained with DAPI (fig. 3C). The overlay of red and green fluorescence clearly demonstrated co-localization of the CCDC33-dsRED fusion protein with PXT1-EGFP in the cytoplasm (fig. 3D).

Functional Analysis of the PTS2 Motifs of the CCDC33 Protein in Mammalian Cells

The co-localization of CCDC33 with PXT1 strongly indicates that PTS2 motifs located in CCDC33 amino acid sequence are responsible for targeting into the peroxisomes. To further prove whether both PTS2 signals are functional, mutations in these motifs were introduced using the CCDC33-dsRED vector as a template. In the first PTS2 at position 484 of the amino acid sequence, the AGGCTG sequence encoding arginine (R) and leucine (L) was mutated into TGGATG sequence encoding tryptophan (W) and methionine (M). In the second PTS2 motif at position 645, the sequence CGGATC encoding arginine (R) and isoleucine (I) was mutated into TGGATG encoding WM. After the first round of site-directed mutagenesis we obtained 2 constructs: m1CCDC33-dsRED with mutation in the first PTS2 and m2CCDC33-dsRED with mutation introduced into the second PTS2. Next the m1CCDC33-dsRED was used as a template for next site-directed mutagenesis to introduce the mutation into the second PTS2 motif and as a result the m1m2CCDC33-dsRED construct was generated. Subsequently, HeLa cells

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were transiently transfected with these different CCDC33-dsRED fusion vectors and analyzed microscopically. The wild-type CCDC33-dsRED fusion protein demonstrated a granular pattern in the cytoplasm of HeLa cells (fig. 4A–C). The m1CCDC33-dsRED mutation did not affect the cellular localization of the CCDC33-dsRED fusion protein (fig. 4D–F). In contrast, the m2CCDC33-dsRED and the m1m2CCDC33-dsRED mutations resulted in a diffused localization of the CCDC33-dsRED fusion protein in cytoplasm of transfected HeLa cells (fig. 4G–L). As a control HeLa cells were transfected with dsRED vector alone, an unspecific overall red fluorescence was present in the cytoplasm as well as in the nucleus of HeLa cells (fig. 4M–O). No red signal was observed in untransfected HeLa cells (fig. 4P–R). In summary, our results obtained from mutation and transfection analyses clearly indicate that the first PTS2 motif is not critical for cellular localization, whereas the second PTS2 is responsible for the correct transport and cellular localization of the CCDC33 protein.

Discussion

In the present study we have demonstrated that mouse Ccdc33 is predominantly expressed in male germ cells and its expression starts at the primary spermatocyte stage. This gene encodes a protein which has a putative peroxisomal targeting signal type 2 (PTS2). We could show that the CCDC33-dsRED fusion protein has punctuate, vesicle-like distribution in the cytoplasm of transiently transfected HeLa cells and when mutations are introduced into the second PTS2, mislocalization of the CCDC33-dsRED fusion protein occurs. Furthermore, the co-localization of CCDC33-dsRED with known peroxisomal protein PXT1 confirmed its peroxisomal localization.

The Ccdc33 gene shows high similarity between mouse, rat, dog, macaque and human. In addition, screening of NCBI and Ensembl databases with mouse cDNA sequence revealed high similarity to: Macaca fascicularis (AB070039.1, 81% similarity), Equus caballus (XM_001494109.2, 79%), Pan troglodytes (XR_021967.1, 81%) and Sus scrofa (AK238150.1, 79%), but no similar sequence was found in Drosophila melanogaster, Caenorhabditis elegans or Saccharomyces cerevisiae. This indicates that Ccdc33 is a mammalian-specific gene. Mouse Ccdc33 is located on chromosome 9 within the syngenic region on human chromosome 15q [Wakana and Imai, 1999]. The human CCDC33 gene is located on 15q24.1, spans a region of 99.8 kb and consists of 20 exons. Genes such as UBL7, SEMA7A, CYP11A1, STRA6, ISLR and ISLR2 are present in this syngenic region in both mouse and human [Pasterkamp et al., 2003; Hsu et al., 2004; Katayama et al., 2005; Kim et al., 2008; Lee et al., 2008]. Interestingly, in mouse the neighbouring gene Cyp11a1 was demonstrated to be important for sex organ development. The XY null mutant of Cyp11a1 reveals sex reversal with external female genitalia but internal small testis and epididymis. Spermatogenesis was arrested at the spermatocyte stage [Hu et al., 2002]. It might be possible that this region contains genes controlling male fertility.

In mouse Ccdc33 gene, the flanking sequence of the start codon (ATG) located in exon 1 represents the typical Kozak consensus, the sequence flanking the second ATG, located in exon 6, represents the rare variant of Kozak consensus, but still consists of all prerequisites for a functional translation start codon [Kozak, 1999].

We have amplified and cloned 4 splice products resulting from the alternative splicing of mouse Ccdc33. Interestingly, also for human CCDC33 4 splice products are reported. Two longer transcripts (ENST00000398814...
and ENST00000321288) contain the entire ORF, whereas 2 shorter transcripts (ENST00000321374 and ENST00000268082) represent only the 5′ region of human CCDC33. This structure is very similar to the mouse splice products. Mouse testis-specific Ccdc33b variant contains only the C2 domain, which was reported to be important for control of spermatogenesis [Linares et al., 2000; Irino et al., 2005; Smith and Wakimoto, 2007]. Four different splice variants in the testis were identified for misfire (MFR) protein of Drosophila melanogaster which contains a C2 domain. The mutation in the Mfr gene resulted in a sperm activation defect [Smith and Wakimoto, 2007]. The Rgs3 gene encoding a protein with the C2 domain was suggested to be involved in control of signalling.

Fig. 4. Subcellular localization of CCDC33-dsRED fusion proteins. HeLa cells were transiently transfected with CCDC33-dsRED constructs and analyzed using fluorescent microscopy. Red signals representing the cellular localization of the CCDC33-dsRED fusion protein demonstrated characteristic punctual pattern (A–C). Next, different mutations were introduced in both PTS2 signals of the CCDC33 protein and HeLa cells were transfected with these modified constructs. The mutation of the first PTS2 motif (m1) did not influence the subcellular localization of the CCDC33-dsRED fusion protein (D–F), whereas the mutation in second (m2) and both PTS2 motifs (m1m2) resulted in diffuse localization of the mutated CCDC33-dsRED fusion protein in the cytoplasm (G–L). As a control, HeLa cells were transfected with the empty dsRED vector and an unspecific localization in the cytoplasm and in the nucleus could be observed (M–O). No red signals were detected in untransfected cells (P–R).
pathways during spermatogenesis. The expression of mouse RGS3 protein in adult animals was found to be restricted to spermatocytes [Linares et al., 2000]. The C2 domain of mouse PLCδ4 protein is responsible for binding with the PDZ6 or PDZ7 domain of GRIP1 and this association plays a role in spermatogenesis [Irino et al., 2005]. Thus, the short Ccdc33b variant might be important for male gametogenesis.

Human CCDC33 was reported as a cancer/testis gene [Chen et al., 2005; Stevenson et al., 2007]. Such genes are normally expressed only in germ cells, but can be also activated in cancer state in different cells [Scanlan et al., 2002, 2004]. Our Northern blot analysis demonstrated a strong testicular expression of mouse Ccde33; however, it should be noted that weak expression detectable only by using the more sensitive RT-PCR technique was also observed in other tissues. This is in agreement with the data from UniGene database (http://www.ncbi.nlm.nih.gov/UniGene) where different cDNA clones representing mouse Ccde33 from various tissues are deposited. Besides the testis, Ccde33 cDNAs were also isolated from mammary gland, brain, embryonic tissue, nasopharynx and olfactory mucosa. According to UniGene the human CCDC33 is also predominantly expressed in the testis, but weak expression is also demonstrated in uterus, trachea, placenta, lung and brain. Moreover, human CCDC33 cDNA clones were also isolated from uterine tumor, lung tumor and germ cell tumor [UniGene; Chen et al., 2005], thus confirming that CCDC33 belongs to CT genes.

The CCDC33 protein contains 2 peroxisomal targeting signals type 2 (PTS2). It is known that the PTS2 motif is recognized by the PEX7 receptor and directs peroxisomal localization [Marziuch et al., 1994]. The mutation changing conserved arginine/leucine (RL) resulted in diffused localization of the CCDC33-dsRED fusion protein. Moreover, CCDC33-dsRED co-localized with a known peroxisomal protein. These results clearly indicate that the second PTS2 sequence is functional and required for the correct localization of CCDC33 to the peroxisomes. During postnatal testis development in mouse, the first pachytene spermatocytes are observed at day 14 or 15 [Silver, 1995]. Expression of mouse Ccde33 starts at 15 dpp and is restricted to germ cells. To date, only one germ cell-specific peroxisomal protein has been reported, namely PXT1. Interestingly, the expression of mouse Pxt1 also starts at the pachytene spermatocyte stage [Grzmil et al., 2007].

Evidence from the knockout mouse models clearly demonstrated that functional peroxisomes are necessary for spermatogenesis. The multifunctional protein 2 (MFP-2)-deficient males have reduced testis size, atrophy of the seminiferous tubules and absence of germ cells [Baes et al., 2000]. Adult males with the targeted disruption of the Pex7 gene demonstrated, besides other phenotypic malformations, infertility and testicular atrophy [Brites et al., 2003]. In another mouse model it was clearly shown that inactivation of peroxisomal enzymes for plasmalogen synthesis leads to infertility due to an arrest in spermatogenesis [Rodemer et al., 2003]. Furthermore, in double knockout mice with null mutations of Pex7 and Abcd1 genes massive germ cell degeneration was observed. Spermatogenesis was disrupted at the pachytene spermatocyte stage and an increased level of apoptosis was noticed [Brites et al., 2009]. The proper function of peroxisomes is obviously crucial for normal spermatogenesis; however, strong heterogeneity of peroxisomal protein content in male germ cells at different spermatogenesis stages was demonstrated [Nenicu et al., 2007]. The abundance and localization of peroxisomes depend on the maturation stage of differentiating germ cells. In addition, the important role of peroxisomes can be underlined by the existence of inherited human peroxisomal disorders, which include the Zellweger syndrome [Gould and Valle, 2000], the infantile Refsum disease (infantile phytanic acid storage disease) [Wanders et al., 1990], classic rhizomelic chondrodysplasia punctata [Braverman et al., 1997; Motley et al., 1997; Purdue et al., 1997] and adrenoleukodystrophy [Libber et al., 1986; Mosser et al., 1993; Brennemann et al., 1997]. Mutations in at least 11 different genes were identified as a cause of peroxisomal biogenesis disorder (PBDs) [Aubourg et al., 1993; Shimozawa et al., 1993], but for several PBD cases genetic causes are still unclear. In this term the identification and characterization of peroxisomal genes expressed in male germ cells seems to be a very important task.

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