
On the Functional Analysis of Murine Peroxisomal Testis Specific 1 (*Pxt1*) Gene



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Karina Paulina Kaczmarek

aus Koło, Polen

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Referent: Prof. Dr. med. Dr. h. c. Wolfgang Engel

Korreferent: Prof. Dr. Sigrid Hoyer-Fender

Tag der mündlichen Prüfung:

*Dedicated to my family,
whose love, support and faith in me were ever present and always appreciated.*

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1. Zusammenfassung

Die Spermatogenese von Säugern ist ein komplexer biologischer Prozess bei dem diploide Spermatogonien in spezialisierte haploide Spermatozoen differenzieren. Die Regulation der männlichen Gametogenese ist verknüpft mit einem hoch organisierten Gen-Netzwerk, welches oft Keimzell-spezifische und/oder Stadien-spezifische Expression zeigt. Kürzlich konnte gezeigt werden, dass Peroxisomen eine essentielle Rolle während der Spermatogenese spielen. Über das murine „*peroxisomal testis specific 1*“ (*Pxt1*) Gen wurde erstmals 2007 berichtet (Grzmil et al., 2007). Zunächst wurde *Pxt1* als ein neues, männliches Keimzell-spezifisches Gen beschrieben, welches für ein peroxisomales Protein codiert. In der vorliegenden Arbeit konnten neue Kenntnisse über die physiologischen Funktionen des PXT1 Proteins in der Spermatogenese, und damit auch über Peroxisomen, erhalten werden.

Im ersten Teil dieser Arbeit wurde ein Interaktionspartner von PXT1, CCDC33, charakterisiert. Die testikuläre Expression des *Ccdc33* Gens ist, ähnlich wie *Pxt1*, auf männliche Keimzellen beschränkt und beginnt in Pachytän-Spermatozyten. Weiterhin konnte gezeigt werden, dass es mindestens vier verschiedene Splicing-Transkripte des *Ccdc33* Gens gibt. Basierend auf *in silico* Analysen wurden für das CCDC33 Protein zwei putative peroxisomale Zielsignale des Typs 2 (PTS2) vorausgesagt, von denen in dieser Arbeit eines der beiden PTS2 als funktionell charakterisiert werden konnte. Es ist verantwortlich für die Einsortierung des CCDC33-dsRED Fusionsproteins in die Peroxisomen. Die Kolo-kalisierung von PXT1 und CCDC33 in Vesikel-ähnlichen Strukturen bestätigt die peroxisomale Lokalisierung von CCDC33.

Im zweiten Teil dieser Arbeit wurde über die Generierung und die Analyse von transgenen Mäusen berichtet, die PXT1 im Testis überexprimieren. Es konnte gezeigt werden, dass die Infertilität der transgenen Männchen auf eine ausgeprägte Apoptose der Pachytän-Spermatozyten zurück zuführen ist. Ausserdem konnte eine BH3-ähnliche Domäne in der PXT1 Proteinsequenz identifiziert werden, die charakteristisch für proapoptotische BCL-2 Familien-Mitglieder ist. Mit Hilfe von Deletionsanalysen konnte die Funktionalität dieser Domäne in transient transfizierten HeLa und NIH3T3 Zellen bestätigt werden. Zusätzlich konnte eine Interaktion von PXT1 mit dem Apoptose-Regulator BAT3 gezeigt und analysiert werden. In den Säugerzellen konnte die

Überexpression von BAT3 die PXT1-induzierte Apoptose unterdrücken und führte zu einer Translokation von PXT1 aus dem Cytosol in den Nukleus.

Ausserdem konnte die PXT1-CCDC33 Interaktion mit verschiedenen molekularen Techniken bestätigt werden. Darüber hinaus konnte in zwei PXT1 transgenen Linien gezeigt werden, dass die endogene 3'UTR von *Pxt1* nicht in die verzögerte mRNA Translation involviert ist. Vorläufige Experimente zeigten, dass die durch PXT1 Überexpression induzierte Apoptose im Testis durch einen Caspase 9 unabhängigen Signalweg vermittelt wird, möglicherweise durch einen extrinsischen Signalweg.

Zusammenfassend liefern die hier erhobenen Daten interessante Einsichten in die Funktion von PXT1 und Peroxisomen im Testis. Dennoch sind weitere Studien, wie zum Beispiel die Analyse von PXT1 knock-out Mäusen, notwendig, um herauszufinden, ob *Pxt1* essentiell für die männliche Fertilität ist. Mithilfe der knock-out Mäuse können auch Erkenntnisse über den molekularen Mechanismus der PXT1-induzierten Apoptose gewonnen werden.

1. Summary

Mammalian spermatogenesis is a complex biological process by which diploid spermatogonia differentiate into specialized haploid spermatozoa. Regulation of male gametogenesis involves a highly organized network of genes, which often show germ cell-specific and/or stage-specific expression. Recently, peroxisomes have been found to play an essential role in spermatogenesis. The identification of the mouse peroxisomal testis specific 1 (*Pxt1*) gene was first reported in 2007 (Grzmil et al., 2007). Initially, *Pxt1* was described as a novel, male germ cell-specific gene that encodes a peroxisomal protein. In the present study, insights into the physiological function of PXT1 protein, and thus peroxisomes, in male germ cells are provided.

In the first part of the thesis, an interaction partner of PXT1, namely CCDC33, is characterized. We showed that testicular expression of the *Ccdc33* gene, similar to the *Pxt1*, is restricted to male germ cells and starts at the pachytene spermatocyte stage. Furthermore, we found that the *Ccdc33* undergoes alternative splicing, giving rise to at least four different transcripts. Based on *in silico* analysis, the CCDC33 protein is predicted to contain two putative peroxisomal targeting signals type 2 (PTS2) and in further study we demonstrated that one of these two PTS2 motifs is functional and responsible for sorting of CCDC33-dsRED fusion protein to peroxisomes. The co-localization of PXT1 and CCDC33 within vesicle-like structures finally confirmed the peroxisomal localization of CCDC33.

In the second part of the thesis, the generation and the analysis of transgenic mice overexpressing PXT1 in the testis are described. The infertility of transgenic males due to wide-spread apoptosis of pachytene spermatocytes was demonstrated. In addition, we identified a BH3-like domain, characteristic for pro-apoptotic BCL-2 family members, in the PXT1 protein sequence. By deletion analysis we validated the functionality of the BH3-like domain in transiently transfected HeLa and NIH3T3 cell lines. Furthermore, the interaction of PXT1 with the apoptotic regulator BAT3 was demonstrated and characterized. We found that in transiently transfected mammalian cells, the overexpression of BAT3 could suppress PXT1-induced apoptosis and led to translocation of PXT1 from the cytosol to the nucleus.

In addition, PXT1-CCDC33 interaction was validated by different molecular techniques. Moreover, using two PXT1 transgenic mouse lines, we could demonstrate that endogenous 3'UTR of *Pxt1* is not involved in the delay of mRNAs translation. Preliminary experiments showed also that apoptosis induced by overexpression of PXT1 in the testes is mediated via caspase 9 independent pathway, thereby indicating that it could be triggered via the extrinsic apoptotic pathway.

In conclusion, the data presented here provide interesting insights into the function of PXT1 and peroxisomes in the testis, however, further studies, including analysis of PXT1 knock-out mouse model, will be necessary to determine whether *Pxt1* is essential for male fertility. From PXT1 knock-out mice we will also learn about the molecular mechanisms underlying PXT1-induced apoptosis.

2. Introduction

2.1. Spermatogenesis, overview

Mammalian spermatogenesis is an elaborate differentiation process in which, through a series of events, diploid spermatogonia become differentiated into highly specialized haploid spermatozoa. The developmental process occurs continuously within the seminiferous tubules of the testes during the reproductive lifetime of the individual and requires 64 days in human and 35 days in mouse (Chubb, 1993). Spermatogenesis can be divided into three functional phases, each involving a different class of germ cells. The initial phase is a proliferative phase, during which spermatogonial stem cells (SSCs) undergo mitosis to produce two types of cells: additional SSCs and differentiating spermatogonia, both of which are located along the base of the seminiferous epithelium. The latter class of cells undergoes rapid and successive mitotic divisions to finally form preleptotene spermatocytes. Mouse spermatogonia can be classified into three types: stem cell ($A_{is/s}$, or $A_{isolated/single}$), proliferative (A_{pr} , or A_{paired} and A_{al} , or $A_{aligned}$) and differentiating [A_1 , A_2 , A_3 , A_4 , In (intermediate), and B] spermatogonia (Russell et al., 1990; Sinha Hikim et al., 2005). A_{is} spermatogonia are single cells that either renew their own population or produce A_{pr} spermatogonia predestined to differentiate. The A_{pr} spermatogonia divide synchronously to form chains of interconnected A_{al} spermatogonia that also proliferate. Subsequently, almost all A_{al} spermatogonia, a few A_{pr} and very few A_{is} cells differentiate into A_1 spermatogonia. The type of A_1 cells divide to give rise to more differentiating (A_2 , A_3 , A_4 , In, B) cells (de Rooij, 2001). At the end of this process, the most mature spermatogonia (specifically type B) proliferate to form the young primary spermatocytes (preleptotene spermatocytes), which migrate upwards from the base of the seminiferous tubule and cross through the Sertoli-Sertoli junctions (blood-testis barrier) (Hess, 1998). In the next phase of spermatogenesis, spermatocytes continue to mature as they go through the subphases of the lengthy meiotic prophase and then they carry out two meiotic divisions to produce haploid cells. In the first meiotic division secondary spermatocytes are formed, which in the second division become round spermatids. The process of meiosis involves among others, DNA synthesis at the preleptotene, synaptonemal complex formation between paired homologous chromosomes at the zygotene and the pachytene, occurrence of genetic recombination through the crossing-over at the pachytene and the

beginning of chromosomal desynapsis at the diplotene stage. The preparation of pachytene spermatocytes to the next phase of spermatogenesis is manifested by an increase in RNA and protein synthesis (Hess, 1998; Sinha Hikim et al., 2005). The final phase of spermatogenesis, called spermiogenesis or differentiation phase, refers to a complex morphogenesis through which round spermatids transform into elongated spermatids and eventually spermatozoa. This is one of the most exciting phases during which cells undergo a dramatic metamorphosis which is necessary for sperm function. Nucleus as well as cytoplasm of round spermatids undergoes coordinated and integrated elongation which composes adaptation to swimming. The elongation process is always accompanied by protamine-mediated DNA compaction, tail formation and growth, development of an acrosome and cytoplasm elimination (Russell et al., 1990; Yan, 2009). Finally, spermiation takes place and mature spermatozoa are released from the Sertoli cells into the lumen of the tubuli seminiferi (Cooke and Saunders, 2002).

Intercellular associations between the germ cells and somatic components (Sertoli cells, Leydig cells and peritubular myoid cells) of the testis are crucial for normal spermatogenesis and contribute to its complexity (Cooke and Saunders, 2002). The cell-cell interactions in the testis may have environmental, nutritional and/or regulatory form. Sertoli cells provide not only physical support for germ cells, but also via the secretion of different regulatory molecules such as growth factors and proteases take part in their biochemical stimulation. Leydig and Sertoli cells are not in direct contact thus essential communication between those cells has androgen-mediated regulatory character. Leydig cells, under the influence of the pituitary-secreted luteinizing hormone (LH), synthesize testosterone, which together with the follicle-stimulating hormone (FSH) acts on Sertoli cells and stimulates gene transcription and the secretion of peptides that promote germ-cell differentiation (Skinner et al., 1991; Griswold, 1998; Kimmins et al., 2004).

The complexity of mammalian spermatogenesis requires a highly organized network of genes, including those with unique expression patterns. Many of genes involved in precise regulation of male gametogenesis have germ cell-specific and/or stage-specific expression (Eddy, 2002). The importance of such factors for proper spermatogenesis was demonstrated in genetically modified male mice. Infertile single CREM, HSP70-2 or TLF knockout mice are such examples. CREM (cyclic AMP-responsive element modulator) is a transcriptional regulator, which is highly expressed in spermatids. Developing spermatids of CREM null mice fail to differentiate into sperm and undergo apoptosis (Blendy et al.,

1996; Nantel et al., 1996). HSP70-2 (heat shock protein 70-2) is a unique 70 kDa male germ cell-specific chaperone for proteins involved in meiosis. Deficiency of HSP70-2 in mice leads to arrest of germ cell development in prophase of meiosis I and apoptotic death of late pachytene spermatocytes (Dix et al., 1997). TLF (also known as TRF2; a homologue of the TATA-binding protein) is strongly and dynamically expressed during spermatogenesis in three types of mouse germ cells: late pachytene spermatocytes, round spermatids and elongated spermatids. Histological analysis of TLF deficient mice testes revealed the presence of many multinucleated giant cells and massive apoptosis of round spermatids. Moreover, in TLF null mice expression of several germ cell-specific genes, which are necessary to initiate the differentiation of round spermatids, is almost completely abolished (Martianov et al., 2001; Martianov et al., 2002).

Unlike most somatic tissues, where transcription and translation are closely linked, both male and female germ cells rely heavily on post-transcriptional regulatory mechanisms. Although many proteins, particularly those which are required for specialized function of spermatozoa, are synthesized during spermiogenesis, the latter part of spermatogenesis proceeds without any transcriptional activity (Schafer et al., 1995; Iguchi et al., 2006). Such temporal disconnection between mRNA transcription and protein synthesis, also known as translational delay, is a consequence of the nuclear condensation in the germ cells. For this reason many of the proteins of spermatozoa must be either synthesized before mid-spermiogenesis or their transcripts must be stored as messenger ribonucleoproteins (mRNPs) and later translationally activated. *Cis*-acting sequences mediating interactions with many RNA binding proteins, and thus controlling translation initiation, were identified both in the 5' as well as in the 3' untranslated regions (UTRs) of many testis specific mRNAs e.g. *Pgk2*, *Tnp1*, *Tnp2*, *Prm1*, *Prm2*, *Acr*, *Smpc* (Gold et al., 1983; Kleene et al., 1984; Braun et al., 1989; Yelick et al., 1989; Kwon and Hecht, 1991; Nayernia et al., 1992; Kwon and Hecht, 1993; Nayernia et al., 1994; Schafer et al., 1995; Nayernia et al., 1996; Fajardo et al., 1997; Hawthorne et al., 2006; Iguchi et al., 2006; Tseden et al., 2007; Xu and Hecht, 2007; Kleene and Bagarova, 2008). *Pgk2* (phosphoglycerate kinase 2) is an example of this large class of genes that undergo translational regulation in the male germ line. The *Pgk2* mRNAs are transcribed and then sequestered as mRNPs in pachytene spermatocytes, whereas protein can be first detected in round spermatids (Gold et al., 1983). The *Smpc* (sperm mitochondria-associated cysteine-rich protein) and *Prm1* (protamine 1) mRNA are translationally repressed in round

spermatids and translationally active in elongated spermatids (Fajardo et al., 1997; Hawthorne et al., 2006). The essentiality of translational control for normal spermatogenesis has been supported by analyses of the transgenic animals expressing *Prm1* or *Tnp2* genes devoid of their 3'UTRs. Lack of 3'UTR-located regulatory sequences resulted in premature translation and consequently in dominant sterility of transgenic males (Lee et al., 1995; Tseden et al., 2007). It is also believed that such timed translation can be a matter of great importance for other genes (Schafer et al., 1995).

2.2. *Pxt1* as a novel male germ cell-specific gene

The mouse *Pxt1* (peroxisomal testis specific 1) cDNA was first identified among genes differentially expressed between the male germ cells and male germ cell/Sertoli cell co-cultures (Kraszucka et al., 1999; Grzmil et al., 2007). High nucleotide and protein sequence conservation as well as similar gene structures in murine and human *Pxt1/PXT1* strongly suggested its important physiological function in male gametogenesis (Table 2.1.).

Table 2.1. The comparison of mouse and human *Pxt1/PXT1* using NCBI, Ensembl databases and ExPASy program. A significant similarity between the mouse and the human *Pxt1* can be found at the nucleotide as well as at the protein level. Both, the mouse and the human *Pxt1/PXT1* contain long 3'UTR sequences suggesting their regulation by translational delay during spermatogenesis. ORF: open reading frame; Mw: molecular weight; PTS1: peroxisomal targeting signal type 1; NHL: asparagine-histidine-leucine.

Feature	<i>Pxt1 Mus musculus</i>	<i>PXT1 Homo sapiens</i>
Gene ID	69307	222659
Locus	17A3.3	6p21.31
Genomic DNA size (bp)	8277	9984
Genomic organization (exon/intron)	3/2	2/1
cDNA length (bp)	1015	1405
5' UTR length (bp)	143	30
3' UTR length (bp)	716	1219
ORF length (bp)	156	156
Protein length (aa)	51	51
Theoretical protein Mw (kDa)	5.93	6.39
PTS1 aa sequence	NHL	NHL
cDNA sequence identity (%)	76	
Protein sequence identity (%)	60	

Expression analysis in mouse has revealed that *Pxt1* mRNA is expressed exclusively in germ cells of the testis. *Pxt1* transcription is first seen in pachytene spermatocytes at day 15 *post partum* (dpp), with higher mRNA levels at more advanced stages of spermatogenesis. *In situ* hybridization experiments have demonstrated the presence of *Pxt1* transcripts within the cytoplasm of primary spermatocytes (Grzmil et al., 2007). Interestingly, both murine and human *Pxt1/PXT1* genes contain long 3'UTR sequences (Table 2.1.), suggesting their regulatory function for translational repression/activation during male gametogenesis. However, to date no information is available on the PXT1 protein expression pattern or its role in spermatogenesis. Grzmil et al. (2007) have shown that murine PXT1 protein contains a functional peroxisomal targeting signal type 1 (PTS1). This motif has been found at the C-terminus of PXT1 and resembles a consensus tripeptide Asn-His-Leu (NHL). The authors have confirmed peroxisomal localization of PXT1 by demonstrating characteristic granular distribution of EGFP-PXT1 fusion protein within the cytoplasm of NIH3T3 cells and co-localization of EGFP-PXT1 with three known peroxisomal markers (CAT, ACOX1 and PMP70). *Pxt1* is, to date, the only known male germ cell-specific gene which encodes a peroxisomal protein.

2.3. Peroxisomes as essential organelles for spermatogenesis

Peroxisomes, originally called microbodies, are 0,1-1 μm (in diameter) cellular organelles, which have a single-limiting membrane surrounding a fine granular matrix and, occasionally, a paracrystalline core. These mysterious organelles are highly dynamic and their size, number, protein composition and biochemical function vary depending on the organism, cell type and/or environmental conditions. There are at least 32 peroxins, proteins encoded by the *Pex* genes, that are involved in the control of peroxisome assembly, division and inheritance (Titorenko and Rachubinski, 2001a; Schrader and Fahimi, 2008). Recently, it has been suggested that peroxisomes have an endoplasmic reticulum (ER) origin in evolutionary history (Gabaldon et al., 2006; Schluter et al., 2006). There is now firm evidence for the existence of two pathways of peroxisomes biogenesis: growth and fission of pre-existing peroxisomes and *de novo* formation from the ER (Hoepfner et al., 2005; Smith and Aitchison, 2009). In contrast to mitochondria, peroxisomes are devoid of DNA and autonomous protein translation machinery. Accordingly, all peroxisomal proteins are encoded by nuclear genes, synthesized on free

polysomes in the cytoplasm and imported to the organelles post-translationally (Schrader and Fahimi, 2008).

Sorting of proteins into peroxisomes occurs via peroxisomal targeting signals (PTS). Basically, there are two types of PTS signals: C-terminal PTS1 and N-terminal PTS2. The majority of the peroxisomal proteins contain PTS1, which resembles a tripeptide consensus: S/T/A/G/C/N–K/R/H–L/I/V/M/A/F/Y (Mohan et al., 2002). The consensus sequence of PTS2 motif corresponds to (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). Peroxisomal import is mediated by either cytosolic or membrane-associated receptors, which interact with the PTS signals of their cargo proteins. PTS1-containing proteins are recognized by and bind to PEX5 and PTS2-containing proteins to the PEX7 receptor. Cargo-loaded PEX5 or PEX7 migrate to the cytosolic surface of the peroxisomal membrane where they interact with a docking complex to trigger the cascade of peroxin-mediated events that ultimately leads to the entrance of cargo proteins into organelle matrix and the recycling of the receptors to the cytosol (Titorenko and Rachubinski, 2001a; Platta et al., 2008). The complexity of this unique machinery is shown for PTS1-containing peroxisomal matrix proteins in figure 2.2.

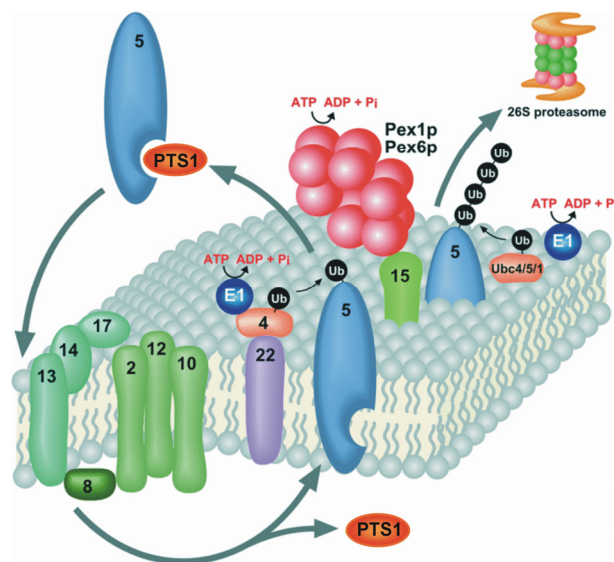


Figure 2.2. The schematic representation of peroxisomal matrix proteins import. Newly synthesized PTS1-containing proteins are recognized in the cytosol by PEX5 receptor (5) that targets them to a docking complex [PEX13 (13), PEX14 (14), PEX17 (17)] at the peroxisomal membrane. Cargo-bound receptor traverses the membrane and PTS1-harboring proteins, probably with the help of PEX8 (8), are released into peroxisomal matrix. Retrotranslocation of the membrane-integrated PEX5 to the cytosol requires ATP

hydrolysis, most likely by the PEX15 (15)-anchored AAA (ATPase associated with various cellular activities) complex consisting of PEX1 (Pex1p) and PEX6 (Pex6p). The recycling of the PEX5 is driven by the PEX22 (22)-anchored PEX4 (4) mediated monoubiquitination. In case of defect in receptor recycling machinery, PEX5 (5) is polyubiquitinated by Ubc4p, Ubc5p and Ubc1p and subsequently degraded by the proteasome. ATP-dependent activation of Ub (ubiquitin) by E1 (ubiquitin-activating enzyme) plays a role in recycling as well as proteosomal disposal of receptor. Moreover, both pathways possibly require the RING (really interesting new gene) peroxins PEX2 (2), PEX10 (10) and PEX12 (12) as putative E3 (ubiquitin-protein ligases) enzymes (modified and adapted from Platta et al., 2008).

Peroxisomes are indispensable for development, morphogenesis and differentiation as they play a role of paramount importance in various metabolic processes. The fundamental functions of these organelles include decomposition of hydrogen peroxide by catalase and fatty acid metabolism. Peroxisomes are involved in: β -oxidation of fatty acids, mostly very-long-chain fatty acids (VLCFAs); lipid biosynthesis (ether phospholipids/plasmalogens, bile acids, cholesterol and dolichol); degradation of amino acids and purines; glyoxylate, dicarboxylate and retinoid metabolism; glycerol synthesis and others. They also participate in the synthesis of poly-unsaturated fatty acids, which are implicated in signaling processes and apoptosis (Schrader and Fahimi, 2008; Luers et al., 2009). Considering this wide range of functions, it is not surprising that peroxisomes are ubiquitous in eukaryotic cells. Just two exceptions to this phenomenon have been found: mature erythrocytes and mature spermatozoa, where peroxisomes seem to disappear during the developmental process (Luers et al., 2006; Nenicu et al., 2007). However, for a long time it was believed that within the testis peroxisomes are restricted to somatic cells. Initially, they were described in the interstitial cells of Leydig (Fawcett and Burgos, 1960; Reddy and Svoboda, 1972; Zini and Schlegel, 1996; Baumgart et al., 1997; Risse et al., 2001) and later in Sertoli TM4 cell line (Lester et al., 1996). In 2000, Figueroa et al. analyzed presence of peroxisomes in rat germ cells. They were able to identify these organelles in the oocyte, but not in spermatozoa. Interestingly, catalase, a peroxisomal protein marker, could be detected within the cytoplasm over the sperm head (particularly over the acrosome region) and tail, but not within microbody-like structures. The first report about existence of peroxisomes in male germ cells was given in 2003 (Luers et al., 2003). The authors demonstrated peroxisomal compartment in immortalized germ cell line GC1spg, which represents the differentiation state of type B spermatogonia or preleptotene spermatocytes (Hofmann et al., 1992). Three years later, evidence appeared for the presence of small peroxisomes in spermatogonia of germinal epithelium in mouse testis (Luers et al., 2006; Huyghe et al., 2006). Furthermore, Luers et al. (2006) suggested that

peroxisomes disappear during the course of germ cell differentiation. In 2007, two groups provided evidence which was contrary to this hypothesis. At first, Grzmil et al. (2007) discovered peroxisomal germ cell-specific *Pxt1* gene expression starting at the spermatocyte stage of spermatogenesis. Subsequently, Nenicu et al. (2007) confirmed the presence of these organelles in most cell types in the human and mouse testis, with the exception of mature spermatozoa. Moreover, the latter group demonstrated marked differences in structure and abundance of these organelles in male germ cells depending on their maturation. Investigators observed that during spermatid maturation, peroxisomes aggregate to large clusters, which consequently leads to a decrease in their total number. During spermatozoa formation large peroxisomal aggregates in residual bodies were demonstrated.

Interestingly, the highest relative expression level of peroxisome-related genes (*PEX1*, *PEX5*, *PEX7*, *PEX10*, *ABCD1*, *ABCD3*, *GNPAT*, *AGPS*, *PXT1*) appeared in the testis rather than in any other organ examined. This observation has suggested that peroxisomes have important functions within the testis (Luers et al., 2009).

The vital importance of peroxisomes in humans is stressed by the existence of an expanding group of genetic diseases in which there is an impairment in one or more of the peroxisomal functions. Based on organelle structure and deficiencies, peroxisomal disorders have been classified into two groups (Table 2.3.). The first group includes peroxisome biogenesis disorders (PBDs), in which the organelle is abnormally formed and lacks several functions. From among at least 32 peroxins regulating peroxisomal biogenesis, mutations in 11 of them cause lethal PBDs in humans. The second group includes peroxisomal single-enzyme/transporter disorders, which result from a deficiency in a single peroxisomal protein, which is not peroxin, therefore they affect a single metabolic pathway and the structure of the peroxisomes is intact (Wanders, 1999; Titorenko and Rachubinski, 2001a; Fidaleo, 2009).

Table 2.3. The classification of peroxisomal disorders: peroxisome biogenesis defects versus single enzyme/transporter deficiencies (modified and adapted from: Schrader and Fahimi, 2008; Fidaleo, 2009).

Peroxisome biogenesis disorders (PBDs)		
Peroxisomal disorders	1. Zellweger syndrome (ZS)	
	2. Infantile type of Refsum disease (IRD)	
	3. Rhizomelic chondrodysplasia punctata type 1 (RCDP type 1)	
	4. Neonatal type adrenoleukodystrophy (NALD)	
	5. Hyperpipecolic acidemia	
	Single enzyme/transporter deficiencies	
	1. Acatalasaemia	
	2. Adult-type Refsum disease	
	3. X-linked adrenoleukodystrophy (X-ALD)/adrenomyeloneuropathy (AMN)	
	4. Rhizomelic chondrodysplasia punctata type 2 or 3 (RCDP type 2/3)	
	5. Pseudo-neonatal ALD (ACOX1 deficiency)	
	6. MFP-2 protein deficiency	
	7. Peroxisomal thiolase deficiency	
8. Glutaric aciduria type 3 (glutaryl-CoA oxidase deficiency)		
9. Mevalonate kinase deficiency		
10. Hyperoxaluria type 1 (alanine glyoxylate aminotransferase deficiency)		
11. Adult-onset sensory motor neuropathy (2-methylacyl-CoA racemase deficiency)		
12. Sterol carrier protein X deficiency		
13. Contiguous ABCD1/DX1357E deletion syndrome		

The existence of infertile patients who suffer from peroxisomal single-enzyme deficiency, like X-linked adrenoleukodystrophy (X-ALD) or adrenomyeloneuropathy (AMN), supports the opinion about essentiality of peroxisomes for normal spermatogenesis. The testicular dysfunction has been noticed in about 50 % of adult males suffering from X-ALD/AMN (Libber et al., 1986; Brennemann et al., 1997). Due to a mutation in peroxisomal membrane ABCD1 (ATP-binding cassette, sub-family D (ALD), member 1) protein, which is involved in the transport of VLCFAs into the peroxisomal matrix, the patients exhibit accumulation of VLCFAs in the cytoplasm of different tissues. Histological analysis of the testis from X-ALD and AMN patients demonstrated among others, hypocellularity in the seminiferous tubules, spermatogenesis arrest at different stages or even the Sertoli/spermatogonia cell only phenotype. On the other hand, severe forms of PBDs lead to cryptorchidism, suggesting involvement of peroxisomes in the development of the testis (Luers et al., 2009).

Considering these, further characterization of the germ cell-specific *Pxt1* gene would help to understand the physiological role of peroxisomes in spermatogenesis. In order to gain more insights into the molecular function of PXT1 protein, the current study is aimed

at determination and characterization of PXT1 interacting partners, generation of transgenic mouse models for evaluating the effects of *Pxt1* overexpression in the testis and verification of *Pxt1* post-transcriptional regulation as well as analysis of *Pxt1* overexpression effect in different mammalian cell lines.

3. Results

- 3.1. *Ccdc33* a predominantly testis expressed gene encodes a putative peroxisomal protein
- 3.2. Overexpression of peroxisomal testis specific 1 protein induces germ cell apoptosis and leads to infertility in male mice

Each chapter within the results starts with a brief description of the aim of the particular manuscript in context of the complete thesis, the status of the manuscript, and the author's contribution to the work.

3.1. *Ccdc33* a predominantly testis expressed gene encodes a putative peroxisomal protein

CCDC33 has been identified as a novel interaction partner of PXT1. In this part of the thesis, the characterization of the previously unknown *Ccdc33* gene is described. We have demonstrated that murine *Ccdc33* gene is predominantly expressed in the testis. Further, we have found that within testis its expression is restricted to male germ cells and starts during meiotic prophase, in pachytene spermatocytes. Moreover, RT-PCR-based assay has revealed that at least four transcripts are generated by alternative RNA splicing from *Ccdc33* gene. Interestingly, *in silico* analysis has predicted two putative peroxisomal targeting signals type 2 (PTS2) in CCDC33 protein sequence. Using site-directed mutagenesis approach we have confirmed functionality of one of the PTS2. Finally, punctate co-localization of CCDC33-dsRED with EGFP-PXT1 within cytoplasm of HeLa cells has validated peroxisomal distribution of CCDC33.

Karina Kaczmarek, Ewa Niedzialkowska, Maja Studencka, Yvonne Schulz, Pawel Grzmil

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1. **Karina Kaczmarek:** Identification of *Ccdc33* transcript. Involved in analysis of *Ccdc33* alternative splicing. Generation of CCDC33-dsRED, EGFP-PXT1 expressing vectors and analysis of the fusion proteins co-localization. Site-directed mutagenesis of PTS2 sequences and analysis of their functionality. Laboratory supervision of E. Niedzialkowska, M. Studencka and Y. Schulz. Involved in the manuscript preparation.
2. Ewa Niedzialkowska: RT-PCR based examination of *Ccdc33* expression profile. Analysis of *Ccdc33* alternative splice variants by RT-PCR and sequencing.
3. Maja Studencka: Examination of *Ccdc33* expression profile by Northern blot. Involved in analysis of alternative splicing of *Ccdc33*.

4. Yvonne Schulz: Assisted in site-directed mutagenesis of PTS2 sequences. Involved in functional analysis of the PTS2 signals.
5. Pawel Grzmil: Concept and research design. Data interpretation and preparation of the manuscript.

Ccdc33 a Predominantly Testis Expressed Gene Encodes a Putative Peroxisomal Protein

K. Kaczmarek¹ E. Niedzialkowska¹ M. Studencka¹ Y. Schulz¹ P. Grzmil^{1,2}

¹Institute of Human Genetics, University of Göttingen, Göttingen (Germany)

²Department of Genetics and Evolution, Institute of Zoology, Jagiellonian University, Krakow (Poland)

Abstract. Many genes crucial for male fertility are often predominantly or exclusively expressed in male germ cells. The analysis of mouse models demonstrated the functional importance of peroxisomes in spermatogenesis. The CCDC33 protein was reported as 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.

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 (Figuroa 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 consensus sequence of 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

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Correspondence: Dr. Pawel Grzmil
Institute of Human Genetics, University of Göttingen,
Heinrich-Düker-Weg 12, D-37073 Göttingen (Germany);
telephone +49 551 3919669; fax +49 551 3993303;
email: pawel.grzmil@med.uni-goettingen.de

current work we characterized the *Ccdc33* gene, which is predominantly expressed in the testis. During mouse spermatogenesis its expression starts at the primary spermatocyte stage. Moreover, we could demonstrate that the CCDC33 contains a functional PTS2 motif responsible for its targeting to the peroxisomes. It undergoes alternative splicing and at least 4 different splice products were identified.

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 BLAST2 (Zhang and Madden, 1997) programs. Prediction of the protein MW and pI was performed using the Protein Calculator (<http://www.scripps.edu/~cdputnam/protcalc.html>). Putative structural/functional motifs were analyzed by PSORT II (<http://psort.nibb.ac.jp/psort/helpwww2.html>) and multi sequence alignment was performed using the ClustalW program (<http://align.genome.jp/>).

Northern Blot Analysis

Total RNA was extracted from different tissues of adult mouse using the peqGOLD TriFast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instruction. After electrophoresis, RNA samples (20 µg per lane) were transferred onto Hybond XL membranes (Amersham, Freiburg, Germany). Mouse *Ccdc33* probe was generated by PCR amplification from testis cDNA using *Ccdc33ex6FP* (5'-GATACCTCCCAGGACCCATA-3') and *Ccdc33ex7RP* (5'-TTCCCGTCTGTGTCCTC-3') primers, located in exons 6 and 7 of the gene, and used for the Northern blot analysis. After washing, radioactive signals were detected on X-ray film. Subsequently, the quality of RNA was verified by rehybridization with a ³²P-labeled human *ACTB* cDNA (GeneID:60) probe (IMAGp998L23787).

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/Wⁿ* mutant mice with no germ cells, *Ins13^{-/-}* mutants with germ cells until the primary spermatocyte stage, *olt/olt* mice with germ cells until the round spermatid stage and *qk/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 cell, 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 (Immolase; Bionline, Luckenwalde, Germany). For the expression analysis of mouse *Ccdc33* gene primers *Ccdc2RTFP* (5'-GAGGACACTGTACGGCACCA-3') and *Ccdc2RTRP* (5'-GAATGGCCAGGTTTGTCTC-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'-AGCCCCAAAATGGTTAAGGTTGC-3' and mHPRT-Rev-Q: 5'-TTGCAGATTCAACTTGCCTCAT-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) 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 *Ccdc33RedN1HindIII*FP (5'-ATGGTAAAGCTTGCCACCATTGGCGAACCAGCTCTCTCTT-3') and *Ccdc33RedN1KpnIRP* (5'-ATGGTACGGTACCGTAATCTGAGAGTCTGAGGGTCTGTT-3'). In this reaction *HindIII* and *KpnI* restriction sites were introduced at the 5' and 3' ends, respectively. The PCR product was digested with *HindIII/KpnI* and cloned in frame in the pDsRed-Mono-N1 vector (Clontech, Heidelberg, Germany). The CCDC33-DsRED fusion vector was then used as a template in subsequent site-directed mutagenesis to introduce different mutations in the *Ccdc33* PTS2 signals. Following primer pairs were designed using the primerX software (<http://www.bioinformatics.org/primerx/>). For introducing mutations into first PTS2 sequence, *Ccdc33PTS2-1LMFP* (5'-GGATATGAAGTGGATGCGAGACAGGGTG-3') and *Ccdc33PTS2-1LMRP* (5'-CACCTGTCTCGCATCCACTTCATATCC-3') were used and for mutation of second PTS2 sequence *Ccdc33PTS2-2IMFP* (5'-GCTCAGAGTTGGATGTTGTCCTGGAAAAC-3') and *Ccdc33PTS2-2IMRP* (5'-GTTTTCCAGGGACAACATCCAACCTGAGC-3') were used. After amplification of the mutant constructs DH5alpha competent cells (Invitrogen, Karlsruhe, Germany) were transformed with mutated plasmids and positive colonies were confirmed by sequencing. Subsequently, the construct harbouring 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

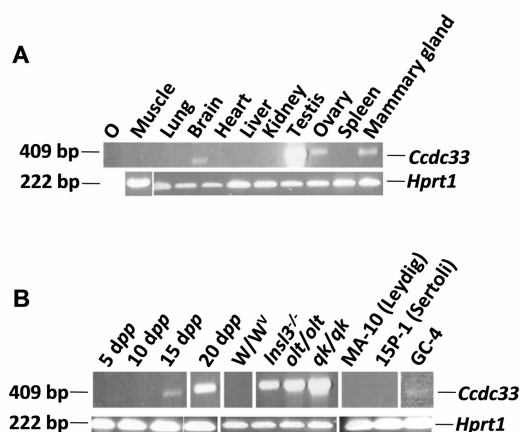


Fig. 1. The expression of mouse *Ccdc33* gene. The RT-PCR analysis on RNA isolated from different adult mouse tissues revealed the strongest expression of *Ccdc33* in the testis and weaker but detectable expression in brain, ovary and mammary gland (A). During postnatal mouse testis development the expression of *Ccdc33* was detected first at 15 dpp. No expression could be detected in RNA derived from the testis of *W/W^v* mutants, whereas the expected 409-bp PCR product was observed in *Insl3^{-/-}*, *olt/olt* and *qk/qk* mutants testis. 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).

cells (ca. 5×10^4) were plated onto the chamber slides 24 hrs before transfection. Then, 1 μ g (for single transfection) or 2 \times 0.5 μ g (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, USA) and observed under a fluorescence microscope (BX-60, Olympus).

Results

The 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 Fig. 1).

The computational analysis revealed the presence of a Ca^{2+} -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 *Ccdc2RTRP* and *Ccdc2RTRP* primers. The strongest expression was detected in the testis but a weaker expression was observed in brain, ovary and mammary gland (Fig. 1A). A strong expression in the testis led us to determine stage-specific expression of the *Ccdc33* during mouse spermatogenesis. For this purpose, the cDNA from testes of mice at different postnatal ages and *Ccdc2RTRP* and *Ccdc2RTRP* primers were used. Expression of *Ccdc33* was observed in the testis of 15 days *post partum* (dpp) males (Fig. 1B). This finding suggests that in the testis, *Ccdc33* transcript appears first when primary spermatocytes are produced (Silver, 1995). To determine the cell type in which *Ccdc33* gene is expressed, RT-PCR using RNA isolated from testis of mutant males with a defined arrest of spermatogenesis at different germ cell stages was performed. *Ccdc33* gene expression was detected in testes of *Insl3^{-/-}* mutant mice (arrested at primary spermatocyte stage), *qk/qk* and *olt/olt* (arrested at post-meiotic germ cells). In contrast, *Ccdc33* transcript was not detectable in testis of a *W/W^v* 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 long 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.

Ccdc33 Undergoes Alternative Splicing

During RT-PCR amplification a shorter fragment of *Ccdc33* transcript was also amplified (data not shown), which led us to analyse alternative splicing forms of this gene. The genomic organization of the mouse *Ccdc33* 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).

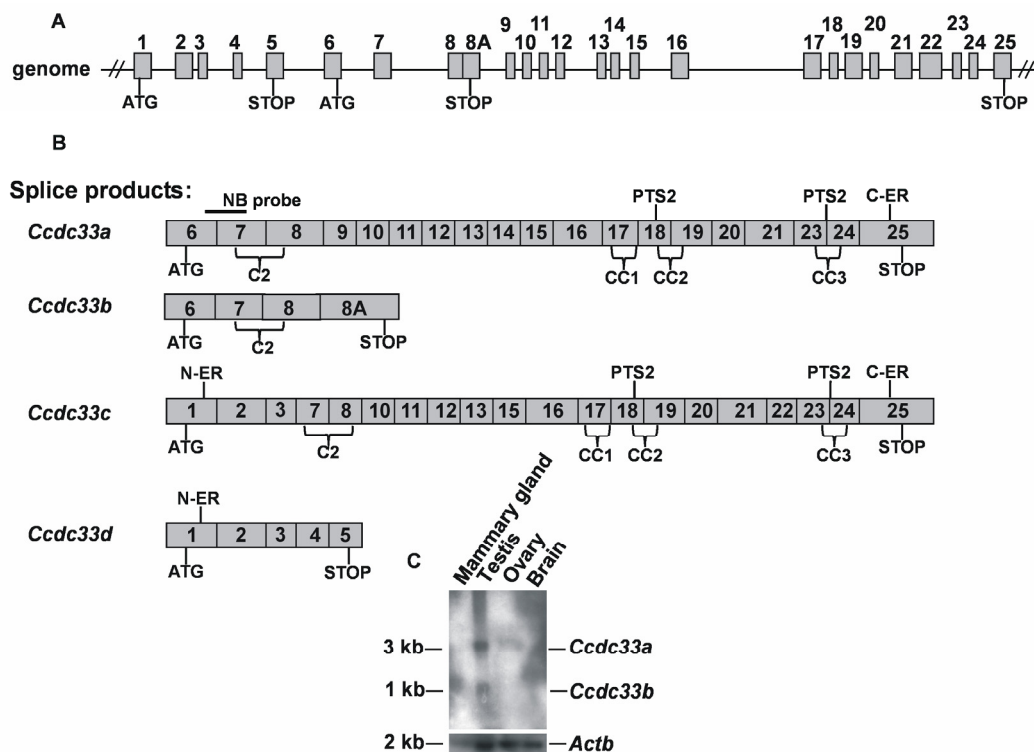


Fig. 2. Genomic organization and alternative splicing of mouse *Ccdc33* gene. Mouse *Ccdc33* gene consists of 25 exons. In addition, exon 8 can be divided into 2 alternative forms (not interrupted by intron sequence) (A). Four alternative splice variants were identified in RT-PCR reaction in different tissues. Two long variants: *Ccdc33a* and *Ccdc33c* encoded a protein containing all characteristic domains. 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. The probe used for NB is given.

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 *Ccdc33* gene (Fig. 2B). The *Ccdc33a* 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 *Ccdc33b* 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 *Ccdc33c* 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 *Ccdc33d*, containing exons 1 to 5 was obtained from a mammary gland cDNA sample.

This variant encodes a protein with the N-terminal ER 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 can not exclude that *Ccdc33* 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 *Ccdc33a* and the shorter testis specific *Ccdc33b* 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 *Ccdc33a* and the 1.0 kb band the *Ccdc33b* transcript. This result corresponds to our RT-PCR findings and confirmed that *Ccdc33* 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).

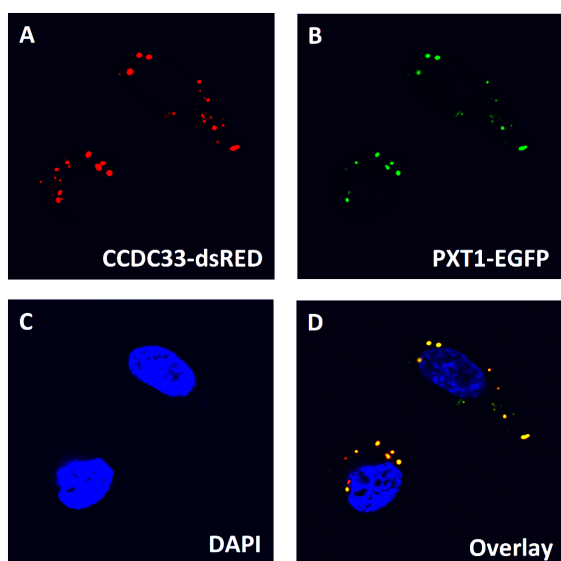


Fig. 3. Co-localization of the CCDC33-dsRED and PXT1-EGFP. HeLa cells were transiently transfected with the CCDC33-dsRED and PXT1-EGFP fusion constructs. Red fluorescence for CCDC33-dsRED fusion protein (A) and green fluorescence for PXT1-EGFP fusion protein (B) could be observed. Nuclei were counterstained with DAPI (C). Overlays of the red and green signals demonstrate a co-localization of the CCDC33-dsRED and PXT1-EGFP fusion proteins (D).

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 *Ccdc33* 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 hours. 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

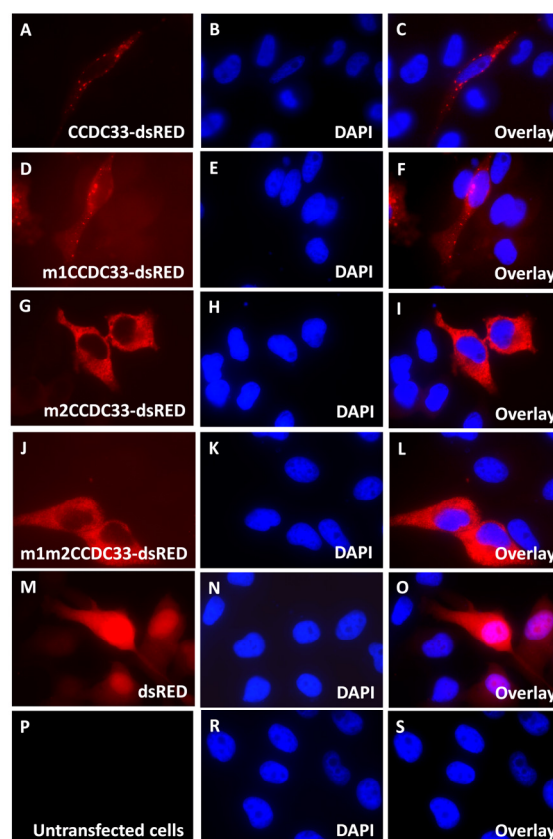


Fig. 4. Subcellular localization of CCDC33-dsRED fusion proteins. HeLa cells were transiently transfected with CCDC33-dsRED constructs and analyzed using fluorescent microscopy. Red signals, representing the cellular localization of the CCDC33-dsRED fusion protein, demonstrated characteristic punctual 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 diffused 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-S).

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 acids 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 for arginine (R) and isoleucine (I) was mutated into TGGATG encoding for 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. Further, 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 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-S). 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 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 PLC δ 4 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 *Ccdc33*, however, it should be noted that weak expression detectable only by the more sensitive RT-PCR technique, was observed also 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 *Ccdc33* from various tissues are deposited. Besides the testis, *Ccdc33* cDNAs were 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 the peroxisomal localization (Marzioch et al., 1994). The mutation changing conserved arginine/leucine (R/L) resulted in diffused localization of *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, first pachytene spermatocytes are observed at day 14 or 15 (Silver, 1995). Expression of mouse *Ccdc33* starts at 15 dpp and is restricted to germ cells. To date only one germ cell specific peroxisomal protein was 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 *Pex7* gene demonstrated, besides other phenotypical 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 mutation 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 disorders (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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Supplemental Materials

Supplementary Table 1. Primers used for the analysis of the alternative splicing of *Ccdc33* gene.

Exons: 1-5

LEFT PRIMER: TGGAGAGAAGACACTGGACCTG
RIGHT PRIMER: AGCTTCCTTTAAATGCCTGCTG

Exon: 3-8

LEFT PRIMER: GGAGGCCCTGAGTGACAAGATA
RIGHT PRIMER: TGTGGCTTTCTCTGATGTGGT

Exon: 5-9

LEFT PRIMER: CCACCTGTGAAATGGATGAGAA
RIGHT PRIMER: CGTAGGACACCAGCTCCTTCTT

Exon: 6-11

LEFT PRIMER: AACCTCCTGCTTAGCTGTCCAT
RIGHT PRIMER: CTACCCGAGCAATTACCACCAT

Exon: 8-15

LEFT PRIMER: ACCACATCAGAGAAAGCCAACA
RIGHT PRIMER: AAAGCAGCTGGAAGGAGAGGTT

Exon: 10-17

LEFT PRIMER: GTTGGCTGTGACCATACAGCTC
RIGHT PRIMER: TCTTCTATGCTCTGCTGGCTCA

Exon: 13-20

LEFT PRIMER: GAACCAGTCCTTCCTCTTCCAA
RIGHT PRIMER: GCCTGTTTGATGGTATTGGTT

Exon: 16-23

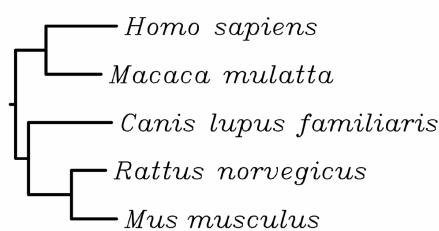
LEFT PRIMER: CCACTCAGGAAATGGAGGAAC
RIGHT PRIMER: CTAGCTTGCCAGGAGGTTAAA

Exon: 18-25

LEFT PRIMER: GATATGAAGAGGCTGCGAGACA
RIGHT PRIMER: GAGAGTCTGAGGGTCTGTTGAGC

Exon: 20-25

LEFT PRIMER: CGAGAAGATGGAGCAGATATTGGA
RIGHT PRIMER: CAGCACATGGCGAAGGAGAT



Supplementary Fig. 1. Phylogenetic tree constructed from multiple alignment of human (*Homo sapiens*), monkey (*Macaca mulatta*), dog (*Canis lupus familiaris*), rat (*Rattus norvegicus*) and mouse (*Mus musculus*) CCDC33 proteins. The tree was generated using ClustalW program, branch length represents the distance in protein similarity.

3.2. Overexpression of peroxisomal testis specific 1 protein induces germ cell apoptosis and leads to infertility in male mice

In the second part of the thesis, the pro-apoptotic properties of PXT1 and interaction between PXT1 and BAT3 are reported. By generating the transgenic mouse model we have shown that the overexpression of *Pxt1* in the testis leads to complete male infertility due to wide-spread apoptosis of pachytene spermatocytes. Moreover, we have shown that overexpression of PXT1 induces apoptosis in different mammalian cell lines. Based on *in silico* analysis we have identified a putative BH3-like domain located at the N-terminus of PXT1 protein and demonstrated that its deletion abolishes most of the PXT1's cell killing ability. Interestingly, the yeast two-hybrid (Y2H) screens of testis cDNA library have identified an apoptotic regulator, BAT3, as a putative PXT1 interacting protein. Using a direct Y2H approach and co-immunoprecipitation (CoIP) method we have confirmed the specificity of this interaction. Moreover, motifs responsible for PXT1-BAT3 association have been determined. Surprisingly, PXT1, which generally shows peroxisomal-cytoplasmic distribution, has appeared to be co-localized with BAT3 mostly within the cell nucleus. Finally, we have reported that overexpression of BAT3 suppresses PXT1-induced apoptosis.

Karina Kaczmarek, Maja Studencka, Andreas Meinhardt, Krzysztof Wieczerzak, Wolfgang Engel, Pawel Grzmil

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Author contributions to the work:

1. **Karina Kaczmarek:** Construction of the transgene, generation of the transgenic mice and evaluation of transgene expression pattern. Phenotypic characterization of transgenic males and quantitative analysis of apoptosis in the testes. Generation of Pxt1pQM-Ntag/A, Pxt1EGFPC1, Bat3dsRED constructs and analysis of apoptosis in HeLa cell line. The Y2H library screens and confirmation of the PXT1-BAT3 interaction by direct Y2H, CoIP and co-localization methods. Generation of PXT1-yeast expression constructs, determination and functional validation of BAT3-

binding site in PXT1 sequence. Involved in experimental design, research concept and data interpretation. Laboratory supervision of M. Studencka. Preparation of the manuscript.

2. Maja Studencka: Generation of Pxt1 Δ BH3pQM-Ntag/A and Bat3pCMV constructs. Involved in quantification of apoptosis in HeLa and NIH3T3 cells.
3. Andreas Meinhardt: Histopathological evaluation of the transgenic testis.
4. Krzysztof Wieczerzak: Generation of BAT3-yeast expression constructs and identification of PXT1-binding region in BAT3 sequence.
5. Wolfgang Engel: Improvement of the experimental concepts, financial support.
6. Pawel Grzmil: Concept and research design. Data interpretation and preparation of the manuscript.

Overexpression of peroxisomal testis specific 1 protein induces germ cell apoptosis and leads to infertility in male mice

Karina Kaczmarek^{*}, Maja Studencka^{*}, Andreas Meinhardt[†], Krzysztof Wiczerzak^{*}, Wolfgang Engel^{*} and Pawel Grzmil^{*‡}

^{*} Institute of Human Genetics, Georg-August-University of Göttingen, Heinrich-Düker-Weg 12, 37073 Göttingen, Germany

[†] Department of Anatomy and Cell Biology, Justus-Liebig-University Giessen, Aulweg 123, 35385 Giessen, Germany

[‡] Department of Genetics and Evolution, Institute of Zoology, Jagiellonian University, Ingardena 6, 30-060 Cracow, Poland

ABSTRACT

Peroxisomal testis specific 1 (*Pxt1*) gene is the only male germ cell-specific gene that encodes a peroxisomal protein known to date. To elucidate the role of *Pxt1* in spermatogenesis, we generated transgenic mice expressing a c-MYC-PXT1 fusion protein under the control of the *PGK2* promoter. Overexpression of *Pxt1* resulted in induction of male germ cells apoptosis mainly in primary spermatocytes, finally leading to male infertility. This prompted us to analyse the pro-apoptotic character of mouse PXT1, which harbours a BH3-like domain in the N-terminal part, in more detail. In different cell lines the overexpression of PXT1 also resulted in a dramatic increase of apoptosis, whilst the deletion of the BH3-like domain significantly reduced cell death events, thereby confirming that the domain is functional and essential for the pro-apoptotic activity of PXT1. Moreover, we demonstrated that PXT1 interacts with apoptosis regulator BAT3. This association protects cells from the PXT1-induced apoptosis by shuttling PXT1 from the cytoplasm to the nucleus. In summary, we demonstrated that PXT1 induces apoptosis via the BH3-like domain and that this process is inhibited by interaction with BAT3.

INTRODUCTION

Programmed cell death (apoptosis) is an active, highly regulated biological process, which enables maintenance of tissue homeostasis by elimination of aged, over-produced or dysfunctional cells. Apoptotic loss of germ cells during testicular development is very

common in both normal and pathological conditions (Hikim *et al.*, 1998), but the mechanisms and genes underlying this important event in the male gonad still remain unclear. One candidate gene predominantly expressed in the testis that is involved in apoptosis is HLA-B-associated transcript 3 (*Bat3*, also known as *Scythe* or *Bag6*) (Wang and Liew 1994; Ozaki *et al.*, 1999). BAT3 is a member of the BCL-2 associated athanogene (BAG) family of proteins that besides a C-terminal BAG domain contains two C-terminal nuclear localization signals, central polyproline and glutamine-rich regions, zinc-finger like motif as well as a N-terminal ubiquitin-like domain (Banerji *et al.*, 1990; Manchen and Hubberstey 2001). It has been demonstrated that BAT3 interacts with the *Drosophila* pro-apoptotic protein reaper and modulates reaper-induced apoptosis (Thress *et al.*, 1998). In addition, the interaction of BAT3 with many other apoptotic regulators like p53, NCR3, AIFM1 and PBF has been reported (Pogge von Strandmann *et al.*, 2007; Sasaki *et al.*, 2007; Desmots *et al.*, 2008; Tsukahara *et al.*, 2009). Interestingly, the targeted disruption of *Bat3* in mice induces apoptosis of meiotic germ cells resulting in complete male infertility (Sasaki *et al.*, 2008). The authors have demonstrated that stabilization of HSPA1B (also known as HSP70-2) by BAT3 is crucial for proper function of HSPA1B during spermatogenesis.

To date, peroxisomal testis specific 1 (*Pxt1*) gene is the only known gene that encodes a male germ cell-specific peroxisomal protein (Grzmil *et al.*, 2007). The expression of *Pxt1* is developmentally regulated during spermatogenesis and the encoded protein consists of 51 amino acids only. It has been demonstrated previously that PXT1 contains a functional peroxisomal targeting signal type 1 (PTS1) at the C-terminus and the EGFP-PXT1 fusion protein co-localizes with known peroxisomal markers (Grzmil *et al.*, 2007). Peroxisomes are important cellular organelles indispensable for cell survival and they are ubiquitously present in eukaryotic cells. However, the existence of peroxisomes in male germ cells was questionable for a long time. The first report about the presence of peroxisomes in a spermatogonial cell line was published in 2003 (Luers *et al.*, 2003). Later, peroxisomes were detected in spermatogonia of mouse testis (Huyghe *et al.*, 2006a; Luers *et al.*, 2006). Recently, using antibodies against different peroxisomal marker proteins Nenicu *et al.* (2007) have demonstrated peroxisomes in all stages of spermatogenesis, except mature spermatozoa. There are many mutant mouse models in which peroxisome-associated spermatogenesis defects have been observed. Among them targeted disruption of the acyl-Coenzyme A oxidase 1 (*Acox1*) gene results in a remarkable

reduction of Leydig cells, hypospermatogenesis and male infertility (Fan *et al.*, 1996). Deficiency of glyceronephosphate O-acyltransferase (*Gnpat*) also leads to testicular atrophy and male infertility (Rodemer *et al.*, 2003). The analysis of knockout mice lacking the peroxisomal protein hydroxysteroid (17-beta) dehydrogenase 4 (HSD17B4, also known as multifunctional protein 2, MFP-2), has revealed that homozygous male mutants exhibit a strongly reduced fertility (Baes *et al.*, 2000; Huyghe *et al.*, 2006a). Although these findings have confirmed the general relevance of peroxisomes for proper spermatogenesis, the biological function of these organelles in the testis still remains poorly understood.

To further elucidate the function of *Pxt1* and peroxisomes in mouse testis, we have generated transgenic mice with male germ cell-specific overexpression of PXT1. In the present work, we show that overexpression of PXT1 induces apoptosis resulting in male infertility. Moreover, we demonstrate that the interaction of PXT1 with BAT3 can inhibit the pro-apoptotic activity of PXT1 in transiently transfected cell lines.

MATERIALS AND METHODS

Animals

All animals were housed in the Animal Facility of the Institute of Human Genetics, Göttingen, Germany, under controlled environmental conditions (21°C, 12h light/12h dark cycle) with free access to standard mouse chow and tap water. All of the experimental procedures were carried out in accordance with the local ethics commission under the license number 33.11.42502-04-096/07.

Generation of the c-myc-Pxt1 transgenic mice

The c-myc-Pxt1 transgenic construct contains: promoter region of human phosphoglycerate kinase 2 (*PGK2*), c-myc tags, the complete open reading frame (ORF) of murine *Pxt1*, 3' untranslated region (UTR) of human growth hormone 1 (*GHI*) and SV40 poly(A) sequence. The transgenic cassette was generated according to Tascou *et al.* (2001) with pBluescript II SK (+/-) vector (Stratagene, La Jolla, CA, USA) containing 1.4-kb region of *PGK2* promoter flanked by *XhoI/HindIII* restriction sites. The 351-bp *EcoRI/NotI* fragment, including leader sequence and five copies of c-myc tag, was amplified by PCR using pCS2-3' mt plasmid (Rupp *et al.*, 1994) as a template, C-mycTag-new-F,

Ad.C-mycTag-R primers and cloned downstream of the *PGK2* promoter. All primer sequences are given in Supplementary File 1. The 179-bp *NotI/SacII Pxt1* coding sequence was amplified by RT-PCR with mouse testis cDNA and adPxt1-ORF-F, adPxt1-ORF-R primers and cloned downstream of the c-myc tag. In this reaction two glycine residues were introduced between the fragment consisting of c-myc tags and the *Pxt1* ORF to facilitate proper and independent folding of this two components in c-MYC-PXT1 fusion protein. The 161-bp 3' UTR of human *GHI* was obtained after PCR on human genomic DNA using hGH-3UTRsacII-F and hGH-3UTRsacII-R primers and afterwards was cloned downstream the *Pxt1* ORF. The 128-bp *SacII/SacI* fragment containing SV40 poly(A) was amplified by PCR using pEGFP-N1 vector (Clontech) as a template and ad.polA-SV40-F, ad.polA-SV40-R primers. Finally, the SV40 polyadenylation signal was included at the 3' end of the transgenic construct. After sequencing, the obtained 2.3-kb transgenic cassette was excised from pBluescript II SK (+/-) vector by *XhoI/SacI* digestion and then purified from agarose gel (QIAquick Gel Extraction Kit, Qiagen). Subsequently, the construct was diluted to a concentration of 30 µg/ml in TE buffer (5 mM Tris, pH 7.4 and 0.1 mM EDTA, pH 8.0) and microinjected into the pronuclei of fertilized oocytes of the FVB/N strain of mice as described (Hogan *et al.*, 1986). Founder transgenic mice were identified by standard PCR on genomic DNA using gen_hPGK2_F1 and gen_Pxt1ex.2-3_R primers. The quality of genomic DNA was verified by PCR with transition protein 2 (*Tnp2*)-specific primers: TP2_F1 and TP2_R1.

Generation of expression constructs

The primer sequences and the cloning strategies for generation of the expression constructs used in this work are given in Supplementary File 2. The following constructs were generated: Pxt1pQM-Ntag/A – encodes the E2-PXT1 fusion protein, Pxt1ΔBH3pQM-Ntag/A – encodes the E2-PXT1-BH3del mutant fusion protein lacking the BH3-like domain of PXT1. Rtn1pQM-Ntag/A – expresses the E2-RTN1 as described previously (Mannan *et al.*, 2006). Bat3pCMV – contains *Bat3* cDNA (exons 7 to 25) fused with c-myc tag and encodes the c-MYC-BAT3 fusion protein. Pxt1EGFPc1 – encodes EGFP-PXT1 fusion protein as previously described (Grzmil *et al.*, 2007). Bat3DsRed – contains *Bat3* cDNA (exons 7 to 25) fused with dsRED and encodes the BAT3-dsRED fusion protein. Pxt1pGBKT7 – contains the complete ORF of *Pxt1*. part1a/part1b Pxt1pGBKT7 –

express the N-terminal parts of PXT1, part2a/part2b Pxt1pGBKT7 – express the C-terminal parts of PXT1. The mut-part1aPxt1pGBKT7 vector encodes the N-terminal part of PXT1 in which mutation of LAPF motif into GAPA sequence was introduced, part1Bat3pGADT7 – contains mouse *Bat3* cDNA “part 1” located between exon 6 and 9, part2Bat3pGADT7 – contains “part 2” of mouse *Bat3* cDNA overlapping exons 9 to 15 and part3Bat3pGADT7 – contains “part 3” of mouse *Bat3* cDNA located between exon 15 and 24.

Cell culture, transfection, immunocytochemistry, apoptotic cell death assay

Human cervical adenocarcinoma (HeLa) and mouse embryonic fibroblast (NIH3T3) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % FCS and 1 % penicillin/streptomycin (PAN, Germany) and in case of NIH3T3 cells with 1x non-essential amino acids (Gibco, Germany) in a humidified atmosphere of 5 % CO₂ at 37°C. Transfection was performed, as described (Grzmil *et al.*, 2007, Kaczmarek *et al.*, 2009), with 1 µg (for single transfection) or 2 × 0.5 µg (for co-transfection) of construct(s) DNA. After 24 hrs of transient transfection, the cells were fixed with 4% paraformaldehyde and 0.1 % Tween 20 for 15 min at RT. Cells expressing EGFP and/or dsRED fluorescence proteins were mounted with Vectashield mounting medium with DAPI (Vector, USA) and proceeded to fluorescent microscopic analysis. The cells transfected with Pxt1pQM-Ntag/A, Pxt1ΔBH3pQM-Ntag/A and Rtn1pQM-Ntag/A vectors, were further washed with DPBS, permeabilized 15-min in 0.1% Triton X-100/DPBS and blocked for 1 h at RT in 5 % BSA/DPBS. In the next step, the cells were incubated for 2 hrs at RT with mouse monoclonal anti-E2 tag antibody (1:500, Abcam) in 1 % BSA/DPBS. Subsequently, the cells were washed with DPBS and incubated for 2 hrs at RT with FITC-conjugated anti-mouse IgG secondary antibody (Sigma-Aldrich) diluted to 1:200 in 1 % BSA/DPBS. Finally, the cells were washed with DPBS, mounted with Vectashield mounting medium with DAPI and observed under a fluorescence microscope. Quantification of apoptosis was performed by analysis of nuclear morphology after DAPI staining. The cells displaying the nuclear morphology typical for apoptosis (half-moon-shaped nucleus, DNA condensation and fragmentation) were related to the total number of positively stained cells by four different investigators. Each investigator performed 2 to 4 independent transfections and counted every transfection two times with a minimum of 60 cells. Data are presented as percent of apoptotic cells ± SD. Detection of apoptosis by

monitoring of phosphatidylserine translocation in plasma membrane was performed on HeLa cells 24 hrs after transfection with Pxt1EGFPC1 or empty EGFPC1 vector. HeLa cells were washed twice with incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl₂) and then subjected to combined Annexin V Alexa 568 (Roche) and DAPI (Vector, USA) labelling for 15 min at RT in the absence of light. Immediately after staining, cells were analysed under a fluorescence microscope (Olympus BX60).

Reverse transcription-PCR (RT-PCR) and Northern blot analysis

Total RNA was isolated from different adult mouse tissues using the peqGOLD TriFast reagent (Peqlab, Germany) according to the manufacturer's recommendations. For RT-PCR, 2 µg of RNA was treated with DNase-I (Sigma, Germany) and reverse transcribed using Superscript II reverse transcriptase system and the Oligo dT₍₁₂₋₁₈₎ primer (Invitrogen, Germany). As a control, the same reaction without reverse transcriptase was prepared simultaneously. Subsequently, an aliquot of cDNA (1 µl) was subjected to 35 cycles of PCR with Taq DNA polymerase (Immolase, Bioline, Germany). Expression of mouse *Pxt1* was determined using Pxt1-ORF-EcoRI-F and Pxt1-ORF-BamHI-R primers yielding a 175-bp long PCR product. *c-myc-Pxt1* transgene expression was detected by RT-PCR with Q-PCR_cmyc_F and Q-PCR_Pxt1ex2-3_R primers generating a 121-bp long product specific for transgenic transcript. The cDNA quality was verified with mHPRT-For-Q and mHPRT-Rev-Q primers amplifying a 222-bp fragment of the mouse *Hprt1* gene (GeneID: 15452). For Northern blot analysis, 20 µg of total RNA was size fractionated by electrophoresis, transferred onto Hybond XL membrane (Amersham, Germany) and hybridised with a ³²P-labelled probe. Mouse *Pxt1*-specific probe was generated by RT-PCR using Pxt1-ORF-EcoRI-F and Pxt1-ORF-BamHI-R primers. *EcoRI/NotI* fragment of the transgenic construct was used as *c-myc* tag-specific probe. After hybridisation, blots were washed at medium to high stringency and radioactive signals were detected on X-ray film. The RNA quality was verified by rehybridization with a ³²P-labelled mouse *Eef1a1* cDNA (GeneID: 13627) probe, generated by PCR using Eef1a1F1 and Eef1a1R1 primers. The *Eef1a1* probe was kindly provided by R. Hahnewald.

Protein extraction, immunoprecipitation and Western blot analyses

Total proteins from mouse tissues were prepared from the organic phase obtained after RNA isolation using peqGOLD TriFast reagent (Peqlab, Erlangen, Germany) according to

the TriReagent protein extraction protocol (Molecular Research Centre), but the protein pellet in 1% SDS was additionally sonicated on ice (Branson Sonifier 250). For immunoprecipitation experiments, proteins were isolated from transiently transfected HeLa cells and precipitated with appropriate antibodies (mouse monoclonal anti-E2 tag or rabbit polyclonal anti-myc tag, 1:100, Abcam) as described (Rzymiski *et al.*, 2008). For Western blot analysis, protein samples were separated on 4-12 % or 12 % NuPAGE Novex Bis-Tris gel (Invitrogen) and electroblotted onto PVDF membranes (0.45- μ m Hybond-P PVDF, Amersham Biosciences or 0.2- μ m PVDF, Invitrogen). After blocking in 5 % non-fat milk TBST (137 mM NaCl, 10 mM Tris-HCl, pH 7.3, 0.1 % Tween 20) for 1 h at RT the membrane was probed overnight at 4°C with the following primary antibodies: mouse monoclonal anti-myc tag (1:2000, Millipore), rabbit polyclonal anti-c-myc (1:1500, Sigma-Aldrich), mouse monoclonal anti-E2 tag (1:1000, Abcam), mouse monoclonal anti- β -actin (1:10000, Abcam). Subsequently, blots were washed three times for 10 min in 2 % non-fat milk TBST and incubated for 1-2 hrs with alkaline phosphatase conjugated anti-mouse/rabbit IgG secondary antibody (1:5000, Sigma-Aldrich). Finally, blots were washed again and signals were visualized using BCIP-NBT system (Roth) according to the manufacturer's instruction.

Immunohistochemistry

Animals were anaesthetized and then perfused with cold DPBS followed by 4 % paraformaldehyde. After overnight incubation at 4°C in 4% paraformaldehyde mouse testes were dissected and cryoprotected by immersion in 30 % sucrose for 16-24 hrs. Subsequently, the testes were frozen in tissue freezing medium (Jung, Leica Microsystems, Germany), sectioned with a cryostat (Leica Microsystems), transferred onto Superfrost slides and stored at -20°C until use. For immunohistochemistry, 5- μ m thick sections were incubated for 1 h at RT in I blocking buffer (7% BSA, 1% Tween 20 in DPBS) and then overnight at 4°C with mouse monoclonal anti-myc tag antibody (Millipore) diluted to 1:200 in II blocking buffer (1.5 % BSA, 1 % Tween 20 in DPBS). Afterwards, slides were washed in DPBS and incubated for 1h at RT with Cy3-conjugated anti-mouse IgG secondary antibody (Sigma-Aldrich) diluted to 1:500 in II blocking buffer. After washing with DPBS, nuclei were counterstained with DAPI in Vectashield mounting medium (Vector, USA) and the slides were observed under a fluorescence microscope (BX-60, Olympus).

Histological analysis and TUNEL assay

Histological and TUNEL analyses were performed as previously described (Burnicka-Turek *et al.*, 2009). Briefly, for morphological examination 5- μ m thick paraffin sections of Bouin fixed testes and epididymides were stained with hematoxylin and eosin (Sigma Aldrich, Germany). For analysis of apoptosis, deparaffinized and rehydrated sections were subjected to TUNEL assay after pretreatment with proteinase K (Roche Diagnostics, Germany) using an ApopTag Peroxidase *in situ* Apoptosis detection kit (Qbiogene, Germany) according to the manufacturer's instruction. Within testis cross-sections apoptosis was quantified by counting the number of TUNEL positive and TUNEL-negative tubules per section and TUNEL positive cells in each tubule. Data are presented as average number of TUNEL-positive cells per tubuli \pm SD. For each animal 10-15 fields were counted. Slides were analysed under a light microscope (BX-60, Olympus).

Yeast two-hybrid screen (Y2H)

To identify putative PXT1 interacting proteins a mouse testis cDNA library, constructed in pGAD10 vector (Clontech, Germany) and kindly provided by I. Adham, was screened with mouse PXT1. The bait construct Pxt1pGBKT7 encoding a GAL4 DNA-binding domain fused with PXT1, was generated as described above. After auto-activation test of the Pxt1pGBKT7 vector the testis cDNA library screening was performed by the sequential transformation according to Matchmaker™ GAL4 Two-Hybrid System 3 & Libraries user manual (Clontech). The yeasts were initially spread on medium stringency plates (SD/-LTH) and then the surviving colonies were verified on high stringency plates (SD/-LTHA + X- α -Gal). Next, plasmid DNA was isolated from blue colonies using Yeastmaker™ Yeast Plasmid Isolation Kit (Clontech). Subsequently, the plasmid DNA was transformed into *E.coli* DH5alpha competent cells (Invitrogen, Germany) and clones containing only the prey-library plasmid were selected on LB/Amp and LB/Kan plates. The cDNA inserts of these clones were amplified by PCR with 5'AD-LD-insert and 3'AD-LD-insert primers and then sequenced. To confirm PXT1-BAT3 interaction determined by library screens, direct reconstruction of Y2H experiment was performed. In this assay the identified Bat3pGAD10 prey vector and the Pxt1pGBKT7 bait construct were co-transformed into AH109 yeast strain by the lithium acetate method according to the Clontech protocol. The co-transformants were selected on SD/-LT plates and the interaction was finally verified on SD/-LTHA + X- α -gal plates. The same method was used for mapping of PXT1 and BAT3

regions essential for the interaction. All bait and prey vectors used in the study were verified for host yeast toxicity and autonomous activation of reporter genes.

Statistical analysis

For statistical analysis, data obtained by counting apoptotic cells were pooled for each cell type and vector used. Likewise, the data collected after TUNEL analyses were pooled for each genotype and age group. The results were expressed as a ratio, thus they were normalized by angular transformation prior to the t-test for independent groups. The *p* value below 0.05 was considered as statistically significant. All statistical analyses were performed using the Statistica 8 software package (StatSoft, Inc., Tulsa, USA).

RESULTS

Generation of the c-myc-Pxt1 transgenic line

The male germ cell-specific expression of *Pxt1* (Grzmil *et al.*, 2007) prompted us to investigate the *in vivo* function of this gene during spermatogenesis. For this purpose the c-myc-Pxt1 transgenic construct (Fig. 1A) expressing *c-myc-Pxt1* fusion transcript under the control of the human *PGK2* promoter was generated. Downstream of the *Pxt1* ORF, the 3' untranslated region (UTR) of human growth hormone 1 (*GHI*) and SV40 poly(A) sequence were located in order to ensure the proper post-transcriptional proceeding of the transgenic mRNA. The transgenic cassette was microinjected into the pronuclei of fertilized FVB/N mouse eggs and founder mice were detected by PCR using genomic DNA and the following transgenic construct-specific primers: gen_hPGK2_F1, gen_Pxt1ex.2-3_R. As can be seen in Figure 1B, the 674-bp fragment was observed only in founder animals. DNA quality was confirmed with *Tnp2*-specific primers (Fig. 1B). Initially, four transgenic male founders were identified and each of them was mated with two wild-type FVB/N females to establish the transgenic line. After three month breeding period no pregnancy could be observed, what suggested the infertility of the male founders. We have also identified a female founder which was fertile and able to transmit the transgene to its progeny. The female founder was mated with wild-type FVB/N male and using the PCR genotyping, we detected the transgenic animals in the F1 generation and established the transgenic line.

Testis-specific expression of the c-myc-Pxt1 transgene

To confirm that the 1.4-kb region of *PGK2* promoter properly conferred the expression of *c-myc-Pxt1* to the testis, total RNA was isolated from various tissues of adult transgenic males. Northern blot experiment with the *Pxt1*-specific probe, which recognizes the full-length of *Pxt1* ORF, revealed that the expected 1.2-kb band representing the endogenous *Pxt1* mRNAs could be observed in both wild-type and transgenic testes. In addition, the same probe detected the 0.8-kb band corresponding to the *c-myc-Pxt1* fusion transcripts exclusively in transgenic testis (Fig. 1C, upper panel). As can be seen in middle panel of Figure 1C, the 0.8-kb band was also identified in the transgenic testis when membrane was hybridized with the *c-myc* tag-specific probe. The integrity of all RNA samples was assessed by rehybridization of the blots with *Eef1a1* cDNA probe (Fig. 1C, the lowest panel). To confirm the Northern blot results a more sensitive RT-PCR method was used. Likewise, the testis-specific expression of the *c-myc-Pxt1* transgene was confirmed (Fig. 1D). To check for DNA contamination in RNA samples, the reaction without reverse transcriptase (-RT) was performed (Fig. 1D). The cDNA quality was proven with *Hprt1*-specific primers (Fig. 1D, lower panel). Next, the expression of c-MYC-PXT1 fusion protein was evaluated by Western blot using anti-myc tag antibody. The expected 17-kDa band corresponding to the size predicted for c-MYC-PXT1 fusion protein was detected in the testis of transgenic mice, but not in the epididymis of transgenic mice or analyzed wild-type tissues (Fig. 1E). Protein quality and integrity was verified using anti- β -actin (ACTB) antibody (Fig. 1E, lower panel). To determine the stage-specific expression of c-MYC-PXT1 during spermatogenesis, an immunohistochemical examination of transgenic and wild-type testes using anti-myc tag antibody was performed. As shown in Figure 1F-H, the specific signals representing c-MYC-PXT1 were detected within cytosol of pachytene spermatocytes in the testis of *c-myc-Pxt1* transgenic mice, but not in the wild type testis (Fig. 1I-K). However, it should be noted that unspecific staining of the interstitial tissues by secondary antibody could not be eliminated and was observed in both, transgenic and wild-type testes (Fig. 1F, H and I, K, respectively).

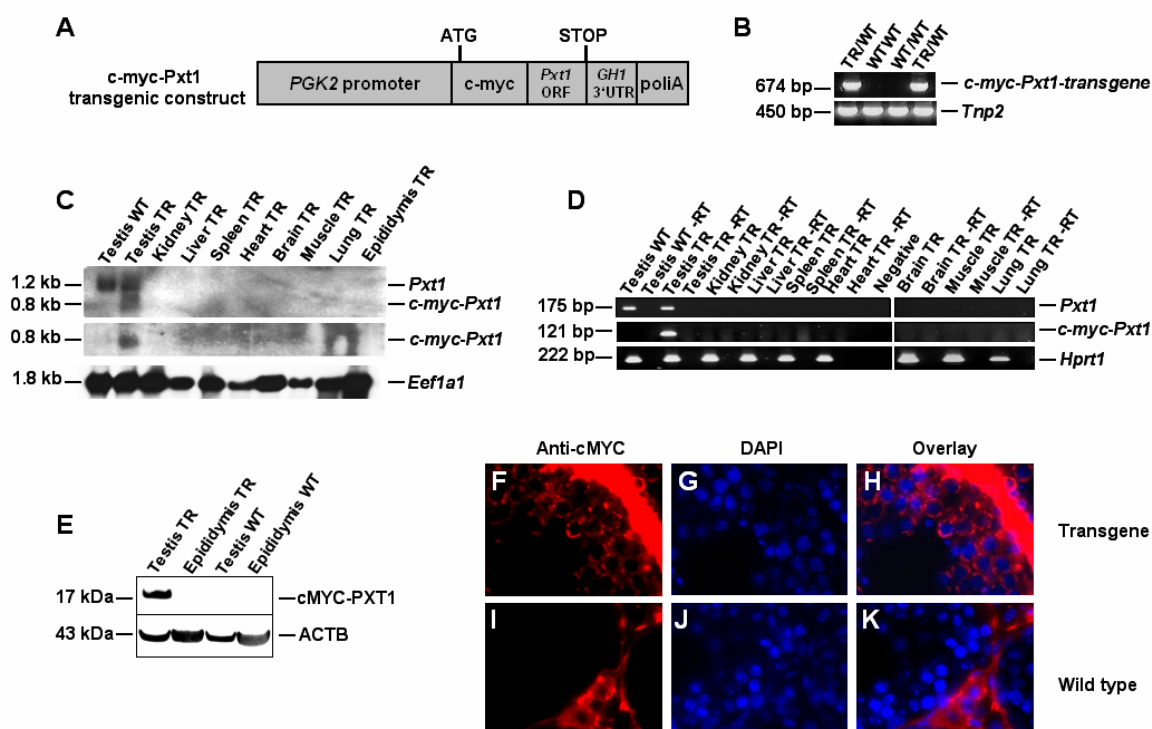


Figure 1. Generation and expression analyses of c-myc-Pxt1 transgenic line. (A) Schematic representation of the c-myc-Pxt1 transgenic construct. The construct consists of a 1.4-kb part of the *PGK2* promoter, c-myc-tag, complete ORF of the mouse *Pxt1* gene, 3'UTR of the *GHI* and poly(A) signal of SV40. Start codon (ATG) and STOP codon are given. (B) Genotyping PCR of the transgenic founders using transgenic construct-specific primers. A 674-bp product could be observed in transgenic founders (TR/WT), but not in wild type (WT/WT) animals. DNA quality was verified with *Tnp2*-specific primers (lower panel). (C) Northern blot analyses of the c-myc-Pxt1 transgene expression in different organs of transgenic males. Using the *Pxt1*-specific probe, we demonstrated a 1.2-kb band, representing the endogenous *Pxt1* in the testis of both wild type (WT) and transgenic (TR) animals and a 0.8-kb band corresponding to the *c-myc-Pxt1* transcript exclusively in testis of transgenic males (upper panel). As expected, the *c-myc* tag-specific probe detected the 0.8-kb band, representing *c-myc-Pxt1* mRNA, only in the transgenic testis (middle panel). RNA quality and integrity were checked using the *Eef1a1*-specific probe (the lowest panel). (D) To confirm the testis-specific expression of the c-myc-Pxt1 transgene the RT-PCR analyses were performed. The *Pxt1*-specific primers amplified the 175-bp fragment in testis of wild type (WT) and transgenic (TR) males, whereas the c-myc-Pxt1 construct-specific primers amplified the 121-bp product only in the testis of transgenic male. The cDNA quality was proven using the *Hprt1*-specific primers. The 222-bp product was detected in all analysed cDNA samples. To exclude any genomic DNA contamination negative control reactions without reverse transcriptase were performed (-RT). (E) Western blot analysis using anti-myc tag antibody demonstrated the expected 17-kDa c-MYC-PXT1 fusion protein in testis of transgenic (TR) but not wild type (WT) animals. To check the protein quality anti- β -actin antibody (ACTB) was used. (F-H) In testicular sections of transgenic animals anti-myc tag antibody detected the c-MYC-PXT1 in seminiferous tubuli, mainly in primary spermatocytes. (I-K) Control reaction on wild type testis confirmed the specificity of the staining. Additional staining of interstitial cells and the basal membrane was found to be unspecific, due to its presence in both transgenic and wild type testes.

Infertility of the c-myc-Pxt1 transgenic male mice

As mentioned, female founder was used to generate the transgenic line, because all four tested male founders were infertile. To examine the fertility of male progeny of the female founder three males were mated each with two wild type females for a period of 3 months. During this time the females were monitored daily for vaginal plugs (VPs). Although, females were positive for VPs (indicating mating) none of them became pregnant. No spermatozoa could be found in uterus and oviduct of VP-positive females. To evaluate the cause of male infertility in the c-myc-Pxt1 transgenic line a morphological and histological examinations of the male reproductive organs were performed. Transgenic males at 103 days *post partum* (dpp) demonstrated testicular atrophy (Fig. 2A) and the histological analysis revealed arrest of spermatogenesis at the level of pachytene spermatocytes (Fig. 2B). Remarkably, only very few degenerating round spermatids and no elongated spermatids were found. The testis displayed massive vacuolization of the seminiferous epithelium, sloughing of immature germ cells into the lumen and formation of numerous multinucleated giant cells. It should be noted that the range of observed abnormalities varied between tubules, but the epididymal lumen contained numerous immature germ cells and multinucleated giant cells, but no or very few spermatozoa (Fig. 2C). The phenotype of transgenic males became even more severe with age and at 151 dpp complete depletion of germ cells resulting in a Sertoli cell-only (SCO) phenotype was evident (Fig. 2D). As mentioned above, the expression of c-myc-Pxt1 transgene was directed by the *PGK2* promoter, thus the c-MYC-PXT1 fusion protein could first be detected in primary spermatocytes (Fig. 1F-H). Accordingly, to elucidate the defective stage of spermatogenesis in transgenic animals, we analyzed testicular histology at different stages. As demonstrated in Figure 2E, spermatogenesis in mice at 10 dpp appeared normal. Only some histological abnormalities were observed at 16 dpp, before the onset of the meiotic divisions. Some of the pachytene spermatocytes within the seminiferous epithelium exhibited abnormal, including pyknotic, nuclear morphology and a few single nucleated giant cells. In contrast, the histological analysis of older animals revealed that the testicular phenotype at 26 dpp and 38 dpp was almost as severe as that observed in 103 days old transgenic mice. To investigate whether the progression of seminiferous tubules atrophy caused by overexpression of *Pxt1* is due to increased apoptosis levels, the number of apoptotic cells per tubuli was quantified followed TUNEL staining. This analysis demonstrated that there was no significant increase in apoptosis in transgenic animals at

10 *dpp* and 16 *dpp* as compared to wild type FVB males at the same age (Fig. 2E, F). However, in older animals a significant increase in apoptosis was observed (Fig. 2E, F). At 30 *dpp* the number of apoptotic cells pro tubuli was approximately 13-fold higher than in wild-type. Light microscopical analysis following TUNEL assay revealed that pachytene spermatocytes were the predominantly labelled cell type. In addition, some of the multinucleated giant cells also appeared to undergo apoptosis.

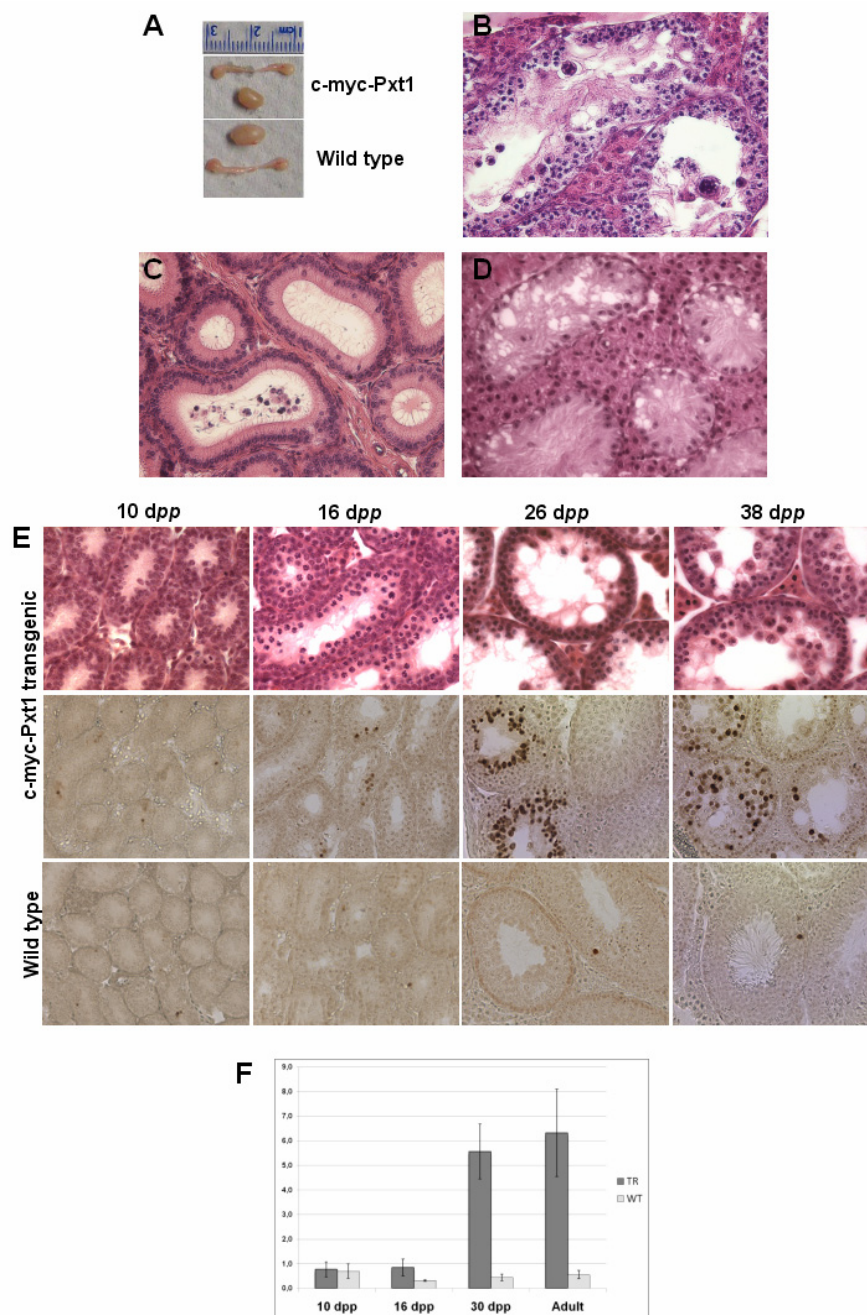


Figure 2. Overexpression of *c-myc-Pxt1* leads to the degeneration of the germinal epithelium and apoptosis of male germ cells. (A) Testis of adult transgenic male shows atrophy, whereas epididymides are normal.

(B) Histological analysis of atrophic testis revealed a prominent degeneration of germ cells in adult animals (103 dpp), especially at the primary spermatocyte stage. Strong vacuolisation of the epithelium, immature germ cells in lumen and giant cells were observed. (C) In epididymides of transgenic animals a very few (or no) spermatozoa were found and often round spermatids and multinucleated giant cells were present. (D) The testis section of transgenic male at 151 dpp shows a Sertoli cell only phenotype. (E) Developmental progression of the male germ cells degeneration. At 10 dpp no obvious changes could be discerned in testis of transgenic males. The first abnormalities were observed at 16 dpp, however, only a few pachytene spermatocytes exhibited pyknotic nuclei. Massive degeneration was observed at 26 and 38 dpp. To investigate whether the degenerated cells undergo apoptosis a TUNEL assay was performed. (E and F) No significant increase in the number of apoptotic cells was observed at 10 dpp and 16 dpp. In contrast, strong apoptosis induction was observed in older transgenic animals (26, 30, 38 dpp and adult, E, F).

Murine PXT1 is a pro-apoptotic BH3-like motif containing protein

The strongly enhanced apoptosis observed in transgenic males overexpressing *Pxt1* prompted us to a more detailed analysis of the PXT1 protein sequence. The *in silico* analysis using the PFAM database (<http://pfam.sanger.ac.uk/>) revealed that the N-terminal fragment of mouse PXT1 contains a putative BH3-like domain (Fig. 3A). To investigate the pro-apoptotic potential of PXT1 protein and verify the functionality of BH3-like region, we analyzed apoptotic event in HeLa and NIH3T3 cells transiently transfected with Pxt1pQM-Ntag/A and Pxt1 Δ BH3pQM-Ntag/A plasmids. As presented in Figure 3B, the Pxt1pQM-Ntag/A encodes a full-length PXT1 protein tagged with N-terminal E2 epitope (E2-PXT1), whereas the Pxt1 Δ BH3pQM-Ntag/A vector encodes a mutant E2-PXT1 fusion protein with a deletion of the BH3-like motif (E2-PXT1-BH3del). Fluorescent microscopic examination showed that the majority of HeLa cells overexpressing E2-PXT1 exhibited cytomorphological alternations typical for apoptosis, including nuclear fragmentation, cell rounding and shrinkage, plasma membrane blebbing and apoptotic body formation (Mund *et al.*, 2003; Nozawa *et al.*, 2009) (Fig. 3C, upper panel). In contrast, overexpression of the E2-PXT1-BH3del protein had much weaker effect on the cell viability and most transfected HeLa cells displayed normal-appearing morphology (Fig. 3C, lower panel). Quantitative analysis of apoptotic events demonstrated a significant decrease in percentage of E2-PXT1-BH3del-positive cells possessing apoptotic morphology as compared to E2-PXT1 expressing cells (32% \pm 1 vs 73% \pm 1, respectively, $p < 0.001$, Fig. 3C). The control transfection using Rtn1pQM-Ntag/A construct resulted in significantly lower percentage of apoptotic cells (6% \pm 2) than the transfections with Pxt1pQM-Ntag/A or Pxt1 Δ BH3pQM-Ntag/A ($p < 0.001$). To confirm that the pro-apoptotic activity of PXT1 was not unique to HeLa cells, we performed similar experiments in NIH3T3 cell line. The quantitative

analysis demonstrated that the kinetics of cell death in NIH3T3 cells were essentially the same as observed in HeLa cells (Supplementary Fig. 1).

One of the early characteristics of apoptosis is the externalization of phosphatidylserine (PS) residues on the outer plasma membrane (Casciola-Rosen *et al.*, 1996). Therefore, to further prove the pro-apoptotic properties of PXT1 we performed Annexin V Alexa 568 assay of EGFP-PXT1 and empty EGFP (mock) transfected HeLa cells. As can be seen in Figure 3D, the majority of EGFP-PXT1 expressing cells were positive for Annexin V and showed typical peripheral staining of the plasma membrane. The quantification revealed that percentage of Annexin V-positive cells was approximately 4-fold higher in EGFP-PXT1 expressing cells than in EGFP mock cells (75%±4 vs 19%±5, respectively, $p < 0.001$).

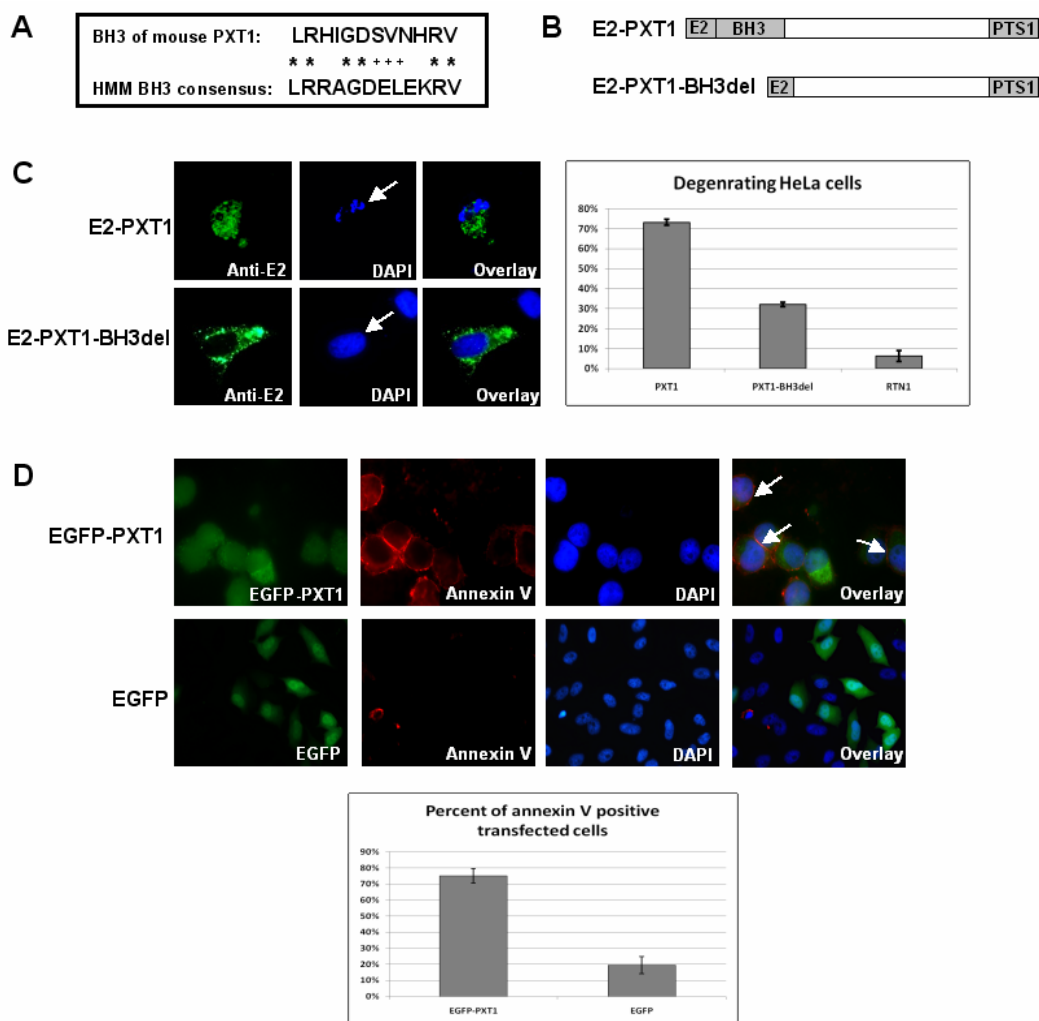


Figure 3. The analysis of the BH3-like domain of mouse PXT1. (A) The PFAM database search using the hidden Markov model (HMM) revealed the presence of BH-like motif at the N-terminus of PXT1. (B) To

analyse whether the BH3 motif is functional two constructs were generated. The first construct expresses E2-PXT1 protein containing the complete ORF of PXT1 fused with E2 tag. The second construct encodes E2-PXT1-BH3del, a truncated PXT1 protein lacking the BH3-like domain. (C) The overexpression of E2-PXT1 protein induces cell death in transfected HeLa cells. Using anti-E2 tag antibody the fusion protein could be detected in the cytoplasm of degenerating cells (upper panel). 73 % of all transfected HeLa cells demonstrated evident signs of degeneration. In contrast, the majority of E2-PXT1-BH3del-positive cells appeared normal (lower panel) and only 32 % of them showed signs of degeneration. As a control, cells were transfected with Rtn1pQM-Ntag/A vector encoding the RTN1 protein. (D) To validate that degenerating cells indeed undergo apoptosis, the EGFP-PXT1 transfected cells were stained with Annexin V Alexa 568. Green fluorescence was observed in cells expressing EGFP-PXT1 fusion protein. Cells positive for Annexin V showed red staining. The analysis demonstrated that the plasma membrane of EGFP-PXT1 expressing cells was positive for Annexin V (overlay, white arrows). The control cells were transfected with EGFP alone. As presented in the diagram, a significant majority of EGFP-PXT1-positive cells showed Annexin V staining in contrast to EGFP transfected cells.

PXT1 interacts with BAT3

To gain further insights into the function of PXT1 in the testis we performed a yeast two-hybrid (Y2H) screen of mouse testis cDNA library using as a bait protein the full-length of murine PXT1. The screen of total 3.77×10^6 transformants yielded two individual positive clones harbouring in-frame sequence of BAT3 protein. The first identified clone (no 184) contained exons 7 to 22 and the second (no 100) exons 7 to 25 of *Bat3* cDNA sequence. Direct Y2H assay validated the interaction of PXT1 with BAT3, as can be seen for clone no 100 in Figure 4A. White yeast colonies growing on medium lacking leucine and tryptophan (-LT) served as co-transformation control and blue colonies growing in high stringency conditions (medium lacking leucine, tryptophan, histidine and adenine, containing X- α -gal (-LTHA+ α -gal)) indicated PXT1-BAT3 interaction. To confirm the specific and physical binding of PXT1 to BAT3 in mammalian cells the co-immunoprecipitation (CoIP) assay was performed. HeLa cells were transiently co-transfected with vectors expressing E2-PXT1-BH3del (6 kDa) and c-MYC-BAT3 (101 kDa) fusion proteins. We intentionally used the plasmid encoding PXT1 protein lacking its BH3-like domain to avoid the induction of apoptosis and facilitate protein isolation. 24 hrs after transfection protein lysates of HeLa cells were subjected to immunoprecipitation using anti-E2 tag or anti-myc tag antibody, followed by Western blot analysis with anti-myc tag or anti-E2 tag antibody, respectively. The samples of total protein extracts (input) not subjected to immunoprecipitation were used as a positive control (Fig. 4B, C). We could efficiently co-precipitate c-MYC-BAT3 and E2-PXT1-BH3del with anti-E2 tag (Fig. 4B) or anti-myc tag (Fig. 4C) antibody. We did not find any unspecific precipitation of

c-MYC-BAT3 protein in lysate of cells transfected with c-MYC-BAT3 vector only using the anti-E2 antibody, although this protein could be detected in input fraction (Fig. 4B). In lysates of untransfected cells, which served as specificity control, no positive signals were detected (Fig. 4B, C). Taken together, our results clearly demonstrate that mouse PXT1 interacts with BAT3.

Determination of the binding regions between PXT1 and BAT3

To characterize the structural requirements of the PXT1-BAT3 association we generated a set of constructs expressing truncated PXT1 and BAT3 proteins and tested them via direct Y2H assay for interactions with Bat3pGAD10 (clone no 100) or Pxt1pGBKT7 (encoding a full-length PXT1) vectors, respectively. For mapping the BAT3-binding site in PXT1 protein sequence we used four different fragments cloned in pGBKT7 vector, two encoding N-terminal, namely 1a (aa 2-30) and 1b (aa 2-26) as well as two encoding C-terminal, namely 2a (aa 24-51) and 2b (aa 20-51) parts of PXT1 (Fig. 4D). Subsequently, the direct Y2H experiments, using as a prey Bat3pGAD10 vector (clone no 100) and as a bait pGBKT7 vector containing sequence encoding one of above mentioned PXT1 fragments, were performed. The blue yeast colonies growing on $-LTHA$, $+\alpha$ -gal plates indicated the interaction, whereas the white yeast colonies growing on $-LT$ plates served as positive control of co-transformation. The analysis demonstrated that the site of PXT1-BAT3 interaction was included in 1a, 2a, 2b but not in 1b part of PXT1 (Fig. 4D). The minimum region of overlap between BAT3-binding fragments of PXT1 was found to be a short motif of Leu-Ala-Pro-Phe (LAPF) at aa position 27-30. To further validate whether this sequence forms a functional core of the interaction motif, we performed an additional direct Y2H assay using as bait a mut-part1aPxt1pGBKT7 vector encoding a mutated 1a fragment of PXT1 in which LAPF was changed into GAPA sequence (Fig. 4E). The mutation of leucine into glycine and phenylalanine into alanine within the LAPF motif in 1a fragment of PXT1, prevented its binding to BAT3, thereby confirming its essentiality for this interaction (Fig. 4E). The localization of PXT1-binding site in BAT3 was performed according to the same strategy. Yeasts were transformed with the Pxt1pGBKT7 vector (encoding a full length PXT1) and one of the three different BAT3 fragments cloned into pGADT7 vector. As illustrated in Figure 4F, fragment 1 of BAT3 was equivalent to aa 169-323, fragment 2 located at aa positions 359-761 contained proline- and glutamine-rich regions and fragment 3 resided at aa positions 756-1119 and included the C-terminal BAG

domain. The direct Y2H assays demonstrated that PXT1-binding site in BAT3 overlaps with proline- and glutamine-rich regions in fragment 2 (Fig. 4F).

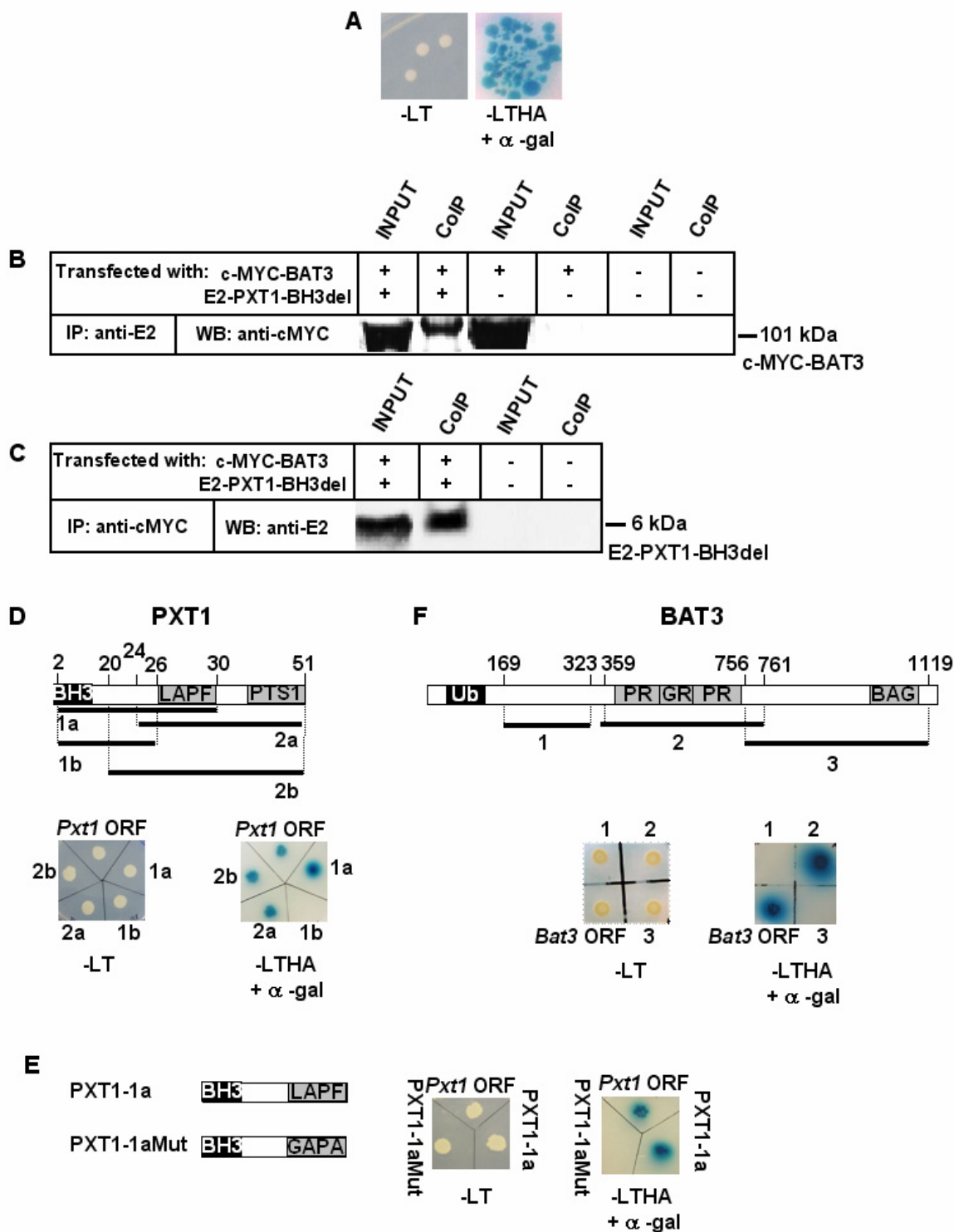


Figure 4. The analysis of PXT1–BAT3 interaction. (A) Confirmation of the association between PXT1 and BAT3 via direct yeast two-hybrid assay using *Pxt1p*GAD10 as a bait and *Bat3p*GAD10 as a prey vector. White yeast colonies growing on nutritional medium lacking leucine and tryptophan (-LT) served as a

positive control of co-transformation. Blue yeast colonies growing on medium lacking leucine, tryptophan, histidine and adenine (-LTHA), but containing X- α -gal (+ α -gal) indicates activation of GAL-4 reporter genes by PXT1-BAT3 interaction. (B-C) Co-immunoprecipitations of murine BAT3 and PXT1. (B) E2-PXT1-BH3del and c-MYC-BAT3 were co-expressed in HeLa cells. As a control, proteins from c-MYC-BAT3 single transfected cells and from untransfected cells were used. Protein extracts were subjected to immunoprecipitation using the anti-E2 tag antibody. A 5 % of the total protein volume was not subjected to immunoprecipitation and used as positive control (input). Next, Western blot analysis using anti-c-myc tag antibody was performed. The 101 kDa-band representing the c-MYC-BAT3 fusion protein is clearly visible in proteins from co-transfected cells. In contrast, no signal can be detected in proteins from single transfected cells with c-MYC-BAT3 only, although a strong band is visible in input. Similar, no unspecific signals were observed in proteins from untransfected cells. (C) The reverse co-immunoprecipitation demonstrated that E2-PXT1-BH3del fusion protein could be efficiently co-precipitated with anti-c-myc tag and detected by anti-E2 tag antibodies. Proteins from untransfected cells served as a negative control and confirmed the specificity of the reaction. (D) Schematic view of truncated fragments of PXT1 used for identification of a domain responsible for the interaction with BAT3 in direct yeast-two hybrid assay. White yeast colonies growing on -LT plate demonstrated successful co-transformation. Blue colonies growing on -LTHA, + α -gal plate represented the interaction of BAT3 with different parts of PXT1. No growth of yeasts expressing BAT3 and part 1b of PXT1, which lacks the LAPF motif, indicates that this sequence is indispensable for the interaction. (E) To check the functionality of the LAPF motif a mutation was introduced into the sequence of part 1a-PXT1. Although efficient co-transformation was demonstrated on -LT plate, yeasts containing BAT3 and part 1a of PXT1 with the GAPA sequence instead of LAPF were not able to grow on -LTHA, + α -gal plate. This result indicates that the LAPF motif is important for PXT1-BAT3 interaction. (F) Schematic representation of truncated fragments of BAT3 generated for identification of BAT3 domain involved in the interaction with PXT1. Yeasts were co-transformed with Pxt1pGBKT7 and pGADT7 vector containing one of three parts of *Bat3*. Only yeasts containing part 2 were able to grow on -LTHA, + α -gal plate indicating that this part contains the domain responsible for the interaction with PXT1. Efficient co-transformation was demonstrated on -LT plate. BH3 – BH3-like domain; PTS1 – peroxisomal targeting signal type 1; Ub – ubiquitin-like domain; BAG – BCL-2 associated athanogene domain; PR/GR – proline-, glutamine-rich, respectively.

Nuclear BAT3-PXT1 co-localization inhibits pro-apoptotic activity of PXT1

In order to localize the intracellular site of PXT1-BAT3 interaction we investigated the co-expression patterns of EGFP-PXT1 and BAT3-dsRED fusion proteins in HeLa and NIH3T3 cells. Surprisingly, the co-localization of the fusion proteins was observed in the nucleus of co-transfected HeLa cells (Fig. 5 A-D). Nevertheless, it should be noted that in few HeLa cells the co-localization could also be found in both nucleus and cytoplasm (data not shown). The co-localization study in NIH3T3 cells demonstrated the overlapping fluorescence signals in the nuclear and/or cytoplasmic compartments (Supplementary Fig. 2). These observations suggest that the nucleus is a main site of interaction between PXT1 and BAT3 and that BAT3 could mediate nuclear import of cytoplasmic PXT1. Interestingly, during this analysis we noticed that cells co-expressing EGFP-PXT1 and BAT3-dsRED fusion proteins displayed a normal, non-apoptotic morphology (Fig. 5 A-D).

As presented in Figure 5E, quantification analysis demonstrated that level of apoptosis in HeLa cells overexpressing both fusion proteins was significantly lower than that observed in cells overexpressing only EGFP-PXT1 ($18\% \pm 2$ vs $73\% \pm 1$, respectively, $p < 0.001$). These findings imply that BAT3 might be involved in regulation of PXT1-induced apoptosis.

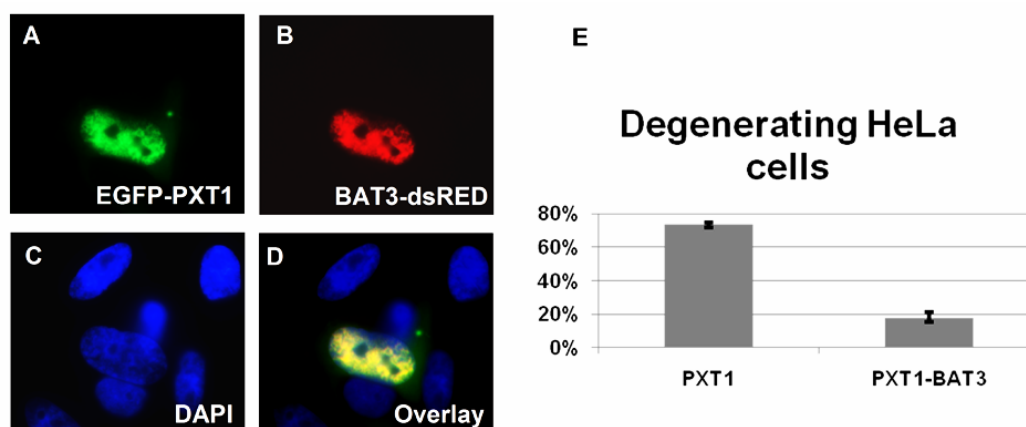


Figure 5. BAT3 translocates PXT1 to the nucleus and protects cells from PXT1-induced apoptosis. HeLa cells were transiently co-transfected with Pxt1EGFP and Bat3DsRed vectors. (A) Green fluorescence was observed for PXT1-EGFP, whereas (B) red fluorescence indicates BAT3-dsRED fusion proteins. (C) Nuclei were counterstained with DAPI and display normal non-apoptotic morphology. (D) Green-red overlay (yellow) is clearly visible in the nucleus of co-transfected cells and represents the co-localization of both fusion proteins. (E) The quantification analysis showed that the level of apoptosis in HeLa cells co-overexpressing PXT1-EGFP and BAT3-dsRED fusion proteins is approximately 4-fold lower than that observed in PXT1-EGFP only overexpressing cells. The experiments demonstrate that PXT1-induced apoptosis is repressed by overexpression of BAT3.

DISCUSSION

The expression of *Pxt1* is restricted to male germ cells and starts at primary spermatocyte stage (Grzmil *et al.*, 2007). To elucidate the role of *Pxt1* in male germ cells we have generated a transgenic line in which the cMYC-PXT1 fusion protein is expressed under the control of the 1.4-kb region of the human *PGK2* promoter. The *PGK2* promoter is known to drive male germ cell specific gene expression beginning at primary spermatocyte stage (Robinson *et al.*, 1989; Tascou *et al.*, 2001). We demonstrated that overexpression of *Pxt1* strongly induces degeneration of germ cells, subsequently leading to disruption of spermatogenesis and finally male infertility. Using the TUNEL and Annexin V assays we have demonstrated that germ cells of transgenic males and transiently transfected cells (HeLa and NIH3T3) overexpressing PXT1 undergo apoptosis. At the N-terminus of PXT1

we identified a putative BH3-like domain and confirmed its importance for the pro-apoptotic activity of this protein. Moreover, we demonstrated the interaction between PXT1 and known apoptosis regulator BAT3. Interestingly, the cells overexpressing both PXT1 and BAT3 showed a strongly reduced apoptosis rate as compared to PXT1-only overexpressing cells. Therefore, we suggest that the PXT1-BAT3 interaction protects cells from PXT1-induced cell death.

Members of the BCL-2 (B cell leukemia/lymphoma-2) family of proteins are well known regulators of apoptosis (Adams and Cory 2001; Spierings *et al.*, 2005; Skommer *et al.*, 2007). Based on the composition of BH (BCL-2 Homology) domains, this family was divided into three subclasses. One of them consists of proteins containing only the BH3 domain, which serve as apoptosis inducer (reviewed in Lomonosova and Chinnadurai 2008). The Pfam-A (<http://www.pfam.sanger.ac.uk/search>) search using the hidden Markov model (HMM) (Sonnhammer *et al.*, 1998) revealed that the N-terminal part of mouse PXT1 contains a putative BH3-like sequence, which is highly conserved between mouse, chimpanzee and human. This sequence follows the consensus of BH3 motif: $\Phi\Sigma\text{XX}\Phi\text{XX}\Phi\Sigma\text{DZ}\Phi\Gamma$, where Φ represents hydrophobic residues, Σ – small residues, Z – acidic residue and Γ is a hydrophilic residue (Day *et al.*, 2008). The core of the BH3 domain consisting of LXXXGDE residues (Lanave *et al.*, 2004) was also found within the BH3 sequence of PXT1 (LRHIGDS). The only difference is that in PXT1 instead of the acidic E the neutral S is present within this sequence. It should be noted that the E residue is not strictly conserved (Lomonosova and Chinnadurai 2008) and that a variant of BH3 motif with S was also found in human BID protein, which has a pro-apoptotic function (Tan *et al.*, 1999; Billen *et al.*, 2009).

Conserved L and D amino acids of the BH3 core sequence are involved in the interaction and neutralization of anti-apoptotic proteins thus triggering apoptosis (Hinds and Day 2005). The overexpression of PXT1 resulted in apoptosis in two transiently transfected cell lines (HeLa and NIH3T3), whilst the deletion of the BH3 domain caused significant reduction of apoptosis rate in transiently transfected cells. However, the decrease in apoptotic cell death did not reach the levels seen in control transfection, suggesting that PXT1 might contain some other domains which could be involved in the apoptotic signaling. The BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) also contains a BH3 motif and can induce apoptosis in transfected cells. The deletion of the sequence encoding the BH3 domain resulted in significant reduction of cell death, however

the apoptotic rate remained above the value for control transfection (Yasuda *et al.*, 1998). Similar results were also obtained for *Bbc3* (Han *et al.*, 2001) and *Spike* (Mund *et al.*, 2003), indicating that this is a relatively common phenomenon. Thus, we can conclude that the BH3 domain of PXT1 protein is functional and responsible for pro-apoptotic activity.

The signalling pathway controlling the apoptosis events mediated by BCL-2 protein family is essential for proper spermatogenesis (reviewed in Sofikitis *et al.*, 2008). Our transgenic model clearly demonstrates that PXT1 protein is also involved in apoptotic regulation of the cellular homeostasis in the testis. We showed that the overexpression of PXT1 induces apoptosis of male germ cells, mainly spermatocytes. Similar progressive germ cells degeneration was observed in mice with gene-trap mutation of *Bcl2l2* (also known as *Bcl2w*) gene (Ross *et al.*, 1998; Russell *et al.*, 2001). In summary, it can be concluded that PXT1 belongs to the BH3-only protein class of BCL-2 family.

HLA-B-associated transcript 3 (*Bat3*) was reported to modulate apoptosis through interactions with other apoptotic regulators (Thress *et al.*, 1998; Minami *et al.*, 2007; Desmots *et al.*, 2005, 2008). Using different techniques, we were able to demonstrate that PXT1 specifically interacts with BAT3, a BAG domain-containing protein. The first characterized BAG protein, termed BAG1, was shown to interact with BCL-2 by its BAG domain (Takayama *et al.*, 1995). However, the interaction of BAT3 with PXT1 is not mediated by the BAG domain, but involves the part of BAT3 between amino acids 359-761. The same region of BAT3 was reported to interact with BORIS protein (Nguyen *et al.*, 2008), thus strongly supporting the finding that a domain responsible for protein-protein interaction must be present in this region. The proline- and glutamine-rich sequences identified in this part of BAT3 protein might represent so called low-complexity region (Wootton and Federhen 1996) reported to be involved in protein-protein interactions (Wright and Dyson 1999; Sonnhammer and Wootton 2001). We also identified the region of PXT1 indispensable for the interaction with BAT3. The data base search using the BLAST program (Altschul *et al.*, 1990) revealed that within the LAPF motif, the L and F amino acids are highly conserved between mouse, human and chimpanzee. Using direct yeast two-hybrid system, we could demonstrate that indeed the L and F represent key residues in the LAPF motif. Their mutation into G and A, respectively, was found to completely abolish the PXT1-BAT3 interaction, hence suggesting that the LAPF motif reflects at least the core of the PXT1 domain which interacts with BAT3.

Full length BAT3 was reported to function as an anti-apoptotic protein (Wu *et al.*, 2004; Kikukawa *et al.*, 2005). Here, we show that the interaction of BAT3 with PXT1 protects cells from PXT1-induced cell death. Moreover, in cells co-transfected with BAT3 and PXT1 the co-localization signals were observed in the nucleus. It has been reported, that BAT3 can interact with another pro-apoptotic protein, namely papillomavirus binding factor (PBF) (Sichtig *et al.*, 2007). Likewise, the BAT3-PBF interaction resulted in a significant decrease in PBF-induced cell death (Tsukahara *et al.*, 2009). The authors also demonstrated that in osteosarcoma cells PBF-induced apoptosis could be suppressed by nuclear co-localization of PBF and BAT3. In contrast, the cytoplasmic BAT3-PBF interaction in 293EBNA cells could not inhibit the PBF-induced cell death (Tsukahara *et al.*, 2009). Our results are in agreement with the conclusion that BAT3 could regulate apoptosis through mediating nucleus-cytoplasm shuttling of proteins, which is well recognized as a regulatory mechanism of apoptosis (Wang and Hung 2005; Chu *et al.*, 2007; Salmena and Pandolfi 2007; Tsukahara *et al.*, 2009).

The expression of *Bat3* was reported to be the strongest in the testis (Wang and Liew 1994; Desmots *et al.*, 2005). In mutants with targeted disruption of *Bat3* the first changes of testicular histology were observed at 14 *dpp*, the phenotype became more severe at 42 *dpp* with increased apoptosis events. The degeneration of germ cells was progressive and only few spermatocytes and no spermatids were observed at 140 *dpp* (Sasaki *et al.*, 2008). The similarity in phenotype of both *Bat3*^{-/-} and PXT1 transgenic line indicates that the PXT1-BAT3 interaction could be important in the regulation of spermatogenesis. We suggest that in PXT1-overexpressing males the balance in concentration of both proteins is disturbed thus endogenous BAT3 expression might not be sufficient to preserve normal spermatogenesis.

Peroxisomes are responsible for different metabolic pathways including plasmalogen synthesis (Brites *et al.*, 2003). In testis plasmalogens protect germ cells from the negative effect of very-long-chain fatty acids (VLCFAs) and the disruption of plasmalogen synthesis leads to germ cell degeneration and apoptosis (Brites *et al.*, 2009). The importance of peroxisomal fatty acids metabolism was also demonstrated in Sertoli cells (Huyghe *et al.*, 2006a, b). It has been also proposed that peroxisomes play a role in acrosome formation (Moreno and Alvarado 2006). Catalase, an enzyme normally present in peroxisomes in somatic cells, was detected in the acrosome region of sperm (Figuroa *et al.*, 2000). However, until now almost nothing has been known about the role of

peroxisomal proteins in controlling apoptosis. A rare exception represents the fission 1 (mitochondrial outer membrane) homolog (*Fis1*) gene, which is localized in both the outer mitochondrial membrane and in peroxisomes (Kuravi *et al.*, 2006). RNAi mediated *Fis1* depletion affected cell sensitivity to apoptosis (Lee *et al.*, 2004). Nevertheless, to our knowledge no peroxisomal protein was reported to act as a pro-apoptotic factor. The function of PXT1 in inducing apoptosis suggests that peroxisomes in male germ cells are involved in programmed cell death indicating a new function of this important cellular compartment.

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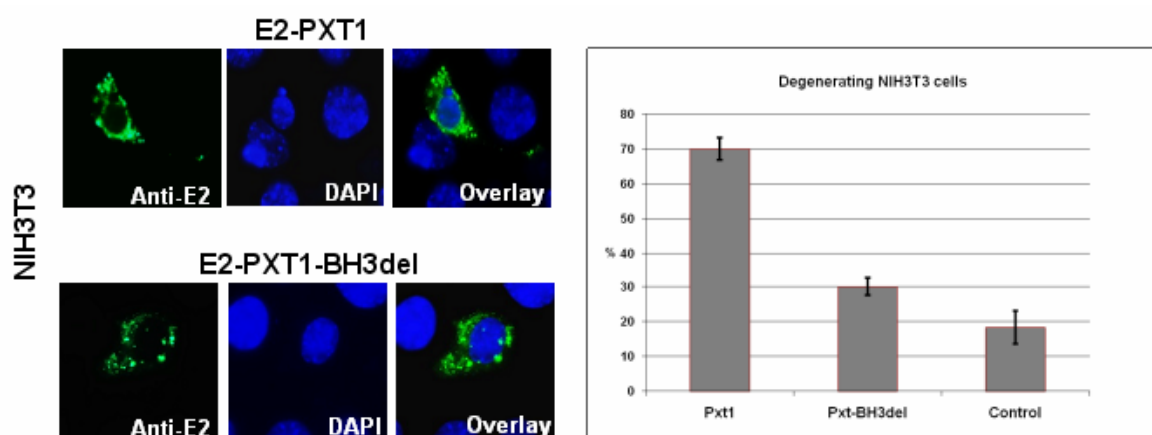
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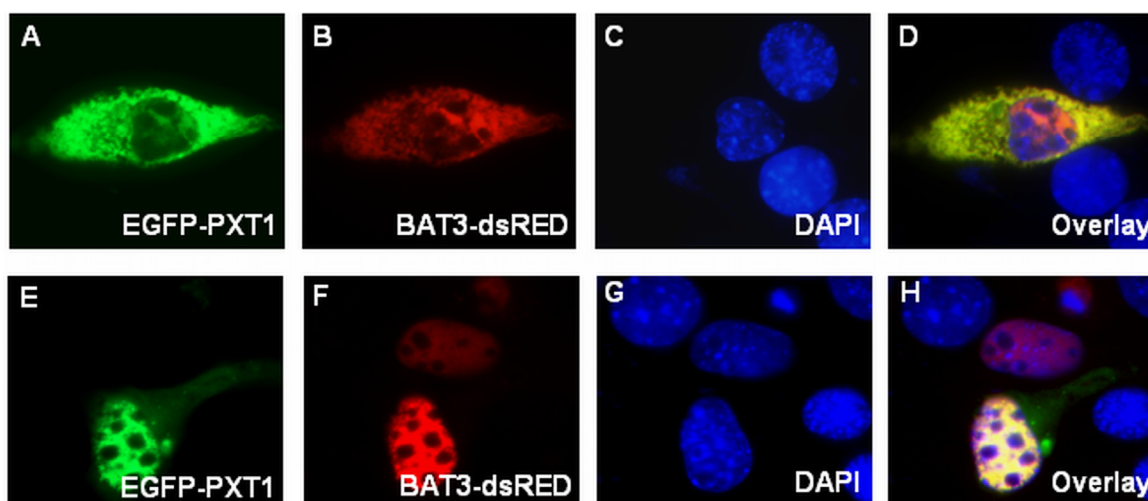
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SUPPLEMENTAL MATERIALS



Supplementary Figure 1. Overexpression of PXT1 protein induces cell death in transfected NIH3T3 cells. The E2-PXT1 fusion protein was detected with anti-E2 tag antibody in cytoplasm of degenerating cells (upper panel). The deletion of BH3 domain strongly reduces the apoptosis of cells transfected with E2-PXT1-BH3del and many of them appear normal (low panel). Quantification of apoptosis in transfected cells clearly demonstrated that the deletion of BH3 domain significantly decreases cell death.



Supplementary Figure 2. NIH3T3 cells were transiently co-transfected with Pxt1EGFPc1 and Bat3DsRed vectors. (A) Green fluorescence representing EGFP-PXT1 was observed in cytoplasm, whereas (B) red fluorescence for BAT3-dsRED-positive cells was present in cytoplasm and nucleus. (C) Nuclei were counterstained with DAPI. (D) Green-red overlay (yellow) was clearly visible in the cytoplasm of some transfected NIH3T3 cells. However, in many cells the green signals were located in nuclei (E) where also red signals were visible (F). (G) Nuclei were counterstained with DAPI and (H) overlay clearly demonstrated co-localization of EGFP-PXT1 and BAT3-dsRED in nuclei of transfected cells. The vast majority of NIH3T3 cells in which both fusion proteins were detected in the nucleus did not show any apoptotic symptoms. This experiment demonstrates that PXT1-induced apoptosis is repressed by overexpression of BAT3.

Supplementary File 1. Sequences of primers used.

C-mycTag-new-F: 5'CCGAATTCCTCGTCCGAGCAAGCTTGATTTA3'
 Ad.C-mycTag-R: 5'TTGC GGCCGCTTTTGCTCCATGGTGAGGTC3'
 adPxt1-ORF-F: 5'GGTGGCGCCGCTGGTGGCATGCAGCTTAGACACATTGG3'
 adPxt1-ORF-R: 5'ATCCGCGGCCTTACAGCAAATGGTTGTTCCAG3'
 hGH-3UTRsacII-F: 5'TTCCGCGGCTGCCCCGGGTGGCATCCCC3'
 hGH-3UTRsacII-R: 5'TACCGCGGCATAACCACCCCTCCAC3'
 ad.polA-SV40-F: 5'TTCCGCGGGTTACAAATAAAGCAATAGCATCAC3'
 ad.polA-SV40-R: 5'TCCGAGCTCCGCTTACAATTTACGCCTTAAGAT3'
 gen_hPGK2_F1: 5'CCAGGAAGTTGGAATCTTCACC3'
 gen_Pxt1ex.2-3_R: 5'GTGCAAGATGCTCCTGAATCAC3'
 TP2_F1: 5'AACCAGTGCAATCAGTGCACC3'
 TP2_R1: 5'ATGGACACAGGAACATCCTGG3'
 Pxt1-ORF-EcoRI-F: 5'ATGGTAGAATCCAGCTTAGACACATTGGGGA3'
 Pxt1-ORF-BamHI-R: 5'ATGGTAGGATCCACAGCAAATGGTTGTTCCAG3'
 Q-PCR_cmyc_F: 5'TTGAATGAAATGGAGAGCTTGG3'
 Q-PCR_Pxt1ex2-3_R: 5'GTGCAAGATGCTCCTGAATCAC3'
 mHPRT-For-Q: 5'AGCCCCAAAATGGTTAAGTTGC3'
 mHPRT-Rev-Q: 5'TTGCAAGATTCAACTTGCCTCAT3'
 Eef1a1F1: 5'AAGCTGAGCGTGAGCGTGGT3'
 Eef1a1R1: 5'TGTGGGCTGTGTGACAATCC3'
 Pxt1FPCAV2FP: 5'TGGTAAGCTTCCCAGCTTAGACACATTGGGGAC3'
 Pxt1FPCAV2RP: 5'TGGTGGTACCACTTACAGCAAATGGTTGTTCCAGA3'
 Bat3FPCAV3FP: 5'TGGTTCTAGAGAAGCTCCTCCTCGAGAGCCC3'
 Bat3FPCAV3RP: 5'TGGTAAGCTTCGGGGTCATCAGCAAATGCC33'
 5'AD-LD-insert: 5'CTATTCGATGATGAAGATACCCACCAAACC3'
 3'AD-LD-insert: 5'GTGAACTTGC GGGGTTTTTTCAGTATCTACGA3'

Supplementary File 2. Expression constructs used in this study.

Pxt1pQM-Ntag/A. The ORF of *Pxt1* gene was amplified by RT-PCR using testis cDNA and Pxt1-ORF-BamHI-F: 5'ATGGTAGGATCCCAGCTTAGACACATTGGGGA3', Pxt1-ORF-KpnI-R: 5'ATGGTAGGTACCTTACAGCAAATGGTTGTTCCAG3' primers. The RT-PCR product was cloned in-frame into *BamHI/KpnI* restriction sites of pQM-Ntag/A intron vector (Abcam).

Pxt1ΔBH3pQM-Ntag/A. The fragment of *Pxt1* ORF lacking the sequence encoding the BH3-like domain was obtained using the same strategy as described above but with different forward primer: Pxt1-BH3del-BamHI-F: 5'ATGGTAGGATCCCACAGGGTGATTCAGGAGCAT3'.

Rtn1pQM-Ntag/A. The expression vector was generated as described previously (Mannan *et al.*, 2006).

Bat3pCMV. Bat3 cDNA sequence identified in Y2H screening and containing exons 7 to 25 flanked by a *NotI* restriction sites was subcloned from Bat3pGAD10 vector into *NotI* site of pCMV-Myc vector (Clontech, Germany) and used for immunoprecipitation experiments.

Pxt1EGFP-C1. The cloning of *Pxt1* coding sequence into the pEGFP-C1 vector (Clontech, Germany) and generation of vector expressing PXT1-EGFP was described previously (Grzmil *et al.*, 2007).

Bat3DsRed. For subcellular co-localization analysis of PXT1 and BAT3 a BAT3-dsRED fusion protein was generated. The cDNA fragment of *Bat3*, corresponding to the sequence identified in Y2H screening, was amplified on testis cDNA by RT-PCR with Bat3RedN1HindIII-FP: 5'ATGGTAAAGCTTGCCACCATGGCCTTGA ACTCACAAAC ATCAGAACC3' and

Bat3RedN1KpnIRP: 5'ATGGTACGGTACCGTATCAGCAAATGCCCGATG3' primers and cloned in-frame into *HindIII/KpnI* restriction sites of the pDsRed-Monomer-N1 vector (Clontech, Germany).

Pxt1pGBKT7. The *Pxt1* insert was obtained using the same primers as for generation of *Pxt1*-specific Northern blot probe and cloned into *EcoRI/BamHI* restriction sites of pGBKT7 vector (Clontech, Germany).

part1a/part1b Pxt1pGBKT7. The same forward primer and the same strategy as for construction of Pxt1pGBKT7 vector and following reverse primers: 2Pxt-1-BamHI_R1: 5'ATGGTAGGATCCAAAAGGAGCCAGCACATCTC3' and 3Pxt-1-BamHI_R1: 5'ATGGTAGGATCCCACATCTCCGACTTCCTGTG3' were used in RT-PCR to generate the *Pxt1* part "1a" and "1b", respectively.

part2a/part2b Pxt1pGBKT7. The same strategy as for construction of Pxt1pGBKT7 vector and following common for both parts reverse primer Pxt-2-BamHI_R2: 5'ATGGTAGGATCCACAGCAAATGGTTGTTCCAGAA3' and different forward primers: 2Pxt-2-EcoRI_F2: 5'ATGGTAGAATTCGGAGATGTGCTGGCTCCTTTT3' for generation of a *Pxt1* part "2a" and 3Pxt-2-EcoRI_F2: 5'ATGGTAGAATTTCGCACAGGAAGTCGGAGATGTG3' for generation of a *Pxt1* part "2b" were used.

mut-part1aPxt1pGBKT7. The same forward primer and the same strategy as for construction of part1aPxt1pGBKT7 vector and following reverse primer: Pxt1LFmutRP: 5'ATGGTAGGATCCTGCAGGAGCACCCACATCTCCGACTTCCTG T3' were used in order to introduce mutation in part 1a of *Pxt1* ORF.

part1Bat3pGADT7. The mouse *Bat3* cDNA "part 1" located between exon 6 and 9 was amplified on testis cDNA by RT-PCR with Bat3-1FP: 5'TGGTCATATGGCTCAGCACATGATTAGG3' and Bat3-1RP: 5'ACCTATCGATGCAACCTCTGGTCCTCCTCAC3' primers and then was cloned into *NdeI/ClaI* restriction sites of pGADT7 vector (Clontech, Germany).

part2Bat3pGADT7. The "part 2" of mouse *Bat3* cDNA containing exons 9 to 15 was obtained in RT-PCR with Bat3-2FP: 5'TGGTCATATGGTTCGGCCTATGTCTCACT3' and Bat3-2RP: 5'ACCTATCGATAGATGTTGCTGGATCCACTG3' primers using the same strategy as described above.

part3Bat3pGADT7. The "part 3" of mouse *Bat3* cDNA located between exon 15 and 24 was generated by RT-PCR amplification with Bat3-3FP: 5'TGGTCATATGAGTGGATCCAGCAACATCTTTG3' and Bat3-3RP: 5'ACCTATCGATGGCTCTCCTGAACCTCTGGTG3' primers according the same strategy as described above.

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Corresponding Author	Dr. Pawel Grzmil (University of Göttingen)
Contributing Authors	Karina Kaczmarek , Maja Studencka , Andreas Meinhardt , Krzysztof Wiczerzak , Wolfgang Engel
Abstract	<p>Peroxisomal testis specific 1 (Pxt1) gene is the only male germ cell-specific gene that encodes a peroxisomal protein known to date. To elucidate the role of Pxt1 in spermatogenesis, we generated transgenic mice expressing a c-MYC-PXT1 fusion protein under the control of the PGK2 promoter. Overexpression of Pxt1 resulted in induction of male germ cells apoptosis mainly in primary spermatocytes, finally leading to male infertility. This prompted us to analyse the pro-apoptotic character of mouse PXT1, which harbours a BH3-like domain in the N-terminal part, in more detail. In different cell lines the overexpression of PXT1 also resulted in a dramatic increase of apoptosis, whilst the deletion of the BH3-like domain significantly reduced cell death events, thereby confirming that the domain is functional and essential for the pro-apoptotic activity of PXT1. Moreover, we demonstrated that PXT1 interacts with apoptosis regulator BAT3. This association protects cells from the PXT1-induced apoptosis by shuttling PXT1 from the cytoplasm to the nucleus. In summary, we demonstrated that PXT1 induces apoptosis via the BH3-like domain and that this process is inhibited by interaction with BAT3.</p>
Highlight Summary for TOC	<p>The overexpression of PXT1 in mouse testis leads to germ cells apoptosis and male infertility. The PXT1 protein contains functional BH3-motif responsible for the apoptosis induction in transiently transfected cells. PXT1 protein interact with BAT3, this association protects cells from PXT1-induced apoptosis.</p>
Monitoring Editor	Assigned
Subject Categories	Differentiation & Development/Gametogenesis, Model Systems/Transgenic Mice, Signal Transduction/Apoptosis, Methodology/Fluorescence/Molecular Microscopy
Reviews and Decision Letter from Another Journal	No editor's disposition letter or peer reviewers' comments from another journal are included.

4. Discussion

Approximately 15 % of couples suffer from infertility and almost half of all cases is due to male infertility factors, often of genetic origin (Feng, 2003). The precise regulation of spermatogenesis via, among others, stage-/cell-specific gene expression and male germ cell apoptosis, is essential for male fertility. Considering the fact that genetic causes of male infertility remain largely unknown it is very important to evaluate the biological function of a new testicular gene, like *Pxt1*. In the present study, the association of PXT1 with CCDC33 protein was determined. We characterized the expression profile of *Ccdc33* and subcellular localization of the protein encoded by this gene. In addition, we demonstrated that PXT1, a novel BH3 like-only protein, can induce apoptosis in both, male germ cells of transgenic mice and transiently transfected mammalian cells. We showed further that testis-specific overexpression of *Pxt1* leads to complete infertility of male mice. Moreover, we identified BAT3 as PXT1-interacting protein that can regulate its pro-apoptotic properties.

4.1. The implication of peroxisomal proteins in spermatogenesis

Murine *Pxt1* was first described by Grzmil et al. in 2007 as a gene that encodes a male germ cell-specific peroxisomal protein. The authors analyzed expression pattern of *Pxt1* by Northern blot technique. Considering the fact that often faint gene expression can not be visualized by Northern blot (Reyes et al., 1998; Jager et al., 2002), we re-investigated the transcription profile of *Pxt1* using a more sensitive mRNA detection method, namely RT-PCR. As can be seen in figure 4.1., we confirmed *Pxt1* testis-specificity limited to male germ cells by demonstrating its expression in wild-type testis, but not in germ cell-deficient testis of W/W^v mutant mice.

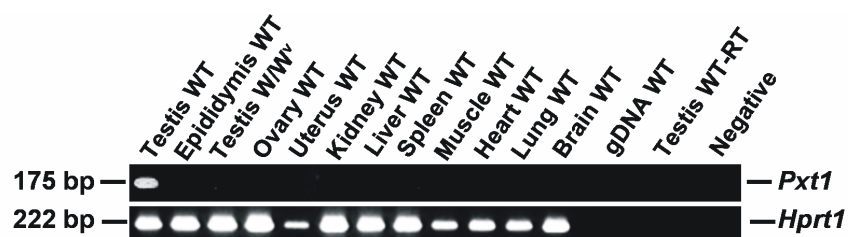


Figure 4.1. RT-PCR analysis of murine *Pxt1* expression profile in different tissues of FVB/N wild-type mice and testis of W/W^v mutant mouse using *Pxt1*-ORF-EcoRI-F and *Pxt1*-ORF-BamHI-R primers (Kaczmarek et

al., 2009b). The cDNA quality was verified by amplification of the *Hprt1* gene. WT: wild-type; W/W^v: mutant mice lacking germ cells in the testis; gDNA WT: wild-type genomic DNA control sample; Testis WT -RT: testis cDNA control sample generated without using reverse transcriptase.

The existence of a germ-cell specific peroxisomal gene, like *Pxt1*, indicates that germ cell peroxisomes might have additional specific functions during spermatogenesis, different to those in somatic cell types of the testis (Grzmil et al., 2007; Luers et al., 2009). Indispensability of peroxisomes for normal spermatogenesis was demonstrated by severe testicular defects, including infertility, induced by peroxisomal dysfunction in numerous knockout mice. Fan et al. (1996) reported that null mutation of ACOX (fatty acyl-CoA oxidase), a key enzyme of peroxisomal β -oxidation pathway, leads to sterility of mice. ACOX knockout males showed absence or marked depletion of Leydig cells and a reduction in spermatid and spermatozoa population. Inactivation of another peroxisomal β -oxidation gene encoding a multifunctional protein-2 (MFP-2) similarly causes male infertility. Histological examination of adult MFP-2 knockout mice showed the presence of lipid inclusions in Sertoli cells, maturation arrest of germ cells and subsequent disintegration of the germinal epithelium. Immature germ cells and debris instead of mature spermatozoa were found in epididymides of such mutant mice (Huyghe et al., 2006). Furthermore, a targeted disruption of dihydroxyacetonephosphate acyltransferase (DAPAT), a fundamental peroxisomal enzyme in the biosynthesis of ether lipids (e.g. plasmalogens), induced the spermatogenic arrest between pachytene spermatocytes and the stage of round spermatids. In adult mutants, testes were atrophic and epididymides were devoid of spermatozoa and contained degenerating spermatids (Rodemer et al., 2003). It was also demonstrated that PEX7 deficient male mice are not fertile (Bites et al., 2003; Bites et al., 2009). Interestingly, different and even more severe pathological alterations in the testis were observed in case of PEX7:ABCD1 double knockout mice, although null mutation of *Abcd1* alone does not lead to any obvious signs of testicular phenotype (Brites et al., 2009). In addition, the Sertoli cell-specific knockout of *Pex13* gene (scsPEX13 ^{-/-}), which is a peroxisomal integral membrane protein with an essential role in both PTS1 and PTS2 protein import, results in male infertility. ScsPEX13 ^{-/-} mice exhibited a later onset and a gradual disruption of spermatogenesis with a loss of germ cells leading to a Sertoli cell only (SCO) phenotype at the age of four months. Moreover, the presence of VLCFA crystals in cytoplasm of strongly proliferating Leydig cells and lipid accumulation in Sertoli cells were observed (Nenicu et al., oral presentation 2009). Similar to the mouse

models, some of male patients suffering from peroxisomal disorders also display fertility problems.

4.2. Peroxisomal CCDC33 is a novel PXT1-interacting protein

To gain insights into the biological function of PXT1 in the testis we screened a testis cDNA library (kindly provided by Prof. Dr. I. Adham, Institute of Human Genetics, University of Göttingen) for PXT1-interacting partners using a yeast two-hybrid approach (Kaczmarek et al., 2009b). Among several putative PXT1-binding proteins, CCDC33 was identified as one of the strongest candidates. As can be seen in figure 4.2., the interaction between PXT1 and CCDC33 was confirmed by direct-Y2H assay and split-EGFP BiFC experiment. The latter analysis is based on the formation of a fluorescent complex by N- and C-terminal non-fluorescent sub-fragments of EGFP, which are brought together by the association of interacting proteins fused to these fragments (Kerppola, 2006). The vector system (FPCAV2 and FPCAV3) used in this study was generated and kindly provided by Prof. Dr. S. Hoyer-Fender (Department of Developmental Biology, University of Göttingen). In HeLa cells co-transfected with the Pxt1FPCAV2 (containing complete ORF of *Pxt1*) and Ccdc33FPCAV3 (containing *Ccdc33* cDNA fragment consisting of the sequence from exons 9 to 20) green fluorescence was visible as a punctate pattern within the cytoplasm, reflecting the peroxisomal localization of interacting proteins (Fig. 4.2. C.).

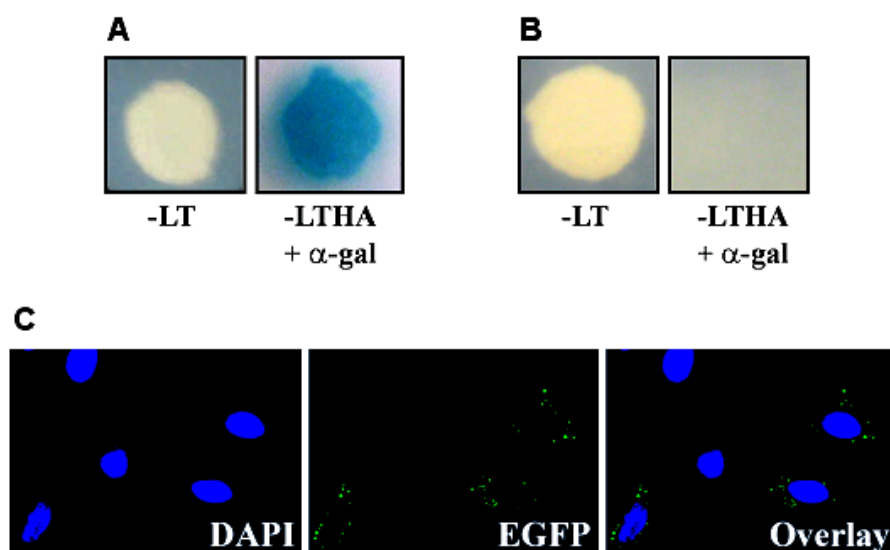


Figure 4.2. Validation of PXT1-CCDC33 interaction. A. Direct-Y2H assay with full-length *Pxt1* in pGBKT7 vector and *Ccdc33* (exons 9-20) in pGAD10 vector. Yeast (AH109 strain) spotted on nutritional medium

lacking leucine and tryptophan (-LT) served as a positive control of co-transformation. Activation of GAL-4 reporter genes via interaction of PXT1 with CCDC33 was demonstrated by robust growth of blue yeast colonies on the high-stringency selection medium lacking leucine, tryptophan, histidine and adenine (-LTHA), but containing X- α -gal (+ α -gal). B. Growth of yeast co-transformed with empty pGBKT7 vector and Ccdc33pGAD10 vector on plate lacking leucine and tryptophan (-LT) but not on high-stringency selection plate (-LTHA + α -gal), confirmed a lack of autoactivation of the prey protein. C. PXT1-CCDC33 association visualized via split-EGFP BIFC assay in HeLa cells. Punctate green fluorescence was observed within the cytoplasm of cells co-transfected with Pxt1FPCAV2 and Ccdc33FPCAV3 vectors. Cell nuclei were counterstained with DAPI. The Pxt1FPCAV2 and Ccdc33FPCAV3 vectors were generated by M. Studencka and E. Niedzialkowska, respectively.

Interestingly, the *Ccdc33* cDNA fragment included in Ccdc33FPCAV3 vector was identical to the sequence identified by Y2H screen and encoded a truncated CCDC33 protein lacking its functional PTS2 signal (see Fig. 2 B., Kaczmarek et al., 2009a). However, it is known that PTS-tagged proteins are able to transport their interacting proteins lacking a peroxisomal targeting signal into the peroxisomes via a “piggy-back” mechanism (Nilsen et al., 2004). Moreover, it should be noted that we intentionally cloned PXT1, which contains a C-terminal PTS1 downstream of the N-terminal EGFP fragment in order to assure its proper subcellular targeting.

To prove physical binding of PXT1 to CCDC33 and *vice versa* by CoIP assay we transiently co-transfected HeLa cells with Pxt1 Δ BH3pQM-Ntag/A (see Materials and Methods, Kaczmarek et al., 2009b) and Ccdc33pCMV (containing *Ccdc33* cDNA fragment encoding exons 9-20, generated by E. Niedzialkowska) vectors. Unfortunately, although set of lysis buffers with gradual increasing detergent concentrations or even sonication have been used, the amount of both, E2-PXT1-BH3del and cMYC-CCDC33 proteins in supernatant (soluble) fraction has never been enough for successful CoIP experiments. As can be seen in figure 4.3., E2-PXT1-BH3del and cMYC-CCDC33 fusion proteins were highly insoluble and almost completely remained in pellet fractions, which are not useful for CoIP analysis. Although CoIP could not be performed, the results from co-localization study (Kaczmarek et al., 2009a), direct-Y2H assay and split-EGFP BiFC experiment clearly demonstrated specificity of the PXT1-CCDC33 interaction.

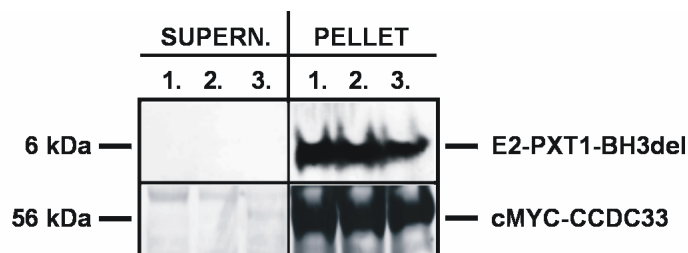


Figure 4.3. Western blot analysis of total protein extracts derived from HeLa cells transiently co-transfected with Pxt1 Δ BH3pQM-Ntag/A and Ccdc33pCMV vectors. Both, E2-PXT1-BH3del and cMYC-CCDC33 fusion proteins were detected exclusively in pellet fractions using anti-E2 tag and anti-myc tag antibody, respectively. Numbers 1-3 represent results from three individual co-transfection experiments.

Moreover, using direct-Y2H assay the CCDC33-binding site in PXT1 sequence was determined. The experiments were performed with Ccdc33pGAD10 as prey vector via identical strategy to that previously described for BAT3 and with the same set of bait vectors, namely part1a/part1b/part2a/part2b/mut-part1a Pxt1pGBKT7 (Kaczmarek et al., 2009b). Surprisingly, the same short motif of Leu-Ala-Pro-Phe (LAPF) within the PXT1 sequence, which was responsible for PXT1-BAT3 association, was found to be involved in PXT1-CCDC33 interaction (Fig. 4.4. A.). As can be seen in figure 4.4. B., we also confirmed the functionality of the LAPF motif by demonstrating that its mutation into GAPA sequence abolished binding of CCDC33 to PXT1.

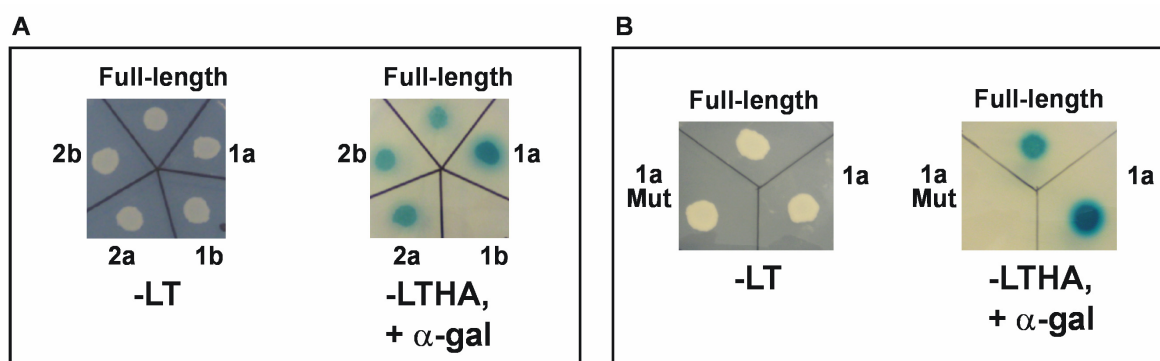


Figure 4.4. Direct-Y2H assays for mapping the CCDC33-binding site in the PXT1 sequence. Different fragments of PXT1 protein, included in pGBKT7 bait vector, were designated as follows 1a (aa 2-30), 1b (aa 2-26), 2a (aa 24-51), 2b (aa 20-51) and 1aMut (1a part containing GAPA motif instead of LAPF sequence). A. Identification of the LAPF motif as a CCDC33-binding site in PXT1 sequence via direct-Y2H assay with pGBKT7 vector expressing 1a or 1b or 2a or 2b fragment of PXT1 sequence and Ccdc33pGAD10 (containing Ccdc33 cDNA fragment encoding exons 9-20) vector. B. Validation of essentiality of the LAPF motif for PXT1-CCDC33 interaction via direct-Y2H assay with GBKT7 vector expressing 1aMut fragment of PXT1 and Ccdc33pGAD10 vector. Yeast (AH109 strain) spotted on nutritional medium lacking leucine and tryptophan (-LT) served as a positive control of co-transformation. Activation of GAL-4 reporter genes

by interaction between specific fragments of PXT1 and CCDC33 was observed as blue colony formation under the high-stringency selection conditions (-LTHA + α -gal).

We determined further that the *Ccdc33* cDNA fragment included in the Ccdc33pGAD10 library clone overlaps exons 9-20. *In silico* analysis predicted that this sequence encodes two putative coiled-coil (CC1, CC2) domains (Fig. 2. B., Kaczmarek et al., 2009a). Typically, the coiled-coil module is composed of two or more α -helices wrapped around each other into a left-handed supercoil (Mason and Arndt, 2004). This common structural motif has been shown to mediate many protein-protein interactions (Wang et al., 2001; Hu and Li, 2002; Biala et al., 2009). Hence, it can be speculated that CC1 and/or CC2 domain(s) of CCDC33 could also be responsible for PXT1-CCDC33 association.

Murine *Ccdc33* appears to be a novel, so far not described gene belonging, like its human ortholog, to the cancer/testis (CT) antigen family (Chen et al., 2005; Stevenson et al., 2007). Therefore, we attempted to characterize its expression profile, alternative splicing and the protein subcellular localization (Kaczmarek et al., 2009a). We showed that *Ccdc33* undergoes alternative splicing resulting in four different splice variants. Interestingly, two of the splice products exhibited expression in the testis. First, the most abundant transcript containing all 25 exons, namely *Ccdc33a*, displayed predominant expression in the testis and very faint in brain, ovary and mammary gland. The shortest splice product, *Ccdc33b*, contained exons 6-8A and was detected exclusively in the testis. Based on the fact that the *Ccdc33* cDNA sequence identified in Y2H screening, included exons 9-20, it can be suggested that the protein encoded by the *Ccdc33a* splice variant is the most likely PXT1-interacting partner. Moreover, we found that testicular expression of *Ccdc33a*, the same as in the case of *Pxt1*, is restricted to germ cells and starts at primary (pachytene) spermatocyte stage. Additionally, we identified the functional PTS2 sequence in CCDC33 and demonstrated the co-localization of EGFP-PXT1 and CCDC33-dsRED fusion proteins within peroxisomes of HeLa cells. All the resulting data support the relevance of PXT1-CCDC33 interaction in the testis.

In 2006, Luers et al. suggested that peroxisomes fulfill other functions in male germ cells than in somatic cells. The authors demonstrated that peroxisomes of spermatogonia differ in their relative protein composition from those of Leydig cells. Nenicu et al. (2007) provided additional evidence supporting this hypothesis. A strong heterogeneity of peroxisomal protein content was shown not only among somatic and male germ cells, but

surprisingly also among distinct germ cell types of the testis (Nenicu et al., 2007). Considering these findings, we propose PXT1 and CCDC33 proteins to be potential candidates determining specificity of peroxisomes in the testis.

4.3. Apoptotic regulator BAT3 is a novel PXT1-interacting protein

Another PXT1-interacting protein that was identified via Y2H screen of murine testis cDNA library is BAT3. Using direct-Y2H, CoIP and co-localization analyses we could clearly demonstrate the association between PXT1 and BAT3 (Kaczmarek et al., 2009b). The *Bat3* gene was originally discovered by Spies et al. (1989) on chromosome 6, within the class III region of the human major histocompatibility complex (MHC). The full-length cDNA clone of human *Bat3* was first determined in 1990 by Banerji et al. Four years later, Wang and Liew (1994), isolated a partial cDNA clone, *Rlc34*, representing the rat ortholog of human *BAT3* and characterized its expression profile in rodents. The *Bat3* was shown to be expressed ubiquitously at a low level and predominantly in the testis of adult rat. Moreover, faint expression of *Bat3* was demonstrated in testis of W/W^v mutant mice as compared to wild-type. The authors concluded that the rodent ortholog of *BAT3* must therefore be predominantly expressed in male germ cells. Interestingly, developmental regulation of *Bat3* expression during spermatogenesis, corresponding largely to that observed for *Pxt1* (Grzmil et al., 2007), was reported. A very weak activity of *Bat3* gene was detected in testis of 2 dpp-, 7 dpp- and 14 dpp- rats. The gradual increase of transcript levels was observed in rat testis from 21 to 180 dpp, indicating that the first significant activation of the *Bat3* occurred during the first meiotic division (approximately between 15-21 dpp). The similar tissue transcription profile of *Bat3* was also demonstrated later, via Northern blot and RT-PCR analyses, in adult mice (Desmots et al., 2005; Sasaki et al., 2008; Tsukahara et al., 2009).

Although, the expression pattern of *Bat3* and its interaction with germ cell-specific PXT1 strongly indicate that BAT3 may play a specific role in the testis, the initial insights into the biological function of this gene were not associated with spermatogenesis. In 1998, Thress et al. demonstrated that *Xenopus* homologue of *Bat3* can interact *in vitro* with reaper (RPR), a very small (65 aa) protein that is a key regulator of developmental apoptosis in *Drosophila melanogaster*. *Bat3* was found to be essential for reaper-induced apoptosis in *Xenopus* egg extracts what was reflected by rapid mitochondrial cytochrome c

release, caspase activation and nuclear fragmentation. In addition, the BAT3C312, a C-terminal 312-aa long fragment of BAT3, effectively promoted apoptosis even in the absence of RPR. Surprisingly, in 1999 Thress et al. demonstrated that excess of full-length *Bat3* suppressed rather than accelerated reaper-induced apoptosis. The investigators suggested that binding of RPR to full-length BAT3 leads to release of BAT3-sequestered apoptotic inducer(s), whereas the excess of BAT3 causes re-binding of those factor(s). Moreover, subsequent analyses confirmed that BAT3 can regulate apoptosis in a variety of settings. It was reported that BAT3 is a novel cellular substrate for caspase 3 and that its C-terminal caspase-cleaved fragment, CTF-131, mediates ricin-induced apoptosis (Wu et al., 2004). In addition, BAT3 was shown to be involved in the genotoxic stress response by modulation of p300-mediated p53 acetylation and in ER stress via regulation of apoptosis-inducing factor (AIFM1) stability (Sasaki et al., 2007; Desmots et al., 2008). Among BAT3-interacting partners are mainly proteins implicated in apoptosis, such as XEF1AO (Minami et al., 2007), NCR3 (Pogge von Strandmann et al., 2007) and PBF (Tsukahara et al., 2009). Recently, the formation of BAT3-SET1A-BORIS complex that modulates H3K4 histone dimethylation and gene expression have been reported (Nguyen et al., 2008).

Preliminary studies of BAT3 knockout mice demonstrated that *Bat3* is critical for viability and normal development, because its inactivation resulted in embryonic (between E13.5-E15.5) or perinatal lethality (Desmots et al., 2005). Surprisingly, the lethal phenotype of *Bat3*^{-/-} mice was partially eliminated in the ICR genetic background and the rescued male mice could serve as a model system for studying *Bat3* role in spermatogenesis (Sasaki et al., 2007; Sasaki et al., 2008). The null mutation of BAT3 led to complete sterility in male, but not in female mice on the ICR genetic background. Histological examination of atrophic testes from *Bat3*^{-/-} mice revealed significant reduction in spermatocyte population as well as absence of spermatids and spermatozoa, thus indicating spermatogenic failure due to arrest at prophase of meiosis I. It was also shown that BAT3 deficiency induced apoptosis of male germ cells, particularly spermatocytes. A similar phenomenon was observed in c-myc-Pxt1 transgenic male mice (Kaczmarek et al., 2009b). We could show that overexpression of pro-apoptotic PXT1 triggered programmed death of pachytene spermatocytes in the testis, which in turn led to severe impairment of spermatogenesis and consequently to male infertility. Interestingly, Sasaki et al. (2008) after extensive analysis of spermatocytes in *Bat3*^{-/-} mice suggested

that *Bat3* is required for proper chromosomal pairing, recombination and desynapsis. Moreover, it was demonstrated that serum levels of FSH, LH and testosterone were not significantly different between *Bat3*^{+/+} and *Bat3*^{-/-} mice. As can be seen in table 4.5., we also could not notice any statistically significant changes in serum testosterone concentrations between c-myc-Pxt1 transgenic and age-matched wild-type males.

Table 4.5. Serum testosterone concentrations in c-myc-Pxt1 transgenic (TR) and wild-type (WT) male mice at different days *post partum* (dpp).

Age (dpp)	Testosterone Mean (\pm SD), ng/ml		p
	WT	TR	
10	0.02 (\pm 0.003)	0.08 (-)	0.08
16	0.25 (\pm 0.134)	0.10 (\pm 0.100)	0.35
30	2.28 (\pm 0.218)	1.46 (\pm 1.076)	0.44
100	6.41 (\pm 7.85)	12.88 (\pm 16.200)	0.72

Interestingly, the interaction of BAT3 with another predominantly testis expressed protein, namely HSP70-2 was reported (Sasaki et al., 2008). It has been found previously that the targeted disruption of HSP70-2 results in increased apoptosis of spermatocytes and complete male infertility (Dix et al., 1996). The authors proposed that HSP70-2 might protect pachytene spermatocytes from apoptosis (Dix et al., 1996). Surprisingly, inactivation of *Bat3* did not affect *Hsp70-2* mRNA levels, however caused significant decrease in the amounts of HSP70-2 protein, therefore acceleration of HSP70-2 protein degradation by BAT3 deficiency was hypothesized (Sasaki et al., 2008). Furthermore, the authors confirmed that BAT3 stabilizes HSP70-2 by inhibiting its polyubiquitination. We demonstrated that overexpression of BAT3 prevents apoptotic cell death induced by overexpression of PXT1. Considering the above information, it is tempting to speculate that among others apoptotic regulators, PXT1, BAT3 and HSP70-2 could directly or indirectly cooperate to regulate the physiological apoptosis during the progress of spermatogenesis. Accordingly, it seems clear that the balance between pro-apoptotic and anti-apoptotic proteins in the testis is crucial for normal spermatogenesis and male fertility.

4.4. Specificity of male germ cell apoptosis

Programmed death of germ cells is considered to be a normal and very common phenomenon during male gametogenesis. In the testis of adult mammals up to 75 % of potential spermatozoa die by the activation of an apoptotic program (Blanco-Rodriguez, 1998). Normally, high levels of spontaneous apoptosis within the testis are seen shortly after birth and later in adult animals (Baum et al., 2005). Among germ cells, spermatogonia and spermatocytes were found to be the main targets of programmed cell death (Royere et al., 2004). Exposition of phosphatidylserine (PS) on the surface of germ cells undergoing apoptosis enables their recognition by Sertoli cells, most probably through the class B scavenger receptor type I, and subsequent elimination via phagocytosis (Nakanishi and Shiratsuchi, 2004).

In the mammalian testes fate of germ cells is regulated by endocrine hormones, such as testosterone and the gonadotrophins, FSH and LH. Withdrawal of these hormones induces germ cell apoptosis, probably in an indirect manner, since the hormone receptors are present on somatic, but not germ cells (Dunkel et al., 1997; Sinha Hikim and Swerdloff, 1999; Print and Loveland, 2000; Baum et al., 2005). In addition, the observation that in adult human testes germ cell apoptosis induced under serum-free conditions is suppressed by testosterone application, indicates that especially testosterone is a critical germ cell survival factor (Dunkel et al., 1997). Although, the mechanism and biological significance of the frequent apoptosis in mammalian testes remain largely unclear, it seems that the programmed cell death contributes to maintenance of an optimal germ cell density (removes excess germ cells that can not be supported by the Sertoli cells) and causes elimination of genetically abnormal or damaged cells (Matsui et al., 1998; Koji and Hishikawa, 2003; Baum et al., 2005).

Some of proteins belonging to the B-cell leukemia/lymphoma-2 (BCL-2) family were found to be involved in apoptosis of spermatogenic cells (Beumer et al., 2000, Tripathi et al., 2009). We demonstrated that PXT1, like some members of BCL-2 family, contains a functional BH3-like domain, which modulates its death promoting activity. However, *Pxt1* appears to be a very unique pro-apoptotic gene, because it encodes a peroxisomal protein. To date, there is no report about another peroxisomal protein involved in apoptosis. Nevertheless, the BCL-Gs protein, encoded by the short alternative variant of human *Bcl-G* (*Bcl-Gonad*) gene, also contains only the BH3 domain that determinates its

pro-apoptotic activity (Guo et al., 2001). In addition, it was demonstrated that BCL-Gs-induced cell death could be suppressed by co-expression of anti-apoptotic BCL-X_L protein. Likewise, the expression of *Bcl-Gs* was shown to be restricted to the testis, but its germ/somatic cells specificity has not yet been determined. Moreover, the BCL-Gs protein displayed a punctate cytosolic distribution, that was only partially co-localized with a mitochondria specific dye. The same like in case of PXT1, the distribution was not disrupted by the deletion of BH3 domain. Unfortunately, the exact subcellular localization of BCL-Gs was not determined. Based on the information about direct relationship between peroxisomes and mitochondria (Neuspiel et al., 2008; Thoms et al., 2009), it can be only speculated that BCL-Gs is somehow connected with peroxisomes. The expression of such unique apoptotic regulators exclusively in the testis provides evidence for the existence of a specific machinery controlling apoptosis during mammalian spermatogenesis.

4.5. Lack of 3'UTR-mediated control of *Pxt1* translation

To explore the physiological role of *Pxt1* during spermatogenesis and verify its translational regulation, a testis-specific “gain-of-function” strategy was undertaken. As mentioned in the introduction, both mouse and human *Pxt1/PXT1* genes have long 3'UTR, 716 bp and 1219 bp, respectively. Considering the fact that many testis-specific transcripts possess long 3'UTRs that contain regulatory sequences delaying mRNAs translation, it was tempting to speculate that *Pxt1* could be controlled in a similar manner. Therefore, two lines of transgenic mice expressing cMYC-PXT1 fusion protein under the control of human phosphoglycerate kinase 2 (*PGK2*) promoter were generated. In both transgenic lines the same 1.4-kb fragment of human *PGK2* promoter was used in order to confer a male germ cell-specific expression of transgenes, starting at the pachytene spermatocytes stage during spermatogenesis (Robinson et al., 1989; Tascou et al., 2001). The first line, destined as P1, carried the construct containing 3'UTR of *Pxt1* gene (Fig. 4.6. A.), therefore we assumed that the translation should be regulated in the endogenous fashion. In contrast, the second line, called P2, harbored a construct containing, instead of endogenous *Pxt1* 3'UTR, the 3'UTR of the human growth hormone 1 (*GHI*) gene (Kaczmarek et al., 2009b), which is known to be neutral for translational regulation (Lee et al., 1995; Tseden et al., 2007). The transgenic line P2 served thus as a control, in which transcription and

translation should be closely linked. As shown in figure 4.6., testis-specific expression of P1 transgene, the same like P2 (c-myc-Pxt1) (Kaczmarek et al., 2009b), was confirmed on both, mRNA as well as protein level. To evaluate the possible role of the endogenous *Pxt1* 3'UTR in post-transcriptional regulation, immunohistochemistry analyses of the c-MYC-PXT1 fusion protein expression in P1 and P2 transgenic testes, using anti-myc tag antibody, were performed. In accordance with our expectations, the expression of c-MYC-PXT1 protein in the testis of P2 transgenic males was found within cytosol of primary spermatocytes at pachytene stage (Kaczmarek et al., 2009b). Surprisingly, exactly the same pattern of stage-specific expression and no delayed translation could be observed in the testis of P1 transgenic males (Fig. 4.6. D.), indicating that 3'UTR of *Pxt1* has no role in regulation of mRNAs translation.

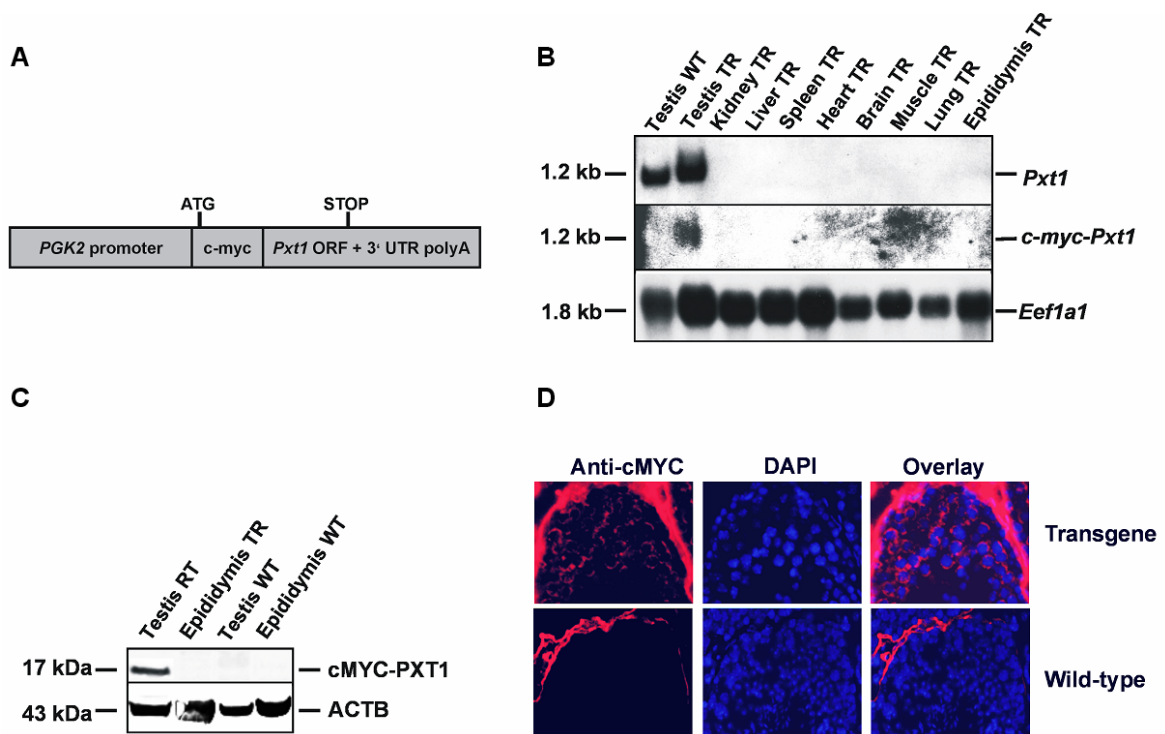


Figure 4.6. Partial characterization of the P1 transgenic mouse line. A. Schematic representation of the P1 transgenic construct, which contains the 1.4-kb region of human phosphoglycerate kinase 2 (*PGK2*) promoter, five copies of c-myc tag, the complete ORF of the mouse *Pxt1* gene, the endogenous 3'UTR of *Pxt1* gene including polyadenylation (polyA) signal. Start codon (ATG) and STOP codon are given. B. Northern blot analysis of the P1 transgene expression in different organs of transgenic males. Using *Pxt1*-specific probe (Kaczmarek et al., 2009b), which recognizes the full-length of *Pxt1* ORF, the expected 1.2-kb band corresponding to the endogenous *Pxt1* could be observed in wild-type (WT) testis (upper panel). The endogenous *Pxt1* is approximately the same in size as the transgenic *c-myc-Pxt1* mRNAs, therefore only a single 1.2-kb band could be detected in transgenic (TR) testes using the *Pxt1*-specific probe (upper panel). The *c-myc* tag-specific probe (Kaczmarek et al., 2009b) detected a 1.2-kb band representing the *c-myc-Pxt1*

fusion transcripts exclusively in transgenic testis (middle panel). The integrity of all RNA samples was assessed by rehybridization of the blots with *Eef1a1* cDNA probe, which was generated by R. Hahnwald (the lowest panel). C. Western blot analysis of the P1 transgene expression. Using anti-myc tag antibody the expected 17-kDa band corresponding to the size predicted for c-MYC-PXT1 fusion protein could be detected in the testis of transgenic mice, but not in the epididymis of transgenic mice or wild-type tissues (upper panel). Protein quality and integrity were verified using anti-ACTB antibody (lower panel). D. The immunohistochemistry analysis of the stage-specific expression of P1 transgene during spermatogenesis. Using anti-myc tag antibody the specific signals representing c-MYC-PXT1 were detected within the cytosol of pachytene spermatocytes in the testis of P1 transgenic mice, but not in the wild-type testis. The unspecific staining of the interstitial tissues by Cy3-conjugated anti-mouse IgG secondary antibody (Sigma) could not be eliminated and was observed in both, transgenic and wild-type testes. Cell nuclei were counterstained with DAPI.

Interestingly, very similar results were found for mouse *Tcp10b* (t-complex protein 10b) gene, which similar to *Pxt1*, is exclusively expressed in male germ cells from the pachytene spermatocyte stage onward (Schimenti et al., 1988; Ewulonu and Schimenti, 1997). Moreover, both *Tcp10* and *Pxt1* are located on chromosome 17 within mouse t-complex, the region containing genes, which have profound effects on spermatogenesis (Ha et al., 1991, Ewulonu and Schimenti, 1997; Grzmil et al., 2007). Analysis of transgenic mice carrying different chimeric constructs with or without *Tcp10b* endogenous 5'UTR and 3'UTR revealed that the presence of either 5'UTR or 3'UTR had no obvious effect upon expression of linked reporter genes (Ewulonu and Schimenti, 1997). Likewise, expression of another testis-specific gene, namely *Ldhc* (lactate dehydrogenase C) starts in pachytene spermatocytes, so at the same stage as expression of LacZ-transgenic construct containing or lacking native *Ldhc* 5'UTR and/or 3'UTR (Kroft et al., 2003). On the contrary, 5'UTR sequence of rat *Acr* (acrosin) gene, whose expression is also restricted to male germ cells, was found to be involved in translational regulation (Nayernia et al., 1992). The authors demonstrated that 2.3-kb region of 5'UTR of rat *Acr* can direct the expression of the chloramphenicol acetyltransferase (CAT) reporter gene to male germ cells so that its transcription starts at pachytene spermatocyte stage, but the translation is first seen in round spermatids (Nayernia et al., 1992; Nayernia et al., 1996). The conclusion therefore must be that *Pxt1* gene in general is not under translational control or such a control may alternatively be determined by its 5'UTR, what requires further investigations. Moreover, the phenotype of transgenic line P1 (data not shown) appeared to be the same as observed for line P2 (Kaczmarek et al., 2009b). Accordingly, the overexpression of the P1 transgene resulted in male infertility due to apoptotic degeneration of germ cells in the testis, especially spermatocytes. These results therefore

provide additional support for the specificity of our previous findings that overexpression of PXT1 induces arrest of spermatogenesis and triggers wide-spread apoptotic cell death in the testis (Kaczmarek et al., 2009b).

4.6. The putative signaling events mediating PXT1-induced apoptosis

Based on the stimuli leading to apoptosis the complex molecular mechanisms underlying this process were categorized into two main pathways: the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway (Elmore, 2007; Bayir and Kagan, 2008). The intrinsic pathway is known to be initiated by intracellular signals that have been produced upon a positive-acting, e.g. radiation, toxins, hypoxia, hyperthermia, viral infections, free radicals or negative-acting, e.g. deprivation of growth factors, cytokines and hormones, stimuli (Elmore, 2007). Proteins belonging to BCL-2 family are involved in the response to these factors, among them pro-apoptotic proteins BAX and BAK, which mediate signal transduction from cytosol to the mitochondria (Brunelle and Letai, 2009). Consequently, the mitochondrial membrane permeabilization occurs and intermembrane space proteins such as: cytochrome c, AIFM1, ENDO G, SMAC/DIABLO are released into cytosol (Bayir and Kagan, 2008). Subsequently, cytosolic cytochrome c promotes the formation of so called apoptosome, which, in addition to cytochrome c, consist of apoptotic protease activating factor 1 (APAF-1), dATP and pro-caspase 9. The construction of this multi-protein complex results in activation of pro-caspase 9 as the initiator caspase, which in turn cleavage the executioner pro-caspase 3.

In contrast, the extrinsic apoptotic pathway is triggered by extracellular signal molecules, known as ligands, which binds to the cell surface death receptors. The best characterized ligand/receptor complexes includes: FasL/FasR, TNF- α /TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Elmore, 2007). Generally, binding of specific ligand to the receptors on the target cell results in a death receptor oligomerization, which consequently leads to amplification of apoptotic signaling and recruitment of additional molecules, namely adaptor proteins e.g. FADD (Fas-associated death domain protein) and finally pro-caspase 8 and/or 10 (Ashkenazi, 2002). All of these proteins assemble together to create the death-inducing signaling complex (DISC). In the DISC, pro-caspase 8 and/or 10 are auto-proteolytically cleaved to the mature forms, which in turn mediate activation of pro-caspase 3. The processing of pro-caspase 3 represents the common step between

intrinsic and extrinsic pathway that initiates the final cascade of reactions, leading inevitably to cell death (Porter and Janicke, 1999; Elmore, 2007; Bayir and Kagan, 2008).

In order to better understand molecular mechanisms underlying PXT1-induced apoptosis, we attempted to categorize this rather particular case of programmed cell death. As mentioned above, the activation of the intrinsic apoptotic pathway is regulated by the BCL-2 family of proteins. We demonstrated that PXT1 is a BH3-only BCL-2 family member, therefore it can be speculated that PXT1 should trigger apoptosis via intrinsic pathway. To verify the hypothesis, the activation of pro-caspase 9 in the testis of the P1 and the P2 transgenic males was examined. However, in contrast to our expectation, no differences in levels of caspase 9 cleaved forms between transgenic and wild-type control testis were found (Fig. 4.7.). Moreover, the activation of pro-caspase 9 was almost not detectable in both cases, while a strong band corresponding to the uncleaved pro-enzyme was clearly visible (Fig. 4.7.).

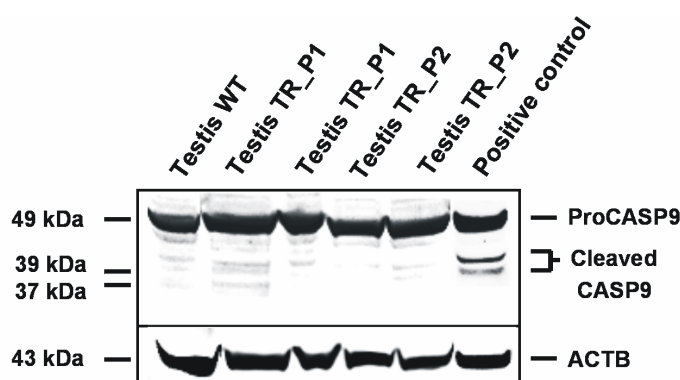


Figure 4.7. Western blot analysis for active pro-caspase 9 in total protein extracts derived from testes of transgenic mouse lines P1 (two individuals) and P2 (two individuals). Using anti-caspase 9 antibody (Cell Signaling), a full-length (49 kDa) pro-caspase 9 (ProCASP9) was detected in all examined samples (upper panel). The 37-kDa and 39-kDa bands representing cleaved fragments of caspase 9 (CASP9), thus indicating pro-caspase 9 activation, were clearly visible only in positive control (total extract from mouse embryonic stem cells (ES RI, Nagy et al., 1993) treated for 24 hrs with 200 μ M citrinin; kindly provided by T. Khromov). The cleaved fragments of caspase 9 were almost not detectable in both FVB/N wild-type (WT) and in the P1 and the P2 transgenic (TR) testes. Protein quality and integrity were verified using anti-ACTB antibody (lower panel).

These results may indicate that PXT1 triggers apoptosis via extrinsic pathway. To characterize the putative signaling pathways activated by overexpression of PXT1, the Mouse Apoptosis RT² Profiler™ PCR Array (SA Biosciences) was used. Interestingly, the preliminary results obtained in this study supported the hypothesis that PXT1 could elicit

extrinsic apoptotic pathway. The quantitative analyses revealed a marked increase in *Tnfrsf10b* (tumor necrosis factor receptor superfamily, member 10b also known as DR5 or TRAIL-R2) mRNA levels in cDNA samples derived from the P2 transgenic testis as compared to those of age-matched wild-type males (Fig. 4.8.).

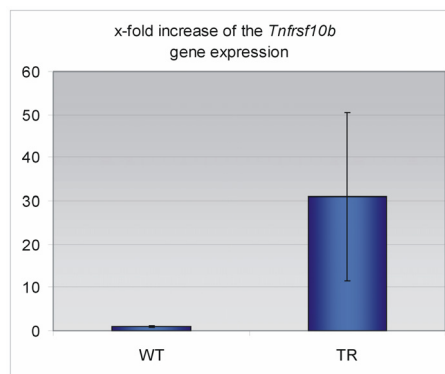


Figure 4.8. Quantitative real-time RT-PCR analysis of *Tnfrsf10b* gene expression in testes cDNA samples of FVB/N wild-type (WT) and the P2 transgenic (TR) males using the Mouse Apoptosis RT² Profiler™ PCR Array (SA Biosciences). Mean values of two technical replicates represents the x-fold increase in *Tnfrsf10b* gene expression. Data were normalized to the following housekeeping genes: *Gusb*, *Hprt1*, *Hsp90ab1*, *Gapdh*, *Actb*.

Murine *Tnfrsf10b* gene, the homolog of the human *TNFRSF10b* (DR5) also closely related to human *TNFRSF10a* (DR4), encodes a protein belonging to the tumor necrosis factor (TNF) receptor (TNFR) superfamily. Members of TNFR superfamily are integral membrane proteins that contain an intracellular death domain (DD) and are capable of transducing an apoptotic signal upon binding to specific ligands. Activation of the TNFRSF10B protein is mediated by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), also known as APO2L and triggers apoptosis via the extrinsic or linked extrinsic-intrinsic pathway (Ashkenazi, 2002). The latter mechanism is designed to amplify the death signal and represents “cross-talk” between both apoptotic pathways. Accordingly, upon the death receptor activation, the BCL-2 pro-apoptotic BH3-only protein BID is processed by active caspase 8 to the truncated form that migrates to the mitochondria causing loss of mitochondrial membrane integrity and consequently cytochrome c release (Ashkenazi, 2002; Wang and El-Deiry, 2003).

Interestingly, although TRAIL was shown to be constitutively expressed in most normal tissues, its apoptotic activity seems to be limited to tumor cells (Wang and El-

Deiry, 2003). Nevertheless, recently McKee et al. (2006) have demonstrated that *in vitro* testicular germ cells, specifically spermatocytes, can undergo TRAIL-mediated apoptosis. It was also shown that not only TRAIL but also its receptor TNFRSF10B are abundantly expressed in testes of mouse, rat and human (Giammona et al., 2002; Grataroli et al., 2002; Grataroli et al., 2004). However, a targeted disruption of TRAIL or TNFRSF10B in mice failed to cause male infertility and in addition did not lead to any morphological alternations in the testis (McKee et al., 2006).

Based on our preliminary results and the available information, it can not be concluded that TRAIL/TNFRSF10B signaling pathway is in fact involved in regulating testicular germ cell apoptosis, especially under physiological conditions. Moreover, to our knowledge, PXT1 is the first BCL-2 family member, that could trigger apoptosis specifically via extrinsic pathway. As mentioned above, to date only BID protein was partially associated with extrinsic pathway, but in contrast to PXT1, as a result of its action the pro-caspase 9 is activated. Taken together, it seems that PXT1 is a quite unique member of BCL-2 family that triggers apoptosis in caspase-9 independent manner and even more interestingly is localized in peroxisomes. To date, peroxisomes have not been directly connected to apoptosis, therefore our results provide new insights into the physiological role of these organelles. Our findings together with other experimental evidence, point towards the existence of new, unexpected functions for peroxisomes (Titorenko and Rachubinski, 2001b). Considering this and the existence of a variety of inherited peroxisomal disorders, it is very important to better decipher the nature and specific role of a novel protein, like PXT1.

4.7. Future endeavors and perspectives

The current study provides the first insights into the physiological role of PXT1 protein in the testis. Our results strongly indicate involvement of PXT1, and thus peroxisomes, in regulation of male germ cell apoptosis during spermatogenesis. Moreover, the results obtained from analysis of PXT1 “gain-of-function” mouse model suggest that PXT1 might control male fertility. However, to verify and better understand these interesting findings further studies are necessary. Therefore, the “loss-of-function” mouse model is currently under investigation in our laboratory. In addition, the characterization of PXT1-interacting partners, namely BAT3 and CCDC33 and examination of signaling

pathways for PXT1-induced apoptosis are continued. It is worth noting that there is a large number of men with possibly genetically caused infertility and that male patients suffering from some of peroxisomal disorders also display impaired fertility. Therefore, it seems important to screen infertile male patients, including those suffering from peroxisomal disorders, for mutations and polymorphisms in *Pxt1* gene. We believe that such examinations will help to verify whether *Pxt1* is a male fertility gene or not and perhaps will provide useful insights into the future medical treatment of male infertility.

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6. Abbreviations

15P-1	mouse Sertoli cell line
aa	amino acid
AAA	ATPase associated with various cellular activities
ACTB	actin beta protein
AMN	adrenomyeloneuropathy
Amp	ampicillin
ATP	adenosintriphosphate
BAG	BCL-2 associated athanogene
<i>Bat3</i>	HLA-B-associated transcript 3 gene
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
BCL-2	B-cell leukemia/lymphoma-2
BH3	BCL-2 homology domain 3
BiFC	bimolecular fluorescence complementation
bp	base pair
BSA	bovine serum albumin
°C	degree Celsius
CASP9	active caspase 9
CAT	chloramphenicol acetyltransferase
<i>Ccdc33</i>	coiled-coil domain containing 33 gene
cDNA	complementary DNA
CoIP	co-immunoprecipitation
CT	cancer/testis antigen
Cy3	indocarbocyanine
DAPI	diamidino-2-phenylindole dihydrochloride
dATP	desoxyriboadenosintriphosphate
DD	death domain
DISC	death-inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease

DPBS	Dulbecco's phosphate buffered saline
dpp	days <i>post partum</i>
DsRED	<i>Discosoma sp.</i> red fluorescent protein
dT	deoxythymidinate
E1	ubiquitin-activating enzyme
E3	ubiquitin-protein ligase
EDTA	ethylene diamine tetra-acetic acid
<i>Eef1a1</i>	eukaryotic translation elongation factor 1 alpha 1 gene
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
et al.	et alii (and others)
ExPASy	expert protein analysis system
Fig.	figure
FITC	fluorescein isothiocyanate
FSH	follicle-stimulating hormone
GC1spg	mouse spermatogonia-preleptotene spermatocyte cell line
GC-4	mouse spermatocyte cell line
Gene ID	gene identification number
<i>GHI</i>	growth hormone 1 gene
HEPES	N-(α -hydroxymethyl)piperazin, α -3-propanesulfoneacid
<i>Hprt1</i>	hypoxanthine guanine phosphoribosyl transferase 1 gene
hr(s)	hour(s)
IgG	immunoglobulin G
<i>Insl3^{-/-}</i>	mouse mutant with germ cells until the primary spermatocyte stage
Kan	kanamycin
kb	kilobase pairs
kDa	kilodalton
LB	Luria-Bertani
LH	luteinizing hormone
MA-10	mouse Leydig tumor cell line
MHC	major histocompatibility complex
min	minute

ml	milliliter
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein
Mw	molecular weight
NBT	4-nitro-blue-tetrazolium chloride
NCBI	national center for biotechnology information
ng	nanogram
NIH3T3	mouse embryonic fibroblast cell line
<i>olt/olt</i>	mouse mutant with germ cells until the round spermatid stage
ORF	open reading frame
p	probability
PAGE	polyacrylamide gel electrophoresis
PBD	peroxisome biogenesis disorder
PCR	polymerase chain reaction
<i>Pex</i>	peroxin gene
<i>PGK2</i>	phosphoglycerate kinase 2 gene
pI	isoelectric point
polyA	polyadenylation
ProCASP9	pro-caspase 9
PS	phosphatidylserine
PTS	peroxisomal targeting signal
PVDF	polyvinilidene difluoride
<i>Pxt1</i>	peroxisomal testis specific 1 gene
<i>qk/qk</i>	mouse mutant with germ cells until the elongated spermatid stage
RCDP	rhizomelic chondrodysplasia punctata
RING	really interesting new gene
RNA	ribonucleic acid
RPR	reaper protein
RT	room temperature
RT-PCR	reverse transcriptase-PCR
SCO	Sertoli cell only
SD	standard deviation

SDS	sodium dodecylsulfate
SSC	spermatogonial stem cell
SV40	<i>Simian virus 40</i>
Taq DNA polymerase	<i>Thermus aquaticus</i> DNA polymerase
TE	Tris-EDTA buffer
TM4	mouse Sertoli-like cell line
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
<i>Tnfrsf10b</i>	tumor necrosis factor receptor superfamily, member 10b gene
<i>Tnp2</i>	transition protein 2 gene
TR	transgenic
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
Tris	trihydroxymethylaminomethane
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
Ub	ubiquitin
UTR	untranslated region
VLCFA	very-long-chain fatty acid
VP	vaginal plug
W/W ^v	mouse mutant lacking germ cells in the testis
WT	wild-type
X-ALD	X-linked adrenoleukodystrophy
X- α -gal	5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside
Y2H	yeast two-hybrid
μ g	microgram
μ l	microliter

The names of nucleic/amino acids are abbreviated in accordance with internationally accepted codes.

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8. Curriculum Vitae

Personal Details:

Name: **Karina Paulina Kaczmarek**
Date of Birth: 11.04.1982
Place of Birth: Koło, Poland
Nationality: Polish
Permanent address: 20-ego Stycznia 20/60
62-600 Koło, Poland
Telephone: 0176/75597309
E-mail: karinakaczmarek@wp.pl

Education:

1989-2001 Primary and Secondary School, Koło, Poland.
2001 High School Diploma.
2001-2006 Nicolaus Copernicus University, Faculty of Biology and Earth Sciences, Toruń, Poland.
2006 Master of Science in Biology, specialisation: General and Molecular Biology. Thesis: "The analysis of *MnSod* gene expression in *in vitro* cultures of *Triticum aestivum* and *Vicia faba* ssp. *minor* under oxidative stress".
Since 2006 Ph.D. studies, Institute of Human Genetics, Georg-August University, Göttingen, Germany. Ph.D. Dissertation: "On the Functional Analysis of Murine Peroxisomal Testis Specific 1 (*Pxt1*) gene".

9. List of Publications

Dąbrowska, G., **Kaczmarek, K.**, Skrzypek, E., and Szechyńska-Hebda, M. (2007). cDNA cloning and expression of gene encoding manganese superoxide dismutase of *Vicia faba* ssp. *minor* in regenerating and non-regenerating explants. *Advances of Agricultural Sciences-Problem Issues*. 523, 83-92.

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