Identification of a novel mouse P4-ATPase family member highly expressed during spermatogenesis

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Summary

P4-ATPases are transmembrane proteins unique to eukaryotes that play a fundamental role in vesicular transport. They have been proposed to act as phospholipid flipases thereby regulating lipid topology in cellular membranes. We cloned and characterized a novel murine P4-ATPase that is specifically expressed in testis, and named it FetA (flipase expressed in testis splicing form A). When expressed in Saccharomyces cerevisiae, FetA localizes partially to the plasma membrane and secretory function, suggesting a crucial role for this novel murine P4-ATPase in spermatogenesis.

Key words: P-type ATPase, Aminophospholipid translocase, Acrosome biogenesis, Phospholipid asymmetry, Spermatogenesis

Introduction

P-type ATPases form a large family of diverse membrane proteins that actively transport substrates across cellular membranes. Based on sequence similarity, the P-type ATPase family is divided into five subfamilies, among which P4-ATPases have been implicated in the translocation of specific phospholipids from the outer to the cytoplasmic leaflet of a membrane. P4-ATPases are unique to eukaryotes where they are found in every sequenced genome. Their intracellular localization is tightly related with acrosome formation, a process that involves intensive intracellular vesicle formation and fusion. Furthermore, loss-of-function of FetA by RNA interference in mastocytoma P815 cells profoundly perturbs the structural organization of the Golgi complex and causes loss of constitutive secretion at lower temperature. Our findings point to an essential role of FetA in Golgi morphology and secretory function, suggesting a crucial role for this novel murine P4-ATPase in spermatogenesis.

Genetic analysis revealed that the five yeast P4-ATPases constitute an essential protein family with overlapping functions in membrane trafficking where their activities are required to support vesicle formation in the secretory and endocytic pathways. Yeast Drs2p has been shown to be involved in the formation of clathrin-coated vesicles at the trans-Golgi network (Chen et al., 1999; Gall et al., 2002). Yeast mutants with deletions in DRS2 produce an abnormally low number of vesicles (Gall et al., 2002). Cells lacking Dnf1p and Dnf2p have a cold-sensitive defect in the formation of endocytic vesicles (Pomorski et al., 2003) and in an early endosome to trans-Golgi network transport pathway (Hua et al., 2002). Inactivation of temperature-sensitive variants of the endosome-associated P4-ATPase Neo1p causes defects in receptor-mediated endocytosis, vacuolar biogenesis and vacuolar protein sorting (Hua and Graham, 2003; Wicky et al., 2004). These findings led to the proposal that the lipid transport could be intimately connected to the process of coat assembly and vesicle budding.

In mammalian cells, 14 P4-ATPases genes have been predicted in both human and mouse genomes by homolog searching with the five yeast members but only a few members have been partially characterized to date. ATP8A1 (previously named ATPase II) was identified as an enzyme that catalyzed the translocation of aminophospholipids across the membrane of chromaffin granules, a specialized class of exocytic vesicles (Tang et al., 1996). Mutations in ATP8B1, a human DRS2 homolog expressed in many epithelial cells of the gastrointestinal tract and liver, are associated with...
hereditary cholestasis, a disease characterized by impaired bile salt secretion from liver into bile (Bull et al., 1998; van Mil et al., 2004). The mouse ortholog of ATP8B3, a putative sperm aminophospholipid transporter (SAPLT), is expressed in the acrosomal region of the head of spermatozoa and has been implicated in the capacitation process, an event in which sperm cells are being prepared for binding to and penetration of the zona pellucida (Wang et al., 2004).

In this work, we identified a novel murine P4-ATPase that is highly expressed in testis. We called this protein flippase expressed in testis splicing form A (FetA). When expressed in yeast, FetA partially localizes to the plasma membrane and facilitates internalization of short-chain fluorescent phosphatidylethanolamine and phosphatidylcholine analogs. During spermatogenesis, FetA is expressed from pachyten spermocytes to mature sperm, with its localization tightly associated with acrosome biogenesis. Silencing FetA expression by RNAi in mouse P815 mastocytes profoundly affects Golgi morphology and inhibits the constitutive secretory activity, suggesting that FetA plays an essential role in acrosome formation.

Results

FetA is a member of the P4-ATPase subfamily
To search for candidate lipid flippases with a potential role in spermatogenesis, we screened the NCBI mouse database using the query sequence of ATP8B1, a human P4-ATPases family member (GenBank accession number NM_001001488). As a result, an undefined sequence AK029992 from an adult testis cDNA library appeared as a promising candidate. Using designed primers corresponding to the candidate sequence, we performed RT-PCR to isolate its open reading frame from mouse testes and confirmed it by sequencing. By 5’ rapid amplification of cDNA ends, its full-length cDNA clone with 4463 base pairs encoding a putative 1183 amino acids protein was obtained (Fig. 1). We named it FetA (flippase expressed in testis splicing form A) to specify the splicing form (see Fig. 5). Sequence analysis indicated that FetA has ten predicted transmembrane segments, and comparison of FetA with other P4-ATPases using the CLUSTALW program (Myers and Miller, 1988) revealed that it shares 43% identity with the murine SAPLT (Atp8b3) and 36% with the S. cerevisiae Drs2p, but does not match any previously predicted murine P4-ATPases flippase sequence. Thus, our result suggests that FetA is a novel member of P4-ATPases family (Fig. 1). The gene sequence has been submitted to GenBank (accession number EF377342).

The members of the P4-ATPase family share functional similarities, implying that they originate from a common ancestor probably through gene duplication, divergence, and selection. To learn more about the evolution of FetA, we searched the NCBI nonredundant protein database with the BLASTP program. All the similar sequences were aligned and an unrooted phylogenetic tree was constructed. We found that FetA orthologs are present in the genomes of various vertebrates, including fish, birds and mammals, and can be classified as a subfamily branch (Fig. 2, blue branches). The FetA subfamily clusters with ATP8B2 and ATP8B4 subfamilies in the tree, representing 1-Φ subclass members (Paulusma and Oude Elferink, 2005). Thus, FetA is highly conserved in evolution.

Characterization of FetA by heterologous expression in yeast
Because FetA is a newly identified member of the P4-ATPases gene family, we first asked whether FetA is required for phospholipid transport across cellular membranes. To address this question we made use of a S. cerevisiae mutant strain deleted in three P4-ATPases (Drs2p, Drs1p and Drs2p). This mutant displays a defect in the ATP-dependent phospholipid transport across the plasma membrane. Thus, even small amounts of functional FetA at the plasma membrane might result in detectable internalization of phospholipid analogues added to the exterior of the cells. FetA was cloned into a yeast expression plasmid under the control of the Cu²⁺-inducible CUP1 promoter. The resulting construct or empty vector was used to transform the yeast Δdrs2ΔdrslΔdrsn2 strain. As an additional control, we generated yeast expressing non-functional FetA by replacing the aspartate (461) in the sequence DKTGT with alanine. This residue, conserved in all P-type pumps, is transiently phosphorylated during the catalytic cycle (Solioz and Vulpe, 1996). Hence any mutation of this aspartate abolishes enzyme activity.

First, we studied the expression and localization of GFP fusions of FetA and FetA (D461A) by fluorescence microscopy. Cells harboring the control vector or expressing free GFP were analyzed as controls. Under induced conditions (+Cu), FetA-GFP and FetA (D461A)-GFP were visible in internal membranes, small intracellular spots and at the plasma membrane, whereas free GFP was homogeneously distributed in the cytoplasm (Fig. 3A). Cells harboring the control vector showed no significant fluorescence. Low expression of FetA was also observed under non-induced conditions (−Cu; data not shown), indicating that the CUP1 promoter is leaky as previously described (Macaulay and McFadden, 1989). Analysis by flow cytometry revealed that in the presence of 50 μM copper 51±4% (n=3) and 58±16% (n=2) of cells expressed FetA-GFP and FetA (D461A)-GFP, respectively, at a high level, whereas the remaining cells showed no or low expression of FetA-GFP. Likewise, untagged FetA and FetA (D461A) were expressed in yeast under induced conditions. Differential centrifugation and fractionation of membranes on a sucrose step gradient confirmed that the bulk of both proteins co-localize with an intracellular membrane marker but a small portion of both proteins co-migrated with a marker for the plasma membrane indicating that untagged FetA reaches the plasma membrane (Fig. 3B).

Lipid transport activity in FetA-expressing yeast
To test for phospholipid transport activity, yeast cells expressing untagged FetA or FetA (D461A) were incubated with fluorescent NBD-lipids for 30 minutes at 30°C and the amount of inwardly transported NBD-lipid, which is protected from BSA extraction, was determined by flow cytometry. Under these conditions, accumulation of NBD-PE was significantly higher in FetA-expressing cells than in empty-vector transformed cells (Fig. 4A). NBD-PC was also translocated but at a lower rate, whereas NBD-PS and NBD-SM were not internalized above background levels. Cells expressing the mutant FetA (D461A) failed to generate any activity that promoted internalization of any of the phospholipid analogues tested (Fig. 4B), proving that catalytically active FetA is required for NBD-PE and NBD-PC uptake.

FetA is specifically expressed in mouse testis
To investigate the expression profiles of FetA in adult mouse tissues, we performed northern blot analysis. During the process of cloning FetA we noticed that there are two splicing forms of Fet in mouse testis and we named them as FetA and FetB. Both splicing forms were sequenced showing that splicing form B is a truncated protein lacking the catalytic domain of P-type ATPases (data not shown). Thus, we focused on FetA in our following experiments. Using a probe recognizing all splicing forms, a major 4.4 kb band was
Fig. 1. Alignment analysis and topology prediction of FetA. Multiple alignment of the FetA with other P4-ATPases. The alignment includes the deduced protein sequence of FetA, SAPLT (Atp8b3), FIC1 (ATP8B1), ATPase II (ATP8A1) and Drs2p. Identical residues are on a black background and similar amino acids are on a gray background. Gaps between amino acids are shown as dashes. The transmembrane domains (TM) are marked according to the prediction by TMHMM2 server.
observed only in the mouse testis with no detectable signal in any other tissues tested, including brain, heart, lung, liver, kidney, spleen, muscle and thymus, indicating that FetA is specifically expressed in adult mouse testis (Fig. 5). A very weak 1.5 kb band representing splicing form B was also detected in testis (Fig. 5, arrowhead).

**FetA mRNA expression during mouse spermatogenesis**

The testis-specific expression pattern suggested that FetA might be associated with spermatogenesis. We therefore studied the spatial and temporal expression of FetA in different developmental stages of postnatal mice testes, by northern blot analysis and in situ hybridization staining. As shown in Fig. 6A, FetA was not detected in testes until 18 days post-partum (d.p.p.). In 22 d.p.p. testes, the expression level of FetA was dramatically increased and remained constant during adulthood. The onset of FetA expression correlated with the accumulation stage of later spermatocytes.

The spatial expression pattern of FetA was analyzed further by in situ hybridization using a FetA-specific probe. Consistent with the results of northern blotting, in situ staining revealed that FetA was first detectable in cells close to the center of the seminiferous tubules in 21 d.p.p. testes (Fig. 6C). This area was occupied by the late pachytene spermatocytes. In fully developed adult testes, FetA was dominantly expressed in pachytene spermatocytes in the middle layer of testis tubule (Fig. 6D). There was no staining in the Sertoli and Leydig cells. The lack of signal in sections of 14 d.p.p. mouse testis tubule, where spermatogonia and somatic cells reside, further confirmed that FetA expression was tightly correlated with spermatocytes differentiation (Fig. 6B). FetA mRNA was exclusively present in pachytene spermatocytes during the spermatogenesis.

**FetA cellular localization during mouse spermiogenesis**

To precisely determine the subcellular localization of FetA in germ cells, we generated polyclonal antibodies against FetA. Various fragment of FetA protein were tested as antigens, and finally one cytoplasmic domain (715-810, not present in splicing form B) yielded a specific antisera. We purified the antisera by affinity chromatography (see Materials and Methods). To characterize the antibody, we analyzed cellular lysates prepared from HEK293T cells expressing HA-tagged FetA by western blotting with the affinity-
purified antibodies. The antibodies clearly recognized a ~110 kDa band in FetA-overexpressing cells, whereas a band with smaller molecular mass was also detected, perhaps resulting from protein degradation or a post-translational modification. No signal was detected in extracts from the parental cells (Fig. 7A). Moreover, there was no cross reaction with preimmune serum (Fig. 7A, lane 4), indicating that the anti-FetA antibodies specifically recognizes FetA.

Western blot analysis using this specific antibody indicate that the antibody detected a band of ~110 kDa in membrane protein extracts of mouse testis (Fig. 7B), whereas in mature sperm extracts FetA migrated at ~120 kDa, suggesting altered post-translational modification of FetA specific to sperm cells. Antibody pre-absorbed with antigen did not react with these bands, providing further evidence for the antibody specificity (data not shown).

The spatial intracellular localization of FetA during mouse spermatogenesis was further studied by immunocytochemistry. FetA was first detected in the cytoplasm of spermatocytes (Fig. 7C), whereas, in round spermatids it was exclusively detected in the developing acrosome vesicle. As shown in Fig. 7C, in the step 2-3 spermatids, FetA was seen as a spot very close to the nucleus. In cap phase spermatids (step 5), the area of positive staining was still very close to the nucleus but had expanded to form an arc over approximately to one-quarter of the nucleus. The subcellular localization largely changed in the elongated spermatids and concentrated in a subcellular domain where the acrosome forms. Moreover, the localization of FetA in mature mice sperm was concentrated in the acrosomal region and colocalized with the acrosome marker PNA-lectin (Fig. 7D,E). Thus, FetA expression is tightly correlated with acrosome formation during spermatogenesis and localizes to the acrosome in the mature sperm.

In search of an appropriate model system to further investigate FetA function, we next screened several mouse cell lines (NIH 3T3, CHO K1, PK136 and P815) and found FetA expressed in the mouse mastocytoma cell line P815 (data not shown and Fig. 8A,B). To

![Fig. 4. Lipid internalization in FetA-expressing S. cerevisiae.](image)

(A,B) Internalization of NBD-lipids by Δdrs2Δdnf1Δdnf2 mutants transformed with empty vector (control) or plasmids expressing FetA or FetA (D461A). Cells grown in the presence of 50 μM CuSO₄ were incubated with NBD-PE for 30 minutes at 30°C. Cell-associated fluorescence was measured by flow cytometry after washing the cells twice with ice-cold BSA-containing medium as described in Materials and Methods. (A) Expression of FetA resulted in cells with increased NBD-PE and NBD-PC uptake. The gray histogram represents control cells, the black histogram represents cells expressing FetA. Data for FetA (D461A) have been omitted, because the values overlapped with the controls. (B) Accumulation of NBD-lipids was expressed as a percentage of fluorescence intensity relative to control Δdrs2Δdnf1Δdnf2 mutant cells. Data are means ± s.e.m. of at least three independent experiments. Significant differences compared with cells harboring the control vector were determined by using the two-tailed Student’s t-test (*P<0.05; **P<0.01).
precisely localize FetA in P815 cells, we performed double-immunofluorescence staining with anti-FetA antibody and different organelle markers. As shown in Fig. 8C, a large part of FetA colocalized with GM130 and the 58K Golgi protein, markers for the cis-Golgi region and general Golgi network, respectively. In addition, FetA exhibited a punctate vesicular staining pattern throughout the cytoplasm, which did not colocalize with markers for endosomes (EEA) or acidic organelles (LysoTracker).

Depletion of FetA in P815 cells causes aberrations in Golgi structure and a defect in protein secretion.

As deletion of the Golgi-associated P4-ATPase Drs2p in yeast is accompanied by the accumulation of aberrant membranes and loss of the normal Golgi tubular networks at lower temperature, we next analyzed the impact of RNAi-mediated depletion of FetA on Golgi morphology in P815 cells at 33°C and 37°C. To this end, we created P815 cell clones stably transfected with one FetA shRNA construct (Fig. 9). After G418 selection, several independent cell clones with reduced FetA expression (knockdown cells) were obtained. Two clones (siFetA-15 and siFetA-16) with substantially reduced (80-90%) FetA protein levels were selected for further analysis (Fig. 9A). The scrambled shFetA cell clone was used as control.

We then analyzed the Golgi morphology of FetA knockdown cells by wheat germ agglutinin (WGA) staining. Silencing of FetA did not influence the Golgi morphology in cell cultured at 37°C (Fig. 9B). However, in cells cultured at lower temperature (33°C)
depletion of FetA resulted in a more dispersed Golgi complex (Fig. 9C). Organelles such as ER and mitochondria (Fig. 9C) remained normal under these conditions. Quantification of the WGA staining confirmed that the Golgi complex was dispersed in at least 40-60% of cells from two independent clones stably transfected with FetA shRNA but not in cells stably transfected with control shRNAs (Fig. 9D). Further electron-microscope analysis of FetA-depleted cells indicated that the cis- and trans-membranes of the Golgi complex could not been distinguished and the mid-membranes were loosely stacked; in addition swollen Golgi membranes were observed (Fig. 9E).

Accumulation of aberrations in Golgi structures in FetA knockdown cells prompted us to analyze the secretory activity in FetA knockdown cells by pulse-chase experiments. Cells were incubated at 33°C for 24 hours, pulse labeled with [35S]methionine and [35S]cysteine and analyzed for release of labeled proteins into the culture medium. In control cells, secretion could be detected after 2 hours and increased over time (Fig. 10). Knockdown of FetA by RNAi in P815 cells nearly completely inhibited the release of labeled proteins into the culture medium. Collectively, these data show that FetA silencing has profound effects on the ultra-structural organization of Golgi complex and the constitutive secretion of cargo proteins at lower temperature.

**Discussion**

P4-ATPases are transmembrane proteins unique to eukaryotes that recently have been linked to lipid transport, the maintenance of membrane structure and vesicle trafficking. We have identified a novel mouse P4-ATPase that is specifically expressed during spermatogenesis and serves an essential role in the maintenance of the structural organization of Golgi complex.

Evidence for a functional assignment of FetA as an inward phospholipid flipase

Phylogenetic analysis of the P4-ATPase family aligns FetA with a cluster that includes S. cerevisiae, mouse and human putative lipid transporters. Evidence for a role of FetA in the inwardly directed transport of phospholipids was provided by heterologous expression in a S. cerevisiae mutant strain deleted in three P4-ATPases (Drs2p, Dnf1p and Dnf2p). Because this yeast mutant lacks an ATP-dependent transport activity for NBD-PE, NBD-PC and NBD-PS from the exoplasmic to the cytosolic leaflet of the plasma membrane, even low inwardly directed transport activities can be analyzed in this system. FetA was detected primarily in intracellular membranes but a minority was present on yeast plasma membranes thus enabling analysis of its lipid uptake activity using fluorescent NBD phospholipids. We observed a small, but reproducible inwardly
A novel mouse P4-ATPase: cloning and characterization

Notably, this transport activity was not observed in yeast cells expressing non-functional FetA (D461A), although this inactive ATPase had similar distribution. Furthermore, we did not observe differences in the accumulation of NBD-PS and NBD-SM between control and FetA-expressing cells, indicating that overexpression of FetA probably does not affect NBD-lipid accumulation by endo- or exocytosis. These results suggest that FetA might function as an inward phospholipid flippase. Notably, Wang et al. (Wang et al., 2004) described a candidate aminophospholipid flippase in mouse sperm cells that is exclusively expressed in the acrosomal region and shares 43% identity with FetA. Mice having a disrupted SAPLT gene expose PS at the sperm surface supporting a role for this P4-ATPase in the regulation of PS distribution across the sperm plasma membrane. However, final proof of a direct role in lipid transport requires studies on purified and reconstituted P4-ATPases.

FetA potential functions in spermatogenesis

Mammalian spermatogenesis includes three phases: germ stem cell (spermatogonia) proliferation, and two rounds of meiosis and spermiogenesis in which the spermatids differentiate to form spermatozoa. One of the major events in spermiogenesis is the formation of the acrosome, a unique membranous organelle that plays an important role in fertilization. This process involves intensive intracellular vesicle formation and fusion (About-Haila and...
Fig. 10. Depletion of FetA in P815 cells causes a defect in protein secretion at 33°C. P815 cells stably transfected with control or FetA siRNA were cultured at 33°C for 24 hours, pulse labeled with [35S]methionine and [35S]cysteine for 2 hours and then chased using non-radioactive amino acids for the indicated time periods. The extent of protein secretion was determined by assaying the radioactivity incorporated into cell-associated or medium TCA-precipitable protein. Values are means of triplicate samples, and error bars represent s.e.m.

Tulsiani, 2000). Our analysis shows that FetA expression tightly correlates with spermatocyte differentiation: FetA is exclusively present in pachytene spermatocytes during spermatogenesis and localizes to the acrosome in mature sperm.

Interestingly, we observed that its apparent molecular mass can differ substantially in testis, sperm and P815 cells. This suggests differences in the post-translational modification between these cell types, which might have important functions in controlling FetA localization and/or activity. For example, many P-type ATPase members such as the plasma membrane Ca2+/ATPase (P2B-type) and the plasma membrane H+ATPase (P3-type) are regulated by phosphorylation of serine residues. Furthermore, we detected a short RNA transcript (1.5 kb) predicted to encode a truncated protein lacking the catalytic domain. Future studies are required to assess expression of this truncated protein and its potential contribution to FetA function and regulation in testis.

Our analysis in mastocytoma P815 cells revealed that silencing of FetA expression has a profound impact on the structural organization of the Golgi complex and protein secretion in these cells at the lower temperature used (33°C). This is in accordance with the comparable observation in the yeast S. cerevisiae, the plant Arabidopsis thaliana, and the worm Caenorhabditis elegans, where loss of P4-ATPases is accompanied by defects in vesicle formation and membrane trafficking in the secretory and endocytic pathways (Chen et al., 1999; Gall et al., 2002). It has been proposed that P4-ATPases might help deform the membrane by moving lipid mass towards the cytoplasmic leaflet, so creating a tension driving vesicle formation required for organelle assembly and maintenance (Graham, 2004). This might be particularly crucial at lower temperatures, when membrane fluidity is decreased. It is thus feasible that FetA might help generate and maintain the Golgi complex by actively supporting vesicle formation, but become indispensable for this process only at lower temperature. As the Golgi structure is maintained by a dynamic balance of anterograde and retrograde membrane flow (Glick, 2002), downregulation of FetA might disrupt this balance resulting in Golgi vesiculation and/or redistribution. Furthermore, P4-ATPases were found to directly interact with cytosolic proteins important in vesicular trafficking (Graham, 2004). Thus FetA might help to concentrate the vesicle budding machinery at specific sites of the Golgi network. In sperm, FetA might also actively participate in the formation of the acrosome that derives from the Golgi complex during spermiogenesis. P4-ATPases might also help create a high local concentration of specific lipids and in the cytosolic membrane leaflet. The enrichment of aminophospholipids in the cytosolic leaflet of the plasma membrane and on the surface of endocytic and exocytic vesicles might help keep these membranes in a fusion-competent state. This might not only be important during spermiogenesis but also during fertilization when the acrosome fuses with the sperm plasma membrane and releases its contents to aid the capacitated sperm to penetrate the egg, a process known as acrosome reaction (Wassarman et al., 2005). Further analysis is warranted to fully uncover the physiological role of this novel P4-ATPase.

Materials and Methods
Database searches and amino acid sequence analysis
Motifs and domains of FetA were predicted using the SMART server (Schultz et al., 1998). Database searches for orthologs of FetA were carried out using BLASTP with standard parameters and the NCBI nonredundant protein database. Blast hits were assigned based on best reciprocal hits. Multiple alignments were performed using the CLUSTALW program. Phylogenetic analysis was carried out with the MEGA program using the neighbor-joining algorithm. Statistical support values for internal branches of the phylogenetic tree were obtained from 1000 bootstrap samples and their analysis. The GenBank accession numbers for the genes constituting the phylogenetic tree are as follows: XP_001069520.1, XP_001062555, XP_001077984, XP_214553, XP_927743, XP_930531, ATP8B1, XP_001085877, XP_00114383, ATP8B2, ATP8B4, ATP8B1, XP_854716, XP_544674, XP_533394, XP_595008, XP_429208, XP_683800, XP_691903.

Isolation of cDNAs and generation of constructs
Reverse transcription PCR (RT-PCR) was performed using mouse adult testis total RNA (2 μg) as template, and FetA cDNA was amplified using primers (forward primer, 5’-GAGTAGGAGGCTCGTGAAG-3’; reverse primer, 5’-GGAGGAAGTTGTA- CATGATG-3’). The 5’ RACE experiment was performed using the SMART RACE kit (Clontech, Palo Alto, CA), using mouse testis total RNA and the gene-specific primers (FetA-5’RACE1 primer, 5’-TGCAGGACCACTGTAGGAAGAC-3’; FetA-5’RACE2 primer, 5’-ACCACGTCGCCT-CACAGCTCTA-3’). The amplified fragment was purified, cloned and sequenced. The sequence has been submitted to GenBank and the accession number is EF377342. A HA-tagged FetA vector was created by ligating the open reading frame sequence of FetA into pcDNA3-HA (provided by Gang Pei, Tongji University, Shanghai, China) between the EcoRI and Xhol sites. To silence FetA expression, three shRNA-expressing plasmids were generated using the pGCU-U6 vector by Genechem Corporation (Shanghai, China). The plasmid containing the target sequences 5’-GCAAGAGACTGCTGTAGAAGAC-3’ corresponding to nucleotides 2189-2207 efficiently knocked down FetA; the scrambled shRNA (GCAAGCGGAAGTGTA-ATAGAAATACGTAAGACCTGT-3’ bearing the Xhol site) and reverse (5’-ggggttgggccgtcatgactgggtgct-3’ bearing the NotI site) primers followed by cloning into the multicopy plasmid pYEXTH-FHC behind CUP1 promoter and tagged with FLAG at the N-terminus. The point mutant of FetA was generated by QuikChange site-directed PCR mutagenesis following the manufacturer’s instructions (Stratagene) and verified by sequencing. To create the FetA tagged with GFP at the N-terminus, FetA was cloned into pPEFPN2 between Xhol and EcoRI sites and subsequently cut out with Xhol and NotI for ligation into pYEXTH-FHC between the XhoI and NotI sites. The multicopy plasmids pYEXTH-FHC and pYEXTH-BN-GFP were provided by Christine Lang (Technical University, Berlin).

Animals, cell culture and 35S pulse-chase experiments
C57BL/6J mice were obtained from the Animal Center for the Chinese Academy of Sciences (Shanghai, China) and maintained at the animal facility in Shanghai Institute of Biochemistry and Cell Biology, CAS. All experimental procedures were performed according to the act of the Institute Animal Care and Use Committee of Shanghai Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences. Human embryonic kidney (HEK293T) cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum; P815 cells were grown in RPMI 1640 containing 5% fetal calf serum (Biochrom). P815 cells were stably transfected with shRNA by electroporation (Cell Line Nucleofector, Program C-005; Amaxa) and selected in medium supplemented with 800 μg/ml G418 (Sigma) for 2 weeks. To assay total proteins secreted into the medium, P815 cells stably transfected with control or FetA siRNA were grown at 33°C for 24 hours and were preincubated with
methionine and cysteine-free medium for 1 hour, metabolically labeled for 2 hours with 200 μCi/ml of EXPRESS protein labeling mix containing [35S]methionine and [35S]cysteine (PerkinElmer Life Sciences), and rinsed twice with PBS. Cultures were incubated with fresh medium containing non-radioactive amino acids and chased for the indicated time. Cells and the culture medium were collected and total proteins were precipitated with 10% trichloroacetic acid (TCA) and analyzed by liquid scintillation counting.

Northern blot
Total RNA (10 μg) was extracted from different tissues of adult mice or from testes of different ages using TRIzol reagent (Invitrogen), fractionated on denaturing gels containing formaldehyde and transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech, Arlington Heights, IL). A PCR fragment corresponding to nucleotides 194-713 of the FetA cDNA was labeled with [α-32P]-deoxyATP using a Primer label kit (Promega) and used as probe. Membrane hybridization, washing and autoradiography, as well as stripping and reprobing were performed according to the manufacturer’s instructions. Blots were stripped and hybridized with a GAPDH probe (Ambion, Austin, TX) to control RNA loading.

In situ hybridization
In situ hybridization was performed as previously described (Brett et al., 2003). Briefly, a fragment of nucleotides corresponding to FetA nucleotides 3783-4237 was subcloned into a pGEM-T vector (Promega). Digestoxigen (DIG)-UTP-labeled sense and antisense probes were generated using a Riboprobe In Vitro Transcription System (Roche Applied Science). Formalin-fixed, paraffin-embedded testes were cut into 10 μm sections, hybridized with probe, incubated with anti-DIG-alkaline phosphate (1:3000), and color reacted by NBT-BCIP substrate according to instructions from Boehringer Mannheim. Sections were fixed and observed with the light microscope.

Generation of antibodies against FetA
To prepare polyvalent anti-mouse FetA antiserum, a chimeric protein comprising two tandem copies of 96-amino acids (715-810) of FetA fused with glutathione S-transferase (GST) was expressed in Escherichia coli, purified by glutathione affinity chromatography and used for immunization of rabbits (Hu et al., 2002). Antibodies were affinity purified using a HIS-FetA fusion protein chromatographic column (Pierce, Rockford, IL) according to the manual. To this end, His-tagged FetA antigen (115 kDa) was expressed in E. coli and purified by metal chelate chromatography (Ni-NTA resin; Qiagen, Inc.) To test the specificity of the FetA antibodies, HEK293T cells were either mock transfected or transfected with pcDNA3-HA-FetA using Fugene 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. After 48 hours in culture, cellular lysates were prepared and subjected to western blot analysis using the affinity purified FetA antibodies.

Western blot
Tissues or cells were homogenized by vortexing with glass beads (425-660 μm) in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.6 M sorbitol) containing 0.5% Triton X-100 (TX-100) and 2 mM benzamide. Cell lysates were centrifuged (500 g, 10 minutes, 4°C), and the resulting supernatant was centrifuged at 100,000 g for 20 minutes at 37°C. Equal amount of sample were separated by SDS-PAGE gel and wet-transferred onto PVDF membrane for 2 hours on ice followed by immunoblotting. Affinity-purified antibodies against FetA were used at a dilution of 1:2000 and incubation was carried out overnight at 4°C. Secondary antibody conjugated with horseradish peroxidase (1:10,000; Santa Cruz Biotechnology) was incubated with PVDF membranes for 1 hour at room temperature. Blots were developed using ECL (Amersham).

Immunofluorescence, confocal microscopy and electron microscopy
Mouse seminiferous tubule segments at defined stages were isolated using the transillumination-assisted microdissection method (Parvinen and Hecht, 1981). Immunofluorescence was performed from staged squash preparations of microdissected tubules from C57 BL/6 mice as previously described (Kotaja et al., 2004; Marianov et al., 2001). Caecal epididymis sperm were collected, washed three times with phosphate-buffered saline, and mounted on slides before being air-dried. Sperm were fixed in 4% paraformaldehyde and 0.1% Triton X-100 for 30 minutes at room temperature, washed and blocked for 1 hour in PBS containing 0.3% normal goat serum. Samples were incubated with anti-FetA (1:100) overnight at 4°C, washed, then incubated with Alexa Fluor-488-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:100; Molecular Probes) for 1 hour. DNA was counterstained with DAPI [2-(4-aminophenyl)-6-indolecarbamidine dihydrochloride, 1 μg/ml] for 1 minute at room temperature. The antibody specificity to anti-FetA was identified using pre-immune serum and anti-serum preincubated with FetA protein. For staining of the Golgi complex, GM130 (1:300; BD Biosciences), S8K (1:50; Abcam), both with Cy3-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:100; Jackson ImmunoResearch Laboratories) and TRITC-conjugated wheat germ agglutinin (TRITC-WGA) lectin (1:50; Invitrogen) were used. Anti-EEA1 (1:500; BD Biosciences) was used as endosomal marker and Lysotracker red DND-99 (Invitrogen-Molecular Probes; 50 nM, 30 minutes) was used to label acidic compartments.

The staining patterns were observed and photographed using a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) and its imaging system. For electron microscopy, cells were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M Sörensen phosphate buffer (pH 7.4), washed, post-fixed in aqueous 2% osmium tetroxide and finally embedded in Epon resin. Electron microscopy was performed with a Joel JEM-1230 transmission electron microscope, at 90 kV, on ultra-thin sections (80 nm) stained with lead citrate and uranyl acetate.

Yeast strains and growth conditions
Saccharomyces cerevisiae strain ZYH709 (MATα his3 leu2 ura3 met15Δ/Δ dna1Δ dna2Δ drs2Δ/LEU2) was used as eukaryotic expression strain (Poulsen et al., 2008). Strain BY4741 (MATα his3 leu2 ura3 met15Δ/Δ EURO3CAREF) was used as a wild-type control. Both strains were provided by Todd R. Graham (Vanderbilt University, Nashville, TN). Unless indicated otherwise, cells were grown at 28°C to 0.3-1.0 optical density (OD600/ml) in selective synthetic dextrose (SD) medium.

Expression and subcellular distribution in yeast
Yeast cells were transformed with the lithium acetate method and selected by uracil prototrophy. Colonies were propagated in selective medium (SD medium lacking uracil) at 28°C. Expression was induced for 3-4 hours by the addition of 50 μM CuSO4 at 0.3-0.5 OD600/ml. Cells were screened for FetA expression by western blotting or analyzed for lipid uptake. For subcellular fractionation, cell lysates were subjected to differential centrifugation (500 g for 10 minutes; 9000 g for 20 minutes). Membrane pellets from the 9000 g centrifugation enriched in the plasma membrane were loaded on top of a step gradient consisting of 53% and 43% (w/w) sucrose in lysis buffer. After centrifugation at 100,000 g in a Beckman SW40T1 rotor (18 hours, 4°C), ten 1-ml fractions were collected from the top. Equal volumes per fraction were used for western blot analysis. pET-3d-FetA plasmids and yeast strains, respectively. We also thank Karin and Peter Mueller for expert comments on the manuscript. The contributions of Nele Alder-Baerens and Hanka Paulitschke during the early stages of this work are greatly appreciated. This work was supported by National Key Project for Basic Science Research of China 2009CB941100, 2007CB947903, 2005CB522701 and National Natural Science Foundation of China 30874114, 30771077 (to X.-Y.D.), National Natural Science Foundation of China 30500276 (to R.-Y.H.), and the Robert Bosch Foundation and the Deutsche Forschungsgemeinschaft Grant Po745/4-4 (to T.G.P.)

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