The Testis Anion Transporter 1 (Slc26a8) is required for sperm terminal differentiation and male fertility in the mouse

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The Slc26 family is a conserved family of anion transporters. In the human, their physiological relevance was highlighted with the discovery of pathogenic mutations in several Slc26 transporters that lead to distinctive clinical disorders (Pendred syndrome, deafness, diastrophic dysplasia, congenital chloride diarrhoea) that are related to the specific distribution of these genes. We previously identified TAT1 as a new family member (Slc26A8), very specifically expressed in male germ cells in both the human and the mouse. To investigate Tat1 function in the male germline, we generated mice with a targeted disruption of the Tat1 gene. Heterozygous and homozygous Tat1 mutant mice were indistinguishable from wild-type littermates concerning survival rate, general appearance and gross behaviour; however, Tat1 null males were sterile due to complete lack of sperm motility and reduced sperm fertilization potential. Ultra-structural analysis revealed defects in flagellar differentiation leading to an abnormal annulus, disorganization of the midpiece–principal piece junction, hairpin bending of the sperm tail with disruption of the axial structures, and abnormal mitochondrial sheath assembly. While ATP levels were normal, ATP consumption was strongly reduced in Tat1 null spermatozoa. Interestingly, Tat1 is located at the annulus, a septin-based circular structure connecting the midpiece to the principal piece. Altogether, our results indicate that Tat1 is a critical component of the sperm annulus that is essential for proper sperm tail differentiation and motility.

INTRODUCTION

The Slc26 family is a conserved family of multifunctional anion exchangers, comprising more than 50 members from bacteria, yeast, plants, nematodes and mammals (1,2). Much of the homology between Slc26 members resides in the transmembrane activity domain and in the regulatory STAS domain (sulphate transporter and anti-sigma antagonist) located near the C-terminus of the protein.

Slc26 members have been shown to transport both monovalent and divalent anions such as sulphate (SO\(_4^{2-}\)), chloride (Cl\(^-\)), iodide (I\(^-\)), or bicarbonate (HCO\(_3^-\)) (2). Their activity is essential for various physiological functions and differentiation processes. In the human, four diseases have been associated to loss-of-function mutations in SLC26 genes (i.e. Pendred’s syndrome, deafness, diastrophic dysplasia, and congenital chloride diarrhoea). Although these disorders display distinct clinical features, they all appear to result from defective anion transport in the tissues where Slc26 transporters are predominantly or specifically expressed (1,3). For example, in patients affected by diastrophic dysplasia due to mutations of the sulphate transporter DTDST (Slc26A2), the sulphate uptake of their chondrocytes is reduced, preventing proper sulphation of the proteoglycans in the extra-cellular matrix of the cartilage, which in turn is
responsible for the defective bone development that is characteristic of these patients' phenotype (4–6). In the same line, the phenotype of patients affected by Pendred's syndrome due to mutations of the iodide/chloride transporter PDS (Slc26A4) is characterised by pre-lingual deafness and an adenomatous goitre that are related to the extremely specific tissue distribution of PDS in the inner ear (cochlea) and the thyroid (7,8).

In recent years, several groups have developed murine models mutant for the Slc26 family members in order to explore the pathophysiological mechanisms of these transporters and to generate useful models for therapeutic experimentation (9–12). Slc26 mouse models have proven useful by replicating many clinical features of homologous human disorders. This was recently illustrated with the knock-in model of the Dtdst gene (Slc26a2) which results in a partial loss of function of the sulphate transporter and reproduces human chondrodysplasia clinically, morphologically, and biochemically (11).

We have previously identified TAT1 (testis anion transporter 1/Slc26A8) as a new member of the human SLC26 family and have shown that, in vitro, TAT1 can transport sulphate in a chloride-dependent manner (13). TAT1 gene expression is restricted to male germ cells at the spermatocyte and spermatid stages, and the TAT1 protein mainly localizes to their plasma membranes (13,14). The remarkable specificity of TAT1 expression in human male germ cells, as evidenced by the analysis of 17 human adult tissues, suggests that TAT1 could play a specific role in germ cell function and/or differentiation. To investigate TAT1 function and relevance in the male germline, we generated mice with a targeted disruption of the Tat1 gene. Our results indicate that the anion transporter Tat1 is a critical factor for proper sperm tail differentiation and motility, which is consistent with the location of the Tat1 protein at the annulus, a septin-based circular structure of the sperm’s flagellum.

RESULTS

Targeted disruption of the Tat1 gene

In the human, TAT1 gene expression has previously been shown to be restricted to the testis and specifically to germ cells at the spermatocyte and spermatid stages. Expression analysis of the murine orthologue revealed the same specificity for adult testes. In addition, analysis in murine pre-pubertal testes shows that Tat1 gene expression begins at 16 days post-partum (dpp), which coincides with the first wave of spermatocytes in the pre-pubertal testis (Fig. 1A). We therefore conclude that like in the human, Tat1 expression in the mouse is specific to post-mitotic germ cells undergoing spermatogenesis.

To investigate Tat1 physiological function in male germ cells, we generated Tat1 deficient mice using standard methods.
homologous recombination procedures. We confirmed successful disruption of the gene by Southern blot (data not shown), RT-PCR and PCR analysis on genomic DNA or RNA, extracted from wild-type, heterozygous, and homozygous mice (Fig. 1B).

Lack of fertility in Tat1 null mice

Heterozygous and homozygous Tat1 mutant mice were viable and indistinguishable from their wild-type littermates in survival rate, general appearance, and gross behaviour (i.e. size, weight, fur, and activity). However, we noticed that over a period of 2 months, and despite normal sexual behaviour confirmed by the presence of vaginal plugs in mated females, Tat1 homozygous mutant males failed to fecundate females.

In order to define the causes of this male infertility, we undertook a broad analysis of the male genital system in Tat1 null mice. Testes, epididymes and seminal vesicles of Tat1 null males appeared macroscopically normal and did not show significant difference in weight when compared to wild-type or heterozygous mutant males (Fig. 2A). Histological analysis of Tat1 null testes and epididymes indicated that spermatogenesis was occurring normally and that all types of germ cells were produced, including spermatozoa (Fig. 2B). Sperm counts from total epididymes did not reveal quantitative defects in mutant mice when compared to wild-type mice (Fig. 2C). We can therefore conclude that the sterility observed in Tat1 null mice was neither due to an immaturity of male reproductive organs nor to an arrest of spermatogenesis.

Lack of motility of Tat1 null sperm

We next proceeded to the microscopic examination of sperm collected from the cauda epididymis, and observed that Tat1−/− sperm were completely immotile. Subsequent quantitative analysis using a Hamilton-Thorne motility analyser indicated that Tat1-deficient males (n = 4) produced 4.6% motile sperm including 3% progressive sperm, whereas wild-type and heterozygous littermates (n = 4) respectively produced 60 and 66.5% motile sperm including 36.25 and 43% progressive sperm (Fig. 3A).

While assessing sperm motility with the microscope, we noticed that sperm collected from Tat1−/− epididymes displayed striking morphological abnormalities consisting of a thinning of the flagella at the end of the midpiece and abnormal angulation of the sperm tail, which was folded at 90 or 180 degrees (Fig. 3B). Consistent with their wild-type appearance and normal breeding performance, Tat1 heterozygous mice did not show any defect in sperm morphology or motility.

In order to stage the onset of the structural defects observed in Tat1−/− sperm, we performed a microscopic analysis of sperm collected from the testes and from the three distinct regions of the epididymis (caput, corpus, and cauda). In the testes, we observed thinning of sperm flagella but only rare cases of angulation (data not shown), thus suggesting that angulation was acquired during the normal transit of the

Figure 2. Tat1 null males have mature reproductive organs and normal sperm counts. (A) Index (weight ratio, WR) of organ weight (in milligrams) per body weight (in grams) calculated for adult testes, epididymes and seminal vesicles reveal no significant difference between the three genotypes (Student t-test; n = 4). (B) Haematoxylin and eosin staining of adult testes and cauda sections showing normal spermatogenesis in Tat1−/− mice. (C) Sperm enumeration showing no quantitative defect in the number of spermatozoa collected from Tat1+/− and Tat1−/− epididymes in comparison with wild-type littermates (Student t-test; n = 5).
spermatozoa in the epididymis. Indeed, precise quantitation of angulated sperm in the three distinct regions of the epididymis clearly showed a progressive increase in the proportion of angulated sperm from the caput to the cauda of Tat1-deficient epididymes (Fig. 3C).

**Tat1 subcellular localisation in spermatozoa**

While Tat1 protein expression at the spermatocyte and spermatid stages is well documented, expression in mature spermatozoa has not been investigated. The striking phenotype of Tat1 null sperm led us to check for the expression of Tat1 in mature spermatozoa. We performed immunofluorescent staining for the Tat1 protein on spread preparations from mouse epididymes. We observed that Tat1 was localised in the end of the midpiece of the flagella, which is easily distinguishable by staining the mitochondrial sheath with a mito-tracker dye. As expected, no expression of the Tat1 protein was found in Tat1−/− spermatozoa (Fig. 4A).

The region labelled for Tat1 expression coincides with the ‘Jensen ring’, a ring-shaped structure which is also called the ‘annulus’ and is located at the junction of the midpiece and principal piece of the tail. To confirm that Tat1 localisation corresponds to the annulus, we attempted to compare the expression pattern of Tat1 with that of Sept4, a recently described marker of the annulus. Since antibodies to Tat1 and human SEPT4 were both raised in rabbits, we were not able to perform a double labelling detection; however, individual staining on human sperm shows an identical pattern of expression for TAT1 and SEPT4 (Fig. 4B). Moreover, analysis of TAT1 and SEPT4 staining by confocal microscopy clearly distinguishes the signal into two symmetrical round spots that are typical of the annulus ring structure (Fig. 4C). We therefore conclude that both in the human and in the mouse, Tat1 expression begins at the spermatocyte stage and that the Tat1 protein persists in spermatozoa where it is entirely localised to the annulus. This has recently been confirmed by a proteomic analysis that showed the presence of Tat1 in the membrane-associated fraction of mouse mature sperm.

**Structural defects of flagella in Tat1 null sperm**

To further characterise the morphological abnormalities observed in Tat1−/− sperm, we performed electron microscopy on sperm preparations from testes or epididymes of wild type, heterozygous and homozygous mutant mice (Fig. 5).

In Tat1−/− testes, spermatogenesis appeared normal until the early stage of elongating spermatids; the first anomalies were observed at the stage of annulus relocation, which failed to reach the midpiece-principal piece junction, leading to an
incomplete mitochondrial sheath. Accordingly, we observed a ‘gap’ between the midpiece and the principal piece that most likely corresponds to the terminal segment of the midpiece without the surrounding mitochondria (Fig. 5A).

In the epididymis, in contrast to normal flagella which are slightly curved at the cytoplasmic droplet (Fig. 5B), Tat1<sup>−/−</sup> flagella were radically bent and displayed a hairpin configuration with the annulus either abnormally shaped, ectopically located, or isolated from other flagellar structures. The retroflexion occurred in the gap region, leading to a disruption of the axial structures (the axoneme and dense fibres) (Fig. 5C).

Normally, the annulus appears as a triangular shaped structure linked to the mitochondria, the fibrous sheath, and the plasma membrane (Fig. 5D). In Tat1<sup>−/−</sup> spermatozoa, the annulus had an oval shape and was linked only to the mitochondria (Fig. 5E).

Finally, the midpiece of Tat1<sup>−/−</sup> spermatozoa appeared disorganised, with unequally sized mitochondria that had failed to establish the usual regular helical pattern (Fig. 5F and G).

**Low ATP consumption in Tat1 null sperm**

We attempted to determine the origin of the lack of motility of Tat1 null sperm. In the literature, many cases of motile folded sperm have been reported; it was therefore unlikely that, on its own, the hairpin formation was responsible for Tat1<sup>−/−</sup> asthenozoospermia. The mechanical defects observed in Tat1 null sperm could have been due to other structural defects (i.e., a disjunction between the midpiece and the principal piece of the flagella) but also to a lack of sperm viability or a defective energy metabolism inherent to the abnormal mitochondrial sheath.

We first assessed sperm viability by staining with ethidium homodimers and flow cytometry analysis. We observed the same proportion of viable cells in Tat1 null cauda epididymis (66.7%, n = 3) and in wild-type and heterozygous cauda epididymis (respectively 66.4%, n = 3 and 76.2%, n = 3) (Fig. 6A).

We then measured ATP content and ATP consumption in sperm collected from the cauda of Tat1<sup>+</sup>/<sup>+</sup> and Tat1<sup>−/−</sup> epididymes. We found that Tat1<sup>−/−</sup> sperm had the same ATP content as wild-type sperm (respectively 3.28 × 10<sup>8</sup> versus 2.73 × 10<sup>8</sup> ATP molecules per spermatozoon at t = 0, n = 4). However, ATP consumption evaluated after a 1 h incubation at 37°C was considerably lower in Tat1<sup>−/−</sup> sperm than in normal sperm (64.5% for wild-type sperm, n = 4, versus 16.5% for Tat1<sup>−/−</sup> sperm, n = 4) (Fig. 6B). The lower ATP consumption cannot be attributed to a reduced proportion of live sperm since we found the sperm viability of both genotypes to be comparable after one hour of incubation.

We conclude that although Tat1<sup>−/−</sup> sperm are viable and able to produce normal levels of ATP, they are unable to consume ATP normally. This low ATP consumption could be
due to a defective expression of motor ATP-dependent proteins of the flagella. However, analysis of epididymides protein content by western blotting showed normal expression of all tested proteins, including dynein (intermediate chain), the major ATP consuming motor protein in the flagellum (data not shown and Fig. 7). Therefore, while the lack of motility of Tat1 null sperm must in some way be linked to reduced ATP consumption, the molecular mechanisms remain to be defined.

**Tat1 null sperm show altered capacitation and reduced acrosomal reaction**

In order to check the fertilization potential of Tat1 null sperm, we performed *in vitro* assays to determine if, despite their lack...
of motility, Tat1 null sperm were subject to the maturation events essential for subsequent capacitation of the sperm in the female genital tract and for the acrosomal reaction.

Sperm capacitation occurs in the female genital tract and leads to hyper-activation of sperm movement associated with a modification of the sperm membrane’s composition and an increase in protein phosphorylation. We used in vitro assays in which sperm isolated from the cauda epididymis were first incubated in capacitating medium then analysed for phosphorylation events as a marker of sperm capacitation. We found that, contrary to Tat1+/+ and Tat1+/− sperm, Tat1−/− sperm did not display the phosphorylation profile that is specific of the capacitation process. This suggests that at least part of the maturation events leading to sperm capacitation are most likely compromised in Tat1 null sperm (Fig. 8A).

We also analysed the potential of Tat1 null sperm to undergo acrosomal reaction by incubating sperm preparations in a capacitating medium containing Ca2+ ionophore, which can artificially induce the acrosomal reaction. We found that although Tat1−/− sperm were indeed capable of undergoing an acrosomal reaction, the percentage of acrosome reacted sperm was significantly lower than in control sperm (Fig. 8B). We therefore conclude that in addition to the lack of motility, some of the maturation events probably do not occur in Tat1−/− sperm, leading to altered capacitation and a lower proportion of acrosomal reactions.

**DISCUSSION**

This study was aimed at investigating the physiological function of Tat1 (Slc26a8) by generating mice with a targeted disruption of the Tat1 gene. Tat1 is an anion transporter that belongs to the Slc26 family, which is expressed in adult testes and shown to be restricted to germ cells at the spermatocyte and spermatid stages. Because of the remarkable tissue specificity of Tat1 expression, we had previously hypothesized that Tat1 played a crucial role during spermatogenesis. In the present study, we demonstrate that Tat1 is indeed a critical factor for male fertility: (i) Tat1 null males are unable to sire offspring although their mating behaviour and reproductive organs are normal; (ii) Tat1 null sperm are completely immotile and have a hairpin-like flagella with associated structural defects; (iii) The sperm tail differentiation of Tat1 null sperm is not normal and leads to an abnormal shape and localisation of the annulus, a shortened intermediate piece, and an irregular mitochondrial sheath. This deficiency in the latest stage of flagellar morphogenesis leads to physical disjunction between the intermediate and the principal piece of the flagella and disruption of the axial structures.

**Origin of Tat1 null male sterility**

We discovered that in addition to its expression in spermatocytes and spermatids, Tat1 is expressed in differentiated sperm where it co-localises with Sept4 in the annulus. The annulus (also known as ‘Jensen’s ring’) is a structure located at the midpiece-principal piece junction of the flagella. Although it was discovered several decades ago, little is known about its function in spermatozoa (15). Some authors suggest it may act as a diffusion barrier along the sperm flagella, or that it may play a role in the formation of the mitochondrial array of the midpiece. In Tat1 null sperm, the annulus is abnormally shaped and ectopically located; we therefore propose that the structural defects of the mitochondrial sheath observed in Tat1 null mice reflect a failure of the midpiece to reach its normal position along the flagella because of a defective annulus.

We checked if a defective energy metabolism inherent to the abnormal mitochondrial sheath could be responsible for the observed asthenozoospermia. We found that despite their abnormal mitochondrial sheath, Tat1 null sperm are viable and have a normal ATP content, suggesting that mitochondria and other ATP producing systems are not deficient. Intriguingly, although dynein—the major ATP-consuming motor protein—is normally expressed in mutant sperm, ATP consumption was found to be low. A plausible hypothesis is that the physical disjunction between the intermediate and the principal piece of the flagella compromises the

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**Figure 8.** Tat1 null sperm show reduced fertilization potential. (A) Phosphorylation profile analysis of Tat1+/+ and Tat1−/− spermatozoa under capacitation conditions. Sperm suspensions from the cauda epididymis were incubated in non-capacitating (NC) or capacitating (C) medium during 60 minutes. Protein extracts (1.5 × 10⁶ cells/lane) were then analysed by immunoblotting with an anti-phosphotyrosine antibody (4G10 antibody, Upstate-Millipore). In contrast to Tat1+/+ and Tat1+/− sperm, Tat1−/− sperm did not display a hyper-phosphorylation profile specific of capacitation events. (B) Flow cytometry analysis of Tat1+/+ and Tat1−/− spermatozoa acrosomal reaction. Sperm suspensions from the cauda epididymis were incubated in capacitating medium and the acrosomal reaction was induced by 20 μM calcium ionophore (calcimycin A23187, Sigma-Aldrich). The percentage of sperms undergoing acrosomal reaction was then measured by FACS analysis using an anti-Izumo antibody. Tat1−/− sperm display a significantly lower acrosomal reaction rate than Tat1+/+ control mice (Student t-test, * P < 0.001; n = 5).
kinesin-mediated intra-flagellar transport, which in turn alters microtubule stability and dynein activation (as has been previously described elsewhere (16)). In addition, during the epididymal transit, a folding occurs in the flagella of Tat1 null sperm at the site of the disjunction associated with a disruption of the axoneme that precludes its sliding motion, which is probably responsible for the severe asthenozoospermia.

The underlying mechanisms responsible for the folding during the epididymal transit are as yet unknown; the folding of the flagella could result from physical pressure applied on the fragile site created by the disjunction and/or from a defective osmotic regulation due to the lack of Tat1 anion transport activity. In support of the latter hypothesis, several studies have highlighted the importance of anion exchanges during sperm differentiation, epididymal maturation, and transit in the female genital tract. In response to changes in osmolarity through these various sites, sperms must regulate their anion transport to preserve normal cell volume and motility; this process has been described as ‘RVD’ and ‘RV1’ (regulatory volume decrease and increase) and is clearly illustrated by the induction of abnormal cell volume, hairpin deformations, and dyskinesia of sperms treated with ion channel inhibitors (17,18). Tat1 transport activity might participate in the anion exchanges involved in these processes during the sperm’s epididymal transit, in which case the lack of Tat1 activity would induce a hairpin deformation, which in turn would, because of the fragility of the site, lead to the disruption of the axoneme and hence to sperm immotility.

Finally, we checked if the severe asthenozoospermia observed in Tat1 null males was the only cause of their sterility by assessing the fertilization potential of Tat1 null sperm. We performed in vitro capacitation assays and observed that, in contrast to Tat1+/+ and Tat1+/- sperm, Tat1-/- sperm did not display the hyper-phosphorylation profile specific of capacitation events. Furthermore, we observed that the proportion of Tat1 null sperms that underwent acrosomal reaction was reduced. We therefore conclude that in addition to the lack of motility, Tat1 null sperms undergo altered maturation events that preclude subsequent capacitation and acrosomal reaction.

The Tat1 pathway

Sept4 is a member of the Septin family, a class of highly conserved GTPases forming heteropolymers and involved in various cellular functions (cytokinesis, yeast septation, vesicular traffic, etc.) (19,20). Recent work from two different laboratories has shown that Sept4, together with Sept1, Sept6 and Sept7 form a septin ring that constitutes the skeleton of the annulus (21,22). Interestingly, Sept4 and Tat1 not only share the same localisation in mature sperm (the annulus) but may also participate in the same flagellar differentiation process. Indeed, both Tat1 and Sept4 null mice display male infertility associated with remarkably similar structural defects of the flagella and sperm immotility (21,22). Like Tat1 null sperm, Sept4 null sperm have an altered midpiece-principal piece junction, a hairpin formation, a disorganised mitochondrial sheath, a low level of ATP consumption, and an alteration of sperm capacitation. There is no data concerning the proportion of Sept4 null sperm acrosomal reactions, but Tat1 and Sept4 mutant mice nevertheless display strikingly similar features.

As previously mentioned, all analysed proteins including septins 1, 4, 6 and 7 were found normally expressed in Tat1 null spermatozoa (Fig. 7), suggesting that the defects observed in the annulus of Tat1-/- spermatozoa are not primarily due to a low amount of septins. Unfortunately, the human Sept4 antibodies that were available to us failed to detect mouse Sept4 in immunofluorescence experiments; we could therefore not localise Sept4 in Tat1 null sperm. Nonetheless, the similarity of Tat1 and Sept4 mutant phenotypes suggests that there must be physical and/or functional interactions between septin(s) and Tat1 in differentiating spermatids and spermatozoa.

In view of the abnormal shape and position of the annulus in Tat1 null sperm (lack of attachment to the plasma membrane, Fig. 5E), Tat1 may be involved in regulating the shape and dynamics of the septin assembly and/or in the interaction of the septin ring with the flagellar membrane. This latter hypothesis is supported by the fact that unlike most other septin rings, the spermatozoa’s annulus is devoid of annilin, a PH-domain protein commonly involved in recruiting septin filaments and mediating their interaction with the plasma membrane (22). Reciprocally, the septin heteropolymer may serve as a scaffolding structure for localizing Tat1 to the annulus and/or regulating Tat1 function. In line with this latter hypothesis, a recent report by Kinoshita’s group indicates that mammalian Sept2 binds to the C-terminal region of the glutamate transporter GLAST and induces the internalisation of GLAST from the surface of glial cells.

Tat1 was initially cloned based on its interaction with MgcRacGAP (RACGAP1), a specific GAP for Rho GTPases (13,23). The role played by MgcRacGAP in the cytokinesis of somatic cells is now very well established by numerous studies, not only in cell cultures but also in vivo by inactivation models in Caenorhabditis elegans and in the mouse (24–26). Despite the abundant expression of MgcRacGAP in male germ cells (spermatocytes and spermatids), investigation of its physiological function in spermatogenesis has been hampered by the embryonic lethality of Mgc homozygous null mice (our unpublished data; (26). We have looked at MgcRacGAP expression in whole testes and epididymides of Tat1 null mice and did not find any protein level modification (data not shown). We also screened MgcRacGAP protein expression on wild-type sperm smear preparations but did not find any expression of the protein at the annulus (data not shown). We therefore assume that if Tat1 and MgcRacGAP cooperate and are part of a Rhod-dependent signalling pathway in germ cells, this must take place in the earlier stages of spermatogenesis where the proteins are co-expressed (i.e. the spermatocyte and spermatid stages). Having generated a Tat1 knockout mouse model and having uncovered the importance of Tat1 during spermiogenesis, we suggest further assessment of MgcRacGAP/Tat1 in vivo interactions by studying Mgc heterozygous mutant mice.

Tat1 implications in human infertility

Based on the results of this study, a crucial point to address is whether Tat1 could also be essential for human sperm differentiation and motility, and whether it is involved in human male infertility. Mutations in other SLC26 family members
have already been involved in human clinical disorders, such as Pendred’s syndrome, deafness, diastrophic dysplasia, and congenital chloride diarrhoea (1,2). These proteins are most often specifically expressed in a particular tissue and, in the absence of redundancy, disruption of essential physiological processes have been associated with loss-of-function mutations (3). The extreme specificity of TAT1 tissue distribution in male germ cells suggests that TAT1 may be involved in cases of human male infertility. A mutation analysis of the Slc26a8/TAT1 gene in oligo/azoospermic patients failed to detect TAT1 mutations in this group of infertile patients (27). The results of the present study suggest the investigation of infertile men with isolated spermiogenesis disorders related to those observed in Tat1 null mice. Interestingly, Iliara have studied septin expression and annulus disorganisation in human patients with asthenozoospermia (21). They reported that 20% of idiopathic asthenozoospermia without oligozoospermia are associated with a defective organisation of the annulus/septin ring. It would be interesting to further investigate the expression and location of TAT1 in sperm in this subset of infertile patients, and to address the question of the genetic involvement of TAT1 in human asthenozoospermia.

MATERIALS AND METHODS

Construction of the targeting vector and generation of Tat1−/− mutant mice

Targeted disruption of the gene was carried out using standard homologous recombination procedures. 129/SvEvBrd ES cells in which the first coding exon of the gene (exon 2) was replaced with an IRES-LacZ sequence followed by the neomycin resistance gene were produced; targeted ES cells were selected by Southern blot analysis and injected into host blastocysts from mouse strain C57Bl/6alb. Mutant mice were then dehydrated, embedded in paraffin, sectioned at 4 μm and stained with haematoxylin and eosin. Slides were examined with a Nikon Eclipse E800 microscope and all images were digitally acquired with a Nikon DXM 1200 digital camera.

Histological analysis

Testes and epididymes were dissected out and immediately fixed in Bouin’s solution for 48 hours at 4°C. Fixed tissues were then dehydrated, embedded in paraffin, sectioned at 4 μm and stained with haematoxylin and eosin. Slides were analysed with a Nikon Eclipse E800 microscope and all images were digitally acquired with a Nikon DXM 1200 digital camera.

Sperm motility analysis

The cauda epididymis was cut into pieces in M16 medium (Sigma-Aldrich Co.) supplemented with 1% BSA; the sperm released into the medium were then incubated 10–15 minutes at 37°C in 5% CO2 to allow sperm diffusion. The sperm suspension was diluted for quantitative assessment of motility using a Hamilton-Thorne motility analyser (Hamilton-Thorne Biosciences). Analysis of sperm movement was performed using the following settings: frame rate, 60 Hz; frames acquired, 30; minimum contrast, 30; minimum cell size, 4 pixels; threshold straightness, 60%; low VAP cut-off, 10 μm/s; low straight-line velocity (VSL) cut-off, 10 μm/s; static size limit, 0.1–2.87; static intensity limit, 0.1 to 2.53; static elongation limit, 1–100. For each mouse (n = 4), 1000 spermatozoa were analysed at 37°C in 100 μm standard counting chambers (Leja B.V).

Immunofluorescence staining

The caput or cauda epididymis were cut into pieces and incubated during 15 minutes in the fixative solution (PBS 3.7% formaldehyde). Smeares were performed on glass slides, briefly dried and rinsed in PBS. After permeabilisation of the cells in cold acetone, slides were blocked in PBS, 1%
BSA for 1 h, and incubated with primary antibodies for 2 h at room temperature. After 3 washes of 5 minutes in PBS, the slides were incubated with secondary antibodies for 1 h at room temperature. After 3 washes of 5 minutes in PBS, the slides were then mounted in Vectashield medium (Vector laboratories) containing 0.5 μg/ml of DAPI.

For the labelling of the midpiece, we added 500 nM of mitotracker dye Red 580 (Molecular Probes) during the incubation with the secondary antibodies.

The slides were analysed with a Zeiss Axioshot epifluorescence microscope; all images were digitally acquired with a cooled charge-coupled device (CCD) camera (Hamamatsu), under identical instrument settings. Confocal analysis was performed using a Leica TCS SP2 AOBS (DMIRE2) microscope.

**Electron microscopy**

For electron microscopy analysis, testes and epididymes (caput, corpus and cauda) were dissected and fixed in 0.1 M phosphate buffer pH 7 containing 3% glutaraldehyde (Grade I; Sigma-Aldrich Co.). After centrifugation, samples were suspended in 0.2 M sodium cacodylate buffer. Secondary fixation was performed using 1% osmium tetra-oxide (Agar Scientific), after which samples were dehydrated in graded alcohol and embedded in Epon resin (Polysciences Inc., Warrington, PA, USA). Semi-thin sections were stained with toluidine blue-Azur II and examined on a Zeiss Axioscope photomicroscope (Carl Zeiss, GmbH, Jena, Germany). Ultra-thin sections (90 nm) were cut with a Reichert OmU2 ultramicrotome (Reichert-Jung AG, Wien, Austria) using a diamond knife, mounted on nickel grids, stained with uranyl acetate and lead citrate, and examined using a JEOL JEM 100CX II electron microscope (Jeol Ltd, Tokyo, Japan). Photographs were taken on a Zeiss Axioscope photomicroscope (Carl Zeiss, GmbH, Jena, Germany).

**Sperm viability**

The cauda epididymis was cut into pieces in M16 medium (Sigma-Aldrich Co) and the released sperm were allowed to diffuse for 10–15 minutes at 37°C in 5% CO2 atmosphere, prior to the addition of ethidium homodimers (EthD) at a final concentration of 400 nM. Spermatozoa were then collected by centrifugation (600 g for 10 min) and re-suspended in PBS-0.1% formaldehyde for flow cytometric analysis (Beckman Coulter Cytometric™ FC 500).

Nucleic acid staining with EthD allowed to distinguish between dead (coloured in red) and live spermatozoa (unstained). The total number of spermatozoa analysed in each sample ranged from 10 000 to 15 000.

**ATP measurement**

For the measurement of spermatozoa whole ATP content, cauda epididymes were cut into pieces in M16 medium, and after a short incubation to allow for sperm diffusion (5 min at 37°C in 5% CO2 atmosphere), we immediately proceeded to measure ATP levels (time point 0) using the CellTiter-Glo luminescent cell-viability assay system (Promega). A second measure was similarly performed after sperm incubation for 1 h at 37°C in 5% CO2 atmosphere. All measures were performed in duplicate for n = 4 animals of each genotype. ATP content was calculated using a standard curve of ATP (range 1–1000 nM).

**Western blot analysis**

Protein lysates from epididymes or testes were obtained by homogenisation in liquid nitrogen and re-suspension in lysis buffer (HEPES 50 mM at pH 7.5; NaCl 100 mM; EDTA 5 mM; Glycerol 10%; Triton 1%) at 10% w/v. Lysates were then denatured and underwent electrophoresis through an SDS/polyacrylamide mini-gel and transferred to a nitrocellulose membrane for immunodetection. The membrane was then blocked (PBS, 0.1% Tween, 5% milk powder) for 1 h at room temperature and incubated 2 h at room temperature with the primary antibody diluted in the blocking solution. After three washes (PBS, 0.1% Tween), the membrane was incubated 45 minutes at room temperature with the secondary antibody. Following three washes (PBS, 0.1% Tween), the signal was revealed by chemiluminescence (West Pico SuperSignal, Pierce Biotechnology, Inc.).

**Capacitation assay**

3 × 10⁶ spermatozoa isolated from caudae epididymes were suspended in 300 μl of capacitating NO medium (120 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 0.36 mM Na₂HPO₄, 5.56 mM glucose, 1 mM sodium pyruvate, 18.5 mM sucrose, 50 mM HEPES, 25 mM NaHCO₃, 3% BSA; pH 7.3) or non-capacitating NO medium (CaCl₂, NaHCO₃, and BSA-free) during 60 min at 37°C in 5% CO₂. Sperm were then washed with the same medium containing 1 mM Na₂VO₃—1 mM PPi. Protein extracts from sperm suspension (1.5 × 10⁶ cells/lane) were then loaded on SDS-PAGE and transferred to nitrocellulose membrane for anti-phosphotyrosine immunoblotting (4G10 antibody, 1:400, Upstate®).

**Acrosomal reaction**

Spermatozoa from cauda epididymes were suspended in capacitating M16 medium (Sigma-Aldrich Co) 3% BSA for 90 min at 37°C in 5% CO₂. Acrosomal reaction was then induced by adding 20 μM calcium ionophore (calcimycin A23187, Sigma-Aldrich) during 90 minutes at 37°C in 5% CO₂. Spermatozoa were stained with 400 nM EthD to check their viability, then washed by centrifugation (600 g for 10 min), re-suspended in PBS, and finally incubated 60 min at room temperature with an anti-Izumo-FITC (1:100) for flow cytometric analysis (Beckman Coulter Cytometric™ FC 500). 10⁴ spermatozoa were analysed in each sample.

**Antibodies**

An antibody directed against the carboxy-terminal region of human TAT1 (aa 664–970) has been previously described.
which recognizes both human and mouse proteins. The rabbit anti-human SEPT4 antibody was a generous gift from Ingrid Bartsch (Zentrale Klinische Forschung, Freiburg, Germany). The anti-Izumo antibody was a generous gift from Masaru Okabe (Genome Information Research Center, Osaka University, Japan).

Antibodies directed against Sept1/6/7, Actin and β-tubulin were purchased from Santa Cruz Biotechnology Inc., the anti-dynein intermediate chain from Sigma-Aldrich Co., the anti-cytochrome C from BD Biosciences, and the anti-phosphotyrosine from Upstate.

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Conflict of Interest statement. None declared.

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