

## Minireview

# Septins at the annulus of mammalian sperm

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## Abstract

The annulus is an electron-dense ring structure connecting the midpiece and the principal piece of the mammalian sperm flagellum. Proteins from the septin family have been shown to localize to the annulus. A septin complex is assembled early in spermiogenesis with the cochaperone DNAJB13 and, in mature sperm, associates with Testis Anion Transporter 1; SLC26A8 (Tat1), a transmembrane protein of the SLC26 family. Studies in mice have shown that the annulus acts as a barrier to protein diffusion and controls correct organization of the midpiece. Consistent with these findings, absence of the annulus is associated with flagellum differentiation defects and asthenozoospermia in humans.

**Keywords:** annulus; asthenozoospermia; DNAJB13; septins; Tat1/SLC26A8.

## Introduction: the sperm cell and the annulus

In mammals, spermatozoa are produced in the seminiferous tubules of the pubertal testis by a complex and highly regulated process including proliferation of the spermatogonial germ cells, meiotic division of the spermatocytes and morphological differentiation of the spermatid cells. This process generates mature sperm, which have two main components: the head and the tail, linked by a connecting structure.

The tail of the sperm consists of a nine plus two microtubular axoneme surrounded by outer dense fibers and can be divided into three main pieces: the midpiece, the principal piece and the terminal piece (Figure 1A). The midpiece consists of a helically arranged mitochondrial sheath around the axoneme, which is replaced by the fibrous sheath in the principal piece. The midpiece and the principal piece are connected by the annulus, also called Jensen's ring, which appears as an electron-dense ring-shaped structure closely associated with the plasma membrane (Figure 1B).

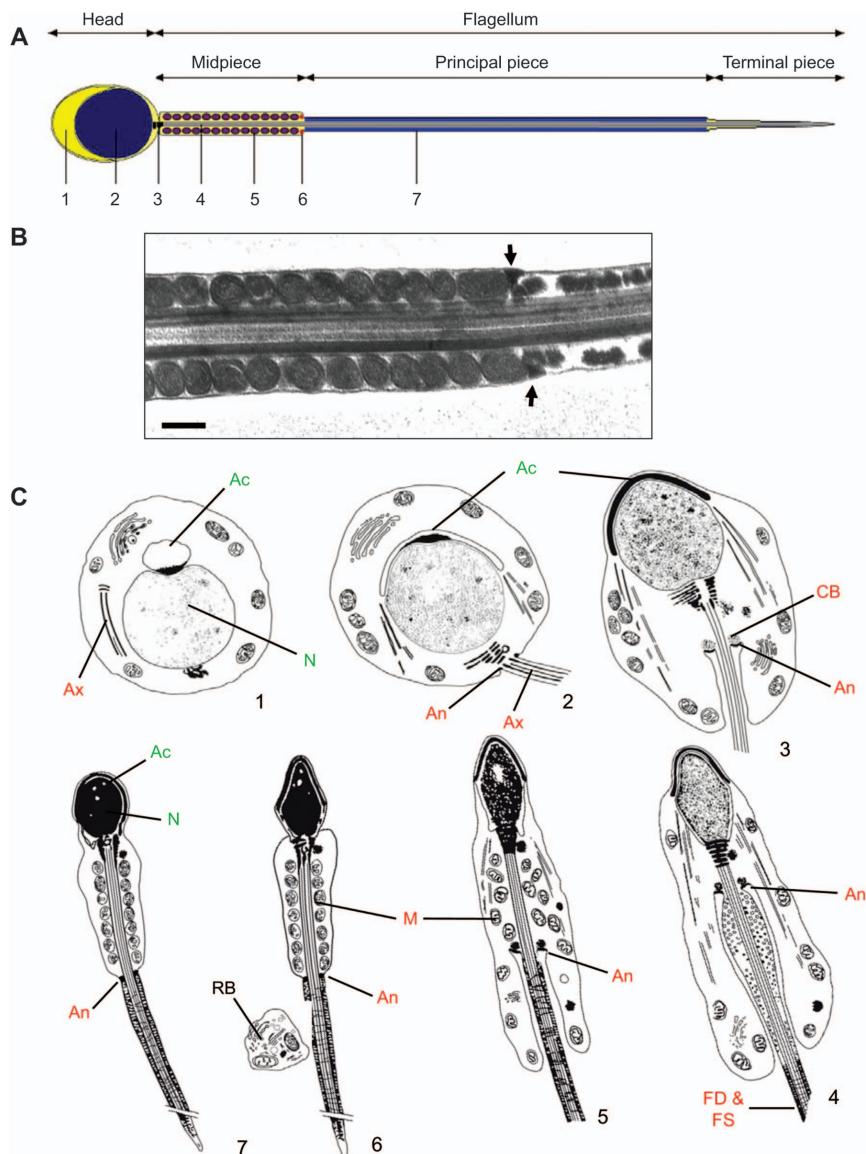
Electron microscopy studies have described emergence of the annulus from the early stages of sperm flagellum development (Holstein and Roosen Runge, 1981). The annulus is already formed when the axoneme starts to extend from the sperm cell. It is initially located at the base of the flagellum. During sperm tail elongation and differentiation, it migrates along the axoneme towards its final position at the junction of the midpiece and the principal piece (Figure 1C). Detailed morphological descriptions of the annulus were available more than four decades ago but the mechanisms underlying the migration of the annulus along the axoneme and the biochemical composition and physiological functions of the annulus remain unclear.

## Composition of the annulus

Important information about the biochemical composition of the annulus was obtained in 2005 with the targeted disruption of the murine *Sept4* gene in the laboratories of Steller and Kinoshita (Ihara et al., 2005; Kissel et al., 2005). Homozygous *Sept4* deletion was shown to result in severe defects in the structure of the sperm flagellum: *Sept4*-null sperm had no annulus, this structure being replaced by a thin segment lacking cortical material, and displayed abnormal bending of the flagellum, conferring a hairpin-like structure. *Sept4*-null males appeared to be sterile due to a lack of sperm motility and capacitation (a maturation process required for fertilization). The molecular basis of these defects was investigated by analyzing the subcellular distribution of *Sept4* and other septins in normal sperm. Immunodetection experiments showed *Sept4* to be present in two distinct dots beneath the plasma membrane of the midpiece-principal piece junction, corresponding to the annulus. *Sept1*, 6 and 7 colocalized with the *Sept4* dots in normal sperm, whereas they were diffusely spread throughout the cytoplasm of *Sept4*-null sperm. Similar associations of septins with the annulus were observed in human and bovine sperm (Ihara et al., 2005).

*Sept12* was subsequently found to be produced in large amounts in mature rat testis and to co-localize with *Sept4* at the sperm annulus (Steels et al., 2007). In humans, *Sept12* was detected in the mitochondria at the connective piece and at the annulus of mature sperm (Lin et al., 2009). Interestingly, *Sept12* forms curved filaments when co-expressed with *Sept4* in Chinese hamster ovary cells and in 293T cells (Steels et al., 2007; Lin et al., 2009), and filament-like structures were observed in the mouse during terminal differentiation of the germ cells (Lin et al., 2009).

Thus, the mammalian sperm annulus is a complex of septins 1, 4, 6, 7 and 12. The nature, subgroup and properties of these septins are consistent with the current model of lin-



**Figure 1** The annulus in terminally differentiating and mature sperm.

(A) Schematic diagram of the structure of spermatozoa. Mature spermatozoa have two main compartments, the head and the tail (flagellum), that are linked by the connective piece. The head comprises the acrosome required for interaction with the oocyte and the highly compacted nucleus. The tail can be subdivided into three parts: the midpiece, the principal piece and the terminal piece. It has a nine plus two microtubular structure, the axoneme, which is surrounded by the mitochondrial sheath (in the midpiece) and the fibrous sheath (in the principal piece). Between the midpiece and the principal piece, the annulus can be seen as a ring-shaped structure beneath the plasma membrane. (1) Acrosome (2) nucleus (3) connective piece (4) axoneme (5) mitochondria (6) annulus (7) fibrous sheath. (B) Electron microscopy analysis of the annulus. The annulus is an electron-dense structure located in the mature sperm at the junction of the midpiece and principal piece of the flagellum (arrowhead). In mouse, it is triangular and lies beneath the last turn of the mitochondrial helix and the plasma membrane. Head of the spermatozoa at the left. Bar: 0.5  $\mu$ m. Taken from Toure et al. (2007). (C) Sperm terminal differentiation (spermiogenesis) in humans. During spermatogenesis, at the end of the meiotic division, the spermatid cells undergo a set of morphological changes that are illustrated in this scheme by steps 1–7 and that lead to mature spermatozoa. This differentiation process is called spermiogenesis. Vesicles from the Golgi apparatus are collected to form the acrosome and the nucleus is highly condensed by the germ cell-specific histones and protamines (steps 1–3). The flagellum is formed by the assembly of the axoneme (a microtubular structure) with the periaxonemal structures (dense fibers and fibrous sheath). The annulus appears as an electron-dense structure assembled in the cytoplasm at very early stages of spermiogenesis when the flagellum starts to extend (step 2). It is associated with another electron-dense structure, the chromatoid body, the composition of which remains unknown (step 3). While the flagellum is growing, the mitochondria align along the axoneme, the excess cytoplasm is removed and the annulus moves towards its final position at the junction of the midpiece and the principal piece (steps 4–7). Adapted from Knobil and Neill (2006).

Ac, acrosome; An, annulus; Ax, axoneme; CB, chromatoid body; RB, residual body; DF, dense fibers; FS, fibrous sheath; M, mitochondria; N, nucleus.

ear hetero-oligomer formation leading to polymerization into higher order structures (filaments or rings) (Weirich et al., 2008). Further studies will have to define the exact architecture of the annulus.

Two other proteins, in addition to septins, have been shown to associate with the sperm annulus: DNAJB13 and Testis Anion Transporter 1; SLC26A8 (Tat1, SCL26A8) (Toure et al., 2007; Guan et al., 2009). DNAJB13 is a member of the HSP40 cochaperone family produced in large amounts in mouse testis and located in the radial spokes of the flagellar axoneme in mouse sperm and similarly in the flagella of *Chlamydomonas reinhardtii* (Yang et al., 2005; Guan and Yuan, 2008). This protein was also recently shown to be transiently associated with the annulus through direct interaction with the Sept4 protein during mouse sperm terminal differentiation. Thus, early in sperm flagellum development, DNAJB13 associates, in the cytoplasm, with a preexisting annular scaffold, which then recruits additional septin complexes to construct a genuine annulus. In the late stage of spermiogenesis DNAJB13 remains co-localized with the annulus, which migrates towards the midpiece-principal piece junction. When the annulus reaches its final position, DNAJB13 gradually disappears from this structure but persists along the length of the flagellum as a radial spoke protein (Guan et al., 2009).

Tat1 is a member of the SLC26 family of anion transporters that has been shown to be very specifically expressed in male germ cells from the spermatocyte stage onwards (Toure et al., 2001; Lohi et al., 2002). Tat1 is an integral membrane protein endowed with anion transport activity *in vitro* but of unknown physiological relevance. Tat1 function and relevance in the male germ line were investigated by generating mice with a targeted disruption of the *Tat1* gene. Sperm from *Tat1*-null males had structural defects very similar to those described in *Sept4*-null sperm including thinning of the flagellum at the midpiece-principal piece junction and hairpin-like bending of the flagellum (Figure 2A). However, unlike *Sept4*-null sperm, which have no annulus, *Tat1*-null sperm had an incomplete oval annulus (the annulus section being normally triangular) that was totally detached from the plasma membrane and the fibrous sheath (Figure 2B). Consistent with this phenotype, immunodetection of the Tat1 protein in normal mouse and human sperm revealed strong staining of the annulus region, coinciding with the distribution of Sept4 (Figure 2C) (Toure et al., 2007).

These findings indicate that the sperm annulus is a permanent septin-rich structure consisting of heteropolymers of septins 1, 4, 6, 7 and 12, the detailed ultrastructure of which remains to be determined. This septin complex appears to assemble early in spermiogenesis, in round spermatids, and is associated with the cochaperone DNAJB13. The spatio-temporal association of this cochaperone with the annulus throughout sperm flagellum development strongly suggests a role in the assembly and/or positioning of the annulus. Although *in vivo*, the functional role of Tat1 as an anion transporter is not yet proved it appears likely that this integral membrane protein plays a critical role in anchoring the annulus to the plasma membrane.

## Role of the annulus in sperm

Two hypothetical functions have long been attributed to the annulus: (i) a diffusion barrier function, confining proteins to distinct compartments of the sperm tail (Myles et al., 1984; Cesario and Bartles, 1994) and (ii) a morphological organizer function guiding the growth of the flagellum and the alignment of the mitochondria along the axoneme (Phillips, 1977). Evidence in support of both of these hypotheses has recently been obtained from analyses of *Sept4*- and *Tat1*-null sperm in particular.

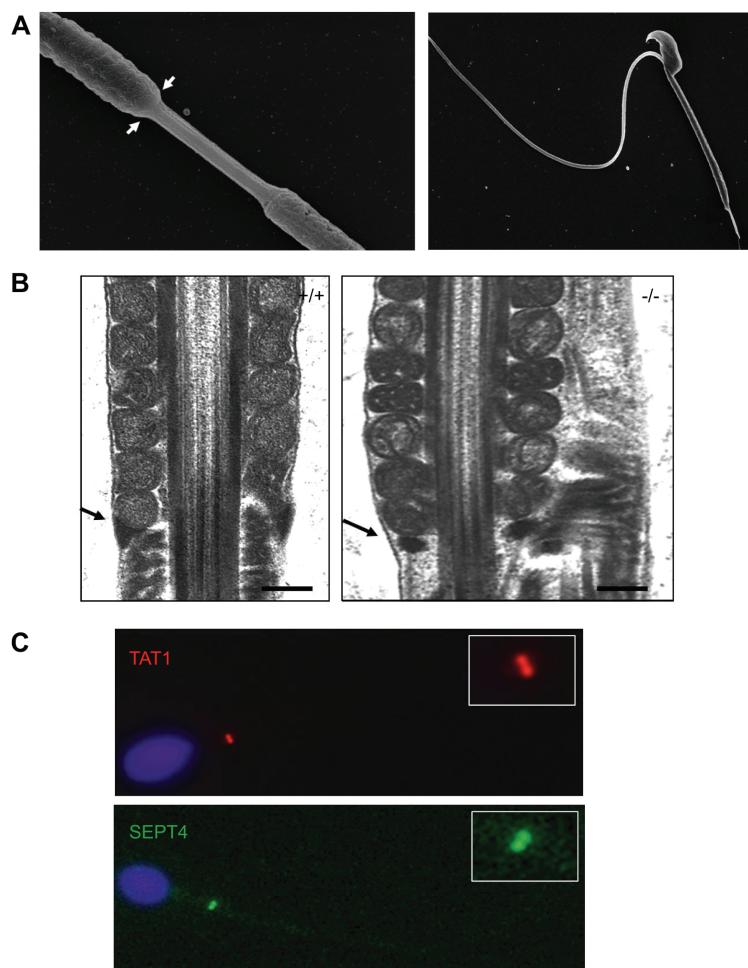
Indeed, the absence of an annulus induced by targeted disruption of the *Sept4* gene was shown to be associated not only with midpiece-principal piece disjunction but also with variable size and heterogeneous distribution of the mitochondria in the midpiece (Ihara et al., 2005; Kissel et al., 2005). Furthermore, the lack of Tat1, a transmembrane protein functionally unrelated to septins but probably involved in the anchoring of the annulus to the membrane has also been shown to cause atrophy of the annulus and similar mitochondrial and midpiece disorganization (Toure et al., 2007), suggesting that the annulus defects were themselves directly responsible for the abnormal sperm tail terminal differentiation observed in both *Sept4*- and *Tat1*-null mice.

It has also recently been shown that the annulus, like other septin rings described at the site of budding in yeast or at the cleavage furrow in mammalian cells, might function as a diffusion barrier. This was the conclusion of an analysis of basigin in the *Sept4*-null model (Kwitny et al., 2010). Basigin is a protein that diffuses over the surface of the cell (it is also called EMMPRIN, CE9, CD147 and MC31) and its location changes with the maturation state of the sperm. Hence, basigin is restricted to the principal piece of the sperm within the testis and in the proximal part of the epididymis (caput). It specifically relocates to the midpiece during sperm transit in the epididymis and next to the head during transit in the female genital tract where sperm undergo the capacitation process. *Sept4*-null sperm dissected from the epididymis have less basigin than normal sperm and this protein is abnormally distributed over the entire plasma membrane of the sperm including the head, a pattern never observed in normal mouse sperm (Kwitny et al., 2010).

The lack of specific confinement of basigin in the absence of the annulus provides clear evidence that in normal sperm the annulus acts as a barrier to diffusion, preventing basigin and, probably other membrane proteins from freely diffusing over the sperm plasma membrane. The mechanisms regulating this diffusion barrier together with their contribution to sperm tail growth and differentiation processes remain unknown.

## Annulus defects in human male infertility

Studies in mice suggest that septins and Tat1 are critical components of the annulus. This conclusion has obviously led to investigations of the role of these proteins in male infertility in humans. Two laboratories have carried out immunodetection studies, analyzing the distribution of Septins and Tat1 at the sperm annulus in infertile patients displaying sperm motility



**Figure 2** Structural defects of the annulus in *Tat1*-null sperm and localization of Tat1 and Sept4 proteins at the annulus of human sperm. (A) At the midpiece-principal piece junction of *Tat1*-null sperm, the axoneme is ‘naked’ and not surrounded by the mitochondria or the fibrous sheath (left); (the head of the spermatozoa is at the left; the annulus is indicated by the arrows). During transit in the epididymis, *Tat1*-null sperm display abnormal bending of the flagella, resulting in a hairpin-like structure (right). (B) Transmission electron microscopy analysis of *Tat1*-null sperm. By contrast to wild-type sperm (left) in *Tat1*-null sperm (right) the midpiece is disorganized and the annulus (arrows) looks incomplete and is not correctly attached to the plasma membrane. Bars: 0.25  $\mu$ m. Taken from Toure et al. (2007). (C) Immunodetection of Tat1 and Sept4 in human sperm. In human sperm and mouse sperm (not shown here), Tat1 and Sept4 immunodetection revealed two dots of staining indicating the localization of these proteins at the annulus. Taken from Toure et al. (2007). Blue, DAPI; red, Tat1; green, Sept4.

defects (asthenozoospermia) (Sugino et al., 2008; Lhuillier et al., 2009). Lhuillier et al. studied 75 asthenozoospermic subjects and reported that in one patient there was no Tat1, Sept4 or Sept7 at the annulus in 97% of the spermatozoa analyzed. Transmission electron microscopy indicated a complete absence of the annulus and severe flagellar disorganization similar to those observed in *Sept4*-null mouse sperm. The molecular defects responsible for the phenotype of this patient remain to be defined as no mutation was identified in either the *TAT1* or *SEPT4* gene (Lhuillier et al., 2009).

Sugino et al. carried out an immunodetection analysis of the distribution of the Sept4 and Sept7 proteins at the sperm annulus of a Japanese cohort of infertile patients. Their findings strongly indicated an association between the absence of an annulus and human asthenozoospermia. Electron microscopy analyses of patients spermatozoa lacking Sept4 and Sept7

staining again indicated an absence of the annulus. However, the frequency of this association was much higher in this study than in that of Lhuillier et al. (Sugino et al., 2008).

Thus, in humans as previously observed in mice, annulus integrity is required for sperm motility and sperm tail terminal differentiation. Annulus staining with septin or Tat1 antibodies could therefore be used as a diagnostic marker for asthenozoospermia.

## Conclusion

The morphology of the mammalian sperm annulus has long been known but the mechanisms underlying its biogenesis, its biochemical composition and its functions remained obscure until very recently.

In the last five years, septins have emerged as constitutive components of the annulus and compelling evidence has been obtained to suggest that a stable septin complex is required for morphological differentiation of the sperm tail and diffusion barrier function. Tat1 was unexpectedly found at sperm annulus and appears to be necessary for anchoring the septin complex to the plasma membrane. Although current evidence suggests that septins bind to plasma membrane via interaction with phosphoinositides (Zhang et al., 1999; Casamayor and Snyder, 2003; Steels et al., 2007; Bertin et al., 2010), this finding suggests that binding to integral membrane proteins could also be involved.

Interestingly the existence of an ‘annulus-like’ structure at the base of the primary cilia in mouse kidney and *Xenopus* cells was revealed last year by two groups (Kim et al., 2010; Hu et al., 2010). These authors described a septin ring at the base of the cilia, probably acting as a diffusion barrier and reported that depletion of Sept2 induces an absence of cilia (Hu et al., 2010). This discovery clearly provides new insight into the possible role of septin in ciliogenesis and in ciliopathies.

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