Formation and function of the manchette and flagellum during spermatogenesis

M S Lehti^{1,2} and A Sironen¹

¹Natural Resources Institute Finland (Luke), Green Technology, FI-31600 Jokioinen, Finland and ²Department of Physiology, Institute of Biomedicine, University of Turku, FI-20520 Turku, Finland

Correspondence should be addressed to A Sironen; Email: anu.sironen@luke.fi

Abstract

The last phase of spermatogenesis involves spermatid elongation (spermiogenesis), where the nucleus is remodeled by chromatin condensation, the excess cytoplasm is removed and the acrosome and sperm tail are formed. Protein transport during spermatid elongation is required for correct formation of the sperm tail and acrosome and shaping of the head. Two microtubular-based protein delivery platforms transport proteins to the developing head and tail: the manchette and the sperm tail axoneme. The manchette is a transient skirt-like structure surrounding the elongating spermatid head and is only present during spermatid elongation. In this review, we consider current understanding of the assembly, disassembly and function of the manchette and the roles of these processes in spermatid head shaping and sperm tail formation. Recent studies have shown that at least some of the structural proteins of the sperm tail are transported through the intra-manchette transport to the basal body at the base of the developing sperm tail and through the intra-flagellar transport to the construction site in the flagellum. This review focuses on the microtubule-based mechanisms involved and the consequences of their disruption in spermatid elongation.

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Introduction

Spermatogenesis is a complex process that begins with mitotic proliferation of spermatogonia, after which spermatocytes undergo meiotic divisions to give rise to haploid round spermatids. During the last phase of spermatogenesis, spermiogenesis, these haploid germ cells undergo substantial changes including condensation and elongation of the sperm head and development of the sperm tail. These morphological changes are crucial for the production of viable sperm and male fertility. In this review, we focus on the role of a transient microtubular platform in elongating spermatids, the manchette and its importance in the nuclear shaping and sperm tail formation. The manchette and core sperm tail structure, the axoneme, are microtubular platforms, which consist of α - and β -tubulin heterodimers; α -tubulin is exposed at the minus-end and β -tubulin at the plus-end. The plus-end is able to grow and shrink rapidly, whereas the minus-end is more stable and anchored to the microtubule-organizing center (MTOC) during nucleation. The current hypothesis is that proteins are transported through the manchette by intra-manchette transport (IMT) to the base of the sperm tail and to the developing sperm tail by intra-flagellar transport (IFT). Both of these transport mechanisms utilize microtubule tracks and

motor proteins for translocation of multi-subunit cargo complexes. The mechanisms of IMT are not well known, but it has been suggested to resemble IFT. IFT is bidirectional movement along the axoneme using motor complexes of kinesin-2 for anterograde and cytoplasmic dynein-2 for retrograde movement. These motor proteins move IFT complex B and A respectively along the axoneme and enable cilia formation and function. Recently, IFT has been postulated also outside the cilium, particularly in targeted delivery of Golgi-derived vesicles (Baldari & Rosenbaum 2010).

Manchette assembly and disassembly

During spermatogenesis in the mouse, the manchette can be first seen in step 8 spermatids, when it rapidly forms a microtubular platform between the perinuclear ring surrounding the nucleus and the elongated sperm axoneme (O'Donnell & O'Bryan 2014). The basic platform of the manchette consists of microtubules and actin filaments. Actin and the associated motor protein myosin play an important role in, e.g., vesicle transport and shaping the spermatid head due to their functional roles in the acroplaxome and manchette (Sun *et al.* 2011, Shen *et al.* 2014). Disassembly of the manchette occurs around steps 13–14 prior to the sperm tail mid-piece formation. The initiation of the manchette microtubule

formation requires a nucleator. The emanation of the manchette microtubules is currently under debate. Two specific nucleation sites have been postulated for the manchette microtubules: the perinuclear ring and the centrosome.

Evidence for the perinuclear ring as the nucleation site

An investigation of microtubular dynamics during mammalian spermatogenesis suggested the appearance of microtubules first to the post-acrosomal region in mouse and bovine spermatids (Moreno & Schatten 2000), where the periodic densities in the perinuclear ring might function as MTOC (Russell *et al.* 1991). The possibility that the perinuclear ring serves as the nucleation site is supported by observations that the free ends of the manchette are more dynamic than the perinuclear ring end (Yoshida *et al.* 1994). Furthermore, abnormal elongation of the manchette in many knockout (KO) mouse models implicates microtubular elongation at the caudal end of the manchette. Such observations are consistent with microtubules growing into the cytoplasm, with growth being regulated by the microtubule severing complex (O'Donnell *et al.* 2012). However, the nucleation of the microtubules at the perinuclear ring is not evident based on the current knowledge. The perinuclear ring lacks the well-known marker for MTOC, γ -tubulin. Thus, alternative nucleation sites should be considered.

Evidence for the centrosome as the nucleation site

The lack of γ -tubulin in the perinuclear ring implies that the centriolar adjunct also serves as the MTOC for manchette microtubules. Consistent with the localization of the nucleator γ -tubulin in the centrosomal adjunct, the microtubule plus-end tracking proteins CLIP-170, EB3 and dynactin components have been located near or at the perinuclear ring (Akhmanova *et al.* 2005, Kierszenbaum *et al.* 2011a, Fig. 1). The treatment with alkylating agent to inhibit the transcription during rat spermatogenesis resulted in a gap between the acrosome and the caudal manchette, and administration of microtubule stabilizing agent resulted in caudally located manchettes indenting into the nucleus (Russell *et al.* 1991). Interestingly, the nucleation of the manchette from the centrosome is also supported by an *in vitro* model, where exogenous taxol and GTP nucleate manchette microtubules with spermatid centrosomes (Kierszenbaum *et al.* 2002).

Alternative hypotheses for the manchette formation

Conflicting reports in the literature require further studies to confirm unequivocally the organization of manchette microtubules. It has also been suggested that

Figure 1 Manchette formation based on acetylated α -tubulin (acTUB) and microtubule plus-end tracking protein EB3 in the mouse. Step-by-step investigation of WT mouse testicular cell spreads stained with acTUB and EB3 revealed a possible model for the manchette assembly. Microtubules (red) are detected near caudal end of the sperm head at spermiogenic steps 6–7. During steps 7–8, when manchette begins to develop, EB3 (green) appears at the plus-ends of the microtubules reaching toward the acrosomal end of the spermatid head. In steps 8–9 spermatids, EB3 localizes at the perinuclear ring and manchette microtubules appear to detach from the centrosomal region. EB3 staining is detected in the perinuclear ring until manchette removal at step 14. Scale bar 5 μ m.

microtubules can be nucleated from existing microtubules (Ishihara *et al.* 2014), while other sites in the cytoplasm have also been implicated. Even a hypothesis that the manchette microtubules are nucleated prior to the pachytene spermatocyte stage and stored until required for manchette formation in elongating spermatids (Moreno & Schatten 2000) has been introduced. However, these hypotheses have not been proven. Centrobins, which is required for microtubule nucleation and stabilization (Gudi *et al.* 2011), has been localized to the acroplaxome, marginal ring, manchette and centrosome during spermatogenesis (Liska *et al.* 2009). A recent study demonstrated a specific localization for centrobins in the cytoplasm outside of the centrosome, which suggest a possible role as a microtubule nucleation site outside of the centrosome (Shin *et al.* 2015). Centrobins C-terminal truncation results in decapitated sperm with head and tail malformations. The perinuclear ring was ectopic, and the microtubule organization of the manchette was asymmetric. Moreover, the caudal end of the nucleus was constricted at the

level of the acroplaxome marginal ring, but the head was not elongated as has been seen in many other microtubule-related gene malfunctions. Thus, the centrobilin truncation does not block the manchette microtubule formation, but instead centrobilin appears to be required for recruitment of KRT5 (keratin 5) to the marginal ring, which may contribute to the sperm head phenotype (Liska *et al.* 2009). Malfunction of KRT9 (keratin 9 (K9)), a structural component of the perinuclear ring (Mochida *et al.* 2000a,b), results with ectopic manchette although the head shaping appears unaffected (Rivkin *et al.* 2005). The manchette is attached to the nucleus, but abnormally placed and therefore appears to be functional for correct head shaping during spermatid elongation. The malformation of the perinuclear ring and manchette microtubules in the KRT9 mouse model may affect the IMT and thus cause the identified phenotype with coiled tails and also influence the observed residual cytoplasm retention, although this may be a secondary effect. Thus, the correct perinuclear ring formation is not crucial for microtubule nucleation, but it is required for correct manchette formation.

The manchette is organized by the perinuclear ring, but nucleated at the centrosomal adjunct?

Although the exact mechanism of the manchette formation is not evident, the formed microtubules are connected and stabilized by the perinuclear ring (Fig. 1). CLIP-50, a short isoform of CLIP-170, has been localized to the perinuclear ring during manchette formation (Tarsounas *et al.* 2001). Tarsounas *et al.* speculated that the manchette microtubules are formed from the perinuclear ring. However, the staining pattern of α -tubulin indicates strong staining at the centrosomal side of the developing manchette (Tarsounas *et al.* 2001). Our experiments show that microtubules can be initially detected close to the basal body (Fig. 1). The microtubule plus-end tracking protein EB3 is localized to the microtubule ends reaching toward the acrosomal region in steps 7–8 murine spermatids (Fig. 1). After formation of the manchette, EB3 concentrates to the perinuclear ring and the microtubules appear to detach from the centrosomal adjunct (Fig. 1). This observation suggests that EB3 may have a role in stabilizing the microtubule plus-ends attached to the perinuclear ring after formation of the manchette. This hypothesis is supported by the localization of microtubule plus-end tracking proteins close to the perinuclear ring end of the manchette in previous studies (Akhmanova *et al.* 2005, Kierszenbaum *et al.* 2011a). Thus, we would like to introduce the hypothesis that the manchette microtubules are nucleated from the centrosomal adjunct with plus-end reaching toward the developing perinuclear ring, and the correct formation of the perinuclear ring is crucial for the organization of the manchette.

Manchette removal

In the mouse, the manchette moves toward the tail neck region around steps 13–14. The zipper-like movement of the manchette has been suggested to contribute to the slight elongation and flattening of the spermatid head (Russell *et al.* 1991). During the spermatid elongation, the nuclear ring and entire manchette move toward the spermatid tail, coincident with the acrosome expansion. During the zipper-like movement toward the caudal site of the spermatid head, the perinuclear ring constricts resulting in the correct sperm head shape, which is maintained after the chromatin condensation. The possible arrangement of microtubule plus-ends at the perinuclear ring would enable the function of kinesin-2 as a possible molecular motor for the zipper-like movement of the manchette. This movement is compromised, but not completely blocked by the depletion of KIF3A. Thus, the kinesin-2 motor has a role in the zipper-like motion of the manchette, but other motors also appear to be involved. It is possible that a malfunction in IMT results in abnormal elongation and delay in manchette clearance, because similar elongated manchette phenotypes have been identified in depletion of proteins important for protein transport such as IFT88 (Kierszenbaum *et al.* 2011a), and CEP131 (Hall *et al.* 2013, Fig. 2). Depletion of HOOK1, a protein-linking microtubule to endosomal trafficking, also results in elongated cylindrical shape nucleus in spermatozoa (Mendoza-Lujambio *et al.* 2002). The interaction between HOOK1 and RIMBP3 has been established, which may represent a link between the manchette microtubules and other cellular organelles, e.g., the nuclear envelope (Zhou *et al.* 2009). *Hook1* and *Rimbp3* KO mouse models also present an ectopic manchette and detached sperm tails. However, the staining pattern in the manchette appears somewhat different for HOOK1 and RIMBP3. HOOK1 is present along the manchette and especially in the perinuclear ring, but RIMBP3 is localized in the caudal part of the manchette, which is not connected to the nucleus. Furthermore, the manchette appears to be connected to the nucleus in *Hook1* KO mice, which is demonstrated by the elongated and cylindrical head shape. The ectopic organization of the manchette suggests that HOOK1 may rather have a linking role between the manchette microtubules. Overall, depletion of proteins involved in IFT and microtubule organization-related proteins appear to primarily affect the disassembly of the manchette causing abnormal elongation and cylindrical shape of the sperm head.

The microtubule severing appears to take place at the caudal site of the manchette as supported by the evidence of Katanin localization to this part of the manchette and the Katanin 80 mouse model (O'Donnell *et al.* 2012). Katanin 80 is a regulatory subunit of the microtubule severing complex, which is important for

Figure 2 The manchette phenotype of depletion of microtubule-associated proteins. (A) Electron microscopic image of the WT manchette. (B) Schematic presentation of the WT elongating spermatid with the skirt-like manchette and sperm tail. Microtubule plus-end proteins such as EB3, CLIP-170, dynactin and CLASP2 are localized at the perinuclear ring side of the manchette, while microtubule severing enzyme Katanin is localized to the free end of the manchette. (C) Schematic presentation of the elongated spermatid head in microtubule-related mutants. Depletion of, e.g., KIF3A, IFT88, Katanin 80, CEP131, CLIP170 and HOOK1 results in abnormal elongation (arrow) of the manchette. The perinuclear ring (arrowhead) constricts during the zipper-like movement of the manchette during normal sperm head formation. In mutants with elongated manchette, the zipper-like movement is delayed, which results in cylindrical shape of the sperm head. (D) Electron microscopic image of the *Kif3A* KO spermatid head with elongated manchette.

microtubule dynamics. Katanin 80 stabilizes Katanin 60, which is the catalytic subunit of the complex. Both proteins are localized to the manchette, in particular the caudal end of the microtubules. As microtubule severing is required for deflagellation and microtubule degradation, it can be assumed that the manchette elongation in depletion of Katanin is caused by malfunction of the microtubule severing at the caudal end of the manchette. The lack of Katanin 80 function results in delayed manchette removal and malformation in the sperm tail structures (O'Donnell *et al.* 2012).

Even though a number of studies have provided observations on the assembly of the manchette, the nucleation sites and exact mechanisms involved remain unclear. The delay in manchette clearance appears in depletion of the anterograde IFT motor protein KIF3A, which suggests that disrupted plus-end directed movement hinders the manchette clearance. On the other hand, the depletion of dynein-related proteins (CLIP-170) results in defects of nuclear shaping. Thus, the established presence of the dynein motor in the connection of the manchette and nucleus (Hayasaka *et al.* 2008) may contribute to the zipper-like movement together with

other molecular motors such as KIFC1 (Yang *et al.* 2006, Wang *et al.* 2010). The interaction between KIFC1 and nucleoporin protein NUP62 indicates a role for KIFC1 in nucleocytoplasmic transport, but the decrease in this transport system during nuclear condensation may suggest a more structural role for this complex at the time of manchette removal. Nevertheless, results from mouse models related to the delays in manchette clearance have underlined the importance of correct disassembly and zipper-like movement to sperm head formation. Mouse models affecting the manchette formation are presented in Table 1.

The manchette is connected to the nucleus through the linker of nucleoskeleton and cytoskeleton complex

Electron microscopy studies have shown that rod-like elements link the manchette microtubules to the nuclear membrane (Russell *et al.* 1991). The manchette appears to be connected to the nucleoskeleton through the linker of nucleoskeleton and cytoskeleton (LINC) complex (Gob *et al.* 2010). In somatic cells, the LINC complex

Table 1 KO mouse models affecting the manchette formation in elongating spermatids.

Gene	Protein	Spermiogenesis phenotype	Interactions	Identified or suggested function in somatic cells	Reference
<i>Azi1/Cep131</i>	CEP131	Short tail, disorganized sperm tail structures, ectopic and elongated manchette	BBS4	Conserved, but non-essential trafficking role in ciliogenesis, localizes to centriolar satellites and the transition zone and traffics along microtubules	Hall <i>et al.</i> (2013)
<i>Cntrob</i>	CNTROB	Ectopic and asymmetric perinuclear ring and manchette, detached centrosome, decapitated and disorganized tails	KRT5, tubulin	Required for centriole duplication and cytokinesis	Liska <i>et al.</i> (2009))
<i>E-Map-115</i>	E-MAP-115	Ectopic manchette along regions of the nucleus that normally do not display manchette and tail appears normal	Kinesin 1	<i>Stabilizing and reorganizing MTs</i>	Komada <i>et al.</i> (2000))
<i>Fused/Axin1</i>	FU	Periaxonemal abnormalities, manchette elongated and malformed, acroplaxome affected	KIF27, ODF1	Constructing or maintaining the central pair apparatus of the vertebrate 9+2 axoneme in multi-ciliated tissues	Nozawa <i>et al.</i> (2014)
<i>Gopc</i>	GOPC	Lack of the acrosome, lack of post-acrosomal sheath and the posterior ring, misplaced perinuclear ring, ectopic and misplaced manchette. Impaired mitochondrial sheath assembly in the epididymal spermatozoa, coiled flagella	Golgi-160, RAB6A, GRID2, BECN1, RHOQ, ACCN3, CFTR, CSPG5	Trafficking of a subset of plasma membrane proteins	Suzuki-Toyota <i>et al.</i> (2004, 2007)
<i>Hook1</i>	HOOK1	Manchette elongated, knobbed-like shape of the head, possibly weak head tail connection and bending of the tail	RIMBP3	Endocytic membrane trafficking to the microtubule cytoskeleton	Mochida <i>et al.</i> (1999)
<i>Ift88</i>	IFT88	No axoneme, disorganized tail components, malformed HTCA, ectopic perinuclear ring and manchette elongated	GMAP210	Part of IFT complex B	Kierszenbaum <i>et al.</i> (2011b)
<i>Iqcg</i>	IQCG	Short tail and disorganized sperm tail structures, irregular nucleus and localized to the manchette	Calmodulin	Not known and expressed in motile cilia	Harris <i>et al.</i> (2014)
<i>Katnb1</i>	KATNB1	Sperm tail motility affected, manchette elongated and knobbed-like	Katanin 60	MT severing	O'Donnell <i>et al.</i> (2012)
<i>Kif3A</i>	KIF3A	No axoneme, disorganized tail components, manchette elongated and knobbed-like shape of the head	KIF3B, KAP, MNS1, KBP	IFT anterograde motor	Lehti <i>et al.</i> (2013)
<i>Lrguk1</i>	LRGUK1	Short tail, acrosome and acroplaxome detached, manchette MTs unevenly distributed and elongated manchette	HOOK2	<i>Role in MT organization</i>	Liu <i>et al.</i> (2015)
<i>Meig1</i>	MEIG1	Disorganized sperm tail structures, disrupted manchette structure reported and round or detached heads	PACRG, SPAG16	<i>Regulation of meiosis</i>	Zhang <i>et al.</i> (2004, 2006)
<i>Pacrg</i>	PACRG	Disorganized sperm tail structures, disrupted manchette structure reported and round or detached heads	MEIG1	Localize to cilia and required for motile cilia function	Li <i>et al.</i> (2015)
<i>Spef2</i>	SPEF2	Short tail, elongated manchette and disorganized sperm tail structures	IFT20	<i>Spef2</i> KO affects the motile cilia motion	Sironen <i>et al.</i> (2010, 2011)
<i>Spem1</i>	SPEM1	Head bend back, mid-piece wrapped around head and retained cytoplasm	RANBP17, UBQLN1	<i>Nucleocytoplasmic transport</i>	Zheng <i>et al.</i> (2007) and Bao <i>et al.</i> (2010)
<i>Sun4</i>	SUN4	Round-headed sperm, severely disorganized manchette and coiled tails	SUN3, Nesprin 1	Expressed only in spermatids and part of the LINC complex	Pasch <i>et al.</i> (2015)
<i>Ube2b</i>	UBE2B	Mislocation of the longitudinal columns of the FS, head shape and MS abnormalities, acrosomal defects and ectopic manchette	RAD18	Ubiquitin pathway and protein degradation	Roest <i>et al.</i> (1996) and Escalier <i>et al.</i> (2003)

MT = microtubule.

consists of conserved KASH–SUN protein bridges. Five of the known KASH proteins (Nesprins 1, 2, 3, 4 and KASH5) are outer nuclear membrane adapters for cytoskeletal components and are reported to interact, e.g., with microtubule motors, actin and intermediate filaments (Luxton & Starr 2014). KASH proteins interact with SUN proteins located in inner nuclear membrane, and this bridge is essential for coupling nuclear structure to cytoskeleton (Crisp *et al.* 2006). Two LINC complexes have been implicated during spermatid elongation; SUN3/Nesprin 1 complex connects the manchette to the nucleus, and SUN1/Nesprin 3 shows an atypical non-nuclear localization at the anterior pole (Gob *et al.* 2010). SUN3 and Nesprin 1 have been localized at the sites where the manchette microtubules contact the nuclear envelope (Gob *et al.* 2010, Calvi *et al.* 2015). Recently SUN4 has been shown to colocalize with SUN3 and Nesprin 1 (Pasch *et al.* 2015). SUN4 appears to have the capacity to interact with SUN3 and Nesprin 1 and thus has been suggested to form a complex, which is required for SUN3/Nesprin 1 localization between the manchette and nucleus. In addition, the SUN1/Nesprin 3 localization appears to be wide spread in depletion of SUN4, which underlines the importance of correct formation of the SUN3/Nesprin 1 complex for precise localization of SUN1/Nesprin 3 at the posterior nuclear envelope (Pasch *et al.* 2015). Interestingly, the SUN3/Nesprin 1 appears to function in manchette/nucleus connection, and SUN1/Nesprin 3 may have a role in basal body attachment to the nucleus. Interaction between Nesprin 1 and the manchette may occur through the microtubule motors dynein or kinesin or via F-actin. The localization of the cytoplasmic dynein close to the nuclear envelope (Hayasaka *et al.* 2008) underlines dynein as a candidate motor protein for the LINC complex. LINC complex has been demonstrated to be involved in nuclear shaping, suggesting a possible involvement in spermatid head modeling.

Dynein as the motor between the manchette and nucleus

The presence of dynein in the manchette has been postulated (Yoshida *et al.* 1994, Hayasaka *et al.* 2008). Cytoplasmic dynein was localized to the manchette, close to nuclear envelope at early steps of manchette formation in rats (steps 7–13), with intense staining observed during the most active steps 11–13 of nuclear shaping. Dynamic studies of CLIP-170, a member of the dynein–dynactin pathway, suggest that the dynein complex is a stable component of the manchette (Akhmanova *et al.* 2005). Cross-sectioned manchette microtubules in *Clip-170* KO mice show a highly irregular arrangement, which may indicate that the formation of cross-bridges between microtubules is

affected (Akhmanova *et al.* 2005). These results indicate a possible role in nuclear shaping rather than in IMT.

Some indications of dynein involvement in protein transport have arisen by results from interacting proteins. A protein of the dynactin complex, ARP1, has been localized to the manchette in addition to the centrosome and Golgi complex (Fouquet *et al.* 2000). ARP1 binds both dynein and cargos (Cheong *et al.* 2014). ADP-ribosylation factor-like 3 (ARL3) and dynein light chain LC8 induce dissociation of dynactin from dynein (Jin *et al.* 2014). A decrease of *Arl3* introduced by siRNA injection induced some abnormalities in head shape, lasso-like coiled tail or decapitation (Qi *et al.* 2013). The lissencephaly protein LIS1 regulates the dynein motility on microtubules (Wang *et al.* 2013, Toba *et al.* 2015). It has been suggested that LIS1 may have a role in nuclear condensation based on the mutant phenotype (Nayernia *et al.* 2003). LIS1 colocalized with dynein along the manchette in elongating spermatids, but not in the axoneme. Furthermore, the acrosome and sperm tail formation was affected. A direct cause and effect of dynein depletion on manchette formation, IMT or nuclear shaping has yet to be established. Although the exact role of dynein motor within the manchette has not been established, it appears to function in connecting the cytoskeleton (manchette) and nucleoskeleton through the LINC complex and may participate in nucleocytoplasmic transport.

The manchette/nucleus connection is crucial for sperm head shaping

The importance of the manchette in sperm head shaping has become evident during recent years. In general, the spermatid nucleus assumes a parallel shape to the manchette, and when the manchette is absent, the nucleus bulges as a round form as seen in models with caudally displaced manchette (Russell *et al.* 1991) and in mutants with lack of the nucleus manchette connection (Calvi *et al.* 2015). When the manchette is abnormally elongated as in many mouse models bearing mutations in IFT or microtubule-related proteins (e.g. KIF3A, IFT88, CLIP-170, HOOK1, FU, KATNB1, PARCG and CEP131), the nucleus is deformed along the elongated manchette as a cylindrical shape (Fig. 2). The connection between the nucleus and the manchette appears unaffected in these mutants causing the elongation of the head along with the manchette. However, the zipper-like movement of the manchette appears affected in these mutants.

The importance of the manchette–nucleus connection has been further demonstrated by the testis-specific LINC component *Sun4* KO (Calvi *et al.* 2015, Pasch *et al.* 2015). In depletion of SUN4, the manchette microtubules are not laterally associated with nuclear envelope causing round headed sperm. Defects appeared during sperm head condensation, when the

acrosome was extended to abnormally large area of the nucleus surface and the manchette was highly disorganized. However, chromatin condensation appeared normal in *Sun4* KO mice suggesting that abnormal manchette was the major cause for the defects in head shaping. Round-headed sperm has also been detected in Golgi-associated PDZ- and coiled-coil motif-containing protein (GOPC)-deficient mice. Manchette microtubules appeared to be attached to the perinuclear ring, but the manchette and perinuclear ring were placed ectopically, and microtubules were assembled in various directions. In addition, occasional invaginations of the manchette in to the nucleus were observed (Ito *et al.* 2004). The connection between the nucleus and manchette is crucial for the shaping of the spermatid head, which is demonstrated by the round-headed shape in lack of this connection.

Nucleocytoplasmic transport and protein degradation

The manchette is involved in transport in and out of the nucleus (nucleocytoplasmic transport) (Kierszenbaum 2002, Kierszenbaum & Tres 2004). Association of KIFC1 motor with the nucleoporin NUP62 suggests a role for this motor in nucleocytoplasmic transport. Prior to localization to the manchette, KIFC1 is associated with vesicles between the Golgi and the spermatid nucleus and in the acrosome. The integrity of the NUP62 complex is dependent on GTP hydrolysis and the GTP state of the small GTPase Ran (Yang *et al.* 2006). RAN is required for transport of proteins in and out of the nucleus and for assembly of microtubules (Kierszenbaum *et al.* 2002, Hermo *et al.* 2010). RAN and a member of RAN-binding protein RANBP17 are localized along the manchette microtubules. RANBP17 is a potential RAN-dependent importin for protein transport in and out of the nucleus. RANBP17 appears to interact with SPEM1, the depletion of which causes bent heads and remnant cytoplasm in mature sperm (Zheng *et al.* 2007). The 'head-bent-back' sperm phenotype has been observed in many mouse lines with targeted inactivation of proteins involved in nuclear condensation (Xu *et al.* 1999, Adham *et al.* 2001, Cho *et al.* 2001, Tanaka *et al.* 2005). However, the sperm head condensation and tail structures appeared unaffected in *Spem1* KO mice, which suggests that SPEM1/RANBP17 interaction may have a role in nucleocytoplasmic transport required for correct removal of the cytoplasm. An additional interacting partner, UBQLN1, has also been proposed for SPEM1. UBQLN1 is part of the ubiquitin-proteasome (UBP) system, which degrades unwanted proteins. Although the exact role for SPEM1 is not known, it may have a role in UBP system through the interactions with UBQLN1. Mutation in murine ubiquitin conjugating enzyme mHR6B (UBE2B) causes

heterogeneous malformations in head shaping and sperm tail formation. In *Ube2b* KO mouse, the manchette was ectopic with microtubule invaginations within the nucleus. Membranous structures were associated with the microtubules, which could represent a possible defect in the transport of vesicular material along the manchette (Escalier *et al.* 2003). This mouse model also underlines the functional involvement of the UBP in the assembly of flagellar components in mammals. The role of the UBP system within the manchette is further supported by the localization of an ubiquitin protein ligase RNF19A and components of 26S proteasome (e.g. PSMC3 and TBP-1) (Mochida *et al.* 2000a,b, Rivkin *et al.* 2009). The evidence points toward a role for the manchette in Ran GTPase-mediated nucleocytoplasmic trafficking of proteins for disposal by the manchette-associated 26S proteasome. The manchette appears to have a role in regulating protein quality and nucleocytoplasmic transport. However, little is known about the activity of these pathways during spermatid elongation.

IMT delivers proteins during spermatid elongation

IMT is carried out by molecular motors, kinesins and dynein, along the microtubule tracks and by myosin along the actin tracks within the manchette. F-actin-related transport proteins myosin VA, MyRIP and RAB27B have been localized to the sperm manchette in humans (Kierszenbaum *et al.* 2003, Hayasaka *et al.* 2008) and may contribute to the short distance transport of proteins. The long distance transport has been suggested to be implemented by the microtubule tracks (Goode *et al.* 2000). It has been hypothesized that proteins can switch between these two types of transport mechanisms (Kierszenbaum & Tres 2004), and the co-operation results in timely delivery of proteins (Gross *et al.* 2000). The suggestion that IMT has molecular homology with IFT (Kierszenbaum 2002) is supported by investigations based on protein localizations and KO mouse models of known IFT proteins KIF3A, IFT20, IFT88 and KIF27 (Sironen *et al.* 2010, Kierszenbaum *et al.* 2011b, Lehti *et al.* 2013, Nozawa *et al.* 2014). All IFT proteins are expressed in spermatogenesis, and the expression appears to increase during the progress of sperm development (Fig. 3). Although the expression indicates that the protein products are required for sperm development, the exact roles for most of the IFT proteins are not known. In addition, KIF17B (Saade *et al.* 2007) and KIFC5A (Navolanic & Sperry 2000) have been associated with the manchette and may contribute to the IMT. KIF17B has been shown to interact with its cargo protein spatial in the manchette and also along the sperm tail principal piece in mature sperm (Saade *et al.* 2007).

Figure 3 IFT genes are expressed during spermatogenesis. IFT motor proteins and complex A and B genes have increasing expression pattern during the first wave of spermatogenesis based on transcriptomic sequencing data (Laiho *et al.* 2013). The X-axis shows the postnatal development days.

Kinesin-2 and IFT complex B proteins are present in the manchette

The *Kif3A* KO demonstrated the consequences of IFT motor protein loss during spermatogenesis. In addition to the axoneme formation, KIF3A depletion affects the elongation and clearance of the manchette (Lehti *et al.* 2013), underlying the importance of kinesin-2 motor to correct head shaping and manchette clearance. A role of kinesin-2 in protein transport through the manchette was also implicated by delayed translocation of MNS1 staining from the acroplaxome to the manchette and finally to the sperm tail. MNS1 depletion only affects sperm tail structures (Zhou *et al.* 2012) suggesting that

MNS1 is a cargo particle of the IMT and transported through the manchette to the developing sperm tail (Lehti *et al.* 2013). Two other IFT complex B proteins, IFT88 and IFT20, have also been localized to the manchette. Studies with the *lft88* KO mouse model provided further evidence of the involvement of IFT in spermatid nuclear shaping and development of the centrosome-derived head–tail coupling apparatus and sperm tail (San Agustin *et al.* 2015). The phenotypes in *Kif3A* and *lft88* KO mouse models were similar, supporting the hypothesis of protein delivery via IMT through the recruitment of IFT complex B.

Delivery of sperm tail proteins through the manchette

IMT has been demonstrated to store and deliver structural sperm tail proteins to the basal body region (Kierszenbaum 2001). Depletion of IFT88 results in the accumulation of a GMAP210-stained vesicle with mitochondrial sheath and outer dense fiber (ODF) material in the manchette, indicative of defects in protein delivery (Kierszenbaum *et al.* 2011a, Fig. 4). In *lft88* KO mice, myosin VA appeared to accumulate in the caudal site of the manchette indicating that inhibition of microtubule-based transport may affect the F-actin motor proteins (Kierszenbaum *et al.* 2011b). This observation also further supports the hypothesis that the microtubule plus-end is in fact at the perinuclear ring of the manchette. A protein required for the central pair assembly in motile cilia, the putative serine–threonine kinase fused (FU), localizes to the manchette and the acrosome–acroplaxome. The depletion of FU affects the formation of sperm tail accessory structures, but the axoneme formation appears unaffected; thus, FU appears to have a slightly different role in sperm tail development than in motile cilia. FU interacts with ODF1 and KIF27 (Nozawa *et al.* 2014) and thus may be transported through the manchette to the developing sperm tail. KIF27-GFP localizes to the perinuclear ring of the manchette and with a weaker signal to the manchette in elongating spermatids. Furthermore, the sperm flagellar 2 (SPEF2) protein, which is required for cilia motility and formation of sperm tail structures, is present in the manchette prior to the localization to the sperm tail mid-piece (Sironen *et al.* 2010, 2011). The interaction between SPEF2 and IFT20 has been postulated; however, it is not clear if SPEF2 is transported through the manchette or if it has a role in the IMT. Nevertheless, the recent findings suggest a role of IFT complex B in transport of sperm tail components through the manchette to the base of the sperm tail (Fig. 4).

The depletion of HOOK1 protein results in elongated manchette and malformed attachment of the connecting piece (implantation fossa) and sperm tail mid-piece anomalies (Mochida *et al.* 1999, Mendoza-Lujambio *et al.* 2002). In addition to the possible microtubule-linking properties of HOOK1, it may function in protein

Figure 4 Identified IFT-related transport complexes during spermatid elongation. (A) KIF3A, IFT88, IFT27 and IFT20 are localized to the spermatid manchette and based on the KO mouse model affect delivery of proteins to the sperm tail. GMAP210 interacts with cargo protein vesicles, which transport to the developing sperm tail is blocked in depletion of IFT88. (B) PACRG/MEIG1 interaction affects the localization of SPAG16L to the sperm tail. (C) IFT complex B proteins IFT27, 81 and 172 have been localized to the sperm tail mid-piece and through interaction with RABL2 appear to be involved in transporting proteins required for glycolysis to the sperm tail FS. However, not all IFT proteins appear to be present in the mid-piece of the sperm tail; thus, the exact role of IFT during late steps of sperm tail development is not known.

transport to the basal body. HOOK1 has been reported to link the endocytic membrane trafficking to the microtubule cytoskeleton (Maldonado-Baez *et al.* 2013). The cargo-specific binding properties of HOOK1 and its ability to bind motor proteins may contribute to transport of cargos to specific locations within the elongating spermatid. HOOK1 belongs to HOOK protein family, which is known to function as cargo-loading proteins onto microtubules for transport. The depletion of an interaction partner of another HOOK protein family member, HOOK2, also causes cylindrical head shaping (Liu *et al.* 2015). The uncharacterized protein called leucine-rich repeats and guanylate kinase domain containing isoform 1 (LRGUK1) is transported via acrosome–acroplaxome–manchette–tail axis in a complex with HOOK2. LRGUK1 dysfunction caused disorganized and uneven distribution of manchette microtubules and lack of the sperm tail axoneme (Liu *et al.* 2015). The involvement of HOOK2/LRGUK1 complex has been suggested in protein transport, but

the exact mechanism is not known. The HOOK family members may represent one cargo-specific link between the motors and cargo within the manchette in addition to acrosome and acroplaxome vesicle transport. However, the elucidation of the exact transport mechanisms and cargo/motor complexes during IMT of sperm tail proteins require extensive further studies.

For IFT proteins, their importance in transport along the developing sperm tail hinders the evaluation of the role of IMT for sperm tail development. However, studies of transport complexes localized only in the manchette underline the crucial importance of IMT for sperm tail development. Meiosis-expressed gene 1 (MEIG1) and Parkin-co-regulated gene (PACRG) form a complex in the manchette, which appears to be required for transport of cargos to the sperm tail (Li *et al.* 2015). PACRG depletion affects the localization of MEIG1 in the manchette indicating that it is an upstream protein in the pathway. Both *Meig1* and *Pacrg* KO are known to affect the localization of axonemal central apparatus protein SPAG16L suggesting that SPAG16L is simply transported through the manchette to the developing sperm tail. In the *Spag16L* KO, sperm tail motility is compromised (Zhang *et al.* 2006).

Protein transport mechanisms during formation of the accessory structures of the sperm tail

The different components of the sperm tail require ordered delivery of particles to the assembly site. After axoneme formation, all accessory structures need to be compiled. The ODFs are constructed along the axoneme, and fibrous sheath (FS) is built in distal to proximal orientation; thus, the proteins need to be transported all the way to the flagellar tip. However, little is known about the exact delivery systems during sperm tail development. IFT has been suggested to be the delivery mechanism in a similar manner as described for cilia formation. Depletion of the kinesin-2 motor resulted in the inability to construct the sperm tail axoneme (Lehti *et al.* 2013). It remains unclear if this is the sole transport mechanism required for sperm tail formation. The fact that the FS and ODFs are assembled in opposite directions implies that there are at least two different transport mechanisms. Although various proteins intended for the sperm tail appear to be stored in the manchette prior to transportation to the sperm tail, the FS proteins have not been identified in the manchette. *Rabl2* structural mutant has been shown to affect the delivery of several components to the developing sperm tail. It has been suggested that RABL2 interacts with IFT proteins IFT27, IFT172 and IFT81 (Lo *et al.* 2012), but the possible interaction site was identified in the mid-piece of the sperm tail (Fig. 4). This led the authors to hypothesize that the lack of RABL2 in the mid-piece affects the localization of ATP6V1E1, glycolytic enzyme

HK1, HSPA4L and lactate dehydrogenase C (LDHC) in the principal piece. Given that the mid-piece is the last structure to appear in the sperm tail, such a transport mechanism is unlikely to be involved in flagella formation, but rather in the delivery of proteins required for sperm motility to the principal piece. Mouse models of HSPA4L and LDHC depletion cause lowered sperm motility without any structural malformations (Held *et al.* 2006, Odet *et al.* 2008). Such observations suggest that RABL2 and the interaction with IFT proteins are specifically required for transport of proteins important for motility pathways, probably for glycolysis, in the principal piece of the sperm tail. It should be noted that this is the first study to identify possible transport pathway for principal piece proteins, which may deliver non-structural proteins from the cytoplasm through the mid-piece after formation of the sperm tail. RABL2 may have a role in delivering proteins to the base of the IFT initiation site, which could be demarcated by the septin ring after formation of the mid-piece. This septin ring moves distally from the base of the sperm tail during the mid-piece formation enabling the mitochondria to be laid down along the developing mid-piece. The kinesin-2 motor protein KIF3A has been localized to the FS in addition to the manchette (Lehti *et al.* 2013), but IFT20 was localized only to the manchette and basal body (Sironen *et al.* 2010). Thus, not all IFT complex B proteins are localized to the mid-piece or principal piece of the sperm tail. The elucidation of the exact role of IFT and related proteins during late steps of sperm tail development require further studies.

Fertile sperm production requires specialized transport mechanisms

Male infertility is caused by various defects during the complex process of spermatogenesis. Recent reports highlight the fact that successful sperm production requires proteins and protein complexes to be transported to the correct assembly site during sperm tail formation. However, the exact role of microtubule and actin-based delivery complexes are not well characterized. Some proteins appear to be transported through the acroplaxome and manchette to the base of the developing sperm tail and may be stored transiently in these structures. However, not all sperm tail proteins have been localized to the manchette, raising the prospect that different mechanisms for delayed translation or protein storage and transport are involved. Functional studies need to be undertaken to provide a more complete understanding of the mechanisms involved in fertile sperm formation, which is important for developing therapies for male infertility. This is particularly important, since the genetic alterations may also affect the ICSI outcome and thus be transferred to the next generation. During spermatid elongation, the

manchette is a crucial structure orchestrating protein distribution and function. Sperm tail development is dependent on both IMT and IFT, with a common molecular system contributing to both transport mechanisms. Although recent studies have shown that disruption of the protein delivery to the correct assembly site during spermiogenesis results in detrimental malformations in mature sperm, the exact mechanism by which the specific cargos are stored, recognized and finally delivered is not known. Thus, to unravel the possible causes for male infertility, we need to investigate the exact mechanism involved in these specific events during spermatid elongation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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