The cytoskeleton in spermatogenesis

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Abstract

As germ cells progress through spermatogenesis, they undergo a dramatic transformation, wherein a single, diploid spermatogonial stem cell ultimately produces thousands of highly specialised, haploid spermatozoa. The cytoskeleton is an integral aspect of all eukaryotic cells. It concomitantly provides both structural support and functional pliability, performing key roles in many fundamental processes including, motility, intracellular trafficking, differentiation and cell division. Accordingly, cytoskeletal dynamics underlie many key spermatogenic processes. This review summarises the organisational and functional aspects of the four major cytoskeletal components (actin, microtubules, intermediate filaments and septins) during the various spermatogenic phases in mammals. We focus on the cytoskeletal machinery of both germ cells and Sertoli cells, and thus, highlight the critical importance of a dynamic and precisely regulated cytoskeleton for male fertility.

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Introduction

Spermatogenesis is a complex process, involving the synchronous production of vast numbers of genetically distinct, haploid spermatozoa capable of surviving and traversing the female reproductive tract. In mammals, the process is spatially restricted to the seminiferous tubules of the testis, wherein germ cells develop in a stratified epithelium.

The spermatogenic cycle initiates with spermatogonial stem cells (SSCs) undergoing mitosis to both renew their own population and to provide cells that become terminally committed to further differentiation. Differentiating spermatogonia subsequently undergo transit amplification, progressing through multiple mitotic divisions, before ultimately entering meiosis. During meiosis, chromosomes are duplicated and undergo homologous recombination to generate genetically diverse tetraploid spermatocytes. Two rapid divisions follow, resulting in the production of haploid round spermatids. During spermiogenesis, round spermatids are then remodelled into streamlined spermatozoa with an acrosome (required for fertilisation) and a flagellum (required for motility), in addition to gaining a species-specific head shape. As germ cells progress through each phase, they are translocated from the base of the seminiferous epithelium to the luminal edge. At the end of spermatogenesis, spermatozoa are released into the lumen via a series of processes collectively called spermiation.

Sertoli cells, the somatic cells of the seminiferous epithelium, provide structural and nutritional support

throughout spermatogenesis and directly facilitate germ cell translocation within the epithelium, as well as spermiation. Fundamentally, spermatogenesis involves numerous germ cell divisions and dramatic changes in both germ and Sertoli cell morphology. Cytoskeletal elements are an integral aspect of these processes and are thus essential for male fertility.

The eukaryotic cytoskeleton is defined as being composed of actin filaments (microfilaments; [Box 1](#)), microtubules ([Box 2](#)), intermediate filaments ([Box 3](#)), and, in more contemporary definitions, septin proteins ([Mostowy & Cossart 2012](#); [Box 4](#)). Each of these elements is fundamental to eukaryotic cell biology and, as a collective, are integral for a diversity of cellular functions, including division, motility, differentiation, migration, adhesion and intracellular trafficking.

This review will focus on the organisation and function of the mammalian germ cell cytoskeleton during the various spermatogenic phases, in addition to addressing the roles of the Sertoli cell cytoskeleton.

Cytoskeletal changes during germ cell development

Spermatogonia and spermatocytes

As is well established for all mammalian cell division, the mitotic and meiotic divisions of spermatogenesis are directly facilitated by the cytoskeleton and many cytoskeletal aspects are generic. Microtubules form the typical machinery of the mitotic bipolar spindle apparatus and the midbody, while F-actin and septins

Box 1 Actin schematic.

Actin

Filamentous actin (F-actin) exists as a two-chained, helical polymer with a diameter of ~ 7 nm. It is formed by the ATP-dependent polymerisation of monomeric or 'globular' actin (G-actin) and new filament formation is typically driven by an actin-nucleating protein, such as ARP2/3, various formins or tandem-monomer-binding nucleators (Pollard 2016). Due to monomer orientation within the filament, F-actin is intrinsically polarised with a fast-growing plus-end known as the 'barbed-end' and a minus-end, which exhibits slower dynamics, known as the 'pointed-end' (Pollard 2016). The basic F-actin polymer is used to generate dynamic cytoskeletal networks, consisting of multiple cross-linked and/or branched actin filaments. Such networks provide mechanical support within the cell, facilitate organelle positioning, form tracks for intracellular trafficking and, via rapid polymerisation at the barbed end, generate forces for dynamic processes such as cell migration, endocytosis and cell division. Together with the actin-associated motor protein myosin, actin generates specialised contractile structures such as the actomyosin ring during cytokinesis and the sarcomere of myofibres (Schwayer *et al.* 2016). Functional specialisation of F-actin is generated by the differential use of actin isoforms (Perrin & Ervasti 2010). Within vertebrates, six actin genes exist, four of which are specific to muscle cells (α_{cardiac} , α_{skeletal} , α_{smooth} and γ_{smooth}), whereas two actins, β_{cyto} and γ_{cyto} -actin, are ubiquitous. Cytoplasmic F-actin can be composed of either or both β_{cyto} - and γ_{cyto} -actin and the ratios of the two actin isoforms can affect polymer characteristics. A large arsenal of interacting proteins and post-translational modifications modulate the dynamics and properties of F-actin (Terman & Kashina 2013, Pollard 2016). For example, F-actin-capping proteins can inhibit polymerisation, cross-linking proteins allow organisation of F-actin into higher order structures and severing proteins modulate filament number and dynamics (Pollard 2016).

are integral components of the actomyosin contractile ring during cytokinesis (for reviews on these aspects; see Wittmann *et al.* 2001, Fung *et al.* 2014, Schwayer *et al.* 2016).

During these phases, a number of unique cytoskeletal features are also seen. The spermatogonial cytoskeleton, for example, is integral for the initial establishment of spermatogenesis. After birth, germ cells migrate to the basement membrane of the seminiferous tubules, where SSCs form a specialised niche between Sertoli cells (Fig. 1). The migration of germ cells into, and the maintenance of cell-cell interactions within, the niche relies on integrin and actin-based adhesion junctions (Kanatsu-Shinohara *et al.* 2008) and key actin regulators (Carlomagno *et al.* 2010, Xu *et al.* 2015). Reports that gonocytes and SSCs exhibit a polarized enrichment of

α -tubulin and acetylated α -tubulin towards the basement membrane (Steger & Wrobel 1994, Luo *et al.* 2010) also suggests microtubules aid in orienting and maintaining cells within the niche, particularly as tubulin acetylation correlates with decreased cell motility (Hubbert *et al.* 2002).

In both rodents and more simple non-mammalian systems, spermatogonia have been reported to undergo 'oriented cell division' in that the mitotic spindle angle is typically positioned perpendicular to the basement membrane (Morrison & Kimble 2006, Lagos-Cabre & Moreno 2008). This type of mechanism is often employed to control cell distribution, and in this instance likely allows one daughter cell to remain attached to the basement membrane, self-renewing the SSC progenitor, while sending the more apical

Box 2 Microtubule schematic.

Microtubules

Microtubules are hollow tubes of ~25 nm in diameter, formed by GTP-mediated polymerisation of α - β -tubulin heterodimers. These $\alpha\beta$ tubulin dimers assemble, head-to-tail, into linear protofilaments, which associate laterally to form the microtubule circumference (Akhmanova & Steinmetz 2015). In vivo, microtubule nucleation is reliant on γ -tubulin, which, in conjunction with γ -tubulin complex proteins, creates a template for microtubule nucleation (Oakley *et al.* 2015). Like actin, microtubules are intrinsically polarised; they have a plus-end (exposing β -tubulin) and a minus-end (exposing α -tubulin) (Akhmanova & Steinmetz 2015). The minus-end is inherently stable and, in vivo, is frequently anchored to a microtubule-organising centre. Conversely, the plus-end exhibits 'dynamic instability', spontaneously switches between states of rapid depolymerisation (catastrophe) and polymerisation (rescue). This instability is powered by GTP hydrolysis; GTP-bound β -tubulin stabilises the plus-end, whereas GDP-bound β -tubulin destabilises the lattice and, if exposed at the plus-end, initiates catastrophe. Dynamic instability allows for the rapid remodelling of microtubule networks and for the generation of pushing and pulling forces using polymerisation and depolymerisation respectively. Within most cell types, microtubules exist as large polarised arrays that extend throughout the cytoplasm, providing infrastructure for organelle positioning and movement, trafficking of proteins and vesicles and establishing and maintaining cell shape and polarity. Microtubules also form the structural basis of centrioles, the mitotic and meiotic spindles and cilia. To function effectively, microtubules rely on a diversity of microtubule-associated proteins (MAPs), including motor proteins, cross-linking MAPs, severing enzymes, nucleators and regulators of dynamic instability (Subramanian & Kapoor 2012). Moreover, post-translational tubulin modifications and the use of different α - and β -tubulin isoforms and paralogues (δ , ϵ , ζ , η , θ , ι , κ) generate a complex 'tubulin code' (Dutcher 2001, Janke 2014), which appears to specify the MAP binding and activity required at specific sites.

daughter into the differentiating pathway. Positioning of the spindle in this way is dependent on the interplay between astral spindle microtubules, the microtubule-associated motor protein, dynein and the cell cortex (Lagos-Cabré & Moreno 2008, Mora-Bermúdez *et al.* 2014, Tame *et al.* 2014).

With the exception of the initial SSC self-renewing divisions, dividing germ cells exhibit incomplete cytokinesis, with cells maintaining stable cytoplasmic intercellular bridges. By allowing gene product sharing between related cells, such bridges likely facilitate the synchrony of spermatogenesis, in addition to

allowing haploid germ cells to remain phenotypically diploid (Haglund *et al.* 2011). The initial formation of these bridges follows the typical events of early cytokinesis wherein an actomyosin contractile ring promotes ingression of the cleavage furrow and compacts equatorial spindle microtubules into a dense midbody. However, instead of undergoing abscission, the midbody is converted into a stable electron-dense bridge. Although microtubules are quickly lost from the developing bridges (Greenbaum *et al.* 2007), actin, δ -tubulin, keratin-5 and multiple cytoskeletal-interacting proteins have been identified as components (Haglund

Box 3 IF schematic.

Intermediate filaments

Intermediate filaments (IF) are a superfamily of six classes of non-polar, 10 nm diameter fibres encoded by over 70 different genes (Szeverenyi *et al.* 2008, Köster *et al.* 2015). IFs are predominantly cytoskeletal with the exception of type V IF proteins (lamin A, B and C), which form the nucleoskeleton (Szeverenyi *et al.* 2008). Although genetically diverse, the basic structure of IFs is largely consistent across the six subfamilies. Unlike the globular actin and tubulin proteins, monomeric IF proteins are fibrous with a central elongated α -helical coil, flanked by non-helical 'head' and 'tail' ends. Dimerisation is not dependent upon nucleotides. Rather, parallel monomers inter-coil their central domains, and typically an IF precursor pool of non-polar tetramers forms by the anti-parallel, lateral half-staggering of two of these coiled-coil dimers. IFs assemble from this pool in a stepwise manner via the lateral association of eight tetramers into unit-length filaments (ULFs), followed by the longitudinal annealing of multiple ULFs. Due to this laterally layered arrangement, the basic IF structure is extremely flexible, providing tensile strength to cytoskeletal structures. Lateral displacement of tetramers and dimers relative to one another via 'subunit gliding', in addition to straightening of the α -helix coils, allows filaments to be elongated by up to 240% of their normal length (Köster *et al.* 2015). Organisation of IFs into complex structures, such as bundles, fibres or mesh-like networks, depends upon IF type, acquisition of post-translational modifications and interactions with binding proteins (bundling and cross-linking proteins, chaperones, kinases, apoptosis-related proteins and nuclear proteins; Köster *et al.* 2015).

et al. 2011), and actin is essential for their continued integrity (Russell *et al.* 1987). In addition, septins 2, 7 and 9 localise to intercellular bridges during their initial formation (Greenbaum *et al.* 2007).

Although male meiosis shares many basic cytoskeletal aspects with mitosis, there are a number of key differences. During the first meiotic prophase, for example, homologous chromosomes pair and exchange genetic material via recombination, to ensure genetic diversity of the gamete. In preparation for pairing, rapid movements converge chromosomes, via their telomeres, at one nuclear pole. Cytoplasmic microtubules drive these movements (Morimoto *et al.* 2012), interacting with telomeres via KASH5-SUN1-based LINC (linker of nucleoskeleton and cytoskeleton) complexes spanning the nuclear membrane (Morimoto *et al.* 2012, Horn *et al.* 2013). The meiotic prophase-specific IF, lamin C2, which is enriched in the nuclear lamina at telomere-LINC attachment sites, is also critical for this process (Table 1; Alsheimer *et al.* 1999, Jahn *et al.* 2010). Indeed, due to its increased mobility compared

to other lamins (Jahn *et al.* 2010), lamin C2 likely allows for greater transmission of microtubule-derived forces across the nuclear envelope. Once converged, chromosome pairing and recombination is facilitated by the formation of specialised protein-based structures termed synaptonemal complexes. The formation of these complexes may rely on actin dynamics, as removal of ALKBH4, a protein that influences the ability of non-muscle myosin II to interact with F-actin, prevents the formation of synaptonemal complexes in spermatocytes leading to apoptosis of these cells (Nilsen *et al.* 2014).

Within the subsequent phases of meiosis, notable peculiarities in spindle microtubule nucleation and size have been reported in *Drosophila*, and preliminary evidence suggests similar peculiarities exist in mammalian spermatogenesis. Unlike female meiotic germ cells, which in most species are acentriolar, spermatocytes predominantly nucleate microtubules from centrioles (Kallio *et al.* 1998, Giansanti *et al.* 2008, Riparbelli & Callaini 2011) and, while confirmation is needed in mammals, research in non-mammalian

Box 4 Septin schematic.

Septins

Septins are a family of GTP-binding proteins capable of polymerising into non-polar filaments that in turn can be organised into bundles, rings and mesh-like structures (Mostowy & Cossart 2012, Fung *et al.* 2014, Valadares *et al.* 2017). Within mammals, 13 septin proteins have been identified (septin 1-12 and 14) and classified into four subgroups SEPT2 (septin 1, 2, 4, 5), SEPT3 (septin 3, 9, 12), SEPT6 (septin 6, 8, 10, 11, 14) and SEPT7 (septin 7). Typically, septins self-assemble into either hexameric or octameric hetero-oligomers with three or four septin subtypes contributing. Within these oligomers, monomers are arranged symmetrically, interacting via alternate G and NC interfaces. A mixture of octamers and hexamers then assemble end-to-end to form filaments. Septin filament composition is thus diverse and cell and function specific, with a number of septin genes showing tissue-specific expression (Mostowy & Cossart 2012). Although the precise mechanisms governing septin assembly, disassembly and architecture are unresolved, interactions with microtubules, F-actin and phospholipid membranes all appear to influence the higher order organisation of septins. Likewise, while septin function is poorly understood relative to other cytoskeletal elements, key roles have been identified in cytokinesis, the formation of diffusion barriers, microtubule dynamics and the release of neurotransmitters (Fung *et al.* 2014, Valadares *et al.* 2017).

systems suggests this pathway is essential for effective male meiotic spindle formation (Giansanti *et al.* 2008, Riparbelli & Callaini 2011). This also differs from typical spindle formation in somatic cells and spermatogonia, wherein nucleation can occur at centrioles but is not dependent on it (Basto *et al.* 2006, Giansanti *et al.* 2008, Riparbelli & Callaini 2011). Interestingly, a large difference in spindle size between mitosis and meiosis has been reported in *Drosophila*, with meiotic spindles consistently the larger of the two (Cenci *et al.* 1994). This reliance on centriole-based nucleation thus potentially accommodates the unusually large amount of tubulin required for male meiosis. The functional significance of these different spindle sizes is unclear; however, a correlation between meiotic spindle size and subsequent sperm flagellum size in *Drosophila* has prompted speculation that tubulins used in meiosis are stored and reutilized for flagellum formation (Lattao *et al.* 2012). However, it is possible that spindle and flagellum formation are simply driven by the same nucleating pathway, and similarities in nucleation efficiency and tubulin quantity arise as a consequence. Indeed, centrosome-nucleating activity and spindle size are also believed to be correlated (Lattao *et al.* 2012).

Spermiogenesis

Following the end of meiosis, haploid round spermatids enter spermiogenesis as small, unpolarised cells with an unremarkable cytoskeletal network. As they undergo progressive remodelling, however, this network is reorganised into multiple complex, and in some cases, unique, cytoskeletal structures that are integral to spermatid remodelling and sperm function.

Acrosome biogenesis

One of the earliest events in spermiogenesis is the formation of the acrosome, an apical nuclear cap, rich in enzymes required for sperm penetration of the oocyte cumulus complex. Acrosome biogenesis begins with trafficking of proacrosomal vesicles to the nucleus from both the trans-Golgi stacks and from an endocytic pathway (Fig. 2; Elkis *et al.* 2015). These vesicles coalesce, adhere to, and progressively spread over, the nuclear envelope. As evidenced by studies wherein various cytoskeletal proteins are disrupted, acrosome biogenesis and integrity is reliant on an intact and diverse cytoskeletal network (Vogl 1990, Moreno

Figure 1 Cytoskeletal organisation of the seminiferous epithelium. Sertoli cells possess a dynamic cytoskeletal network consisting predominantly of actin filaments, IFs and microtubules (Vogl *et al.* 2008). Actin-based adhesion junctions (ectoplasmic specialisations (ESs) interspersed with adherens, gap and tight junctions) are integral in facilitating adhesion and communication between adjacent Sertoli cells (Vogl *et al.* 2014, Stanton 2016), and in physiologically segregating the seminiferous tubules into basal and apical compartments and thus are referred to as the 'blood–testis barrier' (Mital *et al.* 2011, Kaur *et al.* 2014). Dynamic remodelling of this junctional complex by actin-based endocytic tubulobulbar complexes (TBCs) likely allows spermatocytes to move from the basal to the apical compartment (Vogl *et al.* 2014). Sertoli cells also form ES and adherens junctions with spermatocytes and spermatids to allow Sertoli cells to adhere and transmit forces to developing germ cells (Romrell & Ross 1979, Kierszenbaum *et al.* 2007), in addition to forming TBCs with spermatids during spermiogenesis (O'Donnell *et al.* 2011, Vogl *et al.* 2014). IFs are present throughout the Sertoli cytoplasm, and in particular, are concentrated around the Sertoli cell nucleus (Amlani & Vogl 1988, Vogl *et al.* 2008). IFs also contribute to specialised intercellular junctions (desmosomes) and junctions with the basement membrane (hemi-desmosomes; Vogl *et al.* 2008). Microtubules are predominately located in the apical processes of Sertoli cells that surround developing spermatids (Vogl *et al.* 2008), and together with microtubule-based motor proteins (Miller *et al.* 1999, Guttman *et al.* 2000, Vaid *et al.* 2007), are thought to provide tracks for the translocation of spermatids within the depth of the seminiferous epithelium via interactions with the apical ESs.

et al. 2006, Shen *et al.* 2014). Eukaryotic vesicle trafficking is typically mediated by microtubule-based mechanisms for long-range movement followed by actin-based trafficking for more precise local delivery, and several lines of evidence suggest proacrosomal vesicle trafficking adheres to such a model.

Immunolocalisation studies have shown proacrosomal vesicles are decorated with the microtubule-based kinesin-14 motor KIFC1, in addition to the F-actin-based motor myosin-Va and its receptor RAB 27A/B (Yang & Sperry 2003, Kierszenbaum *et al.* 2004).

Consistent with this, reduced KIFC1 expression has been associated with defective acrosome formation in both humans and mice (Zhi *et al.* 2016). Similarly, treatment of spermatids with the microtubule-depolymerising agent nocodazole specifically disrupts the initial steps of acrosome formation (Moreno *et al.* 2006). Multiple microtubule-interacting proteins have also been implicated in proacrosomal vesicle transport, including endocytic sorting regulators USP8/UBPy and HOOK1 (Moreno *et al.* 2006, Berruti *et al.* 2010, Maldonado-Báez *et al.* 2013, Berruti & Paiardi 2015) and GMAP210

Table 1 Mouse models relevant to the function of intermediate and septin filaments in spermatogenesis.

Cytoskeletal type	Gene	Protein	Testis localisation	Spermatogenesis phenotype	Function in somatic cells	References
Intermediate filaments	<i>Lmna</i>	Lamin A and C	Spermatid acroplaxome	siRNA knockdown of <i>Lmna</i> , causing loss of lamin A/C proteins in mice, disrupts acrosome vesicle docking, maintenance of acrosome integrity, and results in abnormal spermatid nuclear morphology	Key component of the nuclear lamina	Shen <i>et al.</i> (2014)
		Lamin C2	Telomere-nuclear envelope attachment sites in spermatocytes during meiotic prophase I	<i>Lamin C2</i> ^{-/-} mice (targeted deletion of the lamin C2 isoform of the <i>Lmna</i> gene) exhibit defective meiotic synapsis, resulting in complete meiotic failure and an absence of postmeiotic male germ cells	Lamin C2 is germline specific, however its ectopic expression in somatic cells results in nuclear deformation and alters distribution of nuclear envelope proteins	Jahn <i>et al.</i> (2010), Link <i>et al.</i> (2013)
	<i>Vim</i>	Vimentin	Present throughout the Sertoli cell cytoplasm	<i>Vim</i> ^{-/-} mice have no obvious spermatogenic defects	Anchors organelles in the cytoplasm and maintains cell shape	Vogl <i>et al.</i> (1996)
Septin filaments	<i>Sept4</i>	Septin-4	Annulus of spermatids and spermatozoa	Spermatozoa of <i>Sept4</i> ^{-/-} mice lack the annulus (and as such substances diffuse between the mid-piece and the rest of the sperm flagella), have mitochondrial abnormalities, retention of the cytoplasmic droplet, are immotile and fail to capacitate. <i>Sept4</i> ^{-/-} mouse testes also have reductions in Septins 1, 2, 6, 7 and 11, β -actin and cytochrome protein levels	Possible roles in exocytosis, cytokinesis and neuronal differentiation and growth	Ihara <i>et al.</i> (2005), Kissel <i>et al.</i> (2005)
	<i>Sept12</i>	Septin-12	HTCA, mid-piece and annulus of spermatids and spermatozoa, and manchette of spermatids	Spermatids and spermatozoa of <i>Sept12</i> ^{+/-} mice exhibit abnormal nuclear morphology and lack of or defective flagella	Not investigated	Lin <i>et al.</i> (2011), Kuo <i>et al.</i> (2013), Yeh <i>et al.</i> (2015)

and IFT88 for Golgi-derived vesicles (Kierszenbaum *et al.* 2011). Recent research has also revealed that microtubules facilitate the correct spatial orientation of the Golgi stacks during acrosome biogenesis (Elkis *et al.* 2015). Specifically, interactions between microtubules and the Golgi-associated protein, TMF/ARA160, are responsible for the unique rotation of the Golgi, so that the trans-Golgi stacks face the spermatid nucleus. However, whether correct Golgi orientation is absolutely required for proacrosomal vesicle delivery requires further clarification. Indeed, there is some contention as to whether microtubules are actually required for delivery of proacrosomal vesicles from the Golgi. Recent data suggest that nocodazole treatment only disrupts the delivery of plasma membrane, not Golgi, derived acrosomal components (Berruti & Paiardi 2015); however, the transport of only one Golgi-derived acrosomal protein was assessed. Given the close proximity of the Golgi to the acrosome, it is possible actin-based mechanisms are more important

for this pathway. Microtubules have also been proposed to facilitate the final shaping of the acrosome in lower order species. Of interest, studies in crustaceans have revealed a novel microtubule-based structure called that acroframosome. It is an 'umbrella-like' scaffold around the acrosome in mid to late spermiogenesis, and the structure is consistent with a possible role in either driving formation or structurally reinforcing the characteristic apical acrosomal spike of crustacean sperm (Li *et al.* 2010). While there has been speculation that a similar structure may exist in mammals (Wei & Yang 2018), this is far from certain and should be explored more closely. Within early round mammalian spermatids, microtubules are present as a randomly arranged, dense cortical array and, in direct contrast to crustaceans, as spermiogenesis proceeds microtubules almost entirely polarise to the caudal half of the spermatid nucleus.

Acrosome biogenesis is further facilitated by the acroplaxome, a dense F-actin/keratin-5 cytoskeletal plate in the subacrosomal space (Kierszenbaum *et al.*

Figure 2 Cytoskeletal aspects of acrosome biogenesis. Vesicles derived from the trans-Golgi stacks and from endocytic pathways drive the formation of the acrosome. During this process, a dense cortical microtubule array is observed in the spermatid cytoplasm, and proacrosomal vesicles are thought to be transported along these microtubules to the site of acrosome biogenesis. Endocytic vesicles are directed to the acrosome by USP8/UBPy, which can bind both the vesicles and microtubules (Berruti *et al.* 2010, Berruti & Paiardi 2015). Golgi-derived proacrosomal vesicles are coated with actin-based motor protein myosin-Va and its receptor Rab 27A/B, in addition to the microtubule-based motor protein, KIFC1, suggesting transport to the acrosome occurs via both microtubule and actin-based mechanisms (Yang & Sperry 2003, Kierszenbaum *et al.* 2004). However, the reliance of Golgi-derived vesicles on microtubule-dependent transport is debated (Berruti & Paiardi 2015). TMF/ARA160-dependent interactions between microtubules and the Golgi, ensure that the trans-Golgi stacks face the acrosome (Elkis *et al.* 2015). MT, microtubule.

2003b; Fig. 3). The acroplaxome appears to adhere to the developing acrosomal sac to the nuclear envelope and facilitates its transverse spreading. Consistent with this, cytochalasin D disruption of F-actin results in nuclear-acrosome detachment and disruption to the expanding edge of the acrosome (Vogl 1990). The presence of myosin-Va between the developing acrosomal sac and the acroplaxome F-actin tract has prompted the hypothesis that it may facilitate acrosome spreading (Kierszenbaum *et al.* 2004; Fig. 3E); however, this requires further investigation. The acroplaxome appears to be anchored to the nuclear envelope by interactions between keratin within the acroplaxome and the IF-based network in the nuclear envelope. Notably, knockdown of lamin A/C-based nuclear IFs disrupts acrosome vesicle docking and the maintenance of acrosome integrity (Table 1; Shen *et al.* 2014). Moreover, electron microscopy has revealed that the caudal marginal ring of the acroplaxome is defined by a desmosome-like structure, consisting of keratin-5-based IFs, which connects the descending edge of the acrosome to the nuclear lamina (Kierszenbaum *et al.* 2003b; Fig. 3B and E).

Flagella formation

The sperm flagellum, formed during spermiogenesis, is a modified motile cilium. It possesses a typical basal body-derived 9+2 central microtubule core, the axoneme, ensheathed along most of its length

by sperm-specific accessory structures (including keratin-like outer dense fibres (ODF), a mitochondrial sheath and a fibrous sheath; Fig. 4). Microtubule dynamics are thus well established to underpin the structure and function of the flagella. More recently, however, the importance of F-actin and septins has been highlighted.

Axoneme formation is initiated early in spermiogenesis, with the polarisation of the centrosome to the caudal pole of the cell. As is typical of ciliogenesis, the spermatid centrosome matures into a basal body that is capable of docking onto the plasma membrane and extending an axoneme. However, unlike typical ciliogenesis, the basal body also docks the nuclear membrane, driving formation of the head-to-tail coupling apparatus (HTCA) (Figs 3 and 4). The distal (or mother) centriole of the basal body, which docks to the plasma membrane, orients along the longitudinal axis of the eventual flagellum and axonemal microtubules nucleate from this template (Fawcett & Phillips 1969).

While the mechanisms driving these events have been given minimal attention in spermatids, insights from somatic cells indicate actin- and microtubule-related processes are integral for these initial phases. Somatic basal body maturation requires several modifications to the microtubule scaffold of the distal centriole, including the acquisition of the distal appendages (Sillibourne *et al.* 2013, Tanos *et al.* 2013, Ye *et al.* 2014), the removal of a CP110-CEP97 distal centriole cap, which inhibits axoneme microtubule extension (Spektor *et al.* 2007), followed by the acquisition of the pre-ciliary vesicle, which ultimately forms the ciliary membrane (Sorokin 1962). Of note, the delivery of the pre-ciliary vesicle to the basal body requires dynein-based transportation along microtubules, followed by myosin-Va-dependent transportation along ARP2/3-associated F-actin networks (Wu *et al.* 2018). Similarly, plasma membrane docking typically requires interactions with cortical F-actin networks to drive docking and correct orientation at the plasma membrane (Dawe *et al.* 2007, Park *et al.* 2008, Werner *et al.* 2011) and focal adhesion proteins, such as FAK, vinculin and paxillin, appear to facilitate these actin-microtubule interactions (Antoniades *et al.* 2014). However, whether this mechanism is conserved in spermatids, particularly in the spermatid-specific process of nuclear envelope-basal body docking requires investigation. Indeed, different mechanisms likely drive basal body-nuclear attachment, with inner nuclear membrane protein SUN5, being essential for the ongoing integrity, although not the initial formation, of the HTCA-nuclear envelope attachment (Fig. 4A; Shang *et al.* 2017). Other proteins such as septin-12 (see below), PMFB1 and ODF1 have also been implicated in HTCA-nuclear envelope attachment (Kefei *et al.* 2014, Yeh *et al.* 2015, Zhu *et al.* 2018). The initial polarisation of the centrosome is also unexplored in spermatids, although

Figure 3 Cytoskeletal structures in spermatid head shaping. Nuclear remodelling in spermatids is thought to be driven by three key elements: (1) the acroplaxome, an actin–keratin-based cytoskeletal plate, (2) extracellular forces from the actomyosin bundles residing in the surrounding Sertoli cell and (3) the manchette, a transient microtubule-based ‘skirt’ (Kierszenbaum *et al.* 2007, Lehti & Sironen 2016). The overall relationship of these three structures, in the context of a mouse elongating spermatid, is illustrated in (A). The structure of the sperm head shaping machinery can be seen in an electron micrograph (B) and a schematic (E) of a mouse spermatid. The transverse aspect of the manchette is also shown via in an electron micrograph in (C). (D) During spermiogenesis the manchette progressively moves down the spermatid head, while concomitantly constricting to sculpt the base of the nucleus (Russell *et al.* 1991, Lehti & Sironen 2016). This is accompanied by expansion of the acrosome and underlying acroplaxome (Kierszenbaum *et al.* 2004, 2007). Ar, acrosome; ES, ectoplasmic specialisation; IF, intermediate filament; MT, microtubule; SA, subacrosomal space.

similar events in somatic cells utilise dynein and the actin motor, non-muscle myosin II (Liu *et al.* 2013).

While many of the aforementioned aspects of somatic centriole and ciliogenesis dynamics are likely conserved in spermatids, it should be noted that evidence indicates that there are at least some spermatid-specific mechanisms. Mouse spermatids lacking the microtubule regulating protein, KATNAL2, exhibit a failure of basal body-plasma membrane docking and axoneme extension; however, aside from spermatogenic defects, the mice are overtly healthy, suggesting somatic ciliogenesis is not catastrophically affected (Dunleavy *et al.* 2017). In addition, after ciliogenesis commences, the spermatid basal body undergoes additional unique remodelling steps. The minus ends of the distal centriole microtubules splay outwards, and specific centriolar proteins become enriched in rod-like formations while other centriole proteins are reduced, resulting in an almost unrecognisable ‘atypical’ centriole (Fishman *et al.* 2018). The functional significance of these unique changes, however, remains to be elucidated.

A role for septins in flagella formation is also emerging. Typical somatic cilia are physiologically segregated into a

cytoplasmic centriole segment and an axonemal segment via a specialised transition zone. A septin-based diffusion barrier at the junction of this zone and the axonemal segment is integral to this compartmentalisation and thus cilia formation. Likewise in sperm, an analogous filamentous septin-based ring known as the annulus forms at the distal tip of the centriole just after centriole docking. In the terminal steps of spermatid maturation, this ring progressively migrates along the axoneme by an unknown mechanism, ultimately residing at the junction between the mid-piece and the principal piece (Fig. 4A; Kissel *et al.* 2005, Sugino *et al.* 2008, Lin *et al.* 2011). Evidence suggests the annulus establishes a diffusion barrier physiologically segregating the mid-piece from the principal and end pieces (Table 1; Kwitny *et al.* 2010), leading to the proposal that the annulus together with the mid-piece represents a modified transition zone. Septin-12 has also been localised to the HTCA of spermatozoa (Lin *et al.* 2011, 2012, Kuo *et al.* 2013) and appears to be important for its structural integrity, potentially linking cytoskeletal elements in the HTCA to SUN4 and the nuclear lamina (Fig. 4A and Table 1; Yeh *et al.* 2015).

Figure 4 Cytoskeletal aspects of the sperm flagellum and its development. The proposed model of mitochondrial sheath and HTCA formation is illustrated in (A). As spermiogenesis proceeds, mitochondria are organised into longitudinal rows in the cytoplasmic lobes of the spermatids in preparation for loading onto the ODF. Given that mitochondria can interact with KLC3, it is possible this organisation is driven via kinesin-based transport up the microtubules of the manchette (Zhang *et al.* 2012). After caudal migration of the annulus, an F-actin helix is observed around the mitochondrial sheath, which may either drive compaction of mitochondria around the ODF or provide structural integrity to the mitochondrial sheath (Gervasi *et al.* 2018). The HTCA is composed of a proximal and distal centriole embedded within a specialised pericentriolar material known as the striated columns. The proximal centriole attaches to the nuclear envelope via the associated dense capitulum. The LINC complex protein, SUN5, connects the nuclear lamina to the capitulum via an unknown mechanism (Shang *et al.* 2017). Septin-12, which is present throughout the HTCA, is also essential for HTCA integrity, likely due to its interactions with SUN4-based LINC complexes in the nuclear membrane (Lin *et al.* 2011, 2012, Kuo *et al.* 2013, Yeh *et al.* 2015). (B) The flagella of mature spermatozoa is structurally characterised by three distinct regions: the midpiece, principal and end pieces. At the transverse aspect, the midpiece is characterised by an outer mitochondrial sheath encasing a ninefold array of ODF and a 9+2 motile axoneme (Fawcett 1975, Lindemann & Lesich 2016). This region connects to the sperm head via the head–tail coupling apparatus (HTCA) (Fawcett & Phillips 1969) and due to the presence of a septin-based diffusion barrier in the midpiece annulus, this region is believed to represent a modified transition zone (Kwitny *et al.* 2010). Within the principal piece, the mitochondrial sheath is absent; instead, a fibrous sheath encases the ODF and axoneme core (Fawcett 1975, Lindemann & Lesich 2016). Moreover, longitudinal columns of fibrous sheath replace ODFs 3 and 8. The ODF and fibrous sheath both terminate at the end of the flagella principal piece. As such, the end piece is the thinnest region of the sperm flagella, containing only the 9+2 axoneme surrounded by a plasma membrane (Fawcett 1975, Lindemann & Lesich 2016). Flagellar movement is dependent on the central axoneme. The dynein arms ‘slide’ adjacent microtubule doublets in relation to one another in an ATP-dependent process (Satir 1968, Summers & Gibbons 1971, Gibbons & Gibbons 1973). Due to both nexin linkers and the attachment of microtubule doublets to the basal body restricting sliding, the action instead results in axoneme bending.

Once axoneme extension initiates in round spermatids, the delivery of constituents required for axoneme and, possibly, fibrous sheath assembly is likely achieved via a modified intraflagellar transport (IFT) pathway (O'Donnell & O'Bryan 2014, Lehti & Sironen 2016). IFT is an evolutionarily conserved mechanism, wherein motor proteins transport large protein complexes along the axoneme microtubules (Rosenbaum & Witman 2002). Axoneme growth occurs exclusively by tubulin addition at the distal tip (Johnson & Rosenbaum 1992); however, protein synthesis is restricted to the cell cytoplasm. IFT thus provides an essential delivery mechanism. Cargoes are transported by IFT-A and IFT-B complexes in association with kinesin-2 and dynein-2 family motor proteins (Rosenbaum & Witman 2002, Taschner & Lorentzen 2016), and notably, various IFT-A and -B complex proteins and the kinesin II protein, KIF3A, are essential for spermatid axoneme formation. *Ift20*, *Ift25*, *Ift27*, *Ift88*, *Ift140* and *Kif3A* mutant mice all exhibit either short, deformed or absent flagella, with disorganised or absent axoneme components and associated ODF and fibrous sheath defects (Kierszenbaum *et al.* 2011, Lehti & Sironen 2016, Zhang *et al.* 2016, Liu *et al.* 2017, Zhang *et al.* 2017a,b). Likewise, a RABL2-dependent IFT pathway has been implicated in the delivery of key components to the fibrous sheath (Lo *et al.* 2012).

While IFT is involved in building the axoneme earlier in spermiogenesis, intramanchette transport (IMT) (Fig. 3E) is likely involved in the final steps of sperm flagellum and accessory structure assembly after spermatid elongation commences. The manchette is a transient microtubule-based sheath present around the spermatid nucleus during the latter half of spermiogenesis (see 'Nuclear remodelling' section). Flagella components, and notably ODF proteins, are transported along manchette microtubules, as are vesicles, IFT complex proteins and kinesin and dynein motors (Rattner & Brinkley 1972, Hall *et al.* 1992, Yoshida *et al.* 1994, Saade *et al.* 2007, Hayasaka *et al.* 2008, Kierszenbaum *et al.* 2011, Lehti & Sironen 2016, Zhang *et al.* 2016). Intriguingly, given that both plus- (kinesin) and minus- (dynein) end directed motors are present on the manchette (Fig. 3E), IMT is likely bidirectional and thus transports proteins from both the apical region and the cytoplasmic lobes to the HTCA. β -actin- and myosin-based vesicles are also present in the manchette (Mochida *et al.* 2000, Kierszenbaum *et al.* 2003a) leading to speculation that F-actin-based transport also contributes to IMT. However, this requires further validation as despite numerous attempts, polymerised actin filaments have never been detected in the manchette (Welch & O'Rand 1985, Halenda *et al.* 1987, Kierszenbaum *et al.* 2003a, Tachibana *et al.* 2005), and thus, the possibility that the β -actin detected is instead part of dynactin complexes, which interact with dynein to facilitate microtubule-based transport, cannot be excluded.

Beyond this, little else is known about the mechanisms that regulate the formation of the sperm axoneme and its accessory structures. The formation of the mitochondrial sheath in particular remains unresolved. Mitochondria are observed to assemble into longitudinal rows in the cytoplasm adjacent to the flagella, and upon the distal migration of the annulus, they load onto the flagella ODFs. Of note, a double-helical F-actin scaffold associated with the mid-piece mitochondria has recently been identified. Given that the F-actin arrangement follows the helical arrangement of the mitochondria, it is possible that F-actin-based mechanisms are involved in the compaction of mitochondria around the flagella and/or in the structural integrity of the sheath (Fig. 4A; Gervasi *et al.* 2018). Microtubules also have a possible role, and indeed, kinesin-facilitated microtubule-based transport of mitochondria occurs in a variety of contexts. In spermatids, kinesin light chain 3 (KLC3) colocalises with mitochondria, and mutant KLC3 appears to disrupt mitochondrial sheath assembly (Zhang *et al.* 2012). Given that KLC3 is a plus-end-directed motor, it is plausible that transport up the inner surface of manchette microtubules is responsible for 'lining up' the mitochondria prior to their loading on to the ODFs (Fig. 4A).

Typical aspects of microtubule regulation likely also contribute to flagella formation. For example, microtubule-severing proteins have emerged as essential for proper axoneme formation in mice (O'Donnell *et al.* 2012; Dunleavy *et al.* 2017). In addition, the axoneme exhibits extensive post-translational modifications, including acetylation, mono-/poly-glutamylation, deetyrosination and mono-/poly-glycylation (Gagnon *et al.* 1996, Kann *et al.* 1998, Huitorel *et al.* 2002, Kierszenbaum 2002), and mutations in tubulin-modifying enzymes cause defective axoneme formation and motility (Ikegami & Setou 2010). Such modifications are thus believed to regulate the formation, stability and function of the axoneme.

Nuclear remodelling

As spermiogenesis proceeds, the spermatid nucleus undergoes extensive compaction and elongation. This coincides with the formation of the manchette around the distal half of the nucleus (Fig. 3A). Within this structure, microtubules arrange into linear arrays, parallel to the long axis of the nucleus (Rattner & Brinkley 1972), with their dynamic plus-ends captured and stabilised by a dense tubulin/keratin-9-based perinuclear ring (Mochida *et al.* 2000, Kato *et al.* 2004, Dunleavy *et al.* 2017). In rodents, at its largest, the manchette contains over 1000 microtubules (Rattner & Brinkley 1972), which form frequent links with adjacent microtubules (Rattner & Brinkley 1972, MacKinnon & Abraham 1972) and the nuclear envelope (Russell *et al.* 1991; Fig. 3C). The manchette is thus extremely rigid

and appears to exert mechanical force, sculpting the base of the spermatid nucleus as it elongates. Indeed, its role in spermatid nuclear shaping is highlighted by consistent observations that genetically modified mouse models displaying manchette abnormalities also exhibit defects in head morphology (Russell *et al.* 1991, Mendoza-Lujambio *et al.* 2002, Zhou *et al.* 2009, Zhang *et al.* 2009, Lehti & Sironen 2016, Dunleavy *et al.* 2017). Collectively, data suggest that the manchette sculpts and tapers the sperm head using a 'ratchet-like' mechanism, forming progressive connections with the nuclear envelope as it slowly moves down the nucleus in a caudal direction, while progressively reducing its diameter (Fig. 3D; Russell *et al.* 1991). However, as the composition of the manchette microtubule–microtubule and microtubule–nuclear envelope linkers is yet to be convincingly determined, the actual mechanisms underlying manchette constriction and movement remain unresolved. Immunolocalisation studies have suggested that manchette–nuclear envelope linkers are LINC complexes, composed of SUN4, SUN3 and KASH protein, Nesprin-1 (Göb *et al.* 2010, Calvi *et al.* 2015, Pasch *et al.* 2015, Yeh *et al.* 2015). Indeed, in other contexts, LINC complexes link the peripheral cytoskeleton to the nucleus and contribute to force transmission across nuclear membranes (Horn 2014). Typically, they comprise a SUN inner nuclear membrane protein, bound to a KASH outer nuclear membrane protein, which can link to microtubule motor proteins (Horn 2014, Kim *et al.* 2015). Given that microtubule motor proteins are observed at the manchette–nuclear interface (Yoshida *et al.* 1994), it is plausible that LINC–microtubule motor protein interactions drive the caudal movement of the manchette. Consistent with this, SUN4 ablation results in the absence of manchette–nuclear linkers, manchette–nucleus detachment and defective sperm head shaping in mice (Calvi *et al.* 2015, Pasch *et al.* 2015). However, Nesprin-1-knockout mice are male fertile (Zhang *et al.* 2007), and of all other known KASH proteins, only KASH-5, which does not localise to the manchette (Horn *et al.* 2013), is required for male fertility (Zhang *et al.* 2007, Horn *et al.* 2013, Ketema *et al.* 2013), suggesting interactions between SUN4 and the manchette are mediated by an as yet unidentified mechanism. Of note, during nuclear shaping, the septin cytoskeletal protein, septin-12, is closely associated with the nuclear envelope and is capable of binding LINC complex proteins, including SUN4 (Yeh *et al.* 2015). Given that several studies have established a role for septin-12 in spermatid nuclear shaping in both rodents and humans (Table 1; Lin *et al.* 2011, 2012, Kuo *et al.* 2013), one attractive hypothesis is that septin-12 is the 'missing link' between the manchette and SUN4 during spermatid remodelling.

As for the manchette microtubule–microtubule linkers, their identity is largely speculative. Strikingly similar inter-microtubule connectors, comprising a

TACC3-ch-TOG-clathrin complex, are observed between adjacent kinetochore microtubules during mitosis (Booth *et al.* 2011, Nixon *et al.* 2015). Whether similar mechanisms participate in manchette microtubule linkage remain to be investigated. Alternatively, microtubule cross-linking and bundling proteins, such as tau and MAP4, localise to the manchette (Ashman *et al.* 1992, Parysek *et al.* 1984), as do the poorly understood tubulins, delta and epsilon (Kato *et al.* 2004, Dunleavy *et al.* 2017) which are predicted to associate laterally with microtubule polymers (Inclán & Nogales 2001). A wide range of MAPs, including microtubule-severing proteins and motor proteins (Dunleavy *et al.* 2017 and older publications reviewed in Lehti & Sironen 2016), also contribute to the movement, constriction and eventual dissolution of the manchette. However, the mechanisms by which these proteins regulate manchette dynamics remain unclear.

The acroplaxome also expands during spermatid nuclear remodelling and a model has been proposed to explain its potential role in shaping the posterior half of the sperm head (Kierszenbaum *et al.* 2004, 2007). In this model, the tensile strength of keratin-5 filaments in the acroplaxome restrains both endogenous (manchette-derived) and exogenous (from an actin-based network within the Sertoli cell, see 'Actin-based structures between Sertoli cells and spermatids' section) forces acting upon the spermatid head, thus preventing abnormal or excessive nuclear deformation (Kierszenbaum *et al.* 2004, 2007); however, the data for this model at present is largely correlative. The acroplaxome has also been proposed to play an active role in generating the endogenous forces that sculpt the spermatid nucleus (Kierszenbaum *et al.* 2004). This is due to observations that the marginal ring of the acroplaxome constricts and descends caudally, mimicking the constriction and migration of the sub-adjacent manchette perinuclear ring. However, in *Sun4*-knockout mice, wherein the manchette fails to associate with the nucleus, although caudal expansion of the acroplaxome–acroplaxome complex still occurs, constriction of the acroplaxome marginal ring is not apparent and spermatid heads remain rounded (Calvi *et al.* 2015). These data instead suggest the reduction in marginal ring diameter is simply a response to, rather than a cause of, the narrowing of the spermatid head.

Similar to acrosome biogenesis, spermatid nuclear remodelling requires an intact IF-based nuclear lamina (Table 1; Shen *et al.* 2014), likely to provide a strong anchor for the manchette and acroplaxome. As discussed earlier, the acroplaxome appears to be tethered to the nuclear lamina via desmosome-like junctions in the marginal ring (Kierszenbaum *et al.* 2003b) and LINC complexes appear to link the manchette microtubules (Calvi *et al.* 2015, Pasch *et al.* 2015, Yeh *et al.* 2015), and possibly the acroplaxome (Göb *et al.* 2010), to the nuclear lamina. Both the desmosome-like junctions and

LINC complexes are anticipated to be involved in nuclear deformation and thus likely facilitate spermatid nuclear remodelling (Fig. 3E). Similar to meiotic prophase I (see 'Spermatogonia and spermatocytes' section), changes in nuclear lamina composition also facilitate nuclear deformation during spermiogenesis. Spermiogenesis is characterised by the upregulation of spermatid-specific lamin B3, which like the meiosis-specific lamin C2, has a shorter, more flexible, central α -helical coil than typical IFs and thus its enrichment reduces stability of the nuclear lamina (Schütz *et al.* 2005a,b, Elkhathib *et al.* 2015).

The dynamic Sertoli cell cytoskeleton

Sertoli cells are the 'nurse' cells of the seminiferous epithelium; they provide the structural framework and physiological environment necessary for effective spermatogenesis. They are polarised, irregularly shaped, columnar epithelial cells that sit on the basement membrane of the seminiferous tubule (Fig. 1). The basal regions of Sertoli cells are 'powerhouses' containing an abundance of organelles. Sertoli cells also have long apical cytoplasmic processes that envelop developing germ cells and, with the exception of cytoskeletal elements and mitochondria, are devoid of organelles. Sertoli cells maintain numerous cytoskeletal-based junctions with germ cells, which both physically tether germ cells into position and contribute to spermatid remodelling (Fig. 1). Moreover, to accommodate and effectuate the structural changes and movements of developing germ cells, Sertoli cell shape is cyclically remodelled. In order to achieve these functions, Sertoli cells possess a dynamic cytoskeleton rich in actin filaments, microtubules and intermediate filaments. The major roles of each of these cytoskeletal structures in Sertoli cells are discussed below.

Actin-based cytoskeleton

When viewed by microscopy, actin filaments are clearly concentrated in particular areas of the Sertoli cell, most notably at adhesion junctions between Sertoli cells at their base, adjacent to spermatids as they elongate during spermiogenesis and at the site of spermiation, when mature spermatids are released from the Sertoli cell.

Actin-based structures between Sertoli cells

Large tracts of actin filaments are present at the junction between the basal aspects of adjacent Sertoli cells towards their base, within a structure known as the ectoplasmic specialisation (ES) (Fig. 1). The actin filaments at this site are bundled into hexagonally packed arrays by the protein espin (Bartles *et al.* 1996). This actin array is sandwiched between the Sertoli cell plasma membrane and a layer of endoplasmic reticulum

(Russell & Peterson 1985, Vogl *et al.* 2008). The Sertoli cell membranes contain proteins that mediate tight intercellular adhesion (Hermo *et al.* 2010). The ES structures at this site are interspersed with other actin-related adhesion junctions – adherens, gap and tight (occluding) junctions (Fig. 1; Vogl *et al.* 2014, Stanton 2016). Actin-based tubulobulbar complexes (TBCs) are also present between Sertoli cells at the basal aspect (Fig. 1); these are unique endocytic structures that can internalise junctional structures (Vogl *et al.* 2014) and are explained in more detail below (see 'Actin-based structures between Sertoli cells and spermatids' section). Thus, ES, TBCs and intercellular adhesion junctions comprise a heterogeneous actin-associated Sertoli cell junctional complex with multiple functions during spermatogenesis.

This junctional complex allows the Sertoli cell to strictly determine the microenvironment of the developing spermatocytes and spermatids. Tight junctions within this complex prevent the free passage of molecules into the adluminal part of the seminiferous tubule (Kaur *et al.* 2014) ensuring that spermatocytes and spermatids develop within an immunologically privileged and strictly regulated microenvironment. The actin-associated Sertoli cell junctional complex contributes to the function of the so-called blood–testis barrier, which prevents the access of factors and cells from the circulation to the inner part of the tubule and protects the immunogenic developing sperm from the immune system (Mital *et al.* 2011, Kaur *et al.* 2014). The Sertoli cell junctional complexes are remodelled during the spermatogenic cycle to allow early spermatocytes to enter the inner compartment of the seminiferous tubule, while maintaining the microenvironment of spermatocytes and spermatids. This remodelling involves various proteins involved in the regulation of F-actin (Li *et al.* 2016) as well as basal TBCs to internalise junctional structures (Vogl *et al.* 2014) (see next section).

Actin-based structures between Sertoli cells and spermatids

Immediately prior to elongation, the centrally located spermatid nucleus polarises to one side of the cell and becomes encircled by a Sertoli cell actin-based ES in the region adjacent to the developing acrosome (Fig. 1). This apical ES confers extremely tight adhesion between the Sertoli cell and developing spermatid (Romrell & Ross 1979) and facilitates movement of spermatids in the epithelium. As these cells begin to elongate, they are enveloped by the Sertoli cell cytoplasm and are pulled towards the base of the epithelium prior to being translocated to the luminal edge in preparation for spermiation. This translocation is facilitated by microtubules interacting with the actin-based apical ES, moving the spermatid basally as it elongates and then apically to the luminal edge prior to its release

at spermiation (Vogl *et al.* 2008). The apical ES is also hypothesised to be involved in spermatid nuclear shaping by exerting anchoring forces on the spermatid head as it elongates (Kierszenbaum *et al.* 2007). However, as actin bundles in the ES are non-contractile (Vogl & Soucy 1985), it is yet to be determined if this structure actively exerts force to sculpt the spermatid head. Conversely, other Sertoli cell actin-based structures have been more conclusively linked to spermatid head shaping (Fig. 3A). Disruption of a Sertoli cortical F-actin meshwork results in a loss of Sertoli-spermatid adherens junctions containing contractile actomyosin bundles, a concomitant increase in non-contractile ES junctions and notably, defective spermatid head shaping (Sakamoto *et al.* 2018).

Actin-based structures during spermiation

During spermiation, the spermatid undergoes its final remodelling and eventual release from the Sertoli cell, prior to its passage to the epididymis, reviewed in O'Donnell *et al.* (2011). The spermatid is situated at the luminal edge of the epithelium while its extensive cytoplasm is 'stripped off' by the Sertoli cell to reveal the flagellum that will ultimately be capable of motility (Fig. 1). During this process, the spermatid must maintain tight adhesion to the Sertoli cell as it is subjected to the flow of fluid through the seminiferous tubule (O'Donnell *et al.* 2011).

At the beginning of spermiation, the spermatid adheres to the Sertoli cell via the apical ES; however, this is gradually removed and replaced by a focal adhesion-like junction (O'Donnell *et al.* 2011). The removal of the extensive apical ES may involve actin regulatory proteins (Qian *et al.* 2014). TBCs between spermatids and Sertoli cells have also been shown to remove adhesion proteins consistent with a role in disassembly of the apical ES (O'Donnell *et al.* 2011, Vogl *et al.* 2014). Based on these data, TBCs are proposed to internalise both Sertoli cell and spermatid plasma membranes along with associated adhesion proteins and ES structures; these structures then 'bud off' and enter the endocytic pathway within the Sertoli cell cytoplasm (Vogl *et al.* 2014). Various proteins involved in F-actin dynamics drive TBC formation and dissolution and ES removal (Young *et al.* 2009, 2012, Vogl *et al.* 2014) and thus successful spermiation relies on the regulation of actin-based processes. It should be noted, however, that recent data have shown that in the absence of TBC formation in mice, the ES disassembles normally suggesting that additional ES disassembly mechanisms may exist (Dunleavy *et al.* 2017). Indeed, given that ectopic induction of a matrix metalloproteinase-2 (MMP-2)-dependent proteolytic pathway causes reductions in $\beta 1$ -integrin and laminin- $\gamma 3$ of the apical ES and the loss of spermatid-Sertoli cell adhesion (Siu & Cheng 2004, Yao *et al.* 2010), it is likely that proteases also contribute to ES disassembly.

Intermediate filament-based cytoskeleton

In adult Sertoli cells, IFs are present in an extensive cytoplasmic network, arranged around the basally placed nucleus (Fig. 1) and emanate out toward specific regions of plasma membrane (Amlani & Vogl 1988, Vogl *et al.* 2008). Sertoli cell IFs are largely vimentin based, in contrast to other epithelia which have predominantly cytokeratin-based filaments (Franke *et al.* 1979). Sertoli cell IFs terminate in desmosome-like junctions between the basal aspects of Sertoli cells (Fig. 1; Vogl *et al.* 2008) and participate in the Sertoli cell junctional complex. They also terminate at the Sertoli cell plasma membrane at junctions with specific types of germ cells: for example, IFs transiently associate with the heads of elongating spermatids that are deeply embedded within the seminiferous epithelium (stage II–IV in mice; Amlani & Vogl 1988). Finally, IFs terminate in hemi-desmosomes between the base of Sertoli cells and the underlying basement membrane (Fig. 1; Vogl *et al.* 2008).

Despite their extensive networks within Sertoli cells, IFs do not appear to be essential for spermatogenesis. Male mice lacking vimentin are fertile with normal testicular histology, despite an absence of IFs within Sertoli cells, suggesting that the role of Sertoli cell IFs may be largely to provide mechanical stability to the seminiferous epithelium, but this function may not be essential for spermatogenesis, reviewed in (Vogl *et al.* 2008).

Microtubule-based cytoskeleton

As is expected of dynamic polarised cells, Sertoli cells have an abundant microtubule cytoskeleton (Vogl *et al.* 2008). As Sertoli cells remodel their shape to respond to the needs of the developing germ cells, their microtubules reorganise accordingly. The most dramatic variations in microtubule architecture are seen in the apical processes (Vogl *et al.* 2008). In processes associated with spermatogonia and spermatocytes cells, microtubules are rarely observed. Conversely, microtubules are abundant in Sertoli cells crypts within which spermatids are embedded as they elongate (Fig. 1). During spermatid elongation, microtubules become concentrated in Sertoli cells in regions surrounding the acrosome and show extensions into regions closely associated with the residual cytoplasm of the spermatid. The observed effects of microtubule-disrupting drugs colchicine and vinblastine (Russell *et al.* 1981, Vogl *et al.* 1983, Allard *et al.* 1993) strongly support the hypothesis that Sertoli cell shape changes are driven by microtubule dynamics. In turn, the dynamic nature of Sertoli microtubules is likely driven by tyrosination. Sertoli cells contain a high concentration of tyrosinated microtubules (Hermo *et al.* 1991, Wenz & Hess 1998), a post-translational modification associated with high turnover rates (Webster *et al.* 1987) and is conducive

to motor-dependent microtubule disassembly (Peris *et al.* 2009). Moreover, microtubule tyrosination levels fluctuate, consistent with stage-specific changes in Sertoli cell architecture and microtubule organisation (Hermo *et al.* 1991, Wenz & Hess 1998).

In a formation typical of polarised cells, Sertoli cell microtubule networks have a linear arrangement, parallel to the longitudinal axis of the cell (Fig. 1; Vogl *et al.* 2008). In other polarised cells, such as neurons, microtubules are predominantly nucleated from a perinuclear centrosome and subsequently released and transported to distal sites. However, interestingly, Sertoli cell microtubules are nucleated in the cytoplasmic processes towards their apex rather than at the site of the Sertoli cell centrosome near the base of the cell (Vogl *et al.* 2008). After induced microtubule depolymerisation, new microtubules are first seen in the Sertoli apical processes (Vogl *et al.* 1995); likewise, overexpression of γ -tubulin, a key element for *de novo* microtubule nucleation, results in the accumulation of microtubules in the apex (Fleming *et al.* 2003). Sertoli cell microtubule minus ends also show no association with the centrosome and are instead uniformly oriented toward the apical cytoplasm (Redenbach & Vogl 1991).

Microtubule-driven kinetics underlie many Sertoli cell functions during spermatogenesis. As discussed earlier, microtubule networks within Sertoli cells make structural links with actin-based ESs and disruption of Sertoli microtubule dynamics results in the failure of spermatids to translocate to the luminal edge in preparation for spermiation (Russell *et al.* 1989, Fleming *et al.* 2003). Taken together, these results support the concept that microtubules, likely in conjunction with dynein and kinesin (Miller *et al.* 1999, Guttman *et al.* 2000, Vaid *et al.* 2007), are used to regulate the position of spermatids within the epithelium via the apical ES (Fig. 1). Following spermatid disengagement, Sertoli cell microtubule networks are also required for translocation of the residual spermatid cytoplasm to the basal surface of the seminiferous tubule lumen (Russell *et al.* 1989, Fleming *et al.* 2003). As residual body phagocytosis typically occurs at the basal region, failure of translocation can prevent residual body elimination. Another key function of microtubules within Sertoli cells is the maintenance of seminiferous epithelium structural integrity. Disruption of microtubule dynamics in Sertoli cells results in a loss of lateral stability and premature shedding of developing germ cells (Russell *et al.* 1981).

Remaining questions and future directions

The seminiferous epithelium is an extraordinarily complex and dynamic epithelium, accompanied by equally extraordinary and dynamic cytoskeletal changes. A variety of actin-, microtubule- and

intermediate filament-based structures enable the Sertoli cell to modify its shape as it responds to the changing complement of germ cells and to control intercellular adhesion at multiple sites simultaneously. Germ cells, too, rely on unique and complex cytoskeletal dynamics to facilitate their development from a round, diploid cell into a haploid, highly specialised spermatozoon. While significant advances in understanding the nature and complexity of these cytoskeletal elements during spermatogenesis have been made, the picture is far from complete. As highlighted throughout this review a number of questions remain, particularly in relation to spermiogenesis. Despite being first described over 60 years ago, the ultrastructure and regulation of the manchette remains poorly understood. Given this structure's role in spermatid head shaping, and the fact that sperm head abnormalities are frequently observed in conditions of infertility, elucidating its structure and function is of clinical relevance. In particular, the identification of the manchette inter-microtubule and microtubule-nuclear linkers is necessary to understand manchette constriction and descent. Indeed, the current LINC complex models of cytoskeletal-nuclear attachment in spermatids are incomplete as they ignore data showing that the proposed KASH domains of these complexes are not required for male fertility. The role of actin in the manchette is also unclear; whether it is present in filamentous form and participates in the transport of myosin-VA-associated vesicles needs to be verified. Likewise, the mysterious process of mitochondrial sheath assembly requires attention, particularly as mitochondrial sheath defects are associated with sterility in mice. Compared to lower order species such as *Drosophila*, there are limited data on the assembly, dynamics and regulation of the male meiotic spindle, and similarly our current understanding of basal body dynamics relies heavily on data from somatic cells.

Most importantly, the robustness of many of the major hypotheses in the field is undermined by the fact they are based on predominantly observational studies of fixed tissues and cells. The roles of many motor proteins are presumed based on their localisation. For example, due to its presence in the acroplaxome, myosin-Va is proposed to facilitate acrosomal spreading; however, no functional data exist to prove this. Moving forward, live imaging studies can provide enhanced insights into dynamic cytoskeletal processes important during spermatogenesis. Given the increasing availability of excellent live cytoskeletal stains compatible with both confocal and super resolution microscopy techniques, such studies are increasingly feasible and their adoption will be integral to the continued advancement of the field. The advent of technologies such as CRISPR will also presumably contribute to the generation of functional data to prove many of the proposed mechanisms.

Declaration of interest

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

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