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Molecular changes and signaling events occurring in sperm during epididymal maturation

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Abstract

After leaving the testis, sperm have not yet acquired the ability to move progressively and are unable to fertilize oocytes. To become fertilization-competent they must go through an epididymal maturation process in the male, and capacitation in the female tract. Epididymal maturation can be defined as those changes occurring to sperm in the epididymis that render the sperm the ability to capacitate in the female tract. As part of this process, sperm cells undergo a series of biochemical and physiological changes that require incorporation of new molecules derived from the epididymal epithelium, as well as post-translational modifications of endogenous proteins synthesized during spermiogenesis in the testis. This review will focus on epididymal maturation events, with emphasis in recent advances in the understanding of the molecular basis of this process.

Keywords

sperm; epididymis; epididymosomes; maturation; signal transduction

1. INTRODUCTION

Spermatogenesis is the process by which functional sperm cells are produced in the testis. After this process is completed, sperm are morphologically highly differentiated; however, they are unable to move progressively and have not yet acquired fertilizing capacity. To become fertilizing competent, sperm need to undergo two extra-testicular maturational processes, one in the male reproductive tract, known as epididymal sperm maturation, which will be the focus of this review, and the second in the female tract, known as capacitation. Both of them are associated with sequential biochemical changes occurring in different sperm compartments. Of these two extra-testicular maturational processes, more is known about capacitation. This process was discovered independently by Chang and Austin in 1951 (Austin 1951, Chang 1951). Soon after, *in vitro* capacitation media were developed and the process extensively studied. The discovery of *in vitro* capacitation was a necessary step for the consequent development of *in vitro* fertilization (IVF) and facilitated the understanding

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of the signaling mechanisms involved in this process. For more information on capacitation, see reviews on this topic (Aitken *et al.* 2013, Buffone *et al.* 2014, Gervasi *et al.* 2016, Miller *et al.* 2015, Nishigaki *et al.* 2014, Santi *et al.* 2013).

On the other hand, the concept of epididymal maturation was developed throughout the years. Changes in motility occurring to spermatozoa during epididymal transit were first described in 1913 (Tournade 1913) and some years later, epididymal transit was linked with the acquisition of fertility (Young 1931). Little was done on this topic until the 1960's when in a series of elegant manuscripts Bedford and other groups demonstrated the relevance of the epididymis and the epididymal milieu for the sperm acquisition of progressive motility and fertilizing capacity (Bedford 1963a, Bedford 1963b, Bedford 1967, Blandau *et al.* 1964, Orgebin-Crist 1967, Orgebin-Crist 1968, Orgebin-Crist 1969). Despite these earlier discoveries, contrary to capacitation, development of *in vitro* models of epididymal maturation had remained elusive. Consequently, the molecular changes regulating this process have been more difficult to investigate than those playing a role during capacitation.

The epididymis is a highly segmented organ that can be divided into four main anatomical regions: initial segment (proximal region, closest to the testis), caput (region between the initial segment and the corpus), corpus (middle portion), and cauda (distal region connected with the vas deferens). Although the initial segment is a hallmark of the epididymis in rodent species, its presence in other mammals has not been clearly described. Each of these segments displays differential expression of genes and maintains distinct luminal ion concentrations, which are essential to regulate the steps of sperm maturation. Most studies on sperm maturation have relied on the comparison between immature sperm taken from caput and mature sperm obtained from the cauda epididymidis. Some studies have also added corpus sperm as an intermediate maturational state. Alternatively, some approaches have used *in vitro* incubation of intact or demembranated caput spermatozoa in the presence of metabolites and pharmacological agents (Lindemann 1978, Mohri *et al.* 1980, Treetipsatit *et al.* 1982). Finally, more recent relevant evidence has been derived from the use of knock-out mouse models (Andersen *et al.* 2003, Huang *et al.* 2005, Joseph *et al.* 2010, Koch *et al.* 2015, Krapf *et al.* 2012, Krutskikh *et al.* 2012, Miyata *et al.* 2015, Xu *et al.* 2014). Altogether, these approaches have provided a starting point towards a working model of epididymal maturation signaling pathways. This review is written from the sperm's perspective; however, the relevance of other aspects of the epididymis should not be overlooked, and excellent supplementary reading can be found in recent reviews (Bedford 2015, Belleannee *et al.* 2012, Da Silva *et al.* 2015, Dacheux *et al.* 2014, Shum *et al.* 2011).

2. SPERM CHANGES OCCURRING DURING EPIDIDYMAL TRANSIT

2.1. Acquisition of progressive movement

Cauda epididymal sperm suspended in appropriate saline buffers display progressive motility, but testicular and caput epididymal sperm do not. However, if immature sperm are demembranated in the presence of low concentrations of the non-ionic detergent Triton X-100, and then reactivated with ATP and cAMP, they become motile (Mohri *et al.* 1980, Yeung 1984). Although the motility achieved by caput sperm in these conditions is similar in intensity to that of cauda sperm, they exhibit different flagellar bending (Vadnais *et al.* 2013,

Yeung 1984). The possibility of inducing motility in demembranated testicular and caput sperm indicates that immature sperm have functional flagellar machinery. In support of this conclusion are epididymal duct ligation experiments showing that spermatozoa retained in the caput region for an extended period become able to move progressively (Bedford 1967, Orgebin-Crist 1967). Despite these findings, it is still unclear which biochemical changes occur to the sperm during epididymal transit that render these cells capable of moving. More recent studies have started to address this problem through comparison and manipulation of signaling pathways in sperm taken from different regions of the epididymis (see Section 3 below).

2.2. Migration of the cytoplasmic droplet (CD)

Although the ability to move progressively is one of the better characterized endpoints, maturation is also associated with other morphological, biochemical and physiological changes occurring to the sperm in their transit through the epididymis (Figure 1). Morphologically, the most obvious change is the migration of the cytoplasmic droplet (CD) from the sperm neck towards the annulus, which connects the mid-piece with the principal piece (Cooper 2011). The CD comprises germ cell cytoplasm remaining from the spermiogenesis process. In recent years, high resolution helium electron-microscopic techniques have shown that spermatozoa in the epididymis may interact with the surrounding epithelium. Images obtained with this technique clearly show the interaction of the sperm CD with many vesicle-like structures that might correspond to epididymosomes (see below) (Paunescu *et al.* 2014).

Depending on the species, mature sperm may lose this organelle upon ejaculation. Whether retention of the CD in mature sperm has a functional role is not known yet (Cooper 2011). Sperm retention of CD has been associated with reduced fertility in boar and bull (reviewed in Sutovsky *et al.* 2010). Despite these results, a recent study (Yuan *et al.* 2013) presented evidence that mouse cauda epididymal sperm bearing CD were more motile that those without the organelle. Proteomic analyses of purified mouse CD revealed the presence of a large number of enzymes associated with energy metabolism (Au *et al.* 2015, Yuan *et al.* 2013). Another protein found in CDs is TEX101, a GPI-anchored protein in the plasma membrane of testicular sperm. This protein has been shown to be essential for processing of the metalloprotease ADAM3, an enzyme required for sperm transport through the utero-tubal junction (UTJ) (Yamaguchi *et al.* 2009). Consistently, knock-out male mice models lacking TEX101 are infertile, they do not have ADAM3 in their sperm plasma membrane and are deficient in sperm transport through the UTJ (Fujihara *et al.* 2013). TEX101 is released from most of the sperm flagellum by tACE before these cells leave the testis (Takayama *et al.* 2005). However, a fraction of this protein remains in detergent-resistant structures from the cytoplasmic droplet (Miranda *et al.* 2009, Sleight *et al.* 2005, Takayama *et al.* 2005, Yuan *et al.* 2013). Whether TEX101 from the CD has a role in sperm maturation is not known.

In addition to metabolism, CDs have also been proposed to regulate sperm ion homeostasis. CDs contain K^+, Cl^- and water channels (Cooper 2011) which have been proposed to play a role in regulation of sperm volume during transport through epididymal regions

characterized by milieus of differing osmolality. Intracellular Ca^{2+} is also accumulated in this organelle (Ecroyd *et al.* 2004). Considering that caput sperm do not undergo tyrosine phosphorylation (Visconti *et al.* 1995), Ecroyd et al. (2004) has hypothesized that high Ca^{2+} found in CD is responsible for maintaining low levels of tyrosine phosphorylation in immature sperm (Ecroyd *et al.* 2004). This hypothesis is consistent with findings that Ca^{2+} has a biphasic role controlling phosphorylation pathways in sperm (Navarrete *et al.* 2015). Finally, although not directly related to a functional aspect of sperm epididymal maturation the CD has been used in the last decade to develop standard electrophysiological methods to measure sperm channels in the whole-cell conformation (Kirichok *et al.* 2006). Although electrophysiological measurements of ion fluxes have been performed in mouse sperm before by patch clamping the sperm head (Espinosa *et al.* 1998), this technique is time-consuming and experimentally challenging. The discovery by Kirichok *et al.* (2006) that the CD can be used to make high resistance seals in sperm from the caput, corpus and most recently cauda, has been essential for a more sophisticated understanding of how sperm channels behave (Figueiras-Fierro *et al.* 2013, Kirichok *et al.* 2006, Lishko *et al.* 2013, Lishko *et al.* 2010, Strunker *et al.* 2011).

2.3. Changes in the sperm proteome

During epididymal transit, sperm undergo changes in their protein, lipid and sugar content. Considering that sperm are translationally silent, proteins appearing in them as a consequence of their maturation in the epididymis are thought to be synthesized by the epididymal epithelium and then incorporated to the sperm cells. The mechanisms involved in the transfer of epididymal proteins to the sperm are not fully understood. Three complementary hypotheses are: 1) absorption of soluble proteins secreted by the epithelium into the epididymal fluid (Cuasnicu *et al.* 2002); 2) transfer of exosomes released from the epididymal epithelium; these are known as epididymosomes and proposed to contain proteins, non-coding RNA and distinct set of lipids to be transferred to the sperm while they pass through different regions of the epididymis (Belleannee 2015, Sharma *et al.* 2016, Sullivan *et al.* 2013); 3) recent high resolution imaging revealed tight connections between the sperm cell and the apical epididymal epithelial surface (Paunescu *et al.* 2014). These results suggest that molecules can be transferred directly from the epididymis to sperm through these contact points by mechanisms not yet described. From these putative mechanisms of molecular transfer from the epididymal epithelium to the sperm, epididymosomes have gained increasing attention during the last decade.

Epididymosomes are proposed to be the major way of transferring proteins, and other molecules to sperm. However, the exact mechanisms by which this transfer of molecules between epididymosomes vesicles and sperm occur is poorly understood (Sullivan *et al.* 2013). In this section we give basic information about epididymosomes. For a better understanding of this topic, we encourage the reader to check excellent reviews published in recent years (Martin-DeLeon 2015, Sullivan *et al.* 2013).

The epididymal epithelium comprises different epithelial cell types including principal cells, narrow cells (found only in the initial segment), clear cells and basal cells (Hermo *et al.* 2002). Each of these cell types has a specific structure and function that varies depending on

their localization along the epididymis (for review see Breton *et al.* 2016, Hermo *et al.* 2002). In addition, communication between these different cellular types generates a luminal microenvironment appropriate for sperm maturation and storage in the epididymis (Shum *et al.* 2011). Principal cells secrete proteins to the epididymal lumen by both merocrine and apocrine mechanisms (Hermo *et al.* 2002). Merocrine secretion involves membrane fusion between Golgi-derived vesicles and the cell plasma membrane allowing the release of the vesicle contents to the extracellular space (Farkas 2015). Conversely, apocrine secretions involve loss of apical protrusions into the lumen containing cytoplasm and secretory materials that can be pre-contained in vesicles or dissolved in the cytoplasm (Farkas 2015). Apocrine secretion is the mechanism by which epididymosomes are believed to be released by principal cells into the lumen of the epididymis (Sullivan *et al.* 2007). Epididymosomes are small membranous vesicles (25–300 nm in diameter) that contain different proteins, lipids and non-coding RNAs (Belleannee *et al.* 2013, Martin-DeLeon 2015, Sullivan *et al.* 2013). Epididymosomes collected from different segments of the epididymis are highly heterogeneous in size and content (Rejraji *et al.* 2006), which may explain differential protein transfer to the sperm. Proteins transferred from epididymosomes to sperm can either be incorporated into the plasma membrane of sperm (Kirchhoff *et al.* 1996) or to intracellular structures (Eickhoff *et al.* 2001, Frenette *et al.* 2005). Nevertheless, the exact mechanism by which epididymosomes transfer proteins to sperm remains elusive (Sullivan *et al.* 2013).

A variety of proteins with diverse proposed functions have been shown to be transferred to the sperm by epididymosomes during epididymal transit, and has been recently reviewed (Sullivan *et al.* 2013). Some of these acquired proteins are involved in the development of sperm functions such as sperm motility (Eickhoff *et al.* 2004, Eickhoff *et al.* 2001, Frenette *et al.* 2003, Frenette *et al.* 2004, Murta *et al.* 2016); sperm capacitation (Krapf *et al.* 2012); the acrosome reaction (Joshi *et al.* 2013); sperm-zona pellucida interaction (Frenette *et al.* 2001) and fertilization (Caballero *et al.* 2012, Gibbs *et al.* 2010, Oh *et al.* 2005). Other acquired proteins are proposed to tag defective sperm; examples of them are: ubiquitin (Sutovsky *et al.* 2001) and epididymal sperm binding protein 1 (ELSPBP1) (D'Amours *et al.* 2012), which are hypothesized to be transferred to defective or dead sperm during epididymal transit. Moreover, it has been recently proposed that epididymosomes can also be part of a mechanism for protein removal. The expression of the protein dicarbonyl L-xylulose reductase (DCXR) is higher in cauda epididymosomes than in those from caput, and is lower in cauda sperm than in caput sperm, suggesting that this protein is removed by epididymosomes from the sperm during sperm maturation (Akintayo *et al.* 2015).

Although the mechanisms of molecule transfer to the sperm are not clear, different methods have been used to characterize proteins secreted by different regions of the epididymis and to identify which of these proteins are found in mature sperm. Recent studies with mass spectrometry have compared proteomes from immature caput sperm with those of mature cauda sperm (Table 1). In some of these studies, proteins were initially separated by using two-dimensional polyacrylamide electrophoresis (PAGE) (Dacheux *et al.* 2014, Ijiri *et al.* 2011, Ijiri *et al.* 2014). In others, mass spectrometry was done on total sperm extracts (Baker *et al.* 2012, Labas *et al.* 2015). These studies have revealed differences in the relative abundance of proteins as well as post-translational modifications occurring to sperm during

epididymal transit (Baker *et al*. 2012, Baker *et al*. 2005, Ijiri *et al*. 2011, Kameshwari *et al*. 2010). Characterization of protein profiles from spermatozoa from different epididymal regions provides grounds for the understanding of the sperm maturation process. However, in most cases, these investigations have been limited by the lack of *in vitro* models to test predictions of the role of particular proteins in sperm function. In some cases, epididymal proteins have been transferred *in vitro* to caput epididymal sperm (Caballero *et al*. 2013); however, functional implications of the *in vitro* transfer have not yet been thoroughly investigated.

In addition to changes in protein content, it has been shown that epididymal maturation is associated with post-translational protein changes, including phosphorylation and oxidation of thiol groups. It has been hypothesized that during sperm maturation oxidation of protein thiol groups gradually stabilizes sperm structures such as the nucleus and tail components, by formation of disulfide bonds (S–S) (Bedford *et al*. 1974a, Bedford *et al*. 1974b, Calvin *et al*. 1971). Most of these studies have focused on the analysis of stabilization by S-S bonds of nuclear sperm protamines (Marushige *et al*. 1975, Pellicciari *et al*. 1983, Saowaros *et al*. 1979) or sperm membrane proteins (Mercado *et al*. 1976) during epididymal transit. Later on, Shalgi et al. (1989) reported that the formation of S-S bonds (with no changes in net content of disulfide and thiols) was increased in sperm proteins from tails and heads of rat sperm during epididymal maturation (Shalgi *et al*. 1989). Consistently, more recent analyses have applied two-dimensional fluorescence difference gel electrophoresis (Ijiri *et al*. 2014) or two dimensional electrophoresis (Dias *et al*. 2014), coupled to proteomic approaches, to assess thiol changes in sperm proteins between caput and cauda epididymal sperm in both the mouse and stallion. Both works identified proteins localized in the sperm tail that increase in S-S during maturation (Dias *et al*. 2014, Ijiri *et al*. 2014). As most of the identified proteins are related to structural and cytoskeletal functions, the stabilization of proteins during sperm maturation by oxidation of thiol groups could have an important function related to the stabilization of the flagellum for the subsequent acquisition of sperm motility.

Besides proteins, epididymosomes deliver fragments of tRNAs to mammalian sperm during sperm maturation (Sharma *et al*. 2016). These tRNA fragments have been shown to affect the metabolism of the offspring and were proposed to participate in paternal epigenetic inheritance (Chen *et al*. 2016, Sharma *et al*. 2016). Interestingly, there is tRNA cleavage in the epididymis and cauda sperm present high levels of these tRNA fragments when compared with immature caput sperm (Sharma *et al*. 2016).

2.4. Changes in the sperm surface

In addition to the acquisition of new proteins, spermatozoa undergo molecular changes on their surface during epididymal maturation. These alterations include addition, removal and/or modification of external sugars and lipids of the sperm plasma membrane. Glycoproteins and polysaccharides form an interface between the sperm and its external environment is known as the glycocalyx (Schroter *et al*. 1999, Tecle *et al*. 2015). Similarly to the strategies used for identification of changes occurring in the sperm proteome, comparative studies of the glycocalyx of sperm from different epididymal regions have been

made. Identification of sugar residues can involve taking advantage of the affinity of different lectins for specific terminal saccharide residues (Cummings 1994). Lectins are macromolecules highly specific for sugar moieties that play a role in biological recognition. This property has been exploited in several cell types to identify terminal saccharides using a variety of approaches including lectin cytochemistry, lectin flow cytometry (Magargee *et al.* 1988), lectin fluorescence (Magargee *et al.* 1988), lectin blots (Srivastava *et al.* 1991), lectin agglutination (Hammerstedt *et al.* 1982, Nicolson *et al.* 1972) and lectin microarrays (Xin *et al.* 2014).

Studies in several species have shown that epididymal maturation is associated with an increase or a decrease in the accessibility of specific lectins to the sperm glycocalyx (Magargee *et al.* 1988, Nicolson *et al.* 1977, Srivastava *et al.* 1991) (see Table 2 for a summary of published data). Epididymal luminal fluid contains high concentrations of soluble glycohydrolases and glycosyltransferases, which are thought to be involved in the alteration of sperm surface glycoconjugates (Tulsiani 2006). A net-negative change in the external sperm surface charge during epididymal maturation has been shown (Bedford 1963a, Yanagimachi *et al.* 1972). It is considered that the increase in negative charge is due to changes in sugar moieties; in particular, the incorporation of negatively-charged sialic acid (Calvo *et al.* 2000, Holt 1980). Consistent with this hypothesis, higher concentrations of sialic acid are present in cauda epididymal luminal fluid when compared to caput luminal fluid (Singh *et al.* 2009, Yanagimachi *et al.* 1972).

On top of the changes observed in surface glycans, epididymal sperm maturation is associated with modifications of lipid homeostasis (see Table 3). In most species, there is an average decrease in the cholesterol:phospholipid ratio between caput and cauda sperm plasma membranes (for review on this topic see: Jones 2002, Saez *et al.* 2011). Consistent with the decrease in cholesterol:phospholipid ratio, fluorescence recovery after photobleaching (FRAP) assays have indicated an increase in sperm plasma membrane fluidity during epididymal maturation (Christova *et al.* 2004, Christova *et al.* 2002, Jones 2002). One of these studies compared caput and cauda sperm from several mammalian species for how changes in pH, osmolality and temperature affected membrane fluidity in different sperm compartments. The FRAP data showed an increase in membrane fluidity occurring as part of epididymal maturation and allowed the authors to propose a model of sperm lipid dynamic movement during this process Christova *et al.* (2004). Changes in membrane fluidity occurring during sperm maturation may be essential for later membrane events required for fertilization such as the acrosome reaction and ability to fuse with the egg oolema.

Other studies on sperm lipid content indicate an epididymal maturation-associated increase in the ratio of polyunsaturated to saturated fatty acids (Awano *et al.* 1993, Haidl *et al.* 1997, Hall *et al.* 1991, Nikolopoulou *et al.* 1985, Parks *et al.* 1985, Pyttel *et al.* 2014, Rejraji *et al.* 2006). Together with cholesterol and phospholipids, these polyunsaturated fatty acids affect sperm membrane fluidity and render these cells more susceptible to oxidative stress (Wathes *et al.* 2007). The relevance of the maturational increase in polyunsaturated fatty acids is highlighted by a recent report of *Dicer1*-conditional knock-out mice (Bjorkgren *et al.* 2012). DICER1 is an RNAase type III involved in processing non-coding RNAs, including micro

RNAs (miRNA) and small interference RNAs (siRNAs) (Li *et al*. 2011a). When this enzyme is eliminated from the epididymal initial segment and caput regions, the mice present a male sterile phenotype (Bjorkgren *et al*. 2012). Sperm from these mice depicted a decrease in long-chain polyunsaturated fatty acids, and presented increased breakage of the neck and acrosomal regions, as well as a reduced ability of cauda sperm to bind to and fertilize oocytes (Bjorkgren *et al*. 2015). Deficiencies in polyunsaturated fatty acids in *Dicer1* epididymis-specific conditional KOs suggest that changes in sperm lipid content occurring during epididymal maturation are due in part to the interaction between the sperm and epididymal epithelial secretions (Bjorkgren *et al*. 2015).

3. SIGNALING PATHWAYS ACTIVATED IN SPERM DURING THEIR EPIDIDYMAL TRANSIT

As a result of their high level of specialization, sperm are transcriptionally and translationally silent (Diez-Sanchez *et al*. 2003). Lack of sperm protein synthesis after leaving the testis suggests that regulation of epididymal maturation is controlled almost exclusively by post-translational modification of their intrinsic protein complement (Vijayaraghavan *et al*. 1996) or by acquisition of exogenous proteins (Caballero *et al*. 2012, Koch *et al*. 2015, Krapf *et al*. 2012, Martin-DeLeon 2015, Sullivan *et al*. 2007, Sullivan *et al*. 2013). One of the better studied post-translational modifications is the addition to and removal of protein phosphate groups. Reversible protein phosphorylation plays a key role as molecular switch in many cellular processes, including transduction of extracellular signals, intracellular transport, and cell cycle progression. In sperm, pharmacological or genetic loss-of-function approaches have conclusively demonstrated the role of phosphorylation cascades in the regulation of capacitation (for reviews see: Buffone *et al*. 2014, Visconti 2009). Less is known about the role and regulation of phosphorylation cascades in sperm during epididymal transit. Recent works have compared caput and cauda spermatozoa (Baker *et al*. 2012) and have shown changes in the phosphorylation status of different proteins. In most cases, further examination will be needed to understand the relevance of the phosphorylation and dephosphorylation events. However, because of the relevance of certain proteins, some of the findings might have significant implications. One of these relevant proteins found to change its phosphorylation status during epididymal transit is IZUMO1, a protein essential for sperm-egg fusion (Inoue *et al*. 2005). One relevant property of this protein is that it changes localization from the anterior head to the equatorial/postacrosomal region during the acrosome reaction (Miranda *et al*. 2009). Acrosome-reacted sperm fuses with the oocyte oolema by their equatorial segment; therefore, IZUMO1 movement is considered essential for its role in sperm-egg fusion. Using mass spectrometry to determine exact phosphorylation sites, Baker et al. recently demonstrated that IZUMO1 is extensively phosphorylated in its cytoplasmic region in cauda but not in caput epididymal sperm (Baker *et al*. 2012). Considering that caput sperm cells are unable to fuse with metaphase II-arrested oocytes, an attractive hypothesis is that IZUMO1 phosphorylation plays a role in IZUMO1 function, either in fusion or in its movement during the acrosome reaction. In this regard, sperm lacking the testis-specific serine/threonine kinase TSSK6 do not depict changes in IZUMO1 localization in acrosome-reacted sperm, they are unable to fuse with zona-pellucida-free oocytes, and the males are sterile *in vivo* and *in vitro* (Sosnik *et al*. 2009).

Although more research is needed, these data are consistent with a model in which IZUMO1 phosphorylation during epididymal maturation plays a role in the acquisition of the ability of sperm to fuse and fertilize.

Besides comparison of sperm from different epididymal regions, several studies have used more direct approaches to study signaling events involved in sperm maturation. In particular, many researchers have used the acquisition of progressive motility, which is one of the most evident changes occurring to sperm during epididymal maturation. Some of these studies used mild detergent treatment to eliminate the sperm plasma membrane while leaving intact the motility machinery. These studies demonstrated that demembranated sperm become motile when ATP and cAMP are added to the incubation media. Therefore, it can be hypothesized that a protein kinase A (PRKA aka PKA) phosphorylation cascade is involved in motility regulation. However, these experiments are silent regarding the reason why intact immature sperm remain immotile in media that allow forward motility of cauda epididymal sperm even when cAMP analogues are added to the media. In 1996, Dr. Vijayaraghavan's group showed that addition of ser/thr phosphatases inhibitors to bovine caput epididymal sperm incubated *in vitro* induced activation of progressive motility (Vijayaraghavan *et al.* 1996). Since flagellar motion patterns of caput sperm treated with the phosphatase inhibitors okadaic acid or calyculin A were similar to those from mature cauda sperm, these experiments are consistent with the hypothesis that inhibition of ser/thr phosphatases play a role in activation of sperm motility.

In eukaryotes, ser/thr phosphatases belong to three main families: PPP (Phospho Protein Phosphatases), PPM (Metallo-dependent Protein Phosphatases) and FCP (transcription initiation factor IIF-stimulated C-terminal domain Phosphatases) (Pereira *et al.* 2011). In sperm, only members of the PPP family have been detected. This family is constituted by seven enzymes (PPP1-PPP7), with a total of 14 catalytic subunits (see table 4). A recent profile of protein phosphatases from human sperm using western blot analyses indicated the presence of five of the catalytic isoforms in these cells: PPP1CB (aka PP1β), PPP1CC2 (PP1γ2), PPP2CA (PP2A), PPP4C (aka PPX), PPP6C (aka PP6) (Fardilha *et al.* 2013). PPP1CC2 has also been reported in mouse, hamster and bull sperm (Chakrabarti *et al.* 2007); and PPP3C (aka PP2B, aka calcineurin) has been found in mouse (Miyata *et al.* 2015, Navarrete *et al.* 2015), bovine (Wasco *et al.* 1984) and human sperm (Castillo Bennett *et al.* 2010). Each PPP subfamily presents a given sensitivity profile to a battery of toxin inhibitors. Therefore, it is possible to predict which type of PPP is involved, by analyzing the effective concentration of a given toxin inhibitor that blocks or enhances an enzyme function. For example, okadaic acid is two orders of magnitude more potent as inhibitor of PPP2A, PPP4 and PPP6 than it is for PPP1 and PPP5 subfamilies (Swingle *et al.* 2007). Therefore, effects observed at low nM ranges suggest a role for the former phosphatases, while those effects only observed at high nM or μM concentrations suggest involvement of PPP1C or PPP5 subfamilies. Regarding the effect of okadaic acid on motility activation of bovine caput sperm is maximal at concentrations of 5 μM suggesting the involvement of PPP1C (Vijayaraghavan *et al.* 1996). This ser/thr phosphatase subfamily has a testis-specific isoform, PPP1CC2, generated by alternative splicing (Sasaki *et al.* 1990).

It has been shown that sperm PPP1CC2 decreases activity during epididymal maturation (Vijayaraghavan *et al.* 1996). Although the molecular basis of this inactivation is not well understood, it has been hypothesized that PPP1CC2 is maintained active by indirect action of glycogen synthase kinase 3 (GSK3) (Vijayaraghavan *et al.* 1996). This hypothesis is consistent with the finding that both GSK3α and GSK3β are present in sperm and with observations of lower GSK3 activity in cauda when compared to caput sperm (Vijayaraghavan *et al.* 1996). Both GSK3 kinases regulate members of the PPP1C family by phosphorylation of the protein phosphatase inhibitor 2 (I2); once phosphorylated, I2 is no longer capable of binding to and inhibiting members of the PPP1C subfamily (Hemmings *et al.* 1982). I2 has been reported in bovine and mouse sperm (Chakrabarti *et al.* 2007).

It is not clear how GSK3 becomes inactivated during epididymal maturation, but it has been proposed that active cauda epididymal sperm PKA phosphorylates GSK3, resulting in the latter's inactivation. This possibility is consistent with experiments with anti-phospho-ser GSK3 in western blots showing that caudal sperm GSK3 has greater phosphorylation of Ser21 (for GSK3α) or Ser9 (for GSK3β) than caput GSK3 (Somanath *et al.* 2004). Phosphorylation of these sites is known to inactivate the respective GSK3, and both sites are known PKA substrates (Fang *et al.* 2000). PKA activation depends on activation of cAMP synthesis by the atypical soluble adenylyl cyclase Adcy10 (aka sAC). In this regard, cauda sperm from mice knock-out genetic models lacking sAC have reduced forward motility (Esposito *et al.* 2004, Hess *et al.* 2005).

On the other hand, it should be taken into consideration that wild type cauda epididymal sperm suspended in media that do not support sAC and PKA activation display progressive motility (Buffone *et al.* 2014). Therefore, the inactivating modification of Ser residues in GSK3 could be the result of phosphorylation by other serine/threonine protein kinases, including Akt (protein kinase B) (Cross *et al.* 1995) and/or serum glucocorticoid kinase (SGK) (Sakoda *et al.* 2003, Wyatt *et al.* 2006). Both Akt and SGK were shown to be present in mouse sperm (Vadnais *et al.* 2013). Their presence suggest that these kinases might play a role in GSK3 inactivation (Vadnais *et al.* 2013) with the consequent decrease in phosphorylated I2 resulting in PPP1CC2 inactivation (see Figure 2A and 2B for a schematic model of putative molecular pathways involved in the regulation of PPP1CC2 in caput and cauda sperm). Remarkably, knock-out mice models lacking either Akt1 or Akt2 are subfertile (Kim *et al.* 2012). It is also known in other biological systems, that Akt1 and SGK can be phosphorylated and activated by the 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Alessi *et al.* 1997, Kobayashi *et al.* 1999). Based on the presence of PDK1 protein in caput and cauda sperm, it has been hypothesized that PDK1 induces activation of Akt1 or SGK, in a molecular pathway alternative to PKA's, for the acquisition of sperm motility during epididymal maturation (Vadnais *et al.* 2013). Even though this is an attractive explanation for inactivation of GSK3 and PPP1CC2 during epididymal maturation, further investigation is required to corroborate this hypothesis.

The increased phosphorylation of GSK3 in cauda sperm can be also explained by inactivation of specific ser/thr phosphatases that have this kinase as a substrate. Phosphorylated GSK3 can be substrate of the protein phosphatase PPP2CA (Hernandez *et al.* 2010). In this regard it has been recently shown that the catalytic activity of the protein

phosphatase PPP2CA is lower in cauda than caput bovine sperm (Dudiki *et al.* 2015). However, solely PPP2CA inactivation is not sufficient to induce sperm motility in immature sperm (Dudiki *et al.* 2015).

An alternative hypothesis about GSK3 inactivation during epididymal maturation has recently arisen. Koch and collaborators proposed that Wnt signaling controls GSK3 inactivation during epididymal maturation independently of β-catenin (Koch *et al.* 2015). In other cell types, Wnt activates LRP6 coreceptors primed by the cyclin-dependent kinase 14 and cyclin Y (Ccny) (Davidson *et al.* 2009). While Ccny is ubiquitously expressed, cyclin Y-like 1 (Ccnyl1) expression is restricted to germ cells in the testis (Koch *et al.* 2015). Genetically modified male mice lacking Ccnyl1 are infertile due to structural and motility sperm defects (Koch *et al.* 2015). This brings the attention to the Wnt pathway in sperm as modulator of sperm maturation and motility. In their recent work, the authors show that Wnt is released from the epididymal epithelium in epididymosomes and sperm are responsive to Wnt via activation of LRP6 (Koch *et al.* 2015). Consistently with these results, incubation with Wnt3a increased the velocity of submotile mouse caput sperm and induced flagellar beating in immotile testicular human sperm (Koch *et al.* 2015). The model proposed is that the activation of the Wnt signaling pathway in the sperm inhibits GSK3 and promotes sperm motility by inactivation of PPP1CC2.

Inactivation of PPP1CC2 can also be explained by alternative mechanisms including binding of proteins to the catalytic subunit of phosphatases and other regulatory post-translational modifications. For example, PPP1CC2 has been shown to bind a protein homologue of the yeast protein phosphatase binding protein sds22 (Huang *et al.* 2002). Formation of the sds22-PPP1CC2 complexes renders the phosphatase inactive (Huang *et al.* 2002). Although sds22 is present in both caput and cauda sperm, sds22 is only able to bind to PPP1CC2 in cauda sperm (Mishra *et al.* 2003). The same group has reported that the protein 14-3-3ξ is expressed in sperm and binds to phosphorylated PPP1CC2 in cauda sperm. However, this pool of phosphorylated PPP1CC2 is active, and additional studies are required to identify the biological relevance of this protein binding (Huang *et al.* 2004). Furthermore, it has been shown that PPP1CC2 forms complexes with the testis-specific serine/threonine kinase 1 (TSSK1) in cauda sperm (MacLeod *et al.* 2014). Members of the TSSK family, including TSSK1, have been found in mouse sperm (Hao *et al.* 2004, Li *et al.* 2011b, Visconti *et al.* 2001). The interaction between PPP1CC2 and TSSK1 is indirect and mediated by a specific kinase substrate known as TSKS (MacLeod *et al.* 2014). Further investigations comparing caput and cauda sperm are required to evaluate if the PPP1CC2/TSSK1 interaction affects the phosphatase activity or participates in the acquisition of motility during sperm maturation

Recently, another ser/thr phosphatase has been proposed to play a role in epididymal maturation. Genetically modified mice lacking the Ca^{2+}- calmodulin-dependent ser/thr phosphatase catalytic subunit PPP3CC (aka calcineurin) are infertile due to failure in penetration of the zona pellucida (Miyata *et al.* 2015). Computer-assisted sperm analysis experiments showed that although the motility of cauda sperm of these knock-out animals is comparable to wild type, null mice present impaired hyperactivated motility due to midpiece rigidness (Miyata *et al.* 2015). Consistently, administration of the calcineurin inhibitors

cyclosporine A or FK506 to wild type mice for at least 5 days induced infertility due to lack of sperm midpiece flexibility and impaired hyperactivation (Miyata *et al.* 2015). Taking in account that the maturational process in the mouse epididymis takes 10 days, these results suggest that sperm calcineurin activity is required during sperm maturation for acquisition of proper midpiece flexibility and development of hyperactivation motility in conditions that support sperm capacitation.

Finally, the role of newly acquired enzymes synthesized by the epididymal epithelium in the regulation of sperm signaling pathways during epididymal maturation should not be ruled out. Recent work from our laboratory presented evidence that cSrc is enriched in epididymosomes and is incorporated into mouse sperm during transit through the epididymis (Krapf *et al.* 2012). This study also showed that cSrc KO mice display deficiencies in cauda epididymal development. Moreover, knock-out male mice of cSrc are sterile (Schwartzberg *et al.* 1997) and cauda sperm of cSrc-null mice have impaired motility (Krapf *et al.* 2010). Altogether these data suggest a role of cSrc in epididymal sperm maturation. Whether cSrc incorporation plays a role in PPP1CC2 down-regulation is not known. However, it is well-established that cSrc can phosphorylate PPP2CA, another ser/thr phosphatase, on tyrosine residues present in this phosphatase C-terminal domain. As mentioned above, PPP1CC2 is a sperm-specific splicing variant of the *Ppp1cc* gene that differs from PPP1CC1 only in the last 14 amino acids of the C-terminus (Fig. 2C). The sperm-specific C-terminal domain contains two tyrosine residues, indicative of a potential differential site of phosphorylation by cSrc or other protein tyrosine kinases. It can be hypothesized that, as for PPP2CA, phosphorylation of these residues changes PPP1CC2 activity. More research will be required to test this possibility.

4. CONCLUDING REMARKS

As stated in the introduction, two extra-testicular maturational processes are required for sperm to gain fertilizing capacity: epididymal maturation and capacitation. Since these are sequential processes, deficient epididymal maturation would prevent sperm to capacitate later on, either in the female tract or *in vitro*. For example, among the functional changes occurring during epididymal maturation, one of the better studied is the acquisition of progressive sperm motility (Yeung *et al.* 2002) which is a necessary step for the ability to undergo hyperactivation when exposed to capacitating conditions. Also related to the capacitation process, as part of their transit through the epididymis, sperm acquire the potential: 1) to undergo an increase in protein tyrosine phosphorylation (Visconti *et al.* 1995); 2) to bind to the zona pellucida (Busso et al. 2007) ; 3) to undergo the acrosome reaction (Burkin *et al.* 2000, Lakoski *et al.* 1988, Sirivaidyapong *et al.* 2001, Williams *et al.* 1991, Yeung *et al.* 1996); 4) to become able to fuse with the oolema (Da Ros *et al.* 2015, Harayama *et al.* 1993a, Harayama *et al.* 1993b, Moore *et al.* 1983); and 5) to fertilize the oocyte. All these events are hallmarks of capacitation which physiologically occur in the female tract. Therefore, epididymal maturation can be defined as all biochemical and physiological changes occurring during epididymal transit that render the sperm competent to undergo capacitation.

An understanding of epididymal sperm maturation at the molecular level has encountered numerous difficulties. First, the absence of methods to fully mature caput sperm *in vitro* has prevented researchers the use of gain- and loss-of-function approaches to analyze the necessity and sufficiency of a given signaling pathway in sperm maturation. Second, because sperm are transcriptionally and translationally inactive, genetic experiments are difficult. Although knock-out mouse models have been useful in the study of sperm signaling pathways, because sperm derive from testicular germ cells which include spermatogonia, spermatocytes and spermatids, it is rather difficult to assign a role of a particular sperm protein to different processes required for fully functional spermatozoa (e.g. spermiogenesis, epididymal maturation, and capacitation). Despite the problems inherent in experimental conditions, it is expected that modern gene editing approaches (Wijshake *et al*. 2014) combined with new state-of-the-art imaging techniques, such as superresolution (Chung *et al*. 2014), or helium ion microscopy (Paunescu *et al*. 2014), will facilitate the study of sperm maturational events at the molecular level.

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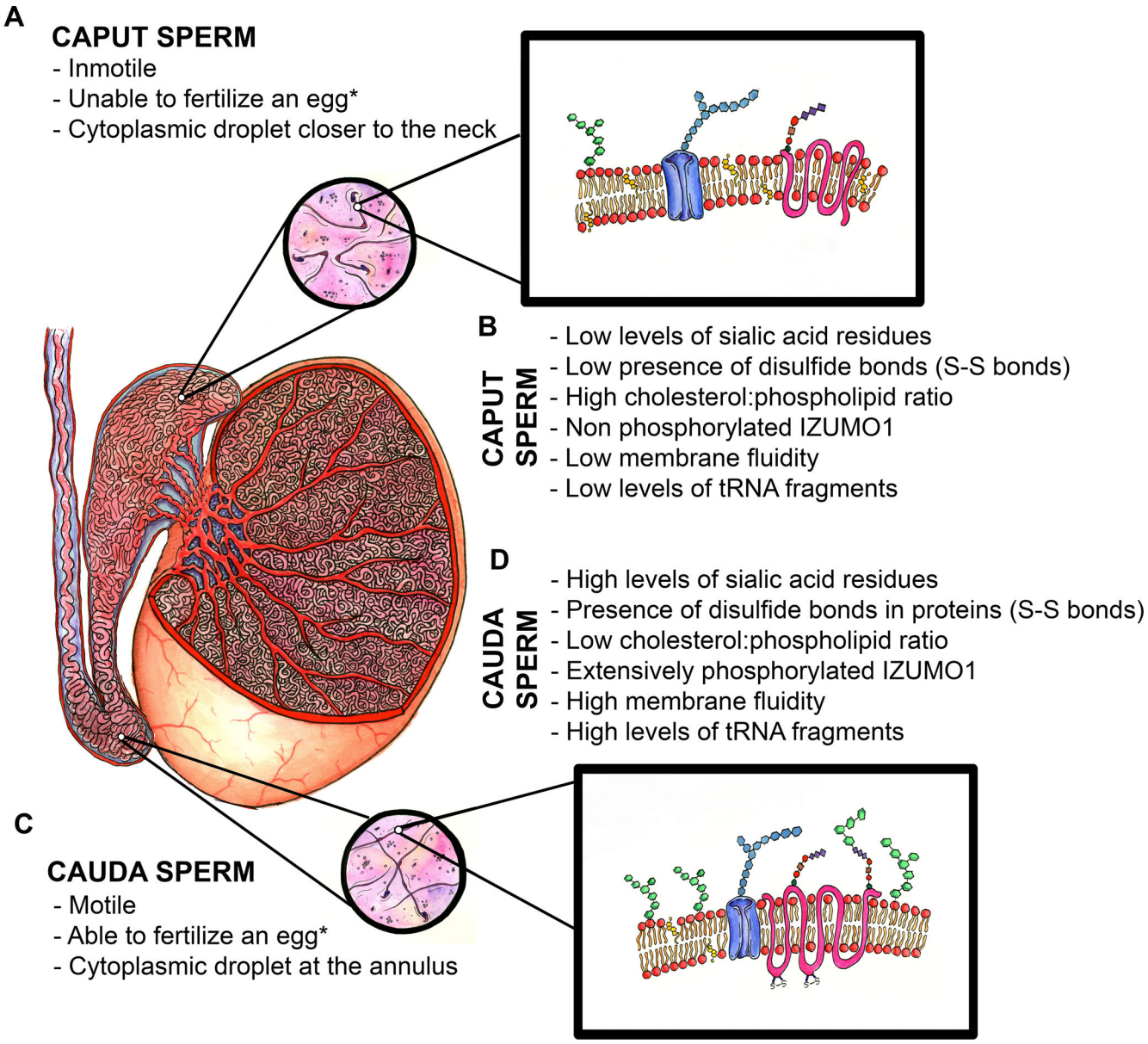
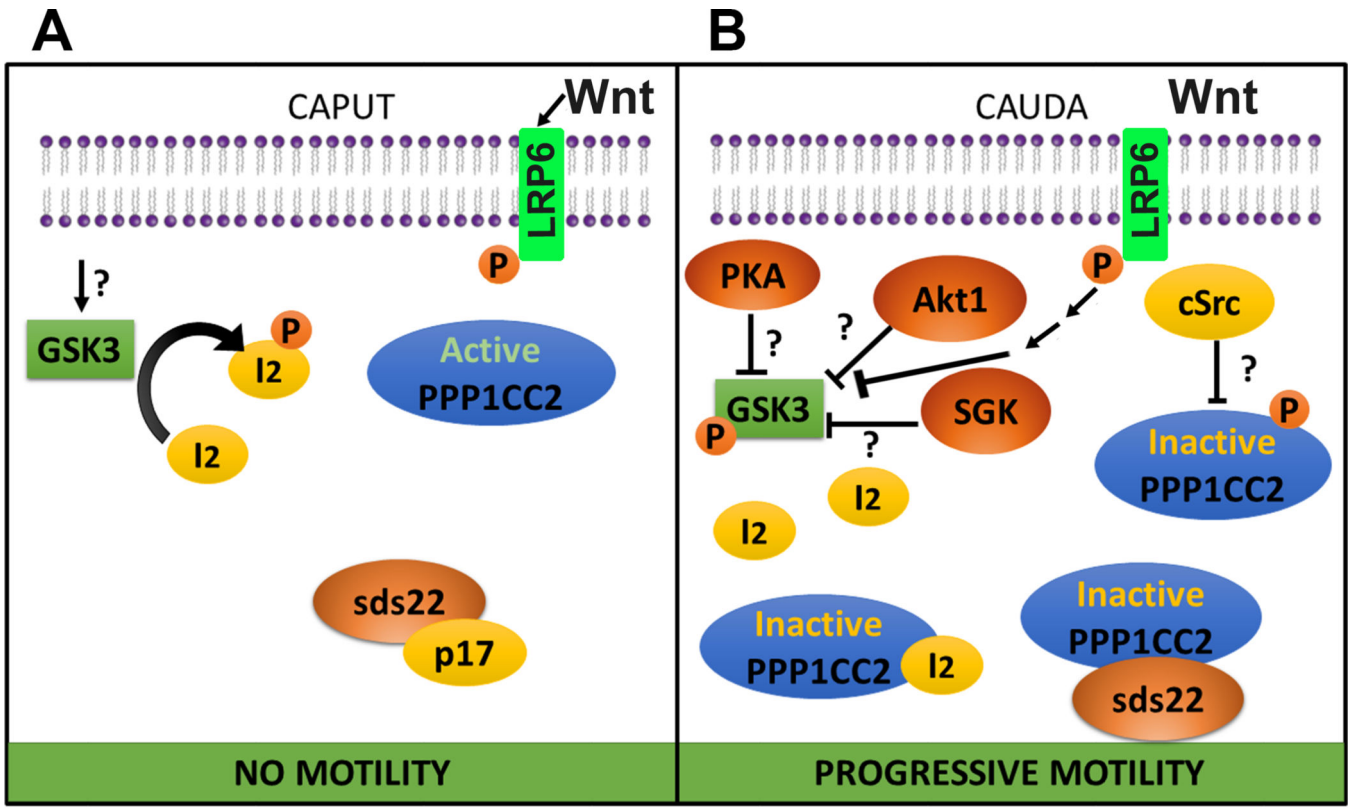


Figure 1.

Schematic representation of a mammalian testis, epididymis and sperm.* after incubation in conditions that support sperm capacitation. A) Principal morphological and functional characteristics of immature caput sperm. B) Molecular characteristics of immature caput sperm. C) Principal morphological and functional characteristics of mature cauda sperm. D) Molecular characteristics of mature cauda sperm.



C

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PPP1CC1          KKKPNATRPVTPPR---GMITKQAKK---------- 323
PPP1CC2          KKKPNATRPVTPPRVASGLNPSIQKASNYRNNTVLYE 337
                 **************    *: ..   *
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Figure 2.

Model of putative molecular pathways involved in the regulation of PPP1CC2 and progressive sperm motility during epididymal maturation. A) CAPUT: glycogen synthase kinase 3 (GSK3) is active and phosphorylates protein I2. Once phosphorylated, I2 is not able to bind to and inactivate the ser/thr phosphatase PPP1CC2. The PPP1CC2 inhibitory protein sds22 has been shown to be complexed with p17 and cannot bind and inhibit the phosphatase. cSrc kinase is absent from caput sperm, PPP1CC2 is active and caput sperm lack motility. Wnt from epididymosomes activates the primed receptors LRP6. B) CAUDA: GSK3 is phosphorylated on ser residues, and rendered inactive by an unidentified serine/threonine kinase. Among the proposed kinases involved in GSK3 phosphorylation and inactivation are protein kinase A (PKA), RACalpha protein kinase 1 (Akt1) and serum and glucocorticoid induced kinase (SGK). All these kinases have been described in sperm. Active Wnt signaling inhibits GSK3. Due to GSK3 inactivation, protein I2 can no longer undergo phosphorylation and consequently binds to PPP1CC2, inactivating its catalytic

activity. On the other hand, sds22 has been found complexed to PPP1CC2 in cauda sperm and can explain PPP1CC2 inhibition. cSrc tyrosine kinase, not present in caput sperm, is incorporated into sperm during epididymal maturation. Because PPP1CC2 is a testis-specific splicing variant containing two unique tyrosine residues in its C-terminal domain, phosphorylation of these residues by cSrc or another tyrosine kinase would also explain PPP1CC2 inactivation. Together, inactivation of PPP1CC2 leads to the ability of the sperm cell to move progressively when exposed to an appropriate medium. C) ClustalW2 sequence alignment of the human protein phosphatases PPP1CC1 and PPP1CC2. These two proteins are products of an alternative splicing and differ only in a small region of the C-terminal, as shown in the Figure. The two unique tyrosine residues (Y) in PPP1CC2 are indicated in red.

Table 1
Comparative proteomic during sperm maturation

Summary of works that present detailed proteomic results comparing caput and cauda sperm of different species.

Identification of abundance of proteins			
Samples analyzed	**Species**	**Technique used**	**Reference**
Cytoplasmic droplets	Mouse	Isolation of cytoplasmic droplets by sucrose gradient followed by MALDI-TOF/TOF MS.	Yuan *et al.* 2013
	Rat	Isolation of cytoplasmic droplets	Au *et al.* 2015
Whole sperm	Boar	Intact cell, detergent-soluble, and detergent-insoluble protein purification. MALDI-TOF MS.	Labas *et al.* 2015
	Hamster	MALDI-MS/MS.	Kameshwari *et al.* 2010
	Mouse	Two-dimensional Fluorescence difference gel electrophoresis (2D–DIGE) followed by MALDI-TOF/TOF MS.	Ijiri *et al.* 2011
Sperm surface proteins	Boar	Labeling and purification of surface protein with sulfo-NHSS-SS-biotin. 1D or 2D electrophoresis followed by MS.	Belleannee *et al.* 2011
Epididymosomes	Human	1D electrophoresis followed by LC-QToF MS.	Thimon *et al.* 2008
	Bull	1D electrophoresis followed by ES-MS/MS	Girouard *et al.* 2011
Identification of post-translational modifications of proteins			
Samples analyzed	**Species**	**Technique used**	**Reference**
Phosphorylation	Rat	TiO_2 enrichment of phosphopeptides followed by LC-MS.	Baker *et al.* 2012
Non-identified posttranslational modification	Rat	2D–DIGE followed by MALDI-TOF MS.	Baker *et al.* 2005
Thiol status of proteins	Mouse	2D–DIGE MALDI-TOF/TOF MS.	Ijiri *et al.* 2014

Table 2

Changes in the composition of sperm glycocalyx during epididymal maturation in different species

Data presented has been reviewed from studies that compared sugar residues present in sperm recovered from caput and cauda epididymis. Arrows are indicative of an increase (↑) or decrease (↓) in the composition of sugars during sperm maturation. ND denotes that published data is not available. When changes in specific proteins were reported, the protein(s) molecular weight (in KDa) is specified.

SPECIES		Bull	Goat	Monkey	Mouse	Rabbit	Ram	Rat	Stallion
Lectin	***Sugar residue***	Comparison of caput vs cauda sperm.							
Con A	D-mannose D-glucose	↓	=	↑	↓[a]	=[a]	=	↓[b]	ND
SBA	D-N-acetyl-galactosamine	=	=	ND	ND	ND	↓	ND	ND
RCA	D-galactose and L-arabinose	=	ND	ND	ND	↓[a]	↓	ND	↓
PNA	D-galactose	↑ (acrosome)	=	ND	ND	ND	↓	↓ (58 KDa)[a]	↓
WGA	Sialic acid and N-acetyl-D-glucosamine	↓	↑(acrosome)	↓	↑[b]	↓ a ↑(acrosome)[b]	↑	↑ (86, 47 and 37 KDa)[a] ↑ (acrosome)[c]	↑
DBA	D-N-acetyl-galactosamine	↓	↑	ND	ND	ND	ND	=	ND
UEA	α-L-fucose	=	↑	ND	ND	ND	ND	=	↑
PSA	D-mannose	ND	ND	ND	ND	ND	ND	↓ (118 KDa)[a] ↓ (58 KDa)[a]	ND
References		Arya *et al.* 1985	Bains *et al.* 1993	Fain-Maurel *et al.* 1984	a: Schlegel *et al.* 1986 b: Kumar *et al.* 1990	a: Nicolson *et al.* 1977 b: Kumar et al. 1990	Magargee *et al.* 1988	a: Srivastava *et al.* 1991 b: Olson *et al.* 1981 c: Kumar et al. 1990	Retamal *et al.* 2000

References in the literature are indicated by letters (a), (b) or (c). Lectin abbreviations: Con A, concanavalin A; SBA, soybean agglutinin; RCA, *Ricinus communis* agglutinin; PNA: peanut agglutinin; WGA, wheat germ agglutinin; DBA, *Dolichos biflorus* agglutinin; UEA, *Ulex europaeus* agglutinin; PSA, *Pisum sativum* agglutinin.

Table 3

Changes in sperm lipid content during epididymal maturation in different species

Data reviewed from studies that compared lipids of whole cells or membrane extracts of sperm recovered from caput and cauda epididymis. Arrows are indicative of an increase (↑) or decrease (↓) in the percentage of lipid during sperm maturation; ND is indicative of no published data.

SPECIES	Boar	Goat	Hamster	Human	Mouse	Ram	Rat
Phospholipids out of Total phospholipids							
Phosphatidylcholine (PC)	↑	↓	=	↑	↑*a* ↓*b*	↑	↓
Posphatidylethanolamine (PE)	↓	↓	=	↓	↓*b*	↓	↓
Phosphatidylinositol (PI)	↓	ND	=	ND	↑*b*	↓	=
Phosphatidylserine (PS)	↓	ND	=	↓	=*b*	↓	↓
Sphyngomyelin (SM)	↑	↓	ND	↓	↑*a,b*	ND	↑
Sterols							
Cholesterol	↓	↑	↓	ND	↓*b*	↓	↓
Desmosterol	↑	↓	↑	ND	ND	↓	↑*b*
Membrane Fluidity	ND	ND	↑	↑	↑*b*	↑*b*	↑
References	Nikolopoulou *et al.* 1985	Rana *et al.* 1991	Awano *et al.* 1993, Awano *et al.* 1989	Haidl *et al.* 1997	a: Pyttel *et al.* 2014 b: Rejraji *et al.* 2006	a: Parks *et al.* 1985 b: Christova *et al.* 2004	a: Aveldano *et al.* 1992 b: Lindenthal *et al.* 2001

Letters (a) and (b) indicate the references.

Table 4
Ser/Thr-specific protein phosphatase families

This table summarizes the catalytic subunit isoforms of human PPPs. PPPs form multi-subunit proteins; nevertheless, the regulatory and scaffolding subunits are omitted. For a more comprehensive analysis of PPPs and their respective subunits, several manuscripts and reviews have been published in the last years (Pereira *et al*. 2011, Zhang *et al*. 2013, Silva *et al*. 2014).

Serine/Threonine-specific Protein Phosphatases				
Family	**Characteristics**	**Phosphatase**	**Catalytic Isoforms**	**Alternative name**
PhosphoProtein Phosphatases (PPPs)	Multi-subunit proteins.	PPP1	PPP1CA PPP1CB PPP1CC1 PPP1CC2	PP1α PP1β PP1γ1 PP1γ2
		PPP2	PPP2CA PPP2CB	PP2A
		PPP3	PPP3CA PPP3CB PPP3CC	PP2B; calcineurin
		PPP4	PPP4C	PPX
		PPP5	PPP5C	PP5
		PPP6	PPP6C	PP6
		PPP7	PPP7CA PPP7CB	PPEF1 PPEF2
Metallo-dependent Protein Phosphatases (PPMs)	Mg^{2+}- or Mn^{2+} dependent enzymes. Single-subunit proteins.	PPM1	PPM1A PPM1B PPM1D	PP2C
Transcription initiation factor IIF-stimulated C-terminal domain Phosphatases (FCPs)	Require Mg^{2+} in the active site for catalysis. Single-subunit proteins.	Small CTD phosphatases (SCPs)	SCP1 SCP2 SCP3	NLI-interacting factor 3 NLI-interacting factor 2 NLI-interacting factor 1
		CTD phosphatases	FCP1	TFIIF-associating CTD phosphatase