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Invited Review

Curating fertility—proteomic remodelling of sperm during epididymal transit

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In brief

The epididymis plays an essential role in promoting the functional maturation of mammalian spermatozoa. This review synthesises the latest proteomic insights into epididymal physiology, highlighting the mechanistic basis of its central role in sperm maturation and key knowledge gaps that remain. Additionally, we introduce *ShinyEpididymis*, a new resource for exploring proteins expressed across this important organ.

Abstract

The epididymis is a highly specialized organ essential for promoting the post-testicular functional maturation of spermatozoa, a process underpinning male fertility. This review examines the latest proteomics advances that have been used to unravel the complex molecular landscape of the epididymis, revealing the dynamic protein networks that shape sperm function beyond their genomic and transcriptomic blueprints. Here, we highlight how high-resolution mass spectrometry has helped to map the proteomic signatures of epididymal tissue, luminal extracellular vesicles (epididymosomes), and spermatozoa at different maturation stages, pinpointing key regulators of motility, capacitation, fertilization competence, and immune regulation. However, critical knowledge gaps remain, including deep protein characterization of the cytoplasmic droplet, epididymal fluid, and relatively underexplored anatomical tissue segments such as the corpus and cauda epididymis. We discuss how integrating global proteomic insights with complementary omics, single-cell proteomics, and advanced imaging is poised to reveal the spatial and temporal refinement of the sperm proteome, providing insights into how its disruption may contribute to idiopathic infertility. To promote data accessibility and accelerate discovery in epididymal biology, we introduce *ShinyEpididymis* (https://reproproteomics.shinyapps.io/ShinyEpididymis/), an interactive, web-based resource integrating

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publicly available proteomic datasets from spermatozoa, epididymosomes, and epididymal tissue. This platform enables researchers to rapidly query proteins of interest, explore spatial patterns of expression, and identify potential biomarkers or therapeutic targets. By consolidating current knowledge and defining future priorities, this review positions proteomics at the forefront of understanding epididymal biology, emphasising its clinical relevance and untapped potential for diagnosing and treating male infertility.

Keywords epididymis, fertility, male reproductive tract, proteomics, sperm, sperm proteome

Introduction

The journey of spermatozoa from their genesis in the testis to their functional competence for fertilization is a complex and highly orchestrated process (Hermo et al., 2010a, 2010b, Nixon et al., 2020). While spermatogenesis within the testis produces a highly specialized, morphologically mature sperm cell, these gametes are functionally immature upon their exit, lacking the biochemical toolkit for progressive motility or the capacity for productive interactions with an oocyte (Nixon et al., 2020). Moreover, the balance of evidence favours the scenario in which these cells leave the testis locked in a state of transcriptional and translational quiescence. This quiescence is imposed by factors such as extensive chromatin condensation and the loss of key organelles including cytoplasmic ribosomes. Notwithstanding this wide-held consensus, the results of a handful of more recent studies have led to the proposal that mature sperm may retain limited, context-dependent translational activity, particularly during capacitation (Gur & Breitbart, 2008, Lymbery et al., 2025, Ren et al., 2017). In particular, the work of Breitbart and colleagues has implicated mitochondrial-type ribosomes in the translation of nuclear-encoded mRNAs via their illustration that spermatozoa can incorporate labelled amino acids into polypeptides during capacitation (Gur & Breitbart, 2006, 2008).Furthermore, the demonstration that inhibition of this process impairs sperm motility, actin polymerization, the acrosome reaction, and fertilization rates, suggests a functional role for de novo protein translation in sperm physiology (Gur & Breitbart, 2006). However, the extent, regulation, and physiological relevance of such activity remains highly debated (Lymbery et al., 2025). As a consequence, all subsequent molecular changes must be driven exogenously (Hermo et al., 2010b). This fundamental functional immaturity underscores the critical role of the epididymis, a highly specialized convoluted ductal system through which sperm must navigate to acquire their functional competence (Nixon et al., 2020, Zhou et al., 2019). The epididymal lumen harbours a dynamic microenvironment, which is created by the lining pseudostratified epithelial cells via the secretion of a diverse array of molecules, including proteins (Nixon et al., 2019), lipids (Girouard et al., 2011), and RNAs (Figure 1) (Trigg et al., 2019). Although several mechanisms of secretion exist, many of the molecules destined for delivery to the maturing sperm population are encapsulated in extracellular vesicles known as epididymosomes (Caballero et al., 2013, Zhou et al., 2018). It is important to note that while epididymosomes deliver a select subset of functionally important proteins to maturing spermatozoa, these additions represent only a minor component of the overall proteome. In parallel, maturing sperm undergo extensive proteome reduction, driven by removal of cytoplasmic droplet (CD)-associated proteins and degradation of residual testis-derived cargo (Skerrett-Byrne et al., 2022). Thus, protein acquisition and protein loss occur concurrently, but the latter is quantitatively far greater, resulting in the net >50% reduction in proteome complexity observed during epididymal transit.

These active regulatory mechanisms in the epididymis drive proteomic changes essential for sperm fertilizing competence. Accordingly, the epididymis epithelium is not uniform, but rather exhibits remarkable segment-specific variations in cellular architecture (Lagarrigue et al., 2020, Rinaldi et al., 2020). This creates a precisely tailored intraluminal milieu that progressively shapes the core sperm proteome and modifies the activity of these proteins via post-translational modifications (PTMs) (Skerrett-Byrne et al., 2022, Zhang et al., 2024b). A deeper understanding of epididymal regulation of sperm function has implications that extend beyond fertilization success to also encompass potential epigenetic implications for offspring (Skerrett-Byrne et al., 2025c). Indeed, emerging evidence indicates that the epididymis acts as a sensory organ, capable of responding to paternal stress or environmental cues and shaping the epigenetic cargo of sperm via the delivery of small non-coding RNAs (sncRNAs). Such epigenetic changes can, in turn, influence early embryo development and even the long-term health of the offspring (Tomar et al., 2024, Trigg et al., 2024, Trigg et al., 2021a). These transgenerational implications arising from a change in the microenvironment of the male reproductive tract extend the significance of epididymal research to broader developmental and evolutionary biology perspectives (Spadafora, 2023).

Comparative proteomic studies across various therian mammalian and reptilian species have revealed a conserved proteomic core in functional, mature sperm cells, highlighting fundamental reproductive mechanisms that are essential across diverse taxa (Pini et al., 2025). Decoding the proteomic basis of epididymal maturation is therefore not only a fundamental scientific endeavour but also holds significant translational promise for informing rational strategies for assisted reproduction in livestock (Jitjumnong et al., 2025) and endangered species (Hildebrandt & Holtze, 2024, Kunkel et al., 2025, Skerrett-Byrne et al., 2021). Moreover, proteomics serves as an indispensable clinical tool for identifying novel diagnostic markers for male infertility, particularly for cases with unknown aetiology (Skerrett-Byrne et al., 2025b, Tüttelmann et al., 2018), uncovering new non-hormonal contraceptive targets and addressing critical unmet needs in reproductive health (Nickels & Yan, 2023), and may even assist in assessing overall male health (Lyons et al., 2025).

In this review we synthesise current knowledge of how proteomic approaches have advanced our understanding of the sperm proteome and PTM remodelling (concurrent acquisition and loss) during epididymal transit. We use this information to explore how segment-specific proteomic profiles of the epididymal epithelium may improve our understanding of secretory function and immune privilege and examine the crucial roles of

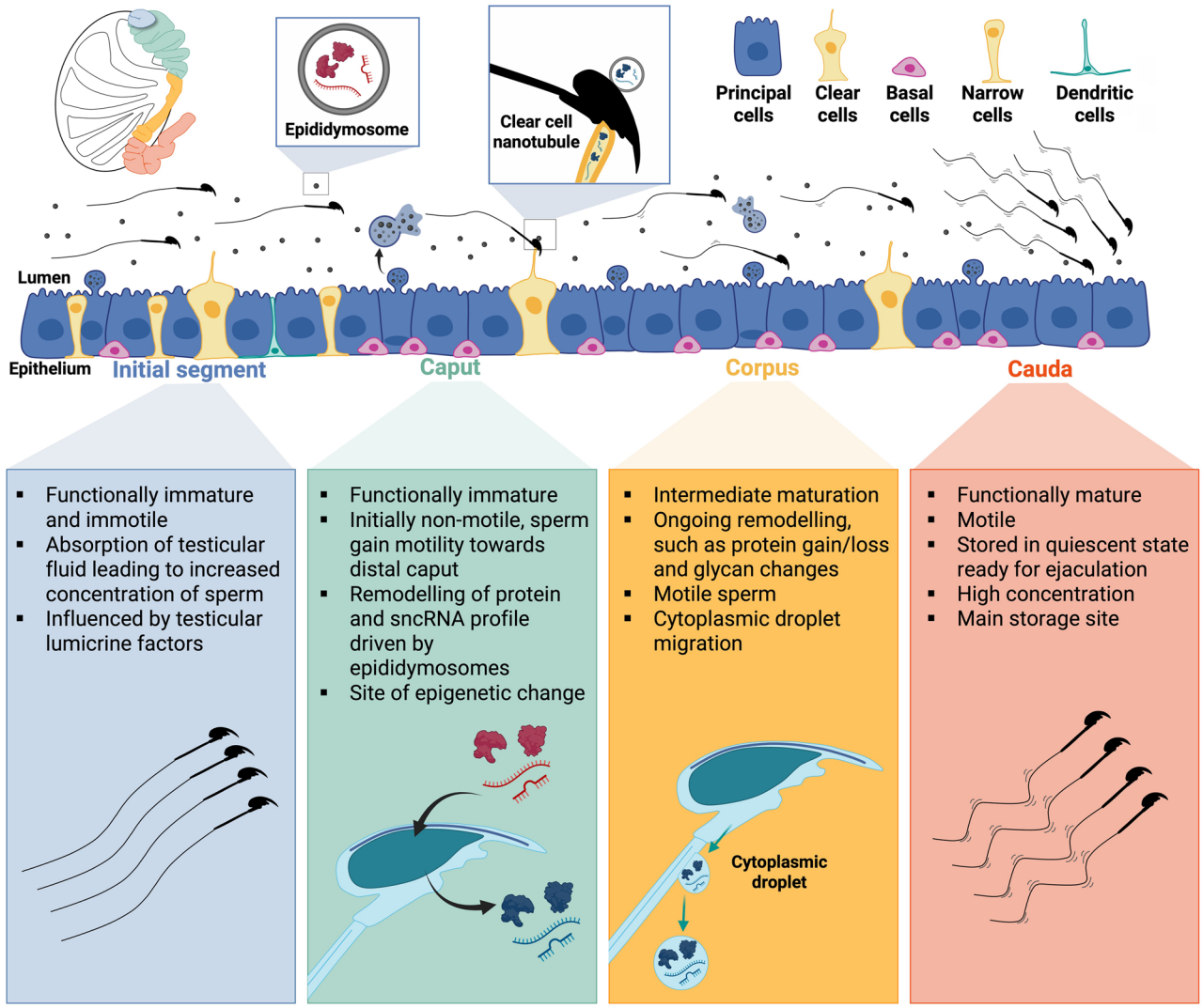

Figure 1 Segment-specific organization and functions of the mammalian epididymis. The epididymis is subdivided into four major segments: initial segment (IS), caput (head), corpus (body), and cauda (tail), each contributing uniquely to sperm maturation and storage. The epididymal epithelium primarily consists of three main cell types, principal cells, clear cells, and basal cells. Additional cell types have also been described and include narrow cells, a morphologically distinct subset of the clear cell lineage found in the proximal epididymis, apical cells, and immunological cells. Initial segment (blue): entry point for immature sperm exiting the testis. A metabolically active segment where testicular fluid components are removed and regulated ion exchange shapes the luminal pH and osmolarity. Caput (green): here, principal cells release epididymosomes that deliver proteins, lipids, and RNAs essential for initiating sperm maturation and shaping the epigenome. Corpus (yellow): transition zone, where sperm undergo fine-tuning of their proteome and surface architecture through proteolytic processing, glycan remodelling, and post-translational modifications. Additionally, progressive shedding of the cytoplasmic droplet begins to emerge. Cauda (orange): terminal segment, serving as a reservoir for fully mature, motility-competent sperm. Here, sperm have shed the cytoplasmic droplet, have a primed functional phosphoproteome, and are maintained in a quiescent state until ejaculation. Aspects of this figure were created with BioRender.com.

epididymosomes as nano-couriers responsible for mediating sperm maturation and soma-germline transfer of epigenetic information. Building on transcriptomic atlases that have provided a comprehensive view of gene expression across the epididymis (Rinaldi et al., 2020, Robertson et al., 2020, Shi et al., 2021), such proteomic studies enable direct assessment of protein abundance and modification, advancing the functional insight of epididymal biology. Over the past two decades, reproduction-focussed proteomics has expanded markedly, with the use of these tools to study spermatozoa and epididymal function increasing ~6.4-fold and 4.3-fold, respectively (Figure 2A). Yet, only a relatively small portion of studies have made their mass spectrometry (MS) datasets publicly available, thus limiting their ability to be mined by others in the field. Such limitations highlight the importance of mandatory data deposition at the time of publication to ensure transparency, reproducibility, and maximal research value (Figure 2B). We also provide perspectives on emerging proteomic technologies that are poised to help resolve long-standing questions in the field of epididymal research. Finally, to enhance accessibility and foster idea generation, we have compiled publicly available proteomic datasets spanning epididymal sperm, epithelium, and epididymosomes into an interactive resource, *ShinyEpididymis.* This will serve as an open access web application allowing rapid querying of proteins, or genes, of interest, facilitating visualization of their localization and relative expression across these key compartments.

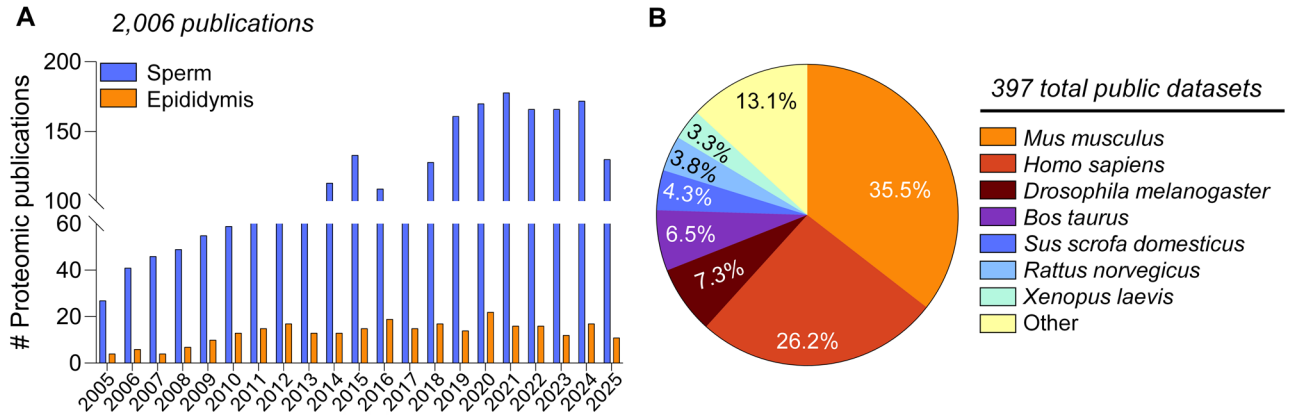

Figure 2. (A) Timeline of publication based on PubMed search using "proteomics" with keywords "sperm" OR "epididymis." (B) Pie chart illustrating the distribution of publicly available (PRIDE repository) sperm proteomic datasets across species. "Other" category (yellow) represents 11 species.

Epididymal sperm proteome—upgrades and offloads

Global sperm proteome remodelling: reduction, acquisition, and modification

Contrary to long-standing dogma, remodelling of spermatozoa during epididymal transit from caput (immature) to cauda (mature) is accompanied by substantial proteomic streamlining rather than an accumulation of proteins. In a recent study conducted by our research group using a mouse model, 6,045 proteins were identified in caput epididymal spermatozoa compared to 2,924 in their mature cauda epididymal counterparts (Skerrett-Byrne et al., 2022), a reduction of >50% in the sperm proteome. In agreement, Skerget *et al.*also report a proportional reduction (42%) in sperm protein complexity coinciding with their transit from the caput to the cauda epididymis. Notably, the sperm proteome was heavily enriched for enzymatic proteins (66.5%), including kinases, phosphatases, and peptidases, whose proportions remained largely stable during epidydimal transit. While multiple protein classes undergo extensive remodelling during epididymal transit, we observed that transcription regulators show a disproportionate reduction in abundance (from 16% to 6.8% in populations of caput versus cauda epididymal sperm). This reflects the progressive elimination of residual testis-associated regulatory machinery as sperm mature. In contrast, although kinases and phosphatases retain similar overall proportional representation across regions, many individual members of these classes still undergo significant quantitative or phosphorylation changes (Skerrett-Byrne et al., 2022, Skerrett-Byrne et al., 2025a). Thus, transcription regulators are one of several protein groups affected during maturation but are not the only class undergoing remodelling. For core proteins that were retained throughout the caput to cauda transition (2,660), substantial remodelling was also observed, with 889 proteins significantly altered in terms of their abundance; the overwhelming majority of which were underrepresented in cauda compared to caput epididymal sperm (832 or 94%).

There are several theories to account for the substantial remodelling of the epididymal sperm proteome. One proposed mechanism for protein removal is shedding of the CD, a remnant of cell cytoplasm residing at the sperm neck after having been stripped from the developing germ cell during testicular spermiogenesis (Hermo et al., 2019). The CD subsequently migrates along the length of the flagellum and is eventually shed via mechanical shearing or enzymatic activities (Figure 1) (Moore et al., 2010). The theory of its shedding is supported by findings associated with the characterization of arachidonate 15-lipoxygenase (ALOX15), an enzyme whose expression is predominately localized to CDs of caput epididymal sperm before becoming significantly depleted in cauda epididymal sperm, as supported by immunoblotting and proteomic analysis (Skerrett-Byrne et al., 2022). Interestingly, *Alox15* knockout mice exhibit impaired fertility associated with significantly increased retention of CDs, implicating a potential regulatory role for ALOX15 in directing organelle degradation during epididymal sperm maturation (Moore et al., 2010). CDs exhibit a defined protein cargo, with gel-based proteomic analysis coupled with MS identifying 105 distinct proteins, with notable enrichment in enzymes involved in energy metabolism (Yuan et al., 2013). Intriguingly our own data has shown loss of proteins for oxidative phosphorylation, cholesterol biosynthesis, and fatty acid β-oxidation in maturing spermatozoa (Skerrett-Byrne et al., 2022), further indicating that the controlled reduction of metabolic enzyme reserves may be an integral component of epidydimal sperm maturation.

Another route of protein refinement may be a consequence of surface adherence: i.e., proteins that exist in high concentration in luminal fluid at the proximal segment remain transiently bound to the surface of the spermatozoon. During epididymal transit, such proteins could be shed by way of proteolytic cleavage. For example, the epididymis-specific trypsin-like serine protease ovochymase 2 (OVCH2) is required for processing of the a disintegrin and metallopeptidase domain 3 protein (ADAM3) on the sperm surface, and in its absence sperm failed to obtain functional competence, resulting in sterility (Kiyozumi et al., 2020). Interestingly, in a recent analysis of OVCH2$^{-/-}$ mice it was shown that the proteome of cauda epididymal sperm was altered (including the accumulation of 30 proteins that would otherwise be downregulated during epididymal transit) and accordingly, the functional maturity of sperm was compromised (Kent et al., 2025). These findings suggest that proteolytic enzymes, such as OVCH2, could contribute to the reduction in proteome complexity observed in sperm as they transit through the epididymal lumen.

Although dwarfed by the volume of protein loss, the enrichment of specific proteins is also key to promoting sperm maturation, a fact illustrated by findings such as the acquisition of proteins related to motility [e.g., adenylate cyclase type 10 and cilia- and flagella-associated proteins 44 and 221 (Esposito et al., 2004, Lee et al., 2008, Schmid et al., 2007, Tang et al., 2017)], capacitation [calcium-binding tyrosine phosphorylation-regulated protein (Naaby-Hansen et al., 2002)], and zona pellucida binding [β-defensin 22 (Tollner et al., 2004, Yudin et al., 2008)]. Although several of these proteins are present in sperm as they enter the epididymis (Jaiswal & Conti, 2001, Li et al., 2010, Noda et al., 2013), their abundance, membrane association, or functional localization increases during epididymal transit. In the absence of active translation in spermatozoa, small membrane-bound extracellular vesicles released from the epididymal epithelium lining, referred to as epididymosomes, have been strongly hypothesised to mediate the acquisition of at least a portion of these proteins (Figure 1) (Nixon et al., 2019, Sullivan, 2015). The proteomic cargo of epididymosomes and their contribution to modifying the composition of the sperm proteome are discussed in detail below.

Beyond traditional model systems, recent cross-species analyses have expanded our understanding of conserved sperm function. By harmonizing 29 publicly available mature sperm proteome datasets across 12 vertebrate species, we have recently been able to define a core sperm proteome, comprising 45 species-level and 135 order-level conserved proteins, including those integral for sperm function and fertility, such as non-selective, voltage-gated ion channel (metabolism) and heat shock-related 70 kDa protein 2 (capacitation) (Pini et al., 2025). Whilst modest in its current size, this conserved proteomic core reflects the deep evolutionary investment in sperm function and integrity. It offers not only insight into the selective pressures that continue to shape sperm protein architecture but also a foundation for developing diagnostics and interventions aimed at universal targets for the regulation of male fertility.

Dynamic phosphorylation cascades underpin the functional regulation of epididymal spermatozoa

The proteomic remodelling of spermatozoa during epididymal transit extends beyond the selective acquisition and loss of proteins (Skerget et al., 2015, Skerrett-Byrne et al., 2022) to encompass extensive PTM-mediated regulation of the activity of its intrinsic proteome. Indeed, of the diverse PTMs currently annotated (Hornbeck et al., 2019), reversible phosphorylation stands out prominently as a critical regulator of sperm function, responsible for orchestrating key events such as the initiation of motility, metabolic activation, signal transduction, and surface remodelling in preparation for fertilization in the female reproductive tract (Asquith et al., 2004, Balbach et al., 2020, Nixon et al., 2010, Porambo et al., 2012, Stival et al., 2016). Historically, sperm phosphorylation research has been largely focused on capacitation-associated tyrosine phosphorylation (Aitken et al., 1995, Visconti et al., 1995a, Visconti et al., 1995b), with the most well-studied signalling cascade featuring soluble adenylyl cyclase production of cyclic adenosine 3′,5′-monophosphate (cAMP) (Gervasi & Visconti, 2016, Visconti et al., 2011). cAMP subsequently activates protein kinase A (PKA), a master orchestrator of the phosphorylation signalling cascade underpinning sperm capacitation (Aitken et al., 1998, Lefièvre et al., 2002), and culminates in the activation of tyrosine kinases including SRC kinase (Baker et al., 2006, Mitchell et al., 2008) and tyrosine-protein kinase Fer (Alvau et al., 2016). However, the indispensability of this canonical cAMP/PKA-dependent phosphorylation pathway may not be universally conserved. In mice, Ca^{2+}-ionophore-induced fertilization can proceed even when cAMP signalling and downstream tyrosine phosphorylation are suppressed (Tateno et al., 2013), indicating that the reliance on specific phosphorylation cascades is species- and context-dependent. These findings highlight the need to map and understand additional phosphorylation networks beyond the classical cAMP/PKA axis that may contribute to sperm maturation within the epididymis and throughout capacitation. To address this, recent advancements in quantitative phosphoproteomics, including improved sample methodology (Humphrey et al., 2018), MS technology (Son et al., 2024), and sophisticated bioinformatic pipelines for spectral deconvolution (Halder et al., 2021), now enable deeper exploration into the epididymal regulation of sperm phosphorylation. Two seminal studies leveraging these advancements have significantly expanded our understanding of phosphorylation dynamics during sperm maturation (Skerrett-Byrne et al., 2025a, Zhang et al., 2024b).

In the first of these studies, Zhang and colleagues employed a chemical-labelling approach, known as tandem mass tags (TMT), to profile sperm collected from three distinct epididymal segments: the proximal caput, the connecting corpus, and the distal cauda (Zhang et al., 2024b). They identified 6,447 phosphorylation sites from 1,407 phosphoproteins and revealed closer phosphoproteomic similarities between corpus and cauda epididymal sperm relative to their caput epididymal counterparts, underscoring progressive maturation through epididymal transit. Functional annotation linked phosphorylation changes between immature caput and mature cauda epididymal sperm to crucial biological processes, including metabolism, motility, and capacitation. Moreover, experimental inhibition of testis-specific serine kinase 2 validated its essential role in regulating sperm motility and ATP homeostasis, underscoring the functional significance of specific kinase pathways in sperm maturation.

One potential limitation of TMT multiplexing and data-dependent acquisition is that this strategy inherently favours detection of highly abundant peptides, which is further compounded in spermatozoa, where a subset of proteins are highly abundant and remain largely conserved during epididymal transit (Skerget et al., 2015, Skerrett-Byrne et al., 2022). As a result, lower abundance phosphopeptides may be inadvertently under-sampled, potentially accounting for why no phosphorylation sites emerged as unique to specific epididymal segments (Zhang et al., 2024b). Consistent with this, in the second study, our complementary label-free phosphoproteomic dataset identified >4,000 phosphorylation sites that were exclusive across the three epididymal sperm populations (Skerrett-Byrne et al., 2025a), underscoring the increased depth achievable when under-sampling constraints imposed by TMT-DDA are avoided. Leveraging this label-free phosphoproteomic approach (Skerrett-Byrne et al., 2025a), we extended the scope of our analysis beyond the epididymis to include capacitated sperm. This strategy has been successful in significantly increasing the depth of phosphoproteomic data pertaining to sperm matu-

ration, with key insights helping to redefine previous mechanistic paradigms of these critical phases of sperm development. Firstly, the largest changes in the sperm phosphoproteome were shown to coincide with epididymal maturation rather than downstream capacitation, as has conventionally been assumed (Aitken et al., 1995, Visconti et al., 1995a, Visconti et al., 1995b). Secondly, akin to the pronounced loss of sperm proteins during maturation (Skerget et al., 2015, Skerrett-Byrne et al., 2022), we identified >3,000 phosphorylation sites that were entirely removed from the sperm proteome between the caput and cauda epididymis. Thirdly, during capacitation, proportionally speaking, phosphorylation was evenly distributed among the three canonical phosphorylated amino acids (serine, threonine, tyrosine), contrary to the prior focus on tyrosine residues (Aitken et al., 1995, Visconti et al., 1995a, Visconti et al., 1995b), spanning proteins that regulate motility and metabolism. Such findings are also complemented by other phosphoproteomic investigations of mature spermatozoa (Ficarro et al., 2003, Martin-Hidalgo et al., 2020, Urizar-Arenaza et al., 2019, Wang et al., 2021), with the combined results reinforcing the evolutionary conservation and biological significance of phosphorylation cascades in regulating critical aspects of sperm function (Nixon et al., 2020, Skerrett-Byrne et al., 2025a). Lastly, combining MS findings with *in silico* prediction identified 343 kinases and 48 phosphatases implicated in functional sperm maturation, data that highlights a robust kinase-driven signalling network supporting the epididymal phase of sperm development. This extensive and differential phosphorylation of proteins during epididymal transit signifies a highly dynamic and finely tuned regulatory network, with >9,000 sites significantly modified across ~2,300 proteins [representing ~45% of the detectable sperm proteome (Skerrett-Byrne et al., 2025a)], priming sperm cells not only for functionality but also ensuring their viability during extended storage within the epididymis (Nixon et al., 2020). Taken together, these findings raise the possibility that certain kinases and phosphatases may exert segment-specific functions within the epididymis, with distinct signalling programmes operating in the caput compared to the cauda. Although this remains speculative, the marked regional differences in phosphorylation signatures and the presence of segment-specific kinase and phosphatase profiles make this an important area for future investigation.

This dynamic regulation of sperm cell protein phosphorylation is governed by the opposing actions of kinases and phosphatases. While our in silico analysis identified the predominant influence of kinases, phosphatases play a critical yet comparatively underexplored role in sperm maturation, balancing kinase-driven phosphorylation events. Notably, the protein phosphatase PP1γ2 is highly expressed in immature caput epididymal sperm and exhibits a decline in activity toward the cauda epididymis, correlating with the onset of sperm motility. Inhibition studies have demonstrated that suppressing PP1γ2 activity initiates premature motility in immature sperm, underscoring its essential role in maintaining sperm in a quiescent state until proper maturation is achieved (Chakrabarti et al., 2007). Similarly, PP2A and PP2B have been identified as integral regulators of sperm phosphorylation dynamics, actively modulating the phosphorylation status of key proteins involved in motility and metabolic pathways during epididymal maturation and capacitation (Ferreira et al., 2022). Thus, the interplay of kinases and phosphatases creates a precisely controlled phosphorylation landscape critical for sperm functional maturation.

Sperm surface remodelling: cross-linking, glycoproteomics, and protein dynamics

Complementing the biochemical changes in intracellular signalling documented above, the surface architecture of the maturing sperm cell is also extensively remodelled, commencing with their release from the seminiferous tubules and continuing throughout all phases of post-testicular maturation including capacitation as the gametes encounter the differing environments of the epididymis, uterus, and oviduct. These remodelling events are an essential requirement for reproductive success, driving structural changes that facilitate oocyte recognition, binding, and fertilization (Gadella & Luna, 2014, James et al., 2020). At the proteome level, these changes manifest as the widespread removal, transient (covalent) or permanent (non-covalent) insertion, adsorption, or modification of plasma membrane proteins (Batra et al., 2024, Batra et al., 2019, Tecle & Gagneux, 2015). Indeed, epididymal proteome data from Skerget et al. (2015) and Skerrett-Byrne et al. (2022) have identified a number of plasma membrane proteins, including several members of the β-defensin, disintegrin and metalloprotease (ADAM), and solute carrier families, that are unique or differentially accumulated between functionally mature (cauda) and immature (caput) sperm cell populations. Complex remodelling of the sperm membrane during epididymal transit is attributed to the action of several key mechanisms, including; (1) enzymatic cleavage/whole protein shedding [i.e., through the action of glycosidases (Kunkel et al., 2025) and peptidases (Gatti et al., 2005) secreted from the epididymal epithelium], (2) modulation of protein homeostasis effectors [i.e., OVCH2, that regulate the structure, stability, and interactions between sperm surface proteins (Kent et al., 2025)], (3) protein loading (James et al., 2020, Marengo, 2008, Nixon et al., 2019) and removal (Leahy et al., 2020) by epididymosomes, and finally, (4) refinement of the sperm glycocalyx (Ji et al., 2025), although, to the authors knowledge, no epididymis-specific glycoproteomic data exploring these global changes currently exist.

It is important to emphasise that despite overall refinement of the sperm proteome throughout maturation, protein dynamics are not unidirectional, with a subset of these proteins acquired by sperm in the epididymis (Skerget et al., 2015, Skerrett-Byrne et al., 2022). Independent of the action of epididymosomes, there are some additional physiological and technical explanations for apparent protein gain. For example, given sperm are transcriptionally and translationally silent and lack protein-synthesis machinery, it has been theorized that changes in glycosylation may facilitate the unmasking of mature sperm proteins. Alternatively, while canonical protein sequences decrease along the epididymis, their annotated splice isoforms (UniProt defined) show reciprocal changes in abundance (Skerrett-Byrne et al., 2022), suggesting that these isoforms are preferentially presented to the cell surface during the latter stages of remodelling or are more easily extracted and/or detected using current MS techniques.

Recent high-resolution epididymal sperm “omics” studies have provided unprecedented insight for the development of protein targets with both contraceptive and therapeutic utility (Skerrett-Byrne et al., 2022). However, to develop effective protein-targeted therapeutics, the surface presentation, lipid interaction, and higher-order architecture (i.e., multimeric protein complexes) of sperm proteins also need to be considered (Bromfield et al., 2015b, Ji et al., 2025, Ravi et al., 2020). Despite knowledge that the

macromolecular organization of proteins and lipids in cell membranes is key to understanding their physiological function, analytical tools to capture these associations have lagged in development (Britt & Robinson, 2025, Jung et al., 2025). Recent advances in native MS (nMS) have demonstrated its remarkable capacity as a key analytical tool to study the organizational state of membrane- and surface-expressed protein complexes and their interaction with lipids (Jung et al., 2025, Zhang et al., 2024a). One caveat is that nMS typically requires the extraction of membrane proteins from their physiological surroundings into detergent-like environments for analysis. However, new platforms with dynamic and expanding mass ranges, and improved resolution for native top-down proteomics, have recently enabled the analysis of drugs binding to membrane proteins directly from their native membranes, an important step for drug development (Jung et al., 2025).

The development of this technology is particularly critical to enhance our understanding of sperm maturation due to the intricate surface-protein assemblies that regulate sperm–zona pellucida interaction (Bromfield et al., 2015a, Hart et al., 2025). In fact, recent work has demonstrated that inactive polypeptide *N*-acetylgalactosaminyltransferase-like protein 5 undergoes epididymal processing, enabling its binding to GalNAc residues on both the uterotubal junction and the zona pellucida, thereby contributing to sperm–uterotubal junction adhesion and ZP binding (Noda et al., 2025). Moreover, nMS and cross-linking MS could be applied to further enrich our understanding of the activity and assembly of ion channels that regulate sperm function and fertility. A notable example of these channels is CatSper, one of the most complex ion channels characterized to date (Young et al., 2024, Zhao et al., 2022), where the extracellular domains of its accessory protein subunits may be essential for modulating channel activity during sperm capacitation (Hwang et al., 2025). This finding was achieved through nanoscale imaging approaches including cryo-electron microscopy (Cryo-EM) and cryo-electron tomography (Cryo-ET), which are often coupled with either nMS or cross-linking MS to yield ultra-high-resolution 3D structural data of protein assemblies (Hwang et al., 2025, Lee & O'Reilly, 2023). Recent applications of Cryo-EM, Cryo-ET, and proteomics to sperm have yielded near atomic-resolution structures of axonemal microtubules (Leung et al., 2023) and revealed important insights into the structural diversity of axonemes across motile cilia in mammals (Leung et al., 2025). To better understand how epididymal maturation primes sperm proteins and protein complexes for fertilization, Cryo-EM approaches could now be applied to study surface protein assemblies acquired during epididymal maturation. Moreover, these approaches can be used to better understand how proteins that are no longer required are removed from sperm via shedding of the CD or epididymosome-mediated loss during epididymal maturation.

Segment-specific proteomics of the epididymal epithelium

Proteomic signatures of caput epididymal tissue

The crucial role the epididymis holds in terms of promoting sperm maturation is achieved through precise spatial compartmentalization of its secretory and absorptive activities (Figure 1). Recent proteomic studies have begun to unravel how such segment-specific molecular landscapes underpin these functions. Unlike the sperm proteome, which has been profiled in sperm populations sampled from different epididymal segments (i.e., caput and cauda epididymis), the epithelial cell proteome has so far been characterized only in the caput and not in other epididymal segments. (Trigg et al., 2021a). This analysis of the caput epithelial cell proteome has revealed a complex inventory of 4,405 proteins, dominated by enzymes (68%), followed by a similar representation of transcription regulators (11%) and transporters (14%). Building on this baseline information, proteomic analyses have also been used to examine the pathophysiological response of the caput epididymis to the administration of acrylamide (Trigg et al., 2021a), a potent male-specific reproductive toxicant known to induce downstream embryo resorption (Katen et al., 2017). This strategy revealed substantial alterations in protein abundances linked to oxidative stress, and notably, acrylamide exposure also led to the upregulation of several transcription factors implicated in the regulation of genes encoding a subset of stress-responsive microRNAs (miRNAs), several of which were pinpointed to increase in abundance in the spermatozoa of acrylamide-exposed male mice. This application of proteomics provided new insight into how the epididymal epithelium responds to paternal environmental insults and thereafter modulates the sperm epigenome through epididymosomes-mediated delivery of an altered sncRNA cargo (Trigg et al., 2021a).

The development of research tools such as an immortalized mouse caput epididymal epithelial cell line, mECap18 (Sipilä et al., 2004), has also helped to provide additional mechanistic insights into epididymal function (Trigg et al., 2021b). In recent studies, our group has characterized the composition of this model cell line, identifying 5,313 mECap18 proteins and confirming a high level of conservation with the proteome of freshly isolated caput epithelial cells (~76% shared proteins), including key regulators of protein synthesis, vesicle transport, and the cellular stress responses (Mulhall et al., 2024). Pathway analysis revealed prominent enrichment of EIF2 signalling and sirtuin signalling, critical for proteostasis and cellular communication (Mulhall et al., 2024, Smyth et al., 2024, Tatone et al., 2018). This work validated mECap18 cells as a robust in vitro surrogate for dissecting epididymal function and epididymosome-associated communication pathways critical for sperm maturation and for relaying environmental information. In harnessing this potential, we have recently reported a phosphoproteomic assessment of mECap18 cellular responses to corticosterone challenge (Skerrett-Byrne et al., 2024a), the major stress hormone in mice and the primary ligand of the glucocorticoid receptor, NR3C1, which itself acts as a master transcriptional regulator of the stress response (Oakley & Cidlowski, 2013). While the global mECap18 proteome was only modestly altered (~1.8%), extensive phosphorylation changes were detected across ~15% of the proteome, highlighting significant activation of pathways involved in DNA damage repair, oxidative stress, and miRNA biogenesis. These data suggest that phosphorylation-driven signalling cascades rapidly recalibrate epididymal epithelial physiology under stress conditions, potentially modifying the sncRNA cargo encapsulated in epididymosomes in preparation for their delivery to spermatozoa (Skerrett-Byrne et al., 2024a). Since there is little phosphoproteomic analysis completed on naïve tissue, it remains to be determined

whether protein phosphorylation along the epididymis also displays discrete regional patterns, adding an additional layer of control over sperm maturation beyond protein abundance.

Spatial proteomics and matrix-assisted laser desorption/ionization imaging: unveiling localized protein dynamics

Transcriptomic approaches have been used to map expressed transcripts to 10 discrete epididymal segments in the mouse (Johnston et al., 2005). Similar resolution has yet to be achieved for the epididymal proteome, largely reflecting the increased accessibility, resolution, and throughput of RNA sequencing compared with current proteomic methodologies. Indeed, investigations have been restricted to the caput epididymis, leaving the corpus and cauda epididymis unexplored. Nevertheless, our collective studies of this segment (Mulhall et al., 2024, Skerrett-Byrne et al., 2024a, Trigg et al., 2021a) highlight that the caput epididymis possesses a distinct, stress-sensitive proteomic and phosphoproteomic profile. Such regional signatures enable dynamic regulation of sperm maturation and epigenetic programming, primarily through epididymosome-mediated communication. However, the current paucity of proteomic studies underscores the need for broader investigation into other epididymal segments to fully elucidate their contributions to sperm maturation and storage, environmental sensing, and reproductive health more broadly. This expanded understanding will be crucial for developing targeted therapeutic interventions aimed at safeguarding male fertility and effective non-hormonal male contraceptives.

While traditional MS approaches accurately measure protein expression, they cannot resolve spatial patterns or cell-type specificity. One of the most widely used spatial proteomic methods, matrix-assisted laser desorption/ionisation (MALDI) MS imaging, is a growing field that enables quantification of biomolecules across tissue sections and reconstructs them as a 2D image. This approach facilitates the precise mapping of protein location profiles and identification of unique regional signatures unavailable in bulk cell MS preparations (reviewed by Moore and Charkoftaki, 2023). MALDI-MS imaging has been used in a reproductive context to investigate testosterone distribution in testicular cells (Cobice et al., 2016); however, at present, the epididymis remains largely overlooked. Importantly, one of the earliest and most detailed applications of MALDI-MS imaging of the mouse epididymis was published over two decades ago, where authors profiled >400 proteins and, utilizing laser capture microdissection, demonstrated regionalized protein expression from the caput down to cauda epididymis (Chaurand et al., 2003). Since then, only a limited number of studies have applied this technology to the epididymis (Aoki et al., 2022, Chaurand & Caprioli, 2002, Cornett et al., 2007). Notably, a recent MALDI-MS imaging study discerned 46 unique lipid profiles in the rat epididymis (Lagarrigue et al., 2020), which largely correlates to previous transcriptome studies in the rat (Jelinsky et al., 2007). Furthermore, in line with the remodelling of the lipid profile of sperm during epididymal maturation (for a review see Serafini and O'Flaherty, 2025), the researchers found that the lipid profile of the epididymal epithelium also displayed significant segment-to-segment variations. In parallel, MALDI has also been applied directly to boar epididymal sperm, where top-down profiling approaches revealed maturation-associated protein changes (Labas et al., 2015). Despite these advances, applications of MALDI imaging to the proteome remain scarce, and thus knowledge of the proteome of specific epithelial cell types (e.g., principal, clear, and basal cells) remains restricted to experimental isolation and shotgun proteomics (Mulhall et al., 2024, Skerrett-Byrne et al., 2024a, Trigg et al., 2021a), without knowledge of the single-cell protein profile. While single-cell RNA-seq studies performed on the epididymal epithelium have highlighted the unique cell-specific gene profile of the different cell types (Rinaldi et al., 2020), proteomics have not yet reached single-cell resolution.

Segment-specific immune landscapes of the epididymis

In addition to various factors required to shape optimal microenvironments for sperm maturation, immunological components are also distributed in a segment-specific manner throughout the epididymis. Unlike the testis, the epididymis is not considered an immune-privileged organ, despite the close contact of millions of maturing sperm with the epididymal epithelium and the dense population of resident immune cells across the tissue (Voisin et al., 2019). Consequently, the epididymal immune environment is uniquely adapted to balance tolerance toward immunogenic spermatozoa with robust protection against pathogens (Hedger, 2011). This balance is achieved through segment-specific immunological adaptations at both cellular and molecular levels. Despite being a single, highly convoluted duct, the epididymis displays a gradually changing epithelial architecture that supports the distinct functional demands of each segment, including sperm maturation and immune surveillance (Battistone et al., 2024, Pleuger et al., 2022). The proximal epididymis, wherein spermatozoa complete the initial stages of their maturation, is densely populated by intraepithelial CX3CR1^{+} mononuclear phagocytes (Battistone et al., 2019a, Da Silva et al., 2011, Pleuger et al., 2022). These mononuclear phagocytes exhibit a homeostatic phenotype, indicative of a sensory "screening" function essential for maintaining tissue integrity and enabling rapid elimination of intraluminal pathogens (Ai et al., 2026, Pleuger et al., 2022). Proteomic data from caput epididymal epithelial cell preparations (Mulhall et al., 2024, Trigg et al., 2021a) suggest that epithelial-derived factors, like interleukin-4, may shape the local immune environment by promoting anti-inflammatory phenotypes in neighbouring macrophages, thereby helping to limit excessive immune activation upon infection (Battistone et al., 2024).

Conversely, the immunological landscape of the distal segment of cauda epididymis reflects its dual function as both a sperm-storage reservoir and as a surveillance zone against ascending pathogens. Pro-inflammatory macrophages, dendritic cells, and lymphocytes (T, NK and B cells) are predominantly located in the interstitial space in close conjunction with the largest vascular hub (Damon-Soubeyrand et al., 2023, Voisin et al., 2018), whereas intraepithelial macrophages are substantially reduced (Pleuger et al., 2022). Accordingly, the cauda epididymis mounts a significantly stronger pro-inflammatory response to immune challenges compared to the proximal epididymal regions, often accompanied by tissue-damaging inflammatory processes that result in long-term fertility impairments (Klein et al., 2019, Pleuger et al., 2022). Intriguingly, emerging evidence suggests that immune and non-

immune cells within the epididymal epithelium act as a coordinated system to regulate mucosal homeostasis and sperm maturation in a region-specific manner (for a review see Battistone et al., 2024).

Emerging evidence indicates that non-immune epithelial cells participate not only in sperm maturation but also in immune surveillance and regulation in healthy and diseased conditions. Among these, clear cells (CCs), proton-secreting cells dispersed throughout the epithelium, appear to play a far more complex role than previously appreciated. CCs contribute to proteome remodelling by transferring proteins to sperm, via direct contact, nanotubes, or epididymosomes, while simultaneously modulating immune cell activity and engaging directly in pathogen defence, as indicated by transcriptomic analyses (Battistone et al., 2019b). Their expression of CRES proteins suggests a major role in generating the epididymal amyloid matrix, which is essential for bacterial trapping and membrane disruption (Battistone et al., 2019b, Whelly et al., 2016), and simultaneously, CCs appear to produce antioxidants protecting sperm and epithelial surfaces (Klein et al., 2019, Pleuger et al., 2022). Beyond these luminal effects, CCs (and other non-immune epithelial cells such as principal cells) help to establish the tolerogenic environment characteristic of the epididymis. This is reflected in their expression of anti-inflammatory mediators such as interleukin 4, IDO1, and region-specific TGF-β ligands [TGF-β1, TGF-β2, TGF-β3; (Battistone et al., 2024, Mulhall et al., 2024, Trigg et al., 2019)], which are canonically involved in promoting anti-inflammatory and immunotolerant immune cell phenotypes (Battistone et al., 2024, Jrad-Lamine et al., 2013, Pierucci-Alves et al., 2018), and thereby maintaining tissue integrity. However, whether and how these immune mediators influence EV biogenesis within male reproductive organs remains poorly understood. Combining transgenic models with EV-focused proteomic and trafficking analyses could provide valuable insights into resolving this complex interplay.

Complementing these cellular functions, the epididymal epithelium establishes a dynamic biochemical barrier via segment-specific secretion of antimicrobial peptides and immunoregulatory proteins {e.g., β-defensins, lipocalins, transforming growth factor-β superfamily members [for reviews see Avellar et al. (2019); Wijayarathna and Hedger (2019)]}. These factors collectively contribute to antimicrobial defence, immune tolerance, and the maintenance of a luminal microenvironment conducive to sperm maturation. Notably, antimicrobial peptides are either constitutively highly expressed or become upregulated in the caput segment following infection, potentially providing early luminal protection against urogenital pathogens (Klein et al., 2020, Pleuger et al., 2022). Together with the actions of CCs and other epithelial cells, these segment-specific secretions of immunomodulatory factors highlight how the epithelium integrates structural, biochemical, and immunoregulatory strategies to simultaneously protect sperm and provide optimal conditions for EV-mediated proteome exchange.

Epididymosomes—epididymal nano-couriers

Mechanisms of sperm–epididymosome interaction

Epididymosomes play a central role in facilitating intercellular communication and delivery of a complex macromolecular cargo essential for promoting sperm maturation and longevity. These small membrane-bound vesicles are released from the epididymal soma via an apocrine secretory pathway (Caballero et al., 2010, Rejraji et al., 2006), and thereafter interact with the luminal population of spermatozoa to deliver their molecular cargo (Nixon et al., 2019). While principal cells were initially considered the primary source of epididymosomes, recent evidence indicates that other epithelial cell types, such as CCs and basal cells, also secrete epididymosomes (Barrachina et al., 2022, Battistone et al., 2019b). In line with this, two distinct epididymosome populations have been identified in the bull, highlighting the potential of different cells of origin (Frenette et al., 2010). Thus, epididymal fluid likely contains a heterogenous population of epididymosomes derived from multiple cell types. The interaction between epididymosomes and sperm is finely tuned by luminal conditions, such as temperature, pH, and the presence of zinc, that mimic the epididymal luminal environment (Frenette et al., 2002, Sullivan et al., 2007). Efforts to dissect the molecular basis of this interaction have identified several key players. Antibody masking and CRISPR-Cas9-mediated disruption of the *Mfge8* gene, have identified its protein product, MFGE8 (lactadherin) as a crucial ligand on the epididymosome surface responsible for mediating the initial interaction with recipient cells (Nixon et al., 2019, Trigg et al., 2021b). Conversely, in sperm, the dynamin 1 mechanoenzyme localizes to the inner leaflet of the sperm membrane where it is required for the internalization of epididymosome cargo. It follows that pharmacological inhibition of dynamin 1 suppresses the transfer of labelled epididymosome proteins to spermatozoa (Zhou et al., 2019). This finding suggests epididymosomes dock and transiently fuse with sperm, performing exchange via a putative “kiss and run” mechanism. This mechanism could theoretically facilitate bi-directional protein transfer, enabling the acquisition of proteins important for fertilization, as well as the shedding of superfluous or inhibitory proteins; although the latter has yet to be experimentally proven (Zhou et al., 2019). Ultimately, the exact mechanism of interaction remains unclear, with additional studies suggesting cargo transfer via membrane fusion and additional modes of epididymosome cargo uptake yet to be examined (Sullivan, 2016, Zhou et al., 2019). While these discoveries shed light on the proteins involved in epididymosome docking and cargo transfer, the characterization of the epididymosome protein cargo and how it influences sperm function and drives the final stages of sperm maturation is also of importance.

Functional significance of epididymosome-delivered proteins

The extent to which epididymosomes shape the proteomic landscape of epididymal spermatozoa has been highlighted across various studies. To date, characterization of the epididymosomes proteome has identified a total of 1,640 proteins carried by these vesicles isolated from mouse epididymal fluid (Nixon et al., 2019) and 849 proteins in vesicles released by an epididymal cell line (Chan et al., 2020). Notably, 88% of these proteins are also represented in the epididymal sperm proteome and include proteins such as zona pellucida binding protein 1, ribonuclease-like protein 10 (RNASE10), and ADAM28 (Skerrett-Byrne et al., 2022); each of which has been identified as key epididymal protein that contributes to sperm functionality and male fertility (Krutskikh

et al., 2012, Lin et al., 2007, Roberts et al., 2006). Although zona pellucida binding protein 1 is expressed in the testis and is essential for spermatogenesis (Lin et al., 2007), its detection in epididymosomes likely reflects supplementary packaging or redistribution rather than de novo acquisition. Moreover, studies employing biotinylated protein labelling, transgenic mouse models, and fluorescent markers, have demonstrated the successful transfer of epididymosome proteins to sperm following co-incubation (Morgan et al., 2023, Nixon et al., 2019, Zhou et al., 2019). Such approaches provide evidence of the ability of epididymosomes to deliver proteins to sperm. Nevertheless, identification of the entire complement of epididymosome-delivered proteins remains to be determined. Recent efforts to elucidate this have used in silico analysis and revealed 25 sperm proteins of potential epididymal origin in humans and mice (Barrachina et al., 2022). Among these proteins were ADAM7, lactotransferrin, and gamma-glutamyltransferase 1 (Barrachina et al., 2022). Notably, ADAM7 is synthesised in the epididymis and found in high abundance in epididymosomes from where it is transferred into the sperm membrane and is required for normal fertility (Choi et al., 2015, Nixon et al., 2019, Su Oh et al., 2009). Moreover, several motility related proteins are encapsulated in epididymosomes, implicating these vesicles as one of the primary drivers of post-testicular sperm maturation. Such proteins include, plasma membrane calcium ATPase 4 and protein Wnt-2b (Björkgren & Sipilä, 2019, Koch et al., 2015, Martin-DeLeon, 2015, Patel et al., 2013).

Given the protective environment epididymosomes provide for their encapsulated cargo, it is not surprising that complementing their diverse proteomic repertoire, these vesicles also harbour alternative cargo, including RNAs and lipids (Girouard et al., 2011, Reilly et al., 2016, Rejraji et al., 2006, Sharma et al., 2018, Zhou et al., 2019). Among the RNA cargo, sncRNAs, such as miRNAs and transfer RNA-derived RNAs are particularly abundant (Reilly et al., 2016, Sharma et al., 2018). These molecules contribute to a sperm-borne epigenetic landscape that is both dynamic and responsive to paternal environmental cues, with downstream consequences for the embryo and offspring (Chan et al., 2020, Rompala et al., 2020, Trigg et al., 2019, Trigg et al., 2021a). Notably, the proteins released by the epididymal epithelium within epididymosomes, such as RNA-binding proteins, may contribute to shaping the RNA payload, influencing the transcripts that are stabilized, trafficked, or delivered to recipient sperm cells (Liu et al., 2024). Hence, epididymosomes deliver a range of biomolecules to sperm that act in concert to support sperm maturation, functional competence, and the transmission of epigenetic information reflective of the prevailing paternal environment.

Other epididymal nano-couriers

While epididymosomes remain the main contenders for facilitating bulk protein transfer during epididymal transit, alternative carriers may complement epididymosome-mediated delivery. Indeed, CCs within the epididymal epithelium exhibit long membrane protrusions, similar to tunnelling nanotubes, that extend into the epididymal lumen and directly connect the cytoplasm of these cells to sperm, allowing for the exchange of molecules (Battistone et al., 2019b, Rustom et al., 2004). While proteomic data of this specific cell population is limited, transcriptomic analyses have identified CC transcripts that encode proteins known to be acquired by sperm during epididymal transit, suggesting this cell population does contribute to the sperm proteome (Battistone et al., 2019b). Accordingly, CC protrusions have been visualized in close proximity to sperm using 3D image reconstruction. However, CCs have also been demonstrated to produce epididymosomes and CC-derived vesicles have been shown to adhere to sperm (Battistone et al., 2019b). Therefore, whether epididymosomes or nanotubes are the main delivery mechanism for CC-derived sperm proteins remains to be determined.

CDs have traditionally been viewed as a mechanism for redundant protein clearance from epididymal sperm (discussed above). However, correlative evidence of RNA species within sperm and the CD has prompted the hypothesis that RNA molecules may be shuttled between sperm and the CD (Wang et al., 2023). Whether such a mechanism could also facilitate the transfer of proteins is yet to be investigated (Skerrett-Byrne et al., 2022, Yuan et al., 2013). Finally, many epididymal-acquired sperm proteins have been shown to be transferred to sperm via a non-vesicular mechanism. The epididymis secretes these proteins in a highly regulated and regionalized manner, enabling both direct and indirect modulation of sperm maturation. While many of these proteins contribute to shaping the unique luminal environment essential for sperm functionality, a subset can directly associate with the sperm surface or be internalized, thereby influencing sperm structure and function (Dacheux et al., 2003). Notable examples include cysteine-rich secretory protein 1, glutathione peroxidase 5, inactive RNASE10, and β-defensin family members [which are implicated in sperm–egg interaction, sperm membrane lipid composition, modifying post-translational protein processing, and sperm calcium signalling, respectively (Belleannée et al., 2011, Björkgren & Sipilä, 2019, Da Ros et al., 2008)]. Importantly, the methods used to prepare epididymal fluid prior to proteomic identification of secreted proteins do not always preclude the presence of epididymosomes, and therefore epididymosome-associated proteins. Thus, to truly discriminate secreted proteins from those encapsulated in epididymosomes, future studies are needed in which the free protein and epididymosome fractions of epididymal fluid are first separated, potentially through density-based fractionation methods. Ultimately, epididymosome-mediated sperm proteome transformation is a key maturation step underpinning fertility potential.

Future perspectives

As outlined in this review, proteomics has fundamentally transformed our understanding of the epididymis, illuminating its critical role in sperm functional maturation and male fertility. This technology has advanced the field beyond morphological descriptions toward an intricate understanding of molecular mechanisms underpinning the development of sperm function. Despite these significant advancements, numerous questions remain, necessitating continued exploration through innovative and integrative proteomic strategies. Namely, human epididymal proteomic data remain extremely scarce, largely due to the invasive nature of sample collection limiting cross-species validation and clinical translation. A further concern is comparability between studies, which is hindered by substantial methodological variability, including differences in sample isolation, sperm purification strategies, proteomic workflows, and MS platform-specific biases.

Encouragingly, the field is beginning to respond to this need for harmonization, with detailed methodological frameworks now available to standardize human sperm phosphoproteomic workflows (Bromfield et al., 2025). Addressing these technical and biological limitations will be essential to fully realize the promise of epididymal proteomics.

A primary knowledge gap highlighted in this review pertains to defining the proteome of distinct functional compartments within the epididymis (Lagarrigue et al., 2020). Proteomic characterization of tissue from the corpus and cauda segments remains relatively unexplored compared to the caput, despite their essential roles in finalizing sperm maturation and storage (Robaire et al., 2006). Similarly, the proteomes of the CD, which is shed during epididymal transit (Bloom & Nicander, 1961, Moore et al., 2010), and epididymal luminal fluid remain to be comprehensively characterized. Further, the protein cargo of epididymosomes and their origins warrant renewed attention. Together, epididymosomes and the CD may act in concert in the refinement of the post-testicular sperm proteome, modulating protein composition through targeted delivery or possibly also disposal. From a clinical perspective, this raises intriguing therapeutic possibilities, whereby epididymosomes could be engineered not only to restore deficient protein cargo in dysfunctional sperm but also to deplete excess or deleterious proteins. Resolving these knowledge gaps is crucial to fully appreciate the regional specialization of different epididymal segments and their overall impact on sperm function. Moreover, interrogating in vitro models, such as 3D epididymal organoids and microfluidic "organ-on-a-chip" systems (Cyr & Pinel, 2022, Leir et al., 2020, Leung et al., 2022) with advanced proteomic techniques such as data-independent acquisition MS, could help accelerate the discovery and validation of novel therapeutics and contraceptives while minimizing animal use. Finally, the absence of proteomic investigations into the epididymal maturation of human spermatozoa, owing to the invasive nature of attaining these samples, continues to limit clinical progress towards defining the contribution of this process to a spectrum of infertility phenotypes.

Expansion of sperm PTM omics

In seeking to build on the important insights provided by global phosphoproteomic studies, additional analyses focussed on more diverse PTMs represent an urgent frontier. PTMs serve as dynamic molecular regulators, influencing protein activity, interactions, and localization. Recent advances in top-down proteomics (Kaulich et al., 2024), which characterize intact proteoforms, have shown exceptional promise for elucidating PTM crosstalk, particularly on structural proteins where subtle modifications critically impact sperm function (Leung et al., 2021, Leung et al., 2023). Glycoproteomics, specifically, is a rapidly advancing field (Jager et al., 2025) critical for unravelling complex glycan structures on the sperm head that are essential for sperm-egg recognition, capacitation, and the subsequent acrosome reaction, as showcased in a recent multi-species comparison (Ji et al., 2025). Additionally, cross-linking MS offers exciting potential, enabling direct insights into protein–protein interactions and structural conformations within the sperm proteome (Piersimoni et al., 2022). In complement, advanced imaging modalities such as Cyro-EM (Leung et al., 2023) and stimulated emission depletion super-resolution microscopy (Oishee et al., 2025) are emerging as powerful tools for spatially resolving protein architecture and localization in maturing sperm cells, offering structural insights into the molecular machinery that underlies sperm function and maturation. Together, such detailed PTM, subcellular resolution, and interactome profiles will substantially refine our molecular comprehension of sperm maturation.

Single-cell proteomics

Emerging single-cell proteomics methods are poised to revolutionize our understanding of epididymal biology by enabling the characterization of proteomes from discrete epididymal cell populations and even individual sperm cells. While single-cell RNA sequencing has yielded valuable transcriptomic atlases of epididymal cell types (Rinaldi et al., 2020), single-cell proteomics offers the direct quantification of epididymal proteins, which are the primary functional molecules of the cell (Sinn & Demichev, 2025). Advanced workflows, such as single-cell proteomics by MS (SCoPE-MS) (Budnik et al., 2018), are pushing the sensitivity of detection limits, and with further MS advances, such as the Astral, has enabled the quantification of >5,000 proteins in a single cell (Bubis et al., 2025). These technologies address inherent challenges in single-cell analysis, including sample loss, sensitivity, and throughput (Bennett et al., 2023). Although technical pitfalls and the complexity of data processing remain, ongoing innovations are rapidly improving data robustness and throughput, with new workflows doubling proteomic depth in single cells and increasing analysis speed (Bubis et al., 2025). Single-cell proteomics will allow researchers to dissect functional heterogeneity and rare subpopulations of epididymal cell types and spermatozoa, previously masked in bulk analyses, fostering precision diagnostics and targeted therapeutic intervention.

Multi-omics integration, clinical and translational outlook

A truly comprehensive understanding of epididymal sperm maturation necessitates integrating proteomic data with other complementary omics modalities, including transcriptomics (gene expression), epigenomics (DNA methylation, histone modifications, sncRNA sequencing), lipidomics (lipid profiles), and metabolomics (small molecule metabolites). Future use of such multiomics approaches will reveal crosstalk between molecular layers, offering holistic insight into sperm function and novel therapeutic targets for male infertility. Further, integrating temporal and spatial omics dimensions of epididymal cell type and sperm populations will be critical for capturing the dynamic nature of sperm maturation.

Clinically, expanded proteomic knowledge holds substantial promise. Approximately 70% of male infertility cases remain idiopathic (Tüttelmann et al., 2018), highlighting the urgent need for improved understanding of post-testicular sperm maturation to identify novel diagnostic biomarkers and therapeutic targets. Proteomic discoveries hold promise to significantly impact clinical diagnostics, revealing molecular signatures indicative of specific epididymal dysfunctions. Proteomic advances can also enhance assisted reproductive technologies by improving patient stratification and fertility treatment outcomes. Moreover,

Table 1 List of all epididymal proteomic studies included in ShinyEpididymis.

Study	PMID	Sample	In vivo or in vitro
(Yuan et al., 2013)	24155961	Cytoplasmic droplet	In vivo
(Chan et al., 2020)	32198406	Epididymosomes	In vitro
(Nixon et al., 2019)	30213844	Epididymosomes	In vivo
(Skerrett-Byrne et al., 2025a)	*BioRxiv*	Sperm (phospho)	In vivo
(Zhang et al., 2024b)	38977202	Sperm (phospho)	In vivo
(Kent et al., 2025)	40155198	Sperm (proteome)	In vivo
(Skerget et al., 2015)	26556802	Sperm (proteome)	In vivo
(Skerrett-Byrne et al., 2022)	36384108	Sperm (proteome)	In vivo
(Mulhall et al., 2024)	37759396	Caput epididymal epithelial cells	In vitro
(Skerrett-Byrne et al., 2024a)	38576152	Caput epididymal epithelial cells	In vitro
(Trigg et al., 2021a)	34610313	Caput epididymal epithelial cells	In vivo

the identification of critical proteins and PTMs essential for sperm function offers promising targets for non-hormonal male contraceptives (Skerrett-Byrne et al., 2025a).

ShinyEpididymis

As a practical step towards addressing these remaining gaps and promoting widespread access and utilization of proteomic data, we have developed a novel interactive web-based resource, building upon previous platforms and blueprints such as ShinySperm (Skerrett-Byrne et al., 2024b) and ShinySpermKingdom (Pini et al., 2025), ShinySpermPlacenta (Skerrett-Byrne et al., 2025c), and inspiration from The Mammalian Reproductive Genetics Database (https://orit.research.bcm.edu/MRGDv2). This new application, *ShinyEpididymis*, integrates publicly available proteomic datasets across mouse sperm, epididymosomes, and epididymal tissue, enabling the research community to rapidly query proteins of interest (Table 1). This resource represents a first-of-its-kind tool, providing a foundational framework for ongoing data integration and future harmonization at the MS level. Currently compiled from 11 datasets (Table 1, see Supplementary File), we anticipate periodic updates, incorporating the latest proteomic findings to continuously refine and expand this valuable research tool.

In conclusion, future proteomic research on the epididymis promises transformative advances. Addressing existing regional proteomic gaps, leveraging cutting-edge single-cell and top-down methodologies, integrating multi-omics approaches, and fostering translational clinical applications will profoundly deepen our understanding of male reproductive biology, substantially benefiting clinical practice and therapeutic development.

Author contributions

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Conflicts of interest

None declared.

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Data availability statement

The full coding script supporting ShinyEpididymis (https://reproproteomics.shinyapps.io/ShinyEpididymis/) can be downloaded from GitHub – https://github.com/DavidSBEire/ShinyEpididymis.

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