Title: Developmental expression of the dynamin family of mechanoenzymes in the mouse epididymis

Running title: Dynamin expression in the mouse epididymis

Summary sentence: The dynamin family of mechanoenzymes are differentially expressed in the mouse epididymal epithelium and selectively regulate protein secretion.

Key words: epididymis; sperm maturation; dynamin; epididymal milieu; protein trafficking; exosomes; epididymosomes; apocrine secretion, merocrine secretion

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ABSTRACT

The mammalian epididymis is an exceptionally long ductal system tasked with the provision of one of the most complex intraluminal fluids found in any exocrine gland. This specialized milieu is continuously modified by the combined secretory and absorptive of the surrounding epithelium and thus finely tuned for its essential roles in promoting sperm maturation and storage. While considerable effort has been focused on defining the composition of the epididymal fluid, relatively less is known about the intracellular trafficking machinery that regulates this luminal environment. Here we characterize the ontogeny of expression of a master regulator of this machinery, the dynamin family of mechanoenzymes. Our data show that canonical dynamin isoforms were abundantly expressed in the juvenile mouse epididymis. However, in peri-pubertal and adult animals dynamin takes on a heterogeneous pattern of expression such that the different isoforms displayed both cell and segment specific localization. Thus, dynamin 1 and 3 were predominately localized in the distal epididymal segments (corpus and cauda) where they were found within clear and principal cells, respectively. In contrast, dynamin 2 was expressed throughout the epididymis, but localized to the Golgi apparatus of the principal cells in the proximal (caput) segment and the luminal border of these cells in more distal segments. These dynamin isoforms are therefore ideally positioned to play complementary, non-redundant roles in the regulation of the epididymal milieu. In support of this hypothesis, selective inhibition of dynamin altered the profile of proteins secreted from an immortalized caput epididymal cell line.

Word count = 246

INTRODUCTION

The mammalian epididymis is of fundamental importance to reproduction owing to its specialized roles in promoting the functional maturation of spermatozoa and their prolonged storage prior to ejaculation [1]. Both functions rely on the production of a complex intraluminal milieu [2] that is continuously modified by the combined secretory and absorptive activity of the epithelium lining this extraordinarily long tubule [3, 4]. This pseudostratified epithelium comprises multiple cell types, each of which possess discrete roles and unique patterns of distribution.

Principal cells dominate along the entire length of epididymis, constituting as much as 80% of the peritubular interstitium [5]. Despite some segment-segment variation in the structural and functional properties of these cells, a defining feature is their highly developed secretory and endocytotic machinery [5]. Such machinery encompasses key elements of the endocytic apparatus including abundant coated pits, endosomes, and lysosomes. Similarly, these cells are also decorated with extensive networks of rough endoplasmic reticulum, Golgi apparatus, small vesicular aggregates and blebs of cytoplasm originating from their apical cell surface [6-8]. The presence of such elaborate trafficking machinery accords with an active role in the synthesis of proteins and their subsequent secretion into the lumen, particularly in the proximal epididymal segments (caput and corpus) where sperm acquire their potential for fertilization [9, 10]. Throughout the epididymis, these cells also display endocytotic activity thus facilitating the recycling of proteins and other luminal contents and contributing to an optimal environment for protracted periods of sperm maturation and storage [6-8]. Such endocytotic activity is also shared with clear cells, the second most abundant cell type in the epididymis [11, 12].

71 Accordingly, clear cells also feature numerous coated pits, vesicles, endosomes, multivesicular
72 bodies, and lysosomes [7, 11]. While comparatively less is known of the function of the
73 remaining subsets of basal, narrow, apical, halo and immunological (macrophage and dendritic)
74 cell types it is widely recognized that the careful integration of their activities is essential to
75 maintain the fidelity of post-testicular sperm development, protection and storage [3, 13].

76 It follows that an understanding of the mechanisms that underpin the creation of the
77 epididymal luminal milieu is of key interest for fertility regulation both in the context of
78 resolving the causes of male factor infertility [14] and as a target for contraceptive intervention
79 [15]. Despite this, our knowledge of the precise molecular machinery and, in particular the
80 vesicle trafficking and fusogenic proteins, that underpin the dynamic secretory and endocytotic
81 activity of these cells is incomplete. In recent studies we have begun to characterize novel roles
82 for the dynamin family of large GTPases in the context of mammalian reproduction [16, 17].
83 Here we have sought to extend this work by examining the spatial and temporal expression of
84 dynamin within the mouse epididymis. Our interest in dynamin reflects the central role the
85 mechanochemical enzyme holds in the coupling of exo- and endocytotic processes [18-21].
86 While dynamin has been best studied in the context of clathrin-coated endocytosis from the
87 plasma membrane [22], it is also implicated in formation and budding of transport vesicles from
88 the Golgi network [23-26], vesicle trafficking [27], orchestrating exocytotic events [28, 29],
89 and in the regulation of microtubular, and actin cytoskeletal dynamics [29-33]. Such diverse
90 functions rely on the ability of dynamin to spontaneously polymerize into high order oligomers
91 in the presence of a variety of tubular templates such as lipid membranes [34], microtubules
92 [35, 36] and actin bundles [37, 38]. In the case of membrane remodeling and scission, this

polymerization leads to the formation of rings and/or helices [20]. In one of the most widely accepted models of action, GTP hydrolysis drives conformational change and constriction of the dynamin helix thus leading to membrane fission and physical separation of nascent vesicles from the parent membrane [18]. It has also recently been shown that dynamin has the potential to fine-tune exocytotic events by virtue of its ability to control the rate of fusion pore expansion, and thus the amount of cargo released from an exocytotic vesicle [28, 39].

In mammals, dynamin is encoded by three different genes (*Dnm1*, *Dnm2*, and *Dnm3*) whose products undergo alternative splicing to generate a several variants [21, 40]. These isoforms are characterized by differential expression within distinct tissues of the body. Thus, dynamin 1 is primarily found within neural tissue [41], dynamin 2 is ubiquitously expressed throughout the body [42], and dynamin 3 (the most structurally divergent of the canonical isoforms) resides mainly within lung, brain, heart and testis tissue [40]. It has also been shown that dynamin 1 and dynamin 2 localize to developing germ cells (spermatocytes and spermatids) as well as nurse Sertoli cells of the murine testes [43-46], leading to speculation of a novel role for the GTPase in the production of spermatozoa during the process of spermatogenesis. The role of dynamin 3 within this tissue appears to center on its participation in the formation of a tubulobulbar structure responsible for the release of spermatozoa from Sertoli cells [47]. Dynamin 1 and 2, but not dynamin 3, have also been implicated in the post-testicular functional maturation of spermatozoa [16, 17, 48], yet to the best of our knowledge there are no reports of any of these dynamin isoforms in the context of the mammalian epididymis. This study was therefore undertaken to characterize the epididymal expression of the canonical dynamin family and investigate their contribution to the function of this important endocrine system.

MATERIALS AND METHODS

Animals

All experimental procedures involving animals were conducted with the approval of the University of Newcastle's Animal Care and Ethics Committee in accordance with the Society for the Study of Reproduction's specific guidelines and standards. Mice were obtained from a breeding colony held at the institute's Central Animal House and raised under a controlled-lighting regime (16 h light: 8 h dark) at 21–22°C and supplied with food and water *ad libitum*. Prior to dissection, animals were sacrificed by CO_2 inhalation.

Antibodies and reagents

Unless otherwise stated, chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of molecular biology or research grade. Rabbit polyclonal antibody against dynamin 1 (ab108458) and PSMD7 (ab11436) were purchased from Abcam (Cambridge, England, UK); rabbit polyclonal antibody against CCT3 (sc-33145), rat monoclonal antibody against CCT8 (sc-13891), goat polyclonal dynamin 2 (sc-6400) and its immunizing peptide (sc-6400 P), IZUMO1 (sc-79543) and ATP6V1B1 (sc-21206) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse monoclonal antibody against dynamin 1 (MA5-15285), sheep polyclonal antibody against dynamin pSer778 (PA1-4621) and rabbit polyclonal antibody against dynamin 2 (PA5-19800) were purchased from Thermo Fisher Scientific (Scoresby, VIC, Australia); rabbit polyclonal antibody against dynamin 3 (14737-1-AP) and its immunizing peptide (ag6381) were from Proteintech Group (Chicago, IL, USA). Rabbit polyclonal antibody against flotillin 1 (F1180), rabbit polyclonal antibody against androgen receptor (SAB4501575) and mouse monoclonal antibody against α tubulin (T5168) were from Sigma-Aldrich. Rabbit

monoclonal antibody against golgin-97 (#13192) was from Cell Signaling Technology (Arundel, QLD, Australia). Alexa Fluor 488-conjugated goat anti rabbit, Alexa Fluor 594 or 488-conjugated donkey anti goat and Alexa Fluor 594-conjugated goat anti mouse were from Thermo Fisher Scientific (Eugene, OR, USA). Anti-rabbit IgG-HRP was supplied by Millipore (Chicago, IL, USA), anti-sheep IgG-HRP was supplied by Abcam, and anti-rabbit IgG-HRP was supplied by Santa Cruz Biotechnology. Cell culture regents (DMEM, L-glutamine, penicillin / streptomycin, sodium pyruvate, Trypsin-EDTA) were from Thermo Fisher Scientific (Scoresby, VIC, Australia), fetal bovine serum (FBS) was from Bovogen (Keilor, VIC, Australia). Nitrocellulose was supplied by GE Healthcare (Buckinghamshire, England, UK), minicomplete protease inhibitor cocktail tablets were obtained from Roche (Sandhoferstrasse, Mannheim, Germany). Bovine serum albumin was purchased from Research Organics (Cleveland, OH, USA). Mowiol 4-88 was from Millipore, paraformaldehyde (PFA) was obtained from ProSciTech (Thuringowa, QLD, Australia). Dynamin inhibitors, Dynasore and Dyngo 4a were purchased from Tocris Bioscience (Bristol, England, UK) and Abcam, respectively.

Immunofluorescent localization

Mouse epididymides were fixed in fresh Bouin's solution, embedded in paraffin and sectioned at 5 μm thickness. Embedded tissue was dewaxed, rehydrated, and then subjected to antigen retrieval under optimized conditions: microwaving in 10 mM sodium citrate at 1,100 W for either 6 min (anti-dynamin 1, ab108458) or 9 min (anti-dynamin 2); microwaving in 50 mM Tris (pH 10.5) for 9 min [anti-dynamin 1 (MA5-15285); anti-dynamin 3; anti-ATP6V1B1; anti-

golgin-97]. After being blocked with 3% BSA/PBS in a humid chamber (1 h at 37ºC), the slides were then incubated with primary antibodies diluted 1:50 in 1% BSA/PBS (4ºC, overnight). After three washes in PBS, slides were incubated with Alexa Fluor 555 and/or Alexa Fluor 488 conjugated secondary antibodies diluted 1:400 in 1% BSA/PBS (37ºC, 1 h). The sections were then washed and counterstained with nuclear dyes; propidium iodide (5 μg/ml) or 4', 6-diamidino-2-phenylindole (2 μg/ml). After an additional wash in PBS, slides were mounted in 10% Mowiol 4-88 (Merck Millipore, Darmstadt, Germany) with 30% glycerol in 0.2 M Tris (pH 8.5) and 2.5% 1, 4-diazabicyclo-(2.2.2)-octane (DABCO) and labeling patterns for all tissue sections recorded using fluorescence microscopy (Zeiss Axio Imager A1, Jena, Thuringia, Germany; Figures 1-5). The wavelengths of the microscopic filters used for excitation and emission are 474 nm and ~527 nm (Alexa Fluor 488 and propidium iodide), 585 nm and ~615 nm (Alexa Fluor 594). Alternatively, confocal microscopy (Olympus IX81, Sydney, Australia) was used for detection of fluorescent labeling patterns observed in mEcap18 cells (Figures 6 and 8) using excitation and emission filters of wavelength 473 nm and 485-545 nm (Alexa Fluor 488), and 559 and 570-670 nm (propidium iodide).

For immunofluorescent staining of mouse caput epididymal (mEcap18) cell cultures [49], the cells were settled onto poly-L-lysine coated coverslips. They were then fixed in 4% PFA for 15 min and permeabilized by incubation in 0.1% Triton X-100 for 10 min. Following washing in PBS, cells were blocked with 3% BSA in PBS and immunolabeled as described for epididymal tissue sections.

All immunolocalization studies were replicated a minimum of 3 times, with epididymal tissue sections being prepared from more than three different male mice or mEcap18 cells being

isolated from three separate cell cultures. The negative controls used in each of these experiments included tissues or cells that were prepared under the same conditions except that the primary antibody was substituted with antibody buffer (i.e. secondary antibody only controls). Where the immunizing peptide was available (i.e., for anti-DNM2 and anti-DNM3 antibodies), an additional control was included in which the antidynamin antibodies were pre-absorbed with excess immunizing peptide prior to use.

mEcap18 cell culture and dynamin inhibition assays

The SV40-immortalized mouse caput epididymal epithelial (mECap18) cells were a generous gift from Dr Petra Sipila (Turku University, Turku, Finland) [49]. Aliquots of 4×10^5 cells were passaged in each well of six well plates and cultured with mEcap18 medium (DMEM supplemented with 1% L-glutamine, 1% sodium pyruvate, 1% penicillin / streptomycin and 50 μM 5α-androstan-17β-ol-3-oneC-IIIN) containing 10% fetal bovine serum (FBS) for 24 h. Cells were then washed three times with DMEM to remove FBS and thus the potential of this protein to bind dynamin inhibitors [50, 51]. Thereafter, equal volumes of mEcap18 medium (FBS free) containing Dynasore, Dyngo 4a, Dyngo-ϴ (an inactive isoform for both Dynasore and Dyngo 4a) or a DMSO vehicle control were added to each well for further incubation. The working concentration of each inhibitor (10 μM for Dyngo 4a and 100 μM for Dynasore) was selected on the basis of effective doses in previous work [52]. After 12 h of incubation, media was carefully aspirated from each of the different treatment groups and centrifuged under 2,000 × g for 10 min to remove all the cellular debris. Proteins released into the media during the incubation were then concentrated via precipitation with one-fifth volume of chilled 100% trichloroacetic acid (4ºC, overnight). The precipitated protein was pelleted by centrifugation

(17,000 × g, 4 ºC, 10 min) and washed twice with chilled acetone prior to being re-centrifuged under identical conditions. The resultant pellet was air-dried before being re-suspended in SDS extraction buffer (0.375 M Tris pH 6.8, 2% w/v SDS, 10% w/v sucrose, protease inhibitor cocktail). To ensure that proteins were not simply released from dead or moribund cells, cell vitality was assessed via a trypan blue exclusion assay prior to, during, and after incubation with dynamin inhibitors. Importantly, none of the treatments used in this study compromised mEcap18 cell viability, which consistently remained >90% across the 12 h of incubation.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), silver staining, and immunoblotting

Epididymal dissection and fluids removal was conducted as previously described [53]. Following treatment, epididymal proteins were separately extracted from the caput, corpus and caudal segments via boiling in SDS extraction buffer at 100°C for 5 min. Insoluble material was pelleted by centrifugation (17,000 × g, 10 min, 4°C) and the soluble proteins present in the supernatant were quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Equivalent amounts of protein were boiled in SDS-PAGE sample buffer (2% v/v mercaptoethanol, 2% w/v SDS, and 10% w/v sucrose in 0.375 M Tris, pH 6.8, with bromophenol blue) at 100°C for 5 min, prior to being resolved by SDS-PAGE and either silver stained or transferred to nitrocellulose membranes. Before detecting proteins of interest, membranes were blocked under optimized conditions of 3% BSA in PBS with 0.5% (v/v) Tween-20 (PBST; dynamin 1), 3% BSA in Tris-buffered saline with 0.1% (v/v) Tween-20 (TBST; IZUMO1, CCT8, α-tubulin, dynamin 3, and dynamin pSer778), 5% skim milk in 0.1%

(v/v) TBST (dynamin 2, FLOT1 and PSMD7) or 5% skim milk in 0.05% PBST (CCT3) for 1 h. Membranes were incubated with primary antibody prepared in either 1% BSA or 1% skim milk in an equivalent diluent to that used for blocking. Blots were subsequently washed with 0.5% PBST (dynamin 1), 0.1% TBST (dynamin 2, dynamin 3, FLOT1, IZUMO1, CCT8, α-tubulin, PSMD7 and dynamin pSer778) or 0.05% PBST (CCT3), followed by incubation with appropriate horse radish peroxidase (HRP) conjugated secondary antibodies (Supplemental Table S1). After three additional washes, labeled proteins were detected using an enhanced chemiluminescence kit (GE Healthcare). The specificity of dynamin 3 antibody was assessed by pre-incubating the antibody with excess immunizing peptide at 4ºC for 2 h prior to immunoblotting. For quantification of dynamin expression, appropriate bands were assessed by densitometry, normalized against an α-tubulin loading control, and nominally expressed relative to the amount of the protein appearing in the caput epididymal tissue within the same developmental time point (Figure 1M, 2M, and 4M). Alternatively, dynamin expression was also quantified based on normalization against the α-tubulin loading control across all epididymal segments and developmental time points examined (Supplemental Figure S2).

Electron microscopy

Samples were fixed and processed for electron microscopy as previously described [54]. Briefly, epididymal tissue and mEcap18 cells were fixed in 4% (w/v) PFA containing 0.5% (v/v) glutaraldehyde. Epididymal tissue and mEcap18 cell [embedded in 2% (w/v) agarose] were processed via dehydration, infiltration and embedding in LR White resin. Sections (80 nm) were cut with a diamond knife (Diatome Ltd, Bienne, Switzerland) on an EM UC6

ultramicrotome (Leica Microsystems, Vienna, Austria) and placed on 200-mesh nickel grids. Sections were blocked in 3% (w/v) BSA in PBS (30 min). Subsequent washes were performed in PBS (pH 7.4) containing 1% BSA. Sections were incubated with primary antibodies (overnight at 4°C) and an appropriate secondary antibody conjugated to 10 nm gold particles (90 min at 37°C). Labeled sections were then counterstained in 2% (w/v) uranyl acetate. Micrographs were taken on a Philips CM12 transmission electron microscope at 120kV.

Statistics

All experiments were replicated a minimum of 3 times, with tissue samples obtained from ≥3 different male mice. Graphical data are presented as mean values ± SEM, which were calculated from the variance between samples. Statistical significance was determined analysis of variance.

RESULTS

Localization and ontogeny of dynamin expression in the mouse epididymis

Dynamin 1 – Low-magnification fluorescence micrographs illustrating the overall expression patterns of dynamin 1 in the initial segment and epididymis are presented in Supplemental Figure S1 and Figure 1A-L, respectively. In the pre-pubertal epididymis (post-natal 10 days), positive dynamin 1 labeling was detected uniformly throughout the epithelium of all epididymal segments. In marked contrast, by peri-pubertal development (30 days) and extending into adulthood (>8 weeks), only weak diffuse dynamin 1 labeling was observed in the cytosol of cells in the initial segment (Supplemental Figure S1D and G) and caput epididymis (Figure 1E and I). Upon transitioning into the distal epididymal regions of the corpus and cauda, the pattern of dynamin 1 expression was abruptly replaced by one in which a majority of cells were

completely devoid of the enzyme. Notably however, dynamin 1 was intensely labeled in a small number of discrete, randomly distributed cells in both the corpus and cauda epididymal segments. The labeling of these large cells generally extended from the apical to the basal surfaces of the tubule, consistent with the distribution pattern expected of clear cells, a possibility that was directly assessed in subsequent experiments. The specificity of antibody labeling was confirmed by the complete absence of labelling in equivalent tissue sections probed with secondary antibody alone (Figure 1D, H, and L).

Immunoblotting of epididymal tissue homogenates confirmed the expression of dynamin 1 in all segments and at all developmental time points examined (Figure 1M). Of note was the labeling of two discrete protein bands of approximate molecular weight ~100 and ~102 kDa in a majority of the tissue samples. The lower of these bands corresponds to the known molecular weight (100 kDa) of dynamin 1, raising the possibility the higher band may reflect the presence of a post-translationally modified form of the parent protein. Such a scenario was assessed through the labeling of tissue homogenates with phospho-specific antibodies that detect dynamin 1 serine 778 phosphorylation. These antibodies consistently labeled the higher molecular weight band only (Figure 1N), a finding that is of potential significance in view of the ability of phosphorylation to modulate dynamin 1 activity [55, 56]. In this context, the higher molecular weight (phosphorylated) form of dynamin 1 predominated in the epididymides of 10- and 30-day-old animals; yet, the lower molecular weight unmodified protein was intensely stained in the epididymis of adult animals. The highest expression of phosphorylated dynamin 1 was recorded in the cauda epididymides of 30-day-old animals (Supplemental Figure S2). Since dynamin 1, and dynamin 2, are known to reside in mouse

spermatozoa, all immunoblots were re-probed with antibodies against IZUMO1 (a protein expressed in spermatozoa but not epididymal tissue) to control for the possibility of sperm contamination. As anticipated, no IZUMO1 was detected in any of our preparations of epididymal tissue (Figure 1M, 2Q).

Dynamin 2 – Similar to the expression profile of dynamin 1, the second isoform of the dynamin family was also readily detected throughout the epithelium of the entire epididymis of pre-pubertal animals (Supplemental Figure S1B; Figure 2A-L). However, in peri-pubertal and adult animals, dynamin 2 was predominantly localized to the supranuclear region of caput epithelial cells where it appeared to be concentrated within dense aggregates most likely corresponding to the Golgi apparatus (Figure 2E and I, asterisks). In the corpus, and particularly the cauda, epididymides of these animals, the majority of staining was detected in the immediate vicinity of luminal border (Figure 2F, G, J, K) and extending into apical blebs that appear to decorate these cells (Figure 2J, see inset in lower panel). Presumably due to issues associated with antigen retrieval [16], luminal spermatozoa were not routinely labeled with dynamin 2 in the adult epididymal sections. Immunoblotting confirmed the abundant epididymal expression of dynamin 2 and revealed that the greatest increase in dynamin 2 expression along the epididymis was detected in the caudal segment of 30-day-old animals (Supplemental Figure S2). In the pre-pubertal and adult stage, the enzyme was expressed at similar overall levels in each epididymal segment examined (Figure 2M; Supplemental Figure S2).

The localization we recorded for dynamin 2, particularly within the caput segment of the adult epididymis, ideally positions the enzyme to contribute to the trafficking of secretory proteins to

316 the luminal environment [57]. We therefore sought to assess the spatial expression profile and
317 the subcellular localization of dynamin 2 within this segment in greater detail. This analysis
318 revealed that in the initial segment (zone 1) [55], dynamin 2 was exclusively restricted to the
319 apical membrane (Figure 3A; Supplemental Figure S1). Notably, supranuclear labeling was
320 first detected immediately distal to the septa delineating the initial segment from that of the
321 caput epididymis (Figure 3A-D; zones 2-5), and appeared most intense within zones 2-3 before
322 gradually declining to be virtually undetectable in this subcellular domain by zone 6 (corpus
323 epididymis). Confirmation that this pattern of supranuclear localization corresponded to the
324 positioning of the Golgi apparatus was afforded by labeling of consecutive epididymal sections
325 with anti-DNM2 (Figure 3E, red) and Golgin-97 (a recognized Golgi marker; Figure 3F, green).
326 This approach was favored over that of dual labeling owing to incompatible antigen retrieval
327 conditions necessary for optimal labeling with these antibodies. Importantly, no such staining
328 was recorded in negative control sections (secondary antibody only; Figure 3G and H).
329 Similarly, pre-absorption of the antidynamin 2 antibody with excess immunizing peptide also
330 effectively eliminated all immunolabeling of epididymal tissue sections (Supplemental Figure
331 S3).

332 Consistent with the localization of dynamin 2 detected by immunofluorescence,
333 ultrastructural analyses confirmed the presence of immunogold labeled dynamin 2 within the
334 cisternae of the Golgi apparatus in the caput epididymis (Figure 3I, arrowheads). In the more
335 distal segments of the corpus and cauda epididymis, immunogold labeled dynamin 2 was not
336 detected within the Golgi apparatus (data not shown), being instead localized to the microvilli
337 and apical blebs extending from the luminal margin of principal cells (Figure 3J and K). Gold

labeled dynamin 2 was also routinely found in the acrosomal region of sperm residing in the epididymal lumen (data not shown). The specificity of immunogold labeling was confirmed through the use of sections stained with secondary antibody alone, none of which revealed any staining (Figure 3L).

Dynamin 3 – Unlike dynamin isoforms 1 and 2, only relatively weak dynamin 3 staining was observed in the cytosol of the pre-pubertal epididymis epithelial cells (Figure 4A-C; Supplemental Figure S1). This labeling pattern subsequently underwent substantial changes in the epididymis of peri-pubertal and adult animals. Thus, dynamin 3 was localized to the apical domain / luminal margin of a small handful of epithelial cells that were randomly dispersed through the tubules of the caput epididymis (Figure 4E and I). Upon entry into more distal epididymal segments, dynamin 3 gradually took on a unique expression profile in which virtually all corpus and cauda epididymal epithelial cells, save those likely to be clear cells, were uniformly stained throughout their cytosol (Figure 4F, G, J and K). Interestingly, dynamin 3 was also labeled in granule-like luminal structures previously referred to as 'epididymal dense bodies' [58] that lie juxtaposed with spermatozoa in the corpus and cauda epididymis (Figure 4K, inset). Few such structures were labeled for dynamin 3 in the epididymis of peri-pubertal animals and similarly, no such labeling was observed in the lumen of the caput epididymis at any developmental time point. Since our previous work has shown that mature mouse sperm do not harbor the dynamin 3 isoform [59], it is unlikely that it features among the proteins that are putatively transferred between dense bodies and the maturing spermatozoa [58, 60, 61]. Importantly, no staining was recorded in negative control sections (secondary antibody only;

Figure 4D, H, and L). Similarly, pre-absorption of the antidynamin 3 antibody with excess immunizing peptide also effectively eliminated all immunolabeling of epididymal tissue sections (Supplemental Figure S3).

Immunoblotting of epididymal tissue homogenates confirmed the expression of dynamin 3 in all segments and at all developmental time points examined (Figure 4M). Similar to the dynamin 1 and dynamin 2 isoforms, increased expression of the dynamin 3 protein was apparent within the epididymides of 30-day-old animals (Supplemental Figure S2). The conserved increase in expression documented at this particular developmental stage may reflect the epididymis preparing for the arrival of first wave of spermatozoa.

Co-localization of dynamin 1 and 3 with ATP6V1B1 in clear cells of the adult mouse epididymis

A notable finding from our immunolocalization studies was that the dynamin isoforms examined did not appear to show a high degree of co-localization. This was particularly true of the labeling patterns of dynamins 1 and 3 within the corpus and cauda epididymis of mature animals (Figure 1J and K; Figure 4J and K). To investigate whether dynamin isoforms are indeed expressed in unique cell populations, dual staining of epididymal tissue was conducted with anti-dynamin 1 and 3 antibodies. This strategy revealed that the distribution of dynamin 1 and 3 perfectly complemented each other with no co-localization apparent in either the corpus (not shown) or cauda epididymis (Figure 5A-C). The most logical explanation for such an expression profile is that dynamin 1 and 3 are exclusively produced in clear and principal cells, respectively. This possibility was examined through co-labeling experiments with ATP6V1B1 (ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B1), a clear cell marker that

mediates the acidification of the luminal environment [62]. As anticipated, dynamin 1 co-localized with ATP6V1B1 in the clear cells of the corpus and cauda epididymis (Figure 5E and F), but was not detected in this cell population in the caput epididymis (Figure 5D). By contrast, dynamin 3 co-localized with ATP6V1B1 in the clear cells of the caput epididymis (Figure 5G) but failed to overlap with the clear cells in more distal epididymal segments (Figure 5H and I).

Selective inhibition of dynamin influences epididymal protein secretion *in vitro*

The existence of unique, non-overlapping profiles of dynamin expression raises the prospect that this family of enzymes may be of fundamental importance in regulating the specialized functions of the epididymis. We therefore sought to document changes in protein trafficking brought about by selective pharmacological inhibition of dynamin. For this purpose, we elected to use a tractable *in vitro* assay employing an immortalized mouse caput epididymal (mEcap18) cell line that has previously been characterized in relation to its ability to faithfully report physiological profiles of epididymal gene and protein expression [49]. Prior to use, these cells were assessed for their expression of dynamin 1, 2 and 3 isoforms (Figure 6A-C) as well as androgen receptor and ATP6V1B1 (Supplemental Figure S4). Consistent with our labeling of caput epididymal tissue sections (Figure 1I and 2I), dynamin 1 was localized throughout the cytosol (Figure 6A) and dynamin 2 was found within the supranuclear domain of a majority of mEcap18 cells (Figure 6B). Dynamin 3 by contrast, exhibited discrete foci of membrane staining in a small number of these cells (Figure 6C), the proportion of which compared favorably to those expressing ATP6V1B1 (Supplemental Figure S4). On the basis of these conserved expression patterns, the mEcap18 cells were deemed a suitable model to explore

dynamin function.

Following incubation of mEcap18 cells in media supplemented with and without the dynamin inhibitors of Dynasore and Dyngo 4a (both of which target dynamin 1 and dynamin 2 with similar efficacy [63, 64]), an equivalent volume of culture medium was recovered for assessment via SDS-PAGE. As shown in Figure 7A, mEcap18 cells readily secreted a number of proteins into the medium during the course of a 12 h incubation. However, the secretion of several of these proteins appeared to be reduced by the introduction of dynamin inhibitors (Figure 7A). This result was confirmed through the quantification of band intensity, normalized against an internal loading control (green arrowhead) (Figure 7B), which illustrated bands of Mr ~26, 30, 34, 42, 45, 47, 65, 80, 110, 115, and 250 kDa were all substantially reduced following dynamin inhibition (Figure 7A, white arrowheads; Figure 7B, green trace). In the majority of instances, this inhibitory effect proved selective such that the proteins were detected at similar levels in the medium sampled from either untreated control populations of cells (Figure 7B, orange trace) or those cells treated with Dyngo-ϴ (an inactive analog of Dynasore and Dyngo 4a; Figure 7B, black trace). From these data, we infer that a subset of epididymal proteins may rely on dynamin-mediated pathways for their secretion.

In support of this hypothesis, we investigated the release of two representative 65 kDa and one 47kDa protein [namely: chaperonin containing TCP1, subunit 3 (CCT3); chaperonin containing TCP1, subunit 8 (CCT8); flotillin 1 (FLOT1), respectively] that are secreted in the caput epididymis (Nixon, unpublished). Using a similar strategy to that reported above, dynamin inhibition was shown to effectively reduce the amount of both CCT3 and CCT8 that was detectable in the incubation media following 12 h of mEcap18 cell culture (Figure 8A).

Notably, dynamin inhibition was also accompanied by an apparent increase in the amount of both proteins detected within the cytosol of mEcap18 cells compared to that of untreated controls (Figure 8B). In a majority of these cells, the staining of the CCT3 and CCT8 appeared to concentrate in numerous punctate foci. While such localization is consistent with that expected of proteins that had been packaged into secretory vesicles, in the absence of direct evidence the precise nature of reaction foci remains to be determined. In contrast, no such inhibition was detected for PSMD7 a protein that has previously been detected in the proteome of bovine caput epididymosomes [65]. Importantly, dynamin inhibition did not have a detrimental impact on mEcap18 cell viability, which remained above 90% in all treatments. In the case of Dynasore, we did note a reduction in the number cells before (~4×10^5) and after (~2.9×10^5) incubation. However, no such reduction was evident in cells treated with Dyngo 4a.

DISCUSSION

The mammalian epididymis holds an essential role in promoting the functional maturation of spermatozoa, in addition to their prolonged storage in a viable state [3, 13]. Both processes are supported by a highly specialized luminal microenvironment that is created, and maintained, by the combined secretory and absorptive activity of the lining epithelium. While elegant ultrastructural studies have defined the key cytological features of this epithelia [6-8], the molecular machinery it employs to regulate the tightly coupled processes of exocytosis and endocytosis remain poorly understood. In this study, we have explored the epididymal expression of dynamin, revealing a number of unique insights into the localization and putative

function(s) of this mechanoenzyme. Namely, we show that the three canonical dynamin isoforms possess different spatial and temporal profiles of expression within the mouse epididymis. Thus, in juvenile animals at a time when the epididymis is undergoing considerable elongation and expansion, dynamins 1 – 3 displayed virtually ubiquitous patterns of localization raising the possibility that they have overlapping roles in regulating the differentiation of the tract. However, with the notable exception of the initial segment, each dynamin partitioned into distinct cells types and/or subcellular compartments prior to entry of the first wave of the spermatozoa, thus suggesting that they may possess fundamentally distinct roles in the secretory and absorptive pathways that dominate the functioning of the adult epididymis. This interpretation is consistent with our ability to selectively manipulate protein secretion through pharmacological inhibition of dynamin in an immortalized caput epididymal cell line.

The exceptional metabolic and secretory activity of the epididymal epithelium is well established, with conservative estimates indicating it is capable of synthesizing and selectively releasing several hundred proteins into the luminal environment [2, 9, 66]. Such activity predominantly resides within the anterior portion of the organ; the principal cells of the caput epididymis being responsible for the synthesis of ~70 - 80% of the overall epididymal secretome [67, 68]. These proteins enter the epididymal lumen via one of two key secretory pathways: (i) a classical merocrine pathway or (ii) an alternative form of apocrine secretion [69]. The former of these is a highly regulated exocytotic process whereby proteins are synthesized in the endoplasmic reticulum before being modified and packaged into large secretory vesicles in the Golgi apparatus [70]. Upon receipt of appropriate physiological stimuli, these vesicles move towards the plasma membrane and release their contents into the

epididymal lumen via the formation of transient fusion pores [70]. On the basis of its localization within the Golgi apparatus of caput principal cells we infer that the dynamin 2 isoform may be a key component of the trafficking machinery involved in regulating the merocrine secretory pathway. Specifically, we postulate that dynamin 2 mediates the production and/or scission of post-Golgi secretory vesicles. Consistent with this notion, independent studies have proven the necessity of dynamin 2 for protein processing in the Golgi apparatus [25, 26] as well in the post-Golgi transportation of secretory proteins [23]. Such roles are also commensurate with our demonstration that dynamin inhibition suppresses the release of a subset, but certainly not all, proteins from an immortalized caput epididymal cell line. It is noteworthy that these proteins include members of the chaperonin containing T-complex protein 1 (TCP1) complex (i.e. CCT3 and CCT8) that have previously been implicated in regulating key aspects of sperm function [71].

Although dynamin 2 retained its association with principal cells in epididymal segments that lie immediate proximal (initial segment) and distal (corpus and cauda) to that of the caput, it was characterized by a marked redistribution to the adluminal border of these cells. Notably, this location is compatible with dynamin 2 fulfilling ancillary roles in either the endocytotic uptake of luminal contents and/or in modulating the fusion of intracellular secretory vesicles with the plasma membrane [8]. In support of the latter mechanism, recent evidence indicates that dynamin can control the rate of fusion pore expansion [39, 72] and thus fine-tune the amount of cargo released to the extracellular space during exocytosis [28]. Nevertheless, the detection of dynamin 2 in apical protrusions extending from the principal cells of the corpus and cauda epididymis, raise the prospect that it may contribute to the apocrine mode of secretion

498 employed by these cells [69]. This pathway serves as a secretory mechanism for proteins
499 synthesized on free ribosomes and lacking an endoplasmic reticulum signal peptide sequence.
500 Such proteins are believed to be either synthesized in or directed to apical blebs; large
501 protrusions that project from the apical cytoplasm into the lumen before detaching from the cell
502 surface and subsequently fragmenting to generate a highly heterogeneous population of small
503 membrane-bound vesicles known as epididymosomes [73]. Although the mechanism(s)
504 underpinning the detachment of apical blebs is yet to be fully resolved, the relatively large areas
505 of continuity that exist between these structures and the apical plasma membrane of principal
506 cells [69] would appear to be incompatible with dynamin mediated scission. Indeed, when
507 assembled in the absence of guanosine triphosphate (GTP), the non-constricted dynamin helix
508 is capable of surrounding a membrane tube with an inner and outer radius of only 10 and 25
509 nm, respectively [18]. Despite this, detailed ultrastructural studies have revealed that the
510 scission of apical blebs is likely to proceed gradually in a process characterized by involution
511 of the plasma membrane and formation of multiple fissures between the blebs and apical
512 cytoplasm [69]. This eventually yields narrow stalk-like attachments, the diameter of which
513 may be more in keeping with the structural characteristics of dynamin helices.

514 In marked contrast to dynamin 2, the localization of dynamin 1 and dynamin 3 isoforms
515 alternated between the principal cell population in some segments and that of the clear cells in
516 other segments of the adult epididymis. Specifically, dynamin 1 was detected throughout the
517 cytosol of caput principal cells before being found exclusively within clear cells in more distal
518 regions (corpus and cauda). Conversely, dynamin 3 was characterized by a reciprocal pattern
519 of expression whereby it was detected in clear cells in the caput epididymis before localizing

throughout the cytosol of corpus and cauda principal cells. This intriguing relationship was confirmed through dual labeling experiments, which demonstrated that the two proteins localized to distinct, non-overlapping cell populations. These data contrast the overlapping localization, and the concomitant redundant functions, that have previously been described for dynamin 1 and 3 in neuronal tissues [74], but are similar to that of dynamin 1 and 3 in mammalian germ cells and their supporting Sertoli cell population in the testes [17, 47, 75]. We remain uncertain why this situation may have arisen in the male reproductive tissue and whether these variants fulfil similar or unique functions in these cells. Nevertheless, we did note that dynamin 3 expression was restricted to the apical membrane and subapical domain of clear cells, whereas this polarity was not shared with dynamin 1; this isoform was instead preferentially located throughout the cytosol of clear cells.

The significance of dynamin expression in clear cells is emphasized by the key role this population of cells play in luminal acidification as well as their pronounced endocytotic activity [11, 76, 77]. The latter of these has been linked to the selective clearance of proteins [77] and other macromolecular entities from the epididymal lumen, including cytoplasmic droplets that are shed from maturing spermatozoa [11]. Thus, the apical membrane / subapical domains of clear cells are known to be populated with a heterogeneous assembly of endocytotic structures including: coated and uncoated pits, numerous small vesicular elements (150 – 200 nm) and larger membrane bound endosomes [11]. It is therefore tempting to speculate that dynamin 3 may contribute to the selective uptake and recycling of luminal material. By contrast, the diffuse cytosolic labeling of dynamin 1 is consistent with that observed for ATP6V1B1, a subunit of the proton-pumping ATPase (V-ATPase) that is highly enriched in clear cells and responsible

for luminal acidification [78, 79]. The positioning of the V-ATPase enzyme complex within the apical pole of cells has previously been shown to be tied to the dynamic remodeling of the actin cytoskeleton [80, 81], as well as being acutely sensitive to inhibition of exocytotic events, such that treatment with microtubule-disrupting agents (colchicine) or cleavage of cellubrevin [a vesicle soluble N-ethylmalemide-sensitive factor attachment protein receptor (v-SNARE)] [82], both lead to a redistribution of the complex throughout the cytosol. Such findings are of interest owing to the fact that dynamin is known to collaborate with SNARE proteins to mediate vesicle trafficking, as well as having been implicated in the regulation of actin cytoskeleton dynamics [27]. Taken together, these data raise the possibility that the cytosolic localization of dynamin 1 in clear cells may be linked to V-ATPase positioning / recycling within these cells, and thus the acidification of the epididymal lumen.

In conclusion, we have shown that three canonical isoforms of dynamin are highly expressed in the mouse epididymis and appropriately positioned to fulfil regulatory roles in vesicle trafficking events that underpin the extraordinary secretory and abortive activity of this specialized region of the male reproductive tract. Despite sharing more than 80% sequence homology, this family of mechanoenzymes were clearly distinguishable on the basis of their cellular and sub-cellular localization thus arguing that they possess unique, rather than overlapping, modes of action within the epididymal epithelium. These results challenge the redundant roles proposed for dynamin isoforms in other tissues and encourage further investigation of the mechanism that regulate the differential expression profiles of dynamin expression within the epididymis. It will also be of considerable interest to determine the functional implications of dynamin in the context of sperm maturation and storage.

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FIGURE LEGENDS

Figure 1. Detection of dynamin 1 in the mouse epididymis. A-L) The spatial and temporal localization of dynamin 1 (arrowheads) was examined in the mouse epididymis at key developmental stages (day 10, 30 and >8 weeks postnatum) by sequential labeling with anti-dynamin 1 (DNM1, green) and the propidium iodide (PI, red) nuclear stain. Representative negative control (Neg, secondary antibody only) images are included to demonstrate the specificity of antibody labeling. ep, epithelial cells; sp, sperm; Int, interstitium; l, lumen. M) The relative levels of dynamin 1 expression were quantified by immunoblotting of tissue homogenates prepared from epididymides at equivalent developmental time points. Blots were subsequently stripped and re-probed with anti - α-tubulin antibody to confirm equivalent protein loading and enable densitometric analysis of band intensity (n=3; * $p < 0.05$). For the purpose of this analysis the labeling intensity of DNM1, or phosphorylated-DNM1 (Phos-DNM1), was

normalized relative to that of α-tubulin. Further, band intensity in caput tissue at each timepoint was nominally set to a normalized value of 1. Prior to protein extraction tissue was cleared of contaminating epididymal fluid and spermatozoa, and the efficacy of this treatment was assessed by labeling with anti-IZUMO1 antibodies (an intrinsic sperm protein that is not expressed in epididymal epithelium). N) The detection of a doublet (of ~100 and 102 kDa) with anti-dynamin 1 antibodies prompted an investigation of the potential for post-translational phosphorylation of the dynamin 1 protein. For this purpose, blots were probed with anti-dynamin 1 pSer778 antibodies, revealing cross-reactivity with the higher molecular weight band only. These experiments were replicated on material from 3 animals and representative immunofluorescence images and immunoblots are presented.

Figure 2. Detection of dynamin 2 in the mouse epididymis. A-L) Immunofluorescence localization of dynamin 2 (arrowheads) was undertaken in the mouse epididymis (day 10, 30 and >8 weeks postnatum) by sequential labeling with anti-dynamin 2 (DNM2, green) and propidium iodide (PI, red). By 30 days post-natum dynamin 2 localization was detected in the supranuclear region of caput epithelial cells (asterisks) and around the adluminal border and extending into apical blebs (ab) (arrows and inset in adult corpus) in the corpus and cauda epididymal segments. Representative negative control (Neg, secondary antibody only) images are included to demonstrate the specificity of antibody labeling (D, H, L). ep, epithelial cells; sp, sperm; Int, interstitium; l, lumen. M) The relative levels of dynamin 2 expression were quantified by immunoblotting of tissue homogenates prepared from epididymides at equivalent developmental time points. Blots were subsequently stripped and re-probed with anti - α-tubulin antibody to confirm equivalent protein loading and enable densitometric analysis of band

intensity (n=3). For the purpose of this analysis the labeling intensity of DNM2 was normalized relative to that of α-tubulin. Further, band intensity in caput tissue at each timepoint was nominally set to a normalized value of 1. Immunoblots were also probed with anti-IZUMO1 antibodies to control for sperm contamination. These experiments were replicated on material from 3 animals and representative immunofluorescence images and immunoblots are presented.

Figure 3. Dynamin 2 localizes to the Golgi apparatus of principal cells in the caput epithelium. A-D) The spatial conservation of dynamin 2 supranuclear localization was assessed throughout zones 1 – 5 (corresponding to the initial segment and caput epididymis, respectively) of the adult mouse epididymis, with the border of different zones being demarcated by dotted lines. This analysis revealed a gradient of supranuclear staining, being initially detected in zone 2 and most intense staining in zones 2 and 3, before gradually decreasing distally in zones 4 and 5, and being undetectable in zone 6 (corpus). E-H) Confirmation that this pattern of supranuclear localization corresponded to the positioning of the Golgi apparatus (arrowheads) was afforded by labeling of consecutive epididymal sections with anti-DNM2 (E, red) and Golgin-97 (a recognized Golgi marker; F, green). This approach was favored over that of dual labeling owing to incompatible antigen retrieval conditions necessary for optimal labeling with these antibodies. G, H) NC: negative controls (secondary antibody only). These experiments were replicated on material from 3 animals and representative immunofluorescence images are presented.

Figure 4. Detection of dynamin 3 in the mouse epididymis. A-L) Immunofluorescence localization of dynamin 3 was undertaken in the mouse epididymis (day 10, 30 and >8 weeks postnatum) by sequential labeling with anti-dynamin 3 (DNM3, green) and propidium iodide

(PI, red). Representative negative control (Neg, secondary antibody only) images are also shown to demonstrate the specificity of antibody labeling. M) The relative levels of dynamin 3 expression were quantified by immunoblotting of tissue homogenates prepared from epididymides at equivalent developmental time points. Blots were subsequently stripped and re-probed with anti - α-tubulin antibody to confirm equivalent protein loading and enable densitometric analysis of band intensity (n=3). For the purpose of this analysis the labeling intensity of DNM3 was normalized relative to that of α-tubulin. Further, band intensity in caput tissue at each timepoint was nominally set to a normalized value of 1. These experiments were replicated on material from 3 animals and representative immunofluorescence images and immunoblots are presented.

Figure 5. Co-localization of dynamin 1 and dynamin 3 with the clear cell marker, ATP6V1B1. A-C) Representative immunofluorescence images of dual staining of dynamin 1 (red arrowhead) and dynamin 3 (green arrowhead) in the cauda epididymis of adult mice. Dynamin 1 and 3 clearly resided in different epithelial cell populations with no co-localization being detected. D-F) Representative immunofluorescence images of dual staining of dynamin 1 (green arrowheads) and ATP6V1B1 (red arrowheads) in the adult mouse epididymis. Dynamin 1 co-localized with ATP6V1B1 in the clear cells of the corpus and cauda but not caput epididymis. G-I) Representative immunofluorescence images of dual staining of dynamin 3 (green arrowheads) and ATP6V1B1 (red arrowheads) in the adult mouse epididymis. Dynamin 3 co-localized with ATP6V1B1 in the clear cells of the caput epithelium, but displayed minimal overlap in the cells and instead occupied a distinct sub-cellular location. This localization pattern was altered in the corpus and cauda epithelium such that dynamin 3 was uniquely

detected in the principal cells in these segments. ep, epithelial cells; int, interstitium; l, lumen.

Figure 6. Mouse mEcap 18 cells and epididymal epithelial tissue possess conserved patterns of dynamin expression. A-C) Immunofluorescence localization was conducted for each dynamin isoform (1 – 3) in fixed mEcap 18 cells (A: dynamin 1; B: dynamin 2; C: dynamin 3). A) Staining for dynamin 1 (DNM1) was localized throughout the cytosol. B) Dynamin 2 (DNM2) localized to the supranuclear domain in the majority of the cells. C) Dynamin 3 (DNM3) localized exclusively to a portion of the plasma membrane in ~11% of the cell population. For A-C, nuclei are labeled with PI (red). Arrowheads indicate representative labeling patterns observed across three independent experiments.

Figure 7. Dynamin inhibitors selectively modulate the secretion of proteins by cultured mouse mEcap18 cells. A) Silver stained gel illustrating the complement of proteins recovered from an equivalent volume of medium after 12 h of mEcap18 cell culture in the absence (control) , or presence of Dyngo-4a (10 μM; an inhibitor of dynamin isoforms 1 and 2) or Dyngo-ϴ (10 μM, inactive isoform for Dyngo-4a). B) The density of the bands were quantified by Image J and normalized to control bands (green arrow head) which exist in the medium..Dyngo-4a treatment selectively inhibited the secretion of a subset of protein bands such that they were substantially reduced or absent (denoted by white arrowheads).

Figure 8. Dynamin inhibitors selectively modulate the secretion of proteins by cultured mouse mEcap18 cells. A) Immunoblotting of three representative epididymal secretory proteins: CCT3, CCT8 and FLOT1 confirmed a significant decrease in abundance within the medium following treatment of mEcap18 cells with either Dynasore or Dyngo-4a dynamin inhibitors. By contrast, the abundance of an alternative epididymal secretory protein, PSMD7,

was not influenced by the presence of dynamin inhibitors. These blots also feature protein recovered from mEcap18 cells treated with the DMSO vehicle control (control) as well as an equivalent volume of cell free medium (medium only). B) Immunofluorescence detection of CCT3, CCT8, and FLOT1 within mEcap18 cells treated with Dynasore, Dyngo-4a or the DMSO vehicle control (control) for 12 h. Substantially more CCT3, CCT8 and FLOT1 were detected in mEcap18 cells treated with Dynasore or Dyngo-4a compared to that of the vehicle controls. In contrast, the abundance of PSMD7 was not influenced by the presence of dynamin inhibitors. These experiments were replicated 3 times and representative gels, immunofluorescence images and immunoblots are presented.

Supplementary Figure S1. Detection of dynamin isoforms in the mouse initial segment of the mouse epididymis. A-I) Immunofluorescence localization of each isoform (1-3) was undertaken in the mouse epididymis (day 10, 30 and >8 weeks postnatum) by sequential labeling with anti-dynamin antibodies (green) and propidium iodide (PI, red). ep, epithelial cells; sp, sperm; Int, interstitium; l, lumen. These experiments were replicated on material from 3 animals and representative immunofluorescence images are presented.

Supplemental Figure S2. Expression levels of dynamin protein in the developing mouse epididymis. The relative levels of dynamin protein expression were quantified by immunoblotting of tissue homogenates prepared from epididymides at key developmental time points (10 days, 30 days, >8 weeks). Blots were subsequently stripped and reprobed with anti-α-tubulin antibody to confirm equivalent protein loading and enable densitometric analysis of band intensity (n = 3). For the purpose of this analysis, the labeling intensity of each dynamin isoform was normalized relative to that of α-tubulin across all epididymal segments and

882 developmental time points examined. In this instance, band intensity in the day 10 caput tissue
883 was nominally set to a value of 1.
884 **Supplemental Figure S3.** Examination of the specificity of dynamin antibodies. (A) The
885 specificity of antidynamin 1, antidynamin 2, and antidynamin 3 antibodies was initially
886 examined by immunoblotting of tissue homogenates prepared from mouse epididymal tissue
887 alongside that of mouse brain (positive control for dynamin expression). In all instances, the
888 antidynamin antibodies labeled a predominant band of the appropriate molecular weight (~100
889 kDa, denoted by arrows) in both brain and epididymal tissue. (B–J) Where available
890 (antidynamin 2 and antidynamin 3), antibody specificity was also assessed by pre-absorption
891 of the antibody with excess immunizing peptide prior to conducting immunolabeling of
892 epididymal tissue sections. (B–E) In the case of antidynamin 2 (DNM2) antibody,
893 immunofluorescence localization in both the caput (B) and corpus epididymis (C) was
894 selectively eliminated (D, E) by preabsorption of the antibody with immunizing peptide (+IP).
895 (F–I) Similarly, in the case of anti-DNM3 antibody, immunofluorescence localization in both
896 the caput (F) and corpus epididymis (G) were also eliminated (H, I) following preabsorption of
897 anti-DNM3 antibody with immunizing peptide (+IP). (J, K) Given the detection of additional
898 cross-reactive bands in antidynamin 3 immunoblots (as shown in A), the specificity of DNM3
899 antibody was further examined by (J) immunoblotting of both epididymis and brain tissue
900 lysates following pre-absorption of DNM3 antibody with immunizing peptide (+IP). While this
901 treatment effectively eliminated labeling of the ~100 kDa protein in both cell lysates, this band
902 was able to be detected once the same membrane was stripped and reprobed with nonabsorbed
903 antibody (K).

Supplemental Figure S4. The mouse mEcap 18 cell line represents a heterogonous culture featuring a predominance of principal cells as well as clear cells that stained positive for ATP6V1B1. Immunofluorescent staining of the mEcap 18 cell line with the clear cell marker ATP6V1B1 (A, green) and the epithelial cell marker androgen receptor (B, green). Nuclei are labeled in red with propidium iodide (PI).

Supplemental Table S1. Details of antibodies used throughout this study.