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Identification and validation of mouse sperm proteins correlated with epididymal maturation

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Abstract

Sperm need to mature in the epididymis to become capable of fertilization. To understand the molecular mechanisms of mouse sperm maturation, we conducted a proteomic analysis using saturation dye labeling to identify proteins of caput and cauda epididymal sperm that exhibited differences in amounts or positions on two-dimensional gels. Of eight caput epididymal sperm-differential proteins, three were molecular chaperones and three were structural proteins. Of nine cauda epididymal sperm-differential proteins, six were enzymes of energy metabolism. To validate these proteins as markers of epididymal maturation, immunoblotting and immunofluorescence analyses were performed. During epididymal transit, heat shock protein 2 was eliminated with the cytoplasmic droplet and smooth muscle γ -actin exhibited reduced fluorescence from the anterior acrosome while the signal intensity of aldolase A increased, especially in the principal piece. Besides these changes, we observed protein spots, such as glutathione S-transferase mu 5 and the E2 component of pyruvate dehydrogenase complex, shifting to more basic isoelectric points, suggesting post-translational changes such as dephosphorylation occur during epididymal maturation. We conclude that most caput epididymal sperm-differential proteins contribute to the functional modification of sperm structures and that many cauda epididymal sperm-differential proteins are involved in ATP production that promotes sperm functions such as motility.

Keywords

caput epididymis; cauda epididymis; sperm maturation; sperm protein; Two-dimensional fluorescence difference gel electrophoresis

1 Introduction

During the course of spermatogenesis, sperm are produced in the seminiferous tubules of the testis. Once mature, the sperm are released into the lumen of the tubules where flow of the fluid transports them to the rete testis and subsequently to the efferent ducts. From there, the sperm pass sequentially through the caput epididymis, the corpus epididymis, and then to the cauda epididymis, where they are stored until ejaculation. As sperm transit through the epididymis, they undergo intrinsic biochemical and functional modifications that result in the acquisition of motility and the ability to become capacitated for fertilization [1]. Thus,

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the epididymis serves three major functions: transport, storage, and, most importantly, maturation.

Sperm from the caput epididymis and cauda epididymis exhibit structural differences. A readily apparent distinction between the two populations is that caput epididymal sperm possess a cytoplasmic droplet at the proximal region of the flagellum that migrates toward the distal region and is eventually shed during epididymal transit [2]. Additionally, remodeling of the acrosomal region may occur in some species during epididymal maturation. For example, the shape of the guinea pig sperm head changes drastically from a planar, tennis racket-shaped structure to a form shaped like a cupped hand or xistera, the hand-held basket used in jai alai [3]. On a biochemical level, acrosomal proteins undergo changes in their states of glycosylation [4–6]. The patterns of protein tyrosine phosphorylation also differ between caput and cauda epididymal sperm [7]. Other biochemical distinctions noted as sperm migrate from the caput to the cauda epididymis include: elevation of cyclic AMP (cAMP) [8], changes in composition of sperm lipids [9], the addition and elimination of sperm proteins [10], an enhancement in the density of surface negatively charged residues [11] and an increase in the oxidation of sulfhydryl groups of sperm nuclear and flagellar proteins [12,13].

The structural differences between caput and cauda epididymal sperm have functional correlates. Cauda epididymal sperm can bind to the zona pellucida after capacitation, however caput epididymal sperm are not capable of fertilization [7]. One of the most obvious differences is that cauda epididymal sperm acquire forward motility when placed in an appropriate medium while caput epididymal sperm exhibit weak, vibrational movements with no forward progress when placed in the same medium [14]. When the motility of hamster caput epididymal sperm is prematurely amplified (by experimentally increasing intracellular cAMP levels) their flagella fold back on themselves in a feature known as angulation. This phenomenon can be prevented in a dose-dependent manner by the addition of the sulfhydryl oxidant diamide prior to blocking cAMP degradation by the introduction of the phosphodiesterase inhibitor theophylline [15]. On the other hand, cauda epididymal sperm do not exhibit angularity when intracellular cAMP levels are raised [16]. These observations had led to the concept that epididymal maturation is correlated with the oxidation of protein sulfhydryls to disulfides that then serve to stabilize the flagellar structure against angulation when motility is stimulated by a rise in internal cAMP.

To extend our studies of sperm flagellar proteins and to address the role of epididymal maturation in promoting sperm motility, we carried out a large-scale proteomic analysis to identify mouse proteins that exhibit differences in pattern profiles between caput and cauda epididymal sperm. Sperm proteins were labeled with cyanine dyes (CyDyes) prior to two-dimensional fluorescence difference gel electrophoresis (2-D DIGE), enabling an accurate fluorescence analysis of differences in protein abundance between samples. As a result, we identified eight caput epididymal sperm-differential proteins and nine cauda epididymal sperm-differential proteins by 2-D DIGE and mass spectrometry analyses. Immunoblot analysis validated the 2-D DIGE results concerning the relative abundances of specific proteins in caput and cauda epididymal sperm. Immunofluorescence was used to determine the subcellular localization of four validated proteins in caput and cauda epididymal sperm to ascertain whether these proteins exhibited maturation-dependent differences in localizations.

2 Materials and Methods

2.1. Isolation of caput and cauda epididymal sperm

All animal procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Sperm were collected from the caput and cauda epididymides of male mice (CD1 retired breeders) (Charles River Laboratories, Wilmington, MA) by cutting the epididymides and extruding the sperm at 37°C into PBS (2.68 mM KCl, 136.09 mM NaCl, 1.47 mM KH₂PO₄, 8.07 mM Na₂HPO₄, pH 7.4) containing protease inhibitors (Roche Applied Science, Indianapolis, IN). Both caput and cauda epididymal sperm were purified by centrifugation at 400 × *g* for 20 min at room temperature through a 35% PureSperm 100 solution (MidAtlantic Diagnostics, Mt. Laurel, NJ) in PBS. Purified sperm were collected from the pellet, resuspended in PBS containing proteinase inhibitors at 4°C, counted, and assessed for purity.

2.2. Two-dimensional fluorescence difference gel electrophoresis

Three groups of six mice each were used to prepare samples of non-reduced proteins from caput and cauda epididymal sperm; likewise, similar sets of mice were used as sources of reduced proteins from caput and cauda epididymal sperm. Prior to lysis, nitrogen gas was layered over every sample and each successive step was carried out under nitrogen. The sperm preparations were lysed by sonication at 4°C in cell lysis buffer [8 M urea, 40 mM Tris-HCl, pH 8.0, 4% (w/v) CHAPS] containing protease inhibitors. The protein lysates were used for procedures immediately.

To identify proteins of caput epididymal sperm that exhibit more disulfide oxidation when extracted from cauda epididymal sperm, we examined sperm proteins from both epididymal regions before and after disulfide reduction. For comparison to extracts of proteins without disulfide reduction, 15 µg aliquots of each protein lysate (1 µg of protein lysate represents ~4 × 10⁴ sperm) were treated with 6 nmol Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) for 1 hr in the dark at 37°C to reduce protein disulfides. TCEP was used to reduce proteins because, at the concentration used, it exhibited negligible interference with the CyDye labeling reactions whereas, according to the manufacturer, other sulfhydryl reagents such as DTT and β-mercaptoethanol significantly reduce labeling. The CyDye DIGE Fluor fluorescent dye saturation labeling protocol (GE Healthcare, Piscataway, NJ) that enables maximal modification of cysteine residues was used to label the protein lysates of the four sperm preparations (caput epididymal sperm protein ± TCEP; cauda epididymal sperm protein ± TCEP). For both unreduced and reduced experimental samples, 5 µg aliquots of individual protein lysates from each sperm preparation (3 groups of 6 mice were used to create protein lysates from caput epididymal sperm and 3 groups of 6 mice for cauda epididymal sperm) were labeled with 4 nmol Cy5 for 30 min in the dark at 37°C. To create an internal control mixture for protein normalization, 10 µg aliquots of each of the protein lysates from the 12 sperm preparations (6 groups ± reduction with TCEP) were pooled for a total of 120 µg protein and labeled with 96 nmol Cy3 for 30 min in the dark at 37°C. Each protein lysate was mixed with equal volumes of DIGE sample buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer (pH 3-11, nonlinear), 130 mM DTT]. For each 5 µg Cy-5 labeled experimental sample, a 5 µg equivalent of the Cy3-labeled internal control mixture was added and stored at -80°C. Excess, unincorporated dye was removed by extracting the protein lysates with chloroform-methanol in the dark [17]. For the analytical electrophoresis, the proteins (5 µg of Cy3-labeled control protein and 5 µg of Cy5-labeled protein per experimental group) were resuspended with 450 µl of rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer pH 3-11 nonlinear, 130 mM DTT and 0.002% bromophenol blue].

Two-dimensional gel electrophoresis was performed with the Ettan IPGphor II and Ettan DALTsix equipment from GE Healthcare. All procedures were performed in the dark. For the first dimension, samples in 450 μ l of rehydration buffer were loaded in the IPGphor strip holder. The 24 cm Immobiline DryStrip (pH 3-11 nonlinear) (GE Healthcare) was placed in a holder and overlaid with ~5 ml of DryStrip cover fluid (GE Healthcare). Strips were hydrated under 50 V for 24 h and focused afterward on the IPGphor II IEF system for a total of 80 kVh at 20°C. After electrophoresis, each strip was incubated with 7.5 ml of equilibration buffer [6 M urea, 100 mM Tris-HCl, pH 8.0, 30% (w/v) glycerol, 2% (w/v) SDS, 0.5% (w/v) DTT and 0.002% bromophenol blue] by rocking for 20 min. For the second dimension, the strips were placed on top of a 10–20% gradient polyacrylamide gel containing SDS with low fluorescence glass plates (Jule Biotechnologies, Milford, CT). Then SDS-PAGE was performed using the Ettan DALTsix electrophoresis system.

2.3. Scanning and image analysis

The Cy3 and Cy5 images were scanned at a resolution of 100 μ m (pixel size) using a Typhoon 9410 scanner (GE Healthcare). The images were analyzed with DeCyder software version 5.01 (GE Healthcare), which allowed gel matching, quantification, and statistical analyses. First, spots were detected with the Differential In-Gel Analysis module of the software. Then all protein-spot maps were matched from 12 gels using the Biological Variation Analysis module. The gel-to-gel variations were normalized using the image of the Cy3-labeled internal control sample, which was consistent from one experimental sample to the next. A one-way analysis of variance (ANOVA) was used to compare changes in protein abundance between all groups and select the spots that undergo a statistically significant ($p < 0.01$) change. The spots with a change of 1.2-fold in volume and Student's t -test $p < 0.05$ were classified as altered.

2.4. Spot picking and in-gel digestion

To identify proteins that displayed differential patterns, the method was scaled up to enable the picking of individual spots by excision, in-gel digestion, and analysis by mass spectrophotometry. For the preparative gel, a 500 μ g aliquot of pooled protein from the four sperm populations (caput epididymal sperm protein $\pm$ TCEP; cauda epididymal sperm protein $\pm$ TCEP) was labeled with 400 nmol Cy3 and separated by 2-D gel electrophoresis as described above. The gel was scanned with a Typhoon 9410 scanner, placed in fixation solution (30% methanol, 10% acetic acid) for 3 days at room temperature, and stored in 5% acetic acid at 4°C until spot selection. Each spot position was determined and a pick list was created using DeCyder software version 5.01. Using an Ettan spot picker (GE Healthcare), the designated gel sections were excised robotically and transferred to microplate wells. Trypsin digestion was carried out according to the method of Strader et al. [18].

2.5. Mass spectrometry and microsequencing

The digested proteins were identified by MALDI-TOF/TOF mass spectrometry using a 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Foster City, CA). MALDI plates were calibrated using six calibration spots as recommended by the manufacturer, resulting in a mass accuracy of approximately ± 50 ppm. Peptide mass maps were acquired in reflectron mode (20-keV accelerating voltage) with 155-ns delayed extraction, averaging 2000 laser shots *per* spectrum. Trypsin autolytic peptides (m/z 842.51, 1045.56, and 2211.10) were used to calibrate each spectrum internally to a mass accuracy within 20 ppm. The MS/MS spectra were acquired with 4000 Series Explorer software version 3.0 (Applied Biosystems).

The spectra were analyzed using GPS Explorer software version 3.5 (Applied Biosystems), which acts as an interface between the Oracle database containing raw spectra and a local

copy of the MASCOT search engine version 1.9.05. Peptide peaks with a signal/noise ratio greater than 5 and a mass between m/z 900 and 4000 were searched against the Swiss Institute of Bioinformatics SwissProt 53.0 database (269,293 sequences; 98,902,758 residues) with *Mus musculus* taxonomy (13,321 sequences). The 10 most intense peaks were automatically selected for MS/MS. Up to one missed trypsin cut was allowed, and the data were searched using oxidation of methionine and carbamidomethylation of cysteine as variable modifications.

For microsequencing, trypsin-digested samples were dissolved in 5 μ l of 0.1% formic acid and injected into a 10 cm long C18 capillary column with an autosampler (Eksigent Technologies, Dublin, CA). Then the peptide samples were eluted by linearly increasing the mobile phase composition to 98% solvent B (0.1% formic acid in 100% acetonitrile) at a flow rate of 200 nl/min for 60 min gradient using a nanoLC system (Eksigent Technologies). Online nanospray (Thermo Fisher Scientific, Waltham, MA) was used to spray the separated peptides into a LTQ mass spectrometer (Thermo Fisher Scientific). The raw data were acquired and analyzed with Xcalibur 2.0 SR2 software (Thermo Fisher Scientific). The protein identification and database search were performed with MASCOT dll script of Xcalibur 2.0 SR2; the combined MS and MS/MS data were used for the MASCOT database search. A protein score of >70 with a protein confidence of identification of >95% was considered acceptable. If a candidate protein was matched with two or more peptides, each peptide score of >30 was used as the threshold. For a low molecular protein (M_r <30,000), matching with a single peptide was also accepted, if the peptide score was defined as >70.

2.6. Antibodies for immunoblot and indirect immunofluorescence analyses

The target antigens and primary antibodies used in this study are as follows with catalogue number, antibody description, and company source: α -tubulin (T5168: mouse monoclonal antibody clone B-5-1-2; Sigma-Aldrich, Saint Louis, MO); HSPA2 (GTX91597: mouse monoclonal antibody clone S51; GeneTex, San Antonio, TX); GRP78 BiP (ab21685: rabbit polyclonal antibody; Abcam, Cambridge, MA); GRP 75 (sc-13967: rabbit polyclonal IgG H-155; Santa Cruz Biotechnology, Santa Cruz, CA); smooth muscle actin (ab40865: mouse monoclonal antibody clone HUC1-1 which recognizes an epitope present on all mammalian muscle actins [19]; Abcam); ALDOA (11217-1-AP: rabbit polyclonal antibody; ProteinTech Group, Chicago, IL); enolase (sc-7455: goat polyclonal IgG C-19; Santa Cruz Biotechnology); PDH-E1 β (sc-65243: mouse monoclonal IgG1 clone 17A5; Santa Cruz Biotechnology); TPI1 (10713-1-AP: rabbit polyclonal antibody; ProteinTech Group); PDC-E2 (sc-16890: goat polyclonal IgG N-20; Santa Cruz Biotechnology). The following three secondary antibodies were used for immunoblot analysis: alkaline phosphatase-conjugated, goat anti-mouse IgG+IgM whole antibody (GE Healthcare), alkaline phosphatase-conjugated goat anti-rabbit IgG whole antibody (GE Healthcare), and alkaline phosphatase-conjugated rabbit anti-goat IgG (H+L) (Vector Laboratories, Burlingame, CA). Three secondary antibodies were used for indirect immunofluorescence analysis: Alexa Fluor 488-conjugated rabbit anti-goat IgG (H+L), Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L), and Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (Invitrogen, Carlsbad, CA).

2.7. Immunoblot analysis and quantification

For immunoblot analysis, the purified sperm were collected by centrifugation at $10,000 \times g$ for 2 min, resuspended in PBS containing protease inhibitors, and sonicated. Then 5 $\times$ SDS sample buffer [290 mM Tris-HCl, pH 6.8, 30% (w/v) glycerol, 8.53% (w/v) SDS, 500 mM DTT and 0.012% bromophenol blue] was added for a final concentration of 1 $\times$ SDS sample buffer. The samples were vortexed, boiled for 5 min, sonicated again, and centrifuged at $10,000 \times g$ for 5 min. The supernatants were saved, snap-frozen in a dry ice-ethanol bath,

and stored at -80°C . The proteins from purified $2 \times 10^4 - 2 \times 10^6$ caput and cauda epididymal sperm were separated by SDS-PAGE in 10% or 12% polyacrylamide gels [20] and transferred to PVDF membranes (Millipore, Billerica, MA) [21]. The membranes were incubated in blocking buffer [Tris-buffered saline-Tween (TBST; 25 mM Tris-HCl, pH 8.0; 125 mM NaCl; 0.1% Tween 20) containing 5% nonfat dry milk] overnight at 4°C . After washing with TBST three times, the blots were incubated with primary antibodies (mouse anti- α -tubulin antibody, 1 $\mu\text{g/ml}$; mouse anti-HSPA2 antibody, 5 $\mu\text{g/ml}$; rabbit anti-GRP78 antibody to HSPA5, 0.1 $\mu\text{g/ml}$; rabbit anti-GRP75 antibody to HSPA9, 0.1 $\mu\text{g/ml}$; mouse anti-smooth muscle actin antibody, 50 $\mu\text{g/ml}$; rabbit anti-ALDOA antibody, 0.115 $\mu\text{g/ml}$; goat anti-enolase antibody, 8 $\mu\text{g/ml}$; mouse anti-PDH-E1 β antibody, 2 $\mu\text{g/ml}$; rabbit anti-TPI1 antibody, 1.05 $\mu\text{g/ml}$; goat anti-PDC-E2 antibody, 0.5 $\mu\text{g/ml}$) in blocking buffer for 1 hr at room temperature. After washing with TBST three times, the blots were incubated with the corresponding alkaline phosphatase-conjugated secondary antibodies (1:5000 or 1:1000) diluted in blocking buffer for 1 hr at room temperature. After washing with TBST three times, the bound enzyme was detected with the Enhanced Chemifluorescence (ECF) Western Blotting Reagent Pack (GE Healthcare) according to the manufacturer's directions. The ECF images were scanned at a resolution of 100 μm (pixel size) using a Storm 860 scanner (GE Healthcare). The pixel volume of each band was quantified with ImageQuant software version 5.2 (GE Healthcare) and then the abundance of each protein was normalized with α -tubulin as an internal control. The volume results are expressed as mean $\pm$ standard deviation from experiments done three times. Statistical analysis was performed using *t*-tests with Microsoft Excel 2004 version 11.2.5 (Microsoft, Redmond, WA). A $p < 0.05$ was considered statistically significant.

2.8. Indirect immunofluorescence analysis

Caput or cauda epididymal sperm collected after extrusion from the epididymis as described above were transferred to another tube on ice, attached to microscope slides for 30 min, and fixed with 4% paraformaldehyde in PBS for 15 min. After washing with PBS three times, the sperm were permeabilized with -20°C methanol for 2 min. The slides were washed with PBS three times and incubated overnight at 4°C with blocking solution [10% goat serum or 10% rabbit serum (in the case of goat anti-PDC-E2 antibody) in PBS]. Then the samples were rinsed with PBS once and incubated with primary antibodies (mouse anti-HSPA2 antibody, 5 $\mu\text{g/ml}$; mouse anti-smooth muscle actin antibody HUC1-1, 5 $\mu\text{g/ml}$; rabbit anti-ALDOA antibody, 1.15 $\mu\text{g/ml}$; goat anti-PDC-E2 antibody, 5 $\mu\text{g/ml}$) in blocking solution for 1 hr at 37°C . For a control, the same concentrations of the corresponding normal, purified IgGs were substituted for the primary antibody mixture. After washing with PBS three times, the samples were incubated for 1 hr at 37°C with the corresponding Alexa Fluor 488-conjugated secondary antibodies (1:500) diluted in blocking solution. After washing with PBS three times, the slides were mounted with coverslips using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL), observed with a Nikon Eclipse TE 2000-U inverted microscope (Nikon Instruments, Melville, NY), and photographed with a CFW-1610C digital FireWire camera (Scion, Frederick, MD) using NIH ImageJ imaging software available online (<http://rsb.info.nih.gov/ij/>). Nomarski differential interference contrast micrographs were taken in parallel with the fluorescence images. Negative controls using secondary antibody alone were also used to check for secondary antibody specificity.

3 Results

3.1. Purity of caput and cauda epididymal sperm

After sperm leave the testis, they pass through the three regions of the epididymis, starting in the caput region, transiting through the corpus segment, and ending up in the cauda epididymis where they are stored until ejaculation (Supporting Information Fig. S1A). As

they transit the epididymis, sperm undergo biochemical and physiological modifications; as a result, they mature and acquire motility and the ability to fertilize eggs. To understand molecular mechanisms of sperm epididymal maturation in the mouse, we identified proteins that showed differences in abundance between caput epididymal sperm and cauda epididymal sperm using 2-D DIGE. For the first step, these sperm were purified. After the collection of sperm cells, particularly from the caput epididymis, contaminants such as tissue particles and red blood cells were apparent (Supporting Information Fig. S1B). A 35% *PureSperm* gradient was used to isolate a pellet of sperm cells and separate out the red blood cells and tissue particles. Purities of >90% and >98% were routinely obtained for caput and cauda epididymal sperm, respectively. Purified caput epididymal sperm after *PureSperm* centrifugation are shown in Supporting Information Fig. S1C.

3.2. Identification of proteins present at different levels in caput and cauda epididymal sperm

The experimental design for the analytical gel phase of 2-D DIGE of samples from the saturation labeling protocol is shown in Supporting Information Fig. S2. Four sperm preparations (caput epididymal sperm protein $\pm$ TCEP; cauda epididymal sperm protein $\pm$ TCEP) were analyzed in triplicate. Each treatment replicate ($\pm$ TCEP) consisted of 6 mice for a total of 18 mice per sample preparation and a grand total of 36 mice for the experiment. In other words, the same 18 mice were used for caput and cauda epididymal sperm protein treated with TCEP, and 18 mice for caput and cauda epididymal sperm protein treated without TCEP, respectively. For use as an internal control, equal amounts of protein from the 12 groups were pooled before labeling as described in the Materials and Methods. Each gel contained 5 μ g of Cy5-labeled sperm preparation and 5 μ g of Cy3-labeled internal control. After spots of interest were determined, 300 μ g of the Cy3-labeled internal control protein was separated on a 2-D DIGE preparative gel. Finally, the protein spots were picked from a preparative gel for their identification.

For DIGE analytical gels, whole cell proteins from four sperm populations (caput epididymal sperm proteins $\pm$ disulfide reduction and cauda epididymal sperm proteins $\pm$ disulfide reduction) were labeled with Cy dyes, mixed, and separated by 2-D gel electrophoresis using the nonlinear pH 3-11 gradient strip in the first dimension followed by SDS-PAGE in the second dimension. Multiple scanned images of Cy3-labeled internal control proteins showed that a highly reproducible pattern of fluorescent protein spots was visualized (Fig. 1). Using DeCyder software to examine and screen the analytical gel images, we identified multiple spots as caput or cauda epididymal sperm-differential proteins. The software first detected approximately 3000 spots in each gel and more than 1500 of these spots were contained in all 12 gels. A one-way ANOVA showed protein abundance changes of 388 spots were statistically significant ($p < 0.01$) between the four protein populations. We performed statistical analysis using the Student's *t*-test and determined that 36 protein spots exhibited differential abundance by 1.2-fold or more ($p < 0.05$) between reduced caput and cauda epididymal sperm populations or between unreduced caput and cauda epididymal sperm populations (Supporting Information Tables S1 and S2). Based on average ratios, these were designated as differential protein candidates in caput epididymal sperm (21 spots) and cauda epididymal sperm (15 spots) (Fig. 2).

Of the differential protein candidates, sixteen and ten spots were recovered from a preparative gel and submitted to the proteomics core for analysis by MALDI-TOF/TOF mass spectrometry and microsequencing. The sequence information for twenty-three spots was obtained and the peptide matches to entries in the database are shown in Supporting Information Tables S1 and S2. Several spots were identified as cytokeratin 10 (KRT10), but we did not pursue analysis of this protein because there are limited data concerning its expression during spermatogenesis or its presence in mature sperm. Multiple spots

corresponding to a specific protein were identified with the same molecular weight but the isoelectric points differed slightly, suggesting that the proteins found in these spots were post-translationally modified [Fig. 1 and Table 1, e.g., spots #1125 and #1127 (HSPA2 and HSPA9), spots #1633, #1634, #1643, and #1649 (ACTG2), spots #1633, #1634, #1649 (SPESP1 and SUCLA2), and spots #1932 and #1940 (TPI1)]. PDHB was identified in three spots, #1932, #1940, and #2303; the latter spot had a lower molecular weight, possibly representing a proteolytically cleaved fragment of the parent protein. Most spots contained peptides that corresponded to multiple proteins (Supplemental Tables S1 and S2). Sequence information from three spots could not be obtained in this analysis, because these (spots #2059, #2081, and #2606) were acidic proteins that probably did not have multiple cleavage sites for trypsin. We did not pursue the analysis of the hemoglobin identified in two spots (spots #2999 and #3008) from caput sperm because its presence probably arose from contamination with red blood cells during sperm purification.

Analysis of the data identified several proteins that exhibited differences between caput and cauda epididymal sperm (Table 1). In total, eight proteins were identified as caput epididymal sperm-differential proteins. Six of these may be involved in the assembly of sperm structures. Three, heat shock protein 2 (HSPA2), heat shock protein 5 (HSPA5), and heat shock protein 9 (HSPA9) are molecular chaperones. Three proteins, cytoplasmic type γ -actin (ACTG1), smooth muscle γ -actin (ACTG2), and sperm equatorial segment protein 1 (SPESP1), are structural and/or cytoskeletal proteins. A total of nine proteins were identified as cauda epididymal sperm-differential proteins. Six are associated with producing energy for sperm functions. Three of the proteins, aldolase 1 and its isozymes (ALDOA, ALDOAV2, ALDOART1, ALDOART2), α -enolase (ENO1), and triosephosphate isomerase (TPI1), are glycolytic enzymes. Pyruvate dehydrogenase E1 β (PDHB) connects the glycolytic pathway to the tricarboxylic acid (TCA) cycle. Other proteins, "ATP synthase, H⁺ transporting, mitochondrial F1 complex, O subunit" (ATP5O) and "NADH-dehydrogenase (ubiquinone) flavoprotein 2" (NDUFV2), play roles in ATP synthesis.

We selected these six caput (HSPA2, HSPA5, HSPA9, ACTG1, ACTG2, and SPESP1) and six cauda epididymal sperm-differential proteins (ALDOA, ENO1, ATP5O, NDUFV2, PDHB, and TPI1) for further analyses. Additionally, two proteins, glutathione S-transferase mu 5 (GSTM5) and pyruvate dehydrogenase E2 (DLAT) were identified as differential proteins in both caput and cauda epididymal sperm. As a consequence of epididymal transit, these proteins shifted in their locations on the 2-D gel. For example, GSTM5 and DLAT spots appeared at lower pI values in the caput epididymal sperm gel and migrated to a higher pI/location in the cauda epididymal sperm gel. (GSTM5: one caput spot #2300 to four cauda spots #2303, #2312, #2350 and #2364; DLAT: two caput spots #1125 and #1127 to one cauda spot #1188) (Supporting Information Fig. S3).

3.3. Validation of protein abundance between caput and cauda epididymal sperm

To validate the 2-D DIGE results, nine out of the twelve selected proteins were analyzed by immunoblotting. We limited our examination to the nine proteins because antibodies for SPESP1, NDUFV2, and ACTG1 were not commercially available. Three independent caput and cauda epididymal sperm populations were examined and similar results were obtained in each case. Although the anti-ATP5O antibody did not result in a strong band (data not shown), the other antibodies recognized specific bands with the ECF system (Fig. 3). Of the four antibodies targeting cauda epididymal sperm-differential proteins, anti-ENO1 and anti-PDHB showed high intensity bands with the expected sizes. As reported in recent studies, anti-ALDOA antibody detected the ubiquitous shorter ($M_r \sim 39,000$) and longer ($M_r \sim 50,000$) male germ cell-specific isoforms [22–24]. Similarly, the anti-TPI1 antibody recognized two longer male germ cell-specific isoforms ($M_r \sim 33,400$ and $\sim 30,800$), as well

as a ubiquitous shorter isoform ($M_r \sim 27,700$). We named these bands TPI1-33, TPI1-31, and TPI1-28, respectively.

We used ImageQuant software to measure the pixel volume of each band to quantify and compare the abundance of each protein between caput and cauda epididymal sperm. To normalize data, α -tubulin was used as an internal control. Normalized volumes were expressed as the caput/cauda ratio, where a value of 1.2 (fold-increase or fold-decrease) was the determinant used to classify a given protein as differentially abundant between the two stages of sperm (Fig. 3D). Three of the four caput epididymal sperm-differential proteins, HSPA2, HSPA5 and ACTG2 (as detected by HUC1-1), were more abundant in caput epididymal sperm (>2.0 -fold). HSPA9 was present in approximately equal amounts in both the caput and cauda epididymal sperm. Of the four cauda epididymal sperm-differential proteins, PDHB was more abundant in cauda epididymal sperm (>2.0 -fold). Similarly, the bands recognized by both anti-ALDOA and anti-ENO1 antibodies were slightly more abundant in cauda epididymal sperm (>1.2 -fold). TPI1-31 was slightly more abundant in cauda epididymal sperm (>1.2 -fold), while TPI1-33 was detected in equal amounts in both caput and cauda epididymal sperm. TPI1-28 was unexpectedly more abundant in caput epididymal sperm (>2.0 -fold). In conclusion, 2-D DIGE results were validated for HSPA2, HSPA5, ACTG2, ALDOA, ENO1 and PDHB proteins. Additionally, DLAT was present in equal amounts in both caput and cauda epididymal sperm (Fig. 3C, D).

3.4. Confirmation of protein location in caput and cauda epididymal sperm

Immunofluorescence analysis was performed with caput and cauda epididymal sperm to investigate whether a change in the abundance of an individual protein was reflected by an alteration in subcellular localization. The locations of four out of the eight previously validated proteins were analyzed by immunofluorescence. Using commercial antibodies directed against HSPA5, HSPA9, ENO1, and PDHB, we could not perform immunofluorescence either because of high background staining or the lack of a difference between a non-immune control IgG and a given antibody. In all other experiments, the IgG controls were negative on caput and cauda epididymal sperm (data not shown). We detected HSPA2 in the cytoplasmic droplet of caput epididymal sperm, although we found no signal in cauda epididymal sperm (Fig. 4A, C). Using mouse monoclonal antibody clone HUC1-1 which recognizes an epitope present on all mammalian muscle actins [19], we found multiple patterns of staining. In about 42% of the caput epididymal sperm, we detected strong staining for the antigen in the anterior acrosome, whereas the staining was moderately intense in the 36% of the remaining sperm and absent in 22% (Fig. 4E, G). However, the staining was moderately intense in about 3% of cauda epididymal sperm but very faint in 42% and absent in 55% of the cells (Fig. 4I, K). The decrease in fluorescence due to anti-HSPA2 and HUC1-1 between sperm recovered from different regions of the epididymis corresponded with the immunoblot defined caput epididymal sperm-differential proteins. ALDOA exhibited very strong fluorescence throughout the entire length of the sperm flagellum, especially in the principal piece of cauda epididymal sperm (Fig. 5C). In caput epididymal sperm, the ALDOA signal was in the cytoplasmic droplet and weak throughout the principal piece (Fig. 5A). ALDOA was not detected in the end piece (data not shown). The immunofluorescence results support the finding that ALDOA was more abundant in cauda epididymal sperm. TPI1 was observed in the flagellum of both caput and cauda epididymal sperm (unpublished data). In addition to our comparison of caput and cauda epididymal sperm-differential proteins, we noted that there were no apparent differences in the localization and abundance of DLAT between caput and cauda epididymal sperm (Fig. 5E, G).

4 Discussion

To date, several proteins have been reported to be either eliminated from or added to sperm during epididymal transit. An example is angiotensin I converting enzyme (ACE), one of the proteins that are gradually released from the sperm surface during epididymal maturation in the ram [25]. In most cases, the disappearance of sperm surface proteins is caused by a specific proteolytic mechanism [10]. P26h, a member of the short-chain dehydrogenase/reductase superfamily, is not detected in caput epididymal hamster sperm, but appears on the acrosomal region in corpus epididymal sperm and accumulates on the acrosomal cap of cauda epididymal sperm [26]. Similarly, P25b is transferred to the sperm head during epididymal transit in the bull [27]. Epididymal secreted protein CRISP1 is a cysteine-rich secretory protein family member and exists in two forms, protein D and E. In rat sperm, protein D associates primarily and transiently to the head while protein E binds the tail [28]. Some proteins are delivered from epididymal cells to sperm via small membranous vesicles, epididymosomes, which are secreted in an apocrine manner into the intraluminal compartment of the epididymis [29]. The list of these proteins is still developing. In hamster sperm, HSPA5 and “heat shock protein 90, beta (Grp94), member 1” (HSP90B1) are caput epididymal specific, while protein disulfide isomerase associated 3 (PDIA3) is caput epididymal abundant [30]. Endoplasmic reticulum protein 29 (ERP29) is more abundant in cauda epididymal rat sperm [31]. Apolipoprotein A-I (APOA1) is transferred into the membrane during mouse sperm transit through the epididymis [32]. In spite of these findings, the entire process of the addition and elimination of sperm proteins has not been elucidated.

Sperm can also undergo various modifications as they mature in the epididymis. Some examples are hyaluronidase/PH20 (sperm adhesion molecule 1: SPAM1) and fertilin/PH30 (a disintegrin and metallopeptidase domain 2 protein: ADAM2). In testicular guinea pig sperm, SPAM1 is found all over the cell whereas ADAM2 is limited to the entire head surface. However, after maturation, these proteins are restricted to the posterior domain of the head of cauda epididymal sperm [33]. Structurally, some proteins undergo post-translational modification during epididymal transit; for example, proacrosin experiences an alteration in oligosaccharide side-chains in the corpus epididymis of the guinea pig [34]. The relocalization of ADAM2 from the whole sperm head to the posterior head (discussed above) is correlated with its proteolytic processing in the epididymis [35].

Of the myriad of sperm protein modifications that occur in the epididymis, we focused on the addition and elimination of specific proteins, using a large-scale proteomic approach to identify caput and cauda epididymal sperm proteins that exhibited differences in amounts or positions using 2-D DIGE and mass spectrometry analyses. This strategy was successful in the identification of eight proteins of caput epididymal mouse sperm and nine proteins of cauda epididymal sperm that showed differences. The amounts of some of these proteins were validated by immunoblotting and we also determined their localizations in sperm by immunofluorescence.

4.1. Caput epididymal sperm-differential proteins

Of the eight caput epididymal sperm-differential proteins identified in this study, three (HSPA2, HSPA5, and HSPA9) are molecular chaperones while another three (ACTG1, ACTG2, and SPESP1) are structural and/or cytoskeletal proteins. Expression of heat shock proteins (HSP) is stimulated when cells are exposed to elevated temperatures or other stresses. To date, eight chaperone proteins have been identified in human sperm [36]. At least seven genes were characterized in the mammalian HSP70 (HSPA) family [37]. HSPA2 is expressed in pachytene spermatocytes of the mouse testis. Male mice with a targeted disruption of the *Hspa2* gene fail to complete meiosis and thus lack postmeiotic spermatids

[38,39]. Additionally, HSPA2 is distributed to the nucleus in mouse spermatids, suggesting that this protein serves as an early chaperone for transition proteins involved in spermiogenic DNA condensation [40]. Immunohistochemistry experiments conducted on nonpermeabilized cells detected HSPA2 throughout the entire tail of ejaculated human sperm [41]. HSPA2 was also identified as a surface protein of ejaculated human sperm using vectorial labeling with biotin or radioactive iodine followed by 2-D gel electrophoresis [42]. In contrast, our results indicate that mouse HSPA2 was located in cytoplasmic droplets of caput epididymal sperm and disappeared from the sperm by the time the cells arrived in the cauda epididymis, suggesting that HSPA2 was eliminated from sperm along with the cytoplasmic droplet as a consequence of epididymal transit. The different localization patterns observed between our findings and the work of others may result from the use of mouse epididymal sperm versus ejaculated human sperm, the examination of nonpermeabilized vs. permeabilized cells, vectorial labeling/2-D gel electrophoresis vs. immunofluorescence, and/or unique properties of the human epididymis compared to the rodent epididymis [1]. Therefore, some sperm proteins involved in epididymal maturation, such as HSPA2, may have different roles in mouse and human.

In addition to HSPA2, HSPA5 (also known as GRP78) and HSPA9 (GRP75) are members of the HSP70 family. HSPA5 and HSPA9 are induced in somatic cells that lack glucose rather than ones that are responding to heat or stress [43,44]. In somatic cells, HSPA5 is localized in the endoplasmic reticulum and is responsible for co-translational folding of nascent polypeptide chains [45]. However, HSPA5 is a surface protein of human sperm [42] and is detected in the neck region of permeabilized, ejaculated human spermatozoa [46]. On the other hand, HSPA5 is localized to the acrosome and principal piece only in caput epididymal hamster sperm during epididymal maturation [30]. In somatic cells, the expression of HSPA9 is predominant in the mitochondrial matrix [47], suggesting that HSPA9 is involved in the refolding of proteins following their transport from the cytosol into the mitochondria [48]. Unfortunately, we were unable to utilize the antibodies we possess for indirect immunofluorescence in our studies. Chaperone proteins have been implicated in epididymal sperm maturation, capacitation, and sperm-egg binding [36,49,50]. In the study by Han et al. [50], mouse HSPA5 was identified in membrane rafts as an interacting protein for ADAM7 (a disintegrin and metallopeptidase domain 7). These authors suggested that ADAM7 functions in fertilization by forming a chaperone complex with HSPA5, calnexin, and integral membrane protein 2B during sperm capacitation. However, further investigations concerning HSPA5 and HSPA9 are required to determine more definitively their localizations in mouse sperm and roles in sperm maturation, capacitation, and fertilization.

Two size classes of actin mRNAs (2.1 kb and 1.5 kb) are found in the mouse testis [51]. The longer size class represents both cytoplasmic β - and γ -actin mRNAs (*Actb*, *Actg1*), which are detected throughout spermatogenesis but decrease towards the end of spermiogenesis. The shorter size class is the smooth muscle γ -actin (*Actg2*) that is first detected in postmeiotic spermatogenic cells and increases during spermiogenesis [52]. In addition to the presence of mRNA, muscle actin protein has also been found in the rat testis [53]. In the present work, we used monoclonal antibody HUC1-1 that detects multiple muscle actins. Due to the fact that spermatogenic cells only contain one mRNA encoding smooth muscle actin [52], any signal detected by the HUC1-1 antibody is representative of only ACTG2. Previous immunolabeling experiments with HUC1-1 showed muscle actin is in the cytoplasm and tails of rat spermatids [53]. Our immunofluorescence experiments detected ACTG2 on the anterior acrosome in caput epididymal sperm but a diminished signal in cauda epididymal sperm. These results suggest that the distribution of ACTG2 changes during passage of the sperm from the testis to caput epididymis. On the other hand, the distribution of actin, detected with a monoclonal antibody AC-40 that recognizes all actin

isoforms, drastically changes during bull sperm maturation. For instance, in permeabilized testicular sperm actin is in the acrosomal region while in permeabilized cauda epididymal sperm it is found in the principal piece [54]. Using probes that do not distinguish specific actin proteins such as ACTG1 or ACTG2, previous studies examining monomeric and polymeric actin among spermatozoa from diverse species (e.g., bull, boar, rabbit, human, rat, mouse, golden hamster, and guinea pig) have yielded a variety of localization patterns [55,56]; thus, it is important to consider that various actin isoforms may have distinct functions in the different compartments within sperm.

We identified SPESP1 as a caput epididymal sperm-differential protein (Table 1). Recently, Fujihara et al. [57] created a *Spesp1* knockout mouse. The *Spesp1*-deficient males yield fewer pups when mated with wild-type females. When examined further, the authors found that *Spesp1*-null males fertilize less eggs *in vivo* because their sperm fail to migrate into the oviducts. Furthermore, the mutant sperm have a lower ability to fuse with eggs, resulting in a decreased rate of *in vitro* fertilization compared to wild-type sperm. In *Spesp1*-deficient sperm, the distributions of some membrane proteins are changed and the plasma membrane of the equatorial segment is absent. Further analysis of SPESP1 may provide additional information concerning the role of this protein in the acquisition of fertility during the course of epididymal maturation.

4.2. Cauda epididymal sperm-differential proteins

Of the nine cauda epididymal sperm-differential proteins, six are important for producing energy to fuel sperm functions. Three of them: ALDOA, ENO1, and TPI1 are enzymes in the glycolytic pathway. PDHB is a component of the pyruvate dehydrogenase (PDH) complex that links the glycolytic pathway to the TCA cycle in the mitochondria of somatic cells. The fifth and sixth proteins, ATP5O and NDUFV2, play roles in oxidative phosphorylation. Glycolysis converts glucose to pyruvate yielding 2 ATPs from a single glucose molecule. Although spermatocytes and spermatids prefer oxidative phosphorylation as a means for ATP production [58,59], studies have suggested that during epididymal maturation sperm of many mammals switch to glycolysis for ATP production [60]. Glycolysis clearly plays an essential role as an energy pathway to fuel basal motility in mouse sperm as is evident by the studies of male mice with genetic deletions of the sperm-specific forms of key glycolytic enzymes such as glyceraldehyde 3-phosphate dehydrogenase-S (GAPDHS) or phosphoglycerate kinase-2 (PGK2) [61,62]. When oxidative phosphorylation is suppressed by carbonyl cyanide m-chlorophenylhydrazone, sperm motility and ATP production are not affected; on the other hand, sperm motility and ATP content are negatively impacted when glucose is replaced in the medium by a non-hydrolyzable analog, 2-deoxyglucose, even though pyruvate or lactate are present to fuel mitochondrial respiration [63]. Several glycolytic enzymes, including ALDOA and ENO1, can interact with tubulin and microtubules [64,65].

Our previous proteomic analysis showed that four glycolytic enzymes, including ALDOA and TPI1, are present in the accessory structures of the mouse sperm flagellum [66]. Refining the subcellular localization further, Krisfalusi et al. [22] demonstrated that three glycolysis-associated enzymes (two variants of ALDOA, GAPDHS, and lactate dehydrogenase A) are tightly bound to the fibrous sheath of the mouse sperm flagellum. These findings indicate that some glycolytic enzymes are organized in specific structural compartments of the sperm flagellum. Recently Vemuganti et al. [23] identified three male germline-specific isozymes of ALDOA and hypothesized that localization to the fibrous sheath may result from amino-terminal extensions specific to the germ cell variants. In other studies, the localization of aldolase using anti-rabbit muscle aldolase is restricted to the acrosomal region as well as the principal piece of permeabilized cauda epididymal mouse sperm [24]. However, we observed a weak signal for ALDOA in the midpiece and a strong

signal in the principal piece of cauda epididymal sperm (Fig. 5C). The discrepancy between our findings and those reported by Arcelay et al. [24] may result from the use of different antibodies.

At least three isoforms (α , β and γ) of enolase have been identified, and α -enolase (ENO1) is expressed in most tissues including the testis. Beside these isoforms, a sperm-specific enolase has been characterized in human, ram and mouse [67]. Enolase, as demonstrated by indirect immunofluorescence, is distributed mostly in the tail of cauda epididymal rat sperm [65].

Male germ cell specific variants of TPI also exist. A testis-specific mRNA for TPI1 has been isolated from the rat testis [68]. Immunofluorescence experiments demonstrated that TPI is localized in the human sperm head [69]; however, further analysis should be performed to confirm the proper localization of the TPI protein using permeabilized sperm. In this report, we demonstrate the presence of three isoforms of TPI1 protein in epididymal mouse sperm. Currently we are investigating the transcripts and proteins of TPI1 in male germ cells. Future investigation of these spermatogenic cell-specific glycolytic isozymes should be conducted to confirm whether they have specific roles in sperm function.

The PDH complex (PDC) contains three main catalytic components – E1 (E1 α and E1 β), E2, and E3 – and catalyzes the irreversible conversion of pyruvate into acetyl coenzyme A (acetyl-CoA) [70]. Phosphorylation and dephosphorylation of somatic E1 α (PDHA1, encoded on the X chromosome) is important for the regulation of PDC [70]. Testis-specific E1 α (PDHA2, encoded on chromosome 3 in the mouse) is localized only in the principal piece of hamster cauda epididymal sperm [71]. PDHB (E1 β) is localized on the principal piece and acrosome as well as the midpiece regions in cauda epididymal mouse sperm [24]. The location of PDHB on the midpiece is logical, because this enzyme is important for ATP production in mitochondria. However the role of PDHB in the acrosome or principal piece is unclear. In hamster sperm flagella, two types of PDHB proteins were identified as 36KA and 36KB [72,73]. Both of these isozymes are similar to PDHB except they lack 30 amino acids at their N-termini and exhibit different isoelectric points. Collectively, these results coupled with our findings indicate that all of the components of the (normally mitochondrial) pyruvate dehydrogenase complex, E1 (E1 α [71] and E1 β [24]), E2 (DLAT (this study Fig. 5E-H)), and E3 [dihydrolipoamide dehydrogenase [74]) are present in the principal piece of mammalian sperm. These results suggest that PDC in rodent sperm functions in the principal piece and may be important for sperm motility.

4.3. Proteins detected in both caput and cauda epididymal sperm

In the present study, some proteins were found in different positions in two-dimensional gels of extracted proteins from caput and cauda epididymal mouse sperm. For example, GSTM5 and DLAT both shifted to more basic isoelectric points in the 2-D gel of cauda epididymal mouse sperm proteins. Generally, it is recognized that the *pI* shift from an acidic to a more basic pH is caused by dephosphorylation [75]. Therefore, we hypothesize that GSTM5 and DLAT proteins in sperm may be dephosphorylated during the course of epididymal maturation.

GSTM5 is a member of the μ -class of glutathione S-transferases and is expressed abundantly in the testis where it plays a role in protecting sperm from oxidative stress [76]. Immunoblot analysis with a polyclonal antiserum to μ -class GSTs detected mouse GSTM5 protein in isolated fibrous sheaths [76]. GSTM5 was also identified in mouse sperm accessory structures by 2-D gel electrophoresis [66]. Furthermore, this protein exists in the head and principal piece of the flagellum of mouse sperm [77]. What would be the consequences of dephosphorylating GSTM5? In gliomas, phosphorylation of another

isozyme of glutathione S-transferase, GSTP1, increases enzymatic activity significantly [78]. Both our results and those presented in the study by Arcelay et al. [24] strongly support the idea that the phosphorylation/dephosphorylation of GSTM5 serves as a mechanism to regulate sperm maturation and acquisition of sperm function. Since a hallmark of epididymal sperm maturation is an increase in protein disulfide bonding [12], the inactivation of GST activity by dephosphorylation may lead to an increase in protein sulfhydryl oxidation. On the other hand, mouse sperm GSTM5 undergoes tyrosine phosphorylation during capacitation [24], suggesting that GST activity could increase, perhaps as part of a mechanism protecting the more metabolically active capacitated sperm from oxidative damage. In our laboratory, experiments to elucidate the role of GSTM5 during sperm maturation are in progress.

The pyruvate dehydrogenase complex is normally located in the matrix of mitochondria in somatic cells and converts pyruvate into acetyl-CoA, which can then be used in cellular respiration, linking glycolysis to the TCA cycle. The E2 subunit of pyruvate dehydrogenase (DLAT) binds to the regulatory PDH kinase and phosphatase and plays roles in the phosphorylation/dephosphorylation of the PDH E1 α component [70]. In the case of astrocytes, phosphorylation of PDHA strongly inhibits PDC activity. PDHA2 is localized to the fibrous sheath of the hamster sperm flagellum and undergoes tyrosine phosphorylation during capacitation [71]. Our experiments indicated that DLAT was also restricted to the principal piece in epididymal mouse sperm and its location did not change during epididymal transit. These observations suggest that PDC functions in the principal piece of rodent sperm flagella.

4.4. General Discussion

For our DIGE experiments, we prepared proteins from whole sperm cells with a standard cell lysis buffer; therefore, the whole sperm lysate should contain most of the proteins from sperm. However, we did not detect differences in the levels of certain proteins, such as ACE and CRISP1, that are components of epididymal sperm cells [25,28]; it is likely they did not fall under the criteria we established in our screening for caput-differential and cauda-differential proteins. Baker et al. [79] identified eight rat sperm proteins that are modified unambiguously during epididymal transit. Compared with these proteins, only two of our identified proteins, ENO1 and HSPA5, corresponded to their protein list. Furthermore, during epididymal rat sperm maturation, ENO1 is more abundant in caput epididymal rat sperm, while HSPA5 is more prominent in cauda epididymal rat sperm [79]. These results are in contrast with our observations in the mouse. Unfortunately, mice that do not express the *Eno1* or *Hspa5* genes due to targeted deletions or gene traps exhibit early embryonic lethality and, therefore, cannot inform us about the functions of these proteins in adult males [80,81]. While preparing this manuscript, an interesting proteomics paper about hamster epididymal sperm maturation was published. Kameshwari et al. (2010) performed 2-D gel electrophoresis using solubilized caput and cauda epididymal sperm proteins, identifying fourteen proteins that decrease and six that increase in intensity during epididymal transit. Of these, six proteins – DLAT, SUCLA2, ENO1, ACTG1, HSPA2, and HSPA5 – were also identified in our study. Their results are generally and remarkably similar to ours; DLAT increases whereas SUCLA2, ACTG1, HSPA2, and HSPA5 decrease in the transition from caput to cauda epididymal sperm. However, as Baker et al. [79] found for the rat sperm, the intensity of ENO1 decreases in hamster during epididymal maturation, suggesting that ENO1 has a unique feature in mouse sperm function because this protein is increased in mouse during epididymal transit. On the other hand, rat HSPA5 increases in cauda epididymal sperm but decreases in hamster and mouse. Therefore, the roles of ENO1 and HSPA5 should be studied further to determine whether they have species-specific functions during epididymal maturation.

Why do some proteins appear to become more abundant in cauda epididymal sperm relative to the caput when sperm are considered transcriptionally and translationally inactive? Recent studies in species such as the bull indicates that some secreted epididymal proteins can be transferred to sperm cells via epididymosomes [29]; however, none of the cauda epididymal sperm-differential proteins identified in our study with mouse sperm corresponded to proteins identified in bovine epididymosomes [82,83] so there may be differences in protein composition between the epididymosomes from diverse species. Alternatively, we have considered other ways to explain the observed differences in protein levels between caput and cauda epididymal sperm. One possibility is that the bulk loss of proteins by the elimination of the cytoplasmic droplet causes a rise in the relative proportion of specific proteins that are not shed from the sperm with the droplet. If this reasoning is correct, some cauda epididymal sperm-differential proteins do not necessarily change in amounts during epididymal maturation but appear to do so because of the bulk loss of other proteins. Currently, investigations concerning the mechanism giving rise to any given cauda epididymal sperm-differential protein are inconclusive and further studies are warranted.

Our investigation provided very clear results that most caput epididymal sperm-differential proteins contribute to the functional modification of sperm structures and that many cauda epididymal sperm-differential proteins are involved in increasing ATP production that promotes sperm functions such as motility. Moreover, these results account for the fact that cauda epididymal sperm are motile and structurally mature. Therefore, this report provides useful information to understand the molecular mechanisms of epididymal maturation. Also, some of validated proteins could be considered as important factors of sperm maturation or biomarkers to evaluate sperm quality. In addition, it will be valuable to study the functions of these proteins to elucidate aspects of male infertility and to establish methods for male contraception.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

acetyl-CoA	acetyl coenzyme A
cAMP	cyclic AMP
CyDyes	cyanine dyes
ECF	enhanced chemifluorescence
PDC	pyruvate dehydrogenase complex
PDH	pyruvate dehydrogenase
TCA	tricarboxylic acid
TCEP	tris-(2-carboxyethyl) phosphine hydrochloride

References

1. Cornwall GA. New insights into epididymal biology and function. *Hum Reprod Update*. 2009; 15:213–227. [PubMed: 19136456]
2. Cooper TG, Yeung CH. Acquisition of volume regulatory response of sperm upon maturation in the epididymis and the role of the cytoplasmic droplet. *Microsc Res Tech*. 2003; 61:28–38. [PubMed: 12672120]
3. Fawcett DW, Hollenberg RD. Changes in the acrosome of the guinea pig spermatozoa during passage through the epididymis. *Z Zellforsch Mikrosk Anat*. 1963; 60:276–292. [PubMed: 13944596]
4. Deng X, Czymmek K, Martin-DeLeon PA. Biochemical maturation of Spam1 (PH-20) during epididymal transit of mouse sperm involves modifications of N-linked oligosaccharides. *Mol Reprod Dev*. 1999; 52:196–206. [PubMed: 9890751]
5. Morin G, Lalancette C, Sullivan R, Leclerc P. Identification of the bull sperm p80 protein as a PH-20 ortholog and its modification during the epididymal transit. *Mol Reprod Dev*. 2005; 71:523–534. [PubMed: 15892045]
6. Tulsiani DRP. Glycan modifying enzymes in luminal fluid of rat epididymis: Are they involved in altering sperm surface glycoproteins during maturation? *Microsc Res Tech*. 2003; 61:18–27. [PubMed: 12672119]
7. Aitken RJ, Nixon B, Lin M, Koppers AJ, et al. Proteomic changes in mammalian spermatozoa during epididymal maturation. *Asian J Androl*. 2007; 9:554–564. [PubMed: 17589795]
8. White DR, Aitken RJ. Influence of epididymal maturation on cyclic AMP levels in hamster spermatozoa. *Int J Androl*. 1989; 12:29–43. [PubMed: 2541086]
9. Nikolopoulou M, Soucek DA, Vary JC. Changes in the lipid content of boar sperm plasma membranes during epididymal maturation. *Biochim Biophys Acta*. 1985; 815:486–498. [PubMed: 3995037]
10. Dacheux JL, Gatti JL, Dacheux F. Contribution of epididymal secretory proteins for spermatozoa maturation. *Microsc Res Tech*. 2003; 61:7–17. [PubMed: 12672118]
11. Yanagimachi R, Noda YD, Fujimoto M, Nicolson GL. The distribution of negative surface charges on mammalian spermatozoa. *Am J Anat*. 1972; 135:497–519. [PubMed: 4674031]
12. Calvin HI, Bedford JM. Formation of disulphide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. *J Reprod Fertil Suppl*. 1971; 13(Suppl 13):65–75. [PubMed: 5292801]
13. Shalgi R, Seligman J, Kosower NS. Dynamics of the thiol status of rat spermatozoa during maturation: analysis with the fluorescent labeling agent monobromobimane. *Biol Reprod*. 1989; 40:1037–1045. [PubMed: 2765609]
14. Gaddum P. Sperm maturation in the male reproductive tract: development of motility. *Anat Rec*. 1968; 161:471–482. [PubMed: 4880105]
15. Cornwall GA, Vindivich D, Tillman S, Chang TS. The effect of sulfhydryl oxidation on the morphology of immature hamster epididymal spermatozoa induced to acquire motility in vitro. *Biol Reprod*. 1988; 39:141–155. [PubMed: 3207793]
16. Cornwall GA, Smyth TB, Vindivich D, Harter C, et al. Induction and enhancement of progressive motility in hamster caput epididymal spermatozoa. *Biol Reprod*. 1986; 35:1065–1074. [PubMed: 3814693]
17. Wessel D, Flügge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem*. 1984; 138:141–143. [PubMed: 6731838]
18. Strader MB, Tabb DL, Hervey WJ, Pan C, Hurst GB. Efficient and specific trypsin digestion of microgram to nanogram quantities of proteins in organic-aqueous solvent systems. *Anal Chem*. 2006; 78:125–134. [PubMed: 16383319]
19. Sawtell NM, Lessard JL. Cellular distribution of smooth muscle actins during mammalian embryogenesis: expression of the alpha-vascular but not the gamma-enteric isoform in differentiating striated myocytes. *J Cell Biol*. 1989; 109:2929–2937. [PubMed: 2687290]
20. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227:680–685. [PubMed: 5432063]

21. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA*. 1979; 76:4350–4354. [PubMed: 388439]
22. Krisfalusi M, Miki K, Magyar PL, O'Brien DA. Multiple glycolytic enzymes are tightly bound to the fibrous sheath of mouse spermatozoa. *Biol Reprod*. 2006; 75:270–278. [PubMed: 16687649]
23. Vemuganti SA, Bell TA, Scarlett CO, Parker CE, et al. Three male germline-specific aldolase A isozymes are generated by alternative splicing and retrotransposition. *Dev Biol*. 2007; 309:18–31. [PubMed: 17659271]
24. Arcelay E, Salicioni AM, Wertheimer E, Visconti PE. Identification of proteins undergoing tyrosine phosphorylation during mouse sperm capacitation. *Int J Dev Biol*. 2008; 52:463–472. [PubMed: 18649259]
25. Gatti JL, Druart X, Guérin Y, Dacheux F, Dacheux JL. A 105- to 94-Kilodalton Protein in the Epididymal Fluids of Domestic Mammals Is Angiotensin I-Converting Enzyme (ACE); Evidence That Sperm Are the Source of This ACE. *Biology of Reproduction*. 1999; 60:937–945. [PubMed: 10084969]
26. Gaudreault C, El Alfy M, Légaré C, Sullivan R. Expression of the hamster sperm protein P26h during spermatogenesis. *Biol Reprod*. 2001; 65:79–86. [PubMed: 11420226]
27. Frenette G, Sullivan R. Prostate-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. *Mol Reprod Dev*. 2001; 59:115–121. [PubMed: 11335953]
28. Roberts KP, Ensrud KM, Hamilton DW. A comparative analysis of expression and processing of the rat epididymal fluid and sperm-bound forms of proteins D and E. *Biol Reprod*. 2002; 67:525–533. [PubMed: 12135891]
29. Sullivan R, Frenette G, Girouard J. Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. *Asian J Androl*. 2007; 9:483–491. [PubMed: 17589785]
30. Kameshwari DB, Bhande S, Sundaram CS, Kota V, et al. Glucose-regulated protein precursor (GRP78) and tumor rejection antigen (GP96) are unique to hamster caput epididymal spermatozoa. *Asian J Androl*. 2010; 12:344–355. [PubMed: 20400973]
31. Guo W, Qu F, Xia L, Guo Q, et al. Identification and characterization of ERp29 in rat spermatozoa during epididymal transit. *Reproduction*. 2007; 133:575–584. [PubMed: 17379652]
32. Asano A, Nelson JL, Zhang S, Travis AJ. Characterization of the proteomes associating with three distinct membrane raft sub-types in murine sperm. *Proteomics*. 2010; 10:3494–3505. [PubMed: 20815087]
33. Phelps BM, Koppel DE, Primakoff P, Myles DG. Evidence that proteolysis of the surface is an initial step in the mechanism of formation of sperm cell surface domains. *J Cell Biol*. 1990; 111:1839–1847. [PubMed: 2229175]
34. Anakwe OO, Sharma S, Hoff HB, Hardy DM, Gerton GL. Maturation of guinea pig sperm in the epididymis involves the modification of proacrosin oligosaccharide side chains. *Molecular Reproduction and Development*. 1991; 29:294–301. [PubMed: 1931047]
35. Blobel CP. Functional processing of fertilin: evidence for a critical role of proteolysis in sperm maturation and activation. *Rev Reprod*. 2000; 5:75–83. [PubMed: 10864851]
36. Mitchell LA, Nixon B, Aitken RJ. Analysis of chaperone proteins associated with human spermatozoa during capacitation. *Mol Hum Reprod*. 2007; 13:605–613. [PubMed: 17595329]
37. Dix DJ. Hsp70 expression and function during gametogenesis. *Cell Stress Chaperones*. 1997; 2:73–77. [PubMed: 9250397]
38. Dix DJ, Allen JW, Collins BW, Mori C, et al. Targeted gene disruption of Hsp70–2 results in failed meiosis, germ cell apoptosis, and male infertility. *Proc Natl Acad Sci USA*. 1996; 93:3264–3268. [PubMed: 8622925]
39. Dix DJ, Allen JW, Collins BW, Poorman-Allen P, et al. HSP70–2 is required for desynapsis of synaptonemal complexes during meiotic prophase in juvenile and adult mouse spermatocytes. *Development*. 1997; 124:4595–4603. [PubMed: 9409676]
40. Govin J, Caron C, Escoffier E, Ferro M, et al. Post-meiotic shifts in HSPA2/HSP70.2 chaperone activity during mouse spermatogenesis. *J Biol Chem*. 2006; 281:37888–37892. [PubMed: 17035236]

41. Huszar G, Stone K, Dix D, Vigue L. Putative creatine kinase M-isoform in human sperm is identified as the 70-kilodalton heat shock protein HspA2. *Biol Reprod.* 2000; 63:925–932. [PubMed: 10952940]
42. Naaby-Hansen S, Herr JC. Heat shock proteins on the human sperm surface. *J Reprod Immunol.* 2010; 84:32–40. [PubMed: 19962198]
43. Shiu RP, Pouyssegur J, Pastan I. Glucose depletion accounts for the induction of two transformation-sensitive membrane proteins in Rous sarcoma virus-transformed chick embryo fibroblasts. *Proc Natl Acad Sci USA.* 1977; 74:3840–3844. [PubMed: 198809]
44. Merrick BA, Walker VR, He C, Patterson RM, Selkirk JK. Induction of novel Grp75 isoforms by 2-deoxyglucose in human and murine fibroblasts. *Cancer Lett.* 1997; 119:185–190. [PubMed: 9570370]
45. Määttänen P, Gehring K, Bergeron JJM, Thomas DY. Protein quality control in the ER: the recognition of misfolded proteins. *Semin Cell Dev Biol.* 2010; 21:500–511. [PubMed: 20347046]
46. Lachance C, Fortier M, Thimon V, Sullivan R, et al. Localization of Hsp60 and Grp78 in the human testis, epididymis and mature spermatozoa. *Int J Androl.* 2010; 33:33–44. [PubMed: 19207617]
47. Bhattacharyya T, Karnezis AN, Murphy SP, Hoang T, et al. Cloning and subcellular localization of human mitochondrial hsp70. *J Biol Chem.* 1995; 270:1705–1710. [PubMed: 7829505]
48. Schneider HC, Berthold J, Bauer MF, Dietmeier K, et al. Mitochondrial Hsp70/MIM44 complex facilitates protein import. *Nature.* 1994; 371:768–774. [PubMed: 7935837]
49. Asquith KL, Baleato RM, McLaughlin EA, Nixon B, Aitken RJ. Tyrosine phosphorylation activates surface chaperones facilitating sperm-zona recognition. *J Cell Sci.* 2004; 117:3645–3657. [PubMed: 15252132]
50. Han C, Park I, Lee B, Jin S, et al. Identification of heat shock protein 5, calnexin and integral membrane protein 2B as Adam7-interacting membrane proteins in mouse sperm. *J Cell Physiol.* 2011; 226:1186–1195. [PubMed: 20945367]
51. Waters SH, Distel RJ, Hecht NB. Mouse testes contain two size classes of actin mRNA that are differentially expressed during spermatogenesis. *Mol Cell Biol.* 1985; 5:1649–1654. [PubMed: 4022010]
52. Kim E, Waters SH, Hake LE, Hecht NB. Identification and developmental expression of a smooth-muscle gamma-actin in postmeiotic male germ cells of mice. *Mol Cell Biol.* 1989; 9:1875–1881. [PubMed: 2747639]
53. Oko R, Hermo L, Hecht NB. Distribution of actin isoforms within cells of the seminiferous epithelium of the rat testis: evidence for a muscle form of actin in spermatids. *Anat Rec.* 1991; 231:63–81. [PubMed: 1750712]
54. Howes EA, Hurst SM, Jones R. Actin and actin-binding proteins in bovine spermatozoa: potential role in membrane remodeling and intracellular signaling during epididymal maturation and the acrosome reaction. *J Androl.* 2001; 22:62–72. [PubMed: 11191089]
55. Flaherty SP, Winfrey VP, Olson GE. Localization of actin in mammalian spermatozoa: a comparison of eight species. *Anat Rec.* 1986; 216:504–515. [PubMed: 2432804]
56. Fouquet JP, Kann ML. Species-specific localization of actin in mammalian spermatozoa: fact or artifact? *Microsc Res Tech.* 1992; 20:251–258. [PubMed: 1543878]
57. Fujihara Y, Murakami M, Inoue N, Satouh Y, et al. Sperm equatorial segment protein 1, SPESP1, is required for fully fertile sperm in mouse. *J Cell Sci.* 2010; 123:1531–1536. [PubMed: 20375058]
58. Nakamura M, Okinaga S, Arai K. Metabolism of pachytene primary spermatocytes from rat testes: pyruvate maintenance of adenosine triphosphate level. *Biol Reprod.* 1984; 30:1187–1197. [PubMed: 6733209]
59. Nakamura M, Okinaga S, Arai K. Metabolism of round spermatids: evidence that lactate is preferred substrate. *Am J Physiol.* 1984; 247:E234–242. [PubMed: 6431825]
60. Storey BT, Kayne FJ. Energy metabolism of spermatozoa. V. The Embden-Myerhof pathway of glycolysis: activities of pathway enzymes in hypotonically treated rabbit epididymal spermatozoa. *Fertil Steril.* 1975; 26:1257–1265. [PubMed: 803042]

61. Miki K, Qu W, Goulding EH, Willis WD, et al. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proc Natl Acad Sci USA*. 2004; 101:16501–16506. [PubMed: 15546993]
62. Danshina PV, Geyer CB, Dai Q, Goulding EH, et al. Phosphoglycerate kinase 2 (PGK2) is essential for sperm function and male fertility in mice. *Biol Reprod*. 2010; 82:136–145. [PubMed: 19759366]
63. Mukai C, Okuno M. Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. *Biol Reprod*. 2004; 71:540–547. [PubMed: 15084484]
64. Volker KW, Knull H. A glycolytic enzyme binding domain on tubulin. *Arch Biochem Biophys*. 1997; 338:237–243. [PubMed: 9028878]
65. Gitlits VM, Toh BH, Loveland KL, Sentry JW. The glycolytic enzyme enolase is present in sperm tail and displays nucleotide-dependent association with microtubules. *Eur J Cell Biol*. 2000; 79:104–111. [PubMed: 10727018]
66. Cao W, Gerton GL, Moss SB. Proteomic profiling of accessory structures from the mouse sperm flagellum. *Mol Cell Proteomics*. 2006; 5:801–810. [PubMed: 16452089]
67. Edwards YH, Grootegeed JA. A sperm-specific enolase. *J Reprod Fertil*. 1983; 68:305–310. [PubMed: 6864646]
68. Russell DL, Kim KH. Expression of triosephosphate isomerase transcripts in rat testis: evidence for retinol regulation and a novel germ cell transcript. *Biol Reprod*. 1996; 55:11–18. [PubMed: 8793052]
69. Auer J, Camoin L, Courtot AM, Hotellier F, De Almeida M. Evidence that P36, a human sperm acrosomal antigen involved in the fertilization process is triosephosphate isomerase. *Mol Reprod Dev*. 2004; 68:515–523. [PubMed: 15236338]
70. Patel, M.; Korotchkina, L. Regulation of the pyruvate dehydrogenase complex. 2006.
71. Kumar V, Rangaraj N, Shivaji S. Activity of pyruvate dehydrogenase A (PDHA) in hamster spermatozoa correlates positively with hyperactivation and is associated with sperm capacitation. *Biol Reprod*. 2006; 75:767–777. [PubMed: 16855207]
72. Fujinoki M, Kawamura T, Toda T, Ohtake H, et al. Identification of 36-kDa flagellar phosphoproteins associated with hamster sperm motility. *J Biochem*. 2003; 133:361–369. [PubMed: 12761172]
73. Fujinoki M, Kawamura T, Toda T, Ohtake H, et al. Identification of 36 kDa phosphoprotein in fibrous sheath of hamster spermatozoa. *Comp Biochem Physiol B, Biochem Mol Biol*. 2004; 137:509–520. [PubMed: 15082001]
74. Mitra K, Rangaraj N, Shivaji S. Novelty of the Pyruvate Metabolic Enzyme Dihydrolipoamide Dehydrogenase in Spermatozoa. *Journal of Biological Chemistry*. 2005; 280:25743–25753. [PubMed: 15888450]
75. He L, Lemasters JJ. Dephosphorylation of the Rieske iron-sulfur protein after induction of the mitochondrial permeability transition. *Biochemical and Biophysical Research Communications*. 2005; 334:829–837. [PubMed: 16023995]
76. Fulcher KD, Welch JE, Klapper DG, O'Brien DA, Eddy EM. Identification of a unique mu-class glutathione S-transferase in mouse spermatogenic cells. *Mol Reprod Dev*. 1995; 42:415–424. [PubMed: 8607970]
77. Dehari H, Tchaikovskaya T, Rubashevsky E, Sellers R, Listowsky I. The proximal promoter governs germ cell-specific expression of the mouse glutathione transferase mGstm5 gene. *Mol Reprod Dev*. 2009; 76:379–388. [PubMed: 18932202]
78. Lo HW, Antoun GR, Ali-Osman F. The human glutathione S-transferase P1 protein is phosphorylated and its metabolic function enhanced by the Ser/Thr protein kinases, cAMP-dependent protein kinase and protein kinase C, in glioblastoma cells. *Cancer Res*. 2004; 64:9131–9138. [PubMed: 15604283]
79. Baker MA, Witherdin R, Hetherington L, Cunningham-Smith K, Aitken RJ. Identification of post-translational modifications that occur during sperm maturation using difference in two-dimensional gel electrophoresis. *Proteomics*. 2005; 5:1003–1012. [PubMed: 15712234]

80. Couldrey C, Carlton MBL, Ferrier J, Colledge WH, Evans MJ. Disruption of murine α -enolase by a retroviral gene trap results in early embryonic lethality. *Dev Dyn*. 1998; 212:284–292. [PubMed: 9626503]
81. Luo S, Mao C, Lee B, Lee AS. GRP78/BiP Is Required for Cell Proliferation and Protecting the Inner Cell Mass from Apoptosis during Early Mouse Embryonic Development. *Mol Cell Biol*. 2006; 26:5688–5697. [PubMed: 16847323]
82. Frenette G, Lessard C, Madore E, Fortier MA, Sullivan R. Aldose reductase and macrophage migration inhibitory factor are associated with epididymosomes and spermatozoa in the bovine epididymis. *Biol Reprod*. 2003; 69:1586–1592. [PubMed: 12826572]
83. Frenette G, Girouard J, Sullivan R. Comparison between epididymosomes collected in the intraluminal compartment of the bovine caput and cauda epididymidis. *Biol Reprod*. 2006; 75:885–890. [PubMed: 16943362]

Figure 1. Scanned image of representative Cy3-labeled mixed internal standard (reference gel) from analytical gels and the position of protein spots that were identified by MS (see Fig. 2 for screening spots). (A) 16 caput epididymal sperm-differential spots and (B) 10 cauda epididymal sperm-differential spots. The horizontal open arrows show the pH gradient from 3 to 11 (nonlinear). Values on the left indicate the approximate molecular weights of four proteins identified from the proteomic analysis: HSPA5 (72,377); ENO1 (47,111); GSTM5 (26,617); and D-dopachrome tautomerase, DDT (13,069). Gradient (10–20%) SDS polyacrylamide gels with reference markers were used.

Figure 2. Differential protein spots between caput and cauda epididymal sperm identified by 2-D DIGE. One-way ANOVA screened 388 spots between four protein populations ($p < 0.01$). Furthermore, Student's *t*-test enabled the selection of protein spots that differed by more than 1.2-fold ($p < 0.05$) between reduced proteins of caput and cauda epididymal sperm populations or between unreduced proteins of caput and cauda epididymal sperm populations, resulting in the designation of 21 caput epididymal sperm-differential and 15 cauda epididymal sperm-differential candidates. Of these, 16 and 10 spots, respectively, were picked for further analysis.

Figure 3.

Validation of 2-D DIGE experiments by immunoblot analyses. (A) Four caput epididymal sperm-differential proteins, (B) four cauda epididymal sperm-differential proteins, and (C) control proteins (α -tubulin and DLAT). Proteins from caput epididymal sperm and cauda epididymal sperm were transferred onto PVDF membranes and incubated with corresponding antibodies. Then blots were detected with the ECF system and their images were analyzed by scanning on a Storm system. The bands shown are representative for three experiments. (D) The pixel volumes of the protein bands were normalized against α -tubulin and the caput/cauda ratio was expressed logarithmically. Mean values are represented $\pm$ SD ($n = 3$). Statistical analysis was performed with a t -test ($* = p < 0.05$). Positive and negative ratios illustrate caput epididymal-sperm differential and cauda epididymal-sperm differential, respectively.

Figure 4.

Indirect immunofluorescence of two caput epididymal sperm-differential proteins (HSPA2 and ACTG2). Caput epididymal sperm were probed with anti-HSPA2 (A) and anti-smooth muscle actin (i.e., ACTG2) (E, G). Cauda epididymal sperm were probed with anti-HSPA2 (C) and anti-smooth muscle actin (I, K). The corresponding Nomarski images are also shown (B, D, F, H, J, L). In negative controls, no signal was detected on caput or cauda epididymal sperm with normal mouse IgG (data not shown). In caput epididymal sperm, two types of ACTG2 intensities were observed: strong (E) and moderate (G) signals, however moderate intensity was minor (I) and a faint signal was detected mostly in cauda epididymal sperm (K). Bar = 10 μ m.

Figure 5.

Indirect immunofluorescence of one cauda epididymal sperm-differential protein (ALDOA) and one protein (DLAT) that was identified as caput epididymal sperm-differential as well as cauda epididymal sperm-differential due to a shift in isoelectric point. Caput epididymal sperm were probed with anti-ALDOA (A) and anti-PDC-E2 (i.e., DLAT) (E). Cauda epididymal sperm were probed with anti-ALDOA (C) and anti-PDC-E2 (G). The corresponding Nomarski images are paired with the fluorescent images (B, D, F, H). In negative controls, no signal was detected on caput and cauda epididymal sperm with normal rabbit IgG and normal goat IgG for ALDOA and DLAT, respectively (data not shown). Bar = 10 μ m.

Summary of the proteins identified as caput epididymal sperm-differential and cauda epididymal sperm-differential proteins by 2-D DIGE.^{a)}

Protein identification ^{b)}	Symbol ^{c)}	Function	Spot no.
Caput epididymal sperm-differential proteins			
Heat shock protein 2	HSPA2	Molecular chaperone	1125, 1127
Heat shock protein 5	HSPA5	Molecular chaperone	1036
Heat shock protein 9	HSPA9	Molecular chaperone	1125, 1127
Actin, gamma, cytoplasmic 1	ACTG1	Structural; Cell motility	1634
Actin, gamma 2, smooth muscle, enteric	ACTG2	Structural; Cell motility	1633, 1634, 1643, 1649
Sperm equatorial segment protein 1	SPSP1	Structural; Membrane fusion	1633, 1634, 1649
Succinate-Coenzyme A ligase, ADP-forming, beta subunit	SUCLA2	Energy metabolism	1633, 1634, 1649
D-dopachrome tautomerase	DDT	Unknown	2993
Cauda epididymal sperm-differential proteins			
A kinase (PRKA) anchor protein 3	AKAP3	Structural; Signal transduction	1940
A kinase (PRKA) anchor protein 4	AKAP4	Structural; Signal transduction	1188
Aldolase A, fructose-bisphosphate	ALDOA	Energy metabolism	1567
ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit	ATP5O	Energy metabolism	2536
Enolase 1, alpha non-neuron	ENO1	Energy metabolism	1567
NADH dehydrogenase (ubiquinone) flavoprotein 2	NDUFV2	Energy metabolism	2312
Pyruvate dehydrogenase (lipoamide) beta	PDHB	Energy metabolism	1932, 1940, 2303
Triosephosphate isomerase 1	TPP1	Energy metabolism	1932, 1940
Diazepam binding inhibitor-like 5	DBIL5	Unknown	3110

^{a)}Detailed information is shown in Supporting Information Tables S1 and S2.

^{b), c)} Protein names and symbols are from Mouse Genome Informatics (<http://www.informatics.jax.org/>)