INTRODUCTION

After spermatogenesis in the testis, spermatozoa pass sequentially through the caput and corpus epididymides and are retained in the cauda epididymis where they are stored until ejaculation (Cornwall, 2009). As spermatozoa migrate 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 (Sullivan et al., 2007). One of these alterations is an increase in the oxidation of sulfhydryl groups of sperm proteins resulting in more disulfide bonds and fewer free sulfhydryls. Earlier studies suggested that most sperm structures, such as the chromatin and tail components, gradually become stabilized by formation of disulfide bonds (Bedford & Calvin, 1974a, b; Calvin & Bedford, 1971). Similarly, labelling with the fluorescent thiol labelling agent mono-bromobimane (mBBr) demonstrates that the protein sulfhydryls of rat sperm structures become more oxidized to disulfide bonds during epididymal transit (Shalgi et al., 1989).

The elevation of the ubiquitous second messenger cyclic AMP (cAMP) is an additional change that spermatozoa undergo as they move through the epididymis (White & Aitken, 1989). The in vitro addition of cell permeant cAMP analogues to hamster caput epididymal spermatozoa results in prematurely amplified motility and flagellar angulation where the sperm tails fold back on themselves. The addition of diamide, a sulfhydryl oxidant, prevents this phenomenon (Cornwall et al., 1986, 1988). On the other hand, hamster cauda epididymal spermatozoa do not exhibit angularity when intracellular cAMP levels are raised (Cornwall et al., 1988). However, these sperm cells will display flagellar angulation when dithiothreitol (DTT) is added (Cornwall & Chang, 1990). These observations have led to the concept that epididymal maturation results in stabilization of flagellar...
structures through oxidation of protein sulphydryls to disulfides thereby preventing against angulation when motility is stimulated by a rise in internal cAMP.

Two classes of adenyl cyclases (ADCYs) convert ATP to cAMP: transmembrane ADCYs and ‘soluble’ ADCY10 (formerly SACY). ADCY10 activity was originally described over 35 years ago in the rat testis and, although present in somatic tissues, is most abundant and its activity most prevalent in the mammalian testis (Braun & Dods, 1975; Sinclair et al., 2000). Most of cAMP in developing germ cells and mature sperm cells is generated by ADCY10, making this enzyme an essential component of cAMP signalling in spermatozoa (Hess et al., 2005). Sperm cells from Adcy10-null males are immotile, owing to the lack of bicarbonate-induced ADCY10 activity, rendering them infertile (Espostito et al., 2004). Their motility can be rescued by the addition of the membrane-permeant cAMP analogue, dibutyryl cAMP (dbcAMP); however, they are still unable to fertilize eggs (Hess et al., 2005). Like caput epididymal spermatozoa of wild-type animals, Adcy10-null spermatozoa show flagellar angularity after the addition of dbcAMP and are not able to attach to the zona pellucida (Hess et al., 2005). From these observations, we hypothesized that ADCY10 is required for flagellar stability and that flagellar angulation of Adcy10-null spermatozoa is caused by diminished disulfide bonding during epididymal maturation. A lack of ADCY10 may limit the ability of the sperm tail to become stabilized in the epididymis.

In this study, we used spermatozoa from Adcy10-null and wild-type mice for examination of flagellar angulation. We identified four sperm proteins: A-kinase anchor protein 4 (AKAP4), fatty acid-binding protein 9 (FABP9), glutathione S-transferase mu 5 (GSTM5) and voltage-dependent anion channel 2 (VDAC2) that exhibited changes in thiol status during epididymal maturation. Although these proteins were present at similar levels in spermatozoa from Adcy10-null and wild-type mice, on the basis of the experiments performed, FABP9 from Adcy10-null mouse spermatozoa contained fewer disulfide bonds.

MATERIALS AND METHODS

Purification of caput and cauda epididymal spermatozoa and protein extraction

All animal procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Spermatozoa were collected from the caput and cauda epididymes of adult C57BL/6 (Charles River Laboratories, Wilmington, MA, USA) and Adcy10-null male mice of similar ages. The Adcy10-null genotype has been previously described (Hess et al., 2005). Caput and cauda epididymal spermatozoa were extracted from the epididymes into phosphate-buffered saline (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, USA) and purified by centrifugation through a 35% (v/v) SupraSperm 100 solution (MidAtlantic Diagnostics, Mt. Laurel, NJ, USA) in PBS. Purified sperm cells were resuspended in PBS containing protease inhibitors at 4°C and counted in a haemocytometer.

For 2-D DIGE, sperm cells were concentrated by centrifugation at 10 000 g for 2 min then lysed by sonication at 4°C in cell lysis buffer (8 mM urea, 40 mM Tris-HCl, pH 8.0, 4% (w/v) CHAPS) containing protease inhibitors. Approximately 1 µg of protein was collected from 4 × 10⁶ spermatozoa. Protein lysates were used immediately.

For SDS-PAGE, sperm cells were concentrated by centrifugation at 10 000 g for 2 min, resuspended in PBS containing protease inhibitors, sonicated for 1 min, and then diluted in sample buffer [58 mM Tris-HCl, pH 6.8, 1.71% (w/v) SDS, 6% (v/v) glycerol, 0.0024% (w/v) bromophenol blue, with or without 100 mM DTT]. Samples were then vortexed, boiled for 5 min, and sonicated a second time. After centrifugation at 10 000 g for 5 min, the supernatants were recovered, snap-frozen and stored at −80°C until used.

Visualization of thiol groups with mBBr

For microscopic observation, 2.5 × 10⁶ caput and cauda epididymal spermatozoa were collected as described above and distributed to four tubes (two caput and two cauda). The fluid volume in each tube was adjusted to 1 mL. DTT (154 µg) was added to epididymal sperm suspensions to a final concentration of 1 mM. All four samples were incubated for 10 min at 37°C. The samples were washed twice by centrifugation at 700 g for 5 min. The sperm pellet was resuspended in 500 µm mBBr (Life Technologies, Carlsbad, CA, USA) in PBS and incubated for 10 min in the dark at room temperature. After being washed with PBS, 200 µL of sperm suspension was spread on a glass slide and allowed to dry for 15 min. Slides were mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, USA) and covered with a coverslip. The samples were observed as described below.

For 1-D gel electrophoresis, 2 × 10⁶ cauda epididymal spermatozoa were collected as described above and divided into two samples, but only one was treated with 5 mM DTT in PBS containing protease inhibitors. Samples were incubated for 10 min at 37°C. After being washed with PBS, 5 mM mBBr was added to the sperm cells for labelling at room temperature for 15 min in the dark. Spermatozoa were prepared for SDS-PAGE as described below. Samples were separated on a 12% (w/v) polyacrylamide gel and then protein fluorescence patterns were visualized by UV light (302 nm) using the Gel Doc 2000 Gel Documentation System (Bio-Rad Laboratories, Hercules, CA, USA). Gels were stained with Coomassie blue to determine protein loading. The values of protein fluorescence were compared with ImageJ software available online (http://rsb.info.nih.gov/ij/) (data not shown).

2-D fluorescence difference gel electrophoresis (2D-DIGE)

A CyDye DIGE Fluor saturation dye labelling protocol (GE Healthcare, Milwaukee, WI, USA) was used to label free cysteine thiols on whole sperm protein extracts. All labelling procedures for 2D-DIGE were carried out under nitrogen. Proteins were extracted as described above from four sperm populations: caput epididymal spermatozoa with or without disulfide reduction with Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) and cauda epididymal spermatozoa with or without disulfide reduction with TCEP. Each of the four sperm populations was analysed in triplicate. Each treatment (caput or cauda epididymal spermatozoa with or without TCEP) was done in triplicate on six mice (18 mice per treatment). The same 18 mice were used for the treatments with caput and cauda epididymal sperm proteins treated with or without TCEP.
To reduce disulfide bonds in sperm cells, 15 μg of each protein lysate were treated with 6 nmol TCEP for 1 h in the dark at 37°C before labelling with fluorescent dye. For both non-reduced and reduced samples, 5 μg of protein lysates from each sperm population were labelled with 4 nmol Cy5 for 30 min in the dark at 37°C. As an internal control, 10 μg of protein lysates from 12 sperm preparations (three samples from caput and cauda epididymal spermatozoa with or without reduction with TCEP) were pooled for a total of 120 μg of protein and labelled 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 (non-linear pH 3–11), 130 mM DTT). Unincorporated dye was removed by extracting the protein lysates with chloroform-methanol in the dark (Wessel & Flügge, 1984).

Five micrograms of Cy3-labelled internal control protein and Cy5-labelled protein from each experimental group were resuspended in 450 μL of rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer (non-linear pH 3–11), 130 mM DTT, 0.002% (w/v) bromophenol blue] and separated by 2-D gel electrophoresis. For the first dimension, samples in 450 μL of rehydration buffer were loaded in the IPGphor strip holder. The 24-cm Immobiline DryStrip (non-linear pH 3–11 gradient) (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 afterwards on the Ettan IPGphor II IEF system for a total of 80 kVh at 20°C. After electrophoresis, each strip was incubated with 7.5 mL equilibration buffer (6 M urea, 100 M Tris–HCl, pH 8.0, 30% (v/v) glycerol, 2% (w/v) SDS, 0.5% (v/v) DTT, 0.002% (w/v) bromophenol blue) by rocking for 20 min. For the second dimension, the strips were placed on top of a 10–20% (w/v) gradient polyacrylamide gel containing SDS with low fluorescence glass plates (Jule Biotechnologies, Milford, CT, USA). SDS-PAGE was performed using the Ettan DALTsix electrophoresis system.

Scanning and image analysis of 2D-DIGE
The Cy3 and Cy5 images were scanned in a Typhoon 9410 scanner (GE Healthcare) at a resolution of 100 μm (pixel size). DeCyder software v5.01 (GE Healthcare) was used to analyse the images. This software performs gel matching, quantification and statistical analyses. Spots were initially detected with the Differential In-Gel Analysis module of the software then protein-spot maps were matched from 12 gels with the Biological Variation Analysis module (Fig. 2). The gel-to-gel variations were normalized against the image of the Cy3-labelled internal control sample. A one-way analysis of variance (ANOVA) was used to compare changes in protein abundance and selected spots that underwent a mobility shift (p < 0.05). Identified spots were further screened using the Student’s t-test to evaluate the low abundance spots in non-reduced cauda epididymal sperm protein samples. Spots were selected when they exhibited a 1.35-fold or more (p < 0.071) difference between non-reduced caput and cauda epididymal sperm proteins (Table S1).

In-gel digestion and mass spectrophotometry analysis of 2D-DIGE spots
To identify proteins that displayed differential patterns, individual spots were excised, in-gel digested, and analysed by mass spectrometry. For the preparative gel, a 500 μg aliquot of pooled protein from the four sperm populations was labelled with 400 nmol Cy3 and separated by 2-D gel electrophoresis as described above. The gel was scanned, placed in fixative (30% (v/v) methanol, 10% (v/v) acetic acid) for 3 days at room temperature, and stored in 5% v/v acetic acid at 4°C until spot selection. Each spot position was determined and a pick list was created using DeCyder software. By the use of an Ettan spot picker (GE Healthcare), the designated gel sections were robotically excised, transferred to microplate wells and digested with trypsin (Strader et al., 2006).

The digested proteins were identified by MALDI-TOF/TOF mass spectrometry in a 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Foster City, CA, USA). MALDI plates were calibrated with six calibration spots, 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 v3.0 (Applied Biosystems).

The spectra were analysed using GPS Explorer software v3.5 (Applied Biosystems), which acts as an interface between the Oracle database containing raw spectra and a local copy of the Mascot search engine v1.9.05. Peptide peaks with a signal/noise ratio greater than 5 and a mass between m/z 900 and 4000 were sought in 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 mined with oxidation of methionine and carbamidomethylation of cysteine as variable modifications.

For microsequencing, trypsin-digested samples were dissolved in 5 μL of 0.1% (v/v) formic acid and injected into a 10 cm long C18 capillary column with an autosampler (Eksigent Technologies, Dublin, CA, USA). The peptide samples were eluted by linearly increasing the mobile phase composition to 98% (v/v) solvent B [0.1% (v/v) formic acid in 100% acetonitrile] at a flow rate of 200 nL/min for 60 min by the use of a nanoLC system (Eksigent Technologies). Online nanospray (Thermo Fisher Scientific, Waltham, MA, USA) was used to spray the separated peptides into a LTQ mass spectrometer (Thermo Fisher Scientific). The raw data were acquired and analysed 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 using the combined MS and MS/MS data. A protein score of >70 with a protein confidence of identification >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 weight protein (Mr < 30 000), matching with a single peptide was also accepted if the peptide score was defined as >70.

Antibodies for immunoblot and indirect immunofluorescence analyses
The commercially available antibodies against AKAP4 and FABP9 did not recognize the proteins in our samples whereas...
commercial anti-GSTM5 (14502-1-AP; Proteintech Group, Chicago, IL, USA) and anti-VDAC (PA1954a; Affinity Bioreagents, Golden, CO, USA) antibodies were successfully used for immunoblotting. Two rabbit polyclonal antibodies were produced for AKAP4 and FABP9. The antibody against AKAP4 was prepared in our laboratory, originally designated as anti-AKAP82, and was raised against the electrophoretically purified AKAP4 protein from the fibrous sheath of the sperm flagellum (Carrera et al., 1994). Anti-perforatorium antibody (PERF15, i.e. FABP9) was a gift from Dr. Richard Oko (Oko et al., 1990). The rabbit polyclonal antibody against the perforatorium was successfully used for immunoblotting, as described below, but did not show specific staining by indirect immunofluorescence. Instead, we used a newly created rabbit polyclonal antibody for mouse FABP9, which was a gift from Dr. Alex Travis, for indirect immunofluorescence (Selvaraj et al., 2010). An antibody for α-tubulin (T5168; Sigma-Aldrich, Saint Louis, MO, USA) was used as an internal control for immunoblotting to compare protein abundance between wild-type and Adcy10-null mice. For immunoblot analysis, the following two secondary antibodies were used: alkaline phosphatase-conjugated, goat anti-mouse IgG + IgM whole antibody (GE Healthcare) and alkaline phosphatase-conjugated, goat anti-rabbit IgG whole antibody (GE Healthcare). For indirect immunofluorescence analysis, Alexa Fluor 488-conjugated goat anti-rabbit IgG (H + L) (Life Technologies) was used as a secondary antibody.

**Immunoblot analysis and quantification**

Wild-type and Adcy10-null cauda epididymal sperm protein extracts from $2 \times 10^4$ (α-tubulin), $1 \times 10^5$ (AKAP4), $5 \times 10^5$ (PERF15), $2 \times 10^5$ (GSTM5) and $1 \times 10^6$ (VDAC) sperm cells were boiled for 5 min then separated by SDS–polyacrylamide gel electrophoresis (PAGE) in polyacrylamide gels (10%: α-tubulin and AKAP4, 12%: GSTM5 and VDAC, 18%: PERF15) (Laemmli, 1970). The gels were then transferred to polyvinylidene difluoride membranes (Towbin et al., 1979). After the membranes were blocked overnight with Tris-buffered saline-Tween (TBST: 125 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.1% (v/v) Tween 20) containing 5% (w/v) non-fat dry milk, they were washed in TBST and incubated for 1 h with the appropriate primary antibody (1 μg/mL mouse anti-α-tubulin antibody, 1: 20 000 rabbit anti-AKAP4 antiserum, 1: 3000 rabbit anti-PERF15 antiserum, 0.175 μg/mL rabbit anti-GSTM5 antibody, 1 μg/mL rabbit anti-VDAC antibody) in TBST containing 5% non-fat dry milk. After being washed with TBST, the blots were incubated for 1 h with the corresponding alkaline phosphatase-conjugated secondary antibody (1: 5000) in TBST containing 5% non-fat dry milk, and after washing with TBST, the bound enzyme was developed with the Enhanced Chemiluminescence (ECL) Western Blotting Reagent Pack (GE Healthcare) according to the manufacturer’s directions. ECL images were scanned in a Storm 860 scanner (GE Healthcare). The pixel volume of each band was quantified with ImageQuant software v5.2 (GE Healthcare) and the abundance of each protein was normalized against α-tubulin. The pixel volume results were analysed with t-tests and expressed as the mean ± standard deviation from experiments done in triplicate. A $p < 0.05$ was considered statistically significant. Negative controls using normal sera for AKAP4 and PERF15, purified IgG, or secondary antibody alone were used to check for specificity.

**Detection of disulfide cross-linking of sperm proteins**

Cauda epididymal spermatozoa from wild-type and Adcy10-null mice were collected as described above. For DTT-treated samples, 100 mM DTT was added, and samples were incubated at room temperature for 10 min. Samples not treated with DTT were incubated at room temperature for 10 min. After the addition of 5 mM iodoacetic acid to DTT-treated and untreated samples, the pH was adjusted to 7.2. Samples were incubated for 30 min in the dark at room temperature. Samples were sonicated then centrifuged at 10 000 g for 5 min, and the supernatants were snap-frozen and stored at −80°C.

SDS-PAGE, immunoblotting, scanning and data quantification were performed as described above with the following modifications: $1.5 \times 10^5$ (AKAP4), $7.5 \times 10^5$ (PERF15), $3 \times 10^5$ (GSTM5) and $1.5 \times 10^6$ (VDAC) cauda epididymal spermatozoa were used. The dilutions and concentrations of the primary antibodies were as follows: 1 : 15000 anti-AKAP4, 1 : 2250 anti-PERF15, 0.233 μg/mL anti-GSTM5 and 1.33 μg/mL anti-VDAC.

**Indirect immunofluorescence analysis**

One million caput or cauda epididymal spermatozoa, collected as described above, were attached to polylysine-coated coverslips for 15 min then fixed and permeabilized with acetone/methanol (1 : 1) for 1 min at −20°C. After extensive washing with PBS, the coverslips were incubated overnight at 4°C with 10% goat serum in PBS (blocking solution). The following day, coverslips were rinsed with PBS and incubated for 1 h at 37°C with anti-FABP9 antibody (1 : 100) diluted in blocking solution. After being washed with PBS, the coverslips were incubated for 1 h at 37°C with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1 : 500) diluted in blocking solution. The coverslips were washed with PBS, mounted on slides with 15 μL of Fluoromount-G, observed with a Nikon Eclipse TE 2000-U inverted microscope (Nikon Instruments, Melville, NY, USA), and photographed with a CFW-1310C digital FireWire camera (Scion, Frederick, MD, USA) with NIH ImageJ. Nomarski differential interference contrast micrographs were photographed in parallel with the fluorescence images. Negative controls with normal sera or secondary antibody alone were also used to check for specificity.

**RESULTS**

A global difference in protein thiol status was not observed between wild-type and Adcy10-null spermatozoa

The fluorescent stain mBBr was used to visualize free sulphydryl groups in whole caput and cauda epididymal spermatozoa from wild-type and Adcy10-null mice. Spermatozoa treated with DTT to reduce disulfide bonds were used as controls. Intense staining was detected throughout the entire sperm cells in control samples (DTT treated). Similar to the controls, caput epididymal spermatozoa displayed fluorescent staining in the heads and flagella (Fig. 1A). However, cauda epididymal spermatozoa had only faint fluorescent signals throughout the cells (Fig. 1B). There were no obvious differences between wild-type and Adcy10-null spermatozoa in these comparisons. Next, we investigated the band patterns of wild-type and Adcy10-null cauda epididymal sperm proteins labelled with mBBr. DTT-treated spermatozoa were used as controls. No difference between the
fluorescence patterns of wild-type and Adcy10-null cauda epididymal spermatozoa were observed (Fig. 1D).

AKAP4, FABP9, GSTM5 and VDAC2 exhibit changes in thiol status during epididymal maturation

We did not detect a change in global disulfide bonding between wild-type and Adcy10-null cauda epididymal sperm proteins, which prompted us to examine more closely the disulfide bond status of individual proteins by proteomic techniques. We used 2-D DIGE to identify proteins in which sulfhydryl groups became oxidized during epididymal maturation. Differences in disulfide bonding were detected by comparing samples from caput and cauda epididymal spermatozoa whose proteins were reduced or not reduced with TCEP before labelling with the fluorescent dye Cy5. As the internal control, Cy3-labelled proteins demonstrated a highly reproducible pattern of fluorescent protein spots in many scanned images (data not shown).

We identified multiple spots as sperm proteins that changed their thiol status during epididymal maturation. Initially, approximately 3000 spots in each gel were identified with more than 1500 of them being present in all 12 gels. Protein abundance changes of 755 spots between the four protein populations were shown to be statistically significant \( (p < 0.05) \) by one-way ANOVA. From these, 57 spots were selected as low abundant spots, only in non-reduced cauda epididymal sperm populations (Table S1). Twenty-three candidate spots for thiol status change were selected from analytical gels; however, three of them were missing from the preparative gel (Fig. 2). Finally, 20 spots were picked from the preparative gel for mass spectrometric analysis. The sequence information for 18 spots was obtained, and the peptide matches to entries in the database are shown in Table S1. Some spots contained peptides that corresponded to more than one protein. GSTM5 and VDAC2 were identified from single spots (#2281 and #1909 respectively). However, AKAP4 was identified from three spots (#2281, #2781 and #2796). All three spots possessed different isoelectric points. Spot #2281 was of higher molecular weight \( (M_r 26,617) \) than the other two spots (#2781 and #2796: \( M_r 15,079 \)) and corresponded by size and peptide sequences to the ‘Pro’ domain of the AKAP4 precursor (Johnson et al., 1997). The peptide from spots #2781 and #2796 aligned 34 amino acids downstream from the pro-AKAP4 cleavage site.

Twelve spots (#2652, #2663, #2672, #2725, #2781, #2796, #2803, #2815, #2816, #2864, #2883 and #2966) were derived from FABP9. These spots showed similar molecular weights \( (M_r \sim 15,079) \) yet could be classified into two major clusters. The first cluster (#2652, #2663, #2672, and #2725) was positioned slightly higher than the second cluster (#2781, #2796, #2803, #2815, #2816, #2864, #2883 and #2966) were derived from FABP9. These spots showed similar molecular weights \( (M_r \sim 15,079) \) yet could be classified into two major clusters. The first cluster (#2652, #2663, #2672, and #2725) was positioned slightly higher than the second cluster (#2781, #2796, #2803, #2815, #2864 and #2883) on the 2-D gel (Fig. 3). #2816 was detected between the first and second clusters whereas #2966 was detected lower than the second cluster. It is important to note that the isoelectric points of FABP9 ranged widely on the 2-D gel. The 12 spots could be divided into seven types that range from acidic to basic:
Six spots (#2008, #2602, #2652, #2749, #2781 and #2966) were identified as keratin 1 (KRT1), keratin 4 (KRT4), keratin 10 (KRT10) or keratin 15 (KRT15). In addition, the α- and β1-subunits of haemoglobin was detected in spot #3089. We did not pursue analysis of these proteins because there are limited data concerning their expression during spermatogenesis and their presence in mature spermatozoa.

**AKAP4, FABP9, GSTM5 and VDAC2 are present at similar levels in wild-type and Adcy10-null spermatozoa**

SDS-PAGE and immunoblotting were used to determine the relative levels of AKAP4, FABP9, GSTM5 and VDAC2 in wild-type and Adcy10-null cauda epididymal spermatozoa. ImageQuant software was used to measure the pixel volume of each protein band, which was then used to quantify and compare the relative abundance of AKAP4, FABP9, GSTM5 and VDAC2. To normalize data, α-tubulin was used as an internal control. Normalized volumes were expressed as the wild-type/Adcy10-null ratio. The pixel volumes of AKAP4, FABP9, GSTM5 and VDAC2 were not significantly different between wild-type and Adcy10-null sperm cells (Fig. 4). Three independent wild-type and Adcy10-null epididymal sperm populations were examined, and similar results were obtained in each case.

**Compared with those from wild-type mice, Adcy10-null cauda epididymal spermatozoa have few disulfide bonds in FABP9**

The presence of disulfide bonds in AKAP4, FABP9, GSTM5 and VDAC2 was evaluated by SDS-PAGE and immunoblotting with non-reduced cauda epididymal sperm proteins from wild-type and Adcy10-null mice. All four proteins in non-reduced sperm samples (arrow heads) were identified at the top of the gel, suggesting that these proteins were present in large disulfide-bonded complexes (Fig. 5). In non-reduced samples, antibodies against FABP9, GSTM5 and VDAC proteins detected faint bands at the expected sizes of $M_r$ 15 000, 26 000 and 31 000
respectively. No bands were observed at $M_r$ 82 000 in non-reduced samples when the AKAP4 antibody was used. The monomeric form of FABP9 was detected at greater levels in the non-reduced Adcy10-null sperm protein extracts than in proteins from non-reduced wild-type spermatozoa indicating that a subset of FABP9 proteins were not cross-linked into high molecular weight complexes in Adcy10-null spermatozoa as was found for wild-type sperm cells.

**FABP9 localizes in the cytoplasmic droplet of wild-type caput epididymal spermatozoa**

Indirect immunofluorescence was used to detect FABP9 in caput and cauda epididymal spermatozoa from wild-type mice. In caput epididymal spermatozoa, FABP9 was detected intensely in the cytoplasmic droplet as well as along the principal piece, midpiece and the non-acrosomal area of the head (Fig. 6). However, this protein localized to the perforatorium, post-acrosomal region and principal piece of cauda epididymal spermatozoa. No signal was observed when normal sera were used (data not shown).

**DISCUSSION**

The pivotal role of disulfide bonds for sperm structure and flagellar straightness has been recognized for over 40 years. A decrease in the fluorescent thiol labelling agent mBBr during epididymal maturation has also been reported in rat (Shalgi et al., 1989) and mouse (Conrad et al., 2005), indicating the oxidation of thiols to disulfides. Hamster sperm proteins of 85–94, 50–55 and 35–40 kDa, which are rich in sulfhydryl groups, are involved in the maintenance of flagellar straightness (Cornwall & Chang, 1990). However, the identity of these proteins has never been analysed. This study is the first comprehensive identification of sperm proteins that change their thiol status during transit through the epididymis.

There are four major hypotheses for the cause of flagellar angulation in spermatozoa: osmolality disturbances (Yeung...
et al., 1999), selenium deficiencies (Schneider et al., 2009), gene mutations in male germ cells (Cheng et al., 2007) and abnormal annulus formation (Ihara et al., 2005). The thiol peroxidase, glutathione peroxidase 4 (GPx4) and the disulfide isomerase/reductase, thioredoxin-glutathione reductase (TGR), have been proposed to play a key role in preventing flagellar angulation through formation of disulfide bonds. Mitochondrial GPx4 disruption in mice results in flagellar angulation and increases the extent of free sulfhydryl groups on epididymal sperm proteins (Schneider et al., 2009). The abundance and status of GPx4 and TGR proteins should be investigated between wild-type and ADCY10-null spermatozoa. Sperm cells from Adcy10-null males are immotile and demonstrate flagellar angularity after the addition of dbcAMP (Hess et al., 2005). We report similar findings with ADCY10-null spermatozoa. Here, we provide evidence that flagellar angulation in ADCY10-deficient spermatozoa is associated with diminished disulfide bond formation involving the FABP9 protein. We speculate that these thiol status changes may result from gene mutations or osmolality disturbances. FABP9 and GSTM5 are downstream targets of TGR in the rat testis (Su et al., 2005).

In the last decade, the combination of 2-D electrophoresis and mass spectrometric techniques has made it possible to identify sperm proteins contained in specific compartments of the sperm cell (Cao et al., 2006). Recently, the more sophisticated 2-D DIGE method has made it feasible to detect minute differences in abundance of protein populations, leading to the identification of sperm proteins that have roles in epididymal maturation and capacitation (Baker et al., 2005, 2006; Ijiri et al., 2011). DIGE Fluor saturation dyes are optimized for the labelling of cysteine residues on reduced proteins. However, in these experiments, we applied this dye for detection of non-reduced as well as reduced caput and cauda epididymal sperm proteins (Fig. 2).

Using this technique, we identified four sperm proteins (Table 1: AKAP4, FABP9, GSTM5 and VDAC2) that displayed differences in disulfide bonds between caput and cauda epididymal sperm cells (Fig. 3). GSTM5 and VDAC2 were identified from single spots. Of the four proteins identified, AKAP4 (M, 82 000) was the only protein identified that corresponded to the size of proteins predicted to be involved in flagellar straightness of hamster spermatozoa (Cornwall & Chang, 1990). FABP9 was recovered from 12 spots that had similar molecular weights (M, 15 000) with a range of various isoelectric points. Because FABP9 was detected in many spots from whole sperm lysate on 2-D gel, it may be possible to detect more candidate proteins using a protein population from the sperm tail alone, excluding FABP9 associated with the sperm head. Fractionation of sperm proteins to eliminate lower molecular weight proteins (<15 kDa) may also yield other candidate proteins that undergo oxidation of sulfhydryl groups during transit from the caput to the cauda epididymis.

Two of the proteins we identified in this study, AKAP4 and VDAC2, were not previously known to undergo further sulfhydryl oxidation during the course of epididymal maturation. AKAP4 is specific to male germ cells and tethers cAMP-dependent protein kinase to the fibrous sheath of the flagellum (Carrera et al., 1994; Fulcher et al., 1995a; Visconti et al., 1997). Immunoelectron microscopy indicates that mouse AKAP4 is present in the longitudinal columns and transverse ribs of the sperm fibrous sheath (Johnson et al., 1997). Akap4-null male mice are infertile; their spermatozoa do not display progressive motility because they do

**Figure 6** Indirect immunofluorescence of wild-type caput and cauda epididymal spermatozoa probed with the anti-FABP9 antibody. This protein was detected intensely in the cytoplasmic droplet as well as along the principal piece, midpiece and the non-acrosomal area of the head in caput epididymal spermatozoa (top). FABP9 localized to the perforatorium, post-acrosomal region and principal piece in cauda epididymal spermatozoa (bottom). The corresponding Nomarski differential interference contrast micrographs are to the right of each fluorescence image.
not form a functional fibrous sheath (Miki et al., 2002). In this study, we identified the proAKAP4 polypeptide that corresponds to the ‘PRO’ domain of the precursor. In previous studies, this 179 amino acid fragment ran with a molecular weight of approximately 26 000, which is similar to our finding here. However, the actual molecular weight is calculated to be 19 994. In our previous work, we rationalized the ~6000 molecular weight estimation to result from anomalous electrophoretic behaviour. However, the finding of GSTM5 peptides in the same spot raises the possibility that the ‘PRO’ domain of proAKAP4 and a fragment of GSTM5 are linked; we think this unlikely as the molecular weight of GSTM5 itself is 26 634 (Fulcher et al., 1995b). VDACs are small pore-forming proteins first identified in outer mitochondrial membranes and play various biological roles in somatic cells (Colombini, 1979). VDAC2 locates to the acrosomal region as well as outer dense fibres of the bovine sperm flagellum (Hinsch et al., 2004; Triphän et al., 2008).

GSTM5 and FABP9 are potential targets for thiol oxidation in spermatozoa. GSTM5 is a germ cell-specific member of the μ-class GST family and serves to protect sperm from oxidative stress. Immunoblot analysis with an antibody to μ-class GSTs indicated this protein in isolated fibrous sheaths from mouse cauda epididymal spermatozoa (Fulcher et al., 1995b). Furthermore, hexokinase type 1 is tethered in the principal piece region by the sperm-specific variant of muscle-type phosphofructokinase, which in turn is bound tightly to GSTM5 in the fibrous sheath, demonstrating the compartmentalization of specific glycolytic enzymes in the sperm cell (Nakamura et al., 2010). FABP9 was originally identified as the major protein of the rat periflagellum between the inner acrosomal membrane and the outer face of the nuclear envelope in the sperm head (Oko & Morales, 1994) and later recognized in the periflagellum of mouse spermatozoa (Korley et al., 1997). In rat spermatogenic cells from mid-pachytene spermatocytes to step 19 spermatids, anti-periflagellum antibodies react to the cytoplasm and nuclei of the sperm head (Oko & Clermont, 1991). We demonstrated that FABP9 was located in the periflagellum and post-acrosomal region in cauda epididymal spermatozoa (Fig. 6). These findings are similar to what has been previously reported (Selvaraj et al., 2010). Interestingly, we detected FABP9 in the midpiece and cytoplasmic droplet of caput epididymal spermatozoa (Fig. 6). Proteomic analysis suggests that the mitochondrial capsule of rat spermatozoa contains minor amounts of FABP9 (Maiorino et al., 2005). The amount of FABP9 in the mitochondria of cauda epididymal mouse spermatozoa may be too low to be detected by indirect immunofluorescence or the epitopes recognized by the antibody may be cryptic under those conditions.

The four proteins identified in this study were all present at similar amounts in wild-type and Adcy10-null sperm cells (Fig. 4). On the basis of the experiments performed here, we demonstrated that only FABP9 contained fewer disulfide bonds in Adcy10-null cauda epididymal spermatozoa than in wild-type sperm cells (Fig. 5). However, AKAP4, GSTM5 and VDAC2 should not be eliminated as candidate proteins displaying thiol status changes. The proportion of disulfide bonds in reduced cauda epididymal spermatozoa may simply be less significant in these proteins, owing to the large number of bonds already present. The overall status of thiols and disulfides was similar between wild-type and Adcy10-null spermatozoa, but a diminution of free sulphhydryl groups was observed between caput and cauda epididymal spermatozoa, regardless of the genotype (Fig. 1). These data suggest the aberration of disulfide bonding in sperm proteins that may affect flagellar morphology between wild-type and Adcy10-null spermatozoa is not global and may be restricted to a limited number of proteins.

Male infertility has various causes, and the majority of cases remain idiopathic. Flagellar angulation has been suggested as one of the causes of male infertility. An example of this is the c-ros knockout and GPX5-Tag2 transgenic mouse models that are infertile owing to flagellar angulation (Cooper et al., 2004). Our results may contribute to the diagnosis and treatment of male infertility caused by sperm flagellar angulation due to diminished disulfide bonds. These results suggest that oxidation of sulphhydril groups during maturation covers a wide area of the spermatozoa and that most of the proteins that contain disulfide bonds are structural or cytoskeletal proteins.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** A detailed statistical analysis of the proteins identified in this study that change their thiol status during epididymal maturation in reduced caput versus non-reduced cauda epididymal sperm cells, non-reduced caput vs. non-reduced cauda epididymal sperm cells, and reduced cauda versus non-reduced cauda epididymal sperm cells. Boxes indicate spots that were identified as two proteins. DIGE analysis was performed. One-way analysis of variance (ANOVA) p-values, Student’s t-test p-values, and average volume ratios were calculated with DeCyder software v5.01 by using the mixed-sample internal standard methodology. ND, not determined. *See text for discussion.*