Clusterin in the mouse epididymis: possible roles in sperm maturation and capacitation

Arpornrad Saewu¹, Suraj Kadunganattil¹, Riya Raghupathy¹, Kessiri Kongmanas^{1,2}, Pamela Diaz-Astudillo¹, Louis Hermo³ and Nongnuj Tanphaichitr^{1,2,4}

¹Chronic Disease Program, Ottawa Hospital Research Institute, Ottawa, Canada, ²Department of Biochemistry, Microbiology Immunology, Faculty of Medicine, University of Ottawa, Ottawa, Canada, ³Department of Anatomy and Cell Biology, Faculty of Medicine, McGill University, Montreal, Canada and ⁴Department of Obstetrics and Gynecology, Faculty of Medicine, University of Ottawa, Ottawa, Canada

Correspondence should be addressed to N Tanphaichitr; Email: ntanphaichitr@ohri.ca

Abstract

Clusterin (CLU) is known as an extracellular chaperone for proteins under stress, thus preventing them from aggregation and precipitation. We showed herein that CLU, expressed by principal cells of the mouse caput epididymis, was present in high amounts in the lumen. In the cauda epididymis, CLU bound tightly to the sperm head surface and its amount on total sperm was similar to that in the bathing luminal fluid. In both immotile and motile caudal epididymal sperm, CLU was localized over the entire sperm head except at the convex ridge, although in the motile sperm population, the CLU immunofluorescence pattern was distinctively mottled with a lower intensity. However, when motile sperm became capacitated, CLU was relocalized to the head hook region, with immunofluorescence intensity being higher than that on the non-capacitated counterparts. Under a slightly acidic pH of the epididymal lumen, CLU may chaperone some luminal proteins and deliver them onto the sperm surface. Immunoprecipitation of epididymal fluid proteins indicated that CLU interacted with SED1, an important egg-binding protein present in a high amount in the epididymal lumen. In a number of non-capacitated sperm, fractions of SED1 and CLU co-localized, but after capacitation, SED1 and CLU dissociated from one another. While CLU moved to the sperm head hook, SED1 translocated to the head convex ridge, the egg-binding site. Overall, CLU localization patterns can serve as biomarkers of immotile sperm, and non-capacitated and capacitated sperm in mice. The chaperone role of CLU may also be important for sperm maturation and capacitation.

Reproduction (2017) **154** 867–880

Introduction

‘Clusterin’ (CLU), first coined by Fritz and colleagues, is an 80-kDa dimeric glycosylated protein isolated from ram rete testis fluid that has the ability to cluster isolated Sertoli cells in suspension (Blaschuk *et al.* 1983, Fritz *et al.* 1983). Immunolocalization and immunoblotting reveal that CLU is expressed in ram and rat Sertoli cells and secreted into the seminiferous tubule lumen (Kissinger *et al.* 1982, Blaschuk *et al.* 1983, Blaschuk & Fritz 1984, Sylvester *et al.* 1984, Griswold *et al.* 1986, Cheng *et al.* 1988, Law & Griswold 1994). CLU is the same protein as SGP-2 (sulfated glycoprotein-2, a major secreted protein of Sertoli cells), TRPM-2 (testosterone-repressed prostate message-2), SP-40, 40, apoJ (apolipoproteinJ) and CLI (complement lysis inhibitor). Studies on the last three proteins indicate that CLU is also present in non-reproductive tissues/fluids (Jenne & Tschopp 1989).

Newly synthesized CLU in rat Sertoli cells and other cells contains 447 amino acids with a molecular mass of ~68–74 kDa. Upon maturation, intracellular cleavage

occurs at Arg226 (numbering based on rat Sertoli cell CLU), yielding CLU- β (34 kDa, Glu22-Arg226) and CLU- α (47 kDa, Ser227-Glu447), which remain linked together by five disulfide bonds (see [Supplementary Fig. 1](#), see section on [supplementary data](#) given at the end of this article) (Blaschuk *et al.* 1983, Collard & Griswold 1987, Sylvester *et al.* 1991). Further biochemical studies strongly indicate the roles of extracellular CLU in lipid transport, membrane remodeling during apoptosis and molecular chaperoning of proteins under physical and chemical stress conditions (Poon *et al.* 2002, Carver *et al.* 2003, Wyatt *et al.* 2009) as well as protective roles against oxidative stress and complement-induced cell lysis (Trogakos 2013), Jenne & Tschopp 1989, Murphy *et al.* 1989, Tschopp & French 1994, Mollnes & Kirschfink 2006). On the other hand, nuclear CLU isoform is a proapoptotic factor (Rohne *et al.* 2016).

In the male rat reproductive system, CLU is also expressed and secreted by epididymal epithelial principal cells, with the highest level in the caput region (Sylvester *et al.* 1984, 1991, Tung & Fritz 1985,

Hermo *et al.* 1991, 2000, Mattmueller & Hinton 1991, Law & Griswold 1994, Robaire & Viger 1995, Ahuja *et al.* 1996). CLU is indeed one of the major proteins in the epididymal lumen of several mammals (e.g., ram, pig, horse, human) (Dacheux *et al.* 2009). CLU in the rat epididymal lumen is exclusively a secreted product of epididymal epithelial cells, since the testicular form of CLU is endocytosed by epithelial cells of the rete testis and efferent ducts (Hermo *et al.* 1991). Rat epididymal CLU is in fact biochemically different from its testicular counterpart, with its molecular mass being lower than the latter (Mattmueller & Hinton 1991, Sylvester *et al.* 1991).

CLU also coats the surface of rat sperm in the descending regions of the epididymis (Hermo *et al.* 1991, Sylvester *et al.* 1991). Since the epididymis is a site of sperm maturation (Robaire & Hinton 2015), CLU in the epididymal fluid and sperm may play roles in this process (Robaire & Viger 1995) as well as the subsequent sperm capacitation event. In particular, we asked whether CLU could act as an extracellular chaperone for proteins present abundantly in epididymal fluid. SED1 is one of such proteins, which we selected for this study, as it also deposits onto epididymal sperm and its essential function in egg binding and male reproduction in mice is well documented (Ensslin & Shur 2003, Shur *et al.* 2006). However, since information on CLU amounts and localization in the epididymal entities (epithelial cells and fluid, and sperm) in mice was unavailable, our initial study was targeted toward this characterization. This study serendipitously indicated that CLU immunolocalization signals could also be used as biomarkers of mouse sperm capacitation.

Materials and methods

Chemicals

Goat polyclonal anti-CLU antibody, directed against the C-terminus sequence EKALQEYRRKSRAE of mouse CLU- α peptide and immunogen-affinity purified, was purchased from Novus Biologicals (Oakville, ON, Canada, Catalog number: NBP1-06027). Immunogen-affinity purified goat polyclonal anti-SED1 (aka MFGE8) IgG antibody generated against recombinant mouse SED1 was from R&D Systems (Catalog Number: AF2805, Cedarlane, Burlington, ON, Canada). Rabbit polyclonal anti-SED1 IgG antiserum generated against recombinant His-tagged SED1 (Ensslin & Shur 2003) was provided by Dr Barry Shur, Emory University School of Medicine. A mouse monoclonal anti-phosphotyrosine IgG antibody, clone 4G-10 (catalog number: 05-321X), was from Millipore Canada. Normal goat IgG (Catalog number: SC-2028) was from Santa Cruz Biotechnology and normal rabbit IgG (Catalog number: 12-370) was from Millipore Canada.

Recombinant mouse CLU (Catalog number: 2747-HS-050) was purchased from R&D Systems (Cedarlane). It was produced in a mouse myeloma cell line as a disulfide-linked heterodimer of CLU- β and CLU- α , with an apparent molecular

mass of 37 kDa and 44 kDa, respectively, upon subsection to reducing SDS-PAGE.

Animals and gamete collection

Male CD-1 (age: 8–10 weeks) and female CF-1 (age: 5–8 weeks) mice (from Charles River Canada) were kept in a temperature-controlled room with 14-h light and 10-h dark photoperiod. They were fed *ad libitum* with Purina rodent chow and water. All experiments involving the use of mice adhered to the Canadian Council on Animal Care guidelines, and the protocols followed were approved by the Committee of the Animal Care & Veterinary Service, University of Ottawa.

Collection of epididymal sperm and fluid

Media

Four types of media were used in sperm preparation and/or incubation. These included: (1) Krebs Ringer Medium containing 4 mM NaHCO₃, buffered with HEPES (KR4B) (112.4 mM NaCl, 4.8 mM KCl, 1.7 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM Mg₂SO₄, 4 mM NaHCO₃, 21 mM HEPES, 25 mM sodium lactate, 1 mM sodium pyruvate, 5.6 mM glucose, 28 μ M phenol red, pH 7.4), (2) KR0B (same ingredients as KR4B, except that NaHCO₃ was omitted and the NaCl concentration was adjusted to 116.4 mM), (3) KR0B-BSA (same ingredients as KR0B but with addition of 0.3% BSA) and (4) KR25B-BSA (same ingredients as KR4B except that HEPES was omitted and the NaHCO₃ concentration was increased to 25 mM). KR0B and KR0B-BSA should not induce capacitation, since they lacked NaHCO₃, an upstream activator of the capacitation-related PKA signaling pathway, whereas KR25B-BSA should support capacitation to the greatest extent due to the presence of both BSA and NaHCO₃ of the highest concentration (Visconti *et al.* 1995, Salicioni *et al.* 2007).

Collection of sperm from the caput and cauda epididymides

The caput and cauda epididymides were dissected out from the animal with special care to eliminate blood vessels and surrounding fat pads. For caput epididymal sperm collection, 6 caput epididymides immersed in 1 mL of KR4B were individually punctured with a 29-G needle and incubated for 15 min at 37°C for sperm to exude into the medium. The mixture of sperm and fluid was passed through a 70- μ m-meshwork cup-type Filcon filter (BD Biosciences, Mississauga, ON, Canada) to remove tissue debris and cellular particulates resulting from tissue puncturing. The suspension of sperm in KR4B-diluted caput epididymal fluid was centrifuged (300g, at room temperature (RT), 5 min) to pellet sperm, which were referred to as washed caput epididymal sperm. In alternate experiments, the sperm suspension in diluted caput epididymal fluid, collected as described earlier, was further diluted with KR4B to 2 mL, and layered on top of 2 mL of 45% Percoll solution (GE Healthcare). The tube was then centrifuged (600g, RT, 30 min). The pelleted sperm were washed once with KR4B (350g, RT, 10 min) and referred to as caput epididymal sperm purified through Percoll centrifugation. On average, ~1 million purified

caput epididymal sperm were obtained from each animal. Sperm were also collected from cauda epididymides and vasa deferentia (Tanphaichitr *et al.* 1993) and diluted in KR4B. They were then subjected to centrifugal washing or 45% Percoll centrifugation in a similar manner to caput epididymal sperm. Consistently, ~50 million caudal epididymal+vas deferens sperm were obtained from each mouse. Luminal fluid from both the caput epididymis and cauda epididymis remaining on top of the 45% Percoll solution was also collected for biochemical characterization.

Motile and immotile caudal epididymal sperm populations were prepared by centrifuging sperm collected in 2 mL of KR4B through a 45%/90% Percoll gradient following our previously described methods (Tanphaichitr *et al.* 1990, Furimsky *et al.* 2005). Upon resuspending the pelleted and Percoll-gradient-interfaced sperm in either KR4B or KR25B-BSA, motility of the former and latter populations was >90% and <15%, respectively. In one set of experiments, both Percoll-gradient pelleted and interfaced sperm were used without further incubation. Alternatively, Percoll-gradient pelleted sperm resuspended in KR4B or KR25B-BSA were further incubated (37°C, 1 h) under air for KR4B or under 5% CO₂ for KR25B-BSA before use for experiments.

Caudal epididymal and vas deferens sperm were also collected in 2 mL of KR0B and subjected to 45%/90% Percoll-gradient centrifugation. Likewise, motile-pelleted sperm resuspended in KR0B were either used immediately or further incubated (37°C, 1 h) under air in KR0B or KR0B-BSA before use.

Sperm motility assessment

A 5-μL aliquot of the sperm suspension was pipetted into a CellVision CV 1020-2cv slide (CellVision Technologies, Heerhugowaard, The Netherlands), which was then video-imaged for motility at 30 frames/second under a Nikon TMS inverted microscope using a 10× objective with a Celestron HD Digital Microscope Imager (Torrance, CA, USA, containing a 30× magnifier lens) attached to an eyepiece.

Immunocytochemical localization of CLU on mouse epididymis sections

Epididymides were fixed in Bouin's fixative solution and processed for paraffin embedding following a standard protocol. For immunocytochemical detection of CLU, sections (5 μm thick) of the embedded tissue were deparaffinized. Successive washing of the sections in PBS containing 0.1% Tween-20 was performed at all steps below when changing the incubation solution. After pre-blocking for non-specific staining in PBS containing 4% BSA (RT, 30 min), the sections were incubated (RT, 1.5 h) with 5 μg/mL anti-CLU IgG in PBS-2% BSA, and then with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (H+L) antiserum (Bio-Rad) at 1:150 dilution in PBS-2% BSA. The antibody-antigen recognition was detected using a Vector peroxidase ABC kit with diaminobenzidine as a substrate (Vector Laboratories Inc, Burlingame, CA, USA). The tissues were counterstained with Mayer's hematoxylin (Sigma-Aldrich Canada Ltd) and 1% methylene blue (Thermo

Fisher Scientific) followed by stepwise dehydration in ethanol and finally in xylene before mounting with Permount (Thermo Fisher Scientific). Images were captured on a Zeiss Axioskop (Carl Zeiss Canada) using a Nikon D5000 digital Camera. In a control experiment, epididymis sections were subjected to the same procedures except that 5 μg/mL normal goat IgG was used in place of anti-CLU IgG.

Immunofluorescence localization of CLU and SED1 on sperm

Sperm from various preparations were washed (500g, 5 min) twice in PBS-0.1% polyvinylpyrrolidone (PVP) and then fixed (RT, 15 min) in 4% paraformaldehyde in PBS. Washing sperm twice with PBS-0.1% PVP was performed at this step and other steps described later when changing the incubation solution. After pre-blocking (RT incubation for 30 min) from non-specific binding with 5% normal rabbit serum made in PBS-0.1% PVP, sperm were incubated with anti-CLU IgG (5 μg/mL in PBS-2% BSA) and then with secondary antibody, Alexa Fluor-488 conjugated rabbit anti-goat IgG (H+L) (Life Technologies, 1:200 dilution in PBS-2% BSA). Incubation for both antibodies was for 45 min at RT. Sperm were then resuspended in PBS+Fluoroshield solution (Sigma-Aldrich Canada Ltd, used as per the manufacturer instruction) and applied onto a microscope slide with a coverslip for imaging under a Zeiss Axio Observer epifluorescence microscope, using excitation and emission wavelengths of 450–490 nm and 520 nm, respectively. Approximately 200 sperm were counted for various patterns of CLU signals in each replicate experiment.

In an alternate experiment, immunofluorescence of CLU was performed on sperm that were bound to the zona(e) pellucida(e) (ZP) layer coated onto the surface of a glass slide. ZP used in this experiment were prepared from mouse ovaries as previously described (Xu *et al.* 2012) and heat solubilized in 0.2M sodium phosphate buffer, pH 6.8 at two ZP/μL. The solubilized ZP solution was plated into the middle of the well of a Thermo Scientific 8-well Diagnostic glass slide (Thermo Fisher Scientific) and allowed to air-dry. After two gentle washes with PBS, the ZP layer was incubated (5 min, 37°C) with 20 μL of sperm pre-incubated for 1 h in KR25B-BSA (prepared as described earlier, but washed once and resuspended in KR4B to a concentration of 10⁶/mL). The zona layer on the glass slide was then washed twice with PBS and semi-dried under air. Sperm bound to the ZP layer were then subjected to CLU immunofluorescence. Approximately 100 sperm were evaluated for CLU signal patterns in each of the duplicate experiments.

Double immunofluorescence for CLU and SED1 was performed on Percoll-gradient pelleted caudal epididymal sperm, which were immediately resuspended in KR4B or further capacitated (37°C, 5% CO₂) in KR25B-BSA for another hour. Sperm were fixed and first subjected to CLU immunofluorescence, following the same protocol as described earlier. However, donkey anti-goat IgG (H+L) conjugated with Alexa Fluor 488 (Life Technologies, 1:200 dilution in PBS-2% BSA) was used as the secondary antibody for CLU signal detection in this experiment. After completing immunofluorescence for CLU, the same sperm samples were

subjected to immunofluorescence for SED1, using the rabbit anti-SED1 IgG antiserum (1:100 dilution) as the primary antibody and goat anti-rabbit IgG (H+L) conjugated with Alexa Fluor 594 (Life Technologies, 1:400 dilution in PBS-2% BSA) as the secondary antibody. Accordingly, 5% donkey serum+2% BSA and 5% goat serum+2% BSA were used in the blocking step (1 h, RT) of the immunofluorescence for CLU and SED1, respectively. Aldehyde fixed sperm incubated with normal goat IgG and normal rabbit IgG in place of anti-CLU IgG and anti-SED1 IgG, respectively, and subjected to the same processing served as negative controls for CLU and SED1 immunofluorescence localization.

Flow cytometry

Relative amounts of CLU on sperm that were subjected to immunofluorescence as described earlier were analyzed by flow cytometry. Sperm were gated based on forward scatter and side scatter profiles in order to exclude debris, and fluorescence was measured in FL1 (510/20 nm band-pass filter). For each sample, a total of 10,000 events were acquired using a Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). Data were analyzed with FCS Express 4.0 software (De Novo Software, Thornhill, ON, Canada).

Co-immunoprecipitation of SED1 and CLU

SED1 and CLU present in the mouse caudal epididymal fluid were co-immunoprecipitated using anti-SED1 IgG captured and cross-linked to paramagnetic Dynabeads Protein G, provided in a Novex Immunoprecipitation Kit (Life Technologies, Catalog Number: 10007D). Sperm+luminal fluid was squeezed out from two sides of the cauda epididymis into 1 mL of PBS+PIC (Roche Diagnostics). Following sperm removal by centrifugation (1000g, 5 min, RT), the supernatant was centrifuged at a high speed (14,000g, 5 min, RT) to remove all particulates. Epididymal fluid in the final supernatant was used in this co-immunoprecipitation experiment. Following methods provided by the manufacturer, 40 µL of prewashed Dynabeads was incubated with 2 µg of goat anti-SED1 IgG, and the Protein G-anti-SED1 IgG complexes obtained were covalently linked using a BS³ linker (Life Technologies). Control beads were also made using normal goat IgG in place of anti-SED1 IgG. These Dynabeads containing protein G-anti-SED1 IgG complexes or protein G-normal goat IgG complexes were then incubated (45 min, RT, with rotation) with 150 µg of caudal epididymal fluid proteins (Input fraction) in 700 µL of Modified Dulbecco's PBS. Unbound proteins were washed off from the beads thrice, each in 200 µL of Washing Buffer (provided in the kit). For each wash, the paramagnetic beads were captured to the wall surface of the tube placed in the holding magnet. Finally, caudal epididymal proteins captured on the anti-SED1-Protein G-Dynabeads were eluted into solution by treatment (95°C, 10 min) with Laemmli's SDS-PAGE sample buffer containing 80 mM DTT. After the beads were removed by the magnetic force, eluted proteins (bound fraction) were subjected to SDS-PAGE and immunoblotting, which was sequentially performed on the same membrane, i.e., first with anti-CLU and then with anti-SED1 antibody.

Protein quantification, SDS-PAGE and immunoblotting

Whole proteins in caput and caudal epididymal fluid were quantified by the Bradford assay (Bio-Rad Laboratories), following the manufacturer's instruction. Aliquots of these samples were treated with Laemmli's sample buffer with or without 80 mM DTT or 1% mercaptoethanol for SDS-PAGE, which was performed on an 8% or 10% polyacrylamide gel following the method described (Laemmli 1970). To extract proteins from caput and caudal epididymal sperm for CLU immunoblotting, sperm were treated with Laemmli's sample buffer, with sonication, followed by centrifugation at 12,000g to pellet the particulates; the supernatants were then subjected to SDS-PAGE. Extraction of caudal epididymal sperm proteins for phosphotyrosine immunoblotting was performed as previously described (Navarrete *et al.* 2015). Following electrophoresis, the proteins were electro-transferred (100V, 1 h) (Towbin & Gordon 1984) onto a nitrocellulose (Bio-Rad Laboratories) or PVDF (GE Healthcare Life Science) membrane (both with a 0.45-µm pore size) for CLU and phosphotyrosine immunoblotting, respectively. In all steps described later, TBS containing 0.1% Tween-20 (TBST) was used as a washing solution (RT, three 5-min washes) for the membrane when changing the incubation solution. After pre-blocking (RT, 1 h) in 5% powdered skim milk in TBST for CLU immunoblotting or in 20% fish skin gelatin (Sigma-Aldrich Canada Ltd) in TBST for phosphotyrosine immunoblotting, the membrane was incubated (overnight, 4°C) with anti-CLU antibody (0.25 µg/mL in TBST containing 5% skim milk) or with anti-phosphotyrosine (monoclonal 4G10, 0.1 µg/mL in TBST), and then with a secondary antibody (1 h, RT) obtained from BioRad (HRP-conjugated rabbit anti-goat IgG, 1:5000 dilution in TBST-5% skim milk for CLU detection, and HRP-conjugated goat anti-mouse IgG, 1:10,000 dilution in TBST for tyrosine phosphate detection). Reactivity of antigen with the antibody was revealed by enhanced chemiluminescence (ECL) on an Amersham Hyperfilm (GE Healthcare Life Sciences), using a Pierce ECL Western blotting Substrate (Thermo Scientific). CLU-α reactive bands were subjected to densitometric analyses using AlphaImager Software (AlphaEase version 5.5, Alpha Innotech Co., San Leandro, CA, USA). Amounts of CLU-α were then determined from the standard curve of rec CLU (3–4 concentrations loaded into the same gel as the samples, and immunoblotted and densitometrically analyzed in parallel).

Similar conditions of immunoblotting for CLU-α as described earlier were used in the co-immunoprecipitation experiments except that anti-CLU antibody was used at 0.5 µg/mL. SED1 was probed using anti-SED1 antibody (1 µg/mL, overnight incubation at 4°C) and HRP-conjugated rabbit anti-goat IgG (1:5000, 1 h, RT). Detection of antigen-antibody reactivity was by chemiluminescence using a regular ECL substrate (as described earlier) and a SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific).

Results

Expression of CLU by epididymal epithelial cells

Mouse Sertoli cells showed immuno-reactive signals for CLU in their cytoplasm (Supplementary Fig. 2). However,

in the epididymis, there was no expression of CLU in the proximal part of the initial segment (Fig. 1A, tubules marked with arrowheads, and Fig. 1E). Only in the distal part of the initial segment, did CLU signals become distinctive in principal cells (Fig. 1A, tubules marked with arrows). From the enlarged image (Fig. 1F), CLU signals were present in the supranuclear reticular network (blue arrows), which was likely the Golgi apparatus, as well as in association with the apical cell membrane and subsurface vesicular structures (red arrows), which were possibly secretory vesicles. In the caput epididymal epithelium, the CLU expression was observed similarly to that of the distal initial segment, although the anti-CLU reactivity was much stronger in the supranuclear reticular network (presumably Golgi apparatus) and the apical cell plasma membrane (Fig. 1B and G). Sperm in the caput epididymal lumen were also recognized by the anti-CLU antibody (Fig. 1B). In the proximal caudal epididymal epithelial cells (Fig. 1C), CLU signals were intense in the area around and beneath the apical cell membrane, but were totally absent in the supranuclear reticular Golgi apparatus. The CLU signal pattern was similarly observed in the distal cauda epididymis, but with a lower intensity (Fig. 1D), and in some areas, the CLU signals were minimal (brackets in Fig. 1D). Low levels of anti-CLU reactivity were observed in subsurface vesicles likely to be endosomes (Fig. 1H, arrows), suggesting the uptake of CLU from the lumen. Nonetheless, sperm in the lumen of both proximal and distal parts of the cauda epididymis were intensely stained with anti-CLU antibody.

Presence of CLU in epididymal lumen and on epididymal sperm

Immunoblotting indicated that CLU was a component of the epididymal luminal proteins in both the caput and cauda epididymides (Fig. 2). Under reducing conditions (Fig. 2A), CLU- α present in the lumen of both epididymal regions possessed a similar apparent molecular mass, i.e., an average of 40 kDa, which was significantly different from that of CLU- α secreted by mouse Sertoli cells in culture (i.e., 44 kDa, Supplementary Fig. 2C). In the caudal epididymal fluid, a faint anti-CLU reactive band of 50 kDa was also observed, possibly reflecting a more heavily glycosylated form of CLU- α , whereas the additional 37-kDa band may be a processed product of the main CLU- α bands (42 kDa and 37 kDa). Caudal epididymal sperm that were centrifuged through a 45% Percoll solution also showed an immuno-reactive CLU band of a 40-kDa molecular mass, a result corroborating their positive CLU immunofluorescence staining (Fig. 2B). In contrast, caput epididymal sperm processed in parallel showed only a trace amount of immuno-reactivity (Fig. 2A). This observation did not agree with the immunocytochemistry results, indicating strong anti-CLU reactivity on sperm inside the caput epididymal lumen (Fig. 1B). Immunofluorescence results of caput epididymal sperm shown in Fig. 2B explained this

disparity. Sperm retrieved from the caput epididymis and simply washed in PBS by centrifugation showed strong immunofluorescence CLU signals on the sperm head and tail (Fig. 2B, left panel), whereas caput epididymal sperm that had been centrifuged through a 45% Percoll solution exhibited only a trace signal of CLU (Fig. 2B, right panel), a result indicating that CLU was loosely bound to caput epididymal sperm.

Immunoblotting of caudal and caput epididymal fluid and caudal sperm, performed on non-reducing SDS-PAGE, revealed CLU bands of a dimeric form, with a molecular mass of 80, 72 and 72 kDa, respectively (Fig. 2C). A slightly lower mass of CLU in caudal epididymal fluid and sperm suggested protein processing occurring in this epididymal area.

Immunoblotting results (Fig. 2A and C) indicated that the CLU level decreased in caudal epididymal fluid, relative to that in the caput fluid. Quantitative analyses indicated that this decrease was 8-fold, from $6.67 \pm 1.01 \mu\text{g}$ in the caput to $0.82 \pm 0.02 \mu\text{g}$ in the cauda in one side of the epididymis. This decrease in the absolute CLU amount together with an increase in total proteins in the caudal epididymal fluid, compared with those in the caput region, further lowered the percentage of CLU over total proteins to only 0.22%, a value 18-fold lower than the corresponding percentage in the caput fluid (3.91%) (Fig. 2D). However, the amounts of CLU in sperm and luminal fluid in one side of the cauda epididymis were of the same range (0.51 ± 0.04 and $0.82 \pm 0.02 \mu\text{g}$) (Fig. 2D).

Immunofluorescence of CLU in mouse caudal epididymal sperm

Different CLU localization patterns between immotile and motile sperm

Percoll-gradient centrifugation of caudal epididymal sperm collected in KR4B generated gradient-interfaced sperm and gradient-pelleted sperm fractions, which were mainly immotile and motile, respectively, upon resuspending in KR4B. The majority of both sperm populations exhibited CLU fluorescence signals but with different patterns, although 12% of immotile sperm and 7% of motile sperm showed no anti-CLU signals (Fig. 3A). In immotile sperm, ~90% showed strong CLU immunofluorescence signals in the head region with 72% displaying the signal over the entire head except at the convex ridge. Another 17% of immotile sperm had the CLU signal at the post-acrosomal region as well as in the concave side of the sperm head hook (Fig. 3A, panels a, b).

Approximately 94% of motile sperm showed positive CLU immunofluorescence signals with mottled patterns. In ~57% of these motile sperm, the mottled pattern appeared to be over the entire sperm head except at the convex ridge, whereas in the remaining 37%, the mottled staining was more confined in the upper part of the sperm head toward the hook region (Fig. 3A,

Figure 1 Immunocytochemistry of CLU in adult mouse epididymis sections. Note different anti-CLU reactivity in various regions of the epididymis. Panel A: The two proximal initial segment tubules (arrowheads) showed no CLU signals, whereas the four distal initial segment tubules (arrows) revealed positive CLU signals (brown color). Panel B: All caput epididymis sections possessed CLU. Panels C and D: Proximal and distal caudal epididymal sections, respectively, also had positive reactivity with anti-CLU. In panel D, some regions of the distal caudal epididymal tubules showed low CLU signals (exemplified by the area under the brackets). Sperm in all epididymis tubules with their epithelial cells containing CLU also showed CLU staining. Zoomed-in images were shown for the proximal initial segment (panel E), the distal initial segment (panel F), the caput epididymis (panel G) and proximal cauda epididymis (panel H). Blue arrows in panels F and G point to the supranuclear reticular network, likely corresponding to the Golgi apparatus, whereas red arrows indicate vesicular structures, some of which were likely secretory vesicles, beneath the apical cell membrane. Black arrows in panel H were small vesicular structures, which could be endosomes. Sections of all epididymis areas, when incubated with normal goat IgG instead of anti-CLU, showed no CLU signals (data not shown). Bar=30 µm. Results shown are representative of two replicate experiments.

panels d, e). Interestingly, flow cytometry indicated that the CLU immunofluorescence intensity of motile caudal epididymal sperm was overall lower than that of the immotile sperm population (Fig. 3B).

Relocalization of CLU during sperm capacitation

Upon incubation for one hour in KR25B-BSA, motile Percoll-gradient pelleted sperm became fully capacitated, as revealed by their hyperactivated whiplash motility patterns and very high levels of protein tyrosine phosphorylation (Supplementary Fig. 3). In the majority (83%) of these fully capacitated sperm, CLU immunofluorescence patterns were markedly different from those that were not capacitated (resuspended in KR4B without further incubation, Supplementary Fig. 3

Figure 2 Presence of CLU in mouse epididymal fluid and on sperm.

Immunoblotting of CLU under (A) reducing and (C) non-reducing SDS-PAGE. CP = caput epididymis, CD = cauda epididymis. $M_r \times 10^{-3}$ of each protein standard was shown on the left of the gel panel. Both caput and caudal epididymal sperm were centrifuged through a 45% Percoll solution to remove loosely bound CLU prior to subjection to protein extraction and immunoblotting. For reducing SDS-PAGE conditions, 5 μg of total proteins from luminal fluid and proteins extracted from 1×10^6 sperm, each from the caput and cauda epididymis, was used for gel loading. For non-reducing conditions, the amounts of proteins loaded were 20 μg and 40 μg of total proteins from caput and caudal epididymal fluid, respectively, and proteins extracted from 3×10^6 caudal epididymal sperm. (B) Immunofluorescence of CLU on caput and caudal epididymal sperm. Sperm collected from the caput or cauda epididymis were simply washed by centrifugation in PBS (left panels), or centrifuged through a layer of 45% Percoll solution (right panels). Results presented in A, B and C were representative of three or more replicate experiments. (D) Quantification of CLU in caput and caudal epididymal fluid and caudal epididymal sperm. Values are given for one side of the epididymis. Data were presented as mean $\pm$ s.d. of replicate data. CLU amounts in the samples were obtained from the rec CLU standard curve after immunoblotting and densitometric analyses. Total proteins in the caput epididymal fluid, caudal epididymal fluid and the 1 N NaOH extract of caudal epididymal sperm in each side of the epididymis, as quantified by the Bradford assay, were 171.3 ± 13.3 , 356.7 ± 20.8 and 2048.0 ± 204.6 μg , respectively. These values were used as denominators for calculation of weight percentages of CLU in the caput and caudal fluid and caudal sperm.

and Video 1). Namely, CLU was present specifically to the head hook region, mostly on the concave side (Fig. 4A, panel b and Fig. 5, bottom plot). The CLU immunofluorescence intensity in these sperm was also higher than that in non-capacitated sperm (Fig. 4A and B). Flow cytometry indicated that an increase in the CLU signal intensity occurred within 30 min of sperm incubation in KR25B-BSA, and reached a plateau at 60 min (Fig. 4B). Only 8% of sperm incubated in KR25B-BSA for 1 h showed an absence of CLU signals, whereas another 7% still possessed mottled CLU localization patterns in the sperm head typical of those in non-capacitated sperm (Fig. 5, bottom plot). However, fully capacitated sperm that bound to mouse ZP coated on the slide showed CLU signals only in the head hook area (Fig. 6).

Figure 3 Immunofluorescence of CLU on the immobile and motile populations of mouse caudal epididymal sperm. (A) Sperm were collected in KR4B and separated into immobile (panels a, b, c) and motile populations (panels d, e, f) by centrifugation through a 45%/90% Percoll gradient. Note higher immunofluorescence intensity of immobile sperm, a result confirmed by flow cytometry (B). Both these sperm populations did not show any fluorescence signals, when incubated with normal goat IgG instead of anti-CLU IgG, and the result of the 'control' immobile sperm was shown in B. Bar = 10 μ m. Results in B are representative of two experiments, whereas the percentages of sperm possessing various CLU immunofluorescence patterns shown in A were obtained from three replicate experiments and expressed as means $\pm$ s.d.

Figure 4 Immunofluorescence (A) and flow cytometry (B) of CLU on non-capacitated and capacitated caudal epididymal sperm. Sperm collected from the cauda epididymis in KR4B were subjected to Percoll-gradient centrifugation. Non-capacitated sperm (A: panel a) were prepared by resuspending Percoll-gradient pelleted sperm in PBS, whereas capacitated sperm (A: panel b) were Percoll-gradient pelleted sperm that were resuspended in KR25B-BSA and further incubated (37°C, 5% CO₂) for 1 h. Note that in 83% of the capacitated sperm population, CLU was relocated to head hook region; the most common CLU pattern was shown in the inset of panel b. The intensity of CLU signal was also noted to be higher in capacitated sperm. No fluorescence signals were observed in both 'control' non-capacitated and capacitated sperm that were treated with normal goat IgG instead of anti-CLU IgG (data not shown). Bar = 10 μ m. (B) Flow cytometry showed increasing CLU intensity levels during the course of capacitation (30 and 60 min). Results shown are representative of more than five replicate experiments.

Video 1

Video records of motility of sperm incubated in four Krebs Ringer media. These included KR0B (containing no NaHCO₃), KR0B-BSA (containing no NaHCO₃ but supplemented with BSA), KR4B (containing 4 mM NaHCO₃) and KR25B-BSA (containing 25 mM NaHCO₃ and 0.3% BSA). Video recording was performed at 0 h of incubation time. The data shown were representative of ten video clips recorded for each sample in one of the three replicate experiments performed on different days.

When sperm were incubated in KR4B for 1 h, ~20% of them gained hyperactivated whiplash motility patterns (Video 2) as well as a slight increase in their protein tyrosine phosphate level (Supplementary Fig. 3).

Figure 5 Localization of CLU in the sperm head hook region was observed in the majority of fully capacitated sperm. Four sperm suspensions incubated for 1 h at 37°C in Krebs Ringer medium with differential amounts of NaHCO₃ and BSA (as described in Materials and methods section) were used to monitor the CLU localization patterns. Three CLU patterns (shown on the top of the four plots) were observed in the 4 sperm suspensions. In pattern A, CLU immunofluorescence signal was not observed on the sperm head. Sperm with positive signals showed CLU localization either on the head with a mottled pattern except at the convex ridge region (pattern B) or exclusively in the head hook region (pattern C). Numbers on top of the bars are means ± s.d. of percentages of sperm in the total population, calculated from results obtained from three replicate experiments.

Figure 6 Immunofluorescence of CLU on capacitated caudal epididymal sperm that bound to the zona pellucida. Solubilized mouse zona pellucidae (ZP) were coated onto the glass slide surface. Sperm were incubated (37°C, 5% CO₂, 1 h) in KR25B-BSA to gain full capacitation prior to applying onto the ZP coated slide. Results shown are representative of two replicate experiments. Bar = 10 μm

Interestingly, 33% of these KR4B-incubated sperm showed CLU localization in the head hook region, concurrently with a decrease in the population with mottled CLU signals (from 94% at 0 h to 62% at 1 h of incubation) (Fig. 5, third plot from the top). In contrast, when Percoll-gradient pelleted (motile) sperm were prepared from caudal epididymal and vas deferens sperm collected in KR0B and further incubated for 1 h in KR0B or KR0B-BSA, CLU localization in the majority (~85%) of these two motile sperm samples was of the same patterns (Fig. 5, first and second plots from the top) as that in motile sperm resuspended in KR4B (0 h incubation) (Figs 3A, panels d and e, Fig. 4A, panel a), i.e., as mottled patterns in either the entire or part of the sperm head except at the convex ridge. Notably, these two motile sperm samples remained non-capacitated, showing no hyperactivated motility patterns and a residual level of protein tyrosine phosphorylation (Supplementary Fig. 3).

Video 2

Video records of motility of sperm incubated in four Krebs Ringer media. These included KR0B (containing no NaHCO₃), KR0B-BSA (containing no NaHCO₃ but supplemented with BSA), KR4B (containing 4 mM NaHCO₃) and KR25B-BSA (containing 25 mM NaHCO₃ and 0.3% BSA). Video recording was performed at 1 h of incubation time. The data shown were representative of ten video clips recorded for each sample in one of the three replicate experiments performed on different days.

Relationship between CLU and SED1

SED1 is present abundantly in the epididymal lumen, with a fraction depositing onto sperm transiting through the epididymis. In solution, SED1 has a propensity

Figure 7 Co-Immunoprecipitation of SED1 and CLU. Paramagnetic beads covalently conjugated with goat anti-SED1 IgG were used in the immunoprecipitation experiment to capture SED1 and CLU from epididymal fluid. Proteins bound to the anti-SED1 beads on were first probed by immunoblotting with anti-CLU (left panel). The same blot was then reprobed with anti-SED1 (middle panel). A parallel aliquot of total epididymal fluid proteins used in this immunoprecipitation experiment (input) was also loaded into the same gel for immunoblotting with anti-CLU and anti-SED1. The blot was developed first using a regular (normal) ECL substrate and then post-developed with a Femto Maximum Sensitivity substrate. See more details in Materials and methods section. The normal and Femto ECL signals of the same 'input' lane for both anti-CLU and anti-SED1 reactivities were then placed next to one another in the left (lanes 1 and 2) and middle (lanes 4 and 5) panels for comparison. Arrowheads in the left and middle panels indicate anti-CLU reactive bands, whereas arrows in the middle panel show anti-SED1 bands. Paramagnetic beads conjugated with normal goat IgG (negative controls) were also incubated with total epididymal fluid proteins under the same conditions as those used for anti-SED1 beads. Note that proteins eluted from these rabbit IgG beads (labeled as 'bound') showed no anti-CLU or anti-SED1 reactive bands (right panel). Results shown are representative of two replicate experiments. IP = immunoprecipitation.

to undergo oligomerization (Ensslin & Shur 2003, Raymond *et al.* 2009, Kongmanas *et al.* 2015), which could lead to precipitation under the slightly acidic pH of the epididymal lumen (Levine & Kelly 1978). Therefore, we asked whether CLU through its chaperone property could interact with SED1, thus preventing SED1 from precipitation. Our immunoprecipitation results indicated that CLU and SED1 in the epididymal fluid were associated with each other. CLU in the epididymal fluid was captured to the paramagnetic beads covalently conjugated with anti-SED1 IgG (Fig. 7, lane 3). The major anti-CLU reactive band had a molecular mass of ~40 kDa, as expected under reducing SDS-PAGE (Fig. 2). The other two minor anti-CLU reactive bands detected in the 'bound' fraction were of 50- and 35-kDa molecular mass. All these anti-CLU bands (Fig. 7, lane 3, arrowheads) were present in the 'input' epididymal fluid (Fig. 7, lanes 1 & 2). When the same protein blot was reprobbed with anti-SED1, three intense reactive bands of 74, 60 and 55 kDa were observed in both bound and input fractions (Fig. 7, lane 6 vs lanes 4 & 5). While the 55-kDa band was as expected based on the known apparent molecular mass of mouse epididymal SED1 (Ensslin & Shur 2003), the identity of the 74- and 60-kDa bands was unclear. They may represent complexes of SED1 with other epididymal fluid proteins, which were resistant to treatment with the SDS-PAGE sample buffer. When the control beads containing protein-G-normal goat IgG complexes were used for incubation with the

same epididymal fluid input (Fig. 7, lane 7), neither SED1 nor CLU was pulled down (Fig. 7, lane 8).

The association between CLU and SED1 on caudal epididymal sperm was further investigated by double indirect immunofluorescence. Approximately 90% of non-capacitated motile caudal epididymal sperm showed reactivity with both anti-CLU and anti-SED1. The SED1 signals were mottled and localized over the entire sperm head except at the convex ridge (Fig. 8A, middle column), similar to the patterns observed for CLU (Fig. 8A, left column; Fig. 3A, panels d, e, and Fig. 4A, panel a). Notably, SED1 and CLU co-localized spots were present in 50% of non-capacitated sperm (Fig. 8A, orange/yellow spots in the 'merged' column), although in these sperm, there were a number of CLU and SED1 signal spots that did not overlap. However, when these motile caudal epididymal sperm were capacitated upon 1-h incubation in KR25B-BSA, ~50% of capacitated sperm showed the SED1 signal mainly in the convex ridge (Fig. 8B, middle column), whereas in the remaining 50%, SED1 signals remained unmoved in the sperm head with the same pattern as in non-capacitated sperm (data not shown). In contrast, ~80% of capacitated sperm possessed the CLU signal at the sperm hook region (Fig. 8B), similar to results described in Fig. 4. In essence, the CLU and SED1 signals were well separated in the majority of capacitated sperm (Fig. 8B). When motile caudal epididymal sperm were incubated with normal goat IgG (control IgG for anti-CLU IgG) or normal rabbit IgG (control IgG for anti-SED1) and subsequently Alexa

Figure 8 Double immunofluorescence of CLU and SED1 on non-capacitated (A) and capacitated (B) caudal epididymal sperm. Immunofluorescence was performed first for CLU and subsequently for SED1. Goat anti-CLU IgG and secondary antibody, Alexa Fluor-488 conjugated donkey anti-goat IgG (green color), were used for CLU detection, whereas rabbit anti-SED1 IgG antiserum and secondary antibody, Alexa Fluor-594 conjugated goat anti-rabbit IgG (red color), were used for SED1 immunofluorescence. (C) Control samples in which non-capacitated sperm were incubated with normal goat IgG or normal rabbit IgG in place of anti-CLU IgG and anti-SED1 IgG antiserum, respectively. DIC=Differential interference contrast. Non-specific staining in the sperm tail was observed in sperm incubated with normal goat IgG and Alexa Fluor-488 conjugated donkey anti-goat IgG. In approximately 50% of non-capacitated sperm (A), co-localized signals were observed for fractions of CLU and SED1 (orange/yellow color), although separate anti-CLU and anti-SED1 signals were also detected. In the majority of capacitated sperm, CLU was located in the hook region of the sperm head, whereas ~50% of capacitated sperm showed SED1 signal in the sperm head convex ridge. Bar=5 μ m. Results shown are representative of two replicate experiments.

Fluor 488-conjugated secondary antibody (for goat IgG) or Alexa Fluor 594-conjugated secondary antibody (for rabbit IgG), no fluorescence signals were detected (Fig. 8C).

Discussion

Presence of CLU in epididymal lumen and sperm: its possible role as a chaperone protein

CLU, a major luminal protein secreted by Sertoli cells and epididymal epithelial cells in various animal species (see Introduction section), is shown herein for the first time for its differential amounts in the mouse caput and caudal epididymis. As well, deposition of CLU from epididymal fluid to only mouse caudal epididymal sperm with appreciable affinity was demonstrated. The level of CLU in the caudal epididymal fluid was 8 $\times$ less than that in the luminal fluid of the caput region (the site of its secretion) and was reaching an equilibrium with the amount of CLU on the surface of total sperm in the cauda epididymis. The decrease in CLU levels in the cauda epididymis might be regulated by endocytic activities of CLU by caudal epididymal epithelial cells, as implicated from the immunocytochemical results (see Fig. 1, panel h).

The high amounts of CLU in the epididymal lumen suggest that it is involved in sperm maturation. Purified CLU is known for its property as an extracellular chaperone preventing precipitation and/or aggregation of proteins under stress (e.g., high temperature or acidic pH and low fluid volume) (Poon *et al.* 2002, Carver *et al.* 2003, Wyatt *et al.* 2009, 2011, Wyatt & Wilson 2010). Since the milieu of the epididymal lumen is slightly acidic (pH 6.5) (Levine & Kelly 1978), the chaperone property of epididymal CLU would be important in preventing luminal proteins that exist in abundance from precipitation. SED1 is one of such proteins secreted by epididymal epithelial cells, which tends to oligomerize and/or form complexes with other proteins in solution (Shur *et al.* 2006, Kiedziarska *et al.* 2007, Raymond *et al.* 2009). A fraction of SED1 in epididymal fluid deposits onto transiting sperm and functions in ZP binding during sperm-egg interaction (Ensslin & Shur 2003, Shur *et al.* 2006). Herein, we demonstrated that SED1 and CLU were associated with each other, and fractions of these two proteins were co-localized on non-capacitated sperm. All these results suggested that CLU may chaperone a pool of epididymal SED1 onto epididymal sperm. ApoE receptor 2 is likely one of the CLU receptors on the mouse sperm surface (Andersen *et al.* 2003, Leeb *et al.* 2014, Riaz *et al.* 2017). CLU also binds to lipids (Gelissen *et al.* 1998, Wilson & Easterbrook-Smith 2000, Nuutinen *et al.* 2009, Mishra *et al.* 2011, Trougakos 2013) which may serve as anchoring molecules during deposition of free CLU and CLU-SED1 complexes onto the sperm surface. SED1 may also have its own receptor(s), and the interaction between CLU receptors and SED1 receptors and

CLU-SED1 complexes would give synergy to the deposition of both proteins onto the sperm surface. However, the relocalization of CLU and SED1 to separate sites on the sperm head following capacitation, i.e., the head hook region for CLU and the convex ridge (the site for ZP interaction) for SED1, would free SED1 to exert its function in ZP binding (see Fig. 9 for a model of the chaperone role of CLU during sperm maturation and capacitation). Notably, association of CLU with two other epididymal luminal proteins, SPAM1 and EPPIN, which deposit onto transiting sperm like SED1, has been documented (Wang *et al.* 2007, Griffiths *et al.* 2009), although it is not clear whether CLU is co-localized with these two proteins on epididymal sperm.

Since the proper transfer of epididymal lumen proteins that are relevant to fertilization to the sperm surface is physiologically important, proteins additional to CLU may also be functioning in this process. Apolipoprotein E with sequence and functional homology to CLU is present in the epididymal lumen (Olson *et al.* 1994, 1995, Law *et al.* 1997) and may be one of these proteins, which can act as a CLU backup. This may explain why *Clu* null male mice are still fertile, although when placed under heat stress, they have an enhanced rate of the formation of apoptotic testicular germ cells and a delay of their clearance (Bailey *et al.* 2002).

In addition to its chaperone role, the protective property of CLU against oxidative stress (Schwochau *et al.* 1998, Trougakos 2013) and complement-induced cell lysis (Jenne & Tschopp 1989, Murphy *et al.* 1989, Tschopp & French 1994, Mollnes & Kirschfink 2006) would likely be beneficial to sperm in the epididymal lumen, which contains reactive oxygen species and complements (Harris *et al.* 2006, Tremellen 2008, Aitken *et al.* 2014).

CLU immunolocalization patterns as potential biomarkers of sperm motility and capacitation

Three main CLU localization patterns belonging to sperm of different physiological properties were

described in this report. The first pattern belonging to immotile caudal epididymal sperm exhibited strong CLU immunofluorescence signals in the head except at the convex ridge. The majority of these immotile sperm, which sediment at the Percoll-gradient interface following Percoll-gradient centrifugation, are known for their abnormal morphology. Their head surface contains membrane vesicles (Tanphaichitr *et al.* 1990), to which CLU would adhere due to its ability to bind to lipids (Gelissen *et al.* 1998, Wilson & Easterbrook-Smith 2000, Nuutinen *et al.* 2009, Mishra *et al.* 2011, Trougakos 2013). In contrast, motile sperm sedimenting as a pellet after Percoll-gradient centrifugation do not have these membrane vesicles, thus explaining their weaker CLU signals as compared with immotile sperm. Our results are in accordance with previous findings that human and stallion sperm of lesser quality have higher amounts of CLU (O'Bryan *et al.* 1994, Novak *et al.* 2010, Muciaccia *et al.* 2012). The increased CLU levels might be for the purpose of protecting these abnormal sperm from further damage.

The majority of non-capacitated motile caudal epididymal sperm exhibited unique mottled patterns of CLU immunofluorescence in the head region. However, CLU immunofluorescence patterns and intensity changed markedly following sperm capacitation. CLU was exclusively localized to the sperm head hook region especially in the concave side in capacitated sperm. In addition, the intensity of CLU immunofluorescence signals in capacitated sperm was higher than that in the non-capacitated counterparts. Since sperm are relatively translationally inactive, the increase in the CLU immunofluorescence signal in capacitated Percoll-gradient-centrifuged sperm was likely from the better exposure of CLU on the sperm surface to the added antibody. This could be a consequence of the separation of CLU from SED1 or other sperm surface proteins that CLU was chaperoning prior to capacitation. Since capacitated sperm bound to the ZP coated on a slide also showed CLU localized to the sperm head hook region, this CLU localization pattern would be a potential biomarker for

Figure 9 Postulated roles of CLU in epididymal sperm maturation and sperm capacitation. As an extracellular chaperone, CLU interacts with certain proteins, which tend to aggregate/multimerize, thus preventing them from precipitation under a slightly acidic condition of the epididymal luminal fluid. SED1, a major ZP-binding protein present in the epididymal fluid, is likely one of such proteins and its association with CLU was shown in this report. SED1-CLU complexes then deposit onto the sperm head mainly in the post-convex ridge area. Following capacitation, all SED1 is released from CLU interaction, trafficking to the convex ridge for the highest efficiency in ZP binding. Free CLU then moves to the sperm head hook region.

mouse sperm with egg-binding ability. The mechanisms of how CLU is separated from proteins that it has chaperoned and how it moves to the sperm head hook region are matters for further investigation. Regardless of these unknown aspects, our results described herein suggest that the unique localization patterns of CLU along with the CLU signal intensity can be used as biomarkers at a single cell level of mouse immotile sperm, motile non-capacitated sperm and motile capacitated sperm. The distribution of immotile sperm and motile non-capacitated and capacitated sperm can be revealed in a single immunofluorescence microscopic and flow cytometry analysis of CLU. In addition, the analysis of sperm of various motility and capacitation states can be conveniently conducted on sperm stored in an aldehyde fixative.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0518>.

Declaration of interest

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

Funding

This research was supported by an NSERC grant RGPIN/183958-2012 and a CIHR grant MOP-119438, both to N T. K K was a recipient of a scholarship from CIHR-Strategic Training Initiative in Health Research (STIHR) in Reproduction, Early Development, and the Impact on Health (REDIH) and also from Development and Promotion of Science and Technology Talented Project (DPST), Ministry of Education, Thailand. P D A has received a scholarship, Emerging leaders in the Americas program (ELAP), from Global Affairs Canada to perform research in male reproductive biology/biochemistry in N T's laboratory.

References

- Ahuja HS, Tenniswood M & Zakeri ZF 1996 Differential expression of clusterin in the testis and epididymis of postnatal and germ cell deficient mice. *Journal of Andrology* **17** 491–501. (doi:10.1002/j.1939-4640.1996.tb01825.x)
- Aitken RJ, Smith TB, Jobling MS, Baker MA & De Iuliis GN 2014 Oxidative stress and male reproductive health. *Asian Journal of Andrology* **16** 31–38. (doi:10.4103/1008-682X.122203)
- Andersen OM, Yeung CH, Vorum H, Wellner M, Andreassen TK, Erdmann B, Mueller EC, Herz J, Otto A, Cooper TG *et al.* 2003 Essential role of the apolipoprotein E receptor-2 in sperm development. *Journal of Biological Chemistry* **278** 23989–23995. (doi:10.1074/jbc.M302157200)
- Bailey RW, Aronow B, Harmony JA & Griswold MD 2002 Heat shock-initiated apoptosis is accelerated and removal of damaged cells is delayed in the testis of clusterin/ApoJ knock-out mice. *Biology of Reproduction* **66** 1042–1053. (doi:10.1095/biolreprod66.4.1042)
- Blaschuk OW & Fritz IB 1984 Isoelectric forms of clusterin isolated from ram rete testis fluid and from secretions of primary cultures of ram and rat Sertoli-cell-enriched preparations. *Canadian Journal of Biochemistry and Cell Biology* **62** 456–461. (doi:10.1139/o84-062)
- Blaschuk O, Burdzy K & Fritz IB 1983 Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. *Journal of Biological Chemistry* **258** 7714–7720.
- Carver JA, Rekas A, Thorn DC & Wilson MR 2003 Small heat-shock proteins and clusterin: intra- and extracellular molecular chaperones with a common mechanism of action and function? *IUBMB Life* **55** 661–668. (doi:10.1080/15216540310001640498)
- Cheng CY, Chen CL, Feng ZM, Marshall A & Bardin CW 1988 Rat clusterin isolated from primary Sertoli cell-enriched culture medium is sulfated glycoprotein-2 (SGP-2). *Biochemical and Biophysical Research Communications* **155** 398–404. (doi:10.1016/S0006-291X(88)81099-4)
- Collard MW & Griswold MD 1987 Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells. *Biochemistry* **26** 3297–3303. (doi:10.1021/bi00386a008)
- Dacheux JL, Belleannec C, Jones R, Labas V, Belghazi M, Guyonnet B, Druart X, Gatti JL & Dacheux F 2009 Mammalian epididymal proteome. *Molecular and Cellular Endocrinology* **306** 45–50. (doi:10.1016/j.mce.2009.03.007)
- Ensslin MA & Shur BD 2003 Identification of mouse sperm SED1, a bimotif EGF repeat and discoidin-domain protein involved in sperm-egg binding. *Cell* **114** 405–417. (doi:10.1016/S0092-8674(03)00643-3)
- Fritz IB, Burdzy K, Setchell B & Blaschuk O 1983 Ram rete testis fluid contains a protein (clusterin) which influences cell-cell interactions in vitro. *Biology of Reproduction* **28** 1173–1188. (doi:10.1095/biolreprod28.5.1173)
- Furimsky A, Vuong N, Xu H, Kumarathan P, Xu M, Weerachatanukul W, Bou Khalil M, Kates M & Tanphaichitr N 2005 Percoll gradient-centrifuged capacitated mouse sperm have increased fertilizing ability and higher contents of sulfogalactosylglycerolipid and docosahexaenoic acid-containing phosphatidylcholine compared to washed capacitated mouse sperm. *Biology of Reproduction* **72** 574–583. (doi:10.1095/biolreprod.104.036095)
- Gelissen IC, Hochgrebe T, Wilson MR, Easterbrook-Smith SB, Jessup W, Dean RT & Brown AJ 1998 Apolipoprotein J (clusterin) induces cholesterol export from macrophage-foam cells: a potential anti-atherogenic function? *Biochemical Journal* **331** 231–237. (doi:10.1042/bj3310231)
- Griffiths GS, Galileo DS, Aravindan RG, Martin-Deleon PA, 2009 Clusterin facilitates exchange of glycosyl phosphatidylinositol-linked SPAM1 between reproductive luminal fluids and mouse and human sperm membranes *Biology of Reproduction* **81** 562–570. (doi:10.1095/biolreprod.108.075739)
- Griswold MD, Roberts K & Bishop P 1986 Purification and characterization of a sulfated glycoprotein secreted by Sertoli cells. *Biochemistry* **25** 7265–7270. (doi:10.1021/bi00371a003)
- Harris CL, Mizuno M & Morgan BP 2006 Complement and complement regulators in the male reproductive system. *Molecular Immunology* **43** 57–67. (doi:10.1016/j.molimm.2005.06.026)
- Hermo L, Wright J, Oko R & Morales CR 1991 Role of epithelial cells of the male excurrent duct system of the rat in the endocytosis or secretion of sulfated glycoprotein-2 (clusterin). *Biology of Reproduction* **44** 1113–1131. (doi:10.1095/biolreprod44.6.1113)
- Hermo L, Xiaohong S & Morales CR 2000 Circulating and luminal testicular factors affect LRP-2 and Apo J expression in the epididymis following efferent duct ligation. *Journal of Andrology* **21** 122–144. (doi:10.1002/j.1939-4640.2000.tb03283.x)
- Jenne DE & Tschopp J 1989 Molecular structure and functional characterization of a human complement cytotoxic inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid. *PNAS* **86** 7123–7127. (doi:10.1073/pnas.86.18.7123)
- Kiedziarska A, Smietana K, Czepczynska H & Otlewski J 2007 Structural similarities and functional diversity of eukaryotic discoidin-like domains. *Biochimica et Biophysica Acta* **1774** 1069–1078. (doi:10.1016/j.bbapap.2007.07.007)
- Kissinger C, Skinner MK & Griswold MD 1982 Analysis of Sertoli cell-secreted proteins by two-dimensional gel electrophoresis. *Biology of Reproduction* **27** 233–240. (doi:10.1095/biolreprod27.1.233)
- Kongmanas K, Kruevaisayawan H, Saewu A, Sugeng C, Fernandes J, Souda P, Angel JB, Faull KF, Aitken RJ, Whitelegge J *et al.* 2015 Proteomic characterization of pig sperm anterior head plasma membrane reveals roles of acrosomal proteins in ZP3 binding. *Journal of Cellular Physiology* **230** 449–463. (doi:10.1002/jcp.24728)
- Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685. (doi:10.1038/227680a0)

- Law GL & Griswold MD 1994 Activity and form of sulfated glycoprotein 2 (clusterin) from cultured Sertoli cells, testis, and epididymis of the rat. *Biology of Reproduction* **50** 669–679. (doi:10.1095/biolreprod50.3.669)
- Law GL, McGuinness MP, Linder CC & Griswold MD 1997 Expression of apolipoprotein E mRNA in the epithelium and interstitium of the testis and the epididymis. *Journal of Andrology* **18** 32–42. (doi:10.1002/j.1939-4640.1997.tb01874.x)
- Leeb C, Eresheim C & Nimpf J 2014 Clusterin is a ligand for apolipoprotein E receptor 2 (ApoER2) and very low density lipoprotein receptor (VLDLR) and signals via the Reelin-signaling pathway. *Journal of Biological Chemistry* **289** 4161–4172. (doi:10.1074/jbc.M113.529271)
- Levine N & Kelly H 1978 Measurement of pH in the rat epididymis in vivo. *Journal of Reproduction and Fertility* **52** 333–335. (doi:10.1530/jrf.0.0520333)
- Mattmueller DR & Hinton BT 1991 In vivo secretion and association of clusterin (SGP-2) in luminal fluid with spermatozoa in the rat testis and epididymis. *Molecular Reproduction and Development* **30** 62–69. (doi:10.1002/mrd.1080300109)
- Mishra VK, Palgunachari MN, Hudson JS, Shin R, Keenum TD, Krishna NR & Anantharamaiah GM 2011 Structure and lipid interactions of an anti-inflammatory and anti-atherogenic 10-residue class G(*) apolipoprotein J peptide using solution NMR. *Biochimica et Biophysica Acta* **1808** 498–507. (doi:10.1016/j.bbame.2010.10.011)
- Mollnes TE & Kirschfink M 2006 Strategies of therapeutic complement inhibition. *Molecular Immunology* **43** 107–121. (doi:10.1016/j.molimm.2005.06.014)
- Muciaccia B, Pensini S, Culasso F, Padula F, Paoli D, Gandini L, Di Veroli C, Bianchini G, Stefanini M & D'Agostino A 2012 Higher clusterin immunolabeling and sperm DNA damage levels in hypertensive men compared with controls. *Human Reproduction* **27** 2267–2276. (doi:10.1093/humrep/des173)
- Murphy BF, Saunders JR, O'Bryan MK, Kirszbaum L, Walker ID & d'Apice AJ 1989 SP-40,40 is an inhibitor of C5b-6-initiated haemolysis. *International Immunology* **1** 551–554. (doi:10.1093/intimm/1.5.551)
- Navarrete FA, Garcia-Vazquez FA, Alvau A, Escoffier J, Krapf D, Sanchez-Cardenas C, Salicioni AM, Darszon A & Visconti PE 2015 Biphasic role of calcium in mouse sperm capacitation signaling pathways. *Journal of Cellular Physiology* **230** 1758–1769. (doi:10.1002/jcp.24873)
- Novak S, Smith TA, Paradis F, Burwash L, Dyck MK, Foxcroft GR & Dixon WT 2010 Biomarkers of in vivo fertility in sperm and seminal plasma of fertile stallions. *Theriogenology* **74** 956–967. (doi:10.1016/j.theriogenology.2010.04.025)
- Nuutinen T, Suuronen T, Kauppinen A & Salminen A 2009 Clusterin: a forgotten player in Alzheimer's disease. *Brain Research Reviews* **61** 89–104. (doi:10.1016/j.brainresrev.2009.05.007)
- O'Bryan MK, Mallidis C, Murphy BF & Baker HW 1994 Immunohistological localization of clusterin in the male genital tract in humans and marmosets. *Biology of Reproduction* **50** 502–509. (doi:10.1095/biolreprod50.3.502)
- Olson LM, Zhou X & Schreiber JR 1994 Immunolocalization of apolipoprotein E in the testis and epididymis of the rat. *Biology of Reproduction* **50** 535–542. (doi:10.1095/biolreprod50.3.535)
- Olson LM, Zhou X & Schreiber JR 1995 Cell-specific localization of apolipoprotein E messenger ribonucleic acid in the testis and epididymis of the rat. *Biology of Reproduction* **52** 1003–1011. (doi:10.1095/biolreprod52.5.1003)
- Poon S, Treweek TM, Wilson MR, Easterbrook-Smith SB & Carver JA 2002 Clusterin is an extracellular chaperone that specifically interacts with slowly aggregating proteins on their off-folding pathway. *Federation of European Biochemical Societies Letters* **513** 259–266. (doi:10.1016/S0014-5793(02)02326-8)
- Raymond A, Ensslin MA & Shur BD 2009 SED1/MFG-E8: a bi-motif protein that orchestrates diverse cellular interactions. *Journal of Cellular Biochemistry* **106** 957–966. (doi:10.1002/jcb.22076)
- Riaz MA, Stammer A, Borgers M & Konrad L 2017 Clusterin signals via ApoER2/VLDLR and induces meiosis of male germ cells. *American Journal of Translational Research* **9** 1266–1276.
- Robaire B & Hinton BT 2015. The epididymis. In *Knobil and Neill's Physiology of Reproduction*, pp 691–771. Eds TM Plant and AJ Zeleznik. New York: Elsevier Inc
- Robaire B & Viger RS 1995 Regulation of epididymal epithelial cell functions. *Biology of Reproduction* **52** 226–236. (doi:10.1095/biolreprod52.2.226)
- Rohne P, Prochnow H & Koch-Brandt C 2016 The CLU-files: disentanglement of a mystery. *Biomolecular Concepts* **7** 1–15. (doi:10.1515/bmc-2015-0026)
- Salicioni AM, Platt MD, Wertheimer EV, Arcelay E, Allaire A, Sosnik J & Visconti PE 2007 Signalling pathways involved in sperm capacitation. *Society of Reproduction and Fertility Supplement* **65** 245–259.
- Schwochau GB, Nath KA & Rosenberg ME 1998 Clusterin protects against oxidative stress in vitro through aggregative and nonaggregative properties. *Kidney International* **53** 1647–1653. (doi:10.1046/j.1523-1755.1998.00902.x)
- Shur BD, Rodeheffer C, Ensslin MA, Lyng R & Raymond A 2006 Identification of novel gamete receptors that mediate sperm adhesion to the egg coat. *Molecular and Cellular Endocrinology* **250** 137–148. (doi:10.1016/j.mce.2005.12.037)
- Sylvester SR, Skinner MK & Griswold MD 1984 A sulfated glycoprotein synthesized by Sertoli cells and by epididymal cells is a component of the sperm membrane. *Biology of Reproduction* **31** 1087–1101. (doi:10.1016/j.biolreprod31.5.1087)
- Sylvester SR, Morales C, Oko R & Griswold MD 1991 Localization of sulfated glycoprotein-2 (clusterin) on spermatozoa and in the reproductive tract of the male rat. *Biology of Reproduction* **45** 195–207. (doi:10.1095/biolreprod45.1.195)
- Tanphaichitr N, Smith J & Kates M 1990 Levels of sulfolactosylglycerolipid in capacitated motile and immotile mouse sperm. *Biochemistry and Cell Biology* **68** 528–535. (doi:10.1139/o90-075)
- Tanphaichitr N, Smith J, Mongkolkeha S, Gradil C & Lingwood CA 1993 Role of a gamete-specific sulfolactosylglycerolipid immobilizing protein on mouse sperm-egg binding. *Developmental Biology* **156** 164–175. (doi:10.1006/dbio.1993.1067)
- Towbin H & Gordon J 1984 Immunoblotting and dot immunobinding-current status and outlook. *Journal of Immunological Methods* **72** 313–340. (doi:10.1016/0022-1759(84)90001-2)
- Tremellen K 2008 Oxidative stress and male infertility – a clinical perspective. *Human Reproduction Update* **14** 243–258. (doi:10.1093/humupd/dmn004)
- Trougakos IP 2013 The molecular chaperone apolipoprotein J/clusterin as a sensor of oxidative stress: implications in therapeutic approaches – a mini-review. *Gerontology* **59** 514–523. (doi:10.1159/000351207)
- Tschopp J & French LE 1994 Clusterin: modulation of complement function. *Clinical and Experimental Immunology* **97** (Supplement 2) 11–14. (doi:10.1111/j.1365-2249.1994.tb06256.x)
- Tung PS & Fritz IB 1985 Immunolocalization of clusterin in the ram testis, rete testis, and excurrent ducts. *Biology of Reproduction* **33** 177–186. (doi:10.1095/biolreprod33.1.177)
- Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P & Kopf GS 1995 Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121** 1129–1137.
- Wang Z, Widgren EE, Richardson RT, O'Rand MG, 2007 Characterization of an eppin protein complex from human semen and spermatozoa. *Biology of Reproduction* **77** 476–484. (doi:10.1095/biolreprod.107.060194)
- Wilson MR & Easterbrook-Smith SB 2000 Clusterin is a secreted mammalian chaperone. *Trends in Biochemical Sciences* **25** 95–98. (doi:10.1016/S0968-0004(99)01534-0)
- Wyatt AR & Wilson MR 2010 Identification of human plasma proteins as major clients for the extracellular chaperone clusterin. *Journal of Biological Chemistry* **285** 3532–3539. (doi:10.1074/jbc.M109.079566)
- Wyatt AR, Yerbury JJ & Wilson MR 2009 Structural characterization of clusterin-chaperone client protein complexes. *Journal of Biological Chemistry* **284** 21920–21927. (doi:10.1074/jbc.M109.033688)
- Wyatt AR, Yerbury JJ, Berghofer P, Greguric I, Katsifis A, Dobson CM & Wilson MR 2011 Clusterin facilitates in vivo clearance of extracellular misfolded proteins. *Cellular and Molecular Life Sciences* **68** 3919–3931. (doi:10.1007/s00018-011-0684-8)
- Xu H, Liu F, Srakaew N, Koppisetty C, Nyholm PG, Carmona E & Tanphaichitr N 2012 Sperm arylsulfatase A binds to mZP2 and mZP3 glycoproteins in a nonenzymatic manner. *Reproduction* **144** 209–219. (doi:10.1530/REP-11-0338)

Received 17 April 2017

First decision 30 May 2017

Revised manuscript received 17 August 2017

Accepted 2 October 2017