FEBS Letters 566 (2004) 136–140 FEBS 28379

Identification of chloride intracellular channel proteins in spermatozoa

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Received 4 March 2004; revised 2 April 2004; accepted 7 April 2004

Available online 22 April 2004

Edited by Peter Brzezinski

Abstract We have identified for the first time the presence of chloride intracellular channel (CLIC) proteins in bovine epididymal spermatozoa. CLIC1 was discovered during microsequencing of proteins that co-purified with protein phosphatase 1, PP1γ2, in sperm extracts. In addition to CLIC1, Western blot showed that two additional CLIC family members, CLIC4 and CLIC5, are also present in spermatozoa. CLIC fusion proteins, GST-CLIC1, GST-CLIC4 and GST-CLIC5, were all able to bind to PP1γ2 in sperm extracts during pull-down assays. Immunofluorescence microscopy revealed that each of the three isoforms occupies a distinct location within the cell. Given that PP1γ2 is a key enzyme regulating sperm motility, PP1γ2-binding proteins, such as the CLIC proteins, are likely to play significant roles in sperm function.

Keywords: Epididymal spermatozoa; Chloride intracellular channel; Sperm motility; Protein phosphatase 1

1. Introduction

Spermatozoa are terminally differentiated cells specialized for efficient delivery of genetic material to the egg. In mammals, testicular spermatozoa are immotile and incapable of fertilization. Acquisition of motility and fertility occur in the epididymis as spermatozoa pass from the caput to the caudal region of this organ. Hyperactivation of motility occurs during capacitation in the female reproductive tract. However, the biochemical mechanisms underlying the acquisition of motility and hyperactivation in spermatozoa are not yet fully understood [1–3].

We previously identified a testis isoform of protein phosphatase 1, PP1γ2, involved in regulating sperm motility [4,5]. PP1γ2 is highly conserved in spermatozoa of all mammalian species studied to date, including humans [6]. Inhibition of PP1γ2 initiates motility in immotile spermatozoa and further stimulates motility in motile spermatozoa [4]. Disruption of the gene for PP1γ in mice causes infertility by arresting spermatogenesis [7], suggesting that one or both of the PP1γ isoforms are required in the final stages of sperm development. Catalytically active PP1γ2 elutes in an 80-kDa fraction during purification from sperm extracts which resolves into two major protein bands in SDS–PAGE, a 39-kDa band, containing PP1γ2, and a 28-kDa band containing protein 14-3-3ζ [6]. We have demonstrated that a binding relationship exists between PP1γ2 and protein 14-3-3 in spermatozoa [6]. Since PP1γ2 is a key protein in the regulation of sperm motility and protein 14-3-3 is involved in signal transduction [8,9], proteins that interact with PP1γ2 or protein 14-3-3 may participate in signaling events in the regulation of motility and other sperm functions.

In this report, we document that a 27-kDa chloride intracellular channel (CLIC) protein, CLIC1, co-purifies with PP1γ2 and protein 14-3-3 in column chromatography. In addition, we have identified two additional CLIC isoforms in spermatozoa, CLIC4 and CLIC5. CLICs constitute a new family of putative chloride channel proteins with several distinct members, including bovine p64 and human CLIC1-6 [10,11]. CLIC proteins are localized to a variety of subcellular compartments in various cell types, and although they have been implicated in chloride ion transport [12,13], several reports show that certain family members can associate with the cytoskeleton, scaffolding proteins, and signaling enzymes [14–19]. The identification of multiple CLIC proteins in spermatozoa, which may interact with PP1γ2, opens a new area of study on the role of CLICs in sperm function.

2. Materials and methods

2.1. Sperm extract preparation

Spermatozoa were isolated from the caput and caudal epididymis of mature bull testes and washed as previously described [4] in 10 mM Tris–HCl (pH 7.2) containing 100 mM NaCl, 40 mM KCl, and 5 mM $MgSO_4$. Sperm pellets were re-suspended in extraction buffer (10 mM Tris (pH 7.2), 1 mM EDTA, 1 mM EGTA supplemented with protease inhibitors (1 mM PMSF, 0.01 mM TPCK, and 5 mM β-mercaptoethanol)) and sonicated. Extracts were centrifuged at $16000 \times g$ for 10 min at 4 °C, resulting in soluble supernatants. The supernatants were ultracentrifuged at $100000 \times g$ for 1 h at 4 °C to produce pellets consisting of membrane vesicles.

2.2. Column chromatography

All column procedures were performed at 4 °C. Bovine caudal epididymal sperm extract (prepared from 5×10^{10} spermatozoa in extraction buffer) was passed through a DEAE-cellulose column (0.5 cm × 13 cm) pre-equilibrated with column buffer (extraction buffer supplemented with 0.05 M KCl and protease inhibitors) [20]. After washing, proteins were eluted with a linear gradient of 0.05–0.65 M KCl in column buffer; PP1γ2 activity [20], and phospho-PP1 and PP1γ2 immunoreactivity were detected in fractions containing 0.185–0.35 M KCl. Pooled gradient fractions were passed through SP-sepharose column (5 ml pre-packed, Pharmacia, Piscataway, NJ) in column buffer. The flow-through fractions containing active and immunoreactive PP1γ2 were concentrated and applied to a Superose 6

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Abbreviations: CLIC, chloride intracellular channel; PP1, protein phosphatase 1

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.04.017

(24 ml, pre-packed high-resolution Pharmacia FPLC, Pharmacia, Piscataway, NJ). Elution through the sizing column was performed at 0.5 ml/min, collecting 0.5 ml fractions. Fractions containing PP1γ2 were identified by dot blot analyses and activity assays utilizing antibodies against PP1γ2 (Zymed Laboratories, San Francisco, CA) and phospho-PP1 [21], respectively. Immunoreactive fractions were pooled and then concentrated using a Centricon-10 filter (Millipore Corp., Bedford, MA). Elution of protein standards under the same conditions produced a standard curve used for molecular weight determination of the PP1γ2-containing fractions.

2.3. Western blot analysis

Sperm extracts and column fractions were boiled in Laemmli sample buffer [22], separated by 12% SDS–PAGE, and then transferred electrophoretically to Immobilon-PVDF membranes (Millipore, Bedford, MA). Blots were incubated in primary antibody overnight at 4 °C, washed, then incubated in peroxidase-labeled anti-rabbit secondary antibody (Amersham, Piscataway, NJ) for 1 h at room temperature. After washing, blots were developed using chemiluminescence (Amersham, Piscataway, NJ).

2.4. Microsequencing

Column fractions enriched in PP1γ2 produced two major bands in a Coomassie blue-stained gel from 12% SDS–PAGE: a 39-kDa band that represented PP1γ2 [6] and a 28-kDa band. The 28-kDa band was excised from the gel and washed with HPLC-grade acetonitrile (Sigma–Aldrich). In-gel digestion and peptide sequencing was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry using a Finnigan LCQ DECA XP quadrupole ion trap mass spectrometer.

2.5. Recombinant CLIC markers and CLIC antibodies

Total bacterial extracts expressing human CLIC1, CLIC4, and CLIC5, which migrate at 31, 29, and 32 kDa, respectively, were described previously [14]. These extracts were used as markers in Western blots to monitor the mobility of endogenous CLIC proteins. Affinity-purified rabbit antibodies that recognize CLIC1 (AP823), CLIC4 (APB134), and CLIC5 (APXB5/6-N) were utilized to identify immunoreactivity in both Western blot and immunofluorescence experiments. AP823 was generated against a 13-amino-acid peptide corresponding to positions 226–238 of human CLIC1 [23]. APB134 was raised against full-length human CLIC4 and affinity-purified using SDS-denatured CLIC4 [14]. APXB5/6-N was raised against a GST fusion protein containing full-length human CLIC5. The resulting antiserum was passed sequentially through columns containing GST and 6His-CLIC4, followed by affinity purification on 6His-CLIC5 using published methods [14]. An antiserum (B61) raised against a 6His-tagged fusion protein containing the C-terminal 157 amino acids of CLIC5 was used in the initial identification of CLIC4 and CLIC5.

2.6. GST pull-down assay

Recombinant GST and full-length human CLIC1, CLIC4, and CLIC5 incorporated into the pGEX-2T vector (Amersham, Piscataway, NJ) were expressed in *Escherichia coli* BL21 and purified. GST-14-3-3 was similarly produced but using a pGEX-4T vector with full-length human 14-3-3ζ inserted (the vector was gift from Michael Yaffe, Department of Biology, Massachusetts Institute Technology). The concentrations of GST and GST fusion proteins were estimated by comparison against protein standards on Coomassie blue-stained gels. Glutathione–Sepharose 4B beads containing equimolar amounts of GST or GST-CLICs were incubated with caudal sperm extract overnight at 4 °C. After washing, proteins were eluted with 50 μl of 20 mM reduced glutathione, 50 mM Tris, pH 8.0, and then denatured by boiling in Laemmli sample buffer. Samples were separated on 12% SDS–PAGE and analyzed by Western blotting with PP1γ2 and protein 14-3-3 antibodies (Zymed Laboratories, San Francisco, CA) or with the CLIC antibodies.

2.7. Immunofluorescence microscopy

Spermatozoa were washed and resuspended in PBS (pH 7.0). Two different procedures were used to fix the cells. Cells were either attached to poly-L-lysine-coated coverslips and then fixed in 100% methanol for 5 min at −20 °C or cells in suspension were fixed with 4% formaldehyde in PBS for 30 min at 4 °C, permeabilized briefly with 0.2% Triton X-100, and then attached to poly-L-lysine-coated coverslips. Cells were washed briefly with Tris-buffered saline containing 0.5% Tween 20 (TTBS), treated with blocking buffer (2.5% BSA and 5% normal goat serum in TTBS) at room temperature for 3 h, and then incubated with primary antibody diluted in blocking buffer at 4 °C overnight. After washing, cells were incubated with goat anti-rabbit IgG conjugated to Cy3 (Jackson Laboratories, West Grove, PA), washed again, and then mounted in Vectashield mounting media (Vector Laboratories Inc., Burlingame, CA). Primary antibodies were omitted in parallel control specimens. Cells were examined by fluorescence and phase-contrast microscopy.

3. Results

3.1. CLIC1 co-purifies with catalytically active sperm PP1γ2

We previously showed that PP1γ2 in sperm extracts eluted as an 80-kDa species in a sizing column [20]. Analysis by SDS–PAGE revealed that this 80-kDa species resolves into two major protein bands migrating at 39 and 28 kDa. Microsequencing and Western blotting identified the 39-kDa band as PP1γ2 and identified one of the proteins in the 28-kDa band as protein 14-3-3ζ [6]. We now show that in addition to protein 14-3-3ζ, the 28-kDa band contains a second protein. Two peptides identified by microsequence analysis of the 28-kDa band were found to match residues 96–113 and 120–131 of human CLIC1 (Fig. 1), which has a calculated mass of 26 900 Da.

To further investigate the presence of CLIC1 in spermatozoa, an antibody against CLIC1 was used. As shown in Fig. 2, Western blot analysis detected a 31-kDa band in the purified 80-kDa column fraction as well as in soluble sperm extracts. This 31-kDa band had the same mobility as the recombinant CLIC1 marker, thus verifying our microsequencing results. With longer exposure times, the CLIC1 antibody also recognized an additional band at 55–66 kDa in soluble sperm extracts (data not shown). In contrast to soluble extracts, no CLIC1 reactivity was seen in membrane pellet fractions (data not shown).

3.2. CLIC4 and CLIC5 are also present in spermatozoa

To investigate the presence of other CLIC species in spermatozoa, we probed blots with an antiserum (B61) that recognizes CLIC4 and CLIC5, which migrate at 29 and 32 kDa, respectively (Fig. 3A). These bands were also detected using affinity-purified antibodies against CLIC4 (Fig. 3B) and CLIC5 (Fig. 3C), confirming their existence in sperm extracts.

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1   maeeqpqvel fvkagsdgak ignсpfsqrl fmvlwlkgvt fnvttvdtkr rtetvqklcp
61  ggqlpflyg tevhtdtnki eefleavlcp prypklaaln pesntagldi fakfsayikn
121 snpalndnle kgllkalkvl dnyltsplpe evdetsaede gvsqrkfldg neltladcnl
181 lpklhivqvv ckkyrgftip eafrgvhryl snayareefa stcpddeeie layeqvakal
241 k
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Fig. 1. Amino acid sequence of human CLIC1 (GenBank Accession No. NP-001279). Peptide sequences obtained from in-gel digestion of the 28-kDa band are highlighted.

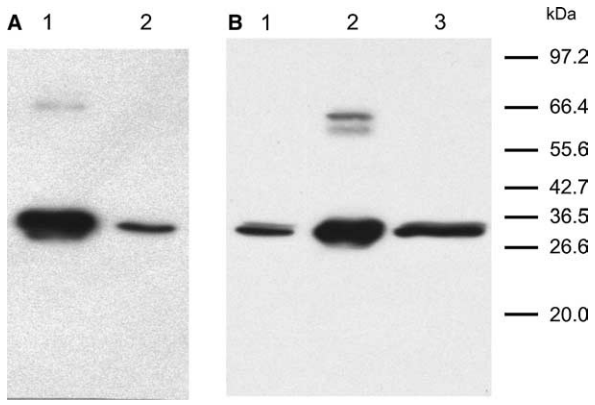

Fig. 2. Western blot analysis of CLIC1. Blots were probed with AP823, a previously described CLICI antibody [22]. (A) Recombinant CLIC1 control marker (lane 1) and PP1γ2-rich fraction from caudal sperm extract (lane 2). (B) A Jurkat cell lysate (lane 1) and recombinant CLIC1 marker (lane 2) were used as positive controls. A soluble extract prepared from caudal epididymal spermatozoa is shown in lane 3.

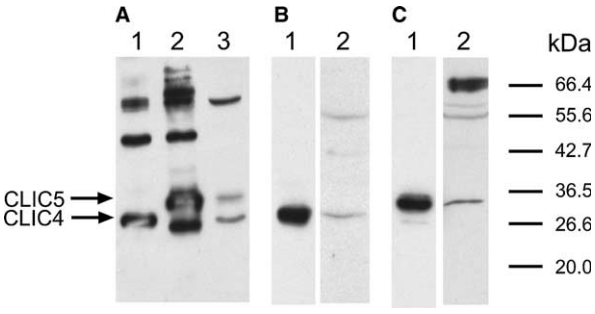

Fig. 3. Identification of CLIC4 and CLIC5 in soluble extracts of epididymal spermatozoa. (A) Blot probed with B61 antiserum. Lanes 1 and 2 contain recombinant markers for CLIC4 and CLIC5, respectively. The bands migrating as intact proteins are indicated by arrows; the faster migrating CLIC5 species in lane 2 is a degradation product. Lane 3 contains caput extract. (B) Blot probed with affinity-purified CLIC4 antibody. Lane 1 contains recombinant CLIC4 and lane 2 contains caput extract. (C) Blot probed with affinity-purified CLIC5 antibody. Lane 1 contains recombinant CLIC5 and lane 2 contains caudal extract.

At least one additional immunoreactive band migrating at 55–66 kDa was seen with each antibody. These slower migrating species were not recognized in control blots incubated with secondary antibody alone. Although CLIC1 was only detected in soluble sperm extracts, CLIC4 and CLIC5 were present in soluble extracts as well as membrane pellets (data not shown).

3.3. Recombinant GST-CLICs bind endogenous PP1γ2 in sperm extracts

To test for possible interactions between co-purifying CLICs, PP1γ2 and protein 14-3-3ζ, we performed co-immunoprecipitation experiments with PP1γ2, protein 14-3-3, and CLIC antibodies. We were unable to co-immunoprecipitate PP1γ2 or protein 14-3-3 with the CLIC antibodies or CLIC proteins with the PP1γ2 or 14-3-3 antibodies, so GST pull-down assays were used as an alternative approach. The pull-down assays were conducted by incubating soluble sperm extracts with immobilized GST-14-3-3ζ or GST-CLIC fusion proteins, and GST alone as a control. Given that a direct interaction between CLIC4 and protein 14-3-3ζ has been demonstrated [15], we first looked for the CLIC proteins in the GST-14-3-3ζ pull-down and for protein 14-3-3 in the GST-CLIC pull-downs. Although GST-14-3-3ζ was unable to bind sperm CLIC proteins, PP1γ2 was readily detected in the same samples, consistent with previous results from our laboratory [6]. Similarly, in the GST-CLIC pull-downs, protein 14-3-3 was not detected but we did observe specific binding of endogenous PP1γ2. As shown in Fig. 4, PP1γ2 was retained by GST-CLIC1, GST-CLIC4, and GST-CLIC5, but not by GST alone.

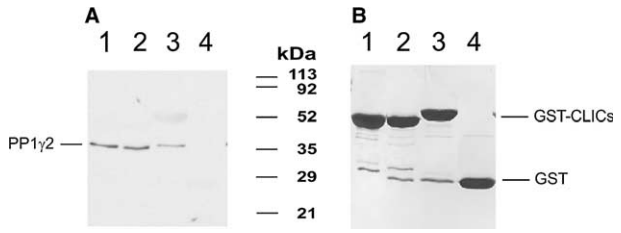

Fig. 4. Western blot analysis of GST-CLIC pull-down assays. (A) GST-CLIC pull-down blot probed with PP1γ2 antibody. Lane 1, proteins eluted from GST-CLIC1 beads; lane 2, proteins eluted from GST-CLIC4 beads; lane 3, proteins eluted from GST-CLIC5 beads; lane 4 contains proteins eluted from GST alone control beads. (B) The same blot stained with Coomassie blue.

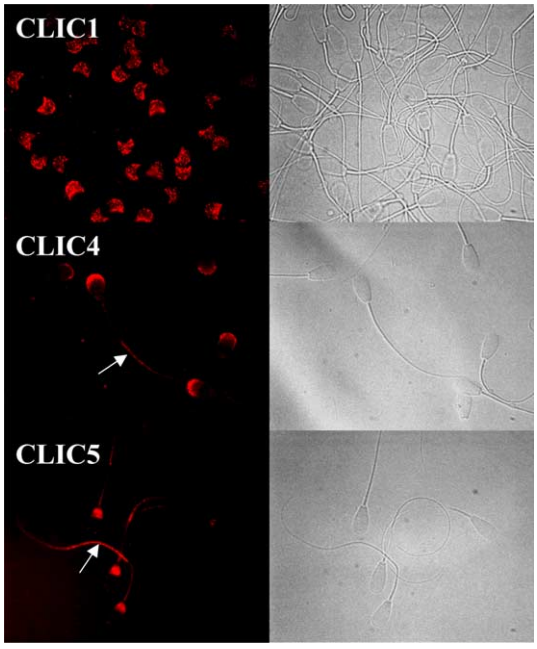

Fig. 5. Immunofluorescence localization of CLIC1, CLIC4, and CLIC5 in spermatozoa. Epididymal spermatozoa were either fixed with methanol (top and middle rows) or fixed with formaldehyde and permeabilized with detergent (bottom row) as described in Section 2. Cells were stained with affinity-purified antibodies against CLIC1 (top row), CLIC4 (middle row), and CLIC5 (bottom row), followed by a fluorescent-labeled secondary antibody. Tail staining with CLIC4 and CLIC5 antibodies is indicated by white arrows. Fluorescence and corresponding phase-contrast images are shown. All micrographs were photographed using a 100× objective.

3.4. CLIC1, CLIC4, and CLIC5 have distinct localizations in spermatozoa

Immunofluorescence microscopy was used to determine the subcellular distribution of CLIC1, CLIC4, and CLIC5 in epididymal spermatozoa using affinity-purified antibodies

against each protein. As shown in Fig. 5, we observed a distinct and specific staining pattern for each antibody. There was minimal background staining in control samples incubated with secondary antibody alone. Staining for CLIC1 was localized to the acrosomal region of the sperm head, whereas CLIC4 staining was confined mainly to the anterior perimeter of the sperm head. In contrast to CLIC1 and CLIC4, staining for CLIC5 was predominantly located in the post-acrosomal region of the sperm head. In addition, CLIC4 and CLIC5 were also localized to the flagellum, mainly in the principal piece.

4. Discussion

This report is the first to identify CLIC proteins in spermatozoa. Microsequencing of proteins that co-purified with testis phosphatase PP1γ2 revealed the presence of CLIC1, and Western blot analyses utilizing affinity-purified antibodies against CLIC proteins confirmed that CLIC1, as well as CLIC4 and CLIC5, are present in spermatozoa. Pull-down assays showed that PP1γ2 in sperm extracts binds specifically to GST-CLIC fusion proteins. Together, these data suggest that CLIC-PP1γ2 complexes exist in vivo.

Interactions between sperm CLIC proteins and PP1γ2, a key enzyme involved in regulating sperm maturation and motility, are consistent with findings from several laboratories that suggest CLIC proteins may play a role in signal transduction. For example, CLIC3 was originally discovered as a binding partner for ERK7, a member of the mitogen-activated protein kinase family [18], and bovine p64 has been shown to associate directly with $p59^{fyn}$, a Src family tyrosine kinase [19]. In addition, multiple CLIC family members can associate with AKAP350, a scaffolding protein that sequesters cAMP-dependent protein kinase and several other protein kinases and phosphatases to particular subcellular sites [16,17]. More recently, CLIC6 has been shown to bind scaffolding proteins, such as radixin and multi-PDZ protein, as well as dopamine D_2-like receptors [24].

PP1γ2 localizes to the posterior region of the sperm head including the equatorial segment and to the entire length of the flagellum including the midpiece and also displays punctuate staining in the acrosomal region [6]. Our immunofluorescence data revealed that CLIC1 is mostly concentrated in the acrosomal region, while CLIC4 is present in the anterior region of the sperm head and the flagellum, and CLIC5 resides in the post-acrosomal region and the flagellum. Although the localization of CLIC5 and PP1γ2 are the most similar, GST-CLIC5 consistently bound less PP1γ2 in pull-down assays compared to GST-CLIC1 and GST-CLIC4. The partial overlap in the distributions of CLIC1 and CLIC4 and PP1γ2 also suggests that only a fraction of the total PP1γ2 might be complexed with the CLIC proteins, at least in epididymal spermatozoa. The striking differences in subcellular localization of CLIC1, CLIC4, and CLIC5 are particularly interesting, because it suggests that each protein may perform a distinct function at specific intrasperm sites. The presence of three distinct CLIC proteins within the sperm head suggests a possible role for these proteins in gamete fertilization events, including sperm–egg membrane fusion and/or the acrosome reaction. Interestingly, a cytosolic chloride ion efflux mediated by ligand-gated chloride channels in the plasma membrane has been shown to be essential to the acrosome reaction [25–27]. If CLIC proteins in spermatozoa function as chloride channels, or as components or regulators of chloride channels, then it is possible that they contribute to the chloride efflux associated with the acrosome reaction. Recent studies have shown that the genes for CLIC1 and CLIC4 are expressed at all stages of development in *Xenopus laevis* embryos, suggesting that they fulfill an essential function during early development [28]. In addition, CLIC1 has been shown to play an important role in cell cycle control [29]. Thus, it is possible that the sperm CLIC proteins are cargo destined for the egg itself. The presence of CLIC4 and CLIC5 in the flagellum also suggests a role in processes controlling axonemal function. Studies have shown that CLIC4 associates indirectly with tubulin in vitro [15,17] and also localizes to microtubule bundles and vesicles closely associated with microtubules in neurons [30].

In addition to the characteristic mobility of CLIC1, CLIC4, and CLIC5 in the 29–32 kDa range in SDS–PAGE, additional slower-migrating bands at 55–66 kDa were consistently observed in Western blots of sperm extracts probed with affinity-purified antibodies. It is unlikely that these bands represent bovine p64, because blots probed with a previously characterized p64 antibody [10] failed to detect proteins in the 55–66 kDa range. One interpretation is that the slower migrating bands represent endogenous CLIC dimers, a possibility that would be consistent with the observation that CLIC proteins can form multimeric complexes [13] and that rat CLIC6 forms homodimers at the carboxyl terminus [24], a region that is highly conserved among CLIC family members. Alternately, the slower migrating species may arise from post-translational modifications, such as phosphorylation or covalent adduct formation.

Further study is required to identify the interactions and functions of CLIC proteins in spermatozoa. PP1γ2 is critical to normal sperm development and motility, thus any proteins that interact with PP1γ2 or with PP1γ2 regulatory proteins are likely to be important regulators of sperm development or function. A thorough understanding of CLIC interactions with other sperm proteins will help elucidate their functional significance in these cells.

Acknowledgements: We express our gratitude to Dr. John Edwards (St. Louis Veterans Affairs Medical Center) for his generous gifts of antibodies and to Dr. Michael Yaffe (Department of Biology, Massachusetts Institute Technology) for his gift of the GST-14-3-3ζ plasmid.

This research was supported by NIH Grant R01 HD38520 (SV) and funds from Ohio University (M.B.).

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