ASSOCIATION OF CATSPER1 OR 2 WITH Ca_{3,3} LEADS TO SUPPRESSION OF T-TYPE CALCIUM CHANNEL ACTIVITY*

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Running Title: CatSper associates with and functionally suppresses Ca_{3,3}

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Sperm-specific CatSper1 and CatSper2 proteins are critical to sperm hyperactivated motility and male fertility. Although architecturally resembling voltage-gated ion channels, neither CatSper1 nor CatSper2 alone forms functional ion channels in heterologous expression systems, which may be related to the absence of yet unidentified accessory subunits. Here we isolated CatSper1 and CatSper2 associated protein(s) from human sperm and analyzed their identities by Multidimensional Protein Identification Technology (MudPIT\(^1\)) approach. We identified the T-type voltage-gated calcium channel Ca_{3,3} as binding to both CatSper1 and CatSper2. The specificity of their interactions was verified by co-immunoprecipitation in transfected mammalian cells. Electrophysiological studies revealed that the co-expression of CatSper1 or CatSper2 specifically inhibited the amplitude of Ca_{3,3}-evoked T-type calcium current without altering other biophysical properties of Ca_{3,3}. Immunostaining studies revealed co-localization of CatSper1 and Ca_{3,3} on the principal piece of human sperm tail. Further, Fluorescence Resonance Energy Transfer (FRET) analysis revealed close proximity and physical association of these two proteins on the sperm tail. These studies demonstrate that CatSper1 and CatSper2 can associate with and modulate the function of Ca_{3,3} channel, which might be important in the regulation of sperm function.

Sperm must swim long distances in the female reproductive tract to reach the site of fertilization. In addition, penetration through the gelatinous zona pellucida layer of the oocyte requires the sperm to swim in hyperactivated state at the time and site of fertilization. This process is characterized by high amplitude and asymmetric beating of the sperm tail. Many studies have indicated that Ca\(^{2+}\) serves as a key regulator in the initiation and maintenance of motility, including the hyperactivated motility (for reviews, see (1,2)).

Extensive efforts have been undertaken to identify and characterize Ca\(^{2+}\) entry pathways, particularly Ca\(^{2+}\) channels, involved in sperm motility processes. Two such ion channel-like proteins, CatSper1 and CatSper2, were shown to be specifically expressed in the principal piece of the sperm tail (3,4). Targeted disruption of CatSper1 led to sterile phenotype in otherwise normal male mice. Further studies revealed that mutant sperm lack hyperactivated motility (5). In vitro fertilization assays revealed that CatSper1 mutant sperm could not fertilize eggs with intact zona pellucida layer, but could fertilize eggs whose outer layers had been enzymatically removed (3). Targeted disruption of CatSper2 also led to male sterile phenotype and the null sperm has identical loss-of-function phenotype as the CatSper1 null sperm (6,7). Therefore, CatSper1 and CatSper2 proteins appear to be essential for hyperactivated motility needed late for the sperm to penetrate zona pellucida.

CatSper1 and CatSper2 represent a unique class of putative ion channel proteins (for a review, see (8)). They contain a single domain comprised of six transmembrane spanning regions, akin to the voltage-gated potassium channels (3). However, their ion selectivity pore sequences between transmembrane regions 5 and 6 are closest to a single domain of the much larger voltage-gated Ca\(^{2+}\)-selective channels. Residues lining the fourth transmembrane region of CatSper resemble a voltage sensor, as described for voltage-gated ion channels. However, recording
ion channel activity following expression of CatSper subunits in heterologous expression systems, including Xenopus oocytes, HEK and CHO-K1 cells have not been successful (3,4). Attempts to measure whole-cell currents from the sperm have proven difficult until very recently, where an alkaline-activated Ca\(^{2+}\) current was recorded from sperm by patch-clamp measurements (9). Interestingly, this current is absent in sperm lacking CatSper1. However, considering the co-dependent expression of CatSper1 and CatSper2 (7), it is still not clear which subunit or subunit complexes mediates this current.

Although several possibilities may be envisaged, the inability to functionally express CatSper subunits may be due to (i) the absence of sperm-specific accessory proteins necessary for a putative ion channel complex in the heterologous systems, as have been observed with other ion channel complexes or (ii) that CatSper may function as an accessory subunit to modulate function of the principal subunit of an undetermined ion channel. Potential for either scenario exist, and it is indeed noteworthy that coiled-coil protein-protein interaction domains are present in the C-terminal regions of each of the CatSper subunits (10). In the present study, we adopted a GST pull-down approach to isolate CatSper-associated proteins from human sperm extracts and have identified the T-type calcium channel subunit Ca\(^{3.3}\) as an interacting protein. Electrophysiological studies revealed that the co-expression of either CatSper1 or CatSper2 specifically inhibited the amplitude of Cav3.3-evoked T-type Ca\(^{2+}\) current without altering other biophysical properties of Cav3.3. Considering that CatSper1 and Ca\(^{3.3}\) subunits are co-expressed and associated with each other on the tail of the human sperm, our observations suggest that CatSper-Ca\(^{3.3}\) interactions could play an important role in regulating sperm functions such as hyperactivated motility.

**EXPERIMENTAL PROCEDURES**

**Cloning**---To generate flag-tagged human CatSper1 (GenBank™ accession number: AF407333) and CatSper2 (GenBank™ accession number: AF411818) expression constructs, the coding sequences of CatSper with an in-frame flag tag sequence were amplified by PCR and cloned into the NotI site of pIRESngeo3 (Clontech, Palo Alto, CA). To generate pGEX-4T-1-Cat1-C to express the C-terminus of CatSper1 as a GST fusion protein, coding sequence covering the C-terminal 111 amino acids of human CatSper1 was amplified by PCR and cloned in-frame with GST in pGEX-4T-1 vector (Amersham, Buckinghamshire, England). To prepare pGEX-4T-1-Cat2-N and pGEX-4T-1-Cat2-C constructs for the expression of the N- and C-termini of CatSper2 as GST fusion proteins respectively, coding sequences covering the N-terminal 103 amino acids and C-terminal 190 amino acids of human CatSper2 were amplified by PCR and cloned in-frame with GST in pGEX-4T-1.

**Antibodies**---To generate antibody against human CatSper1, the C-terminal 111 amino acids of CatSper1 as a GST fusion protein was purified from bacteria and used to immunize rabbits. The CatSper1 C-terminal fragment, which was released from the GST fusion protein by thrombin cleavage, was used to affinity-purify antibodies against the CatSper1 part from the crude serum. The strategy as described by Quill et al. (4) was adopted to generate rabbit antibody against a peptide derived from the C-terminal 27 amino acids of mouse CatSper2. The resulting antibody recognized the human CatSper2 as well. The following antibodies were obtained from the sources indicated: human Ca\(^{3.3}\) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, and Alomone Labs, Israel), Na\(^{+}/K^{+}\)-ATPase antibody (Abcam, Cambridge, England), PMCA4 antibody (Sigma, St Louis, MO), human 14-3-3\(\epsilon\) antibody (Assay Designs, Ann Arbor, MI), V5 tag antibody (Invitrogen), flag tag antibody (Sigma).

**GST pull-down Assay**---To prepare GST fusion proteins, pGEX-4T-1, pGEX-4T-1-Cat1-C, pGEX-4T-1-Cat2-N, and pGEX-4T-1-Cat2-C were transformed into BL21 Star(DE3) pLysS (Invitrogen) and the expression of recombinant
proteins were induced by 0.5 mM IPTG. GST fusion proteins were purified from bacteria using glutathione-Sepharose 4B beads (Amersham) according to the protocol recommended by the manufacturer. The purified proteins were eluted from the beads by a buffer containing glutathione, dialyzed, and re-conjugated on fresh beads to obtain purer fractions.

To prepare human sperm extract, cryo-preserved sperm samples were washed to remove the seminal fluid and lysed in lysis buffer (50 mM Tris 7.5, 250 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 2 mM EDTA, 0.5 mM DTT and protease inhibitor cocktail from Sigma) for 1 hour. The extract was centrifuged to remove cell debris and combined with equal volume of dilution buffer to bring down NP-40 and sodium deoxycholate concentration to 0.5%. The sperm extract was precleared by incubation with glutathione-Sepharose 4B beads for 1 hour. To pull down CatSper-associated proteins, the sperm extract was divided into equal parts and beads conjugated with GST (as a negative control), GST-Cat1-C, or a mixture of GST-Cat2-N and GST-Cat2-C proteins, was added. After incubation at 4°C with gentle rotation overnight, the beads were washed three times with 10 ml ice-cold IP buffer (50 mM Tris 7.5, 250 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 2 mM EDTA, 0.5 mM DTT and protease inhibitor cocktail from Sigma). The GST fusion proteins along with associated proteins on the washed beads were eluted in a buffer containing glutathione (100 mM Tris 8.0, 150 mM NaCl, 20 mM glutathione, 0.2% Triton X-100).

MudPIT Analysis of Protein Identities---
Half of the eluted protein mixtures from GST pull-down experiments were resolved by SDS-PAGE and visualized by silver staining. Selective protein bands on the gel were excised and the gel slices were destained and chopped into 1-mm size cubes. A slightly modified procedure originally developed by Shevchenko et al. (11) was employed for in-gel digestion. The extracted peptides were lyophilized and resuspended in 15-20 µl of 5% formic acid until further analysis. The other portion of the eluted protein mixtures were subjected to in-solution digestion (12) resulting in complex peptide mixtures. Peptide digests resulting from in-gel digests or in-solution digests were then individually loaded on to a 3-phase MudPIT (RP-SCX-RP) column. A 3-step MudPIT analysis was used for analyzing the in-gel digests and a 6-step MudPIT analysis was used for in-solution digests (13).

Transfection and Cell Culture---
Human embryonic kidney (HEK) cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified 5% CO₂, 95% O₂ incubator at 37°C. Cells were transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. To generate a cell line stably expressing Ca₃.3, pcDNA3.1-Ca₃.3–V5/His was linearized by SalI and transfected into HEK cells. Stable cell line was generated by antibiotic selection (1 mg/ml G418) 48 hours post-transfection. Single cell colonies were selected 14 days post-transfection, amplified, and the expression of Ca₃.3 was assessed.

Co-immunoprecipitation---
HEK cells in 10 cm dishes were transiently transfected with equal amounts of the expression constructs (totally 10 µg of DNA) using LipofectAMINE 2000. When only a single construct was transfected, pcDNA3.1 vector alone was included to maintain the same final amount of DNA. Cells were lysed 48 hours post-transfection with IP buffer (50 mM Tris 7.5, 250 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 2 mM EDTA, 0.5 mM DTT and protease inhibitor cocktail from Sigma). The cell lysates were then centrifuged to remove cell debris and the antibody used for immunoprecipitation was added to the supernatant. After overnight incubation at 4°C with gentle agitation, immune complexes were precipitated with protein A or protein G agarose beads (Invitrogen) followed by washes in 1 ml IP buffer for three times. After the final wash, the pellet was resuspended in Laemmli sample buffer, and proteins were resolved by SDS-PAGE (4-12% gel) and transferred to PVDF membrane for immunoblot analysis.

Whole Cell Patch Clamp---
HEK cells stably expressing Ca₃.3 were transfected with either pcDNA3.1 vector, CatSper1 or CatSper2 expressing constructs using LipofectAMINE 2000 (Invitrogen). Cells were also cotransfected with a plasmid encoding a GFP reporter (in a 1:5 ratio) to allow identification of positively transfected cells for whole cell patch clamp measurements. 24 to 48
hours post-transfection, whole cell currents were recorded at room temperature using the standard patch clamp technique with an Axopatch 200B amplifier (Axon Instruments, Union City, CA), controlled with a PC computer using pCLAMP6 software (Axon Instruments). Data were filtered at 5 KHz using the built-in filter of the amplifier. Borosilicate pipettes with a typical resistance of 2–3 MΩ were filled with a solution containing: 110 mM CsCl, 10 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, 0.6 mM GTP (pH adjusted to 7.2 with CsOH). Extracellular solution contained 5 mM CaCl₂, 155 mM tetraethyl ammonium (TEA) chloride, 10 mM HEPES (pH adjusted to 7.4 with TEA-OH).

Data Analysis--- In electrophysiology measurements, peak currents were determined using Clampfit 8.0 software (Axon Instruments). The conductance-voltage relationship for activation was deduced by the chord conductance method, wherein conductance was obtained by normalizing peak current at each pulse against driving force, plotted as function of voltage, fit with a single Boltzmann function and normalized against maximal value. Average data are presented as mean ± SEM, and statistical differences between data sets was assessed by Student’s t-tests, and significance accepted at the p< 0.05 level.

Biotinylation of Cell Surface Proteins for Expression Analysis--- Cells were transfected with a fixed amount of DNA (10 µg) in 10 cm dishes using LipofectAMINE 2000. 0.5 µg of a plasmid expressing a HA-tagged protein was included in each transfection to allow normalization of transfection efficiencies among various samples. 48 hours post-transfection, cells were harvested, washed with PBS, and then incubated in PBS containing 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) for 45 minutes at 4 °C to label cell surface proteins. The biotinylation reaction was quenched with 50 mM NH₄Cl for 10 min. The cells were washed with PBS and incubated in lysis buffer (0.5% NP-40, 0.5% sodium deoxycholate, 1 mM DTT, protease inhibitor cocktail in PBS). The cell lysates were then centrifuged to remove cell debris and proteins in the supernatant were quantitated. Equal amount protein lysates were aliquoted, one part for the measurement of total protein expression, and the rest were incubated with beads coated with Streptavidin (Pierce) for affinity-capture and purification of biotinylated proteins. The proteins were resolved by SDS-PAGE (4-12% gel) and transferred to PVDF membrane for immunoblot analysis. The intensities of protein bands within linear range of detection on the scanned blot were quantitated by ImageQuant image analysis software and the expression levels of protein were normalized to that of the transfection control protein.

Immunofluorescence--- Cryo-preserved human sperm samples were thawed, diluted, spotted onto chamber slides, and air-dried. The sperm were fixed and permeabilized with 4% paraformaldehyde for 10 minutes. After rinsing twice in PBS, the slides were incubated in blocking buffer (2% FBS, 2% BSA in PBS) for 30 minutes to reduce non-specific binding. The slides were then incubated with either rabbit anti-Ca,3.3 antibody (Alomone labs, 1:50 dilution) or rabbit anti-CatSper1 antibody (1:50 dilution) diluted in the blocking buffer for 2 hours. To assess specific binding, 2 µg competing peptide for anti-Ca,3.3 antibody or 25 µg purified CatSper1 C-terminus fragment for competing anti-CatSper1 antibody were preincubated with the primary antibodies in the blocking buffer for 30 minutes and then applied to the slides. After incubating with primary antibodies, the slides were washed once in PBS with 0.25% NP-40 followed with twice in PBS. The slides were then incubated with FITC-conjugated Donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in blocking buffer (1:200 dilution) for 1 hour. After washing once in PBS with 0.25% NP-40 followed by twice in PBS, the slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) for observation under a fluorescent microscope.

FRET Analysis--- Anti-Ca,3.3, anti-CatSper1 and anti-PMCA4 antibodies were labeled with Alexa Fluor 488 dye or Alexa 555 dye by Zenon IgG labeling kit (Invitrogen) according to the manufacturer’s protocol. These labeled antibodies were employed to immunostain sperm samples as described for indirect immunofluorescence, but without using the secondary antibody. Before mounting, the stained sperm on the slide were fixed again with 4% paraformaldehyde for 10 minutes. Fluorescent images were acquired using a LSM 5 PASCAL
laser scanning confocal imaging system (Carl Zeiss, Thornwood, NY). All images were taken with an oil immersion objective with appropriate filter sets (donor, excitation 488 nm, emission filter BP 505-530 nm; acceptor, excitation 543 nm, emission filter LP 560 nm; FRET, excitation 488 nm, emission filter LP 560 nm). The quantitative FRET analysis was performed using the PASCAL software (Zeiss) according to manufacturer’s sensitized emission protocol, which is based on the conventional three-filter method described by Xia and Liu (14). The FRET filter raw image contains the FRET signal, as well as the bleed-through of direct donor and acceptor emissions into the FRET channel. To determine bleed-through and background corrections, the three track images of sperms stained with don or only and acceptor only were separately obtained. The donor and acceptor images of the samples were then multiplied with the respective correction factor and subtracted from the raw FRET image, and a normalized FRET (NFRET) image was calculated by PASCAL software according to Xia and Liu (14). NFRET values of at least 10 regions on the tails of 3-5 stained sperm were measured and expressed as means ± SEM.

RESULTS

Identification of Ca\textsubscript{v}3.3 as a CatSper1 and CatSper2 Associated Protein from Human Sperm Extracts by GST Pull-down Approach---

To identify sperm proteins that associate with CatSper1, we initially tried to immunoprecipitate CatSper1 along with its binding partners from the sperm extract using CatSper1 antibody. However, this approach failed due to the poor solubility of sperm CatSper1 protein under conditions that maintain protein-protein interactions. Since CatSper proteins have relatively large N- and C-terminal segments and short loops between the transmembrane domains, we hypothesized that CatSper might associate with other proteins through their N- and C-terminal segments. Especially, the C-terminal segments of both CatSper1 and CatSper2 contain coiled-coil domains that could mediate protein-protein interactions (10). We, therefore, generated the N- and C-terminal segments of CatSper as GST fusion proteins and utilized them in pull-down experiments to identify proteins in sperm that may

GST fusion protein constructs were prepared and expressed in bacteria. The N-terminus of CatSper1 as a GST fusion protein expressed poorly and was largely insoluble, whereas good expression of the C-terminus of CatSper1 and the N- and C-termini of CatSper2 as GST fusion proteins (GST-Cat1-C, GST-Cat2-N, GST-Cat2-C, respectively) were obtained. These GST fusion proteins were affinity purified to apparent homogeneity and conjugated to glutathione sepharose 4B beads. To pull down CatSper-associated proteins, human sperm extract was incubated with beads conjugated with GST (negative control), GST-Cat1-C, or a mixture of GST-Cat2-N and GST-Cat2-C proteins, respectively. Aliquots of the pull-down products were resolved by SDS-PAGE and visualized by silver staining. GST fusion proteins used in pull-down appeared as major protein bands on the gel. Besides, less abundant protein bands appeared in all three lanes (Fig. 1). Some of these protein bands, with identical molecular weights across all lanes, were apparent non-specific contaminants during purification. Nonetheless, distinct protein bands were observed specifically in GST-CatSper1 and 2 pull-down lanes, but not in GST pull-down controls. We applied Multidimensional Protein Identification Technology (MudPIT), which incorporates on-line two-dimensional capillary chromatography coupled to tandem mass spectrometry to determine the identities of proteins in the pull-down mixtures. As expected, several peptides derived from either GST or CatSper were identified due to the presence of the GST fusion proteins in the pull-down mixtures. To identify proteins specifically associated with CatSper, we subtracted, \textit{in-silico}, proteins identified in GST alone pull-down from those obtained from GST-CatSper1 and 2 pull-downs. This subtractive analysis identified a few proteins present/unique to the GST-Cat1-C, or mixture of GST-Cat2-N and GST-Cat2-C pull-down. Some identified proteins were non-specific contaminants from semen, including apolipoprotein and semenogelin. One peptide (RTFRLLRVLKLVRFMPALRR) derived from the T-type calcium channel Ca\textsubscript{v}3.3 was consistently identified (from two separate experiments) in both in-gel and in-solution
digestion of GST-CatSper1 and GST-CatSper2 pull-down complexes, but not from pull-down product by GST alone. These findings suggested that Ca3.3 might be a likely associated protein for both CatSper1 and CatSper2.

Co-immunoprecipitation of CatSper1 and CatSper2 with Ca3.3 in Mammalian Cells— In order to confirm the association of Ca3.3 with the CatSper1 and 2 observed in GST pull-down, co-immunoprecipitation experiments were conducted to investigate their interactions in mammalian cells. CatSper1 with a Flag tag was transiently transfected into HEK cells either alone (negative control) or along with a construct expressing Ca3.3 with a V5 tag. Forty-eight hours post-transfection, the expression of transfected Ca3.3 and CatSper1 could be detected by Western blot analysis of products immunoprecipitated with the corresponding antibodies (Fig. 2A). When Ca3.3 antibody was used in the immunoprecipitation, we observed that CatSper1 co-immunoprecipitated with Ca3.3 in the case when both were co-transfected, but not when CatSper1 alone was transfected (Fig. 2A). To determine the specificity of interactions between Ca3.3 and CatSper1, we examined whether other unrelated proteins, such as Na+/K+ ATPase and 14-3-3 ε, could also co-immunoprecipitate with Ca3.3. Although these two proteins were abundantly expressed, they were not present in the products immunoprecipitated by the Ca3.3 antibody (Fig. 2A). Similar co-immunoprecipitation studies revealed that CatSper2 also associates with Ca3.3 in mammalian cells (Fig. 2B). In reciprocal experiments, when antibodies recognizing the cloned CatSper proteins were used in immunoprecipitation, we observed that Ca3.3 could be co-immunoprecipitated along with either CatSper1 or CatSper2 (Fig. 2C).

CatSper1 and CatSper2 Reduce Ca3.3-Mediated Ca2+ Currents in Transfected Mammalian Cells and Xenopus Oocytes— The physical association of Ca3.3 with CatSper1 and 2 raised the possibility of functional interactions between these proteins. We generated HEK cells stably expressing CatSper1 and CatSper2, and in consistent with earlier reports (3,4), neither CatSper1 nor CatSper2 alone elicit functional responses as measured by whole cell patch clamp studies (data not shown). We next generated a cell line stably expressing Ca3.3 and transiently transfected either a pcDNA3.1 vector (control), or CatSper1 or 2 expression constructs, along with a GFP reporter construct. Whole cell patch clamp experiments were performed on selected GFP-positive cells. By using 300 ms voltage pulses ranging from −100 to +50 mV in 5 mV increment from a holding potential of −110 mV, we observed voltage-dependent current responses in cells expressing Ca3.3 alone, or in combination with CatSper1 or CatSper2. Representative current traces are shown in Fig. 3A. These traces displayed typical features of T-type currents such as activation at low voltages and crossing over of current traces at certain potentials (15). Interestingly, co-expression CatSper1 or 2 with Ca3.3 significantly reduced current amplitudes as evidenced by the current-voltage (I-V) relationship (Fig. 3B). The maximal current densities were reduced significantly from control (Ca3.3 alone) density of −124.9 ± 10.1 pA/pF to −66.5 ± 9.8 pA/pF (46.7%) and −75.4 ± 4.4 pA/pF (39.6%) for Ca3.3/Catsper1 and Ca3.3/CatSper2 respectively. To examine effect on the activation parameters, the conductance-voltage relationship for activation was derived from current-voltage relationship curves (Fig. 3C). The V1/2 and slope factors for Ca3.3 were −44.5 ± 0.5 mV and 7.8 ± 0.5 mV (n = 9) respectively. The V1/2 values were slightly shifted by +1.3 mV and +2.9 mV for Ca3.3/CatSper1 and Ca3.3/CatSper2 combinations respectively (p< 0.1).

The voltage dependence of channel availability was determined by a two-pulse protocol (Fig. 4A). From a holding potential of −110 mV, a 3-s pulse ranging from −110 to −20 mV was applied to allow channel inactivation, and a second pulse to −30 mV was applied to assess relative channel availability. Peak currents elicited by second pulse were plotted as function of voltage of the first pulse and fit with Boltzmann function to determine the maximal peak currents. The normalized currents against maximal peak currents were plotted and fit with Boltzmann function to derive voltage dependent inactivation. Neither the V1/2 nor slope factors were significantly altered by co-expression with CatSper1 or 2 (Fig. 4B).

We also investigated the effects of co-expression of CatSper1 on the electrophysiological properties of Ca3.3 in another expression system, viz., Xenopus oocytes. Current responses were
measured following co-injection of Ca,3.3 and CatSper1 cRNA (90 ng each). Similar to effects observed in HEK cells, co-injection of CatSper1 reduced peak currents at −25 mV by 42 ± 6% (n = 22) whereas other biophysical properties of Ca,3.3 were largely unaffected (data not shown). To exclude the possibility that the expression of CatSper1 might nonspecifically affect other channels besides Ca,3.3, we co-expressed CatSper1 with HCN2, which encodes the hyperpolarization-activated cyclic nucleotide-gated channel. Over-expression of CatSper1 does not affect the expression level of HCN2 (data not shown), supporting the idea that the inhibition of Ca,3.3 currents is due to its specific interaction with CatSper1.

Effect of Co-expression of CatSper1 on the Surface and Total Expression levels of Ca,3.3 in transfected cells--- A possible explanation for the effects of CatSper expression on the amplitude of the Ca,3.3-evoked current is that CatSper proteins might affect the expression level of Ca,3.3 on the cell surface. To explore this possibility, Ca,3.3, either with or without CatSper1, along with a control plasmid (to normalize transfection efficiencies among different samples) were transfected into cells. Forty-eight hours post-transfection, surface membrane proteins of viable, intact cells were labeled with an impermeable biotin probe followed by affinity purification of the biotinylated proteins using the Streptavidin beads. The detection of plasma membrane marker Na+/K+ ATPase, but not the cytoplasmic protein 14-3-3ε, in the biotinylated protein fraction validated the use of biotinylation technique for assessing cell surface expression of proteins (Fig. 5A). The expression levels of Ca,3.3 in the biotinylated cell surface fractions and in total cell were examined by Western blot analysis (Fig. 5A) and then quantitated and normalized against the expression levels of a transfection control protein (Fig. 5B). The normalized surface and total expression of Ca,3.3 in the absence of CatSper1 were 75 ± 10 and 138 ± 12 respectively, while in the presence of CatSper1, the normalized surface and total expression were reduced to 61 ± 12 and 119 ± 15 respectively (n = 3; Fig. 5B). Thus, a modest reduction in the levels of surface or total expression of Ca,3.3 was observed when co-expressed with CatSper1 (although this did not achieve statistical significance p<0.05).

Co-expression of CatSper1 and Ca,3.3 on the Principal Piece of Human Sperm Tail--- To investigate whether CatSper1 and Ca,3.3 proteins are co-expressed in human sperm, indirect immunofluorescence technique was used. Unlike the observations by Trevino et al. (16), we could not conclusively determine Ca,3.3 expression using the same anti-Ca,3.3 antibody (purchased from Santa Cruz) since immunostaining was only partially blocked by the corresponding antigen. We instead relied on an anti-Ca,3.3 antibody from a different source (Alomone Labs) in our immunostaining experiments (17). Western blot analysis revealed that this antibody not only recognize cloned Ca,3.3 expressed in HEK cells, but also detected Ca,3.3 expressed in human sperm and cerebral cortex (Fig. 6A). When this anti-Ca,3.3 antibody was used in immunostaining experiments, robust staining on the principal piece and a slightly weaker staining on the middle piece of the sperm tail was observed (Fig. 6, B2). The staining was totally blocked by pre-incubation of the primary antibody with the corresponding antigen peptide (Fig. 6, B4). We next examined the expression of CatSper1 in human sperm. We observed robust fluorescence signals on the principal piece of sperm tail with the antibody raised against the C-terminus of human CatSper1 (Fig. 6, C2). This staining was blocked by pre-incubation of the primary antibody with the recombinant CatSper1 C-terminus fragment used to raise the antibody (Fig. 6, C4), indicating specific staining of CatSper1 expression on the principal piece. This is consistent with the observation of CatSper1 localization on mouse sperm (3). The staining of both Ca,3.3 and CatSper1 on human sperm tail demonstrates colocalization of these two proteins in this region.

FRET Analysis of CatSper1 and Ca,3.3 Interaction on Human Sperm Tail--- We employed the Fluorescence Resonance Energy Transfer (FRET) microscopy to assess whether CatSper1 physically associates with Ca,3.3 in sperm. FRET consists in radiationless energy transfer between one fluorophore (the donor) in the excited state and another fluorophore (the acceptor) when in close proximity. Simple colocalization of two proteins is not sufficient to yield energy transfer, which requires the proximity of the two fluorophores at distances that are to the order of a few nanometers. Thus, the presence of FRET
between two proteins is an indicator of close proximity and protein-protein interaction (18,19).

We labeled anti-Ca_v3.3 antibody with Alexa Fluor 488 dye as the donor (Ca_v3.3-488) and anti-CatSper1 antibody with Alexa 555 dye as the acceptor (CatSper1-555). These two labeled antibodies were mixed to immunostain human sperm and FRET analysis was performed using the sensitized emission method (14). A set of representative donor, acceptor and raw FRET image are shown in Figure 7. A normalized FRET (NFRET) image, which shows FRET intensities with high spatial resolution, was calculated from the raw FRET image after correction for the effects of bleed-through of direct donor and acceptor emissions into the FRET channel. We observed robust NFRET signals along the sperm tail but not in the head (Fig. 7), indicating a region-specific close molecular association between CatSper1 and Ca_v3.3. Quantitative measurement of 10 regions on the tails of different sperm samples revealed a mean NFRET value of 0.394 ± 0.017. As a positive control, we labeled anti-CatSper1 antibody with either Alexa 488 dye as the donor or Alexa 555 dye as the acceptor and mixed them to immunostain human sperm (Fig. 7). Both labeled antibodies should be in close proximity by recognizing the same protein and indeed as expected, we detected robust NFRET signal (mean value of 0.416 ± 0.012), which is comparable to that from the experimental group.

To assess the specificity of the FRET analysis, we also immunostained sperm with anti-PMCA4 antibody labeled with Alexa 488 dye and anti-Ca_v3.3 antibody labeled with Alexa 555 dye (Fig. 7). Although PMCA4, a Ca^{2+}-ATPase, is expressed in the sperm tail just like the CatSper1 and Ca_v3.3 (20), Only marginal NFRET signals (mean value of 0.075 ± 0.009) were observed in the stained sperm, indicating lack of interaction between Ca_v3.3 and PMCA4. Collectively, these results indicate that Ca_v3.3 specifically associates with CatSper1 on the sperm tail.

**DISCUSSION**

This study reports the identification and analysis of interactions of the voltage-gated T-type calcium channel Ca_v3.3 with putative sperm ion channel proteins CatSper1 and CatSper2. Initial evidence for such protein-protein interactions stems from MudPIT analysis of proteins from human sperm extracts that associate with CatSper1 C-terminus or a mixture of CatSper2 N- and C-terminus as GST-fusion proteins. In both cases, a peptide derived from Ca_v3.3 was identified. Subsequent co-immunoprecipitation experiments confirmed the physical interactions between Ca_v3.3 and CatSper1 or CatSper2 in mammalian cells. Furthermore, our FRET analysis demonstrates that Ca_v3.3 and CatSper1 interactions do occur physiologically and suggests that these two proteins associate with each other on the tail of human sperm.

To our knowledge, Ca_v3.3 is the first protein identified that associates with CatSper1 and 2. The presence of coiled-coil domains on the C-terminus of CatSper1 and CatSper2 suggests that the C-terminal segments of CatSper proteins likely mediate Ca_v3.3-CatSper interactions. However, the potential promiscuity of coiled coil interactions with other proteins should be acknowledged, as it is not known whether other proteins with coiled-coil segments associate with Ca_v3.3 as well. The association of both CatSper1 and CatSper2 with Ca_v3.3 initially raised the possibility that these three proteins might form a triple complex. However, when we co-expressed all three proteins in mammalian cells, we failed to detect CatSper2 in the product immunoprecipitated by CatSper1 antibody or vice versa (unpublished observations). This implies that CatSper1 and CatSper2 may bind competitively to Ca_v3.3, perhaps through a region common in Ca_v3.3.

The physical association of Ca_v3.3 with CatSper1 and 2 suggested functional interactions between these proteins. However, we attempted, but failed, to elicit any currents other than the T-type calcium current from cells expressing both Ca_v3.3 and CatSper1 or 2, suggesting that co-expression of Ca_v3.3 still cannot facilitate the functional expression of CatSper in heterologous systems. It is likely, however, that Ca_v3.3 may not be the sole accessory protein that interacts with CatSper and that we could have missed the identification of other binding partners for CatSper1 or CatSper2 in our GST pull-down experiments. Other proteins in the sperm membrane might associate with CatSper proteins so tightly as a protein complex that precludes its capture by GST-CatSper in the pull-down
procedure. We also noticed multiple distinct protein bands specifically in GST-CatSper pull-downs; however, we failed to resolve their identities. This might be due to dynamic range limitation of the MudPIT analysis, considering that the peptides derived from the GST fusion proteins are overwhelmingly abundant and may affect the sampling efficiency of the peptides from other relatively low abundant proteins.

Although co-expression of Ca,3.3 with CatSper did not reconstitute a novel or distinct ion channel complex, we observed net reduction in Ca,3.3-evoked T-type Ca\(^{2+}\) currents upon co-expression with either CatSper1 or CatSper2 in heterologous expression systems. The whole cell current amplitudes of Ca,3.3 showed significant reductions of 47% and 40% when co-expressed with CatSper1 and CatSper2, respectively. Interestingly, other biophysical properties of Ca,3.3 were largely unaltered except for a modest (1-3 mV) shift in activation V_{1/2} of Ca,3.3 towards the depolarizing direction. The effects of CatSper1 on Ca,3.3 were replicated using the Xenopus oocyte expression system and are specific because over-expression of CatSper1 does not affect the expression of another channel, viz., HCN2. These studies demonstrate that the association of CatSper proteins with Ca,3.3 predominantly affects the current amplitude of Ca,3.3 without altering other channel properties.

Recombinant T-type Ca,3 channel subunits generally do not require accessory proteins for functional expression in a variety of heterologous expression systems, which is in contrast to L-type calcium channels that are profoundly modulated by \(\beta\) and \(\alpha_2\delta\) subunits and function as multimeric complexes. It has, however, been reported that \(\beta_{1b}\) and \(\alpha_2-\delta_1\) subunits, typically associated with L-type calcium channels, can also modulate T-type calcium channels including Ca,3.3 (21). Unlike CatSper, these auxiliary subunits enhance the current amplitudes of Ca,3 channels by increasing their cell surface expression. However, physical interactions between T-type Ca\(^{2+}\) channel subunits and \(\beta_{1b}\) or \(\alpha_2-\delta_1\) have not been established. In another report (22), it was shown that co-expression of calcium channel \(\gamma_5\) subunit, but not the \(\gamma_4\) or \(\gamma_7\) subunits, with Ca,3.1 in HEK cells significantly decreases current density without changing the kinetic properties and the protein expression of Ca,3.1. To date, CatSper1 and CatSper2 remain as the first identified proteins shown to physically associate with and functionally modulate Ca,3.3 channels. It would be interesting to determine whether CatSper proteins also modulate other T-type Ca\(^{2+}\) channel types such as Ca,3.1 and Ca,3.2 that are also expressed in sperm.

How does the association of CatSper1 modulate Ca,3.3 current? It is known that the macroscopic current is proportional to the product of the single channel conductance, the number of channels, the open probability and the effective driving force. Since we did not see significant changes in the kinetic properties of Ca,3.3, our data do not support the possibility that the decrease in current density is due to changes in Ca,3.3 biophysical properties, although this cannot be entirely eliminated without detailed biophysical analysis at a single channel level. In a number of cases, it has been reported that auxiliary proteins may modulate the current density by altering the amount of channel protein on the cell surface (21,23). To assess whether the reduced amplitude could be attributed to reductions in the number of Ca,3.3 channels, surface membrane proteins of viable, intact cells were labeled with biotin and quantified. We observed a tendency that co-expression of CatSper1 reduced the surface expression of Ca,3.3 by about 20%, suggesting that reduction in expression of Ca,3.3 might contribute to the decreased current density.

The low-voltage-activated T-type Ca\(^{2+}\) channels produce low-threshold spikes that have been shown to trigger burst firing in various cell types. They play important physiological roles in diverse tissues, especially in central and peripheral nervous systems and in the heart (for a review see (24)). In mammalian germ cells and sperm, the expression of all three members of the T-type Ca\(^{2+}\) channels have been reported (16,25) and electrophysiological studies have documented the existence of T-type Ca\(^{2+}\) current in mouse spermatogenic cells (26). The most prominent role attributed for T-type Ca\(^{2+}\) channels is in the acrosome reaction. Blockade of T-type Ca\(^{2+}\) channels during gamete interaction inhibited zona pellucida-dependent Ca\(^{2+}\) elevation and acrosome reaction (26). However, although T-type Ca\(^{2+}\) channels are present in the sperm head (16), the
expression of neither CatSper1 nor CatSper2 was localized in the acrosome region (3,4). Therefore, it is unlikely that Ca\textsubscript{v}3.3-CatSper interactions could play a role in sperm acrosome reaction. Instead, the co-expression and association of Ca\textsubscript{v}3.3 and CatSper1 on human sperm tail suggests a role for their interactions in sperm motility.

Ca\textsuperscript{2+} is a key regulator in the initiation and maintenance of hyperactivated motility of the sperm (27). During this process, Ca\textsuperscript{2+} concentration in the cytoplasm rises which regulates the movement of axoneme (28). The release of Ca\textsuperscript{2+} from membrane-bound internal Ca\textsuperscript{2+} stores, such as the redundant nuclear envelope, is critical for hyperactivated motility (29). Besides, the increase of cytoplasmic Ca\textsuperscript{2+} can also result from the influx of extracellular Ca\textsuperscript{2+} through the plasma membrane; however, the type of Ca\textsuperscript{2+} channels mediating this process remains elusive. Knockout studies revealed that mice lacking either CatSper1 or CatSper2 have defects in sperm hyperactivated motility which is accompanied by a reduction in Ca\textsuperscript{2+} concentration in sperm tail (3,6), indicating a crucial role for these CatSper proteins in Ca\textsuperscript{2+} influx during hyperactivated motility. The detection of Ca\textsubscript{v}3.3 expression on sperm flagellum, and more importantly, its association with CatSper, imply that Ca\textsubscript{v}3.3 may also play a role in mediating calcium influx required for hyperactivated motility. However, studies with Ca\textsubscript{v}3.3 knockout or with selective T-type Ca\textsuperscript{2+} channel blockers are currently unavailable to directly assess the role of Ca\textsubscript{v}3.3 in sperm function. It has been reported that weak T-type Ca\textsuperscript{2+} channel inhibitors, mibebradil and gossypol, did not significantly affect sperm basal motility at low concentrations, but did cause motility alterations at higher concentrations at which high-voltage activated Ca\textsuperscript{2+} channels may also be blocked (16). Accordingly, the assessment of Ca\textsubscript{v}3.3 role in sperm function awaits the identification of potent and selective Ca\textsubscript{v}3.3 blockers or knockout studies.

Should Ca\textsubscript{v}3.3 indeed contribute to Ca\textsuperscript{2+} influx during sperm hyperactivated motility, CatSper1 could modulate Ca\textsubscript{v}3.3 function by physical interactions in a region-specific manner. Interestingly, our studies demonstrate a decrease, rather than increase, in the amplitude of Ca\textsubscript{v}3.3-mediated Ca\textsuperscript{2+} currents upon co-expression of CatSper1 or CatSper2 in heterologous systems. Although it remains to be proven whether this holds true in native sperm environment, this observation suggests that CatSper1 and 2 may “fine tune” Ca\textsubscript{v}3.3-mediated current to maintain an optimal level of Ca\textsuperscript{2+} in the principal piece during hyperactivated motility. Indeed, recent studies reveal the presence of both major Ca\textsuperscript{2+} influx and efflux mechanisms in the principal piece. PMCA4, which is a plasma membrane Ca\textsuperscript{2+}-ATPase that acts as an extrusion pump to mediate the efflux of excess Ca\textsuperscript{2+} from the cytosol, is highly expressed in the principal piece just like CatSper1 and Ca\textsubscript{v}3.3. Targeted ablation of PMCA4 in mice resulted in defects in sperm hyperactivated motility and male fertility (20,30). Abnormal mitochondria was observed in PMCA4\textsuperscript{-/-} sperm, which was attributed to Ca\textsuperscript{2+} overload. Therefore, perhaps a controlled regulation of Ca\textsuperscript{2+} levels, rather than mere increase, could be important for regulating hyperactivated motility of the sperm, and it may be speculated that Ca\textsubscript{v}3.3 and CatSper proteins partner together to regulate Ca\textsuperscript{2+} influx needed for hyperactivated motility.

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FOOTNOTES

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1 The abbreviations used are: MudPIT: Multidimensional Protein Identification Technology; HEK: human embryonic kidney; CHO-K1: Chinese hamster ovary K1; GST: glutathione s-transferase; PCR:
polymerase chain reaction; RT: reverse transcription; IPTG: Isopropyl-β-D-thiogalactopyranosid; GFP: green fluorescent protein; TEA: tetraethyl ammonium; PBS: phosphate buffered saline; SDS-PAGE: SDS polyacrylamide gel electrophoresis; PVDF: Polyvinylidene fluoride; FBS: fetal bovine serum; BSA: bovine serum albumin; FRET: Fluorescence Resonance Energy Transfer; NFRET: normalized FRET.

**FIGURE LEGENDS**

**Fig. 1.** Identification of CatSper-associated proteins from sperm extracts by GST pull-down. Human sperm extracts were incubated with beads conjugated with GST (negative control), GST-Cat1-C, or a mixture of GST-Cat2-N and GST-Cat2-C proteins, respectively, to isolate CatSper-associated proteins. Shown is the silver staining gel of aliquots of the pull-down products resolved by SDS-PAGE. GST fusion proteins used in pull-down (arrows) appeared as major protein bands on the gel, along with other less abundant protein bands in all three lanes.

**Fig. 2.** CatSper proteins co-immunoprecipitate with Ca,3.3 in transfected HEK cells. A, CatSper1-flag construct was transiently transfected in HEK cells either alone (negative control) or along with a construct expressing Ca,3.3-V5. Western blot (WB) of products immunoprecipitated (IP) by the anti-Ca,3.3 antibody (Santa Cruz) recognized Ca,3.3 in transfected cells. Western blot using anti-CatSper1 antibody revealed comparable CatSper1 expression in both transfections by analyzing the proteins immunoprecipitated by the anti-flag antibody. CatSper1 was detected in the proteins immunoprecipitated by anti-Ca,3.3 antibody only when Ca,3.3 and CatSper1 co-expressed in the cells. Western blot analysis using anti-Na+/K+ ATPase antibody or anti-14-3-3ε antibody revealed abundant expression of these two proteins in the cell lysates; however, these were not detected in the products immunoprecipitated by Ca,3.3. B, Similar co-immunoprecipitation experiments demonstrating the association of CatSper2 with Ca,3.3 in HEK cells. C, In reciprocal experiments, Ca,3.3 were co-immunoprecipitated along with either CatSper1 or CatSper2, utilizing anti-flag or anti-CatSper2 antibody respectively in immunoprecipitation experiments.

**Fig. 3.** Co-expression of CatSper1 or 2 reduces Ca,3.3-evoked current amplitudes. A, Voltage protocol and representative current traces from cells expressing Ca,3.3 alone, Ca,3.3/CatSper1 and Ca,3.3/CatSper2. Currents were elicited by voltage steps ranging from −100 mV to +50 mV in 5 mV increment from a holding potential of −110 mV. B, current-voltage (I-V) relationships were obtained by measuring the peak currents during the 300 ms pulses and plotted as function of voltage (n = 9, 6 and 6 for Ca,3.3, Ca,3.3/CatSper1 and Ca,3.3/CatSper2 respectively). Currents peaked at −25 mV. C, The conductance-voltage relationship for activation was deduced by the chord conductance method, wherein conductance was obtained by normalizing peak current at each pulse against driving force, plotted as function of voltage, fit with a single Boltzmann function and normalized against maximal value. The mean values were plotted and fitted with Boltzmann function to obtain V1/2 and slope factor k. Ca,3.3 (n= 9): V1/2 = −44.5 ± 0.5 mV, k= 7.8 ± 0.5 mV; Ca,3.3/CatSper1 (n= 6): V1/2 = −43.2 ± 0.3 mV, k= 8.4 ± 0.4 mV; Ca,3.3/CatSper2 (n= 6): V1/2 = −41.6 ± 0.9 mV, k= 7.7 ± 0.2 mV.

**Fig. 4.** Co-expression of CatSper1 or 2 does not alter the inactivation properties of Ca,3.3. A, Representative Ca,3.3 currents evoked by two-pulse protocol used to estimate voltage dependence of inactivation. The upper panel shows voltage protocol and the lower panel shows currents recorded during the interval highlighted by arrows in upper panel. B, Relative peak currents for Ca,3.3, Ca,3.3/CatSper1 and Ca,3.3/CatSper2 during the second 300 ms pulse at −30 mV were fitted with Boltzmann function to determine half-maximal (V1/2) and slope factor (k) values for each channel type. Ca,3.3 (n= 6): V1/2 = −75.9 ± 0.2 mV, k= 5.2 ± 0.3 mV; Ca,3.3/CatSper1 (n= 4): V1/2 = −75.2 ± 0.2 mV, k= 5.4 ± 0.2 mV; Ca,3.3/CatSper2 (n= 4): V1/2 = −75.7 ± 0.3 mV, k= 5.4 ± 0.2 mV.
Fig. 5. Effects of co-expression of CatSper1 proteins on surface and total expression levels of Ca,3.3. HEK cells were transfected with Ca,3.3 either with or without CatSper1 as indicated, together with a control plasmid to normalize for differences in transfection efficiencies between samples. A, Representative Western blots showing the expression of Ca,3.3, CatSper1, Na+/K+ ATPase, and 14-3-3ε in the biotinylated surface protein fractions (surface) and the total cell lysates (total). B, Normalized relative surface and total expression of Ca,3.3 is plotted as means ± SEM of three separate transfection experiments.

Fig. 6. Immunolocalization of Ca,3.3 and CatSper1 on human sperm tail. A, Anti-Ca,3.3 antibody (Alomone Labs) recognized Ca,3.3 expressed in human sperm and cerebral cortex, as well as cloned Ca,3.3 expressed in HEK cells by Western blot analysis. B, Shown are immunofluorescence images of mature human sperms stained with anti-Ca,3.3 antibody preincubated without (2) or with (4) competing antigenic epitope. The corresponding phase contrast images (1, 3) were shown on the left. C, Shown are immunofluorescence images of mature human sperms stained with anti-CatSper1 antibody preincubated without (2) or with (4) competing antigen. The corresponding phase contrast images (1, 3) were shown on the left.

Fig. 7. FRET analysis of the association of CatSper1 with Ca,3.3 on human sperm tail. Human sperm were immunostained either with Alexa 488-labeled anti-CatSper1 antibody (CatSper1-488) and Alexa 555-labeled anti-CatSper1 antibody (CatSper1-555) as a positive control, or with Alexa 488-labeled anti-Ca,3.3 antibody (Ca,3.3-488) and Alexa 555-labeled anti-CatSper1 antibody (CatSper1-555), or with Alexa 488-labeled anti-PMCA4 antibody (PMCA4-488) and Alexa 555-labeled anti-Ca,3.3 antibody (Ca,3.3-555) as a negative control. FRET analysis was performed and donor (Alexa 488), acceptor (Alexa 555) and raw FRET images were taken using corresponding excitation and filter settings. A set of representative images along with the calculated NFRET image are shown. Color scales for NFRET intensity are displayed. Red and blue indicate high and low intensity, respectively.
Fig. 1

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- GST-Cat2-N
- GST-Cat2-C
- GST-Cat1-N
- GST-Cat2-N
- GST-Cat2-C
- GST-Cat1-N
- GST-Cat2-N
- GST-Cat2-C
- GST-Cat1-N
- GST-Cat2-N
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- GST-Cat2-C
- GST-Cat1-N
- GST-Cat2-N
- GST-Cat2-C
- GST-Cat1-N
- GST-Cat2-N
- GST-Cat2-C
- GST-Cat1-N
- GST-Cat2-N
- GST-Cat2-C
- GST-Cat1-N
- GST-Cat2-N
- GST-Cat2-C
- GST-Cat1-N
Fig. 2

A.

| CatSper1-flag | + | + |
|---------------|---|---|
| Lysate        | IP: α-CatSper1 | WB: α-Ca,3.3 |
|               | IP: α-flag     | WB: α-Ca,3.3 |
|               | IP: α-Ca,3.3   |               |

B.

| CatSper2-flag | + | + |
|---------------|---|---|
| Lysate        | IP: α-Ca,3.3 | WB: α-Ca,3.3 |
|               | IP: α-Ca,3.3 | WB: α-Ca,3.3 |
|               | IP: α-Flag    |               |

C.

| CatSper1-flag | + | + |
|---------------|---|---|
| Lysate        | IP: α-Ca,3.3 | WB: α-Ca,3.3 |
|               | IP: α-Flag    |               |
|               | IP: α-14-3-3 e |

| CatSper2-flag | + | + |
|---------------|---|---|
| Lysate        | IP: α-Ca,3.3 | WB: α-Ca,3.3 |
|               | IP: α-Ca,3.3 | WB: α-Ca,3.3 |
|               | IP: α-Ca,3.3 | WB: α-Ca,3.3 |
|               | IP: α-Flag    |               |
|               | IP: α-Ca,3.3 |               |
|               | IP: α-Ca,3.3 |               |

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Fig. 3

A. 

CaV3.3

CaV3.3/CatSper1

CaV3.3/CatSper2

Voltage (mV)

-110 mV -100 mV, 300 ms

50 mV

1 nA

B. 

Current densities (pA/pF) Normalized currents

Voltage (mV)

16
Fig. 4

A. [Graph showing voltage (mV) and current (0.5 nA) relationships with time (20 mV, 3 s, -30 mV, 0.3 s)]

B. [Graph showing normalized currents vs. voltage (mV) for CaV3.3, CaV3.3/CatSper1, and CaV3.3/CatSper2]
Fig. 5

A.

|               | surface | total |
|---------------|---------|-------|
| CatSper1-flag | +       | +     |
| Ca,3.3-V5     | + +     | + +   |
| Ca,3.3        |         |       |
| CatSper1      |         |       |
| Na+/K+ ATPase |         |       |
| 14-3-3 ε      |         |       |

B.

![Bar graphs showing normalized expression of Cav3.3 and Ca,3.3 with and without CatSper1-flag.](image)
Fig. 6

A.  

|   |    | HEK: Cav3.3 |
|---|---|-------------|
| sperm | cortex | kDa |
|       |       | 200 |

B.  

1.  

2.  

3.  

4.  

Ca\textsubscript{v}3.3

C.  

1.  

2.  

3.  

4.  

CatSper1
Fig. 7

|                | Donor | Acceptor | FRET | NFRET |
|----------------|-------|----------|------|-------|
| CatSper1-488   | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| CatSper1-555   | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| Ca,3.3-488     | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| CatSper1-555   | ![Image](image13) | ![Image](image14) | ![Image](image15) | ![Image](image16) |
| PMCA4-488      | ![Image](image17) | ![Image](image18) | ![Image](image19) | ![Image](image20) |
| Ca,3.3-555     | ![Image](image21) | ![Image](image22) | ![Image](image23) | ![Image](image24) |
Association of CatSper1 or 2 with Cav3.3 leads to suppression of T-type calcium channel activity
Di Zhang, Jun Chen, Anita Saraf, Steven Cassar, Ping Han, John C. Rogers, Jorge D. Brioni, James P. Sullivan and Murali Gopalakrishnan

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