Cysteine-rich secretory protein 4 is an inhibitor of transient receptor potential M8 with a role in establishing sperm function

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The cysteine-rich secretory proteins (CRISPs) are a group of four proteins in the mouse that are expressed abundantly in the male reproductive tract, and to a lesser extent in other tissues. Analysis of reptile CRISPs and mouse CRISP2 has shown that CRISPs can regulate cellular homeostasis via ion channels. With the exception of the ability of CRISP2 to regulate ryanodine receptors, the in vivo targets of mammalian CRISPs function are unknown. In this study, we have characterized the ion channel regulatory activity of epididymal CRISP4 using electrophysiology, cell assays, and mouse models. Through patch-clamping of testicular sperm, the CRISP4 CRISP domain was shown to inhibit the transient receptor potential (TRP) ion channel TRPM8. These data were confirmed using a stably transfected CHO cell line. TRPM8 is a major cold receptor in the body, but is found in other tissues, including the testis and on the tail and head of mouse and human sperm. Functional assays using sperm from wild-type mice showed that TRPM8 activation significantly reduced the number of sperm undergoing the progesterone-induced acrosome reaction following capacitation, and that this response was reversed by the coaddition of CRISP4. In accordance, sperm from *Crisp4* null mice had a compromised ability to undergo to the progesterone-induced acrosome reaction. Collectively, these data identify CRISP4 as an endogenous regulator of TRPM8 with a role in normal sperm function.

cysteine-rich secretory proteins | antigen 5 | pathogenesis-related 1 | epididymal maturation | fertility

Male infertility affects about 1 in 20 young males in Western societies (1). Conversely, there is a pressing need for additional contraceptive options in the majority of societies and for several species. In this regard, a greater understanding of the processes of male fertility offers potential. Male fertility is dependent upon successful spermatogenesis within the testis and two phases of posttesticular sperm maturation, known as epididymal maturation and capacitation (2). Epididymal maturation involves, among other processes, protein absorption onto the sperm membrane, and the establishment of ionic gradients (3, 4). Capacitation normally occurs in the female reproductive tract and broadly involves the shedding of proteins, membrane reorganization, changes in ion permeability, and the activation of signal transduction pathways. Collectively, these events lead to sperm hyperactivated motility, and the ability to bind to and fuse with the oocyte complex and undergo the acrosome reaction (AR) (5, 6). Establishing a highly condensed sperm genome during spermatogenesis is believed to prevent sperm from transcribing mRNAs or translating proteins. Thus, pathways required for posttesticular maturation are controlled largely through post-translational protein modifications, the addition of epididymal-derived products, and the shedding of testicular proteins.

With the goal of increased contraceptive and infertility treatment options in mind, those proteins with an expression bias to the male reproductive tract are of particular interest. One such protein is the epididymal protein cysteine-rich secretory protein (CRISP) 4 (7, 8).

CRISPs are a subgroup of the CRISP, antigen 5, pathogenesis-related 1 (CAP) superfamily, which is characterized by the presence of an N-terminal CAP domain (9). CRISPs are vertebrate-specific, contain a C-terminal CRISP domain (10, 11), and are found primarily in the mammalian male reproductive tract and in reptile venom (9, 12). Reptile CRISPs can regulate a range of ion channels including voltage-gated K^+ (K_V) and Ca^{2+} (Ca_V) channels, cyclic-nucleotide gated A channels and ryanodine receptors (12, 13). Consistent with this, mouse CRISP2 is capable of altering ryanodine receptor function in vitro (10).

Within the mouse there are four CRISPs, and all are incorporated into or onto sperm (9, 14). CRISP2 is incorporated into the sperm acrosome and tail (15–17). CRISP1 and CRISP4 are secreted from epididymal principal cells and surround sperm in high concentrations (7, 8, 18). CRISP3 is produced by the seminal vesicles and prostate and found in seminal fluid (19). CRISP3 has also been implicated in the progression of human prostate cancer (20, 21). Importantly, phylogenetic analyses have shown that mouse CRISP4 is the likely ortholog of human CRISP1 (7, 9). As opposed to the situation in the mouse, humans contain three *CRISPs*, of which CRISP1 is the only epididymial CRISP (8, 19).

To further define the function of CRISPs, and in particular mouse CRISP4, we have used patch-clamping of testicular sperm, lipid bilayer experiments, and a heterologous cell-expression system to identify the transient receptor potential (TRP) M8 channel as a target channel for CRISP4. We have shown that the selective activation of sperm TRPM8 alters acrosome function and that this change can be reversed by the coaddition of the CRISP4 CRISP domain. Furthermore, using a knockout mouse model, we have shown that an absence of CRISP4 suppressed the ability of sperm to undergo the AR in a manner consistent with that seen in in vitro assays.

Results

CRISP4 CRISP Domain Can Inhibit Cationic Currents in Testicular Spermatozoa. To assess the potential for CRISP4 CRISP domain to regulate ion currents in sperm, testicular sperm were isolated and patch-clamped via the cytoplasmic droplet. The addition of

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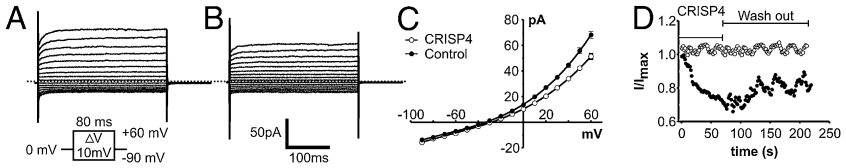

Fig. 1. CRISP4 CRISP domain inhibited whole-sperm cationic currents (I). (*A*) A representative macroscopic control current recorded using physiological buffer conditions and substituting most Cl^- by an impermeable anion, on a testicular sperm patch-clamped at the cytoplasmic droplet using the pulse protocol shown (10 mV per step). (*B*) Current inhibition by addition of 10 μM CRISP4 applied with a picospritzer. (*C*) Current-voltage (I-V) relationship of currents shown in *A* and *B*. Current inhibition at +60 mV was 25% ($n = 4$). The negative reversal potential (−35 mV) showed that cation-selective channels contributed predominantly to the current. (*D*) Perfusion of the recording chamber showed the reversible nature of CRISP4 function. One micromolar CRISP4 CRISP domain inhibition was reverted to 85% of control after 100 s of perfusion (●). Cysteine-alkylated and heat-inactivated CRISP4 CRISP domain was used as a negative control and showed no effect on cell currents (○).

10 μM CRISP4 CRISP domain to testicular sperm attenuated the rapidly activating cation current revealing the presence of CRISP4-sensitive ion channels (Fig. 1 *A* and *B*). Specifically, between −20 and +60 mV, 10 μM CRISP4 reduced the conductance; inhibition at 60 mV was 25% (Fig. 1*C*). Current recovery of 85% was observed 100 s after removal of CRISP4, demonstrating the reversible nature of its action (Fig. 1*D*). The slow return to control current levels indicated a moderate-to-high affinity with a receptor. These data were consistent with previous results for CRISP2 inhibition of ryanodine receptor-2 (10). No inhibition was evident in control experiments performed with 1 μM reduced, alkylated, and heat-inactivated CRISP4 CRISP domain, indicating that inhibition required natively folded CRISP4.

Increased $[Ca^{2+}]_i$ is an essential signaling mechanism for many aspects of posttesticular sperm biology, including the phosphorylation of proteins associated with capacitation (22), the manifestation of hyperactivated motility (23), and the ability of sperm to undergo the AR when they reach the oocyte complex (24). These data raise the potential for CRISP4, via the regulation of ion channels, to impact upon several aspects of sperm function. In the present study, we chose to focus on the TRP channel subset of Ca^{2+} conducting channels in mammalian sperm (25–27).

As outlined in *Materials and Methods*, Cs^+ was used as the main charge carrier as it can permeate efficiently through TRPs, but blocks most K^+ and other ion channel classes, including ENacs (28), thus allowing a more transparent analysis of TRP channel activity. Under these ionic conditions we recorded whole-cell currents from patch-clamped testicular sperm following addition of CRISP4 CRISP domain at a range of concentrations. This ionic substitution explains the changes in the kinetic profile of the currents and the increase in the percentage of inhibition by CRISP4 CRISP domain (Fig. 2 *A* and *B*). The normalized current-voltage (I-V) relationship showed a concentration-dependent inhibition of total current (1, 3, and 10 μM) by the applied CRISP4 CRISP domain. The reversal potential of these curves (∼ −10 mV) was consistent with Cs^+ being the major ion carrying the currents (Fig. 2*C*). Between −100 and +130 mV, current inhibition by CRISP4 did not appear voltage-dependent. Ten micromolars of CRISP4 achieved 61 ± 2% inhibition of maximal current. The percentage of blocked channels, normalized to the maximal inhibition by 10 μM CRISP4 CRISP domain, displayed a sigmoidal relationship, which was fitted to the Hill equation and gave a calculated IC_{50} of 1.96 μM (Fig. 2*D*) and a Hill slope of 0.31.

CRISP4 Antagonized TRPM8 Channels in Sperm. Several TRP channels have been localized to mouse or human sperm, including TRPC1-6 and TRPM8 (25, 29, 30). Given our recent data demonstrating the localization and functional analysis of TRMP8 on mouse sperm (27) and the considerable similarity of the current data to earlier investigations (26), we chose to analyze the affect of CRISP4 on TRPM8 in the first instance.

To explore the effect of CRISP4 on TRPMP8 function, patch-clamped sperm were stimulated with the TRPM8 ion channel agonist menthol (300 μM), and the effect of CRISP4 CRISP domain assessed. As shown previously, menthol addition resulted in the activation of current relative to the control (27), demonstrating TRPM8 stimulation (Fig. 3 *A*, *B*, and *D*). The addition of 10 μM CRISP4 CRISP domain to menthol-activated (TRPM8) sperm strongly inhibited current to levels below the control (Fig. 3 *C*–*E*). Furthermore, the perfusion of unstimulated sperm with the TRPM8 antagonist BCTC [*N*-(4-t-butylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide] (3 μM) inhibited current by ∼20% and the addition of CRISP4 did not further inhibit ion flow (Fig. 3*F*). Collectively, these data are consistent with the presence of the TRPM8 ion channel on sperm and strongly support that the CRISP4 CRISP domain can inhibit ion flow through TRPM8. Control experiments over 300 s with low CRISP4 CRISP domain concentrations (10 nM) showed no effect on current, demonstrating that the effect was attributed to individual treatments and not because of the effect of the patch pipette (Fig. S1).

As an additional preliminary assessment of the characteristics of the individual CRISP4-regulated ion channels, membrane vesicles were prepared from A23187 or progesterone acrosome-reacted sperm and incorporated into a lipid bilayer. Purified sperm-derived lipid vesicles were fused into a painted lipid bilayer and single-channel activity in the absence or presence of the CRISP4 CRISP domain was measured. The heterogeneous nature of proteins within the purified sperm membrane vesicles dictated that channel incorporation could not be controlled. Reproducible data ($n = 3$ for hybrid vesicles generated with A23187and $n = 2$ with progesterone) were obtained for two distinct channels. Recording of control currents showed incorporation of a cationic channel, with a reversal potential of −36 mV and a conductance of 77 pS, and an anionic channel with a reversal potential of 23 mV and a conductance of 108 pS (Fig. 4*A*). Amplitude distributions at 80 mV indicated two current peaks with normal distributions of current centered at 5.1 and 11.2 pA, which likely correspond to each of the two channels identified. The cationic single-channel conductance is consistent

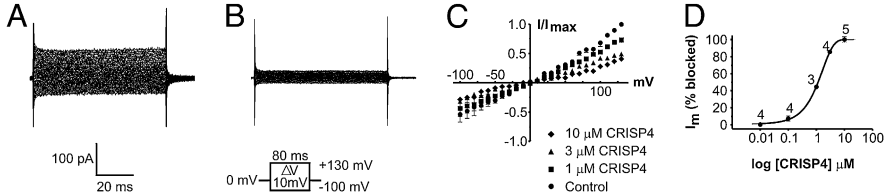

Fig. 2. CRISP4 CRISP domain inhibited whole-sperm cationic currents (I). (*A*) Macroscopic cationic current from voltage-clamp recordings of a control sperm cell. (*B*) Current inhibition after addition of 10 μM CRISP4 CRISP domain applied onto the cell with a picospritzer. Currents were elicited by the voltage-step protocol shown (10 mV per step). (*C*) The normalized current-voltage relationship showed the concentration-dependent inhibition of the whole-cell current by 10 μM, 3 μM, and 1 μM CRISP4 CRISP domain. (*D*) An EC_{50} of 1.96 μM was calculated from the sigmoidal dose-response curve for the CRISP4 CRISP domain inhibition of macroscopic I_m. Solutions are as in Fig. 1 but substituting Na^+ and K^+ by Cs^+. The number of replicates at each concentration is shown above each datapoint.

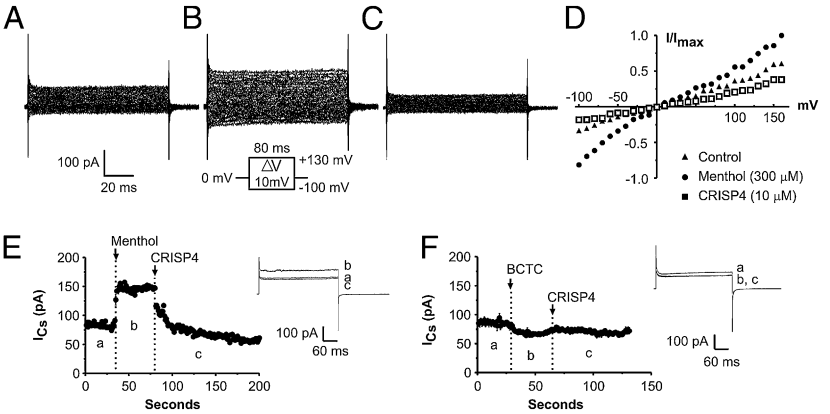

Fig. 3. CRISP4 CRISP domain inhibited menthol and BCTC-sensitive current (TRPM8). (*A*) Representative macroscopic whole-sperm currents (I) in control sperm elicited by the voltage protocol shown in the inset below *B*. (*B*) Current after perfusion of 300 μM menthol into the bath solution, and (*C*) current after direct application of 10 μM CRISP4 CRISP domain. Results showed current activation by menthol with inhibition of menthol and basal current by CRISP4 CRISP domain. (*D*) The I-V relationship of control, menthol, and CRISP4 current normalized to the maximal menthol current (n = 3). (*E*) Train stimulation showed an increase in current following perfusion of 300 μM menthol into the bath solution. Application of 10 μM CRISP4 CRISP domain onto the cell inhibited menthol-activated outward cell current. (*F*) Train stimulation showed a decreased whole-cell current following perfusion of 3 μM BCTC into the bath solution indicating inhibition of TRPM8. Ten micromolars of CRISP4 CRISP domain did not further inhibit I, confirming CRISP4 inhibition of TRPM8. In both *E* and *F*, the inset shows representative current traces obtained at the points signaling a, b, and c. Cells were stimulated with 120 mV for 250 ms at one pulse per second.

with TRPM8 channels (26). A definitive identification of the second channel is not possible at this time.

In planar bilayers with incorporated A23187-generated sperm hybrid vesicles, the CRISP4 CRISP domain was added to the *cis* solution at 11 nM as a control, a concentration that did not inhibit whole testicular sperm currents (Fig. 4); thereafter, its concentration was elevated to 111 and 611 nM (Fig. 4*B*). The open probability measured during 60 s decreased from ~0.03 at 11 nM to 0.022 at 111 nM, and to almost 0 at 611 nM. Open-state O_1 remained open most of the time at all CRISP4 CRISP domain concentrations, except at 611 nM (Fig. 4 *C*–*E*).

CRISP4 CRISP Domain Inhibited TRPM8 in Transfected CHO Cells. To further assess the activity of CRISP4 on TRPM8 in a defined heterologous expression system, we used a fluorometric assay measuring intracellular Ca^{2+} ($[Ca^{2+}]_i$) in a CHO cell line stably producing mouse TRPM8 (31). In these cells, application of the TRPM8 agonist icilin (100 nM) (32) showed a typical sigmoidal dose-response curve with an EC_{50} of 77.6 nM (Fig. 5*A*) (31). Consistent with the data from patch-clamped sperm, the addition of 100 μM CRISP4 CRISP domain to TRPM8-CHO cells stimulated with 100 nM icilin inhibited 98% of Ca^{2+} influx through TRPM8 (Fig. 5*B*). The inhibition of TRPM8 activity was CRISP4 CRISP domain concentration-dependent and gave a sigmoidal dose-response curve and a calculated IC_{50} of 32 μM. These data confirm that CRISP4 CRISP domain is capable of inhibiting Ca^{2+} through TRPM8 channels.

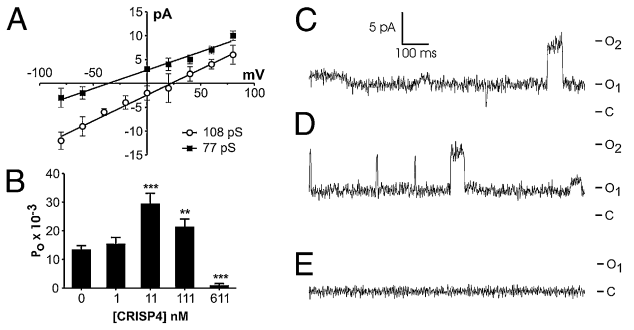

Fig. 4. CRISP4 CRISP domain modulates the activity of mouse sperm single channels in lipid bilayers. (*A*) I-V curves of single channel transitions of bilayers containing sperm-derived ion channels revealed the presence of a cationic channel (77 pS) and anionic channels (108 pS). Currents were recorded under asymmetrical buffer conditions (*cis*:*trans*, 600 mM KCl:100 mM KCl) from a holding potential of 0 mV. (*B*) Channel open probability (P_o) decreased as a function of the concentration of CRISP4 CRISP domain in the *cis* chamber. As sperm patch-clamp experiments showed no effect at 11 nM CRISP4 CRISP domain, this concentration was used as control; 111 and 611 nM decreased P_o over the 60-s recordings. Inhibition of P_o was significant ($P < 0.001$) and complete at 611 nM (n = 3). Representative current traces at 80 mV containing (*C*) 11 nM, (*D*) 111 nM, and (*E*) 611 nM CRISP4 CRISP domain in the *cis* chamber.

TRPM8 Activation Inhibited the Sperm AR. To explore the potential involvement of the TRPM8-CRISP4 interaction in sperm function, we added exogenous CRISP4 CRISP domain and icilin separately, and in combination, to sperm under capacitating conditions and monitored motility, global protein tyrosine phosphorylation levels (as a marker of capacitation status), and the ability of sperm to undergo the progesterone-induced AR. The incubation of sperm in the presence of icilin or CRISP4 did not result in decreased sperm viability compared with control-untreated sperm.

Sperm incubated under capacitation-permissive conditions in the presence of 100 nM icilin showed a significant ($P < 0.05$) decrease in the ability to undergo the progesterone-induced AR compared with control vehicle treated sperm (29 vs. 52%) (Fig. 6). Although the addition of 15 μM CRISP4 CRISP domain alone to capacitation-permissive medium showed no effect on the percentage of AR sperm, the coincubation of sperm with 100 nM icilin plus 15 μM CRISP4 CRISP domain, reversed the icilin-induced suppression of the AR and returned levels to those seen in control untreated sperm (Fig. 6). These data were confirmed using a comparable dose of menthol (2 μM) (Fig. S2).

Sperm incubated under capacitation-permissive conditions in the presence of either CRISP4 CRISP domain or icilin alone had motility characteristics indistinguishable from nontreated control sperm (Fig. S3). Sperm incubated in the presence of both CRISP4 and icilin displayed normal progressive and hyperactivated motility characteristics with a small, but significant, decrease in the average motility and the smoothed path velocity (VAP) (Fig. S3). The significance of this change is currently not known.

Fertility in Crisp4-Null Mice. To define the function of CRISP4 in vivo, we analyzed a *Crisp4* knockout mouse line. The *Crisp4* floxed mice and ultimately those carrying null alleles were produced as outlined in Fig. S4. The removal of exon 3 and the introduction of a frame shift and a premature stop codon was confirmed by sequencing and the elimination of CRISP4 protein was confirmed by Western blotting and immunohistochemistry on wild-type vs. knockout epididymides using the 17G10 antibody (Fig. 7). CRISP4 staining was completely absent in *Crisp4* knockout mice (Fig. 7).

The body and testis weights of knockout and wild-type mice did not differ (Fig. S5*A*). *Crisp4* knockout males (and females) were fertile. *Crisp4* knockout mice had normal mating behavior and produced litters of comparable size to wild-type littermates (Fig. S5*B*). Histological examination of the caput, corpus and cauda regions of the epididymis revealed no obvious histological abnormalities (Fig. S6).

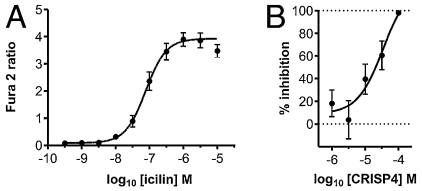

Fig. 5. CRISP4 CRISP domain inhibited TRPM8 in transfected CHO cells. (*A*) CHO cells expressing TRPM8 showed an icilin concentration-dependent increase in $[Ca^{2+}]_i$ measured by Fura-2 fluorescence. (*B*) CRISP4 CRISP domain inhibited TRPM8 stimulated with 100 nM icilin in a concentration-dependent fashion. Inhibition was 98% at 10 μM CRISP4 (n = 6).

A closer examination of sperm from *Crisp4* knockout and wild-type mice, however, revealed a subtle fertility abnormality consistent with the in vitro data. Although sperm from wild-type and *Crisp4* knockout males displayed comparable levels of tyrosine phosphorylation following in vitro capacitation (Fig S5*C*), sperm from *Crisp4* knockout mice have a decreased ability to undergo the AR in response to progesterone compared with sperm from wild-type littermates (Fig. 8) (31% vs. 51.25%, $P < 0.01$). Of note, sperm from *Crisp4*-null males retained both the presence of TRPM8 and the ability to respond to icilin or menthol (Fig. S7), indicating that the phenotype was a result of the absence of CRISP4 rather than altered TRPM8 processing.

Discussion

CRISP4 is produced in the proximal regions of the epididymis, where it surrounds the transcriptionally silent sperm throughout epididymal transit (7, 8, 33). Herein, we have confirmed the presence of functional TRPM8 on mouse sperm and identified CRISP4 as a physiologically relevant inhibitor of TRPM8 activity using both in vitro and in vivo methods. These data strongly suggest that CRISP4, via the regulation of Ca^{2+} flow through TRPM8 channels, is involved in defining the functional competence of the sperm acrosome. Electrochemical gradients (K^+, Na^+, Ca^{2+}, H^+) are established in sperm throughout epididymal maturation at or around the transition from caput to the corpus epididymis in the mouse (34–36). Changes in ion flow have been correlated with the acquisition of specific sperm functions normally manifest during capacitation (3). The data presented herein, using sperm from wild-type and CRISP4-deficient mice, suggests that CRISP4, acting at least in part through TRPM8, is involved in the establishment of an epididymal environment conducive with normal sperm function. Furthermore, the icilin (or menthol) stimulation of TRPM8 in cauda sperm can be reversed in a dose-dependent manner by CRISP4 CRISP domain, suggesting a CRISP4-sensitive pathway remains patent in mature sperm.

We have shown that the addition of CRISP4 CRISP domain to mouse testicular sperm eliminated a component of the ion current in a dose-dependent manner, that it eliminated menthol-stimulated currents in sperm, and that it did not further inhibit current in BCTC-treated sperm. Collectively, these data suggested the main component of the Cs^+ current inhibited by CRISP4 CRISP domain was TRPM8.

At this point in time it is not possible to definitively state whether CRISP4 is specifically involved in sperm epididymal maturation or simply involved in keeping TRPM8 in a quiescent state until the time of ejaculation and CRISP4 dilution in the female tract.

These data extend the physiological characterization of TRPM8 in mouse sperm (25, 27), and in doing so, we have described only the second endogenous protein antagonist of TRPM8 (37). TRPM8 is an outward rectifying cation channel of the TRP family and is permeable to Ca^{2+}, Cs^+, Na^{2+}, and K^+. Within tissues exposed to the external environment, TRPM8 is the primary sensor of environmental cold and is activated at temperatures below ~23 °C (38–40). TRPM8 is, however, also produced in a range of tissues that are unlikely to be regulated by temperature, including sensory neurons (41) and the malignant prostate (42). The mechanism by which TRPM8 activity is regulated in these tissues remains to be fully determined; however, several regulatory mediators have been identified, including Ca^{2+}, pH, PIP2 and phosphorylation (43–47). In those tissues where CRISP4 and TRPM8 are coexpressed (33), our data suggests that CRISP4 is also likely to modulate TRPM8 function.

Mounting evidence suggests that CRISPs regulate a range of ion channel types (10, 11, 48), with our data here and previously (10) showing that this is likely to occur via the CRISP domain. The relatively low specific inhibitory activity of the CRISP4 CRISP domain in both electrophysiological recordings and transfected CHO cell assays herein, indicated that high localized concen-

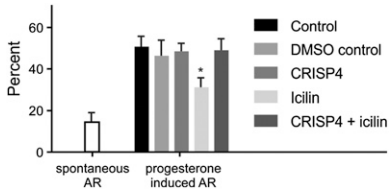

Fig. 6. TRPM8 contributes to signaling leading to the AR. Incubation of sperm with icilin (100 nM) under capacitation-permissive conditions resulted in a significant ($P < 0.05$) inhibition of the number of sperm undergoing the progesterone-induced AR compared with control untreated sperm or sperm treated with denatured CRISP4 CRISP domain. Incubation of the CRISP4 CRISP domain (15 μM) had no effect on induced AR. Coincubation of sperm with CRISP4 and icilin resulted in normal levels of induced AR, demonstrating that CRISP4 inhibited icilin mediated activation of TPRM8. In all experiments, n = 3.

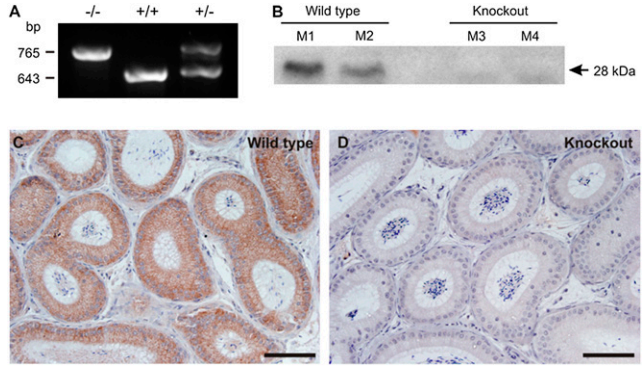

Fig. 7. Validation of the *Crisp4* null mouse line. (*A*) Genotyping showing the amplified PCR product from homozygous null (–/–), wild type (+/+) and heterozygous (+/–) litter mates. (*B*) Western blot showed CRISP4 immunoreactivity in epididymal protein extracts from wild-type adult mice (M1, M2), but not in knockout tissue (M3, M4). (*C* and *D*) Immunohistochemical staining of caput epididymal sections from adult wild type and knockout mice. (Scales bars, 100 μM.)

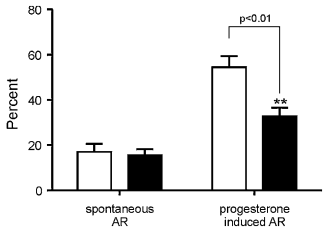

Fig. 8. The ability of sperm to acrosome react was modified in the *Crisp4* knockout mice. After 90 min of in vitro capacitation, there was no significant difference between the spontaneous AR in the wild-type (white bar) or *Crisp4* knock-out (black bar) sperm. However, a significant reduction in the percent of *Crisp4* knockout sperm undergoing the progesterone-induced AR was apparent. n = 4.

trations are required for the inhibition of TRPM8 to be biologically relevant. Although this is undoubtedly the case in the epididymal lumen, this conclusion cannot immediately be drawn for other tissues where CRISP4 has been demonstrated, namely skeletal muscle, spleen, thymus, and lacrimal glands (7, 8, 33). Supporting the possibility of a functionally relevant role for CRISP4 within these tissues, however, it has recently been shown that the full-length venom CRISP PsTx has at least a 120-fold higher activity against cyclic-nucleotide gated A2 in comparison with its ICR region alone (CRISP domain), indicating a dramatic enhancement of activity because of the presence of both domains. Assuming that this holds true for all members of the CRISP family, CRISP4 may be of physiological relevance in all tissues where expression is observed. Unfortunately, and as indicted in *Materials and Methods*, full-length, soluble recombinant mouse CRISP4 has proven to be extremely difficult to produce, so we have been unable to confirm these observations. Regardless, through the use of a *Crisp4* knockout mouse lines we have proven that CRISP4 is a physiologically relevant regulator of sperm maturation within the epididymis.

The data contained within this article and our previous work (27) suggest a complex role for TRPM8 in sperm. For example, the activation of TRPM8 with either low (as used herein) or higher concentrations of icilin or menthol (as used in ref. 27), differentially stimulate the AR in wild-type sperm. Specifically, high concentrations of menthol stimulate sperm to undergo the AR in the absence of progesterone (27). The potential also exists that the timing of TRPM8 stimulation may influence the propensity of sperm to acrosome react. As indicated herein, exposing sperm to a TRPM8 agonist throughout the duration of capacitation resulted in fewer sperm reacting to progesterone. This inhibition was reversed by coincubation with agonist plus CRISP4 CRISP domain during capacitation, suggesting that CRISP4 is a competitive inhibitor of TRPM8 function. The effect of TRPM8 later during the capacitation process or at the time of zona interaction has, however, not been tested and may vary as sperm become functionally more mature.

Our knockout model clearly shows that although CRISP4 has a role in establishing normal acrosomal function, it is not in and of itself an essential requirement for fertility. We believe that this is likely to be because of functional redundancy from the closely related family member CRISP1, which is also produced at high concentrations within the mouse epididymis. Unlike other species examined to date, the mouse contains four rather than three CRISPs (9). Within the human for example, there is a single epididymal-expressed CRISP, *CRISP1*. Human CRISP1 is the likely ortholog of mouse CRISP4; however, we hypothesize that from a fertility perspective, it is likely that human CRISP1 maybe the functional equivalent to the sum of CRISP4 and CRISP1 in the mouse (14). In support of this hypothesis, a *Crisp1* knockout mouse has been produced (49) and male mice, although grossly fertile, possess a subtle fertilization phenotype.

Herein we have shown that CRISP4 is a regulator of ion channel signaling in sperm and the CRISP4 CRISP domain is a specific antagonist of TRPM8. These data are unique in providing direct evidence of the molecular function of CRISP4 on the cell it is likely to regulate in vivo, and provide strong support for the role of CRISPs in establishing and maintaining molecular pathways involved in sperm storage and function. Deregulated ion channel expression/function is associated with many diseases (50, 51) and ion channels are a major class of therapeutic drug target (52). As such, the characterization of endogenous protein ion channel regulators with expression beyond the reproductive tract is of broad significance (33).

Materials and Methods

Animals. Animal procedures were conducted in accordance with the Animal Care and Bioethics Committee of the Instituto de Biotecnología Universidad Nacional Autónoma de México and the National Health and Medical Research Council's Guidelines on Ethics in Animal Experimentation, and approved by the Monash Medical Centre and Monash University Animal Experimentation Ethics Committees. Electrophysiological assessments using testicular spermatozoa were carried out on sperm from CD1 mice. Sperm function assays were carried out on sperm from 10- to 12-wk-old C57Bl6 × CBA (F1) mice.

Generation of Recombinant CRISP4. *Crisp4* cDNA was amplified from the mouse epididymis and the CRISP4 CRISP domain expressed and purified as described previously (10) (*SI Materials and Methods*). Full-length recombinant CRISP1 to -4 was produced as described and was insoluble (33).

Electrophysiology of Testicular Spermatozoa and Single-Channel Recordings in Planar Bilayers. Sperm were harvested from the seminiferous tubules of adult CD1 mice as previously described (28) and whole-cell currents in patch-clamped sperm using established methods (53). We used testicular sperm to avoid prior exposure of cells to endogenous (epididymal) CRISP4. In addition, the larger cytoplasmic droplet on testicular sperm facilitated a good voltage clamp. Our preliminary results using epididymal sperm indicated that the currents we recorded in testicular sperm in this work were similar. Initially, total whole-cell currents were recorded using the following solutions: external solution (in mM): 118 Na-$MetSO_4$, 8 NaCl, 2.5 $CaCl_2$, 2 KSO_4, 1 $MgCl_2$, 10 Hepes, 3.3 dextrose, (pH was adjusted to 7.4 with NaOH), and internal solution (in mM): 122 K-$MetSO_4$, 8 KCl, 20 KF, 2.5 $CaCl_2$, 1 $MgCl_2$, 5 EGTA, 10 Hepes (pH was adjusted to 7.3 with KOH). The osmolality of all solutions was adjusted with dextrose. Thereafter, to focus on whole-cell currents mainly through TRP channels, we used Cs^+ as the main current carrier in symmetrical conditions (in mM): 100 CsOH, 40 CsF, 0.2 $CaCl_2$, 10 *N*-methyl-D-glucamine (NMDG, an impermeant cation), 5 EGTA, 10 Hepes, 20 dextrose (pH 7.3). Further details concerning the sperm electrophysiological recordings can be found in *SI Materials and Methods*. Single-channel recordings were performed by established methods (54) (see details in *SI Materials and Methods*).

CHO Cell TRPM8 Flexstation II Assay. CRISP4 CRISP domain antagonism of TRPM8 was tested in CHO cells expressing HA-tagged TRPM8 using a fluorometric plate assay. Calcium-dependent Fura-2 fluorescence was measured using a Flexstation II (Molecular Devices), as previously described (31). Nontransfected CHO cells were used as a negative control in all experiments.

Icilin and CRISP4 CRISP domain were added to each well at 15 s and recordings were carried out for an additional 105 s. The peak value of Fura-2 fluorescence was used to establish the TRPM8 channel activity. Peak values were subtracted from baseline values recorded from 0 to 15 s. Positive controls included ATP to stimulate intracellular calcium mobilization as a measure of cell viability, which was effective on both transfected and nontransfected cells (Fig. S8). Icilin was used to ensure appropriate TRPM8 function in TRPM8 transfected cells. Negative controls treatments included vehicle controls (0.5% DMSO) and the addition of CRISP4 CRISP domain alone without icilin stimulation. NT-CHO cells never showed increased fluorescence following icilin or CRISP4 treatment (Fig. S7). TRPM8 transfected cells never showed a difference to unstimulated control cells. Each assay was completed in duplicate and assays were completed a minimum of six times.

Sperm Capacitation and Motility Assessment. The functional competence of caudal epididymal sperm was tested as outlined in detail in the *SI Materials and Methods*. Treatments included 100 nM icilin, 0.1% DMSO vehicle control, and 15 μM CRISP4 CRISP domain from the beginning of capacitation. Sperm were incubated at 37 °C/5% CO_2 for up to 90 min. In all cases when icilin was used, sperm were assessed alongside a 0.1% DMSO vehicle control and in normal conditions (no vehicle). The DMSO control results were not different to the normal control.

Mouse sperm motility was measured using a Hamilton-Thorne computer assisted sperm analyzer, as described previously (55) (*SI Materials and Methods*). A minimum of 300 sperm were scored per animal ($n = 5$ per group). The ability of sperm to undergo the progesterone-induced AR was tested as described in *SI Materials and Methods*. Acrosomal contents was labeled with FITC-labeled *Arachis hypogaea* and nuclear DNA was counter stained with DAPI (Invitrogen). Two-hundred sperm cells were assessed for acrosome integrity per treatment. Sperm capacitation status was assessed using global tyrosine phosphorylation Western blotting using the 4G10 (Millipore) antibody, as described previously (56), and the percentage of live:dead sperm was assessed using ethidium labeling (*SI Materials and Methods*).

Generation and Fertility Testing of Crisp4 Knockout Mice. The *Crisp4* knockout construct was designed for the generation of both null and conditional knockout alleles. The *flox* allele carrying an insertion of loxP sites flanking exon 3 and a third loxP site in intron 2 (Fig. S4) was generated through homologous recombination in C57BL/6-derived ES cells. Excision of exon 3 could be achieved by either breeding mice carrying heterozygous *flox* allele with ubiquitous-*Cre* or tissue-specific-*Cre* transgenic mice. Removal of exon 3 resulted in a deletion of 60 bp from the mature *Crisp4* transcript and the creation of a premature stop codon within exon 4 (ENSMUST00000115344). We generated *Crisp4*-null mice by breeding mice carrying heterozygous *flox*

allele with CMV-*Cre* transgenic mice for a complete excision of exon 3 in all tissues. Heterozygous knockout mice were intercrossed to generate homozygous knockout mice. Genotyping was performed as described in *SI Materials and Methods*. CRISP4 production in the epididymis of CRISP4 in knockout and wild-type mice was examined by immunohistochemistry and Western blot using the 17G10 antibody. The 17G10 antibody was generated as described in *SI Materials and Methods*. *Crisp4*-null and wild-type mouse fertility testing was conducted as previously described (57) and in *SI Materials and Methods*.

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