CRISP2 Is a Regulator of Multiple Aspects of Sperm Function and Male Fertility

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The cysteine-rich secretory proteins (CRISPs) are a group of proteins that show a pronounced expression biased to the male reproductive tract. Although sperm encounter CRISPs at virtually all phases of sperm development and maturation, CRISP2 is the sole CRISP produced during spermatogenesis, wherein it is incorporated into the developing sperm head and tail. In this study we tested the necessity for CRISP2 in male fertility using *Crisp2* loss-of-function mouse models. In doing so, we revealed a role for CRISP2 in establishing the ability of sperm to undergo the acrosome reaction and in establishing a normal flagellum waveform. *Crisp2*-deficient sperm possess a stiff midpiece and are thus unable to manifest the rapid form of progressive motility seen in wild type sperm. As a consequence, *Crisp2*-deficient males are subfertile. Furthermore, a yeast two-hybrid screen and immunoprecipitation studies reveal that CRISP2 can bind to the CATSPER1 subunit of the Catsper ion channel, which is necessary for normal sperm motility. Collectively, these data define CRISP2 as a determinant of male fertility and explain previous clinical associations between human *CRISP2* expression and fertility. **(*Endocrinology* 160: 915–924, 2019)**

CRISP2 is a member of the cysteine-rich secretory protein (CRISP) clade of the cysteine-rich secretory protein/antigen 5/pathogenesis (CAP) superfamily (1). Its expression is highly enriched within the testis, and it is specifically localized to the sperm acrosome, accessory structures of the sperm tail, and the junction between germ and Sertoli cells within the seminiferous epithelium (2–5). Like all CRISPs, CRISP2 consists of a CAP domain, which is implicated in cell-cell adhesion and is capable of steroid binding (5–7), and a CRISP domain. The CRISP domain can be further subdivided into a hinge region, which is necessary to maintain spatial separation between the CAP and CRISP domains, and an ion channel regulatory region, which in the case of CRISP2 can specifically regulate calcium flow through ryanodine receptors (8, 9). Based on the functional plasticity of reptile CRISPs, however, it is plausible that CRISP2 may regulate other ion channels in addition to ryanodine receptors (10). Unlike the other three members of the mammalian CRISPs, CRISP2 is usually an intracellular protein. Of clinical relevance, decreased CRISP2 content in sperm is associated with human male infertility (11, 12), and likely pathogenic mutations are found within human populations (13) and associated with male fertility in horses (14).

Despite these compelling pieces of information, both the molecular function of CRISP2 and its role in male fertility are poorly characterized. Therefore, in this study

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Abbreviations: CAP, cysteine-rich secretory protein/antigen 5/pathogenesis; CRISP, cysteine-rich secretory protein; GCKO, germ cell–specific conditional knockout; hKO, hypomorphic knockout; qPCR, quantitative PCR.

we aimed to determine the overall necessity for CRISP2 in mammalian male fertility and its specific cellular functions. Specifically, we hypothesized that CRISP2 plays a role in the function of the acrosome or sperm tail function. Consistent with this hypothesis, our data show that optimal CRISP2 production in mice is necessary for maximal fecundity. Specifically, CRISP2 is involved in manifestation of the acrosome reaction and for establishing the normal sperm flagella waveform. Data also reveal that CRISP2 can bind to the CATSPER1 subunit of the CATSPER calcium channel. These data establish CRISP2 as a determinant of qualitatively normal spermatogenesis and provide a molecular basis for the correlation between CRISP2 expression and readouts of human male fertility.

Materials and Methods

Mouse production

All animal experimentation conducted at the Australian site was approved by the Monash University Animal Experimentation Ethics Committee (approval numbers BSCI/2017/45 and BSCI/2017/27/BC) in accordance with National Health and Medical Research Council guidelines. All experiments conducted at the German site were done in accordance with the German Animal Welfare Act and with the approval of the district veterinary office, Landesamt für Natur, Umwelt und Verbraucherschutz, of the state of North Rhine-Westphalia, Germany (approval number AZ 84-02.05.20.13.115).

For production of the *Crisp2* knockout mouse line, an embryonic stem cell line carrying a targeted gene trap allele inserted into intron 5 of the *Crisp2* gene (reporter-tagged insertion with conditional potential, project ID 37433) was purchased from KOMP and used to generate mice through the Australian Phenomics Network by standard methods (15). Insertion of the cassette was predicted to result in a truncated protein whereby the *N*-terminal 62 amino acid residues will be fused to LacZ. Cells were injected into C57BL/6N blastocysts to generate chimeric mice, and the knockout line (herein referred to as $Crisp2^{hKO}$) was maintained on a C57BL/6N background. All primers for the characterization of genetic deletions are listed in an online repository (16).

A germ cell–specific knockout line was produced as described previously (17). In brief, $Crisp2^{hKO/hKO}$ males (where hKO indicates a hypomorphic knockout) were mated with a CMV-Flp transgenic line to excise the gene trap cassette, thus generating a *Crisp2* flox allele whereby exon 6 (ENSMUSE00000136444) was flanked by loxP sites. The flox line was subsequently mated with a *Stra8*-cre transgenic line (18) via a two-step breeding strategy: homozygous flox females were mated with *Stra8*-cre males, and $Crisp2^{flox/WT}$, *Stra8*-Cre males were then mated with homozygous flox ($Crisp2^{flox/flox}$) females to produce mice carrying *Crisp2* ablation after a Cre-mediated recombination (referred to as GCKO) (16). *Crisp2* exon 6 (ENSMUSE00000136444) excision would result in the production of frame-shifted mRNA with the creation of terminal codon in exon 7 (ENSMUSE00000136441), thus resulting in a truncated protein carrying the *N*-terminal 63 amino acid residues. Based on the *Stra8* expression time point during spermatogenesis (18), we predicted that germ cells from late spermatogonia onward would carry *Crisp2* exon 6 excision. The efficiency of exon 6 excision was assessed via quantitative PCR (qPCR) with primers listed in an online repository (16). For $Crisp2^{hKO/hKO}$ males, $Crisp2^{WT/WT}$ males were used as wild type controls. For $Crisp2^{GCKO/GCKO}$ males, $Crisp2^{flox/flox}$ males were used as wild type controls. Mice were genotyped by a commercial genotyping service (Transnetyx, Cordova, TN).

Fertility analysis

Male fertility status was assessed as previously described (19). Briefly, 10- to 12-week-old $Crisp2^{WT/WT}$ and $Crisp2^{KO/KO}$ males were mated with 7-week-old wild type females ($Crisp2^{WT/WT}$ n = 5; $Crisp2^{hKO/hKO}$ n = 8; $Crisp2^{flox/flox}$ n = 4; $Crisp2^{GCKO/GCKO}$ n = 6) and litter sizes recorded. Plugging was monitored as an indication of normal mating behavior, and the size of the first two litters only was recorded to avoid mouse-to-mouse bias. Litter size was averaged between the two litters. Daily sperm outputs and total epididymal sperm content were determined as described previously (n = 5 per genotype) (20, 21). Basic sperm motility parameters were determined with a computer-assisted sperm analysis system (MouseTraxx, Hamilton Thorne) as described previously (22). Rapid sperm motility was defined as velocity >35 μm/s, medium motility was defined as sperm velocity between 10 and 35 μm/s, and slow motility was defined as sperm velocity <10 μm/s. Electron microscopy and assessment of the ability of sperm to undergo the progesterone-induced acrosome reaction were performed as previously described (23) (n = 3 mice per genotype, >100 sperm per mouse). Caudal epididymal sperm morphology was examined by hematoxylin and eosin staining (n = 3 per genotype).

For the analysis of fertility, groups were compared via *t* test, where $P < 0.05$ was considered significant. To ascertain whether *Crisp2* expression was a quantitative determinant of a particular aspect of male fertility, *Crisp2* expression in the testes of individual males, of all genotypes, was determined via qPCR methods described below. Expression data were then plotted relative to the fertility parameter of interest and the correlation assessed by Pearson correlation test.

Sperm preparation and high-resolution motility analysis

Cauda epididymides and vas deferentia were dissected and transferred into a well of a four-well plate (Nunclon Delta Surface) containing 500 μL of modified TYH medium (135 mM NaCl, 4.8 mM KCl, 2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1 mM $MgSO_4$, 5.6 mM glucose, 0.5 mM Na-pyruvate, 10 mM L-lactate, and 10 mM HEPES, pH 7.4). To isolate sperm, small incisions were made in the tissue to allow sperm to swim out into the media. After 10 minutes, the incised tissue and sperm suspension were transferred to a 2-mL tube. After 15 minutes of incubation at 37°C, the uppermost 1.8 mL was collected and sperm concentration determined. Sperm motility was studied in custom-made observation chambers with a depth of 150 μm. Briefly, two strips of three-layer adhesive tape were affixed to a glass slide ($0.15 \times 24 \times 24$ mm^3) 16 mm apart. Then 40 μL of sperm suspension (1×10^5 sperm/mL in TYH supplemented with 0.3 mg/mL BSA to facilitate sperm tethering) was pipetted between the two strips and a coverslip ($0.15 \times 18 \times 18$ mm^3) placed on top to seal the chamber. For analysis, only head-tethered

sperm with a free-beating flagellum were selected. Sperm motility was studied at room temperature, with an inverted microscope (IX73; Olympus, Tokyo, Japan) equipped with a condenser (IX2-LWUCD; Olympus) housing a custom-made dark-field adaptor. Sperm were illuminated with a red LED (M660L4; Thorlabs) that was controlled with a custom-made power supply. Images were collected at 200 frames per second for 2.5 seconds with a 10× objective (UPlanFLN, NA 0.3; Olympus) and a high-speed sCMOS camera (Zyla 4.2 Plus; Andor). Image processing and analysis were performed in ImageJ (US National Institutes of Health, Bethesda, MD). For each image sequence, the contrast was first enhanced via the ImageJ function "Enhance Contrast" (0.1% to 0.5% pixel saturation with normalization). Next, background was removed with the "Subtract Background" function with a radius of 10 pixels, followed by subtraction of a medium-intensity projection of the entire sequence. To generate stop motion images illustrating a flagellar beat cycle, the sperm head in each frame was aligned along a horizontal axis. To that end, each frame was accordingly rotated and then translated in the ImageJ function "Template Matching." To determine the main beat frequency, the position of the initial portion of the midpiece was followed over time. The regular fluctuations in position revealed the main beat frequency.

Testicular germ cell isolation

Spermatocytes (>90% pachytene) and round spermatids were purified from 28-day-old and 10-week-old testes via the Staput method as described previously (24). Spermatocytes had a purity of ≥90% and round spermatids ≥95%.

Assessment of the interaction between CRISP2 and CATSPER1

Potential CRISP2 binding partners were identified via a yeast two-hybrid screen of an adult mouse testis library as described previously (25, 26). One of the potential CRISP2 interactors identified was the CATSPER1 subunit of the CATSPER ion channel. The identified clones contained the full-length *Catsper1* cDNA. To scrutinize the ability of CRISP2 and CATSPER1 to interact, full-length mouse *Crisp2* and *Catsper1* were amplified from cDNA of murine testis and cloned into the pEGFP-C1 and pmCherry-C1 expression vector (Takara Bio, Mountain View, CA), respectively, as described previously (25, 26). Constructs were cotransfected into HEK293 cells with Lipofectamine 3000 Reagent (catalog no. L3000008; Life Technologies, Carlsbad, CA). Empty pEGFP-C1 vector was used as a negative control. After the protein was extracted from the transfected cells, CRISP2 and associated proteins were immunoprecipitated with anti-GFP-Trap-A beads according to the manufacturer's instructions (catalog no. gta-100; Chromotek, Munich, Germany), and CATSPER1 (27) was detected via Western blotting as described previously (17). The presence of recombinant CRISP2 and CATSPER1 proteins were determined with anti-GFP (28) and anti-mCherry antibodies (29).

Western blotting

Testes were homogenized in radioimmunoprecipitation assay buffer containing protease inhibitors (1/200; Calbiochem) and quantitated via DC protein assay (BioRad, Hercules, CA). Then 15 μg of protein from each sample was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Blots were blocked with 5% skim milk/PBS and incubated in rabbit CRISP2 antibody (30, 31) overnight at 4°C. Goat anti-rabbit horseradish peroxidase (32) was added for 1 hour at room temperature. Chemiluminescence was detected with Clarity Western ECL substrate kit (BioRad). A rabbit actin antibody (33) was used as a loading control.

Quantitative PCR analysis

Total RNA from testis and isolated germ cells were extracted with Trizol reagent (Life Technologies) and converted to cDNA via SuperScriptIII reverse transcription and oligo dT (Life Technologies). *Crisp2* transcript expression level was quantitated via TaqMan qPCR assay (Mm01269289_g1) and normalized against *Ppia* expression (Mm02342429). Statistical analysis was performed via two-tailed unpaired Student *t* test.

Results

CRISP2 is necessary for qualitatively normal male fertility

To test the necessity of CRISP2 for male fertility, conditional-ready embryonic stem cells were used to produce mice wherein *Crisp2* was deleted in a body-wide fashion (C57BL/6N background). An additional strain of mice was produced wherein *Crisp2* was deleted specifically in male germ cells as directed by *Stra8-cre* (referred to as $Crisp2^{GCKO}$, mixed C57BL/6N and FVB background). In $Crisp2^{GCKO}$ mice, testis showed an average reduction in testicular *Crisp2* mRNA expression of 92.4% (Fig. 1A). As indicated below, there was mouse-to-mouse variation in expression, indicative of "leaky"

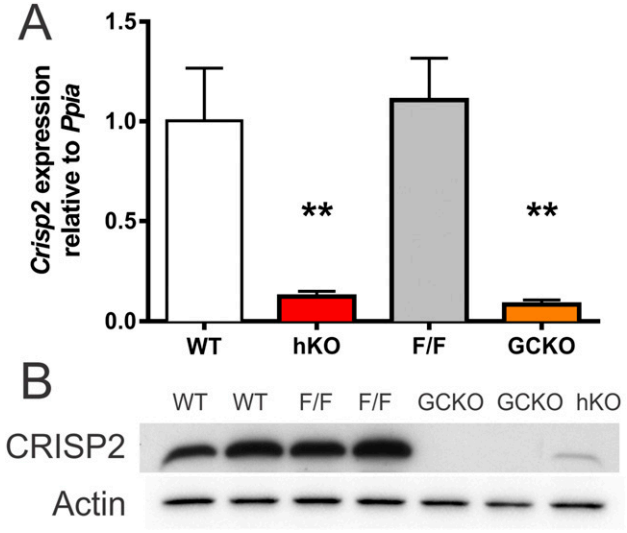

Figure 1. *Crisp2* expression in wild-type (WT) and genetically modified mouse models. (A) Quantitative PCR (n = 6 to 7 per group). (B) Western blot demonstrating a reduction in CRISP2 protein levels in the hKO mouse testes and the GCKO purified germ cells compared with WT and flox/flox (F/F) controls, respectively. For the Western blot, protein loading was normalized with actin content. $^{**}P < 0.01$.

expression in the body-wide deletion. Therefore, these mice are considered as having *Crisp2* loss-of-function, or hypomorphic ($Crisp2^{hKO}$). Consistent with the qPCR results, testicular CRISP2 protein production was greatly suppressed in both models (Fig. 1B).

$Crisp2^{hKO/hKO}$ and $Crisp2^{GCKO/GCKO}$ mice were indistinguishable from wild type and heterozygous littermates in appearance, general behavior, survival rate, and mating behavior. However, $Crisp2^{hKO/hKO}$ and $Crisp2^{GCKO/GCKO}$ males, but not females, were subfertile (Fig. 2A).

To define the origin of the subfertility, we examined the histology of the male reproductive tract and sperm function. $Crisp2^{hKO/hKO}$ and $Crisp2^{GCKO/GCKO}$ males had normal testis weight and daily sperm production (16) and qualitatively normal histology (Fig. 2B). Furthermore, sperm from *Crisp2*-deficient males were morphologically normal at both light and electron microscopic level (16).

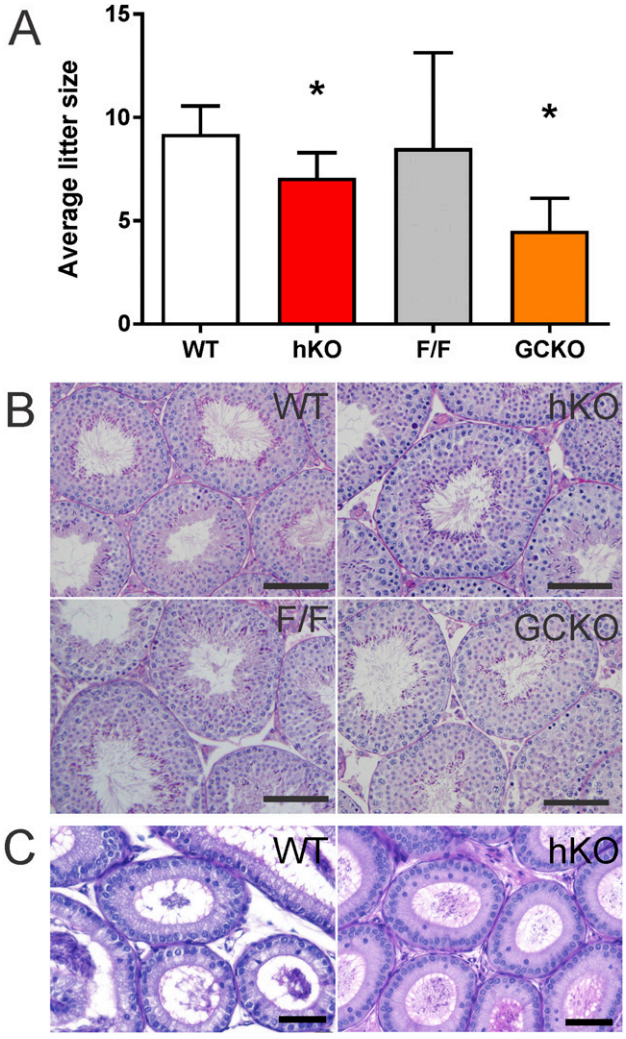

Figure 2. CRISP2 is necessary for optimal male fertility. (A) Reduced litter size in hKO vs wild-type (WT) (n = 8 and 5, respectively) and GCKO male mice vs flox/flox (F/F) controls (n = 6 and 4, respectively). (B) Representative periodic acid-Schiff stained testis sections from GCKO and hKO mice showing normal testis morphology in GCKO and hKO mice. Scale bar, 100 μm. (C) Representative hematoxylin and eosin stained epididymal section from WT and hKO males. Scale bar, 50 μm.

Because CRISP2 has been proposed to be involved in adhesion between Sertoli and germ cells (5, 34), we also investigated whether decreased *Crisp2* expression led to the precocious release of germ cells from the seminiferous epithelium into the epididymis. As illustrated in Fig. 2C, the presence of immature germ cells within the epididymis was rare and comparable between $Crisp2^{hKO/hKO}$, $Crisp2^{GCKO/GCKO}$, and wild type males, arguing against an essential role for CRISP2 in Sertoli–germ cell adhesion (5, 34).

In wild type sperm, CRISP2 is localized to the acrosome and the sperm tail (3, 7, 31). Therefore, we assessed whether the deletion of *Crisp2* affected acrosomal exocytosis in response to progesterone stimulation. In $Crisp2^{hKO/hKO}$ and $Crisp2^{GCKO/GCKO}$ sperm, the percentage of sperm that underwent the progesterone-evoked acrosome reaction was lower (Fig. 3A) (16), whereas the percentage of spontaneously acrosome-reacted sperm, and those that were partially reacted, was similar between $Crisp2^{hKO/hKO}$, $Crisp2^{GCKO/GCKO}$, and their corresponding wild type littermates (Fig. 3A). Of interest, a more fine-grained analysis of the percentage of sperm undergoing the acrosome reaction within individual males positively correlated with the amount of residual testicular *Crisp2* expression, thus indicating that CRISP2 is a quantitative determinant of the ability of sperm to undergo the acrosome reaction (Fig. 3C). These data thus suggest that the subfertility seen in CRISP2-deficient mice was due, at least in part, to the inability of a proportion of sperm to manifest a normal acrosome reaction and thus fertilize an oocyte.

Next, we assessed sperm motility by using a computer-assisted sperm analyzer in a manner analogous to what would occur in a human infertility clinic. Compared with their wild type littermates, sperm populations from $Crisp2^{hKO/hKO}$ and $Crisp2^{GCKO/GCKO}$ mice showed a 16.1% and 28.7% reduction in the number of sperm displaying any form of motility (*i.e.*, total motility) (Fig. 4A). For $Crisp2^{hKO/hKO}$ males, this reduction correlated with a significant reduction in the percentage of sperm displaying progressive motility (Fig. 4B). For both loss-of-function lines, we also observed a significant reduction in the percentage of sperm displaying rapid forms of progressive motility compared with wild type littermates (Fig. 4C); that is, reduced *Crisp2* expression led to a decreased percentage of sperm displaying rapid motility (>35 μm/s) and an increase in the number of static sperm. These results indicate that CRISP2 is necessary for normal sperm motility.

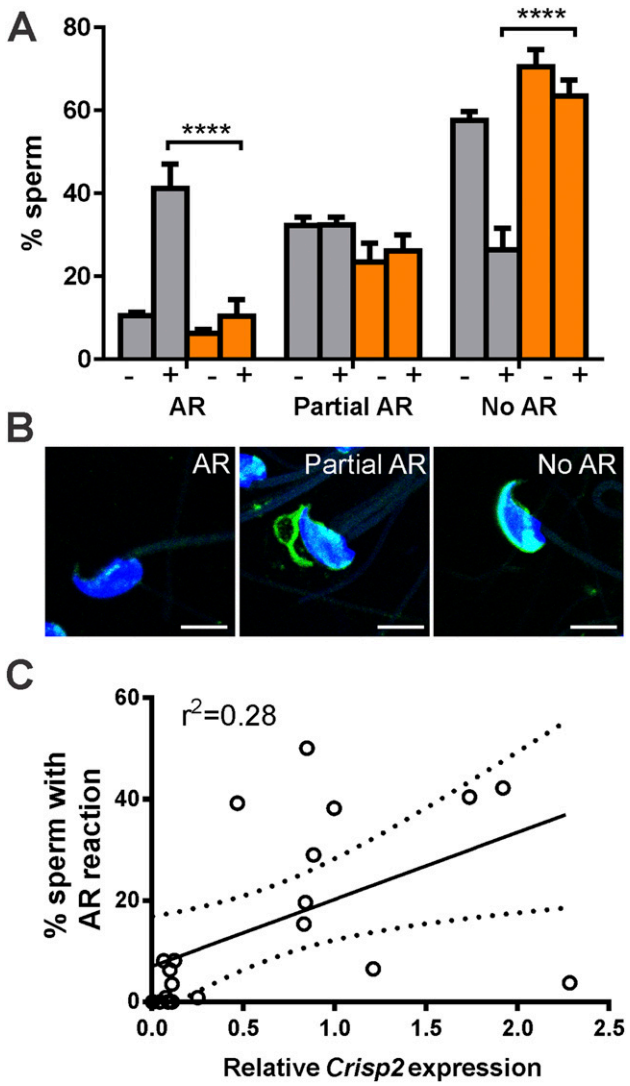

Figure 3. Compromised acrosomal reaction (AR) in *Crisp2* GCKO mice. (A) Decreased AR in sperm from GCKO mice (orange) compared with sperm from flox/flox littermates (gray) (n = 6). Plus and minus signs indicate the presence or absence of progesterone as an AR stimulator. Gray bars indicate flox/flox control samples, and orange bars indicate GCKO samples (n = 20 per genotype). (B) Immuno-histological images illustrating an AR sperm, a partially AR sperm, and a non-AR sperm. (C) The reduction in the percentage of sperm undergoing the AR from an individual mouse plotted relative to the level of *Crisp2* mRNA expression in the testis. $^{****}P < 0.0001$.

To gain insight into the origin of the motility defect, we studied the beating pattern of head-tethered $Crisp2^{bKO/bKO}$ sperm. The beat frequency of sperm from $Crisp2^{bKO/bKO}$ and wild type mice was similar (3.2 Hz vs 3.5 Hz; Fig. 5A). However, we observed that in many but not all $Crisp2^{bKO/bKO}$ sperm, the distal part of the midpiece was stiff, thus leading to an abnormal beating pattern (Fig. 5B and 5C) (16). To demonstrate this defect, we aligned and superimposed successive frames to yield quasi stop-motion images illustrating a representative flagellar beating cycle (Fig. 5B).

In summary, these data reveal that CRISP2-deficient males are subfertile as the result of a compromised ability of sperm to manifest normal motility and acrosomal exocytosis. The corresponding human condition would be classified as asthenozoospermia.

CRISP2 is a CATSPER1 binding protein

To gain further insight into the function of CRISP2 in sperm, we undertook a yeast two-hybrid screen of an adult testis expression library. One of the candidate binding proteins identified was CATSPER1. CATSPER1 is a pore-forming subunit of the sperm-specific CatSper Ca^{2+} channel. CatSper is absolutely necessary for sperm function and fertilization (35, 36). Catsper channels control the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and, thereby, sperm motility (36–41). The *Catsper1* cDNA sequences contained with the yeast two-hybrid clones contained the full-length *Catsper1* sequence. To scrutinize this interaction, mCherry- and GFP-tagged CATSPER1 and CRISP2, respectively, were exogenously expressed in HEK293 cells, then immunoprecipitated with respective tag antibodies. Analysis of the coimmunoprecipitates by Western blotting indicates that CATSPER1 and CRISP2 can indeed interact to form a protein complex (Fig. 6). Although both CRISP2 and CATSPER1 are known to localize to the principal piece of the sperm tail (26, 31, 36), the absence of specific CATSPER1 antibodies precludes a more in-depth analysis of this interaction *in vivo*. However, these data are consistent with a recent publication indicating that the closely related extracellular protein CRISP1 can interact with the CatSper channel (42).

Discussion

The analysis of two *Crisp2*-deficient mouse lines shows that appropriate CRISP2 expression is necessary for optimal sperm function and male fertility. These data are consistent with clinical observations in humans (11, 12) and the localization of CRISP2 *in vivo* (3, 26, 31) and specifically defines a role for CRISP2 in sperm tail motility and in establishing the acrosome reaction, and thus fertilization. The loss of CRISP2 compromises the ability of sperm to undergo the acrosome reaction, in response to the stimulant progesterone, and a shift toward slower motility. The latter is probably a consequence of stiff midpiece syndrome. Data also revealed that CRISP2 can bind to the CATSPER1 component of the key sperm ion channel Catsper, in addition to the previously defined ability of CRISP2 to regulate calcium flow through ryanodine receptors (8).

Stiff midpiece syndrome is a rare subtype of human infertility (43) wherein the midpiece of the sperm flagellum behaves as a stiff rod, thus disrupting the flagellar waveform. This defect is probably underdiagnosed in

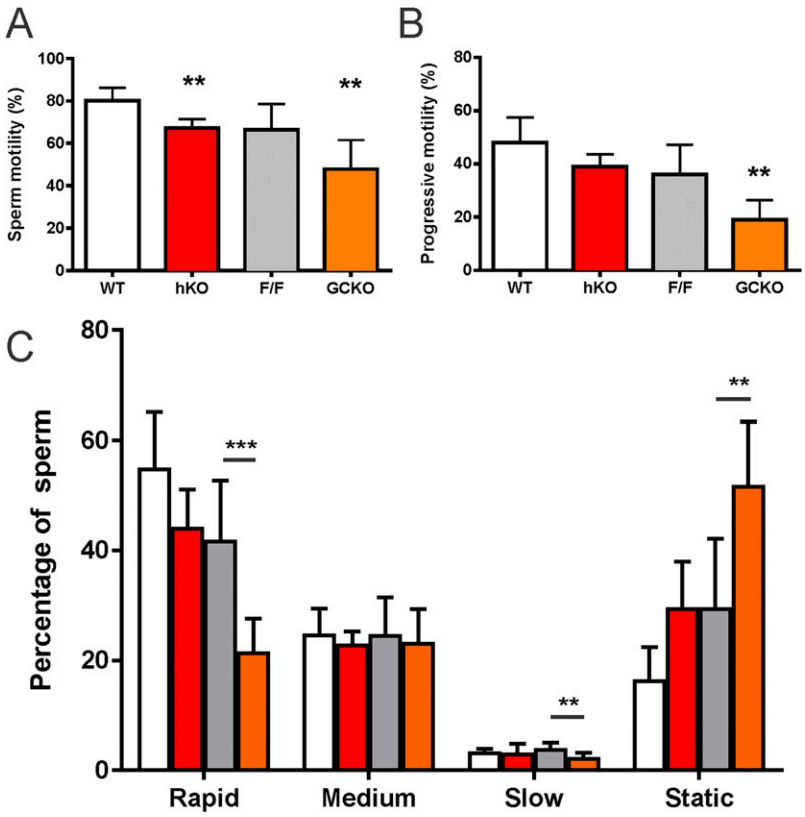

Figure 4. Reduced sperm motility in hKO and GCKO mice. Computer-assisted sperm analysis demonstrated reduced (A) sperm total motility and (B) progressive motility in hKO (n = 6) and GCKO (n = 10) mice compared with wild-type (WT) (n = 6) and flox/flox (F/F) (n = 6) littermates. (C) A more fine-grained analysis of sperm motility vs genotype, where rapid sperm motility was defined as velocity >35 μm/s, medium motility was defined as sperm velocity within the range of 10 to 35 μm/s, and low motility was defined as sperm velocity <10 μm/s. Static sperm did not display progressive motility. White bars represent WT mice, red bars represent hKO mice, gray bars represent F/F mice, and orange indicates GCKO mice. $^{**}P < 0.01$; $^{***}P < 0.001$.

infertile men because sperm waveform is rarely analyzed at high spatial and temporal resolution and thus would generally be classified as asthenozoospermia. However, stiff midpiece syndrome has been observed in several mouse models (44–46). Though mechanistically poorly understood, it is thought to result from altered calcium regulation or defects in the ultrastructure of either the microtubules of the axoneme or, more likely, their interaction with the outer dense fibers. Although it is difficult to rule out definitely, the electron microscopic analysis (16) indicates that the loss of CRISP2 does not alter sperm ultrastructure.

CATSPER is the principal calcium channel in sperm and absolutely necessary for sperm hyperactivation and

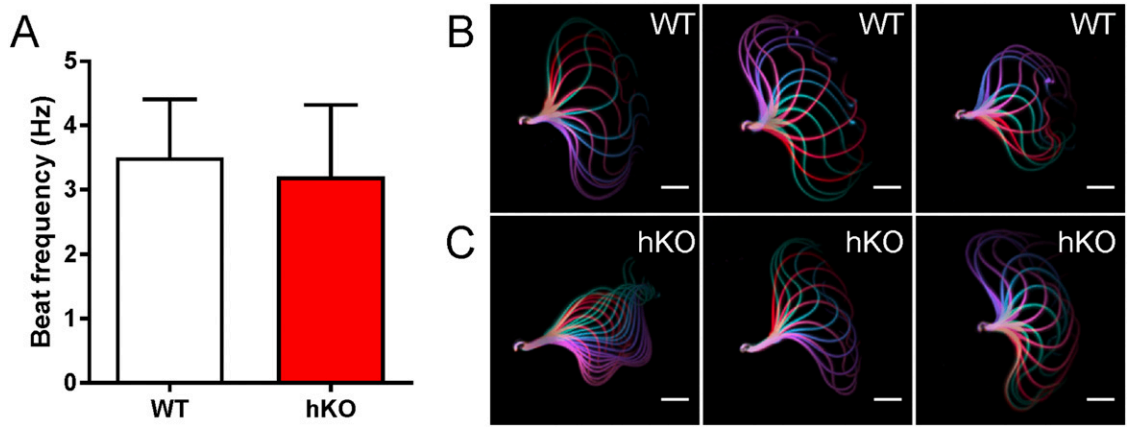

Figure 5. The loss of CRISP2 leads to an altered flagellum beating pattern. (A) Flagellar beat frequency of wild-type (WT) and *Crisp2*$^{hKO/hKO}$ sperm (mean ± SD; n = 11). (B, C) Representative quasi–stop-motion images illustrating one flagellar beating cycle constructed from successive, aligned, and superimposed images from three WT and *Crisp2*$^{hKO/hKO}$ sperm (each from different mice). Scale bars, 20 μm.

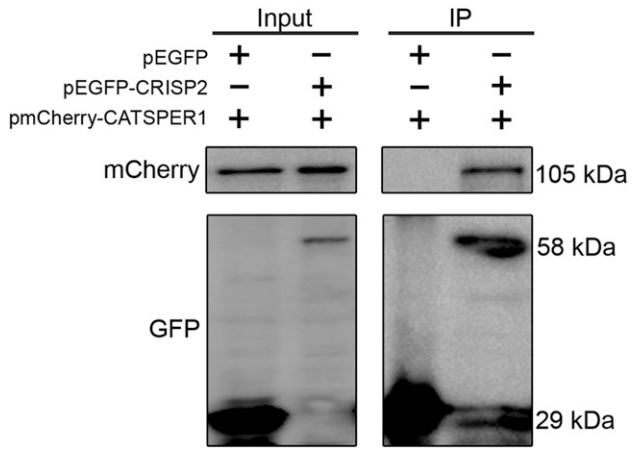

Figure 6. Heterologous CRISP2 and CATSPER1 interact. Pulldown experiments showing that pEGFP-CRISP2 can bind to pmCherry-CATSPER1 in HEK293 cells. Input equals the total cell lysate. IP, immunoprecipitated material.

fertility (36). We found that heterologous CRISP2 and CATSPER1 interact, in addition to the previously defined ability of CRISP2 to regulate calcium flow through ryanodine receptors. CRISP2 and CATSPER both localize to the principal piece of the sperm tail (26, 36), suggesting that CRISP2 binds to the native channel in sperm. The lack of a suitable CATSPER1 antibody precluded the analysis of a potential interaction *in vivo*. However, the presence of the stiff midpiece within sperm lacking CRISP2 is consistent with perturbed calcium flow and the previously defined role of CRISP2, and several other mammalian and reptile CRISPs, in ion channel regulation (8, 10, 23, 47–50). At this point, however, it is unclear whether the stiff midpiece is caused by altered CATSPER function, as suggested by the current data, or by altered calcium flow from ryanodine receptor–regulated calcium stores localized in the connecting piece at the junction of the sperm nucleus and flagellum (8, 51), or potentially a combination of both. The precise role of CRISP2 in ion channel regulation within sperm and the kinematics of flagellum function should be defined in future studies.

Data presented here also support a role for CRISP2 in establishing the ability of sperm to undergo the acrosome reaction. The acrosome reaction is a modified form of exocytosis that is necessary for sperm to penetrate the outer vestments of the oocyte before fertilization (50). The loss of CRISP2 leads to a greatly decreased ability to undergo the acrosome reaction. The ability of some CRISP2-deficient sperm to undergo the acrosome reaction, and thus achieve some residual fertility, suggests functional redundancy with other CRISPs in sperm. Although loss of either CRISP1 or CRISP4 also independently leads to a compromised ability to undergo the acrosome reaction (22, 23, 51–54), it is unlikely that there is compensation between CRISP2 and CRISP1 or CRISP4 *in vivo*. CRISP2 is an intracellular protein incorporated into sperm during spermatogenesis, whereas CRISP1 and CRISP4 are extracellular proteins added onto sperm during epididymal maturation (55, 56). However, previous research has raised the possibility that immediately before loss of the plasma and outer acrosomal membranes during the acrosome reaction, they become partially porous, and acrosomal proteins, including CRISP2, are released and may thus act extracellularly (57, 58). However, the precise biochemical pathways, either intracellular or extracellular, through which CRISP2 functions remain unknown.

In contrast to the now demonstrated roles for CRISP2 in sperm motility and the acrosome reaction, this study does not support an essential role for CRISP2 in germ cell–Sertoli cell adhesion, as had been suggested previously (5, 34). However, the possibility of an accessory role for CRISP2 in cell-cell adhesion remains, and as suggested by the work of Maeda *et al.* (34), it is probably mediated by the amino terminal 101 amino acids of the CAP domain. Also of note, although a hypothesized role for CRISP2 in sperm-oocyte adhesion does support a role in cell-cell adhesion (7), the data in the current manuscript suggest that the latter cannot be mechanistically distinguished from the compromised ability of CRISP2-deficient sperm to undergo the acrosome reaction.

Lastly, the results presented here are in partial contrast to those published from a separate *Crisp2* knockout model. Brukman *et al.* (59) reported comparable conception rates between knockout and heterozygous males but decreased fertilization rates in hemivasectomized males or males mated with hormone-treated females. Although the precise reasons for these differences are difficult to define, the original publication was confounded by two primary factors. First, heterozygous males were used as controls in natural mating experiments, whereas data presented herein indicate that any loss of CRISP2 will compromise fertility parameters. Second, vasectomized males were used to model physiological stress, despite the known inflammatory effects that will ultimately lead to the development of antisperm antibodies, thus plausibly impeding sperm function independent of a genetic variation (60–63). Furthermore, the use of a mixed background strain [mixed C57BL/6J, DBA/2J (D2), C57BL/6N] compared with the pure C57BL/6N and mixed C57BL/6N, FVB background used herein may have led to subtle differences in phenotypes. Regardless, both studies indicate a role for CRISP2 in establishing maximal fecundity.

The CRISPs are a clade of the CAP superfamily of proteins and collectively show a notable enrichment of expression to the male reproductive tract wherein they

either are incorporated into sperm, in the case of CRISP2, or interact with the plasma membrane, as in the case of CRISP1, CRISP3, and CRISP4. Although no individual CRISP is necessary for male fertility, evidence increasingly suggests that at least CRISP1, CRISP2, and CRISP4 are necessary for optimal sperm function (22, 23, 52, 59, 64). For a number of cellular functions, but not all, CRISPs appear to act redundantly (22). Based on this evidence, we propose that in species wherein polyandrous mating is the norm, or in situations of physiological stress, the presence of high CRISP concentrations may offer sperm a competitive advantage and may be the difference between successful fertilization or none. This hypothesis is consistent with the positive evolution of CRISP genes (65) and the correlation between high semen CRISP3 content and male fertility in horses (66). These data also explain a previous correlation between *CRISP2* expression in spermatozoa and human male fertility parameters (11, 12). In summary, the data presented in this article define a role for CRISP2 in optimal sperm motility and acrosome function.

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