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Functional redundancy and compensation: Deletion of multiple murine *Crisp* genes reveals their essential role for male fertility

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Abstract

Mammalian Cysteine-RIch Secretory Protein (CRISP) family includes four members present in sperm and reported to regulate Ca^{2+} channels and fertilization. Based on our previous observations using single knockouts models and suggesting the existence of functional compensation among CRISP proteins, we investigated their relevance for male fertility by generating multiple *Crisp* gene mutants by CRISPR/Cas9 technology. Whereas targeting of *Crisp1* and *Crisp3* yielded subfertile males with early embryo developmental defects, the same deletion in zygotes from fertile *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ mice led to the generation of both triple and quadruple knockout mice exhibiting a complete or severe disruption of male fertility due to a combination of sperm transport, fertilization, and embryo developmental defects linked to intracellular Ca^{2+} dysregulation. These observations reveal that CRISP proteins are essential for male fertility and organize in functional modules that contribute distinctly to fertility success, bringing insights into the mechanisms underlying functional redundancy/compensation in protein families and emphasizing the importance of generating multiple and not just single knockout which might be masking the true functional relevance of family genes.

KEYWORDS

development, embryo, fertilization, protein family, sperm

Abbreviations: Cas9, CRISPR associated protein 9; CASA, computer assisted sperm analysis; CatSper, Cation channel of Sperm; COC, cumulus-oocyte complexes;; CRD, Cysteine-Rich Domain; CRISP, Cysteine Rich Secretory Proteins; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; DKO, double knockout; P4, progesterone; PI, propidium iodide; PR-1, Pathogenesis-related-1 domain; QKO, quadruple knockout; MFI, mean fluorescence intensity; sgRNAs, single guide RNAs; TKO, triple knockout; UTJ, utero-tubal junction; ZP, zona pellucida.

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1 | INTRODUCTION

Understanding of human physiology and disease has significantly benefited from the generation of genetically modified mice which allows to elucidate mammalian gene function in vivo. This type of studies became more feasible in recent years due to the availability of the novel CRISPR/Cas9 technology which represents a quick and efficient system of genome editing.[1] In particular, the development of mutant mice has revolutionized the reproduction field due to the finding that only few of the proteins previously known to have critical roles in the fertilization process resulted indispensable for animal fertility in gene knockout studies.[2-4] A possible explanation for these observations may rely on the existence of functional redundancy among members of the same family with partial overlapping functions which may compensate for each other's loss[5] and significantly contribute to strengthen reproductive success and preservation of species. This could be especially important in sperm which are transcriptionally and translationally silent cells.[6]

Functional redundancy and compensation have been recently proposed to exist among members of the highly evolutionarily conserved Cysteine-RIch Secretory Proteins (CRISP)[7] characterized by the presence of 16 conserved Cys, 10 of which are located in the C-terminal region containing both a Cysteine-Rich Domain (CRD) and a hinge that connects it to the plant Pathogenesis-related-1 (PR-1) domain located in the N-terminus of the molecule.[8] Evidence indicates that these two domains have evolved to perform a variety of functions that rely on these different domains. Whereas the PR-1 domain has been implicated in membrane interaction and protease activity, the C-terminal region has been found to exhibit ion channel regulatory properties.[9-14] In mammals, four members of the CRISP family have been described which are distributed along the male and female reproductive tracts[8] and reported to be involved in different stages of the fertilization process.[15,16] CRISP1, the first described member of the family,[17] is an epididymal glycoprotein that associates with the surface of sperm during maturation.[18,19] Different biochemical approaches revealed roles for CRISP1 in both sperm interaction with the zona pellucida (ZP) and gamete fusion through its binding to complementary sites localized in the ZP and egg plasma membrane, respectively.[20-24] Interestingly, results revealed that whereas the sperm-ZP binding ability of CRISP1 depends on the conformation of the protein,[24] the egg binding ability resides in a region of only 12 amino acids within the PR-1 domain that corresponds to a highly conserved motif of the CRISP family.[25] The functional roles of CRISP1 in fertilization were subsequently confirmed by the impaired in vitro fertilizing ability of sperm obtained from mice lacking CRISP1.[26] However, in spite of these sperm functional defects, CRISP1-deficient males were fertile, at least under standard laboratory mating conditions.[26] Parallel studies from our group revealed that another member of the CRISP family, sperm testicular protein CRISP2, is also involved in sperm-egg fusion through its binding to the same egg complementary sites of CRISP1.[23] Moreover, we observed that the ability of *Crisp1*$^{-/-}$ sperm to fuse with the egg in vitro was affected by the presence of CRISP2, suggesting the existence of a functional cooperation between CRISP1 and CRISP2 during gamete fusion.[26] Consistent with this idea, mice lacking *Crisp2* were also fertile in spite of exhibiting several sperm fertilizing deficiencies under in vitro conditions.[27,28] However, when the reproductive performance of these animals was challenged by either reducing the number of sperm in their ejaculates (through unilateral vasectomy) or increasing the number of oocytes to be fertilized in vivo (using superovulated females), *Crisp2*$^{-/-}$ males showed significantly lower fertility rates compared to their wild-type littermates.[27] These observations support the idea that both CRISP1 and CRISP2 play important roles in the fertilization process that are partially masked in each individual gene knockout mouse model by compensatory mechanisms and become evident only under more demanding conditions that better reflect the natural environment.

The mouse CRISP family also includes CRISP3 present in submandibular and lacrimal gland, uterus, ovary, and prostate[29-31] as well as CRISP4, principally expressed in the epididymis.[32] While there are no loss-of-function studies for CRISP3, results from different groups showed that male mice bearing a deletion in the *Crisp4* gene are fertile[7,14,33] although their sperm failed to interact with the ZP and fuse with the oolemma[7,33] as previously described for CRISP1, suggesting that CRISP4 could be partially compensating the impaired sperm fertilizing ability of *Crisp1*$^{-/-}$ mice, and vice versa. Together, our observations using knockout models for individual CRISP support the involvement of each CRISP in more than one stage of fertilization and the participation of more than one CRISP in each fertilization step.[15,16,34] Considering the possible functional redundancy and compensation among CRISP family members, we then generated double mutant mice simultaneously lacking *Crisp1* and *Crisp4*. Because these two genes are located in different chromosomes, we and others generated *Crisp1*$^{-/-}$.*Crisp4*$^{-/-}$ mice by cross-breeding the corresponding single knockout mouse colonies.[7,35] Interestingly, *Crisp1*$^{-/-}$.*Crisp4*$^{-/-}$ males were subfertile supporting the existence of functional redundancy and compensation between the two epididymal CRISP family members.[7]

Of note, CRISP proteins are also expressed in humans.[29] Unlike mice, there are only three instead of four CRISP proteins in men,[8] having been proposed that human epididymal CRISP1 represents the combination of mouse epididymal CRISP1 and CRISP4 based on sequence homology, tissue distribution, and in vitro functional roles.[7,21,22,24,32,36] As human *CRISP* genes (*CRISP1*, *CRISP2,* and *CRISP3*) are typically clustered within less than 200 kb, a possible chromosome aberration may hamper the three genes simultaneously with

potential clinical implications. Based on this, and considering the possible existence of functional redundancy and compensation among CRISP family members, in the present study, we investigated the relevance of this family for animal fertility by CRISPR/Cas9-mediated targeting of multiple *Crisp* genes.

2 | MATERIALS AND METHODS

2.1 | Chemicals

All the chemicals employed were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

2.2 | Animals and ethical approval

Animals were housed at 23°C under a 12/12 hours light/dark cycle with ad libitum access to food and water. Approval for the study protocol was obtained from the IACUC of the Institute of Biology and Experimental Medicine (protocol N° 26/2018). All protocols were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH).

2.3 | Generation of mutant mice

The double mutant mouse line *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ was obtained by successive cross-breeding of the individual *Crisp2*$^{-/-}$ and *Crisp4*$^{-/-}$ C57BL/6;DBA (B6;D2) lines.[7,27] Some of the animals from this colony carry, in an independent *locus*, a transgenic EGFP under the actin promoter fused to the proacrosin signal peptide.[37]

The CRISPR/Cas9 system was used to generate deletions encompassing *Crisp1* and *Crisp3* coding sequences. Single guide RNAs (sgRNAs) were selected using crispr.mit.edu website of Zhang Lab. Guides 5′-CAGCACAAGCATTAATGCCA-3′ and 5′-GTTATCTTGAAGAAGGGATG-3′ were selected because they fell next to the ATG site in the second exon of both genes, and had a high quality score and thus low chances of producing off-target mutations. Each guide was separately subcloned in plasmid DR274 (Addgene Plasmid #42250) and synthesized using MEGAshortscript T7 Transcription Kit (Ambion, Cat# AM1354). Cas9 mRNA was synthesized from plasmid MLM3613 (Addgene Plasmid #42251) using mMESSAGE mMACHINE T7 Transcription Kit (Ambion, AM1344) and Poly(A) Tailing Kit (Ambion, AM1350). Cas9 mRNA (50 ng/μL) and sgRNAs (50 ng/μL each) were injected in the cytoplasm of zygotes obtained from wild-type C57BL/6J or *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ colonies.

Genotyping was carried out by PCR using mouse genomic DNA obtained from ear punch biopsies and the primers listed in Table S1. The mutations were confirmed by sequencing of PCR products. In all cases, animals from the same colony that resulted either wild-type or heterozygous for mutations in *Crisp* genes were used as controls.

2.4 | Fertility tests

Fertility was tested by caging a male mouse (2-6 month old) with a control female (2-6 month old) for 5 days and scoring the number of born pups 21 days after observation of copulatory plug.

2.5 | In vivo fertilization assays and in vitro embryo development

Males were caged individually for one night with either a natural estrus or superovulated female. The estrous cycle stage of mature females was determined by vaginal smear examinations.[38] For ovulation induction, adult females were treated with an i.p. injection of equine chorionic gonadotropin (eCG; 5 IU; Syntex SA, Buenos Aires, Argentina), followed by an ip injection of human chorionic gonadotropin (hCG; 5 IU, Syntex SA) 48 hours later. Mating was evaluated the following morning and considered successful by the presence of copulatory plugs. In those cases, the eggs were recovered from the oviducts, placed in KSOM medium[39] supplemented with 0.1% (w/v) of bovine serum albumin (BSA), covered with paraffin oil (Ewe, Sanitas SA, Buenos Aires, Argentina), and incubated overnight at 37°C in an atmosphere of 5% (v/v) CO_2 in air. Eggs were considered fertilized when they reached the two-cell stage. In some experiments, embryos were incubated for additional 4 days to evaluate their development under a stereoscopic microscope.[40]

2.6 | Assessment of uterine and oviductal sperm

Male mice expressing a transgene for an acrosomal EGFP were mated with superovulated females to detect sperm within the oviduct, as previously described.[41] Briefly, a wild-type female, subjected to superovulation, was caged for 30 minutes with a transgenic male 12 hours after hCG administration. After 4 hours detection of copulatory plug, the uterus and oviduct were recovered and placed in a dish for subsequent evaluation of the contained sperm. Uterine sperm were recovered by incising the uterine walls and allowing the release of the uterine fluid that was then directly observed under a microscope (Nikon ECLIPSE E200; Basler acA-78075gc

21517342 camera; pylon5 viewer) at 40× magnification. For examination of oviductal sperm, the oviducts were placed in KSOM medium supplemented with 0.3% (w/v) of BSA, mounted on slides, covered with coverslips and immediately observed under an Olympus IX83 Spinning Disk microscope (Olympus Corp., Tokyo, Japan) at ×40 and ×100 magnification. The number of fluorescent sperm within the oviduct was evaluated subjectively.

2.7 | Epididymal sperm collection and in vitro capacitation

Sperm were collected by incision of the two cauda epididymides in 300 µL of capacitation media (Fraser and Drury, 1975) supplemented with 0.3% (w/v) of BSA and covered with paraffin oil. The cells were transferred to 300 µL of fresh medium under paraffin oil, at a final concentration of 1-10 × 10^6 sperm/mL, and capacitation was carried out for 90 minutes at 37°C in an atmosphere of 5% (v/v) CO_2 in air.

2.8 | In vitro fertilization assays

Cumulus-oocyte complexes (COC) were collected from superovulated females 12-15 hours post-hCG administration. When needed, cumulus cells were removed by incubating the COC for 3-5 minutes in 0.3 mg/mL hyaluronidase (type IV). In some cases, the ZP was dissolved by treating the eggs with acid Tyrode solution (pH 2.5) for 10-20 seconds.[42] COC and ZP-intact eggs were inseminated with capacitated sperm (final concentration: 1-5 × 10^5 cells/mL) and the gametes coincubated in capacitation media for 3 hours at 37°C in an atmosphere of 5% (v/v) CO_2 in air. For fusion assays, ZP-free eggs were inseminated with capacitated sperm (final concentration: 1-5 × 10^4 cells/mL) and the gametes coincubated for 1 hour. In all cases, eggs were fixed with 2% of paraformaldehyde in PBS, washed, stained with 10 µg/mL Hoechst 33342, mounted on slides and finally analyzed under a Nikon Optiphot microscope (Nikon, Tokyo, Japan) equipped with epifluorescence optics (×200). Eggs were considered fertilized when at least one decondensing sperm nucleus or two pronuclei were observed in the egg cytoplasm.

2.9 | Analysis of sperm functional parameters

2.9.1 | Sperm count and viability

Epididymal sperm concentration was determined using a hemocytometer. Viability was assessed by staining sperm with prewarmed 0.5% (v/v) eosin Y and dye exclusion (indicative of sperm viability) analyzed under light microscopy (×400).

2.9.2 | Acrosome reaction

Spontaneous and progesterone-induced acrosome reaction was analyzed by Coomassie Brilliant Blue staining. For progesterone induction, sperm were exposed to 30 µM of the hormone during the last 15 minutes of capacitation. Once the incubation period was completed, sperm were fixed with 1 vol of 8% of paraformaldehyde in PBS and washed with 0.1 M of ammonium acetate, pH 9, mounted on slides and air-dried. For Coomassie Brilliant Blue staining, slides were washed by successive immersions in water, methanol, and water (5 minutes each) and then, incubated in 0.22% (w/v) Coomassie brilliant blue G250 solution (50% methanol and 10% acetic acid) for 3 minutes. After staining, slides were thoroughly washed with distilled water, mounted and observed under a light microscope (×400). Sperm were scored as acrosome-intact when a bright blue staining was observed in the dorsal region of the head or as acrosome-reacted when no labeling was observed.

2.9.3 | Motility

Sperm aliquots (15 µL) were placed between prewarmed slides and cover slips (22 × 22 mm) to create a chamber with 30 mm depth, and were examined at 37°C using the ISASw (Integrated Semen Analysis System) v1.2 Computer-assisted sperm analysis (CASA) system (Proiser R&D, SL, Valencia, Spain) or the Sperm Class Analyzer system (SCA v.6.2.0.1., Microptic SL, Barcelona, Spain) for *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ and *Crisp1*$^{-/-}$.*Crisp2*$^{-/-}$.*Crisp3*$^{-/-}$.*Crisp4*$^{-/-}$ (QKO) colonies, respectively. In both colonies, for each sample, at least two hundred cells were evaluated. For *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$, cells distributed in a minimum of twenty different microscope fields were evaluated (30 frames acquired at 60 Hz for each measurement) and sperm were considered motile when showing straight line velocity (VSL, µm/s) > 0, and hyperactivated when presenting curvilinear velocity (VCL, µm/s) ≥ 271, linearity (LIN, %) < 23.6 and amplitude of lateral head displacement (ALH, µm) ≥ 5. For QKO sperm, cells distributed in a minimum of ten different microscope fields were evaluated (30 frames acquired at 60 Hz for each measurement) and sperm were considered hyperactivated when presenting VCL ≥ 238.5, LIN < 33, and ALH ≥ 4.22. These custom cutoffs were calculated based on the values corresponding to control animals in each colony, according to our previous experience[7,27] and recommendations.[43]

2.9.4 | Intracellular Ca^{2+} levels

Cytoplasmic Ca^{2+} levels in sperm were measured by flow cytometry as previously described.[27,44] Briefly, after 60 minutes of incubation in capacitation medium, sperm were loaded with 2 mM of Fluo-4 AM (Invitrogen, Carlsbad, California, USA) diluted in 10% (w/v) of Pluronic F-127 (Invitrogen) and incubated for an additional 30 minutes. Samples were washed to remove the excess of probe, resuspended in BSA-free medium, and exposed to 2.5 μg/mL of propidium iodide (PI) just before measurement. Fluorescence was detected using a BD FACSCantoTM II analyzer following the manufacturer's indications. Data analysis was performed by FlowJo 10 software (FlowJo LLC, Ashland, OR, USA).

2.10 | Protein extract preparation and Western blot analysis

Organ samples were homogenized in ice-cold lysis buffer (phosphate-buffered saline (PBS) containing 5 mM EDTA, 1% (w/v) IGEPAL CA360, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 5 mM MgCl2, and 10 mM HEPES, pH 7.2) with 0.2 mM of phenylmethylsulfonyl fluoride. The homogenates were then centrifuged at 10 000 *g* for 20 minutes at 4°C and an aliquot of the supernatant diluted in Laemmli sample buffer.[45] Sperm aliquots (1×10^6 spermatozoa) obtained before or after capacitation were washed with PBS and resuspended in Laemmli sample buffer. In all cases, samples were boiled, centrifuged at 5000 *g* for 5 minutes and the supernatants were recovered. For tyrosine phosphorylation assessment, the supernatants were boiled again in the presence of 70 mM 2-β-mercaptoethanol.

Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 2% skim milk in PBS, the membranes were probed with either anti-mouse CRISP1 (1:1000; #AF4675; R&D Systems, Minneapolis, MN, USA) or anti-human CRISP2 (1:1000; #AF2575; Proteintech, Chicago, IL, USA) or anti-mouse CRISP4 (1:1000; #AF5017; R&D Systems) polyclonal antibodies, or anti-phosphotyrosine monoclonal antibody (1:10 000; clone 4G10; Upstate, Lake Placid, NY, USA). Protein loading was analyzed by β-tubulin immunoblot (anti-β-tubulin antibody; 1:5000; clone D66) or anti-actin monoclonal antibody (1:2000; Santa Cruz sc-8432). Next, the membranes were incubated with the corresponding peroxidase-conjugated secondary antibody (1:4000). The immunoreactive proteins were detected by ECL Western Blotting kit (GE Healthcare UK Ltd, Buckinghamshire, England) and images captured with G:BOX GENI (Syngene, Synoptics Ltd, Cambridge, England) according to the manufacturer's instructions.

2.11 | Statistical analysis

Data represent the mean ± SEM of at least three independent experiments and "n" indicates the number of animals analyzed in each group and included in each figure legend. Calculations were performed using the Prism 6.0 software (GraphPad Software, La Jolla, CA). Comparisons between two experimental groups were made by the Student's *t* test and comparisons among three or more were made by one-way analysis of variance (ANOVA) and Bonferroni's post-test. As an exception, spontaneous and induced acrosome reaction percentages were evaluated by two-way ANOVA followed by Bonferroni's test. For Contingency tables analysis, Fisher's exact test was used. Linear correlation analysis was tested calculating Pearson's correlation coefficient. Differences were considered significant at a level of $P < .05$.

3 | RESULTS

To generate multiple (double, triple, and quadruple) *Crisp* mutants, we used a CRISPR/Cas9 based strategy to simultaneously inactivate *Crisp1* and *Crisp3 loci* in wild-type zygotes or in zygotes taken from a *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ colony. The premise of this strategy relied on the high similarity between *Crisp1* and *Crisp3* coding sequence which allowed us to target these two genes (located in the same chromosomal *locus*) with the same sgRNA, and the possibility of generating mice lacking both *Crisp2* and *Crisp4* genes (located in different chromosomes) due to the availability of the individual colonies in our laboratory.[7,27]

3.1 | Generation and characterization of *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ mice

Double *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ mice were generated by successive cross-breeding of *Crisp2*$^{-/-}$ and *Crisp4*$^{-/-}$ single colonies. DNA analysis by PCR from ear biopsies confirmed the presence of homozygous null mutations in *Crisp2* and *Crisp4* alleles whereas Western blot studies failed to detect CRISP2 and CRISP4 proteins in sperm extracts (Figure S1). To evaluate fertility, *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ males individually caged were mated with wild-type females for 5 days and the number of born pups were analyzed 21 days after detecting copulatory vaginal plugs. No differences were found in the average litter size compared to control males even when double mutant males were mated with double mutant females (Figure 1A). However, when fertilization in the oviduct was evaluated under more challenging conditions such as mating double mutant males with superovulated wild-type females,[27] a marked decrease in fertilization

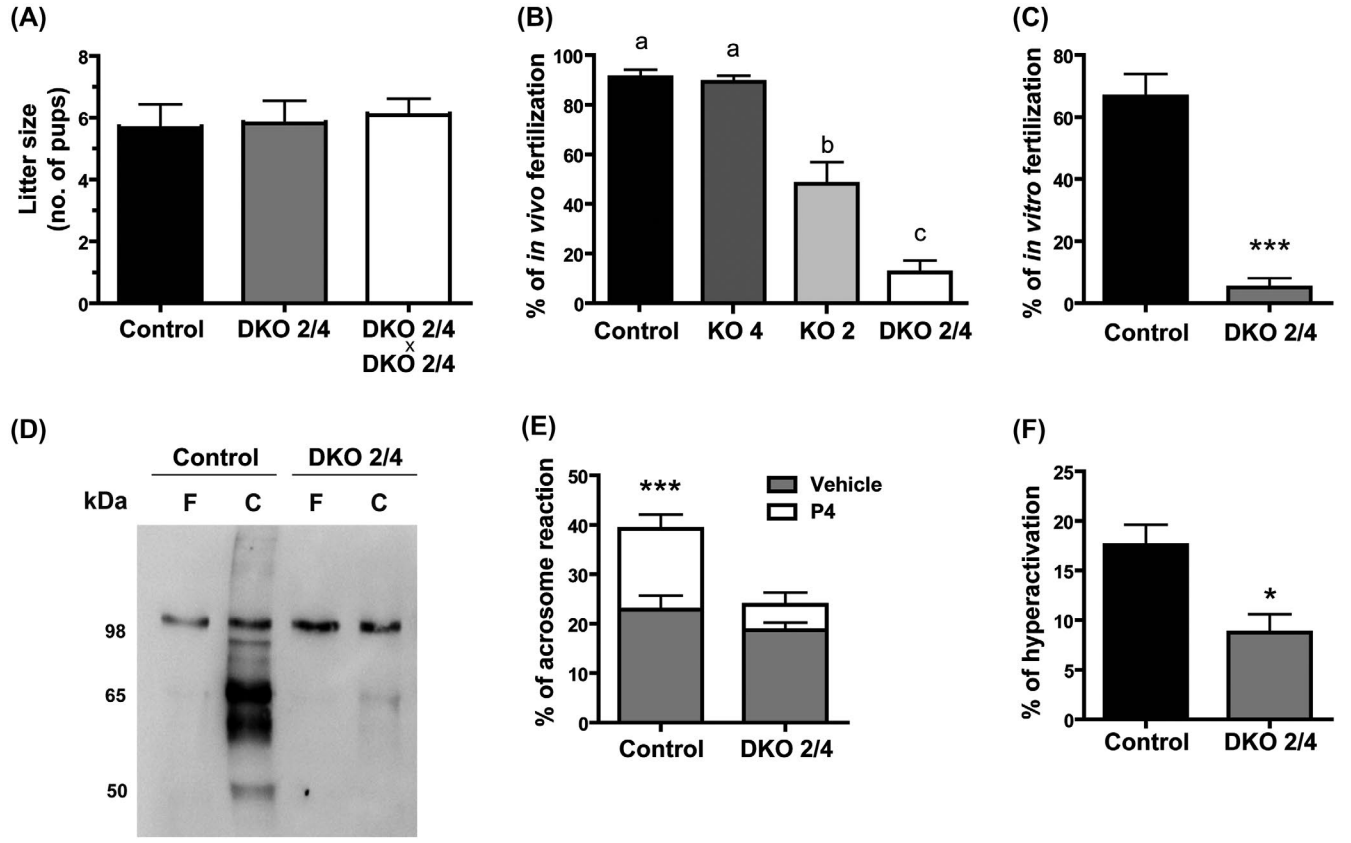

FIGURE 1 **Reproductive phenotype of *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ mice**. A, Control and *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ (DKO 2/4) males were housed with control or DKO 2/4 females and litter size was determined. Data are mean ± SEM. n = 10 for control and DKO 2/4 groups and n = 6 for DKO 2/4 × DKO 2/4 group; not statistically significant. B, Control, *Crisp2*$^{-/-}$ (KO 2), *Crisp4*$^{-/-}$ (KO 4) and DKO 2/4 males were mated with control superovulated females and the percentage of fertilized eggs recovered from the ampulla was evaluated the following day. Data are mean ± SEM. n ≥ 4; different letters indicate statistical significance ($P < .05$). C, Control and DKO 2/4 sperm capacitated for 90 minutes were coincubated with cumulus-intact eggs for 3 hours and the percentage of fertilization determined. Data are mean ± SEM. n = 4; ***$P < .001$. D-F, Control and DKO 2/4 sperm were incubated under capacitating conditions for 90 minutes and different functional parameters were evaluated. D, Protein tyrosine phosphorylation analyzed by western blotting using an anti-phosphotyrosine antibody. A representative blot is shown. n = 4. E, Percentage of acrosome reaction determined by Coomassie Brilliant Blue staining in sperm exposed to progesterone (P4) or dimethyl sulfoxide alone (vehicle) during the last 15 minutes of capacitation. Data are mean ± SEM. n = 8; ***$P < .001$ vs vehicle. F, Percentage of hyperactivation evaluated by CASA. Data are mean ± SEM. n = 5; *$P < .05$

rates was observed in *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ males compared to controls or to either *Crisp2* or *Crisp4* single mutant males (Figure 1B). Consistent with these results and the reported roles for CRISP2 and CRISP4 in fertilization,[7,27,28,35] sperm from *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ mice showed severe defects in their ability to fertilize cumulus-oocyte complexes in vitro (Figure 1C) as well as in several capacitation-associated parameters such as tyrosine phosphorylation, progesterone-induced acrosome reaction and hyperactivation (Figure 1D-F).

3.2 | Generation and characterization of *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ mice

Based on the high DNA sequence identity of *Crisp1* and *Crisp3*, we were able to introduce null mutations simultaneously into these two syntenic genes by using a pair of sgRNAs designed to target exon 2 coding sequences separated by approximately 40 bp, with one of the cutting positions next to the translation initiation site (Figure S2A). The sgRNAs together with Cas9 mRNA were first microinjected in wild-type zygotes to generate a *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ colony not developed so far. From a total of 259 C57BL/6 zygotes microinjected embryos, 168 were transferred to the oviduct of 9 foster mothers and 34 live pups (F0) were born (Table S2).

Mutations in F0 mice were assessed by PCR of genomic DNA using primers (Table S1) flanking the targeted exon 2 of each gene, followed by gel electrophoresis. Results revealed that 12 out of 34 F0 mice presented mutations in both *Crisp1* and *Crisp3* (Figure S2B; Table S2). Mating of one F0 female exhibiting both mutations (ie, animal #27) (Figure S2B) with a wild-type male generated nine pups (F1) that, when analyzed by PCR, showed different mutations in *Crisp1* and *Crisp3* genes (Figure S2C), indicating

the presence of a high level of mosaicism in the F0 germline. Three out of the nine F1 pups presented mutant alleles in heterozygosity for both *loci* detectable by agarose gel electrophoresis (Figure S2C). Considering that *Crisp1* and *Crisp3* are closely located within the same chromosome having low chances of segregation, the presence of mutations in both genes in F1 mice suggested these mutations were located in the same homologous chromosome inherited from the F0 rather than the WT founder. Sequencing of PCR products of one of the three F1 pups with mutations in the two genes confirmed the deletion of regions of 55 bp in *Crisp1* and of 73 bp in *Crisp3*, both of which included the translation initiation sites and the signal peptide sequences (Figure S2D) thus, eliminating any possibility of expression of the secreted full-length proteins. Consistent with this, the mutation size for each allele was comparable to the size of the bands detected in agarose gels. Based on these observations, that animal was finally selected as founder of the $Crisp1^{-/-}.Crisp3^{-/-}$ colony, whereas none of the available commercial antibodies against murine CRISP3 was capable of specifically detecting the protein in any of the control tissues tested, analysis by Western blot confirmed that $Crisp1^{-/-}.Crisp3^{-/-}$ males lack CRISP1 (Figure S2E). Mating of $Crisp1^{-/-}.Crisp3^{-/-}$ and control males with wild-type females showed a significant reduction in the number of born pups corresponding to the mutant mice when compared to controls (Figure 2A). With the aim of investigating the mechanisms underlying this subfertility, $Crisp1^{-/-}.Crisp3^{-/-}$ males were mated with control estrus or superovulated females, and the percentage of fertilized eggs recovered from the oviduct was determined. We found no differences in the fertilization rates between mutant and control males under the two mating conditions (Figure 2B), suggesting the existence of postfertilization defects as potential responsible for the subfertility of this colony. To investigate this possibility, fertilized eggs recovered from the ampulla of superovulated females mated with either mutant or control males were incubated in vitro for 4 days to analyze their ability to reach the blastocyst stage. In this case, results revealed that the percentage of blastocysts was significantly lower for $Crisp1^{-/-}.Crisp3^{-/-}$ than for control males (Figure 2C), supporting that CRISP1 and/or CRISP3 are relevant for early embryo development.

3.3 | Generation of triple and quadruple *Crisp* knockout mice by CRISPR/Cas9

As proposed, we next microinjected the *Crisp1/Crisp3* sgRNAs into zygotes from the $Crisp2^{-/-}.Crisp4^{-/-}$colony. Of a total of 111 microinjected zygotes, 65 were transferred to 3 foster mothers, one of which gave birth to 4 female pups. Analysis of mutations in these pups (F0) by PCR of genomic DNA using the primers described before (Table S1) showed that two of them (ie, animals #2 and #4) presented large deletions in both *Crisp1* and *Crisp3 loci* that were visible in an agarose gel (Figure S3A, Table S2). When these two animals were mated with wild-type partners, only female #2 gave birth to pups (F1). PCR analysis using another set of primers flanking exon 2 (Table S1) revealed that whereas the four F1 born animals contained a mutant allele for *Crisp1* and *Crisp3* in heterozygosity, two of them (#16 and #18) exhibited larger deletions than the remaining ones (#15 and #17) (Figure S3B). Sequencing of PCR products confirmed the presence of deletions in each allele of sizes comparable to those corresponding to the bands detected in the agarose gel (Figure S3C). As mentioned before, *Crisp1*, *Crisp2,* and *Crisp3* genes are closely located within the same chromosome having low chances of segregation and behaving like a single allele. Based on this, and considering that the female progenitor was null for CRISP2, it can be concluded that the mutations in *Crisp1* and

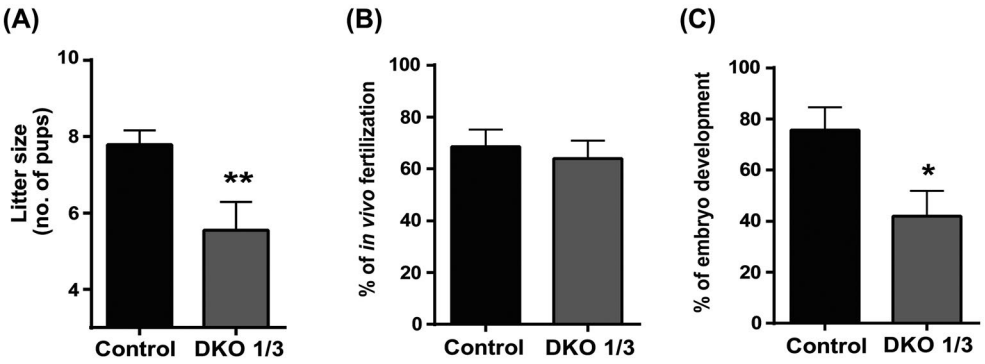

FIGURE 2 **Reproductive phenotype of $Crisp1^{-/-}.Crisp3^{-/-}$ mice**. A, Control and $Crisp1^{-/-}.Crisp3^{-/-}$ (DKO 1/3) males were housed with wild-type females and litter size was determined. Data are mean ± SEM, n = 8 for control and n = 7 for DKO 1/3 males; $^{**}P < .01$. B, Control and DKO 1/3 males were mated with superovulated females and the percentage of fertilized eggs recovered from the ampulla the following day was determined. Data are mean ± SEM. n = 7 for control and n = 8 for DKO 1/3 males; not statistically significant. C, Control and DKO 1/3 males were mated with superovulated females and the percentage of in vitro embryo development obtained 4 days later was determined. Data are mean ± SEM. n = 5 for control and n = 6 for DKO 1/3 males; $^{*}P < .05$

Crisp3 must be located within the same homologous chromosome as *Crisp2* inherited from the F0 founder. On the contrary, as *Crisp4* is located in another chromosome, its independent segregation would allow the generation of both triple knockout (TKO) mice lacking *Crisp1, Crisp2,* and *Crisp3* genes as well as quadruple knockout (QKO) mice deficient in the four members of the CRISP family.

The new colony was established employing mice carrying relatively large deletions in *Crisp1* and *Crisp3* (162 and 71 bp, respectively) which included the translation initiation sites and signal peptide sequences (Figure S3C). Genotyping of these animals was carried out by PCR using the same primers described above (Table S1). The absence of both epididymal CRISP1 and CRISP4, and testicular CRISP2 in males from the new colony (Figure S3D) confirmed the success of the strategy employed.

TABLE 1 Reproductive phenotype of triple (TKO) and quadruple (QKO) *Crisp* knockout males

	Control	TKO	QKO
Litter size (N° pups)	7.0 ± 0.5	0.9 ± 0.5****	0.9 ± 0.4****
Sterile mice	0 (0%)	3 (50%)	4 (50%)
In vivo fertilization (%)	89.3 ± 3.0	21.2 ± 9.3****	17.9 ± 7.8****

Note: Fertility is expressed as the average of n° of pups per litter with each male being evaluated at least twice. Those males that sired no offspring, but left copulatory plug, were considered sterile. Data are mean ± SEM, n = 17 for control, n = 6 for TKO and n = 8 for QKO; ****$P < .0001$. For in vivo fertilization assays, Control, TKO and QKO males were mated with estrus females and the percentage of fertilized eggs recovered from the ampulla was evaluated the following day. Data are mean ± SEM; n = 15 for control, n = 6 for TKO males, n = 6 for QKO; ****$P < .0001$.

3.4 | Characterization of triple and quadruple *Crisp* knockout mice

3.4.1 | Fertility and in vivo fertilization

Mating quadruple heterozygous male and female mice yielded TKO as well as QKO mice following the expected Mendelian frequency (TKO: 14.98% vs expected 18.75% and QKO: 7.12% vs expected: 6.25%, n = 267, n.s.). Examination of mutant adult mice revealed they were normal in terms of growth, general health and behavior, indicating that CRISP proteins are not essential for overall animal development under laboratory conditions. As previously reported for $Crisp1^{-/-}.Crisp4^{-/-}$ mice,[7] a subgroup of TKO and QKO mice showed an epididymo-orchitis phenotype with dead sperm in their epididymis, the reason why subsequent studies were carried out using only those mutant males which did not exhibit this phenotype. These animals were identified based on our observations revealing that males with epididymo-orchitis leave no sperm within the uterus after their mating with superovulated females. Fertility studies showed that whereas all control males were fertile, both TKO and QKO males exhibited an average of less than one born pup and a high proportion of sterile males (Table 1), confirming that CRISP family members play an essential role in male fertility.

Based on previous findings in $Crisp1^{-/-}.Crisp4^{-/-}$ males[7] and present observations in $Crisp1^{-/-}.Crisp3^{-/-}$ males, fertility failure in TKO and QKO males could be due to impaired in vivo fertilization and/or embryo development. To investigate these possibilities, we next determined the percentage of fertilized eggs recovered from the oviduct of estrus females mated with mutant or control males. Consistent with fertility rates, TKO and QKO males led to significantly lower in vivo fertilization rates compared to controls (Table 1). As the decrease in in vivo fertilization could be a consequence of sperm defects to reach and/or to fertilize the eggs, we then examined sperm transport within the female tract as well as sperm fertilizing ability. Sperm transport studies were carried out by mating TKO and QKO males with hormone-stimulated females and examining the presence of sperm within both the uterus and oviduct, 4 hours after confirmation of mating. As controls, the same parameters were analyzed in females mated with either wild-type or $Crisp1^{-/-}.Crisp3^{-/-}$ males which showed normal in vivo fertilization rates (see Figure 2B). Direct observation of uterine fluids under the microscope showed that whereas most wild-type sperm were moving within the fluid, $Crisp1^{-/-}.Crisp3^{-/-}$, TKO and QKO uterine sperm were either mostly immotile or moving in a very viscous fluid. Furthermore, most of the few motile TKO and QKO uterine sperm showed an abnormal flagellar beating not detected in either $Crisp1^{-/-}.Crisp3^{-/-}$ or control sperm cells. A more careful examination of TKO and QKO sperm recovered from the uterus revealed they exhibited stiffness in the midpiece region of their tails (Video S1 and S2).

The presence of sperm within the oviduct was analyzed by video fluorescence microscopy taking advantage of the expression of the EGFP transgene in TKO and QKO mice and the consequent presence of fluorescent labeling in the sperm acrosomes.[37] Results revealed that whereas TKO and QKO sperm were capable of reaching the oviduct, they both exhibited defects in migrating within the organ as judged by the clearly fewer fluorescent labeled cells detected in the lower/middle isthmus of females mated with mutant compared to control males (Figure 3).

3.4.2 | In vitro fertilization and early embryo development

As another approach to elucidate the mechanisms leading to in vivo fertilization defects, the fertilizing ability of

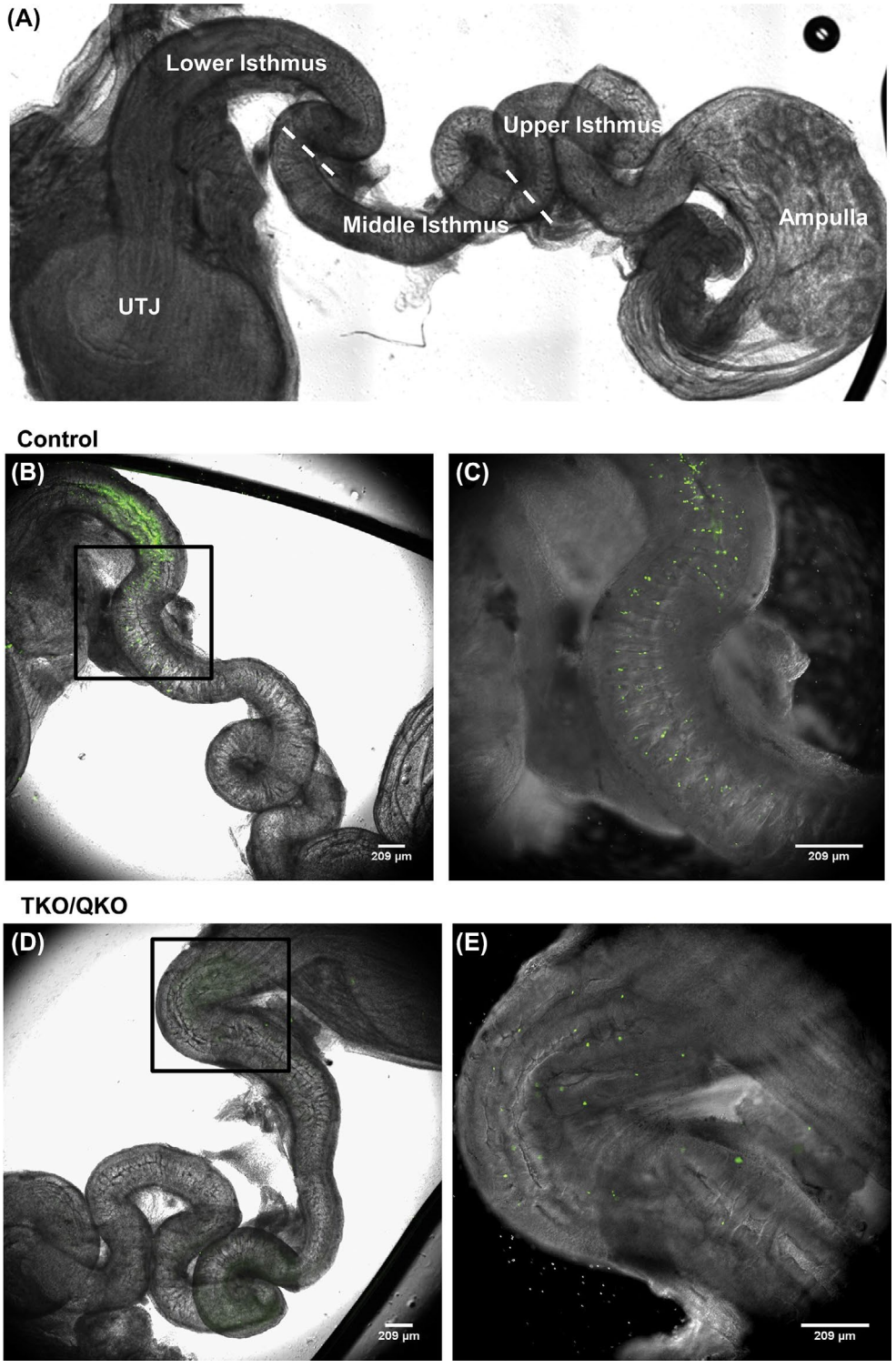

FIGURE 3 **Sperm migration in the female reproductive tract**. A, Representative diagram showing the different regions of the oviduct. Adult superovulated females were housed with control or mutant (TKO or QKO) males and, 4 hours after mating, sperm were analyzed inside the oviduct by fluorescence microscopy. UTJ, utero-tubal junction. B, Merged image of the oviduct of a female mated with a control male. C, Magnification of the region boxed in (B) corresponding to the lower/middle isthmus. D, Merged image of the oviduct of a female mated with a mutant male. E, Magnification of the region boxed in (D) corresponding to the lower isthmus. The figures are representative of at least four independent experiments

mutant sperm was analyzed by in vitro fertilization assays using either cumulus oocyte complexes (COC), oocytes surrounded just by the ZP or oocytes without any coat. Results revealed that whereas epididymal TKO sperm exhibited a dramatic decrease to penetrate COC or ZP-intact eggs and had a significantly lower ability to fuse

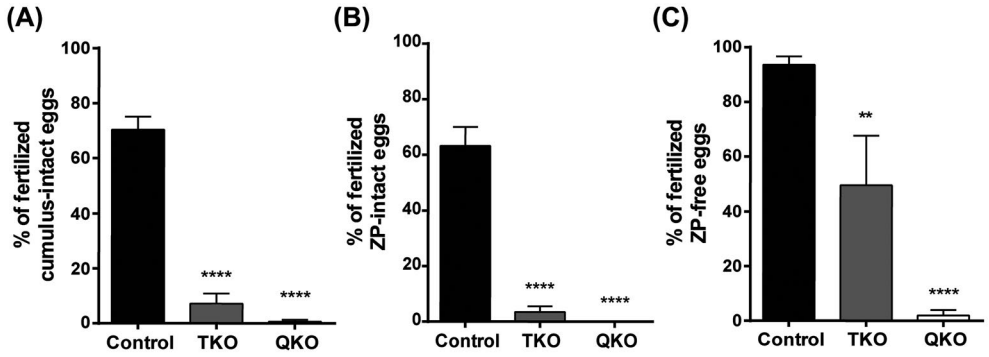

FIGURE 4 In vitro fertilizing ability of triple (TKO) and quadruple (QKO) *Crisp* knockout sperm. Control, TKO and QKO sperm capacitated for 90 minutes were coincubated with (A) cumulus-intact eggs for 3 hours (n = 5), (B) ZP-intact eggs for 3 hours (n = 5) and (C) ZP-free eggs for 1 hour (n = 5). At the end of all incubations, the percentage of fertilization was evaluated by the presence of decondensing sperm heads within the egg cytoplasm. In all cases, data are mean ± SEM; $^{**}P < .01$

with zona-free eggs, QKO were completely unable to fertilize the eggs under all the conditions tested (Figure 4A-C). No accumulation of sperm in the perivitelline space was observed under any of the conditions tested, indicating that fertilization failure was mostly due to sperm defects to interact with the egg coats. Further examination of different sperm parameters carried out in QKO sperm showed that whereas no differences compared to controls were found in the number, viability, or progressive motility of fresh epididymal sperm (Figure S4A-C), capacitated mutant cells were unable to acrosome react in response to progesterone (Figure 5A) and exhibited significant differences in the percentage of hyperactivated cells compared to controls (Figure 5B) as well as in two motility parameters (Table S3). Interestingly, QKO sperm also exhibited the midpiece rigidity described above for ejaculated sperm. Considering that all mammalian CRISP proteins have been described as Ca^{2+} channel regulators[9-14] and that Ca^{2+} plays major roles in fertilization,[46,47] we measured intracellular Ca^{2+} levels by flow cytometry in both fresh and capacitated mutant sperm to elucidate the molecular mechanisms leading to fertilization failure, whereas Ca^{2+} levels in fresh QKO sperm were not different from controls, capacitated QKO sperm did not exhibit the characteristic increase in Ca^{2+} levels during capacitation observed in control sperm (Figure 5C).

Having investigated the different mechanisms that could lead to the low in vivo fertilization rates of TKO and QKO males, and based on the results obtained for *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ males, we decided to analyze the possibility that embryo development failure could also contribute to the severely disrupted fertility of TKO and QKO males. For this purpose, the very few fertilized eggs that could be recovered from the ampulla of females mated with TKO or QKO mutant males were incubated under in vitro conditions and the percentage reaching the blastocyst stage determined for each group. Results showed that embryo development rates corresponding to TKO and QKO were significantly lower than controls (Table 2), confirming that the great decrease in fertility rates observed in TKO and QKO mutant males is also due to defects in early embryo development. This was further supported by the observation that the number of born pups showed a correlation slope closer to one when analyzed as a function of the number of in vivo fertilized eggs that develop into blastocysts than as a function of just the number of fertilized eggs (Figure S5).

Collectively, our observations show that males lacking multiple CRISP proteins exhibit severe defects in their fertility due to a combination of defects in sperm migration, gamete interaction, and early embryo development.

4 | DISCUSSION

Previous studies involving deletions of individual *Crisp* genes by homologous recombination showed normal fertility in mutant males despite of several defects found when sperm were tested in in vitro fertilization assays.[7,14,26-28,33] These results, together with the finding that *Crisp1*$^{-/-}$.*Crisp4*$^{-/-}$ mutant males exhibited a mild subfertility[7] supported the possible existence of partially redundant and compensatory mechanisms among CRISP family members. Based on these observations, in the present study, we investigated the relevance of CRISP proteins for male fertility by generating mutant mice carrying simultaneous null mutations in several *Crisp* genes using CRISPR/Cas9 technology. Our results showing that males lacking more than two CRISP proteins exhibited a dramatic inhibition in their fertility revealed, for the first time, the essential role of CRISP proteins for animal fertility and confirmed the existence of redundancy and compensation among CRISP family members.

The strategy used for generating mice lacking several *Crisp* genes at the same time involved the microinjection of sgRNAs targeting *Crisp1* and *Crisp3* in zygotes from

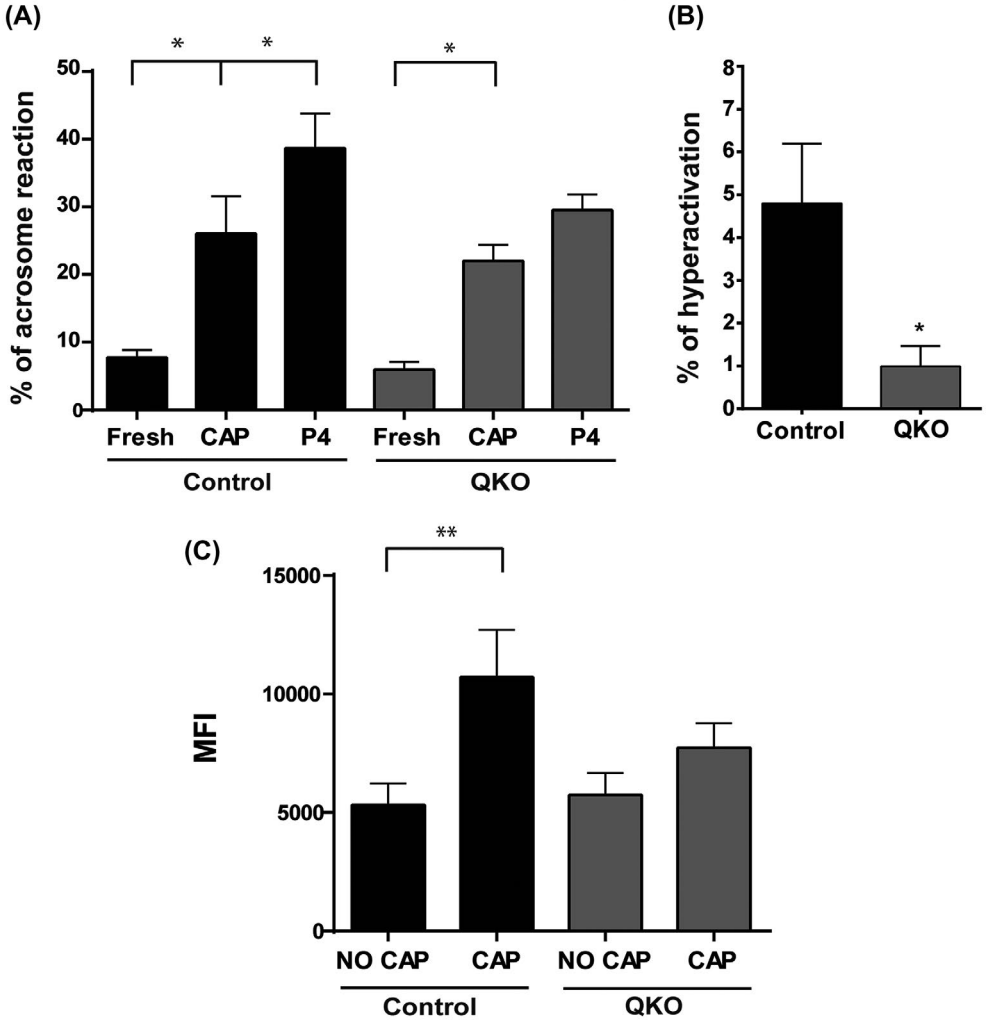

FIGURE 5 **Capacitation-associated parameters in quadruple (QKO) *Crisp* knockout sperm**. Epididymal control or QKO sperm were incubated under capacitating conditions for 90 minutes and different functional parameters were evaluated. A, Percentage of acrosome reaction determined by Coomassie Brilliant Blue staining in fresh sperm and capacitated sperm (CAP) exposed to progesterone (P4) or dimethyl sulfoxide alone (CAP) during the last 15 minutes of capacitation. n = 5. B, Percentage of hyperactivation evaluated by CASA n = 3 for control and n = 5 for QKO males. C, Intracellular Ca^{2+} levels determined by flow cytometry using Fluo-4, AM. Results are shown as mean fluorescence intensity (MFI) for noncapacitated (NO CAP) and capacitated (CAP) sperm. n = 5. In all cases, data are mean ± SEM; $*P < .05$, $**P < .01$

TABLE 2 Analysis of embryo development corresponding to triple (TKO) and quadruple (QKO) *Crisp* knockout males

	Control	TKO	QKO
Blastocyst (N°)	96	8	16
Total two-cell embryos (N°)	104	12	25
Embryo development (%)	92.3	66.7*	64.0***

Note: Percentage of two-cell embryos that reach the blastocyst stage after 4 days of in vitro culture in KSOM medium.

n = 5. $*P < .05$; $***P < .005$ vs Control.

the $Crisp2^{-/-}.Crisp4^{-/-}$ colony obtained by crossbreeding $Crisp2^{-/-}$ and $Crisp4^{-/-}$ mutants in our laboratory. In contrast to the subfertility observed in $Crisp1^{-/-}.Crisp4^{-/-}$ mutants,[7] the simultaneous absence of testicular *Crisp2* and epididymal *Crisp4* did not impair male fertility under normal laboratory reproductive conditions. Although the reasons for the different fertility phenotypes in the two double mutant mouse models are unknown, it is possible that the lack of epididymal CRISP4 in $Crisp2^{-/-}.Crisp4^{-/-}$ is partially compensated by the presence of the other epididymal CRISP protein (ie, CRISP1) whereas in $Crisp1^{-/-}.Crisp4^{-/-}$, the simultaneous lack of the two epididymal CRISP proteins cannot be compensated by the remaining family members (ie, CRISP2 and CRISP3). In spite of being fertile, $Crisp2^{-/-}.Crisp4^{-/-}$ males showed in vivo fertilization rates significantly lower than those observed for $Crisp2^{-/-}$[27] or $Crisp4^{-/-}$[7] males when mated with superovulated females probably due to the fact that $Crisp2^{-/-}.Crisp4^{-/-}$ sperm exhibited a combination of the capacitation-associated phenotypes observed in sperm

from *Crisp2*$^{-/-}$ and *Crisp4*$^{-/-}$ single mutant mice such as impaired tyrosine phosphorylation, progesterone-induced acrosome reaction and hyperactivation.[7,14,27,28,33]

Taking advantage of the possibility of multiple gene deletions by the CRISPR/Cas9 technology, we successfully mutated *Crisp1* and *Crisp3* genes by employing sgRNAs for these two genes in both wild-type and *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ colonies. We first introduced the *Crisp1* and *Crisp3* sgRNAs in wild-type zygotes with the aim of producing and characterizing a *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ model not yet available. Functional analysis of these mice revealed that disruption of *Crisp1* and *Crisp3* produced a subfertility that had not been observed in mice lacking just *Crisp1* in two different genetic backgrounds.[26,48] Notwithstanding our limitations to assess CRISP3 expression due to the lack of reliable commercial antibodies against this protein, our observations showing subfertility in *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ but not in *Crisp1*$^{-/-}$ males support a disruption of CRISP3 expression in the male reproductive tract. Surprisingly, in spite of their subfertility, *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ mice exhibited normal levels of in vivo fertilization even when mated with superovulated females, likely due to a functional compensation between the two epididymal proteins (CRISP1 and CRISP4) as proposed for *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ and for *Crisp1*$^{-/-}$ and *Crisp4*$^{-/-}$ single mutant mice.[7] Regardless of the reasons for the normal levels of in vivo fertilization, these observations indicated that *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ male subfertility was associated with postfertilization defects. Subsequent studies showing significantly lower percentages of blastocysts for *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ than for control males showed, for the first time, the potential role for the combined presence of male CRISP1 and CRISP3 in early embryo development. In this regard, it has been proposed that alterations in the epididymal maturation process could lead to a delayed fertilization and subsequent embryo developmental defects.[49] Interestingly, recent evidence shows that microinjection of caput but not cauda sperm into oocytes results in embryo development defects that can be reverted by microinjection of cauda-specific small RNAs[50] supporting the role of epididymal maturation beyond the well-known acquisition of sperm motility and fertilizing ability. In addition, embryo developmental defects could be associated with an impaired sperm capacitation process based on a recent report showing that capacitation conditions have impact not only on fertilization but on embryo development as well.[51] The mechanisms by which the simultaneous lack of *Crisp1* and *Crisp3* genes leads to impaired embryo development are currently under investigation and will provide interesting information on how paternal factors are critical for developmental success.

Having confirmed the generation of mutations in *Crisp1* and *Crisp3* which led to subfertile males, we next introduced the same targeting sgRNAs in zygotes from the *Crisp2*$^{-/-}$.*Crisp4*$^{-/-}$ colony. This strategy led to the generation of males with mutations in either three (ie, *Crisp1/Crisp2/Crisp3*) or the four genes of the CRISP family which represent excellent models to study the relevance of CRISP proteins for male fertility. TKO and QKO males exhibited a severely impaired fertility phenotype including a high proportion of sterile males. Consistent with their severe fertility failure, TKO and QKO males showed in vivo fertilization rates significantly lower than controls, suggesting defects in sperm transport through the female tract and/or in gamete interaction. Examination of sperm recovered from the uterus of mated females revealed that, in contrast to the fully motile wild-type sperm population, TKO and QKO mutant sperm were mostly immotile or moving into a very viscous fluid. Whereas the reason for this phenotype is still unknown, it might be associated with an alteration of the recently reported ability of CRISP proteins to form amyloid-like structures known to trap damaged sperm within the uterus.[52] In addition, considering the reported presence of CRISP3 in human and equine seminal fluid,[53,54] the possibility that the lack of CRISP3 in the seminal plasma of mutant males leads to coagulation/liquefaction defects within the uterus cannot be ruled out. Although comparison between TKO/QKO and *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ phenotypes needs to consider their different genetic backgrounds, it is interesting to note that *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ males with normal in vivo fertilization rates exhibited the same phenotype in the uterus arguing against the idea that these defects were responsible for the in vivo fertilization failure observed for TKO and QKO males. Microscopy studies aimed to analyze sperm transport within the oviduct showed the existence of migration defects in both TKO and QKO males as judged by the clearly lower number of fluorescent sperm detected in the different oviductal regions compared to controls, suggesting that sperm migration defects within the oviduct might contribute to the severe fertility failure in multiple *Crisp* gene mutants. Nevertheless, unlike male mice deficient in other proteins (ie, ADAM 3 or RNAse 10) which are infertile due to the inability of sperm to cross the utero-tubal junction,[55-58] TKO and QKO sperm reached the oviduct, making it unlikely that sperm migration defects alone could lead to the high proportion of sterility of the mutant males.

In addition to sperm transport defects, in vitro fertilization studies revealed that sperm from TKO and QKO males exhibited severe deficiencies in their fertilizing ability, supporting the contribution of gamete interaction defects to the infertility of TKO and QKO males. In this regard, whereas both TKO and QKO sperm showed major defects to fertilize COC and ZP-intact eggs, QKO but not TKO were unable to fertilize the denuded eggs. The finding that TKO exhibit gamete fusion rates not different from those previously reported for individual CRISP1[26] or CRISP2[27] knockout males indicates that CRISP3 may not have a critical role in gamete fusion. This, together with the inability of QKO sperm to fuse with the eggs supports the crucial role of CRISP4 in

gamete fusion observed in all male models lacking CRISP4 (ie, *Crisp4*$^{-/-}$ and *Crisp1*$^{-/-}$.*Crisp4*$^{-/-}$[7] and likely mediated by its interaction with egg complementary sites[26,59] and/or its role in acrosome reaction.[7] Interestingly, however, the lack of the four CRISP proteins did not result in a stronger fertility phenotype compared to that observed for TKO males, supporting the notion that in vivo fertilization failure is mainly due to sperm defects in those events that precede gamete fusion such as sperm migration within the oviduct and penetration of the egg coats. The impaired ability of multiple mutant sperm to penetrate the egg coats might well be explained by their defects to undergo the progesterone-induced acrosome reaction and to develop hyperactivation, two events known to be essential for penetration of the resilient ZP.[46] In this regard, the finding that TKO and QKO sperm exhibited tail midpiece rigidity supports this defect as responsible for their impaired hyperactivation and egg coat penetration ability. Moreover, it is possible that the midpiece rigidity detected in uterine TKO and QKO sperm affects the development of hyperactivation in the oviduct, preventing the detachment of sperm from the isthmus epithelium and thus, their migration within the organ as described for CatSper knockout males.[60] Interestingly, midpiece rigidity was detected in TKO and QKO but not *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ sperm, indicating this defect is linked to the lack of testicular CRISP2, as previously proposed.[28] Although the mechanism leading to midpiece stiffness in TKO and QKO sperm is unknown, the presence of this phenotype not only in ejaculated but also in epididymal sperm together with the finding that sperm normally develop midpiece flexibility during epididymal transit[61] supports this phenotype appears as a consequence of epididymal maturation defects in sperm lacking CRISP2. The possibility that midpiece rigidity affects hyperactivation development and thus, in vivo fertilization and fertility, is further supported by previous reports showing this flagellar phenotype in several other mouse models deficient in sperm proteins (ie, CatSper z, EFCAB9, calcineurin, Nsum 7), all of which exhibit severe fertility problems as well as intracellular Ca^{2+} dysregulation.[61-64] In this regard, given the critical role of Ca^{2+} for most fertilization events including hyperactivation and acrosome reaction, and the reported ability of CRISP proteins to regulate several Ca^{2+} channels,[9-13] we analyzed intracellular Ca^{2+} levels in mutant sperm to gain insights into the molecular mechanisms underlying their functional defects. Interestingly, sperm from QKO males did not show the characteristic Ca^{2+} increase that takes place during capacitation, supporting the idea that a dysregulation in intracellular Ca^{2+} homeostasis could be responsible for several of the functional defects detected in mutant sperm. In this regard, epididymal CRISP1 and CRISP4 are peripheral proteins that regulate TRPM8 and CatSper Ca^{2+} channels located in the head and tail plasma membrane [13,14] whereas testicular CRISP2 is an intracellular protein located in the redundant envelope[65] and reported to regulate ryanodine channels[12] present in this region of the tail.[66] Considering that sperm lacking CRISP1 exhibited no significant changes in intracellular Ca^{2+} levels compared to wild-type cells[48] whereas those lacking CRISP2 or CRISP4 showed either an increase[27] or a decrease (unpublished observations) in Ca^{2+} concentration during capacitation, respectively, we believe that the decrease in Ca^{2+} levels observed in sperm from QKO mice reflects the final balance of the individual contribution of each CRISP to the regulation of Ca^{2+} homeostasis during sperm maturation and/or capacitation.

The finding that *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ mice showed embryo developmental defects opened the possibility that TKO and QKO males which lack CRISP1 and CRISP3, exhibit postfertilization defects in addition to their impaired in vivo fertilization. As expected, the very few eggs fertilized by sperm from TKO or QKO mice showed deficiencies to reach the blastocyst stage, indicating that embryo developmental defects were also contributing to the severe fertility decrease of these mutant males. Supporting this conclusion, fertility rates showed a much better correlation with early embryo development than with in vivo fertilization rates.

Together, our results indicate that the severe fertility disruption observed for TKO and QKO males results from the combination of defects in sperm migration, gamete interaction and early embryo development, likely linked to a dysregulation in sperm intracellular Ca^{2+}. According to our observations, simultaneous mutations in *Crisp1* and *Crisp3* results in both uterine sperm motility and early developmental defects while alterations in *Crisp2* and *Crisp4* are mainly associated with gamete interaction defects due to the key roles of CRISP2 in sperm midpiece flexibility and of CRISP4 in gamete fusion. In addition, sperm motility defects within the uterus and/or midpiece rigidity may lead to the sperm migration defects observed in TKO and QKO mice (Figure 6). These observations suggest that CRISP family members have evolved to perform redundant as well as specialized functions. In this regard, whereas epididymal CRISP1 and CRISP4 exhibit overlapping functions (ie, in acrosome reaction, gamete fusion) that allow them to compensate to each other, testicular CRISP2 and seminal CRISP3 seem to be associated with functional roles not compensated by the epididymal CRISP members (ie, sperm tail flexibility, sperm behavior within the uterus), suggesting the existence of different functional modules within the CRISP family. This, together with the finding that mice lacking three or four CRISP proteins exhibit more severe phenotypes than single or double CRISP knockout mice supports the notion that the combined mutations of *Crisp* members lead to disruption of multiple independent pathways that contribute distinctly to fertility success.

Considering that human *CRISP1*, *CRISP2,* and *CRISP3* are located close to each other in a region of less than 200 kb within chromosome 6, a chromosome rearrangement such as

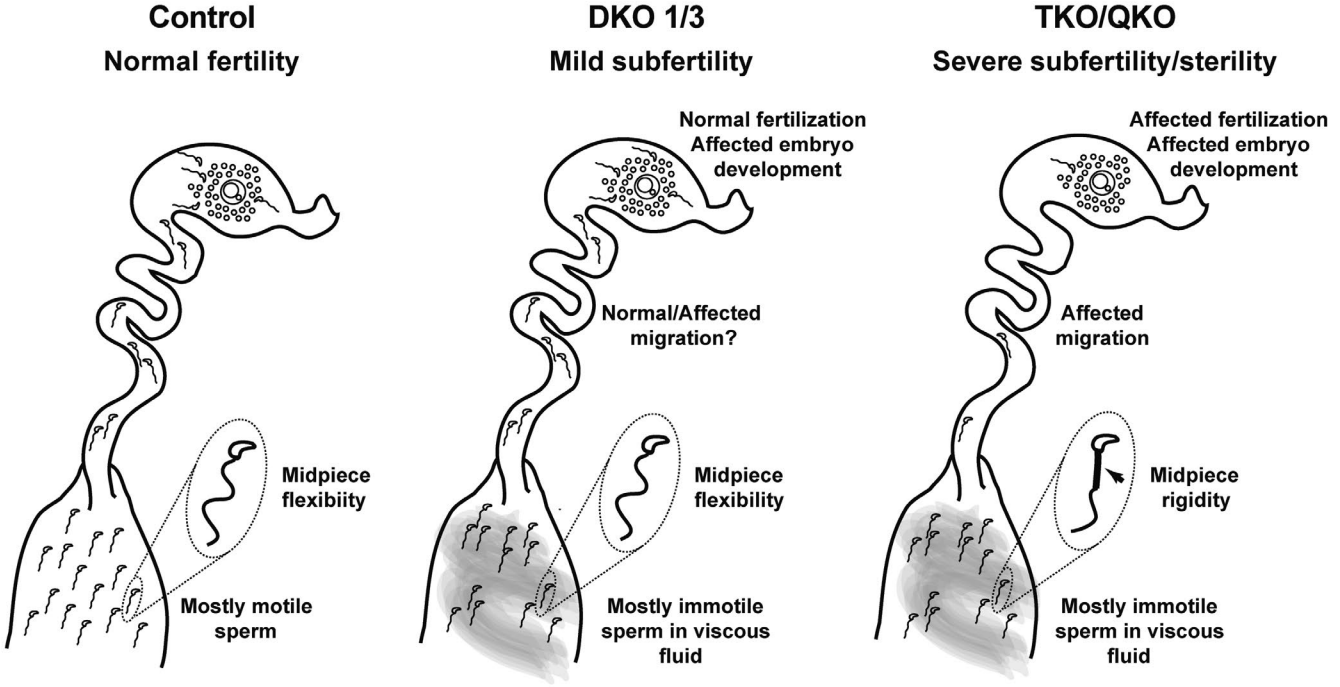

FIGURE 6 Schematic diagram showing possible causes of impaired fertility in multiple *Crisp* knockout males. Diagram summarizing our observations for control (left panel), *Crisp1*$^{-/-}$.*Crisp3*$^{-/-}$ (DKO 1/3) (middle panel) and TKO/QKO (right panel) sperm within the uterus and oviduct (isthmus and ampulla) after mating. DKO 1/3 males showed a mild subfertility compared to controls that can be attributed to defects in early embryo development. In spite of the finding that DKO 1/3 sperm were mostly immotile within the uterus, in vivo fertilization was not different from controls, suggesting no major defects in the ability of DKO 1/3 sperm to migrate to the oviduct. TKO and QKO exhibited a severe subfertility/sterility compared to controls. In this case, in addition to the immotility of sperm within the uterus and the affected early embryo development already observed for DKO 1/3 males, TKO/QKO sperm exhibited midpiece rigidity and defects to both migrate to the oviduct and fertilize the eggs in the ampulla

the one already described in this region[67,68] could affect the expression of all human *CRISP* genes simultaneously making it possible that some cases of unexplained infertility are due to the absence of more than one human CRISP protein. Our results, therefore, may have clinical implications and provide relevant information for the diagnosis and treatment of human infertility as well as for the development of new nonhormonal contraceptive options. Moreover, we believe our observations in mutant mice lacking different numbers and combinations of *Crisp* genes reveal how CRISP members are functionally organized within the family bringing insights into the mechanisms underlying functional redundancy and compensation within other protein families involved in different biological processes.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

L. Curci, N.G. Brukman, M. Weigel Muñoz, D. Rojo, M. Rubinstein, V.G. Da Ros, and P.S. Cuasnicu designed research. L. Curci, N.G. Brukman, M. Weigel Muñoz, D. Rojo, G. Carvajal, V. Sulzyk, S.N. Gonzalez, and V.G. Da Ros performed research. L. Curci, N.G. Brukman, M. Weigel Muñoz, V.G. Da Ros, and P.S. Cuasnicu analyzed data. L. Curci, N.G. Brukman, M. Weigel Muñoz, D. Rojo, M. Rubinstein, V.G. Da Ros, and P.S. Cuasnicu wrote the paper.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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