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Relevance of CRISP proteins for epididymal physiology, fertilization, and fertility

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ABSTRACT

Background: The molecular mechanisms involved in the acquisition of mammalian sperm fertilizing ability are still poorly understood, reflecting the complexity of this process.

Objectives: In this review, we describe the role of Cysteine RIch Secretory Proteins (CRISP1–4) in different steps of the sperm journey to the egg as well as their relevance for fertilization and fertility.

Materials and Methods: We analyze bibliography reporting the phenotypes of CRISP KO mice models and combine this search with recent findings from our team.

Results: Generation of individual KO for CRISP proteins reveals they are key mediators in different stages of the fertilization process. However, in spite of their important functional roles, KO males for each of these proteins remain fertile, supporting the existence of compensatory mechanisms between homologous CRISP family members. The development of mice lacking epididymal CRISP1 and CRISP4 simultaneously (DKO) revealed that mutant males exhibit an impaired fertility due to deficiencies in the sperm ability to fertilize the eggs in vivo, consistent with the proposed roles of the two proteins in fertilization. Interestingly, DKO males show clear defects in both epididymal epithelium differentiation and luminal acidification known to be critical for sperm maturation and storage. Whereas in most of the cases, these epithelium defects seem to specifically affect the sperm fertilizing ability, some animals exhibit a disruption of the characteristic immune tolerance of the organ with clear signs of inflammation and sperm viability defects.

Discussion and Conclusion: Altogether, these observations confirm the relevance of CRISP proteins for male fertility and contribute to a better understanding of the fine-tuning mechanisms underlying sperm maturation and immune tolerance within the epididymis. Moreover, considering the existence of a human epididymal protein functionally equivalent to rodent CRISP1 and CRISP4, DKO mice may represent an excellent model for studying human epididymal physiology and pathology.

INTRODUCTION

Spermatozoa produced in the testes are considered functionally immature because they are unable to move progressively and to recognize and fertilize the egg. Spermatozoa acquire these abilities during a maturation process that takes place while they are passing through the epididymis (Robaire & Hinton, 2015). To express their fertilizing ability, spermatozoa need to undergo an additional process that occurs while they are ascending through the female tract known as capacitation (Chang, 1951; Austin, 1952), which allows spermatozoa to undergo the acrosome reaction, an exocytotic event that takes place in the head as well as to develop a vigorous and intermittent motility called hyperactivation, both absolute requirements for fertilization (Florman & Fissore, 2015).

Sperm epididymal maturation is a complex process that involves the continuous interaction between the spermatozoa and the luminal fluid composed not only of inorganic ions and proteins but also of small non-coding RNAs (Belleannée *et al.*, 2012; Nixon *et al.*, 2015), non-pathological amyloid (Whelly *et al.*, 2012), and epididymosomes (exosome-like vesicles) (Sullivan *et al.*, 2005). Each of the main anatomical regions of the epididymis, that is the initial segment, the caput, the corpus, and the cauda (Robaire & Hinton, 2015), has a different fluid composition and functionality produced by a combination of the secretory and absorptive activities of a pseudostratified epididymal epithelium that contains different highly specialized cells (Robaire & Hinton, 2015; Breton *et al.*, 2016). Principal cells, the most abundant cell type, are present throughout the organ and are mainly involved in both the synthesis of proteins that are either retained within the cells or secreted into the epididymal lumen and the endocytosis of luminal components (Hermo & Robaire, 2002). Clear cells are expressed in all epididymal regions except the initial segment and have an important role in luminal acidification, endocytosis, and uptake of the

cytoplasmic droplets released by spermatozoa during epididymal transit (Hermo *et al.*, 1988; Robaire & Hinton, 2015). Narrow cells are found only in the initial segment and are involved in endocytosis and luminal acidification (Adamali & Hermo, 1996), whereas basal cells reside in all regions of the epididymal tubule, extend long projections into the initial segment lumen known as axiopodia (Shum *et al.*, 2008), and have a predominant role in sperm protection (Robaire & Hinton, 2015). In this regard, given that male germ cells appear after the establishment of the immune tolerance, the epididymal epithelium is also the major component of the blood-epididymal barrier which contributes to maintain the luminal microenvironment and to avoid an immune response to sperm antigens that could lead to chronic inflammation and/or infertility (Gregory & Cyr, 2014; Hedger, 2015). The luminal microenvironment and, particularly, luminal pH are critical for both the acquisition of the sperm fertilizing ability during maturation and the storage of mature spermatozoa in the epididymal cauda (Shum *et al.*, 2009).

Although the molecular mechanisms involved in the acquisition of sperm fertilizing ability are still far from being completely understood, reflecting the complexity of this process, it is known that sperm maturation involves numerous changes, most of which occur as a result of the association of epididymal proteins with the sperm plasma membrane (Robaire & Hinton, 2015). Two such proteins are CRISP1 and CRISP4 (Kohane *et al.*, 1980a,1980b; Jalkanen *et al.*, 2005; Nolan *et al.*, 2006), which belong to a highly conserved and widely distributed family among vertebrates known as Cysteine RIch Secretory Proteins (CRISP). CRISP proteins are characterized by the presence of sixteen conserved cysteines, ten of which are located in the C-terminal region or cysteine-rich domain (CRD) connected to the plant pathogenesis-related 1 (PR-1) domain located in the N-terminus (Guo *et al.*, 2005; Gibbs *et al.*, 2008). In mammals, four proteins mainly expressed in the male reproductive tract have been described: CRISP1, identified by our laboratory, synthesized by the epididymis (Cameo & Blaquier, 1976); CRISP2 of testicular origin (Hardy *et al.*, 1988; Kasahara *et al.*, 1989); CRISP3 with a wide distribution that includes reproductive and non-reproductive organs (Haendler *et al.*, 1993; Kjeldsen *et al.*, 1996; Udby *et al.*, 2005); and CRISP4 also expressed by the epididymis (Jalkanen *et al.*, 2005). In the present review, we describe the role of mammalian CRISP in different steps of the sperm journey to the egg as well as their relevance for fertility analyzed through the development of different knockout (KO) mice models.

CYSTEINE RICH SECRETORY PROTEIN 1

Rat CRISP1, the first member of the family identified by our group (Cameo & Blaquier, 1976), is a glycoprotein expressed by the principal cells of the epithelium in response to androgens and secreted into the lumen where it associates with the sperm surface (Kohane *et al.*, 1980a,1980b; Garberi *et al.*, 1982; Eberspaecher *et al.*, 1995) with two different affinities (Cohen *et al.*, 2000a). Whereas one population is loosely bound to spermatozoa and it is released during capacitation acting as a decapacitating factor (Cohen *et al.*, 2000a; Roberts *et al.*, 2003), the other population is tightly bound to the gamete, remains on capacitated/acrosome-reacted spermatozoa, and participates in both sperm-zona pellucida (ZP) binding and gamete fusion through its interaction with egg-complementary sites (Rochwerger *et al.*, 1992; Cohen *et al.*, 2000b, 2001; Busso *et al.*, 2007). Rodent CRISP1 exhibits significant homology with human epididymal CRISP1 (hCRISP1) (Hayashi *et al.*, 1996; Kratzschmar *et al.*, 1996) also reported to be involved in sperm-ZP interaction and gamete fusion through complementary sites in the egg surface (Cohen *et al.*, 2001; Maldera *et al.*, 2014). In vitro fertilization studies using recombinant fragments as well as synthetic peptides revealed that the egg-binding ability of CRISP1 resides in a 12-amino acid region located in the PR-1 domain which corresponds to one of the two consensus sequences of the CRISP family named Signature 2 (S2) (Ellerman *et al.*, 2006).

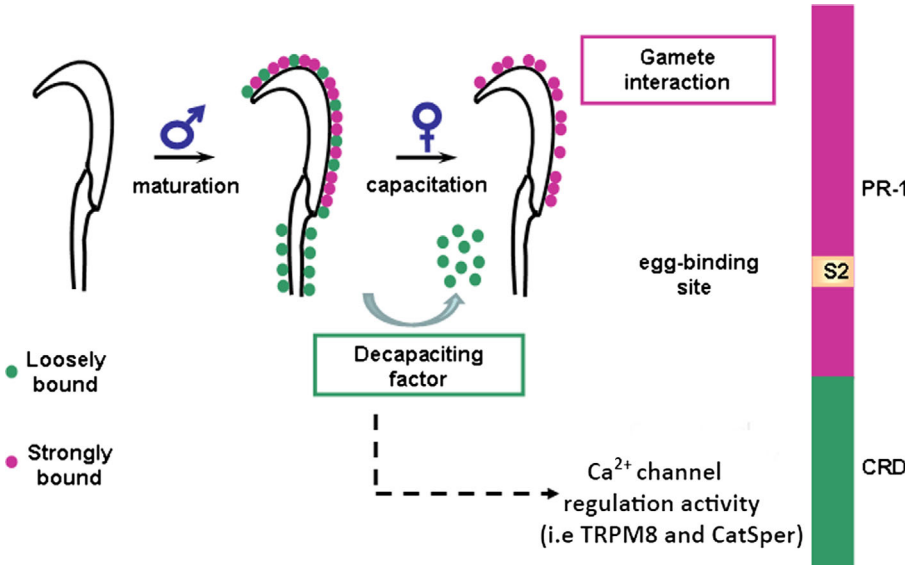
The potential relevance of CRISP1 for fertility was first indicated by experiments showing that immunization of rats with CRISP1 produced a specific and significant decrease in male and female fertility without eliciting pathological effects (Cuasnicú *et al.*, 1990; Pérez Martínez *et al.*, 1995; Ellerman *et al.*, 1998, 2008; Muñoz *et al.*, 2012). As another approach to investigate the relevance of CRISP1 for fertilization and fertility, we developed mice lacking CRISP1, which constituted the first KO model for a CRISP family protein (Da Ros *et al.*, 2008). Results showed that CRISP1 mutant spermatozoa exhibited defects in capacitation-associated parameters as well as in their ability to bind to the ZP and to fuse with the egg, confirming the roles of CRISP1 in sperm-ZP binding and gamete fusion previously described (Rochwerger *et al.*, 1992; Cohen *et al.*, 2000b; Busso *et al.*, 2007). However, in spite of the important roles of CRISP1 in fertilization, CRISP1 KO males were as fertile as controls (Da Ros *et al.*, 2008) even in mice from different genetic backgrounds (Hu *et al.*, 2018; Weigel Muñoz *et al.*, 2018). Considering the high sequence homology among CRISP family members, these observations support the existence of compensatory mechanisms between homologous CRISP.

Interestingly, results from our group showed that CRISP1 is also expressed by the cumulus cells that surround the egg where it plays a role in sperm orientation by modulating sperm hyperactivation (Ernesto *et al.*, 2015). More specifically, our observations revealed that CRISP1 is able to inhibit both TRPM8 channels as well as CatSper (Ernesto *et al.*, 2015), the main sperm calcium channel involved in the development of hyperactivation and essential for male fertility (Ren *et al.*, 2001; Smith *et al.*, 2013). This finding, together with the detection of lower intracellular calcium levels in spermatozoa exposed to CRISP1 (Ernesto *et al.*, 2015), led us to speculate that whereas the strongly bound CRISP1 population participates in gamete interaction through the S2 located within the PR-1 (Ellerman *et al.*, 2006), the weakly bound population of CRISP1 acts as a decapacitating factor by inhibiting CatSper and hyperactivation through the CRD domain reported to be involved in ion channel regulation (Guo *et al.*, 2005; Gibbs *et al.*, 2006, 2011) (Fig. 1).

CYSTEINE RICH SECRETORY PROTEIN 2

CRISP2 is expressed almost exclusively in the testis, and differently from CRISP1, it is not glycosylated and its expression is not under androgen regulation (Haendler *et al.*, 1997; Gibbs *et al.*, 2008). Whereas CRISP2 was originally described as a guinea pig autoantigen capable of eliciting autoimmune orquitis (Hardy *et al.*, 1988; Foster & Gerton, 1996), immunization of rats with CRISP2 did not produce an autoimmune response (Muñoz *et al.*, 2012), opening the possibility that CRISP2 is not a causative agent of orchitis in other mammalian species either. In testicular

Figure 1 Relationship between CRISP1 behavior and functionality. There are two populations of CRISP1 in spermatozoa: a weakly bound population (green dots) released from spermatozoa during capacitation and proposed to act as a decapacitating factor by regulating calcium channels through the CRD domain, and a strongly bound population (pink dots) which remains on spermatozoa after capacitation and participates in gamete interaction through the S2 region located within the PR-1 domain.



spermatozoa, CRISP2 is localized in the outer dense fibers of the tail and inside the acrosome (O'Bryan *et al.*, 1998). During capacitation, CRISP2 relocalizes from the acrosomal compartment to the equatorial segment (Busso *et al.*, 2007; Nimlamool *et al.*, 2013) and participates in gamete fusion through its ability to bind to the same egg-complementary sites than CRISP1 (Busso *et al.*, 2007). Like CRISP1, CRISP2 has a functional homologue in humans which is also involved in gamete fusion (Busso *et al.*, 2005).

The generation of Crisp2$^{-/-}$ mice revealed that although mutant males mice exhibited no histological alterations in the testes and were fertile (Brukman *et al.*, 2016), unilateral vasectomy reduced their fertility compared to control males (Brukman *et al.*, 2016), supporting the relevance of CRISP2 for fertility. This phenotype seems to be a consequence of the participation of CRISP2 in the fertilization process since lower fertilization rates were observed in the ampulla of those females mated with the unilaterally vasectomized Crisp2$^{-/-}$ males compared to controls (Brukman *et al.*, 2016). In vitro experiments revealed that CRISP2 KO exhibited not only a deficient sperm fusion ability consistent with its previously reported role in gamete fusion (Busso *et al.*, 2007) but also an impaired ability to penetrate both the cumulus oophorus and the ZP very likely associated with their lower levels of hyperactivation (Brukman *et al.*, 2016). Moreover, considering the high intracellular calcium levels observed in CRISP2 mutant spermatozoa (Brukman *et al.*, 2016), the defects in hyperactivation might be linked to the reported ability of CRISP2 to regulate calcium channels (Gibbs *et al.*, 2006). Interestingly, motility and fertility disorders were also reported in patients with aberrant expression of CRISP2 (Du *et al.*, 2006; Zhou *et al.*, 2015) as well as in a recently reported KO for CRISP2 (Lim *et al.*, 2019).

CYSTEINE RICH SECRETORY PROTEIN 3

CRISP3 exhibits a wide tissue distribution being expressed predominantly in exocrine glands such as salivary gland, pancreas, and prostata, and with lower levels in the epididymis, ovary, timo, colon (Kratzschmar *et al.*, 1996), and uterus (Evans *et al.*, 2015). Little information exists, however, regarding the presence of CRISP3 in spermatozoa and its relevance for fertilization and fertility (Da Ros *et al.*, 2015). CRISP3 has been reported to be located on the surface of horse spermatozoa, and a polymorphism of the gene associated with fertility has also been found (Hamann *et al.*, 2007). In humans, a glycosylated and a non-glycosylated form have been identified not only in the secretory epithelium of the entire male reproductive tract but also in seminal plasma and spermatozoa (Udby *et al.*, 2005). Moreover, results from our group indicated that, as rodent CRISP1, human CRISP3 associates with the sperm surface with two different affinities. While the glycosylated form is weakly bound and released during capacitation acting probably as a decapacitated factor, the non-glycosylated form is tightly bound and remains on the spermatozoa even after the acrosome reaction (Da Ros *et al.*, 2015). Although our results do not support the participation of human CRISP3 in gamete fusion (Da Ros *et al.*, 2015), the potential involvement of this protein in sperm-ZP interaction remains to be explored. Whereas no evidence of the presence of CRISP3 in mouse spermatozoa has been reported so far, the development of CRISP3-deficient mice might contribute to unveil the functional relevance of this protein for both fertilization and fertility.

CYSTEINE RICH SECRETORY PROTEIN 4

Like CRISP1, CRISP4 is predominantly expressed in the principal cells of the epididymal epithelium in an androgen-dependent manner and associates with spermatozoa during epididymal maturation (Jalkanen *et al.*, 2005; Turunen *et al.*, 2011). Differently from CRISP1, however, CRISP4 is not expressed in the female tract (Reddy *et al.*, 2008; Turunen *et al.*, 2011). Recent observations from our laboratory in which spermatozoa were exposed to different extraction treatments show that whereas CRISP4 cannot be released from either fresh or

capacitated spermatozoa by high ionic strength, it can be removed by a non-ionic detergent treatment (Triton X-100) from both types of cells (Fig. 2). These results showing the lack of a loosely bound population as the one described for CRISP1, together with the finding that exposure of spermatozoa to the C-terminal domain of CRISP4 during capacitation did not affect capacitation-associated parameters (i.e. sperm tyrosine phosphorylation or progesterone-induced acrosome reaction) (Gibbs *et al.*, 2011), do not support CRISP4 as a decapacitating factor. Extraction experiments revealed, however, the existence of a tightly bound CRISP4 population in mature spermatozoa that remains in the cells after capacitation (Fig. 2) and which could, therefore, be involved in fertilization.

The potential role of CRISP4 in fertilization and fertility was investigated by different groups using KO models (Gibbs *et al.*, 2011; Turunen *et al.*, 2011; Carvajal *et al.*, 2018; Hu *et al.*, 2018). Whereas males lacking CRISP4 were fertile in different genetic backgrounds (Gibbs *et al.*, 2011; Turunen *et al.*, 2011; Carvajal *et al.*, 2018; Hu *et al.*, 2018), several sperm capacitation-associated parameters as well as the sperm fertilizing ability were differently affected in each strain (Turunen *et al.*, 2011; Carvajal *et al.*, 2018; Hu *et al.*, 2018). Spermatozoa from CRISP4 KO mice generated by our group failed to undergo protein tyrosine phosphorylation and the progesterone-induced acrosome reaction during capacitation and were unable to fertilize cumulus-invested, ZP-intact and ZP-free eggs (Carvajal *et al.*, 2018). As CRISP4 mutant spermatozoa did not exhibit defects in either cumulus penetration or sperm-ZP binding, their lower fertilizing ability under the different conditions assayed would be mainly due to defects at ZP penetration and gamete fusion, likely associated to their impaired ability to undergo the acrosome reaction. Moreover, considering the reported ability of CRISP4 to regulate TRPM8 (Gibbs *et al.*, 2011), a calcium channel involved in the acrosome reaction (Martínez-López *et al.*, 2011), it is possible that the failure of spermatozoa to acrosome react is related to defects in TRPM8 regulation. Finally, although there is no *Crisp4* in humans, the high sequence and functional homology of rodent CRISP4 and CRISP1 with human CRISP1 suggests that the human epididymal CRISP1 protein may be equivalent to a combination of rodent epididymal CRISP1 and CRISP4 (Maldera *et al.*, 2014; : Gibbs *et al.*, 2011).

CRISP1/CRISP4 DOUBLE KNOCKOUT

The results described above indicate that CRISP are multifunctional proteins that cooperate with each other in the different stages of the fertilization process. According to these observations, the lack of fertility defects in the KO for each protein might be due to the existence of a functional compensation between homologous CRISP in each stage of fertilization, making it possible that the lack of more than one CRISP simultaneously leads to a stronger phenotype at fertility level. To explore this possibility, we recently generated double KO (DKO) mice for the two epididymal proteins CRISP1/CRISP4 and analyzed their reproductive phenotype. In this regard, whereas epididymal CRISP1 is involved in both sperm-ZP binding and gamete fusion through its interaction with egg-complementary sites in a ligand-receptor manner, CRISP1 synthetized by the cumulus cells participates in the first stage of cumulus penetration by modulating sperm orientation and hyperactivation through its ability to regulate calcium channel CatSper (Fig. 3). Epididymal CRISP4, on the other hand, is only expressed in the male and participates in both ZP penetration and gamete fusion, very likely by modulating the acrosome reaction through its ability to regulate TRPM8 (Fig. 3). Our results revealed that, differently from single CRISP1 or CRISP4 KO mice, DKO males exhibited clear fertility defects (Carvajal *et al.*, 2018), confirming both the relevance of CRISP

Figure 2 Association of CRISP4 with spermatozoa. Spermatozoa were recovered from young adult males by incising cauda epididymides in 300 μL of capacitation medium (Fraser & Drury, 1975) supplemented with 0.3% of bovine serum albumin and incubated under paraffin oil at 37 °C in an atmosphere of 5% CO_2 in air. After 10 min, aliquots of the suspension (fresh spermatozoa) were added to 300 μL of capacitation medium (final concentration of 0.1–1 $\times 10^7$ cells/m) and spermatozoa incubated at 37 °C in an atmosphere of 5% CO_2 in air for 90 min (capacitated spermatozoa). Fresh and capacitated spermatozoa were then washed and exposed for 1 h to high ionic strength (0.6 M NaCl), non-ionic detergent (1% Triton X-100), or PBS as control. After treatment, spermatozoa were centrifuged and supernatants (Sn) and pellets (Pl) were analyzed by SDS-PAGE followed by Western Blot using anti-mCRISP4 antibody (R&D Systems #AF5017) to determine the release or permanence of CRISP4 in spermatozoa. Anti-beta tubulin (clone D66, Sigma) was used for loading controls.

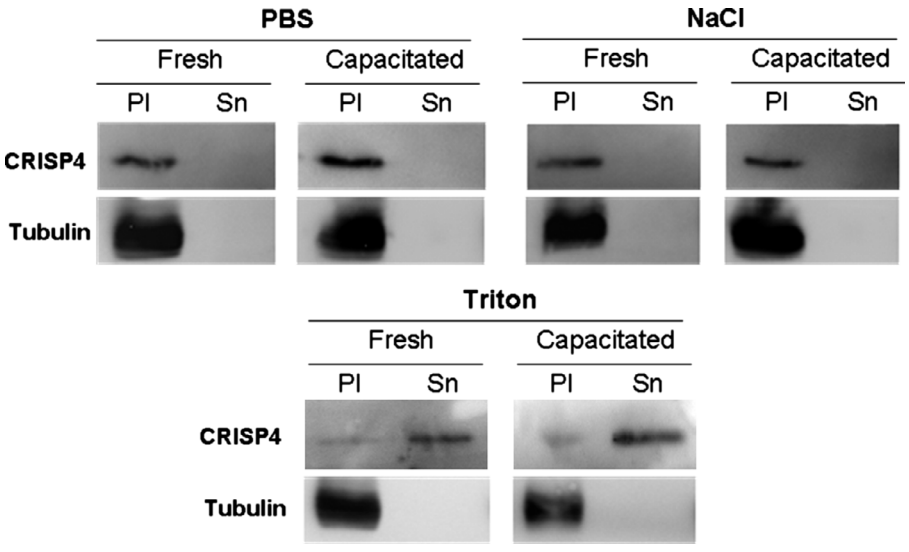


Figure 3 Involvement of CRISP1 and CRISP4 in fertilization. Epididymal CRISP1 participates in sperm-ZP binding and gamete fusion through ligand–receptor interactions, whereas cumulus CRISP1 has a role in sperm orientation by modulating sperm hyperactivation through its ability to regulate calcium channel CatSper. Epididymal CRISP4 participates in ZP penetration and gamete fusion by modulating the acrosome reaction likely through its ability to regulate calcium channels (i.e. TRPM8).

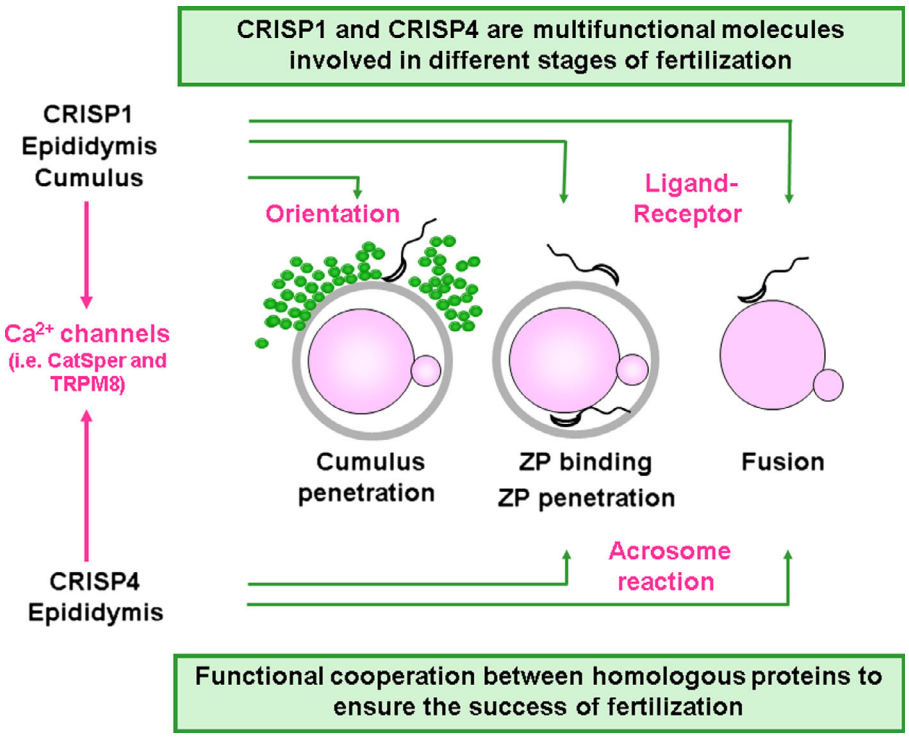
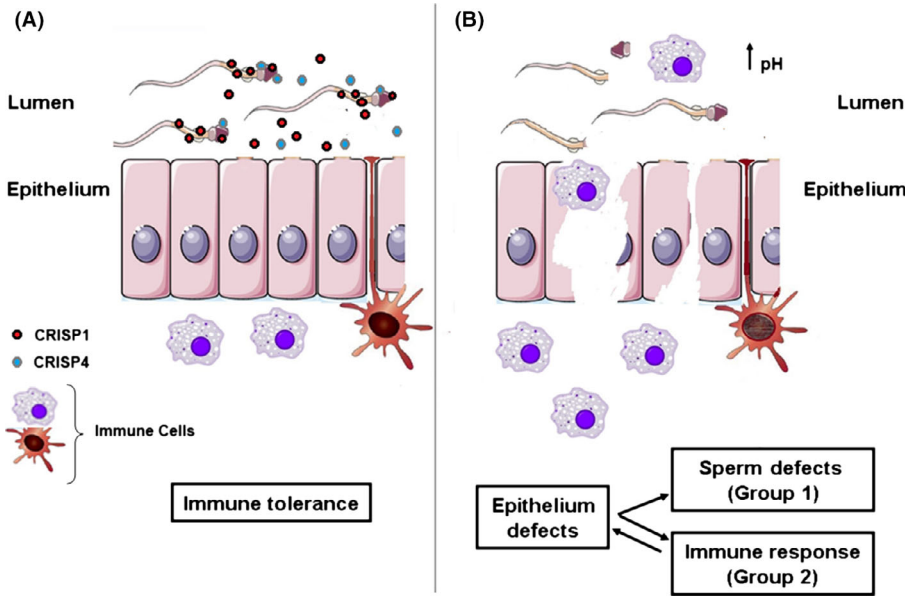


Figure 4 Schematic representation of functional defects in DKO males. (A) In the presence of CRISP1 and CRISP4, the epididymal epithelium as well as spermatozoa and the immune tolerance of the epididymis are normal. (B) The absence of CRISP1 and CRISP4 produces both epididymal epithelium and luminal acidification defects that may just affect sperm function (group 1) or also lead to an immune response which affects sperm viability and further compromises epithelium integrity (group 2).



proteins for animal fertility and the existence of a functional compensation among CRISP family members. Moreover, the number of born pups correlated with the number of fertilized oviductal eggs in females mated by the same mutant males, indicating that DKO fertility defects were due to deficiencies in the in vivo fertilization process (Carvajal *et al.*, 2018).

Interestingly, when the testes and epididymides were removed for sperm functional analysis, we observed that whereas most of the animals exhibited normal epididymides and testes (group 1), more than one third of the DKO males exhibited bigger epididymides and testes either unilaterally or bilaterally compared to controls (group 2). Subsequent studies revealed that while

group 1 males showed significantly lower fertility rates compared to controls, mice from group 2 exhibited severe fertility defects or were infertile when unilaterally or bilaterally affected, respectively (Carvajal *et al.*, 2018). These fertility defects were not found in recently reported DKO model for CRISP1 and CRISP4 (Hu *et al.*, 2018) very likely due to differences in the genetic context of the animals and/or environmental conditions.

Analysis of different sperm functional parameters revealed that whereas spermatozoa from group 1 were morphologically normal and as viable and motile as controls, those from group 2 had impaired motility and viability. Subsequent in vitro studies using viable spermatozoa from group 1 showed that mutant spermatozoa exhibited defects to undergo capacitation-associated events such as progesterone-induced acrosome reaction and hyperactivation as well as deficiencies to penetrate the cumulus mass, to bind to the ZP, and to fuse with the egg, confirming that DKO spermatozoa show deficiencies at several stages of the fertilization process. The accumulation of functional defects produced by the lack of each CRISP protein may explain why DKO spermatozoa exhibit in vivo fertilization defects not observed in single mutant spermatozoa.

Histopathological and immunohistochemical studies of the testes and epididymides showed that group 2 mice exhibited tissue damage and a clear immune response indicative of an epididymo-orchitis (Carvajal *et al.*, 2018). Nevertheless, as testicular and epididymal inflammation was moderate, the higher weight of the organs might be due not only to an immune infiltrate but also to edema. Further analysis of group 2 epididymal tissues revealed altered levels of the immunomodulator Gal-1 as well as of several cytokines such as Il-6, Il-10, and TGF-β, supporting a disruption of the characteristic immunological tolerance of the epididymis associated with epididymitis. The fact that this phenotype was neither observed in group 1 DKO mice nor in the single KO for CRISP1 or CRISP4 supports the relevance of these epididymal proteins for normal epididymal function as well as the existence of compensatory mechanisms between the two molecules.

Examination of the epididymal epithelium known to participate in the immune control of the organ revealed that DKO mice from both groups exhibited defects in all epithelial cell types with a more severe phenotype in group 2. More specifically, we observed that basal cells in the initial segment lacked their characteristic axiopodias, that principal and clear cells from all the regions (i.e. caput corpus and cauda) expressed less AQP9 and V-ATPase, respectively, and that clear cells from the cauda exhibited an immature 'narrow' and 'packed' phenotype (Carvajal *et al.*, 2018), supporting the relevance of CRISP1 and CRISP4 for a proper epididymal epithelium differentiation. As epithelial cells are involved in maintaining the blood-epididymal barrier that prevents sperm antigens from escaping the duct (Gregory & Cyr, 2014; Hedger, 2015), it is possible that the more severe epididymal epithelium defects of group 2 compromise the structure and/or function of the barrier allowing exposure of spermatozoa to the immune system.

Accompanying the epithelial defects, DKO mice from both groups showed an increase in luminal pH known to be critical to create a proper environment that maintains sperm quiescent during sperm maturation and storage in the epididymis (Shum *et al.*, 2011, 2013). This increase in luminal pH is consistent with the lack of axiopodias able to sense the luminal content and to regulate clear cell function as well as with the observed defects in both clear and principal cells that actively participate in luminal acidification and bicarbonate reabsorption/proton secretion, respectively (Park *et al.*, 2017). As luminal acidification involves an increase in cAMP concentration in both clear and principal cells, it is possible that luminal pH defects are linked to the recently reported ability of CRISP1 to regulate intracellular cAMP (Weigel Muñoz *et al.*, 2018). Moreover, as both CRISP1 and CRISP4 are expressed in epithelial cells and regulate calcium channels, defects in the cross-talk between calcium and cAMP signaling pathways may affect epididymal epithelium differentiation and, thus, the luminal microenvironment and sperm functionality. In addition, given the reported ability of CRISP1 and CRISP4 to inhibit CatSper/TRPM8 activity (Gibbs *et al.*, 2011; Ernesto *et al.*, 2015), the lack of these proteins might also directly affect sperm intracellular calcium levels leading to the different functional defects observed in DKO spermatozoa.

In conclusion, our results show that deletion of both epididymal CRISP1 and CRISP4 affects epididymal epithelium differentiation and luminal acidification leading to male fertility defects. Whereas in most of the cases, the epithelium and luminal defects specifically affected sperm function (group 1), in some animals there was a disruption of the characteristic immune tolerance of the organ with clear signs of an immunological reaction and severe sperm viability defects (group 2) (Fig. 4). Considering that DKO were not generated in an inbred strain, individual variabilities in the genetic background might be responsible for the different phenotypes observed for group 1 and group 2 DKO mice. Although the mechanisms underlying the different phenotypes observed in DKO mice remain to be elucidated, the finding that both epithelium and luminal acidification defects are observed in males that do not exhibit signs of inflammation indicates that these defects do not occur as a consequence of the immune reaction.

We believe these results will contribute to a better understanding of the fine-tuning mechanisms underlying both sperm maturation and immune tolerance within the epididymis with clear clinical implications for male infertility and fertility regulation. Moreover, as human epididymal CRISP1 has been proposed to be equivalent to the combination of rodent CRISP1 and CRISP4, DKO mice may represent an excellent model for studying the mechanisms underlying epididymal physiology and pathology in men (Mayer *et al.*, 2016; Fijak *et al.*, 2018).

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