Developmental Biology

Contribution of the epididymis beyond fertilization: relevance of CRISP1 and CRISP3 for sperm DNA integrity and early embryo development

Reviewed Preprint
v2 • March 14, 2025
Revised by authors

Reviewed Preprint
v1 • May 21, 2024

Valeria Sulzyk, Ludmila Curci, Lucas N González, Abril Rebagliati Cid, Mariana Weigel Muñoz, Patricia S Cuasnicu

Instituto de Biología y Medicina Experimental (IByME-CONICET), Buenos Aires, Argentina

eLife Assessment

This **valuable** study reports that epididymal proteins are required for embryogenesis after fertilization. The data presented are generally supportive of the conclusion and considered **solid**. This work will be of interest to reproductive biologists and andrologists.

https://doi.org/10.7554/eLife.97105.2.sa3

Abstract

Numerous reports show that the epididymis plays a key role in the acquisition of sperm fertilizing ability but less information exists on its contribution to embryo development. Evidence from our laboratory showed that mammalian CRISP (Cysteine-Rich Secretory Proteins), known to be expressed in the epididymis, to regulate calcium (Ca^{2+}) channels and to participate in fertilization, may also be relevant for embryo development. More specifically, we found that males with simultaneous mutations in *Crisp1* and *Crisp3* genes exhibited normal *in vivo* fertilization but impaired embryo development. In the present work, aimed to investigate the mechanisms underlying this reproductive phenotype, we observed that embryo development failure was not due to delayed fertilization as no differences in sperm transport within the female tract nor in *in vivo* fertilization were found shortly after mating. The observation that impaired embryo development was also found in eggs fertilized by epididymal sperm either after uterine insemination or *in vitro* fertilization, revealed that the defects were already present at epididymal level. Of note, eggs fertilized *in vitro* by mutant sperm exhibited impaired meiotic resumption not due to defects in Ca^{2+}oscillations during egg activation, prompting us to examine potential sperm DNA defects. Interestingly, DNA fragmentation was found in cauda but not caput epididymal mutant sperm revealing that DNA integrity defects appear during epididymal maturation. Moreover, exposure of control sperm to mutant epididymal fluid significantly increased DNA fragmentation, indicating the relevance of the luminal environment for sperm DNA integrity. The finding that incubation of sperm with control epididymal fluid in the presence of Ca^{2+} also increased DNA fragmentation together with the higher intracellular Ca^{2+} levels detected

in mutant sperm supports a dysregulation of Ca^{2+} homeostasis as the main responsible for DNA fragmentation and subsequent early development failure of mutant males. Together, our results support the contribution of the epididymis beyond fertilization, identifying CRISP1 and CRISP3 as novel male factors relevant for DNA integrity and early embryo development. Given the existence of human functional homologues of CRISP and the incidence of DNA fragmentation in infertile men, we believe these findings not only provide relevant information on the impact of epididymal factors on embryonic development but will also contribute to a better understanding, diagnosis and treatment of human infertility.

Introduction

Mammalian sperm that leave the testes do not have the ability to fertilize an egg and must undergo different processes to become fertilizing competent. Initially, they need to mature while they pass through the epididymis which provides a suitable environment for acquisition of fertilizing ability, storage and protection of sperm (Robaire *et al*., 2015). Subsequently, sperm must undergo another process known as capacitation while ascending through the female reproductive tract which will allow them to undergo the acrosome reaction in the head and to develop a hyperactivated motility in the tail, both essential for fertilization (Florman *et al*., 2015). Once they reach the site of fertilization in the oviduct, sperm penetrate the different coats that surround the egg (i.e. cumulus oophorus and zona pellucida (ZP)) and fuse with the plasma membrane of the egg, resulting in the formation of a single-cell zygote which then embarks on its development into a multicellular organism through a highly complex and tightly regulated process (Ramathal *et al*., 2015). Whereas numerous reports show the relevance of post-testicular maturation for fertilization, less information exists on the contribution of epididymal transit to embryo development.

The epididymis, the main location where sperm maturation takes place, has a very specialized epithelium involved in ion transport, protein matrix formation, and both protein and vesicle secretion (Robaire *et al*., 2015). Among such secretory proteins are CRISP (Cysteine-RIch Secretory Proteins), a group of evolutionarily conserved proteins mainly expressed in the male reproductive tract (Gibbs et al, 2008; Gonzalez *et al*., 2021). In mammals, four members have been described which escort sperm during their transit through both the male and female reproductive tracts. CRISP are characterized by the presence of 16 conserved Cys and two domains that have evolved to perform a variety of functions. Whereas the N-terminal domain of CRISP proteins (i.e. PR-1) is involved in cell to cell interaction (Ellerman *et al*., 2006; Maeda *et al*., 1998), proteolytic processes (Milne *et al*., 2003) and amyloid-like-aggregation activity (Olrichs *et al*., 2014, Sheng *et al*., 2019), the C-terminal domain (i.e. CRD) has the ability to regulate ion channels, such as ryanodine (RyR), CNGs, TRPM8 and Catsper (Ernesto *et al*., 2015; Gibbs *et al*., 2006; Gibbs *et al*., 2011; Yamazaki *et al*., 2002).

Biochemical, molecular and genetic approaches from our group and others revealed that CRISP proteins play key roles in sperm maturation, capacitation and fertilization (Gonzalez *et al*., 2021). Epididymal CRISP1, first described by our laboratory (Cameo and Blaquier, 1976), (Cohen *et al*., 2000a) associates with sperm during maturation and both inhibits CatSper (Ernesto *et al*., 2015), the primary sperm Ca^{2+} channel essential for male fertility (Ren *et al*., 2001; Sun *et al*., 2017), and participates in gamete interaction through egg-complementary sites (Busso *et al*., 2007; Cohen *et al*., 2000b; Rochwerger *et al*., 1992). CRISP4, almost exclusively synthesized in the epididymis, also associates with sperm during maturation (Jalkanen *et al*., 2005; Nolan *et al*., 2006, Weigel Muñoz *et al*., 2019), inhibits Ca^{2+} channel TRPM8 (Gibbs *et al*., 2011), and participates in the fertilization process (Carvajal *et al*., 2018; Gibbs *et al*., 2011; Turunen *et al*., 2012). CRISP3 exhibits a wider expression distribution including the epididymis and accessory glands within the male reproductive tract (Haendler *et al*., 1997; Schwidetzky *et al*., 1995; Udby *et al*., 2005) as well as organs and cells with immunological

functions (Reddy *et al.,* 2008). Whereas CRISP3 was found to bind to sperm with no roles in fertilization described so far (Da Ros *et al*., 2015), several reports support its association with male fertility in different species including humans (Chen *et al*., 2020; Doty *et al*., 2011; Gholami *et al.,* 2021).

In spite of clear evidence supporting the relevance of CRISP proteins for fertilization, knockout (KO) male mice for each individual CRISP remain fertile (Brukman *et al*., 2016; Carvajal *et al*., 2018; Da Ros *et al*., 2008; Gibbs *et al*., 2011; Turunen *et al*., 2012; Volpert et *al*., 2020; Weigel Muñoz *et al*., 2018), suggesting the existence of compensatory mechanisms among homologous CRISP family members. This idea was later confirmed by experiments from our group showing that simultaneous modifications in more than one CRISP gene can significantly impair male fertility (Curci *et al*., 2020). Whereas males lacking epididymal CRISP1 and CRISP4 (C1/C4 DKO) were subfertile due to a significant decrease in *in vivo* fertilization (Carvajal *et al*., 2018), males lacking CRISP1 and CRISP3 (C1/C3 DKO) were subfertile but exhibit normal *in vivo* fertilization, (Curci *et al*., 2020), supporting the notion that fertility inhibition in this colony resulted as a consequence of post-fertilization defects. Interestingly, subfertility in *Crisp1-/-Crisp3-/-* colony was associated with a failure of *in vivo* fertilized eggs to reach the blastocyst stage, revealing the potential relevance of CRISP1 and CRISP3 for early embryo development (Curci *et al*., 2020). Moreover, examination of ejaculated sperm within the uterus of control mated females showed that while control sperm were freely moving in the uterine fluid, *Crisp1-/-Crisp3-/-* sperm were mostly immotile and trapped into aggregates within a viscous uterine fluid (Curci *et al*., 2020), suggesting the relevance of these two proteins for sperm survival within the female reproductive tract. Considering the expression of CRISP1 and CRISP3 in the male reproductive tract, it is possible that defects during and/or following epididymal passage are responsible for the early embryo development defects observed for the mutant males. In view of this, in the present work, we explored possible mechanisms leading to *Crisp1-/-Crisp3-/-* male phenotype and provide novel evidence supporting the contribution of the epididymis and the relevance of both CRISP1 and CRISP3 for sperm DNA integrity and early embryo development.

Results

Embryo development defects associated with the lack of CRISP1 and CRISP3 are not due to a delayed fertilization

To analyze whether defects in early embryo development were specifically linked to mutations in C*risp1* and *Crisp3* genes or could also be contributing to the subfertility of C1/C4 DKO males (Carvajal *et al*., 2018), superovulated females were mated with mutant or control males from each DKO colony and those eggs recovered from the ampulla and reaching the two-cell stage *in vitro* (i.e. fertilized eggs) were further incubated to analyze the percentage progressing to blastocysts. Results revealed that whereas two-cell embryos from the C1/C3 DKO group showed a significant decrease in the percentage of blastocysts, those corresponding to C1/C4 DKO mice showed no differences in the percentage of blastocyst compared to controls (**Table 1**), supporting that early embryo development defects were caused by the simultaneous mutations of *Crisp1* and *Crisp3* genes.

Considering that delays in the time of *in vivo* fertilization could lead to embryonic development defects (Brackett *et al*., 1978; Lacham-Kaplan and Trounson, 1991, 1994; Orgebin-Crist, 1968; Orgebin-Crist and Jahad, 1977), and given the presence of aggregates of immotile sperm in the uterus of females mated with C1/C3 DKO males (Curci *et al*., 2020), we next investigated whether the early embryo development failure in this colony was due to a delayed fertilization caused by an impaired sperm transport within the female reproductive tract. To this aim, we analyzed both sperm migration within the oviduct and *in vivo* fertilization shortly after mating (i.e 4hs) as a way to avoid the possibility that the prolonged stay of sperm within the female tract

	Control	C1/C3 DKO	Control	C1/C4 DKO
Total two-cell embryos (N°)	53	53	80	76
Blastocysts (N°)	38	23	52	46
Embryo development (%)	75,2 ± 8,3%	37,4 ± 10,7% **	67,7 ± 12,1%	66,9 ± 13,4%

*Note: Percentage of embryo development was calculated as the mean of at least 5 independent experiments n = 5. ** p< 0.01 vs control.*

Table 1.

Analysis of embryo development corresponding to C1/C3 and C1/C4 DKO males

corresponding to the conventional mating schedule (18 hs), could be giving defective sperm enough time to reach the ampulla and fertilize the eggs. Using Acrosine-GFP (Green Fluorescent Protein)-tagged C1/C3 DKO or control males, we first examined sperm migration within the oviduct via fluorescence microscopy 4 hs after observation of copulatory plugs. Results indicated that both mutant and control sperm exhibited no difficulties to pass the uterotubal junction and migrate within the oviduct as judged by the presence of labeled sperm in both the lower and middle isthmus (**Figure 1**). The observation of very few fluorescent mutant or control sperm beyond the isthmus (**Figure 1B, C**) is due to the reported loss of the acrosome in sperm after reaching the middle/upper isthmus (La Spina *et al*., 2016; Muro *et al*., 2016). Consistent with these observations, examination of fertilization in the ampulla 4 hs after mating showed no significant differences between groups in the percentage of eggs recovered from the ampulla that develop to two-cell embryos *in vitro* (**Figure 2A**), confirming no defects in the time of arrival of sperm to the ampulla. However, once again, the eggs fertilized by mutant sperm exhibited clear defects to reach the blastocyst stage *in vitro* compared to controls (**Figure 2B**). Together, our observations suggest that factors other than a delayed fertilization due to transport defects were responsible for the observed embryo development phenotype of the mutant colony.

Mutant epididymal sperm already carry defects leading to embryo development failure

Given that our results had been obtained by natural mating, we next investigated whether the embryo developmental defects observed for C1/C3 DKO males appear during or after epididymal transit. To address this question, we inseminated C1/C3 DKO mature cauda epididymal sperm into one uterine horn and control cauda sperm in the contralateral horn of superovulated females, and then analyzed the percentage of eggs recovered from the ampulla capable of reaching the two-cell and blastocyst stages *in vitro*. Results showed that although no differences between groups were observed in the percentage of two-cell embryos (**Figure 2C**), the percentage of two-cell embryos progressing to blastocysts was significantly lower for mutant than for control sperm (**Figure 2D**), revealing that sperm defects contributing to embryo development deficiencies in C1/C3 DKO males were already present at the epididymal level.

It is known that fertilization under *in vitro* conditions provides a controlled environment for gamete interaction, avoiding potential sperm selection mechanisms or delays in sperm arrival to the egg that may occur *in vivo*, and allowing a more precise analysis of the kinetics of both fertilization and embryo development. In view of this, we next conducted a series of *in vitro* fertilization (IVF) studies using capacitated epididymal sperm and eggs surrounded or devoid of their coats. Results showed that whereas no differences in either fresh or capacitated sperm were found in sperm count, viability and progressive motility between groups (**Supp Table 1**), co-incubation of cumulus-oocyte complexes (COC) with mutant sperm led to significantly lower percentages of both fertilized eggs evaluated by Hoechst staining (i.e. decondensing heads or two pronuclei within the ooplasm) (**Figure 3A**) and eggs capable of progressing to two-cell embryos (**Figure 3B**). Interestingly, besides these fertilization defects, when two-cell embryos continued their incubation *in vitro* and the percentage of embryos at different stages of development was analyzed, a significant decrease in the percentage of embryos reaching the morula and blastocyst stages was observed for the mutant group (**Figure 3C**), confirming that defects leading to embryo development failure were already present in epididymal cells and could be detected even outside the female tract environment.

To investigate whether difficulties in penetration of the egg coats that surround the egg could generate a potential delay in fertilization that finally leads to embryo development failure, *in vitro* fertilization assays were carried out using eggs devoid of both cumulus cells and ZP, and the percentage of fertilized ZP-free eggs analyzed. Under these conditions, there was a lower but still

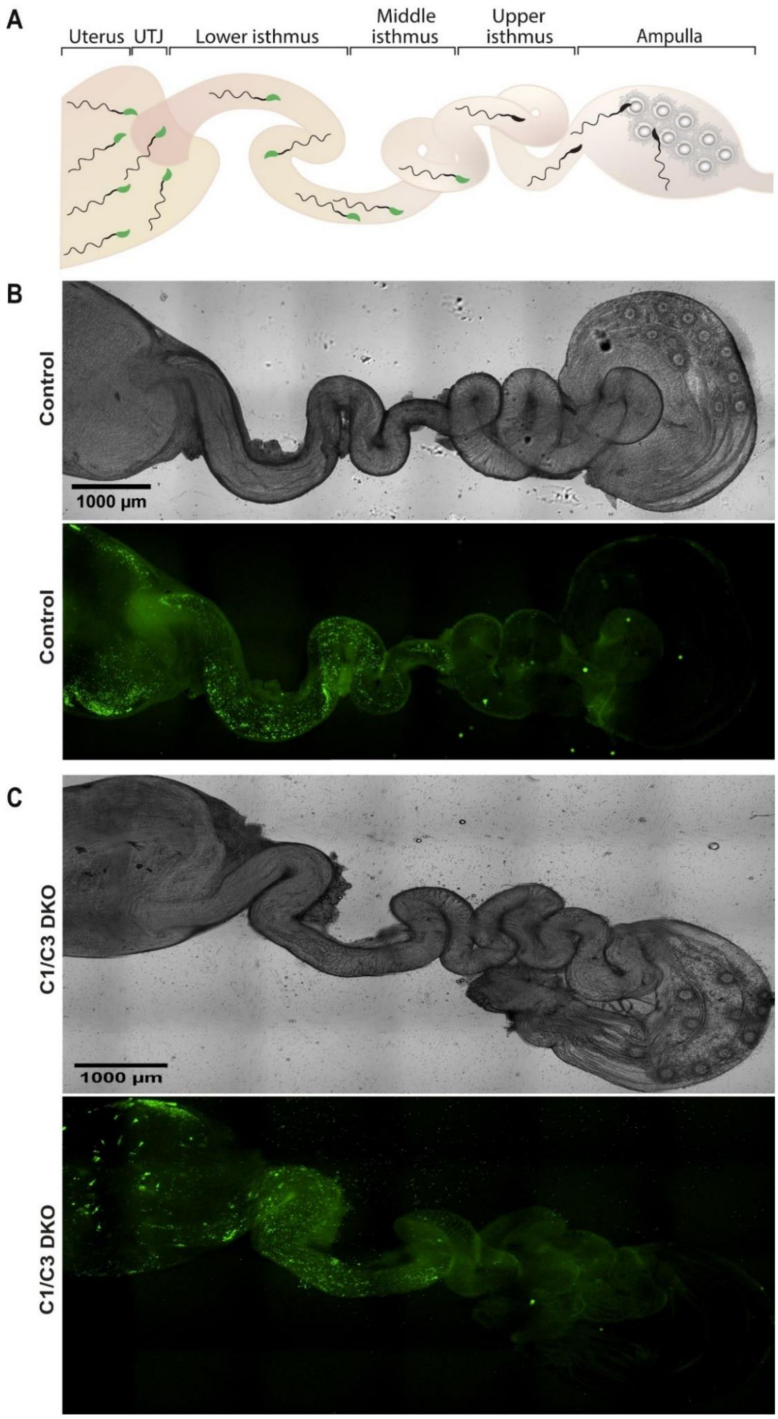

Figure 1.

Sperm migration through the female reproductive tract.

A. Representative diagram showing sperm within the different regions of the female reproductive tract. UTJ, utero-tubal junction. Superovulated females were mated with Acrosine-GFP control or C1/C3 DKO males and, 4 hours after mating, sperm were analyzed inside the tract by fluorescence microscopy. **B.** Bright field (upper panel) and fluorescence (lower panel) images of the reproductive tract of a female mated with a control male (x40). **C.** Bright field (upper panel) and fluorescence (lower panel) images of the reproductive tract of a female mated with C1/C3 DKO male (x40). Figures are representative of at least 3 independent experiments.

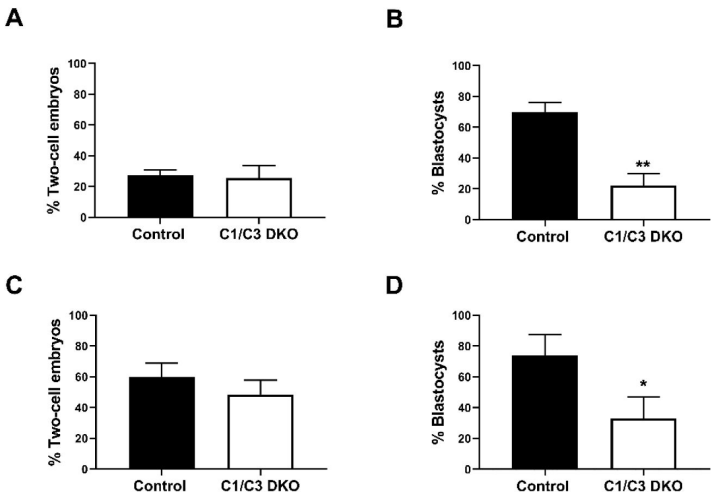

Figure 2.

In vivo fertilization and embryo development

A. Control and C1/C3 DKO males were mated with superovulated females and the percentage of fertilized eggs recovered from the ampulla 4 hours after mating was evaluated. Eggs were considered fertilized when they reached the two-cell embryo stage 24 h after *in vitro* incubation. **B.** Two-cell embryos from **A** were incubated *in vitro* for an additional 3 days, and the percentage reaching the blastocyst stage was determined. **C.** Control or C1/C3 DKO cauda epididymal sperm were inseminated into the uterus of superovulated females. After 15 h, eggs were recovered from the ampulla, incubated for 24 hours *in vitro* and were considered fertilized when reaching the two-cell embryo stage. **D.** Two-cell embryos from **C** were incubated *in vitro* for an additional 3 days and the percentage reaching the blastocyst stage was determined. Data are mean ± SEM; n=5, *p<0.05; **p<0.01. Percentages of two-cell embryos were determined by dividing the number of two-cell embryos by the total number of eggs examined and percentages of blastocysts as the number of eggs reaching the blastocyst stage divided by the total number of two cell embryos recovered.

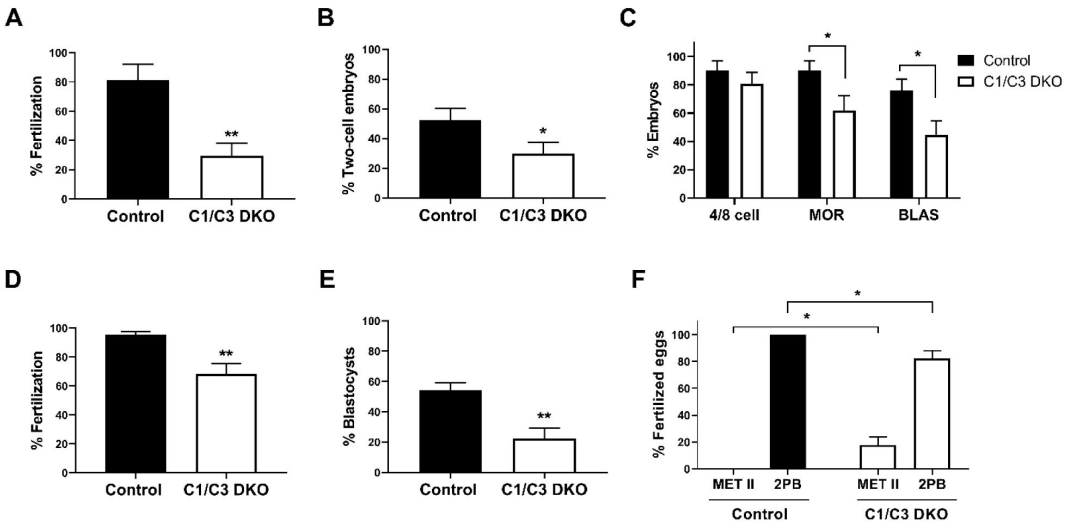

Figure 3.

In vitro fertilization and embryo development.

A, B. *In vitro* capacitated control or C1/C3 DKO cauda epididymal sperm were co-incubated with cumulus oocyte complexes (COC) for 3.5 hs. Eggs were either analyzed at that moment and considered fertilized when at least one decondensing sperm nucleus or two pronuclei were found in the ooplasm (n=3) (**A**) or incubated for additional 24 hs to determine the percentage reaching the two-cell embryo stage (n=7) (**B**). **C.** Two-cell embryos from **B** were incubated for 3 days *in vitro* and the percentage reaching 4/8 cells (day 1), morula (day 2) or blastocyst (day 3) stages determined (n=7). **D.** *In vitro* capacitated control or C1/C3 DKO epididymal sperm were co-incubated with ZP-free eggs for 1 h and fertilization was evaluated by DNA staining. Eggs were considered fertilized when at least one decondensing sperm nucleus was found in the ooplasm (n≥5). **E.** Fertilized ZP-free eggs obtained as in D were incubated for an additional 3 days *in vitro* and the percentage progressing to blastocysts was determined. (n=5). **F.** Fertilized eggs from **D** were analyzed for maternal DNA status and classified as arrested in Metaphase II (Met II) or exhibiting 2nd polar body (2PB) (n≥5). Data are the mean ± SEM; *p< 0.05; **p < 0.01. Percentages of fertilization were determined by dividing the number of fertilized eggs by the total number of eggs examined. Percentages of two-cell embryos were determined by dividing the number of two-cell embryos by the total number of eggs examined. Percentages of either 4/8 cell embryos, morula and blastocysts were calculated as the number of embryos reaching each of these stages divided by the total number of two cell embryos obtained.

significant decrease in the percentage of eggs fertilized by mutant sperm accompanied again by significantly lower rates of blastocysts (**Figure 3D, E**), indicating that defects in egg coat penetration were not responsible for embryo development failure.

To further analyze the mechanisms leading to embryo development defects, ZP-free eggs were co-incubated with capacitated sperm as above and both sperm and egg DNA status within the ooplasma of fertilized eggs were analyzed by Hoechst staining. Of note, results showed that whereas all eggs with decondensing heads had already extruded the 2nd polar body in controls, in 4 out of 6 experiments, a proportion of eggs with decondensing heads corresponding to the mutant group were still at Metaphase II (Met II) (**Figure 3F**), revealing defects in epididymal sperm affecting early post-fertilization events as the potential cause of the phenotype observed in the mutant colony.

Mutant epididymal sperm exhibited higher levels of both DNA fragmentation and intracellular Ca^{2+}

The finding that a proportion of eggs fertilized by epididymal mutant sperm *in vitro* were still at Met II, opened the possibility of defects in the meiotic resumption event that occurs during egg activation. Based on this, we next monitored the characteristic repetitive series of changes in intracellular Ca^{2+} concentration (Ca^{2+} oscillations) known to underpin release from meiotic arrest during egg activation and initiation of embryo development in mammalian eggs (Miyazaki *et al*., 2006, Wakai *et al*., 2019). For this purpose, ZP-free eggs were stained with Fluo-4 AM, co-incubated *in vitro* with control or mutant capacitated epididymal sperm and subjected to live confocal fluorescence imaging. Results showed no differences between the two genotypes in either the pattern of Ca^{2+} oscillations (**Figure 4A**) nor in a series of associated parameters such as the number of oscillations within 90 minutes, time until first oscillation (considered the time to gamete fusion), oscillation frequency, first transient area or first transient duration (**Figure 4B-F**). Together, these observations indicate that the presence of eggs still at Met II among those fertilized by mutant sperm was not due to defects in Ca^{2+} dynamics known to be critical for meiotic resumption during egg activation.

Considering that delays in early embryo development may result from the time taken by the egg to repair damaged paternal DNA (Esbert *et al*., 2018; Newman *et al*., 2022, Nguyen *et al*., 2023), we next decided to analyze possible defects in DNA integrity in mutant cauda epididymal sperm. These studies were carried out using the sperm chromatin dispersion assay (SCD) which is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo of dispersed DNA loops observed in sperm with non-fragmented DNA (Fernández *et al*., 2003). Interestingly, results showed a significantly higher level of DNA fragmentation in mutant than control sperm as indicated by the distribution of individual cells as a function of their DNA halo area and the percentage of cells failing to produce the halo (**Figure 5A**). To investigate whether the higher DNA fragmentation levels observed in mutant cauda sperm appear during epididymal transit, DNA fragmentation was also analyzed in immature sperm recovered from the caput region using cauda sperm from the same epididymis as control. Results showed that caput cells exhibited no differences in DNA fragmentation levels between groups, indicating that defects in DNA integrity in mutant sperm develop within the epididymis (**Figure 5B**). Given the well-established relationship between DNA fragmentation and oxidative stress, we next analyzed reactive oxygen species (ROS) levels in cauda epididymal sperm from control and mutant mice. Interestingly, results showed that ROS levels in mutant sperm were not higher than those observed in the control group (**Figure 5C**), not favoring the idea that higher sperm DNA fragmentation levels were due to an increase in oxidative stress in mutant cells.

To assess the impact of the epididymal environment on sperm DNA fragmentation, WT cauda epididymal sperm were exposed to epididymal fluid recovered from mutant mice, and sperm DNA integrity analyzed by the SCD assay. Results showed that, under these conditions, WT sperm

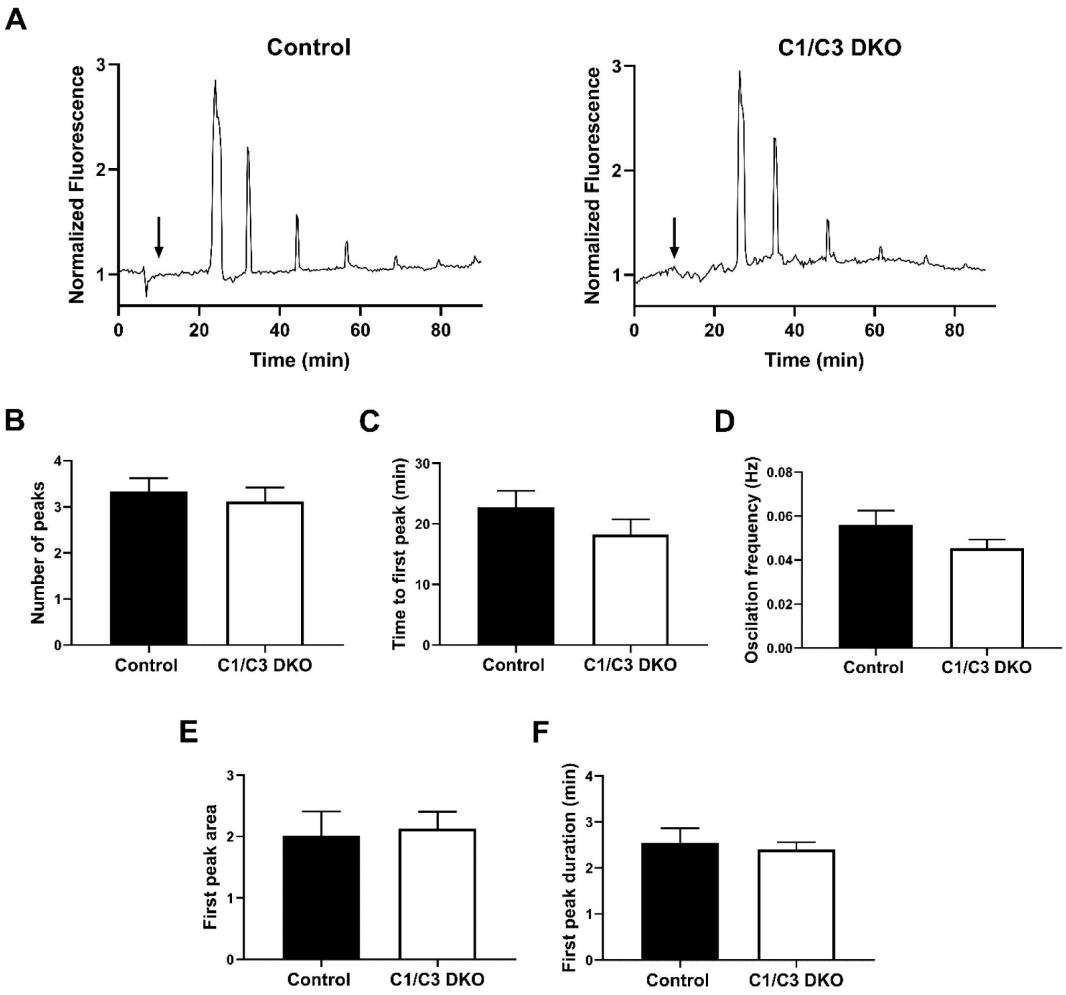

Figure 4

Egg Ca^{2+} oscillations in *in vitro* fertilized eggs.

A. Representative traces of Ca^{2+} oscillation patterns following *in vitro* fertilization of ZP-free eggs with control or C1/C3 DKO sperm. Arrows indicate the time of sperm addition. **B.** Number of peaks within 90 minutes, **C.** Time to first peak, **D.** Oscillation frequency, **E.** First peak area under the curve and **F.** First transient duration. Values were normalized to basal Ca^{2+} levels recorded prior to sperm addition. Data are mean ± SEM of at least 9 oocytes from 3 independent experiments; A-F: ns.

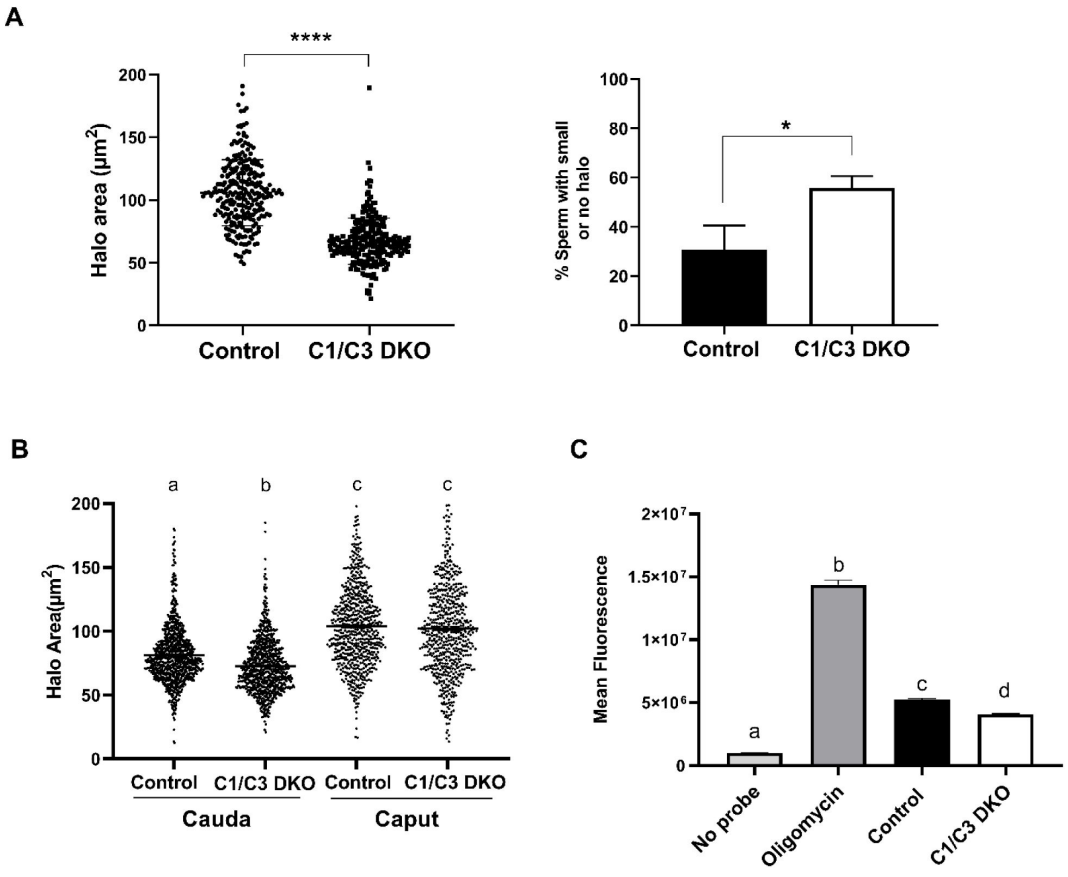

Figure 5.

Sperm DNA fragmentation and ROS levels.

A. DNA fragmentation of control or C1/C3 DKO cauda epididymal sperm was analyzed by SCD assay and both the area of DNA halo in each individual cell (left panel) and the percentage of sperm heads with small or no halo (right panel) determined. **B.** DNA fragmentation of cauda and caput epididymal control or C1/C3 DKO sperm was analyzed by SCD assay and the area of DNA halo in each individual cell determined. **C.** ROS levels in control and C1/C3 DKO cauda sperm analyzed by fluorescence confocal microscopy. Absence of probe and presence of Oligomycin were used as negative and positive controls, respectively. In all cases n=4. *p<0,05; ****p < 0,0001, Different letters indicate significant differences between treatments, p<0.0001.

exhibited significantly higher levels of DNA fragmentation, supporting the relevance of epididymal fluid for sperm DNA integrity. Exposure of mutant sperm to epididymal fluid from WT mice, on the other hand, did not modify the higher DNA fragmentation levels exhibited by mutant cells (**Figure 6A**). Considering both reports showing that sperm DNA fragmentation can be induced by divalent cations in the presence of epididymal fluids (Gawecka *et al*., 2015; Shaman *et al*., 2006), and that CRISP proteins are Ca^{2+} channel regulators (Ernesto *et al*., 2015; Gibbs *et al*., 2006; Gibbs *et al*., 2011), we next analyzed the possibility that higher Ca^{2+} levels in the epididymis might contribute to the impaired DNA integrity of mutant sperm. For this purpose, WT cauda epididymal sperm were incubated *in vitro* with WT epididymal fluid in the presence of Ca^{2+} (10 mM), detecting significantly higher levels of DNA fragmentation in these cells compared to controls incubated in the absence of the cation (**Figure 6A**). As another approach to explore the involvement of Ca^{2+} in sperm DNA fragmentation, we next analyzed intracellular Ca^{2+} in cauda epididymal sperm by flow cytometry detecting significantly higher Ca^{2+} levels in mutant than control sperm either before or after capacitation (**Figure 6B**). Together, these observations support a dysregulation of Ca^{2+} within the epididymis and sperm as the main responsible for the higher sperm DNA fragmentation levels and, thus, the subsequent embryo development failure observed for mutant males.

Discussion

Substantial evidence supports the involvement of the epididymis in the acquisition of sperm fertilizing ability (Björkgren and Sipilä, 2019). However, limited information exists on the contribution of epididymal transit to the early stages of embryo development known to be crucial in determining the overall fitness of the organism. In this regard, whereas the molecular mechanisms underlying the impact of paternal factors on embryo development remain to be fully elucidated, the present work provides novel findings supporting the contribution of the epididymis to early embryo development and identifying CRISP1 and CRISP3 proteins as novel male factors relevant to this process.

Previous observations from our laboratory (Curci et al, 2020) showed that females mated with males with mutations in *Crisp1* and *Crisp3* genes (C1/C3 DKO) exhibited normal *in vivo* fertilization rates but significantly lower percentages of embryo development, supporting the notion that male-derived factors are required to allow for correct development of the embryo (Vallet-Buisan *et al*., 2023). Moreover, we observed that triple knockouts lacking CRISP1, CRISP2 and CRISP3 as well quadruple knockouts lacking the four members of the family exhibited both fertilization and embryo development defects, supporting the lack of CRISP1 and CRISP3 as relevant for the embryo development phenotype. Expanding on those observations, in the present study we confirmed an association between embryo development failure and mutations in *Crisp1* and *Crisp3* as indicated by the finding that eggs fertilized by sperm from *Crisp1*-/-*Crisp4*-/- males did not exhibit evidence of embryo development failure. Thus, whereas males from both double knockout colonies are subfertile and share the lack of CRISP1, the mechanisms underlying their subfertility are different depending on the other simultaneously deleted protein, providing information on the different functional modules that operate within the CRISP family (Curci *et al*., 2020, Gonzalez et al, 2021), and establishing two models that uncouple fertilization from early embryo development. In this way, whereas C1/C4 DKO mice exclusively exhibiting defects in fertilization contribute to a better understanding of the mechanisms involved in gamete interaction, C1/C3 DKO males showing only defects in egg progression to blastocysts become an excellent model for elucidating the molecular mechanisms inherent to the early embryo development process.

Our findings showing the presence of aggregates of immotile sperm within the uterus of females mated with C1/C3 DKO males (Curci *et al*., 2020) opened the possibility that a delayed fertilization due to a retarded arrival of sperm to the ampulla (the site of fertilization) could be the reason for embryo development failure as previously reported for immature sperm (Brackett *et al*.,

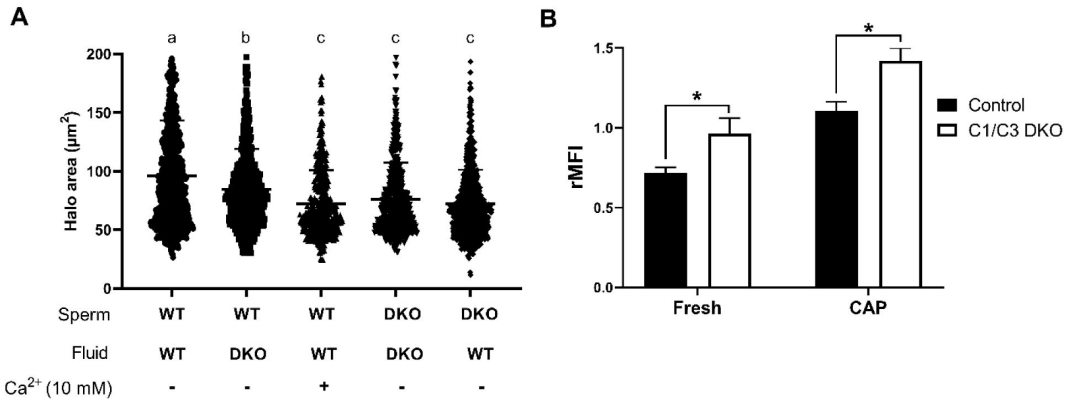

Figure 6.

Influence of epididymal fluid on sperm DNA integrity and intracellular Ca^{2+} levels.

A. DNA fragmentation of control or C1/C3 DKO cauda epididymal sperm incubated for one hour with their own or the other genotype epididymal fluid in the presence or absence of 10mM Ca^{2+} was analyzed by SCD assay and the area of DNA halo in each individual cell determined. Different letters indicate significant differences between treatments ($p<0.05$). **B.** Intracellular Ca^{2+} levels were evaluated by flow cytometry using Fluo-4-AM probe. Results are shown as normalized mean fluorescence intensity (rMFI) of Fluo-4-AM compared to the control condition in each experiment for non-capacitated (fresh) and capacitated (CAP) sperm. Data are mean ± SEM, n= 5; *p<0,05.

1978; Lacham-Kaplan and Trounson, 1991; 1994; Orgebin-Crist, 1968; Orgebin-Crist and Jahad, 1977). Evaluation of sperm migration within the female tract shortly after mating to avoid a delayed fertilization showed, however, that mutant sperm could pass through the uterotubal junction and reach the lower/middle isthmus just like control sperm, indicating that embryo development failure in mutant mice is unlikely to be due to defects in sperm transport from the uterus to the oviduct and/ or migration within the oviduct. Moreover, although the loss of the acrosomes due the occurrence of the acrosome reaction in the isthmus (La Spina *et al*., 2016; Muro *et al*., 2016) did not allow the detection of labeled sperm within the ampulla, our results indicated that the time of sperm arrival to the ampulla was comparable to that of control sperm as evidenced by fertilization rates not different from controls at 4 hs post copulation. However, in spite of the lack of differences in sperm arrival to the ampulla and fertilization rates observed shortly after mating, the fertilized eggs still exhibited an impaired ability to reach the blastocyst stage, not favoring the idea that a delayed fertilization and/or a consequently compromised oocyte quality could be responsible for the early embryo development defects observed in the mutant colony.

Considering various reports showing that male accessory glands contribute to proper embryo development without affecting fertilization rates (Jodar, 2019; Ma *et al*., 2022; Vallet-Buisan *et al*., 2023; W. S. *et al*.,1988), the possibility existed that the sperm defects leading to the embryo development phenotype were associated with the lack of CRISP1 and CRISP3 in the accessory gland secretions. Interestingly, however, when epididymal sperm were directly inseminated into the uterus, we observed again normal fertilization rates in the ampulla accompanied by impaired embryo development, revealing that defects were already present in epididymal sperm. This conclusion was further supported by IVF studies showing that cumulus oocyte complexes fertilized by mutant epididymal sperm also exhibited embryo developmental defects. Unlike *in vivo assays*, however, IVF studies revealed fertilization defects in mutant sperm not detected by mating. This difference is likely due to the effective mechanism of sperm selection within the female reproductive tract that allows only high-quality sperm to reach the eggs (Cummins and Yanagimachi 1982) even in males with reproductive deficiencies, thus, masking sperm defects detected under *in vitro* conditions (Brukman *et al*., 2016). Nevertheless, the finding that C1/C3 DKO sperm exhibit *in vitro* fertilization capabilities similar to those reported for sperm lacking only CRISP1 (Da Ros *et al*., 2008), does not support a major role for murine CRISP3 in gamete interaction in agreement with our previous observations for human CRISP3 (Da Ros *et al*., 2015).

The observation that ZP-free oocytes fertilized *in vitro* by mutant epididymal sperm also exhibited a lower ability to reach the blastocyst stage excluded potential delays generated by deficiencies in egg coat penetration as a cause of embryo development failure, indicating the existence of defects other than those associated with gamete interaction *per se* as responsible for the mutant colony phenotype. Interestingly, a proportion of ZP-free eggs fertilized by mutant sperm were still at Met II in contrast to all eggs exhibiting 2nd polar body in controls, supporting that immediate post-fertilization timing defects could be contributing to embryo development failure. In this regard, incorporation of the time lapse technology in human IVF treatments has highlighted the relevance of the timing of post-fertilization events and early cleavage as robust predictors of both embryo development to blastocyst and implantation success (Basile *et al*., 2015, Esbert *et al*., 2018; Meseguer *et al*., 2011).

Our observations showing defects in meiotic resumption in eggs fertilized by mutant sperm opened the possibility that an impaired egg activation could be the cause of embryo development failure. In this regard, the well-established significance of egg Ca^{2+} oscillations in predicting the developmental competence of mouse zygotes and their pivotal role in meiosis resumption (Miyazaki *et al*., 2006, Wakai *et al*., 2019), led us to analyze egg Ca^{2+} oscillations following fertilization by mutant sperm. Results showing no difference in the pattern of oscillations nor in

the time to the first peak which is indicative of gamete fusion confirmed immediate post-fertilization defects as responsible for egg development failure, dismissing alterations in Ca^{2+} oscillations as the cause of embryo development impairment in the mutant mice.

Evidence showing that time is needed before the first embryonic cell division for activation of the egg DNA repairing machinery (Martin *et al*., 2019; Newman *et al*., 2022) together with the known association between high levels of DNA fragmentation and abnormal embryonic development (Marinaro *et al*., 2023; Nguyen *et al*., 2023; Simon *et al*., 2014), raised the possibility that meiotic resumption failure may result from defects in sperm DNA integrity. In this regard, as spermatozoa harboring DNA damage have no mechanism for its repair but retain their ability to negotiate the female reproductive tract, reach the site of fertilization and fertilize oocytes, DNA-repairing activity relies on the oocyte once fertilization takes place (González Marin *et al*., 2012, Martin *et al*., 2019), representing a critical step in the generation of viable embryos. Our findings showing significantly higher levels of DNA fragmentation in mutant than control cauda epididymal sperm support sperm DNA damage as the potential cause of the early post-fertilization defects. In agreement with this conclusion, several embryo kinetics studies showed a significant delay in the early stage of second polar body extrusion in human eggs fertilized by sperm with DNA fragmentation (Casanovas *et al*., 2019; Esbert *et al*., 2018; Wdowiak *et al*., 2015). Together, our observations underscore the intricate relationship between sperm DNA integrity and the regulatory role of CRISP proteins in shaping early embryonic outcomes.

How the lack of male CRISP1 and CRISP3 proteins could lead to higher levels of sperm DNA fragmentation? The finding that defects leading to embryo development in the mutant colony were present in epididymal sperm and that neither CRISP1 nor CRISP3 are expressed in the testes, supports the appearance of mutant sperm DNA fragmentation within the epididymis. In this regard, detection of these defects in cauda but not caput sperm supports that DNA integrity defects develop during epididymal transit and/or storage in agreement with the idea that the main pathways leading to DNA damage are triggered during epididymal transit (Okada *et al*., 2020; Sakkas *et al*., 2010; Suganuma *et al*., 2005), probably to allow elimination of defective sperm (Gawecka *et al*., 2015). Based on this, we can speculate that the lack of CRISP1 and CRISP3 in the epididymis renders sperm more susceptible to DNA degradation i.e., by reactive oxygen species. However, in spite of the commonly accepted association between DNA fragmentation and oxidative stress, our results did not show higher levels of ROS in mutant sperm, not favoring oxidative stress as the mechanism underlying DNA fragmentation in our mutant colony. These results differ from those reported for mice lacking epididymal antioxidant GPX5 (Glutathione Peroxidase 5) which showed higher incidence of embryo development and increased sperm DNA fragmentation but accompanied by higher levels of ROS in mutant sperm (Chabory *et al*., 2009). Nevertheless, as these mice also exhibited changes in small RNAs within the epididymis (Chu *et al*., 2020), we do not exclude the possibility that the lack of CRISP1 and CRISP3 in the epididymis may also affect the profile of small RNAs known to change during epididymal transit and to be important for embryonic development (Yuan *et al*., 2016).

Previous reports showing induction of DNA fragmentation by exposure of mouse sperm to divalent cations (Ca^{2+} and Mn^{2+}) in the presence of luminal fluids concluded that DNA fragmentation is largely controlled by the luminal fluids with a contribution from sperm that is acquired during maturation (Gawecka *et al*., 2015, Shaman *et al*., 2006). This, together with the fact that all CRISP are Ca^{2+} channel regulators, opened the possibility that changes in Ca^{2+} regulation within the epididymis could be responsible for the higher fragmentation levels observed in our mutant sperm. Consistent with this idea, our results showed a significant increase in DNA fragmentation when WT sperm were exposed to either mutant epididymal fluid or WT epididymal fluid in the presence of Ca^{2+}. These observations, together with the higher intracellular Ca^{2+} levels detected in mutant sperm, support a dysregulation in Ca^{2+} homeostasis in the epididymis as the main responsible for the observed sperm DNA integrity defects, probably

through the activation of Ca^{2+}-dependent nucleases present within the epididymal fluid and/or sperm cells (Shaman *et al*., 2006, Sotolongo *et al*., 2005, Boaz *et al*., 2008, Dominguez and Ward, 2009).

In summary, our observations provide strong evidence supporting the contribution of the epididymis beyond the acquisition of sperm fertilizing ability, identifying CRISP1 and CRISP3 as novel male factors relevant for sperm DNA integrity and early embryo development. Moreover, to our knowledge, this is the first report showing the relevance of epididymal proteins for early post-fertilization events. Given the existence of homologous human CRISP proteins in the epididymis and sperm playing equivalent roles to their rodent counterparts (Curci *et al*., 2020, Gonzalez *et al*., 2021), it is possible that CRISP are also involved in human embryogenesis through similar mechanisms. Considering the incidence of sperm DNA fragmentation in male infertility (Agarwal *et al*., 2020; Esteves *et al*., 2020), we believe our findings not only provide key information on the impact of epididymal factors on mammalian embryo development but will also contribute to a better understanding, diagnosis and treatment of human infertility.

Materials and Methods

Animals and ethical approval

Adult males (3-5 months old) from *Crisp1-/-Crisp3-/-* (Curci *et al*., 2020) or *Crisp1-/-Crisp4-/-* colonies (Carvajal, *et al*., 2018) and control young (3-5 weeks old) or adult (2-5 months old) females were used. Animals were maintained with food and water *ad libitum* in a temperature-controlled room with a 12:12-h light:dark cycle. 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).

In vivo fertilization assays and in vitro embryo development

Males were caged individually for one night with superovulated females. For ovulation induction, females were treated with an i.p. injection of equine chorionic gonadotropin (5 UI, Zoetis, Buenos Aires, Argentina), followed by an i.p. injection of human chorionic gonadotropin (hCG; 5 IU, Zoetis, Buenos Aires, Argentina) 48 hours later. Mating was evaluated the following morning and considered successful by the presence of copulatory plugs. Eggs were then recovered from the oviducts, placed in KSOM médium (Erbach *et al*., 1994) 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 embryo stage. For evaluation of their development to blastocyst, two-cell embryos were incubated for an additional 3 days under the same conditions.

Sperm transport and migration within the female tract

Male mice expressing a transgene for an acrosomal EGFP were mated with superovulated females to detect sperm within the oviduct, as previously described (La Spina *et al*., 2016; Curci *et al*., 2020). Briefly, a wild-type female subjected to superovulation was caged for 45 minutes with a transgenic male 12 hours after hCG administration. After 4 hours of detection of copulatory plug, the uterus and 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 microscope (Olympus Corp., Tokyo, Japan) at ×40. The number of fluorescent sperm within the oviduct was evaluated subjectively.

Epididymal sperm collection and in vitro capacitation

Mouse sperm were recovered by incising the cauda epididymis in 150 µl of capacitation medium (Fraser and Drury, 1975; Giaccagli *et al*., 2021) supplemented with 0.3% (w/v) bovine serum albumin (BSA), pH: 7.3–7.5, allowing motile sperm to swim-out of the cauda for 10 minutes at 37°C in an atmosphere of 5% (v/v) CO_2 in air. For *in vitro* capacitation, aliquots of the swim-out suspension were added to 300 µl of capacitation medium to a final concentration of 1–10 × 10^6 spermatozoa/ml and incubated for 90 min under the same conditions.

Intrauterine insemination

For intrauterine insemination young females were superovulated as previously described. Eight hours after hCG injection, the females were anesthetized with an i.p. injection of xylazine/ketamine (10:100 mg/kg), and an incision was made in the abdomen, exposing both uterine horns. Using a syringe, 50 µl of either mutant or control swim-out sperm suspensions (1×10^7 spermatozoa/ml) were introduced into one uterine horn followed by immediate ligation, whereas the remaining sperm suspension was introduced in the contralateral horn. After approximately 15 hours, oocytes were recovered from the ampulla, placed in KSOM medium, incubated at 37°C and 5% v/v CO_2, and the percentage of cells reaching the two-cell embryo stage was analyzed the following day. Two-cell embryos were then incubated for additional 3 days under the same conditions to evaluate their development to blastocyst.

In vitro fertilization assays

Gamete interaction assays were carried out as previously reported (Curci *et al*., 2020). Briefly, cumulus-oocyte complexes (COC) were collected from superovulated females 12-15 h after hCG administration. When needed, cumulus cells were removed by incubating the COC in 0.3 mg/ml hyaluronidase (type IV) for 3-5 min and zona pellucida (ZP) was dissolved by treating the eggs with acid Tyrode solution (pH 2.5) for 10-20 s (Nicolson *et al*., 1975). COC were inseminated with a final concentration of 1-5 × 10^5 cells/ml and gametes were co-incubated for 3.5 h at 37°C in an atmosphere of 5% (v/v) CO_2 in air. For gamete fusion assays, ZP-free eggs were inseminated with a final concentration of 1-5 × 10^4 cells/ml and gametes co-incubated for 1 h under the same conditions. In all cases, eggs were recovered at the end of incubation, washed, fixed with 2% (w/v) paraformaldehyde in PBS and stained with 10 µg/ml Hoechst 33342 for evaluation of fertilization under epifluorescence microscope (× 200). Eggs were considered fertilized when at least one decondensing sperm nucleus or two pronuclei were observed in the egg cytoplasm. Alternatively, ZP-intact or ZP-free eggs were recovered at the end of incubation and placed in KSOM medium for 24 hs to determine the percentage reaching the two-cell embryo stage or for 3 additional days for evaluation of eggs in blastocyst stage. To avoid sticking, ZP-free eggs were incubated in individual droplets. For evaluation of progression to different stages of embryo development, two cell embryos obtained from COC were incubated for 3 days in KSOM and the percentage reaching each stage (i.e. 4/8 cells, morula or blastocyst) determined.

Analysis of sperm functional parameters

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). For progressive motility assessment, sperm suspensions (15 µl) were placed between prewarmed slides and coverslips (22 mm x 22 mm) to create a chamber with 30 mm depth and sperm movement was recorded by video microscopy under a light microscope (Nikon ECLIPSE E200; Basler acA-78075gc) at 400x magnification for subsequent analysis. The percentage of progressive motile sperm was calculated by analyzing a minimum of 300 cells distributed in at least 20 different microscope fields.

Oocyte Ca^{2+} *oscillations*

ZP-free eggs were incubated with 1 µM Fluo-4 AM, 0.02% (p/v) pluronic acid and 15 µg/ml Hoechst 33342 in capacitation medium for 25 min at room temperature. Eggs were then extensively washed in fresh medium, mounted in 100 µl of medium covered with paraffin oil and analyzed on an Olympus IX83 Spinning Disk microscope (Olympus Corp., Tokyo, Japan) (x100), equipped with an environmental chamber sustaining a temperature of 37.5 °C and 5% CO_2. Images were taken every 20 seconds. Basal Ca^{2+} was recorded for 10 minutes. Then, *in vitro* capacitated sperm were added and image recording continued for at least 1.5 hours. In all cases, fertilization was analyzed by the presence of at least one decondensing sperm head within the ooplasm. Polyspermic eggs were excluded from the analysis. Intracellular Ca^{2+} was determined in a single equatorial plane of each egg by measuring the fluorescence intensity using the ImageJ software (*http://imagej.nih.gov/ij*) and normalized to basal fluorescence.

Sperm chromatin dispersion (SCD) assay

Sperm DNA integrity was assessed as described before (Fernández *et al*., 2003). Briefly, aliquots of 200 µl of sperm from swim-out in capacitating medium were mixed with 1% low-melting-point aqueous agarose (to obtain a 0.7% final agarose concentration) at 37°C. In those cases in which caput sperm were used, cells were centrifuged for 3 minutes at 1500 rpm prior to the addition of agarose. Aliquots of 50 µl of the mixture were pipetted onto a coverslip and then placed over a glass slide precoated with 0.65% standard agarose and left to solidify at 4°C for 10 minutes. Coverslips were carefully removed, and slides were immediately immersed horizontally in a tray with freshly prepared acid denaturation solution (0.08 N HCl) for 14 minutes at room temperature in the dark to generate restricted single-stranded DNA (ssDNA) motifs from DNA breaks. Then, proteins were removed by transfer of the slides to a tray with neutralizing and lysing solution 1 (0.4 M Tris, 0.8 M β-mercaptoetanol, 1% SDS, and 50 mM EDTA, pH 7.5) for 20 minutes at room temperature, which was followed by incubation in neutralizing and lysis solution 2 (0.4 M Tris, 2 M NaCl, and 1% SDS, pH 7.5) for 15 minutes at room temperature. Slides were thoroughly washed in TBE buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 7.5) for 12 minutes, dehydrated in sequential 70%, 90%, and 100% ethanol baths (2 minutes each), and air dried. Cells were stained with Hoechst (10 µg/ml) in Vectashield (Vector Laboratories, Burlingame, CA) and halo surface analyzed by fluorescence microscopy on an Olympus IX83 Spinning Disk microscope (Olympus Corp., Tokyo, Japan) (x600, AN 1.42). Pictures of at least 200 sperm heads were taken and the halo area analyzed by ImageJ software (*http://imagej.nih.gov/ij*). Sperm DNA was considered fragmented when no halo was observed or when the halo area was smaller than twice the area corresponding to non-dispersed sperm.

Analysis of Reactive Oxygen Species (ROS)

ROS levels in sperm were measured by confocal microscopy. Briefly, after swim-out in capacitating medium without BSA, Hoechst 33342 (40 µg/ml) and CellROX-Gren (25 µM) were added and sperm incubated under these conditions for 30 minutes at 37 °C and 5% CO_2. Samples were then fixed with 4% paraformaldehyde for 10 minutes, exposed to 100mM ammonium acetate (pH:9.0), centrifuged at 3000 rpm for 3 minutes two times and, finally, placed over a glass slide, mounted with glycerol and analyzed on an Olympus IX83 Spinning Disk microscope (Olympus Corp., Tokyo, Japan) (x600). Images of at least 200 sperm heads for each condition were analyzed and fluorescence intensity calculated by ImageJ software (*http://imagej.nih.gov/ij*).

Incubation of sperm with epididymal fluids

After 10 min of swim-out in capacitating medium without BSA, sperm were centrifuged 2 min at 1500 rpm and the recovered supernatants containing diluted cauda fluid stored at 37°C in an atmosphere of 5% (v/v) CO_2 in air. Sperm were then washed two times with 1 ml of capacitating

medium without BSA followed by a 2 min centrifugation at 1500 rpm. After the second centrifugation, cauda fluids from either the same or the other genotype were added and incubation continued for 1 hour at 37°C in an atmosphere of 5% (v/v) CO_2. For evaluation of the effect of Ca^{2+}, sperm were incubated as described above in the presence of 10 mM Ca^{2+} prepared from a 100X $CaCl_2$ stock solution.

Sperm Intracellular Ca^{2+} measurement

Cytoplasmic Ca^{2+} levels in sperm were measured by flow cytometry as previously described (Brukman *et al.*, 2016; Curci *et al.*, 2020). 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 and at least 10,000 events were analyzed per sample. Data analysis was performed by FlowJo 10 software (FlowJo LLC, Ashland, OR, USA). In each condition, the fluorescence mean was normalized to basal Fluo-4-AM fluorescence.

Statistical analysis

Data represents the mean ± SEM of at least three independent experiments and "n" indicates the number of animals analyzed in each group for all experiments except for oocyte Ca^{2+} oscillations where "n" indicates the number of oocytes analyzed. Calculations were performed using the Prism 8.0 software (GraphPad Software, La Jolla, CA). Comparisons between two experimental groups were analyzed by one-way t-student test whereas comparisons among three or more groups were analyzed by two-way analysis of variance (ANOVA) followed by Fisher LSD for embryo development progression and Holm-Sidak's for both resumption of meiosis and sperm intracellular Ca^{2+}. In those cases in which data did not meet the assumptions required to perform two-way ANOVA (i.e. caput sperm DNA fragmentation, ROS levels and incubation with fluids), non-parametric Kruskal-Wallis followed by Dunńs test were used. In all cases differences were considered significant at a level of $p<0.05$.

Acknowledgements

The authors would like to thank Dr Cuasnicu laboratory members, especially Dr Debora Cohen, for their helpful comments and critical input. This study was partially supported by the National Research Council of Argentina (CONICET) grant (PIP 2022) to PSC and MWM and by the National Agency for Scientific and Technological Promotion (ANPCyT) grants (PICT 2019, No 3588) to MWM and (PICT 2021. No 00765) to PSC.

Additional information

Author contributions

V. Sulzyk, L. Curci, M. Weigel Muñoz, and P.S. Cuasnicu designed research. V. Sulzyk, L. Curci, M. Weigel Muñoz, A. Rebagliati Cid and Gonzalez L. performed research. V. Sulzyk, M. Weigel Muñoz, and P.S. Cuasnicu analyzed data. M. Weigel Muñoz, V. Sulzyk and P.S. Cuasnicu wrote the paper.

	Control		C1/C3 DKO	
	Fresh	Capacitated	Fresh	Capacitated
Sperm count (10^6/ml)	48,35 ± 3,83	---	59,87 ± 12,03	---
Viability (%)	66,22 ± 5,25	61,04 ± 2,78	65,54 ± 2,29	58,21 ± 4,31
Progressive motility (%)	63,99 ± 1,14	57,91 ± 2,39	62,65 ± 2,37	54,27 ± 3,19

ns.

Table Supp. 1.

Analysis of different parameters in fresh and capacitated sperm

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Author information

Valeria Sulzyk
Instituto de Biología y Medicina Experimental (IByME-CONICET), Buenos Aires, Argentina

Ludmila Curci
Instituto de Biología y Medicina Experimental (IByME-CONICET), Buenos Aires, Argentina

Lucas N González
Instituto de Biología y Medicina Experimental (IByME-CONICET), Buenos Aires, Argentina

Abril Rebagliati Cid
Instituto de Biología y Medicina Experimental (IByME-CONICET), Buenos Aires, Argentina

Mariana Weigel Muñoz*
Instituto de Biología y Medicina Experimental (IByME-CONICET), Buenos Aires, Argentina

*These authors have contributed equally to this work and share last authorship

Patricia S Cuasnicu*
Instituto de Biología y Medicina Experimental (IByME-CONICET), Buenos Aires, Argentina
ORCID iD: 0000-0002-4784-488X

For correspondence: pcuasnicu@gmail.com
*These authors have contributed equally to this work and share last authorship

Editors

Reviewing Editor
Wei Yan
Washington State University, Pullman, United States of America

Senior Editor
Wei Yan
Washington State University, Pullman, United States of America

Reviewer #1 (Public review):

Summary:

The main observation that the sperm from CRISP proteins 1 and 3 KO lines are post-fertilization less developmentally competent is convincing. The data showing progressive acquisition of the sperm defects during epididymal transport and the exchange fluid studies showing the altered epididymal environment are important. However, the molecular characterization of the mechanism(s) that leads to these defects requires additional studies.

Strengths:

The generation of these double mutant mice is valuable for the field. Moreover, the fact that the double mutant line of Crisp 1 and 3 is phenotypically different from the Crisp 1 and 4 line suggests different functions of these epididymis proteins. The methods used to demonstrate that developmental defects are largely due to post-fertilization defects are also a considerable strength. The initial characterization that these sperm have altered intracellular Ca2+ levels, and increased rates of DNA fragmentation are valuable. The increase fragmentation of control sperm DNA when exposed to mutant epididymal fluid is significant and an excellent platform for future studies.

Weaknesses:

The study is mechanistically incomplete because evidence of how these proteins alter the environment is not shown. What are the target(s) of these proteins that result in increased Ca2+?

https://doi.org/10.7554/eLife.97105.2.sa2

Reviewer #2 (Public review):

Summary:

The study highlights the role of CRISP1 and CRISP3, two epididymal proteins, in early embryo development through DNA integrity. The authors demonstrate that C1/C3 DKO sperm exhibit defects in the DNA integrity, probably due to Ca2+ dysregulation in the epididymis. However, direct evidence for this mechanism requires further experiments. The finding of the involvement of the epididymal environment in embryogenesis is significant, but some results on sperm fertilizing ability of C1/C3 DKO mice were similar to the previous report. Thus, this point raises concern about the perspective of novelty.

Strengths:

The authors demonstrate that CRISP1 and CRISP3 regulate Ca2+ in the epididymal fluid, and loss of CRISP1 and CRISP3 disrupts Ca2+ regulation in the epididymal fluid, leading to sperm DNA fragmentation and impaired embryonic development after fertilization. This proposed mechanism is both novel and intriguing, offering valuable insights into the epididymal control of sperm quality.

Weaknesses:

The evidence supporting the mechanism of CRISP1 and CRISP3 in calcium regulation within epididymis and its contribution to the sperm DNA damage remains limited.

Major comments:

The data provided in this manuscript (Figure 2A and B) appear to overlap with data in previously published paper (PMID:33037689), despite differences in the duration of in vivo fertilization after mating. The results in both studies show similar findings, raising concerns about potential data redundancy.

As shown in Figure 6A, while wild-type sperm were exposed to the epididymal fluid of C1/C3 DKO mice, the wild-type sperm exhibited DNA fragmentation. Additionally, when wild-type sperm were exposed to the epididymal fluid of wild-type mice with 10 mM Ca2+, DNA fragmentation is still observed. Therefore, the authors conclude that the DNA fragmentation in C1/C3 DKO sperm is due to the increased level of the Ca2+. However, the connection between the DNA damage in wild-type sperm exposed to the epididymal fluid of C1/C3 DKO mice and the increased levels of Ca2+ remains unclear. To clarify this, it is suggested that intracellular calcium levels in the wild type sperm should be analyzed before and after exposure to the epididymal fluid of C1/C3 DKO mice (or before and after adding 10 mM Ca2+ into wild-type fluid). Furthermore, the author should explain detailed information on epididymal fluid collection, because Ca2+ levels vary between different sections of the epididymis.

In lines 321-323, the authors mention the selection system of the female reproductive tract that only allows high-quality sperm to reach the eggs (Cummins and Yanagimachi 1982), but this paper is not listed in the bibliography. It is important to ensure proper referencing.

The discussion section is too long and difficult to follow well because there is redundancy of the results in many parts. It is recommended to shorten it by focusing only on relevant and important information.

https://doi.org/10.7554/eLife.97105.2.sa1

Author response:

The following is the authors' response to the previous reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *The main observation that the sperm from CRISP proteins 1 and 3 KO lines are postfertilization less developmentally competent is convincing. However, the molecular characterization of the mechanism that leads to these defects and the temporal appearance of the defects requires additional studies.*

We thank the reviewer for the valuable comments. As requested, additional experiments were carried out to analyze both the molecular mechanisms and the temporal appearance of the observed defects. Our results showed that DNA integrity defects appear during epididymal maturation and/or storage (see Figure 5B), that the epididymal fluid contributes to sperm DNA fragmentation defects (See Figure 6A) and that these defects seem not to be due to an increase in oxidative stress (Figure 5C) but rather to a dysregulation in Ca^{2+} homeostasis within the epididymis (Figure 6A,B).

> *Strengths:*
>
> *The generation of these double mutant mice is valuable for the field. Moreover, the fact that the double mutant line of Crisp 1 and 3 is phenotypically different from the Crisp 1 and 4 line suggests different functions of these epididymis proteins. The methods used to demonstrate that developmental defects are largely due to post-fertilization defects are also a considerable strength. The initial characterization of these sperm has altered intracellular Ca^{2+} levels, and increased rates of DNA fragmentation are valuable.*

We thank the reviewer for the positive comments on our work.

> *Weaknesses:*
>
> *The study is mechanistically incomplete because there is no direct demonstration that the absence of these proteins alters the epididymal environment and fluid, wherein during the passage through the epididymis the sperm become affected. Also, a direct demonstration of how the proteins in question cause or lead to DNA damage and increased Ca^{2+} requires further characterization.*

The new experiments included in the revised version (see Figure 6A) showed that exposure of control WT sperm to epididymal fluid form mutant mice leads to an increase in sperm DNA fragmentation levels, confirming that the absence of CRISP1 and CRISP3 alters the epididymal fluid wherein the sperm become affected. In addition, new observations showing that WT sperm exposed to WT epididymal fluid in the presence of Ca^{2+} also exhibit higher DNA fragmentation levels (Figure 6A) together with the finding that mutant sperm exhibit higher intracellular Ca^{2+} levels (Figure 6B) but no higher levels of ROS, strongly support a dysregulation in Ca^{2+} homeostasis within the epididymis and sperm as the main responsible for DNA integrity defects.

> ***Reviewer #2 (Public Review):***
>
> *The authors showed that CRISP1 and CRISP3, secreted proteins in the epididymis, are required for early embryogenesis after fertilization through DNA integrity in cauda*

epididymal sperm. This paper is the first report showing that the epididymal proteins are required for embryogenesis after fertilization. However, some data in this paper (Table 1 and Figure 2A) are overlapped in a published paper (Curci et al., FASEB J, 34,15718-15733, 2020; PMID: 33037689). Furthermore, the authors did not address why the disruption of CRISP1/3 leads to these phenomena (the increased level of the intracellular Ca^{2+} level and impaired DNA integrity in sperm) with direct evidence. Therefore, if the authors can address the following comments to improve the paper's novelty and clarification, this paper may be worthwhile to readers.

We thank the reviewer for the constructive comments. Regarding the data included in Table 1 and Figure 2A, it is important to note that Table 1 includes data on embryo development corresponding to C1/C4 DKO mice not published before in which the data on embryo development corresponding to C1/C3 DKO was used as simultaneous control. Figure 2A showed *in vivo* fertilization results at short times after mating (4h instead of 18 h) that have been neither reported before.

Regarding studies to address why the disruption of CRISP1 and CRISP3 leads to defects in DNA integrity and Ca^{2+} levels, we have carried out new experiments showing that mutant sperm do not exhibit higher levels of ROS (see Figure 5C), not favoring oxidative stress as the mechanism underlying mutant sperm defects. In addition, we found that DNA integrity defects develop during epididymal transit (Figure 5B) and that exposure of WT sperm to epididymal fluid from mutant mice leads to an increase in sperm DNA fragmentation levels (Figure 6A), confirming that the absence of CRISP1 and CRISP3 alters the epididymal fluid. Finally, our new results showing that WT sperm exposed to WT epididymal fluid in the presence of Ca^{2+} also exhibit higher DNA fragmentation levels (Figure 6A) together with the higher intracellular Ca^{2+} levels detected in mutant sperm (Figure 6B) strongly support a dysregulation in Ca^{2+} homeostasis within the epididymis and sperm as the main responsible for DNA integrity defects.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Overall comments:

This manuscript investigates the mechanisms whereby the absence of the epididymal CRISP proteins 1 and 3 (Cysteine-Rich Secretory Proteins) causes infertility and lower embryo developmental rates. This strain's infertility seems to have a post-fertilization origin because the rates of in vivo fertilization are like the controls, but the development to the blastocyst stage is decreased. The results of this study show that (1) mutant sperm viability, progressive motility, and morphology are normal;

(2) in vivo fertilization rates are comparable to controls, but embryo development is reduced;

(3) in vitro fertilization studies found reduced fertilization rates and activation rates even in zona-free studies;

(4) additional functional studies showed increased rates of DNA fragmentation and elevated Ca^{2+} levels in mutant sperm.

The results presented are credible and hint that the epididymis might play a role before and after fertilization and directly affect embryo development. However, the study is mechanistically incomplete, as there is no direct demonstration that the absence of these proteins alters the epididymal environment and fluid, wherein the passage through the epididymis the sperm become functionally defective, and whether mutant or control epididymal fluid or purified CRISP proteins can change, either reduce or overcome,

> *respectively, the developmental competence of the control or mutant sperm and induce functional changes in the counterpart sperm. In summary, the main observation that the sperm from CRISP proteins 1 and 3 KO lines are post-fertilization less developmentally competent is significant and important, but the molecular characterization of the defects and the temporal appearance of defects requires additional studies.*
>
> *Specific comments:*
>
> *(1) Introduction.*
>
> *It is too long. The description of the function of the epididymis should be reduced. The functional properties of the Crisp genes should also be substantially shortened.*

As requested, the Introduction has been revised and descriptions of the epididymis and CRISP have been shortened

> *(2) Results.*
>
> *• Lines 140 to 142. Remove these initial lines. Start directly addressing the results of the C1/C3 strain, which is the mutant under consideration here. Referring to the C1/C4 results detracts from the focus of the study.*

As suggested by the reviewer, lines 140 to 142 have been removed.

> *• Table 1. Move the two-cell embryo line to the top of the Table and place the Blastocyst line below it. This organization is the conventional method to present this type of data.*

As suggested, the order of the lines in Table 1 has been modified to align with the conventional presentation method.

> *• Figures 1 and 2A and B data are solid and support the notion that enough sperm reach the site of fertilization, and that the sperm are defective in their capacity to support embryo development. Figures 2C and D have interesting data, although additional information would strengthen these results. The authors concluded that the sperm were defective in the epididymis. Where in the epididymis? These sperm were all from the cauda. Could the authors collect sperm from the upper portion of the cauda, or midportion, and compare if the defects manifest gradually?*

We appreciate this interesting and appropriate comment from the reviewer. In this regard, all the studies in our work were carried out using sperm from the whole cauda epididymis, the reason why we could not answer where defective sperm appear in the epididymis. In view of this, we have now conducted a comparative DNA fragmentation analysis between caput and cauda sperm from both genotypes. Our findings indicate that while cauda mutant sperm showed once again higher DNA fragmentation levels than controls, caput sperm exhibited levels of DNA damage not significantly different between genotypes. These results confirm that defects in DNA appear following sperm passage through the epididymal caput, supporting the hypothesis that defects in DNA fragmentation manifest during sperm transit through the epididymis and /or during storage in the cauda. These results have been included in the revised version of the manuscript (see lines 235-240/Figure 5B of the revised version)

> *• Figure 3 displays the results of in vitro fertilization, either COCs A-C or zona-free fertilization D-F. The results are important and differ from those produced by fertilization in vivo. The authors indicate that these confirm that the in vivo conditions overcome in vitro defects. However, this study never addresses the reason behind it. Is there less expression of proteins related to these functions, or the function of some proteins is*

> *compromised? The authors should advance a hypothesis or a rationale to explain these results.*

As indicated by the reviewer, our results showed differences between the fertilization rates observed for mutant mice under *in vivo* and *in vitro* conditions, as previously observed for all our single and multiple KO models (Da Ros et al., 2008; PMID: 18571638, Brukman et al., 2016; PMID: 26786179, Weigel Muñoz, 2018; PMID: 29481619, Ernesto et al., 2015; PMID: 26416967, Carvajal et al,. 2018; PMID: 30510210) and also reported by other groups (Okabe et al., 2007; PMID: 17558467). In this regard, it has been well established that, although millions of sperm are ejaculated into the female tract, only a few (approximately one per oocyte) reach the fertilization site (i.e. the ampulla) (Cummins and Yanagimachi, 1982; doi:10.1002/mrd.1120050304). This efficient selection system by the female reproductive tract leads to the arrival of only the best sperm at the fertilization site, even in males with reproductive deficiencies, thereby "masking" sperm defects that can be detected under *in vitro* conditions due to the competition between good and bad quality sperm for the egg. Thus, although we can not exclude other mechanisms to explain the commonly observed differences between *in vivo* and *in vitro* fertilization rates, our rationale is that the natural and efficient sperm selection process that takes place within the female reproductive tract masks sperm defects that can, otherwise, be detected under the competitive *in vitro* conditions. This explanation is now included in the discussion of the revised version of the manuscript (see lines 320-325).

> *• Data in Figures 4 and 5 support the interpretation of the authors. However, it is necessary to establish the level of oxidative stress in the mutant sperm vs. the controls. Also, a question to explore is for how long does the sperm need to reside in that mutant environment to start undergoing the DNA fragmentation reported?*

In response to the valuable request from the reviewer regarding the level of oxidative stress in sperm, we have analyzed reactive oxygen species (ROS) levels in mutant and control epididymal sperm. Our results showed that ROS levels in mutant sperm were not higher than those observed in the control group, supporting the idea that mechanisms other than oxidative stress may be leading to the increased DNA fragmentation observed in mutant sperm. These results are now included in the revised version of the manuscript (see Figure 5C).

Regarding the question on how long the sperm need to reside in the mutant environment to undergo DNA fragmentation, recent experiments carried out in response to this reviewer in which we analyzed DNA fragmentation in caput sperm led us to conclude that DNA fragmentation develops during epididymal transit and/or storage in the cauda. While these observations do not precisely define the time within the epididymis that sperm require for exhibiting DNA fragmentation, our additional new *in vitro* experiments analyzing the effect of epididymal fluids on sperm DNA integrity showed that exposure of WT sperm to DKO fluid for only 1 hr already leads to an increase in DNA fragmentation (see Figure 6A of the revised manuscript), suggesting that sperm do not need long periods within the mutant environment to be affected.

> *(3) The length of the Discussion section should be shortened, especially by not recapitulating data presented in the Results section.*

As requested by the reviewer, sections recapitulating results have been modified.

> *Minor comments:*
>
> *(1) The sentence in lines 171 and 172 is unclear, "However, despite the short time after mating, once again, the in vivo fertilized eggs corresponding to the mutant group*

> *exhibited clear defects to reach the blastocyst stage in vitro compared to controls." What do the authors mean by short time? It is the expected time, correct?*

It is well established that after copulatory plug formation, most oocytes are fertilized within 2 to 8 hours, with fertilization rates that increase over time: 0–5% at 1.5 hours post-mating; 40% at 4 hours post-mating and more than 90% at 7 hs after mating (Muro et al., 2016; PMID: 26962112, La Spina et al., 2016; PMID: 26872876). In order to examine whether the embryo development defects observed for mutant mice were due to a delayed arrival of sperm to the ampulla, we decided to analyze the percentage of fertilized eggs recovered from the ampulla at "short times" (4 hs) after mating to avoid the possibility that the prolonged stay of sperm within the female tract corresponding to the usual "overnight mating" schedule could be giving defective sperm enough time to reach the ampulla and, finally, fertilize the eggs (i.e. delayed fertilization). Our results showed that, despite the expected lower fertilization rates observed for both control and mutant males when analyzed just 4 hs after mating, the fertilized eggs corresponding to the mutant group were still exhibiting clear defects to develop into blastocysts compared to controls, not favoring the idea that embryo development defects were due to a delayed fertilization. The sentence in lines "171 and 172" has been modified in the revised version of the manuscript to better explain this conclusion (see lines 152-155 of the revised version).

> *(2) Line 177. Mutant epididymal sperm already carry defects leading to embryo development failure. Under this subheading, the authors compare within the same female the ability of mutant and control sperm delivered into different horns to support fertilization and embryo development. They show that the embryo development induced by mutant sperm is diminished vs. controls under very similar conditions, confirming the previous results of post-fertilization failure. The data also answers the question raised by the authors of whether the fertilization defects appear during or after epididymal transit; the interpretation of the results is the functional defects in the sperm are present before the transport into the female tract. Important unaddressed questions are, could these defects begin even earlier before arriving at the cauda? Did the authors try to incubate the mutant sperm with the epididymal fluid of WT mice to examine if the sperm defects could be rescued? The opposite experiment could also be performed, where WT sperm are incubated with the epididymal fluid of mutant mice, and the treated sperm examined for altered Ca^{2+} levels or DNA fragmentation.*

First of all, we would like to clarify that our question about whether the fertilization defects appear "during or after epididymal transit" was in fact referring to whether defects appear during epididymal maturation or later on, at the moment of ejaculation. In this regard, our *in vivo* and *in vitro* fertilization studies allowed us to conclude that defects were already present in epididymal sperm without excluding the possibility that additional defects could appear at the vas deferens or at the moment of ejaculation due to the contribution of seminal plasma secretions.

Regarding whether sperm defects could appear even earlier before arriving to the cauda, we have now analyzed DNA fragmentation defects in caput vs cauda both mutant and control sperm observing differences between genotypes only for cauda sperm. Based on these observations, we conclude that DNA integrity defects appear within the epididymis after sperm passage through the caput either when sperm reach the corpus or the cauda epididymis, or during their storage within the cauda region.

Also, as suggested by the reviewer, we incubated in vitro WT sperm with epididymal fluid from DKO mice (and vice versa) and then analyzed DNA fragmentation levels. Results showed that exposure of control sperm to the mutant epididymal fluid for 1 hr significantly increased DNA fragmentation levels. When mutant sperm (exhibiting higher levels of DNA fragmentation than control sperm), were exposed to epididymal fluid from WT mice, no

differences between groups were observed. Together, these results confirm both that the epididymal fluid from mutant mice contributes to the higher DNA fragmentation levels detected in mutant sperm, and that normal epididymal fluid would not be able to rescue the DNA fragmentation present in mutant cells. These results are now included in the revised version of the manuscript (see Figure 6A).

> *(3) Lines 203 to 216. In these paragraphs the authors indicate "that mutant sperm had a lower percentage of fertilization and lower rates of blastocysts (Figure 3D, E), indicating that defects in egg coat penetration were not responsible for embryo development failure. Later, they indicated that a few eggs fertilized by mutant sperm failed to activate. It is shown that Ca^{2+} oscillations are normal, indicating that the defects lie elsewhere. Could the authors propose a mechanism based on their sperm DNA defects?*

As described in the Result and Discussion sections of the original manuscript, we decided to investigate the existence of possible defects in sperm DNA fragmentation based on evidence indicating that delays in early embryo development may result from the time taken by the egg to repair damaged paternal DNA (Esbert *et al*., 2018; PMID: 30259705, Newman *et al*., 2022; PMID: 34954800, Nguyen *et al*., 2023; PMID: 37658763). In this regard, it is known that time is needed before the first embryonic cell division for activation of the egg DNA repairing machinery (Martin et al., 2019; PMID: 30541031, Newman et al., 2022; PMID: 34954800) and that increased sperm DNA damage may necessitate more time for repair by the oocyte (Martin et al., 2019; PMID: 30541031, Newman et al., 2022; PMID: 34954800). Based on this, we decided to examine possible DNA damage in sperm. Our finding that, in fact, sperm DNA fragmentation was clearly increased in mutant sperm led us to propose that delays in early embryo development in our mutant colonies may result from the time required by the egg to repair sperm DNA fragmentation.

> *(4) The demonstration that C1/C3 sperm have abnormal rates of DNA fragmentation and Ca^{2+} levels is significant. Additional studies would strengthen the findings reported here. For example, what are the levels of oxidative stress in these sperm? Are there other changes related to oxidative stress? Performing a TUNNEL assay will strengthen the notion of DNA damage demonstrated here with the chromatin dispersion assay.*

As mentioned previously, we analyzed oxidative stress by evaluating ROS levels in control and mutant sperm observing no differences between genotypes. These results have been included in the revised version of the manuscript (See Figure 5C). We appreciate the suggestion of performing TUNNEL assay for future studies.

> ***Reviewer #2 (Recommendations For The Authors):***
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> *Major comments:*
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> *(1) There are some reports small RNAs gained during the epididymal transition of sperm are essential for embryonic development (e.g., Conine et al., Dev Cell, 46, 470480, 2018; PMID: 30057276), suggesting that the luminal changes in Crisp1/3 double KO (dKO) epididymis lead to the phenotype in this study. In fact, there is no evidence whether CRISP1/CRISP3 secreted from an epididymis exists in cauda epididymal sperm and directly controls the observed phenomena. Also, the authors wrote there is no strong evidence to exclude the possible role of small RNA in Crisp1/3 dKO sperm (lines 370-372). Therefore, it is at least necessary to measure small RNA abundance in dKO mice.*

As mentioned by the reviewer and as cited in our manuscript, there is a report indicating that the small RNAs gained during epididymal transit may play a role in embryonic development (Conine et al., 2018; PMID: 30057276). However, the need of small RNAs for embryonic development still remains a topic of debate (Wang et al. 2020; PMCID: PMC7799177). In this

regard, clear evidence indicating that sperm DNA fragmentation is associated with embryo development defects together with the increase in sperm DNA fragmentation levels observed in mutant sperm support sperm DNA damage as one of the causes leading to the observed phenotype in our mutant mice. Moreover, recent experiments carried out in response to Reviewer 1 comments revealed that exposure of control sperm to epididymal fluid from mutant mice significantly increases DNA fragmentation levels, confirming that the absence of CRISP1 and CRISP3 proteins in epididymal fluid contributes to sperm DNA damage in mutant sperm. Finally, whereas oxidative stress might also lead to embryo development impairment as mentioned in our original manuscript, recent evaluation of ROS levels in control and mutant sperm carried out in response to Reviewer 1's comments did not show higher ROS levels in mutant sperm. Thus, although as mentioned in the manuscript, we do not exclude the possibility that small RNAs may also contribute to embryo development defects, our observations support DNA fragmentation and a dysregulation in Ca^{2+} homeostasis within the epididymis and sperm as the main responsible for embryo development failure in our mutant males. The experiments using epididymal fluid (Figure 6A) and those evaluating ROS levels (Figure 5C) have been included in the revised version of the manuscript and discussed accordingly.

> *(2) Lines 245-248 and 354-374: According to Figure 5C, the intracellular Ca^{2+} level significantly increased in Crisp1/3 dKO sperm compared to control. The author hypothesized that this increase could destroy sperm DNA integrity, causing defects in early embryogenesis. However, the authors did not show the direct evidence.*
>
> *Specifically, as CRISP1 inhibits CatSper (line 95), the authors believed the increased Ca^{2+} level in Crisp1/3 dKO sperm was observed. Crisp1/3 dKO and Crisp1/4 dKO mice share the disruption of Crisp1, but the phenotype is totally different. Thus, the authors should also examine the CatSper activity in Crisp1/3 dKO sperm.*

We appreciate the reviewer's insightful comments. In this regard, whereas C1/C3 and C1/C4 DKO colonies shares the disruption of Crisp1, the intracellular Ca^{2+} levels in these two colonies are different as no increase in sperm intracellular Ca^{2+} was detected in Crisp C1/C4 DKO mice. Thus, this difference in intracellular Ca^{2+} levels might explain the different embryo development phenotype observed in our two DKO colonies. In this regard, our results revealed that sperm intracellular Ca^{2+} levels are different depending on the Crisp gene being deleted. Whereas the lack of Crisp1 did not affect intracellular sperm Ca^{2+} levels (Weigel Munoz et al, 2018; PMID: 29481619), there was an increase in Ca^{2+} levels in CRISP2 KO sperm (Brukman et al., 2016; PMID: 26786179) and a decrease in sperm when Crisp4 was deleted (Carvajal 2019, Ph.D Thesis). Thus, although the ability of CRISP3 to regulate sperm Ca^{2+} channels has not yet been reported, the existence of functional compensations between homologous CRISP members (Curci et al., 2020; PMID: 33037689) makes it complicated to draw straightforward conclusions based on the behavior of each individual protein in Ca^{2+} regulation. In fact, while the lack of CRISP1 and CRISP4 does not affect sperm Ca^{2+} concentration (Carvajal 2019, Ph.D Thesis), the simultaneous lack of CRISP1 and CRISP3 produced an increase in Ca^{2+} levels and the lack of the four CRISP proteins showed a decrease in the intracellular levels of the cation after capacitation (Curci et al, 2020). Based on these observations, we conclude that the absence of CRISP1 may or may not lead to altered intracellular Ca^{2+} levels depending on the other simultaneously-deleted gene/s.

> *The authors make a hypothesis that the increased Ca^{2+} level may lead to damaged DNA integrity by citing a published paper (lines 360-363). In the published paper, the authors examined the influence of the luminal fluid of the epididymis and vas deference on sperm chromatin fragmentation (Gawecka et al., 2015). However, they did not mention the increased DNA fragmentation in epididymal sperm when these sperm were incubated with Ca^{2+} or Mn2+. So, the authors' hypothesis is over discussion. Thus, the correlation*

> *between the intracellular Ca^{2+} level and DNA integrity in sperm is still unclear. So, the authors should show why the increased Ca^{2+} level leads to DNA fragmentation with direct evidence.*

We appreciate the reviewer's comment regarding the work by Gawecka et al., (2015), and the opportunity to clarify the proposed mechanism underlying our observations. In the above mentioned paper, the authors reported that when mouse epididymal or vas deferens sperm were incubated with divalent cations (Ca^{2+} and Mn^{2+}) in the presence of luminal fluid, they were induced to degrade their DNA in a process termed sperm chromatin fragmentation (SCF). The fact that both the ejaculated and epididymal mutant sperm used in our studies had been exposed to epididymal fluid lacking CRISP proteins known to regulate sperm Ca^{2+} channels, opened the possibility that changes in Ca^{2+} levels within the epididymal fluid and/or sperm could be responsible for the higher DNA fragmentation levels observed in mutant cells. In this regard, it is important to note that, as requested by Reviewer 1, we performed additional *in vitro* experiments in which WT epididymal sperm were exposed to mutant or WT epididymal fluid in the presence or absence of Ca^{2+} and DNA fragmentation analyzed at the end of incubation. Results showed a significant increase in DNA fragmentation in WT sperm exposed to either mutant epididymal fluid or WT fluid in the presence of Ca^{2+} (Figure 6A). We believe these observations together with the higher intracellular Ca^{2+} levels detected in DKO sperm (Figure 6B) provides strong evidence supporting changes in Ca^{2+} homeostasis in the epididymis and sperm as the main responsible for the observed sperm DNA integrity defects. This could be mediated by the activation of Ca^{2+}-dependent nucleases present within the epididymal fluid and/or sperm cells as previously suggested (Shaman *et al.*, 2006; PMID: 16914690, Sotolongo *et al.*, 2005; PMID: 15713834, Boaz *et al.*, 2008; PMID: 17879959, Dominguez and Ward, 2009; PMID: 19938954). These observations have now been included and discussed in the revised version of the manuscript (see lines 245-265 and 427-439).

> *Minor Comments:*
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> *(3) Standards for measuring rates should be clarified, such as two-cell rates are determined by dividing the number of two-cell embryos by the total number of eggs.*

As requested, standards for measuring rates have now been clarified in the corresponding figure legends

https://doi.org/10.7554/eLife.97105.2.sa0