An update on the characterization of human sperm CRISP2: Analysis of its localization, post-translational modifications, and oligomerization.

Thibault Masai[1], Marie Dendievel[1], Jean-François Simon[2], Vanessa Arcolia[2], Pascale Lybaert[3] & Elise Hennebert[1]

[1] Laboratory of Cell Biology, Research Institute for Biosciences, Research Institute for Health Sciences and Technology, University of Mons, Place du Parc 20, 7000 Mons, Belgium

[2] Fertility clinic, CHU Ambroise Paré Hospital, Boulevard Kennedy 2, 7000 Mons, Belgium

[3] Research laboratory on Human Reproduction, Université Libre de Bruxelles, Route de Lennik 808, 1070 Bruxelles, Belgium

State of the art

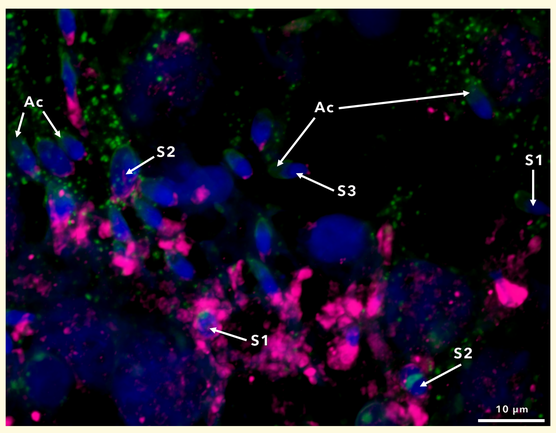
Cystein-RIch Secretory Proteins (CRISPs) are well known for their role in sperm function and in fertilization [1, 2]. Humans have 3 of them [3]. Unlike CRISP1 and 3, which are secreted by the epididymal epithelium and associate with the sperm surface [2-4], **CRISP2 is an intracellular protein expressed during spermatogenesis** [1]. Several functions have been described for CRISP2: (a) it **interacts with Sertoli cells** during spermatogenesis [5], (b) it mediates **interaction with the oocyte** [6], and (c) it **regulates** some **ion channels** such as CatSper and RyR, involved in sperm motility [7-9]. CRISP2 **localization** has been studied in various species. It is systematically detected in the connecting piece (or neck) as well as in the acrosome, the perinuclear theca, and/or flagellum, depending on the study [6, 10-14]. Unlike other CRISPs, which are glycosylated, CRISP2 does not exhibit this **post-translational modification (PTM)** [15]. However, the presence of other PTMs has not yet been investigated. Interestingly, in boars, it was shown that CRISP2 function would be linked to its capacity to form **protein complexes** through oligomerization and interaction with other sperm proteins [13].

Study aim

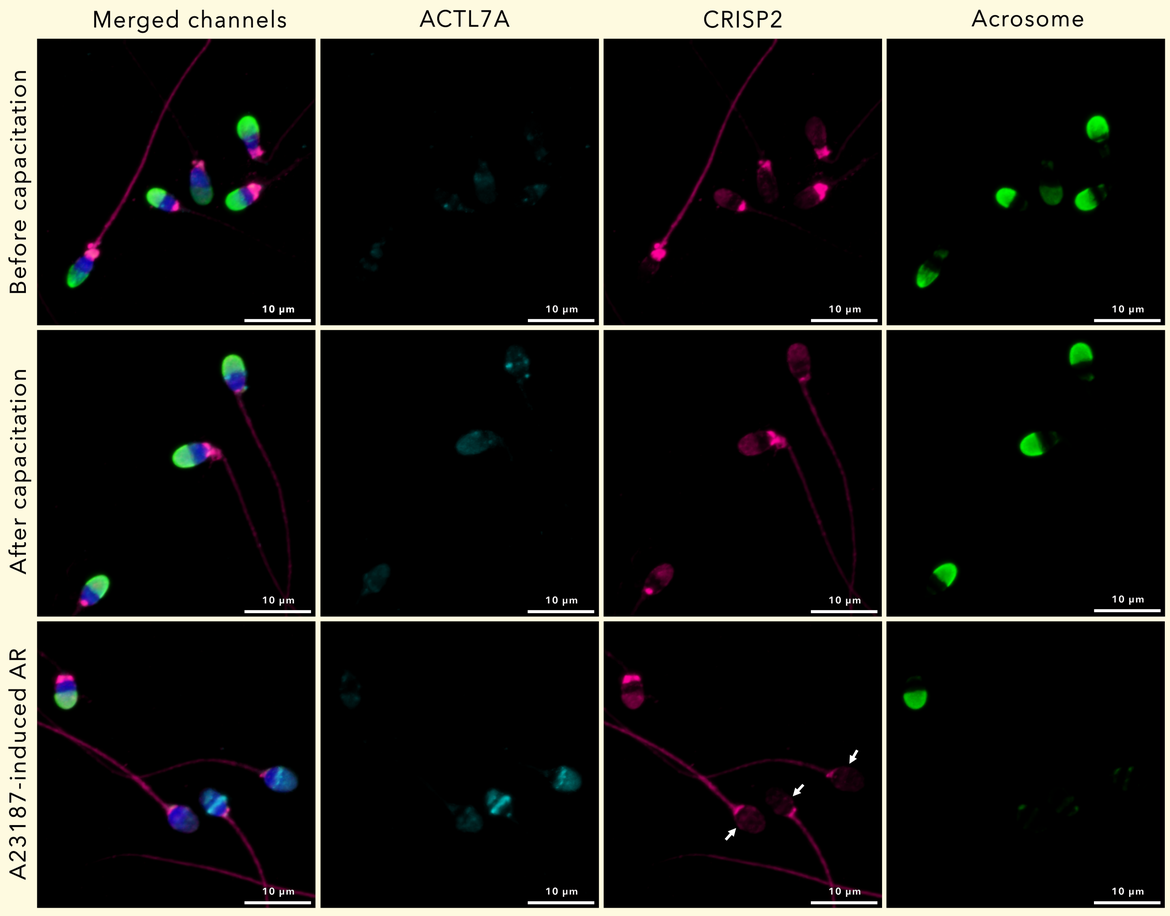
In the present study, we contributed to the **characterization of CRISP2 in human spermatozoa**. First, using **immunofluorescence confocal imaging**, we investigated the localization of CRISP2 on testis sections and ejaculated spermatozoa. In the latter, co-labelling with acrosome and perinuclear theca markers was performed to study CRISP2 sub-cellular localization. Then, we analyzed CRISP2 quaternary organization by **native-PAGE**. Finally, we investigated the presence of phosphorylation by **Western blot** and re-assessed the presence of glycosylation by **lectin blot**.

Localization in testis sections and ejaculated spermatozoa

In testis sections, CRISP2 labelling was observed in spermatids and spermatozoa, in the form of a dense spot located opposite the acrosome (Fig. 1). **In ejaculated spermatozoa** (Fig. 2), for all investigated maturation stages (non-capacitated, capacitated, and acrosome-reacted spermatozoa), an intense labelling was observed at the level of the neck. The flagellum was also labelled, with varying intensity depending on the spermatozoon. A faint labelling at the level of the acrosome was observed in non-capacitated and capacitated spermatozoa. This labeling disappeared after AR, leaving only a very faint labeling at the level of the equatorial segment. CRISP2 localization did not match exactly that of ACTL7A, a marker of the perinuclear theca.



↑ **Figure 1:** Localization of CRISP2 in human testis section in immunofluorescence. **Magenta**: CRISP2. **Dark blue**: DAPI staining of the nuclei. **Green**: PSA-FITC staining (used to visualize sperm acrosomes). Scale = 10 µm. Images are maximum-intensity projections (MaxIP) obtained from z stack images using Nikon NIS Elements software.



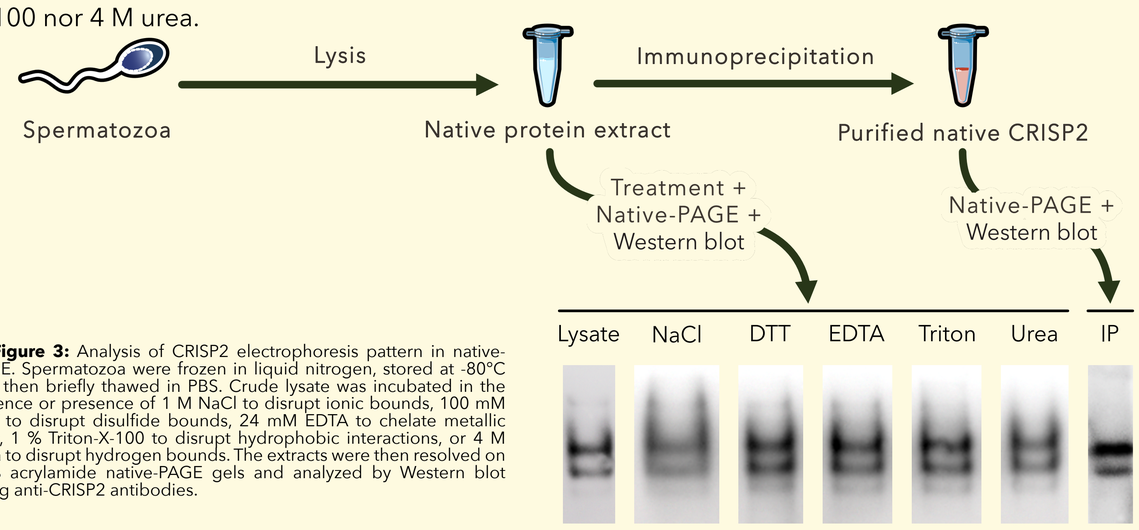
↑ **Figure 2:** Colocalization between CRISP2 and perinuclear theca markers within human spermatozoa, before and after capacitation as well as after acrosomal reaction (AR). Spermatozoa were capacitated for 4 h in supplemented Earle's balanced salt solution (sEBSS). Acrosomal reaction was induced by addition of 10 µM A23187 for 1 h. **Magenta**: CRISP2. **Dark blue**: DAPI staining of the nuclei. **Green**: PSA-FITC staining (used to visualize sperm acrosomes). **Cyan**: ACTL7A, a perinuclear theca marker. Arrows : equatorial segment. Scale = 10 µm. Images are maximum-intensity projections (MaxIP) obtained from z stack images using Nikon NIS Elements software.

What is next?

Regarding CRISP2 localization, only the intense labelling at the level of the sperm neck was common between testis sections and ejaculated spermatozoa. This could correspond to **CRISP2 localization within the redundant nuclear envelope (RNE)**, consistent with its known function as a RyR regulator. Contrary to what was recently described in boar [13,14], human CRISP2 does not appear to be localized in the perinuclear theca. This is supported by the fact that CRISP2 appears to be a soluble protein, as it was easily extracted with freeze-thawing in PBS. **The faint labelling at the level of the acrosome** in non-capacitated and capacitated spermatozoa, and at the level of the **equatorial segment after AR**, is consistent with CRISP2's role in oocyte interaction [6]. We also showed that **CRISP2 forms highly stable protein complexes.** We are currently performing mass spectrometry analysis to identify the proteins forming these complexes (i.e., CRISP2 alone, or associated with other proteins). Finally, we showed that, under the experimental conditions used, **CRISP2 does not seem to be glycosylated or phosphorylated.**

Quaternary organization

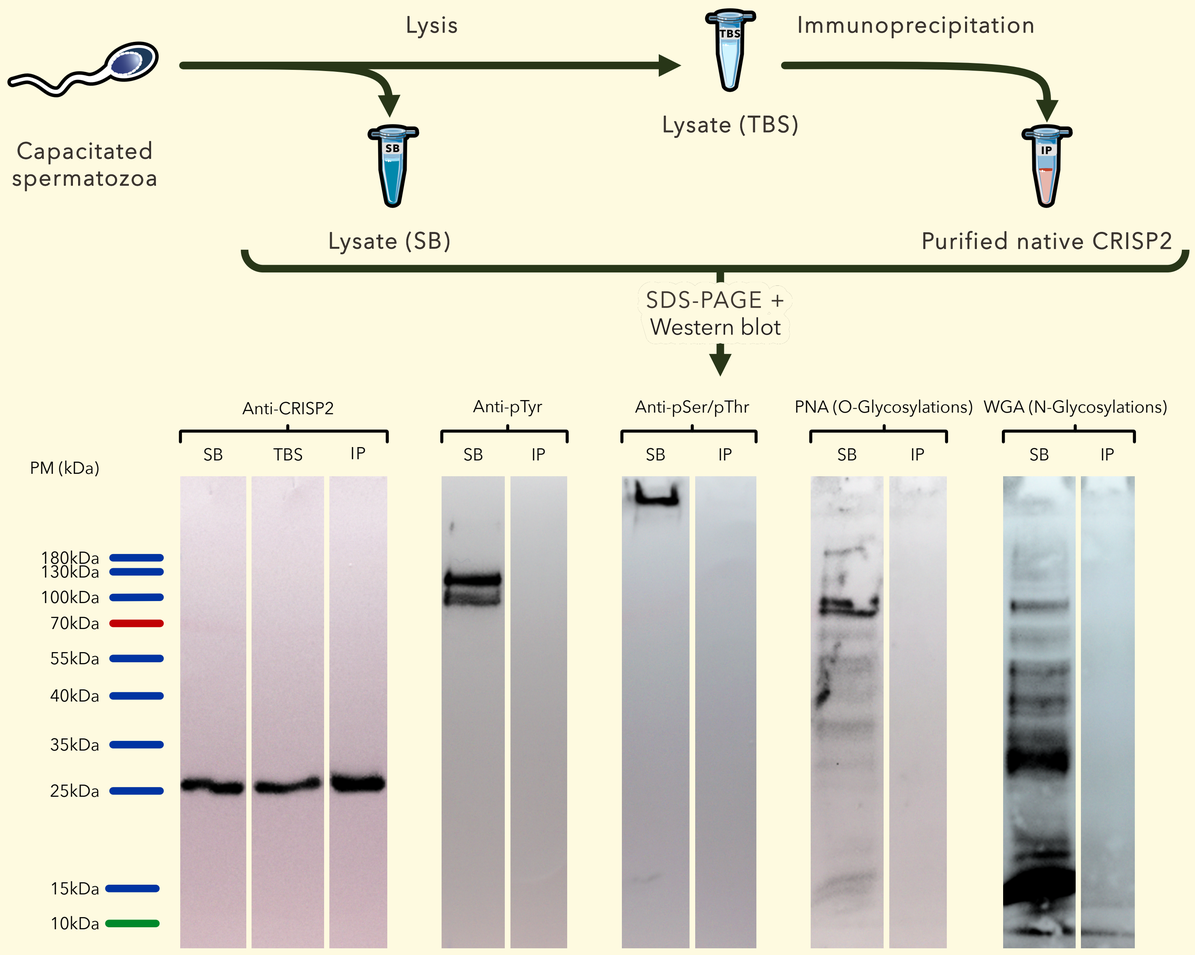
CRISP2 was easily extracted from spermatozoa by freeze-thawing in PBS. Under these conditions, it separated as three distinct bands in Native-PAGE, both in the crude sperm extract and after immunoprecipitation, indicating that it forms protein complexes. In the crude lysate, these complexes were not dissociated in the presence of 1 M NaCl, 100 mM DTT, 24 mM EDTA, 1% Triton-X-100 nor 4 M urea.



→ **Figure 3:** Analysis of CRISP2 electrophoresis pattern in native-PAGE. Spermatozoa were frozen in liquid nitrogen, stored at -80°C and then briefly thawed in PBS. Crude lysate was incubated in the absence or presence of 1 M NaCl to disrupt ionic bounds, 100 mM DTT to disrupt disulfide bounds, 24 mM EDTA to chelate metallic ions, 1 % Triton-X-100 to disrupt hydrophobic interactions, or 4 M urea to disrupt hydrogen bounds. The extracts were then resolved on 12% acrylamide native-PAGE gels and analyzed by Western blot using anti-CRISP2 antibodies.

Post-translational modifications

CRISP2 PTMs were analyzed by Western blot and lectin blot on sperm lysates (obtained after extraction of capacitated spermatozoa in SDS sample buffer (SB) or Tris buffered saline buffer (TBS)) and after CRISP2 immunoprecipitation (IP). No labeling was observed in the IP eluate with WGA and PNA lectins, targeting glycosylations, or antibodies targeting protein phosphorylation, indicating that CRISP2 does not display such PTMs. The labelling obtained in the crude sperm lysate for other proteins demonstrates that the absence of labelling of CRISP2 is not due to the inability of the antibody or lectin to recognize its target.



↑**Figure 4:** Analysis of CRISP2 PTMs. Capacitated spermatozoa were extracted in SDS sample buffer (SB) or Tris buffered saline (TBS), and CRISP2 was immunoprecipitated from the TBS extract. The crude extracts and IP eluate were separated by SDS-page (12%) and then analyzed by Western blot using the lectin Peanut Agglutinin (PNA) to detect O-glycosylations, the lectin Wheat Germ Agglutinin (WGA) to detect N-glycosylations, and anti-phosphotyrosines (pTyr) and anti-phosphoserines/phosphothreonines (pSer/pThr) antibodies to detect phosphorylations.

References

[1] Gaikwad, A.S., et al.. Hum Reprod Update, 2020. 26(5): p. 689-723. **[2]** Gonzalez, S.N., et al.. Front Cell Dev Biol, 2021. 9: p. 800351. **[3]** Kratzschmar, J., et al.. Eur J Biochem, 1996. 236(3): p. 827-36. **[4]** Thimon, V., et al.. Hum Reprod, 2008. 23(8): p. 1698-707. **[5]** Maeda, T., J. Nishida, and Y. Nakanishi. Dev Growth Differ, 1999. 41(6): p. 715-22. **[6]** Busso, D., et al.. Mol Hum Reprod, 2005. 11(4): p. 299-305. **[7]** Gibbs, G.M., et al.. J Biol Chem, 2006. 281(7): p. 4156-63. **[8]** Brukman, N.G., et al.. Mol Hum Reprod, 2016. 22(4): p. 240-51. **[9]** Lim, S., et al.. Endocrinology, 2019. 160(4): p. 915-924. **[10]** Busso, D., et al.. Biol Reprod, 2007. 76(4): p. 701-8. **[11]** Busso, D., et al.. Biol Reprod, 2007. 77(5): p. 848-54. **[12]** Anklesaria, J.H., et al.. J Pept Sci, 2016. 22(6): p. 383-90. **[13]** Zhang, M., et al.. Biol Reprod, 2021. 105(5): p. 1160-1170. **[14]** Zhang, M., et al.. Front Cell Dev Biol, 2022. 10: p. 836208. **[15]** Hardy, D.M., et al.. Biol Reprod, 1988. 38(2): p. 423-37.

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