1 **SPINK3-sperm interaction determines a stable sperm subpopulation with intact**
2 **CatSper channel**
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50   **GRAPHICAL ABSTRACT**
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56   **ABSTRACT**
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Sperm capacitation involves proteolytic remodeling of membrane proteins, including
components of the CatSper calcium channel, which is essential for hyperactivation and male
fertility. Here, we identify the seminal protease inhibitor SPINK3, a known decapacitation factor
that suppresses premature capacitation in the female tract, as the first physiological inhibitor
of CATSPER1 processing. In mouse sperm, SPINK3 blocks capacitation-induced CATSPER1
cleavage, preserving a subpopulation with intact CatSper channels and lacking pTyr
development in the flagellum. SPINK3 localizes to the outer surface of the sperm principal
piece membrane in a CatSper-dependent but non-quadrilateral pattern, stabilizes membrane

organization, and delays cholesterol efflux. These results reveal SPINK3 as a multifunctional
regulator of capacitation, shaping sperm subpopulations in the female reproductive tract.
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69 **Keywords:** sperm capacitation, SPINK3, CATSPER1, protease inhibition, membrane
70 stabilization, decapacitation factors
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72 **SIGNIFICANCE STATEMENT**
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This study unveils a novel physiological regulator from the male reproductive tract, SPINK3,
which extracellularly controls the processing of CATSPER1, a key subunit of the CatSper
calcium channel essential for sperm capacitation in mice. We show that SPINK3 binds to the
sperm surface and prevents the processing of CATSPER1 during capacitation. SPINK3-
sperm interaction is dependent on the presence of the sperm-specific CatSper channel. Our
findings reveal that SPINK3 stabilizes sperm membranes providing new insights into the
molecular regulation of sperm function and fertility.
81
82 **INTRODUCTION**
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The sperm journey begins in the male reproductive tract, where millions of sperm undergo
surface modifications induced by male secretory molecules (1). After ejaculation, mammalian
sperm must undergo biochemical and functional changes within the female reproductive tract,
a process collectively known as capacitation (2, 3). This process involves membrane
remodeling and the activation of intracellular signaling pathways leading to the acquisition of
fertilizing ability. Decapacitation factors, typically originating from the male reproductive tract
or seminal plasma, coat and stabilize the sperm surface after ejaculation, preventing or
delaying capacitation until the sperm reaches an appropriate environment in the female
reproductive tract (1, 4, 5).
CatSper is the primary Ca^{2+} channel in mammalian spermatozoa, consisting of four
pore-forming subunits (CATSPER1, 2, 3, 4) and at least ten additional auxiliary subunits (6).
This channel is specifically located in the flagellar membrane of the principal piece, exhibiting
pH sensitivity and weak voltage dependence (7, 8) and is essential for the development of
hyperactivated motility of sperm (9, 10). Infertility has been observed in male mice lacking the
Catsper genes encoding the transmembrane subunits (7, 9–13). Capacitation induces specific
cleavage and processing of CATSPER1, one of the pore-forming subunits, in the cytosolic N-
terminus by unknown sperm enzyme(s), resulting in a graded distribution of heterogeneous
sperm populations with varying degrees of CATSPER1 integrity along the female reproductive
tract (14, 15). Spermatozoa that reach the ampulla, where fertilization takes place, mostly
have intact CATSPER1 signal compared to those that remain behind the utero-tubal junction
(UTJ). Recently, it was reported that another CatSper transmembrane subunit, CATSPERθ,
which is essential for the CatSper channel assembly and serves as a checkpoint for flagellar
trafficking during spermiogenesis, is also processed during sperm capacitation (12). This
highlights the proteolytic regulation of the CatSper channels as a sperm selection mechanism.
Although the cell-permeable potent proteasome inhibitor MG-132, as well as calpain inhibitors
and the phosphatase 1/2A inhibitor calyculin A, have been shown to inhibit CATSPER1
processing (14, 15), physiological inhibitors responsible for modulating the processing of
CatSper subunits remains unidentified.
SPINK3 (also known as P12 or SPINK1) is a small and highly conserved protein
consisting of 80 amino acids, naturally secreted by the male seminal vesicles of most

mammals (16–18). It contains a Kazal domain that acts as a substrate analogue by
competitively and stoichiometrically binding to the active site of its target protease, forming a
stable protease–protease inhibitor complex, such as kallikreins, trypsin and chymotrypsin-like
proteases (19). SPINK3 also has a sperm binding domain (20) and attaches to sperm during
ejaculation (21). SPINK3 is a known decapacitation factor regulating SRC tyrosine kinase
activation and membrane potential, apparently without affecting the tyrosine kinase or PKA
activity. (18). The BSA-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was
demonstrated to depend on CatSper channels. This $[Ca^{2+}]_i$ increase and CatSper activity are
inhibited by the presence of SPINK3 (18), suggesting an either direct or indirect blockade of
calcium entry through the CatSper channel. Similar to other decapacitation factors (22–24),
SPINK3 has been demonstrated to bind sperm during ejaculation until detachment in the
female tract, following a specific but asynchronous spatiotemporal kinetics, and contributing
to sperm heterogeneity (21). The protease TESP1 is the only natural target reported for
SPINK3 on the sperm head and this interaction was found to be blocked by the soybean
trypsin inhibitor, SBTI (25).
In this study, we show that SPINK3 regulates CATSPER1 processing and contributes
to the generation of sperm subpopulations with different capacitation states. This action is
accompanied by the stabilization of the sperm plasma membrane, suggesting that its
regulatory effect may involve protease inhibition and membrane-stabilizing mechanisms that
prevent protease access to CATSPER1 cleavage sites. These findings highlight a multifaceted
role for SPINK3 in modulating sperm capacitation.

RESULTS

SPINK3 inhibits CATSPER1 processing induced by *in vitro* capacitation
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It has previously been reported that the cytoplasmic N-terminal domain of the
CATSPER1 undergoes proteolytic processing in sperm cells during capacitation, and the
progression of this processing is negatively correlated with the appearance of tyrosine
phosphorylation at single cell level *in vitro* and *in situ* (15). More recently, another CatSper
subunit, CATSPERθ, which plays a role in CatSper channel assembly and serves as a
checkpoint for CatSper flagellar trafficking, was also found to undergo capacitation-induced
degradation (12).
SPINK3 has previously been shown to inhibit CatSper channel activity (18). As this
small protein is not only a decapacitation factor but also a protease inhibitor, we hypothesize
that SPINK3 might be related to the regulation of CATSPER1 and/or CATSPERθ processing.
To test this idea, we evaluated capacitation-induced CATSPER1 and CATSPERθ protein
degradation in mouse sperm incubated under capacitating conditions, with or without SPINK3.
Interestingly, we found that the presence of SPINK3 abolished the degradation of CATSPER1,
but not CATSPERθ (**Figure 1A**). Consistent results were seen with anti-CATSPER1
immunostaining in mouse spermatozoa (**Figure 1B, Supp. Figure 1**). As sperm contains
several proteases (26), we assessed the proteolytic activity of sperm lysates using synthetic
substrates specific for trypsin, chymotrypsin, and elastase, in order to identify the protease
family targeted by SPINK3. Our results confirmed that trypsin-like activity was predominant,
and that this activity was inhibited by SPINK3, by seminal vesicle lysates (which contain native
SPINK3), and by commercial inhibitors such as PMSF (phenylmethylsulfonyl fluoride) and
SBTI (soybean trypsin inhibitor) (Figure 1C). As a control, we capacitated mouse spermatozoa

in the presence of SBTI and observed the same blocking effect on CATSPER1 processing
(**Supp. Figure 2**). Heterologously expressed full-length recombinant CATSPER1 has also
been validated for processing by sperm lysate *in vitro* due to the presence of specific
endogenous protease(s) (15). Similarly, this *in vitro* CATSPER1 processing was also inhibited
by the presence of SPINK3 (**Figure 1D**). Taken together, these results show that SPINK3
interferes with proteolytic processing of CATSPER1, by inhibiting sperm trypsin-like
protease(s). Furthermore, when sperm were preincubated with recombinant SPINK3 under
capacitating conditions, only spermatozoa lacking SPINK3 showed protein tyrosine
phosphorylation (pTyr) development in the flagella (**Figure 1E**), consistent with the previously
reported inverse correlation of pTyr and CatSper channel integrity (6, 14, 15). These results
support the idea that SPINK3 binding results in a population of sperm with intact CATSPER1
and SPINK3 bound to the flagellar surface.

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172   **SPINK3 binds to the sperm flagellar membrane in a CatSper-dependent manner**

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The CatSper channel forms four linear nanodomains along the flagellar membrane in
which the lipid raft marker caveolin1 resides (14). Recombinant SPINK3 can bind to raft
membrane domains in both acrosomal and tail membranes of non-capacitated mature
epididymal spermatozoa and this union is avoided by choleratoxin B supporting binding to
GM1 (18, 21). Given that SPINK3 is a secretory protein, we tested whether SPINK3 and
CatSper co-localize in the quadrilinear nanodomains. Confocal microscopy shows that, unlike
CATSPER1, which is localized exclusively to the principal piece, SPINK3 is present in both
principal piece and end piece of the flagellum (**Figure 2A, upper panel**). The distinct linear
CatSper nanodomain arrangement visualized by Structured Illumination Microscopy (SIM)
was not seen for SPINK3 in the flagellar membrane (**Figure 2B**). These observations,
combined with our finding that SPINK3 inhibits the processing of the N-terminal cytoplasmic
domain of CATSPER1, prompted us to further investigate the spatial distribution of SPINK3 in
relation to the plasma membrane. Thus, SPINK3 immunostaining was performed together with
membrane labelling using the specific plasma membrane probes FM4-64 and CellBrite.
Images from Airyscan microscopy and MSSR indicate that SPINK3 is externally associated
with the plasma membrane (**Figure 2C, Supp. Movie 1**). Accordingly, SPINK3 signal spans
a wider area than the measured membrane-to-membrane distances (**Figure 2C, MSSR
Images**). Nevertheless, we observed that the SPINK3 association with sperm is compromised
in *Catsper1* KO sperm and there is also a pattern shift (**Figure 2A (lower panel), D; Supp.
Figure 3**). It is possible that in *Catsper1* KO sperm the loss of CatSper complex from flagellum
changed the membrane property and/or protease localization, thus compromising the
attachment of SPINK3. These results suggest that SPINK3 interacts with the flagellar
membrane in a CatSper-dependent manner but does not directly bind to CatSper.

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198   **SPINK3, but not SBTI, specifically affects sperm capacitation events**

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Given that protection of CATSPER1 from proteolytic processing (*i.e.*, intact CATSPER-
mediated Ca^{2+} signaling) is required to control the timing of capacitation, we analyzed whether
the protease inhibitory activity of SPINK3 accounts for its function as a decapacitation factor.
To this end, we first tested whether the trypsin inhibitor SBTI could mimic the known effects of
SPINK3 on capacitation since SBTI can also inhibit CATSPER1 processing (**Supp. Figure 2**).
In contrast to SPINK3, there was no difference in the BSA-induced increase in $[Ca^{2+}]_i$ between
SBTI-treated and untreated spermatozoa as assessed by single-cell Ca^{2+} imaging with Fluo-
3 AM (**Figure 3A**), nor in the percentage of acrosome-reacted spermatozoa (**Figure 3B**).

Notably, neither SPINK3 nor SBTI affected intrasperm alkalinization (**Figure 3C**), which is
known to increase CatSper currents (8, 27, 28). All these results suggest that among trypsin-
like protease inhibitors, SPINK3 specifically inhibits capacitation events such as rise in $[Ca^{2+}]_i$
and AR likely through a mechanism distinct from its inhibition of CATSPER1 cleavage.
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213 **SPINK3 alleviates capacitation-induced membrane perturbations**
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Cholesterol efflux from the sperm plasma membrane is a crucial step in capacitation.
These changes promote an increase in membrane fluidity or decrease stability (29) and can
be measured using BODIPY-cholesterol (BODIPY/Ch), a fluorescent compound that can
replace endogenous cholesterol in the membrane without disturbing the lipid acyl chain region.
Therefore, its loss can be measured by changes in fluorescence intensity (30, 31). By contrast,
the subsequent membrane remodeling can be assessed by the incorporation of anionic
lipophilic fluorescent dye Merocyanine 540 (M540) as the amount of dye per unit surface area
increases in less-ordered membranes (32, 33). Using these lipid indicators, we show that
capacitated spermatozoa contain lower cholesterol content (**Figure 4A**) and increased lipid
disorder (**Figure 4B**) compared to non-capacitated sperm, as previously reported. In contrast,
pre-incubation with SPINK3 blocked the capacitation-associated cholesterol efflux and lipid
disorder (**Figure 4A**, **B**), suggesting that SPINK3 binding to the membrane hinders the
membrane reordering during capacitation.
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229 **SPINK3-exposed spermatozoa during capacitation reduce *in vitro* fertilization**
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Pharmacological inhibition of CatSper during *in vitro* capacitation compromises sperm
fertilizing ability *in vitro* (34). *In vivo*, decapacitation factors are gradually removed from the
sperm surface within the female reproductive tract, thereby allowing capacitation to proceed.
In contrast, conventional *in vitro* fertilization (IVF) assays are performed with epididymal
sperm, which naturally lack seminal plasma proteins such as SPINK3, and membrane-bound
proteins might not be efficiently detached under these conditions. To specifically evaluate
whether exogenously added SPINK3 exerts an inhibitory effect in vitro, epididymal sperm were
incubated under capacitating conditions in the absence or presence of 13 µM recombinant
SPINK3 and subsequently used for inseminating cumulus–oocyte complexes (COCs). We
found that SPINK3 produced a significant decrease in the percentage of fertilized eggs (**Figure
5A, B**). Most sperm that were preincubated with SPINK3 retained SPINK3 immunostaining in
the flagellum when recovered 4 hours after insemination (**Figure 5C**).
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244 **DISCUSSION**
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246 **SPINK3 is involved in the regulation of CATSPER1 processing protease in mice**
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We have previously reported that the small vesicular protein SPINK3, found in mouse
male secretions, acts as a decapacitation factor by inhibiting CatSper activity and the increase
of $[Ca^{2+}]_i$ during the early stages of capacitation induced by BSA and HCO_3^- (18). We have
also shown that SPINK3 functions as a Kazal-type serine protease inhibitor that competitively
inhibits trypsin-like proteases (19). Concurrently, it has been shown that both CATSPER1 and
CATSPERθ undergo capacitation-induced degradation (12, 14, 15). However, endogenous
protein(s) responsible for this physiological regulation remains unidentified. In this study, we
show that SPINK3 inhibits sperm trypsin-like proteases and specifically inhibits the

capacitation-associated cleavage of CATSPER1, but not CATSPERθ, suggesting a
sophisticated regulatory role for SPINK3 in controlling CatSper channel activity.

It has been suggested that inhibitors of calpain, a family of calcium-dependent cysteine
proteases, also block CATSPER1 processing (15). However, SPINK3, as a serine protease
inhibitor, is unlikely to directly inhibit calpains. Given that SPINK3 specifically inhibits serine
proteases and interferes with CATSPER1 processing, if this effect results from a direct action
of SPINK3 on a sperm protease, then the protease responsible for CATSPER1 cleavage is
likely a serine protease. Alternatively, CATSPER1 N-terminal domain cleavage could be a
multi-protease event involving different types of proteases. Other sperm serine proteases have
been reported in mouse sperm, such as ACROSIN (Q3ZB06_MOUSE), PRS55
(Q14BX2_MOUSE), and TESP1 (Q3U132_MOUSE), but all are localized in the sperm head,
either as part of the membrane fraction (GPI-anchored or transmembrane included) or soluble
in the acrosome. However, no protease has been described specifically in the sperm flagella,
where CATSPER1 processing is thought to occur. This requires further investigation.

SPINK3 modulates capacitation through mechanisms that extends beyond its
protease inhibitory activity
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While the finding that SPINK3 inhibits CATSPER1 processing does not fully explain
the mechanism by which this protein regulates capacitation, we used soybean trypsin inhibitor
(SBTI) as an alternative trypsin-like protease inhibitor to further investigate this process. SBTI
had previously been shown to block the SPINK3 binding site on the sperm head in mouse
sperm (25), suggesting a potential shared target between these molecules in this region.
Additionally, SBTI has been used to detect acrosin exposure in other mammalian sperm (35),
indicating its interaction with acrosomal proteases. Given these findings, we selected SBTI to
determine whether inhibiting serine proteases alone is sufficient to regulate capacitation. Our
results show that, despite SBTI's ability to inhibit sperm proteases involved in CATSPER1
degradation, it does not modulate capacitation as SPINK3 does, suggesting that SPINK3's
role in capacitation extends beyond its inhibitory function on serine proteases.

Our *in vitro* results demonstrate that both SPINK3 and seminal vesicles extracts
(containing native SPINK3) strongly inhibit sperm trypsin-like proteases, however evidence
shows that SPINK3 acts extracellularly. Therefore, it is possible to consider a role for surface
attached SPINK3 in stabilizing the membrane and CatSper arrangements in the principal
piece, by binding a membrane protease, whose substrate is CATSPER1, and likely to be
differently compartmentalized outside of CatSper nanodomains before capacitation. Although
there is no strict co-localization of SPINK3 and CATSPER1, sperm from *Catsper1* KO mice,
which lack the entire channel complex in the spermatozoa, displayed a different SPINK3-
sperm binding ability and pattern, suggesting that SPINK3 binds to the sperm flagellum in a
CatSper dependent manner. The different and compromised SPINK3 binding with sperm may
be because lipid domain arrangement was disrupted due to the lack of CatSper channel in the
sperm flagella. Given that TESP1 has previously been reported as the ligand for SPINK3 in
the acrosomal region (25), it is possible that the mechanisms governing SPINK3 binding to
sperm vary across membrane subregions. In sperm lacking the CatSper channel—and
consequently exhibiting defective molecular organization along the flagellum—SPINK3
attachment may be restricted to the head, as the principal piece may no longer provide a

suitable binding site. Additionally, SPINK3 might play distinct roles in different sperm regions:
one regulating calcium influx to modulate hyperactivation and the other controlling the
acrosome reaction. However, the latter is beyond the scope of this study and requires further
investigation.

SPINK3 might be involved in the development of sperm subpopulations in the
oviduct

SPINK3 is bound to the majority of sperm during ejaculation, and it is progressively
detached along the oviduct; however, this detachment is asynchronous, as it does not occur
simultaneously in all sperm (21). Besides, it is not clear what modulates CatSper activity and
how CatSper channel containing N-terminally processed CATSPER1 (15) alters its activity.
As most sperm found in the ampulla has a molecular barcode of acrosome reacted (AR +),
tyrosine phosphorylation negative (pY -), and CATSPER1 intact (CATSPER1 +), and our
results show that pY- sperm are also SPINK3+, one can speculate that late release of SPINK3
from the flagellar membrane contributes to avoid premature cleavage of CATSPER1 and thus
premature degeneration. Proteolysis of membrane protein ectodomains acts as a post-
translational modification that determines the levels and activity of membrane proteins (36).
Would CATSPER1-cleaved sperm show increased or reduced CatSper activity? Would intact
CATSPER1 be maintained in the fertilizing sperm or just until right before? In this work, our
evidence shows that SPINK3 prevents this proteolysis that occurs in capacitating media and
also prevents membrane reorganization, increase in $[Ca^{2+}]_i$, and acrosome exocytosis, but it
is unclear whether early or late release constitutes the destiny of sperm for fertilization.
Recently, it has been reported that seminal plasma spermine reversibly inhibits CatSper's
temperature gating, protecting against premature activation (37). May heterogeneity reflects
a compilation of the time- and space-dependent changes that sperm undergo along the
oviduct? Could the importance of tightly regulating CatSper function be such that multiple
redundant molecules operate in parallel to ensure fertilization success? All these questions
serve ongoing and future research directions.

While SPINK3 is gradually removed from sperm surface *in vivo* in female's
reproductive tract (21), the detachment is less efficient *in vitro*, as evidenced by the intense
SPINK3 labeling in the sperm recovered from the co-culture and fertility significantly reduced,
suggesting that the detachment of SPINK3 from sperm needs specific physiological conditions
or specific molecules in female's reproductive tract.

In conclusion, we demonstrate a role for SPINK3 in stabilizing the membrane and
preventing CATSPER1 processing, either indirectly or directly acting as an inhibitor of the
responsible protease that cleaves CATSPER1. Our findings provide molecular insights into
the dynamic regulation of capacitation by male-derived SPINK3 and provide the first evidence
of a natural molecule involved in modulating the CatSper channel. This work helps to explain
the mechanism for the graded subpopulations of sperm along the female reproductive tract.

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339 **MATERIALS AND METHODS**
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    Animals

    Female and male mice (*Mus musculus*) of BALB/c and C57BL/6 strains and sexually
    mature (BALB/c x C57BL/6) F1 hybrids were maintained at 22°C with a photoperiod of 12 h
    light:12 h darkness, food and water *ad libitum*. Sexually mature (2–3 months) male and female
    mice were euthanized by cervical dislocation.
    Catsper1-null mice were previously generated (7) and maintained on a C57BL/6
    background.
    Mice were cared for in compliance with the guidelines approved by local Animal Care
    and Use Committees (UNMdP RD 104/22), following the National Institutes of Health Guide
    to the Care and Use of Laboratory Animals.

    Antibodies and reagents

    Rabbit polyclonal antibodies against CATSPER1 (7), CATSPERθ (12) and SPINK3
    (Sigma HPA027498) were used. A monoclonal anti-HA antibody was purchased from Cell
    Signaling Technology (#3724), and a monoclonal anti-tubulin antibody (T6094, 1:5000) was
    obtained from Sigma-Aldrich. HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG
    were used as secondary antibodies. Recombinant SPINK3 protein was produced in *E. coli*
    and purified using HiTrap IMAC HP (GE Healthcare Life Sciences).
    Additional reagents included: Soybean Trypsin Inhibitor (SBTI) (1 µM), Fluo-3 AM
    (Invitrogen USA, Molecular Probes, F1242), Pluronic acid (Invitrogen, USA), Laminin (100
    µg/ml, BD Biosciences), PNA Alexa Fluor 594 lectin (1 µg/ml, Molecular Probes, L-32459),
    Bodipy-Cholesterol (15 µM, Cayman, 24618), M540 (2.7 µM, Sigma-Aldrich, 323756), FM4-
    64FX (100 µM, Invitrogen, F34653), CellBrite Fix 640 (2.5 µM, Biotium, 30089-T), Yo-Pro1 (50
    nM, Invitrogen, Y3603), Trypsin (347 USP-u/mg, Biological industries, 4192025), PMSF
    (Phenylmethylsulfonyl fluoride, 500µM, ICN Biomedicals Inc., 195381) and fluorogenic
    protease substrates Z-CGGR-AMC (7-Amino-4-methylcoumarin), Suc-LLVY-AMC, and Z-
    SLY-AMC (2 µM each; all from Sigma-Aldrich, Merck, Darmstadt, Germany) .

    Expression and purification of recombinant SPINK3

    For the production of SPINK3, the cDNA encoding the mature SPINK3 from *M.*
    musculus (NCBI ID: NM 009258.5) was cloned into the pET-24b(+) (Novagen, Madison, WI,
    USA) expression vector. Overexpression of SPINK3 was performed in *Escherichia coli*
    Rosetta cells (Novagen) and the recombinant protein was purified to apparent homogeneity
    as described (19, and Supplementary Information S1).

    Sperm preparation

    Epididymal spermatozoa from adult male mice were collected by swim-out from caudal
    epididymis in M2 medium (EMD Millipore, MR-015-D) or HM medium (Modified Krebs Ringer
    Bicarbonate medium: 20 mM Hepes, 119.3 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 5.6 mM
    glucose, 1.2 mM KH_2PO_4, 0.5 mM Na pyruvate and 1.7 mM $CaCl_2$; pH 7.4). Collected sperm
    were washed with fresh HM or PBS.
    For capacitation, sperm were incubated in HMB (HM plus 15 mM $NaHCO_3$ and 5 mg
    mL^{-1} BSA) or human tubular fluid HTF medium (EMD Millipore, MR-070-D) at (2-7 x 10^6 cells

mL^{-1}) w/o purified SPINK3 at 37°C, 5% CO_2 for 1 or 2 hr. SPINK3 concentrations (5-13 µM)
varied according to the assay.
In a set of experiments capacitation was performed in the presence of SPINK3 (13 µM)
or   SBTI   (1   µM)   (38,   39).
For the preparation of sperm lysates, sperm samples (10×10^6 cells) were
resuspended in 1 mL of 0.1% Triton X-100 in PBS. The suspension was sonicated three times
for 1 second each using a small ultracentrifuge tube. Samples were then agitated for 1 hour
at 4 °C, followed by centrifugation at 15,000 × g for 10 minutes at 4 °C. The supernatant was
collected and used immediately. Lysates were always prepared freshly before use.
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398   ***Seminal vesicle lysates***
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Seminal vesicle homogenate was prepared from seminal vesicles obtained from adult
male mice. The tissues were placed in ice-cold PBS (pH 7.4) and homogenized using a IKA
T10 basic homogenizer under cold conditions. The homogenate was then centrifuged at
10000 × g for 10 minutes, and the supernatant was filtered (0.22µm) and stored on ice until
use. The homogenate was subsequently used in activity assays, adjusting the volume based
on the estimated concentration of native SPINK3 in seminal vesicle extracts.
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407   ***Proteolytic activity of sperm lysates and inhibitory assay***
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Proteolytic activity of sperm lysates was assessed over fluorogenic substrates: Z-Arg-
Gly-Arg-AMC (for trypsin-like proteases), Suc-LLVY-AMC (for chymotrypsin-like proteases),
and Z-SLY-AMC (for elastase-like proteases), each at a final concentration of 2 µM. All
reactions were performed in 96-well black plates (Nunc) with a final volume (Fv) of 100 µl per
well.
Reaction mixtures were prepared at room temperature with 40 µl of sperm lysate as
protease source. The reactions (Fv=100 µl) contained 50 mM Buffer phosphate pH 7.4, w/o
the following inhibitors: SPINK3 (1.3 µM final), SBTI (2 µg/ml final), seminal vesicle
homogenate as a control of native SPINK3 (6 µl) and PMSF (500 µM). The reaction was
initiated by adding 2 µM CGGR-AMC substrate, and fluorescence (Ex 355 nm / Em 436 nm)
was measured every minute for 30 min at 37 °C with agitation (Fluoroskan Ascent microplate
reader, Thermo Electron Corporation, Waltham, MA, USA). Blank reaction was conducted
including 40 µl 0,1% Triton. Enzymatic activity was calculated as Δfluorescence over time and
expressed relative to the sperm lysate fluorescence. Inhibition is evidenced as a decrease in
proteolytic activity.

In vivo proteolysis of CATSPER1

Capacitation was performed w/o 13 µM SPINK3 or 1 µM SBTI for 2hrs in HTF media.
Western blots with whole sperm lysates (1 or 2×10^6 cells/lane) were performed and
developed with anti-CATSPER1 (7), anti-CATSPERθ (12), anti-HA (CST, #3724) or anti-
acetylated Tubulin (Sigma, T7451), followed by the corresponding HRP-conjugated goat anti-
rabbit or anti-mouse IgG.
430
In vitro proteolysis of CATSPER1

HEK293T cells were transiently transfected with construct encoding CATSPER1-HA
with Lipofectamine 2000 (Invitrogen), following the manufacturer's instruction. CATSPER1-
HA was solubilized with 0.1% Triton X-100 PBS (PBST) buffer, containing EDTA-free protease
inhibitor cocktail and pulled-down with anti-HA magnetic beads (Pierce, 88836) for 1 h. at RT.
The enriched CATSPER1-HA protein beads were incubated with 30µL of sperm lysates
solubilized from 3.0×10^5 sperm cells w/o 13 µM SPINK3 at 37ºC for the indicated times. After
incubation, the mixture was denatured by LDS sampling buffer supplemented with 50 mM DTT
at 75ºC for 15 min. Western Blot was performed as described before.

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441   ### Time-lapse live cell calcium Imaging
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Mouse epididymal spermatozoa (n=3) (10×10^6 cells/ml) were loaded with Fluo-3 AM
(10 µM) (Invitrogen USA, Molecular Probes F1242) and 0.02% (v/v) pluronic acid (Invitrogen,
USA) as a surfactant at 37°C for 20 minutes in a non-capacitating medium (NC). Non-
capacitated sperm were then incubated w/o SPINK3 (13 µM) or SBTI (1 µM) for 15 minutes
at 37°C and then, adhered to a fluorescence compatible 96 well plate previously covered with
laminin (100 µg/ml, BD Biosciences). HM medium supplemented with 15 mM $NaHCO_3$ was
added to the chamber and basal fluorescence was monitored under confocal microscope
(excitation 488 nm; emission 526 nm, Nikon C1SiR) at 400x magnification. BSA (5 mg/ml) in
a minimum volume was applied, and changes in the calcium signal were recorded throughout
the process. The viability of the spermatozoa was determined through the motility of the
flagellum. The intensity of the fluorescence signal in the head of spermatozoa with motile
flagella was quantified using the free software ImageJ 1.43 (National Institutes of Health,
Bethesda, Maryland, United States). Data was normalized using the equation (F-F0)/F0,
where F is the fluorescence intensity at time t and F0 is the mean fluorescence taken during
the period before the addition of BSA (initial 10 seconds). Then, the total series of (F-F0)/F0
was plotted against time, as well as the percentage of positive response to the stimulus.
459
460   ### Acrosomal reaction (AR) assay
461
Mouse epididymal spermatozoa (n=3) were capacitated (7.5×10^6 cells/ml) for 60
minutes w/o SPINK3 (13 µM) or SBTI (1 µM). To induce the acrosomal reaction, spermatozoa
were incubated with progesterone (50 µM) (18) or Ca^{2+} ionophore A23187 (10 µM) (Sigma-
Aldrich, United States) for 15 min at 37°C after capacitation. The cells were then incubated for
15 minutes at 37°C with the PNA Alexa fluor 594 lectin (1 µg/ml, Molecular Probes, L-32459),
which allows AR to be detected by binding to glycoproteins of the internal acrosomal
membrane. The fluorescence was observed under a microscope at a magnification of 400x.
Sperm that lost their acrosomes (red fluorescence) were expressed as a percentage of 100
cells per replicate.

471
472   ### Cholesterol efflux assay
473
Mouse epididymal spermatozoa (n=3) (15×10^6 cells/ml) were loaded with Bodipy-
Cholesterol (15 µM) (Cayman, 24618) for 30 minutes at 37°C and incubated 15 minutes at
37°C w/o SPINK3 (13 µM) or SBTI (1 µM). Cells were washed to remove excess reagent and
exposed for 60 minutes to either capacitation (CAP) or non-capacitation (NC) conditions. The
fluorescence of the labeled Bodipy cholesterol was observed in a Nikon Eclipse T2000

epifluorescence microscope with a magnification of 400x, and the percentage of cells that
preserved the labeled cholesterol was quantified.
481
482 ### *Determination of lipid disorder*
483
Mouse epididymal sperm (n=3) ($10x10^6$ cells/ml) were incubated in the presence or
absence of rSPINK3 (13 µM) for 15 minutes at 37°C in HM medium. Subsequently, they were
incubated under CAP or NC conditions. After capacitation, 2.7 µM M540 (Ex 555/Em 578 nm,
Sigma-Aldrich, 323756) was added to the sperm suspension ($1x10^6$ total cells) and incubated
for 15 minutes at room temperature in the dark. Cells were washed and Yo-Pro1 was added
at a concentration of 50 nM (Ex 491/Em 509 nm., Invitrogen, Y3603). The samples were
analyzed by flow cytometry. The results were analyzed using the open access Flowing 2
software. During acquisition, sperm were discriminated from debris. The results are expressed
as the percentage of cells with lipid disorder over the total live cells (M540+/Yo-Pro1-).
493
494 ### *Immunolabeling of SPINK3 and CATSPER1*
495
Epididymal spermatozoa from WT and *Catpser1*-/- mice (n=3) were incubated in non-
capacitating medium w/o SPINK3 (13 µM) for 15 minutes at 37°C. Each condition was
centrifuged twice at 500 × g for 5 minutes, and re-suspended in the same volume of fresh PBS
medium without SPINK3. Cells were seeded on 8-well glass slides and allowed to air-dry.
Sperm were fixed with 3.7% paraformaldehyde in PBS for 15 minutes at room temperature,
washed with PBS and permeabilized with 0.5% Triton X-100 for 5 minutes. After washing with
PBS, cells were blocked with 5% BSA in PBS for 1 h at room temperature and followed by
overnight incubation with anti-SPINK3 (1:50 in TBS supplemented with 1 % BSA and 0.1 %
tween, Sigma HPA027498) at 4 °C. After washing with PBS, anti-rabbit IgG-Alexa 488
(1:1000, Abcam, ab15105) was added for 2 hours at room temperature. Finally, the cells were
washed with PBS and mounted to acquire images with the fluorescence microscope NIKON
ECLIPSE E800. Images from both conditions (WT and *Catpser1*-/-) were equally processed
and analyzed with ImageJ (v1.38, NIH). For data analysis, the percentage of SPINK3-positive
sperm under each condition was adjusted by subtracting the count of false positive sperm
observed in the basal immunofluorescence controls (i.e., WT or *Catpser1*-/- sperm incubated
without SPINK3).
To assess tyrosine-phosphorylated proteins (anti-pY), sperm were incubated w/o
SPINK3 (13 µM), incubated under CAP or NC conditions and then the cells were fixed with
4% formaldehyde, washed, and placed on slides. Once dried, they were fixed with 96%
ethanol, permeabilized with 10 µl of T-PBS (1x PBS, 0.5% Tween 20%) with 0.5% Triton 100
for 20 minutes. After washing, they were blocked with 3% BSA in T-PBS for 1 hour. The
samples were washed and incubated for 1 hour with the primary antibody anti-tyrosine mouse
monoclonal (anti-pY) (1:500, Clone 4G10, Millipore) or anti-SPINK3 (1:50, Sigma Aldrich,
HPA027498) in blocking buffer, followed by the secondary antibodies anti-rabbit IgG-Alexa488
(1:1000, Invitrogen A11070) and anti-mouse IgG-Alexa455 (1:500, Invitrogen, A21425). The
preparations were mounted with glycerol:PBS (9:1) and observed under a fluorescence
microscope with a magnification of 400x (Ex 480/Em 525 nm).

Structured illumination microscopy

2D structured illumination microscopy (SIM) imaging was performed with Zeiss
LSM710 Elyra P1 using alpha Plan-APO 100X/1.46 oil objective lens. Samples were prepared
as described in the previous section (Sperm labelling with SPINK3 and immunostaining with
CATSPER1 antibody). Raw images were processed and rendered using Zen 2012 SP2
software (Carl Zeiss).

Plasma membrane labeling
530
After obtaining the sperm sample and co-incubating it with SPINK3 as previously
described, the sample was incubated with 100 µM FM4-64 FX for 3 minutes at room
temperature or with 2.5 µM CellBrite Fix 640 for 15 minutes at 37°C, followed by the SPINK3
immunolabeling protocol as described above. Confocal images were acquired using a Zeiss
LSM-880 microscope equipped with an Alpha Plan-Apochromat 100x/1.46 objective. Images
were also captured in Super-Resolution mode using an Airyscan detector. A total of 10 frames
were acquired, and the Mean-Shift Super Resolution (MSSR) algorithm (40) was applied to
enhance resolution.

539
540   ***In vitro fertilization***
541
F1 hybrid females (female Balb/c x male C57BL/6) were superovulated by
intraperitoneal injection with 7.5U of PMSG and 48 hours later with 5U of hCG. Cumulus-
oocyte complexes (COCs) were obtained from the ampulla 15 hours post hCG and placed in
G-IVF plus medium droplets (LifeCell) under mineral oil (M8410, Sigma-Aldrich).
Subsequently, groups of COCs were transferred to a clean medium drop (90 µl) under oil.
Spermatozoa were obtained from C57BL/6 males in HM medium, incubated w/o SPINK3 (13
µM) for 15 minutes at 37°C and capacitated in G-IVF plus medium (LifeCell) (5 x 10^6 cells/ml).
COCs were inseminated with 10 µl of the sperm suspension and incubated at 37°C, 5% CO2
for 4 hours. Afterward, COCs were washed, and putative zygotes were incubated for 24 hours
and 5 days (post-IVF) to assess the two-cell and blastocyst stages under an inverted
microscope with a magnification of 400x (Nikon Eclipse Ti-S). Remanent sperm were prepared
for immunostaining. Bar charts show the percentage of two-cell embryos and blastocysts over
the total fertilized oocytes.

Statistical analysis
556
Data were analyzed by GLMM (generalized linear mixed effect model) to determine
statistical significance between treatments and control (41). The model included the male
variable as a random effect. Normality of residuals was assessed by plotting theoretical
quantiles vs standardized residuals (Q–Q plots). Homogeneity of variance was evaluated by
plotting residuals vs fitted values. A post hoc analysis was conducted with the 'lsmeans''
package (42). In all cases, the analysis was performed using R software version 3.3.33, with
the 'nlme' package for Gaussian models (43). Statistically significant differences were
determined at $P < 0.05$. Graph bars indicate mean ± s.e.
565

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data availability statement

All datasets used for this study are available upon request.

Author contribution statement

AC and JJ contributed to the conceptualization of the study and supervision. AN, XH, CS, GD, AR and LG contributed to the design and development of the methodology. AN, XH, DK, MB, LZ, AC and JJ contributed to the writing and editing of the manuscript. All the co-authors were involved in the formal analysis, validation, visualization of the results and revised the manuscript.

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REFERENCES

1. A. R. Nicolli, A. Cesari, The other side of capacitation: role of mouse male molecules in the regulation of time and place of capacitation. *Reproduction* **166** (2023). DOI: 10.1530/REP-23-0188.

2. M. C. Chang, Fertilizing capacity of spermatozoa deposited into the fallopian tubes [9]. *Nature* [Preprint] (1951). DOI: 10.1038/168697a0.

3. C. R. AUSTIN, The capacitation of the mammalian sperm. *Nature* **170** (1952). DOI: 10.1038/168697b0.

4. W. R. Dukelow, H. N. Chernoff, W. L. Williams, Properties of decapacitation factor and presence in various species. *J Reprod Fertil* **14** (1967). DOI: 10.1530/jrf.0.0140393.

5. T. Leahy, B. M. Gadella, Sperm surface changes and physiological consequences induced by sperm handling and storage. *Reproduction* [Preprint] (2011). DOI: 10.1530/REP-11-0310

6. J. Y. Hwang, J. J. Chung, CatSper Calcium Channels: 20 Years On. *Physiology* [Preprint] (2023). DOI: 10.1101/2024.11.18.624146.

7. D. Ren, *et al.*, A sperm ion channel required for sperm motility and male fertility. *Nature* **413** (2001). DOI: 10.1038/35098027.

8.   Y. Kirichok, B. Navarro, D. E. Clapham, Whole-cell patch-clamp measurements of
spermatozoa reveal an alkaline-activated Ca2+ channel. *Nature* **439** (2006). DOI:
10.1038/nature04417.

9.   T. A. Quill, *et al.*, Hyperactivated sperm motility driven by CatSper2 is required for
fertilization. *Proc Natl Acad Sci U S A* **100** (2003). DOI: 10.1073/pnas.2136654100.

10. H. Qi, *et al.*, All four CatSper ion channel proteins are required for male fertility and
sperm cell hyperactivated motility. *Proc Natl Acad Sci U S A* **104** (2007). DOI:
10.1073/pnas.0610286104.

11. J. J. Chung, B. Navarro, G. Krapivinsky, L. Krapivinsky, D. E. Clapham, A novel gene
required for male fertility and functional CATSPER channel formation in spermatozoa.
Nat Commun **2** (2011). DOI: 10.1016/j.bpj.2010.12.698.

12. X. Huang, *et al.*, A CUG-initiated CATSPERθ functions in the CatSper channel
assembly and serves as a checkpoint for flagellar trafficking. *Proc Natl Acad Sci U S A*
120 (2023). DOI: 10.1073/pnas.2304409120

13. J. Y. Hwang, Analysis of Ca2+-mediated sperm motility to evaluate the functional
normality of the sperm-specific Ca2+ channel, CatSper. *Front Cell Dev Biol* **12** (2024).
DOI: 10.3389/fcell.2024.1284988.

14. J. J. Chung, *et al.*, Structurally distinct Ca2+ signaling domains of sperm flagella
orchestrate tyrosine phosphorylation and motility. *Cell* **157** (2014). DOI:
10.1016/j.cell.2014.02.056.

15. L. Ded, J. Y. Hwang, K. Miki, H. F. Shi, J. J. Chung, 3D in situ imaging of female
reproductive tract reveals molecular signatures of fertilizing spermatozoa in mice. *Elife*
9 (2020). DOI: 10.7554/eLife.62043.

16. A. Dematteis, S. Miranda, … M. N.-B. of, undefined 2008, Rat caltrin protein modulates
the acrosomal exocytosis during sperm capacitation. *academic.oup.com*. DOI:
10.1095/biolreprod.107.067538.

17. R. Tiwari, *et al.*, Androgen deprivation upregulates SPINK1 expression and potentiates
cellular plasticity in prostate cancer. *Nat Commun* **11** (2020). DOI: 10.1038/s41467-
019-14184-0.

18. L. Zalazar, *et al.*, Male Decapacitation Factor SPINK3 Blocks Membrane
Hyperpolarization and Calcium Entry in Mouse Sperm. *Front Cell Dev Biol* **8** (2020).
DOI: 10.3389/fcell.2020.575126.

19. D. M. Assis, *et al.*, Novel inhibitory activity for serine protease inhibitor Kazal type-3
(Spink3) on human recombinant kallikreins. *ingentaconnect.com* **20**, 1098–1107
(2013). DOI: 10.2174/0929866511320100003.

20. H. Lin, C. Luo, C. Wang, Y. C.-B. and Biophysical, undefined 2006, Epitope topology
and removal of mouse acrosomal plasma membrane by P12-targeted
immunoaggregation. *Elsevier*. DOI: 10.1016/j.bbrc.2006.08.045.

21. A. Nicolli, C. Alonso, C. Otamendi, … M. C.-, undefined 2022, How, where and when is
SPINK3 bound and removed from mouse sperm? *rep.bioscientifica.com*. DOI:
10.1530/REP-21-0195

22. A. A. S. Silva, *et al.*, Dissecting EPPIN protease inhibitor domains in sperm motility and
fertilizing ability: Repercussions for male contraceptive development. *Mol Hum Reprod*
27 (2021). DOI: 10.1093/molehr/gaab066.

23. M. Lin, *et al.*, SPINKL, a Kazal-type serine protease inhibitor-like protein purified from
mouse seminal vesicle fluid, is able to inhibit sperm capacitation. *rep.bioscientifica.com*.
DOI: 10.1530/REP-07-0375.

24.   J. Li, J. Roca, C. Pérez-Patiño, … I. B.-A. reproduction, undefined 2018, Is boar sperm
660   freezability more intrinsically linked to spermatozoa than to the surrounding seminal
661   plasma? *Elsevier*. DOI: 10.1016/j.anireprosci.2018.05.002.

662   25.   S. Ramachandran, … R. B.-M. H., undefined 2021, A mouse testis serine protease,
663   TESP1, as the potential SPINK3 receptor protein on mouse sperm acrosome.
664   *academic.oup.com*. DOI: 10.1093/molehr/gaab059.

665   26.   A. Cesari, M. de los Angeles Monclus, G. P. Tejón, M. Clementi & M. W. Fornes,
666   Regulated serine proteinase lytic system on mammalian sperm surface: There must be
667   a role. *Theriogenology*, **74** (2010). DOI: 10.1016/j.theriogenology.2010.03.029.

668   27.   J. J. Ferreira, P. Lybaert, L. C. Puga-Molina, C. M. Santi, Conserved Mechanism of
669   Bicarbonate-Induced Sensitization of CatSper Channels in Human and Mouse Sperm.
670   *Front Cell Dev Biol* **9** (2021). DOI: 10.3389/fcell.2021.733653.

671   28.   J. Y. Hwang, *et al.*, Dual Sensing of Physiologic pH and Calcium by EFCAB9 Regulates
672   Sperm Motility. *Cell* **177** (2019). DOI: 10.1016/j.cell.2019.03.047.

673   29.   F. M. Flesch, *et al.*, Bicarbonate stimulated phospholipid scrambling induces cholesterol
674   redistribution and enables cholestrol depletion in the sperm plasma membrane. *J Cell
675   Sci* **114** (2001). DOI: 10.1242/jcs.114.18.3543.

676   30.   S. Sankaranarayanan, *et al.*, A sensitive assay for ABCA1-mediated cholesterol efflux
677   using BODIPY-cholesterol. *J Lipid Res* **52** (2011). DOI: 10.1194/jlr.D018051.

678   31.   N. C. Bernecic, *et al.*, BODIPY-cholesterol can be reliably used to monitor cholesterol
679   efflux from capacitating mammalian spermatozoa. *Sci Rep* **9** (2019). DOI:
680   10.1038/s41598-019-45831-7.

681   32.   L. McEvoy, R. A. Schlegel, P. Williamson, B. J. Del Buono, Merocyanine 540 as a flow
682   cytometric probe of membrane lipid organization in leukocytes. *J Leukoc Biol* **44** (1988).
683   DOI: 10.1002/jlb.44.4.363.

684   33.   D. Steckler, T. A. E. Stout, C. Durandt, J. O. Nöthling, Validation of merocyanine 540
685   staining as a technique for assessing capacitation-related membrane destabilization of
686   fresh   dog   sperm.   *Theriogenology*   **83**   (2015).   DOI:
687   10.1016/j.theriogenology.2015.01.019.

688   34.   L. Curci, G. Carvajal, V. Sulzyk, S. N. Gonzalez, P. S. Cuasnicú, Pharmacological
689   Inactivation of CatSper Blocks Sperm Fertilizing Ability Independently of the
690   Capacitation Status of the Cells: Implications for Non-hormonal Contraception. *Front
691   Cell Dev Biol* **9** (2021). DOI: 10.3389/fcell.2021.686461.

692   35.   S. Sumigama, *et al.*, Progesterone accelerates the completion of sperm capacitation
693   and activates catsper channel in spermatozoa from the rhesus macaque. *Biol Reprod*
694   **93** (2015). DOI: 10.1095/biolreprod.115.129783.

695   36.   S. F. Lichtenthaler, M. K. Lemberg, R. Fluhrer, Proteolytic ectodomain shedding of
696   membrane proteins in mammals—hardware, concepts, and recent developments.
697   *EMBO J* **37** (2018). DOI: 10.15252/embj.201899456.

698   37.   D. K. Swain, C. Vergara, J. Castro-Arnau, Lishko, P. V., The essential calcium channel
699   of sperm CatSper is temperature-gated. *Nature communications,* **16** (2025). DOI:
700   10.1038/s41467-025-58824-0.

701   38.   L. Samanta, R. Parida, T. R. Dias, A. Agarwal, The enigmatic seminal plasma: A
702   proteomics insight from ejaculation to fertilization. *Reproductive Biology and
703   Endocrinology* [Preprint] (2018). DOI: 10.1186/s12958-018-0358-6.

704   39.   T. Noda, M. Ikawa, Physiological function of seminal vesicle secretions on male
705   fecundity. *Reprod Med Biol* [Preprint] (2019). DOI: 10.1002/rmb2.12282.

706   40.   E. Torres-García, *et al.*, Extending resolution within a single imaging frame. *Nat*
707   *Commun* **13** (2022). DOI: 10.1038/s41467-022-34693-9.
708   41.   Zuur, *Mixed Effects Models and Extensions in Ecology with R by Zuur* (2019).
709   42.   R. V. Lenth, Least-squares means: The R package lsmeans. *J Stat Softw* **69** (2016).
710   DOI: 10.18637/jss.v069.i01
711   43.   J. Pinheiro, *et al.*, Package nlme: Linear and Nonlinear Mixed Effects Models. *R*
712   *package* (2020). https:// CRAN.R-project.org/package=nlme.
713

FIGURES AND LEGENDS

Figure 1. Capacitation-induced CATSPER1 processing by sperm proteases is inhibited by SPINK3 _in vitro_. A) Mouse epididymal spermatozoa were incubated w/o SPINK3 (13 µM) and exposed to capacitating conditions for either 1 or 2 hr., as indicated. Immunoblot analysis of non-capacitated (NC) or capacitated (CAP) sperm lysates was performed with anti-CATSPER1 antibody raised against the 1–150 aa region of CATSPER1 (7) and anti-CATSPERθ antibody, raised against the C-terminus of CATSPERθ (12). Acetylated tubulin (AC-TUB) was probed as loading control (n=3). **B)** Immunostaining with anti-CATSPER1 antibody was performed on non-capacitated sperm or capacitated sperm that were previously incubated w/o SPINK3 for 30 min. Sperm with intact CATSPER1 signal were quantified (n=3). **p < 0.001. **C)** Proteolytic activity of sperm lysates was measured over three fluorogenic substrates—Z-Arg-Gly-Arg-AMC (CGGR; trypsin substrate), Suc-LLVY-AMC (LLVY; chymotrypsin substrate), and Z-SLY-AMC (SLY; elastase substrate) in the presence or absence of SPINK3 (13 µM), seminal vesicle homogenate (native SPINK3 source), SBTI

(soybean trypsin inhibitor) (2 µg/ml), or PMSF (500µM) (serine protease inhibitor) (n=3).
Enzymatic activity was calculated as Δfluorescence over time and expressed relative to the
sperm lysate. **D)** FL-CATSPER1-HA was expressed in 293T cells and pulled-down using
magnetic beads conjugated with anti-HA antibody. The recombinant protein was then
incubated with sperm lysates w/o SPINK3 (13 µM) at 37℃ for 0, 10, 30, or 60 min and
subjected to immunoblot with anti-HA antibody. **E)** Tyrosine phosphorylated proteins and
sperm-bound SPINK3 were detected by immunofluorescence, with anti-pY (clone 4G10,
green) and anti-SPINK3 (HPA027498, red) antibodies, respectively. Arrows indicate
spermatozoa exclusively stained with anti-pY or anti-SPINK3. Scale bar: 10 µm.
786

Figure 2. Immunodetection of recombinant SPINK3 binding to epididymal spermatozoa
in wild type (WT) and *Catsper1* KO (*Catsper1-/-*) mice. A) Non-capacitated (NC) epididymal
spermatozoa from WT and *Catsper1-/-* mice (n=3) were preincubated with SPINK3 (5 μM),
and immunostaining was performed to detect SPINK3 (red) and CATSPER1 (green). Nuclei
were counterstained with Hoechst (blue). Representative images from three independent
experiments are shown. Scale bar: 10 μm. **B)** 2D Structured illumination microscopy (SIM)
images reveal the plasma membrane localization of SPINK3 (red) on the WT NC sperm cells.
The immunostaining of CATSPER1 (green) was used to indicate the boundary of the plasma

membrane. Three of the four lines of CatSper nanodomains (as indicated by CATSPER1,
green) were seen in this confocal microscopy image. **C)** (Left panel) Airyscan- and MSSR-
processed images show SPINK3 (red) localized on the plasma membrane of the sperm
principal piece, labeled with FM4-64 FX (green). At the level of the yellow line in the FM4-64
FX MSSR image, the distance measured for FM4-64 FX is 0.409 μm, whereas for SPINK3, it
is 0.736 μm. Orthogonal projections from a Z-stack are shown. Scale bar: 1 μm. (Right panel)
Airyscan- and MSSR-processed images show SPINK3 (red) on the plasma membrane of the
sperm principal piece, labeled with CellBrite Fix (cyan). The midpiece plasma membrane
exhibits stronger staining than the principal piece. At the level of the yellow line in the CellBrite
FixMSSR image, the distance measured for CellBrite is 0.442 μm, whereas for SPINK3, it is
0.752 μm. Orthogonal projections from a Z-stack are shown. Scale bar: 1 μm. **D)** (Left panel)
Quantification of SPINK3-positive cells in WT and *Catsper1*-/- spermatozoa, analyzing both
head and tail labeling. Data represent the mean ± SEM (n=3). Significant differences (p < 0.05)
between WT and *Catsper1*-/- groups are indicated. (Right panel) Distribution of SPINK3
labeling in spermatozoa head and tail regions, expressed as percentages for WT and
Catsper1-/- samples.

Figure 3. The inhibitory effect of SPINK3 on capacitation is not mimicked by a standard trypsin inhibitor A) Non-capacitated (NC) sperm loaded with Fluo-3 AM were exposed to SPINK3 (13 µM) or SBTI (1 µM). After washing with fresh media containing $NaHCO_3$, sperm cells were attached on laminin-coated slides and calcium signal (F0) was monitored before and after BSA was added (left panels). The fluorescence increments of individual live cells (F-F0)/F0 after 24 sec after BSA addition were plotted for each condition: control (-), SPINK3 or SBTI (right panels). *p < 0.05 significant differences in the percentage of responsive cells (>0.5 fold), compared to control untreated sperm. **B)** Acrosome reaction was induced by 50 µM progesterone (P4) after capacitation. As control, NC sperm cells were incubated with the Ca^{2+} ionophore A23187 (10 µM). Data represent mean ± SE; N = 3. *p < 0.05 significant differences compared to NC sperm. **C)** Cells under capacitating (CAP, 60 min) or non-capacitating (NC)

 conditions were loaded with the pH-sensitive probe BCECF-AM (2 μM), and fluorescence was
measured (Ex 520/Em 605 nm). *p < 0.05 significant differences compared to NC sperm.

Figure 4. SPINK3 blocks capacitation-associated cholesterol efflux and membrane
destabilization. Mouse epididymal spermatozoa were loaded with Bodipy Cholesterol
(BODIPY/Ch) **(A)** or M540 and Yo-Pro1 **(B)** with or without SPINK3 (13 μM) and incubated
under capacitating (CAP) or non-capacitating (NC) conditions. n=3 LD: low disordered
membrane (Yo-Pro1$^-$/M540$^-$). HD: high disordered membrane (Yo-Pro1$^-$/M540$^+$). Percentage
of M540$^+$ cells is expressed relative to the total live cells (Yo-Pro1$^-$). (*) Significant differences
among treatments, p < 0.05. Scale bar: 10 μm.

A

B

C

834
Figure 5. Effect of SPINK3 on *in vitro* fertilization. Mouse sperm were incubated in the
absence or presence of rSPINK3 (13 µM) under capacitating conditions (CAP). After

capacitation, sperm (5 thousand total cells, 10 µl) were added to groups of COCs (5-6 COCs,
90 µl) obtained from superovulated females, (n=4). **A)** Representative images of two-cell
embryos and blastocysts obtained 24 hs. or 5 days post-IVF, respectively. **B)** Quantification
of two-cell embryos and blastocysts relative to the total oocytes (n=113). (*) Significant
differences compared to the control without SPINK3, $p < 0.05$. **C)** Sperm recovered after 4 hs.
of co-culture were incubated with α-SPINK3 antibodies to detect sperm-bound SPINK3 bound
to sperm by immunofluorescence. Scale bar: 10 µm.

Supplementary Figure 1. Immunostaining was performed with anti-CATSPER1 antibody on
capacitated sperm that were previously incubated w/o SPINK3 for 30 min. Representative
images from three independent experiments are shown. Scale bar: 10 µm.

Supplementary Figure 2. *Effect of SPINK3 and SBTI on the proteolytic degradation of*
CATSPER1 in vivo. Mouse epididymal sperm (n=3) were incubated with or without SPINK3
(13 µM) or SBTI (1 µM) and exposed to CAP conditions. Cell lysis was performed on aliquots
of different treatments at 0 and 60 min. Proteins (0.5 x 10^6 cells/ml) were separated on a 10%
polyacrylamide gel and revealed by Western blot, using anti-CATSPER1 followed by anti-
Tubulin.

BF   α-SPINK3

WT
NC + SPINK3

CATSPER1 -/-
NC + SPINK3

Supplementary Figure 3. *Immunodetection of recombinant SPINK3 incubated with*
epididymal spermatozoa from wild type and Catsper1 KO mice. Mouse epididymal NC
spermatozoa were incubated in the presence of SPINK3 (13 µM) and immunofluorescence
assays were performed using rabbit anti-SPINK3 (α-SPINK3) followed by anti-IgG-rabbit-
Alexa488 (green). (n=3) Scale bar: 10 µm. Images are representative of three independent
experiments. BF, bright field.

Supplementary Movie 1. *3D projection of SPINK3 and plasma membrane markers FM4-64*
FX and CellBrite Fix on the sperm flagellum, related to Figure 2. The video shows a rotating
view along the y-axis, providing a spatial visualization of SPINK3 (red) localization on the
plasma membrane of the sperm principal piece. The membrane is labeled with FM4-64 FX
(green) and CellBrite Fix (cyan), highlighting differences in staining intensity between the
midpiece and the principal piece. https://drive.google.com/drive/folders/1nl2lqIkbBCWcofPsq-
asTwwj_lwKv36C?usp=drive_link