Zinc ion flux during mammalian sperm capacitation

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Sperm capacitation, the ultimate maturation event preparing mammalian spermatozoa for fertilization, was first described in 1951, yet its regulatory mechanisms remain poorly understood. The capacitation process encompasses an influx of bicarbonate and calcium ions, removal of decapacitating factors, changes of pH and sperm proteasomal activities, and the increased protein tyrosine phosphorylation. Here, we document a novel biological phenomenon of a unique zinc (Zn2+) ion redistribution associated with mammalian sperm in vitro capacitation (IVC). Using image-based flow cytometry (IBFC), we identified four distinct types of sperm zinc ion distribution patterns (further zinc signature) and their changes during IVC. The zinc signature was altered after sperm capacitation, reduced by proteasomal inhibitors, removed by zinc chelators, and maintained with addition of external ZnCl2. These findings represent a fundamental shift in the understanding of mammalian fertilization, paving the way for improved semen analysis, in vitro fertilization (IVF), and artificial insemination (AI).
A zinc spark (an exocytic event releasing billions of zinc ions) issued from the oocyte induced by the spermatozoa at fertilization is implicated as a novel biomarker of mammalian embryo quality and developmental potential. To date, zinc ion (Zn$^{2+}$) fluxes have not been well characterized in mammalian spermatozoa, though sperm-flagellar voltage-gated proton channel HVCN1, negatively regulated by Zn$^{2+}$, has been implicated as the main proton extrusion mechanism during mammalian sperm capacitation. This channel regulates intracellular pH and as the main proton extrusion mechanism during mammalian sperm capacitation. Furthermore, the 26S proteasome, a multi-subunit ubiquitin-dependent protease, regulates fertilization at multiple steps from spermiogenesis to sperm penetration of the oocyte zona pellucida (ZP) including certain aspects of sperm capacitation. In particular, the A-kinase anchoring protein AKAP3 is degraded by the ubiquitin-proteasome system (UPS) during bull sperm capacitation, and the E1-type ubiquitin-activating enzyme (UBA1) inhibitor PYR-41 alters acrosomal remodeling. Additionally, proteasomal inhibitors hinder the capacitation-associated shedding of acrosin-inhibitor serine peptidase inhibitor kazal type 2 (SPINK2) and spermadhesin AQN1 in the boar (see review).

Here, we use image-based flow cytometry (IBFC) to document four distinct types of sperm zinc signature and their changes during in vitro capacitation (IVC) of domestic boar, bull, and human spermatozoa, altered after sperm capacitation, further reduced under proteasome inhibiting IVC conditions, removed by zinc chelators, and maintained with addition of external ZnCl$_2$. The zinc shield established by the oocyte following fertilization could derail such sperm zinc signaling as an added barrier to pathological polyspermic fertilization. This all together supports a new role of zinc ions during capacitation and fertilization. Such findings represent a fundamental shift in the understanding of mammalian fertilization, paving the way for a more accurate semen analysis to ameliorate the methodology of in vitro fertilization (IVF) and artificial insemination (AI).

**Results**

**Mammalian spermatozoa possess four distinct zinc signatures.** We used state-of-the-art IBFC and epifluorescence microscopy to trace the sperm zinc signature using Zn-probe FluoZin™-3 AM (FZ3), DNA stain Hoechst 33342, acrosomal remodeling detecting lectin PNA (Arachis hypogea/peanut agglutinin) conjugated to Alexa Fluor™ 647 (PNA-AF647), and live/dead cell, plasma membrane integrity reflecting DNA stain propidium iodide (PI), which is taken up exclusively by cells with a compromised/remodeled plasma membrane. The IBFC, which combines the fluorometric capabilities of conventional flow cytometry with high speed-multi-channel image acquisition, proved to be advantageous due to the high presence of Zn$^{2+}$ in sperm cytoplasmic droplets and seminal debris, which otherwise would distort traditional flow cytometry results. We developed a unique gating and masking strategy to ensure unbiased data analysis (Supplementary Fig. 1). Analyses were performed using the initial, pre-sperm-rich fraction of ejaculates, which had highest sperm viability/plasma membrane integrity, repeatability, and sensitivity to proteasomal inhibition compared to secondary, sperm-rich fraction that appeared more prone to spontaneous capacitation and loss of plasma membrane integrity.

We identified four distinct types of sperm zinc signatures conserved across boar (Fig. 1a–d), bull (Fig. 1e–h), and human spermatozoa (Fig. 1i–l): high Zn$^{2+}$ presence in the sperm head and whole-sperm tail (signature 1; Fig. 1a, e, i), medium-level (based on relative intensity of fluorescence in FlowSight measurements) Zn$^{2+}$ presence in both the sperm head and...
sperm tail midpiece (signature 2, spermatozoa undergoing capacitation; Fig. 1b, f, j), Zn²⁺ presence in the midpiece only (signature 3/capacitated state signature in spermatozoa that underwent capacitation and may be dying; Fig. 1c, g, k), and no Zn²⁺ presence (signature 4, spermatozoa with compromised/remodeled plasma membrane; Fig. 1d, h, l). Spermatozoa after 72 h of storage in Beltsville thaw solution (BTS semen extender) show varied zinc signatures (Fig. 1m).

Zinc signature is indicative of capacitation status in vitro. A drawback to commonly used 15 mM sodium bicarbonate IVC media is rapid sperm death (as compared to in vivo sequential capacitation10), illustrated in the time course study by a shift to PI⁺ cell death flow cytometry gating (Fig. 2a) and rapid acrosomal modification (Fig. 2b). In the interest of emulating in vivo sperm lifespan and sequential capacitation as a fertility diagnostic method, we used a previously described capacitation medium11 with low (2 mM) sodium bicarbonate and increased sodium pyruvate (5 mM) that prolonged sperm viability (Fig. 2c) and elicited similar hyperactivation (Supplementary Movie 1) while achieving hallmark acrosomal modification (Fig. 2d; discussed further in Methods, in vitro capacitation section).

Most spermatozoa in zinc signature 1 and 2 states had no capacitation-like acrosomal remodeling (93.0 ± 6.8% and 95.0 ± 2.6%, data presented as mean ± s.d.; 10,000 cells analyzed per treatment, n = 3 biological replicates) compared to spermatozoa signature 3 and 4 (11.1 ± 5.8% and 7.0 ± 9.9%; P < 0.0001, as determined by the general linear model (GLM) procedure). Capacitation-like acrosomal remodeling was most prevalent with zinc signatures 3 and 4 (81.0 ± 8.5% and 62.2 ± 12.9%) compared to zinc signatures 1 and 2 (4.0 ± 4.7% and 3.4 ± 2.9%; P < 0.0001, as determined by the GLM procedure). Acrosome exocytosis occurred within the subpopulation of spermatozoa with zinc signature 4 (30.7 ± 3.0%) and was greater than zinc signatures 1, 2, and 3 (3.0 ± 2.6%, 1.6 ± 1.2%, 7.9 ± 2.9%; P < 0.001, as determined by the GLM procedure; Fig. 3a; Table 1). As sperm plasma membrane integrity decreased, signaled by increased PI labeling, the zinc patterns progressed to signatures 3 and 4 (Fig. 3b). Hyperactivated spermatozoa, capable of recognizing and binding the oocyte ZP have zinc signature 2 (Supplementary Movies 2–4), in which the transition from signature 1 to 2 occurs within the first 30–60 min of IVC (Supplementary Fig. 2m).

26S proteasome modulates zinc signature capacitation shift. Fresh, ejaculated boar spermatozoa mostly had signature 1, (83.8 ± 3.1%; data presented as mean ± s.e.m.; 10,000 cells analyzed per treatment, n = 3 biological replicates; Fig. 4a, e; Table 2). A small portion of spermatozoa incubated in non-IVC media for 4 h at 37 °C progressed to signature 2 (Fig. 4b) as compared to spermatozoa in the same media incubated at room temperature to emulate the conditions of AI (Fig. 4a), suggesting that some spermatozoa undergo temperature-induced, early-stage capacitation. When proteasome inhibitor MG-132 was added to IVC conditions to reduce sperm proteasome activity as previously described12,13, a significantly higher portion of spermatozoa retained signature 1 when using the pre-sperm-rich fraction (Fig. 4c, d, e) as compared to IVC+ vehicle (P = 0.0271; when signatures 1 and 2 combined P = 0.0008, as determined by Duncan’s multiple range test). After 4 h of IVC, the zinc signature changed to mostly signature 3 (49.4 ± 7.9%), with a small portion of spermatozoa having signature 2 (31.3 ± 12.3%; Fig. 4c). Remarkably, manipulation of sperm Zn²⁺ content during IVC reset the zinc signature (Fig. 4f, h, i). Spermatozoa retained signature 1 with addition of 1 mM ZnCl₂ to IVC medium (Fig. 4f). Cell-permeant Zn²⁺ chelator N,N,N’,N’-Tetakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN) removed a majority of FZ3 fluorescence to signature 3 and 4 states (Fig. 4g) compared to TPEN vehicle (Supplementary Fig. 3h), but TPEN did not reduce Fluo-4 calcium probe intensity compared to vehicle (Supplementary Fig. 3i). Remaining FZ3 fluorescence is likely due to Zn presence, but ions tightly bound within the mitochondrial sheath, as zinc has been previously detected there by electron microscopy14. With the exception of the midpiece, zinc ions appeared to be associated with the sperm surface, as the stepwise extraction removed Zn²⁺ tracer fluorescence early in treatment procedure (Supplementary Fig. 3j–l).
Zinc signature associated with varied fertility in AI boars. We examined possible individual variability in sperm zinc signature in AI boars with acceptable but varied fertility. In a small preliminary fertility trial (n = 4 boars with known fertility in AI service; fertility records in Supplementary Table 1), zinc signatures differed between high- and low-fertile boars both after IVC (Fig. 5a; original histograms located in Supplementary Fig. 4). Boars with high fertility have double the amount of signature 3 spermatozoa prevail after IVC (as percentage of population) as opposed to minimal signature 3 increase in low-fertility boars (Fig. 5b).

**Discussion**

The inhibition of the zinc signature shifts may be related to the presence of Zn-binding UPS enzymes in spermatozoa, such as the RING-finger E3-type ubiquitin ligase UBR7. The RING (really interesting new gene) finger ubiquitin ligases contain an amino acid motif that binds two zinc cations, allowing interaction with other proteins/enzymes, including establishment of a stable E3-substrate interaction required for protein ubiquitination. Other Zn-binding/Zn-containing proteins are likely present in spermatozoa since UBR7 is only detectable in the acrosomal region of fully differentiated spermatozoa and in the centriolar vault of the sperm tail-connecting piece. For example, sperm DNA-binding
Sperm capacitation, although required for fertility, is a terminal maturation event that leads to rapid cell death unless fertilization occurs. The superimposition of zinc ion labeling and PI labeling in flow cytometric scatter plots allows us to subdivide spermatozoa within the boar ejaculate into four subpopulations (Fig. 3b). Thus, the disproportional representation of signatures 3 and 4, associated with sperm capacitated state and death, may indicate low-fertility ejaculates. Cell membrane changes heralded by PI incorporation in the sperm head at capacitation are concomitant with acrosomal remodeling signaled by lectin PNA binding, even though they occur at the opposite poles of the sperm head (Fig. 3c). As indicated in our time-lapse study, PI intensity changes over the course of capacitation and there are two subgroups of PI⁺ spermatozoa: PI⁺ live with plasma membrane change and PI⁺ cell death (Fig. 2c). Although the signature 2 spermatozoa are the hyperactivated and ZP-interacting ones in Supplementary Movie 2, it is likely that a rapid transition occurs through these last stages of capacitation that cannot be distinguished within limitations of today’s technology. After this rapid transition from zinc signature 2 to 3 and associated acrosomal changes, we hypothesize that the final cell death occurs rapidly if fertilization is unsuccessful. This might be the reason for sequential sperm capacitation observed within the oviductal sperm reservoir.

Comparison of zinc signature patterns in boars with varied fertility indicates potential of Zn probes in the evaluation of livestock sperm quality. While such findings with a small group of boars are preliminary, Zn fluorometry could be also given consideration in human andrology and infertility diagnostics. For instance, the sperm content of flagellar voltage-gated proton channel HVCN1 varies between human donors. The HVCN1 regulates human CatSper Ca²⁺ channel localized at the flagellum. With CatSper activation required for hyperactivation and ultimately male fertility, it seems reasonable that zinc signature changes are reflective of this biological event and necessary for preparing the spermatozoa for hyperactivation. Other studies have shown that spermatozoa with higher fertilizing ability have increased changes in capacitation-induced biomarkers. Altogether, this encourages dedicated trials with high statistical power sperm sample sets, aspiring to validate zinc signature as a candidate fertility marker. Such findings not only indicate the existence of sperm subpopulations capable/incapable of fertilizing the oocyte, but even more so that sequential capacitation and resulting waves of sperm release from the sperm reservoir, originally thought to be primarily driven by female reproductive tract-issued signals, are rather co-dependent of sperm subpopulation (Fig. 6a, b). Other recent studies hint at the significance of Zn²⁺ for sperm structure and function/fertility. A decrease in Zn content of human seminal plasma has been associated with infertility stemming from accidental Chernobyl radiation in Ukraine and individuals with high levels of asthenozoospermic spermatozoa (low or no motility) have reduced seminal plasma Zn level. These findings are consistent with other studies reporting fertile males having increased seminal plasma Zn levels compared to infertile men. Optimization of semen Zn²⁺ and/or zinc containing protein(s) levels could thus improve the outcomes of AI in livestock and assisted reproductive therapy in humans.

The signature we describe here is likely representative of Zn²⁺ being involved in multiple steps of sperm capacitation. At ejaculation, sperm motility is highly dependent upon ionic environment. It is well understood that Zn²⁺ can modulate cellular signaling, as well as protein kinase and phosphatase activities, and inhibit proteasomal activity. Porcine seminal plasma reportedly contains 1.6–3.6 mM Zn²⁺, the highest known levels of Zn²⁺ found in any bodily fluid, thus likely to be serving some biological function. Further, Zn-binding seminal plasma proteins may protect the sperm plasma membrane against cold shock. As mentioned earlier, Zn²⁺ negatively regulates HVCN1 channel, the main proton extrusion mechanism in human spermatozoa. Certain aspects of early capacitation events such as Ca²⁺ influx require HVCN1 activation by Zn²⁺ removal. The Zn²⁺ content in the human sperm flagella is negatively correlated to sperm motility; however, this may be due to capacitation-induced hyperactivation being inhibited in the presence of high Zn²⁺. Further, Zn²⁺-chelator DEDTC has been shown to immobilize spermatozoa, reaffirming the role of Zn²⁺ in sperm motility. Beyond sperm capacitation, the zinc signature or sperm zinc signaling of spermatozoa bound to the oocyte ZP could be altered by the Zn spark triggered by the first fertilizing spermatozoon as well be altered by the 300% increase of zinc content in the ZP matrix following the Zn spike. Altogether, such massive release of extracellular Zn²⁺ and increased ZP Zn content could establish a combined zinc shield. Consequently, this zinc shield could derail Zn signaling in the spermatozoa surrounding the oocyte as an added barrier to polyspermic fertilization. Such mechanisms seem plausible in consideration that Zn has been shown to be chemorepulsive to fertilization-competent human, mouse, and rabbit spermatozoa. Furthermore, acrosin and matrix metalloproteinase MMP2, two of the proteinases implicated in sperm-ZP

### Table 1: Statistical analysis of zinc signature and acrosomal status

| No remodeling | Remodeled | Excyotosed |
|---------------|-----------|------------|
| Signature 1   | 93.0 ± 6.8%⁶⁶⁶ | 4.0 ± 4.7%⁶⁶⁶ | 3.0 ± 2.6%⁶⁶⁶ |
| Signature 2   | 95.0 ± 2.6%⁶⁶⁶ | 3.4 ± 2.9%⁶⁶⁶ | 1.6 ± 1.2%⁶⁶⁶ |
| Signature 3   | 11.1 ± 5.8%⁶⁶ | 9.0 ± 8.5%⁶⁶⁶ | 7.9 ± 2.9%⁶⁶⁶ |
| Signature 4   | 7.0 ± 9.9%⁶⁶ | 62.2 ± 12.9%⁶⁶⁶ | 30.7 ± 3.0%⁶⁶⁶ |

Data are presented as mean ± s.d. (three biological replicates). Values with different uppercase and lowercase superscripts (A,B,C,D) indicate significance of the acrosomal status (P-value ≤0.0001) and lower-case superscripts (a,b,c) indicate significance of differences in sperm signatures (P-value ≤0.0002) as determined by the GLM procedure in SAS 9.4. Both PI+ and PI- cells were included in this analysis. A total of 10,000 cells were measured for each replicate.
Increased Zn$^{2+}$ concentration in bovine IVF media has previously been shown to inhibit fertilization adding further support to the effective role of the zinc shield. Based on the present data, sperm zinc signature likely changes as the spermatozoa advance through the female reproductive tract and progress.
Table 2 Effect of proteasomal inhibition on zinc signature

| Treatment                      | Signature 1 | Signature 2 | Signature 3 | Signature 4 | P-value |
|--------------------------------|-------------|-------------|-------------|-------------|---------|
| Fresh, ejaculated              | 83.8 ± 1.8% | 9.2 ± 0.9%  | 6.1 ± 1.2%  | 0.9 ± 0.4%  | < 0.0001|
| Incubation, non-IVC            | 70.3 ± 2.5% | 17.3 ± 3.0% | 10.4 ± 2.7% | 2.0 ± 2.7%  | < 0.0001|
| 100 μM MG132 + IVC             | 30.8 ± 13.1%| 32.9 ± 19.5%| 31.0 ± 4.9% | 5.4 ± 4.9%  | 0.3755  |
| 10 μM Epox, CLBL, MG132 + IVC  | 13.5 ± 4.0% | 33.7 ± 8.8% | 46.8 ± 3.6% | 6.1 ± 3.6%  | < 0.0001|
| “100 μM” Vehicle + IVC        | 9.6 ± 3.3%  | 31.3 ± 7.0% | 49.4 ± 4.5% | 9.8 ± 4.5%  | 0.0006  |
| “10 μM” Vehicle + IVC         | 11.2 ± 2.5% | 25.8 ± 3.6% | 53.1 ± 3.8% | 9.9 ± 3.8%  | 0.0001  |

P-value: A, B, C, D indicate significant differences between the control (fresh, ejaculated sperm) and vehicle controls and treatment groups and lowercase superscripts (a, b, c) indicate significant differences between signatures as determined by Duncan’s multiple range test. Both PI127 and PI128 cells were included in this analysis. Treatment column refers to proteasomal inhibitors MG132, clasto-lactacystin β-Lactone (CLBL), and epoxomicin (Epox.). A total of 10,000 cells were measured for each data point.

Methods

Reagents. All reagents unless otherwise noted were from Sigma. FluoZin-3, AM (FZ; zinc probe) from ThermoFisher (F24195) was reconstituted with DMSO to a stock solution of 500 μM. Lectin PNA (A. hypogea/peanut agglutinin) conjugated to Alexa Fluor™ 647 (PNA-AF647) was from Invitrogen™ (L32460). Fluo-4 NW (calcium probe) from ThermoFisher (F36260) was reconstituted using kit provided assay buffer. Hoechst 33342 (H33342) from Calbiochem (382065) was reconstituted with DMSO to a stock solution of 20 mM; epoxomicin (Epox, BML-P1127) was reconstituted to a stock solution of 20 mM (using MG132 stock); and clasto-lactacystin β-Lactone (CLBL, BML-P1108) was reconstituted with DMSO to a stock solution of 5 mM. Zn-chelator TPEN from Tocris (16858-02-9) was reconstituted to a stock solution of 18 mM. PI from Acros Organics (AC440300010) was reconstituted with H2O to a stock solution of 18 mM. PI from Acros Organics (AC440300010) was reconstituted with H2O to a stock solution of 1 mg mL–1. Proteasomal inhibitors were from Enzo Life Sciences: MG132 (BML-P1102) was reconstituted with DMSO to a stock solution of 20 mM; epoxomicin (Epox, BML-P1127) was reconstituted to a stock solution of 20 mM (using MG132 stock); and clasto-lactacystin β-Lactone (CLBL, BML-P1108) was reconstituted with DMSO to a stock solution of 5 mM. Zn-chelator TPEN from Tocris (16858-02-9) was resuspended with 1:100 EtOH:H2O to a stock solution of 1 mM. Bovine serum albumin was from Sigma (A4503). Anti-phosphotyrosine antibody, clone 4G10™ was from EMD Millipore (05-321).

Semen collection and processing. Boar semen collection was performed under the guidance of approved Animal Care and Use (ACUC) protocols of the University of Missouri-Columbia. Boar semen for the fertility trial was collected, extended, and shipped by overnight parcel from a private boar stud following their extended, and shipped by overnight parcel from a private boar stud following their...
Proposed zinc signature population segregation: 16% of fresh, ejaculated spermatozoa had undergone early-stage capacitation upon semen collection (lightest blue working to darkest); 14% of spermatozoa spontaneously undergo early-stage capacitation during incubation without IVC inducers; 60% of spermatozoa remained capacitation competent with IVC inducers, with 21% sensitive to proteasomal inhibition; remaining 10% of sperm were capacitation incompetent under IVC conditions (darkest blue) (s.e. bars included). Proposed zinc signature changes throughout female reproductive tract and oocyte zinc spark interference with sperm zinc signature as a combined polyspermy defense mechanism, the zinc shield performed every 60 min. Control incubations under non-IVC conditions used NCM. Proteasome inhibitors (100 μM MG-132 and 10 μM Epox/CLBL/MG-132) were mixed with IVC media prior to sperm pellet resuspension. A 100 μM MG-132 and “100 μM” vehicle contained 0.5% (v/v) DMSO. A 10 μM Epox/CLBL/MG-132 and “10 μM” vehicle contained 0.3% (v/v) DMSO. PVA helped to reduce sperm aggregation and spermatozoa were pipetted repeatedly to dissociate sperm aggregates in a satisfactory manner prior to IBFC data acquisition. To confirm normal capacitation in our experimental IVC media, compared to 15 mM sodium bicarbonate-containing medium to display the prolonged lifespan of spermatozoa as seen in in vivo capacitation.

Zn2+ chelation. Zn2+ chelation was performed using TPEN (membrane permeable). Ten micromolar TPEN was incubated with 40 million sperm per mL for 1 h. Stock TPEN: 1 mM in 1:100 EtOH:H2O.

Multiplex fluorescence probing. Upon 4 h of IVC, a sample size of 100 μL (4 million spermatozoa) were incubated 30 min with 1:200 H33342, 1:200 PI, and 1:100 FZ3 for epifluorescence microscopy. Lower probe concentrations were necessary for IBFC due to camera detection differences, thus 1:1000, 1:10000, and 1:100000.
1:500 were used, respectively, with inclusion of 1:1000 PNA-AF647. For Flu-4 calcium probe, we followed manufacturer protocol using identical cell concentra-
tions. Spermatozoa were then washed of probes once and resuspended in corre-
sponding IVIC treatment media to allow complete de-esterification of intracellular AM esters, as suggested by Thermofisher’s FZ3 protocol, followed by an additional wash and resuspended in 100 µL PBS for IBFC analysis (or added to a slide for epifluorescence microscopy imaging).

**Epifluorescence microscopy imaging.** Live spermatozoa were imaged using a Nikon Eclipse 800 microscope (Nikon Instruments Inc.) with Cool Snap camera (Roper Scientific, Tuscon, AZ, USA) and MetaMorph software (Universal Imaging Corp., Downington, PA, USA). Images were adjusted for contrast and brightness in Adobe Photoshop CS5 (Adobe Systems, Mountain View, CA) to match the fluorescence intensities viewed through the microscope eyepieces.

**Image-based flow cytometric data acquisition.** IBFC data acquisition was performed following previous methodology. Specifically, using a FlowSight flow cytometer (FS) fitted with a ×20 microscope objective (numerical aperture of 0.9) with an image rate up to 2000 events per s. The sheath fluid was PBS (without CaCl2 or MgCl2). The flow-core diameter and speed was 10 µm and 66 mm per s, respectively. Raw image data were acquired using INSPIRE software. To produce the highest resolution, the camera setting was at 1.0 µm per pixel of the charged-device. In INSPIRE FS data acquisition software, two bright-field channels were collected (channels 1 and 9), one FZ3 image (channel 2), one PI image (channel 3), one side scatter (SSC; channel 6), one H33342 (channel 7), and one PNA image (channel 8), with a minimum of 10,000 spermatozoa detected. The following lasers and power settings were used: 405 nm (to excite H33342): 10 mW; 488 nm (to excite FZ3): 60 mW; 642 nm (to excite PNA-AF647): 25 mW; and 785 nm SSC laser: 10 mW.

**IBFC data analysis.** Data were analyzed using IDEAS® analysis software from AMNIS EMD Millipore. Gates were used to identify events that met criteria for sperm gating, which was defined as described previously. Flow cytometry analysis was done to distinguish signature 1 and 2 based on whole-cell FZ3 intensity, therefore creating a mask that only analyzes the sperm tail proved to be key in distinguishing these two populations. Two populations were created by combining specific morphological properties that were obtained by the specific sperm gating used. Finally, this gating was used to identify the sperm head, where FZ3 signal was high. This combined gating and masking strategy provided robust clean data, where spermatozoa lost F23 signal as determined using the image gallery.

**Sequential sperm extraction treatment.** Approximately 200 million washed spermatozoa were used per single treatment, which was conducted by adding 100 µL of a relevant reagent; protease inhibitors included. In the first step, PBS was added to the sperm pellet, allowed to incubate on ice for 30 min and spun. In second step, the pellet was resuspended in 0.75 M KCl in PBS and added and incubated on ice for 30 min and spun down. The pellet was washed once with PBS to remove residual salt and reused in the third step for treatment with 30 mM n-octyl-β-D-glucopyranoside (OGG) in PBS. The sperm after each treatment step were analyzed for their zinc signature.

**Western blotting.** Sperm pellets (15 million spermatozoa per pellet) were mixed with reducing SDS-PAGE loading buffer, boiled for 5 min and briefly spun at 5000xg. The SDS-PAGE was carried out on a 4–20% gradient gels (PAGer Precast gels; Lonza Rockland, Rockland, ME, USA) as previously described. The molecular mass of the separated proteins was estimated by using prestained PreCise protein color-coded markers (Lonza Rockland) run in parallel. After SDS-PAGE, proteins were electro-transferred onto a PVDF Immobilon Transfer Membrane (Millipore, Bedford, MA, USA) using an Owl wet transfer system (Fischer Scientific) at a constant 50 V for 4 h for immunodetection.

**Statistics.** All results are presented as mean ± s.e. SAS 9.4 GLM procedure and Duncan’s multiple range test was used to analyze the replicates. Bartlett and Leven tests found the sample set to be homogeneous.

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Author contributions
K.K. and P.S. designed the research; K.K., M.Z., E.Z.D., and P.S. performed the research; K.K., M.Z., and P.S. analyzed the data; K.K., M.Z., E.Z.D., M.S., and P.S. wrote the paper.

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