

## Supplementary Materials for

### **Ca<sup>2+</sup> Signaling Tools Acquired from Prostrasomes Are Required for Progesterone-Induced Sperm Motility**

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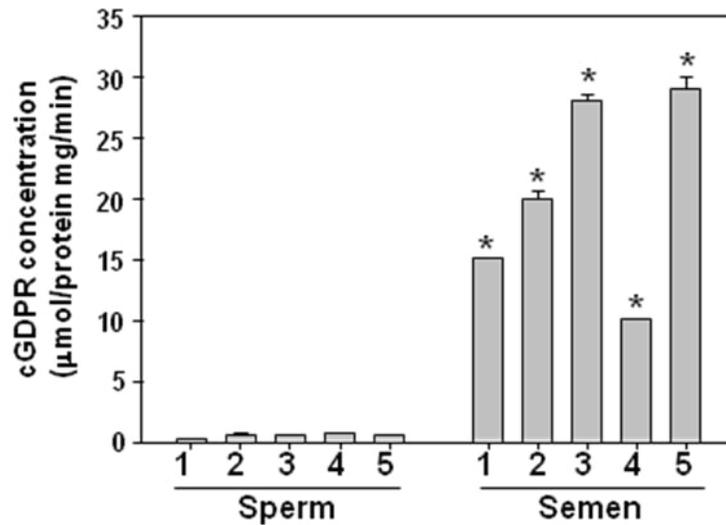
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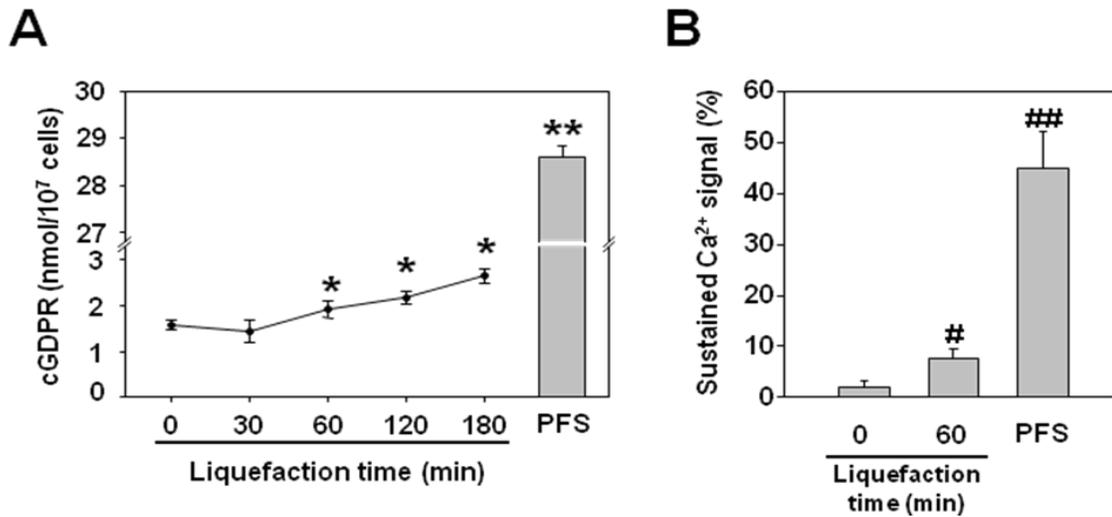
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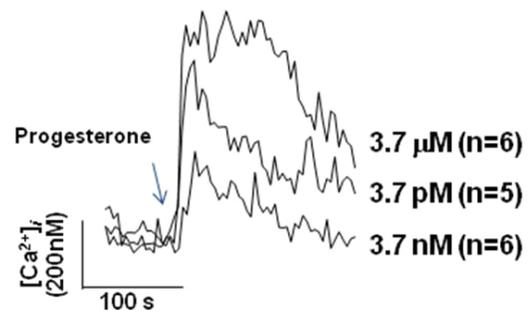


**Fig. S1. ADPR-cyclase activity in sperm and semen of human subjects.** Semen samples were obtained from 5 volunteers. ADPR-cyclase activity was measured using nicotinamide guanine dinucleotide (NGD). To measure ADPR-cyclase activity in sperm and semen, sperm were washed with BWW (Bigger, Whitten, and Whittingham) media and the activity was measured after lysis with 1% Triton X-100-PBS at 4°C. Semen was lysed with the above lysis buffer. ADPR-cyclase activity in sperm and semen was measured in samples containing the same amount of protein. Data are expressed as mean  $\pm$  SEM from three independent experiments. \* $<0.001$  versus spermatozoa from the same subject



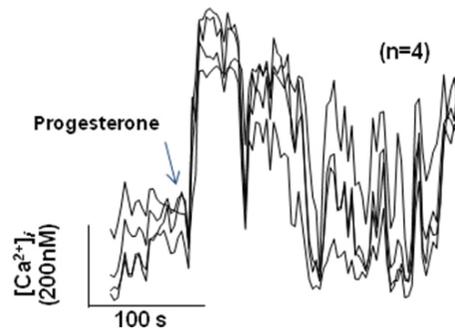
**Fig. S2. Transfer of ADPR-cyclase activity by liquefaction.**

(A) Spontaneous transfer of ADPR-cyclase activity was measured in spermatozoa at various times following semen liquefaction. Semen was maintained at 37°C and enzyme activity was measured immediately after washing with warmed BWW media containing 1% BSA at the indicated liquefaction time. As a positive control, prostasomes were incubated in fusion buffer (20 mM MES, 0.32 M sucrose, 1% BSA (w/v), 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 5.0) with washed spermatozoa of equal volume from the same semen, and then enzyme activity was measured after lysis with 1% Triton X-100-PBS at 4°C for 30 min. N=3. (B) Proportion of sperm with a sustained Ca<sup>2+</sup> signal 180 s after treatment with progesterone is shown as a percent of total. \*p < 0.05 and \*\*p < 0.001 versus zero time. #p < 0.05 and ##p < 0.005 versus sperm at zero liquefaction time. N=4 experiments.



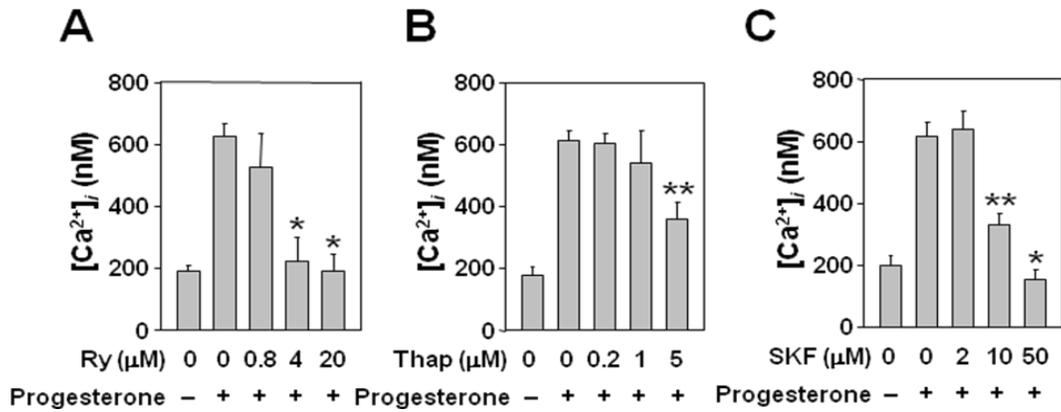
**Fig. S3. Progesterone concentration dependency of  $Ca^{2+}$  signaling in PFS.**

The response to a given concentration of progesterone was similar in approximately 70% of responsive sperm. N=3 experiments.



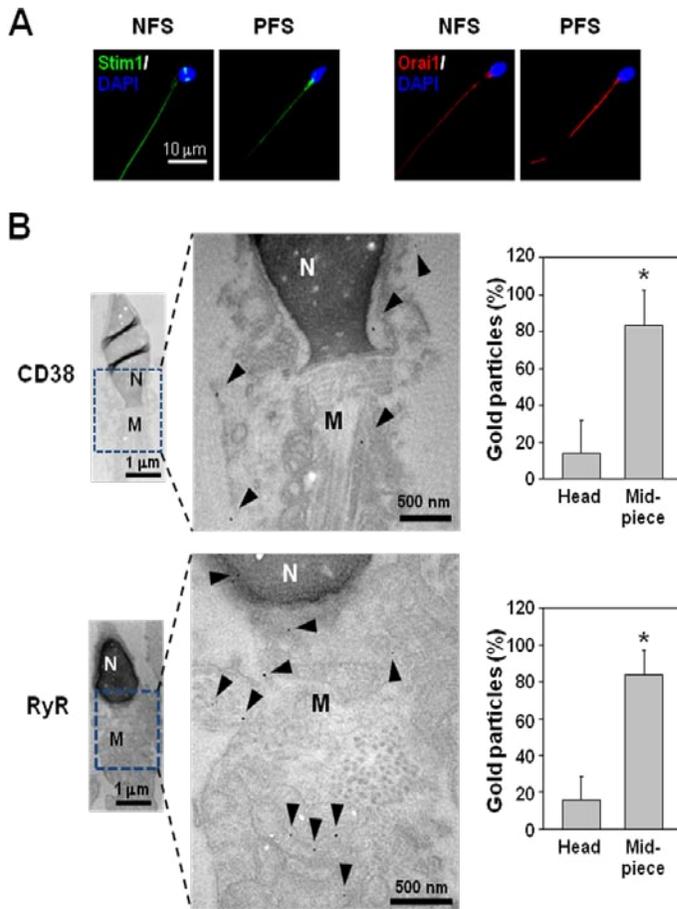
**Fig. S4. Occurrence of progesterone-induced  $Ca^{2+}$  oscillations**

4.1  $\pm$  0.7% of cells (n=21; 510 sperm total in five experiments) showed oscillations with a period of 1-5 min after treatment with 3.7  $\mu$ M progesterone.

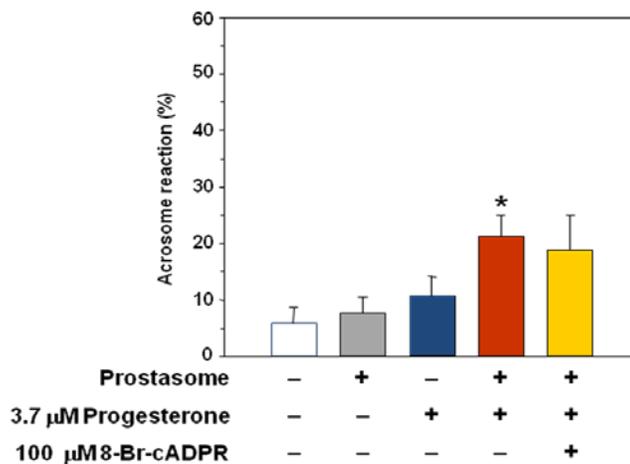


**Fig. S5. Characteristics of progesterone-induced Ca<sup>2+</sup> signaling in PFS**

Inhibitory effect on progesterone-induced Ca<sup>2+</sup> signaling in PFS various concentrations of inhibitors. (A) ryanodine (Ry), (B) Thapsigargin (Thap.), and (C) SKF96365 (SKF). A-C. N=4 experiments. \*p<0.01 and \*\*p<0.05 versus no inhibitor + progesterone group.

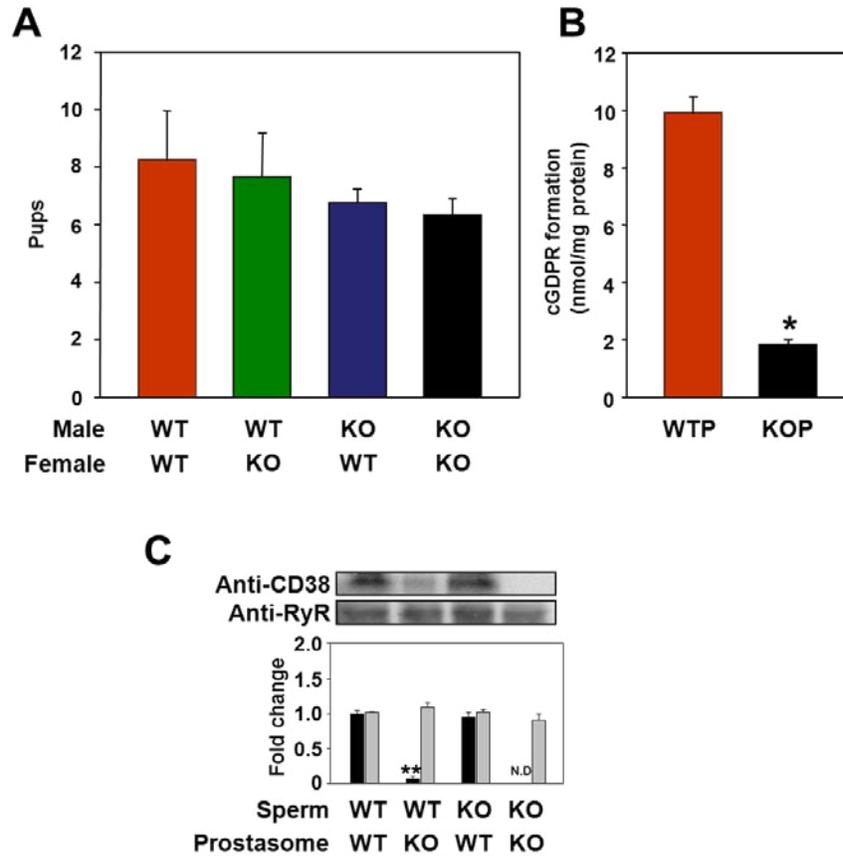


**Fig. S6. Expression and transfer of  $Ca^{2+}$  signaling molecules in sperm** (A) Confocal microscopic images of Stim1 and Orai1 in sperm before and after fusion with prostasomes. Sperm were fixed, stained, and examined with a confocal microscope as described in Materials and Methods. N=3. (B) Localization of CD38 and ryanodine receptor (RyR) by immunogold labeling. Immunogold labeling was performed following incubation of PFS with specific antibodies directed against CD38 and RyR. Longitudinal sections through the nucleus (N) and midpiece (M) are shown. Arrow heads indicate CD38 and RyR. Results (Bar graphs; proportion of gold particles in the midpiece and the posterior region of the head) are expressed mean  $\pm$  SEM of six (CD38) and eight (RyR) independent experiments. \* $p < 0.01$  versus head region.



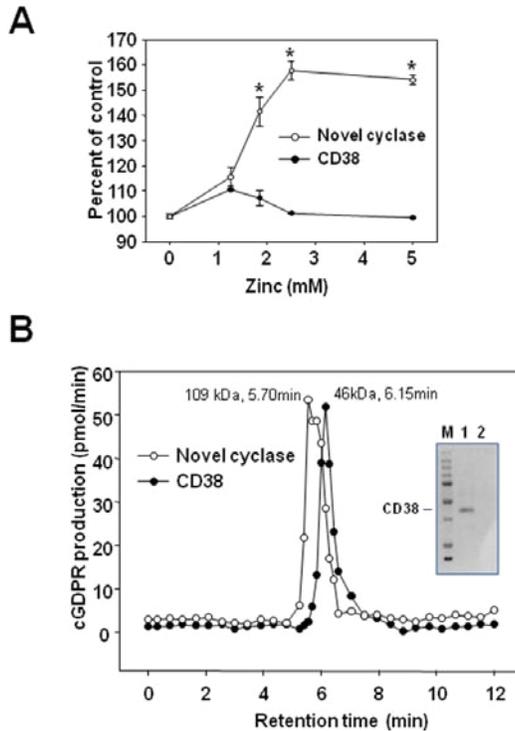
**Fig. S7. Progesterone-induced acrosome reaction in NFS and PFS**

Acrosome reactions were induced by 3.7  $\mu\text{M}$  progesterone in NFS and PFS with a cADPR antagonist, 8-Br-cADPR. The presence or absence of the acrosome was determined by Coomassie brilliant blue staining and microscopic observation (magnification:  $\times 400$  or  $\times 630$ ). Percent of total sperm that underwent acrosome reaction was determined—in randomly selected microscopic fields. Results represent mean  $\pm$  SEM of four independent experiments. \* $p < 0.01$  versus NFS without progesterone.



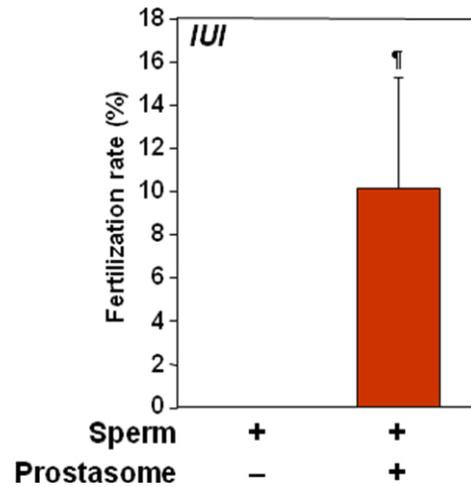
### Fig. S8. Effect of CD38 knockout on mouse fertilization

(A) Mating experiments between  $CD38^{+/+}$  or  $CD38^{-/-}$  mice. All females became pregnant and their litter size was counted (4 females per group, N=2 experiments). (B) Comparison of cGDPR formation activity in prostasome-like vesicles prepared from  $CD38^{+/+}$  mouse prostate gland (WTP) or  $CD38^{-/-}$  mouse prostate gland (KOP). N=3 experiments. (C) Epididymal sperm and prostasome-like vesicles were incubated in fusion buffer (pH 5.0) at 37°C for 15 min. Lower panel: Densitometric analysis of CD38 (black bar) and RyR (gray bar) abundance (from immunoblot) of mouse sperm fused with prostasome-like vesicles. Data are expressed as mean  $\pm$  SEM from three independent experiments. \* $p < 0.01$  versus  $CD38^{+/+}$ . WT and KO represent wild type and  $CD38$  knockout mice, respectively. \*\* $p < 0.001$  versus  $CD38^{+/+}$ . N.D: Not detected.



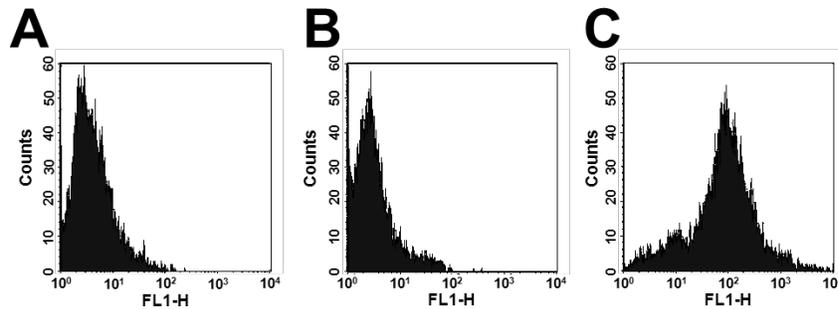
**Fig. S9. Characterization of a novel ADPR-cyclase from prostasomes.**

(A) Effects of  $Zn^{2+}$  on the activity of the previously-unidentified ADPR-cyclase and CD38. Partially purified ADPR-cyclase from prostasomes or immuno-affinity purified human CD38 from prostasome lysate was incubated with various concentrations of  $ZnCl_2$  at  $37^\circ C$  for 10 min and ADPR-cyclase activity was measured using  $NGD^+$  as the substrate. Mean  $\pm$  SEM of three independent experiments. (B) Determination of the molecular weight of the previously-unidentified ADPR-cyclase. ADPR-cyclase and CD38 were separated by HPLC. Molecules were separated with Bio-Sil SEC 125-5 (300 x 7.8 mm)(Bio-Rad) equilibrated with separation buffer (20 mM Tris, 0.5% Triton X-100, 1 mM  $Na_2VO_4$ , 1 mM NaF) by a HPLC system (Gilson, WI). Flow rate: 1 ml/min, Pressure: 750 psi, Fraction size: 0.15 min/fraction. (Inset) Western blot data of the two peaks using an anti-human CD38 antibody (lane 1: CD38; lane 2: previously unidentified ADPR-cyclase). Representative of 2 Western blots, \* $p < 0.01$  versus CD38 group..



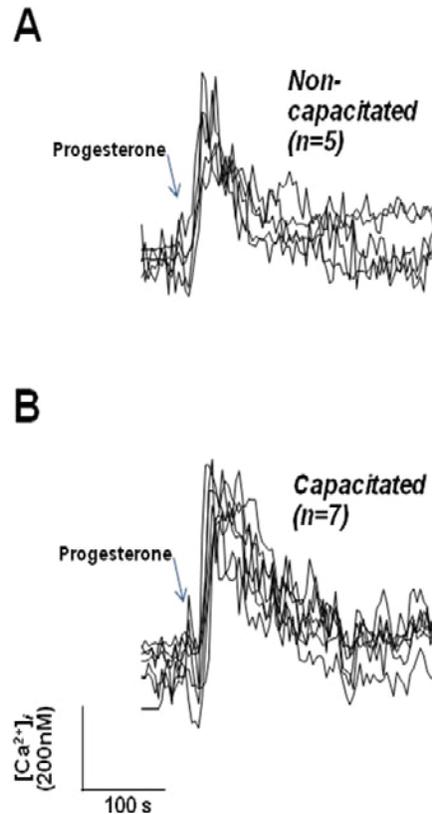
**Fig. S10. IUI before and after fusion of sperm with prostasomes**

Comparison of the percentage of fertilized two-cell stage embryos per total recovered oocytes 30-32 hours after intrauterine insemination. Fertilization rate was expressed as a percentage (number of 2-cell stage oocytes/total recovered oocytes). n=10 mice per group. Eight to twelve oocytes were recovered from each inseminated mouse. Mean ± SEM of three independent experiments. ¶p<0.01 versus control.



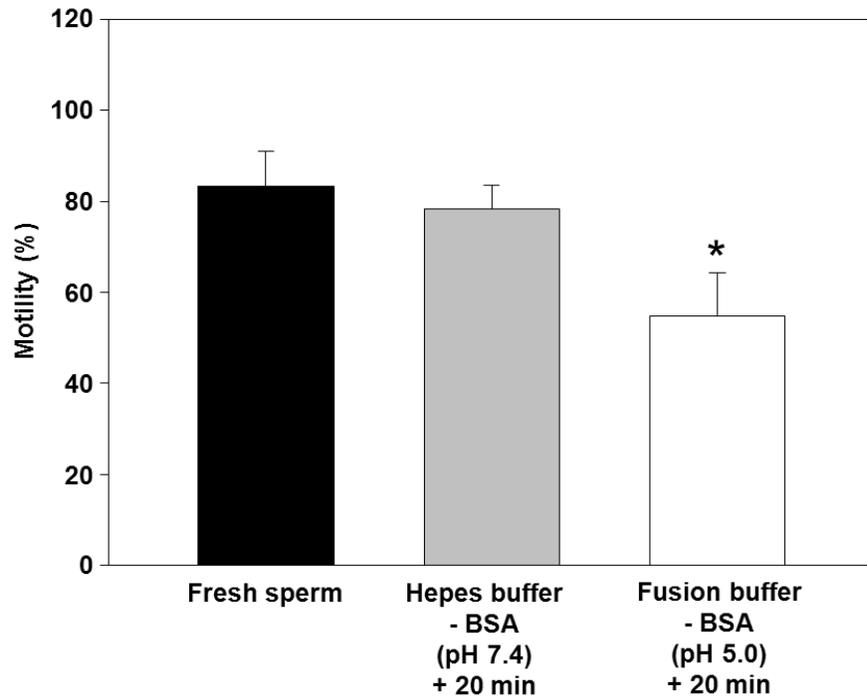
**Fig. S11. Expression of nongenomic membrane progesterone receptor in PFS.**

Progesterone 3-(O-carboxymethyl)oxime-bovine serum albumin–fluorescein isothiocyanate (P–BSA–FITC, Sigma–Aldrich, St. Louis, MO) was pre-cleared by treatment with dextran-charcoal to remove free P and FITC. The P–BSA–FITC was diluted in BWW (1 mg/ml) and added to sperm suspensions to give a final concentration of 10  $\mu$ g/ml. For flow cytometry, sperm in BWW media were incubated in the absence (A) or presence of P–BSA–FITC (B, NFS and C, PFS), followed by centrifugation (700  $\times$ g for 10 min) and resuspension of the resulting pellet with PBS. This step was repeated twice and the resuspended samples (100  $\mu$ l) were then added to isotonic-9% formalin solution (400  $\mu$ l) for fixation (adjusted to  $1 \times 10^6$  cells/ml). Sperm suspensions were analyzed with a Becton Dickinson FACSCalibur (San Jose, CA) with FITC settings (excitation at 488 nm; emission detected in FL1-H channel). The sperm cell window was gated in a dot plot distribution of cells, and fluorescence intensity of at least 10,000 cells was monitored. N=3 independent experiments.



**Fig. S12. Comparison of progesterone-induced  $[Ca^{2+}]_i$  rise in noncapacitated and capacitated sperm.**

(A) Non-capacitated sperm and (B) capacitated sperm were stimulated with  $3.7 \mu\text{M}$  progesterone. Non-capacitated sperm were collected following liquefaction of human semen. For capacitation, highly motile sperm were harvested from liquefied semen by Percoll density gradient, and left for 6 h at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  incubator. n = number of representative traces from non-capacitated and capacitated sperm (416 and 448 cells analyzed, respectively in five experiments). Arrows indicate the time point of the addition of progesterone.



**Fig. S13. Effect of acidic condition on mouse epididymal sperm motility.**

Mouse epididymal sperm were isolated from two cauda epididymis of male mice (10-12 weeks old), The organs were minced in IVF medium, and sperm were allowed to disperse by incubating the tube at 37°C for 15 min. Sperm ( $3 \times 10^6$  cells/ml) were further incubated with 1% BSA (w/v), 150 mM NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 5 mM glucose with either 2 mM HEPES (pH 7.4: Hepes buffer) or 20 mM MES (pH 5.0: Fusion buffer) at 37°C for 20 min. The motility of the incubated sperm was assessed by CASA methods. Instruments were set at: temperature: 37°C; minimum cell size: 4 pixels; video frequency: 60; VAP cutoff: 10.0  $\mu\text{m/s}$ ; VSL cutoff: 0  $\mu\text{m/s}$ . Motile sperm were defined as the velocity which is  $\text{VCL} \geq 60 \mu\text{m/s}$  as instruction of manufacturer for mouse sperm (Hamilton Thorne Biosciences, MA). Results are mean  $\pm$  S.E.M of three independent experiments. \* $p < 0.05$  versus fresh sperm group.