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ORIGINAL RESEARCH

# Multivariate analysis of male reproductive function in *Inpp5b*$^{-/-}$ mice reveals heterogeneity in defects in fertility, sperm–egg membrane interaction and proteolytic cleavage of sperm ADAMs

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**ABSTRACT:** Past work indicated that sperm from mice deficient in the inositol polyphosphate 5-phosphatase *Inpp5b* have reduced ability to fertilize eggs *in vitro* and reduced epididymal proteolytic processing of the sperm protein A Disintegrin and A Metalloprotease 2 (ADAM2). On the basis of these data, our central working hypothesis was that reduced ADAM cleavage would correlate with reduced sperm–egg binding and fusion and in turn with reduced male fertility in *Inpp5b*$^{-/-}$ mice. Multiple endpoints of reproductive functions [mating trials, *in vitro* fertilization (IVF) assays and ADAM2 and ADAM3 cleavage] were investigated on a male-by-male basis, with pair-wise correlation analysis used to assess the relationships between these various parameters. Motile sperm from *Inpp5b*$^{-/-}$ mice showed significantly reduced fertilization of zona pellucida-free eggs due to reduced binding to the egg plasma membrane and subsequent fusion. Localization of a mouse sperm protein required for gamete fusion, IZUMO1, appears normal in *Inpp5*b-null sperm. To our surprise and differing from previous reports, we found that ADAM cleavage was only modestly impaired in numerous *Inpp5b*-null males and varied between individual animals. Performance in mating trials also differed from past reports. The pair-wise correlation analysis revealed that ADAM2 and ADAM3 cleavage was positively correlated, suggesting that processing of these proteins occurs by related/identical mechanisms, but otherwise, there were few correlations between the reproductive endpoints examined here. Nevertheless, this work provides detailed analysis of the *Inpp5b*$^{-/-}$ phenotype and also a blueprint for multivariate analysis to examine relationships between molecular characteristics and *in vitro* and *in vivo* physiological functions.

**Key words:** A Disintegrin and A Metalloprotease / fertilization / inositol polyphosphate 5-phosphatase knockout mice / sperm–egg interaction / IZUMO1

## Introduction

Phosphoinositides play important roles in a variety of cellular functions, acting as substrates for second messenger generation and in signal transduction pathways involved in protein trafficking, apoptosis, ion channel regulation and exocytosis (Hellsten *et al.*, 2001). Mammals have 10 enzymes known as 5-phosphatases that hydrolyze the 5-phosphate from inositol rings that are phosophorylated at the 5-position (Jefferson and Majerus, 1995; Hellsten *et al.*, 2002; Ooms *et al.*, 2009). In humans, a deficiency in the type II 5-phosphatase *Ocrl1* causes the oculocerebrorenal syndrome of Lowe, which is characterized by renal Fanconi syndrome, congenital cataracts and developmental delays (Hellsten *et al.*, 2001). Surprisingly, *Ocrl1*-deficient mice are phenotypically normal (Janne *et al.*, 1998), and it was speculated that another type II inositol polyphosphate 5-phosphatase, INPP5B, would contribute to a murine equivalent of Lowe Syndrome. OCRL1 and INPP5B have 53% amino-acid identity and 71% similarity, share a similar domain structure, and have the same phosphoinositide substrates, except OCRL1 hydrolyzes PtdIns(3,5)$P_2$ and INPP5B does not (Attree *et al.*, 1992; Jefferson and Majerus, 1995; Hellsten *et al.*, 2001; Ooms *et al.*, 2009). However, neither the *Inpp5b*-deficient mice nor the mice deficient

in both *Inpp5b* and *Ocrl1* exhibit Lowe syndrome-like phenotypes. Instead, the *Inpp5b*$^{-/-}$ *Ocrl1*$^{-/-}$ mice were embryonic lethal (Janne *et al*., 1998) and male *Inpp5b*$^{-/-}$ mice were found to have greatly reduced fertility, with multiple defects in sperm function and maturation (Hellsten *et al*., 2001, 2002). *Inpp5b* RNA is detected in the epididymis and testis, specifically in Sertoli cells, types A and B spermatogonia, and leptotene and zygotene primary spermatocytes (http://mrg.genetics.washington.edu/), although INPP5B is not detected by immunoblot in mature sperm (Hellsten *et al*., 2001). A conditional knockout of *Inpp5b* in spermatids with a *PrmCre*$^{+}$ mouse line did not cause the fertility defects seen in the *Inpp5b*$^{-/-}$ mice, suggesting that loss of INPP5B in somatic cells supporting sperm function could be the cause of infertility seen in the knockout males (Hellsten *et al*., 2001).

The purpose of this work was to extend the understanding of the defects in sperm function in *Inpp5b*-deficient mice. The original studies used *Inpp5b*$^{-/-}$ males in two genetic backgrounds, 129S6 and mixed 129S6 × FVB/N (hereafter referred to as FVBS6), and found that only 1 out of 17 of 129S6 *Inpp5b*$^{-/-}$ males were able to sire a single litter of only three pups, whereas 9 out of 20 FVBS6 *Inpp5b*$^{-/-}$ males were able to sire pups with reduced numbers of pups per litter (Hellsten *et al*., 2001, 2002). Sperm from *Inpp5b*$^{-/-}$ males in both backgrounds underwent capacitation and acrosome exocytosis, but had reduced motility (Hellsten *et al*., 2001). Use of these sperm in inseminations of zona pellucida-intact eggs resulted in virtually no fertilization, and inseminations of zona pellucida-free eggs resulted in delayed and reduced incidence of second polar body emission (Hellsten *et al*., 2001). Finally, epididymal proteolytic processing of the A Disintegrin and A Metalloprotease (ADAM) protein ADAM2 was observed to be abnormal, and preliminarily associated with male infertility. This was of interest because mice deficient in *Adam2* exhibit multi-faceted male infertility phenotypes, including reduced ability of sperm to interact with the egg plasma membrane (Cho *et al*., 2000), and because the proteolytic cleavage of ADAM2 is associated with the redistribution of ADAM2 during epididymal maturation, which in turn is speculated to be important for the ADAM2 function in guinea pig sperm (Lum and Blobel, 1997).

On the basis of this previous work, the working model that we speculated would connect these different components of male reproductive function was reduced ADAM cleavage would correlate with reduced sperm–egg interaction, which in turn would correlate with reduced male fertility. With this as our central hypothesis, the work here had several interrelated goals. We performed mating trials in order to have detailed measures of male fertility of each animal studied and also examined epididymal proteolytic processing of ADAM2 and also ADAM3. We also sought to understand the cause(s) of reduced fertilization of zona pellucida-free eggs, using sperm in *in vitro* fertilization (IVF) assays from the males that had also been assessed in the mating trials and for ADAM cleavage. The goal of these IVF studies was to determine whether the failure of sperm from *Inpp5b*$^{-/-}$ males to fertilize zona pellucida-free eggs was due to defects in sperm–egg binding, sperm–egg fusion and/or egg activation. Finally, in testing our central hypothesis, we examined these different endpoints of reproductive function using multiple statistical approaches to determine if there were correlations between male reproductive functions on a physiological level (performance of males in mating trials), a cellular level (performance of sperm in IVF assays) and a molecular level (cleavage of sperm ADAMs during epididymal transit). Two-way nested ANOVA analysis was used to analyze IVF assay data because this method takes into account male-to-male variability, thus leading to more accurate views of the data. Pair-wise correlation analysis was used to assess the relationships between the multiple readouts of reproductive function. Assessing multiple reproductive parameters for each individual male within this population of mice enabled us to determine if there were correlations between the parameters measured, and allowed testing the hypothesis that the success or failure in one reproductive readout is correlated to another reproductive readout, despite the male-to-male variability in the various individual endpoints of reproductive function.

We found, to our surprise, that performance in mating trials differed from past reports and that ADAM cleavage was not as severely affected as would have been expected from past reports. Nevertheless, *Inpp5b*$^{-/-}$ males had fewer pups, fewer litters and fewer pups per litter than *Inpp5b*$^{+/+}$ or *Inpp5b*$^{+/-}$ males did. We also determined that the failure of sperm from *Inpp5b*$^{-/-}$ males to fertilize zona pellucida-free eggs were due a significant reduction in their ability to bind to and fuse with the egg plasma membrane. However, whereas sperm from *Inpp5b*$^{-/-}$ mice did have defects in ADAM2 and ADAM3 cleavage, these defects varied significantly from male to male, from significantly deficient cleavage to completely normal cleavage. Furthermore, pair-wise correlation analysis revealed that there were few correlations between the reproductive functions examined. Taken together, these data indicate that there is significant complexity in how the *Inpp5b* deletion produces an overall reproductive phenotype.

# Materials and Methods

## Mice and mating trials

*Inpp5b*$^{+/-}$ FVBS6 and 129S6 mice, which have been described previously (Hellsten *et al*., 2001, 2002), were provided by Dr Robert Nussbaum (formerly of the National Human Genome Research Institute, NIH; currently University of California-San Francisco, School of Medicine) and breeding colonies were established. Genotyping was performed using the appropriate PCR primers as previously described (Hellsten *et al*., 2001, 2002). Mating trials consisted of individual *Inpp5b*$^{-/-}$ males being housed with two females at a time for 21–28 days and then with a second pair of females for a second 21–28 day period. All males were mating with the females based on copulatory plugs being consistently observed.

## Assessment of ADAM proteolytic cleavage

Cauda epididymal sperm for immuoblots were collected in parallel with sperm for IVF (below) from *Inpp5b*$^{-/-}$ mice and from controls (*Inpp5b*$^{+/+}$ and *Inpp5b*$^{+/-}$) by mincing the cauda epididymis in Whitten's medium [109.5 mM NaCl, 4.7 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 5.5 mM glucose, 0.23 mM pyruvic acid, 4.8 mM lactic acid hemicalcium salt (Whitten, 1971) with 22 mM $NaHCO_3$] containing 15 mg/ml bovine serum albumin (BSA). The sperm were allowed to swim out of the tissue and then the tissue was removed. The sperm for the protein lysate were washed three times with PBS (800 μl each wash, following by brief centrifugation at 500*g*) and then lysed in Laemmli's sample buffer with 0.5% β-mercaptoethanol, heated at 100°C for 10 min, briefly centrifuged and separated by electrophoresis on a 10% SDS-polyacrylamide gel, with 250 000–500 000 sperm equivalents per lane.

Following gel electrophoresis, proteins were transferred to an Immobilon membrane (Millipore, Billerica, MA, USA). After transfer, the membrane

was blocked for 2 h with shaking at room temperature in 10% cold water fish gelatin in PBS containing 0.1% Tween-20 (PBS-T). After blocking, the membrane was rinsed three times in PBS-T and then incubated with 1 μg/ml anti-ADAM2 (Chemicon International/Millipore, Billerica, MA, USA) antibody in PBS-T containing 3% BSA for 1 h with shaking at room temperature. The membrane was then washed three times, 10 min each with PBS-T, and then incubated with 0.7 μg/ml horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG (GAM-HRP; Sigma-Aldirch) in PBS-T containing 3% BSA for 1 h with shaking at room temperature. Following this incubation, the membrane was washed four times, 15 min each in PBS-T. ADAM2 was detected by incubating the membrane with Supersignal Chemiluminescent Substrate (Pierce Protein Research Products, Rockford, IL, USA) for 5 min, followed by exposure on X-ray film. In cases where the blot was re-probed, the membrane was stripped with Restore Western Blot Stripping buffer (Pierce Protein Research Products) for 15 min at room temperature, then washed three times for 10 min each with PBS-T; the membrane was then incubated with Supersignal Chemiluminescent Substrate, followed by a 5 min exposure on X-ray film ensure the stripping had been effective. The membrane was then incubated 1 μg/ml anti-ADAM3 (Chemicon International/Millipore) antibody in PBS-T, 3% BSA for 1 h with shaking at room temperature and treated as above. Images of scanned X-ray films were analyzed using ImageJ Software (http://rsb.info.nih.gov/ij/). The rectangular selection tool was used to outline each lane and the peak intensity determined. The density of both the uncleaved and cleaved forms of the ADAMs was added together to get the total ADAM density and then the density of the uncleaved and cleaved bands were divided by the total ADAM density to determine the fraction of ADAM cleaved.

## IVF assays

Female 6–8-week-old CF-1 mice (Harlan, Indianapolis, IN, USA) were primed with 5 i.u. of pregnant mare's serum gonadotrophin (PMSG; Sigma-Aldirch, St. Louis, MO, USA) and then induced to ovulate with 5 i.u. of human chorionic gonadotrophin (hCG; Sigma-Aldirch) 46–48 h later. Cumulus enclosed–egg complexes were collected from oviducts 12–13 h after hCG administration. Cumulus cells were removed by briefly (<5 min) incubating the eggs in Whitten's medium [109.5 mM NaCl, 4.7 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 5.5 mM glucose, 0.23 mM pyruvic acid, 4.8 mM lactic acid hemicalcium salt (Whitten, 1971) with 8 mM $NaHCO_3$ and 15 mM HEPES (hereafter referred to as Whitten's-HEPES) and 0.025% Type IV-S Hyaluronidase (Sigma-Aldrich) and 30 mg/ml BSA (Albumax I; Gibco-BRL, Gaithersburg, MD, USA)]. After cumulus cell removal, eggs were washed through Whitten's medium with 22 mM $NaHCO_3$ (hereafter referred to as Whitten's-Bicarbonate). Next, the zona pellucida were removed by brief incubation (~10 s) in acidic medium-compatible buffer (10 mM HEPES, 1 mM $NaH_2PO_4$, 0.8 mM $MgSO_4$, 5.4 mM KCl, 116.4 mM NaCl, pH 1.5) and then allowed to recover for 60 min in Whitten's-Bicarbonate containing 15 mg/ml BSA. Eggs were cultured in 5% $CO_2$ in air at 37°C.

Sperm were prepared from *Inpp5b*$^{-/-}$ mice and from controls (*Inpp5b*$^{+/+}$ and *Inpp5b*$^{+/-}$) by a swim-up preparation. The cauda epididymis and vas deferens were minced in 125 μl of Whitten's-Bicarbonate containing 15 mg/ml BSA. Sperm were allowed to swim out of the tissue for 10–15 min and then the tissue was removed from the medium. The sperm-containing medium was then collected and carefully pipetted into the bottom of a tube containing 750 μl Whitten's-Bicarbonate containing 15 mg/ml BSA. After 45 min of culture at 37°C in 5% $CO_2$ in air, 220 μl was pulled from the top of the medium ('swim-up sperm'), transferred to a small petri dish and covered with oil. The sperm were then cultured at 37°C in 5% $CO_2$ in air for a total of 2.5–3 h (1.5–2 h after the swim-up preparation was complete) to allow for capacitation and spontaneous acrosome exocytosis. Sperm were counted using a hematocytometer.

After the recovery period following zona pellucida removal, eggs were inseminated with various sperm concentrations (see Results for details; 13 000–100 000 sperm/ml in 10 μl drops with 10 eggs per drop) for 60 min following methods previously described (Wortzman *et al.*, 2006). To assess sperm binding, eggs were washed three times with a pulled pipette of ~100–120 μm in diameter. Eggs were then fixed in 3.7–4.0% paraformaldehyde (Sigma-Aldrich) in PBS for 15–60 min, permeabilized in 15 min in PBS containing 0.01% Triton X-100, washed through three drops of indirect immunofluorescence (IIF) blocking solution (PBS, 0.1% BSA, 0.01% Tween-20, 0.02% $NaN_3$), and blocked for 15–60 min. The eggs were mounted in 8–10 μl of Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 1.5 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI), which allowed visualization of the decondensing DNA of the fused sperm head in the egg cytoplasm (Wortzman *et al.*, 2006). We used this method preferentially over Hoechst- or DAPI-loaded eggs to examine dye transfer to the fused sperm (Conover and Gwatkin, 1988); that method works well for live eggs but not as well for fixed eggs, as dye leakage can occur from fixed eggs. For this study, it was critical to fix the eggs at the end of the insemination time, to insure that all eggs and all samples were precisely time-matched for post-insemination time. Eggs were scored for the number of sperm bound, sperm fused and exit from metaphase II arrest.

## IZUMO1 immunofluorescence

Caudal epididymal sperm were collected from *Inpp5b*$^{-/-}$ mice or heterozygote control mice were collected by placing the two caudae epididymides and vasa deferentia in 900 μl of Whitten's-Bicarbonate containing 4 mg/ml BSA under mineral oil. The sperm were then cultured at 5% $CO_2$ in air at 37°C for 2.5–3 h to allow for capacitation and spontaneous acrosome exocytosis. Sperm were washed three times with 800 μl of PBS to wash away BSA. Sperm were counted using a hematocytometer and 25 000–50 000 cells were pipetted onto Fisherbrand SuperFrost slides (Fisher Scientific) and allowed to dry at room temperature for 1.5 h. The sperm were then fixed with 3.7% formaldehyde for 10 min at room temperature. After fixation, the slides were washed with PBS with 0.1% Tween-20 (PBS-T) and the cells were permeabilized with 0.5% Triton X-100 in PBS-T for 5 min at room temperature. The slides were washed again and then blocked overnight at 4°C in 0.5% BSA in PBS-T. After blocking, the slides were incubated 2 h at room temperature with 10 μg/ml of either anti-IZUMO1 rabbit polyclonal antibody (gift of Masaru Okabe, Osaka University, Japan) or non-immune rabbit IgG (diluted in 0.5% BSA in PBS-T for 2 h at room temperature). The slides were washed three times 10 min with PBS-T and then incubated for 2 h at room temperature in PBS-T containing 0.5% BSA, FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated Peanut Agglutinin (PNA; 100 μg/ml; Vector Laboratories). The slides were then washed again three times 10 min with PBS-T and then mounted with in 20 μl of Vectashield (Vector Laboratories) containing 1.5 μg/ml DAPI.

## Statistical analysis

All statistical analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org).

# Results

## Assessment of fertility of *Inpp5b*$^{-/-}$ male mice

A total of 29 *Inpp5b*$^{-/-}$ males were assessed in these studies. Each individual *Inpp5b*$^{-/-}$ male (or *Inpp5b*$^{+/-}$ control) was first housed

with two females for 21–28 days and then with a second pair of females for a second 21–28 day period. All males were mating with the females based on copulatory plugs being consistently observed. The number of pups, number of litters and number of pups per litter were determined for each individual male tested. Table I summarizes the results, showing the percentage of males that sired pups, the average number of pups and litters over the 6–8 week trial and the average number of pups per litter. These mating trials revealed that 21% (3/14) of FVBS6 *Inpp5b*$^{-/-}$ males pups and 67% (10/15) of 129S6 *Inpp5b*$^{-/-}$ males sired pups, whereas 100% of FVBS6 and 129S6 *Inpp5b*$^{+/-}$ males sired pups (Table I). The average numbers of pups sired during the mating trial and the average numbers of litters were reduced in *Inpp5b*$^{-/-}$ mice in both genetic backgrounds as compared with heterozygote controls (Table I). Comparison of the number of pups per litter (from mice that were able to sire at least one litter, excluding the males that sired no pups/litters) revealed that despite the fact that some *Inpp5b*$^{-/-}$ males were able to sire a litter, these males still had a reduced litter size when compared with the litter sizes produced by *Inpp5b*$^{+/-}$ males (Table I). The number of fertile males was statistically significantly different in the FVBS6 background (Fisher's exact test; $P = 0.001$ for 3/14 FVBS6 *Inpp5b*$^{-/-}$ males as compared with 7/7 FVBS6 *Inpp5b*$^{+/-}$ males). Evidence was weaker for a significant difference in the number of fertile males in the 129S6 background (Fisher's exact test; $P = 0.12$ for 10/15 129S6 *Inpp5b*$^{-/-}$ males as compared with 9/9 192S6 *Inpp5b*$^{+/-}$ males).

Comparisons of the mating trial outcomes with knockout males in the two genetic backgrounds revealed that the number of pups and number of litters sired by the FVBS6 *Inpp5b*$^{-/-}$ males and 129S6 *Inpp5b*$^{-/-}$ males were statistically significantly different (*t*-test, $P = 0.02$ and 0.01, respectively). However, litter size (the average number of pups per litter) was not statistically significantly different between FVBS6 *Inpp5b*$^{-/-}$ males and 129S6 *Inpp5b*$^{-/-}$ males (*t*-test, $P = 0.29$). The number of fertile males, 3/14 in the FVBS6 background as compared with 10/15 in the 129S6 background, was statistically significantly different (Fisher's exact test; $P = 0.03$).

These results on the fertility of *Inpp5b*$^{-/-}$ males differed somewhat from previous work, although it is worth noting that this previous work had assessed fertility very differently, with reproductive outcomes observed with *Inpp5b*-null males that were housed with at least one female over 2–9 months (Hellsten *et al.*, 2001, 2002). These past observations determined that 6% (1/17) of 129S6 *Inpp5b*$^{-/-}$ males sired pups (differing from the 67% observed in these studies) and 45% (9/20) FVBS6 *Inpp5b*$^{-/-}$ males sired pups (differing from the 21% observed here; Hellsten *et al.*, 2001, 2002). Most notably, this previous work suggested that the 129S6 *Inpp5b*$^{-/-}$ males were nearly completely infertile, whereas the mating trials here showed that two-thirds of 129S6 *Inpp5b*$^{-/-}$ males tested here sired pups.

## Proteolytic cleavage of sperm ADAMs during epididymal transit

Several sperm ADAMs, including ADAM2 and ADAM3, are proteolytically cleaved during sperm transit through the epididymis, resulting in a reduction in electrophoretic mobility from a larger form on testicular sperm to a smaller form on cauda epididymal sperm (Fig. 1A and B; Lum and Blobel, 1997, Yuan *et al.*, 1997). ADAM2 and ADAM3 are implicated in sperm–egg membrane interactions (Cho *et al.*, 2000, Nishimura *et al.*, 2001), but there are no clear data on whether deficient ADAM2 or ADAM3 cleavage can affect fertility. Previous work suggested that ADAM2 cleavage was impaired in *Inpp5b*$^{-/-}$ mice (Hellsten *et al.*, 2001); therefore, we examined ADAM2 cleavage in more detail and also examined the cleavage of ADAM3.

Sperm from *Inpp5b*$^{+/+}$ and *Inpp5b*$^{+/-}$ mice in both genetic backgrounds had 100% of ADAM2 and ADAM3 cleaved to the smaller, mature form, with none of the larger immature form detected on the blots (Fig. 1A and B). To our surprise and in contrast to previous reports, cleavage of ADAM2 and ADAM3 was only modestly impaired in cauda epididymal sperm from 129S6 *Inpp5b*$^{-/-}$ mice; these sperm had 88 ± 2% of ADAM2 and 94 ± 2% of ADAM3 cleaved to the mature forms (Fig. 1C and D). Sperm from FVBS6 *Inpp5b*$^{-/-}$ males had more dramatic perturbation of ADAM cleavage, with 19 ± 8% of ADAM2 and 65 ± 13% of ADAM3 cleaved to their mature forms (Fig. 1E and F). There was also a considerable range from male to male in ADAM2 and ADAM3 cleavage in both genetic backgrounds. In the 129S6 *Inpp5b* males, ADAM cleavage ranged from 100% cleaved (i.e. normal) to 70% cleaved. In the FVBS6 *Inpp5b*$^{-/-}$ males, ADAM cleavage ranged from 100% cleaved (i.e. normal) to 0% cleaved (i.e. completely deficient ADAM cleavage; note the X-axes in the graphs in Fig. 2).

These observations of male-to-male variability in ADAM cleavage and of a more severely compromised extent of ADAM cleavage in the FVBS6 *Inpp5b*$^{-/-}$ mice and less severely compromised ADAM cleavage in 129S6 *Inpp5b*$^{-/-}$ mice raised the possibility that the extent of ADAM2/ADAM3 cleavage could be associated with male fertility in these animals. The male-to-male variability in both ADAM cleavage and fertility outcomes allowed us to test this hypothesis; specifically, we examined if the extent of ADAM2 or ADAM3 cleavage was correlated with the reproductive endpoints of the number of pups sired and the number of pups per litter, on a male-by-male basis. We tested this hypothesis using pair-wise correlation analysis, examining

**Table I Fertility of *Inpp5b*$^{+/-}$ and *Inpp5b*$^{-/-}$ male mice.**

| Strain | Genotype | # Males that sired pups per total males (%) | Avg. # total pups sired in the 6–8 week mating trial | Avg. # of litters in the 6–8 week mating trial | Pups/litter |
|---|---|---|---|---|---|
| FVBS6 | *Inpp5b*$^{+/-}$ | 7/7 (100%) | 14.1 ± 1.9 | 3.1 ± 0.3 | 4.4 ± 0.3 |
| | *Inpp5b*$^{-/-}$ | 3/14 (21%) | 0.4 ± 0.2 | 0.2 ± 0.1 | 1.7 ± 0.3 |
| 129S6 | *Inpp5b*$^{+/-}$ | 9/9 (100%) | 14.6 ± 3.2 | 3.1 ± 0.7 | 5.0 ± 0.5 |
| | *Inpp5b*$^{-/-}$ | 10/15 (67%) | 3.1 ± 1.2 | 1.2 ± 0.4 | 2.2 ± 0.3 |

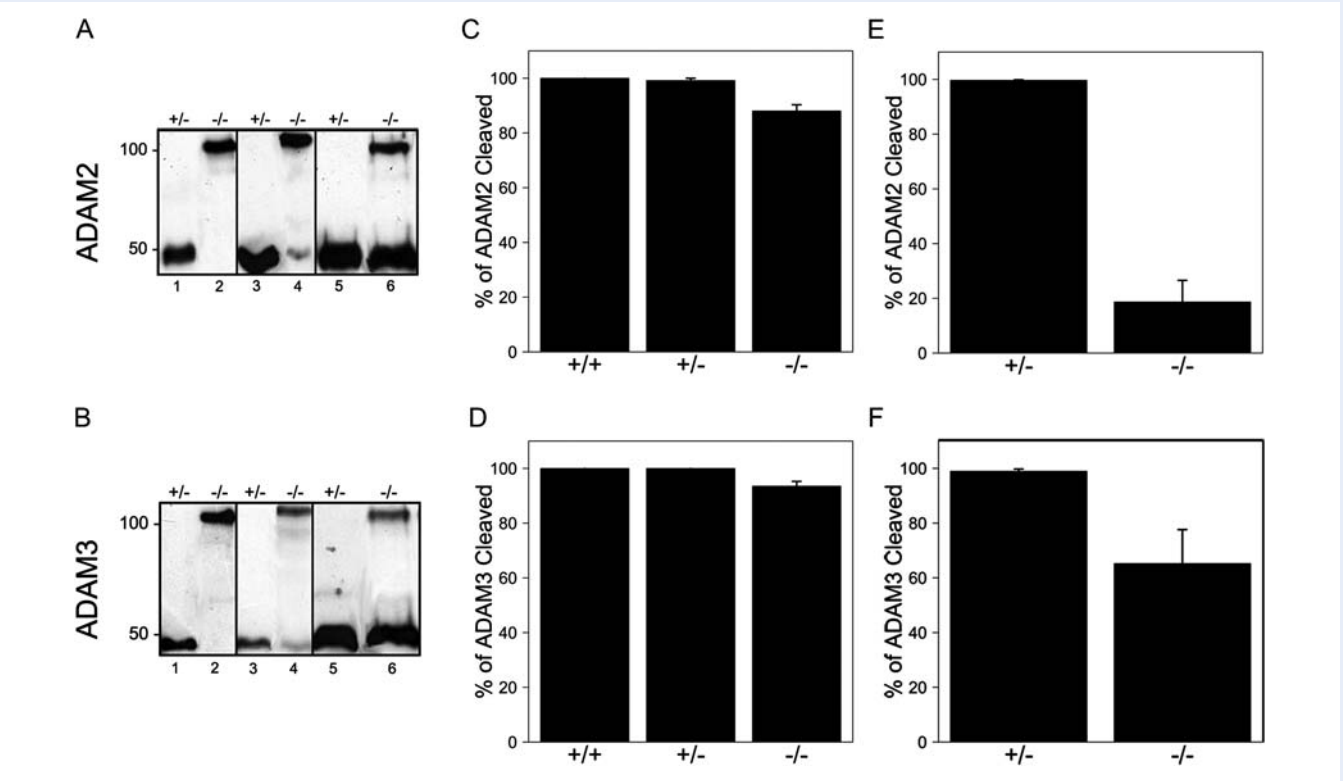



**Figure 1** ADAM2 and ADAM3 cleavage in sperm from *Inpp5b*$^{+/-}$ and *Inpp5b*$^{-/-}$ mice. (**A** and **B**) Representative western blots of ADAM2 (A) and ADAM3 (B) in lysates from cauda epididymal sperm from *Inpp5b*$^{+/-}$ (lanes 1, 3 and 5) and *Inpp5b*$^{-/-}$ (lanes 2, 4 and 6) mice. For ADAM2, the uncleaved form migrates at $M_r = \sim 100\,000$ and the cleaved, mature form migrates at $M_r = \sim 45\,000$. For ADAM3, the uncleaved form migrates at $M_r = \sim 110\,000$ and the cleaved, mature form migrates at $M_r = \sim 40\,000$. These blots show examples of *Inpp5b*$^{-/-}$ males that had no ADAM cleavage (lanes 2), moderate ADAM cleavage (lanes 4) and significant ADAM cleavage (lanes 6). Lysates of sperm from *Inpp5b*$^{+/-}$ show complete ADAM cleavage (lanes 1, 3 and5). (**C**–**F**) The percentages of ADAM2 (C, E) and ADAM3 (D, F) that were cleaved to the mature form were analyzed in multiple individual *Inpp5b*$^{-/-}$ males (126S6 background, C and D; FVBS6 background, E and F).

paired experimental outcomes using a covariance test for association with Pearson's product moment correlation coefficient. We plotted the mating trial endpoints (number of total pups for all males; number of pups per litter for the subset of males that produced offspring) versus the extent of ADAM2 and ADAM3 cleavage for each individual male tested (Fig. 2), speculating that *Inpp5b*$^{-/-}$ males that had a higher extent of ADAM2 and ADAM3 cleaved to the mature form would have a greater number of pups per litter and/or total pups. However, this was not the case with *Inpp5b*-null males in either genetic background, with the exception of the subset of 129S6 *Inpp5b*$^{-/-}$ males that had sired pups. In FVBS6 *Inpp5b*$^{-/-}$ males, the extent of ADAM2 and ADAM3 cleavage had no correlation to the number of pups (Fig. 2A and B). In 129S6 *Inpp5b*$^{-/-}$ males, the extent of ADAM2 and ADAM3 cleavage on the sperm did not correlate to the number of pups sired (Fig. 2C and D). However, for the subset of 129S6 *Inpp5b* males that had sired pups (i.e. omitting the five males that sired no pups), there were statistically significant correlations between the extent of ADAM2 and ADAM3 cleavage and the number of pups per litter (Fig. 2E and F; $P = 0.02$ for ADAM2, $P = 0.05$ for ADAM3). This analysis was not performed with the FVBS6 *Inpp5b*$^{-/-}$ males due to the low number of litters; only three FVBS6 *Inpp5b*$^{-/-}$ males produced one litter apiece, and thus it was not possible to calculate a correlation coefficient for the extent of ADAM2 or ADAM3 cleavage and the number of pups per litter.

## Ability of *Inpp5b*-null sperm to undergo sperm–egg interactions and induce egg activation

Although these analyses above did not identify a correlation between ADAM cleavage and the endpoints of male fertility, we also investigated the performance of sperm from *Inpp5b*$^{-/-}$ males in IVF assays with zona pellucida-free eggs. This was because sperm from *Adam2*$^{-/-}$ and *Adam3*$^{-/-}$ males are defective in sperm–egg membrane interactions (Cho *et al.*, 2000; Nishimura *et al.*, 2001), and therefore we wanted to investigate if defects in ADAM cleavage (Fig. 1) could correlate with deficiencies in sperm–egg interactions. Second, zona pellucida-free eggs inseminated with *Inpp5b*-null sperm show very low rates of and delays in second polar body emission (Hellsten *et al.*, 2001), and therefore we sought to determine if this was due to defects in sperm–egg binding, sperm–egg fusion and/or egg activation.

These assays used motile sperm collected by a swim-up preparation. Additionally, these assays used a range of sperm

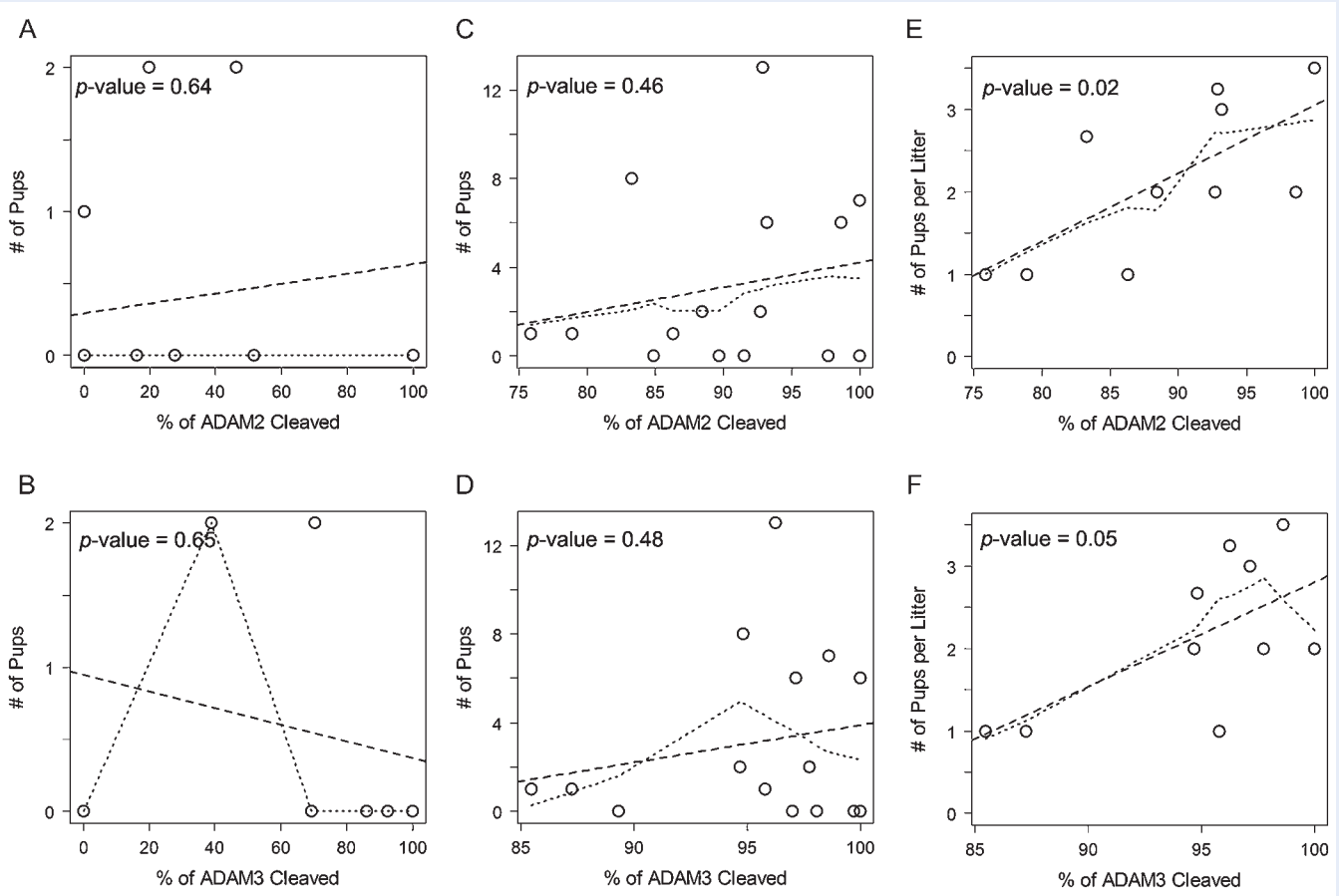



**Figure 2** Pair-wise correlation analysis of ADAM cleavage and reproductive endpoints (pups sired and pups per litter) in *Inpp5b*$^{-/-}$ mice. Bivariate scatter plots between various endpoints of male reproductive function, with a linear fit line (dashed line) and Lowess curve (dotted line). Each open circle shows a data point for the two indicated parameters for an individual mouse. (**A** and **B**) show data from FVBS6 *Inpp5b*$^{-/-}$ males: (A) number of total pups versus percentage of cleaved ADAM2; (B) number of total pups versus percentage of cleaved ADAM3. (**C**–**F**) show data from 129S6 *Inpp5b*$^{-/-}$ males (C) number of total pups versus percentage of cleaved ADAM2; (D) number of total pups versus percentage of cleaved ADAM3; (E) number pups per litter versus percentage of cleaved ADAM2; (F) number of pups per litter versus percentage of cleaved ADAM3.

concentrations since our experience has shown that challenging zona pellucida-free eggs with different sperm:egg ratios can reveal different deficiencies in sperm–egg interaction and gamete function (Evans *et al*., 1995; Glazar and Evans, 2009; Vjugina *et al*., 2009), in agreement with past work (e.g. Fraser and Drury, 1975; Amann and Hammerstedt, 2002). For data analysis, we grouped the studies by ranges of sperm concentrations: high (94 000–100 000 sperm/ml), medium (52 000–80 000 sperm/ml) and low (13 000–30 000 sperm/ml) (Supplementary Table SI). Overall, the IVF assay results showed comparable trends for all sperm concentration ranges tested for both genetic backgrounds, namely that the eggs inseminated with sperm from *Inpp5b*$^{-/-}$ mice showed reduced levels of sperm–egg binding and fusion as compared with eggs inseminated with sperm from *Inpp5b*$^{+/+}$ or *Inpp5b*$^{+/-}$ males (Fig. 3, Supplementary Fig. S1 and Table SII). For example, eggs inseminated with the high concentration of sperm from 129S6 *Inpp5b*$^{-/-}$ mice had an average of $1.8 \pm 0.2$ sperm bound and $0.2 \pm 0.0$ sperm fused per egg, whereas wild-type and heterozygote controls showed much higher levels of binding and fusion to eggs ($8.9 \pm 0.6$ sperm bound per egg and $1.7 \pm 0.1$ sperm fused per egg for sperm from *Inpp5b*$^{+/+}$ mice; $6.1 \pm 0.5$ sperm bound per egg and $1.6 \pm 0.1$ sperm fused per egg for sperm from *Inpp5b*$^{+/-}$ mice; Fig. 3, Supplementary Fig. S1). Two-way nested ANOVA was used to analyze the differences between the genotypes because it takes mouse-to-mouse variability into account and gives a more accurate statistical representation of the data. Nested ANOVA analysis of the IVF data gave strong evidence that 129S6 *Inpp5b*-null sperm are deficient in binding and fusion to zona pellucida-free eggs [*P*-values for sperm binding, $3.8 \times 10^{-4}$ (*Inpp5b*$^{+/+}$ versus *Inpp5b*$^{-/-}$), $2.0 \times 10^{-3}$ (*Inpp5b*$^{+/-}$ versus *Inpp5b*$^{-/-}$); for sperm fusion, $8.0 \times 10^{-4}$ (*Inpp5b*$^{+/+}$ versus *Inpp5b*$^{-/-}$), $2.7 \times 10^{-7}$ (*Inpp5b*$^{+/-}$ versus *Inpp5b*$^{-/-}$)].

The analyses of FVBS6 males revealed that sperm from the FVBS6 males, both heterozygotes and knockouts, performed less well in IVF than did sperm from 129S6 males (Fig. 3, Supplementary Fig. S1 and Table SII). Combining data from all the IVF experiments performed here, only 2% of eggs (5/274) were fertilized by sperm from FVBS6 *Inpp5b*-null mice, as compared with 13% of eggs (93/736) fertilized by 129S6 *Inpp5b*-null sperm (see Supplementary Table SII for full

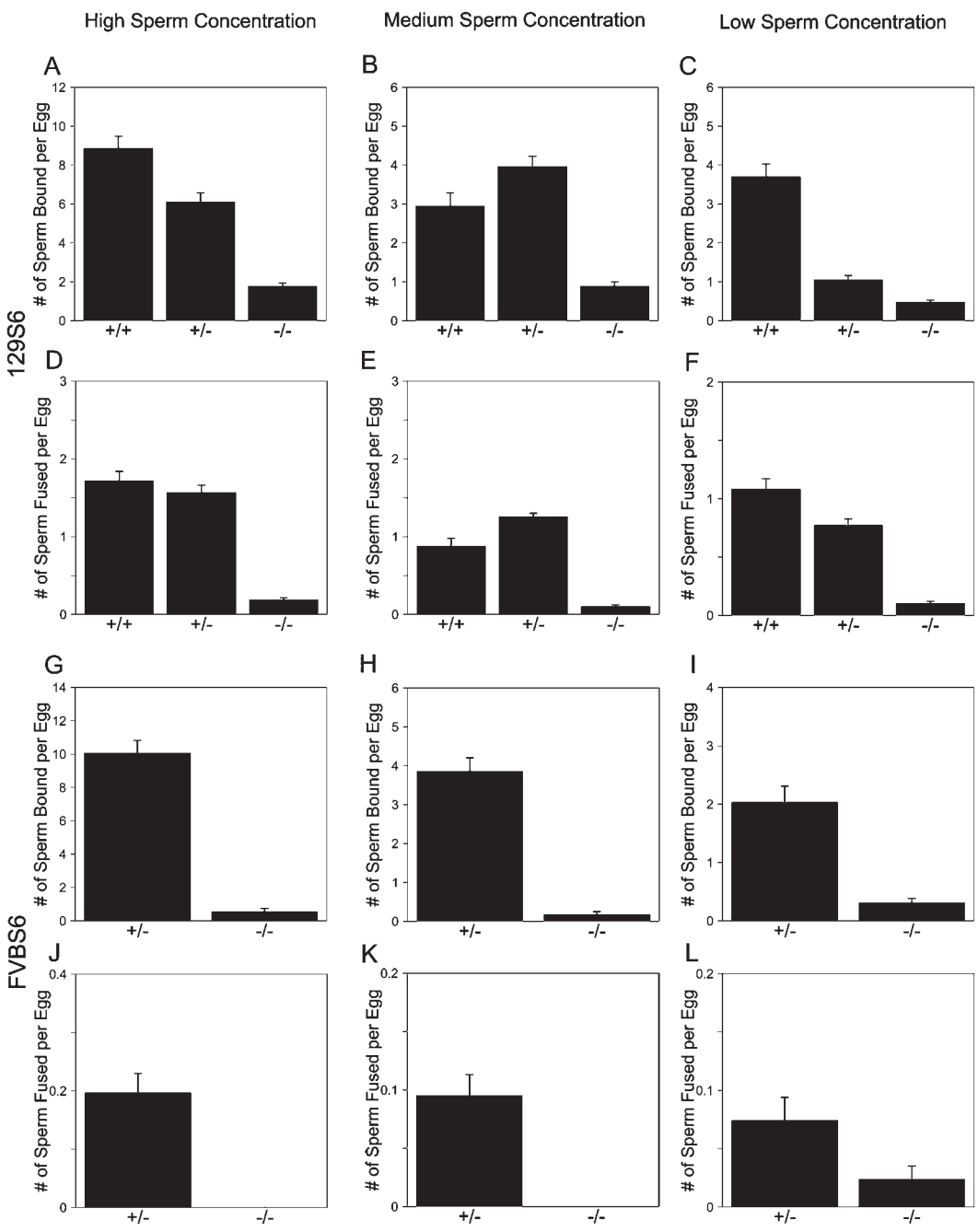



**Figure 3** Sperm–egg binding and fusion in inseminations of zona pellucida-free eggs with sperm from *Inpp5b*$^{+/+}$, *Inpp5b*$^{+/-}$ or *Inpp5b*$^{-/-}$ mice. Zona pellucida-free wild-type eggs were inseminated with sperm from *Inpp5b*$^{+/+}$, *Inpp5b*$^{+/-}$, or *Inpp5b*$^{-/-}$ mice for 60 min and then washed, fixed and assessed for sperm–egg binding (**A**–**C**, **G**–**I**) and fusion (**D**–**F**, **J**–**L**). Three different ranges of sperm concentrations were used: high (94 000–100 000 sperm/ml; A, D, G and J), medium (52 000–80 000 sperm/ml; B, E, H and K) and low (13 000–30 000 sperm/ml; C, F, I and L). (A–F) show data from 129S6 males and (G–L) show data from FVBS6 males.

details). Sperm from heterozygous FVBS6 males also performed poorly; only 11% of eggs (70/643) eggs were fertilized by sperm from FVBS6 *Inpp5b*$^{+/-}$ mice, as compared with 80% of eggs (421/518) fertilized by sperm from 129S6 *Inpp5b*$^{+/-}$ mice (see also Supplementary Table SII). The low variance and high numbers of eggs with no sperm bound or fused did not allow for the accurate use of nested ANOVA with these studies of FVBS6 sperm. Instead, to determine if there was a difference in sperm binding and fusion between FVBS6 *Inpp5b*$^{+/-}$ and *Inpp5b* mice, we used $\chi^2$ analysis to compare the number of eggs that had at least one sperm bound to the number of eggs that had no sperm bound; the same analysis was done for sperm–egg fusion. This $\chi^2$ analysis showed that more

eggs inseminated with FVBS6 *Inpp5b*-null sperm had no sperm bound as compared with eggs inseminated with control sperm from heterozygotes [$P = 6.3 \times 10^{-15}$ (inseminations with the high sperm concentration), $3.0 \times 10^{-12}$ (medium sperm concentration), $9.8 \times 10^{-8}$ (low sperm concentration)]. There was strong evidence to indicate that, compared with eggs inseminated with sperm from FVBS6 *Inpp5b*$^{+/-}$ males, there were more eggs that had no FVBS6 *Inpp5b*-null sperm fused from inseminations with the high and medium sperm concentrations ($P = 0.003$ and 0.04, respectively), and slight evidence at the low concentration range ($P = 0.07$). These results indicate that eggs inseminated with sperm from FVBS6 *Inpp5b*$^{-/-}$ mice had fewer sperm bound and fused per egg than eggs inseminated with sperm from FVBS6 *Inpp5b*$^{+/-}$ mice.

Previous analyses of fertilization of zona pellucida-free eggs by *Inpp5b*-null sperm assessed second polar body emission over time (Hellsten *et al.*, 2001); the delay in and lower extent of second polar body emission could have been due to deficiencies in the ability of sperm to trigger egg activation. Our assays here showed that sperm from *Inpp5b*$^{-/-}$ males that were able to penetrate eggs were able to initiate the egg's exit from metaphase II arrest. Ninety-one of the 93 eggs (98%) that were fertilized by sperm from 129S6 *Inpp5b*$^{-/-}$ mice showed progression to anaphase or telophase II, which was not different from the extent of cell cycle resumption observed in eggs penetrated sperm from 129S6 *Inpp5b*$^{+/+}$ or *Inpp5b*$^{+/-}$ mice [262/264 (99%) and 419/421 (99%) for *Inpp5b*$^{+/+}$ and *Inpp5b*$^{+/-}$, respectively]. This was more difficult to assess with FVBS6 *Inpp5b*$^{-/-}$ mice, as only five eggs were fertilized by FVBS6 *Inpp5b*-null sperm, but of these five eggs, four had clearly exited from metaphase II arrest [4/5 (80%)]; for sperm from FVBS6 *Inpp5b*$^{+/-}$ mice, the percentage of activated eggs was 99% (69/70). There appears to be no significant defect in *Inpp5b*-null sperm in the ability to induce egg activation.

## Pair-wise correlation analysis of male reproductive function parameters in *Inpp5b*$^{-/-}$ mice

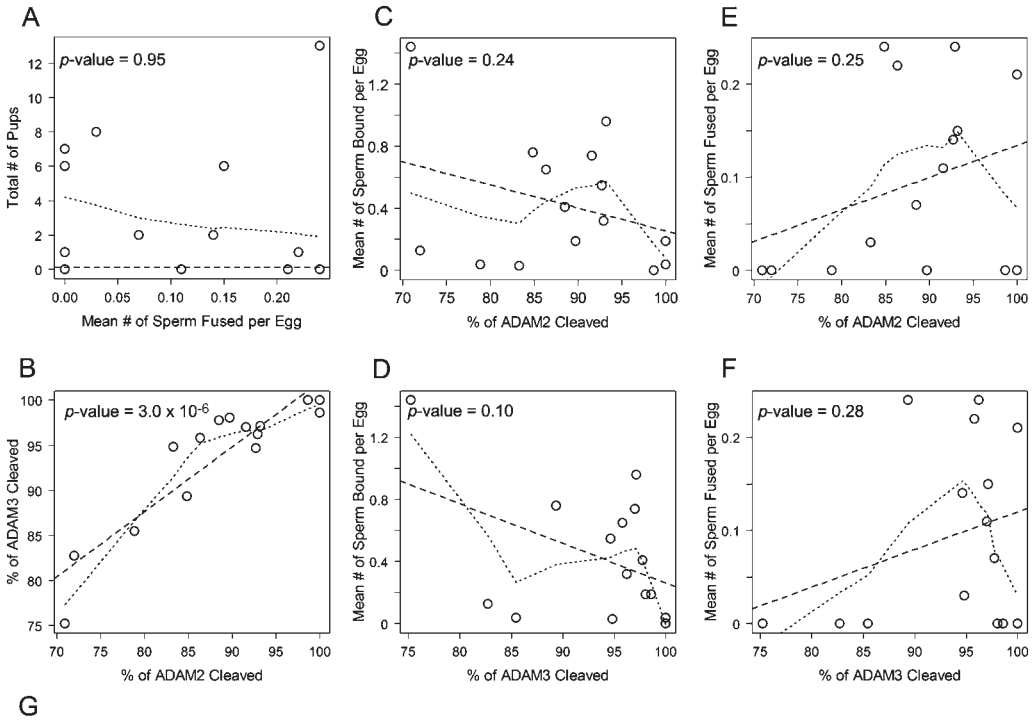
With these data on sperm–egg binding and fusion in hand, we extended the pair-wise correlation analysis from the assessments of possible correlations between ADAM cleavage and male fertility (shown in Fig. 2) to examine if there was a correlation between any of the endpoints of male reproductive function: physiological (performance of males in mating trials), cellular (performance of sperm in IVF assays with zona pellucida-free eggs) or molecular (cleavage of sperm ADAMs during epididymal transit). There was variability between the *Inpp5b*$^{-/-}$ males in all of these assays, including the IVF assays, as well as the mating trial assays and ADAM cleavage assays shown above (Fig. 2); this male-to-male variability in outcomes from individual assays led us to hypothesize that there would be correlations in performance in these various assays for individual *Inpp5b*$^{-/-}$ males. In other words, we speculated that the *Inpp5b*$^{-/-}$ males that had a poor performance in one assay would also have poor performances in other assays, whereas males that had a strong performance in one assay would have strong performances in other assays. One specific hypothesis tested was that sperm binding and/or fusion to the egg plasma membrane would correlate with the extent of ADAM2 and ADAM3 cleavage; to date, there are no clear data on whether ADAM cleavage affects the ability of sperm to interact with eggs.

We used pair-wise correlation analysis, as was done for the analyses of ADAM cleavage and mating trial data above (Fig. 2), plotting the experimental outcome for one assay versus the experimental outcome for another assay for each of the individual males tested, accompanied by statistical analyses (Figs 4 and 5). Analyses were performed on data separated by genetic background and by the experimental design of the IVF assays (i.e. high, medium or low sperm concentration) (Fig. 4, Supplementary Figs S2, S3, S4). We also performed a cross-strain comparison, using all the animals in both strains [Fig. 5; this was done with the IVF data from the low sperm concentration only, since only a small number *Inpp5b*$^{-/-}$ FVBS6 males had sufficient numbers of sperm to perform the IVF assays with the medium and high sperm concentrations (Supplementary Table SI)]. This analysis includes 15 129S6 mice (blue circles) and six FVBS6 mice (red triangles; Fig. 5A–F). Representative plots for the 129S6 *Inpp5b*$^{-/-}$ males and IVF assays using the low sperm concentration (13 000–30 000 sperm/ml) are shown in Fig. 4, and for the 129S6 and FVBS6 *Inpp5b*$^{-/-}$ males combined in Fig. 5(Panels A–F), and the full set of results (*P*-values and correlation coefficients) from these analyses of *Inpp5b*$^{-/-}$ males and IVF assays using the low sperm concentration are shown Figs 4G and 5G. In the interest of space, we show only the analyses from the IVF assays using the low sperm concentration, which was the largest dataset (see Supplementary Table SI for complete details on the number of males tested in the IVF assays). Identical analyses were done using IVF data from assays using the medium and high sperm concentrations from 129S6 *Inpp5b*$^{-/-}$ males and low sperm concentrations from the FVBS6 *Inpp5b*$^{-/-}$ males, with similar results (Supplemental Figs S2–S4).

This pair-wise correlation analysis demonstrated, somewhat surprisingly, that relatively few metrics examined here had a strong correlation with each other. The number of pups was correlated to both the number of litters and the number of pups per litter, and the extent of ADAM2 cleavage was correlated to extent ADAM3 cleavage (Figs 4B, G and 5B, G). However, few other correlations were observed. For example, to determine if sperm function in IVF assays was related to mating trial outcomes, we plotted the total number of pups versus the mean number of sperm fused per egg; this analysis revealed that there was no correlation between these two endpoints (Fig. 4A and G). One specific hypothesis being tested in these analyses was that the extent of ADAM2 or ADAM3 cleavage would be correlated with the extent of sperm–egg binding and/or fusion. However, in the analysis of all *Inpp5b*$^{-/-}$ animals (FVBS6 and 129S6), there was only weak evidence that sperm fusion was correlated with the extent of ADAM2 cleavage ($P = 0.07$, $r = 0.39$; Fig. 5E and G), but this did not extend to ADAM2 cleavage and sperm binding (Fig. 5C and G), to ADAM3 cleavage and sperm binding or fusion (Fig. 5D, F, G), or to the analyses of just the FVB animals (Supplemental Fig. S4) or just the 129S6 animals (Fig. 4, Supplemental Figs S2 and S3).

## Localization of IZUMO1 is normal in 129S6 *Inpp5b*-null sperm

Although the reduced sperm–egg binding and fusion did not correlate with ADAM cleavage, it nonetheless was clear that 129S6 *Inpp5b*-null sperm had an impaired ability to interact with the egg plasma membrane

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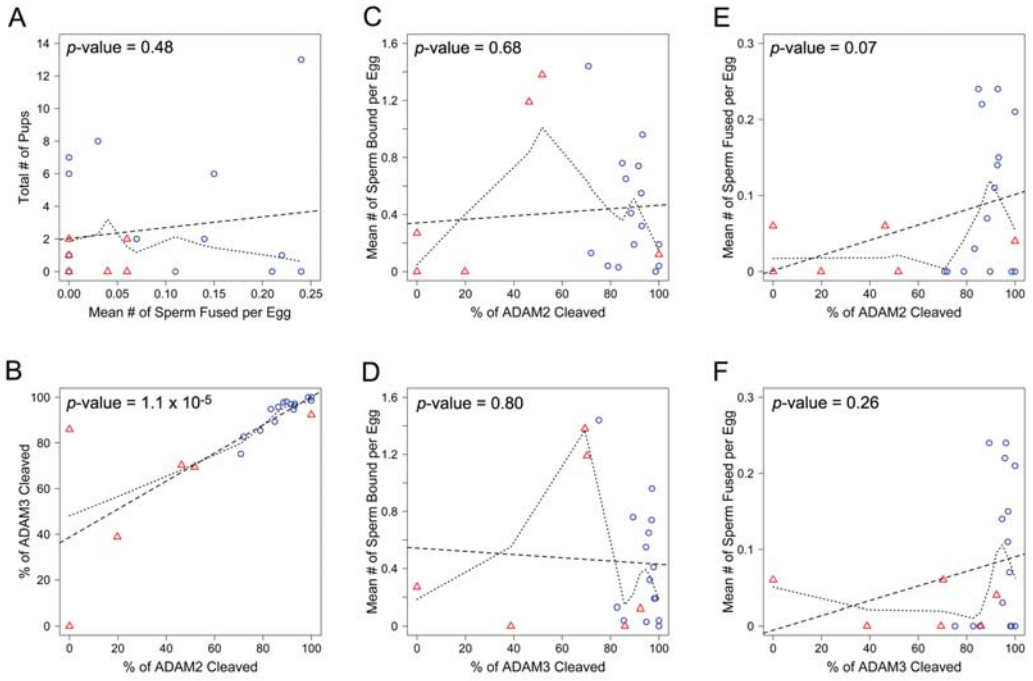
| Cor. Coef. / p-value | Pups | Litters | Pups per Litter | Sperm Bound | Sperm Fused | % of Fert. Eggs | ADAM2 Cleavage | ADAM3 Cleavage |
|---|---|---|---|---|---|---|---|---|
| Pups | | 0.96* | 0.81* | 0.20 | 0.02 | 0.05 | 0.24 | 0.22 |
| Litters | $3.1x10^{-7}$* | | 0.64 | 0.28 | 0.11 | 0.05 | 0.15 | 0.17 |
| Pups per Litter | 0.01* | 0.07 | | 0.05 | 0.03 | 0.05 | 0.61 | 0.53 |
| Sperm Bound | 0.52 | 0.36 | 0.89 | | 0.24 | 0.03 | 0.32 | 0.44 |
| Sperm Fused | 0.95 | 0.72 | 0.94 | 0.39 | | 0.35 | 0.32 | 0.30 |
| % of Fert. Eggs | 0.88 | 0.86 | 0.90 | 0.93 | 0.20 | | 0.01 | 0.24 |
| ADAM2 Cleavage | 0.44 | 0.62 | 0.08 | 0.24 | 0.25 | 0.96 | | 0.91* |
| ADAM3 Cleavage | 0.48 | 0.59 | 0.14 | 0.10 | 0.28 | 0.39 | $3.0x10^{-6}$* | |

**Figure 4** Pair-wise correlation analysis of male reproductive function parameters in 129S6 *Inpp5b*$^{-/-}$ mice. Representative graphs (**A**–**F**) and full analysis (**G**) of putative correlations between various endpoints of male reproductive function, presented in bivariate scatter plots, with a linear fit line (dashed line) and Lowess curve (dotted line), showing selected variables for each 129S6 *Inpp5b*$^{-/-}$ mouse tested. The data shown here from IVF assays (sperm bound, sperm fused and % of fertilized eggs) come from the experiments done with the low sperm concentration range (13 000–30 000 sperm/ml). (A–F) Each open circle shows a data point for the two indicated parameters for an individual mouse. (A) Number of total pups versus mean number of sperm fused per egg. (B) Percentage of cleaved ADAM3 versus percentage of cleaved ADAM2. (C) Mean number of sperm bound per egg versus the percentage of cleaved ADAM2. (D) Mean number of sperm bound per egg versus the percentage of cleaved ADAM3. (E) Mean number of sperm fused per egg versus the percentage of cleaved ADAM2. (F) Mean number of sperm fused per egg versus the percentage of cleaved ADAM3. (G) Pair-wise comparisons, with correlation coefficients in the upper boxes and *P*-values in the lower boxes, for all variables. The paired endpoints that showed a correlation are indicated with an asterisk.

as compared with sperm from the *Inpp5b*$^{+/-}$ and *Inpp5b*$^{+/+}$ males (Fig. 3A–F and Supplemental Fig. S1A–F). This led us to speculate that there could abnormalities with the sperm protein IZUMO1 (also called simply Izumo) in 129S6 *Inpp5b*-null males, as the absence of IZUMO1 [i.e. in knockout mice (Inoue *et al.*, 2005)] or abnormalities in the redistribution of IZUMO1 during acrosome exocytosis (Sosnik *et al.*, 2009) are associated a loss in the sperm's ability to undergo sperm–egg fusion and to fertilize eggs. Immunofluorescence with an anti-IZUMO1 antibody was used to examine IZUMO1 localization in acrosome-intact and spontaneously acrosome-reacted sperm from 129S6 *Inpp5b*$^{+/-}$ and *Inpp5b*$^{-/-}$ mice (Fig. 6). We found that IZUMO1 was present in sperm from 129S6 *Inpp5b*$^{+/-}$ and *Inpp5b*$^{-/-}$ males and underwent normal redistribution upon acrosome exocytosis (Fig. 6; Okabe *et al.*, 1987; Miranda *et al.*, 2009; Sosnik *et al.*, 2009).

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| Cor. Coef. / p-value | Pups | Litters | Pups per Litter | Sperm Bound | Sperm Fused | % of Fert. Eggs | ADAM2 Cleavage | ADAM3 Cleavage |
|---|---|---|---|---|---|---|---|---|
| Pups | | 0.96* | 0.82* | 0.12 | 0.17 | 0.09 | 0.33 | 0.27 |
| Litters | $4.0\times10^{-11}$* | | 0.66* | 0.18 | 0.09 | 0.03 | 0.30 | 0.28 |
| Pups per Litter | $1.2\times10^{-3}$* | 0.02* | | 0.12 | 0.17 | 0.05 | 0.47 | 0.23 |
| Sperm Bound | 0.61 | 0.45 | 0.71 | | 0.19 | 0.13 | 0.09 | 0.06 |
| Sperm Fused | 0.48 | 0.69 | 0.60 | 0.40 | | 0.29 | 0.39 | 0.26 |
| % of Fert. Eggs | 0.97 | 0.92 | 0.88 | 0.58 | 0.20 | | 0.016 | 0.25 |
| ADAM2 Cleavage | 0.15 | 0.20 | 0.13 | 0.68 | 0.07 | 0.94 | | 0.81* |
| ADAM3 Cleavage | 0.26 | 0.24 | 0.50 | 0.80 | 0.26 | 0.28 | $1.1\times10^{-5}$* | |

**Figure 5** Pair-wise correlation analysis of male reproductive function parameters in 129S6 and FVBS6 *Inpp5b*$^{-/-}$ mice. Representative graphs (**A**–**F**) and full analysis (**G**) of putative correlations between various endpoints of male reproductive function, presented in bivariate scatter plots, with a linear fit line (dashed line) and Lowess curve (dotted line), showing selected variables for each FVBS6 *Inpp5b*$^{-/-}$ mouse (red triangles, $n = 6$) or 129S6 *Inpp5b*$^{-/-}$ mouse (blue circles, $n = 15$) tested. The data shown here from IVF assays (sperm bound, sperm fused and % of fertilized eggs) come from the experiments done with the low sperm concentration range (13 000–30 000 sperm/ml). (A–F) Each open circle shows a data point for the two indicated parameters for an individual mouse. (A) Number of total pups versus mean number of sperm fused per egg. (B) Percentage of cleaved ADAM3 versus percentage of cleaved ADAM2. (C) Mean number of sperm bound per egg versus the percentage of cleaved ADAM2. (D) Mean number of sperm bound per egg versus the percentage of cleaved ADAM3. (E) Mean number of sperm fused per egg versus the percentage of cleaved ADAM2. (F) Mean number of sperm fused per egg versus the percentage of cleaved ADAM3. (G) Pair-wise comparisons, with correlation coefficients in the upper boxes and *P*-values in the lower boxes, for all variables. The paired endpoints that showed a correlation are indicated with an asterisk.

# Discussion

This study was initiated with the plan to use *Inpp5b*$^{-/-}$ mice to examine ADAM cleavage as part of epididymal function and the relationship of this component of reproductive tract function to sperm function and male fertility. The putative mechanism that we speculated would connect these parameters was reduced ADAM cleavage correlating with reduced sperm–egg interaction, which in turn would correlate with reduced fertility. However, in the course of this research we uncovered differences in the overall reproductive phenotype from what was in the past reports (Hellsten *et al.*, 2001, 2002).

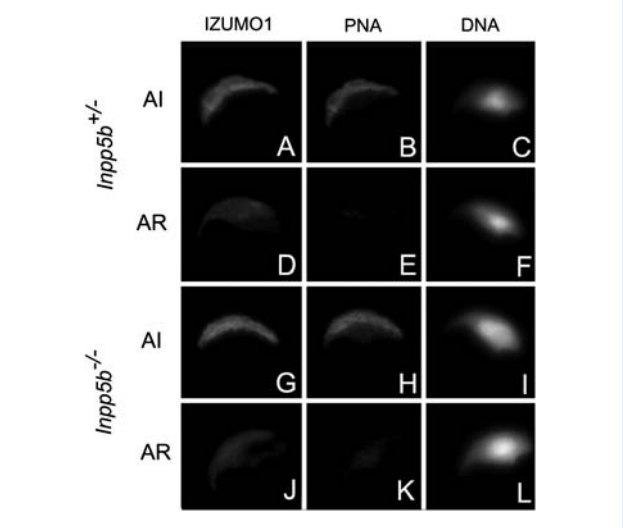


**Figure 6** IZUMO1 localization in sperm from 129S6 *Inpp5b*$^{+/-}$ and *Inpp5b*$^{-/-}$ mice. Indirect immunofluorescence analysis of IZUMO1 in sperm from 129S6 *Inpp5b*$^{+/-}$ mice (**A**–**F**) and *Inpp5b*$^{-/-}$ mice (**G**–**L**). Images show the sperm head, with labeling with an anti-Izumo antibody (A, D, G, J), peanut agglutinin (PNA) to label the acrosome (B, E, H, K) and DAPI to label DNA (C, F, I, L). Both acrosome-intact sperm (AI; A–C, G–I) and acrosome-reacted (AR, D–F, J–K) are shown. IZUMO1 protein is appropriately localized in sperm from heterozygote and knockout animals.

The first difference was in overall fertility, although a likely explanation of this that the methods used to assess fertility were very different. In the previous studies, *Inpp5b*-null males were housed with at least one female for a period of 2–9 months; this revealed significantly impaired fertility of 129S6 *Inpp5b*$^{-/-}$ males [6% (1/17) males sired a litter], and moderately compromised fertility of FVBS6 *Inpp5b*$^{-/-}$ males [45% (9/20) sired a litter] (Hellsten *et al*., 2001, 2002). In the work here, fertility was assessed in more rigorous mating trial experiments; each male was housed with a total four females over 6–8 weeks (two females for 3–4 weeks, then two new females for a second 3–4 week period). These mating trials identified 67% (10/15) of 129S6 *Inpp5b*$^{-/-}$ males and 21% (3/14) FVBS6 *Inpp5b*$^{-/-}$ males were able to sire a litter.

One speculation that was put forward previously was that there was a genetic modifier responsible for what appeared to be rescue of the male infertility phenotype in the FVBS6 *Inpp5b*$^{-/-}$ mice (Hellsten *et al*., 2001). Indeed, one rationale for this study was to characterize the phenotype in more detail as a springboard to progressing to pursue this genetic modifier, but then our mating trials here indicated that the 129S6 *Inpp5b*$^{-/-}$ males were not as infertile as expected and the FVBS6 were not as fertile as expected compared with past work. We also observed variability between the 129S6 and FVBS6 strains in male reproductive functions. There is extensive evidence for strain-to-strain variability in reproductive functions, including performance in IVF (e.g. Fraser and Drury, 1976; Parkening and Chang, 1976; Byers *et al*., 2006; Vasudevan *et al*., 2010). In our studies, *Inpp5b*$^{+/-}$ and *Inpp5b*$^{-/-}$ FVBS6 animals' sperm performed poorly in IVF as compared with 129S6 sperm. Thus, the low sperm binding and fusion in the FVBS6 *Inpp5b*$^{-/-}$ may be due to the deletion of *Inpp5b*, but may also be affected by the genetic background as the FVBS6 heterozygotes also showed significantly reduced sperm binding and fusion as compared with the 129S6 heterozygotes. Nevertheless, the findings that some *Inpp5b*$^{-/-}$ males were not completely infertile and that there was noteworthy male-to-male variability prompted us to take advantage of the ranges of severity of the reproductive performance parameters for each individual male in this population. Therefore, we examined if there were correlations between reproductive function on a physiological level (performance of males in mating trials), a cellular level (performance of sperm in IVF assays with zona pellucida-free eggs) and a molecular level (cleavage of sperm ADAMs during epididymal transit).

A second difference that we observed was in ADAM cleavage and its putative association with fertility. Previous work indicated that ADAM2 was only partially processed in infertile 126S6 and FVBS6 *Inpp5b*$^{-/-}$ males, and was fully processed in the FVBS6 *Inpp5b*$^{-/-}$ males that were fertile (Hellsten *et al*., 2001). We were very interested in ADAM2 and ADAM3 cleavage because (i) mice deficient in *Adam2* or *Adam3* exhibit multi-faceted male infertility phenotypes, including reduced ability of sperm to interact with the egg plasma membrane (Cho *et al*., 2000; Nishimura *et al*., 2001), and (ii) the proteolytic cleavage of ADAM2 in guinea pig sperm has been linked with the movement of ADAM2 on the sperm head during epididymal maturation, which in turn is speculated to be important for the functionality of this protein (Lum and Blobel, 1997). However, our examinations of 129S6 and FVBS6 *Inpp5b*$^{-/-}$ animals for ADAM2 and ADAM3 cleavage and performance in mating trials revealed that there was not a significant correlation of cleavage of either ADAM with the mating trial endpoints (*P*-values, depending on the exact mating trial endpoint, ranged from 0.13 to 0.50; Fig. 5G). One difference between the past work (Hellsten *et al*., 2001) and our study is we quantified the extent of ADAM cleavage and found this was highly variable from male to male; it could be that range of ADAM cleavage went underappreciated. In 129S6 *Inpp5b*$^{-/-}$ mice, the majority of ADAM2 and ADAM3 was cleaved to the mature form, with 71–100% of ADAM2 cleaved and 75–100% of ADAM3 cleaved. ADAM cleavage was more adversely affected in the FVBS6 *Inpp5b*$^{-/-}$ mice, although it ranged from 0% of ADAM2 or ADAM3 cleaved to 100% cleaved, depending on the animal. Some of these males had very low levels of ADAM cleavage (Fig. 2 and Supplemental Fig. S4), but also one FVBS6 *Inpp5b*$^{-/-}$ mouse had normal ADAM cleavage and failed to sire any pups in the mating trial experiment, and three of these FVBS6 *Inpp5b*$^{-/-}$ mice had severely impaired normal ADAM cleavage and did sire pups (Fig. 2A and B). Thus, there is significant male-to-male variability in the extent of ADAM cleavage, and there is a lack of clear correlation of ADAM2 or ADAM3 cleavage with male fertility (*P*-values 0.46–0.65; Fig. 2A–D). Nevertheless, this study makes the *Inpp5b*-null mouse model one of the best-characterized cases of sperm with defects in epididymal ADAM cleavage. To our knowledge, only one other knockout model, the Niemann–Pick C1-deficient mouse, has been reported to have reduced epididymal cleavage of a sperm ADAM (Fan *et al*., 2006), although this was apparently only examined in one mouse. On the basis of the findings here, it is valuable to examine multiple animals. Prohormone proprotein convertase subtilisin/kexin-like 4 (PCSK4, also known as PC4) has been suggested to be a protease that

mediates ADAM2 processing (Basak *et al*., 2004), but epididymal cleavage of sperm ADAM2 in *Pcsk4*$^{-/-}$ mice appears to be normal (Gyamera-Acheampong *et al*., 2009).

These studies of male reproductive function in *Inpp5b*$^{-/-}$ mice included analysis of sperm–egg binding and fusion assessed in IVF assays, and also extended our pair-wise correlation analyses to examine if there was a correlation between the numerous endpoints of male reproductive function that were assessed here. There was male-to-male variability between the *Inpp5b*$^{-/-}$ mice in all of these assays. The number of pups was correlated to both the number of litters and the number of pups per litter; these would be expected to be correlated, and even could be viewed as positive controls. The proteolytic cleavage of ADAM2 and of ADAM3 was positively correlated, suggesting that these proteins could be processed by related or identical mechanisms. Otherwise, there were very few correlations detected in our statistical analyses. For the subset of 129S6 *Inpp5b*$^{-/-}$ males that had sired pups (i.e. omitting the five males that sired no pups), there were statistically significant correlations between the extent of ADAM2 and ADAM3 cleavage and the number of pups per litter (Fig. 2E and F). However, we interpret this cautiously, and do not necessarily take this as strong evidence connecting ADAM cleavage with fertility or the ability of sperm to fertilize eggs. Although the association between ADAM2 and ADAM3 cleavage and the number of pups per litter in the *Inpp5b*$^{-/-}$ 129S6 mice is statistically significant, it is difficult to know if this will extend to biological significance. It seems likely that if this had biological significance, we would have observed a correlation of ADAM cleavage in the analyses with all the males, including the infertile ones (i.e. ADAM cleavage compared with total number of pups or litters), although additional confounding factors with the infertile males cannot be ruled out.

Despite the lack of correlation between the molecular (ADAM cleavage), cellular (sperm–egg binding and fusion) and physiological (mating trial) endpoints assessed here, there nevertheless are some important discoveries here with regard to the phenotype of the *Inpp5b*$^{-/-}$ mice. The *Inpp5b*-null males assessed here that did sire offspring were subfertile, having significantly fewer offspring than wild-type or heterozygote controls. Even though the number of fertile 129S6 *Inpp5b*$^{-/-}$ males was higher than in the original study [1/17 (Hellsten *et al*., 2001) as compared with 10/15 (Table I), $P = 0.001$, $\chi^2$], it is clear that both FVBS6 and 129S6 *Inpp5b*$^{-/-}$ male mice have a defect in the ability to reproduce. This work also advances our understanding of the defects of *Inpp5b*$^{-/-}$ sperm function. Past work showed that inseminations of zona pellucida-free eggs had delays in and reduced incidence of second polar body emission (Hellsten *et al*., 2001), but it was unknown if this was due to defects in sperm binding, sperm fusion and/or the sperm inducing egg activation. We show here that sperm from *Inpp5b*$^{-/-}$ mice show reduced levels of sperm–egg binding and fusion; the reduction in sperm–egg fusion is most likely a result of the decrease in sperm binding to the egg.

Although these data do not provide strong evidence that in this *Inpp5b*-null model there is a connection between ADAM cleavage and sperm–egg interaction and/or male fertility, the data do not necessarily rule out such a connection either. It is crucial to note that the reduction of ADAM cleavage observed here was fairly modest in most of the *Inpp5b*$^{-/-}$ males examined (especially 129S6), and thus sperm from these *Inpp5b*$^{-/-}$ animals would have a significant amount of properly cleaved ADAM2 and ADAM3. It is possible that the deficiency in ADAM2/ADAM3 cleavage in these mice was not sufficiently extensive to cause a defect in sperm function (e.g. decreased sperm binding), either because cleavage was only partial and/or because other molecules on the sperm are sufficient to support interaction with the egg. One candidate for this is the sperm protein IZUMO1, which is essential for sperm–egg fusion in mice (Inoue *et al*., 2005). However, IZUMO1 is not overtly affected in 129S6 *Inpp5b*$^{-/-}$ males, as IZUMO1 is present and localized properly in both acrosome-intact and acrosome-reacted *Inpp5b*-null sperm. It should be noted, however, that the effects of *Inpp5b* deficiency do differ from those of *Izumo1* deficiency or another knockout, *Tssk6*, which also shows defects in sperm–egg fusion and has an abnormality in the localization of IZUMO1 (Sosnik *et al*., 2009). The main defect in sperm from *Inpp5b*$^{-/-}$ mice is a reduced ability to bind to the egg membrane, in contrast to the normal binding observed with *Izumo1*$^{-/-}$ sperm (Inoue *et al*., 2005). Sperm from *Izumo1*$^{-/-}$ males and *Tssk6* males are completely unable to fuse with eggs (Inoue *et al*., 2005; Sosnik *et al*., 2009), whereas sperm from *Inpp5b* males are able to undergo sperm–egg fusion to some extent. Finally, an additional contributor to sperm function may be sperm surface protein complexes and membrane order. It has been reported that ADAMs and IZUMO1 are in complexes with other proteins and that ADAM deletion or IZUMO1 deletion can alter the sperm surface proteome (Ellerman *et al*., 2009; Han *et al*., 2009). This raises the possibility that although *Inpp5b*-null sperm have generally moderate defects in ADAM cleavage and appear to have normal IZUMO1, there more be more subtle factors at work that perturb normal sperm membrane order and thus impair normal sperm function.

In summary, this work demonstrates that mice deficient in *Inpp5b*$^{-/-}$ have reduced fertility and severe reductions in sperm–egg binding and fusion. Although nuances in the phenotypes revealed here suggest that *Inpp5b*$^{-/-}$ mice are a complex model for investigating ADAM2 and ADAM3 cleavage during epididymal maturation, the *Inpp5b*-null mice are still an interesting model to advance understanding of potential causes of male infertility. One important aspect for the future of reproductive medicine is to progress from observing a physiological phenotype, such as infertility or subfertility, to associating this physiological dysfunction with a cellular or molecular dysfunction. Some cases can be reasonably straightforward, such as in the case of mice lacking *Izumo1* produce sperm that appear to be normal in all ways except for their inability to fuse with the egg plasma membrane (Inoue *et al*., 2005), or mouse knockouts that are infertile as a result of azoospermia caused by spermatogenic failures (Matzuk and Lamb, 2002; Yatsenko *et al*., 2009). Analyses such as the work here show that other cases are more complex, particularly when the gene knockout does not produce the exact same identical phenotype in every single animal. These models are still worthy of study since, as we have noted previously (Vjugina and Evans, 2008; Vjugina *et al*., 2009), a gamete function deficiency that has modest effects in the mouse could translate to a significant effect in the human, and thus these cases of subfertility in mouse knockout models can prove to be very significant for reproductive success in humans. Moreover, multivariate analyses such as described here have the potential to be highly informative for the study of complex cases requiring appreciation of subfertility to infertility, for handling analysis of animal-to-animal variability, and to examine the

relationships between molecular characteristics and *in vitro* and *in vivo* physiological functions, all which could be broadly useful for studies of knockout mice.

## Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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