A polycystin-1 controls postcopulatory reproductive selection in mice

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***Pkdrej*, a member of the polycystin-1 gene family, is expressed only in the male germ line. Male mice that are homozygous for a targeted mutation in the *Pkdrej* allele (*Pkdrej*$^{tm/tm}$) are fertile in unrestricted mating trials, but exhibit lower reproductive success when competing with wild-type males in sequential mating trials and in artificial insemination of mixed-sperm populations. Following mating, sperm from *Pkdrej*$^{tm/tm}$ mice require >2 h longer than those of wild-type males to be detected within the egg/cumulus complex in the oviduct. Sperm from mice of both genotypes are able to capacitate *in vitro*. However, one of the component processes of capacitation, the ability to undergo a zona pellucida–evoked acrosome reaction, develops more slowly in sperm from *Pkdrej*$^{tm/tm}$ animals than in sperm from wild-type males. In contrast, a second component process of capacitation, the transition to hyperactivated flagellar motility, develops with a similar time course in both genotypes. These two behavioral consequences of capacitation, exocytotic competence and altered motility, are therefore differentially regulated. These data suggest that *Pkdrej* controls the timing of fertilization *in vivo* through effects on sperm transport and exocytotic competence and is a factor in postcopulatory sexual selection.**

capacitation | evolution | fertilization | polycystin | sexual selection

Initial interest in the polycystin-1 proteins followed the realization that mutations of *Pkd1* were associated with ≈85% of cases of autosomal dominant polycystic kidney disease (1, 2). It is now recognized that there is a family of polycystin-1 proteins that includes Pkd1 as well as Pkd1l1, Pkd1l2, Pk1l3, and Pkdrej and that these are found in tissues outside the kidney. These proteins share a domain architecture that includes a GPS proteolytic cleavage site in the extracellular N terminus and a PLAT/LH2 lipase/lipoxygenase motif in the first intracellular loop. Other common features include 11 transmembrane domains, an intracellular C terminus, and, in most members of this family, a REJ domain in the extracellular N-terminal region (3, 4). Functional studies indicate that polycystin-1 proteins are components of mechanosensory or chemosensory signal transduction mechanisms (4, 5). In the renal tubule epithelium Pkd1 is located in the primary cilium and acts as a fluid flow sensor (6, 7), whereas Pkd1l3 is associated with sour taste transduction (8, 9). In contrast, the functions of other family members are poorly understood. In the case of *Pkdrej*, hints are provided by observations that the expression of this gene has been detected only in the mammalian male germ lineage and the protein is present in the anterior sperm head (10, 11). In addition, the Pkdrej protein sequence is evolving rapidly and exhibits positive selection (12), characteristics that are shared with many reproductive proteins (13). These observations suggest a role for Pkdrej in sperm function.

Sperm of many animal species must complete a secretory event, or acrosome reaction, to penetrate the egg coat and fuse with the egg plasma membrane (14–16). The sea urchin acrosome reaction is triggered as sperm contact the jelly coat surrounding the egg by fucose sulfate polymer, a polysaccharide component of egg jelly (17). suREJ1, a polycystin-1-related protein in sea urchin sperm, binds fucose sulfate polymer and is a candidate subunit of the receptor that initiates acrosome reactions (18, 19). Additional polycystin-1 proteins are also present and may participate in the signal transduction mechanism that leads to exocytosis (19, 20). An acrosome reaction is also required for mammalian fertilization. In this case, sperm first undergo a process of physiological reprogramming, or capacitation, within the female reproductive tract (16, 21). Acrosome reactions are triggered following contact of capacitated sperm with the egg coat, or zona pellucida (ZP), by the agonist glycoprotein ZP3 (16, 22). Given the proposed role of polycystin-1 proteins in echinoderm acrosome reactions, it was suggested that Pkdrej is a candidate component of a ZP3-activated signaling pathway that drives mammalian sperm acrosome reactions (12). This hypothesis is consistent with the localization of Pkdrej in the acrosomal region of the sperm head (11), where sperm interact with the ZP (14), and with the demonstration that Pkdrej can account for some elements of ZP3 signal transduction (23). However, the function of Pkdrej has not been determined.

Here, we analyzed the reproductive phenotype of male mice that are homozygous for a targeted mutation in the Pkdrej allele (*Pkdrej*$^{tm/tm}$). These studies suggest that Pkdrej is not required for the ZP-evoked acrosome reaction but, rather, controls the preliminary processes of capacitation by which sperm develop fertility as well as sperm access to the egg/cumulus complex.

Results

The Pkdrej allele was disrupted by replacement of the first six transmembrane domains by an internal ribosome entry site–LacZ/neomycin-resistance cassette. Mice that were homozygous for this targeted mutation did not express full-length *Pkdrej* mRNA in testis, as demonstrated by Northern blotting, quantitative RT-PCR (Fig. 1*A* and *B*), and gene chip array analysis (data not shown). Disruption of the *Pkdrej* locus did not result in compensatory overexpression of other polycystin-1 family genes in the testis, as compared with wild-type (*Pkdrej*$^{+/+}$) littermates (Fig. 1*B*).

Pkdrej$^{tm/tm}$ mice were viable and initial observations did not reveal a male reproductive phenotype. On reaching sexual maturity, homozygous mutant mice and wild-type littermates were similar with regard to testis weight, the numbers of sperm recovered from cauda epididymis, sperm morphology as indicated by light microscopic analysis, and levels of spontaneous acrosome reaction immediately after release from cauda epididymis (Table 1). Similar fractions of the population from each genotype activated flagellar motility in culture medium, and there were no significant differences in movement characteristics

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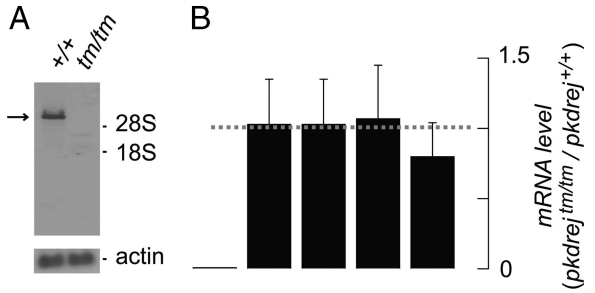

Fig. 1. Disruption of the *Pkdrej* gene. The region encoding transmembrane domains 1–6 of this intronless gene were replaced with an IRES-LacZ/neomycin-resistance cassette. (*A*) Northern blot analysis of mouse testis polyA$^+$-RNA. (*Upper*) A single band was seen in RNA from *Pkdrej*$^{+/+}$ (+/+), corresponding to the size of the *Pkdrej* transcript (arrow), and was absent in RNA from *Pkdrej*$^{tm/tm}$ (*tm/tm*) mice. (*Lower*) Loading control, *actin*. (*B*) Quantitative RT-PCR analysis of mouse testis RNA. Levels of polycystin-1 transcripts were determined in RNA from mice with either the wild-type (+/+) or homozygous mutant (*tm/tm*) *Pkdrej* allele. Data for each polycystin-1 are expressed as a ratio of the value in *Pkdrej*$^{tm/tm}$ testis relative to that in *Pkdrej*$^{+/+}$ (mean ± SD). Values for *Pkdrej* represent the lower limit of detection for this system. Dashed line represents a ratio of 1.0, indicating no difference in transcript level as a result of mutation of the *Pkdrej* allele. *Pkdrej* mutation resulted in a significant reduction of *Pkdrej* transcripts ($P < 0.05$), whereas no other polycystin-1 transcripts were affected ($P > 0.1$).

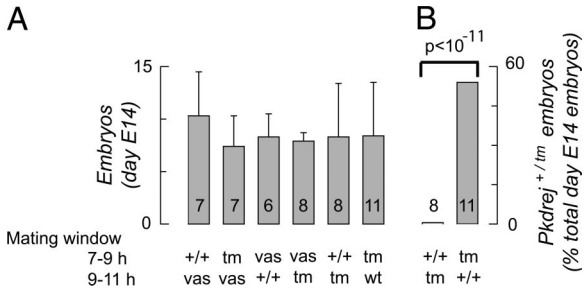

Fig. 2. *Pkdrej* regulates sperm competitiveness *in vivo* in sequential mating experiments. (*A*) Fertility of *Pkdrej*$^{+/+}$ and *Pkdrej*$^{tm/tm}$ mice *in vivo* during sequential mating studies. Litter sizes were not significantly different. (*B*) *Pkdrej*$^{+/+}$ males produced all embryos when provided access to females during the first mating window. However, when *Pkdrej*$^{tm/tm}$ males were present during the first mating window, they fertilized only 54% of eggs (49 of 91), and wild-type males still competed efficiently. *Inset* in bars indicate number of litters. Data were analyzed by χ^2 test ($P < 10^{-11}$). vas, vasectomized; +/+, sperm from *Pkdrej*$^{+/+}$ mice; tm, sperm from *pkdrej*$^{tm/tm}$ mice.

of uncapacitated sperm as assessed by computer-assisted motion analysis (Table 1). Moreover, when males were housed continuously with wild-type females for 24–72 h, there were no differences between wild-type and homozygous mutant males in the sizes of litters sired (Table 1).

This unrestricted breeding protocol permits multiple intromissions by a single male and provides optimal conditions for reproduction. In contrast, in natural populations outside the laboratory, female mice are polyandrous and mate with multiple males during an estrus cycle (24–26). A second breeding protocol was used to mimic these aspects of mating behavior. Ovulation time was synchronized by gonadotropin injection, resulting in egg/cumulus complexes first being detected in the oviducts at 10.5 h after human chorionic gonadotropin (hCG) injection and reaching maximal values at 11.5 h after hCG. Males were given access to wild-type females for 2-h time windows, either 7–9 h or 9–11 h after hCG. Mouse sperm require a mean time of 1 h to reach the site of fertilization in the ampulla of the oviduct (27). Thus, mating during the first time window resulted in sperm that arrived in the oviduct before the entry of eggs, whereas in the second window, these events occurred at approximately the same time. Control experiments, in which females were mated with males of only a single genotype, showed that the fertility of *Pkdrej*$^{+/+}$ and *Pkdrej*$^{tm/tm}$ males was similar, as assessed by litter size, irrespective of the time window used (Fig. 2*A*).

Table 1. Reproductive phenotype of *pkdrej*$^{+/+}$ and *pkdrej*$^{tm/tm}$ mice

Parameter	Phenotype	
	pkdrej$^{+/+}$	*pkdrej*$^{tm/tm}$
Testis weight, g	0.18 ± 0.03 (10)	0.18 ± 0.02 (10)
Sperm number (× 10^7)	1.0 ± 0.2 (12)	1.2 ± 0.6 (12)
Sperm motility		
Motile, %	71 ± 7 (6)	69 ± 6 (6)
Curvilinear velocity, μm/sec	165.8 ± 11.2 (6)	169.1 ± 19.9 (6)
Linearity, %	39.2 ± 3.8 (6)	38.3 ± 2.1 (6)
Straightness, %	62.8 ± 5.1 (6)	62.3 ± 1.6 (6)
Spontaneous acrosome reaction, %	6.0 ± 4.3 (6)	3.6 ± 2.7 (6)
Fertility, no. in litter	8.8 + 1.8 (36)	7.1 + 2.0 (36)

Data represent means ± SD, based on the number of observations (shown in parentheses). Sperm were extruded from the cauda epididymis and used for total cell count, motility analysis, and determination of spontaneous acrosome reaction levels. Motility analysis was carried out by using computer-assisted sperm analysis. Values for all parameters did not differ significantly between phenotypes ($P > 0.1$, Student *t* test).

This sequential mating protocol revealed that mutation of *Pkdrej* resulted in a fertility defect (Fig. 2*B*). When wild-type males were provided access to females during the first time window, and homozygous mutant males were introduced during the second period, all embryos were *Pkdrej*$^{+/+}$, as assessed by genotyping day E14 embryos, and homozygous mutant sperm introduced during the second time window failed to generate embryos (0 of 66, 0%). However, homozygous mutant males were not similarly successful when presented during the first mating window. If *Pkdrej*$^{tm/tm}$ males were allowed initial access and *Pkdrej*$^{+/+}$ males secondary contact, we observed that only 54% of the embryos were *Pkdrej*$^{+/tm}$, and so were derived from eggs fertilized by the homozygous mutant males (49 of 91) and the wild-type sperm present only during the second time window nevertheless were able to produce approximately one-half of the embryos ($P < 10^{-11}$ by χ^2 test; Fig. 2*B*). These differences are due to sperm and cannot be attributed to genotype-dependent effects on male reproductive tract fluids, as shown in control experiments with vasectomized males (Fig. 2*A*).

These results suggest that a functional Pkdrej protein provides sperm with a fertility advantage when sperm from two males are present within the female reproductive tract simultaneously. This hypothesis was tested directly in sperm competition experiments *in vivo*. Sperm were collected from the cauda epididymis and introduced into wild-type females by artificial insemination. First, preliminary experiments found that fertilization, as assessed by the presence of cleavage-stage embryos (≥2-cell stage) at 40 h after insemination, was proportional to the sperm number inseminated (Fig. 3*A*). This was anticipated from previous studies (28). Mice typically deposit >10^7 sperm into the female reproductive tract during natural mating (29), and, at those high sperm numbers, there is no fertility defect associated with mutation of the *Pkdre* locus (Table 1). Yet, sperm from homozygous mutant mice are subfertile when lower numbers of sperm are used (Fig. 3*A*).

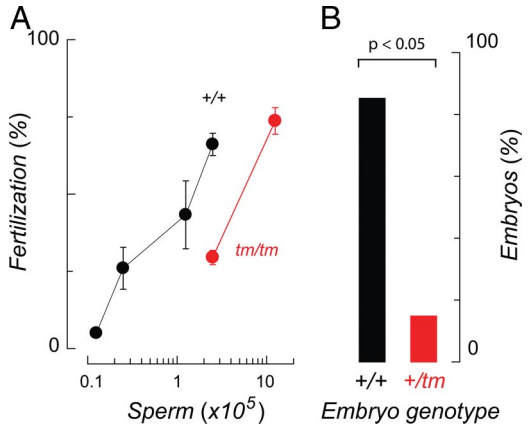

Fig. 3. *Pkdrej* regulates sperm competitiveness *in vivo* in artificial-insemination experiments. (*A*) The proportion of eggs fertilized *in vivo* after artificial insemination with sperm of a single genotype (*Pkdrej*$^{+/+}$, black circles; *Pkdrej*$^{tm/tm}$, red circles) was proportional to the insemination dose. (*B*) Females were artificially inseminated with mixed populations containing 2.5×10^5 sperm from wild-type males and 2.5×10^5 sperm from homozygous mutant males. The embryo genotype (*Pkdrej*$^{+/+}$, black bar; *Pkdrej*$^{+/tm}$, red bar) differed significantly from that expected based on fertility of unmixed populations ($P < 0.05$).

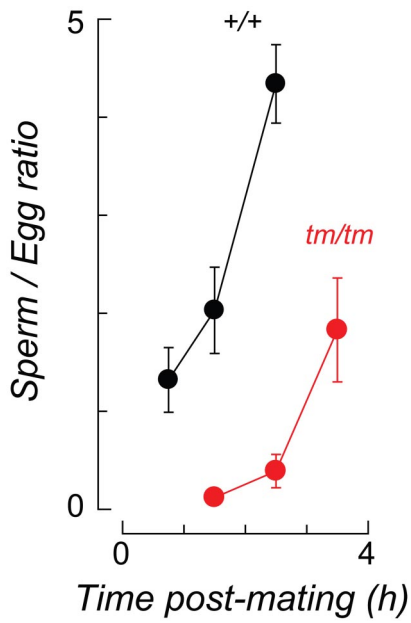

Fig. 4. *Pkdrej* regulates the time course of sperm entry into the egg–cumulus complex *in vivo*. Cumulus–egg complexes were removed at the indicated time after a 2-h mating time window and sperm within the cumulus were counted (*Pkdrej*$^{+/+}$, +/+, black circles; *Pkdrej*$^{tm/tm}$, *tm/tm*, red circles). Data represent the mean (+SD) of three to six separate experiments. Values at 1.5 and 2.5 h differed significantly between genotypes ($P < 0.05$).

Next, *in vivo* competition studies were performed by coinseminating 250,000 sperm from each genotype. In these experiments, successful fertilization was assessed by the genotype of the implanted embryo at 14 days after insemination. Based on single genotype studies in the absence of competition, it was expected that 66% of the embryos would have been *Pkdrej*$^{+/+}$ and so would have been fertilized by wild-type sperm (Fig. 3*A*). Yet, in competition experiments, 85% of the embryos were *Pkdrej*$^{+/+}$, whereas only 15% were heterozygous and so were fertilized by sperm from *Pkdrej*$^{tm/tm}$ males (29 of 34 and 5 of 34 total embryos, respectively; $n = 4$; $P < 0.05$ by χ^2 test; Fig. 3*B*). These data are consistent with sequential mating studies and indicate that mutation of the *Pkdrej* locus results in sperm that are fertile but that cannot compete efficiently with those of wild-type males.

We next examined whether these fertility differences were due to a genotype-dependent difference in the arrival of sperm in the vicinity of eggs within the oviduct. Males were housed with wild-type females for a 2 h period (7–9 h after hCG), oviducts were isolated at the indicated time points after the end of this mating period, and the numbers of sperm within the egg/cumulus complex was determined. Sperm from homozygous mutant males were delayed by >2 h, relative to wild-type sperm, in entering the cumulus complex *in vivo* (Fig. 4).

This phenotype was also examined in a series of *in vitro* experiments. The results of *in vivo* studies suggested that wild-type sperm might be able to fertilize eggs more rapidly than those from homozygous mutant males. In this regard, mammalian sperm are released by the male in an infertile state and must be functionally reprogrammed, or capacitated, within the female reproductive tract in order to fertilize (16, 21). Capacitation occurs in a time-dependent fashion and alters sperm behavior in at least two ways: Sperm acquire the ability to undergo a ZP-evoked acrosome reaction (30, 31); and sperm develop hyperactivated motility, a form of asymmetric flagellar bending that is characteristic of sperm found near the site of fertilization *in vivo* (32).

We found that mutation of the *Pkdrej* allele resulted in sperm that acquire competence to undergo ZP-induced acrosomal exocytosis more slowly during incubation under capacitating conditions *in vitro* (Fig. 5*A*). Sperm from wild-type males develop the ability to acrosome react in response to ZP stimulation during a capacitating incubation with a half time of 38 ± 4 min, whereas those from homozygous mutant males require 84 ± 5 min (Fig. 5*A*). There was only a small effect of genotype on the maximal response to ZP after a prolonged capacitation incubation (>2 h), with calculated maximal acrosome reaction responses for *Pkdrej*$^{+/+}$ and *Pkdrej*$^{tm/tm}$ sperm of 58 ± 5% and 64 ± 6%, respectively. This *Pkdrej*-

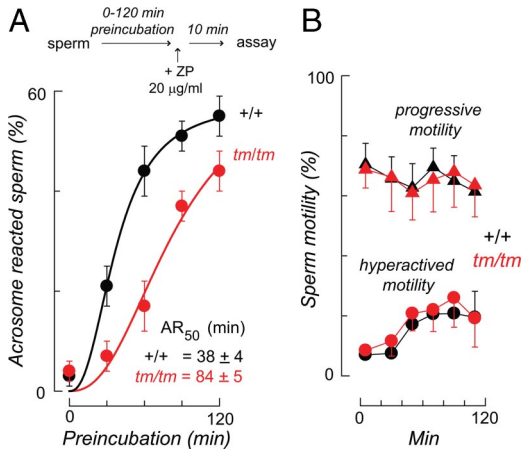

Fig. 5. *Pkdrej* regulates sperm capacitation *in vitro*. (*A*) Mutation of *Pkdrej* slows the rate at which exocytotic competence develops during capacitation. Experimental design is shown above the figure. Data were fit to a single-site binding equation (*Methods*). The half-time for acquisition of competence by sperm from *Pkdrej*$^{+/+}$ mice was 38 ± 4 min and increased to 84 ± 5 min after mutation of *Pkdrej* ($P < 0.05$). Data represent the mean (±SD) of three to five separate experiments, with >200 sperm assessed in each experiment. (*B*) Mutation of *Pkdrej* has no effects on the proportion of motile sperm in a population or on hyperactivation of flagellar motility during capacitation ($P > 0.1$). *Pkdrej*$^{+/+}$ sperm, +/+ (black symbols); *Pkdrej*$^{tm/tm}$ sperm, *tm/tm* (red symbols).

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dependent difference cannot be attributed either to an altered sensitivity of sperm to ZP stimulation, as shown by dose–response relationships [supporting information (SI) Fig. S1*A*], or to differences in the speed of the exocytotic process itself, as shown by time-course experiments (Fig. S1*B*). Thus, Pkdrej is not required directly for the acrosome reaction, but rather controls the timing with which sperm become responsive to signals from the ZP during capacitation.

In contrast, Pkdrej did not regulate the modifications of flagellar motility that are coincident with capacitation. Sperm from both *Pkdrej*$^{+/+}$ and from *Pkdrej*$^{tm/tm}$ males developed hyperactivated flagellar motility with similar time courses and to a similar extent (20%–25% of the population) during capacitation *in vitro* (Fig. 5*B*). Several movement characteristics have been linked to hyperactivation using computer-assisted motion analysis, including track linearity (LIN), velocity along a curvilinear path (VCL), and the amplitude of lateral head displacement (ALH) (33). Pkdrej had no effect on the time-dependent changes in these parameters (Fig. S2), consistent with observations that hyperactivated motility was not affected.

Discussion

Mice that are homozygous for a mutant *Pkdrej* allele are fertile when tested in a standard mating protocol yet are unable to compete efficiently with wild-type males for fertilization of eggs when sperm of both genotypes are present in the female reproductive tract at the same time. This indicates that the unrestricted mating paradigm that is often used to evaluate the fertility of genetic animal models, but which does not mimic natural reproductive behavior of the mouse, may not be adequate to reveal all reproductive phenotypes.

We found that one aspect of the phenotype of homozygous mutant mice is related to the regulation of the ZP-induced acrosome reaction. The relevance of this phenotype is indicated by observations that disruption of ZP-evoked exocytosis *in vitro* is linked to idiopathic male infertility in humans (34). It had been speculated that Pkdrej acts in the reception or transmission of ZP3 signals during the initiation of the mammalian sperm acrosome reaction that follows contact with the ZP (35, 36). However, our results indicate that this protein does not play a direct role in the ZP-induced acrosome reaction but, instead, controls exocytosis through the process of capacitation. Yet, this represents a conserved role of polycystin-1 proteins in the regulation of acrosome reactions between sea urchins, where sperm are spawned in the vicinity of eggs and can undergo the acrosome reaction within seconds (15) and where suREJ1 participates directly in the induction of exocytosis, and in mice, where sperm undergo a protracted period of capacitation to develop the ability to respond to ZP stimuli and where Pkdrej functions indirectly in the regulation of exocytosis as a part of the timing mechanism.

Pkdrej was also linked to a second reproductive phenotype; sperm from homozygous mutant mice required a longer period before being detected within the cumulus matrix surrounding the egg *in vivo*. To access the cumulus, sperm must enter the uterotubal junction region of the oviduct from the uterus, ascend the oviduct to the site of fertilization, and target the egg/cumulus complex (32). Capacitation is completed within the oviduct (14, 32) and controls several of those component steps by which sperm access the cumulus, including exit from the uterotubal junction (14, 32), the chemotactic response of sperm to egg/cumulus-derived attractants (37, 38), and sperm penetration into the cumulus matrix (14, 39). The precise role of Pkdrej in this process is presently the subject of active investigation. However, hints of a role of polycystin-1 proteins in sperm transport are provided by studies in *Drosophila*. Polycystin-1 proteins form a functional complex with polycystin-2 proteins (4). The recognition that a polycystin-2 family member controls oriented motility of fly sperm within the female reproductive tract (40, 41) suggests a role for a polycystin-1 in this process in dipterans and, possibly, of related proteins in mammalian sperm transport.

Given that capacitation controls both the development of exocytotic competence and aspects of sperm transport, the most parsimonious explanation of our data is that Pkdrej modulates the speed at which sperm capacitate. Mouse sperm require ≈1 h to reach the site of fertilization *in vivo* (27) (Fig. 4) and to develop ZP responsiveness in *in vitro* assays (Fig. 5*A*). This is also the minimum estimated time for capacitation *in vivo* (42). Mutation of *Pkdrej* results in a delay in both sperm transport and the acquisition of ZP responses and so account for the advantages of wild-type sperm in sequential mating and competition artificial insemination experiments. These phenotypes point to a role of *Pkdrej* as a chronoregulator of capacitation, with mutations of this locus affecting the timing of sperm behavioral changes rather than a complete failure of those processes.

As noted earlier, capacitation alters the behavioral repertoire of sperm in at least two ways: sperm acquire the ability to undergo ZP-evoked exocytosis, which is essential for sperm penetration of the ZP and for fusion with eggs; and sperm develop hyperactivated motility, which is required for ascent of the oviduct and penetration of the ZP (14, 16, 21). It is not known whether these functional changes were regulated coordinately or independently. Previously, media conditions or pharmacological treatments dissected the development of hyperactivated sperm motility *in vitro* from such component events of capacitation as the increased frequency of spontaneous (that is, ZP agonist-independent) acrosome reaction (43, 44) or the enhanced tyrosine protein phosphorylation that accompanies capacitation (45, 46). Similarly, hyperactivation depends on the function of the Catsper family of cation channels and of Pcma4, the Ca^{2+}-conducting ATPase (47–49). However, the effects of such treatments or gene disruption on ZP-evoked exocytosis were not addressed. Pkdrej controls a rate-limiting step in the development of exocytotic competence but does not modulate the appearance of hyperactivated motility during capacitation, thereby isolating these pathways and demonstrating the distinct regulation of these elements of capacitation. Hence, Pkdrej is not a master regulator of capacitation but rather controls a distinct subset of component pathways.

Finally, a role for Pkdrej was revealed only under conditions where postcopulatory sperm competition operates, that is, when sperm from multiple males are present simultaneously within the female reproductive tract, either as a result of sequential mating protocols designed to mimic the behavior of natural populations, or after artificial insemination of mixed sperm populations. Sexual selection was first formulated with regard to precopulatory processes (50), but it was subsequently recognized that competition and selection continues after copulation in polyandrous situations (51). In this regard, capacitation represents a time delay between insemination and fertilization. As noted previously, the time requirement for the onset of sperm fertility corresponds to the time of sperm arrival in the vicinity of the egg, suggesting that the reproductive process has evolved to coordinate these events. It has been speculated that, under conditions of postcopulatory sperm competition, genes that control the duration of capacitation could provide a selective advantage for paternity and so be targets of positive-selection mechanisms (52, 53). The present study shows that single genetic loci can modulate the timing of capacitation and provides an experimental system for testing the molecular basis for positive selection in mammalian reproduction.

Methods

Animal Experiments. *Pkdrej*$^{tm/tm}$ mice were generated at Deltagen and backcrossed onto a C57BL6 background (The Jackson Laboratory). All data were collected from animals at the F_{13} generation or later, and *Pkdrej*$^{+/+}$ littermates

were used as controls. To synchronize ovulation, *Pkdrej*$^{+/+}$ females were injected with 5 units of pregnant mare serum gonadotrophin, followed 48 h later by 5 units of hCG. In sequential mating experiments, data included only those females that mated, as evidenced by copulatory plugs, with both males.

For artificial insemination experiments, sperm were collected from the cauda epididymis, adjusted to an initial concentration of 10^7 per milliliter in Whitten's medium (54), and further diluted such that the desired numbers of sperm are delivered in 50-μl volumes. In competition experiments, 25 μl (250,000 sperm) each of *Pkdrej*$^{+/+}$ and of *Pkdrej*$^{tm/tm}$ sperm were mixed and coinseminated. ZP were isolated from ovarian homogenates and solubilized as described (54).

Sperm Function Assays. Sperm were recovered from the cauda epididymis, diluted to a concentration of 10^6 per milliliter in Whitten's medium supplemented with $NaHCO_3$ (20 mM) and BSA (10 mg/ml) and incubated (37°C in air) for up to 2 h. This medium supports sperm capacitation *in vitro* (54). Fertilization assays were performed as described (55) to confirm that sperm from both wild-type and homozygous mutant mice capacitate *in vitro*. However, this assay lacks the time resolution necessary to detect differences in capacitation time between sperm from these genotypes.

Hyperactivation was assessed by a trained observer. In addition, sperm movement characteristics were evaluated by using a CEROS Sperm Analyzer (Version 12; Hamilton Thorne, recorded for >45 frames at 60 Hz). CEROS determined the fraction of motile cells as well as movement parameters that are indicators of hyperactivated motility, including linearity of swimming paths (LIN), velocity along a curvilinear path (VCL), and amplitude of lateral head displacement (AHL). Values correlated to hyperactivation of mouse sperm include LIN ≤38, VCL ≥180, and AHL ≥9.5 (56).

Acrosome reactions were assessed after stimulation with soluble extracts of the ZP, as described (57). Separate experiments determined the time course of the development of sperm responsiveness to ZP (Fig. 3*A*), dose–response relationships for induction of acrosome reactions by ZP (Fig. S1*A*), and the time course of the acrosome reaction itself (Fig. S1*B*). Data were fitted to the single-site binding equation (58) $AR_i = AR_{max}/(1 + 10^{(\log AR50 - \chi)} \times n)$. AR_i, AR_{max}, and AR_{50} are the acrosome reaction responses at ZP dose or incubation time point *i*, the maximal response, and the ZP dose or incubation time point giving a half-maximal response, respectively; χ is log of either ZP protein concentration (dose–response experiments, Fig. S1*A*) or incubation time (rate experiments, Fig. 3*A* and Fig. S1*B*); and *n* is the Hill slope, reflecting cooperativity. Data were fit by using SigmaPlot 10.0 (Systat Software).

Molecular Assays. cDNA for quantitative PCR assays were synthesized (QuantiTech Reverse Transcription kit; Qiagen) from testis RNA. Amplification, detection, and quantification were carried out on five *pkdrej*$^{+/+}$ and five *pkdrej*$^{tm/tm}$ males (40 pg RNA per assay, duplicate samples per animal) by using QuantiTech SYBR PCR (Qiagen) performed with an MH Research Opticon real-time PCR machine. The following primer sets were used (cat. nos. *pkdrej*, QT00262458; *pkd1*, QT00158501; *pkd1l1*, QT00280882; *pkd1l2*, QT00141120; *pkd1l3*, QT00169421; *actin*, QT00095242; (Qiagen). Specificity of the probe sets was validated by control reactions lacking reverse transcriptase and by sequencing to confirm primer specificity.

Data Analysis. All data are presented as means (±SD), and differences were evaluated by using a two-tailed Student *t* test, except for sequential mating and artificial insemination studies, which were analyzed by the χ^2 method.

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