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The Acrosomal Protein Dickkopf-Like 1 (DKKL1) Is Not Essential For Fertility

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Abstract

Objective—To determine the role of *Dkkl1* on mouse development, viability and fertility

Design—Prospective experimental study

Setting—Government research institution

Animals—Mice of C57BL/6 and 129X1/SvJ strains, as well as transgenic mice of mixed C57BL/6 and 129X1/SvJ strains were used for the studies.

Intervention(s)—Mice were constructed that lacked a functional *Dkkl1* gene.

Main Outcome Measure(s)—Deletion of the gene was confirmed by DNA, RNA, and protein analyses; *in vivo* fertility was examined by continuous mating scheme.

Result(s)—Previous studies have shown that *Dkkl1*, a gene unique to mammals, is expressed predominantly, if not exclusively, in developing spermatocytes, and the DKKL1 protein accumulates in the acrosome of mature sperm. Subsequent studies (reported in the accompanying paper) demonstrate that *Dkkl1* also is expressed in the trophectoderm/placental lineage. Taken together, these results strongly suggested that DKKL1 protein is required for terminal differentiation either of trophoblast giant cells or of sperm, both of which are directly involved in fertility. To challenge this hypothesis, conditional targeted mutagenesis was used to ablate the *Dkkl1* gene in mice. Surprisingly, *Dkkl1* nullizygous embryos developed into viable, fertile adults, despite the fact that they failed to produce any portion of the DKKL1 protein.

Conclusion(s)—DKKL1 is a mammalian-specific acrosomal protein that is not essential either for development or fertility.

Keywords

Fertilization; Acrosome; Sperm; DKKL1; conditional knock-out

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Capsule: Ablation of the *Dkkl1* gene, which is expressed predominantly, perhaps exclusively, in developing spermatocytes and trophoblast giant cells, does not prevent production of viable, fertile mice.

INTRODUCTION

Dickkopf-Like 1 (*Dkkl1*) was identified independently as a distant homologue to the Dickkopf (DKK) family of proteins that modulate WNT/β-catenin signaling (1), and as a gene located 3.8 kb upstream of the transcription factor *Tead2* [Fig. 1A; (2)]. Tead2 RNA is present in oocytes and eggs, but *Dkkl1* RNA is not. Both are detectable soon after the onset of zygotic gene expression, but then the expression patterns of these two genes diverge. At the blastocyst stage, *Dkkl1* expression is localized to the trophectoderm whereas *Tead2* expression occurs at similar levels in both the trophectoderm and inner cell mass (3), suggesting that DKKL1 functions in placental development. In adult mice, *Dkkl1* RNA is expressed abundantly and almost exclusively in developing spermatocytes where DKKL1 accumulates first in developing acrosomes and then in the acrosome of mature sperm (2,4). DKKL1 is an N-glycosylated protein that is processed during maturation of spermatocytes into sperm (4), raising the possibility that DKKL1 exists in multiple active forms.

The most direct approach to understanding gene function is to mutate the gene using gene targeting methodology (5). Given the possibility that deletion of *Dkkl1* may result in lethality and/or sterility, a conditional *Dkkl1* knockout mouse was constructed so that *Dkkl1* could be ablated either in all mouse tissues or in selected tissues. Subsequent analysis of the *Dkkl1* gene locus (RNA and protein products) confirmed the complete absence of DKKL1 protein in mice nullizygous for the *Dkkl1* gene. Surprisingly, *Dkkl1*−/− mice were as fertile as wild-type mice. Therefore, *Dkkl1* is not essential for development, viability or fertility.

MATERIALS AND METHODS

Construction of conditional knockout allele and RNA/protein analyses

The conditional *Dkkl1* knockout allele was constructed in a manner similar to that described for a *Tead2* conditional knockout allele (6). The targeting vector (Fig. 1A) was constructed using recombinant DNA methods [(7); described at http://recombineering.ncifcrf.gov/] using BAC clone 75.6i [Genome Systems (2)] as template for all PCR reactions]. Briefly, gap repaired plasmid was made by PCR amplification using primers D/E (see below) and restriction endonucleases NotI/HindIII as well as PCR amplification using primers F/G and HindIII/SpeI. The two fragments were cloned into NotI/SpeI-digested pL253. This plasmid was digested with HindIII, electroporated into heat-induced EL350 *E. coli* containing BAC 75.6i, and selected for ampicillin resistance. LoxP site was introduced into intron 1 (Fig. 1B) in the gap repaired plasmid as described (6). The “right-arm” was constructed by PCR using primers H/I and digested with NotI/EcoRI. The “left-arm” was constructed by PCR using primers J/K and digested with EcoRI/XhoI. The two arms were mixed and ligated to NotI/SalI digested pBluescript (Stratagene). LoxP flanked PGK-Neo cassette was ligated into EcoRI/BamHI sites of the above mini-targeting vector. This mini-targeting cassette was excised from the vector by digestion with NotI/Acc65I and along with gap repaired plasmid, was electroporated into heated-induced EL350 *E. coli*. Neo cassette was removed from resulting plasmid (leaving a single loxP site within intron 1) as described (6). A second mini-targeting vector was constructed as follows. The ‘left arm’ was constructed by PCR using primers L/M and digested with NotI/EcoRI. The ‘right arm’ was constructed by PCR using primers N/O and digested with SalI/EcoRI. The two fragments were ligated into NotI/SaI digested pBluescript. The FRT flanked loxP/PGK-Neo cassette (digested with EcoRI/BamHI) was then ligated into the EcoRI/BglII-digested mini-targeting vector. NotI/SalI digested mini-targeting cassette along with gap repaired plasmid containing the loxP in intron 1 was electroporated into heat-treated EL350. The final targeting vector was linearized by NotI and electroporated into the GSI-1 ES line (Genome Systems). ES selection and injection of appropriately cloned ES lines into blastocysts were done exactly as previously described (6).

Multiplex PCR genotyping was done as described (6) using primers A/B/C. Southern, Northern, Western, immunofluorescence and histological analyses were done as described (4,6).

PCR Primer Sequences (5'→3')

(A) acacattgcaggtgggtgggag, (B) ccagaactggatattaactgtccg, (C) ccctttgacacgttacact, (D) ataagcggccgcaagtggctcctatcccactta, (E) gtcaagcttctgtcccaggatagacagag, (F) gtcaagcttgcttgggtacctattaatgcgg, (G) ggactagttatcggcagctttcttctgcg, (H) ataagcggccgcagggcgtggtgagacaattag, (I) gtcgaattcgagctcggcgttctctccatttcagg, (J) ggaattcggatcctacagaatccaggggggatgc, (K) ccgctcgagttggagaagcctctgaaggc, (L) ataagcggccgcttccagtttcctctctgctg, (M) gtcgaattcgagctccagatatctcaagga, (N) ggaattcagatctagtgtaacgtgtcaaaagggag, (O) acgcgtcgactttctggaacgaccaaaacgc, (P) aagtagaagcaaaagagcccc, (Q) cctgaggatgtagagaaagtgtgtc, (R) tatgcacagttccagctgcg, (S) aggtggcagacagatggtgt

RESULTS

Construction Of A *Dkkl1* Conditional Knockout Allele

To determine whether or not *Dkkl1* is required during either mammalian development or reproduction, a conditional knockout allele of *Dkkl1* was constructed to allow inactivation by expression of Cre-recombinase in specific tissues. *EIIa-Cre*-mediated excision in the progeny removed exons 2 through 4, encoding 56% of the protein (Fig. 1). This deletion removed the first in-frame ATG and the putative signal peptide, both of which are encoded by exon 2. The deletion also removed the next two downstream ATGs present in exon 3 and the putative *Dkkl1* transcription enhancer element in intron 2 (Kaneko K.J., Vassilev, A. and M.L. DePamphilis, unpublished results). This deletion should prevent production of full-length functional DKKL1 with a signal peptide.

The wild-type *Dkkl1* allele (Fig. 1A) was mutated by homologous recombination in ES cells using a genetically altered targeting vector that contained a loxP site within intron 1 and another loxP site upstream of exon 5 (Fig. 1B). Cre-recombinase then directs excision of the intervening sequence. The targeting vector also contained the phosphoglycerate kinase promoter driving a neomycin-resistance gene (PGK-Neo) in intron 4 upstream of the loxP site to allow selection of recombinants. The *Neo* gene was flanked by FRT sites (the target of FLP-recombinase) to allow deletion of *Neo* (6,8).

Genomic DNA prepared from tail clippings of agouti offspring (indicative of germline transmission) were screened by PCR for the presence of the mutant allele (data not shown), and the results were confirmed by Southern blotting-hybridization using either 5'-/3'-specific DNA probes (Fig. 1C). Both assays confirmed that the mutant allele was transmitted through the germline. These mice were then mated with *EPEIIa-Cre*$^{+}$ mice (9) to delete exons 2 through 4 by Cre-dependent recombination (Fig. 1B/C). Since *EIIa* promoter activity in mice is restricted to oocytes and preimplantation embryos (10), mating with *EIIa-Cre*$^{+}$ mice produced a constitutive knock-out allele.

Inactivation Of *Dkkl1* Did Not Prevent Development Of Viable Adult Animals

Dkkl1+/− F1 mice were mated and their offspring genotyped both by Southern blotting-hybridization and PCR analyses (Fig. 2A). Both assays confirmed Cre-mediated deletion of the region flanked by the two loxP sites ('floxed' gene, Fig. 1B). The genotypic ratios of *Dkkl1* +/+, *Dkkl1*+/−, *and Dkkl1*−/− offspring were consistent with Mendelian segregation. Of the 173 mice produced by mating *Dkkl1*+/− mice, 28% were +/+, 45% were +/− and 27% were −/−. Moreover, the ratio of males to females was 1.5, 1.2 and 1.8, respectively, indicating that

Dkkl1 did not influence sex determination. Furthermore, the size, morphology, and behavior of *Dkkl1* nullizygous mice were indistinguishable from wild-type mice with the same mixed genetic background. Therefore, despite the strong expression of *Dkkl1* in placental tissues (11), *Dkkl1* is not required for development of fertilized eggs into healthy adult animals.

Functional *Dkkl1* Protein Was Absent In *Dkkl1* Knockout Mice

To confirm that *Dkkl1* expression was indeed absent in *Dkkl1*−/− mice given their unexpected viability, the levels of *Dkkl1* specific mRNA were examined by Northern blotting-hybridization with RNA collected from testes. The results confirmed that *Dkkl1* mRNA was reduced in *Dkkl1* heterozygous mice, and absent in *Dkkl1*−/− mice (Fig. 2B). A smaller band was generated by the deleted allele, but its level was significantly reduced compared with the wild-type allele, consistent with the fact that the *Dkkl1* mutant allele retains the native *Dkkl1* promoter, but it lacks the enhancer found in intron 2 (Fig. 1).

To confirm that *Dkkl1* exons 2, 3 and 4 were deleted from any *Dkkl1* mRNA that may have been produced from the residual exons in *Dkkl1*−/− mice, total RNA was prepared from testis and splenocytes, the only tissues in adult mice that produce detectable levels of *Dkkl1* (3), and the presence of exons 2−4 was assayed for by RT-PCR, as previously described (2). As expected, *Dkkl1* mRNA containing exons 2−4 was detected in both tissues either from *Dkkl1* wild-type or from heterozygous mice, but exons 2−4 were absent in mRNA from both testis and splenocytes of *Dkkl1* nullizygous mice (Fig. 2C; data not shown). Hence, any mRNA produced from the *Dkkl1* mutant allele could not produce a functional DKKL1 protein. Of equal importance is the fact that the mutated *Dkkl1* allele did not affect expression of either *Tead2* or *CD37* (Fig. 2C), two genes that lie in closest proximity to *Dkkl1* (2).

To confirm that DKKL1 protein was absent in *Dkkl1*−/− mice and examine the effects of DKKL1 loss on acrosome structure, tissue sections of testis were stained either with anti-DKKL1 antiserum to detect DKKL1 protein, or with Periodic acid-Schiff (PAS) to detect glycoproteins (12). No differences were detected between testis from *Dkkl1*+/+ *and Dkkl1*−/− males stained with PAS (Fig. 3, “testis”/PAS). In contrast, DKKL1 protein was abundant in testis from wild-type males, but absent in testis from *Dkkl1*−/− males (Fig. 3, “testis”/DKKL1). Testis sections from *Dkkl1*+/− males exhibited an intermediate level of staining (data not shown).

Similarly, anti-DKKL1 antiserum revealed that DKKL1 protein in wild-type sperm localized to the acrosome (4), whereas DKKL1 protein was absent in sperm from *Dkkl1*−/− males (Fig. 3 “sperm”). Interestingly, a relatively weak DKKL1 signal was consistently detected at the base of the head in wild-type sperm (Fig. 3, white arrow). Visual inspection of sperm by phase contrast microscopy failed to detect any consistent morphological differences between $Dkkl1^{+}$ and $Dkkl1^{-}$ sperm (Fig. 3; data not shown).

Although mRNA transcripts from the ablated *Dkkl1* locus could not produce a normal DKKL1 protein, they might still produce a truncated protein made from the open reading frame (ORF) within exon 5, the only coding exon remaining after Cre-mediated excision (Fig. 1). Such a truncated protein might contain partial DKKL1 activity, thereby masking the phenotype of *Dkkl1*−/− mice. To address this possibility, we first determined whether or not the polyclonal DKKL1 antibody recognized truncated proteins.

Expression vectors were constructed that contained either full-length or partial sequences of the *Dkkl1* ORF (including one that contained only exon 5 with an in-frame ATG) fused to enhanced green fluorescence protein (EGFP) attached at the DKKL1 C-terminus. NIH3T3 cells were then transfected with these plasmids. All of the chimeric proteins were detected by Western immuno-blotting (Fig. 4A, left panel), and each chimeric protein produced *in vivo* co-

migrated with [^{35}S]methionine-labeled proteins that had been translated *in vitro* (Fig. 4A, right panel). Similarly, immuno-fluorescent staining detected both full-length and partial fragments of DKKL1 in EGFP expressing cells (data not shown). These results confirmed that the polyclonal DKKL1 antibody recognized epitopes throughout the DKKL1 protein, including an epitope specific to exon 5, and therefore would have detected a partial protein produced from the deleted allele, if it was present.

No DKKL1 protein or protein fragment detected either in testis or in sperm from either *Dkkl1* +/+ or *Dkkl1*+/− males was detected either in testes or in sperm from *Dkkl1*−/− males (Fig. 4B; 4C). Moreover, sperm from *Dkkl1*+/− males contained about half as much DKKL1 as wild-type sperm. Taken together, these results demonstrate that all forms of DKKL1 protein were effectively absent from *Dkkl1* nullizygous mice.

DKKL1 Protein Was Post-Translationally Modified In Wild-Type Mice

Western immuno-blotting further demonstrated that DKKL1 protein was post-translationally modified. For example, DKKL1 from testis migrated during SDS-PAGE with a molecular weight of approximately 34 kDa, but after treatment with a glycosidase, it migrated with a molecular weight of approximately 29 kDa. This was similar in size to DKKL1 protein translated *in vitro* (4). Therefore, DKKL1 in testis is a glycoprotein containing predominantly, if not exclusively, N-linked carbohydrates. With increased resolution, DKKL1 in testis migrated as two bands with approximate molecular weights of 37 kDa and 35 kDa (Fig. 4B). DKKL1 in mature sperm migrates predominantly as a 22 kDa protein before treatment with glycosidase, and as an 18 kDa protein after treatment (Fig. 4C). In addition, a minor fraction migrated at 17 kDa before treatment, and 13 kDa after treatment. Since none of these proteins were detected in *Dkkl1* nullizygous sperm, all of them must be forms of DKKL1. Thus, two modifications occur during sperm maturation; DKKL1 protein is N-glycosylated at multiple sites, and it is truncated.

Fertility Does Not Require DKKL1 Protein

To determine whether or not *Dkkl1* expression affected fertility, wild-type C57BL/6 males and C57BL/6 females were continually mated with mice that were either *Dkkl1*+/+, *Dkkl1*+/− or *Dkkl1*−/−, and both the number of pups born per litter and the number of litters produced in six months were recorded. Surprisingly, ablation of the *Dkkl1* gene did not affect the ability of these mice to reproduce by natural matings (Fig. 4D).

DISCUSSION

In previous studies, we have shown that *Dkkl1* expression is restricted to developing sperm, trophectoderm and T-lymphocytes (2-4). In a companion study, we extended the expression pattern to the trophoblast cells that give rise to the placenta (11). To determine whether or not Dkkl1 affected mammalian development, viability or fertility, we constructed a mouse line that permitted complete ablation of the Dkkl1 gene either throughout the animal or in specific tissues. Comparisons of wild-type and Dkkl1 deficient mice revealed that between spermatogenesis in the testis and sperm maturation in the epididymis, N-glycosylated DKKL1 protein undergoes what appears to be partial deglycosylation and protease cleavage that reduces its apparent molecular weight by about 40%. Hence, the DKKL1 protein in mature sperm is a different form of the protein than that in developing spermatocytes. The biological significance of this transition remains to be elucidated.

Remarkably, mice lacking any form of the DKKL1 protein developed into what appear to be normal, healthy, viable, fertile adults. Given that the Dkkl1 gene is unique to mammals, we assume that its function, particularly in fertility, is too subtle to be revealed under normal

laboratory conditions. Presumably, it imparts an evolutionary advantage to mammals in the wild. In fact, as revealed in the accompanying manuscript (11), *Dkkl1* is, in fact, required for efficient sperm penetration of the zona pellucida.

While this manuscript was in preparation, another study appeared that also reported that *Dkkl1* −/− mice were fertile (13), although no analysis of fertility was included. This study concluded that DKKL1 induces apoptosis in post-pubertal spermatocytes, thereby regulating their population density. We did not detect differences in levels of apoptosis in testis sections of *Dkkl1*−/−males compared to wild-type or heterozygous males. One explanation for this difference may be that our studies were done on 6-month old animals, whereas the conclusions of Dakhova et al. were based on younger animals. Other possibilities arise from the fact that their *Dkkl1*−/− mice were created by insertional mutagenesis which is susceptible to artifacts (5). Their construction retained two downstream ATGs that may produce truncated forms of DKKL1 (2) from mRNAs that read through the polyA stop signal. Since DKKL1 exists in different forms (Fig. 4C), different forms of DKKL1 may have different functions. Their construction also contained a PGK promoter and the Dkkl1 enhancer, elements that may alter expression of other genes that are responsible for their observed phenotype.

Acknowledgments

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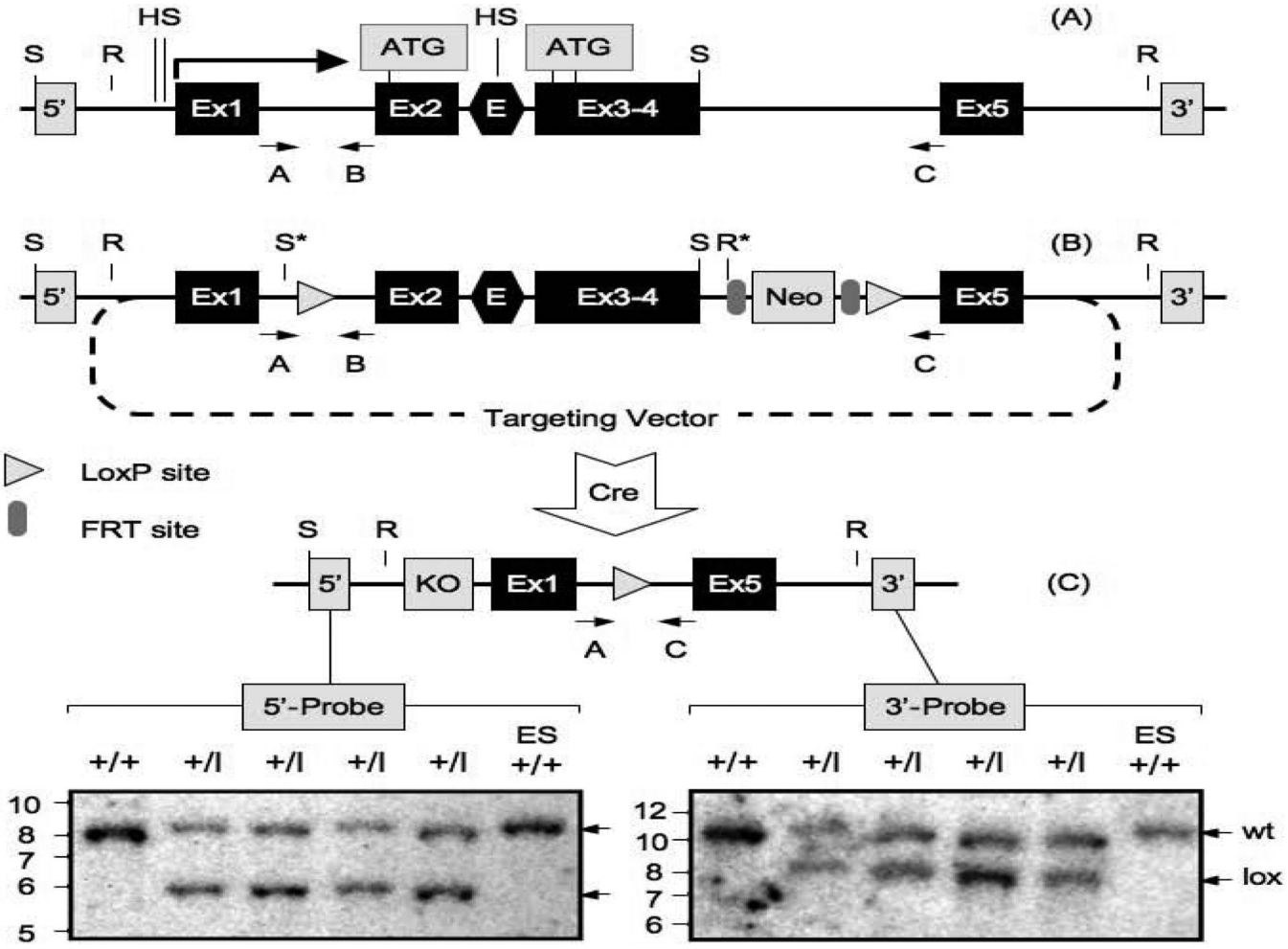

Figure 1.

Construction of a *Dkkl1* conditional knock-out mouse. (A) The *Dkkl1* gene contains five exons (Ex) that encode 230 amino acids. The transcription start site is indicated by a long arrow (2), while three in-frame translation start sites (ATGs) are located in exons 2 and 3. A putative *Dkkl1* gene enhancer (E) is located within intron 2. Two DNase I hypersensitive sites (HS) are located in the *Dkkl1* promoter and one in the enhancer (3). Also indicated are EcoRV (R) and SacI (S) restriction endonuclease sites, 5'- and 3'-sequence specific DNA probes used in Southern blotting-hybridization analysis (shaded 5' and 3' boxes), and PCR primers A, B and C (see Materials and Methods) used for multiplex genomic DNA PCR assays. (B) The 'floxed gene' within the targeting vector was used to introduce a mutated *Dkkl1* locus by homologous recombination, and spans the region from just upstream of the *Dkkl1* promoter (EcoRV site) through exon 5. Lox P sites were inserted within introns 1 and 4. A neomycin resistance gene (Neo), driven by the phosphoglycerate kinase (PGK) gene promoter and flanked by Flip recombinase target (FRT) sites, was inserted into intron 4 just upstream of the 3'-loxP site. Newly created restriction sites are indicated with an asterisk. (C) Cre recombinase excised the region between the two lox P sites to generate the *Dkkl1* knock-out allele. The probe (shaded KO box) used to detect DNA fragments containing this allele was just upstream of exon 1. Genomic DNA from the tails of pups born from matings between chimeric males and C57BL/6J females was digested either with SacI (5'-probe, nucleotides 6135−7514, Genbank AF274313.1) or EcoRV (3'-probe, nucleotides 190309−189131, Genbank AC149868.2) restriction endonuclease and then subjected to Southern blotting-hybridization. SacI digested

DNA was detected with the 5'-probe as indicated, and EcoRV DNA was detected with the 3'-probe. SacI produced a 7.9kb DNA fragment from the wild-type allele (wt), and a 5.9kb DNA fragment from the mutant allele (lox). EcoRV produced a 10.8kb fragment from the wild-type allele (wt) and an 8.1kb fragment from the lox allele (lox). DNA from wild-type ES cells was included as a positive control. DNA from agouti pups are in lanes 2−5 (+/lox), and DNA from a black littermate (+/+) is in lane 1.

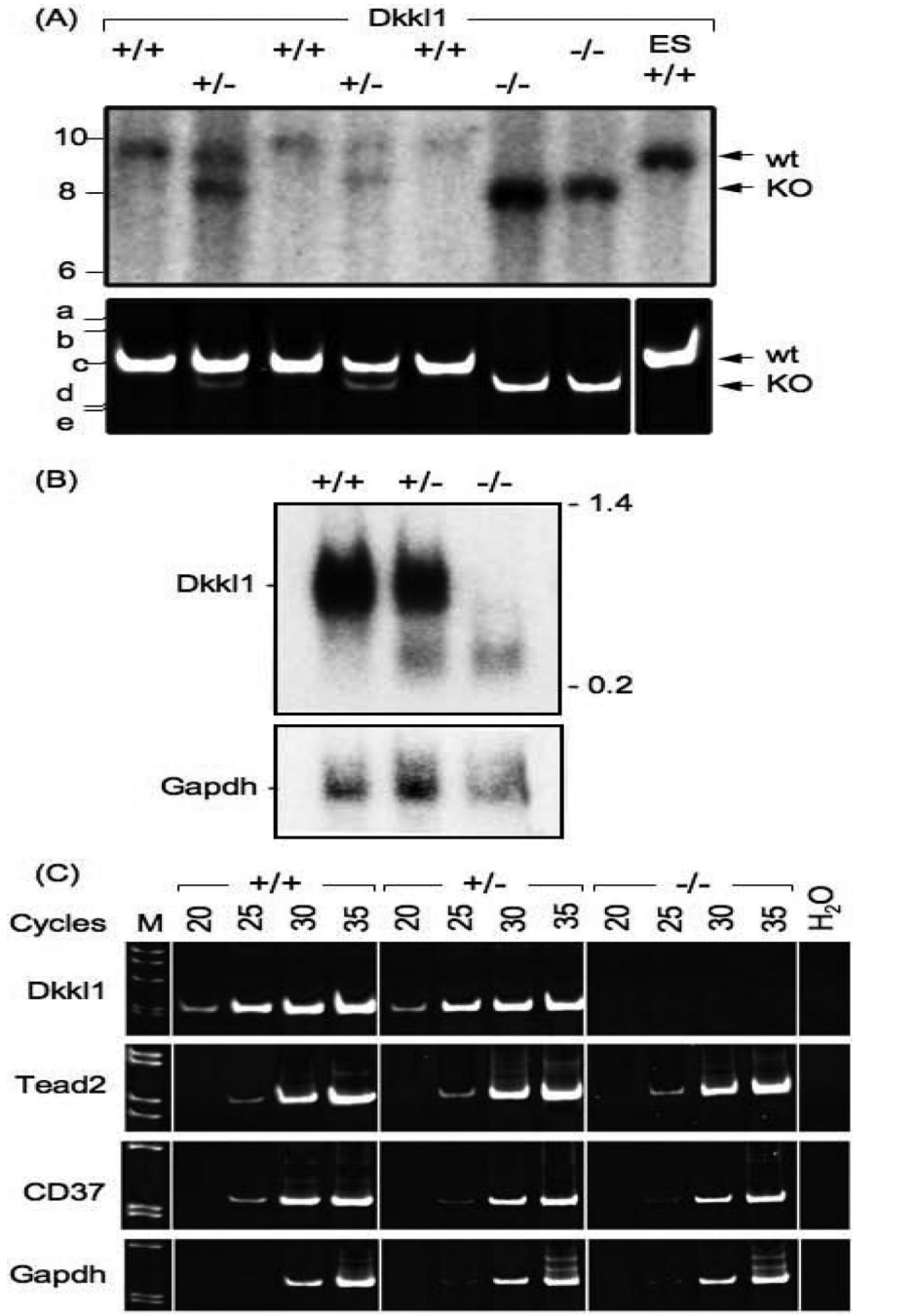

Figure 2.

Absence of *Dkkl1* mRNA in *Dkkl1*−/− mice. Total RNA was isolated (14) and subjected to Northern blotting-hybridization and reverse transcription-polymerase chain reaction (RT-PCR) (2). (A) Mice containing the mutant allele were mated to EIIa-Cre^{+} mice, and resulting pups were assayed for the deleted allele (KO). *Dkkl1*+/− mice were then mated to each other, and their litter of seven pups was assayed for the presence of the KO allele. Top panel: DNA was digested with EcoRV, subjected to Southern blotting-hybridization, and the DNA detected with the 'KO probe' (nucleotides 2168−3034 Genbank AF274313.1) indicated in Figure 1C. The wild-type allele generated a 10.8kb DNA fragment (wt) whereas the deleted allele generated a 8.8kb DNA fragment (KO). Bottom panel: The same DNA samples also were

subjected to multiplex PCR analysis. Primers A and B (Fig. 1A) amplified a 297bp fragment from the wild-type allele (wt), and primers A and C (Fig. 1C) amplified a 271bp fragment from the KO allele (KO). (B) Total RNA (15μg) was prepared from adult testes that were either wild-type, heterozygous, or nullizygous for *Dkkl1* and subjected to hybridization-blotting as in Figure 3 using *Dkkl1* ^{32}P-cDNA as a probe. Position of full-length *Dkkl1* mRNA is indicated. The blot was stripped and rehybridized with *Gapdh* ^{32}P-cDNA as control. The 0.24−9.5kb RNA ladder from Invitrogen was used to determine migration of RNA according to molecular size. (C) RNA from panel B was used as a template for cDNA synthesis to determine whether or not *Dkkl1* exons 2 and 3 were present. RT-PCR was performed for the indicated number of cycles using *Dkkl1* primers that amplified the region from exons 2 and 3, as described (2). The same aliquot of RNA also was used to amplify *Tead2* and *CD37* specific sequences (11). The 1kb DNA ladder from Invitrogen (M) was used to determine the size of the RT-PCR products. RT-PCR of *Gapdh* mRNA confirmed that equal amount of RNA samples were amplified. Water (H_2O) was used as a negative control. Probes and PCR primers for *Tead2*, *Dkkl1* and *Gapdh* are described in (3). Probe for *Dkkl1* Exon5 was generated using PCR primers P and Q (Materials and Methods). Primers R and S were used to amplify a 617bp DNA fragment from the *CD37* gene.

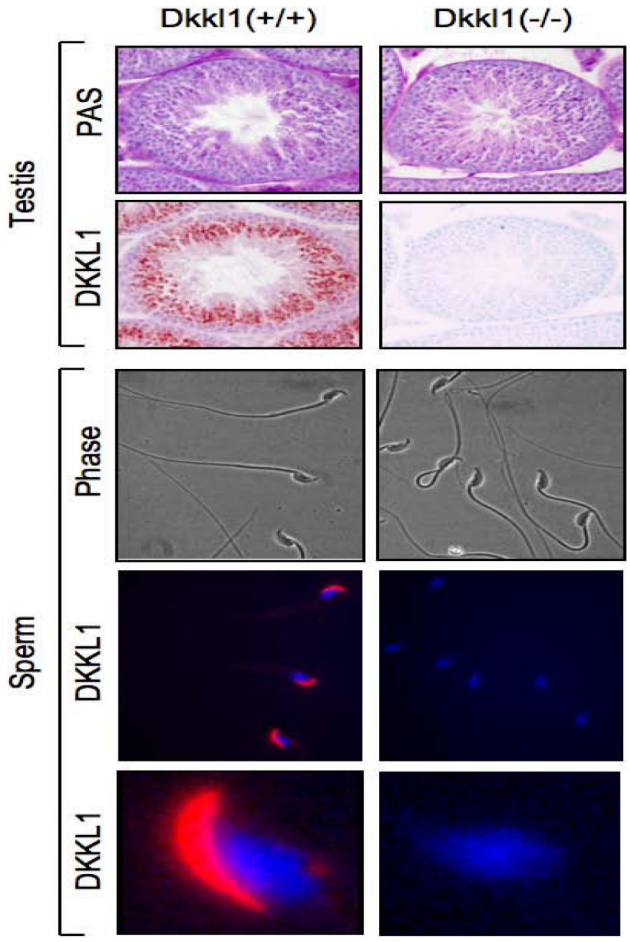

Figure 3.

DKKL1 protein was absent in *Dkkl1*−/− testis and sperm. Testes from wild-type and *Dkkl1*−/− males at six months of age were fixed overnight in Bouin's fixative, sectioned and stained with periodic Acid Schiff (PAS) to visualize glycoproteins. In both cases, sections were counterstained with hematoxylin. The same tubule is shown in both top and bottom images from neighboring sections. Sperm from cauda epididymides was collected from wild-type and *Dkkl1* nullizygous males, washed several times with phosphate buffered saline and spread on glass slides. DKKL1 protein was detected in tissue sections using a goat polyclonal antibody (AF1508, R&D Systems), followed by Cy3-coupled anti-goat secondary antibody (Jackson Immunologics) or biotinylated anti-goat secondary antibody (Invitrogen) (4). PAS staining was performed according to the manufacturer's protocol (Polysciences). Sperm from wild-type and nullizygous males were stained for DKKL1 protein (red). Nuclei were visualized with DAPI (Blue). One of the sperm heads from above panel was enlarged to show DKKL1 specific staining at the base of the sperm head (bottom, white arrow). Phase contrast images corresponding to above immunostaining show normal morphology of sperm (top).

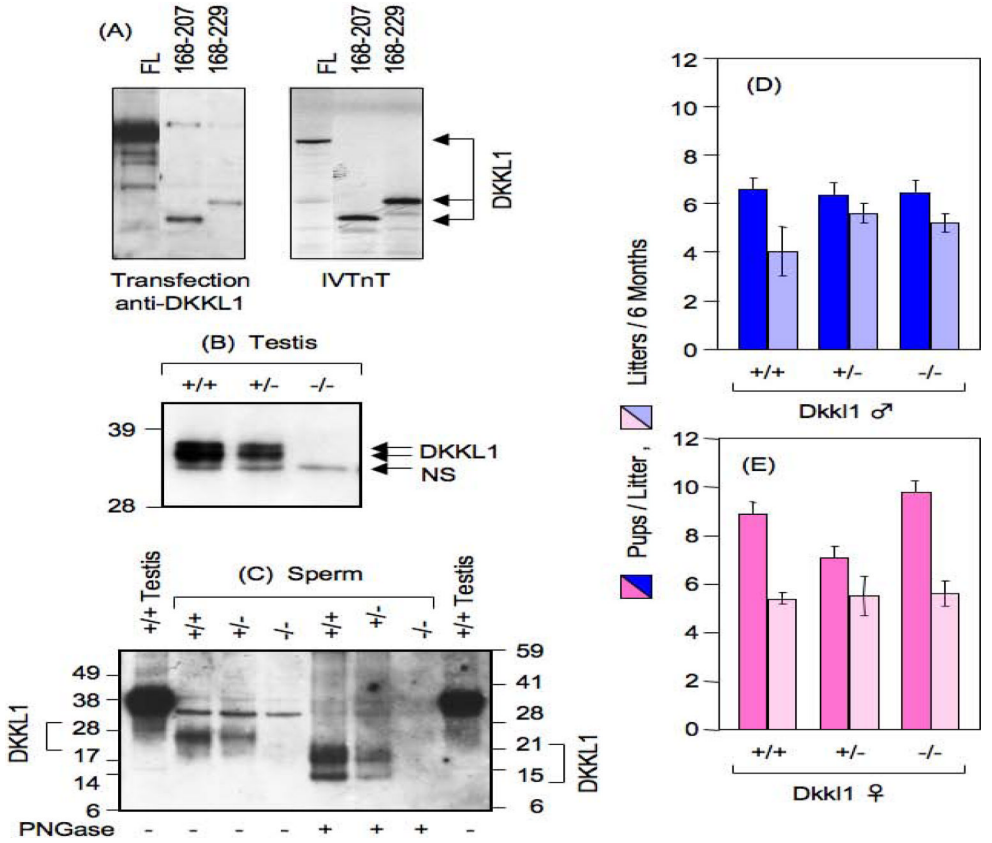

Figure 4.

DKKL1 protein and its effect on mouse fertility. (A) To determine whether or not polyclonal *Dkkl1*-specific antibody can recognize truncated DKKL1 protein, an EGFP fusion expression vector containing full-length (FL) or potential open reading frames within Exon 5 (corresponding to amino acid residues 168−207 or 168−229) were driven by a CMV promoter *in vivo* or by a bacteriophage T7 promoter *in vitro*. Plasmids were transfected into mouse NIH3T3 cells, and 48 hours later the cells were probed with the antibody by Western immuno-blotting (left panel). The antibody recognized the same size DKKL1 products as those produced from ^{35}S-labeled *in vitro* translation (right panel). To determine whether or not DKKL1 was absent in *Dkkl1*−/− testis and sperm, whole cell extracts of testis (B) or sperm (C) prepared from mice that were wild-type (+/+), heterozygous (+/−), or nullizygous (−/−) for the *Dkkl1* allele were denatured and fractionated on SDS-PAGE followed by Western immuno-blotting (4) using the same polyclonal antibody specific to DKKL1. DKKL1 products are highlighted with bracket/arrows, whereas a non-specific background band is indicated by "NS". DKKL1 from testis migrated ~34 kDa, as previously reported (4). Sperm samples were treated with N-glycosidase-F (PNGase) to reveal the presence of N-glycosylation (4). Sperm extracts from wild-type CD-1 males (labeled "+/+ Testis") were used as controls in panel C. To determine the effect of DKKL1 on mouse fertility, *Dkkl1*+/+, or +/− or −/− males (♂) were mated with C57/B16 females (D), and Dkkl1+/+, or +/− or −/− females (♀) were mated with C57/B16 females (E). The number of pups born per litter (dark blue and red bars) and the number of litters produced per 6 months (light blue and pink bars) were recorded. Error bars indicate the SEM. N = 5 for all animals except for *Dkkl1* wild-type males (N = 2) and heterozygous females (N = 4). An average of 20 litters per mating were analyzed.