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American Journal of Pathology, Vol. 164, No. 6, June 2004

Male Infertility and DNA Damage in Doppel Knockout and Prion Protein/Doppel Double-Knockout Mice

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The prion protein (PrP) and Doppel (Dpl) have many structural and biochemical properties in common, leading to the suggestion that the lack of an obvious phenotype in PrP-deficient mice maybe because of compensation by Dpl. To test this hypothesis and also investigate the function of Dpl we have generated *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ mouse lines. Both develop normally and display an identical male sterility phenotype that differs from that reported for another *Prnd*$^{-/-}$ mouse line. Sperm from both our mutant lines were present at normal concentrations, had normal motility, and no morphological abnormalities. Despite only rarely fertilizing oocytes *in vivo*, because of an inability to perform the acrosome reaction, mutant sperm were capable of fertilization *in vitro*, albeit at reduced rates compared to wild type. Elevated levels of oxidative DNA damage were found in both types of mutant sperm and resulting embryos failed at an early stage. Therefore we found no evidence that Dpl compensates for the loss of PrP function in mutant mouse lines, but it does have an important anti-oxidant function necessary for sperm integrity and male fertility. *(Am J Pathol 2004, 164:2279–2288)*

An abnormal isoform of PrP (PrP^{Sc}) accumulates in the central nervous system in the transmissible spongiform encephalopathies, which are fatal neurodegenerative disorders occurring in man and animals. The cellular prion protein (PrP^{C}), encoded by the *Prnp* gene, is a cell surface sialoglycoprotein expressed preferentially in the central nervous system and at lower levels in a number of nonneural tissues. To gain insight into the function of PrP^{C}, a number of *Prnp*$^{-/-}$ mouse lines have been generated. The first knockout lines, ZrchI and Npu, were generated by disrupting the PrP coding region located in exon 3 of the *Prnp* gene.[1,2] Studies on these lines indicated only a mild phenotype associated with PrP deficiency.[3,4] Subsequently, in the Ngsk, Rcm0, and ZrchII lines, the entire coding region and part of intron 2 were deleted.[5–7] Surprisingly, a late-onset ataxia, because of cerebellar Purkinje cell loss, developed in Rcm0, Ngsk, and ZrchII, but not ZrchI or Npu. This prompted the discovery of the *Prnd* gene, located 16 kb downstream of *Prnp*, encoding the prion-related protein, Doppel (Dpl).[8] Because of the structure of the targeted *Prnp* allele, intergenic splicing between *Prnp* and *Prnd* led to ectopic expression of Dpl in the brains of the ataxic, but not the nonataxic, PrP-null mouse lines.

Evidence that ectopic Dpl expression is responsible for Purkinje cell death in the ataxic *Prnp*$^{-/-}$ lines has been provided by the generation of *Prnp*$^{-/-}$/Dpl-overexpressing transgenic strains that show an inverse correlation between levels of Dpl in the brain and the age of onset of ataxia.[7,9] The abrogation of the ataxic phenotype in the Ngsk and ZrchII lines by the introduction of a PrP-overexpressing transgene suggests that the absence of PrP^{C} function is required for Dpl-induced neuronal death and that PrP^{C} functions antagonistically with respect to Dpl neurotoxicity.[7,9,10] This suggests some shared biological but distinct biochemical activities between PrP^{C} and Dpl and it seems plausible that the toxicity of ectopically expressed Dpl is at least in part because of it interfering with PrP^{C} -dependent pathways in the absence of that protein.

The amino acid sequence of Dpl is 23% homologous with PrP and has an N-terminal signal peptide sequence and C-terminal consensus sequences for Asn-linked glycosylation and GPI anchor addition.[11] Both proteins bind copper [Cu(II)] ions[12,13] and the solution structure of recombinant Dpl displays a similar topology to PrP^{C} such that it closely resembles an N-terminally truncated PrP^{C}

Supported by the Medical Research Council (grant G9818420).

Accepted for publication February 2, 2004.

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lacking the octapeptide repeat region.[14] It is of note that N-terminally truncated PrP^C is incapable of rescuing Dpl-dependent ataxia and can indeed cause a similar ataxic phenotype in the absence of the wild-type PrP^C protein.[15,16]

In mice, Dpl is normally expressed at high levels in adult testis and heart, but is only detectable in the brain during embryogenesis and in neonates.[17] Dpl overexpression in the brain does not effect disease progression in scrapie-challenged Rcm0 PrP-null mice.[18] In another study, after inoculation with scrapie prions, Dpl-deficient neural grafts showed spongiosis, gliosis, and normal PrP^{Sc} accumulation and infectivity.[19] Polymorphisms in the human *Prnd* gene have been described, but no association between these and the development of human prion diseases has been identified.[20,21] Thus, substantial evidence exists that Dpl is not required for susceptibility to prion diseases and PrP^{Sc} production.

In the adult mouse testis, Dpl immunoreactivity was found in both round and elongated spermatids.[22] In humans, Dpl expression was also found in the testis, but in this case localized to Sertoli cells and also on the flagellum of ejaculated mature spermatozoa and in the seminal plasma.[23] Behrens and colleagues[22] generated Dpl-deficient mice and found that they develop normally to adulthood, but males are infertile. A partial blockade of spermiogenesis, resulting in reduced numbers of spermatozoa in mutant seminiferous tubules and epididymes was reported. Furthermore, Dpl-deficient spermatozoa had reduced motility and displayed both sperm head and flagellar morphological abnormalities. Dpl-deficient sperm were found to be incapable of fertilization *in vitro* unless the zona pellucida was partially dissected. This observation led the authors to propose that Dpl function regulates the development of the acrosome, a sperm head-specific structure required for the penetration of the zona pellucida.

The expression of PrP^C has been reported in the male reproductive system of both mice and humans.[23,24] PrP^C is found in epididymal cells and truncated isoforms have been identified on ejaculated human spermatozoa, although the nature of the truncation is unclear, with the first report describing a C-terminally truncated isoform and the second an N-terminal truncation. All $Prnp^{-/-}$ mouse lines reproduce normally, suggesting that PrP^C expression is not essential for normal reproductive function, or that a related protein compensates for the loss of PrP^C function in these mice. However it has been shown that PrP^C can protect sperm from the detrimental effects of copper toxicity and associated oxidative stress,[24] and because of the biochemical similarities between PrP^C and Dpl it is not unreasonable to suggest Dpl may play a similar role.

To investigate the relationship between PrP^C and Dpl we have generated a $Prnp^{-/-}Prnd^{-/-}$ mouse line as well a new $Prnd^{-/-}$ line by gene targeting and found that $Prnp^{-/-}/Prnd^{-/-}$ animals develop normally to adulthood and display a male sterility phenotype indistinguishable from that of our $Prnd^{-/-}$ line. Surprisingly, this sterility phenotype differs in a number of ways from that of the $Prnd^{-/-}$ line produced by Behrens and colleagues.[22] Mutant spermatozoa from our lines were capable of fertilization *in vitro*, albeit at a significantly reduced frequency than wild-type control sperm, despite an inability to undergo the acrosome reaction in response to a calcium ionophore. Embryos arising from mutant sperm failed at an early stage and we found elevated levels of oxidative DNA damage in mutant sperm. We find no evidence that Dpl compensates for PrP^C activity in the development or integrity of the nervous system, but Dpl does play a critical role in multiple areas of sperm function.

Materials and Methods

Generation of $Prnd^{-/-}$ *and* $Prnp^{-/-}/Prnd^{-/-}$ *Mice*

Gene targeting was performed using the *Hprt*-deficient strain 129/Ola ES cell line, HM-1.[25] The *Hprt* mutation was crossed out before the establishment of targeted lines and all mouse lines were maintained on a 129/Ola inbred background. The 1.25-kb 5′ homology arm of the *Prnd*-targeting construct was generated by polymerase chain reaction (PCR) using primers F4521 (*Bam*HI linker then 5′ GCTGCCTCAGTATTCCTATGCTCTGATG, positions 34,237 to 34,264; GenBank accession no. U29187) and F4522 (5′ GCCTCCCACCCTCCAACAAGTGACAG, positions 36,164 to 36,139) to generate a 1.9-kb fragment that was subsequently trimmed at a naturally occurring *Eco*RI at the 3′ side. The 2.5-kb 3′ homology arm was generated using primers F3984 (*Eco*RI linker then 5′GCTGGGTTTCGTTTGGTTCATTGTGAAG, positions 36,721 to 36,748) and F3985 (*Cla*I linker then 5′ AGTCACTTGCAGCACATCTGTACTGTGG, positions 39,241 to 39,214). The two arms were cloned together into *Bam*HI/*Cla*I-cut pBluescriptII SK(+) (Stratagene, La Jolla, CA) and the *Hprt* minigene PGK-HPRT(RI)[26] was cloned into the central *Eco*RI site in the opposite transcriptional orientation to *Prnd*. An HSV-TK marker for negative selection was cloned adjacent to the 5′ homology arm as a *Not*I/*Bam*HI fragment and the vector was linearized with *Cla*I. Gene targeting was performed as described[27] and targeted clones were identified by PCR, using primers f11976 (5′ GATGCTAGGAGCCTGCTCATTCATTCC, positions 34,208 to 34,234) and 262W (located in the 3′ end of the Hprt marker[6]) (see Figure 1a). Targeting was confirmed by Southern analysis using a 147-bp probe generated by PCR (positions 34,088 to 34,234; GenBank accession no. U29187). A 3.7-kb wild type *Prnd Sac*I fragment was replaced with a 5.2-kb targeting-specific fragment, whereas a 2.0-kb *Pst*I fragment was replaced with a 5.7-kb targeting-specific fragment (see Figure 1a, data not shown).

The *Prnp/Prnd* targeting construct contained the same 5′ *Pst*I/*Eco*RI fragment of *Prnp* sequence used in making the Rcm0 PrP-null mouse line,[6] whereas the 3′ homology region and cloning of the selectable markers were the same as in the *Prnd* targeting construct (described above). Targeted clones were identified by PCR, using the primers G1176mod (5′ CTGCCGCACTTCTTGTGAATGCAGC) and 262W in the reaction previously de-

scribed for the identification of the Rcm0 line.[6] Targeting of the *Prnd* locus was confirmed by Southern analysis using a 171-bp 3′ probe generated by PCR (positions 39,510 to 39,680; GenBank accession no. U29187). This probe detected the replacement of a 7.5-kb wild-type *Prnd Xho*I/*Bam*HI fragment with a 5.0-kb targeting-specific fragment (see Figure 1b, data not shown). Successful targeting of the *Prnp* locus was confirmed by Southern analysis using a *Bam*HI/*Pst*I probe fragment to detect 8.5-kb wild-type *Bam*HI and 3.8-kb targeted *Bam*HI/*Xho*I fragments, as described previously (see Figure 1b, data not shown).[6]

Western Blotting

Tissue homogenates were prepared (10% w/v) in lysis buffer [10 mmol/L Tris-HCl, pH 7.6, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 100 mmol/L NaCl, 0.5% v/v Nonidet P-40, and 0.5% w/v sodium deoxycholate, 0.2 mmol/L phenylmethyl sulfonyl fluoride] and clarified by centrifugation. Protein concentrations of the sample supernatants were determined (DC Protein Assay kit, Bio-Rad, Hercules, CA) and samples (20 μg total protein) were mixed with gel-loading buffer, boiled for 3 minutes, cooled, and electrophoresed through a 12% sodium dodecyl sulfate-polyacrylamide gel. Proteins were then transferred by electroblotting onto Immobilon-P polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Blots were incubated in primary antibodies [polyclonal anti-Dpl, Rb2 (a kind gift from I. Sylvester, Institute of Animal Health, Compton, UK); monoclonal anti-PrP, 6H4 (Prionics, Schlieren, Switzerland); monoclonal anti-GAPDH (Chemicon International, Temecula, CA)], followed by the appropriate horseradish peroxidase-conjugated secondary antibody. Blots were developed using the ECL+ Plus detection kit and exposed to Hyperfilm ECL (both Amersham Pharmacia Biotech).

Sperm Analysis

The cauda epididymes of 6- to 10-week-old male mice were dissected into 1 ml of activation buffer,[28] diced, and incubated at 37°C for 30 minutes to allow sperm to disperse into the medium. Aliquots of the epididymal suspension were fixed in 5% paraformaldehyde and counted using a hemocytometer. Sperm motility was assessed by counting the number of sperm that showed tail motion[29] and expressing this as a percentage of the total number of sperm (minimum of 100) counted. To assess sperm morphology a minimum of 200 sperm were counted with the numbers of morphologically normal sperm and those with abnormal heads or flagellae being recorded. Computer-assisted sperm analysis of motility was performed as described.[30]

In Vitro *Fertilization*

Female 129/Ola mice were superovulated by intraperitoneal injections of 5 IU pregnant mare serum gonadotrophin, followed by 5 IU of human chorionic gonadotrophin (hCG) 48 hours later. Oocytes were collected 8 hours after hCG administration, washed, and held in T6 medium.[31] Spermatozoa were isolated from the cauda epididymes and vas deferens and allowed to capacitate in drops of T6 medium overlaid with silicone fluid for 2 hours at 37°C in 5% CO_2. Sperm (1 to 2 $\times$ 10^6) were added to 30 to 40 oocytes held in a 0.5-ml drop of T6 medium and incubated for 4 to 5 hours at 37°C in 5% CO_2 to allow fertilization to occur. Oocytes were then transferred to 200-μl drops of KSOM medium,[32] overlaid with silicone fluid, and incubated at 37°C in 5% CO_2 to allow development to proceed. The following day two-cell embryos were scored as a measure of successful fertilization and transferred to smaller drops (2 μl of medium per embryo) of KSOM and left in the incubator to develop further. Developmental progress to the morula stage was scored.

Acrosome Reaction

This was performed essentially as described.[33] Briefly, spermatozoa were squeezed from the cauda epididymes and vas deferens of 8- to 10-week-old animals into 0.5 ml of human tubule fluid using a pair of fine forceps. Spermatozoa were incubated for 1 hour at 37°C in 5% CO_2 to allow capacitation. Capacitated sperm were counted and adjusted to a concentration of $\sim$1 $\times$ 10^6 sperm/ml in human tubule fluid medium. Aliquots (25 μl) were added to 25 μl of medium, either with or without, the calcium ionophore A23187 (40 μmol/L). After a further 30-minute incubation at 37°C in 5% CO_2, sperm samples were fixed in 100 μl of 5% paraformaldehyde for 10 minutes. After two rounds of centrifugation and resuspension in 0.1 mol/L of ammonium acetate (pH 9.0), sperm aliquots (10 μl) were dried onto glass slides and stained for 2 minutes with 0.22% Coomassie Blue G-250 in 50% methanol/10% glacial acetic acid. Slides were rinsed with H_2O and mounted before scoring at least 300 sperm from each sample as being either acrosome-reacted or acrosome-intact. Intense staining on the anterior aspect of the sperm head in acrosome-intact sperm was lost in acrosome-reacted sperm.

Sperm Chromatin Structure Assay (SCSA)

Sperm samples from the cauda epididymes were adjusted to a concentration of 2 $\times$ 10^6 cells/ml with TNE (0.15 mol/L NaCl, 0.1 mol/L Tris, 1 mmol/L EDTA, pH 7.4). One hundred μl of the sample was taken and 200 μl of acid detergent solution (0.1% Triton X-100, 0.15 mol/L NaCl, 0.08 N HCl, pH 1.2) added and left at room temperature. After 30 seconds 600 μl of acridine orange staining solution was added (37 mmol/L citric acid, 126 mmol/L Na_2HPO_4, 1 mmol/L EDTA, 0.15 mol/L NaCl, pH 7.4 with acridine orange added fresh to 6 μg/ml) and left at room temperature for 150 seconds. The cells were then immediately analyzed in a Coulter Epics XL Flow Cytometer. Red fluorescence (from single-stranded DNA) was measured using a 675-nm detector and green fluorescence (from double-stranded DNA) measured using a

525-nm detector. The Epics XL was calibrated for each run by adjusting the wild-type control samples to give a mean fluorescence value of 145 ± 10 at 675 nm and 445 ± 10 at 525 nm. Ten thousand events were collected for each sample and each was run in triplicate. Raw data were then exported in Listmode format and analyzed using FlowJo (Tree Star, Inc., Ashland, OR). Background contamination (bacterial contaminants and cells other than spermatozoa) was removed by gating and the results expressed in terms of αt [= red/(red + green) fluorescence, expressed as a percentage value] and COMP αt.[34] COMP αt (cells outside the main population) was calculated as the percentage of cells in the population exhibiting an αt value greater than 26%.

Terminal dUTP Nick-End Labeling (TUNEL) Assay

Sperm samples were measured for DNA strand breaks using the TUNEL assay.[35] Ten thousand events were counted for each sample.

Results

Prnd$^{-/-}$- *and* Prnp$^{-/-}$/Prnd$^{-/-}$*-Null Animals Are Viable but Males Are Infertile*

To investigate the function of Dpl we generated a *Prnd*$^{-/-}$ mouse line by gene targeting in embryonic stem cells. The Dpl ORF (except the last 31 bases) and a further 713 bases upstream were replaced by a selectable *Hprt* minigene (Figure 1a). To test for possible compensation by Dpl for PrP^{C} we also generated a *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ mouse line in which both the PrP- and Dpl-coding regions were ablated. Owing to the small distance between the two genes (~16 kb), we deleted both coding regions in a single targeting event. Eighteen kb of sequence, spanning the *Prnp* and *Prnd* loci, was replaced by an *Hprt* minigene (Figure 1b). Immunoblotting confirmed the ablation of Dpl expression in the *Prnd*$^{-/-}$ line and the absence of both PrP and Dpl expression in the *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ line (Figure 2, a and b).

Animals from both lines developed normally well into adulthood. Despite Dpl expression in wild-type mouse brain during embryonic development and in neonates, and strong neuronal expression of PrP throughout development and during adulthood, *Prnd*$^{-/-}$ (n = 6) and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ animals (n = 8) of older than 90 weeks displayed no obvious neurological abnormalities.

Although *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ females reproduced normally, male nulls from both lines never reproduced successfully despite displaying normal mating behavior, as judged by the production of copulation plugs. Furthermore, spermatozoa were recovered from the uteri of females after copulation with *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ males, confirming that these animals are capable of ejaculation.

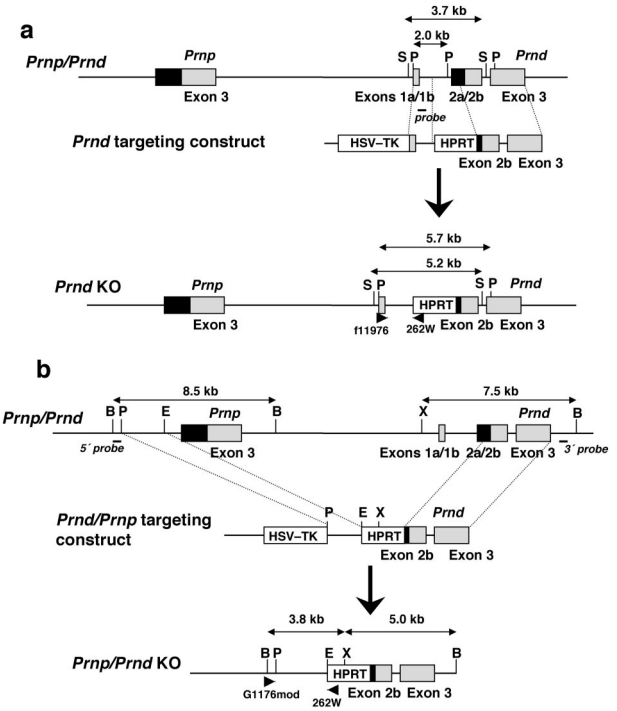

Figure 1. Generation of *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ mouse lines by gene targeting. **a**, *Prnd*; **b**, *Prnp/Prnd*. The structure of the *Prnp* and *Prnd* loci, targeting constructs, and targeted loci are shown schematically. Exons for each gene are indicated: **filled boxes**, coding regions; **shaded boxes**, untranslated regions. In the targeting constructs the positive (HPRT) and negative (HSV-TK) selectable markers are indicated. The locations of PCR primers, probes, and restriction fragments used to identify targeted clones are shown. Restriction sites: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sca*I; X, *Xho*I.

Normal Number, Motility, and Morphology of Prnd$^{-/-}$ *and* Prnp$^{-/-}$/Prnd$^{-/-}$ *Spermatozoa*

The morphological appearance of *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ testis was normal, as judged by histological analysis (Figure 3a), with all spermatogenic cell types present at normal levels. Analysis of sperm extracted from the cauda epididymes revealed no significant differences between wild type and both mutant lines in

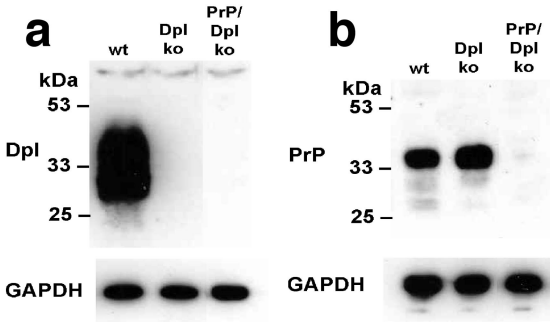

Figure 2. Ablation of Dpl in *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ mouse lines. **a:** Immunoblotting of wild-type (wt), *Prnd*$^{-/-}$ (Dpl ko) and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ (PrP/Dpl ko) testis lysates using an anti-Dpl antibody only detected a diffuse band of Dpl immunoreactivity in the wild-type sample. The mobility of molecular weight markers on the same gel is shown. Immunoreactivity against GAPDH in all samples provided an internal control for protein loading. **b:** Ablation of PrP in the *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ mouse line. Immunoblotting of wild-type (wt), *Prnd*$^{-/-}$ (Dpl ko) and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ (PrP/Dpl ko) brain lysates with an anti-PrP antibody only detected PrP immunoreactivity in the wild-type and *Prnd*$^{-/-}$ samples.

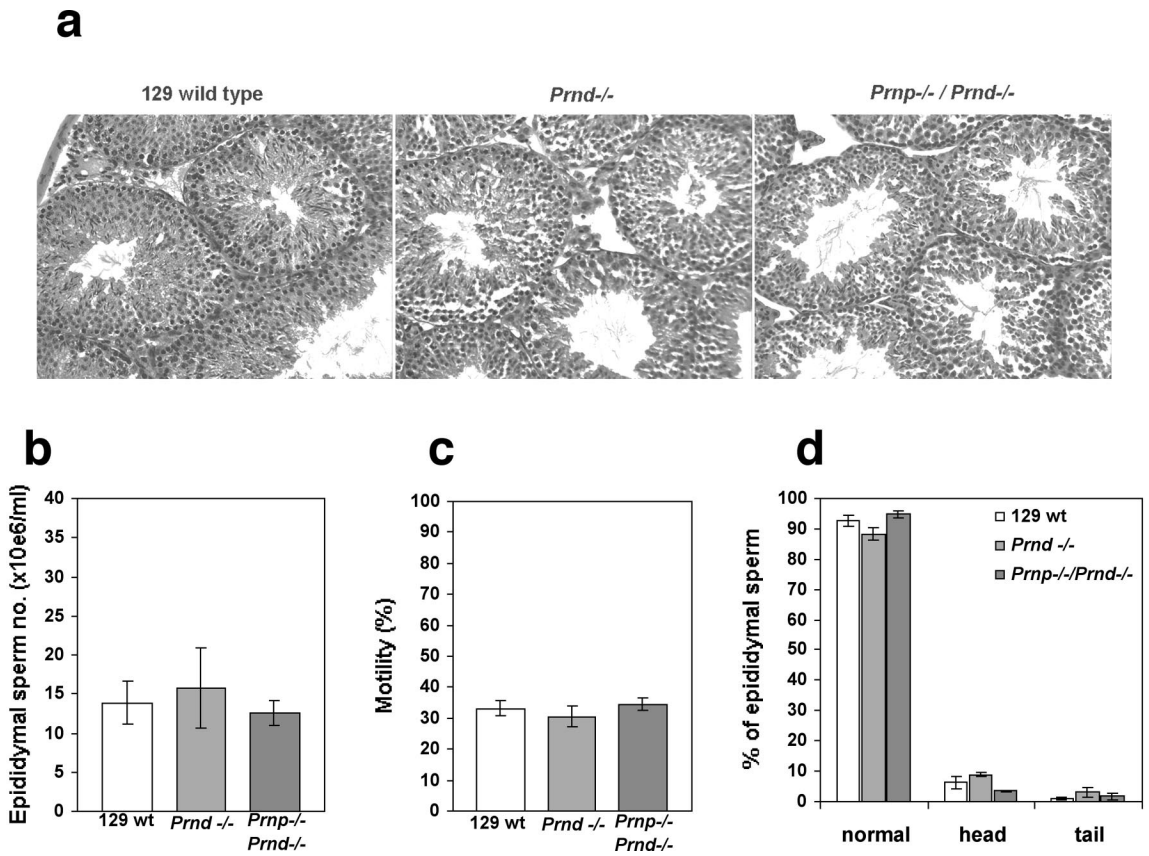

Figure 3. Spermatogenesis in $Prnd^{-/-}$ and $Prnp^{-/-}/Prnd^{-/-}$ mouse lines. **a:** H&E-stained testis sections from wild-type, $Prnd^{-/-}$, and $Prnp^{-/-}/Prnd^{-/-}$ mice, showing normal histology with all spermatogenic cell types present. **b:** Epididymal sperm counts. Normal counts in wild-type ($n = 4$ animals examined), $Prnd^{-/-}$ ($n = 2$), and $Prnp^{-/-}/Prnd^{-/-}$ ($n = 4$). **c:** Sperm motility. Normal motility in wild-type ($n = 6$), $Prnd^{-/-}$ ($n = 4$), and $Prnp^{-/-}/Prnd^{-/-}$ ($n = 4$). **d:** Sperm morphology. Normal morphology with low levels of head and tail abnormalities in wild-type, $Prnd^{-/-}$, and $Prnp^{-/-}/Prnd^{-/-}$ (all $n = 4$).

terms of number, progressive motility, and the presence of head or tail malformations (Figure 3; b to d). In the case of $Prnd^{-/-}$ sperm, these results contrast directly with those reported for an independently generated $Prnd^{-/-}$ mouse line.[22] For this reason and because we were concerned that our simple observations may be missing more subtle motility differences, we also performed a computer-assisted sperm analysis. With the exception of a (just) significant increase in the amplitude of lateral head displacement in $Prnd^{-/-}$ sperm, no significant differences were found between either type of mutant and control sperm for any of the parameters of sperm motility measured (Table 1).

Prnd$^{-/-}$ *and* Prnp$^{-/-}$/Prnd$^{-/-}$ *Sperm Fertilize* in Vitro *but Only Rarely* in Vivo

To gain further insight into the exact nature of the male infertility in our mutant animals, timed matings were set up between wild-type females and wild-type, $Prnd^{-/-}$ or $Prnp^{-/-}/Prnd^{-/-}$ males. Uteri were flushed 1.5 days *post coitum* and the number of two- and four-cell embryos and unfertilized oocytes counted (Figure 4a). No cleaved embryos were recovered from matings with $Prnd^{-/-}$ males and only a single embryo from $Prnp^{-/-}/Prnd^{-/-}$ males, compared to 44% cleaved embryos from control males. Thus, $Prnd^{-/-}$ or $Prnp^{-/-}/Prnd^{-/-}$ spermatozoa only rarely successfully fertilize oocytes *in vivo*.

To test whether mutant spermatozoa are actually incapable of fertilization, or that some other defect is responsible for the lack of successful fertilization *in vivo* (eg, an inability of mutant sperm to reach the site of fertilization), we performed *in vitro* fertilization using wild-type, $Prnd^{-/-}$ and $Prnp^{-/-}/Prnd^{-/-}$ spermatozoa with wild-type oo-

Table 1. Computer-Assisted Sperm Analysis*

Genotype†	Wild type	$Prnd^{-/-}$	$Prnp^{-/-}/Prnd^{-/-}$
VAP (μm/seconds)	31.1 ± 0.4	39.2 ± 4.1	36.8 ± 3.3
VSL (μm/seconds)	25.8 ± 0.8	33.6 ± 3.2	31.9 ± 3.6
VCL (μm/seconds)	45.9 ± 1.2	57.7 ± 6.2	49.6 ± 4.4
ALH (μm)	7.7 ± 0.4	9.1 ± 0.4‡	7.9 ± 0.3
BCF (Hz)	4.7 ± 0.2	4.7 ± 0.3	5.0 ± 0.6
STR (%)	84.8 ± 1.4	84.8 ± 1.5	86.0 ± 1.2
LIN (%)	60.5 ± 2.1	60.5 ± 1.6	65.3 ± 1.5

*Mobility parameters measured were: VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; STR, straightness; LIN, linearity.[30]

†Sperm was assayed from four animals of each genotype.

‡$P = 0.05$ by Student's *t*-test. No other significant differences between mutant and wild-type sperm were found.

a

		In-vivo 2-4 cell stage/total oocytes	*In vitro* 2 cell stage
Genotype	129 wt	43/97 (44%)	56.50 ± 4.5 (n=2)
	Prnd$^{-/-}$	0/88 (0%)	39% ± 1.0 (n=2)
	Prnp$^{-/-}$/ *Prnd*$^{-/-}$	1/60 (2%)	42% ± 1.5 (n=2)

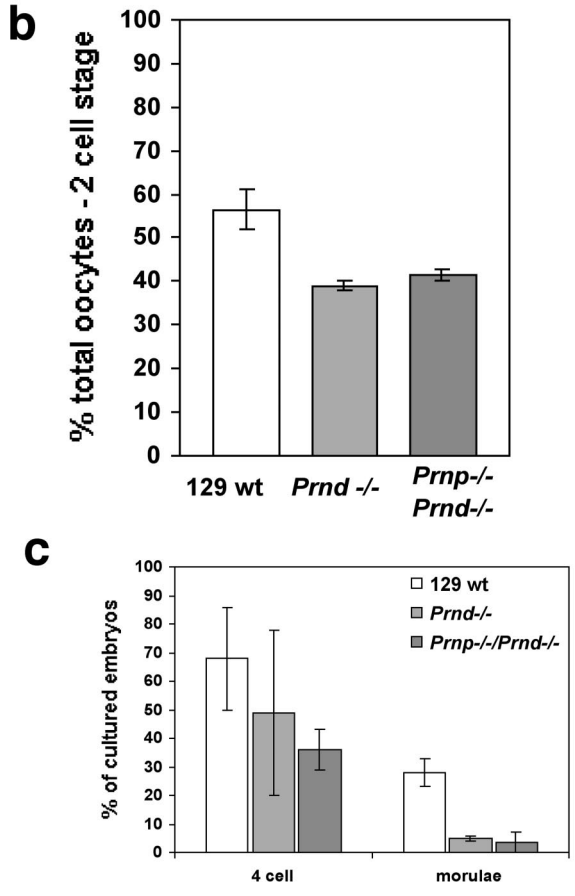

Figure 4. Fertilization potential of *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm. **a:** Frequency of *in vivo* and *in vitro* fertilization by wild-type, *Prnd*$^{-/-}$, and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm. **b:** Frequency of two-cell stage embryos after *in vitro* fertilization. Two separate determinations were performed for wild-type, *Prnd*$^{-/-}$, and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm. **c:** Frequency of two-cell stage embryos, produced by *in vitro* fertilization, that develop to four-cell and morula stages. Two separate determinations were performed for wild-type, *Prnd*$^{-/-}$, and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$.

cytes. Both *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm were able to produce two-cell stage embryos, indicative of successful fertilization (Figure 4, a and b). The rate of fertilization with mutant sperm was reduced compared to wild type, but this difference was not significant. Two-cell embryos were allowed to develop further in culture and the number developing to the four-cell and morula stages was recorded (Figure 4c). The percentage of embryos generated from *Prnd*$^{-/-}$ or *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm that developed to these later stages *in vitro* was again reduced in comparison to wild-type controls, this time significantly in the case of development to the morula stage ($P = 0.04$ for *Prnd*$^{-/-}$; $P = 0.05$ for *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ by Student's *t*-test). Thus, in addition to a greatly reduced ability to successfully fertilize *in vivo*, *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm have a further defect that prevents embryos generated *in vitro* progressing beyond all but the earliest stages of development. This early embryonic failure could also contribute to the infertility observed.

Prnd$^{-/-}$ *and* Prnp$^{-/-}$/Prnd$^{-/-}$ *Sperm Do Not Undergo the Acrosome Reaction in Response to a Calcium Ionophore*

The ability of sperm from our *Prnd*$^{-/-}$ line to fertilize oocytes *in vitro* contrasts with a previous report on an independently generated *Prnd*$^{-/-}$ mouse line, which found that mutant sperm was only capable of fertilization *in vitro* after partial dissection of the zona pellucida.[22] This led the authors to propose that a defect in acrosome biogenesis, leading to an inability to perform the acrosome reaction, was the basis of the male infertility. Consequently, we tested the ability of sperm from our *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ lines to undergo the acrosome reaction in response to treatment with the calcium ionophore A23187. Sperm from both mutant lines underwent a spontaneous acrosome reaction to the same extent as that from wild-type controls (Figure 5, a and b). However, unlike controls, mutant sperm failed to show increased levels of the acrosome reaction after exposure to A23187 and this difference was significant ($P = 0.02$ for both *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ by Student's *t*-test). Thus, as proposed by Behrens and colleagues,[22] the acrosome reaction is indeed defective in *Prnd*$^{-/-}$ sperm.

Prnd$^{-/-}$ *and* Prnp$^{-/-}$ /Prnd$^{-/-}$ *Spermatozoa Show Increased Levels of DNA Strand Breaks and Altered Chromatin Structure*

Any explanation for the early embryonic failure resulting from *in vitro* fertilization by *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm must take into account that the principal contribution of the sperm to the embryo is the paternal genome. If this DNA were damaged it could explain the observed developmental failure that occurs soon after activation of embryonic gene expression. To investigate whether there was altered chromatin structure and DNA damage within the spermatozoa from the mutant lines, the same spermatozoa used for the computer-assisted sperm analysis were assayed for DNA integrity using the SCSA and for DNA strand breaks using the TUNEL assay. In the SCSA the extent of DNA denaturation in each sperm head is quantified by the calculated parameter αt and the COMP αt value gives a measure of the number of sperm in the population with increased levels of DNA denaturation. Very strong correlations between COMP αt values and conventional DNA strand break assays (TUNEL and COMET) have shown that the DNA denaturation measured in the SCSA is primarily because of DNA strand breaks.[36,37] The SCSA (Figure 6a) showed significant increases in DNA damage for the two mutant lines over wild type ($P = 0.04$ for *Prnd*$^{-/-}$ and $P = 0.01$ for *Prnp*$^{-/-}$/*Prnd*$^{-/-}$), but no significant difference between the mutant lines ($P = 0.37$). Similarly, when DNA strand breaks were measured directly by

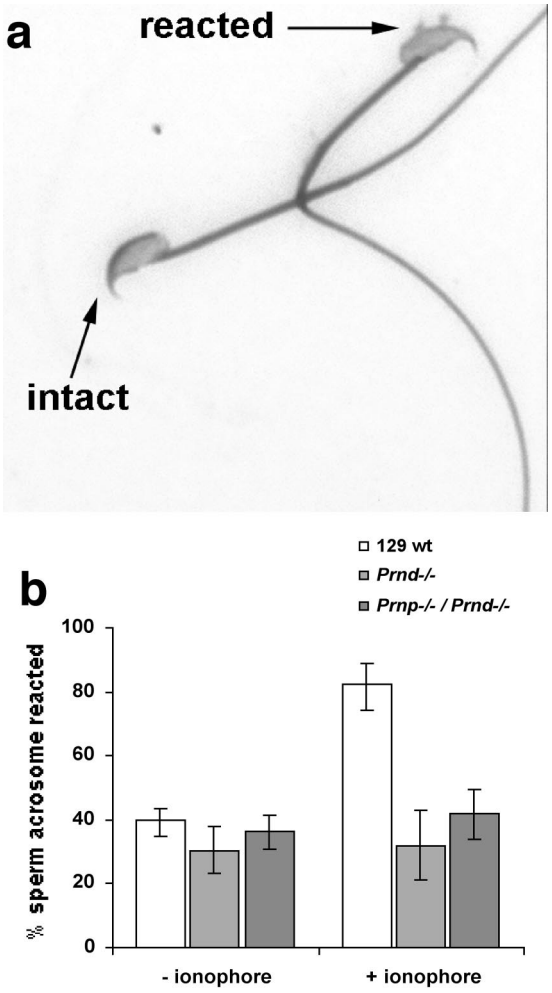

Figure 5. Defective acrosome reaction in $Prnd^{-/-}$ and $Prnp^{-/-}/Prnd^{-/-}$ sperm. **a:** Illustration of acrosome-reacted and nonreacted (intact) sperm heads stained with Coomassie blue G-250. **b:** Frequency of acrosome-reacted sperm in the presence and absence of calcium ionophore A23187 for wild-type (n = 3 separate determinations), $Prnd^{-/-}$ (n = 2), and $Prnp^{-/-}/Prnd^{-/-}$ (n = 3) sperm.

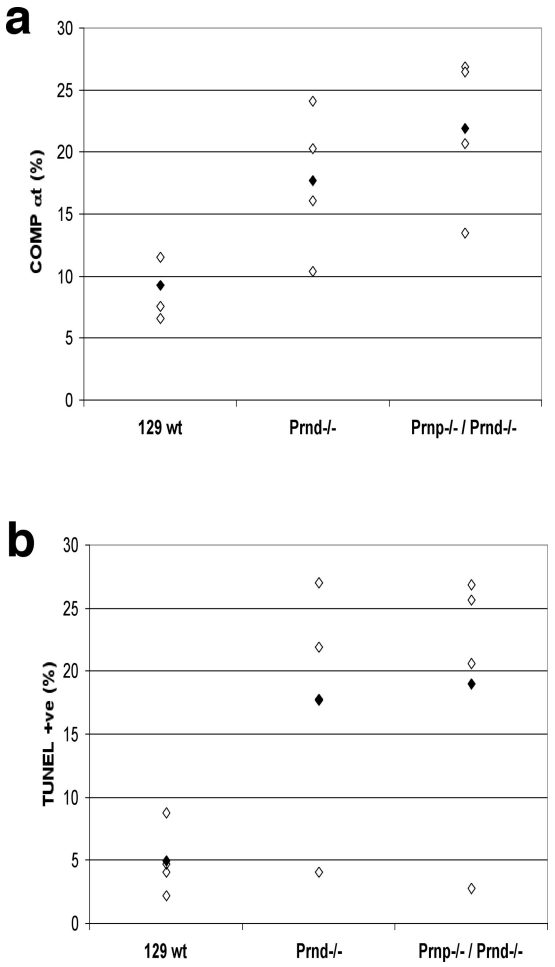

Figure 6. a: Levels of DNA damage measured by the SCSA. $Prnd^{-/-}$ and $Prnp^{-/-}/Prnd^{-/-}$ samples show a significant increase in COMP αt values greater than the wild-type control. **Open points** represent individual data, **filled points** represent the means for each genotype (n = 4 independent samples for each genotype). **b:** Levels of DNA damage measured by the TUNEL assay. $Prnd^{-/-}$ and $Prnp^{-/-}/Prnd^{-/-}$ samples show a significant increase in the frequency of TUNEL-stained sperm greater than the wild-type control. **Open points** represent individual data, **filled points** represent the means for each genotype (n = 4 independent samples for each genotype).

TUNEL assay (Figure 6b), a significant increase in the level of damage was observed for the two mutant lines over wild-type (P = 0.05 for both $Prnd^{-/-}$ and $Prnp^{-/-}/Prnd^{-/-}$), but there was no significant difference between the mutant lines (P = 0.87). Thus, sperm from $Prnd^{-/-}$ and $Prnp^{-/-}/Prnd^{-/-}$ lines has elevated levels of DNA strand breaks and this is because of the lack of Dpl.

Discussion

Only mild phenotypic changes have been attributed to the loss of PrP^C function in gene-targeted mouse lines. Often the ablation of a protein can be compensated for by the expression of a related protein, thus masking the development of a phenotype. It has been suggested that a PrP-like protein compensates for the loss of PrP in the various $Prnp^{-/-}$ mouse lines, thus preventing the manifestation of a more striking phenotype. If this is the case then our finding that PrP/Dpl double-knockout mice lack any overt neurological phenotype well into adulthood excludes Dpl from having such a role.

Behrens and colleagues[22] reported the production of a $Prnd^{-/-}$ mouse line and found that Dpl is essential for male fertility. They discovered a partial blockade of spermiogenesis leading to a reduction in numbers of spermatozoa in the seminiferous tubules and cauda epididymes. Mutant epididymal sperm also had significantly reduced levels of motility and abnormal head and flagellar morphology. In addition, these sperm were found to be incapable of fertilizing oocytes *in vitro* unless the zona pellucida was partially dissected.

The male sterility phenotypes reported in our $Prnd^{-/-}$ and $Prnp^{-/-}/Prnd^{-/-}$ lines are indistinguishable. This, combined with no reports of reproductive defects in any $Prnp^{-/-}$ lines generated to date, strongly suggests that PrP^C does not have a fundamental role in male reproductive function, although it can protect sperm cells from environmental damage under certain circumstances.

Surprisingly though, the nature of the infertility reported in our *Prnd*$^{-/-}$ (and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$) males differs in a number of important aspects from that described by Behrens and colleagues.[22] Firstly, we find no differences between mutant and control animals in epididymal sperm concentration, motility (even using computer-assisted sperm analysis), and morphology. Secondly, although mutant sperm only rarely fertilize oocytes *in vivo*, they can (unlike those of Behrens and colleagues[22]) successfully fertilize *in vitro*, albeit at reduced frequencies compared to control sperm. The calcium ionophore A23187 is commonly used to induce the acrosome reaction in capacitated sperm. We found that treating sperm from our mutant lines with A23187 failed to induce the acrosome reaction. Based on the observation that sperm from their *Prnd*$^{-/-}$ line displayed sperm head abnormalities and were incapable of fertilization *in vitro* unless the zona pellucida was partially dissected, Behrens and colleagues[22] proposed that Dpl was involved in acrosome biogenesis. Our results support this hypothesis, and explain the reduced rate of fertilization observed *in vitro* for our *Prnd*$^{-/-}$ sperm. Studies on other male sterile mouse mutants have found that an inability to perform the acrosome reaction in response to A23187 correlates with a reduced rate of fertilization *in vitro* eg, *Tnp2*- and *Dax-1*-deficient mouse lines.[33,38]

The reasons for the differences in the infertility phenotype between the different *Prnd*$^{-/-}$ lines may lie in their different genetic backgrounds. All of our mice are kept on an inbred 129/Ola background, whereas those of Behrens and colleagues[22] are on a mixed C57BL6/CBA background. Background related differences in male infertility phenotypes have been reported in other targeted mouse mutants, including the sperm mitochondria-associated cysteine-rich protein, transition proteins 1 and 2, and desert hedgehog genes.[38–41]

In addition to a greatly reduced ability to fertilize *in vivo*, *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm are unable to support the development of embryos generated *in vitro* beyond the morula stage. This suggests that Dpl plays an important role in a second area of sperm function. What could this be? In the mouse the centrosome is provided by the oocyte, not the sperm.[42] Thus, the contribution that a sperm makes to embryonic development is primarily restricted to providing the paternal genome. If this DNA is damaged then early embryonic failure might be anticipated soon after the activation of embryonic gene transcription, because many forms of damage will prevent transcription. In the mouse many embryonic genes are switched on at the two-cell stage.[43] Alternatively, unrepaired DNA damage could lead to genome instability and embryonic failure during the rapid early cleavage divisions. Targeted disruption of DNA repair genes, *Rad51* and *Brca1*, leads to preimplantation and early postimplantation lethality, respectively.[44,45] We demonstrated increased levels of DNA damage in *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm using two independent assays. The simple SCSA, in which increased levels of DNA denaturation correlate closely with increased DNA strand breaks measured by the more direct, but laborious TUNEL and COMET assays.[36,37] These results were confirmed by TUNEL assay. In human fertility clinics a high level of DNA damage in sperm (measured as a high percentage of sperm in the sample with high levels of DNA denaturation outside the normal range) is the best predictor for infertility.[34] It is tempting to speculate that the elevated levels of DNA strand breaks in *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm are the cause of the early embryonic failure we observed after *in vitro* fertilization. We have observed a very low level of *in vivo* fertilization with mutant sperm, but have never seen live births. The failure of these embryos to develop could also be because of the DNA damage in *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm.

Endogenously generated reactive oxygen species are a potent cause of DNA strand breaks and exposure of sperm to hydrogen peroxide causes increased DNA denaturation in the SCSA.[34] The involvement of PrP in antioxidant defenses against reactive oxygen species in the central nervous system and reproductive system has been suggested. Studies *in vivo*, on the brains of PrP-null mouse lines, and *in vitro*, on primary neuronal cultures derived from these lines, have revealed a reduction in the activities of the antioxidant enzymes SOD1 and glutathione reductase in the absence of PrP.[46,47] Additionally, cultured PrP-deficient neurons were found to have increased susceptibility to hydrogen peroxide or oxygen-free radical toxicity. Furthermore, we have found evidence of oxidative stress-induced damage in the form of increased levels of protein oxidation and lipid peroxidation in the brains of the Dpl-expressing ataxic, Rcm0, line as compared with the nonataxic, Npu, line.[48] The oxidative stress proteins, heme-oxygenase-1 and nitric oxide synthase were also induced in the brains of these mice. The abrogation of the ataxic phenotype in the Ngsk line by the introduction of a PrP-expressing transgene suggests a possible antagonistic interaction between PrP^{C} and ectopically expressed Dpl with respect to their involvement in oxidative stress in the central nervous system.[10] The situation may be different in the testis with Dpl rather than PrP^{C} having the leading role in protection from oxidative stress. This would certainly be compatible with the elevated levels of oxidative DNA damage observed in *Prnd*$^{-/-}$ and *Prnp*$^{-/-}$/*Prnd*$^{-/-}$ sperm.

In summary, we have found no evidence that Dpl compensates for the loss of PrP expression in *Prnp*$^{-/-}$ targeted mouse lines, but it is required for spermatozoa to perform the acrosome reaction and therefore for fertilization *in vivo*. Furthermore Dpl is also required for sperm to contribute to embryonic development beyond the morula stage and the elevated levels of DNA damage observed in *Prnd*$^{-/-}$ sperm indicate that it is involved in protection from oxidative stress. Further insight into the function of Dpl could be important for two reasons. Firstly, it might facilitate a greater understanding of the role of the structurally similar PrP, which could have major implications for treatment of transmissible spongiform encephalopathies. Secondly, human mutations in Dpl may be responsible for a subset of male infertility cases. The *Prnd*$^{-/-}$ mouse strain could thus provide an invaluable tool for the understanding of male infertility and the development of much needed therapies.

Acknowledgments

We thank Alison Murray and Norah Spears (Division of Biomedical and Clinical Laboratory Sciences) for their assistance with the *in vitro* fertilization work; Haley Scott and Jenny Templeman (Medical Research Council Human Reproductive Sciences Unit) for computer-assisted sperm analysis; and Dr. Iain Sylvester (Institute of Animal Health, Compton) for kindly providing the anti-Dpl antisera.

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