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Loss of R2D2 Proteins ROPN1 and ROPN1L Causes Defects in Murine Sperm Motility, Phosphorylation, and Fibrous Sheath Integrity[1]

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ABSTRACT

The fibrous sheath (FS) is a flagellar cytoskeletal structure unique to sperm that surrounds the outer dense fibers and axoneme. Its primary components are A-kinase anchoring proteins (AKAPs) 3 and 4, which suggests that the FS affects flagellar beating via the scaffolding of signaling pathways necessary for motility. Sperm proteins ROPN1 and ROPN1L bind AKAP3. To determine the role of ROPN1 and ROPN1L in sperm function, we created mice deficient in ROPN1 (RKO), mice deficient in ROPN1L (RLKO), and double knockout mice (DKO). All three strains of mice had normal testicular morphology and spermatogenesis. Only the DKOs had obvious defects in sperm morphology (thinning and shredding of the principal piece), which was accompanied by a reduction in AKAP3 levels. RLKO mice had slightly reduced sperm motility and increased levels of ROPN1. RKO mice had moderately impaired motility and increased levels of ROPN1L. DKO sperm were immotile. We have previously determined that RKO male mice are subfertile, and DKO males are infertile. Together these data indicate that ROPN1L and ROPN1 compensate for each other in the absence of the opposing protein, possibly to maintain AKAP3 incorporation in the FS. Sperm from mice lacking ROPN1L exhibited reductions in both cAMP-dependent protein kinase (PKA) phosphorylation of a 270-kDa protein (perhaps FSCB), and in capacitation-induced tyrosine phosphorylation. Sperm from mice lacking ROPN1 had reduced levels of FSCB and increased tyrosine phosphorylation of noncapacitated sperm. These data demonstrate that mutations in ROPN1 and ROPN1L can cause defects in FS integrity, sperm motility, and PKA-dependent signaling processes, leading to male infertility.

AKAP, fibrous sheath, OmniBank, PKA, ROPN1, sperm

INTRODUCTION

Most proteins that participate in cellular signaling networks contain modular protein interaction domains. Many kinases and phosphatases use docking interactions where a short peptide motif in target partners is recognized by a groove on the catalytic domain that is distinct from the active site. Docking is particularly prevalent in serine/threonine kinases and phosphatases, and is a versatile organizational tool for building complex signaling networks. The high degree of modularity may make these systems more evolvable, with novel circuits arising from relatively simple genetic events such as recombination, deletion, or insertion [1]. The cAMP-dependent protein kinase (PKA)/A-kinase anchoring protein (AKAP) interactions are a prime example of signaling molecules that use these docking groove/peptide motifs. The PKA holoenzyme consists of two regulatory (R) and two catalytic subunits. In addition to binding catalytic subunits, the R subunit of PKA contains a dimerization/docking domain that interacts with a short peptide region on AKAPs. The binding domain on each AKAP consists of approximately 18 amino acids that form an amphipathic helix structure. The docking groove on PKA that recognizes AKAPs is more complex and consists of approximately 50 amino acids on the N-terminus of the R subunit. Dimerization of this region is required for formation of a hydrophobic groove [2].

Docking grooves are generally less modular than peptide motifs [3]. However, our lab has identified four proteins in addition to PKA that contain the RII dimerization/docking (R2D2) domain. These proteins are ROPN1 (ropporin 1), ROPN1L (ROPN1-like protein, formerly known as ASP), CABYR (calcium binding tyrosine-[Y]-phosphorylation regulated), and SPA17. Based on binding studies and mutational analysis, two of these proteins (ROPN1 and ROPN1L) appear to interact with AKAPs in a manner similar to PKA; similar single-amino acid substitutions that alter either the dimerization/docking domain or the amphipathic helix domain prevent both RII and ROPN1 from interacting with AKAP3. Also, addition of Ht31 (a peptide that encompasses the amphipathic helix region of an AKAP and binds to the regulatory subunit of PKA with nanomolar affinity) disrupts ROPN1 binding to AKAPs in vitro [4–6]. We have shown that these proteins do not bind cAMP. Outside of the docking groove they bear little or no similarity to PKA, and they do not appear to have any intrinsic enzyme activity. Taken together these data suggest that ROPN1/ROPN1L have different functions than PKA but might act as regulatory subunits, similar to RII [6]. ROPN1/ROPN1L orthologs are conserved in a variety of species of animals, including chickens, *Xenopus*, fish, sea urchin, and ciliated parasites such as *Chlamydomonas reinhardtii*, suggesting these proteins serve a vital function. Collectively, these data indicate that AKAPs may have a dual signaling function, supporting both cAMP/PKA-dependent processes in addition to the predicted PKA-independent interaction with R2D2 proteins.

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The fibrous sheath (FS) is a flagellar cytoskeletal structure unique to sperm that surrounds the outer dense fibers and axoneme in the principal piece of the flagella. Its full function is still being defined, but the FS likely provides support and flexibility to the flagella, and provides a scaffold for glycolytic and signaling enzymes. Because glycolysis is necessary for both hyperactivated motility and the protein tyrosine phosphorylation events that take place during capacitation, it follows that a properly formed FS is critical for normal sperm function [5, 7–10]. Although the kinetics and gross structural mechanics of FS formation have been described elegantly, the proteomic mechanisms driving FS formation are scarce, and they remain limited primarily to data concerning the two most abundant FS structural proteins: AKAP4 and AKAP3 [5, 7, 11, 12]. ROPN1 has also been localized to the FS. Because ROPN1 binds to AKAP3 in a phospho-regulated manner and has also been shown to complex with (FS protein) RHPN1 (previously known as rhophilin) and RHO [5, 13–15], it surely contributes to FS function, but whether its role(s) are developmental, structural, and/or signaling related is unknown.

It has long been known that PKA signaling is critical to capacitation/fertilization, and yet many basic gaps still exist in our specific knowledge of the enzymes, proteins, and pathways that mediate its end results (global tyrosine phosphorylation, hyperactivated motility, capacitation). In Burton and McKnight [16], data gathered from mice null for various PKA subunits (Cα, Cα2, RIα, and RIIα) and AKAPs (AKAP1 and AKAP4) are summarized nicely in an attempt to gain some clarity on the role of PKA-dependent processes in fertility. Two interesting conclusions are drawn in this review. First, that PKA is not playing an essential role in sperm development, and in fact unregulated PKA activity can be toxic to sperm during development. Second, the authors conclude that although AKAPs appear to have a functional role during sperm maturation, this role is not dependent on the presence of RIIα.

Ht31 disrupts interactions between PKA and all cellular AKAPs [2, 17]. Adding Ht31 to vigorously motile sperm inhibits and ultimately arrests motility in a time- and dose-dependent manner. (Control peptide, Ht31-P, containing a proline substitution designed to prevent formation of an amphipathic helix structure within the peptide, has no effect on motility.) However, addition of potent PKA inhibitor H-89 to bovine sperm has only a minor effect on sperm motility [18]. In similar experiment using boar spermatozoa, Ht31 also arrests motility and has only a small effect on the overall levels of PKA phosphorylation [19]. Additionally, mutant mouse sperm lacking RIIα are motile and the mice are fertile, despite the fact that the catalytic subunit is concentrated in the cytoplasmic droplet rather than located along the flagellum [20]. One hypothesis consistent with these data is that in addition to PKA interactions, AKAPs interact with R2D2 proteins via the amphipathic helix domain to regulate sperm motility.

There is a significant body of literature related to R2D2 protein localization and in vitro interactions, particularly for CABYR (which is phosphorylated during capacitation and is known to bind calcium and GSK3β) and SPA17 [6, 21–32]. However, to our knowledge the only previously described in vivo mammalian R2D2 functions are a role for SPA17 in zona pellucida binding [27] and our recent report demonstrating that loss of ROPN1L (but not ROPN1) decreases ciliary motility [33]. In the present study, we use these previously developed mutant mouse lines (one lacking ROPN1, one lacking ROPN1L, and a cross that lacks both proteins) to test the hypotheses that the R2D2 proteins ROPN1 and ROPN1L are necessary for proper FS structure and/or function, and that they participate in regulation of sperm motility via PKA-dependent signaling pathways.

MATERIALS AND METHODS

Chemical Sources

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich.

Care and Breeding of Mice

These studies were approved by the Portland Veterans' Affairs Medical Center (PVAMC) Institutional Animal Care and Use Committee, which follows the guidelines of the Office of Laboratory Animal Welfare and sets policies according to the *Guide for the Care and Use of Laboratory Animals* and the *Department of Veterans Affairs Handbook 1200.7, Use of Animals in Research*. Mice were housed at the PVAMC Veterinary Medical Unit. Mutant mouse lines that contain insertional mutations in *Ropn1l* and *Ropn1* that interrupt protein expression were purchased from Lexicon Pharmaceuticals (see Zambrowicz et al. [34] for methodology). To obtain double-mutant animals, we crossbred the two lines to double homozygosity. Production and genotyping of these mice were described previously [33]. In this manuscript, we will refer to mice with a mutation in *Ropn1l* as ROPN1L knockouts (or RLKO), those with a mutation in *Ropn1* as ROPN1 knockouts (or RKO), and the crossbred mice with mutations in both *Ropn1l* and *Ropn1* as double knockouts (or DKO).

Isolation and Preparation of Sperm and Testes for Western Blot Analysis

Immediately after CO_2 euthanization, wild-type (WT), RLKO, RKO, and DKO mice were weighed, and testes and epididymides were removed from mice. Testes were weighed, and caudal epididymides were minced in a 12-well plate in PBS (EMD Chemicals) and then placed in a 37°C incubator/5% CO_2 for at least 15 min to allow sperm to swim up prior to counting, washing in PBS, and lysis in boiling SDS sample buffer (1 μl of sample buffer per 30 000 sperm; 62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.05% bromophenol blue). Testes were lysed in lysis buffer (1 ml per 100 mg of tissue: 10 mM Tris [pH 7.6], 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 5 mM ethylene diamine tetraacetic acid) and sonicated. The insoluble and soluble fractions were separated by centrifugation (16 000 × *g*, 10 min at 4°C) and SDS sample buffer was added. The SDS-PAGE and Western blot analyses using rabbit polyclonal antibodies to AKAP3, ROPN1L, ROPN1, CABYR, SPA17 (antibodies characterized in Newell et al. [6]), AKAP4, and outer dense fiber 2 (ODF2; Santa Cruz Biotechnology) were performed as previously described [6]. Anti-tubulin Western blot analyses (mouse monoclonal, diluted 1:10 000 in Tris-buffered saline with Tween 20 [TBST]; Zymed, now Life Technologies) were performed to confirm equal protein loading.

Immunohistochemistry

Immunohistochemical staining was performed as described previously [6, 33]. Briefly, ROPN1L antibody was diluted 1:500 and ROPN1 antibody was diluted 1:2000. All primary antibody incubations were for 1 h. To make valid comparisons of staining between WT and mutant animals in Figure 2 (compensation), we tightly controlled for variables in staining and image capture. First, WT, RLKO, and RKO sections were affixed to a single slide to ensure that all three sections were stained with identical conditions. Second, imaging parameters on the microscope (lighting, brightness, contrast, exposure, and color balance) were set on WT sections and then maintained for all subsequent pictures. Finally, any adjustments made in Photoshop (brightness/contrast) were applied to all images. Pictures were taken with a Zeiss AxioCam connected to an AxioPlan2 epifluorescence microscope.

Immunocytochemistry

AKAP3 staining of spermatozoa was performed as previously described [4] with minor modifications as follows. Following acetone permeabilization, sperm were permeabilized for 5 min in 0.1% Triton X-100 in PBS, and then washed once prior to blocking, which was for 30 min. Primary antibody incubation was overnight at 4°C, and secondary antibody was Texas Red conjugated (Jackson Immunoresearch Laboratories). Pictures were taken as described above.

Motility Analyses

Caudal epididymides were harvested as described above and minced in motility buffer (PBS, 5 mM glucose, 1.7 mM $CaCl_2$, and 4 g/L bovine serum albumin [BSA; fraction V]). Sperm were counted, diluted to approximately 2 ×

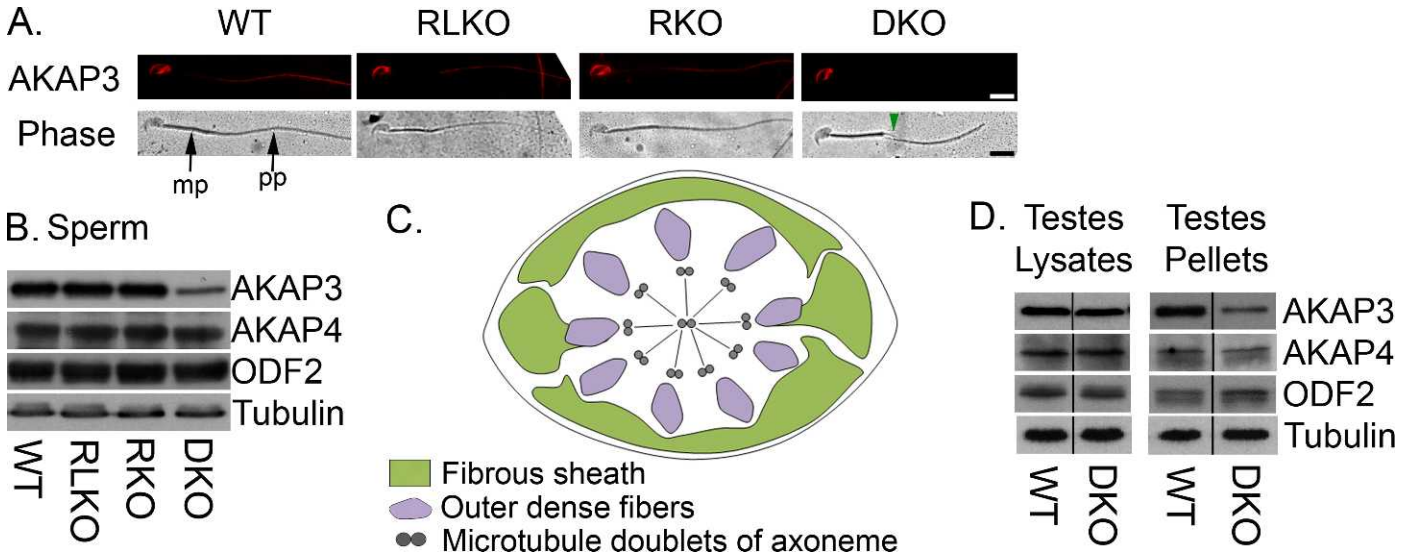

FIG. 1. The principal piece of mature double-knockout (DKO) spermatozoa exhibits structural defects, including loss of FS protein AKAP3. **A**) Sperm from WT, RLKO, RKO, and DKO mice were fixed and stained with rabbit polyclonal AKAP3 antibody and examined at original magnification ×100. In WT panels: mp, midpiece; pp, principal piece. Arrows in DKO phase point to principal piece defects. Insets are enlargements of dashed boxes to show detail. **B**) AKAP3, AKAP4, ODF2, and tubulin Western blots of caudal epididymal sperm. **C**) Diagram of the principal piece. **D**) AKAP3, AKAP4, ODF2, and tubulin Western blots of soluble (lysates) and insoluble (pellets) testis proteins from WT and DKO mice. These are representative of at least three independent experiments, each using unique sets of animals. Bars = 10 μm.

10^6 per milliliter in motility buffer, and incubated at 37°C/5% CO_2. Thirty-microliter aliquots were removed and loaded into Leja 100 micron 2 chamber counting slides (Spectrum Technologies). Motility of each sample was recorded three times within 3 h of prep at 10× under darkfield illumination using a JAI CV-730 CCD camera and a Panasonic DMR-EA18 DVD Recorder connected to an AxioPlan2 epifluorescence microscope (Zeiss). Sperm motility was assessed with the aid of computer-assisted motility analysis (CASA) from Hamilton Thorne Biosciences installed with the Ceros version 12.2g software. The analysis was performed on images captured from three random fields per sample each of 60 frames at 60 frames per second. The settings used for the motility parameters were as follows: minimum contrast 30, minimum cell size 4 pixels, static cell size 8 pixels, static cell intensity 60, low-size gate 0.17, high-size gate 2.26, low-intensity gate 0.35, high-intensity gate 1.84, minimum static elongation gate 0, maximum static elongation gate 90, minimum average path velocity (VAP) 50 μm/sec, minimum straightness (STR) 50%, VAP cutoff 10 μm/sec, and VSL cutoff 0 μm/sec.

Sperm Treatments and Western Blot for Phosphorylation Studies

Caudal epididymal sperm were harvested as described above but placed in either noncapacitating M2 or capacitating M16 media (Sigma-Aldrich) and incubated for 60 min at 37°C/5% CO_2 as described previously [35]. After incubation, sperm were washed twice in PBS, counted, and lysed as described above, then subjected to SDS-PAGE. Western blot analyses were performed with: 1) anti-phospho-(Ser/Thr) PKA substrate antibody (rabbit polyclonal; Cell Signaling Technology; detects Ser/Thr residues that are phosphorylated by the catalytic subunit of PKA) diluted 1:500 in TBST; 2) FSCB (fibrous sheath CABYR-binding protein) antibody (goat polyclonal; Santa Cruz Biotechnology) diluted 1:250 in TTBS with 5% BSA; or 3) 4G10 antibody, kindly supplied by Dr. Brian Druker, Oregon Health & Sciences University (mouse monoclonal, detects phosphorylation on tyrosine residues; commercially available from EMD Millipore), diluted 1:4000 in TBST with 5% nonfat milk and 1% BSA. Anti-tubulin Western blot analyses were also performed as described above to confirm equal protein loading. Densitometry measurements of changes in phosphorylation were made using ImageJ version 1.44p (National Institutes of Health).

Statistical Analyses

One-way ANOVAs for Figures 3, 4, and Supplemental Figure S1 were performed on the VassarStats website (http://vassarstats.net), and the difference between any two sample means was determined using the Tukey honestly significant difference test.

RESULTS

Testis Structure and Spermatogenesis Are Normal in Mice That Lack ROPN1 and ROPN1L

We have previously shown that mRNA expression of R2D2 proteins is highest in testes. We have also localized ROPN1 protein expression to the flagella of human sperm, and more specifically to the principal piece of mature mouse sperm. Additionally, we localized ROPN1L in the cytoplasm of spermatocytes in the seminiferous tubules of human testes [4, 6]. As such, we hypothesized that there might be developmental and/or structural defects in the testes and sperm of mice lacking ROPN1 and ROPN1L. To begin to explore this possibility, we compared the ratio of testis weight to body weight, and the epididymal sperm counts from WT, RLKO, RKO, and DKO mice, but we found no significant differences (Supplemental Fig. S1; all Supplemental Data are available online at www.biolreprod.org). To further investigate whether there are defects in testes and spermatogenesis in the absence of ROPN1L and ROPN1, we fixed testes from WT and double-knockout mice in formalin, embedded the tissues in paraffin, sectioned them, and stained them with hematoxylin and eosin. The staining showed no differences between WT and double-mutant testes, indicating that testicular structure and spermatogenesis are not affected by the lack of ROPN1L/ROPN1 (Supplemental Fig. S2, arrows point to sperm heads). It should be noted here that (as we have previously published) ROPN1L-knockout mice produce a very small amount of ROPN1L—protein levels are knocked down more than 98% but not completely knocked out [33].

Mice That Lack Both ROPN1L and ROPN1 Have Structural Defects in the Principal Piece of the Sperm Flagellum

ROPN1 is located in the principal piece of the mature sperm flagellum, specifically in the FS [15]. AKAP3 is a major protein of the FS that colocalizes with ROPN1 and binds it in a phospho-regulated manner in vitro [4, 14]. To

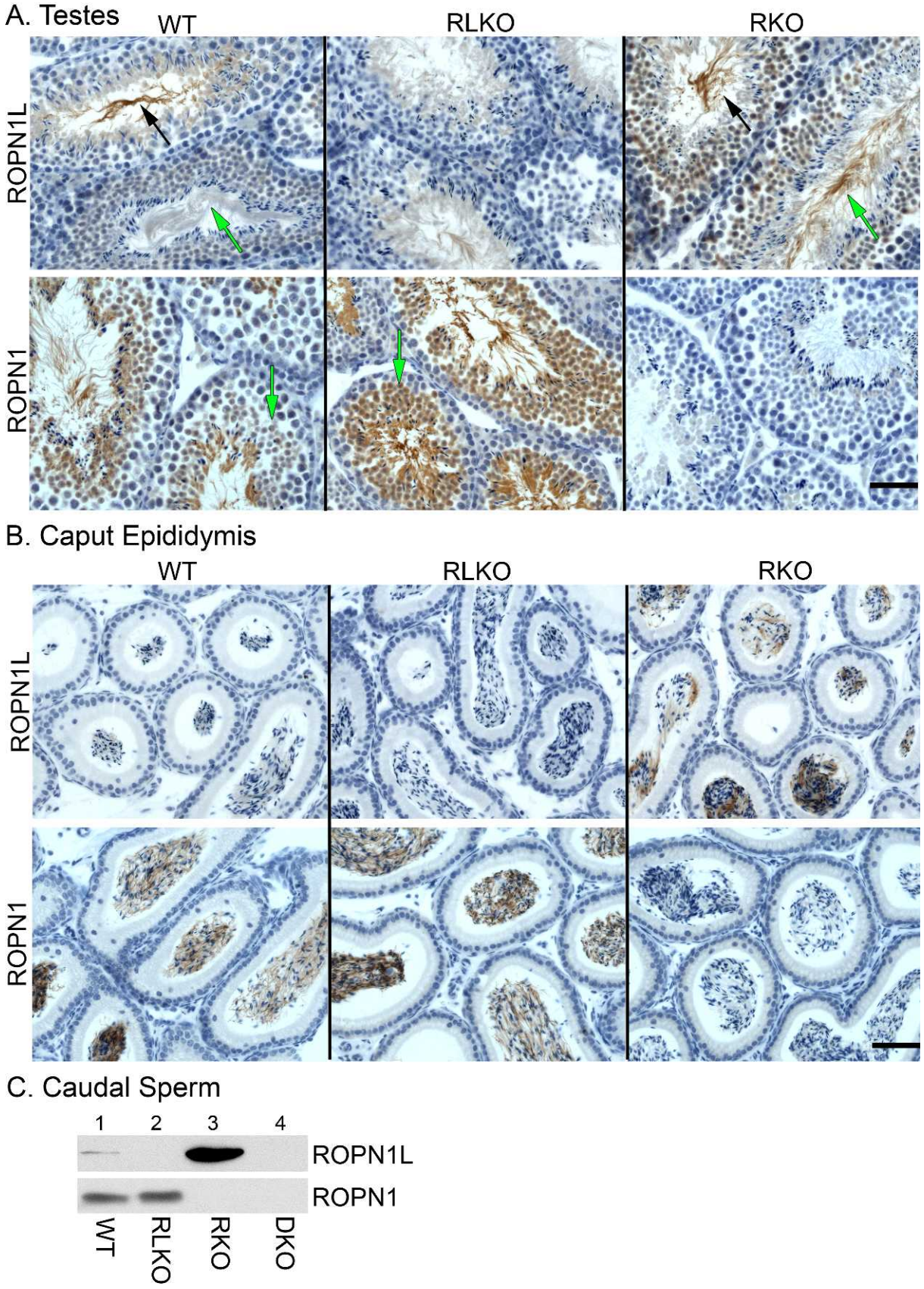

FIG. 2. ROPN1L and ROPN1 compensate for each other during spermatogenesis in opposing gene trap animals through additional localization and increased expression. Immunohistochemical staining of WT, RLKO, and RKO testes (**A**), and caput epididymal sections (**B**) using rabbit polyclonal antibodies to ROPN1L and ROPN1 shows diaminobenzidine-positive (brown) staining. Blue hematoxylin counterstain stains nucleic acid-rich regions. Arrows in **A** point to areas of common (black arrows) or differential (green arrows) staining between WT and knockout tissues. In **C**, these antibodies were also used to demonstrate a large increase in ROPN1L levels in RKO caudal epididymal sperm (upper panel, compare lanes 1 and 3). These pictures are representative of three independent experiments using three unique sets of animals. Bars = 60 μm.

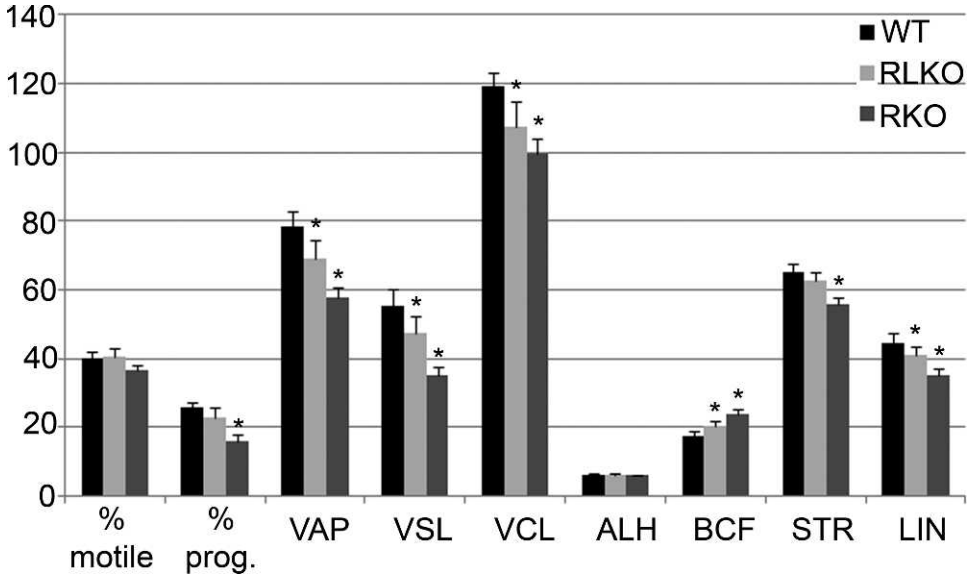

FIG. 3. CASA of motility parameters from WT, RLKO, and RKO mice. Motility parameters include percentage of sperm that are motile (% motile), percentage of sperm that exhibit progressive motility (% prog.), VAP, VSL, VCL, amplitude of lateral head displacement (ALH), BCF, and ratios of velocity for linearity VSL:VCL (LIN) and straightness VSL:VAP (STR). Error bars are ± SEM. Statistical significance is indicated by $*P < 0.05$. These pictures are representative of three independent experiments using three unique sets of animals.

determine whether there are defects in the principal piece not visible in the staining above, we isolated and fixed epididymal sperm, followed by staining for AKAP3 in each of our four mouse lines. ROPN1L-knockout and ROPN1-knockout sperm appeared to have normal flagellar structure and exhibited a typical AKAP3 staining pattern in the head and principal piece but not the midpiece (Fig. 1A, compare staining in WT panel with RLKO and RKO panels; WT staining has been published previously [4]); additionally, AKAP3 levels in single-knockout animals were equal to that of WT sperm (Fig. 1B, compare WT with RLKO and RKO). However, sperm from double-knockout animals had clear defects in the principal piece (Fig. 1A, green arrow in phase image of DKO panel points to areas of thinning and shredding; see also Supplemental Fig. S3 for more examples of DKO principal piece defects). Additionally, AKAP3 staining was undetectable in the flagella (Fig. 1A, DKO panel), in accordance with a major reduction in overall AKAP3 levels as measured by Western blot analysis (Fig. 1B, compare WT with DKO). Expression levels in DKO spermatozoa of other proteins, including another major FS protein (AKAP4), a protein expressed in ODF2, and a major axonemal protein (tubulin), remained relatively unchanged (Western blots in Fig. 1B, diagram of sperm tail principal piece cross section in Fig. 1C). These data suggest that major sperm cytoskeletal structures are present in double-knockout sperm, despite the visible defect and loss of AKAP3 from the FS. Interestingly, the level of AKAP3 in the soluble fraction of DKO testes lysates was normal, but it was significantly reduced in the insoluble fraction (Fig. 1D). Because the FS is a highly insoluble structure, these data support the supposition that although AKAP3 is produced in the testes, it is not successfully incorporated into the FS during spermatogenesis when both ROPN1L and ROPN1 are missing. Collectively, these data suggest that one function of R2D2 proteins is the proper assembly and/or maintenance of the FS via binding to AKAP3.

ROPN1L and ROPN1 Compensate in the Testes and Sperm of Opposing Knockouts Through Increased Protein Levels and Additional Localization

Although there was clearly a loss of AKAP3 and associated structural defects in the principal pieces of DKO sperm, the single-knockout animals (RLKO and RKO) produced sperm with normal expression levels of all proteins examined and without visible structural defects (Fig. 1, B and A, respectively). Because ROPN1L and ROPN1 not only share an AKAP-binding domain but are also 39% identical, we hypothesized that the lack of defects in single-knockout spermatozoa is due to cross compensation. To test this hypothesis and to further define whether potential compensation occurs via increased expression, additional localization, or both, we examined expression of ROPN1L in ROPN1-knockout testes, epididymides, and caudal sperm (and vice versa). We fixed testes and epididymides from WT, ROPN1L-knockout, and ROPN1-knockout mice in formalin, embedded the tissues in paraffin, sectioned them, and stained them with rabbit polyclonal antibodies against ROPN1L and ROPN1. In WT testes, ROPN1L is expressed in pachytene spermatocytes, similar to axonemal proteins, such as dynein and radial spoke proteins [36]. ROPN1L was detected in the flagella of elongated spermatids, but as the sperm matured (heads move towards the center lumen), ROPN1L staining was reduced (Fig. 2A, compare black and green arrows in testes for the WT panel). However, in ROPN1-knockout mice, ROPN1L staining remained visible in mature testicular sperm (Fig. 2A, green arrow in the RKO panel). More strikingly, there was little or no ROPN1L staining of epididymal sperm in WT animals, whereas in ROPN1-knockout epididymides there was clear flagellar staining in caput epididymal sperm (Fig. 2B, RKO panel). This indicates that additional ROPN1L is being expressed in a new location (most likely the outer FS) to compensate for the loss of ROPN1 in RKO sperm. Because alterations in immunohistochemistry staining levels are not always indicative of changes in protein expression levels, we

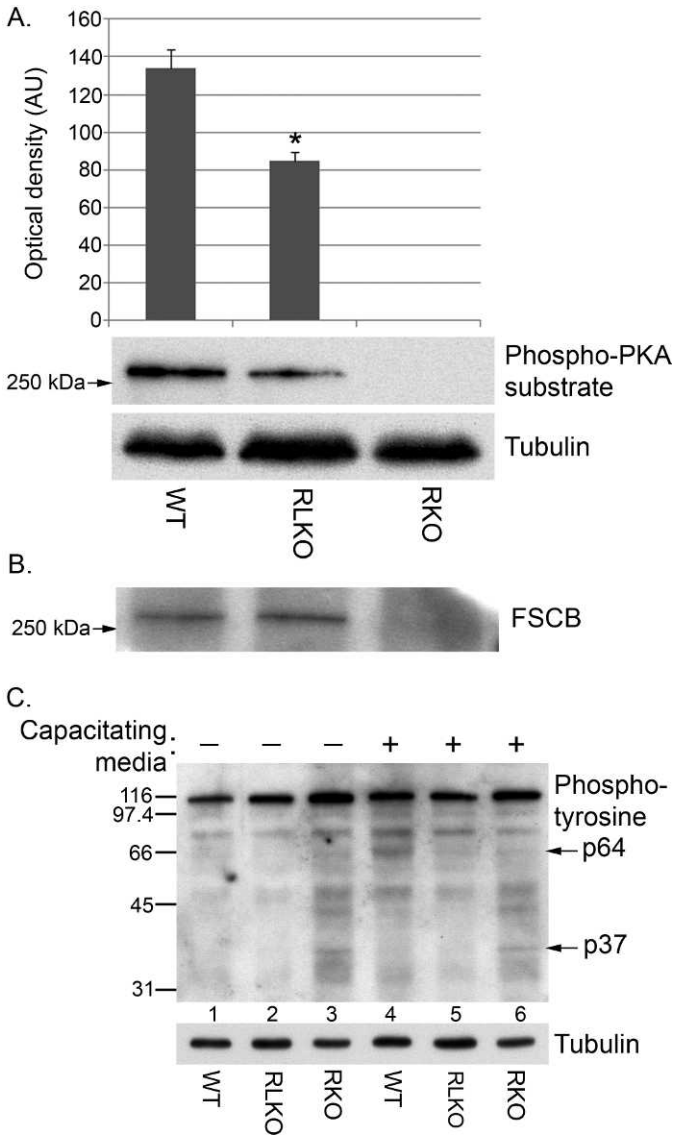

FIG. 4. PKA and tyrosine phosphorylation of caudal epididymal sperm proteins is altered in the absence of ROPN1L and ROPN1. Caudal epididymal spermatozoa were isolated from WT, RLKO, and RKO mice in noncapacitating M2 media (capacitating media [–], lanes 1–3) or capacitating M16 media (capacitating media [+], lanes 4–6) and were incubated for 60 min at 37°C/5% CO_2. Sperm were then collected, counted, and lysed in boiling SDS sample buffer prior to SDS-PAGE, transfer, and Western blot analyses. In **A**, rabbit polyclonal antibody that detects substrates phosphorylated by PKA was used to look for alterations in PKA signaling, and differences were quantitated by densitometric analyses with ImageJ. Error bars are ± SEM. Statistical significance is indicated by $^*P < 0.05$. In **B**, Western blot analysis using goat polyclonal antibody to FSCB, and in **C** using mouse monoclonal antibody 4G10, which detects proteins phosphorylated on tyrosine residues. Tubulin Western blot analyses were performed as loading controls. These pictures are representative of at least three independent experiments, each using unique sets of animals.

also performed Western blot analysis of ROPN1L and ROPN1 expression in spermatozoa. Mirroring immunohistochemistry data, there was a large increase in ROPN1L in ROPN1-knockout sperm (Fig. 2C, compare lanes 1 and 3 of ROPN1L panel).

ROPN1 expression in WT mice does not start until the round spermatid stage, similar to other FS proteins like AKAP3 and AKAP4 [36]. ROPN1 staining was detectable in the flagella of sperm at all stages of development in the testis and epididymis (Fig. 2, A and B). In ROPN1L-knockout testes, there appears to have been a general increase in ROPN1 staining intensity (suggesting a possible increase in expression), particularly at earlier stages of spermatogenesis (Fig. 2A, compare lower WT and RLKO panels). This is consistent with the increase in ROPN1 levels seen by Western blot analysis in the testes (published previously in Fiedler et al. [33] and included here in Supplemental Fig. S4) but not the sperm (Fig. 2C). Together, these data indicate that ROPN1L and ROPN1 compensate for each other in the absence of the opposing protein to maintain AKAP3 incorporation, and thus the structural integrity of the principal piece of single-knockout spermatozoa.

To determine whether the other R2D2 proteins are compensating for loss of ROPN1/ROPN1L in double-knockout mice we also performed CABYR and SPA17 Western blot analyses using WT and double-knockout testes. Results indicate that there may be a very slight increase in the level of higher-molecular weight isoforms of CABYR in DKO testis lysates (Supplemental Fig. S5, compare lanes 1 and 2 of upper panel; note that CABYR has six isoforms that can all be posttranslationally modified, leading to a wide range of bands). It appears that SPA17 may increase slightly in the pellet fraction of DKO testes compared with WT testes (Supplemental Fig. S5, compare lanes 3 and 4 of the middle panel) and decrease slightly in the lysate fraction (Supplemental Fig. S5, compare lanes 1 and 2 of the middle panel), indicating a change in solubility of the protein that could be due to increased incorporation into the insoluble FS in DKO sperm.

Motility Parameters Are Impaired in ROPN1-Knockout Spermatozoa

We have previously published observational data indicating that ROPN1L-knockout sperm appear to exhibit motility analogous to that of WT sperm, whereas ROPN1-knockout sperm exhibit varying degrees of reduced motility. In accordance with these observations, it was shown that ROPN1L-knockout mice had normal fertility, whereas ROPN1-knockout male mice were subfertile—only 40% of males produced litters, and those litters had significantly fewer pups than WT litters [33]. To further define the motility defect in ROPN1-knockout sperm, and also to determine whether there are more subtle motility defects in ROPN1L-knockout sperm that were not visible in our observational analyses, we performed CASA. Because double-knockout sperm are completely immotile (likely because of structural defects in the principal piece, as discussed above), we omitted them from these analyses. As expected, ROPN1-knockout sperm showed significant impairment, particularly in progressive motility, which was approximately half that of WT sperm. In contrast, ROPN1L-knockout sperm did not exhibit significantly lowered progressive motility compared with WT sperm, although there were reductions in VAP, straight-line velocity (VSL), curvilinear velocity (VCL), beat-cross frequency (BCF), straight-line velocity (VSL), and linearity (LIN). Of note, none of these reductions was as great as those seen in ROPN1-knockout sperm (Fig. 3), and in fact VAP, VSL, VCL, STR, and LIN in ROPN1 knockouts were all significantly lower ($P < 0.05$) than ROPN1L knockouts.

PKA-Substrate and Protein Tyrosine Phosphorylation Patterns Are Altered in the Absence of ROPN1L or ROPN1

Because ROPN1L and ROPN1 can both occupy the PKA-binding domain present on the FS AKAPs (AKAP3 and

AKAP4), we hypothesized that the absence of the R2D2 proteins would cause alterations in PKA signaling. PKA-dependent increases in protein tyrosine phosphorylation are a hallmark of normal sperm capacitation processes, during which a spermatozoan acquires the ability to fertilize an egg [37]. Therefore, in order to begin to elucidate the mechanism(s) by which sperm motility and male fertility are impaired in ROPN1L- and ROPN1-knockout mice, we examined patterns of PKA-substrate and protein tyrosine phosphorylation in WT, ROPN1L-knockout, and ROPN1-knockout spermatozoa in both noncapacitating and capacitating media.

Mirroring results seen in the motility analyses above and in accordance with previously published fertility data, defects were seen in both ROPN1L and ROPN1 sperm, but, again, alterations in ROPN1 sperm were more significant than those in ROPN1L sperm. Western blot analyses of capacitated caudal epididymal sperm using an antibody that detects phosphorylation of PKA substrate revealed a phospho-protein band that runs slightly above the 250-kDa marker in WT sperm. This band was reduced in intensity by approximately 37% in ROPN1L-knockout sperm ($P < 0.05$) and was absent in ROPN1-knockout sperm (Fig. 4A). FSCB is a 270-kDa sperm protein localized in the FS that has recently been identified as a direct PKA substrate during capacitation [30, 35, 38]. To investigate whether the PKA substrate that is seen to be phosphorylated in Figure 4A could be FSCB, and also to determine whether the decrease in phospho-bands is due to decreased phosphorylation levels or, alternatively, due to decreased FSCB expression, we performed Western blot analyses with FSCB antibody. The FSCB bands migrated at a molecular weight identical to those in the phospho-PKA substrate Western blots (compare upper blot in Fig. 4A with FSCB bands in Fig. 4B). Combined with coimmunoprecipitation results presented by Liu et al. [38] that identify FSCB as a PKA-phosphorylated substrate during sperm capacitation, our data strongly suggest that the identity of the p270 PKA-phosphorylated bands we detected is FSCB (although immunoprecipitation followed by mass spectrometry analysis would be necessary to prove this point). Additionally, these data show that FSCB was expressed at normal levels in ROPN1L-knockoutsperm, indicating that the decrease in cross-reaction with the phospho-PKA antibody was due to a decrease in the level of phosphorylation (Fig. 4B, compare WT with RLKO). In contrast, the FSCB band in ROPN1-knockout sperm was greatly reduced (Fig. 4B, compare WT with RKO), Assuming that p270 is indeed FSCB, the absence of a band in the phospho-PKA substrate Western blot in RKO sperm was primarily due to decreased FSCB levels rather than a reduction in PKA phosphorylation of the protein.

To determine whether the changes seen in PKA-phosphorylated p270 were correlated with alterations in downstream capacitation-associated protein tyrosine phosphorylations, we performed Western blot analyses using the monoclonal 4G10 antibody, which detects tyrosine-phosphorylated sites. These analyses revealed several differences in sperm from mice lacking ROPN1L or ROPN1 compared with WT sperm, which are shown in a representative blot in Figure 4C. First, an unidentified protein of approximately 64 kDa that became highly phosphorylated during in vitro capacitation in WT sperm appeared to be only slightly elevated in RLKO and RKO sperm (Fig. 4C, compare bands at p64 arrow). Second, irregularly elevated basal tyrosine phosphorylation of many proteins (arrow in Fig. 4C points to p37 as one example) was exhibited in ROPN1-knockout caudal epididymal sperm that were incubated in noncapacitating media (Fig. 4C, upper panel; compare WT and RLKO lanes with RKO in noncapacitating media [lanes 1–3]). Finally, unlike WT sperm, RKO sperm did not respond to incubation in capacitating media with any further increases in tyrosine phosphorylation (Fig. 4C, compare changes between WT [−] and [+] capacitating media [lanes 1 and 4] with lack of changes between RKO [−] and [+] [lanes 3 and 6]). In contrast, ROPN1L KO sperm did respond to incubation in capacitating media with increases in tyrosine phosphorylation, although the increases were less robust than WT and largely excluded p64 phosphorylation (as discussed above; Fig. 4C, compare changes between WT [−] and [+] capacitating media [lanes 1 and 4] with changes between RLKO [−] and [+] [lanes 2 and 5]). These data demonstrate significant signaling alterations in both ROPN1L- and ROPN1-knockout sperm, although defects were greater in the absence of ROPN1 compared with ROPN1L. The absence of PKA-phosphorylated p270 coupled with improper protein tyrosine phosphorylation under noncapacitating conditions is likely part of the mechanism that contributes to the decreased motility and fertility of ROPN1-knockout male mice.

DISCUSSION

The FS is a flagellar cytoskeletal structure unique to sperm that surrounds the outer dense fibers and axoneme in the principal piece of the flagella, providing support and flexibility to the flagella. Its primary components are AKAP3 and AKAP4, which suggests that the FS also affects flagellar beating via the scaffolding of signaling pathways necessary for motility. ROPN1 is also present in the FS, and it binds to AKAP3, which in turn binds AKAP4 [5, 13, 15]. AKAP3 levels are greatly reduced or absent in the flagella of our double-mutant sperm, but not in single mutants. Taken together with the thinning and shredding of the principal piece in these sperm, we believe that double-knockout (ROPN1L and ROPN1) sperm lack a fully formed FS. Interestingly, ROPN1 knockout mice do not appear to share the FS phenotype, although they do exhibit a significant reduction in progressive motility (accompanied by significant alterations in VAP, VSL, VCL, BCF, STR, and LIN). Although ROPN1L and ROPN1 are 39% identical, they do not have any homology in the RHPN1-binding region of ROPN1. One hypothesis that fits these data is that in ROPN1-knockout mice, ROPN1L is able to compensate for the R2D2 function of ROPN1 (binding AKAPs), but it is not able to bind RHPN1 and anchor the RHO signaling pathway to regulate motility. If this is true, it would indicate that in sperm, ROPN1 participates in the proper assembly of the FS via (R2D2 domain-mediated) interactions with AKAPs. Adding weight to this theory, AKAP4-knockout sperm display thinning and shredding of the principal piece similarly to double-mutant sperm, which was identified by further analysis to be due to incomplete FS formation [10]. Our data showing normal flagellar morphology and both increased protein levels and new localization of ROPN1L in mature ROPN1 knockout sperm (to compensate for the loss of ROPN1) also support the hypothesis that R2D2-AKAP interactions are critical for FS integrity. It remains to be determined whether increased ROPN1L expression levels are the result of additional protein production or stabilization of the protein through new interactions with AKAPs in the FS.

The normal testicular development, spermatogenesis, and counts of caudal epididymal sperm in the absence of both ROPN1L and ROPN1 indicate that these R2D2 proteins do not play an essential role in these developmental processes. However, as we have previously published, ROPN1L-knockout mice produce a very small amount of ROPN1L (protein expression is knocked down greater than 98% but not

completely knocked out), so we cannot entirely rule out the possibility that this small amount of ROPN1L protein is sufficient to perform a critical developmental function in double-knockout animals [33]. Additionally, small alterations in the levels and solubility of the R2D2 proteins CABYR and SPA17 in double-knockout testes may be indicative of compensation for some ROPN1/ROPN1L-dependent developmental process. We also speculate that these alterations may reflect a role for CABYR and/or SPA17 in maintaining AKAP4 levels in the absence of ROPN1/ROPN1L, which struck us as curious given the nearly complete loss of AKAP3 in DKO sperm.

Although it is widely accepted that cAMP-dependent PKA signaling plays critical roles in sperm motility, capacitation, and acrosome reaction, very little is known about the identities of PKA substrates in sperm, how phosphorylation changes their activities, or the role of these substrates in subsequent processes [5]. Recently, two separate groups have characterized a 270-kDa sperm protein that appears to be a direct target for PKA phosphorylation both in vitro and in vivo during capacitation, with one group identifying the protein as FSCB [30, 35, 38]. CABYR, like ROPN1, is an R2D2 protein located in the FS; during in vitro sperm capacitation it is phosphorylated on both tyrosine and serine/threonine residues [31, 32, 39, 40]. Like the regulatory subunits of PKA, the R2D2 proteins form homodimers that bind to AKAPs via their shared dimerization/docking domains. It is not known whether either the PKA regulatory subunits or the R2D2 proteins form heterodimers with each other in vivo, although there is some indication (from yeast-two hybrid) that ROPN1 may interact directly with both CABYR and SPA17 (another R2D2 protein) in vitro [28, 31, 39]. The 37% reduction of Ser/Thr phosphorylation of a 270-kDa protein (perhaps FSCB) in capacitated sperm that lack ROPN1L indicates that PKA signaling in these sperm is altered. Downstream tyrosine phosphorylation in these sperm in response to in vitro capacitation is present, but it is generally less robust than WT phosphorylation. However, despite these changes, motility impairment in RLKO sperm is minor (slight changes in various parameters but no reduction in progressive movement) and males retain normal fertility.

In contrast to mice lacking ROPN1L, ROPN1-knockout males exhibit a significant reduction in fertility [33]. Sperm lacking ROPN1 fare worse than those lacking ROPN1L in several ways. First, more motility parameters are altered significantly; in particular, progressive motility is impaired in RKO sperm. Second, rather than simply exhibiting a reduction in PKA phosphorylation of what appears to be FSCB, sperm lose expression of the protein. FSCB is located primarily on the outer surface of the FS and is incorporated late in FS formation. Previously published studies indicate that ROPN1 and CABYR (which binds FSCB) are part of a shared complex in vivo, although whether the interaction between the two is direct or mediated through AKAPs is not certain [30, 38, 39]. Our data indicating that FSCB is lost in sperm lacking ROPN1 whereas expression of the major sperm AKAPs 3 and 4 is unchanged suggest that either ROPN1 binds directly to FSCB to tether it to the FS, or it binds to CABYR directly (perhaps through heterodimerization) to accomplish the same.

The final manner in which ROPN1-knockout sperm are more highly compromised than those that lack ROPN1L is in alterations to tyrosine phosphorylation. RKO sperm exhibit increases in protein tyrosine phosphorylation in M2 media, which does not contain sufficient sodium bicarbonate to support capacitation. This increase in tyrosine phosphorylation is similar (though not identical) to that seen in capacitated WT sperm. Additionally, incubation of these RKO sperm in M16 media (which supports capacitation) does not increase protein tyrosine phosphorylation beyond that seen in M2 media, as it does in WT and RLKO sperm, perhaps because phosphorylation is already at maximum levels in the absence of capacitation. This premature phosphorylation in the absence of ROPN1 hints at a negative regulatory role for the protein, perhaps in functioning to regulate PKA signaling in sperm. The mechanism of this possible regulation may be as simple as competitive binding (i.e., ROPN1 blocks RII access to certain AKAPs until it is appropriate for PKA to be localized and activated). Alternatively, ROPN1 (or ROPN1L) may serve as a negative regulatory subunit to an enzyme—perhaps a Ser/Thr phosphatase that dephosphorylates proteins such as FSCB when active.

It has long been known that spermatids and early epididymal (i.e., caput) sperm lack the characteristic cAMP responses of caudal sperm (hyperactivation, protein tyrosine phosphorylation, acrosome reaction) [41–46]. However, the reasons for this, as well as the mechanisms involved in the acquisition of cAMP sensitivity during epididymal passage are incompletely understood. Discovery of the R2D2 proteins, which share the AKAP-binding site of PKA, raises the intriguing possibility that they are regulating PKA activity by preferentially binding AKAPs during spermatogenesis, not only participating in FS assembly as discussed above, but also thus blocking PKA from being anchored in prime localization for activation. This type of mechanism of PKA regulation has previously been described for AMY-1, which binds the amphipathic helices of AKAP1 and AKAP8 to block RII binding and suppress PKA activity [47]. In future studies we will seek to characterize the regulation of interactions between sperm AKAPs, PKA, and R2D2 proteins, as well as examine the kinetics of cAMP responsiveness in our mutant sperm during epididymal maturation.

Although few genetic factors have been directly linked to human male infertility, AKAP3 and AKAP4 gene deletions were found in men with dysplasia of the FS, which causes severe asthenozoospermia (reduced motility) or total immotility [48–50]. Of note, AKAP3 and AKAP4 protein expression may be normal in at least some of these patients, raising the possibility that there are other AKAP-binding proteins involved in this condition [51]. It is also interesting to note that even though the axonemes in cilia and sperm flagellum are very similar structures, the signaling pathways that regulate their motility are unique. Work in our lab looking at airway cilia has demonstrated that loss of ROPN1L reduces basal ciliary motility, whereas loss of ROPN1 has no effect [33]. Because ROPN1 appears to have a critical role in regulating sperm function but no role in cilia, it could be especially interesting as a potential target for a male contraceptive.

AKAPs have been shown to be critical components of the biochemical machinery controlling a variety of physiological functions. We have shown that AKAPs play a critical role in regulating sperm functions; indeed, adding peptides that disrupt AKAP interactions (Ht31) can arrest sperm motility [18]. In addition, we have illustrated a previously undefined function for AKAPs in the sperm flagellum, demonstrating that the amphipathic helix domain of several AKAPs binds the R2D2 proteins ROPN1L and ROPN1 [6]. The exciting discovery of proteins that contain a conserved RII-like AKAP-binding domain necessitates rethinking of the AKAP model in sperm. Results presented in the present study demonstrate for the first time that loss of R2D2 protein function alters PKA-dependent signaling processes. Therefore, although cAMP/PKA signaling is crucial for regulation of sperm function, a new model needs

to account for the added complexity of R2D2/AKAP protein interactions. Just how complicated is this AKAP scaffolding system? PKA, CABYR, SPA17, ROPN1L, and ROPN1 are all present in sperm, may heterodimerize with each other, and share the same AKAP binding site (which is present in both AKAPs 3 and 4 in the FS). ROPN1 is not only associated with CABYR and FSCB (which are both likely involved in the calcium signaling pathways in sperm), but it is also linked to the RHO signaling pathway via RHPN1 binding [6, 14, 27, 30, 33, 38, 39]. The results presented in this manuscript coupled with our ongoing studies of these R2D2 proteins repeatedly place them at the nexus of the signaling pathways known to be most critical for sperm motility, and thus normal male fertility. The incredible intricacies of these biochemical mechanisms will surely require much work to fully unknot, but given the growing rate of (largely idiopathic) male infertility, an improved understanding of these pathways seems well worth the effort.

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