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Letter to the Editor

CRISPR/CAS9-mediated amino acid substitution reveals phosphorylation residues of RSPH6A are not essential for male fertility in mice[†]

Dear Editor,

Capacitation, the physiological changes that occur in mammalian spermatozoa in order to gain the ability to fertilize the egg, is an event that occurs only after spending several hours in the female reproductive tract. Although it is well known that phosphorylation events that start with protein kinase A (PKA) are essential for capacitation [1], downstream phosphorylated molecules and their functions are not well understood. Using phospho-proteomic and subsequent biochemical analyses, we previously identified phosphorylation residues (S17 and S20) of radial spoke head 6 homolog A (RSPH6A) that were associated with capacitation [2]. RSPH6A is an evolutionarily conserved and testis-enriched protein, and thought to be a component of the radial spokes in the flagellar axoneme [2–4]. In a previous study, we generated *Rsph6a* knock-out (KO) mice to analyze its function; however, because *Rsph6a* KO spermatozoa exhibited abnormal flagellar formation [4], this hampered the analysis of RSPH6A in sperm capacitation. To overcome this, we substituted two serine residues of mouse RSPH6A to alanine using the CRISPR/CAS9 system.

We electroporated a crRNA/tracrRNA/Cas9 ribonucleoprotein and a single-stranded oligonucleotide containing the S17A and S20A mutations into the fertilized eggs (Figure 1A and Supplementary Methods). In addition to amino acid substitutions, several silent mutations were introduced to prevent the mutated region from being recognized and recut by the ribonucleoprotein (Figure 1A). Of the 97 fertilized oocytes that were electroporated, 62 two-cell embryos were transplanted into the oviducts of three pseudopregnant female mice. Ten pups were born and one of the ten pups had the intended mutation. Subsequent breeding resulted in a knock-in (KI) mouse with the S17A and S20A mutations, which was confirmed by PCR and *NarI* digestion (Figure 1B) as well as sequencing analysis (Figure 1C). Although point mutations could lead to decreased or diminished amounts of targeted protein [5, 6], a comparable amount of RSPH6A in spermatozoa collected from the cauda epididymis was observed in *Rsph6a*^{KI/KI} mice with immunoblotting (Figure 1D).

In contrast to impaired flagellar formation observed in *Rsph6a* KO spermatozoa [4], no abnormal morphology was observed with phase-contrast microscopy in spermatozoa collected from the cauda epididymis of *Rsph6a*^{KI/KI} mice (Figure 1E). We then mated *Rsph6a*^{KI/KI} males with three wild-type females for 2 months. No significant difference was observed between *Rsph6a*^{WT/WT} and *Rsph6a*^{KI/KI} mice (Figure 1F). Next, we analyzed sperm motility with a computer-assisted sperm analysis system. The percentage of motile spermatozoa was comparable between *Rsph6a*^{WT/WT} mice (10 min: $87.9 \pm 4.4\%$, 2 h: $75.1 \pm 11.2\%$, $n = 4$ males) and *Rsph6a*^{KI/KI} mice (10 min: $84.5 \pm 1.5\%$, 2 h: $70.2 \pm 5.0\%$, $n = 4$ males). Furthermore, no significant differences were observed in velocity parameters of non-capacitated (10 min) and capacitated (2 h) spermatozoa between *Rsph6a*^{WT/WT} and *Rsph6a*^{KI/KI} mice (Figure 1G). Taken together, these results indicate that S17 and S20 phosphorylation are not essential for sperm formation, motility, and male fertility.

Super-resolution imaging with stochastic optical reconstruction microscopy suggests that the catalytic PKA subunit can be localized in the sperm axoneme [7]. RSPH6A localized in the axoneme was identified as a PKA substrate candidate using phospho-proteomic analysis [2]; however, this study reveals that S17 and S20 of RSPH6A are not essential for male fertility. As is the case for RSPH6A [4], disruption of axonemal proteins often leads to impaired flagellar formation [8], which makes it challenging to analyze their function in mature spermatozoa using a straight KO approach. To overcome this problem, amino acid substitution can be introduced with the CRISPR/CAS9 system, which can be conducted more easily in a shorter period of time than the conventional method that uses homologous recombination in embryonic stem cells [5]. In addition, CRISPR/CAS9 enables us to manipulate the genome without introducing selection cassettes such as drug resistance. Considering the difficulty in manipulating the genome of spermatozoa and eggs in vitro, CRISPR/CAS9-mediated amino acid substitution in an organismal level will be the future of the reproduction field and could uncover the hidden mysteries of PKA downstream effectors in sperm capacitation.

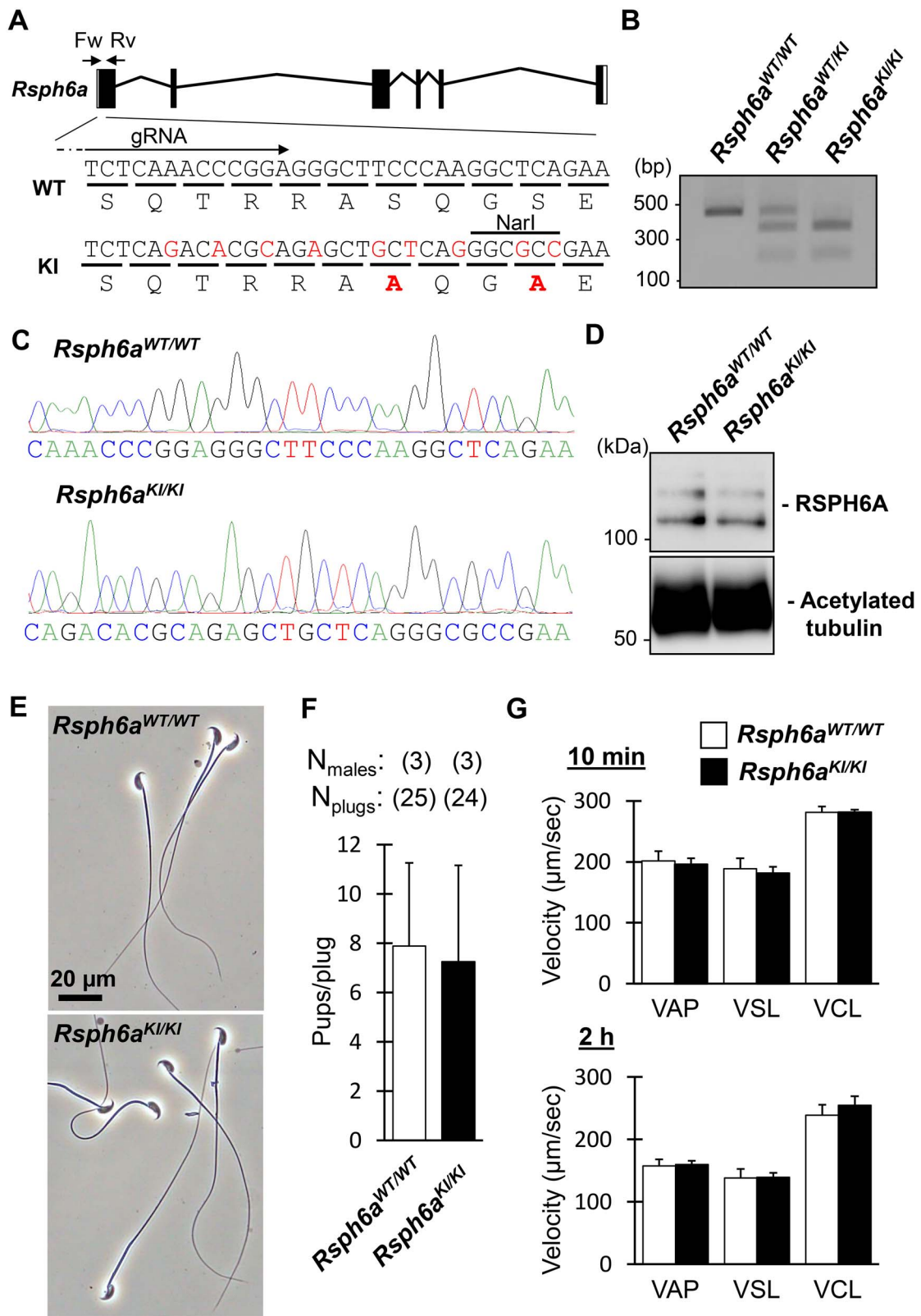


Figure 1. Phosphorylation of RSPH6A S17 and S20 is not essential for male fertility. **A.** CRISPR/Cas9 targeting scheme. gRNA was designed to target Exon1. Primers (Fw, Rv) used for genotyping are shown. Mutated nucleotides and amino acids are written in red. **B.** Genotyping of *Rsph6a*^{KI/KI} mice using NarI. **C.** Wave pattern sequence of *Rsph6a* confirming the mutated allele. **D.** Protein expression of RSPH6A in cauda epididymal spermatozoa. Acetylated tubulin as a loading control. **E.** Observation of spermatozoa obtained from cauda epididymis. **F.** The fertility of *Rsph6a*^{KI/KI} male mice. **G.** VAP (average path velocity), VSL (straight line velocity) and VCL (curvilinear velocity) were analyzed 10 min (non-capacitated) and 2 h (capacitated) after sperm incubation; *n* = 4 males each for *Rsph6a*^{WT/WT} and *Rsph6a*^{KI/KI} mice.

Supplementary data

Supplementary data is available at *BIOLRE* online.

Author Contributions

H.M, P.E.V, and M.I designed research; H.M, and F.A performed research; H.M, F.A, P.E.V, and M.I analyzed data; and H.M, F.A, P.E.V, and M.I wrote the paper.

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Conflict of interest

Dr. P.E.V. owns equity interest in Sperm Capacitation Technologies Inc. a company with goals in improving assisted reproductive technologies. All other authors declare that they have no conflict of interest with the contents of this article.

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