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LETTER TO THE EDITOR

Sperm Biology

Ultrastructural changes and asthenozoospermia in murine spermatozoa lacking the ribosomal protein L29/HIP gene

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Dear Editor,

We present here the first report linking a specific gene, ribosomal protein L29 (*Rpl29*), also known as *Hip* (heparin/heparan sulfate interacting protein), to the mammalian sperm flagellar morphological anomaly termed the “dag” defect. In addition, we show that loss of *Rpl29* invariably results in low sperm motility with infertility accompanying this abnormality.

Ribosomal protein L29 is one of 79 *Rps* that regulate the efficiency of protein synthesis. Expressed in a variety of tissues, including the testis, it is a constituent of membrane-associated and cytoplasmic translationally-active ribosomes.¹ It has been suggested that along with *Rpl10*, it participates in the process of coupling of the 40S and 60S subunits during translation initiation.² Abnormalities in expression of *RPs* are known to manifest in various ribosomopathies.³ However, neither human *RP* spontaneous mutations nor specific gene-targeting strategies reported in mice, have been previously associated with sperm or fertility abnormalities.

Previous work characterizing a loss-of-function mutation of the murine *Rpl29* showed that it leads to global growth defects and male infertility.¹ Null females are markedly subfertile with reduced litter size and significant postnatal fatality.¹ *Rpl29*^{-/-} (null) males, generated as described,¹ were fed a high-energy diet to alleviate growth deficiencies and maximize reproductive competence. The diet successfully restored the body weights of the animals, maintained under husbandry procedures approved by the Institutional Animal Care/Use Committee at the University of Delaware. A Student's *t*-test showed no significant difference between the mean body weights of the nulls and that of wild-type (WT) or *Rpl29*^{+/-} heterozygotes (Table 1). Fifteen of these null males were used for fertility studies, each housed with three proven-fertile WT females for at least three cycles each. In ~100 matings (detected by vaginal plugs) no pregnancies occurred, confirming that infertility in *Rpl29* nulls males is not specifically a result of growth retardation.

To determine the basis of the infertility, we used 14 WT controls, 18 *Rpl29*^{+/-} (Het), and 16 body weight-restored *Rpl29*^{-/-} (all age-matched) on C57BL6 background, for andrological and ultrastructural investigations. Caudal epididymal sperm were recovered from sexually mature males, as described.⁴ Student's *t*-tests showed no statistical difference between nulls

or Hets and WT (Table 1) for testicular weights, a measure of total sperm production. However, in analyses of a minimum of 200 sperm per animal, gross motility (80% reduced) and progressive motility (91% reduced) were significantly ($P < 0.001$; Table 1) impaired in null sperm, compared to WT. The Het sperm showed 35% and 32% decrease ($P < 0.001$) in gross and progressive motility ($P < 0.001$), compared with WT. In addition, when sperm were acrosome-reacted, using the physiologically relevant progesterone-induced enhanced assay, nulls ($P < 0.001$) and Hets ($P < 0.01$) showed significantly lower positive numbers than WT (Table 1). Thus the severity of the defects was related to the number of *Rpl29* alleles deleted.

The striking feature of *Rpl29* gene deletion observed in these mice was the morphology of the sperm flagella: ~80% of caudal sperm from each null, and ~40% from Het sperm exhibited flagellar abnormalities characteristic of “dag” defects^{5,6} (Figure 1a), significantly ($P < 0.001$) different from the 3% seen in WT controls (Figure 1a). Despite the very low motility and high incidence of “dag” defects, null sperm were alive, as evident from eosin-nigrosin dye exclusion test (Figure 1b) and normal mitochondrial membrane potential (Figure 1c), both performed as previously described.⁴ Testicular morphology seminiferous tubular architecture and germ cell associations were similar in WT and nulls (data not shown).

Characterization of the “dag” defects (Figure 1d–1q) revealed a wide range of severity, as reported by others.^{5,6} The angle of flagellar bends ranged from acute to mild (Figure 1d), with the tails either tightly enclosed (Figure 1e and 1f) or loosely bound (Figure 1g) within the plasma membrane. Frequent presence of cytoplasmic droplets in nulls (Figure 1h–1j) suggested incomplete epididymal maturation. The usual 9 + 2 organization of the microtubules and outer dense fibers in normal WT sperm (Figure 1m) was often found disrupted in nulls with either missing (Figure 1n–1p) or disorganized elements (Figure 1q). While the flagellar effects could directly account for the motility loss

Table 1: Physiological attributes of WT and *Rpl29* null male mice

Attribute	WT	Het	<i>Rpl29</i> -null
Body weight (g)	26.5±1.3	27.2±0.8	23.6±1.6
Testes weight (mg)	206.2±4.8	191.7±3.6	162.3±28.4
Sperm motility (%)	89.7±2.7	58.2±5.6**	17.6±3.4**
Sperm progressive motility (%)	68.3±1.7	45.8±5.4*	6.2±3.3**
Acrosome reaction (%)	58.9±3.2	46.6±3.5*	4.4±2.2**

Using the *t*-test, ±s.e.m. * $P < 0.01$; ** $P < 0.001$. s.e.m.: standard error of mean; WT: wild-type; *Rpl29*: ribosomal protein L29

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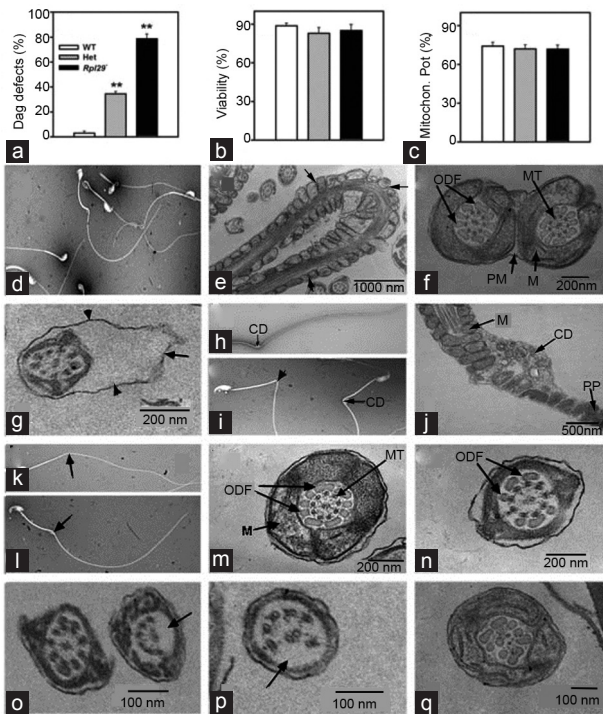


Figure 1: Light and transmission electron microscopy showed that deletion of ribosomal protein L29 gene results in sperm flagellar “dag” defects in mice. (a) The percentages of “dag” defects in both Het and null were significantly greater than that in wild type sperm (** $P < 0.001$), using a t -test, and the cells were alive as evidenced by lack of differences in dye-exclusion viability test. (b) Mitochondrial membrane potential evaluation. (c) The severity and extent of dag defects in null sperm varied from severe to mild kinks in the midpiece. (d) In many of the cells with acute bends in the flagellum, the kinks were enveloped within a distended, but intact, plasma membrane (PM) despite an otherwise well-organized axoneme, evidenced in longitudinal. (e, arrows) Transverse. (f) Sections of flagella giving the appearance of “doublets”; ODF: outer dense fiber, MT: microtubule, M: mitochondria. (g) Occasionally, the PM was found loose (arrowheads) and ruptured (arrow), possibly at the “postdoublet” portion of the principal piece. Often, the “dag” defects were associated with the presence of cytoplasmic droplets (CD, h–j), or were severe enough to appear fractured (i, arrowhead); M: midpiece; PP: principal piece. Most of the sperm with defects, however, exhibited only mild, but characteristically distinct, bends of the mid-piece (k–l). (m) Ultrastructure of normal axonemal architecture of wild-type sperm flagella with their 9 + 2 organization of MT doublets flanked by 9 ODFs inside the mitochondrial helix (M); (n–p) Transverse sections through null sperm principal piece showing disrupted and missing ODFs and MTs (arrows); (q) cross-section of null sperm midpiece showing disorganization and loss of characteristic circular arrangement of ODFs and MTs.

and infertility, it is unclear how they relate to the decrease in acrosome reactions, which were detected after a blind analysis.

Interestingly, the Het males lacking one *Rpl29* allele are indistinguishable from WT in terms of growth and phenotype,¹ except for the “dag” defects, which are seen in ~40% of their sperm. This percentage in the absence of severe effects on fertility is consistent with the report that an adverse effect on fertility requires levels >50% of “dag” defects in the sperm population.⁵ It also suggests that *Rpl29* is haploid-expressed in male germ cells with the lack of transcript sharing during spermiogenesis, such that one-half of the meiotic products are without the transcript, as seen for genes such as *Spam1* (sperm adhesion molecule 1) and *Hyal5*.⁷ Although an array of gene ablations are known to compromise fertility as a collateral consequence, it is likely that *RPL29* may serve an extra-ribosomal sperm-specific function as increasingly extra-ribosomal roles are being reported for a variety of RPs.

It has been reported that fully differentiated testicular sperm do not exhibit “dag” defects which arise at the distal part of the caput and

progressively manifest during transit through the corpus and caudal epididymal regions.⁶ Because even mature sperm contain nearly 3000 messenger ribonucleic acids, including those encoding RPs,⁸ and their mitochondrial 55S ribosomes are able to translate the transcripts,⁹ albeit sparingly, it is conceivable that the architectural foundation for the expression of the abnormality is likely laid during early spermiogenesis where the translational machinery is more robust.

Our study suggests that in clinical idiopathic symptomatic situations,¹¹⁰ a simple semen analysis for “dag” defects could be a useful tool for diagnosing certain ribosomopathies involving abnormal *RPL29* expression. Though a predominance of “dag” defects in semen may not be expected unless *RPL29* is completely ablated, as in the current study, an atypical and consistent occurrence of these defects may be indicative of increased risk for disease.¹¹⁰ Further studies to gain insights in the mechanism by which the loss of *Rpl29* results in the “dag” effect and infertility await the availability of an effective *RPL29* antibody.

AUTHOR CONTRIBUTIONS

RGA first observed “dag” defects, performed all experiments, and interpreted the results; CBK-S generated *Rpl29*-targeted mutant mice and carried out fertility studies; MAS performed acrosome reactions in sperm; PAM-D assisted with light microscopy, provided laboratory facilities and reagents, participated in project coordination, and wrote the letter.

COMPETING INTERESTS

The authors declare no competing interests.

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