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Letter to the Editor
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Letter to the Editor

RNF216 is essential for spermatogenesis and male fertility†

Dear Editor,

Mutations in *Rnf216* have been found in families with Gordon Holmes syndrome, a human disease characterized by hypogonadism and ataxia [1, 2]. This led us to the hypothesis that RNF216 plays essential roles in mammalian reproduction. To investigate the physiological function of *Rnf216*, we first examined the tissue distribution of the RNF216 protein in mice by Western blotting. RNF216 is broadly expressed in various tissues with a high expression in the testis (Figure 1A). During postnatal spermatogenesis, RNF216 expression was detected at 1 week of age and elevated at 2 weeks through adulthood (Figure 1B). To elucidate the function of *Rnf216*, we generated mutant mice harboring a targeted *Rnf216*

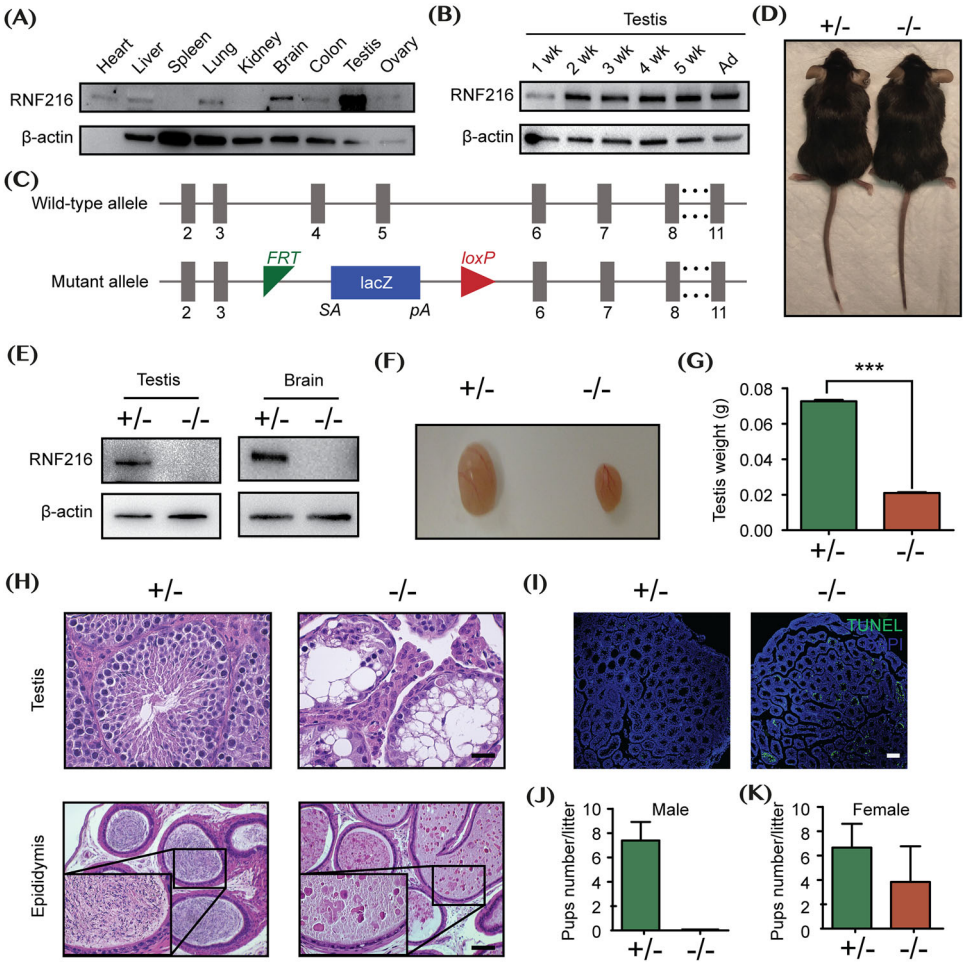

Figure 1. RNF216 is required for spermatogenesis and male fertility. (A), Western blotting showing expression levels of RNF216 in different mouse tissues. β-actin served as a loading control. (B), Western blotting showing expression levels of RNF216 at different stages of testis development. β-actin served as a loading control. (C), *Rnf216* gene targeting strategy. *Rnf216* mutant allele was generated by replacing exons 4 and 5 with a LacZ cassette. (D), Comparison of the body appearance of $Rnf216^{+/-}$ and $Rnf216^{-/-}$ mice. (E), Western blotting showing the absence of RNF216 protein in $Rnf216^{-/-}$ brain and testis. β-actin served as a loading control. (F), Gross morphology of adult $Rnf216^{+/-}$ and $Rnf216^{-/-}$ testes. (G), Testes weight of adult $Rnf216^{+/-}$ and $Rnf216^{-/-}$ mice. ***, $P < 0.001$; paired *t*-test. Error bars represent s.e.m. (H), Hematoxylin and eosin staining of adult $Rnf216^{+/-}$ and $Rnf216^{-/-}$ testes and epididymides. Upper, scale bar indicates 25 μm; lower, scale bar indicates 100 μm. (I), TUNEL assay of testis sections of 3-week-old $Rnf216^{+/-}$ and $Rnf216^{-/-}$ *mice*. Scale bar indicates 100 μm. (J-K), Fertility tests of male (J) and female (K) $Rnf216^{+/-}$ and $Rnf216^{-/-}$ *mice*. Error bars represent s.e.m.

mutation in which exons four and five were replaced by a LacZ cassette (Figure 1C and Supplementary Figure S1A) [3, 4]. *Rnf*216 homozygous mutant mice are viable and grossly indistinguishable from heterozygous littermates (Figure 1D). Moreover, we found that RNF216 was absent in the testis and brain of homozygous mutant mice, indicating that the *Rnf216* mutant allele (*Rnf216*$^{-}$) is a protein null allele (Figure 1E).

Although the body sizes were similar, the testis size of *Rnf216*$^{-/-}$ mice was significantly smaller than that of *Rnf216*$^{+/-}$ mice (Figure 1F-G). This suggests that germ cell development is compromised due to RNF216 deficiency. Histological analysis revealed severe germ cell loss in adult *Rnf216*$^{-/-}$ testes. The seminiferous tubules displayed an array of germ cell degeneration phenotypes, ranging from those with degenerating spermatocytes or round spermatids to Sertoli-cell-only tubules (Figure 1H). As a result, *Rnf216*$^{-/-}$ cauda epididymides lacked normal spermatozoa, but contained cellular debris (Figure 1H). These results indicate that *Rnf216* is a critical gene for spermatogenesis.

We further examined the onset and progression of germ cell degeneration in *Rnf216*$^{-/-}$ mice at different developmental stages of spermatogenesis. At 1 week of age, no gross differences were observed between control and *Rnf216*$^{-/-}$ testes (Supplementary Figure S1B). However, germ cell degeneration of spermatocytes started to occur at around 2 weeks of age and peaked at 3 to 4 weeks (Supplementary Figure S1C-E). Additionally, we found elevated germ cell apoptosis in 3-week-old *Rnf216*$^{-/-}$ testes by the TUNEL assay (Figure 1I), which correlates with the onset of germ cell degeneration. Consistent with this, very few elongated spermatids were generated in *Rnf216*$^{-/-}$ testes. To test the effect of RNF216 deficiency on fertility, we performed a 4-month fertility test for male and female *Rnf216*$^{-/-}$ mice. Results showed that *Rnf216*$^{-/-}$ males were sterile (Figure 1J), but *Rnf216*$^{-/-}$ females were fertile (Figure 1K), indicating that RNF216 is critical for male fertility.

RNF216 is an E3 ubiquitin ligase involving ubiquitination and stability of a spectrum of proteins in different cellular settings [5–9]. We did not observe obvious ataxia disorder in *Rnf216*$^{-/-}$ mice as identified in human Gordon Holmes syndrome. This could be explained by the normal function of OTUD4 in mice, another gene mutation identified in Gordon Holmes syndrome patients [1]. Nonetheless, we cannot rule out that there could be specific neurological defects following detailed neurological and behavioral tests in *Rnf216*$^{-/-}$ mice. Since RNF216 is broadly expressed in various tissues including the brain and the testis, the disruption of spermatogenesis by RNF216 deficiency could be caused by testis-intrinsic defects, testis-extrinsic (hypothalamus-pituitary-gonad axis) defects, or a combination of both. Using LacZ staining we show that in the testis RNF216 is most likely predominantly expressed in meiotic and postmeiotic germ cells and Leydig and Sertoli cells did not appear to have LacZ staining (Supplementary Figure S2). Future in depth characterization of RNF216 expression in wild-type testis using other approaches should be done to validate this finding. Moreover, germ cell-specific ablation of RNF216 in mice will enable to confirm a direct role for germ cell expressed RNF216 in spermatogenesis.

In summary, our findings reveal for the first time the physiological function of *Rnf216* in mice by demonstrating that RNF216 is essential for spermatogenesis and male fertility. Together with *Rnf216* gene mutations identified in men with hypogonadism, these data strongly suggest an important role for RNF216 in human male fertility.

Supplementary data

Supplementary data are available at *BIOLRE* online.

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