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Mice lacking Ran binding protein 1 are viable and show male infertility

Masahiro Nagai [a,b], Tetsuji Moriyama [a], Rashid Mehmood [a], Keizo Tokuhiro [c], Masahito Ikawa [d], Masaru Okabe [d], Hiromitsu Tanaka [c], Yoshihiro Yoneda [a,b,e,*]

[a] Biomolecular Dynamics Laboratory, Department of Frontier Biosciences, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565-0871, Japan
[b] Department of Biochemistry, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan
[c] TANAKA Project, Center for Advanced Science and Innovation, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan
[d] Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan
[e] JST, CREST, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565-0871, Japan

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ABSTRACT

The small GTPase Ran plays important roles in multiple aspects of cellular function. Maximal RanGAP activity is achieved with the aid of RanBP1 and/or presumably of RanBP2. Here, we show that RanBP1-knockout mice are unexpectedly viable, and exhibit male infertility due to a spermatogenesis arrest, presumably caused by down-regulation of RanBP2 during spermatogenesis. Indeed, siRNA-mediated depletion of RanBP2 caused severe cell death only in RanBP1-deficient MEFs, indicating that simultaneous depletion of RanBP1 and RanBP2 severely affects normal cell viability. Collectively, we conclude that the dramatic decrease in "RanBP" activity impairs germ cell viability and affects spermatogenesis decisively in RanBP1-knockout mice.
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1. Introduction

Small GTPase Ran [1] plays important roles not only in fundamental cellular activities (nucleo-cytoplasmic transport, mitotic spindle assembly, and nuclear envelope formation [2–8]), but also in tissue activities such as nerve cell response to axon injury [9]. The asymmetric distribution of RanGTP in the nucleus and RanGDP in the cytoplasm is regulated by nuclear RCC1 (RanGEF) [1] and cytoplasmic RanGAP (=GTPase activating protein) [10]. In addition, however, biochemical analyses have shown that the activity of RanGAP is achieved with the aid of a Ran binding protein, RanBP1 [11]. Cytoplasmic RanBP1 serves as a crucial cofactor for stimulating RanGAP activity [12] by dissociating RanGTP-containing complexes that exit from the nucleus [13] (Fig. S1). Although *Caenorhabditis elegans* and *Drosophila melanogaster* do not have direct homologues of *RanBP1* [6], *Saccharomyces cerevisiae* possesses a *RanBP1* homologue (*Yrb1*) as an essential gene. Temperature-sensitive mutants of *Yrb1* show deficiency in nuclear import and poly A^+ RNA export activity [14,15]. Among identified Ran binding proteins in mammals, only Ran binding protein 2 (RanBP2 [16]; also called Nup358, a giant nucleoporin) contains RanBP1-like domains that show high affinity to Ran, so RanBP2 is biochemically predicted to be the sole functional substitute for RanBP1 [2–8]. In addition, it has recently been demonstrated that the null allele of the mouse *RanBP2* gene causes embryonic lethality [17,18]. Although the functions of RanBP1 have been well investigated at the cellular level [2–8,19,20], its roles at the level of the organism remain to be understood.

2. Materials and methods

2.1. Cell culture

Mouse embryonic fibroblasts (=MEFs) were cultured in DMEM supplemented with 10% FBS, at 37 °C, 5% CO_2.

2.2. Preparation of tissue and cell lysates

Fresh tissues obtained from 12-week old male mice or MEFs were lysed with buffer (4 M Urea, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1 mM EDTA with 10 μg/ml each of leupeptin, aprotinin, and pepstatin), and after centrifugation at 15 000 rpm for 10 min, the supernatant was used.

2.3. Generation of RanBP1-knockout mice

The targeting vector was constructed with the use of pMulti-ND 1.0 vector (a kind gift from Dr. J. Takeda and T. Ijiri) containing the

* Corresponding author at: Biomolecular Dynamics Laboratory, Department of Frontier Biosciences, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamada-oka, Suita Osaka 565-0871, Japan. Fax: +81 6 6879 4609.
E-mail address: yyoneda@anat3.med.osaka-u.ac.jp (Y. Yoneda).

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Neo-resistance gene (*neo*^{r}) as a positive selection marker and diphtheria toxin A chain (DT) as a negative selection marker. The 5′ short arm spanned exon 1 and the 3′ long arm included exon 3 and exon 4. The short and long arms were cloned into the pMulti-ND-1.0 plasmid. The targeting construct was electroporated into D3 embryonic stem (ES) cells. *RanBP1* mutant cells obtained by homologous recombination were identified by PCR and Southern blot analysis. Targeted ES cell clones were microinjected into C57BL/6 blastocists to generate chimeric mice. Chimeras were then mated to C57BL/6 mice for germline transmission.

2.4. Southern blot and PCR analysis

Southern blot hybridization was used to confirm short-arm recombination in the embryonic stem cells using standard protocols. Specifically, targeted clones were identified using a 5′ external probe, which detects the change of a 9.6 kb WT BamHI/NdeI fragment to a novel 3.5 kb BamHI/NdeI fragment. To confirm long-arm recombination, long arm PCR was performed using LA-Taq™ (TaKaRa) according to the manufacturer's instructions, with the shared primer (see Fig. 1A) **1**(5′-GTTTCTCCTCAGACTTCTCTTCAGCCTCC-3′) and three pairs of primers. **2**(5′-TCTGTTGTGCCCAGTCATAGCCGAATAGCC-3′) to amplify a ~8 kb PCR product, **3**(5′-TTGGCGCCTACCGGTGGATGTGGAATGTGTG-3′) to amplify a ~7.6 kb PCR product, **4**(5′-GCTACTTCCATTTGTCACGTCCTGCACGACG-3′) to amplify a ~7.7 kb PCR product, respectively.

PCR products were resolved on 1% agarose gels.

2.5. Genotyping of the offspring was performed to detect the WT and mutant alleles

For genotyping PCR (see Fig. 1A), the shared primer (3) (5′-ACCCATTGATGTTTGAGTGTGTAGGAGTGTC-3′) was designed upstream of the deleted region. This primer can pair with WT allele-specific primer (2) (5′-CTCATCTGCATTCTCTGTGGAAGTATCATGG-3′) to amplify a ~300 bp PCR product or with the targeted allele-specific primer (1) (5′-GCCTTCTATCGCCTTCTTGACGAGTTCTTC -3′) in the *neo* cassette to amplify a ~430 bp fragment from the mutant allele. PCR was performed with rTaq™ (TaKaRa) according to the manufacturer's instructions, and PCR products were resolved on 2% agarose gels.

2.6. Continuous breeding assay

(Male) 8-week old *RanBP1*$^{+/-}$ and $^{-/-}$ male mice were mated with two wild-type (=WT) female mice and the cumulative number of progeny was counted for the period indicated.

(Female) 8-week old *RanBP1*$^{+/-}$ and $^{-/-}$ female mice were mated with three RanBP1$^{+/-}$ male mice and the cumulative number of progeny was counted for the period indicated.

2.7. Histology of testis sections

Fresh mouse testis and epididymis were fixed in 10% formalin neutral buffer solution, embedded in paraffin. Sections (7 μm) were stained with Hematoxilin-Eosin (HE).

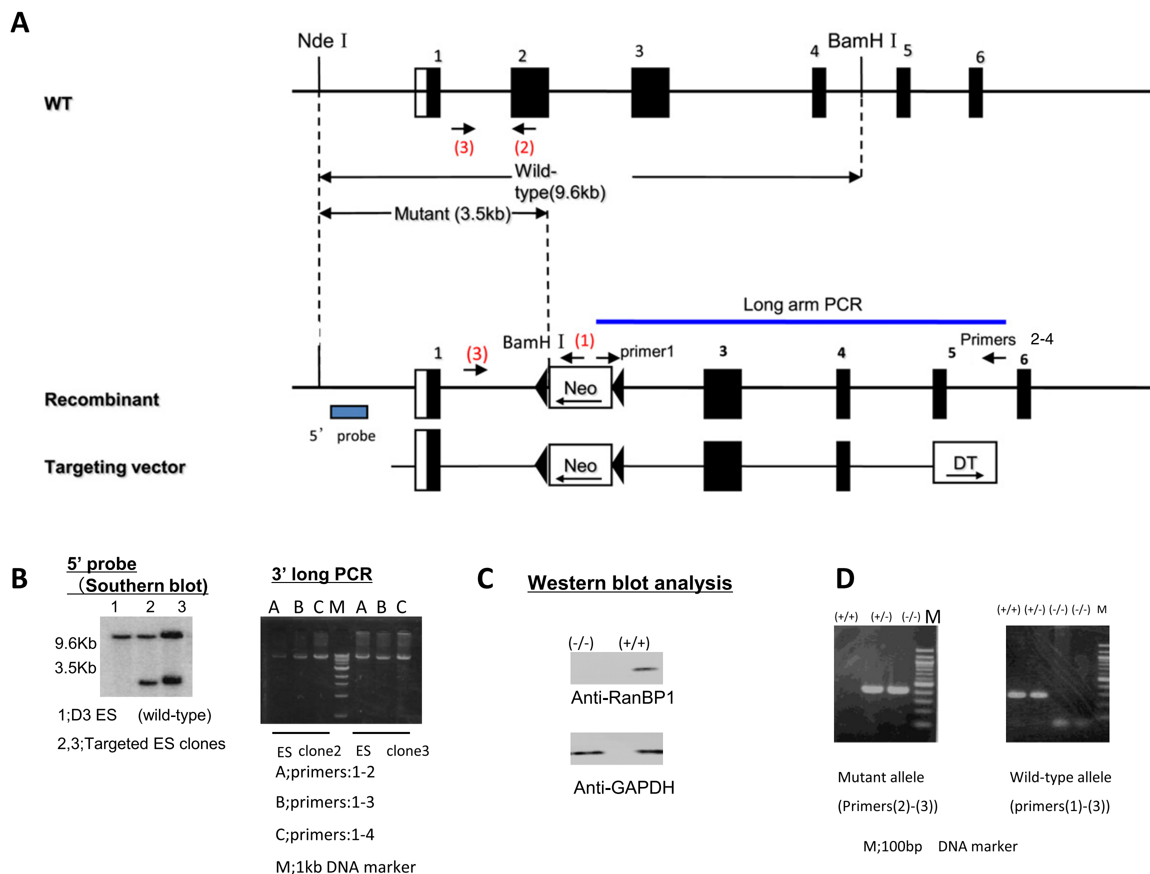

Fig. 1. Generation of RanBP1-knockout mice. (A) Genomic organization and targeting strategy for *RanBP1* gene disruption. (B) Southern blot analysis (5′ arm) and PCR-based analysis (3′ arm) of WT and *RanBP1*$^{+/-}$ ES cells. (C) Immunoblot of brain lysates from WT and *RanBP1*$^{-/-}$ mice (GAPDH as a loading control). (D) PCR-based genotyping of *RanBP1*$^{+/+}$, $^{+/-}$, and $^{-/-}$ mice.

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