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LETTER TO THE EDITOR

Mouse RING finger protein Rnf133 is a testis-specific endoplasmic reticulum-associated E3 ubiquitin ligase

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Dear Editor,

Spermatogenesis, the process by which sperms are generated within the male gonads, involves a number of events that occur only in the testis. Examples include the nucleus condensation as a result of histone sequential replacement by transition proteins 1, 2 and protamin, the formation of sperm-specific organelles such as acrosomes and sperm tails, and the shedding of majority of the cytoplasm as residual bodies. To support the accomplishment of these events, it is hypothesized that a large number of testis-specific genes need to be expressed, which is consistent with the estimation that about 4% of the genes in the mouse genome are specifically expressed in the testis [1]. Identification of these genes and their biological functions will not only help us to elucidate the mechanisms of spermatogenesis but also provide potential targets for novel non-hormonal contraceptive drugs [2].

In this study, we identified a potential testis-specific gene named *Rnf133* (NCBI: Mm.436547). It belongs to the RING finger protein (RNF) family, whose members are very divergent in terms of their sequences and functions except for sharing a RING domain (CX2CX(9-39)CX(1-3)HX(2-3)C/HX2CX(4-48)CX2C) [3]. A number of RNFs have been reported to be expressed in the testis, but their functions are poorly understood [4]. Multiple sequence alignment of Rnf133 proteins from five mammalian species (*Mus musculus*, *Homo sapiens*, *Pan troglodytes*, *Rattus norvegicus*, *Macaca mulatta*) indicated that they are highly conserved (~70% identity among all species, Supplementary information, Figure S1). All these proteins possess a C3H2C3 RING finger domain, a protease-associated (PA) domain, a signal peptide (SP) and a transmembrane (TM) domain. These features are typical of the GREUL (for Goliath Related E3 Ubiquitin Ligase) proteins, a group of RNFs with some of its members being identified as E3 ubiquitin ligases [5].

Northern blotting analysis of 14 adult mouse tissues indicated that Rnf133 was detected only in the testis (Figure 1A). RT-PCR results indicated that Rnf133 mRNA started to appear in the testis of day 21 mice and increased dramatically from day 28 and thereafter (Figure 1B). As round spermatids appear by day 21 during spermatogenesis in mice, Rnf133 seemed to be expressed by round spermatids. This was supported by the results of in situ hybridization in which strong signals were detected in the adluminal regions of the seminiferous tubules where haploid germ cells are located (Figure 1C). The interesting observation that signal was not observed in all of the seminiferous tubules strongly implied stage-specific expression of Rnf133 during spermatogenesis. Indeed, immunohistochemical staining using a rabbit polyclonal antiserum developed against the region from aa 209 to aa 382 (see the Supplementary information, Materials and Methods for procedures of the production of the antiserum and Figure S2 for its specificity characterization) revealed that Rnf133 was present in the cytoplasm of the step 14-16 elongated spermatids in the stage II-VI seminiferous tubules (Figure 1D).

The putative SP and TM domains in Rnf133 (Supplementary information, Figure S1) are indicative of a membrane-bound protein. As shown in Figure 1E1, the GFP-tagged full-length Rnf133 was localized, in a reticular,

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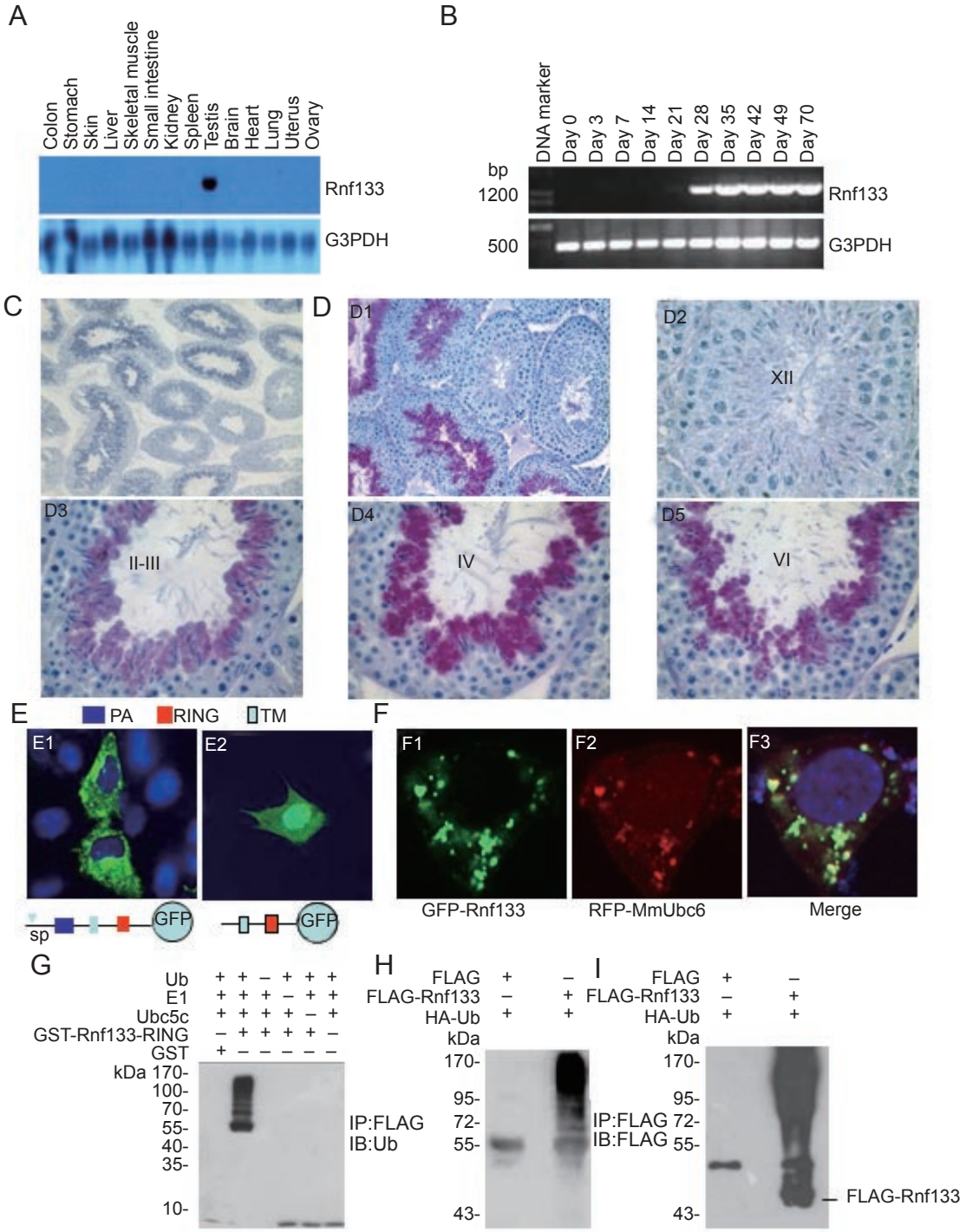

Figure 1 (A) Northern blotting analysis of Rnf133 mRNA expression in different tissues. **(B)** RT-PCR analysis of Rnf133 mRNA in the testis on different days after birth. **(C)** In situ hybridization indicated that Rnf133 mRNA expression was confined in the adluminal regions of the seminiferous tubules where haploid germ cells are located (magnification 100 ×). The detection of signals in some but not all of seminiferous tubules is indicative of stage-specific expression during spermatogenesis. **(D)** Immunohistochemical localization of Rnf133 protein in the adult mouse testis. The Roman letters denote the stages of the seminiferous tubules during a cycle of spermatogenesis (D1, 100 ×; D2-5, 600 ×). **(E)** Subcellular localization of GFP-fusion proteins of full-length Rnf133 and a truncated form in transfected GC-2 cells (400 ×). Nuclei were stained with Hoechst 33258. **(F)** Co-localization of GFP-Rnf133 with RFP-MmUbc6 in transfected GC-2 cells (800 ×). **(G)** Self-ubiquitination of recombinant GST-Rnf133-RING protein in an in vitro ubiquitination assay. **(H, I)** Ubiquitination of FLAG-Rnf133 in transfected 293T cells.

speckled manner, to the cytoplasm of the GC-2 cells, an immortalized mouse spermatocyte cell line [6]. In contrast, a truncated form with the putative SP and the PA domain removed was evenly distributed in the whole cell (Figure 1E2). More convincingly, GFP-Rnf133 co-localized with the RFP-tagged E2 conjugating enzyme MmUbc6, an endoplasmic reticulum (ER)-localized protein [7] (Figure 1F). These data indicated that Rnf133 was most likely an ER-associated protein.

To test whether Rnf133 is an E3 ubiquitin ligase as predicted, recombinant GST-fusion protein of the region containing the RING domain after the TM domain (aa 209-382) (GST-Rnf133-RING, expected size 45 kDa) was produced from E. coli. An in vitro ubiquitination assay was carried out in which GST-Rnf133-RING was used as a ubiquitin E3 ligase. As was shown, polyubiquitin chains were observed only in the presence of GST-Rnf133-RING (Figure 1G). We next tested for its E3 activity in vivo by overexpressing FLAG-tagged Rnf133 (expected size 42 kDa) together with the HA-tagged ubiquitin in 293T cells. As can be seen in Figure 1H and 1I, immunoblotting of FLAG-Rnf133 immunoprecipitates with anti-FLAG and anti-ubiquitin Abs revealed the presence of high-molecular-weight smears representing polyubiquitinated Rnf133. The blot using the anti-FLAG Ab also revealed several bands smaller than 42 kDa, which could be degraded Rnf133 containing the FLAG tag (Supplementary information, Figure S3). These results indicated that Rnf133 was indeed an E3 ubiquitin ligase, which itself could be poly-ubiquitinated both *in vitro* and *in vivo*.

The above-described features of Rnf133 are reminiscent of two well-studied ER-bound E3 ligases Hrd1p and Doa10p, although these proteins share no similarities in their primary sequences. Hrd1p and Doa10 are the key players in two ER-associated degradation (ERAD) pathways [8]. ERAD is widely used to destruct misfolded or mutant proteins or to regulate the steady-state levels of proteins in response to physiological cues [9]. The stage-specific expression of Rnf133 during spermatogenesis, its ER-localization, and its E3 ligase activity altogether imply that Rnf133 may play a role in sperm maturation through an ERAD pathway.

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(**Supplementary Information** is linked to the online version of the paper on the Cell Research website.)