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Rpn10-Mediated Degradation of Ubiquitinated Proteins Is Essential for Mouse Development▽

Jun Hamazaki,[1,2] Katsuhiro Sasaki,[1] Hiroyuki Kawahara,[3] Shin-ichi Hisanaga,[2] Keiji Tanaka,[1] and Shigeo Murata[1]*

Laboratory of Frontier Science, Core Technology and Research Center, Tokyo Metropolitan Institute of Medical Science, Bunkyko-ku, Tokyo 113-8613,[1] Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, Hachiohji, Tokyo 192-0397,[2] and Department of Biochemistry, Graduate School of Pharmaceutical Science, Hokkaido University, Sapporo 060-0812,[3] Japan

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Rpn10 is a subunit of the 26S proteasome that recognizes polyubiquitinated proteins. The importance of Rpn10 in ubiquitin-mediated proteolysis is debatable, since a deficiency of Rpn10 causes different phenotypes in different organisms. To date, the role of mammalian Rpn10 has not been examined genetically. Moreover, vertebrates have five splice variants of Rpn10 whose expressions are developmentally regulated, but their biological significance is not understood. To address these issues, we generated three kinds of Rpn10 mutant mice. Rpn10 knockout resulted in early-embryonic lethality, demonstrating the essential role of Rpn10 in mouse development. Rpn10a knock-in mice, which exclusively expressed the constitutive type of Rpn10 and did not express vertebrate-specific variants, grew normally, indicating that Rpn10 diversity is not essential for conventional development. Mice expressing the N-terminal portion of Rpn10, which contained a von Willebrand factor A (VWA) domain but lacked ubiquitin-interacting motifs (Rpn10ΔUIM), also exhibited embryonic lethality, suggesting the important contribution of UIM domains to viability, but survived longer than Rpn10-null mice, consistent with a "facilitator" function of the VWA domain. Biochemical analysis of the Rpn10ΔUIM liver showed specific impairment of degradation of ubiquitinated proteins. Our results demonstrate that Rpn10-mediated degradation of ubiquitinated proteins, catalyzed by UIMs, is indispensable for mammalian life.

The ubiquitin-proteasome system is the main nonlysosomal apparatus for intracellular protein degradation that is conserved in all eukaryotes from *Saccharomyces cerevisiae* to mammals (10, 15). Short-lived proteins as well as abnormal proteins are mostly recognized by the ubiquitin system and are tagged with ubiquitin chains as degradation signals. The polyubiquitinated proteins are then targeted for degradation by 26S proteasomes.

The 26S proteasome is composed of one proteolytically active 20S proteasome (also called the core particle) and two 19S regulatory particles (RP), each attached to one end of the 20S proteasome (1). The 19S RP plays an essential role in the degradation of ubiquitinated proteins. The 19S RP can be divided into two subcomplexes, known as the "base" and the "lid" (11). Structurally, the base subcomplex is made up of six different ATPases (Rpt1 to Rpt6) and two large subunits called Rpn1 and Rpn2, which act as scaffolds for molecules that modulate proteasome functions, such as Rpn13, Ubp6 (an USP14 orthologue), and Rad23 (an mHR23A/B orthologue) (4, 5, 13, 17, 24, 29, 35, 50). The base binds to the α-ring of the 20S proteasome and opens its narrow gate in an ATP-dependent manner (39). In addition, the ATPase subunits supply energy for unfolding target proteins, so that they can be translocated into the interior cavities of 20S proteasomes, where the active sites are located. The lid subcomplex consists of multiple non-ATPase subunits (Rpn3, Rpn5 to -9, Rpn11, Rpn12, and Rpn15). The role of the lid complex is less well understood, but it is reported to be essential for the degradation of ubiquitinated proteins, at least through the function of Rpn11, which deubiquitinates ubiquitin chains of proteasome substrates prior to degradation (44, 49).

In the ubiquitin-proteasome pathway, the process through which the polyubiquitin chains are recognized by the proteasome remains elusive. To date, several proteins have been identified as receptors that bind ubiquitinated proteins to ferry them to proteasomes for degradation. The UBL-UBA proteins, which contain ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains, can interact with the proteasome through their N-terminal UBL domains as well as with polyubiquitin chains through their C-terminal UBA domains and are thought to shuttle ubiquitinated substrates to the proteasome and to facilitate their degradation (2, 8, 34). There are three UBL-UBA proteins in budding yeast called Rad23, Ddi1, and Dsk2 (2, 8, 34). Furthermore, several UBL-UBA proteins are also found in mammals, and some of them act in a manner similar to that of their yeast counterparts (12, 21).

Polyubiquitinated proteins are also recognized directly by the 19S proteasome subunit Rpn10 (3). Rpn10 is composed of one N-terminal von Willebrand factor A (VWA) domain and one or two C-terminal ubiquitin-interacting motifs (UIM). Rpn10 was the first protein recognized to bind to polyubiquitin chains through UIM domains. Surprisingly, genetic studies

* Corresponding author. Mailing address: Laboratory of Frontier Science, Core Technology and Research Center, Tokyo Metropolitan Institute of Medical Science, Bunkyko-ku, Tokyo 113-8613, Japan. Phone and fax: 81-3-3823-2237. E-mail: smurata@rinshoken.or.jp.

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TABLE 1. Genotyping PCR primers[a]

Primer name	Sequence (5′–3′)
a	CAAGTAGTGCCTCTGGCTGCAAGA
b	TCCTGTCATCTCACCTTGCTCCTG
c	CTTGGGAGGCAGAGACAGATGGAT
d	AGGGACAAGAACAGCCCATGTCTGATTC
e	GATGCAATGCGGCGGCTGCATACGCTTG
f	ATGGCCGCTCACAACTGTCTGCAACTCC
g	CCTGTGTAGGATACCACAGCATCGACT
h	ATTGCTGGGAGTATGAACCACCATGCTG

[a] Primers a, b, and c were used for genotyping of Rpn10 knockout mice. Primers d, e, and f were used for Rpn10a knock-in mouse genotyping. Primers f, g, and h were used for Rpn10ΔUIM mouse genotyping.

TABLE 2. PCR primers for Rpn10 splicing variants

Primer name	Sequence (5′–3′)
Rpn10a,c,d,e forward	CAAAGGCAAGATCACCTTCTGCACTGGCA
Rpn10b forward	TACACCTGGGACTGAAGGTGAAAGA
Rpn10a,b reverse	GTTCTCTAGGACGCTCTG
Rpn10c reverse	AAGTGTTTCCTTCTGTTGCTCCAAG
Rpn10d reverse	GGCCCTGCCACCCAAGCCATGGCCCAC
Rpn10e reverse	TCGTGAAATGGCTAGCAC

with yeast showed that deletion of the gene resulted in little loss of viability, in contrast to most other proteasome genes, which are essential for life (6, 43). The degradation defect of the *rpn10*Δ strain is modest, and the defect becomes evident when the strain is crossed with strains that lack UBL-UBA proteins such as Rad23 and Dsk2 (23, 31). In vitro analysis of the degradation of polyubiquitinated Sic1 showed that while its degradation was defective in *rpn10*Δ proteasomes and *rad23*Δ proteasomes, simultaneous addition of Rad23 protein and the VWA domain of Rpn10 restored the degradation of ubiquitinated Sic1, suggesting a "facilitator" activity within the VWA domain of Rpn10 (45). Finally, a yeast strain that has mutations in the UIM domain of Rpn10 showed a clear deficiency in ubiquitin chain recognition, and this *rpn10-uim* mutation confers synthetic sensitivity to canavanine when combined with either a *rad23*Δ or a *dsk2*Δ mutation, indicating that the UIM domain of Rpn10 and UBL-UBA proteins work redundantly (4). It is now suggested that UBL-UBA proteins and Rpn10 define a layer of substrate selectivity, which might depend on the length of polyubiquitin chains (6, 20, 30, 45).

Although Rpn10 is not essential for life or for overall ubiquitin-mediated protein turnover in yeast and worm (6, 36, 41, 43), Rpn10-deficient mutants of *Physcomitrella patens*, *Arabidopsis thaliana*, and *Drosophila melanogaster* showed more-severe phenotypes such as developmental arrest and lethality (9, 38, 40). These results suggest that higher eukaryotes depend on Rpn10-mediated degradation of polyubiquitinated proteins for their development.

Vertebrates have acquired a diversity of proteasomes by creating new subunits. For example, the gamma interferon-inducible subunits β1i, β2i, and β5i evolved to produce immunoproteasomes, which enable viral proteins to be presented on major histocompatibility complex class I molecules more efficiently than constitutive proteasomes (42). Vertebrates have also acquired a diversified Rpn10 subunit, which was accomplished by developmentally regulated alternative splicing, resulting in the generation of five isoforms named Rpn10a to Rpn10e (see Fig. 1A) (18). It is reported that Rpn10c in *Xenopus laevis* associates with Scythe/BAG-6 and regulates apoptosis (19). However, the significance and distinct functions of these isoforms in mammals are not understood at all. In addition, the importance of the UIM and VWA domains of Rpn10 for mammalian development has not been examined.

In the present study, we generated and analyzed three types of Rpn10 mutant mice in order to understand the role(s) of Rpn10 in mammals. Our results provide genetic evidence for the in vivo significance of Rpn10 in mammals.

MATERIALS AND METHODS

Gene targeting of Rpn10. A targeting vector for Rpn10-null mice was constructed by replacing exons 2 to 8 with a neomycin resistance gene (*neo*) cassette. For Rpn10a knock-in mice (with, at the same time, conditional deletion of UIM domains), exons 7 to 10 were replaced with the corresponding cDNA of Rpn10a, with a polyadenylation signal attached at its 3′ end. A *neo* cassette was inserted at the 3′ end of the cDNA. LoxP sequences were inserted at the 5′ end of the cDNA and the 3′ end of the *neo* cassette. TT2 embryonic stem cells were screened as described previously (27). For Southern blot analysis, genomic DNA was digested with EcoRI for Rpn10 knockout or with EcoRV for Rpn10a knock-in and was hybridized with the probes shown in Fig. 1A and 2A, respectively. EIIa-Cre and Alb-Cre were purchased from The Jackson Laboratory. PCR primers used for mouse genotyping are listed in Table 1. Mice were housed in pathogen-free facilities, and the experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Tokyo Metropolitan Institute of Medical Science.

RNA isolation, reverse transcription, and real-time PCR. Expression levels of Rpn10 variants were determined by reverse transcription-PCR (RT-PCR) as described previously (18). Specific primers for each variant are listed in Table 2. For real-time PCR analysis, total RNAs were isolated from the livers of 5-week-old mice by using an RNAspin minikit (GE Healthcare), reverse transcribed to cDNA using a Transcriptor first-strand cDNA synthesis kit (Roche), and subjected to real-time PCR using the LightCycler 480 system (Roche). PCR primers and universal probes (Roche), which are listed in Table 3, were designed according to the Universal Probe Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). Glucuronidase beta (GUSβ) was used for normalization. Real-time PCR data were analyzed by the $\Delta\Delta C_T$ method.

Immunological analysis. Mouse livers were homogenized and subjected to immunoblotting and immunoprecipitation as described previously (13). The antibodies against Rpn1, Rpn3, Rpt6, the VWA domain of Rpn10 [Rpn10(N)], USP14, polyubiquitin, and actin have been described previously (13). Polyclonal antibodies against a6, a7, Rpn6, Rpn7, Rpt3, Rpt5, the UIM domain of Rpn10 [Rpn10(C)], and mHR23B (all sequences were derived from mice) were raised in rabbits by using recombinant proteins expressed in and purified from strain BL21RIL (Novagen) as His_6 fusion proteins of α6 (residues 152 to 263), α7 (residues 157 to 255), Rpn6 (residues 1 to 162), Rpn7 (residues 1 to 137), Rpt3 (residues 1 to 100), and Rpt5 (residues 1 to 131) and as glutathione *S*-transferase fusion proteins of Rpn10 (residues 255 to 376) and mHR23B (full length), respectively. For immunoprecipitation, a liver homogenate from an $Rpn10^{a/a}$ or an $Rpn10^{a/a:Alb}$ mouse (the two mice had equal Suc-LLVY hydrolyzing activities) was immunoprecipitated with an anti-Rpt6 antibody.

Glycerol gradient analysis. Mouse liver homogenates were clarified by centrifugation at 20,000 × *g* and subjected to 10 to 40% (vol/vol) linear glycerol gradient centrifugation (22 h, 83,000 × *g*) as described previously (16).

Assay of proteasome activity. The assays of proteasome chymotryptic peptidase activity, degradation of recombinant ^{35}S-labeled ornithine decarboxylase (ODC), and degradation of polyubiquitinated ^{35}S-labeled cIAP1 protein have been described previously (13, 16).

Histological examination. Embryos in utero were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Sections were stained with Mayer's hematoxylin, followed by eosin staining.

Culture of blastocysts. Blastocysts were flushed out from pregnant female uteri at embryonic day 3.5 (E3.5) and were cultured in M16 medium (Sigma-Aldrich) at 37°C under 5% CO_2 on a gelatin-coated chambered coverglass (Nalgene).

TABLE 3. PCR primers and universal probes for real-time PCR

Gene	Probe no.	Forward primer (5′–3′)	Reverse primer (5′–3′)
GUS	6	GATGTGGTCTGTGGCCAAT	TGTGGGTGATCAGCGTCTT
α5	25	TCGCTCATCATCCTCAAGC	AAATTCTGACCAGGCTGCAC
α6	80	CCGTTCTCAATCAGCTCGTA	ACCAGTTCATCCAAATTGCAC
Rpn3	32	TCCACAACATGTCTGTCAAGG	CCTTGGCAAACTCCAGGTC
Rpn7	29	TAGGTCATTAACCCTCGGCTAT	CTTCCAGCAGCAATAAACCTG
Rpt3	22	GTCGCCAGAAGAGGTTGATT	ATCTGGACGGGCCACATA
Rpt5	74	GGGTTGGACATGCTTGGT	CCTGGGCAACGTGTTTCT
mHR23B	2	CTGGAAGTGGGCACATGAAT	TTCAGGAAATCCTAATGCCTTT
USP14	18	GGCGAACAAGGGCAGTATC	TCTGTTGCAGGACTCTCATCA

RESULTS

Loss of Rpn10 causes early embryonic lethality in mice. To determine the significance of Rpn10 in mammals, we generated Rpn10-null mice by replacing exons 2 to 8 with a neomycin resistance gene (Fig. 1B to D). Rpn10-heterozygous ($Rpn10^{+/-}$) mice were born without any gross abnormality and were fertile. These mice were intercrossed to produce $Rpn10^{-/-}$ mice. The progeny did not contain surviving $Rpn10^{-/-}$ pups, suggesting that the absence of Rpn10 during embryogenesis is lethal. To investigate this issue further, we examined, at various stages of development (mainly E6.5 to E7.5), embryos that had been produced in timed intercrosses. In normal E6.5 embryos, a cylinder-like two-layered cellular structure was observed. However, Rpn10-deficient embryos failed to form this structure at E6.5 (Fig. 1E, left) and were resorbed by E7.5 (Fig. 1E, right).

To analyze further the defects associated with Rpn10 deficiency, we isolated blastocysts from $Rpn10^{+/-}$ intercrosses at E3.5. $Rpn10^{-/-}$ blastocysts were identified by PCR at the expected Mendelian frequency (Fig. 1F, left; also data not shown), representing relatively mild phenotypes compared to those of Rpt3 and Rpt5 knockout mice, which did not develop beyond the 8-cell stage (33). When these blastocysts were cultured in vitro, most of the $Rpn10^{-/-}$ blastocysts hatched from the zona pellucida, spread trophoblastic cells with proliferating inner cell masses (ICMs), which form the future embryonic ectoderm, and grew on the gelatin-coated glass, like wild-type blastocysts (Fig. 1F, center). However, ICMs of $Rpn10^{-/-}$ blastocysts could not expand beyond 48 h of culture and detached from the trophoblastic cells before 96 h of culture, in contrast to wild-type ICMs (Fig. 1F, right). These results indicate that Rpn10 is essential for embryonic development beyond blastocyst formation; presumably it is involved in the expansion of the embryonic ectoderm after implantation.

Rpn10a is sufficient for the development of mice. In vertebrates, Rpn10 has five splice variants named Rpn10a to Rpn10e. Rpn10a is the conventional isoform expressed throughout development and throughout the body, while Rpn10b to Rpn10e are expressed at specific developmental stages or in specific organs (Fig. 1A). These facts raise the possibility that the diversity of Rpn10 plays a role in development in vertebrates. To test this hypothesis and to clarify the roles of these vertebrate-specific isoforms, we generated Rpn10a knock-in mice. The Rpn10 isoforms are generated by different splice acceptor and donor usages of a genomic locus that corresponds to exons 7 to 10 of the Rpn10a isoform, which encode the major part of the two UIM domains of Rpn10 (18). Therefore, a targeting vector was designed to replace a genomic locus with the corresponding cDNA sequences of Rpn10a and to disrupt the expression of other isoforms (Fig. 2A to C). The inserted Rpn10a cDNA was flanked with *loxP* sequences to enable the generation of mice expressing Rpn10 lacking UIM domains (Rpn10ΔUIM). Mice heterozygous for the Rpn10a knock-in allele ($Rpn10^{a/+}$ mice) were born healthy and fertile without noticeable pathological phenotypes. $Rpn10^{a/a}$ mice, obtained by intercrossing $Rpn10^{a/+}$ mice, were born healthy at Mendelian frequency, were fertile, and grew apparently normally without any gross abnormality (data not shown). RT-PCR analysis of RNAs from newborn mice demonstrated loss of Rpn10b to Rpn10e isoforms in $Rpn10^{a/a}$ mice, while all the isoforms were expressed in wild-type mice (Fig. 2D). The protein levels of Rpn10 as well as other proteasome subunits in $Rpn10^{a/a}$ mice were similar to those in the wild type (Fig. 2E), and the expressed Rpn10a was incorporated normally into 26S proteasomes, like that expressed in wild-type mice (Fig. 2F, right). The proteasome activity of the $Rpn10^{a/a}$ liver, assessed with fluorogenic peptides, was nearly equal to that of the wild-type liver (Fig. 2F, right). The proteasome activities of adult brains were also comparable in $Rpn10^{a/a}$ and wild-type mice (data not shown). These results indicate that vertebrate-specific isoforms of Rpn10 do not play an important role in development and that the conventional isoform Rpn10a is sufficient for life, at least under normal circumstances. However, it is possible that isoforms Rpn10b to Rpn10e are involved in the degradation of specific target proteins or play a role in a process other than ubiquitin-mediated proteolysis, defects in which might become apparent only under certain conditions.

Mice deficient in UIM domains exhibit embryonic lethality but survive longer than Rpn10-null mice. In genetic analyses using yeast and moss, lack of the UIM domains of Rpn10 displayed modest phenotypes compared to null mutations, thus questioning the physiological significance of the UIM domains of Rpn10 (6, 9). To examine the role of these domains in mice, we generated Rpn10ΔUIM-expressing mice by Cre recombinase-mediated excision of the UIM domain-coding region (Fig. 2A, bottom, and Fig. 3A). By crossing $Rpn10^{a/a}$ mice with EIIa-Cre transgenic mice, in which the expression of Cre recombinase appears from the zygote stage (22), we obtained mice harboring an Rpn10 gene encoding Rpn10ΔUIM protein throughout the body, including germ cells ($Rpn10^{\Delta UIM/+}$ mice). $Rpn10^{\Delta UIM/+}$ mice were born at the expected Mende-

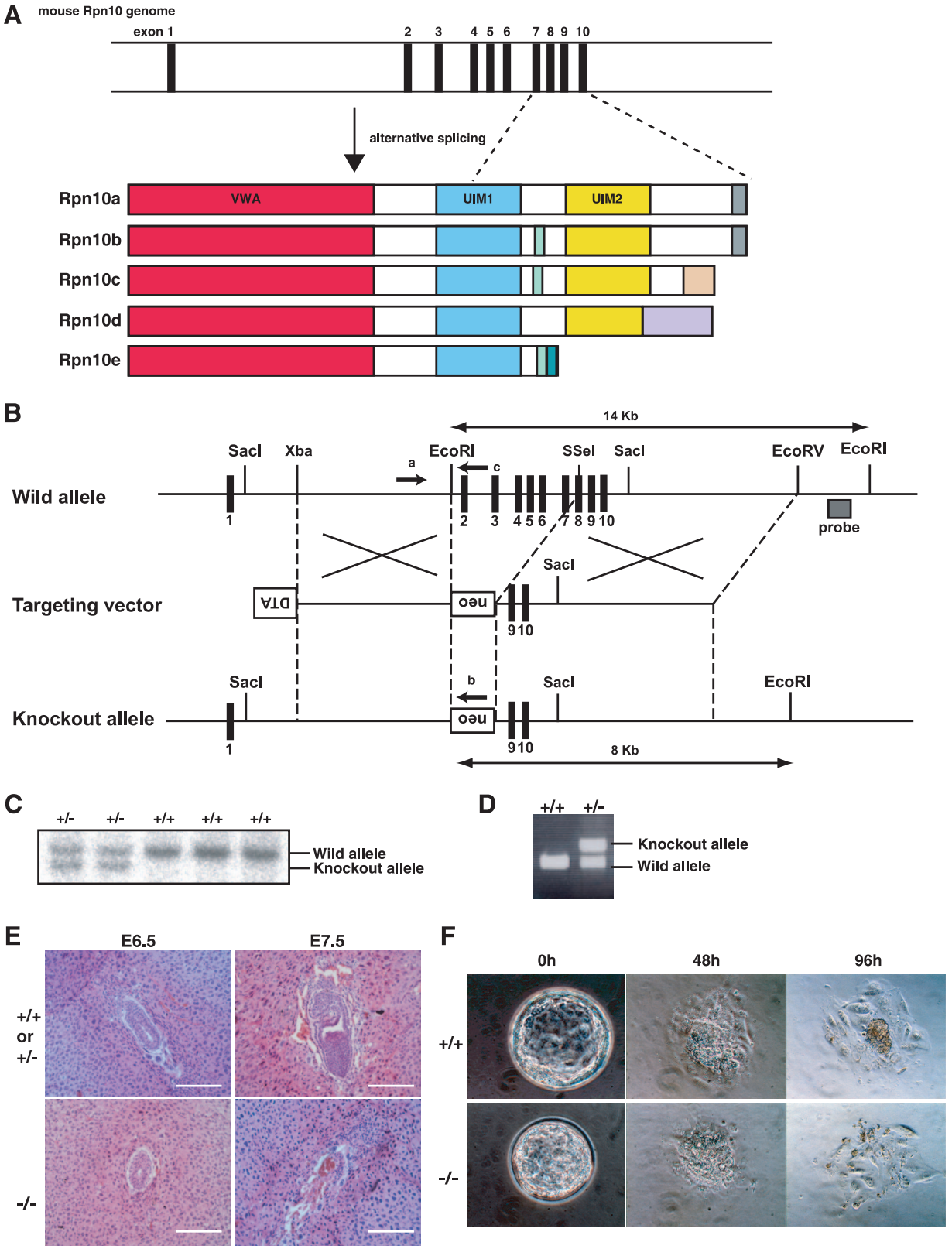

FIG. 1. Death in utero of Rpn10-deficient mouse embryos at E6.5. (A) Schematic representation of the Rpn10 gene and a family of mouse Rpn10 proteins. (Top) Physical map of the Rpn10 gene. Exons are indicated by filled rectangles and are numbered from 1 to 10 (upper panel). (Bottom) The structures of multiple Rpn10 proteins (Rpn10a to Rpn10e) generated by alternative splicing are shown schematically. Red, blue, and yellow represent the VWA, UIM1, and UIM2 domains, respectively. The sequences specific to each variant are represented by various colors. For details of the generation of the variants listed, see reference 18. (B) Schematic representation of the targeting vector and the targeted allele of the

lian frequency but exhibited slightly retarded growth and maturation compared to wild-type mice or even to $Rpn10^{+/-}$ mice (data not shown), implying that incorporation of Rpn10 protein lacking UIM domains into 26S proteasomes exerted a somewhat dominant-negative effect. However, these mice were fertile and showed no obvious phenotypes other than slow growth. $Rpn10^{\Delta UIM/+}$ mice were intercrossed to produce $Rpn10^{\Delta UIM/\Delta UIM}$ mice. The progeny did not contain any surviving $Rpn10^{\Delta UIM/\Delta UIM}$ pups, suggesting that the absence of the UIM domains of Rpn10 was incompatible with embryogenesis. Examination of embryos at various developmental stages revealed that the development of $Rpn10^{\Delta UIM/\Delta UIM}$ embryos was normal before E6.5 (data not shown) but appeared to be delayed at E8.5 (Fig. 3B and C). At E9.5, development arrested at a stage corresponding to E8.5 of the wild type; the turning process that results in a fetal position, normally seen at the transition from the 6-somite to the 8-somite stage, was not initiated (Fig. 3B and C). However, we could not find specific morphological defects in the embryos, such as disturbed formation of heart tubes, which are often associated with the failure of turning seen in other knockout mice such as GATA4 knockout mice (47). Intriguingly, $Rpn10^{\Delta UIM/\Delta UIM}$ embryos developed to an advanced stage compared to $Rpn10^{-/-}$ embryos, indicating that the VWA domain of Rpn10 rescued development from E6.5 to E9.5. These results suggest that the VWA domain alone plays some roles in proteasome function but that the UIM domain-dependent function of proteasomes is still required for mouse development, especially at the turning stage.

UIM domain deficiency in the liver is associated with impaired degradation of ubiquitinated proteins. To determine the biochemical basis of the significance of the UIM domain of Rpn10, we generated mice that expressed Rpn10ΔUIM exclusively in postnatal hepatocytes by crossing $Rpn10^{a/a}$ mice with transgenic mice that expressed Cre recombinase under the control of the albumin (Alb) promoter (28). $Rpn10^{a/a:Alb}$ mice, which expressed Rpn10ΔUIM proteins instead of Rpn10a proteins in the liver postnatally, were born without any abnormal appearance or developmental defect. We first confirmed the deletion of the UIM domains of Rpn10 in the liver. In the $Rpn10^{a/a:Alb}$ liver, no full-length Rpn10a proteins were detected. Instead, as expected, a truncated form of Rpn10 appeared, which could be detected by an anti-Rpn10 antibody raised against the VWA domain of Rpn10 [Rpn10(N)] but not by an anti-Rpn10 antibody raised against the UIM domain of Rpn10 [Rpn10(C)] (Fig. 4A). This Rpn10ΔUIM species was incorporated correctly into 26S proteasomes, consistent with the findings of studies with yeast (Fig. 4B). Interestingly, immunoblot analysis of liver lysates revealed that protein levels of subunits of the 20S proteasome (α6, α7), the base (Rpn1, Rpt3, Rpt5, Rpt6), and the lid (Rpn3, Rpn6, Rpn7), as well as levels of some proteasome-interacting proteins (mHR23B, USP14), were all increased in the $Rpn10^{a/a:Alb}$ liver (Fig. 4A). The up-regulation of proteasome subunits led to approximately twofold increases in the levels of 20S and 26S proteasomes and in proteasome-specific peptidase activities in $Rpn10^{a/a:Alb}$ liver lysates relative to those for $Rpn10^{a/a}$ liver lysates (Fig. 4C). To examine the reason for the increased proteasome levels, we quantified relative mRNA levels of proteasome subunits by real-time PCR analysis. We noted 1.8- to 2.5-fold increases in levels of mRNAs of proteasome subunits and proteasome-interacting proteins in the $Rpn10^{a/a:Alb}$ liver relative to those in the $Rpn10^{a/a}$ liver, indicating that transcription of overall proteasome-related genes was up-regulated in the $Rpn10^{a/a:Alb}$ liver (Fig. 4D). Despite the elevated amounts of proteasomes and the consequently increased peptidase activities, accumulation of polyubiquitin-conjugated proteins was noted in the $Rpn10^{a/a:Alb}$ liver (Fig. 4E). To test whether degradation of native proteins was impaired in the $Rpn10^{a/a:Alb}$ liver, we measured the degradation rates of two types of proteasome substrates in vitro. One is ODC, which is degraded by 26S proteasomes in a ubiquitin-independent but antizyme-dependent manner (26). The other is cIAP1 protein, a RING finger type ubiquitin ligase that ubiquitinates itself for degradation by 26S proteasomes in a ubiquitin-dependent manner (37). The degradation rate of ODC was increased in lysates of the $Rpn10^{a/a:Alb}$ liver, and this increase correlated with the increase in the level of the 26S proteasome (Fig. 4F, left). In contrast, the degradation rate of ubiquitinated cIAP proteins was markedly reduced in the $Rpn10^{a/a:Alb}$ liver, although the amounts of 26S proteasomes and mHR23B were larger than those in the $Rpn10^{a/a}$ liver (Fig. 4F, right). These results indicate that the UIM domain of Rpn10 plays an important role in the recognition and degradation of ubiquitinated proteins in the mouse liver. It is likely that increased transcription of proteasome-related genes is a feedback regulation mechanism to compensate for the impaired degradation and accumulation of ubiquitinated proteins, as was also observed for the Rpn10-deficient fly (40, 48).

Previous reports showed that human homologues of yeast Rad23 bind to proteasomes via the second UIM domain of human Rpn10 (7, 17, 25, 46), whereas Rad23 binds directly to Rpn1 in yeast (5, 32). To assess the significance of the UIM domains of Rpn10 in recruiting Rad23 species to proteasomes in mammals, we immunoprecipitated 26S proteasomes from liver lysates with an anti-Rpt6 antibody and compared the amount of proteasome-associated mHR23B (a mouse homologue of yeast Rad23) in Rpn10ΔUIM liver to that in $Rpn10^{a/a}$ liver by immunoblotting (Fig. 4G). Since Rpn10ΔUIM liver contained increased levels of proteasomes and proteasome activity (Fig. 4A and C), the amounts of proteasomes loaded were adjusted for the peptide-hydrolyzing activities of the ly-

Rpn10 gene. Exons 1 to 10 are shown as solid rectangles. The probe for Southern blot analysis is shown as a gray box. The positions of PCR primers are depicted as arrows. neo, neomycin-resistant cassette; DTA, diphtheria toxin gene. (C) Southern blot analysis of genomic DNAs extracted from mouse tails. Wild-type and knockout alleles were detected as 14-kb and 8-kb bands, respectively. (D) PCR analysis of genomic DNAs extracted from wild-type and $Rpn10^{+/-}$ mouse tails. (E) $Rpn10^{+/+}$ or $Rpn10^{+/-}$ (top) and $Rpn10^{-/-}$ (bottom) embryos at E6.5 and E7.5 were sagittally sectioned and stained with hematoxylin and eosin. (F) Impaired development of $Rpn10^{-/-}$ blastocysts in in vitro cultures. Genotypes were determined by PCR.

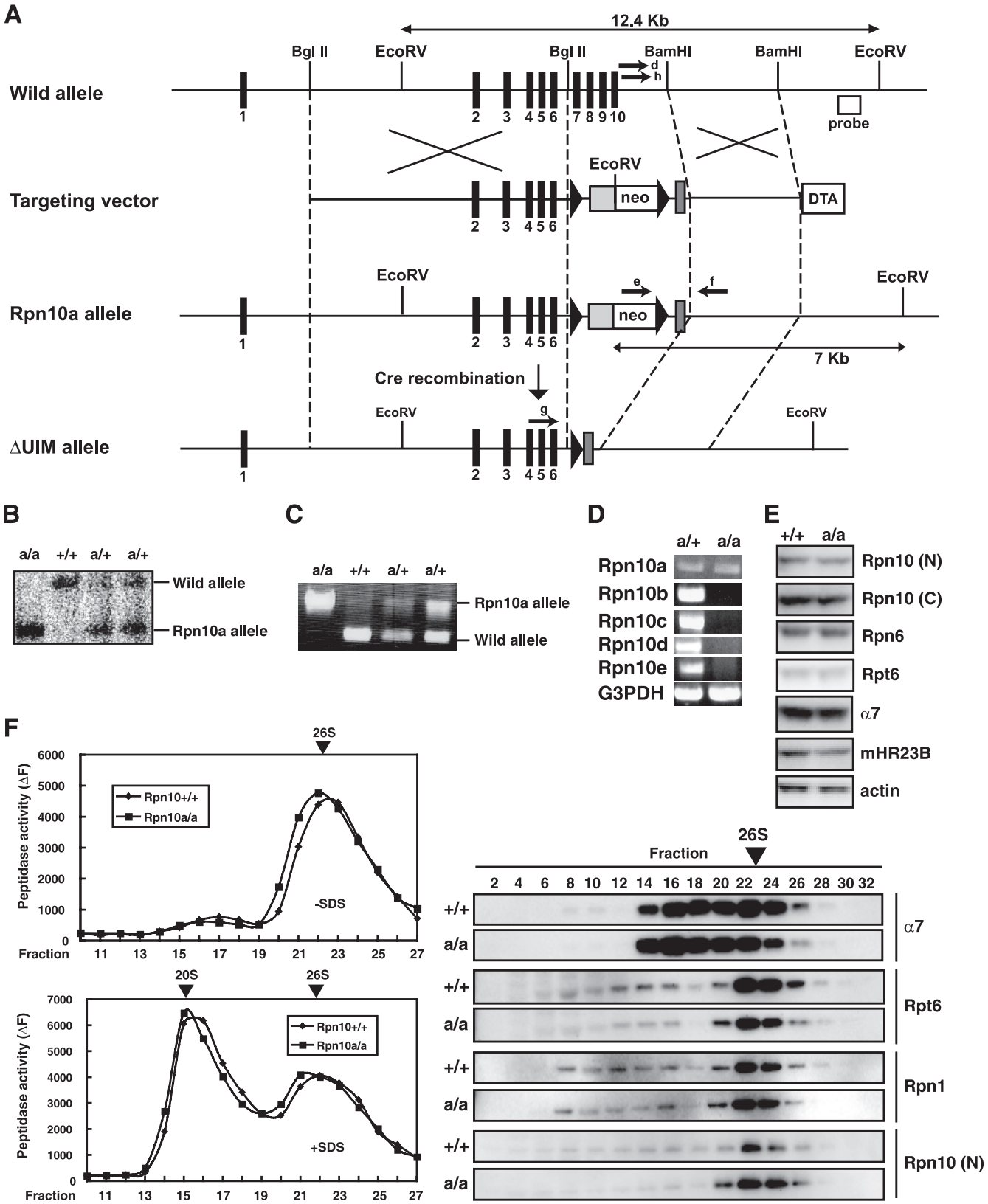

FIG. 2. Generation and analysis of Rpn10a knock-in mice. (A) Schematic representation of the targeting vector and the targeted allele of the Rpn10 gene. Light shaded box, the Rpn10a cDNA fragment corresponding to amino acids 219 to 376 with a polyadenylation signal. Dark shaded box, Rpn10a exon 7 splicing acceptor sequences followed by a stop codon and polyadenylation signal. Triangles, *loxP* sequences. Open box, probe used for Southern blot analysis. Arrows indicate positions of PCR primers. (B) Southern blot analysis of genomic DNAs extracted from mouse tails.

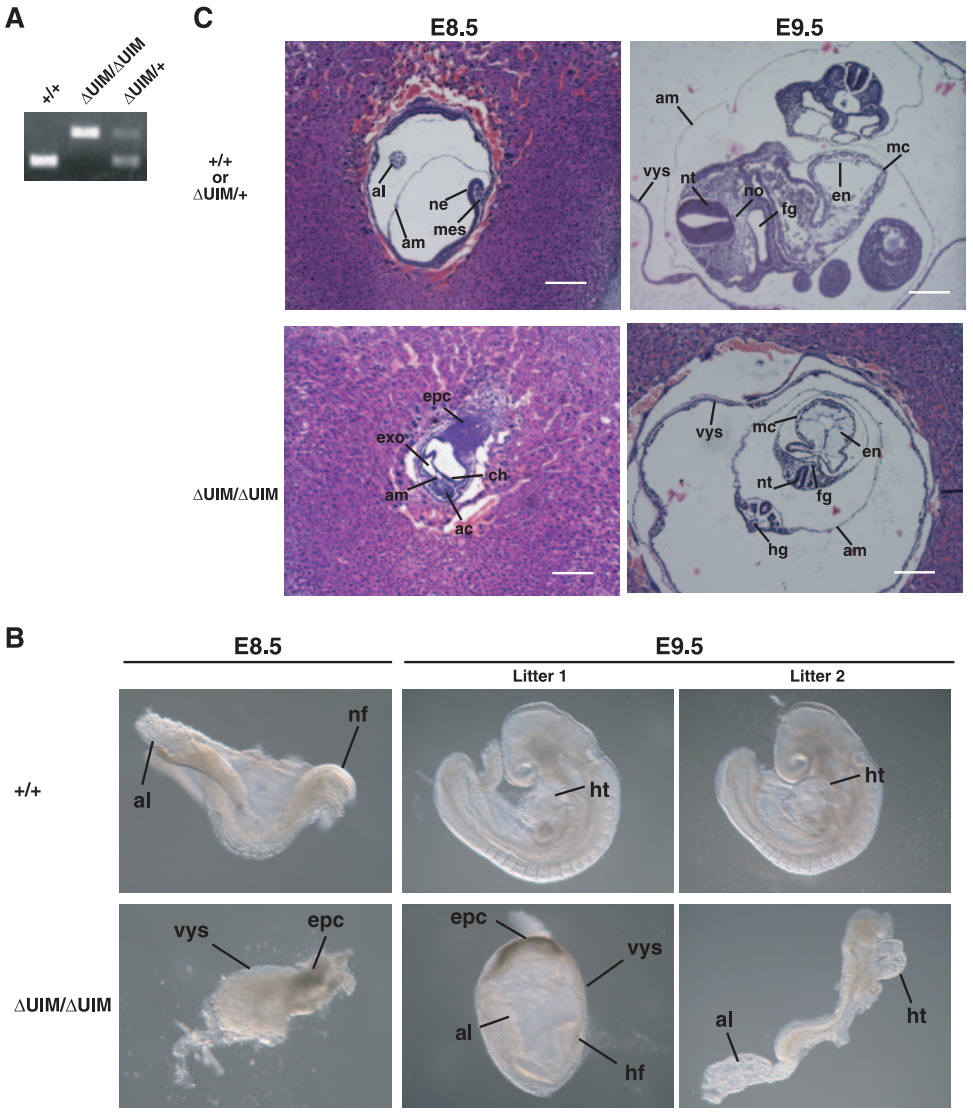

FIG. 3. Developmental arrest of mice deficient in the UIM domains. (A) PCR analysis of genomic DNA extracted from wild-type, Rpn10$^{\Delta UIM/\Delta UIM}$, and Rpn10$^{\Delta UIM/+}$ embryos at E8.5. The amplified fragments derived from wild-type, ΔUIM/+, and ΔUIM/ΔUIM alleles are indicated. (B) Morphology of Rpn10 embryos. Genotypes of embryos were determined by PCR. The longitudinally arranged panels represent littermates. Note the lack of turning of mutant embryos at E9.5. (C) Rpn10$^{+/+}$ (top) and Rpn10$^{\Delta UIM/\Delta UIM}$ (bottom) embryos at E8.5 and E9.5 in utero were sagittally (E8.5) and transversely (E9.5) sectioned and stained with hematoxylin and eosin. Genotypes of embryos were deduced morphologically. Scale bars, 200 μm. ac, amniotic cavity; al, allantois; am, amnion; ch, chorion; en, endocardium; epc, ectoplacental cone; exo, exocoelom; fg, foregut; hf, head fold; hg, hindgut; ht, heart tube; mc, myocardium; mes, mesoderm; ne, neuroectoderm; nf, neural fold; no, notochord; nt, neural tube; vys, visceral yolk sac.

sates. Although the band intensities of proteasome subunits (Rpt6, Rpn1, Rpn2, Rpn6) were comparable between the genotypes, the amount of mHR23B in Rpn10ΔUIM proteasomes was approximately 60% lower than that in Rpn10$^{a/a}$ proteasomes (Fig. 4G), consistent with the previous observations that the UIM domain of Rpn10 recruits Rad23 species in mammals (7, 17, 25, 46). However, this result also indicates that the UIM domain is not the sole receptor for Rad23 species in mammals, because a portion of mHR23B remained associated with Rpn10ΔUIM proteasomes (Fig. 4G). It should be noted that

Wild-type and mutant alleles are detected as 12.4- and 7-kb bands, respectively. (C) PCR analysis of genomic DNA extracted from the tails of wild-type, Rpn10$^{a/+}$, and Rpn10$^{a/a}$ mice. The amplified fragments derived from wild-type and Rpn10a alleles are indicated. (D) RT-PCR analysis of Rpn10 splice variant transcripts. (E) Liver lysates from 13-week-old mice were immunoblotted with antibodies against the indicated proteins. (F) Lysates from Rpn10$^{+/+}$ and Rpn10$^{a/a}$ livers were fractionated by glycerol gradient centrifugation (10 to 40% glycerol from fraction 1 to fraction 30). (Left) An aliquot of each fraction was used for an assay of chymotryptic activity of proteasomes using succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (Suc-LLVY-AMC) as a substrate in the absence (top) or presence (bottom) of 0.025% sodium dodecyl sulfate (SDS). (Right) Immunoblot analysis of each fraction was performed using antibodies against the indicated proteins.

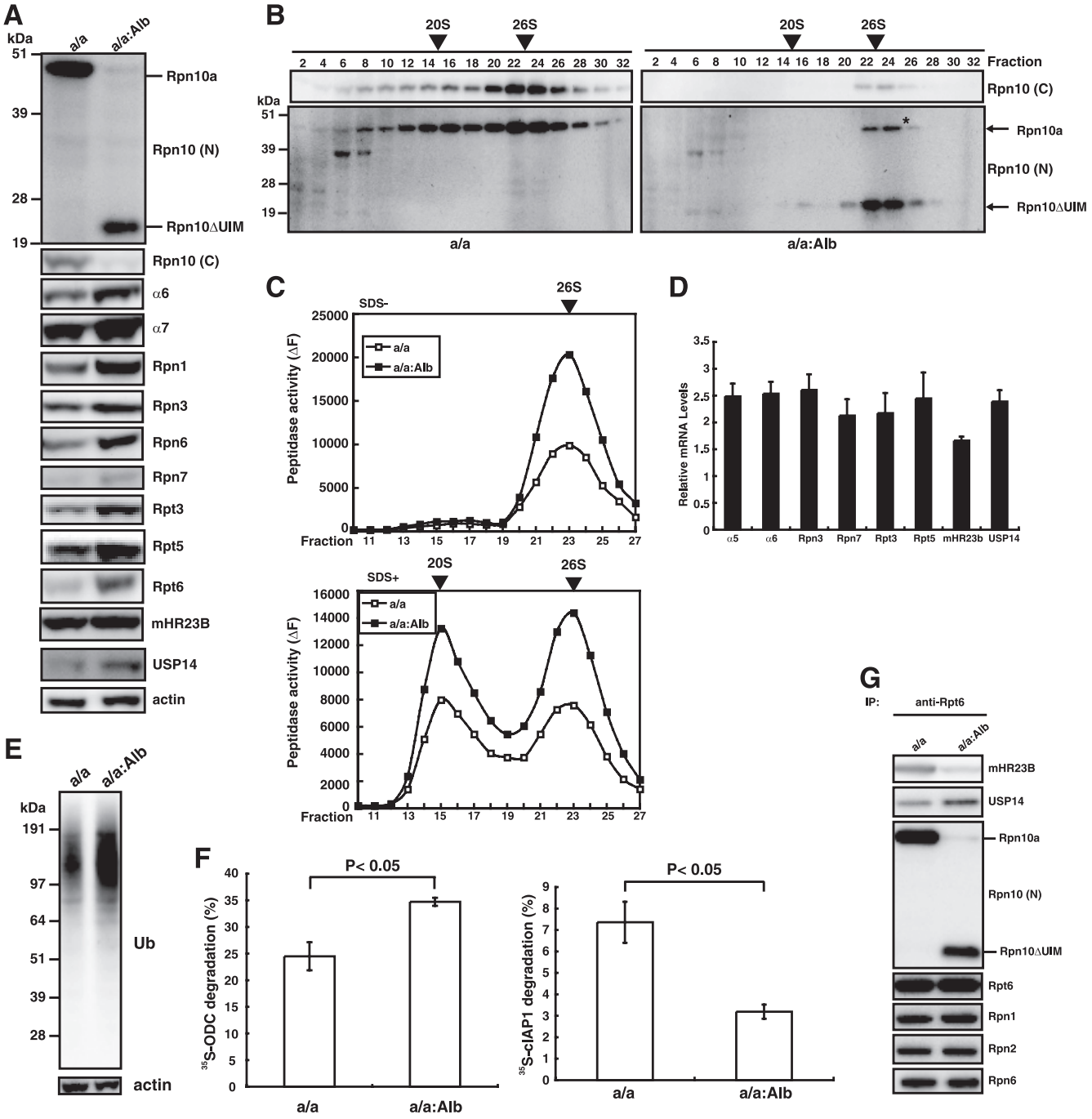

FIG. 4. UIM domain deficiency in the liver causes impaired degradation of ubiquitinated proteins. (A) Homogenates from 6-week-old $Rpn10^{a/a}$ and $Rpn10^{a/a:Alb}$ mouse livers were immunoblotted with the indicated antibodies. (B) The homogenates for which results are shown in panel A were fractionated by 10 to 40% glycerol gradient centrifugation and immunoblotted with the indicated antibodies. The faint Rpn10a bands detected in blots with anti-Rpn10(C) and anti-Rpn10(N) (asterisk) in the panels for $Rpn10^{a/a:Alb}$ liver are presumably derived from non-albumin-expressing cells in the liver. (C) The peptide-hydrolyzing activity of each fraction from panel B was measured as for Fig. 2F. (D) Real-time RT-PCR was used to measure the expression of transcripts encoding proteasome-related genes in the livers of 6-week-old $Rpn10^{a/a}$ and $Rpn10^{a/a:Alb}$ mice. Data represent levels of transcripts in $Rpn10^{a/a:Alb}$ liver relative to those in $Rpn10^{a/a}$ liver and are means ± standard deviations from experiments with three pairs of littermates. (E) The homogenates for which results are shown in panel A were immunoblotted with an anti-ubiquitin (Ub) antibody. (F) Ubiquitin-independent and -dependent protein-degrading activities of proteasomes. Homogenates from 6-week-old mouse livers were subjected to an in vitro protein degradation assay. Antizyme-dependent degradation of ^{35}S-labeled ODC (left) and ubiquitin-dependent degradation of ^{35}S-labeled cIAP1 (right) were measured. Data are means ± standard deviations from triplicate experiments. (G) Homogenates from $Rpn10^{a/a}$ and $Rpn10^{a/a:Alb}$ mouse livers were immunoprecipitated with an anti-Rpt6 antibody and subjected to immunoblotting with the indicated antibodies.

an increased amount of USP14 was detected in Rpn10ΔUIM proteasomes, consistent with the observation with yeast that ubiquitin stress enhances the loading of proteasomes with Ubp6, which is known to bind to 26S proteasomes via Rpn1 (14, 24). It is possible that USP14 competes with mHR23B for binding to proteasomes, specifically to Rpn1, although the binding of USP14 and mHR23B to mammalian Rpn1 has yet to be established. Therefore, we cannot evaluate the exact contribution of the UIM domain in accepting Rad23 in mammalian proteasomes. Whether ubiquitinated proteins are recognized directly by the UIM domains of Rpn10 or delivered to the UIM domain by Rad23 species, these results demonstrate that deletion of the UIM domains of Rpn10 causes insufficient delivery of ubiquitinated proteins to proteasomes, resulting in impairment of their degradation in mammals.

DISCUSSION

To date, genetic analysis of Rpn10 has been conducted on yeast, moss, plant, and fly (6, 9, 36, 38, 40, 41, 43). In the present study, we extended the genetic analysis of Rpn10 to vertebrates, employing gene-targeting techniques on mice. One of the aims of our study was to clarify the role of vertebrate-specific splice variants of Rpn10 (18). This was achieved by generating mice with Rpn10a knock-in and Rpn10b-to-Rpn10e knockout. Contrary to our expectation, $Rpn10^{a/a}$ mice did not show any obvious defect throughout development, suggesting that the constitutive form of Rpn10 (i.e., Rpn10a) was sufficient at least for conventional development of mice. However, a previous report that analyzed Rpn10 variants in *Xenopus* suggested that specific interaction between Rpn10c and Scythe, a regulatory factor of Reaper-induced apoptosis, plays an important role in embryonic development (19). While our study was not designed to identify specific interacting molecules for mouse Rpn10 variants, it is possible that Rpn10 variants play some roles in more specific situations in mice.

Another aim of our study was to explore the role of Rpn10 in mice. Rpn10 deficiency is known to cause different phenotypes in different organisms. The severe developmental defects observed for Rpn10-deficient mice are in contrast to the viability of Rpn10-deficient yeast and worms but similar to the phenotypes observed for moss and flies, further confirming an essential role in higher eukaryotes. However, based on the phenotypes of ATPase subunit knockout mice, which exhibited an earlier halt in the developmental process than that for Rpn10 knockout mice (33), it is suggested that loss of Rpn10 could be compensated for to some degree, presumably by increasing transcription levels of proteasomes and UBL-UBA proteins as a feedback circuit in response to the accumulation of ubiquitinated proteins (40, 48).

The roles of the VWA and UIM domains of Rpn10 are also issues of debate. Rpn10ΔUIM mice, which express Rpn10 proteins lacking UIM domains but with the intact VWA domain, died in utero around E8.5, suggesting that the UIM-mediated recognition of ubiquitinated proteins is essential for mammalian development. Biochemical analysis of mice with liver-specific deletion of the UIM domains demonstrated accumulation of ubiquitinated proteins and defective proteolysis of ubiquitinated proteins. Considering that Rpn10ΔUIM mice survived longer than Rpn10-null mice, the VWA domain of Rpn10 might act as a "facilitator," as proposed for yeast (45). As shown in Fig. 4G, we detected a decreased amount of mHR23B in Rpn10ΔUIM proteasomes. Therefore, deletion of the UIM domains of Rpn10 impairs both direct binding and mHR23B-mediated delivery of ubiquitinated proteins to proteasomes. At present, the specific cause of the lethality of these mice in utero is not clear. Further studies are necessary to clarify whether such lethality is due to excessive general stress caused by accumulation of ubiquitinated proteins or to accumulation of some specific proteins that might essentially regulate mouse development, and in the latter case, what types of proteins these are.

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