Generation and identification of a conditional knockout allele for the PSMD11 gene in mice

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Abstract  

Background: Our previous study have shown that the PSMD11 protein was an important survival factor for cancer cells except for its key role in regulation of assembly and activity of the 26S proteasome. To further investigate the role of PSMD11 in carcinogenesis, we constructed a conditional exon 5 floxed allele of PSMD11 (PSMD11\(^{\text{flx}}\)) in mice.  

Results: It was found that homozygous PSMD11\(^{\text{flx}/\text{flx}}\) mice showed normal and exhibited a normal life span and fertility, and showed roughly equivalent expression of PSMD11 in various tissues, suggesting that the floxed allele maintained the wild-type function. Cre recombinase could induce efficient knockout of the floxed PSMD11 allele both in vitro and in vivo. Mice with constitutive single allele deletion of PSMD11 derived from intercrossing between PSMD11\(^{\text{flx}/\text{flx}}\) and CMV-Cre mice were all viable and fertile, and showed apparent growth retardation, suggesting that PSMD11 played a significant role in the development of mice pre- or postnatally. No whole-body PSMD11 deficient embryos (PSMD11\(^{−−}\)) were identified in E7.5–8.5 embryos in utero, indicating that double allele knockout of PSMD11 leads to early embryonic lethality. To avoid embryonic lethality produced by whole-body PSMD11 deletion, we further developed conditional PSMD11 global knockout mice with genotype Flp\(\text{FSF-R26CAG-CreERT2/}^{+/+}\); PSMD11\(^{\text{flx}/\text{flx}}\), and demonstrated that PSMD11 could be depleted in a temporal and tissue-specific manner. Meanwhile, it was found that depletion of PSMD11 could induce massive apoptosis in MEFs.  

Conclusions: In summary, our data demonstrated that we have successfully generated a conditional knockout allele of PSMD11 in mice, and found that PSMD11 played a key role in early and postnatal development in mice, the PSMD11\(^{\text{flx}/\text{flx}}\) mice will be an invaluable tool to explore the functions of PSMD11 in development and diseases.

Background  

In living mammalian cells, it has been estimated that more than 80% of the protein degradation is catalyzed by the 26S multiprotein complex proteasome [1], thus the proteasome has significant role in many of the biological processes, such as apoptosis, cell cycle regulation, antigen presentation and DNA damage repair [2]. The 26S proteasome consists of the 670 kDa proteolytic core particle (CP) or the 20S proteasome, and the 900 kDa regulatory particle (RP), or the 19S complex. The RP is made up of a lid comprising 9 subunits including PSMD11(Proteasome 26 s subunit, non-ATPase, 11), and a base which contains 6 ATPases and several other components [3, 4]. The 19S regulatory subunit is responsible for recognizing and binding the ubiquitin-protein conjugates and transporting the proteins into the CP to be degraded [5, 6].
In recent years, more and more diseases have been found to be associated with the dysfunction of proteasome, such as neurodegenerative diseases, cancer and aging [7, 8]. In order to understand the role of proteasome in these diseases, knockout studies for several subunits of proteasome have been conducted in mice, including Psmc3 and Psmc4 [9], Psmc1 [10], and Psmd4 [11], and found that proteasome play an indispensable role in early-embryonic development of mice and pathogenesis of some of the neurodegenerative diseases [10, 12].

The PSMD11 (Proteasome 26s subunit, non-ATPase, 11) protein (also known as RPN-6 in Caenorhabditis elegans) [13–15] is a component of the lid of the 19S RP, and plays a key role in the regulation of assembly and activity of the 26S proteasome [16, 17]. Additionally, PSMD11 protein was also found to be an important survival factor for cancer cells [18, 19]. Quick degradation of PSMD11 protein has been found to be associated with acute apoptosis in cancer cells, and its knockdown by siRNA can promote acute apoptosis in pancreatic cancer cells [18], suggesting that PSMD11 may be a multifunctional protein, and may be a novel therapeutic target for cancer, whether it can be targeted to treat cancer deserve further investigation.

To further delineate the role of PSMD11 in the process of carcinogenesis, we generated a conditional exon 5 floxed allele of PSMD11 (PSMD11\textsuperscript{flx}), and demonstrated that Cre recombinase could induce efficient knockout of the floxed PSMD11 allele both in vitro and in vivo in a temporal and tissue-specific manner. This novel conditional knockout mice of PSMD11 (PSMD11\textsuperscript{flx/flx}) will be very helpful for us to understand the function and mechanism of PSMD11 in physiological and pathological conditions.

**Results**

**Construction and identification of a conditional floxed allele of PSMD11 in mice**

The murine PSMD11 gene, about 44.62 kb in length, is situated on the reverse strand of chromosome 11, and consists of 14 exons in which the ATG start codon is in exon 1 and TAG stop codon in exon 13 (Fig. 1a). To construct a functional conditional knockout allele of PSMD11 in mice, we selected exon 5 as conditional knockout target by insertion of flanking lox\textsuperscript{P} sequences for its 100% sequence homology with the human PSMD11 exon 5, which indicated a highly conserved function for this exon. In addition, deletion of exon 5 can lead to a formation of a stop codon in the chimeric transcript of exon 4 and 6 (Fig. 1b). To construct the floxed allele of PSMD11 (PSMD11\textsuperscript{flx}) in mice, two relevant genomic DNA fragments in the murine BAC library was selected; a lox\textsuperscript{P} sequence and a frt-flanked Neo expression cassette for positive selection were inserted 220 bp downstream from exon 4, another lox\textsuperscript{P} sequence was inserted 549 bp downstream from exon 5. After confirmed by restriction analysis with restriction endonuclease ApaLI, Drdl, EcoRI, FspI5 or NotI (Fig. 1c and d) and sequencing (Additional file 1: Figure S1) with S14 primers (Table 1), the linearized targeting vector was electroporated into C57BL/6 embryonic stem cells, then G418 treatment, PCR (Fig. 2a) and Southern blotting (Fig. 2b) were performed to screen the correctly recombined ES cell colonies. Three correctly recombined clones (1B11, 1D5, 2H7) were injected into C57BL/6 blastocysts. Germline transmission was obtained in 3/3 clones. Then, the Neo cassette in the germline was removed through intercrossing F1 PSMD11\textsuperscript{flx-neo/+} mice with the FLPe deleter mice [20]. Then the resulted PSMD11\textsuperscript{flx/flx} offspring were interbred to obtain homozygous PSMD11 floxed (PSMD11\textsuperscript{flx/flx}) mice. The homozygous PSMD11 \textsuperscript{flx/flx} mice can be detected at the expected Mendelian ratio by Genotyping (Fig. 2c).

**Floxed PSMD11 gene could be deleted efficiently in vitro with Cre-mediated gene recombination**

Homozygous PSMD11\textsuperscript{flx/flx} mice appeared normal and fertile, and had a normal life span. Roughly equivalent endogenous expression of PSMD11 could be detected in various tissues such as liver and pancreas at both protein and mRNA level in both PSMD11\textsuperscript{flx/flx} mice and age matched WT mice (Fig. 3a and b). To demonstrate whether the floxed allele of PSMD11 could be knocked out by Cre in vitro, we isolated MEFs (mouse embryonic fibroblasts) from E14.5 embryos of intercrosses between PSMD11\textsuperscript{flx/flx} mice and infected with Adeno-associated virus (AAV) expressing either ZsGreen or Cre. Genomic PCR genotyping and sequencing confirmed the recombination of the floxed PSMD11 allele after 5 days following infection (Fig. 3c and Supporting Information 1). Near complete loss of mRNA and protein expression of PSMD11 could also be found by qRT-PCR and Western blot respectively (Fig. 3d-f). The loss of PSMD11 mRNA indicated that deletion of exon 5 could probably lead to premature transcription termination or mRNA decay due to the premature stop codon [21].

**Constitutive ablation of PSMD11 leads to early embryonic lethality (PSMD11\textsuperscript{+/−}) and growth retardation (PSMD11\textsuperscript{−/−}) in mice**

To verify if the floxed allele of PSMD11 could be knocked out by Cre in vivo, the PSMD11\textsuperscript{flx/flx} mice were crossed with cytomegalovirus (CMV)-Cre mice (Fig. 4a) to induce constitutive deletion of the floxed allele of PSMD11 in whole body [22]. The resultant heterozygous male and female PSMD11\textsuperscript{+/−} mice could be identified at the expected Mendelian ratio, and they appeared normal
Fig. 1 (See legend on next page.)
PSMD11 could be conditionally depleted in vitro and in vivo could induce massive apoptosis in mouse embryonic fibroblasts (MEFs)

To avoid embryonic lethality caused by whole-body PSMD11 deletion, we further developed conditional PSMD11 global knockout mice with genotype FLP;FSF-R26\textsuperscript{Δ}\textsuperscript{-\textsuperscript{CreERT2}/\textsuperscript{gfp}};PSMD11\textsuperscript{flx}\textsuperscript{/+} (FCP\textsuperscript{flx}Ftp) by crossing the human beta-actin FLPe deleter strain of mice with the FSP-R26\textsuperscript{Δ}\textsuperscript{-\textsuperscript{CreERT2}/\textsuperscript{gfp}} mice [24] and homozygous PSMD11\textsuperscript{flx}/\textsuperscript{flx} mice, in which FLP induced deletion of the FRT-stop-FRT (FSF) cassette could activate the latent tamoxifen-inducible allele CreERT2 (Fig. 5a and b). The FCP\textsuperscript{flx} mice appeared normal and fertile. For validating PSMD11 depletion, MEFs were isolated from mouse E14.5 embryos with genotype FLP;FSF-R26\textsuperscript{Δ}\textsuperscript{-\textsuperscript{CreERT2}/\textsuperscript{gfp}};PSMD11\textsuperscript{flx}/\textsuperscript{flx}, and treated with 0.5\textmu M 4-hydroxytamoxifen or vehicle (ethanol) for 5 days to induce deletion of the floxed allele of PSMD11. As shown in Fig. 5c, the deleted allele of PSMD11 (PSMD11\textsuperscript{Δ}, 643 bp) could be detected in the 4-hydroxytamoxifen treated MEFs, loss of protein expression of PSMD11 in the cytoplasm and nucleus of the majority of MEFs could be found by immunofluorescence staining (red arrows in Fig. 5d), qRT-PCR further confirmed that PSMD11 transcripts (Fig. 5e) could be depleted significantly.

For it had been shown that knockdown of PSMD11 by siRNA could promote acute apoptosis in pancreatic cancer cells [18], we also detected if depletion of PSMD11 could induce apoptosis in MEFs. As shown in Fig. 5f and g, treatment with 0.5\textmu M 4-hydroxytamoxifen for 5 days could gradually deplete the protein expression of PSMD11. The intracellular level of ubiquitinated proteins was found to be increased following the treatment with 4-hydroxytamoxifen, suggesting that the proteasomal function was compromised. No change of expression of PSMD4 was found, another component of the he 19S RP of the 26S proteasome. The apoptosis-specific cleaved-PARP and cleaved caspase 3 increased with the depletion of PSMD11, and a great deal of cells with nuclear condensation and fragmentation could be found by Hoechst 33342 staining, demonstrating that depletion of PSMD11 could induce apoptosis in MEFs.

To further characterize the effect of PSMD11 depletion on the functionality of the 26S proteasome, the chymotrypsin like activity of both 20S and 26S complexes was measured for 180 min by measuring directly the degradation rate of the peptide Suc-LLVY-AMC in whole-cell extracts from vehicle and tamoxifen treated MEFs. No significant differences was found (Fig. 5h), suggesting that PSMD11 depletion had no obvious effect on the peptide hydrolysis in the 20S proteolytic core, at least in this time point.

To detect whether PSMD11 could be depleted in a tissue-specific manner in vivo, the FCP\textsuperscript{flx} mice were fed with tamoxifen-containing food (400 mg/kg) for 3 days, then we detected the expression of PSMD11 in pancreas, liver and kidney. As shown in Fig. 5i, tamoxifen could induce efficient PSMD11 depletion in pancreas and liver, and partial depletion in kidney, suggesting that
the conditional knockout system also works in a tissue-specific manner in vivo. Meanwhile, it was found that the P53 protein was up-regulated following down-regulation of PSMD11, which is consistent with the findings that proteasome inhibition could up-regulate p53 [23]. The biologic effect of PSMD11 global knockout in adult mice is under investigation.

Discussion

To further delineate the function of PSMD11 in vivo, we generated a conditional exon 5 floxed allele of PSMD11 (PSMD11$^{flx/flx}$) in mice. It was found that homozygous PSMD11$^{flx/flx}$ mice were normal and fertile, and showed normal expression of PSMD11 in various tissues, suggesting that the floxed allele maintained the wild-type function. Cre could induce efficient knockout of the floxed allele of PSMD11 both in vitro and in vivo. Mice with constitutive single allele deletion of PSMD11 obtained through intercrossing between PSMD11$^{flx/flx}$ and CMV-Cre mice showed apparent growth retardation compared with the age-and sex-matched WT mice, suggesting that PSMD11 have important role in the development of mice pre- or postnatally. No whole-body PSMD11 deficient E7.5–8.5 embryos (PSMD11$^{-/-}$; FSF−) were identified in utero, indicating that double allele knockout of PSMD11 led to early embryonic lethality. To avoid embryonic lethality elicited by whole-body PSMD11 deletion, we further developed conditional PSMD11 global knockout mice with genotype Flp$^{FSF}$;CreERT2/+.PSMD11$^{flx/flx}$, and demonstrated that PSMD11 could be depleted in a temporal and tissue-specific manner. Meanwhile, it was found that depletion of PSMD11 could induce massive apoptosis in MEFs, further indicating that PSMD11 was a significant survival factor for cells, whether it could be targeted to treat cancer deserve further investigation.

Proteasomes have important role in many of the biologic processes such as survival, DNA repair and

Table 1 Oligonucleotides used in this study

| Name   | Sequence | Purpose                        |
|--------|----------|--------------------------------|
| Region | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| 1-F    | 5′-gcagcttgactctct GG-3′       | Gene targeting                 |
| Region | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| 1-R    | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| Region | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| 2-F    | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| Region | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| 2-R    | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| Neo-F  | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| Neo-R  | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| 3-F    | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| Region | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| 3-R    | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| Region | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| 4-F    | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| Region | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| 4-R    | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| CRE-F  | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| CRE-R  | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| F1     | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| R1     | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| F2     | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| R2     | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| F3     | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| S1     | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| S2     | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| S3     | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| S4     | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| GAPDH- | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| F      | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| GAPDH- | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| R      | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| PSMD11- | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |
| F      | 5′-tcagctgtggcctctcatacttc-3′ | Gene targeting                 |

PSMD11$^{flx/flx}$

Flp-F
Flp-R
Probe for neo
proliferation of malignant cells, and RAS mutant cancer cells have been shown to be selectively addicted to proteasome activity [25–27]. However, proteasome inhibitors (PIs) are only effective in hematologic malignancies including multiple myeloma and mantle cell lymphoma [28]. For solid tumors, PIs only showed marginal effect [29, 30], the reason was not clear. For the proteasome inhibitors current in use all work primarily at the chymotrypsin-like site on β5 subunit of the 20S catalytic core of proteasome [31], one of the reasons may be that the chymotrypsin-like site on β5 is not an appropriate therapeutic target for solid cancers, thus whether other components of proteasome can be targeted to treat cancer deserve further investigation.

PSMD11 is a component of the lid of the 19S RP, it can hold the 20S CP and 19S RP together [14] like a molecular clamp through protein-protein interactions with Rpt-6 of the ATPase ring of the 19S catalytic core of proteasome [31], one of the reasons may be that the chymotrypsin-like site on β5 is not an appropriate therapeutic target for solid cancers, thus whether other components of proteasome can be targeted to treat cancer deserve further investigation.

PSMD11 is a component of the lid of the 19S RP, it can hold the 20S CP and 19S RP together [14] like a molecular clamp through protein-protein interactions with Rpt-6 of the ATPase ring of the 19S RP and the a-2 subunit of the 20S CP [32]. In this study, it was found that deletion of PSMD11 could lead to increased intracellular levels of ubiquitinated proteins, but had no obvious effect on the peptide hydrolysis activity of the 20S proteolytic core, further confirmed that PSMD11 played an essential role in the RP to recognize, unfold and translocate the ubiquitinates-proteins into the 20S proteolytic cavity of the proteasome [33]. For depletion of PSMD11 could induce massive apoptosis in MEFs, it also indicated that the 20S proteolytic CP alone was unable to maintain survival of the MEFs.

PSMD11 constitutively deficient mice have never been reported previously so far. In this study, it was found that PSMD11 single allele deleted mice (PSMD11+/−) showed evident growth retardation, whereas double allele deleted mice (PSMD11−/−) showed early embryonic lethality, indicating that PSMD11 had important role in the development of mice pre- or postnatally. Meanwhile, it was found that depletion of PSMD11 led to up-regulation of P53 in the conditional knockout mice of PSMD11, suggesting that regulation of the expression level of some of the critical proteins might be one of the possible mechanisms for regulation of development by PSMD11 or proteasome. In the following study, we will try to identify these critical proteins with proteomics.

Fig. 2 Generation of mice with floxed allele of PSMD11. a PCR amplification of region 1 and 2 with primer A1 and A2, or B1 and B2 to identify appropriately targeted embryonic stem cells. Size of the resulting PCR products was 6 kb (MT) for primer A1 and A2, and 310 bp (MT) and 240 bp (WT) for primer B1 and B2 respectively. b reconfirmation of targeted embryonic stem cell clones by southern blotting. Nde I and Hind III digested DNA from three individual clones that had passed all controls was electrophoretically separated on a 0.5% agarose gel, after transfer to a nylon membrane, a probe targeted against the Neo cassette was hybridized with the digested DNA, which showed two bands at 10.1 kb, and 8.3 kb respectively. c Genotyping of mice with genomic DNA from tail biopsies. Primers F2 and R2 were used to distinguish the floxed allele (PSMD11flox, 463 bp) from the wild-type allele (WT, 335 bp)
One of the limitations of our study is that we did not characterize the time and reason of embryo death of PSMD11 double allele deleted mice. Taking into account the findings that hESCs (human embryonic stem cells) were highly sensitive to proteasome inhibition [16], and depletion of PSMD11 could induce massive apoptosis in MEFs, we speculate that the death time of embryo may be at the zygote stage, further study with our conditional PSMD11 global knockout mice will be helpful to resolve this question.

Conclusions
In summary, our data demonstrated that we have successfully generated a conditional knockout allele of PSMD11 in mice, and found that PSMD11 played a key role in early and postnatal development in mice. This novel conditional knockout mice of PSMD11 will be very helpful for us to understand the function and mechanism of PSMD11 in physiological and pathological conditions.

Methods
Materials
Cell culture reagents were all purchased from Invitrogen (Groningen, the Netherlands). Primers were synthesized by Sangon Biotech (Shanghai, China). Restriction endonucleases were got from New England BioLabs (Mannheim, Germany). Transformation and plasmid amplification was performed with TOP10 and Stbl3 E. coli strains (Invitrogen, Groningen, the
Netherlands). Tamoxifen Citrate (S1972) and 4-Hydroxytamoxifen (S7827) were purchased from Sell-eck (Shanghai, China).

Fig. 4 Constitutive ablation of PSMD11 leads to early embryonic lethality (PSMD11+/−) and growth retardation (PSMD11+/−) in mice. a Methods to generate PSMD11 constitutive KO mice. b Genotyping of E7.5→8.5 embryos from intercrossing between PSMD11+/− heterozygous mice. The 643 bp band indicate the exon 5 deleted allele of PSMD11 (PSMD11Δ). c Representative smaller body size of PSMD11+/− mice compared with WT littermates. d Growth curve of PSMD11+/− mice (n = 9) and age- and sex-matched male and female WT littermates (n = 9). **P < 0.01, *P < 0.05. e Representative image of E7.5→8.5 embryos of mice under stereomicroscope. f Representative HE images of E7.5→8.5 WT and PSMD11+/− embryos of mice. g and h Representative images of Arcturus Histogene™ staining and Laser Capture Microdissected tissues of E7.5→8.5 embryos of mice.

Mouse strains
CMV-Cre mice (Jackson lab, stock#: 006054) (ref. [15]), PSP-R26CAG-CreERT2/+ mice (ref. [17]), the human beta-
Fig. 5 (See legend on next page.)
actin FLPe deleter strain of mice (B6;SJL-Tg (ACTF LPe)9205Dym/J, the Jackson Laboratory, Stock Number: 003800) and R26 $^{CAG-CreERT2/+}$ mice [24] have been described previously [20]. The mice were all on a mixed C57BL/6;129S6/SvEv genetic background. All mice were kept at a SPF environment, no mice were excluded from analyses. Experiments with mice were performed with protocols approved by the Shandong University Institutional Animal Care and Use Committee, complying with the rules of Regulations for the Administration of Affairs Concerning Experimental Animals (Approved by the State Council of China). Mice after the study were all euthanized by cervical dislocation.

### Targeting vector construction for PSMD11

The floxed allele of PSMD11 was generated with traditional gene targeting methods in collaboration with Cyagen Biosciences (Guangzhou, China). The targeting vector was constructed with positive BAC clone RP23-307G3 and RP23-276 J12 from the C57BL/6 J library. In the targeting vector, the long homology arm (LA) including exon4 (4.93 kb), the short homology arm (SA, 2.85 kb), and the conditional knockout region (cKO) including exon 5 were generated by PCR with high fidelity Taq DNA polymerase, and then sequentially integrated into the targeting vector with recombination sites and selection markers.

A loxP/frt Neo cassette including exon 5 was inserted 220 bp downstream from exon 4, two flr sites flanked the Neo cassette for later Flp (Flipase) recombinase excision, it also contained the promoter of the mouse phosphoglucokinase gene and a synthetic polyadenylation sequence. The cassette was flanked by two loxP sites, one outside the flr sites, one outside the exon 5. The targeting vector was 17.3 kb totally including the vector backbone and Neo cassette. Restriction analysis with restriction endonuclease ApaLI, Drdl, EcoRI, FspI5 and NotI was performed to confirm the structure of the targeting vector respectively. Sequence of connection region between vector backbone and homology arm or the conditional knockout region (cKO) was confirmed by sequencing with $S_{1-4}$ primers (Table 1).

### Targeted embryonic stem (ES) cells

The linearized targeting vector (10 μg) by NotI was electroporated into C57BL/6 ES cells, then the ES cells were selected with G418, total 175 drug-resistant clones were obtained. After expansion of the surviving clones, genotyping by PCR was applied to identify correctly recombinant clones with primer A1 and A2 for region 1, B1 and B2 for region 2 (Table 1). Size of the PCR bands were 6 kb (MT) for primers A1 and A2, and 310 bp (MT) and 240 bp (WT) for primers B1 and B2 respectively. Three ES clones which passed all the controls were further reconfirmed by Southern blotting. DNA digested with Nde I and Hind III was separated on a 0.5% agarose gel, then transferred onto a PVDF membrane, a probe targeting the Neo cassette was hybridized with the digested DNA, which resulted in two bands at 10.1 kb, and 8.3 kb respectively. DNA from wild-type C57Bl/6 (B6) mice were used as controls.

### Generation of floxed allele of PSMD11 in mice

Three correctly targeted ES clones were used to microinject into C57BL/6 blastocysts. Germline transmission was gained in 3/3 clones harboring the targeted allele. Then, the Neo cassette in the germine was removed by crossing the F1 PSMD11 $^{flx-neo/+}$ mice with the FLPe deleter mice. Then the resulted PSMD11 $^{flx/ne}$ offspring were interbred to obtain homozygous PSMD11 floxed (PSMD11 $^{flx/flx}$) mice. PCR using F1, R1 and F2, R2 primers (Table 1) was used to genotype mice.

### Generation of PSMD11 constitutively knocked out (KO) mice

Intercrossing between homozygous PSMD11 $^{flx/flx}$ mice and CMV-Cre mice was performed to generate the
constitutively knocked out (KO) mice of PSMD11. The CMV-Cre mice express Cre ubiquitously, and can delete floxed gene in all the tissues, including germ cells. The obtained heterozygous mice with single allele deletion (PSMD11\(^{+/−}\)) were then intercrossed to generate PSMD11 global knockout mice (PSMD11\(^{−/−}\)). The genomic sequence of the PSMD11\(^{+/−}\) mice after deletion of exon 5 was confirmed by sequencing with F3 and R1 primers (Table 1).

**DNA extraction, genotyping and sanger sequencing**

One-tube General Sample DNAup kit for PCR was used to extract DNA following the manufacturer’s protocol (Sangon Biotech, Shanghai, China). PCR genotyping was performed using the primers in Table 1. PCR bands were separated by 1% agarose gel electrophoresis or purified by an agarose gel extraction kit (TransGen Biotech, Beijing, China) for Sanger sequencing (Sangon Biotech, Shanghai, China).

**Protein extraction and Western Bloting**

Protein was extracted from tissues or cells on ice using RIPA with 1% proteinase inhibitor cocktail (Sigma-Aldrich, Shanghai, China) and 1% phenylmethane sulfonyl fluoride. Protein concentration was determined with a BCA assay kit. 30-50 μg of total protein was resolved with a 10% SDS-PAGE gel, then transferred to nitrocellulose membranes (Millipore). The membranes were blocked with 5% nonfat milk in TBS and 0.1% Tween20, then probed with antibodies against k48-linkage specific polyubiquitin (#12805), anti-cleaved-caspase 3 (#9664) and anti-k48-ubiquitinated β-actin and GAPDH. Biotinylated secondary antibodies (Zhongshan Goldenbridge Biotechnology Co., LTD) were used to probe corresponding proteins. The membranes were washed twice with PBS, it was blocked for 1 h PBS containing 3% (w/v) bovine serum albumin (BSA), 1% (w/v) Saponin and 1% (v/v) Triton X-100, then incubated with rabbit anti-PSMD11 antibody (1:100, 14,786–1-AP, Proteintech Group, Rosemont, USA) for 24 h at 4°C in the dark followed by FITC conjugated goat anti-rabbit antibodies (#0311, Zhongshan Goldenbridge Biotechnology Co., LTD) for 1 h at room temperature. Nuclei were counterstained with DAPI (1:1000, Invitrogen) for 5 min at room temperature. After three rinses in PBS, slides were mounted with antifading Mounting Medium (Solarbio, Beijing, China) and imaged with fluorescent microscopy with appropriate excitation wavelengths.

**Real-time RT-PCR for PSMD11 mRNA**

Real-time RT-PCR was carried out as described previously [19]. Briefly, RNA was extracted with Trizol (Qiagen) and purified by an agarose gel extraction kit (TransGen Biotech, Beijing, China). PCR genotyping was performed using the primers in Table 1. PCR bands were separated by 1% agarose gel electrophoresis or purified by an agarose gel extraction kit (TransGen Biotech, Beijing, China) for Sanger sequencing (Sangon Biotech, Shanghai, China).

**Laser-capture microdissection and DNA extraction**

Formalin-fixed, paraffin-embedded tissue sections (8 μm thick) of mouse E7.5–8.5 embryos were dewaxed, stained with Histogene™ staining solution (Applied biosystems by Thermo Fisher Scientific, Lithuania) and microdissected using an Arcturus XT laser-capture microdissection system (Appliedbiosystems by Thermo Fisher Scientific, Shanghai, China). The Rapid Animal Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China) was used to isolate DNA from the micro-dissected cells.

**Immunofluorescence staining**

MEFs were fixed in 4% buffered formalin for 20 min, after washing twice with PBS, it was blocked for 1 h PBS containing 3% (w/v) bovine serum albumin (BSA), 1% (w/v) Saponin and 1% (v/v) Triton X-100, then incubated with rabbit anti-PSMD11 antibody (1:100, 14,786–1-AP, Proteintech Group, Rosemont, USA) for 24 h at 4°C in the dark followed by FITC conjugated goat anti-rabbit antibodies (#0311, Zhongshan Goldenbridge Biotechnology Co., LTD) for 1 h at room temperature. Nuclei were counterstained with DAPI (1:1000, Invitrogen) for 5 min at room temperature. After three rinses in PBS, slides were mounted with antifading Mounting Medium (Solarbio, Beijing, China) and imaged with fluorescent microscopy with appropriate excitation wavelengths.
Apoptosis detection with Hoechst 33342 staining and confocal laser-scanning microscopy

MEFs were treated with 0.5μM 4-hydroxytamoxifen or vehicle (ethanol) for 5 days to induce deletion of the floxed allele of PSMD11, after that, the cells were incubated with 1 μg/mL Hoechst 33342 in culture media for 20 min, then photos with a frame size of 1024 × 1024 pixels were taken with Zeiss LSM 800 with airyscan technology (Carl Zeiss, Jena, Germany) with a x 10/0.45 and a x 20/0.8 Plan-APOCHROMAT objective. Images were collected and processed with ZEN 2009 software.

Detection of proteasome activity in crude protein extracts

The chymotrypsin-like activity of proteasome was detected with 0.1 mM Suc-LLVY-AMC peptide (HY-P1002, MedChemExpress LLC, Shanghai, China) in 100 μl ATP/DTT lysis buffer (Tris-HCl, pH 7.8, 0.5 mM DTT, 5 mM ATP, MgCl2 5 mM) at 37 °C in 30 μg whole-cell extracts from control and tamoxifen treated fibroblasts for 4 days. The fluorescence of released amino-methylcoumarin was monitored with a plate reader at an excitation wavelength of 360 nm and emission wavelength of 460 nm over a period of 180 min.

Tamoxifen induction deletion of PSMD11 in mice

Tamoxifen-containing food (400 mg/kg) was fed to mice for 3 days to activate CreERT2.

Statistical analysis

All data was obtained from at least three independent assays and shown as mean ± SEM. Prism 5.0 (Graphpad) was used to create graphs and to do statistics. Student’s t-test or ANOVA was used to detect the significance of differences, p < 0.05 was considered to be statistically significant. Only representative results are shown.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12861-020-00233-1.

Additional file 1: Figure S1. Sequence of the final targeting vector. Homology arms are in green, cKO region is in blue. Flt sites are in violet, LoxP sites are in red. Exon 4 and 5 are underlined. Sequence confirmed regions are highlighted in yellow. Sequence in lower-case letters is sequence deleted after Flp and Cre-mediated recombination.

Additional file 2.

Abbreviations

PSMD11: Proteasome 26 s subunit, non-ATPase, 11; RPN-6: Regulatory particle non-ATPase 6; CP: Core Particle; RP: Regulatory Particle; BAC library: Bacterial Artificial Chromosome; hESCs: Human Embryonic Stem Cells; MEFs: Mouse Embryonic Fibroblasts; CMV: Cytomegalovirus; cKO: Conditional Knockout; AAV: Adeno-associated virus; Flp: Flippase; ACTB: Actin Beta; DAPI: 4',6-diamino-2-phenyl indole; RT-PCR: Reverse Transcription-quantitative Polymerase Chain Reaction; ANOVA: Analysis of Variance; SEM: Standard Error of the Mean; Pls: Proteasome Inhibitors; PARP: Poly ADP Ribose Polymerase; Cre: Cyclization recombination enzyme; Floxed: Flanked by LoxP; LoxP: Locus of X-overP1; frt: flippase recognition target; Neo: Neomycin; ZsGreen: Reef coral Zoanthus sp. green fluorescent protein; KO: Knockout; WT: Wild Type; SPF: Specific Pathogen Free; BSA: Bovine Serum Albumin; LSM: Laser-scanning microscopy; DTT: Dithiothreitol; ATP: Adenosine triphosphate; DNA: DeoxyriboNucleic Acid; PSMC: 3: Proteasome 26S subunit; ATPase 3; PSMC: 4: Proteasome 26S subunit; ATPase 4; PSMD: 4: Proteasome 26S subunit, non-ATPase 4; siRNA: Small interfering RNA; RFU: Relative Fluorescence Units; SD: Standard Deviation

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Authors’ contributions

L.Z. and J.Z. contributed equally to this work. T.Q. planned and coordinated the study and performed final data analysis. L.Z., J.Z., Y.Z., L.W., A.N., W.Z., X.X., S.Z., C.S., J.W., Z.B., X.Z., C.W. performed the experiments, data analysis and interpretation. D.S. and B.S. kindly provided the FSF-r26CreERT2+ mice. T.Q. and L.Z., J.Z. wrote the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study is available from the corresponding author on reasonable request. The sequencing data is available in the NCBI SRA database: [https://www.ncbi.nlm.nih.gov/sra/PRJNA668444]. Accession ID: PRJNA668444.

Ethics approval and consent to participate

Experiments with mice were performed with protocols (# 20140139) approved by the Shandong University Institutional Animal Care and Use Committee, complying with the rules of Regulations for the Administration of Affairs Concerning Experimental Animals (Approved by the State Council of China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Bochtler M, Ditzel L, Groll M, Hartmann C, Huber R. The proteasome. Annu Rev Biophys Biomol Struct. 1999;28:295–317.
2. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem. 1998; 67:425–79.

3. Hoffman L, Rechsteiner M. Molecular cloning and expression of subunit 9 of the 26S proteasome. FEBS Lett. 1997;404(2–3):179–84.

4. Saito A, Watanabe TK, Shimada Y, Fujitani T, Slaughter CA, DeMartino GN, Tanahashi N, Tanaka K. cDNA cloning and functional analysis of p41.5 and p55, two regulatory subunits of the 26S proteasome. Gene. 1997;203(2):241–50.

5. Thrower JS, Hoffman L, Rechsteiner M, Pickart CM. Recognition of the polyubiquitin proteolytic signal. EMBO J. 2000;19(1):94–102.

6. Kohler A, Ciscio P, Leggett DS, Woo KM, Goldberg AL, Finley D. The axial channel of the proteasome core particle is gated by the Rpt2 ATPase and controls both substrate entry and product release. Mol Cell. 2001;7(6):1143–52.

7. Schmidt M, Finley D. Regulation of proteasome activity in health and disease. Biochim Biophys Acta. 2014;1843(1):13–25.

8. Klöll-Hermitz A, Ebstein F, Stotzel C, Geoffroy V, Schaefer E, Scheidecker S, Bär S, Takamiya M, Kawakami K, Zieba BA, et al. Proteasome subunit PSMC3 variants cause neurosensory syndrome combining deafness and cataract due to proteinotoxic stress. EMBO Mol Med. 2020;12(7):e11861.

9. Sakao Y, Kawai T, Takeuchi Q, Copeland NG, Gilbert DJ, Jenkins NA, Takeda K, Akira S. Mouse proteasome ATPases Pimc3 and Pimc4: genomic organization and gene targeting. Genomics. 2000;67(1):1–7.

10. Bedford L, Hay D, Devoy A, Paine S, Powe DG, Seth R, Gray T, Topham I, Fone K, Rezvani N, et al. Depletion of 26S proteasome in mouse bone marrow causes neurodegeneration and Lewy-like inclusions resembling human pale bodies. J Neurosci. 2008;28(3):1899–908.

11. Hamazaki J, Sasaki K, Kawahara H, Hisanaga S, Tanaka K, Murata R. Rpn10-mediated degradation of ubiquitinated proteins is essential for mouse development. Mol Cell Biol. 2007;27(29):6629–38.

12. Tashiro Y, Urushitani M, Inoue H, Koike M, Uchiyama Y, Komatsu M, Tanaka K. The proteasome regulatory subunit p55, two regulatory subunits of the 26S proteasome. J Biol Chem. 2003;278(9):6687–95.

13. Isono E, Saito N, Kamata N, Sairi Y, Toh EA. Functional analysis of Rpn6, a molecular clamp holding the core and regulatory subcomplexes together. Proc Natl Acad Sci U S A. 2012;109(1):149–54.

14. Santamaria PG, Finley D, Ballesta JP, Remacha M. Rpn6p, a proteasome subunit from Saccharomyces cerevisiae, is essential for the assembly and activity of the 26 S proteasome. J Biol Chem. 2003;278(9):6687–95.

15. Vilchez D, Boyer L, Morantte I, Lutz M, Merkwirth C, Joyce D, Spencer B, Page L, Masleh A, Berggren WT, et al. Increased proteasome activity in human embryonic stem cells is regulated by PSMD11. Nature. 2012;489(7413):304–8.

16. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. Cell. 2009;136(5):823–37.

17. Chun S, Wu J, Lu Y, Ma YB, Lee BH, Yu Z, Ouyang Q, Finley DJ, Kirchner MW, Mao Y. Structural basis for dynamic regulation of the human 26S proteasome. Proc Natl Acad Sci U S A. 2016;113(46):12991–6.

18. Tanaka K. The proteasome: overview of structure and functions. Jpn J Pharmacol. 2007;104(3):537–47.

19. Pathare GR, Nagy I, Bohn S, Unverdorben P, Hubert A, Komer R, Nickell S, Lasker K, Sale A, Tamura T, et al. The proteasomal subunit Rpn6 is a molecular clamp holding the core and regulatory subcomplexes together. Proc Natl Acad Sci U S A. 2012;109(1):149–54.

20. Chen S, Wu J, Lu Y, Ma YB, Lee BH, Yu Z, Ouyang Q, Finley DJ, Kirchner MW, Mao Y. Structural basis for dynamic regulation of the human 26S proteasome. Proc Natl Acad Sci U S A. 2016;113(46):12991–6.

21. Manasanch EE, Ofori RW. Proteasome inhibitors in cancer therapy. Nat Rev Clin Oncol. 2017;14(7):417–33.

22. Chen S, Wu J, Lu Y, Ma YB, Lee BH, Yu Z, Ouyang Q, Finley DJ, Kirchner MW, Mao Y. Structural basis for dynamic regulation of the human 26S proteasome. Proc Natl Acad Sci U S A. 2016;113(46):12991–6.

23. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. Cell. 2009;136(5):823–37.

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