SUPPLEMENTARY MATERIALS

MATERIALS AND METHODS

Real-Time RT-PCR Conditions

The RT reaction was performed with the following parameters: 60 minutes at 44 °C followed by 15 minutes at 70 °C. The reaction contained 2.5 ng/μL random hexamer, 25 ng/μL oligo dT 18 mer (Integrated DNA Technologies, Coralville, IA), 500 μM dNTPs (Bioline, Taunton, MA), 1X RT Buffer, 10 mM dithiothreitol (DTT), 10 U superase inhibitor (Ambion, Austin, TX), and 100 U Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a 20 μL total reaction volume. Quantification of cDNA was performed with the Quant-iT PicoGreen dsDNA assay kit (Invitrogen). The cDNA (6 ng) was then used as the template for Real-Time PCR performed in an iQ5 Multicolor Real-Time PCR detection system (BioRad, Hercules, CA). PCR reactions were carried out using a two-step amplification with a melt curve using the following cycle parameters: 7 minutes at 95 °C, followed by 35 cycles of 10 s at 95 °C and 30 s at 55 °C. For each gene, the reaction was conducted in triplicate in a 25 μL reaction volume containing 1X PCR buffer, 3 mM $MgCl_2$, 800 μM dNTPs (Bioline), 10 nM fluorescein (USB Corporation, Cleveland, OH), 0.025X SYBR Green (Invitrogen), 0.1% Triton X-100, 100 ng/μL bovine serum albumin (Sigma, St. Louis, MO), 5% DMSO (Sigma), 0.03 U/μL Immolase DNA polymerase (Bioline), and 200 nM of each primer set (Integrated DNA Technologies) (see Table S1).

Proteasome-enriched homogenates from spleen

Single spleen homogenates from 2 mo. mice were prepared similar to methods described previously (Husom et al., 2004). Frozen spleen was homogenized in buffer containing 20mM Tris (pH 7.4), 20% (wt/vol) sucrose, 2 mM $MgCl_2$, 10 mM glucose, and 2% (wt/vol) CHAPS. Tissue homogenate was centrifuged at 4600 x g for 15 min at 4 ^{o}C. The pellet was

rehomogenized and centrifuged as above. Supernatants were combined and centrifuged at 12,000 x g for 15 min at 4 ^{o}C. The final supernatant was centrifuged at 100,000 x g at 4 ^{o}C for 16 h. Pellets enriched for proteasome were resuspended in 50 mM Tris-HCl, 5mM $MgCl_2$ (pH 7.5). Protein concentration was determined using the BCA assay.

Western Blotting

Western blot membranes were incubated with a primary antibody (Table S2) for 14-16 h at 4 ^{o}C. The appropriate secondary antibody conjugated to HRP (Pierce) was applied to the membrane. Membranes were developed using SuperSignal West Dura Extended Duration chemiluminescence substrate (Pierce). Images were taken using a ChemiDoc XRS (Bio-Rad) and densitometry was performed using Quantity One (Bio-Rad). Samples were normalized to a standard run on each blot. To confirm incorporation of proteasome β-subunit in the proteasome complex, Western blot antibody reactions were aligned with the 20S proteasome purified from mouse spleen that was run on each blot to ensure that only the processed form of the subunit was measured.

Supplementary Table 1. Primers used for Real-Time RT-PCR.

Gene	Accession #	Product (bp)	Primer Sequence
LMP7	NM_010724	101	For 5'-GGGACAAGAAGGGACCAGGA-3'
			Rev 5'-TGCCGGTAACCACTGTCCATCA-3'
LMP2	NM_013585	133	For 5'-CATCATGGCAGTGGAGTTTG-3'
			Rev 5'-TGAGAGGGCACAGAAGATG-3'
MECL-1	NM_013640	120	For 5'-AAGACCGGTTCCAGCCAAACATGA-3'
			Rev 5'-TGATCACACAGGCATCCACATTGC -3'
β5	NM_011186	145	For 5'-CGAATCGAAATGCTTCACGG -3'
			Rev 5'-GAAGGTACGGGTTGATCTCT -3'
β1	NM_008946	132	For 5'-GCCTTAGCTGTTCGTCGAG -3'
			Rev 5'-TAGAACCACGCCCCCATTAAA -3'
β2	NM_011187	122	For 5'-GTGTCGGTGTTTCAGCCAC -3'
			Rev 5'-GTGCCAGTTTTCCGAGCTTTC -3'
α7	NM_011184	143	For 5'-AAACAGTAGTACAGCGATTGGG-3'
			Rev 5'-CTGCAACTGCCATTCCAACA-3'
ARBP	NP_031501	102	For 5'-CTTTCTGGAGGGTGTCCGCAA -3'
			Rev 5'-ACGCGCTTGTACCCATTGATGA -3'

Accession number is from NCBI database. For, Forward; Rev, Reverse; ARBP, acidic ribosomal phosphoprotein P0.

Supplementary Table 2. Antibodies used for Western blotting.

Antibody	Type	Dilution	Company
α7	M	1:1000	Biomol, Plymouth Meeting, PA
LMP2	M	1:1000	Biomol
LMP7	R	1:1000	Biomol
β1	R	1:1000	Affinity BioReagents, Golden, CO
β5	R	1:1000	Affinity BioReagents
PA28α	R	1:1000	Biomol
PA700 S4	R	1:1000	Affinity BioReagents
Ubiquitin	R	1:650	Stressgen, Ann Arbor, MI

All antibodies were isotype IgG. Monoclonal, host species mouse (M); polyclonal, host species rabbit (R).

Supplementary Figure 1

mRNA expression of proteasome subunits in 2 mo. mouse retina. (A) Representative 2% agarose gel of PCR products for immunoproteasome subunits (LMP2, LMP7, and MECL-1), α7 proteasome subunit, and ARBP (reference gene). 1=WT, 2=L7M1, 3=L7. Size marker units are in base pairs. Summary of qRT-PCR results for the (B) α7 subunit, immunoproteasome subunits (LMP2, MECL-1), and (C) standard catalytic proteasome subunits (β5, β1, β2). Values are mean ± SEM and are expressed relative to WT. WT n=5-8; L7 n=4-9; L7M1 n=9.

Supplementary Figure 2

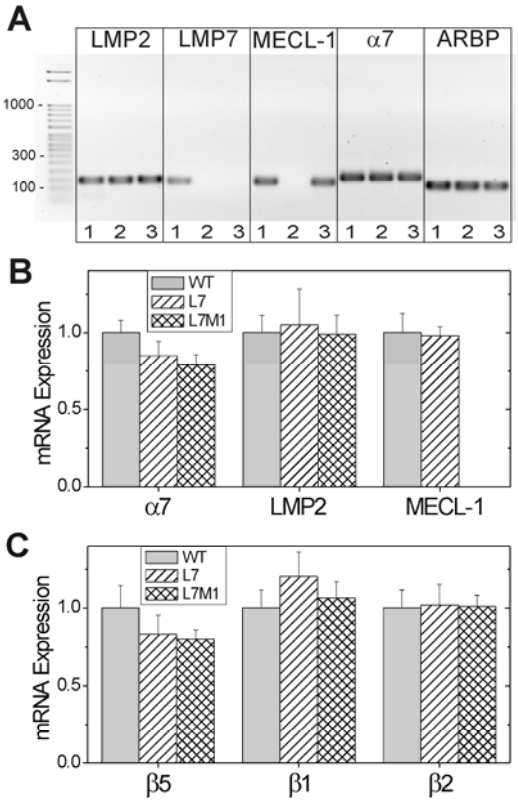
Age- and strain-related content of ubiquitin-modified proteins. Summary of Western blot densitometry for WT (■), L7 (○) and L7M1 (Δ) retina. Two-way ANOVA results showed no significant difference with age or between strains of ubiquitin-modified proteins. All values are mean ± SEM and are shown relative to WT 2 mo. WT n=4-6; L7 n=3-7; L7M1 n=3-6 per group.

Supplementary Figure 3

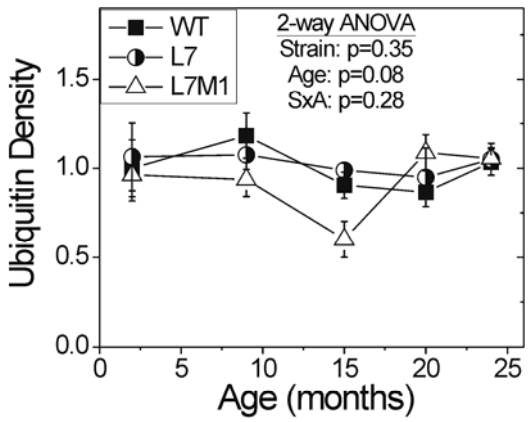
Age- and strain-related measures of content of proteasome activators. Summary of Western blot densitometry for WT (■), L7 (○) and L7M1 (Δ) retina. Two-way ANOVA results are provided in panels A and B. (A) PA700 S4 subunit content. No significant difference with age or between mouse strains. (B) PA28α subunit content. No significant difference with age or between mouse strains. All values are mean ± SEM and are shown relative to WT 2 mo. WT n=3-15; L7 n=4-7; L7M1 n=2-7 per group.

Supplementary Figure 4

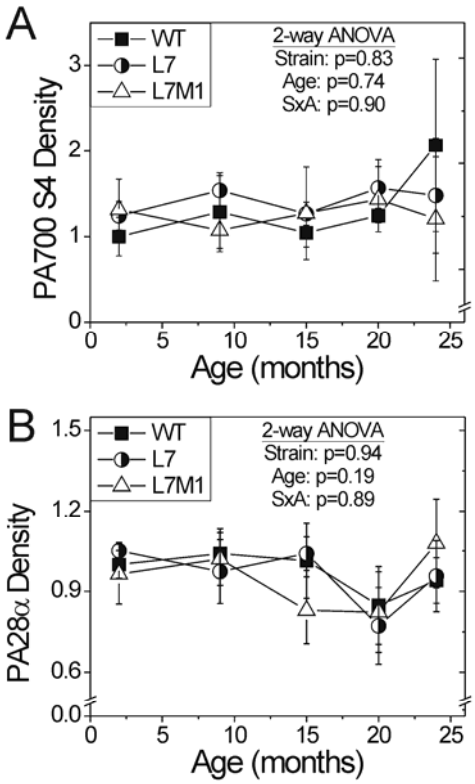
Catalytic activity of standard and immunoproteasome. (A) Representative Western blot of proteasome-enriched homogenates from WT or L7M1 spleen. (B) Catalytic activity of proteasome-enriched homogenates from WT (solid) or L7M1 (cross-hatched) spleen using fluorogenic peptide substrates to test chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C-L) activities. Two-sample T-tests were performed; *, indicates significance at $p<0.05$. All values are mean ± SEM, n=4 per group.



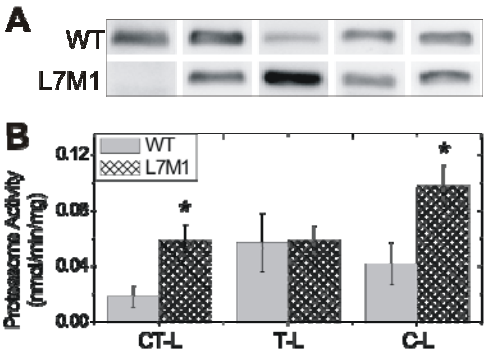
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4