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Uncoupling phototoxicity-elicited neural dysmorphology and death by insidious function and selective impairment of Ran-binding protein 2 (Ranbp2)

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Abstract

Morphological disintegration of neurons is coupled invariably to neural death. In particular, disruption of outer segments of photoreceptor neurons triggers photoreceptor death regardless of the pathological stressors. We show that *Ranbp2*$^{-/-}$*::Tg-Ranbp2*^{CLDm} mice with mutations in SUMO-binding motif (SBM) of cyclophilin-like domain (CLD) of Ranbp2 expressed in a *null Ranbp2* background lack untoward effects in photoreceptors in the absence of light-stress. However, compared to wild type photoreceptors, light-stress elicits profound disintegration of outer segments of *Ranbp2*$^{-/-}$*::Tg-Ranbp2*^{CLDm} with paradoxical age-dependent resistance of photoreceptors to death and genotype-independent caspase activation. *Ranbp2*$^{-/-}$*::Tg-Ranbp2*^{CLDm} exhibit photoreceptor death-independent changes in ubiquitin-proteasome system (UPS), but death-dependent increase of ubc9 levels. Hence, insidious functional impairment of SBM of Ranbp2's CLD promotes neuroprotection and uncoupling of photoreceptor degeneration and death against phototoxicity.

1. Introduction

Morphological disintegration of neurons invariably triggers neural death and is a hallmark feature of neurodegeneration [1–6]. Photoreceptors neurons are the primary photon-capturing neurons of the retina. In particular, the outer segment (OS) structures of photoreceptors undergo degeneration owing to intrinsic (e.g. genetic mutations) or extrinsic (e.g. environmental) pathological stimuli [5, 6]. Light-stress is a powerful and extrinsic deleterious stressor that promotes the dysplasia of the OS of photoreceptors and ultimately photoreceptor death [7–13]. However, the factors, molecular pathways, and mechanisms underpinning the degeneration of photoreceptors and their death are poorly understood and such understanding is needed to develop neuroprotective approaches against risk factors

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(e.g. light damage) that potentiate photoreceptor degeneration in disease conditions, such as age-related macular degeneration and retinitis pigmentosa [14–20].

The Ranbp2 is a vital, large, mosaic, pleiotropic and cytoplasmic peripheral nucleoporin, which among other functions, controls the nuclear-cytoplasmic trafficking and proteostasis of selective substrates in a cell-type dependent manner [21–27]. Despite the broad expression of Ranbp2, heterogeneous genetic changes in *Ranbp2* underlie the insidious or pervasive expressions of diseases or traits affecting restricted cell types or tissues. For example, asymptomatic and selective semi-dominant mutations in *RANBP2* cause necrotic encephalopathy upon exposure to selective infectious agents [28–31], whereas haploinsufficiency of *Ranbp2* promotes carcinogenesis [24], deficits in glucose metabolism upon glucose challenge [27], MPTP-elicited parkinsonism [32], and neuroprotection of photoreceptor neurons to light damage in mice [10, 33]. However, the modular activities of Ranbp2 that contribute to stress-dependent and cell-type restricted expressions of these pathological traits remain to be defined.

Here, we show that mutations in a purported SUMO-binding motif (SBM) in the cyclophilin-like domain (CLD) of Ranbp2 [34, 35], a domain which associates with subunits of the 19S cap of the proteasome [36, 37], preordains outer segment of photoreceptors to profuse structural disruption upon light-stress, while causing the paradoxical suppression of death of photoreceptors by mechanisms independent of caspases' activations and overall function of the ubiquitin-proteasome system (UPS).

2. Materials and methods

2.1. Transgenic mice

The generation of *Ranbp2*$^{+/-}$ (*Ranbp2*$^{Gt(pGT0pfs)630Wcs/-}$) mice was previously described [27]. *Ranbp2*$^{-/-}$ are embryonic lethal [24, 27]. *Tg-Ranbp2*^{CLDm} mice were generated by BAC recombineering with a BAC construct comprising the complete murine *Ranbp2* gene and the I2471K and V2472A mutations in the sequence encoding its CLD as described elsewhere [26]. *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice were obtained by intercrossing *Ranbp2*$^{+/-}$ and *Tg-Ranbp2*$^{CLDm\text{-}HA}$. *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice were pigmented, hemizygous for the transgene allele and they were in a heterozygous *Rpe65*$^{L450/M450}$ background. Mice were reared in a pathogen-free transgenic barrier facility at <70 lux in the cages and 12 hr light-dark cycles (6:00 AM – 6:00 PM). Mice were given *ad libitum* access to water and chow diet 5LJ5 (Purina, Saint Louis, MO). The Institutional Animal Care and Use Committee at Duke University approved the animal protocols and all procedures adhered to the National Academy of Sciences and ARVO guidelines for the Use of Animals in Vision Research.

2.2. Light-exposure treatment

Light experiments were carried out as described elsewhere [26]. Briefly, mice were first dark-adapted for 18 hours. Then, mice pupils were dilated by applying 1% atropine sulfate and 10% phenylephrine hydrochloride (HUB Pharmaceuticals, LLC, Rancho Cucamonga, CA) onto their eyes. Mice were placed individually in white reflective cages with bedding, food, water, and they were exposed to the illuminance of 5,000 lux of continuous white

light-emitting diode (LED) by a dimmable LED panel mounted at the top of the cage for 24 hours. Following the light-exposure treatment, mice were placed in the dark for 24 hrs, killed, and the eyeballs were immediately collected and processed for immunohistochemistry or biochemical analyses.

2.3. Semithin Sections and Transmission Electron Microscopy (TEM)

Semithin sections of posterior eyecups were processed for light and TEM of mice as described elsewhere [10, 38]. Briefly, mice were perfused with 2% paraformaldehyde/phosphate buffered saline (PBS); then, eyeballs were fixed with 2% glutaraldehyde-paraformaldehyde, 0.1% cacodylate buffer, pH 7.2, 0.5-μm sections along the vertical meridian were mounted on glass slides and stained with 1% methylene blue. Images were captured with an Axioplan-2 light microscope controlled by Axiovision release 4.6 and coupled to an AxioCam HRc digital camera (Carl Zeiss). For electron microscopy, specimens were postfixed in 2% osmium tetraoxide in 0.1% cacodylate buffer and embedded in Spurr resin. 60-nm-thick sections were cut with Leica Ultracut S (Leica Microsystems); stained with 2% uranyl acetate, 4% lead citrate and imaged with a JEM-1400 transmission electron microscope (JEOL) coupled with an ORIUS 1000CCD camera.

2.4. Antibodies

The following primary antibodies were used for immunohistochemistry: rabbit monoclonal anti-cleaved caspase-3 Asp175 (Cell Signaling), rabbit polyclonal anti-cleaved-caspase-7 Asp353 (Cell Signaling), rabbit anti-cleaved caspase-8 Asp391 (Cell Signaling), rabbit anti-cleaved caspase-9 Asp 330 (Cell Signaling), mouse anti-ubiquitin (Santa Cruz Biotechnology), rabbit anti-hsc70 (ENZO Life Science), rabbit anti-S1 subunit of the 19 S proteasome (Thermo Scientific); rabbit anti-HDAC4 (Santa Cruz Biotechnology); mouse anti-Ubc9 (BD Biosciences), Alexa-conjugated secondary antibodies (408, 488, 568 and Cy5) were from Invitrogen (Carlsbad, CA).

2.5. Immunohistochemistry and confocal microscopy

The immunohistochemistry and confocal microscopy procedures employed were similar to those described elsewhere [26, 38]. Briefly, eyecups were fixed in 2% paraformaldehyde (PFA)/phosphate buffered saline (PBS), washed with 1xPBS and placed sequentially in 5% and 30% sucrose solutions until complete submersion of the eyecups was reached. The eyes were embedded in optimal cutting temperature (OCT) compound and immediately frozen on dry ice and stored at −80°C before sectioning. 12μm-thick cyrosections were collected for immunohistochemistry, blocked with blocking solution (1xPBS, 0.1% Triton X-100, and 10% normal donkey serum) at room temperature overnight followed by treatment with proteinase K (20μg/ml, Promega) for 8 minutes and standard immunostaining protocols. Specimens were viewed under 60x magnification using a Nikon C90i C1 Plus confocal laser-scanning microscope controlled by the EZ-C1.3.10 confocal software (v6.4). Two images were taken from the central and peripheral superior and inferior regions of the eyecup, for a total of six images per section. The central region was defined as the retinal area within a radius of 215μm of the optic nerve head, while the peripheral region was defined as any region outside the central region.

2.6. TUNEL assay

Sections were washed for 30 minutes with PBS, treated with proteinase K for 8 minutes and washed with PBS [38]. Apoptotic retinal neurons were labeled by the TdT-mediated dUTP nick end-labeling (TUNEL) assay (DeadEnd Fluorimetric TUNEL system; Promega) for 1 hour in a 37°C incubation chamber as described by the manufacturer and elsewhere [10, 38]. After washing with PBS for 30 minutes, the specimens were mounted with Fluoromount G (Southern Biotech) and a glass cover slip.

2.7. Morphometric and statistical analysis

Morphometric analyses of immunostained photoreceptors were carried out with Nikon Elements AR software (v4.0). $TUNEL^+$-cell bodies in the outer nuclear layer (ONL) area were counted. Seven 12μm-thick cryosections were analyzed per eye globe. The total number of $TUNEL^+$ and $caspase^+TUNEL^+$ cell bodies was counted throughout the entire retinal sections, converted to a percentage with the total number of $TUNEL^+$ nuclei being 100%. Statistical Mann-Whitney U tests were performed with $p \leq 0.05$ considered significant.

2.8. Immunoblot and ubiquitin-proteasome assays

Retinas were frozen immediately on dry ice after sacrificing mice. The amount of poly-ubiquitylated substrates, diubiquitin, S1, ubc9 and HDAC4 were measured by western blot and densitometric analysis of RIPA-solubilized retinal homogenates resolved in premade 4–15% Criterion (BioRad) SDS-polyacrylamide gels and immunostained with respective antibodies as described elsewhere [26]. Blots were reprobed with hsc70 antibody. Band intensities were quantified with Metamorph v 7.0 (Molecular Devices), integrated density values (idv) of ubiquitylated smears or protein bands were normalized to the background and idv of hsc70. Data were analyzed by the two-tail *t*-test and a *p* value of ≤0.05 was considered significant. To measure the proteolytic activities of the 20S proteasome, retinas were re-suspended in 60 μl of homogenization buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5mM $MgCl_2$, 2mM ATP,1mM DTT, 0.5mM EDTA and 0.0025% digitonin), incubated on ice for 5 min and then, they underwent standard protocols for 20S proteasome assays as described elsewhere [26]. Briefly, supernatants (3 μg) from retinal extracts were incubated with 100 μM 7-aminomethylcoumarin-conjugated substrates [Suc-LLVY-AMC (Boston Biochem), Ac-RLR-AMC (Boston Biochem) or Ac-nLPnLD-AMC (ENZO Life Science)] in the absence or presence of epoxomicin (20 μM, CalBiochem) for 45 minutes at 37°C. Proteolytic activities were determined by measuring free AMC at excitation/emission/cutoff =380/460/455 with a SpectraMax M5 spectrophotometer (Molecular Devices). Protein concentration for supernatant was measured by BCA method. Data were analyzed by the two-tail *t*-test and a *p* value of ≤0.05 was considered significant.

3. Results

3.1. The outer segments of photoreceptors of mice with mutations in SBM of CLD of Ranbp2 are preordained to profuse degeneration by light-stress

Ranbp2 has two internal repeats (IR1 and IR2) and a cyclophilin-like domain (CLD) with low homology to its C-terminal cyclophilin domain (CY) (Fig. 1) [34]. The first internal

repeat (IR1) of Ranbp2 partially overlaps with CLD (Fig. 1) [34, 39]. The overlapping region of CLD and IR1 contains a purported SBM, V/I-X-V/I-V/I, and mutations in SBM reduce or abolish *in vitro* the interaction of SUMOylated RanGAP (SUMO-1-RanGAP) to Ranbp2 [35]. Prior studies showed that IR1 of Ranbp2 is sufficient to bind the E2-conjugating enzyme, ubc9 [39], and that the formation of the Ranbp2-SUMO-1-RanGAP-ubc9 complex induces the formation of a multisubunit E3 ligase *in vitro* and cell lines [40]. By contrast, CLD of Ranbp2 associates with the S1 (also called Rpn2 in yeast or Psmd1) and other subunits of the 19S cap of the 26S proteasome in cell lines and retinal extracts [36, 37]. Mutations in SBM of CLD selectively relieve the suppression of the degradation of a properly folded and ubiquitylated reporter substrate by ubiquitin-proteasome system (UPS) and without impairing the binding of subunits of the 19S cap of the 26S proteasome to CLD in cell lines [37].

To interrogate the physiological role(s) of CLD of Ranbp2 and its SBM, we generated *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice with the mutations, I2471K and V2472A (CLDm), in SBM of CLD of the *Ranbp2* gene in a bacterial artificial chromosome (BAC) and expressed in a *null Ranbp2* background (Fig. 1) [26]. *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice present similar expression levels and subcellular localization of the transgenic protein, *Tg-Ranbp2*$^{CLDm\text{-}HA}$, compared to endogenous Ranbp2 [26]. *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice reared in cyclic-light and low luminance harbor decreased steady-state levels of SUMO-1-RanGAP and of another substrate, HDAC4, which is prone to sumoylation and degradation by Ranbp2 [41, 42], but these mice do not harbor changes of S1 levels in the retina [26]. Although *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice present incomplete penetrance of embryonic lethality, surviving and adult *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice do not exhibit overt morphological, developmental or behavioral phenotypes [26].

Ranbp2 haploinsufficiency confers neuroprotection to photoreceptors against light damage [10, 33] and light-stress is thought to induce proteotoxicity owing to increased oxidative stress and deregulation of the UPS and protein homeostasis (aka “proteostasis”) [10, 33, 43–46]. Hence, we investigated what domains of Ranbp2 conferred protection of photoreceptors to light-stress by ascertaining the role of CLD of Ranbp2 and its SBM in neuroprotection to phototoxicity. We compared by light and ultrastructural microscopy the overall morphologies of photoreceptors of *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ and wild-type mice reared under cyclic/low light and light-stress conditions. As shown in figure 2, there were no overt differences in retinal morphology and photoreceptor structures by both light and ultrastructural microscopy between *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ and wild-type mice reared under cyclic/low light conditions (Figs. 2A–D). Under light-stress however, the OS of photoreceptors of wild-type mice presented overall well-stacked disks but with the formation of duet vesicles which disrupted focally disk stacking, a feature that is typically observed in OS by phototoxicity (Figs. 2E–G) [10]. By contrast, *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice had broad disorganization of the OS of photoreceptors (Fig. 2H) with profuse structural erosion of their disks and some sparing of the rim structures (Figs. 2I, J).

3.2. Photoreceptors of Ranbp2$^{-/-}$::Tg-Ranbp2$^{CLDm\text{-}HA}$ undergo decreased apoptosis by light stress

Light-stress elicits strongly the apoptosis of photoreceptors [10]. The effect of phototoxicity in photoreceptor cell death between genotypes was examined by TUNEL assays. We did not observed differences in the development of apoptotic cell bodies of photoreceptors between genotypes at 8 weeks of age in the absence and presence of light stress (data not shown). At 24 weeks of age, the amount of apoptotic photoreceptor cell bodies was very low in mice reared under low light conditions and no differences in cell death were detected between genotypes (Figs. 3A, B). However, light-stress elicited a strong increase of apoptotic cell bodies of photoreceptors in 24-week-old wild-type mice, whereas such increase in cell death was blunted in age-matched *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice (Figs. 3C, D).

3.3. Genotype-independent caspases' activations in photoreceptors by light-stress

Intrinsic and extrinsic apoptotic stimuli are known to initiate the activation (cleavage) of caspases 9 and 8, respectively, and these promote the activations of downstream effector caspases (e.g. caspases 3, 6, 7) [47]. However, the conservation and modulation of these apoptotic pathways across pathophysiological stimuli and photoreceptor degenerations remain controversial [43, 48–53]. Hence, we extended the analysis of the modulatory role of CLDm of Ranbp2 in cell death by examining the activation of initiator and effector caspases in cell bodies of photoreceptors. Unlike the suppression of light-induced apoptosis in *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$, we found no differences of activated (cleaved) caspases 3, 7, 8 and 9 in apoptotic photoreceptors between *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ and wild-type mice (Figs. 4A, B). Further, all caspase^{+}-cell bodies were TUNEL^{+}, but only a subset of TUNEL^{+} were caspase^{+} (Fig. 4A).

3.4. Age-and genotype-dependent, but photoreceptor death-independent, modulation of the ubiquitin-proteasome system (UPS) by light stress

Light-stress is thought to induce proteotoxicity owing to increased oxidative stress and deregulation of the UPS [10, 33, 43–46]. In this regard, haploinsufficiency of *Ranbp2* was shown to promote the age-dependent neuroprotection of photoreceptors to light damage [10] and elicit phototoxicity-dependent up-regulation of UPS activity and down-modulation of selective partners of Ranbp2, such as the S1 subunit of the 19S cap and ubc9 [33]. By contrast, *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice reared under cyclic-light and low luminance lack untoward retinal effects in the accumulation of ubiquitylated substrates, ubiquitin proteostasis and 20S proteasome activities except for a mild reduction in chymotrypsin-like activity [26]. To ascertain the role of CLD in UPS activity upon light-stress, we examined first the amount of ubiquitylated substrates between genotypes of 8 and 24-week old mice as an indicator of the activity of the UPS. As shown in figures 5A and C, 8-week-old *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ compared to wild type mice had decreased and increased levels of ubiquitylated substrates and free diubiquitin, respectively, in retinal homogenates. However, there were no significant changes in the levels of ubiquitylated substrates between genotypes at 24 weeks of age, whereas free diubiquitin was undetectable in either genotype (Figs. 5B and C). Then, we determined the proteolytic activities of the 20S proteasome of *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ and wild-type mice reared under light-stress. There were

no changes in chymotrypsin and trypsin-like activities between genotypes; however, the caspase-like activity was mildly reduced in *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ compared with wild-type mice (Fig. 5D).

3.5. Age-and genotype-dependent modulation of the proteostasis of the S1 subunit of the 19S cap and ubc9 by light-stress

The CLD sequence overlaps partially with the IR1 of Ranbp2 [34]. CLD and IR1 associate with the S1 subunit of the 19S cap of the 26S proteasome and the E2 SUMO-conjugating enzyme, ubc9, respectively (Fig. 6A), and SBM mutations have a limited effect in CLD binding to subunits of the 19S cap in cell culture [36, 37, 39]. Physiologically and compared with wild type mice, light-stress down-modulates the levels of S1 and ubc9 in *Ranbp2* haploinsufficient mice [33]. Hence, we examined the effect of CLDm of Ranbp2 and light-stress on the steady-state levels of S1 and ubc9 (Figs. 6B, C). As shown in figure 6B, *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ had increased levels of S1 subunit than wild type mice at 8 weeks of age, but by 24 weeks of age, there were no differences between genotypes. By contrast and in comparison to wild type mice, 24-week-old *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice had increased levels of ubc9, but ubc9 levels were similar between genotypes at 8 weeks of age (Fig. 6C).

We also examined HDAC4 proteostasis, because its levels are decreased in retinas of *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ in the absence of light stress [26]. In cell lines, HDAC4 is also a substrate for degradation by Ranbp2 [42] and sumoylation by a Ranbp2 construct comprising the IR1+2 and the C-terminal region of CLD (~103 residues) [41]. Further, over-expression and pharmacological inhibition of HDAC4 in *rd1* mice paradoxically lead to the protection of photoreceptors [54, 55], whereas HDAC4 nuclear accumulation promotes neurodegeneration in ataxia telangiectasia (ATM) [56]. However, as shown in Fig. 6D, comparisons of HDAC4 levels between *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ and wild-type mice of 8 and 24 weeks of age showed that light-stress, genotype and age lacked untoward effects in HDAC4 proteostasis.

4. Discussion

This study uncovers that mice with insidious functional expression of I2471K and V2472A mutations (CLDm) in SBM of CLD of Ranbp2 lead to two contrasting and counterintuitive manifestations of photoreceptors upon exposure to phototoxicity: the photoreceptors undergo massive disruption of their outer segments, while the cell bodies of photoreceptors become strongly resistant to apoptosis in a manner which is independent of activations of prodeath caspases and overall UPS functions.

The paradoxical disruption of OS and suppression of apoptosis by the light stress-dependent insidious function of CLDm of Ranbp2 is counter to expectation. Hence, the molecular underpinnings linked to these manifestations will shed light to novel targets and biological mechanisms that are critical to sustain the integrity of OS of photoreceptors and their survival against deleterious stressors. Two alternative models for the light-dependent insidious expression of CLDm function of Ranbp2 in photoreceptors are worth further investigation. The first model is based on the findings that Ranbp2 localizes to two

subcellularly and functionally distinct regions of the photoreceptors: the connecting cilium and the cytoplasmic fibrils emerging from nuclear pores [26, 57, 58]. These distinct subcellular structures are thought to share substrates and transport mechanisms that are regulated by Ranbp2 [59–61]. In this regard, CLDm of Ranbp2 may impair the intercompartmental trafficking of limiting substrate(s) at the cilium that are highly prone to oxidative damage and essential to the maintenance of OS, while suppressing the nucleocytoplasmic transport and signaling of selective light-induced factors which promote photoreceptor death. The second model entails the existence of a crosstalk mechanism mediated by the nucleocytoplasmic shuttling of light-induced and pleiotropic factor(s), whose pathological functions hinge on the Ranbp2-dependent partitioning of such factors(s) between the nuclear and cytosolic or ciliary compartments. In this regard, it is possible that light-stress activates nuclear signaling and photoreceptor death by factor(s), whose delocalization(s) in the cytosol by CLDm of Ranbp2 promotes the activation (e.g. posttranslational) of proteolytic factors which cause the acute erosion of the OS of photoreceptors. Regardless, the elucidation of the insidious functions of CLD of Ranbp2 under light-stress will likely rest on the identification of CLD partner(s) whose light-dependent function(s) and association to CLD are impaired by SBM mutations in CLD of Ranbp2.

The SBM of Ranbp2 overlaps with the C-and the N-terminal sequences of CLD and IR1, respectively [34, 35, 39]. CLD and IR1 associate with subunits of the 19S cap of the proteasome and ubc9, respectively [36, 37, 39]. Further, haploinsufficiency of *Ranbp2* promotes the down-regulation of the S1 subunit of the 19S cap, ubc9, and ubiquitylated substrates in retinas exposed to light-stress [33]. However, this and other studies showed that there were no differences in the levels of ubiquitylated substrates and free diubiquitin between wild-type and $Ranbp2^{-/-}::Tg\text{-}Ranbp2^{CLDm\text{-}HA}$ mice at 24 weeks of age, when photoreceptors become neuroprotected to light-stress in $Ranbp2^{-/-}::Tg\text{-}Ranbp2^{CLDm\text{-}HA}$ and haploinsufficient *Ranbp2* mice [10, 33]. Hence, these data support that the light-dependent control of proteostasis of ubiquitylated substrates by Ranbp2 is likely not a major contributor to neuroprotection against phototoxicity and that insidious function(s) of SBM of CLD of Ranbp2 exerts a neuroprotective role via another partner(s), whose function is stress-elicited and unrelated to the overall UPS activity. This notion is in apparent contrast to recent studies suggesting that there is induction of broad cytoprotective mechanisms by the modest down-regulation of the proteostasis of 19S cap subunits against chemical toxic insults targeting the 20S proteasome [62] and that SUMOylation of S1 subunit (also called Psmd1) of the 19S cap, a modification which has not been observed in our physiological studies, may control 26S proteasome activity and composition [63]. These apparent discrepancies may be caused by differences in regulatory signaling activities converging to the 26S proteasome owing to differences in the nature of stress stimuli, biological systems, and/or developmental stages employed between these studies.

Our study suggests that the age-dependent up-regulation of ubc9 proteostasis by insidious SBM function(s) of CLD of Ranbp2 may contribute to the age-dependent neuroprotection of photoreceptors against selective stressors, such as phototoxicity. Notably, ubc9 is reported to have dual activities. Ubc9 acts as an E2 enzyme by modifying Ranbp2 and some of its

partners, such as RanGAP and Ran GTPase [64–66], with SUMO-1, but ubc9 also acts as a co-transcriptional regulator independently of its E2 activity [67–70]. Contrary to *ubc9*$^{-/-}$ mice [71], loss of SBM of CLD of Ranbp2 and SUMO-1 do not produce overt morphological and physiological phenotypes in adult mice [26, 72, 73], although loss of SBM of Ranbp2 cannot be fully compensated during development [26]. Further, ubc9 controls cell death [74] and its activity is exquisitely sensitive to oxidative stress [75]. Hence, ubc9 proteostasis and functions are likely under tight regulation by Ranbp2, aging and oxidative stress. Selective deregulation of ubc9 by SBM loss of Ranbp2 and phototoxicity may underlie paradoxical and insidious manifestations, such as the OS disruption and death resistance of *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice. In this regard, the *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice emerge as unique physiological platforms to enable the discovery of novel factors and mechanisms that control the maintenance of critical subcellular structures and the age-dependent survival of selective neural-cell types, such as photoreceptors, against light damage.

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Abbreviations

CLD	cyclophilin-like domain
CLDm	cyclophilin-like domain mutant
SBM	SUMO-binding motif
Ranbp2	Ran-binding protein 2
Tg	transgenic
UPS	ubiquitin-proteasome system
ubc9	ubiquitin carrier protein 9
SUMO	small ubiquitin-like modifier
RanGAP	Ran GTPase-activating protein
HDAC4	histone deacetylase 4
HA	hemagglutinin
BAC	bacterial artificial chromosome

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Highlights

- SBM mutations in CLD of Ranbp2 cause photoreceptor dysmorphology by light-stress
- SBM mutations in CLD of Ranbp2 suppress photoreceptor death by light-stress
- Caspase activation by light-stress is independent of Ranbp2 CLD function(s)
- Light and Ranbp2 CLD-dependent photoreceptor death lack untoward effects in the UPS
- Insidious loss of SBM function of CLD of Ranbp2 confers neuroprotection

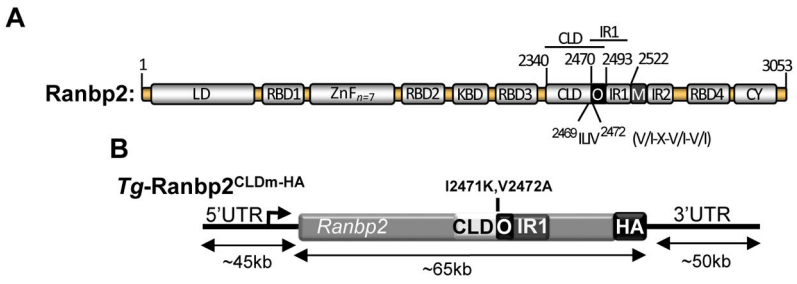

Fig. 1.

(A) Structural domains of Ranbp2. V/I-X-V/I-V/I is the consensus SUMO-binding motif (SBM) in the overlapping region (O) of CLD and IR1 of Ranbp2. Numbering refers to residues of the primary mouse sequence of Ranbp2 and positioning of selective domains and motifs of Ranbp2. Domains are not drawn to scale. (B) Transgenic BAC construct (~160 kb) of *Ranbp2*, *Tg-Ranbp2*$^{CLDm\text{-}HA}$, with the mutations, I2471K andV2472A, in the SBM of CLD, and a C-terminal hemagglutinin (HA) tag insertion at the end of the terminal exon encoding the CY domain of *Ranbp2*. Note drawing not to scale. Legend: LD, leucine-rich domain; $RBD_{n=1–4}$, Ran GTPase-binding domains, n=1–4; $ZnF_{n=7}$, zinc finger-rich domains; KBD, kinesin-1-binding domain; CLD, cyclophilin-like domain; IR1 and IR2, internal repeats 1 and 2, respectively; M, middle domain between IR1 and IR2; O, overlapping region between CLD and IR1; CY, cyclophilin domain; UTR, untranslated region.

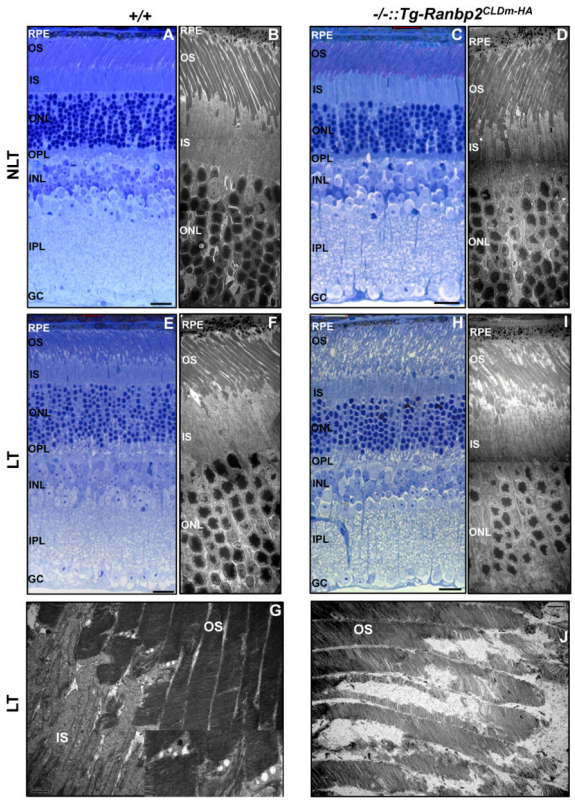

Fig. 2.

Morphological changes of photoreceptor neurons between wild-type and *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice reared in the absence and presence of light-stress. There are no visible morphological changes under light (A, C) and ultrastructural microscopy (B, D) of photoreceptors of wild-type (A, B) and *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice (C, D) reared under non-light treatment (NLT) at 24 weeks of age (A–D). Under light-treatment (E–J), the OS of photoreceptors of 24-week-old wild-type mice show mild disruption primarily at the base of the OS as shown by light (E) and ultrastructure microscopy (F, G), whereas light (H) and ultrastructure microscopy (I, J) show profuse derangement and erosion of the OS of photoreceptors, respectively, of age-matched *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice. Inset picture is magnified dashed box in (G). Legend: OS, outer segments of photoreceptors; IS, inner segments of photoreceptors; ONL, outer nuclear (cell bodies) layer of photoreceptors; OPL, outer plexiform layer; INL, inner nuclear (cell bodies) layer of retinal neurons; IPL, inner plexiform layer; GC, ganglionic neurons; RPE, retinal pigment epithelium; NLT, non-light treatment; LT, light treatment (light-stress); +/+, wild type; −/−*::Tg-Ranbp2*$^{CLDm\text{-}HA}$, *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$. Scale bars: 20 μm (A, C, E, H), 5 μm (B, D, F, I), 1 μm (G, J).

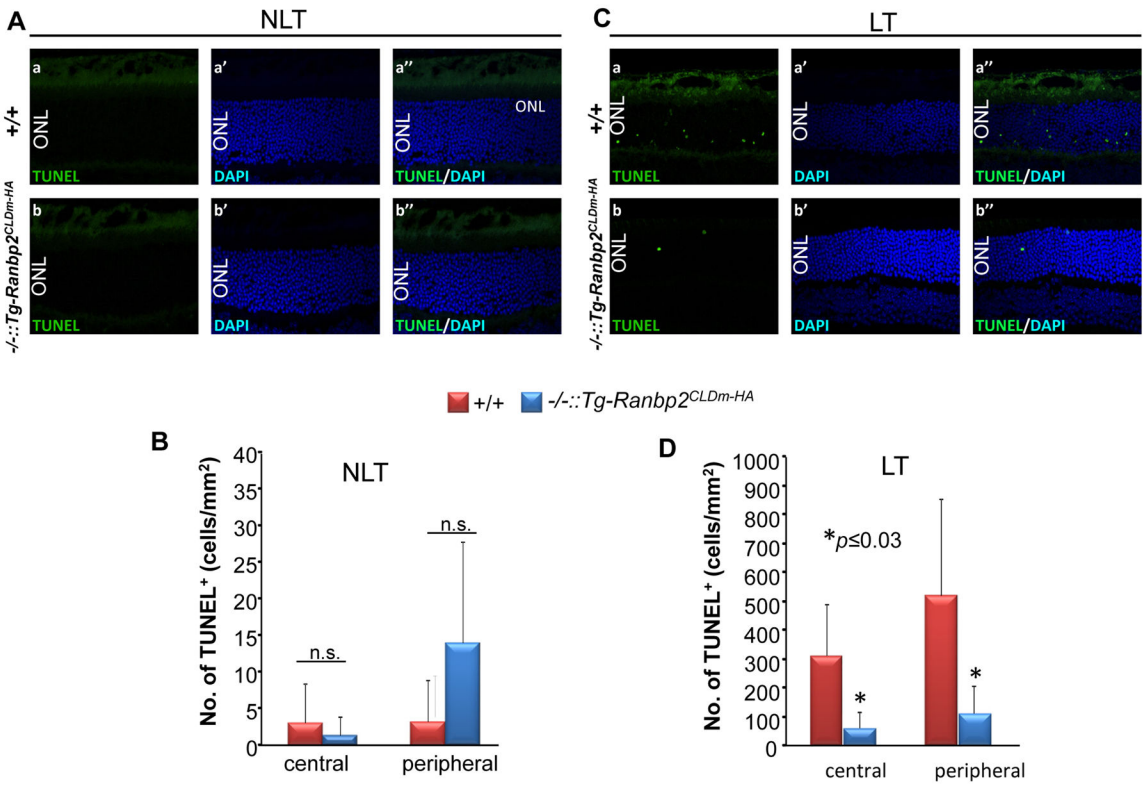

Fig. 3.

Ranbp2$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ suppresses the development of apoptotic cell bodies by light-stress. The number of apoptotic cell bodies in 24-week-old *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ and wild type mice are scarce in the absence of light-stress and there is no difference between genotypes (A, B). In the presence of light stress (C, D), 24-week-old *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ show strong suppression of TUNEL^{+}-apoptotic cell bodies (arrowheads) of photoreceptors in the central and peripheral regions of the retina compared to age-matched wild type mice. (B) and (D) are quantitative analyses of representative images in (A) and (C), respectively. Retinal sections were counterstained with DAPI. Data shown represent the mean ± S.D., *n*=5–6. Legend: NLT, non-light treatment; LT, light treatment (light stress); ONL, outer nuclear (cell bodies) layer of photoreceptors; +/+, wild type; −/-::*Tg-Ranbp2*$^{CLDm\text{-}HA}$, *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$; n.s., non-significant; Scale bar: 20 μm.

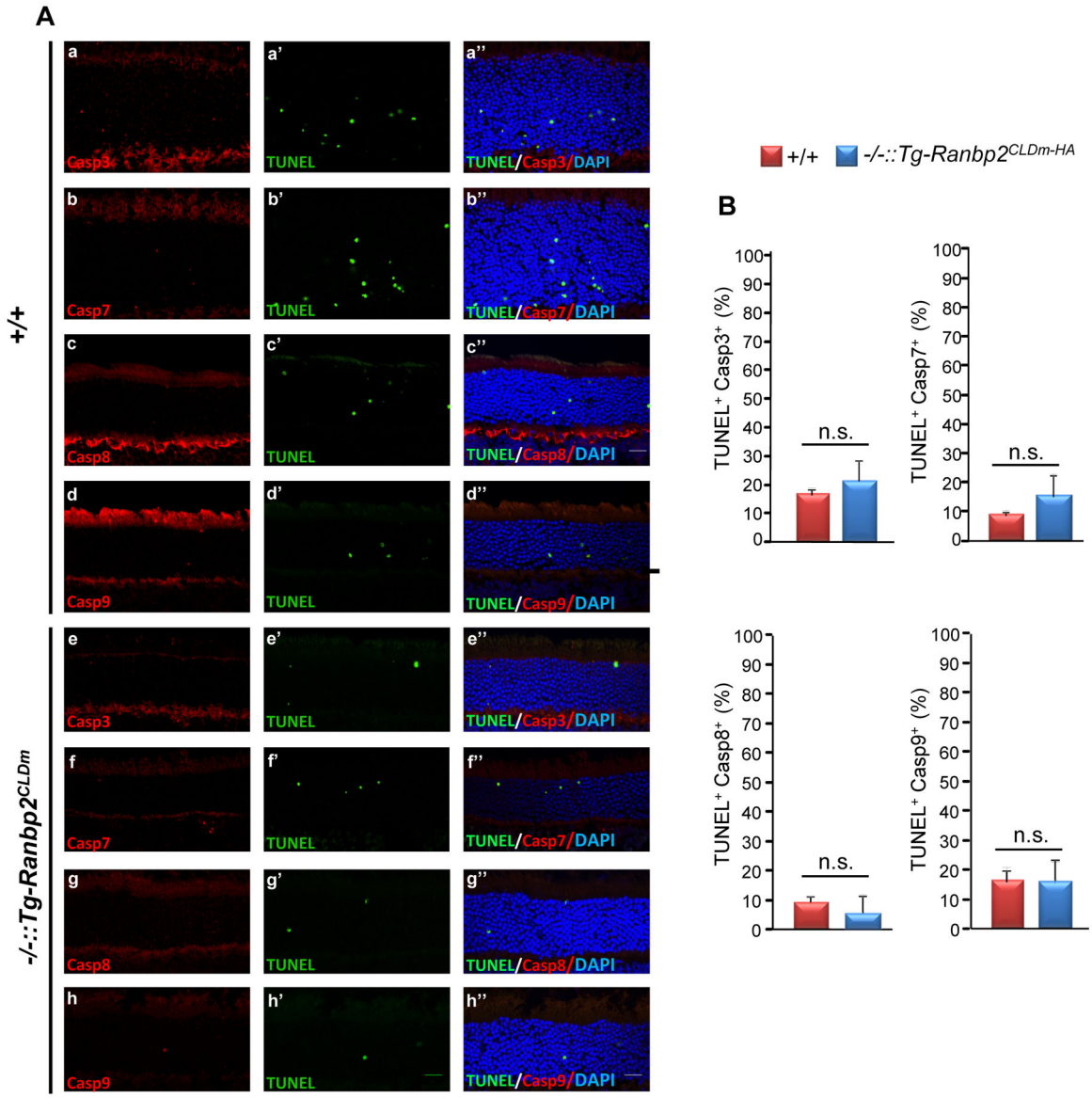

Fig. 4.

Apoptotic cell bodies of photoreceptors of 24-week-old *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ and wild type mice lack differences in caspases' activations (cleaved caspases) induced by light-stress. There are no changes in TUNEL^{+}Casp3^{+}, TUNEL^{+}Casp7^{+}, TUNEL^{+}Casp8^{+}, TUNEL^{+}Casp9^{+} in cell bodies of photoreceptors between age-matched genotypes by light stress (A, B). Arrows show TUNEL^{+} cell bodies without caspases' activations, whereas arrowheads show TUNEL^{+}Casp^{+} cell bodies. (B) are quantitative analyses of representative images in (A). Retinal sections were counterstained with DAPI. Data shown represent the mean ± S.D., *n*=3. Legend: +/+, wild type; −/−*::Tg-Ranbp2*$^{CLDm\text{-}HA}$, *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$; n.s., non-significant; Scale bar: 20 μm.

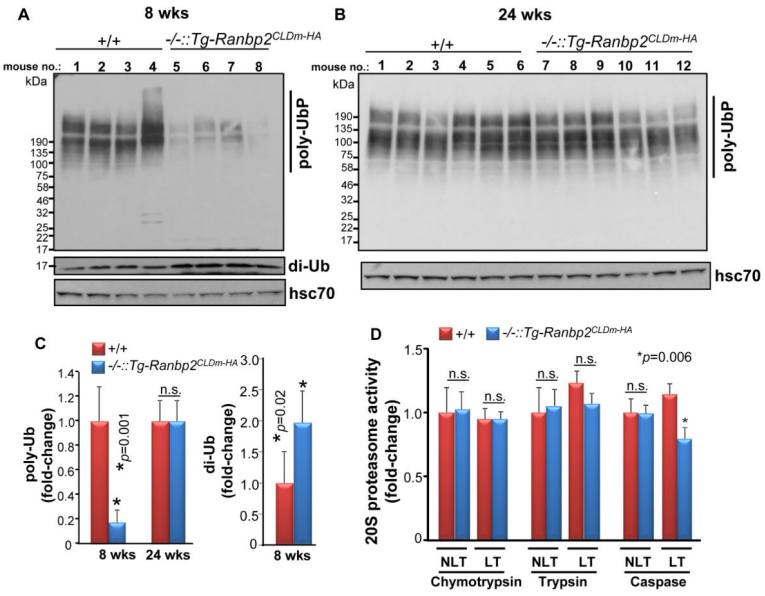

Fig. 5.

Age-dependent accumulation of ubiquitylated substrates and diubiquitin, and 20S proteasome activities, between *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ and wild type mice exposed to light-stress. In comparison to wild type mice, *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice present decreased and increased levels of ubiquitylated substrates and diubiquitin, respectively, at 8 weeks of age (A, C), but their levels are similar between genotypes at 24 weeks of age (B, C). (D) Retinal extracts of 24 week-old *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ and wild type mice have similar chymotrypsin and trypsin-like activities of the 20S proteasome, but *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ have modestly lower caspase-like activity than wild type mice. Data shown represent the mean ± S.D., *n*=4–6. Legend: +/+, wild type; *−/−::Tg-Ranbp2*$^{CLDm\text{-}HA}$, *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$; di-Ub, diubiquitin; poly-UbP, poly-ubiquitylated proteins; hsc70, heat shock cytosolic protein 70; n.s., non-significant.

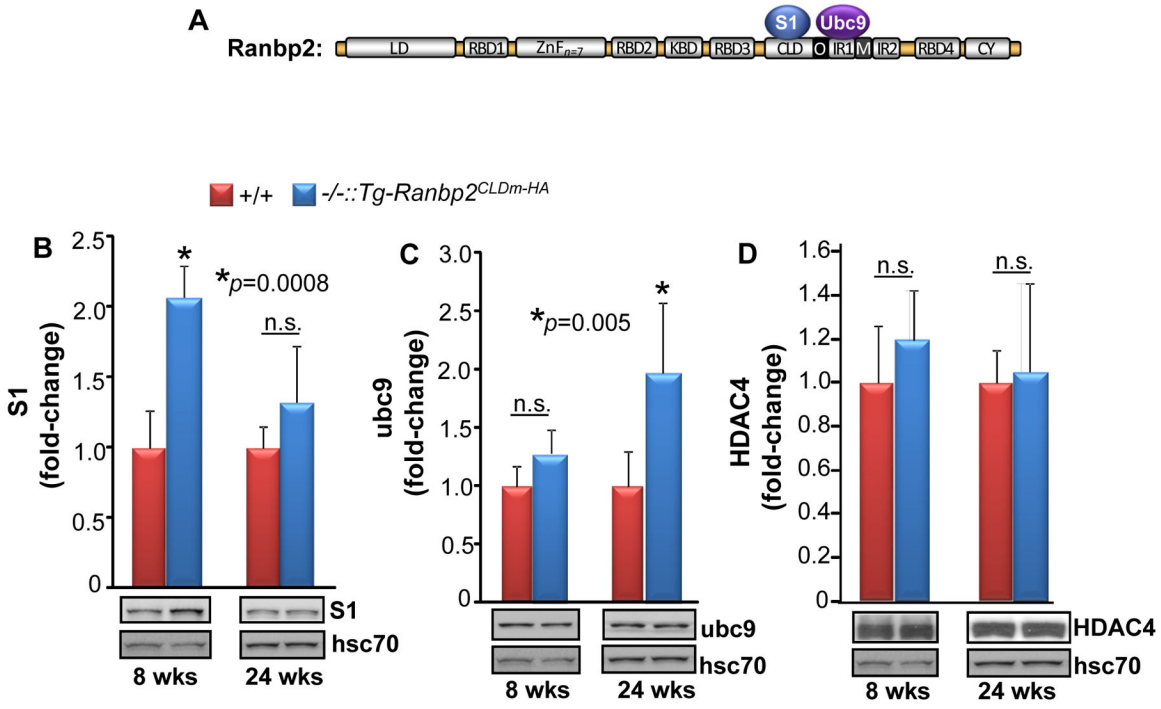

Fig. 6.

Age-dependent changes in S1 subunit and ubc9 proteostasis between *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ and wild type mice exposed to light-stress. (A) Diagram of the S1 and ubc9 partners of CLD and IR1 of Ranbp2, respectively. In comparison to wild type mice, *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$ mice have increased levels of S1 subunit of the 19S cap of the 26S proteasome (B) and ubc9 (C) selectively at 8 and 24 weeks of age, respectively, whereas the levels of HDAC4 remain unchanged between genotypes at 8 and 24 weeks of age (D). Data shown represent the mean ± S.D., *n*=4. Legend: +/+, wild type; −/−*::Tg-Ranbp2*$^{CLDm\text{-}HA}$, *Ranbp2*$^{-/-}$*::Tg-Ranbp2*$^{CLDm\text{-}HA}$; hsc70, heat shock cytosolic protein 70; n.s., non-significant.