

Balance between autophagic pathways preserves retinal homeostasis

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Balance between autophagic pathways preserves retinal homeostasis

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Figure 5: Colour/12 X 17.5 cm

Summary

Aging contributes to the appearance of several retinopathies and is the largest risk factor for aged-related macular degeneration, major cause of blindness in the elderly population. Accumulation of undegraded material as lipofuscin represents a hallmark in many pathologies of the aged eye. Autophagy is a highly conserved intracellular degradative pathway that plays a critical role in the removal of damaged cell components to maintain the cellular homeostasis. A decrease in autophagic activity with age observed in many tissues has been proposed to contribute to the aggravation of age-related diseases. However, the participation of different autophagic pathways to the retina physiopathology remains unknown. Here we describe a marked reduction in macroautophagic activity in the retina with age, which coincides with an increase in chaperone-mediated autophagy (CMA). This increase in CMA is also observed during retinal neurodegeneration in the *Atg5^{lox/lox}*; *nestin-Cre* mice, a mouse model with downregulation of macroautophagy in neuronal precursors. In contrast to other cell types, this autophagic cross-talk in retinal cells is not bi-directional and CMA inhibition renders cone photoreceptor very sensitive to stress. Temporal and cell-type specific differences in the balance between autophagic pathways may be responsible for the specific pattern of visual loss that occurs with aging. Our results show for the first time a cross-talk of different lysosomal proteolytic systems in the retina during normal aging and may help the development of new therapeutic intervention for age-dependent retinal diseases.

Introduction

Autophagy is a catabolic process by which cells degrade intracellular components inside lysosomes (Mizushima *et al.* 2008). Three main autophagic pathways have been described in mammalian cells, macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). During macroautophagy parts of the cytosol including whole organelles are enclosed in a double membrane structure named autophagosome that then fuses with lysosomes to degrade the enclosed material (Mizushima *et al.* 2008). In CMA, specific cytosolic soluble proteins bearing a targeting motif are recognized by a chaperone that delivers them to the lysosomal receptor the lysosome-associated membrane protein type 2A (LAMP-2A) for translocation into the lysosomal lumen (Cuervo 2010). Macroautophagy and CMA are maximally activated under stress conditions but basal activity for these pathways is also detected in most cell types. Previous studies in cultured cells have suggested the existence of a bidirectional crosstalk between these two pathways as macroautophagy is upregulated after CMA blockage (Massey *et al.* 2008) and CMA is induced in cells with macroautophagy compromise (Kaushik *et al.* 2008). While the role of autophagy in the maintenance of cell and tissue homeostasis is well documented (Mizushima *et al.* 2008), the relative contributions of the different autophagic pathways in retinal pathophysiology remains unknown (Boya In press).

Genetic inhibition of autophagy induces degenerative changes in mammalian tissues that resemble those associated with aging, and normal and pathological aging are often associated with a reduced autophagic potential (Cuervo 2008). The role of autophagy in the nervous system is a matter of intense investigation as these pathways are often missregulated during neurodegenerative conditions and autophagy

manipulation could represent a new approach to cure these devastating diseases (Harris & Rubinsztein 2012). Little is known on the role of autophagy in the retina. We have recently shown that autophagy protects retinal ganglion cells after axonal damage in vivo (Rodriguez-Muela *et al.* 2012) and that helps to maintain ATP levels during retinal development (Boya *et al.* 2008; Mellén *et al.* 2008).

Retinal aging is often associated to a decrease in visual acuity, ocular accommodation and dark adaptation. In addition, this aging phenotype is aggravated by several diseases such as age-dependent macular degeneration (AMD), cataracts, glaucoma and diabetic retinopathy, causing blindness. AMD, the leading cause of vision loss in the elderly world-wide (Lim *et al.* 2012), is characterized by morphological and functional abnormalities in the retinal pigmented epithelium (RPE), that often lead to their cell death causing the secondary adverse effects in the neural retina and ultimately, the loss of vision (Kaarniranta *et al.* 2009). Recent evidence demonstrating an increased autophagy activity in the aging RPE and the presence of autophagy markers in the extracellular protein deposits in the eyes of human donors with AMD (Wang *et al.* 2009a) has led to the hypothesis that autophagy plays a pathogenic role in AMD (Wang *et al.* 2009b). Conversely, impaired autophagy in RPE cells also results in increased lipofuscinogenesis in vitro (Krohne *et al.* 2010). However, the impact of autophagy in age-related retinal dysfunction is still unknown.

The most abundant cell types in the retina are photoreceptor cells, rods and cones, which are in charge of sensing light and start the phototransduction cascade. Rods are used for low-light vision and cones for daylight, bright-colored vision. Damage to photoreceptors contributes to the gradual loss of sight associated with physiological aging and retinopathies resulting in blindness. Age-related alterations in the ubiquitin-proteasome system have been extensively described in the central nervous

system, including the retina (Shang & Taylor 2012). However, concomitant changes in the lysosomal-autophagic system, the other major system for cellular quality control, are less well characterized.

In this work, we have identified a marked reduction in macroautophagy activity with age in the retina, which coincides with an increase in levels of several limiting components for chaperone-mediated autophagy (CMA). Inhibition of macroautophagy in retinal cells *in vivo* and *in vitro* induces robust upregulation of CMA in the compromised cells. However, in contrast to other cell types, the autophagic cross-talk in retinal cells is not bi-directional. We propose that cell type-dependent differences in the interplay in between autophagic pathways in the retina may be responsible for the specific pattern of visual loss in aging.

Results

Macroautophagy is the best characterized autophagic pathway in mammals (Mizushima *et al.* 2008). Using mouse retinas, a highly accessible part of the central nervous system, we compared levels of macroautophagy at different ages. No significant differences in the basal steady-state levels of LC3-II, an integral component of the autophagosome membrane (Tanida *et al.* 2005), were observed in aged animals (Fig. 1A). However, when studying the autophagy flux -determined as the change in the levels of LC3-II in the presence and in the absence of lysosomal inhibitors- we observed that in the older animals (12 and 22 months old) LC3-II levels did not significantly increase after the lysosomal blockage compared to the young ones (Figure 1A, B). The inferred age-related decrease in autophagosome formation was supported by a reduction in mRNA levels of *Beclin1*, a regulatory component of the autophagy initiation complex, and *Atg7*, the rate-limiting enzyme mediating elongation of the autophagosome membrane (Fig. 1C). This decrease in the mRNA expression is correlated with changes in Beclin1 protein levels by immunofluorescence in animals of 22 months of age in comparison with young animals (Fig. 1D). Moreover, we observed a concomitant transcription-independent increase in protein levels of p62, a well-described macroautophagy substrate (Fig. 1E-G). Ultrastructural analysis of the aged retinas revealed no significant expansion of autophagy-related compartments (Supp. Fig. 1A), further supporting the data that the main defect in macroautophagy occurs at the level of autophagosome formation and not during the degradation phase. Aged retinas exhibited lipofuscin-loaded lysosomes and electron-dense aggregates, compatible with inefficient removal of cytosolic cargo by macroautophagy and severe alterations in cellular quality control (Fig 2A and Supp. Fig. 1B, Fig 2A). Some of the inclusions were of proteinaceous nature, as revealed by ubiquitin immunostaining, which was detected throughout all

layers of aged retinas (Fig 2B). The observed reduction in macroautophagy in the retinas of the old animals was accompanied by several degenerative features, including reduced cell density of photoreceptors in the ONL as an increase in the area devoid of cells (Supp. Fig. 1C), altered structure of the outer segments of photoreceptors (Supp. Fig. 1D), and increased numbers of apoptotic cells (Supp. Fig. 1E). Although a significant compromise of macroautophagy (reduced LC3 flux and p62 accumulation) was only evident in the 22 months old group, we found a gradual trend toward declined activity in the middle age group. The fact that the ultra structural analysis did not reveal marked changes between 3 and 12 months old mice (Fig. Sup 1 C, E) supports that cells may activate responses to compensate for this gradual loss in autophagic function and to preserve cellular homeostasis.

Generalized lysosomal failure has been associated with age-related neurodegeneration (Dehay *et al.* 2010; Wong & Cuervo 2010). Unexpectedly, we detected a dramatic increase in total proteolysis of long-lived proteins in retinal explants from aged animals (Fig. 3A). This increase in degradative capacity was independent of the proteasome, as even in the presence of proteasome inhibitors, higher proteolysis rates were still evident in aged retinas as compared with younger ones (Supp. Fig. 2A). These observations suggest the upregulation of a lysosomal pathway other than macroautophagy in aged retinas. Upregulation of chaperone-mediated autophagy (CMA), the other stress-induced autophagic pathway in mammals (Cuervo 2010), has been previously described *in vitro* in macroautophagy-deficient cell lines (Kaushik *et al.* 2008). We thus analyzed the levels of LAMP-2A, a limiting component required for CMA. Levels of both LAMP-2A and Hsc70, the chaperone that participates lysosomal delivery of CMA substrates, were increased in aged versus young retinas (Fig 3B, and Supp. Fig. 2B,C). This finding supports that the observed increase in lysosomal-

dependent proteolysis may indeed be a result of the upregulation of CMA in aged retinas.

The age-related increase in retinal CMA markers noticeable contrasts with the decreased activity of this pathway that occurs in other organs with age (Cuervo & Dice 2000; Zhang & Cuervo 2008), and may indicate a prominent role for CMA in the retina when macroautophagy is compromised. To address this possibility, we downregulated the expression of Atg7 or LAMP-2A, essential proteins for macroautophagy and CMA respectively, by shRNA-mediated knock-down (KD) in 661W photoreceptor cells. CMA blockade resulted in a marked reduction in basal proteolysis rates in 661W cells, while compromised macroautophagy (Atg7 KD) resulted in increased proteolysis as compared with control cells, in agreement with our *in vivo* findings (Fig. 3C). This increase in proteolysis is likely due to the upregulated CMA activity, measured by means of a photo-switchable fluorescent reporter KFERQ-PA-mCherry1 (Koga *et al.* 2011), that we found in Atg7 KD cells. Blockage of macroautophagy in 661W cells results in a significant increase on basal CMA activity but not further activation when this pathway is upregulated by removal of nutrients in the culture media (Fig. 3D). As expected, LAMP-2A KD cells displayed reduced CMA activity, confirming the validity of this reporter assay (Fig. 3D; prolonged serum removal, a well-characterized stimulus of CMA, was used as a positive control). As expected Atg7 knockdown reduced autophagic flux by westernblot and by counting LC3 dots after immunofluorescence (Fig 3E, F).

Analysis of possible changes in macroautophagy in response to the CMA blockage in these cells revealed that, contrary to the upregulation of macroautophagy observed in most cell types in response to CMA blockage (Massey *et al.* 2008), CMA down-regulation in 661W cells did not increase macroautophagy activity (Fig. 3E, F). This

inability to upregulate macroautophagy upon CMA blockage may explain the higher sensitivity to oxidative stress observed in the 661W L2AKD cells as increased cell death after paraquat treatment (Fig. 3G). Moreover, this high dependence on proper functioning of autophagic pathways for maintenance of homeostasis appeared to be specific to photoreceptor-derived cells. Thus downregulation of Atg7 or LAMP-2A in RGC-5 immortalized retinal ganglion cells had no significant effect on proteolysis (Fig. 3C), supporting a possible higher contribution of the activity of the ubiquitin/proteasome system to intracellular degradation in these cells. Interestingly, despite this lower participation of autophagic pathways to proteolysis, and as a difference with 661W cells, RGC-5 cells showed the expected bi-directional crosstalk between macroautophagy and CMA. RGC-5 cells increased autophagic flux after L2A knockdown (Sup Fig 3A) and respond to blockage of macroautophagy by upregulating CMA under basal conditions (Suppl. Fig. 3B).

Overall, these results support the existence of cell type-dependent differences on the contribution of autophagic and non-autophagic pathways to retinal homeostasis and in the cross-talk among these pathways. Thus, we have demonstrate that whereas ablation of macroautophagy in cone photoreceptor cells results in the expected increased CMA activity, CMA downregulation does not lead to increased macroautophagy, demonstrating that the crosstalk between autophagic pathways in this cell type is unidirectional.

To further assess the impact of macroautophagy downregulation in the retina *in vivo* and to determine whether the observed age-related changes in CMA were secondary to altered macroautophagic function, we studied conditional *Atg5* knockout mice generated by crossing of *Atg5^{flox/flox}* with *nestin-Cre* mice. These mice display ubiquitin-positive inclusions in several neuronal types, as well as motor deficits and

neurodegeneration (Hara *et al.* 2006), though their retinal phenotype has not been characterized to date. Like aged animals, conditional *Atg5* knockout mice displayed increased levels of p62 and ubiquitinated proteins in the retina (Fig. 4A). Moreover, levels of both LAMP-2A and Hsc70, were also increased in the retina of *Atg5*-deficient mice (Fig. 4 A-C), supportive of compensatory activation of CMA.

Next, we analyzed the consequences of macroautophagy blockade in retinal homeostasis. TUNEL-positive apoptotic nuclei were observed in the photoreceptor layer of conditional *Atg5* knockout mice (Fig. 4D, E), as well as elevated levels of phosphorylated Tau (Fig. 4F), reproducing the increase observed in the aged retinas (Sup Fig. 4). Together with the histological signs of neurodegeneration, *Atg5* deficient mice also presented a clear decline in their visual function (Fig. 5A). Electroretinograms (ERG) selectively associated with scotopic tests (STR, b-scot, a-mixed and OP), indicative of rod function, revealed a reduction in scotopic/dim-light vision in these mice by 7 weeks of age, comparable with that described in aged mice (Kolesnikov *et al.* 2010). We postulate that cone function is preserved in these animals due to the increased contribution of CMA in these cells. In support of this view, TUNEL-positive cells in the retinas of conditional *Atg5* knockout mice were negative for three different cone markers (Fig. 5B, C).

In summary, our studies have identified the existence of cross-talk between the two principal types of autophagy, macroautophagy and CMA in the retina *in vivo*. Communication between these two autophagic systems helps eliciting compensatory mechanisms, which contribute to maintain cellular homeostasis when one of the pathways is compromised.

Discussion

In this work, we describe a primary dysfunction of macroautophagy in the retina of aged mice, which contributes to the age-associated reduction of visual function and to retinal dystrophy. We have also confirmed, for the first time *in vivo*, the existence of cross-talk between different autophagic pathways and provide an example of the physiological relevance of the intercommunication between macroautophagy and CMA. Thus, the robust compensatory activation of CMA observed in cone retinal cells when macroautophagy is experimentally blocked is likely behind the higher resistance of these cells to the functional loss of macroautophagy observed with age (Kolesnikov *et al.* 2010).

A combination of defects in autophagosome formation and clearance contributes to the reduced macroautophagy activity with age described in organs such as liver, kidney, heart and some brain regions (Cuervo & Dice 2000). Ultrastructural analysis of the aged retinas revealed no significant expansion of autophagy-related compartments suggesting that, in clear contrast with these other organs, the main defect in macroautophagy occurs at the level of autophagosome formation and not during the degradation phase. Interestingly, although the defect in macroautophagy function is already detectable at 12 months of age, the degenerative phenotypes in the retina are not fully evident until later ages, supporting the importance of the activation of compensatory mechanisms, such as the CMA upregulation described in this work, that help preserving retinal homeostasis even when macroautophagy is defective. Future studies are necessary to determine the cause of the transcriptional downregulation of essential autophagy components such as Beclin-1 and Atg7 observed in the aged retina and whether they are solely responsible for the reduction in autophagosome biogenesis in this tissue.

It is noteworthy that contrary to other cell types, the cross-talk between autophagic pathways in photoreceptor cells is unidirectional. Whereas macroautophagy downregulation leads to a robust enhancement of CMA activity, in agreement with our observations in aged retina, macroautophagy is not activated in these cells in response to CMA blockade, leaving these cells vulnerable to stressors such as oxidative stress. We have only found a similar inability to upregulate macroautophagy in response to CMA inhibition in the case of cancer cells, which coincidentally show high basal levels of CMA activity, similar to the ones that we describe here for retinal cells (Kon *et al.* 2011). Disruption of the autophagic cross-talk may be necessary to support the observed constitutive activation of CMA in retinal cells.

The increase in CMA activity observed in the aging retina is unprecedented, as in most organs the activity of this autophagic pathway declines with age (Cuervo & Dice 2000; Zhang & Cuervo 2008). Upregulation of CMA in the retina of old animals, could be reactive to a loss of macroautophagic activity with age, and may exert a protective effect, as the one that we observed in the cone cells of conditional Atg5 knockout mice. Alternatively, CMA activation in the old retina may reflect a generalized response of retinal cells to stress; indeed, transcriptional upregulation of LAMP-2A was recently described in a model of retinitis pigmentosa (Punzo *et al.* 2009). Both, the inability of some retinal cells to upregulate CMA in response to macroautophagic blockage, and the higher dependence of some cell types on macroautophagy, could make CMA upregulation insufficient to compensate for the gradual loss of macroautophagy with age, eventually leading to their functional failure.

We have found that 661W cells, a cone-derived cell line, are very dependent on CMA for survival after oxidative stress (Fig. 3G). *In vivo*, cone markers never colocalize with TUNEL staining after genetic ablation of macroautophagy. It is thus

tempting to speculate that a compensatory increase in CMA would make cones more resistant to stress in comparison with other retinal cell types, such as rods. Indeed, rods are the first to degenerate in most retinal dystrophies (Organisciak & Vaughan 2010) and after light induced damage in albino mice (Kunchithapautham *et al.* 2011). Moreover, being responsible for night vision and dark adaptation, photoreceptor rod loss is one of the first consequences of physiological aging (Kolesnikov *et al.* 2010). We find a reduction in rod-associated electroretinograms after macroautophagy ablation in agreement with the increased vulnerability of those cells in many pathological settings, including aging. Thus the differential capability of retinal cells to upregulate CMA or macroautophagy may be behind the observed decrease in retinal function during physiological and pathological conditions.

In this work we have characterized for the first time the retinal phenotype of an autophagy-deficient animal model. Our data show the presence of protein aggregates as has been observed in other parts of the central nervous system in those animals at similar ages such as the cerebral cortex, the hippocampus and the thalamus (Hara *et al.* 2006). In addition, TUNEL-positive cells are found in the nuclear layer of photoreceptors at P52. Other cell types shown to be affected by apoptotic cell death in these autophagy-deficient mice are the granular cells of the cerebellum at 6 weeks of age (Hara *et al.* 2006). Interestingly, although the gross morphology of the retina is maintained, we find severe deficits in night vision indicating that proper autophagy is essential to keep retinal function in the absence of major structural abnormalities. This is in contrast to other types of retinal degeneration where a marked reduction in the number of photoreceptor cells does not have a great impact in electroretinographic responses (Corrochano *et al.* 2008).

In conclusion, our study reveals the occurrence of tissue-dependent, and even of cell type-dependent differences in the autophagic pathway primarily affected with aging and in the capability to compensate for this defect by upregulating a different autophagic pathway. In the case of the retina, these differences provide a plausible explanation for the specific pattern of sight loss with age.

For Peer Review

Experimental procedures

All animal procedures were approved by the local ethics committee for animal experimentation, under animal study protocols approved by the Albert Einstein College of Medicine Animal Institute Animal Care and Use Committee and by the CSIC and were carried out in accordance with the American and European Union guidelines. C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *Atg5^{flox/flox}* mice (Hara *et al.* 2006) were kindly provided by Noboru Mizushima (Tokyo Medical and Dental University, Japan). Nestin-*Cre* mice were provided by Marcos Malumbres at CNIO, Spain. Control animals are considered *Atg5^{+/flox}*; nestin-*Cre* the *Atg5^{flox/flox}* and the *Atg5^{+/flox}* as described in (Hara *et al.* 2006). Animals of either sex were used for this study. For the aging studies, 3- 12 and 22-month-old mice from the National Institute on Aging age-controlled colony were employed. Mice were maintained on a 12-h light/dark cycle in a temperature-controlled barrier facility, with free access to water and food.

ERG Recordings

Mice were dark adapted over night, and subsequent manipulations were performed in dim red light. Mice were anesthetized with intraperitoneal injections of ketamine (95 mg/kg) and xylazine (5 mg/kg) solution and maintained on a heating pad at 37°C. Pupils were dilated with a drop of 1% tropicamide (Colircusii Tropicamida; Alcon Cusi, Barcelona, Spain). To optimize electrical recording, a topical drop (2% Methocel; Hetlingen, Switzerland) was instilled on each eye immediately before situating the corneal electrode. Flash induced ERG responses were recorded from the right eye in response to light stimuli produced with a Ganzfeld stimulator. Light intensity was measured with a photometer at the level of the eye (Mavo Monitor USB; Nürnberg, Germany). Four to 64 consecutive stimuli were averaged with an interval between light

flashes in scotopic conditions of 10 seconds for dim flashes and of up to 60 seconds for the highest intensity. Under photopic conditions, the interval between light flashes was fixed at 1 second. ERG signals were amplified and band filtered between 0.3 and 1000 Hz with an amplifier (CP511 AC amplifier; Grass Instruments, Quincy, MA). Electrical signals were digitized at 20 kHz with a power laboratory data acquisition board (AD Instruments, Chalgrove, UK). Bipolar recording was performed between an electrode fixed on a corneal lens (Burian-Allen electrode; Hansen Ophthalmic Development Laboratory, Coralville, IA) and a reference electrode located in the mouth, with a ground electrode located in the tail. Under dark adaptation, scotopic threshold responses (STR) were recorded to light flashes of $-4 \log \text{cd}\cdot\text{s}\cdot\text{m}^{-2}$; rod responses were recorded to light flashes of $-2 \log \text{cd}\cdot\text{s}\cdot\text{m}^{-2}$ and mixed responses were recorded in response to light flashes of $1.5 \log \text{cd}\cdot\text{s}\cdot\text{m}^{-2}$. Oscillatory potential (OP) was isolated using white flashes of $1.5 \log \text{cd}\cdot\text{s}\cdot\text{m}^{-2}$ in a recording frequency range of 100 to 10,000 Hz. Under light adaptation, cone-mediated responses to light flashes of $2 \log \text{cd}\cdot\text{s}\cdot\text{m}^{-2}$ on a rod-saturating background of $30 \text{cd}\cdot\text{m}^{-2}$ were recorded. Wave amplitudes of the STR, rod responses (b-rod), mixed responses (a-mixed and b-mixed) and OP were measured off line by an observer masked to the experimental condition of the animal.

Cryosections, Immunofluorescence and detection of apoptosis

Animals were euthanized by an overdose of sodium pentobarbital. Cryosections and immunofluorescence in retinal sections were performed as previously described (Rodriguez-Muela *et al.* 2012). Immunofluorescence in 661W cells was performed as previously described (Vazquez *et al.* 2012). Primary antibodies used in this study were LC3 (MBL, MA, USA), p62 (Enzo, NY, USA), LAMP-2A (Invitrogen, CA, USA), Hsc70 (Stressgen, NY, USA), Ubiquitinated proteins (Santa Cruz), Opsin red/green (Chemicon), Opsin blue (Chemicon), Cone transducin (Cytosignal) and P-Tau

(Thermoscientific, IL, USA). Sections were visualized by confocal microscopy (TCS SP2; Leica Microsystems, Wetzlar, Germany). Apoptosis was detected by TUNEL as described (Mellén *et al.* 2008) using the Apoptosis Detection System; Promega, Madison, WI, USA.

Transmission electron microscopy

TEM in whole retinas was performed as previously described (Rodriguez-Muela *et al.* 2012). Sectioning for electron microscopic examination followed was accomplished with an ultramicrotome (Vitracut E, Reichert-Jung, Austria) and electron microscopy was performed with a Zeiss EM 902 transmission electron microscope (Germany), at 90 kV, on ultra-thin sections (50 nm) stained with uranyl acetate and lead citrate.

Semiquantitative and quantitative RT-PCR

RNA was isolated from individual retinas using Trizol (Invitrogen). Reverse transcription was performed on 1 µg of total RNA using Oligo (dT) and the Superscript III enzyme (Invitrogen) following the manufacturer's instructions. For q-PCR, 100 ng of the obtained cDNA were used and the assays were performed using the TaqMan Universal PCR Master mix (Roche Applied Biosystems, Basel, Switzerland), and probes were obtained from the Universal ProbeLibrary Set (Roche Applied Science). Amplifications were run in a 7900 HT-Fast Real-Time PCR System (Roche Applied Biosystems). Each value was adjusted by using GAPDH RNA levels as a reference. The primer sequences used were: beclin-1F 5'-caggcgaaccaggagag-3'; beclin-1R 5'-cgagttcaataaatggctcct-3'; lamp2aF 5'-gtgacaaaaggacagtattctacagc-3', lamp2aR 5'-ccaataaaataagccagcaaca-3'; atg7F 5'-ccggtggttctactgtta-3', atg7R 5'-aaggcagcgttgatgacc-3'; p62F 5'-gctgccctatacccacatct-3', p62R 5'-cgcttcatccgagaaac-3'; 18SF 5'-tgcgagtactcaacaccaaca-3', 18sR 5'-ttctcaacaccacatgagc-3

Western blot

Western blot in retinas were performed as previously described (Mellén *et al.* 2009). Briefly, after removal of the eyes, neuroretinas were dissected free of other tissues and lysed in a buffer containing 50 mM Tris-HCl pH 6.8, glycerol 10% (v/v), 2% SDS (w/v), 10 mM DTT, and 0.005% bromophenol blue. Thirty micrograms of protein was resolved on a 15% SDS-PAGE gel and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The antibodies used were LC3 (MBL), p62 (Enzo), ubiquitinated proteins (Santa Cruz Biotechnology), LAMP-2A (Invitrogen), Hsc70 (Stressgen), β -actin (Sigma) and GAPDH (Abcam, Cambridge, UK). Antibodies were detected with the appropriate horseradish peroxidase-labelled secondary antibodies (Pierce, Rockford, IL, USA) and were visualized with the SuperSignal West Pico chemiluminescent substrate (Pierce). Densitometric analysis was performed with Quantity One software (Bio-Rad).

Cell culture

661W murine photoreceptor-derived cell line (Tan *et al.* 2004) was provided by Dr Muayyad Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Center, OK, USA). Mouse retinal ganglion cell line (RGC-5) (Krishnamoorthy *et al.* 2001; Van Bergen *et al.* 2009) and 661W cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) 10% FBS, 1% penicillin/streptomycin in a 5% CO₂ incubator. LAMP-2A and Atg7-knockdown cells were generated by using a vector expressing GFP and shRNA and control cells are transfected with a non-silencing lentiviral vector expressing the unrelated protein PGK coupled to GFP to correct for the effects of lentiviral infection as previously described (Singh *et al.* 2009). For western blot analysis, 2×10^4 cells were plated in 6 well plates and exposed to the treatments for 24 h. Serum removal was performed by thoroughly washing the cells with Hanks' Balanced Salt Solution (Invitrogen) and placing them in serum-free complete medium.

Cells were treated with the combination of 100 μ M leupeptin (Thermo Fisher Scientific, MA, USA) plus 20 mM ammonium chloride (Sigma) for 4h to inhibit lysosomal proteolysis.

Intracellular Protein Turnover

Rates of protein synthesis were measured in retinal explants or in confluent cells as the incorporation of [3 H]leucine [10 mCi/ml (1 Ci = 37 GBq)] into acid-insoluble material in the presence of an excess (2.8 mM) of unlabeled leucine in the medium (to minimize differences due to alteration of amino acid transport and/or intracellular amino acid pool sizes). To measure degradation of long-lived proteins, retinas or confluent cells were labelled with [3 H]leucine (2 mCi/ml) for 48 h at 37°C and extensively washed. Retinas were maintained in R16 medium (provided by Dr P. A. Ekstrom, Wallenberg Retina Centre, Lund University, Lund, Sweden) and cell lines in DMEM containing an excess of unlabelled leucine, and treated with 5 μ M lactacystin (Calbiochem, Darmstadt, Germany) or 20 mM ammonium chloride (Sigma) plus 100 μ M leupeptin when indicated. Aliquots of the medium at different times were precipitated with trichloroacetic acid, and proteolysis was expressed as the percentage of the initial acid-insoluble radioactivity (protein) transformed into acid-soluble radioactivity (amino acids and small peptides) at the end of the incubation. Total radioactivity incorporated into cellular proteins was determined as the amount of acid-precipitable radioactivity in labeled cells immediately after washing.

CMA activity assays, construction of the reporter plasmids, photoconversion and imaging procedures.

The pKFEFQ-PA-mCherry plasmid construction and the establishment of stable cell lines expressing the CMA reporter was performed by using lentiviral transfer vectors as

described (Koga *et al.* 2011). Photoconversion of cells grown on coverslips was carried out with a 405/20 nm LED array (Norlux) for 10 min using 50 mW cm⁻² light intensity. More than 90% of the cells were viable after the photoconversion. All images were acquired with an Axiovert 200 inverted fluorescence microscope (Zeiss, Oberkochen, Germany). A sufficient number of fields were acquired to analyze at least 40 cells per well. Images were analyzed with Image J software (NIH).

Quantification of LC3 puncta by image analysis

LC3 puncta were quantified after immunofluorescence for LC3 (Nanotools, Teningen, Germany) using the web-based image analysis tool WimAutophagy of Wimasis (www.wimasis.com) on at least 300 cells per treatment. This online software tool is able to recognize fluorescent puncta and associate them to each nucleus as well as to recognize GFP-positive (knockdown cells) and GFP-negative cells (non-transfected cells). Graphs display the number of puncta per cell in GFP-positive cells.

Statistical analysis

Results are expressed as mean \pm SE. Statistical analysis was performed using the JMP IN 4.0.3 software. For most analyses we used one-way ANOVAs with treatment as a fixed factor. For analysis in figure 1B and 3E, D and Supp. Fig 3C 2-way ANOVAs were performed with the factors treatment and age or cell type respectively. All interactions were non-significant. For significantly different factors, comparisons among factor levels were performed using posthoc Tuckey tests. If normality and homoscedasticity assumptions were not met, we applied non-parametric tests. For all tests the significance level was $p < 0.05$ (2-tailed) and multiple testing was accounted for using Bonferroni correction

Acknowledgements

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Author's contributions

NRM performed most of the research and wrote the first draft of the manuscript, HK performed research and helped to write the manuscript, LGL performed research, PdV performed the electroretinograms, EJdR helped to write the manuscript, AMC and PB coordinated the research, supervised the project and wrote, edited and revised the manuscript.

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Supplementary information listing

Includes 4 supplementary figures

For Peer Review

Figure legends

Figure 1: Impaired macroautophagy in the aged mouse retina. (A) Autophagic flux as determined by western blot of LC3 in whole retinal extracts from mice of different ages in the absence/presence of the lysosomal inhibitors ammonium chloride and leupeptin (N/L). (B) Densitometric quantification of LC3-II in A and autophagic flux determined as the ratio of LC3-II in the presence/absence of lysosomal inhibitors (dotted lines) (n = 10). (C) *Beclin1* and *Atg7* mRNA expression as determined by qPCR. GAPDH is used as housekeeping gene (n = 6). (D) Beclin1 immunostaining (red) in retinal sections from 3 and 22 months old animals. DAPI is used as nuclear stain (blue). (E, F) Western blot for p62, and its densitometric quantification, of retinal extracts from 3, 12 and 22 month-old mice showing increase of p62 levels with age (n = 10). (G) mRNA expression by qPCR of *p62* in retinal extracts from 3, 12 and 22 month-old animals (n = 6). *p<0.05.

Figure 2: Presence of lipofuscin and ubiquitin aggregates in aged retinas. (A) Accumulation of lipids, lipofuscin and proteinaceous material in the INL of 22 month-old retinas. Lower panels in A represent the area highlighted inside the white box. N, nucleus; Nc, nucleolus; lf, lipofuscin; L, lysosome; P, proteinaceous material. Scale bars as depicted. (B) Representative immunofluorescence for ubiquitin in retinal cryosections from 3, 12 and 22 month-old mice. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment. Scale bar 45 μ m.

Figure 3: Perturbation of macroautophagy results in increased CMA. (A) Retinal proteolytic rates of long-lived proteins determined by metabolic labeling of the indicated ages mice retinas (n = 5), *p<0.05. (B) Western blot for LAMP-2A in the same samples and densitometric quantification (n = 10); *p<0.05. (C) Proteolysis

determined as in A in 661W and RGC-5 cells in control (Co) and after Atg7 or LAMP-2A knockdown (KD) at the indicated times of the chase period (n=4). (D) CMA activity as determined with the fluorescent reporter KFERQ-PA-mCherry in control, Atg7 and LAMP-2A KD 661W cells maintained in the presence of serum (+) or in the absence of serum (-) for 16h. Representative images (left) and quantification of the number of puncta per cell (right) are shown. Scale bar as depicted. *p<0.05, ** p<0.01, ***p<0.005. (E) Autophagic flux as determined by western blot of LC3 in the same cell lines maintained in the presence or absence of serum and of the lysosomal inhibitors ammonium chloride and leupeptin (N/L). Left: representative immunoblot (#) lower exposure time immunoblot. Right: Densitometric quantification of LC3-II in A and autophagic flux determined as the ratio of LC3-II in the presence/absence of lysosomal inhibitors (n = 3). ***p<0.005. (F) Immunofluorescence of LC3 in the same cells maintained in the presence or absence of N/L. Left: top panels show LC3 (red) and DAPI (blue) channels. Bottom panels include GFP channel (green) to track the knocked down cells. Right: Quantification of the number of LC3 positive puncta per cell (n>300 cells). ***p<0.005. (G) Cell viability as determined by propidium iodine (PI) staining and flow cytometry in control, Atg7 and LAMP-2A KD 661W treated with or without 150 μ M paraquat (PQ) (n = 10). *p<0.05.

Figure 4: Increased expression of CMA markers and signs of retinal damage in *Atg5^{fllox/fllox}; nestin-Cre* mice at 7 weeks of age. (A) Western blot for LAMP-2A, p62 and ubiquitin of total retinal extracts from control (Ctr) and *Atg5^{fllox/fllox}; nestin-Cre* (*Atg5^{-/-}*) mice (n = 6). (B-C) Staining for LAMP-2A and Hsc70. (D-E) TUNEL staining and its quantification in retinal sections. (F) Staining for p-Tau in representative retinal cryosections from control and *Atg5^{fllox/fllox}; nestin-Cre* mice. White boxed areas are shown at higher magnification below of each panel. GCL, ganglion cell layer; INL,

inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment. * $p < 0.05$. Scale bars as depicted.

Figure 5: Decreased rod function and apoptotic cell death in the photoreceptor layer of *Atg5^{fllox/fllox}; nestin-Cre* mice (A) Electroretinograms in control (Ctr) and *Atg5^{fllox/fllox}; nestin-Cre* mice (*Atg5^{-/-}*) (n = 5). STR, scotopic threshold potential; OP, oscillatory potentials. * $p < 0.05$ vs. Ctr. (B-C) Representative immunostaining for cones in retinal cryosections from control (Ctr) and *Atg5^{fllox/fllox}; nestin-Cre* (*Atg5^{-/-}*) mice at 7 weeks of age by means of three different antibodies: (B) transducin (red) and (C) the combination of blue and red/green opsins (red). Apoptotic cells are stained by TUNEL (green). OS, outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; and GCL, ganglion cell layer. Scale bars as depicted.

Supplementary legends

Supplementary Figure 1: Ultrastructural age-related changes in the mouse retina. Ultrastructure of retinas from 3, 12 and 22-month-old mice showing pathological features in the oldest group: (A) Increased presence of lysosomes but not of autophagosomes in the INL. Damaged mitochondria (black “m”) and swollen ER are also detected in the aged retinas. (B) Lipofuscin deposits in retinal pigment epithelial cells. Electron-dense structures (black arrows) are melanin granules in RPE cells. (C-E) (C) decrease in the photoreceptor density in the ONL (the round and electron-dense structures correspond to photoreceptors nuclei), (D) disorganization in the disposition of the OS, (E) and cell death in the GCL. c, centrosome; N, nucleus; N* apoptotic nucleus; G, golgi; ER, endoplasmic reticulum; m (white), normal mitochondria; lf, lipofuscin; L,

lysosome; P, proteinaceous material; ONL, outer nuclear layer; OS, outer segment, RPE retinal pigment epithelium. Scale bars as depicted.

Supplementary Figure 2: Increased levels of CMA components in the aged retina. (A) Retinal proteolytic rates of long-lived proteins determined by metabolic labeling of the indicated ages mice retinas after pharmacological inhibition of proteosomal activity with lactacystin (n = 3). (B-C) (B) Representative immunostaining for LAMP-2A (red), and Hsc70 (cyan) in the outer nuclear layer (ONL) and (C) in the other layers of the retina from 3 and 22 month old mice. OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; and GCL, ganglion cell layer. * p<0.05 vs. 3 month-old mice. Insets show higher magnification areas. Scale bars 20 μ m.

Supplementary Figure 3: Macroautophagy and CMA activity in RGC-5 cells. (A) Autophagic flux as determined by western blot of LC3 in RGC-5 control (Co) or knock-down (KD) for LAMP-2A (L2A) or Atg7 maintained in the presence or absence of serum and of the lysosomal inhibitors ammonium chloride and leupeptin (N/L). Left: representative immunoblot. Right: Densitometric quantification of LC3-II in A and LC3-II levels in the presence/absence of lysosomal inhibitors (n=3). (B) CMA activity as determined with the fluorescent reporter KFERQ-PA-mCherry in control, Atg7 and LAMP-2A KD RGC-5 cells maintained in the presence of serum (+) and in the absence of serum (-) for 16h. Values show quantification of the number of puncta per cell (n>50 cells). *p<0.05.

Supplementary Figure 4: P-Tau staining in mouse retinal cryosections at 3, 12 and 22 months of age. OS, outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; and GCL, ganglion cell layer. Lower panels display higher magnification insets. Scale bar 45 μ m.

Figure 1

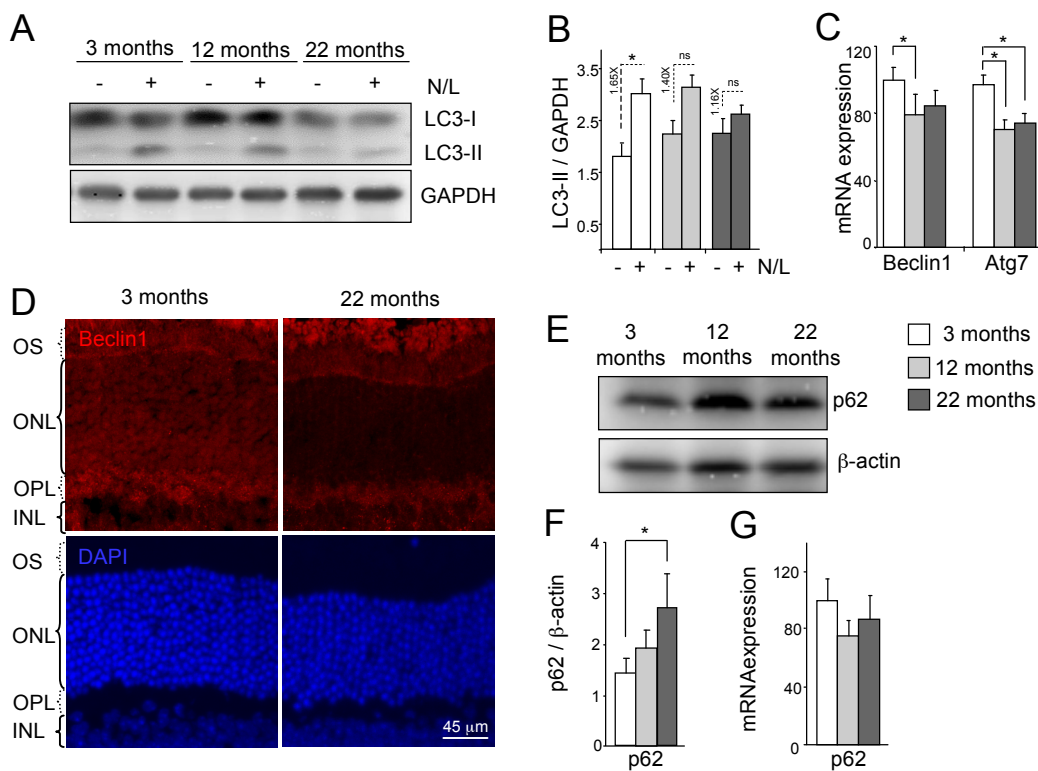


Figure 2

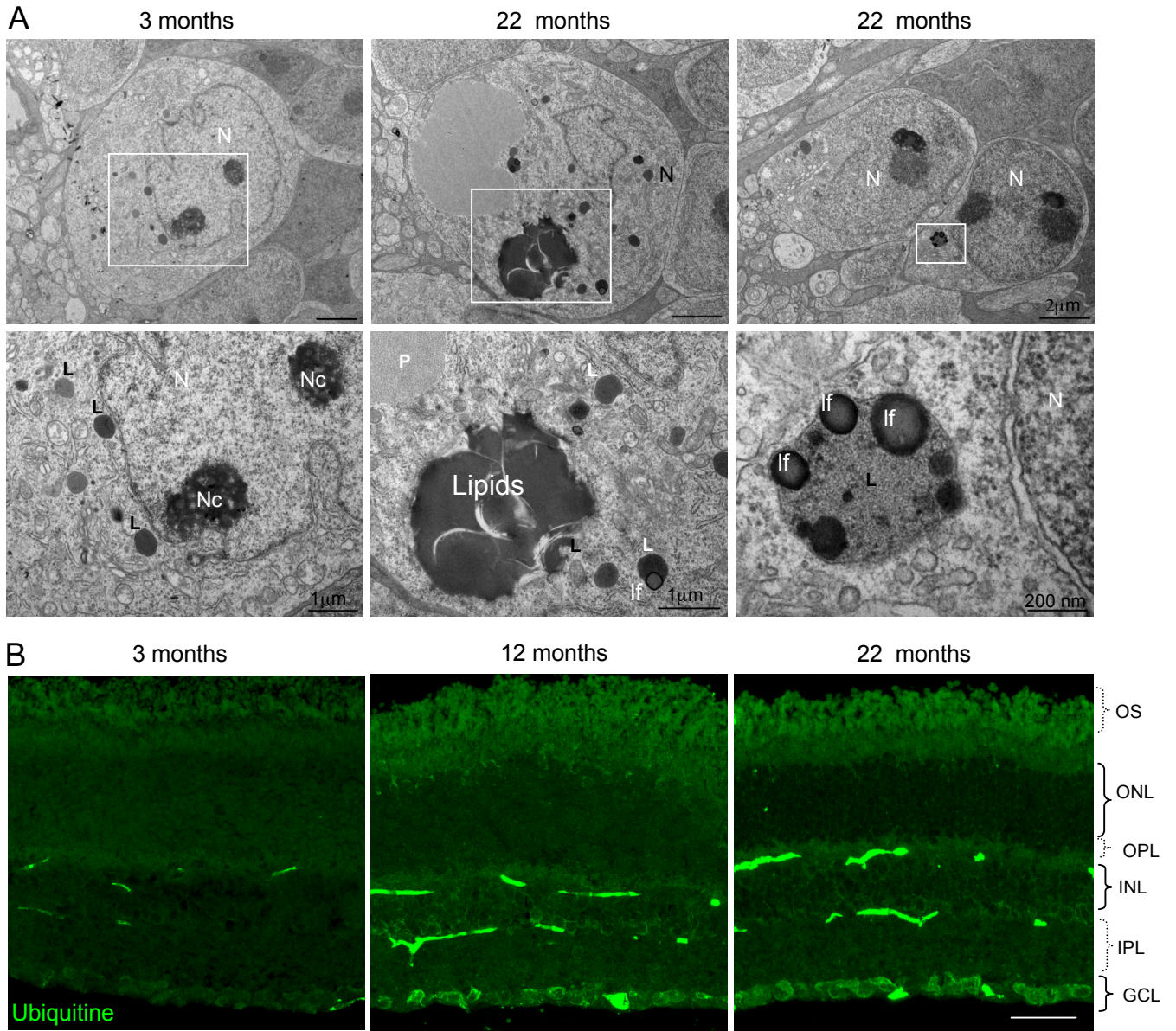


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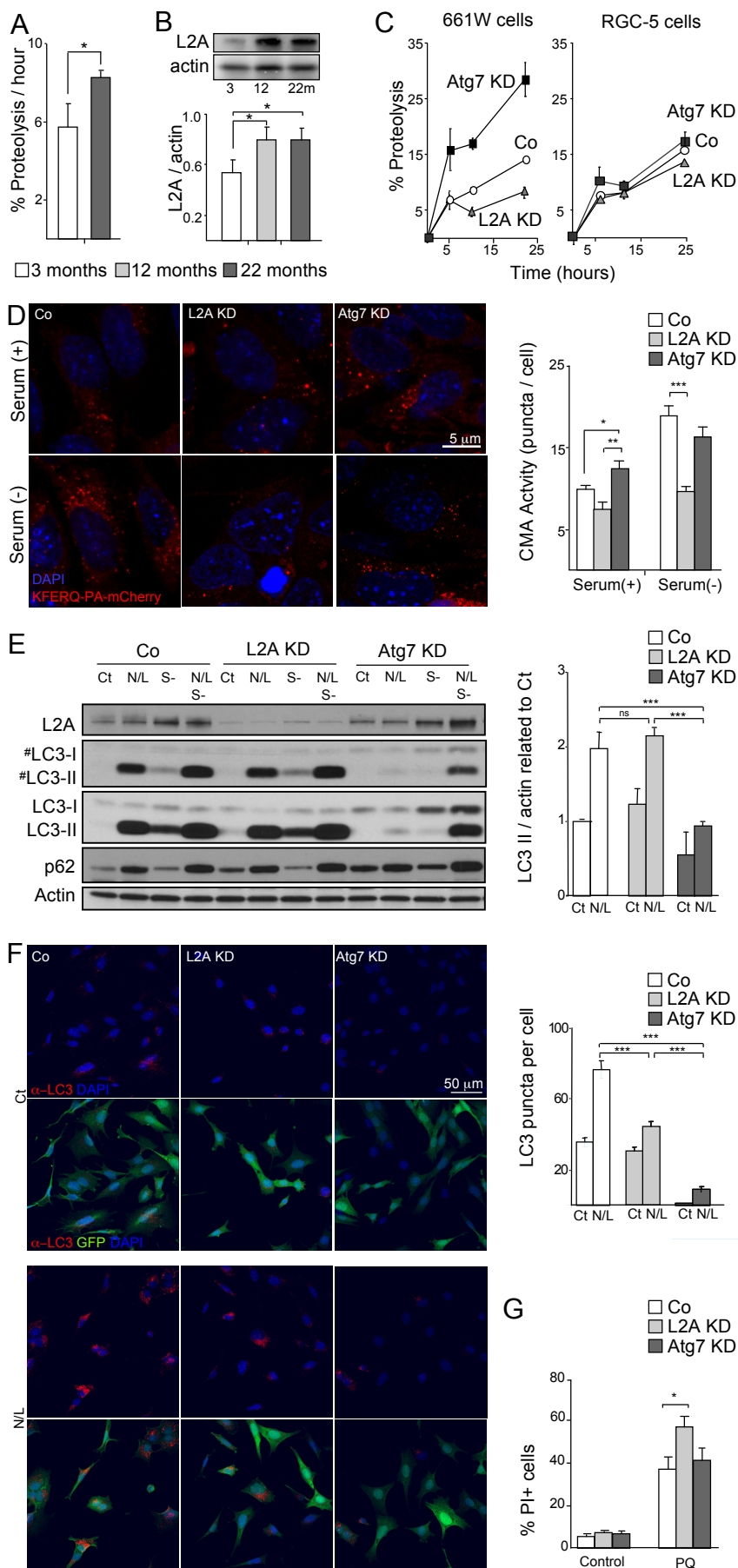


Figure 4

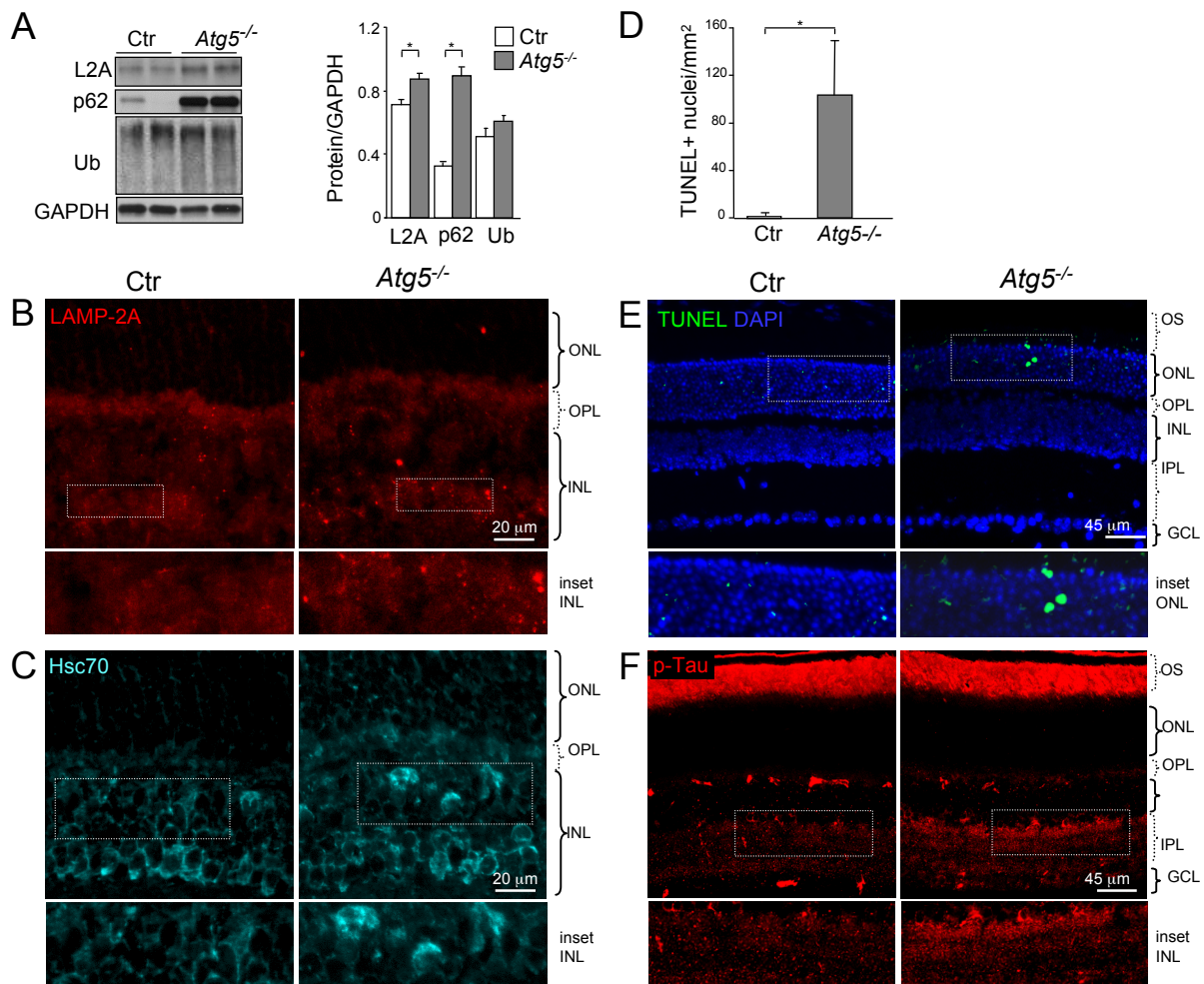
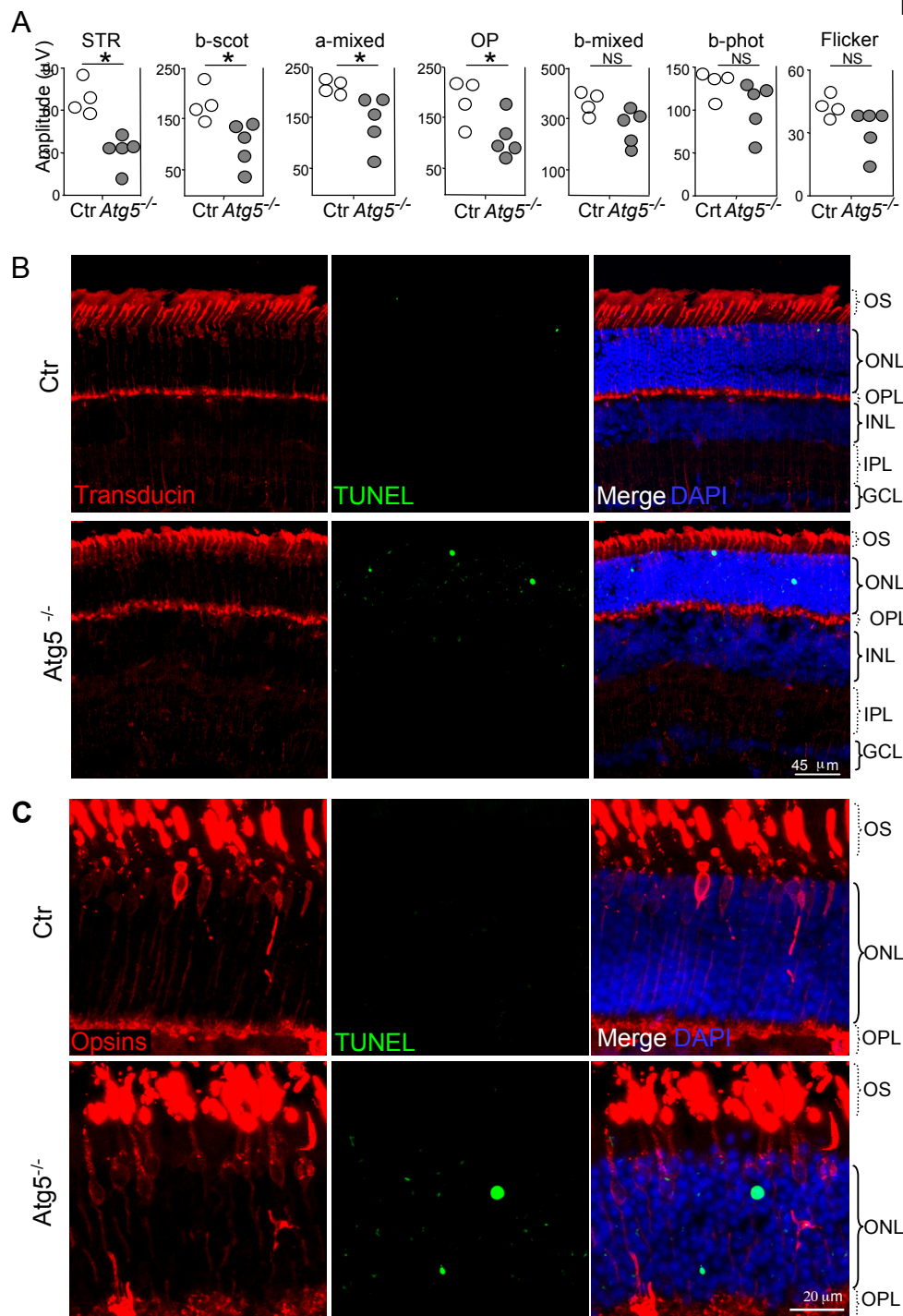
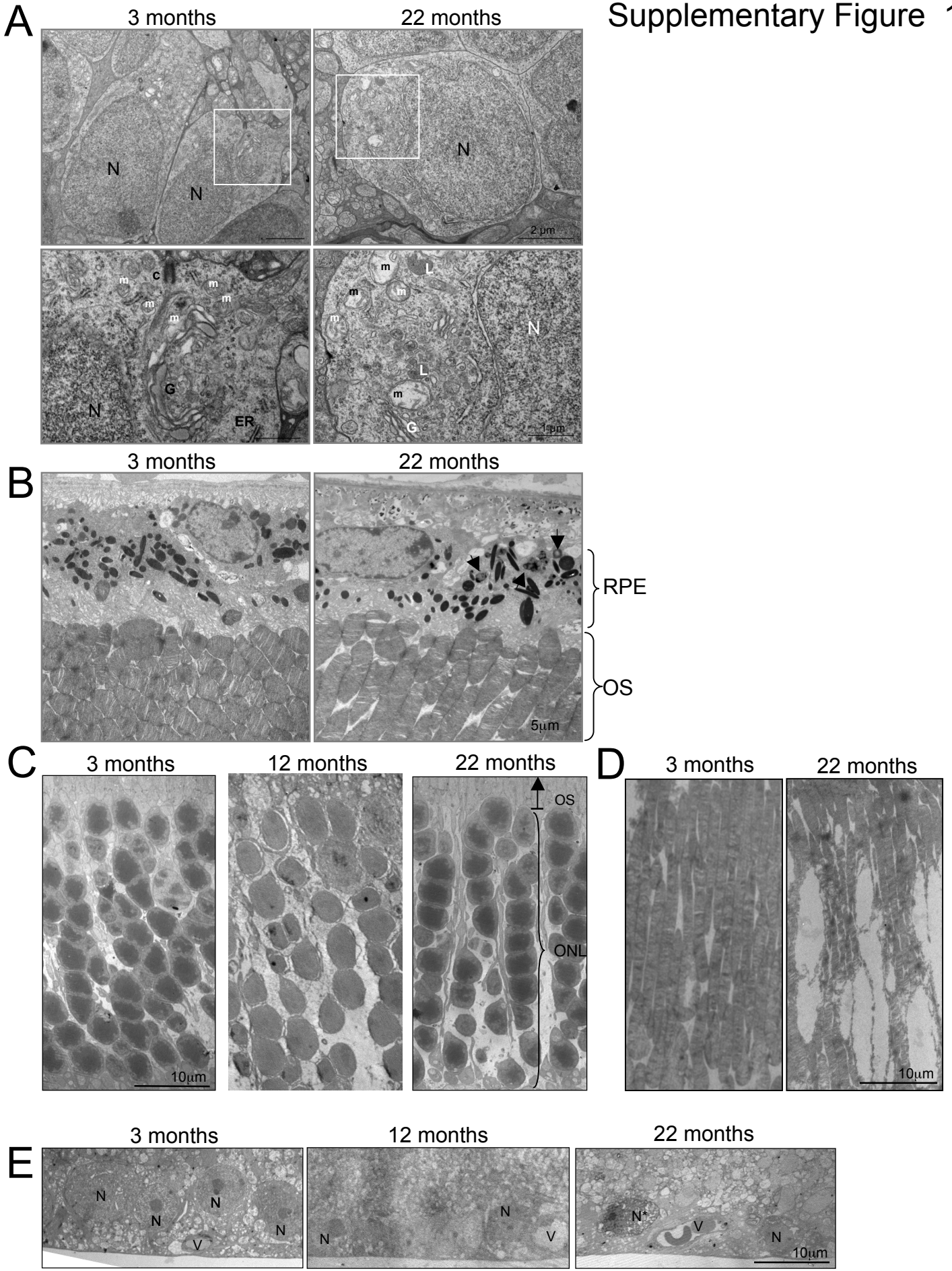


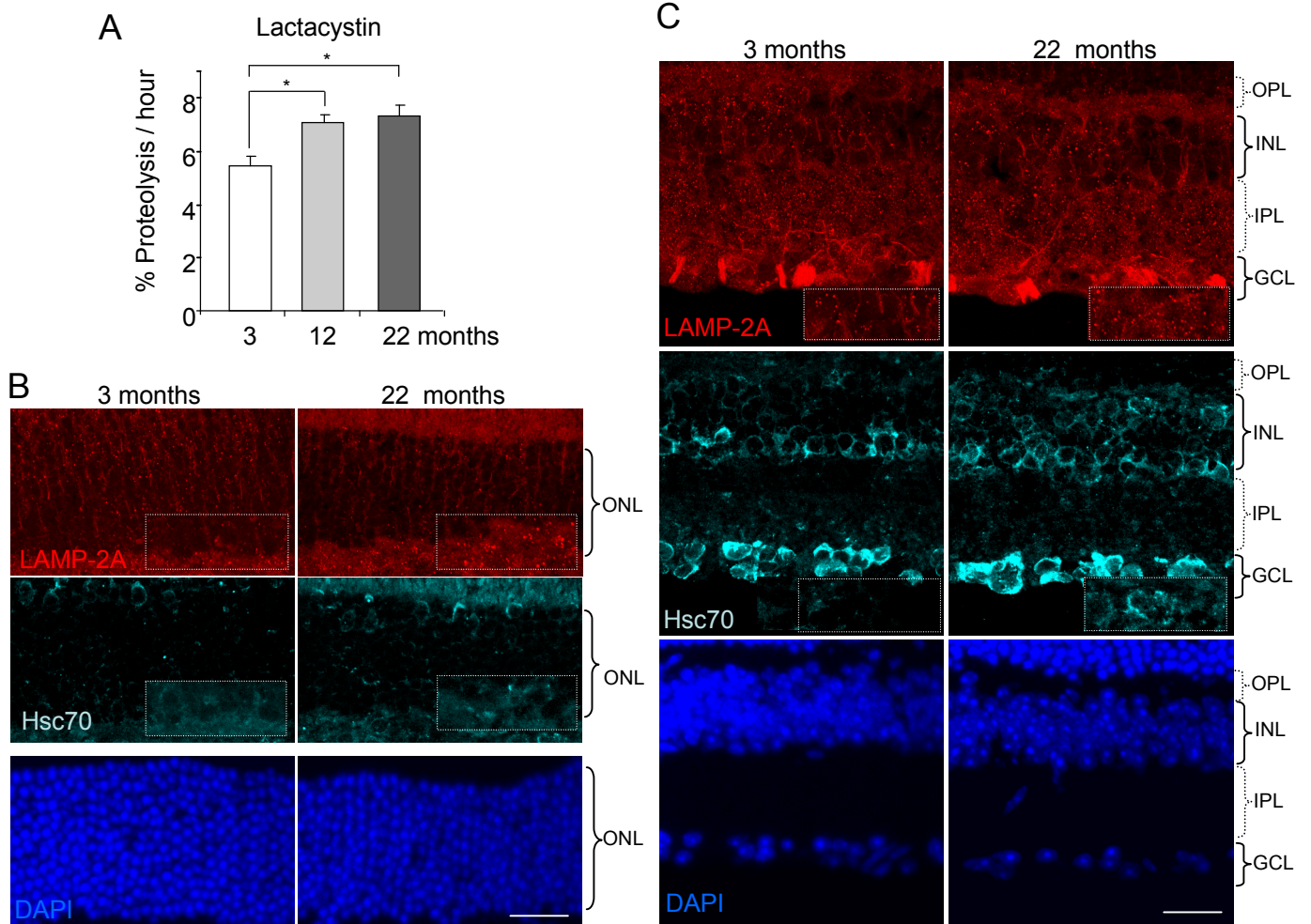
Figure 5



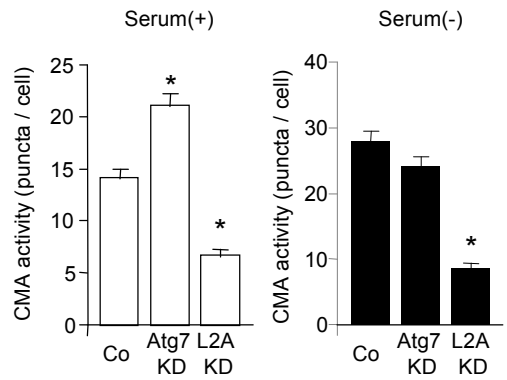
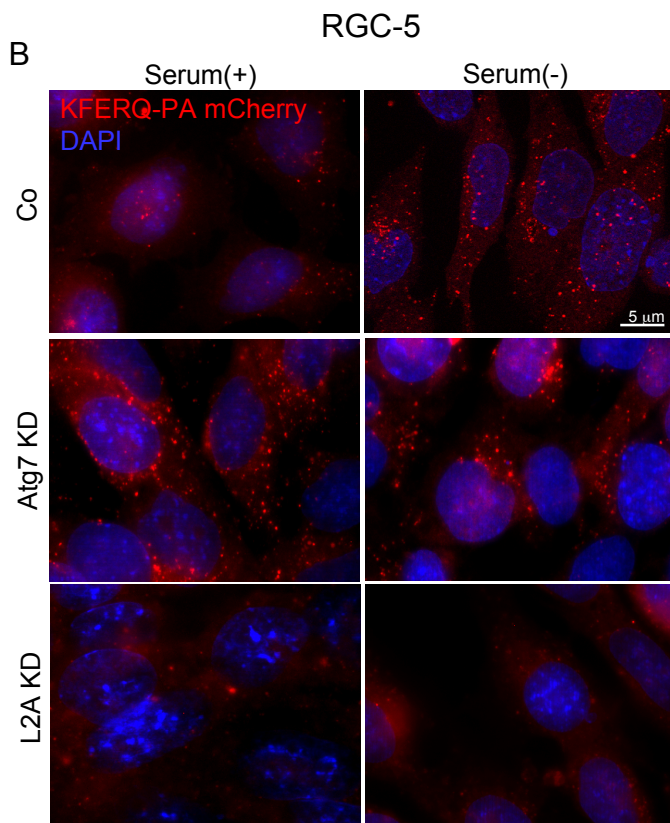
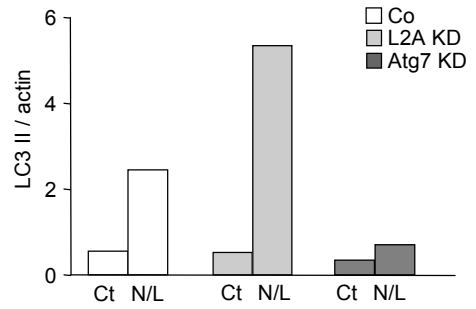
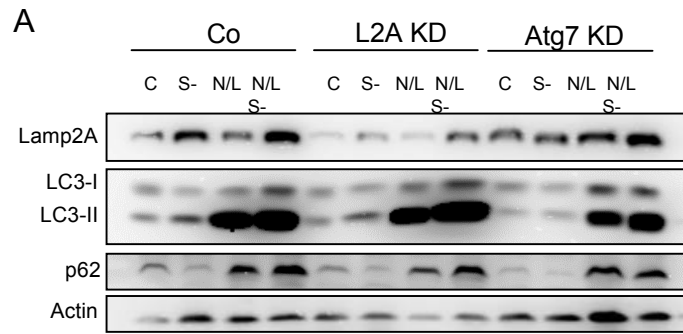
Supplementary Figure 1



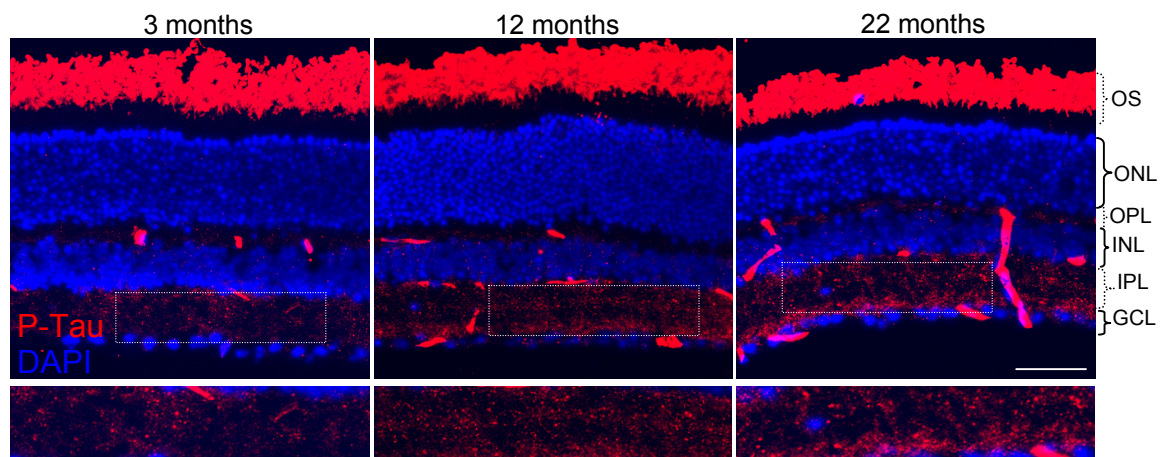
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Point by point response to manuscript ACE:12-0216

We thank the reviewers and the Supervising editor for the very useful comments on the manuscript and their constructive suggestions. This is our point-by-point response to their recommendations, which we have introduced in the revised version of the manuscript.

Reviewer: 1

Comments to the Author

The authors present evidence suggesting that macroautophagy is decreased in retina with aging, while at the same time chaperone-mediated autophagy increases. Further, the data suggests that old retina is not able to induce macroautophagy even in starvation conditions. Increased CMA in retina is also demonstrated in mice where the autophagy gene Atg5 is conditionally knocked down. The manuscript deals with a very timely topic. The role of autophagy in retinal degeneration is suggested by many recent studies, but nevertheless, this role is still poorly understood. Overall, the study seems sound and the experiments are well controlled. However, the presentation of the results could be improved, and some more experimental work may be necessary to acquire data that fully supports the conclusions.

We thank this reviewer for his/her enthusiastic comments on the novelty of our study. We have now added the requested additional experiments and have modified text and figures to improve the presentation of the results.

Specific comments

1. On page 8, the authors write: “Decreased levels of LC3-II, ..., were observed in aged animals (Fig. 1A).” However Fig. 1B, which shows the quantification of the Western blotting experiments presented in Fig. 1A, shows that the level of LC3-II is increased, not decreased, in the older animals (age groups 12 months and 22 months). The only thing that differs between young (3 months) and older animals (12 and 22 months) seems to be that the LC3-II levels do not significantly increase with the lysosomal inhibitors in the older animals. This point should be explained in more detail in the text and Figure legend.

Our response: We thank the reviewer for rising this important point that we have now clarified better in the text. Indeed, as noted by the reviewer, the differences in levels of LC3-II between young (3 months) and old animals (12 and 22 months) became only evident in the presence of lysosomal inhibitors that are used to determine autophagic flux as a functional read out of the autophagic process. Although steady-state levels of LC3-II are not apparently different across ages, the use of the functional test has allowed us to unveil a severe compromise in the formation of autophagosomes in the older groups. Young animals increase 1.65 fold the levels of LC3-II in the presence of lysosomal inhibitors while in older animals this capacity is not statistically differentially increased. We have now added the fold change in the autophagic flux in the graph in new Figure 1B for further clarification and discuss better this important point in the text in page 7.

2. On page 10, the authors state: “CMA down-regulation in 661W cells did not increase macroautophagy levels (Supp. Fig. 3A; see LC3 flux)”. The LC3-II bands in Suppl. Fig. 3A are very much overexposed, therefore, it is not possible to see if the levels differ or not between the control, LAMP-2A KD, and Atg7 KD samples.

Our response: We apologize for the selection of a high exposure of the immunoblot that was intended to also make visible levels of LC3-I in the same samples. We have now included both a high exposure (to show LC3-I) and the requested lower exposure image that allows now appreciating better changes in LC3-II levels upon inhibitor treatment. We have also included a quantification of LC3-II (done in the unsaturated images) to better illustrate that, contrary to most cells analyzed before, autophagic flux is not increased in 661W cells upon CMA blockage. This data is in agreement with our observations in the Immunofluorescence studies for LC3 (panel F of the new Fig. 3). We discuss these changes in more detail in the text in page 9.

Further, taking into account how much this result is emphasized in the manuscript, the authors should use another autophagy assay, such as LC3 immunostaining (+quantification of dots per cell) or quantitative electron microscopy, to estimate macroautophagy levels in the 661W cells.

Our response: We agree with the referee that this is a very important point in our study. As suggested by the reviewer and explained above we have now performed LC3 immunofluorescence experiments in control L2A KD and Atg7 KD untreated or in the presence of lysosomal inhibitors. These studies, shown now in the new Fig. 3F have confirmed the absence of upregulation of macroautophagy in 661W cells in response to CMA blockage.

3. Figure 1C. Panel C is claimed to demonstrate a “decrease in the photoreceptor density in the ONL”. For a cell biologist who is not an expert in retinal histology, the decrease is not at all obvious in panel C. Do the dark roundish objects represent photoreceptors? Could these be quantified? The authors should explain this more clearly.

Our response: We thank the referee for this observation. Indeed the dark roundish objects are the photoreceptor cells in the outer nuclear layer. The darker areas are the nuclei of these cells. The decrease in cell density may be better appreciated by an increase in the area devoid of cells. A better explanation of these data has now been added in the revised version of the manuscript in pages 7 and 8.

4. Figure 4B, C, and Suppl. Figure 2B,C. The authors should use arrows or higher magnification inserts to indicate in which region of each image the differences are located between control and Atg5^{-/-} tissue, or between the young and older animals. Especially the difference in TUNEL staining is unclear (Fig. 4C).

Our response: We have now included insets at higher magnification to improve Figure

4B,C (LAMP-2A and Hsc70) and 4E,F (TUNEL and p-Tau) and Supp. Fig 2. Moreover we have now provided quantitative results for TUNEL staining in new Figure 4D.

5. The statistical significance levels in the Figures are marked with different symbols, which are then explained in the Legend. It would be much easier for the readers if the significance levels were marked in the panels in a way that is self explanatory, and would also indicate which two values are compared in each case.

Our response: We have now depicted which values are compared for all graphs in the manuscript and used a more consistent annotation for the statistical significances.

6. On page 9, the authors write: “we detected a dramatic increase in total proteolysis of long-lived proteins in retinal extracts from aged animals (Fig. 3A)”. However, in materials and methods the authors describe (page 19) that proteolysis was measured using cultured retinas (retinal explants), not retinal extracts.

Our response: We apologize for the mislabeling and thank the referee for this remark that now has been amended in the revised version of the manuscript.

Reviewer: 2

Figure 1,2 and Supplemental figure 1: These figures set up the manuscript, suggesting a loss of macroautophagy in the aging retina. This section could be further developed by improving the description of the figures in the text and figure legend, as well as the presentation and description of the statistics that have been used. For example, for Figure 1A and B, the text simply states that there are changes ‘observed in aged animals’. Does this refer to the 22 mo mice alone? In Figure 1A, there does not seem to be a difference at 12 mo. How was the ANOVA designed here? Based on the methods section, is this based on a one-way ANOVA? If the goal is to determine whether autophagic flux is altered by age, perhaps there should be two variables, age and treatment, and an examination of whether there is an interaction between age and treatment, and the posthoc performed on the different ages? For Figure 1D and E, the image suggests more of a difference between 3 and 12 months, and thus the statistics again become important. Is the comparison between the different ages the product of a posthoc? If so, was there overall a significant difference in age? By providing this information, it will make it easier for the reader to interpret this figure.

Our response: We thank the referee for these remarks that has helped us to describe now in a more accurate manner the results presented in this figure. We have now indicated the groups compared in each statement and provide a better explanation of the statistical tests used to compare the data for each figure.

Only mRNA is examined for Beclin 1 and Atg7, but their protein levels would also be welcome in this figure.

Our response: We have now included protein staining for Beclin1 in retinal sections in new Figure 1D. Unfortunately, the antibodies against Atg7 currently available display poor immunoreactivity for this protein and that is one of the reasons why knock-down efficiency is often determined only by RT-PCR alone or, as in our study, in combination with measurement of LC3 flux rather than analysis of Atg7 levels by immunoblot or immunostaining. We thank the reviewer for the suggestion to include the staining for Beclin1 that illustrates better that changes in the levels of this protein with age are particularly pronounced in the ONL (new Fig. 1D).

Finally, if possible, was optineurin examined? As the authors are most likely aware, this protein has a potential of being an autophagic substrate (as an autophagic receptor like p62), with stronger potential relevance to the retina. Changes in optineurin may provide a provocative point to enhance the importance of this work, although it is not required.

Our response: We agree with the referee that optineurin is a very interesting protein to study since it is often mutated in glaucoma patients and has been recently described as a new autophagy receptor for selective autophagy. As continuation of this first study, we indeed intent in the future to analyze more in detail age-related changes in the different types of autophagy (mitophagy, lipophagy etc) and agree with this reviewer that inclusion

of these other cargo-recognition molecules would be highly relevant for those studies. However, it is our humble opinion that inclusion of a single staining for these proteins in these study, without a complete functional analysis that is beyond the current scope of this work, would increase complexity and create confusion, as the analysis in the present manuscript refers to in bulk macroautophagy. In addition, we do not have still information in other tissues/organs of possible differences in the autophagic cross-talk between CMA and specific macroautophagy subtypes. We agree with the reviewer that these are questions of great importance and will make them a priority in our future studies.

Finally, Supplemental Figure 1 shows only 3 and 22 months. Can 12 months be provided? The decreases observed in Figure 1 seem to be strongest where there are profound degenerative changes in the retina. There is an exception of p62 and the increased proteolysis in Figure 3A, but the data would be strengthened if changes could be correlated to an earlier age range. For example, Beclin 1 and Atg 7 are changed at 12 months of age, at which point the degenerative events might be preceded by decreased macroautophagy.

Our response: The reviewer raises a very interesting point that we had also entertained in our assessment of the data. As correctly pointed out by this reviewer, we have observed changes in mRNA expression of the autophagy regulators already at 12 months of age and a trend for decline in LC3 flux at that age, whereas degenerative changes are not observed until later time points. As previously stated we have not observed major morphological or functional differences at 12 months in many of the parameters studied. These data would suggest, in agreement with our overall message in this study, that the early deficit in macroautophagy does not become evident until later times, thanks to the successful activation of compensatory mechanisms for the loss of macroautophagy function. We agree with this reviewer that decreased autophagy may impact retinal function in the longer term in agreement with the housekeeping effects of autophagy in the nervous system. Following the reviewer suggestion we have now included new data on EM at 12 months and discussed on the lines suggested by the reviewer in page 12.

Figure 3 and Supplemental Figure 2 represent a very interesting transition in this work, and some improvements will help strengthen its presentation.

Our response: As the referee suggest we have now changed figure 3 by adding data from Supp. Fig 3 and improving its presentation.

Supplemental Figure 2: Based on the images alone, it is difficult to observe the upregulation of Hsc70 or Lamp-2A, even though based on Figure 3B, the upregulation of protein is quite evident. Perhaps arrows pointing to where the changes are observed would be helpful. Moreover, grayscale can also improve the ability of the reader to distinguish levels, rather than color.

Our response: Following the referees suggestion we have now included as insert in the whole field images enlargements of the relevant areas in the staining for Hsc70 or LAMP-2A in Supp. Fig. 2.

page 8, figure 3C: Is the line labeled as 'Atg 7' suppose to be 'Atg7KD', as to which it is referred in the text? The Figure 3C figure legend should read 'knockdown' rather than 'down regulation'.

Our response: We thank the referees for this suggestion and have accordingly improved now the labeling of that figure.

Figure 3C: The Lamp2A knockdown suggests that most of the proteolysis in this cell type is driven by CMA, and macroautophagy has little to no impact. If that is the case, why is there this apparent dependence and a strong response to a loss of macroautophagy?

Our response: We thank the referee for these remarks that have made us to attempt clarify now better this essential point in our manuscript. We were also surprised about the pronounced decrease in protein degradation observed in these cells when CMA is compromised, and this reviewer stands correct that this is an unusually high contribution when compared to other autophagic pathways in the same cell. However, we want to point out that the experimental design excludes measurement of the degradation of the short-lived proteins (mainly proteasome substrates) so we cannot discard that the proteasome may be the other system contributing to degradation in these cells, that as highlighted by the reviewer have a remarkable dependence on CMA.

The strong response to macroautophagy blockage, in the form of increased protein degradation, may reflect the additive upregulation of inducible CMA over cells that already have considerably high rates of basal CMA. We have evidence supporting that regulation of basal and inducible CMA is different and that the cross-talk with macroautophagy is preferentially with the inducible form of CMA. We hypothesize that although these cells depend less on macroautophagy the downregulation of this pathway will still create a stress situation (as organelles and aggregate proteins cannot be removed by CMA). Consequently the marked increase in degradation in the Atg7 KD is likely the result of the already high basal level of CMA in these cells plus the activation of inducible CMA (in response to the stress). We have incorporated these clarifications in the text for the description of the new Fig. 3 and Supplementary Fig. 3 (pages 9, 10 of the results).

Are the control cells transfected with a scramble or non-silencing siRNA (it is unclear from the text or figure legend)? This may help to rule out the possibility that the Atg7KD effect is due to an issue with the siRNA transfection. Could there also be included a 3-methyladenine (3-MA) control, or perhaps another siRNA against another core protein such as Beclin 1?

Our response: Control cells are transfected with a non-silencing lentiviral vector expressing the unrelated protein PGK coupled to GFP to correct for the effects of lentiviral infection. This is now clearly stated in the methods section. We tried the pulse and chase experiments in the presence of 3-MA. However this treatment resulted in high rates of cytotoxicity for the assay times required for these experiments.

The comparison presented in Supplemental Figure 3 and Figure 3 is very interesting. Is there a reason why the two different cell types are not presented side by side in Figure 3? The cell type specificity is a very interesting finding.

Our response: Following the recommendations of this and other reviewers we have modified Figures 3 and supplementary Fig. 3 to accommodate the new requested additional LC3 analysis, and include now similar type of plotting for data on 661W and RGC-5 cells to allow for easier comparison.

Figure 4, 5: Characterization of retina from *Atg5* conditional KO mice.

This is a very nice addition to this work. Perhaps adding text that explains when these changes in the retina are observed relative to the already published work on these cKO mice will help the reader better appreciate the larger context in which the work should be placed.

Our response: we thank the reviewer for this comment. We have now added in the discussion section a paragraph explaining the phenotype of the *Atg5*^{flox/flox} X *nestin-Cre* mice and discuss it in relation to the retinal phenotype we have observed (pages 15,16).

Figure 4C: quantification of cell death could help, as well as arrows that may point out where the phospho-tau increase is present (is it in the IPL?). Which neurons are dying (also in the context of Figure 5).

Our response: Following the referee's suggestion we have now quantified the number of TUNEL-positive cells and depicted that data in Figure 4D.

In addition we have now highlighted the area with increased p-Tau expression in the new figure 4F and indeed the increase is clear in the IPL as the reviewer states. For a complete response of the cell type affected please see below in the answers to comments from the third referee.

page 4: miss regulated should be misregulated; theses should be these

page 7: 'some of the inclusions were proteinaceous in nature as revealed by ubiquitin'- which inclusions?

Figure 2: Should be 'ubiquitin' rather than 'ubiquitine'

Our response: All these changes have now being included in the revised version of the manuscript.

Reviewer: 3

1. The authors strongly suggest the temporal, cell type-specific compensation by CMA when macroautophagy is impaired, particularly in cone cells. However, the upregulation of CMA seems to occur in many cell types of retina from either aged or *Atg5*^{-/-} mice. It is unclear whether upregulation of CMA markers occurs only in cone or rod cells. Therefore, it would be helpful if the authors can show IHC or IF staining that demonstrate the difference in CMA activity in cone vs. rod (or other cell types) in order to support their hypothesis.²

Our response: We thank the reviewer for pointing out the need for this important clarification that we feel will help to convey our message in a more effective manner. As noticed by the reviewer upregulation of CMA occurs in many cell types when macroautophagy is compromised, and it is the magnitude of this response and the intrinsic contribution that CMA has in the maintenance of homeostasis in specific cell types what would determine the beneficial effect of this upregulation.

We apologize if in the previous version it was not clear that the statement on the “unidirectionality of the cross-talk between macroautophagy” referred to the inability of the photoreceptor cells to upregulate macroautophagy in response to CMA blockage rather than the other way around. In any case, we agree with the reviewer that it was important to show that upregulation of CMA still occurs in the other cells. Consequently, we have now performed new experiments to determine CMA activity in the retinal ganglion cell line RGC-5 after macroautophagy blockage and compare the data with the results in the photoreceptor cell line 661W. As it is now shown in supplementary figure 3B, knocking down *Atg7* in RGC-5 still results on upregulation of CMA, at least under basal conditions in these cells. We also present now comparative analysis of the changes in macroautophagy in RGC-5, which contrary to our observations in 661W are able to upregulate autophagic flux in response to the CMA blockage (supplementary Fig. 3A). We have now incorporate the requested experiment in Suppl Fig. 3 and describe it in page 10

2. Have the authors examined the CMA activity outside retina in *Atg7* conditional KO mice? Whether is the upregulation of CMA unique to retina as the author suggested?

Our response: Published and unpublished data from our laboratories shows that so far the retina is the only tissue that shows an increase of CMA with aging and it is this finding what separates retina for the rest of organs analyzed in the context of CMA and aging. Upregulation of CMA in response to macroautophagy blockage occurs in many cells and tissues of the knock-out animals that include among others mouse embryonic fibroblasts hepatocytes, T-cells, dendritic cell, macrophages, fibroblasts and kidney epithelial cells. We have now included a sentence clarifying this point in the discussion (pages 14, 15).

3. In figure 3C, the kinetics of proteolysis is hard to understand when Atg7 or macroautophagy is compromised. If inhibition of macroautophagy stimulates CMA as a compensatory mechanism, it is expected that there is a reduction of long-lived protein proteolysis at initial stage due to autophagy impairment, followed by an increase in proteolysis due to upregulation of CMA. However, the figure 3C does not seem to have the initial downward phase.

Our response: We agree with the referee and have a methodological explanation for this observation. The kinetics of the graph does not represent time since the knockdown. It corresponds to time since we remove the labeling and start the chase. It is not technically possible to measure the changes in degradation with the progression of the knockdown because of the washes required for the infection and the fact that L2A is a long lived protein and takes several days (7) to achieve a complete knockdown. CMA is already upregulated at the time that chase starts and that is why even after normalizing time 0 at value 0% degradation in the next time point the differences are already visible and persist for the totality of the experiment. We have clarified in the legend of the figure that the time in X axis correspond to time of chase and not to time of knock-down.

AGING CELL AUTHOR CHECKLIST. Authors should submit this checklist together with their manuscript. Please ensure that you have read the Author Guidelines in detail before submission.

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|---|---|------------------|------------------------|-----------------------------|------------------------------|---|--|
| Title | Balance between autophagic pathways preserves retinal homeostasis | | | | | | |
| Authors | Rodriguez-Muela et al. | | | | | | |
| Manuscript Type | Primary research paper | | | | | | |
| Character Count of Title (including spaces)¹ | 65 | | | | | | |
| Total Character Count (including spaces)² | 41479 | | | | | | |
| Word count of Summary³ | 233 | | | | | | |
| Number of papers cited in the References⁴ | 34 | | | | | | |
| Listing of all Tables (Table1, Table 2 etc)⁵ | | | | | | | |
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| | | | | | | | |
| Figure specifications (please complete one row per figure)⁶ | Colour | Greyscale | Black and white | Single column (80mm) | Double column (167mm) | Size of figure at full scale (mm x mm) | Smallest font size used in the figure at full scale (minimum 6pt) |
| Figure no. | <i>(yes/no)</i> | <i>(yes/no)</i> | <i>(yes/no)</i> | <i>(yes/no)</i> | <i>(yes/no)</i> | <i>(insert details)</i> | <i>(insert details)</i> |
| Figure 1 | yes | | | | | 13.5 X 10.5 cm | 8 |
| Figure 2 | yes | | | | | 16.5 X 14.5 cm | 8 |
| Figure 3 | yes | | | | | 11 X 22 cm | 8 |
| Figure 4 | yes | | | | | 16 X 5.5 cm | 8 |
| Figure 5 | yes | | | | | 12 X 17.5 cm | 8 |
| | | | | | | | |

¹ Titles should be no more than three typeset lines (approximately 126 characters including spaces).

² The maximum character count allowed is 50,000 (incl. spaces) for Primary Research Papers, 10,000 for Short Takes and 20,000 for Reviews and is imposed to all sections with the exception of Title Page, Checklist, Figures and Tables. Figure Legends and References are included in the character count.

³ Summary should not exceed 250 words.

⁴ Primary Research Papers can contain a maximum of two tables. If more are needed they should replace some of the Figures or can be placed in the Supporting Information.

⁵ A maximum of 45 references is allowed for Primary Research Papers and 20 references for Short Takes.

⁶ A Primary Research Paper may contain up to 6 figures and a Short Take up to 2 figures. Authors are encouraged to provide figures in the size they are to appear in the journal and at the specifications given.