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Venkatesh A, Cheng S, Punzo C. (2017). Loss of the cone-enriched caspase-7 does not affect secondary cone death in retinitis pigmentosa. University of Massachusetts Medical School Faculty Publications. Retrieved from https://escholarship.umassmed.edu/faculty_pubs/1474

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Loss of the cone-enriched *caspase-7* does not affect secondary cone death in retinitis pigmentosa

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Purpose: The apoptotic mechanisms responsible for secondary cone death in retinitis pigmentosa (RP) remain largely unknown. The cone-enriched apoptotic protease *caspase-7* (*Casp7*) is thought to be triggered by endoplasmic reticulum (ER) stress and plays a pivotal role in mice deficient in the cone cyclic nucleotide-gated channels, a deficiency that causes achromatopsia in humans and in mice with autosomal dominant rhodopsin mutations, in particular the T17M mutation. Thus, we tested in two mouse models of RP whether the cone-enriched *Casp7* plays a role during secondary cone death.

Methods: *Casp7* knockout mice were crossed to two different RP mouse models with significantly different rod and cone death kinetics: the *rd1* mouse model, which carries a mutation in the *Pde6b* gene, and the rhodopsin knockout mouse model (*Rho-KO* or *Rho*^{-/-}). In both models, cone survival was assessed on retinal flat mounts by quantifying the percentage of cone arrestin staining over the retinal surface area. The analyses were performed at two different time points for each model.

Results: Loss of *Casp7* did not alter cone survival in either of the two mouse models tested regardless of the time point analyzed. Rod survival was also not affected in either model nor did loss of *Casp7* affect rod or cone function in a wild-type background as assessed with electroretinogram analyses.

Conclusions: Secondary cone death in retinitis pigmentosa is unlikely to be triggered by ER stress and is likely independent of *Casp7* activity.

Retinitis pigmentosa (RP) is an inherited photoreceptor degenerative disease that leads to blindness. The disease is often caused by mutations in rod photoreceptor (PR) specific genes resulting in initial loss of night vision. However, once a certain threshold of rod death is breached [1], secondary cone death follows leading to the loss of daylight, color, and high-acuity vision [2]. Understanding the underlying cause of secondary cone death may allow for the development of treatments that are applicable to all individuals with mutations in rod-specific genes. For example, the identification of a cell death mechanism could be exploited to prevent the execution of cone death itself.

Caspases belong to a family of cysteine proteases that control and execute apoptosis, which is a form of controlled cell death that leads to the removal of compromised cells within a tissue [3]. Caspases can be subdivided into initiator and executioner caspases and are generally activated by specific cell intrinsic or extrinsic signals or insults [4-6]. Thus, identifying a specific caspase as part of a cell death mechanism can give insights into the underlying cause for

cell death. For example, we have previously proposed that secondary cone loss in RP is caused by a glucose and thus, NADPH, shortage in cones, which is elicited by structural changes induced by the loss of the overabundant rods [1]. By improving cell metabolism in cones through activation of the mammalian target of rapamycin complex 1 (mTORC1), we showed that cone survival is improved, and retinal NADPH levels are increased [1,7-10]. Consistent with that result, we found that loss of the NADPH-sensitive initiator *Caspase-2* delays cone death in the *rd1* mouse model of RP, a mouse model that carries a mutation in the rod PR specific phosphodiesterase 6-beta gene (*PDE6b*) [7,11]. Similarly, loss of the executioner *Casp7* delays rod degeneration in the autosomal dominant *Rhodopsin* T17M mouse model of RP [12]. Interestingly, *Casp7* is enriched in cones and expressed in the inner nuclear layer but is absent in adult rods [13]. However, in the case of the T17M mutant, which triggers endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR), *Casp7* is upregulated in rods [12,14]. Other autosomal dominant mutations in *Rhodopsin*, such as the S334ter and the P23H, have also been shown to lead to ER stress, yet the protective effect of loss of *Casp7* in these mutants has not been tested [15,16]. Activation of *Casp7* has also been shown to occur for some cone-specific mutations, such as mutations in *cyclic nucleotide-gated channel subunits alpha 3* and *beta 3* (*CNGA3* and *CNGB3*) [17]. Mutation in

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either gene has also been associated with ER stress and activation of the UPR. The fact that *Casp7* appears to be involved in rod and cone cell death when either cell experiences ER stress led us to test whether *Casp7* may also play a role during the periods of secondary cone degeneration in RP, particularly because *Casp7* is cone enriched and ER stress has been predicted to contribute to cone death in RP. Further, loss of *caspase-2* did not completely prevent cone death; instead, the loss delayed cone death [7,13,18]. Thus, other yet unidentified cell death mechanisms are likely involved in triggering cone death in RP. Such a mechanism could involve ER stress-mediated activation of CASP7, because ER stress can also be triggered by increased oxidative stress, a condition known to be present during cone degeneration in RP and when alleviated, delays cone death [19-24].

METHODS

Animals: All procedures involving animals were in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Massachusetts Medical School. Animals were maintained on a 12 h:12 h light-dark cycle with unrestricted access to food and water. Lighting conditions were kept constant in all cages, with illumination ranging between 10 and 15 lux. The *Pde6b*^{-/-} (*rd1*, FVB strain), *C57BL/6J*, *Casp7*^{-/-}, and *Casp2*^{-/-} mice were purchased from Jackson Laboratories (Bar Harbor, ME) [11,25,26]. Because the FVB strain that carries the *rd1* mutation is albino, it was first backcrossed to *C57BL/6J* for ten generations to generate the *rd1_C57BL/6J* line [7]. The *Casp7*^{-/-} was already on a *C57BL/6J*, and the *Rho*^{-/-} mice, which have been described previously by Janis Lem [27], are also maintained on a *C57BL/6J* background. However, to avoid any possible strain background differences and to be able to directly compare littermates, we first generated heterozygous *Casp7* siblings (F1: e.g., *Casp7*^{+/-}*_Rho*^{-/-}) that were then crossed to each other to obtain the two (e.g., *Casp7*^{-/-}*_Rho*^{-/-} versus *Rho*^{-/-}) of the three possible different genetic combinations of the F2 cross that were then used for analysis. For the triple loss of function of *Casp7*^{-/-}*_Casp2*^{-/-}*_rd1*, we generated the desired genotype, analyzed the data at 20 weeks of age, and compared the data to our previously published data for the *Casp2*^{-/-}*_rd1* genotype [7]. Genotyping was performed as described in the original publications. All mice were genotyped for absence of the *rd8* allele with a mutation in the *Crumbs 1 gene* [28]. Electroretinography (ERG) was performed using the Espion E3 console in conjunction with the Color Dome (both sold by

Diagnosys LLC, Lowell, MA) as described previously [29] with a minimum of six animals per genotype and age.

Histological methods: Antibody stainings on retinal flat mounts were performed as described previously [7,30]. CASP7 activity on retinal whole mounts was detected using the FAM-FLICA Caspase 3 & 7 Assay Kit (Cat# 93) from ImmunoChemistry Technologies (Bloomington, MN) as previously described [7] following the manufacturer's instructions. The following primary antibodies and concentrations were used: rabbit α -cone arrestin (1:500; Cat# 15,282) from EMD Millipore (Now: MilliporeSigma, Burlington, MA), fluorescein-labeled peanut agglutinin lectin (PNA; 1:500; Cat# FL-1071) from Vector Laboratories (Burlingame, CA), and α -cleaved caspase 3 (1:300; Cat# 9662) from Cell Signaling (Danvers, MA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Cat# 9542) from Sigma-Aldrich (Now: MilliporeSigma, Burlington, MA). All secondary antibodies (donkey) were purchased from Jackson ImmunoResearch (West Grove, PA) and were purified F(ab)₂ fragments that displayed minimal cross-reactivity with other species. Quantification of cone survival was performed as previously described by calculating the surface area of the retina that is covered by the cone arrestin signal versus the entire retinal surface area to extrapolate the percentage of cone survival [1,7,9,31]. A minimum of six animals were used per genotype and age. Colocalization between the cone arrestin staining and the retinal surface area was calculated using CoLocalizer Pro software [32]. All images were acquired on a Leica DM5500 fluorescence microscope Leica (US Branch: Buffalo Grove, IL) equipped with an automated stage for tiling.

Molecular biology and cell culture methods: Protein extractions and western blot analysis were performed as described previously [7]. The following primary antibodies were used: rabbit α -caspase-7 (1:1,000; Cat# 9492) from Cell Signaling and mouse α - β -actin (1:2,000; Cat# A5316) from Sigma. To clone the *Casp7* cDNA for overexpression *Casp7* was amplified by PCR (For: 5'-GCG GCC GCC ACC ATG ACC GAT GAT CAG GAC TG-3'; Rev: 5'-CTA ACG GCT GAA GTA CAG CT-3'; PCR conditions: 95°C for 2'; 25 times: 95°C for 15'', 53°C for 30'', 68°C for 1') with TaKaRa (US Branch: Mountain View, CA) LA Taq (Cat# RR002A) from a *C57BL/6J* retinal cDNA library that was generated as previously described [1]. The PCR product (994 bp) was sub-cloned into the p-GEMT-Easy vector (Cat# A1360) Promega (Madison, WI) and sequence verified. The *Casp7* cDNA was then cloned into a recombinant adenoassociated virus (rAAV2) plasmid carrying a *cytomegalovirus (CMV) promoter* and the SV40 poly-adenylation site. The human

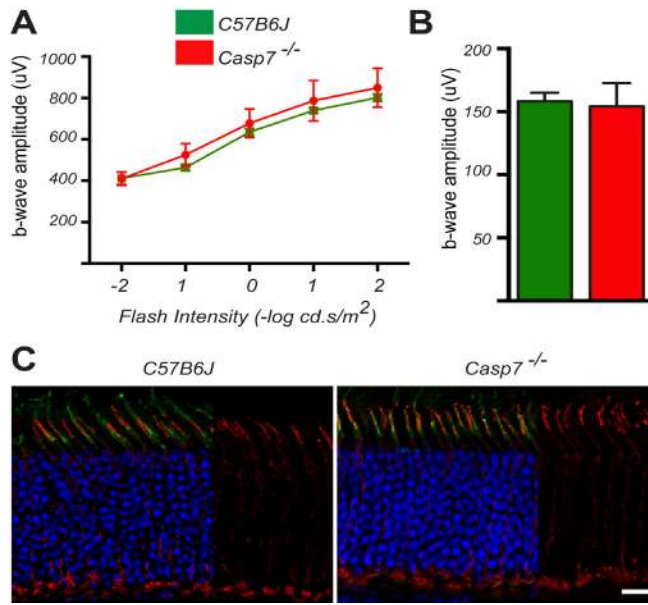


Figure 1. Loss of *Casp7* does not affect rod and cone function in a wild-type background. **A**, **B**: Electoretinogram (ERG) recordings at 20 weeks of age showing averages of b-wave amplitudes of scotopic ERGs over five different light intensities (**A**) and average of b-wave amplitudes of photopic ERGs (**B**) n=6 in **A** and **B**; Error bars: SEM. **C**: Immunofluorescence analysis on a cross section at 20 weeks of age showing normal expression of cone arrestin (red signal) and normal labeling of cone segment sheets with PNA (green signal). Blue: nuclear

4',6-diamidino-2-phenylindole (DAPI). (Blue and green signals were removed from one third of each panel to better visualize the cone arrestin staining.) Scale bar: 20 μ m.

embryonic kidney (HEK 293) cells were either treated with 100 nM rapamycin or transfected with *Casp7* using PEI (Polyscience Inc, Warrington, PA; Cat# 23,966). For all conditions, cells were maintained in regular Dulbecco's Modified Eagle Medium (DMEM; Cat# 11965092) containing 10% fetal bovine serum (FBS; Cat# 16140071) and a penicillin/streptomycin mixture (Cat# 15140122) all from Gibco by Life Technology (now Thermo Fisher Scientific; Grand Island, NY). Twelve hours post rapamycin treatment and 24 h post transfection with *Casp7*, protein extractions were performed as described [7].

Statistical analysis: The Student *t* test was used for statistical analyses. P values of less than 0.05 were considered statistically significant. None of the comparisons showed any statistically significant difference. All error bars represent the standard error of the mean (SEM).

RESULTS

Loss of *Casp7* does not affect rod and cone function in a wild-type background: To study the long-term effect of *Casp7* loss on secondary cone death in RP, we first assessed whether *Casp7* loss alone has any negative effect on cones and rods in a wild-type background. The *Casp7*^{-/-} mice showed no appreciable difference in rod and cone function by 20 weeks of age when compared to the wild-type *C57BL/6J* mice (Figure 1A,B). The expression of cone-specific proteins, such as cone arrestin, was unchanged in the *Casp7*^{-/-} mice, and the retinal structure appeared indistinguishable from that in the

wild-type mice (Figure 1C) indicating that the loss of *Casp7* has no overt effect on retinal development and function.

***Casp7* loss does not alter rod survival in the *rd1* and *Rho-KO* mouse models of RP:** The endoplasmic reticulum stress induced *Casp7* has been implicated in several autosomal dominant mouse models of RP [15,16], and the loss of this protein has been shown to delay rod death in the T17M rhodopsin mouse model of autosomal dominant RP [12]. Endoplasmic reticulum stress has also been proposed to contribute to retinal degeneration in the *rd1* mouse model of retinal degeneration [18]. Because rod survival positively affects cone survival [1], we wanted to ensure that any effect of *Casp7* loss on cones was not due to increased rod survival due to *Casp7* loss in rods. Thus, we analyzed rod survival in *rd1* and *Rho-KO* mice lacking *Casp7* at 3 and 17 weeks of age, respectively. These time points were chosen as they coincide with the onset of cone death in these two mouse models of RP [1]. Therefore, any substantial increase in the number of surviving rods at the onset of cone death would inevitably delay cone death. Because at 3 and 17 weeks, respectively, the outer nuclear layer thickness in both models is reduced to one row of cells in the central retina, any difference in thickness would be clearly evident at those ages. No difference was seen in outer nuclear layer thickness on the retinal cross sections between the *rd1* mice and the *Casp7*^{-/-}*rd1* mice at 3 weeks of age indicating that rod survival was not altered in the absence of *Casp7* (Figure 2A). Retinal flat mounts revealed a uniform distribution of cones as assessed with cone arrestin antibody

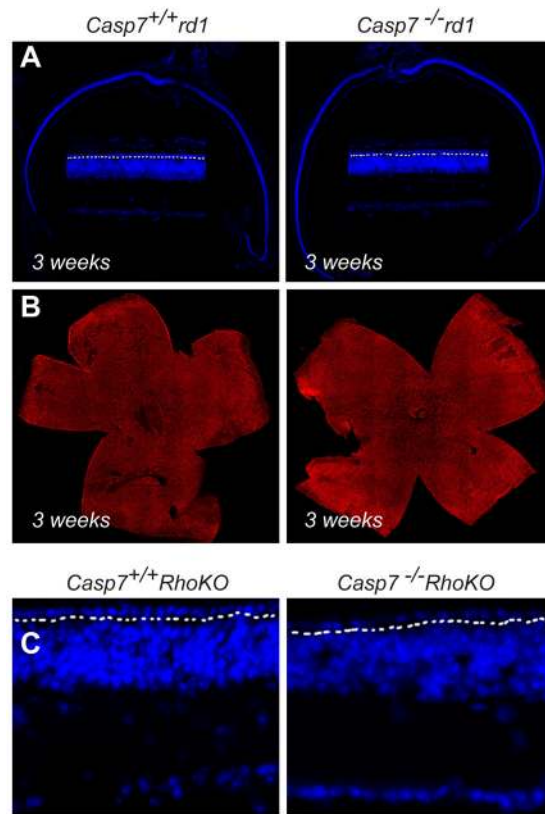


Figure 2. Loss of *Casp7* does not affect rod degeneration. **A**: Retinal cross section of 3-week-old *Casp7*^{+/+}*rd1* and *Casp7*^{-/-}*rd1* mice stained with 4',6-diamidino-2-phenylindole (DAPI) showing no difference in the thickness of the outer nuclear layer in the central retina (n = 3 animals; the zoomed-in view shows one row of cells above the dotted line which separated the outer nuclear layer from the inner nuclear layer). **B**: Retinal flat mounts stained with cone arrestin (red signal) showing no difference in the distribution of cones at the onset of cone death (genotype and age same as in panel A). **C**: Retinal cross section of 17-week-old *Casp7*^{+/+}*Rho-KO* and *Casp7*^{-/-}*Rho-KO* mice showing no difference in the thickness of the outer nuclear layer in the central retina (n = 3 animals; the dotted line separates the outer nuclear layer from the inner nuclear layer).

staining consistent with 3 weeks being the onset of cone death (Figure 2B). Similarly, in the *Casp7*^{-/-}*Rho-KO* mice the outer nuclear layer was also reduced to one row of cells at 17 weeks of age indicating that also in the *Rho-KO* background the loss of CASP7 had no effect (Figure 2C). This is significantly different from what was seen in the T17M *Rhodopsin* mouse model where the thickness of the outer nuclear layer was three times wider [12]. In summary, loss of *Casp7* did not affect rod survival in the *rd1* and *Rho-KO* mouse models of RP.

Secondary cone death is not affected by loss of Casp7 in the rd1 and Rho-KO mouse models of RP: To determine whether *Casp7* is activated in cones during the period of secondary cone death, we first used western blot analyses to test for cleaved *Casp7*. Executioner caspases are commonly activated by cleavage [33]; thus, the presence of a cleaved product is an indicator of an activated caspase. We did not detect any cleaved *Casp7* in the *rd1* mice (Figure 3A) although we readily identified the cleaved product in the HEK293 cells treated with rapamycin. However, *Casp7* is the exception among the executioner caspases in that dimerization of *Casp7* is sufficient for activation albeit at lower levels [34,35]. Consistent with that, overexpression of *Casp7* in the HEK293 cells was sufficient to cause enough dimerization of *Casp7*

resulting in autocatalytic cleavage of the caspase (Figure 3A). Interestingly, the levels of CASP7 declined with the demise of cones over time consistent with it being enriched in cones. Alternatively, the decline could also be due to a decline in the number of cones in conjunction with an age-related decline in the inner nuclear layer and possibly in cones. To further test whether *Casp7* was activated without cleavage, we used a fluorescein isothiocyanate (FITC)-labeled peptide with the target sequence of *Casp7* (DEVD). The peptide becomes covalently bound to *Casp7* only when the active site of the protease is formed. The fluorescent label thus allows determining if there is any in situ activity of *Casp7* in cones. Although labeling of cones was seen in the 6-week-old *rd1* mice, labeling was not abolished in the *Casp7*^{-/-}*rd1* mice (Figure 3B). Interestingly, most of the labeled cell had a more condensed nucleus with almost no expression of the cone-specific protein cone arrestin consistent with the labeled cells undergoing apoptosis (Figure 3C). However, the same peptide can also bind to activated *Casp3*; thus, cross-reactivity cannot be excluded [36], although we were not able to detect any cleaved *Casp3* in cones (data not shown) with an antibody that readily detected cleaved *Casp3* in rods during the period of rod degeneration [1]. Finally, quantification of cone survival at 6 weeks and 20 weeks of age did not show any statistically

significant difference between the *rd1* mice and the *rd1* mice with *Casp7* loss (Figure 3D,E). Similarly, loss of *Casp7* in the slower progressing *Rho-KO* mouse model of RP also did not result in any statistically significant difference in the distribution of cones at the onset of cone death, meaning at 17 weeks of age and at 30 weeks of age (Figure 4A,B). The 30 week time point, which is roughly equivalent to 10 weeks of age in the *rd1* mouse model [1], was chosen as the second time point because by then cone death has progressed significantly in the *Rho-KO*. Cone function was also not changed although there was a slight reduction in the *Casp7*^{-/-}*_Rho-KO* mice (Figure 4C). Overall, the data suggest that *Casp7* likely does not contribute to secondary cone death in RP.

DISCUSSION

We set out to understand whether *Casp7* is involved in facilitating secondary cone death during the periods of cone degeneration in RP. Because *Casp7* is cone enriched and has been shown to play a role in PRs that experience ER stress, and ER stress has been proposed to be a contributing factor to PR death in RP, *Casp7* was chosen as a rational candidate to identify additional cell death mechanisms that contribute to secondary cone death in RP [12,13,16-18]. Although we were able to detect activated *Casp7* with the FITC-labeled activity-peptide assay, we did not identify any cleaved CASP7 protein product nor was the peptide-activity assay negative in the *Casp7*^{-/-}*_rd1* background. Because the same peptide sequence used here also recognizes activated CASP3, cross-reactivity to CASP3 cannot be excluded [36]. However, we failed to detect activated CASP3 in cones with immunofluorescence although in our previously published work

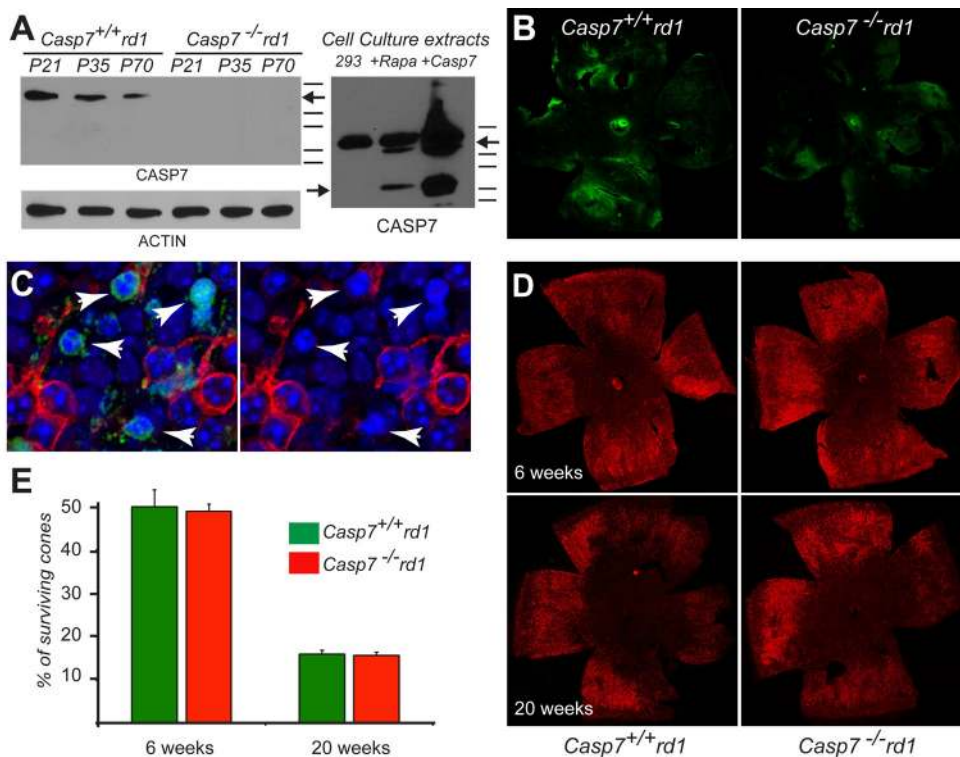
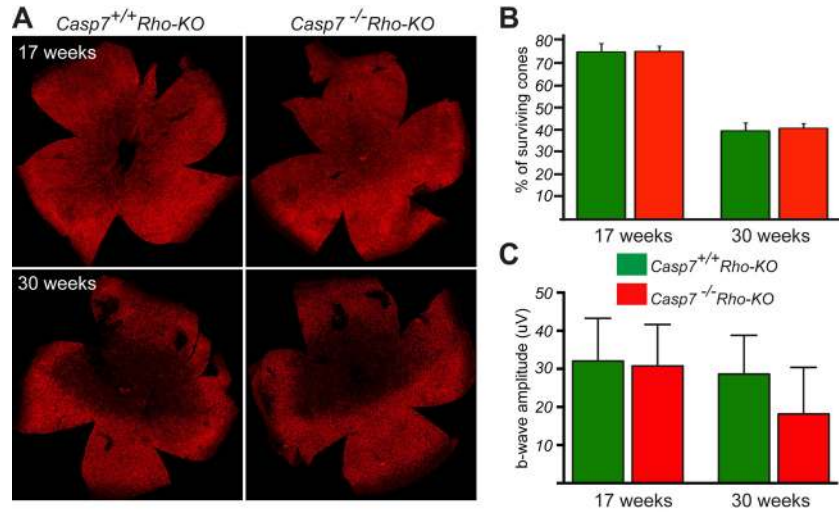


Figure 3. Loss of *Casp7* does not affect cone death in the *rd1* mouse model of RP. **A:** Western blot analysis at postnatal (P) days 21, 35, and 70 with retinal extracts from *Casp7*^{+/+}*_rd1* and *Casp7*^{-/-}*_rd1* mice showing on the left no cleaved CASP7 protein in *Casp7*^{+/+}*_rd1* mice, a decline in the CASP7 protein over time, consistent with its enrichment in cones, and a lack of detectable CASP7 protein in *Casp7*^{-/-}*_rd1* mice. To the right, the western blot analysis with protein extracts from HEK293 cells show cleavage of the CASP7 protein after rapamycin treatment or overexpression of *Casp7*, indicating that the antibody is able to recognize the cleaved product and that dimerization by overexpression is sufficient to activate CASP7 (the lines on the right of both western blots indicate molecular weight markers in kDa;

starting from the top line, their respective sizes are 37, 25, 20, 15, 10; arrows on right side of the western blot point to pro-CASP7 while the arrow on the left of the western blot points to the cleaved CASP7 product). **B:** Immunofluorescence on retinal flat mounts to detect active CASP7 (the green signal indicates the fluorescein isothiocyanate (FITC)-labeled CASP7 activity peptide) in the *Casp7*^{+/+}*_rd1* and *Casp7*^{-/-}*_rd1* mice. Because the FITC signal is still present in *Casp7*^{-/-}*_rd1* mice, the peptide is likely cross-reacting. **C:** Higher magnification of the assay in (B) showing that cone cells strongly labeled by the FITC CASP7 activity peptide (arrowheads) tend to have pyknotic nuclei and less cone arrestin immunoreactivity (red signal), suggesting that the peptide was labeling dying cones (blue: nuclear 4',6-diamidino-2-phenylindole [DAPI]). **D:** Examples of retinal flat mounts of *Casp7*^{+/+}*_rd1* and *Casp7*^{-/-}*_rd1* mice stained for cone arrestin (red signal) expression at 6 and 20 weeks of age. **E:** Quantification of cone survival at 6 and 20 weeks of age showed no statistically significant improvement in cone survival upon loss of *Caspase-7* in *rd1* mice. (n=6 per genotype and time point; Error bars: SEM).



photopic electroretinogram recordings showed also no statistically significant improvement in cone function upon loss of *Casp7*. (n=6 per genotype and time point in B and C; Error bars: SEM).

the antibody was able to detect activated CASP3 during the periods of rod degeneration [1]. This does not exclude that the FITC-labeled peptide-activity assay may have reacted with low levels of activated CASP3. Alternatively, the chemically activated FITC-labeled peptide could have reacted with other proteases or protein degradation products produced during the cell death process, because labeling with the FITC-labeled peptide was preferentially seen in cells undergoing apoptosis. We favor this hypothesis over cross-reactivity with CASP3, because although CASP3 and CASP7 were for a long time believed to be functionally redundant, more and more research indicates that there are clear functional differences in vivo and regarding substrate recognition, indicating that these two proteases occupy distinct non-redundant roles within the cell death machinery [36-40].

We previously identified *Casp2* as the first caspase that plays a pivotal role during the periods of secondary cone death in RP [7]. Thus, if *Casp7* plays any role in secondary cone death, one could envision that in the absence of *Casp7* cones increase CASP2 activity. Such a scenario could offer an explanation for the cross-reactivity of the FITC-labeled peptide and the fact that loss of *Casp7* did not result in improved cone survival. To determine whether in the absence of *Casp7* *Casp2* plays a more prominent role in secondary cone cell death, we also generated the double loss of function in an *rdl* background and analyzed cone survival at 20 weeks of age, when we saw a clear improvement in our previously published data in the absence of *Casp2* [7]. However, although at 20 weeks of age concomitant loss of *Casp2* and *Casp7* in an *rdl* background resulted in improved cone survival when

Figure 4. Loss of *Casp7* does not affect secondary cone death in *Rho-KO* mice. A: Examples of retinal flat mounts of *Casp7*^{+/+}*Rho-KO* and *Casp7*^{-/-}*Rho-KO* mice stained for cone arrestin (red signal) expression at the onset of cone death (17 weeks) and a later time point (30 weeks) that corresponds to roughly 10 weeks in *rdl* mice. B: Quantification of cone survival at 17 and 30 weeks of age of *Casp7*^{+/+}*Rho-KO* and *Casp7*^{-/-}*Rho-KO* mice. No statistically significant improvement in cone survival is seen upon loss of *Casp7*. C: B-wave amplitudes of

compared to the loss of *Casp7* alone (30.9%±2.90% versus 15.7%±1.00%, respectively), the double loss of function did not result in a statistically significant improvement in cone survival when compared to our previously published data [7] with the loss of *Casp2* alone (31.5%±1.00%), suggesting that *Casp2* does not compensate for the lack of *Casp7*.

The *rdl* mouse model of RP is one of the fastest degenerating mouse models used to study the disease. Thus, it is quite possible that some mechanisms of cone cell death may be quite specific to this model and not apply to slower models of cone degeneration. To address this issue, we also used the much slower degenerating mouse model of RP, the *Rho-KO* mouse. Nonetheless, there was no difference in cone degeneration in the *Rho-KO* mouse model in the absence or presence of *Casp7*. In summary, *Casp7* is either not involved in secondary cone death in RP or the effect of *Casp7* is negligible. This is surprising given that *Casp7* is expressed in cones and is absent from rods, albeit rods are perfectly capable of upregulating *Casp7* under stress conditions that cause ER stress [12,14]. This raises the question, why would cones express *Casp7* rather than upregulating it when needed? One possible explanation could be that CASP7 can cleave CASP12. In its uncleaved form, CASP12 can bind CASP1 and thus prevent CASP1-mediated activation of proinflammatory cytokines, such as interleukin (IL)-1β and IL-18 [41]. Thus, the presence of CASP7 in cones could accelerate the release of proinflammatory cytokines from cones after ischemia, light damage, or infection. However, the present findings allow us only to conclude that CASP7 is not required for secondary cone death in RP suggesting

that under these conditions cone death is unlikely due to an increase in ER stress. Additionally, the findings suggest that the mechanism(s) used to execute secondary cone death in RP may be the same across all disease-causing mutations that are in genes exclusively expressed in rods, meaning the mechanisms are independent of the rod death kinetics.

ACKNOWLEDGMENTS

We would like to thank Janis Lem for the *Rho-KO* mice. We acknowledge Eric Baehrecke, Francis Chan, Daryl Bosco, Hemant Khanna, Lolita Petit and Marina Zieger for insightful discussions and critical reading of the manuscript. This work was supported in part by grants from the NIH R01EY023570.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 15 December 2017. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.