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Activated mTORC1 promotes long-term cone survival in retinitis pigmentosa mice

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Retinitis pigmentosa (RP) is an inherited photoreceptor degenerative disorder that results in blindness. The disease is often caused by mutations in genes that are specific to rod photoreceptors; however, blindness results from the secondary loss of cones by a still unknown mechanism. Here, we demonstrated that the mammalian target of rapamycin complex 1 (mTORC1) is required to slow the progression of cone death during disease and that constitutive activation of mTORC1 in cones is sufficient to maintain cone function and promote long-term cone survival. Activation of mTORC1 in cones enhanced glucose uptake, transportation, and utilization, leading to increased levels of the key metabolite NADPH. Moreover, cone death was delayed in the absence of the NADPH-sensitive cell death protease caspase 2, supporting the contribution of reduced NADPH in promoting cone death. Constitutive activation of mTORC1 preserved cones in 2 mouse models of RP, suggesting that the secondary loss of cones is caused mainly by metabolic deficits and is independent of a specific rod-associated mutation. Together, the results of this study address a longstanding question in the field and suggest that activating mTORC1 in cones has therapeutic potential to prolong vision in RP.

Introduction

The interneuronal relationship between rod and cone photoreceptors in humans and mice is such that rod death always leads to cone death, while conversely, loss of cones has only a minimal effect on rods (1–4). This phenomenon plays a central role in retinitis pigmentosa (RP), since mutations in a few rod photoreceptor–specific genes affect a disproportionately large number of patients (5) (RetNet: https://sph.uth.edu/retnet/). Because cones are essential for daylight, color, and high-acuity vision, it is their loss that leads to blindness. The fact that cone death always follows rod death independently of the underlying mutation in a rod-specific gene suggests that the reason(s) for cone death might be similar across various forms of RP. Thus, targeting the common mechanism(s) of cone death may allow for the development of vision therapies with broad clinical significance.

Many theories have been proposed to explain the dependence of cones on rods. Such theories include the production of toxic byproducts from dying rods, the loss of rod trophic support as rods die (6–11), overactive microglia recruited to remove the dying rods, and increased oxidative stress (12–16). We recently proposed a different theory suggesting that cones suffer from a nutrient shortage induced by disruption of the retinal architecture once most rods have been lost (17). Because rods outnumber cones at a greater than 20:1 ratio and photoreceptors account for approximately 75% of all retinal cells, loss of rods severely alters the retinal architecture (18, 19). Thus, we hypothesized that once rod death progresses beyond a critical threshold, cone death initiates as a cell-autonomous event due to a reduction in nutrient flow from the adjacent cell layer, the retinal pigmented epithelium (RPE) (17). This hypothesis was based on the following findings: First, cone death always initiates only after approximately 90% of rods have died, irrespective of the rod death kinetics. Second, during the period of cone degeneration, cones display signs of prolonged starvation. Finally, during cone death, we observed gene expression changes in many metabolic genes and genes of the insulin/mTOR pathway, a key pathway controlling cell metabolism (17). To test whether activation of the insulin/mTOR pathway alters cone survival, we treated the fast-progressing rd1 mouse model (20) of RP with daily systemic injections of insulin (17). While cone survival did improve, the therapeutic effect of insulin lasted only for a period of 4 weeks, possibly due to the negative-feedback loop within the insulin/mTOR pathway (Figure 1 and refs. 17, 21). Our findings, though encouraging, left many questions unanswered regarding the role of mTOR and its potential as a therapeutic target to promote cone survival in RP. As such, it remained unclear whether insulin acts directly on cones to improve cone survival, or whether it stimulates other cells such as RPE cells or retinal Müller glia cells to release neuroprotective factors, as its administration was systemic. It was also unclear whether the protective effect of insulin requires mTOR activity and whether, by circumventing the feedback mechanism within the insulin/mTOR pathway, cone survival can be prolonged to the point of being therapeutically relevant to humans. Last, it remained to be tested whether the effect of insulin can be extended to other mouse models of RP, giving it a broader clinical significance.

To evaluate the long-term therapeutic potential of the insulin/mTOR pathway on cone survival and to test whether insulin
acts directly on cones through this pathway, we have now constitutively activated this pathway in cones. Through the use of various conditional alleles of genes downstream of the insulin receptor (Figure 1) that were deleted using the same cone-specific Cre recombinase line (ref. 22 and Supplemental Figure 1, A–E; supplemental material available online with this article; doi:10.1172/JCI79766DS1), we show that mTORC1 activity in cones is required to slow the progression of the disease and that constitutively active mTORC1 in cones is sufficient to significantly prolong cone survival and maintain cone function. This mechanism of protection functions in the fast-progressing rd1 mouse model of RP and in the slow-progressing rhodopsin-KO (Rho-KO) (23) model. Activated mTORC1 increases the expression of genes that improve glucose uptake, retention, and utilization, resulting in increased NADPH levels. Because low NADPH levels can trigger cell death by activation of the initiator caspase, caspase 2 (CASP2) (24, 25), we crossed caspase 2–/– mice (Casp2–/–) (26) with the fast-progressing rd1 model of the disease, which slowed the progression of cone death. In summary, our results show that cell-autonomous activation of mTORC1 in cones is sufficient to promote long-term cone survival and that high glucose and NADPH levels are crucial for cone survival, indicating that the secondary loss of cones in RP is mainly due to a nutrient imbalance. Therapies aimed at increasing mTORC1 activity in cones or increasing the expression of key metabolic target genes of mTORC1 are thus plausible strategies to prolong vision in humans suffering from RP.

Results
mTORC1 is required and sufficient to promote cone survival in RP.

To evaluate whether insulin acts directly on cones through the insulin/mTOR pathway and to test whether continued stimulation of the pathway significantly prolongs cone survival, we constitutively activated the pathway in cones of rd1 mice by conditional deletion of the tumor-suppressor gene phosphatase and tension homolog (Pten) using the Cre-lox system (21, 27) (rd1 M-opsin-Cre Ptenfl/fl mice, herein referred to as rd1 Ptenfl/fl mice. cKO denotes cone KO; in all instances, Cre denotes cKO of the gene indicated; the same cone-specific Cre driver line [M-opsin-Cre mice] [ref. 22] that was tested for efficient recombination [Supplemental Figure 1, A–E] was used in all experiments that used conditional alleles]. Pten is a phosphatase that counteracts the action of growth factors such as insulin by decreasing intracellular levels of the second messenger phosphatidylinositol-triphosphate (PIP3) (Figure 1). Therefore, activation of the pathway by loss of Pten is not sensitive to the negative-feedback loop of the insulin/mTOR pathway, as PIP3 levels remain high even after growth factor receptors are turned off by the feedback loop (28). As a consequence of sustained pathway activity, we found that cone survival was significantly improved in retinae of rd1 Ptenfl/fl mice up to 8 months of age when compared with cone survival in Cre littermate control retinae (Figure 2, A and B, and Supplemental Figure 2A) or with that observed in retinae following insulin injections in our previous study (17). The data suggest that sustained cell-autonomous activation of the pathway in cones can serve as a long-term therapeutic strategy to prolong cone survival in RP. Loss of Pten activates various downstream signaling pathways and kinases. Two key kinases downstream of PTEN that are central to the insulin/mTOR pathway are protein kinase B (PKB), also known as AKT, and mTOR (21, 28–30). AKT has 3 isoforms that are often cell-type specific and a multitude of targets. In contrast, mTOR is found in 2 large protein complexes referred to as mTORC1 and mTORC2, each with a small, distinct set of known targets and characterized by their unique accessory proteins RAPTOR and RICTOR, respectively (21). Both AKT and mTORC2 respond mainly to growth factor changes and control many prosurvival mechanisms, while mTORC1 is growth factor and nutrient sensitive and regulates mainly cellular growth processes (21) (Figure 1). To identify which kinase(s) downstream of PTEN promote(s) cone survival, we first assessed the phosphorylation status of AKT and other bona fide targets of both mTORC1 complexes. Western blot analyses from whole retinal extracts at the onset of cone death (17) (P21) showed no appreciable phosphorylation changes, a result that could be explained by the low proportion of cones in the retina (3%) (18, 19, 31) and/or the high expression levels of these phosphorylated proteins in other retinal cell types (Supplemental Figure 2, B and C). However, immunofluorescence on retinal flat mounts at P21 revealed an increase in the number of cones positive for 3-phosphosinositide–dependent protein kinase (PKD1) phosphorylation of AKT at Thr308 and a decrease in the number of cones positive for mTORC2-mediated phosphorylation of AKT at Ser473 (Figure 2C and ref. 21). Consistent with this finding, the number of cones positive for phosphorylated serum/glucocorticoid-regulated kinase 1 (p-SGK1), another mTORC2 target, was also reduced, while the number of cones phosphorylated on ribosomal protein S6 (p-S6), an indirect mTORC1 target (21), was increased (Figure 2C). Since phosphorylation of AKT on both sites is required for most AKT-mediated prosurvival functions, but phosphorylation on Thr308 is sufficient to activate mTORC1, the data suggest that cone survival upon loss of PTEN is mediated mainly by increased mTORC1 activity (21, 29, 32). The reduction in mTORC2 activity upon loss of PTEN is likely due to the inhibitory effect of mTORC1 on mTORC2 (33, 34).

To test whether increased mTORC1 activity is responsible for the survival effect mediated by loss of PTEN, we generated rd1 mice with simultaneous deletion of Pten and Raptor in cones (rd1 M-opsin-Cre Ptenfl/fl Raptorfl/fl allele; ref. 35 and Figure 1) and quantified cone survival in these mice at 2 months of age, a time point at which the difference in cone survival was quite large following loss of PTEN. Interestingly, concurrent loss of Pten and RAPTOR not only abolished the survival effect seen upon loss of PTEN, but also accelerated cone death (Figure 3, A and B). A similar acceleration of cone death was seen upon loss of RAPTOR alone (Figure 3, A and B), while loss of RAPTOR in cones of WT mice did not affect cone survival at 2 months of age (data not shown). In contrast, loss of RICTOR (Rictorfl/fl allele; ref. 36) in cones had no effect on cone survival in rd1 (Figure 3, A and B) or WT mice (data not shown) at 2 months of age. Loss of RAPTOR and RICTOR was verified by phosphorylation changes in their downstream targets (21) (S6, AKT, and SGK1 (Figure 3, C and D)). The results show that mTORC1 activity is not only required for the improved cone survival seen upon loss of PTEN, but is also critical for cone survival under the stress conditions encountered during disease. Furthermore, the data suggest that
mTORC1 in rd1 Tsc1<sup>loxo</sup> retinae (Figure 4D). Loss of the protective effect by simultaneous ablation of TSC1 and RAPTOR, but not TSC1 and RICTOR, confirmed that mTORC1 activity was both required and sufficient to promote cone survival (Figure 5, A–C). Importantly, while mutations in TSC cause benign tumors in tissues such as the kidney and brain (37), loss of TSC1 in cones did not induce cone proliferation as assessed by the proliferation marker Ki67, indicating that the protection was due to improved cone survival and not cone numbers (Figure 5, D and E).

**mTORC1 prolongs cone survival by improving cell metabolism.** Photoreceptors are among the most metabolically active cells in the human body (41), since they need to replenish membranes and proteins lost due to the daily shedding of their OSs (42). Consequently, photoreceptors require large quantities of glucose to synthesize sufficient amounts of NADPH to keep up with daily membrane synthesis. mTORC1 is at the center of cell growth and proliferation, regulating genes involved in glycolysis, the pentose phosphate pathway (PPP), and de novo lipid synthesis (43). We therefore investigated whether sustained mTORC1 activity increases the expression of key metabolic target genes that increase NADPH production in cones, thereby improving survival and function. As such, we analyzed the expression of glucose transporter 1 (GLUT1), which increases glucose uptake, hexokinase 2 (HK2), which phosphorylates glucose more effectively, and glucose-6-phosphate dehydrogenase (G6PD), which shunts glucose into the pentose phosphate pathway (PPP) for NADPH synthesis (44). Immunofluorescence analysis revealed an increase in the expression of all 3 aforementioned genes in cones of 2-month-old rd1 Tsc1<sup>loxo</sup> mice (Figure 6A and Supplemental Figure 3A). We did not observe this increase in immunofluorescence upon the concurrent loss of TSC1 and RAPTOR (Figure 6A), indicating that the increase was mTORC1 dependent. Other glycolytic genes that contribute to an increase in NADPH production (45), such as pyruvate kinase muscle isoform 2 (PKM2) and malic enzyme 1 (ME1), were also revealed by immunofluorescence to be upregulated in cones of rd1 Tsc1<sup>loxo</sup> mice at 2 months of age, as was an increase in the transcription factor hypoxia-inducible factor 1α (HIF-1α), which regulates
the transcription of many glycolytic genes (ref. 46, Supplemental Figure 3A, and Supplemental Figure 4). The data suggest that glucose uptake, retention, and divergence into the PPP are improved. In agreement with these findings, we detected significantly higher levels of NADPH in whole retinal extracts from retinal extracts from WT mice at 2 months of age showing more central cones in Cre+ animals (red signal indicates cone arrestin). Scale bar: 1 mm (left); 200 mm (right). (B) Quantification of cone survival at the indicated time points. Numbers in bars represent the number of retinae analyzed. **P < 0.01 and ***P < 0.005 by Student’s t test. (C) Immunofluorescence analyses at P21 of retinal flat mounts to detect phosphorylation of sites on the indicated proteins (red signal). Upper right of each panel shows magnification of the area indicated by an arrow. Cone layer is identified by PNA staining (green signal indicates PNA; blue signal indicates nuclear DAPI). Scale bars: 20 mm (original magnification for insets, x2.5).

To further test whether NADPH levels are crucial for cone survival during disease, we examined the role of the initiator caspase, CASP2, a protease that initiates apoptotic cell death and has been shown to be activated under low intracellular NADPH levels but not low ATP levels (24, 25). Caspases are commonly activated by cleavage, thus the identification of a cleavage product is generally used to assess caspase activity (49). Western blot analysis using retinal extracts from rd1 mice revealed no cleaved CASP2 products, while cleaved CASP2 was readily detectable after transfection of HEK239 cells with full-length Casp2 (Figure 7A), suggesting that CASP2 is not cleaved during cone degeneration. However, because dimerization of initiator caspases is sufficient to activate the protease, albeit to a lesser extent, the absence of a cleavage product does not necessarily mean that a caspase is not active (49). To further test whether CASP2 was activated in cones, we performed immunofluorescence analyses using 6 different CASP2 antibodies, none of which revealed any specific pattern of loss of CASP2 expression in the retinae of Casp2−/− mice, making it difficult to determine whether CASP2 was actually expressed and activated in cones (data not shown).
mice did not affect photoreceptor survival (Figure 7E). In summary, the data suggest that removal of an NADPH-sensitive cell death mechanism can delay cone death in RP, further supporting the notion that cone death in RP is intimately linked to glucose and NADPH levels and indicating that this cone death is likely a result of nutrient deprivation.

Constitutively activated mTORC1 delays cone death in rhodopsin-KO mice. To test whether improved cone survival mediated by increased mTORC1 activity is applicable to other models of RP, we used the rhodopsin-KO (Rho−/−) (23) mouse, which displays slower degeneration kinetics. Cone death initiates at around 17 weeks of age, and by 30 weeks of age, Rho−/− retinae show a degree of cone degeneration equivalent to that of 2-month-old rd1 mouse retinae (17). Similar to the observations made in the rd1 mouse model, loss of TSC1 in the cones of Rho−/− mice was able to significantly prolong cone survival at 30 weeks of age (Figure 8, A and B). Moreover, increased immunoreactivity against metabolic genes such as HK2 and PKM2 in cones, as well as an increase in the number of cones positive for p-S6 (Figure 8C), indicates that the mechanism of protection is similar to that observed in rd1 cones. These findings suggest that this approach is independent of the mutation in a rod-specific gene, allowing for therapeutic intervention at the mTORC1 level to prolong vision in RP.

Figure 3. Raptor but not Rictor is required for loss of Pten-mediated survival. Data shown are from rd1-mutant mice harboring the indicated conditional alleles. (A and B) Representative retinal flat mounts (A) and quantification of cone survival (B) at 2 months of age (red signal indicates cone arrestin). Numbers in bars represent the number of retinae analyzed. **P < 0.005 by Student’s t test. (C) Immunofluorescence analyses (red signal) of retinal flat mounts at P21 to detect phospho-specific sites on the indicated proteins (green signal indicates PNA; blue signal indicates nuclear DAPI).
Discussion

In this study, we show that cell-autonomous activation of metabolic genes downstream of mTORC1 is sufficient to prolong cone survival in RP, suggesting that improving nutrient uptake, retention, and utilization in cones delays cone death during disease. Our data showing an almost WT distribution of cones upon loss of TSC1 in mice at 2 months of age hold great promise, as the rd1 mouse model is one of the fastest-progressing models of RP. Because cone death is also significantly delayed in the slow-progressing Rho–/– model of RP, the approach is predicted to be mutation independent and thus to improve cone survival across a wide spectrum of RP diseases in which the primary mutation is in a rod-specific gene.

Loss of the TSC has been shown in cell culture systems to render cells sensitive to complete glucose withdrawal, since mTORC1 fails to balance demand with supply (50). However, low nutrient conditions such as reduced glucose levels confer a survival advantage to cells that lose the TSC (51). This is because sustained mTORC1 activity under nutrient-restrictive conditions maintains protein synthesis and therefore allows for increased expression of transporters and other metabolic genes that help counterbalance the nutrient shortage. Similar to the findings in cell culture, loss of TSC in mouse cones confers a survival advantage during disease and increases the expression of transporters and metabolic genes. Therefore, increased mTORC1 activity helps starved cones to balance nutrient demand with supply. Consistent with this idea, loss of mTORC1 activity accelerates cone death in RP as cones fail to adapt to the nutrient shortage, while its activity is not required to keep cones alive in WT mice up to 2 months of age, since cones in these mice do not experience a nutrient shortage. Thus, the basal expression of many of these metabolic genes does not seem to be regulated by mTORC1 in cones, since its loss does not affect cones of WT mice up to 2 months of age. Our data suggest that the induction of mTORC1 target genes upon loss of TSC1 functions as an adaptive response in cones to counter nutrient stress. This idea is further supported by the finding that the expression of these metabolic genes increases gradually from P21 onward and is seen only in rd1 and not WT retinae upon loss of TSC1. While this may appear at odds with in vitro data, in which loss of the TSC automatically increases the expression of mTORC1 targets (43), it is important to note that our experiments were carried out in vivo in postmitotic neurons within the context of a tissue. Thus cell
OS length, which can improve photoreceptor function. Photoreceptor shedding rates exceed the synthesis rate, thereby maintaining lipid and thus OS synthesis, increased levels can help prevent the two different mechanisms. First, because NADPH is required for activity and decreased p-AKT (Ser473) in cones. Similarly, loss of PTEN, which generally increases the activity of both mTOR complexes and AKT (21), results in decreased mTORC2 activity and decreased p-AKT (Ser473) in cones.

Cellular redox potential is tightly linked to nutrient availability. For example, NADPH is used to reduce oxidized glutathione, which serves to scavenge many free radicals. Increased NADPH levels upon loss of TSC1 likely contribute to cone survival through 2 different mechanisms. First, because NADPH is required for lipid and thus OS synthesis, increased levels can help prevent the OS shedding rates exceed the synthesis rate, thereby maintaining OS length, which can improve photoreceptor function. Photoreceptor function is also directly linked to NADPH, since NADPH is required to recycle the visual chromophore (47, 48). An overall healthier and better-functioning photoreceptor is likely to survive longer. Second, because oxidative stress is known to occur during the periods of cone degeneration, increased NADPH levels can directly reduce oxidative stress by increasing glutathione availability (52). Therefore, starvation of cones in RP is consistent with a model of increased oxidative stress as a contributing factor to cone death (52). However, oxidative stress itself is unlikely to be the sole cause for cone death in RP, since antioxidant treatments have had limited success in prolonging cone survival (13-16).

An understanding of the cell death mechanisms for cone death in RP remains largely elusive. Identifying a cell death mechanism may shed light on the overall cause of cell death, as some mechanisms require specific stress conditions or triggers. In this regard, the identification of Casp2 as the first apoptotic mechanism for cone death in RP further corroborates the notion that low NADPH levels are a contributing factor to cone death and supports the overall idea of a nutrient shortage in cones. However, a delay in cone death is only seen at later stages. This could be due in part to the involvement of necrosis during the initial phase of cone death. A recent report showed that early cone death in the rd10 mouse model of RP includes necrotic cell death (53). The rd10 model harbors a different mutation in the same gene as that in the rd1 model; however, rod death progresses slightly slower than in the rd1 model, yet is still faster than in the Rho−/− model. The electron microscopic analyses performed in that study revealed several cone nuclei with necrotic features at early stages of cone death. Additionally, the study showed that inhibition of necrosis reduces the number of necrotic nuclei and delays early cone death. Nonetheless, a greater number of cone nuclei showed signs of apoptotic cell death, even at early stages. Early necrotic cone death could be due to the rapidly occurring structural insult on the retina because of the rather rapid loss of rods. Once the necrotic phase ceases, the remaining cones may die by apoptotic mechanisms, including the activation of CASP2 induced by low NADPH levels. Early necrosis could explain why cone death progresses rapidly within the first days (P21-P28; Supplemental Figure 5), before activation of mTORC1 turns on the metabolic transcriptional network that delays cone death upon loss of TSC1. In combination with early necrosis, there are likely additional, as yet unidentified cell death mechanisms that contribute to cone death. These could explain why loss of CASP2 does not result in a model of increased oxidative stress as a contributing factor to cone death (52). However, oxidative stress itself is unlikely to be the sole cause for cone death in RP, since antioxidant treatments have had limited success in prolonging cone survival (13-16).
targets in cones may provide a feasible approach for future treatments in humans. This approach rests on the identification of the correct combination of target genes required to prolong cone survival. Alternatively, drugs that increase mTORC1 activity could be delivered to the retina through slow-releasing eye implants.

It is of interest to note that the most common form of photoreceptor degeneration and thus the major cause for blindness in the industrialized world, age-related macular degeneration (AMD), may result in photoreceptor death due to the same proposed mechanism by which cones die in RP. In AMD, RPE cell function and health are affected, as the accumulation of drusen between the RPE and choriocapillary impinges on nutrient transfer from the bloodstream to RPE cells (54). Because RPE cells are the main source of nutrients for photoreceptors, a reduction in nutrient availability to RPE cells inevitably affects photoreceptors, too. Thus AMD and RP, although having different etiologies, may both result in blindness caused by starving cones. Therefore, improving photoreceptor metabolism by increasing mTORC1 activity may be applicable not only to RP, but also holds great promise for treating the most common form of retinal degeneration in the industrialized world.

**Methods**

**Animals.** Mice were maintained on a 12-hour light/12-hour 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<sup>rd1/rd1</sup> (rd1), Casp2<sup>−/−</sup>, C57BL/6J, Pten<sup>fl/fl</sup>, Tsc1<sup>fl/fl</sup>, and Ai9 Cre reporter mice were purchased from The Jackson Laboratory (20, 26, 27, 38, 55). The M-opsin-Cre (cone-specific Cre line using the human medium wavelength promoter), Raptor<sup>fl/fl</sup>, Rictor<sup>fl/fl</sup>, and Rho<sup>−/−</sup> mice have been described previously by Yun Z. Le, Michael N. Hall, Markus A. Rüegg, and Janis Lem, respectively (22, 23, 35). Genotyping was performed as described in the original publications. None of the mice used for analysis were on an albino background, and all mice were genotyped for absence of the rd8 mutation (56). To dissect the role of the insulin/mTOR pathway in cone survival, the conditional allele was first crossed with the cone-specific Cre-driver line and then with the rd1 line to generate Cre<sup>−</sup> and Cre<sup>+</sup> rd1–conditional allele (cKO) lines. In some cases, double-conditional alleles were also generated. The Cre<sup>−</sup> and Cre<sup>+</sup> lines were crossed with each other (e.g., rd1 Pten<sup>fl/fl</sup> Pten<sup>fl/fl</sup> M-opsin-Cre<sup>−/−</sup> Pten<sup>fl/fl</sup>) to generate the Cre<sup>+</sup> and Cre<sup>−</sup> littermates that were used for analyses. Because various strains were interbred, strain background differences may account for the slight variations in cone survival in Cre<sup>−</sup> animals at 2 months of age. Nonetheless, our data clearly show that mTORC1 was required for cone survival during disease, since loss of RAPTOR always accelerated cone death, while increasing mTORC1 activity consistently improved cone survival. Because loss of Casp2 did not allow us to compare Cre<sup>−</sup> and Cre<sup>+</sup> littermates, we generated a congenic rd1 Casp2<sup>−/−</sup> rd1 mice at the indicated time points. Numbers in the bars represent the number of retinas analyzed. *P < 0.05 by Student’s t test. (E) Immunofluorescence analyses to detect cone arrestin (red signal) and PNA (green signal; blue signal indicates nuclear DAPI) in retinal cryosections from 20-week-old WT and Casp2<sup>−/−</sup> mice.
by an i.p. injection of a ketamine-xylazine (100 mg/kg and 10 mg/kg, respectively) mixture. One drop each of phenylephrine (2.5%) and tropicamide (1%) was applied for pupil dilation 10 minutes before to recording. Animals were kept on a warming plate during the entire ERG procedure to maintain body temperature at 37°C. Photopic ERGs were recorded after light adaptation with a background illumination of 34 cd/m² (white 6500 K, produced by a Ganzfeld stimulator) for 8 minutes. Flashes were presented at 1-minute intervals with a pulse length of 4 ms, and each recording consisted of a single flash of 35 cd × s/m². Six to nine trials were averaged for single-flash responses.

**Histological methods.** Antibody stainings on retinal cryosections and retinal flat mounts were performed as described previously (17, 57). CASP2 activity on retinal whole mounts was detected using the Green FLICA Caspase 2 Activity Kit (catalog 918; ImmunoChemistry Technologies) according to the manufacturer’s instructions. Briefly, retinæ were incubated in DMEM media with the detection reagent at 37°C for 30 minutes, followed by 2 washes of 5 minutes each in apoptosis wash buffer. Retinæ were then fixed and processed for antibody staining as described (17, 57). Antibody stainings were performed in TBS buffer for phospho-specific antibodies and in PBS buffer for all other antibodies. The following primary antibodies and concentrations were used: rabbit α-p-AKT (Thr308) (1:1,000; catalog 2965); rabbit α-p-AKT (Ser473) (1:1,000; catalog 4060); rabbit α-p-S6 (Ser24) (1:300; catalog 5364); rabbit α-p-S6 (Ser235/236) (1:300; catalog 4856); rabbit α-PKM2 (1:300; catalog 4053); rabbit α-HK2 (1:300; catalog 2867) (all from Cell Signaling Technology); rabbit α-p-SGK (Ser422) (1:300; catalog ab55281); rabbit α-G6PD (1:300; catalog ab993); rabbit α-ME1 (1:300; catalog ab97445); rabbit α-Ki67 (1:300; catalog ab15580) (all from Abcam); rabbit α-HIF-1a (1:300; catalog ab1536; R&D Systems); rabbit α-GLUT1 (1:30; catalog GT11-A; Alpha Diagnostics); mouse α-Cre (1:500; catalog MMS-106P; Covance); rabbit α-cone arrestin (1:300; catalog AB15282; EMD Millipore); rabbit α-red-green opsins (1:300; catalog AB5405; EMD Millipore); and fluorescein-labeled peanut agglutinin lectin (PNA) (1:500; catalog FL-1071; Vector Laboratories). All secondary antibodies (donkey) were purchased from Jackson ImmunoResearch and were purified F(ab), fragments that displayed minimal cross-reactivity with other species.

**Quantification of cone survival.** The A9 Cre-reporter line was used to validate uniform expression of Cre recombinase across the retina (Supplemental Figure 1, A–E, and ref. 55). Quantification of cone survival by colocalization was performed using a method similar to one described previously (17). In brief, the percentage of surviving cones is based on a calculation of the percentage of the retinal surface area that is covered by cones. To determine the area that was covered by cones, we used the staining detected by the cone arrestin antibody (Supplemental Figure 1, F and G: red signal) as a proxy for surviving cones. To determine the retinal surface area, retinæ were stained with PNA-coupled FITC (Supplemental Figure 1, F and G: green signal) and retinal flat mounts (gray scale signal in the third column) at a dilution that was 3 times higher than that used for all other staining procedures, with the goal to only elicit sufficient background fluorescence to highlight the entire retinal surface area rather than detect cone OSs. The ratio of red to green pixels, which was calculated using CoLocalizer Pro software (CoLocalization Research Software) (58) with its integrated background correction, thus directly representing the percentage of the retinal surface area that was covered by the cone arrestin antibody staining. Before calculating the percentage of colocalization of red and green, the upper and lower thresholds of each signal were adjusted, such that only cones were visible in the red signal (Supplemental Figure 1, H and I), and the entire retina was visible in the green signal. To calibrate the percentage of colocalization of red and green signals on an rd1-mutant background to the actual percentage of surviving cones, we performed a real cone count rather than determining the percentage of cone arrestin staining that covered the retina in WT mice. The reason for this is that during degeneration, cone arrestin was also found in the cell body, resulting in a different pattern of expression; thus, the percentage of colocalization was different when compared with that in retinae of WT mice (Supplemental Figure 1, J and K). A cone count was performed across 3 WT retinæ and across 3 rd1 Tsc+/– retinæ at P21 (Supplemental Figure 1, L–N). The mutant strain and time point were chosen at P21, since at this point, cone death had just started,
and the percentage of colocalization of red and green signals was the highest upon loss of TSC1 (52.7%). The actual cone count in WT retinae was in agreement with published data (59) and revealed that at P21, 95.2% of cones were still present in the rd1 Tsc1−/− retina when compared with those in WT retinae, meaning that 52.7% of colocalization (red to green signal) corresponds to 95.2% of cones. These numbers were used to calibrate all colocalization data representing cone survival. To validate the method, we counted cones in retinae of rd1 Tsc1−/− mice at 2 months of age (Supplemental Figure 1L). This time point was chosen because upon loss of TSC1, cone survival at 2 months of age is still quite homogenous, rather than demonstrating a patchy pattern of survival, thus making it more accurate to determine the number of cones across the entire retina by counting individual fields. The colocalization and actual cone count data for mice at 2 months of age were in agreement, with no statistically significant difference between them (77.6% ± 3.9% vs. 75.8% ± 3.7%, respectively), indicating that the colocalization method can be used to determine the percentage of surviving cones. The method of quantifying cones by colocalization over the entire retinal surface, rather than counting the number of cones in a few individual fields, was chosen because cone degeneration is quite patchy, progressing from the center to the periphery. Therefore, counting a few individual fields, even if always done in the exact same location, is not likely to properly represent the number of surviving cones. Thus, this method is independent of the degeneration pattern and the orientation of the retina.

For quantification of cones by cone count, retinae were first divided into 5 different sectors with increasing radiiues of 500 mm per sector (Supplemental Figure 1L). Cones were then counted manually in 4 squares per sector (20 squares/retina), each square measuring 40,000 mm², to determine the average cone density per sector (cones/mm²). The average cone density per sector was then multiplied with the sector surface area to obtain the number of cones per sector. The surface area of each sector was calculated as the sector surface area of a sphere. The total number of cones per retina was then obtained by adding the number of cones across the 5 sectors. The numbers shown represent an average of 3 retinae per genotype and age. Retinal flat-mount images for the colocalization analyses and cone count were acquired by tiling individual images taken at ×16 magnification (Leica DM5500) over the entire retinal surface area with an automated scanning stage.

Western blot analysis. Retinae from 2 animals were pooled and homogenized by sonication in ristocetin-induced platelet aggregation (RIPA) buffer containing protease and phosphatase inhibitors (Complete Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail; both from Roche Diagnostics). Protein concentration was assessed using a Protein Assay Kit (Bio-Rad), and 10 μg protein was loaded per lane. Proteins were separated on a 4%–20% Tris-Glycine gradient gel (Bio-Rad) at 170 V and transferred onto a nitrocellulose membrane for 2 hours at 4°C with a current of 150 mA. Membrane was blocked for 1 hour at room temperature in 5% fat-free dry milk powder, incubated with primary antibody overnight at 4°C, washed 3 times for 20 minutes each wash at room temperature, incubated with an HRP-coupled secondary antibody (1:10,000; Santa Cruz Biotechnology Inc.) for 2 hours, and washed 3 times for 20 minutes each wash at room temperature. Signal was detected with SuperSignal West Dura (Pierce Biotechnology). All incubations were performed in the presence of 0.1% Tween-20 and 5% fat-free dry milk powder. Incubations were performed either in TBS buffer for phospho-specific antibodies or in PBS buffer for all other antibodies. The following primary antibodies were used: rabbit α-p-akt(308) (1:1,000; catalog 2965); rabbit α-p-akt(473) (1:1,000; catalog 4060); mouse α-akt (1:1,000; catalog 2967); rabbit α-p-s6 (1:1,000; catalog 5364); and rabbit α-s6 (1:1,000; catalog 2217) (all from Cell Signaling Technology); mouse a-β-actin (1:2,000; catalog A5316; Sigma-Alrich); mouse a-CRE (1:2,000; catalog 69050-3; Novagen); rabbit a-cone arrestin (1:1,000; catalog AB15282; EMD Millipore); and rat a-CASP2 (1:1,000; catalog ALX-804-556; clone 11B4; Enzo Life Sciences).

qRT-PCR. RNA was isolated using TRizol (Life Technologies) as described previously (17). Three biological samples of each genotype were analyzed, and each sample consisted of retinae from 2 different animals. cDNAs were generated using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics), and SYBR green–based qRT-PCR (Kapa Biosystems) was performed using the Bio-Rad CFX96 Real-Time System. Samples were run in duplicate and normalized to β-actin using the 2−ΔΔCt method. The primer sets used are provided in Supplemental Table 1.

NADPH measurement. NADPH was measured using the FluoroNAP/NADP kit from Cell Technology Inc. (NADPH100-2). The assay was performed in triplicate using 3 biological samples, with each biological sample consisting of 3 retinae. Retinae were dissected in DMEM media, rinsed with 1X PBS, and processed following the manufacturer’s instructions. Retinae were sonicated after the addition of the lysis buffer to ensure efficient dissociation.

Cell culture. Casp2 was amplified by PCR (forward: 5′-ATGGC-GGCGCGAGCGGAGGTGC-3′; reverse: 5′-TACAGTGGTGTTGGG-TAGCCTGGG-3′) from a C57BL/6j retinal cDNA library that was generated as described previously (17). The PCR product (1359 bp) was subcloned into the pGEM-T easy vector (Promega) and sequence verified. The Casp2 cDNA was then cloned into a rAAV2 plasmid carrying a CMV promoter and the SV40 polyadenylation site. HEK293 cells were transfected with PEI (29366; Polyscience Inc.) and maintained in regular DMEM media containing 10% FCS and a penicillin-streptomycin mixture. The same media were used to maintain the Y79 cell line. Protein extractions were performed as described above.

Statistics. The Student’s t test was used for statistical analyses. P values of less than 0.05 were considered statistically significant. If not otherwise noted in the figure legend, all error bars represent the SEM.

Study approval. The IACUC of the University of Massachusetts Medical School approved the animal study. All procedures involving animals were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

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