HHS Public Access

Author manuscript

Cell Rep. Author manuscript; available in PMC 2017 October 19.

Published in final edited form as:

Cell Rep. 2017 October 17; 21(3): 692–706. doi:10.1016/j.celrep.2017.09.069.

The circadian clock gene *Bmal1* controls thyroid hormone-mediated spectral identity and cone photoreceptor function

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SUMMARY

Circadian clocks regulate various aspects of photoreceptor physiology, but their contribution to photoreceptor development and function is unclear. Cone photoreceptors are critical for color vision. Here, we define the molecular function of circadian activity within cone photoreceptors and reveal a role for the clock genes *Bmal1* and *Per2* in regulating cone spectral identity. ChIP analysis revealed that BMAL1 binds to the promoter region of the thyroid hormone-activating enzyme type 2 iodothyronine deiodinase (*Dio2*) and thus regulates the expression of *Dio2*. Thyroid hormone treatment resulted in a partial rescue of the phenotype caused by the loss of *Bmal1*, thus revealing a functional relationship between *Bmal1* and *Dio2* in establishing cone photoreceptor identity. Furthermore, *Bmal1* and *Dio2* are required to maintain cone photoreceptor

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Accession Number

The accession number for the data reported in Figure S7 is GSE103484.

AUTHOR CONTRIBUTIONS

O.B.S., and S.R. designed the research. Experiments and investigations were conducted by O.B.S., A.M.H., O.F.Z., R.C., V.L.B., I.S.S., and S.R. Data analysis was done by O.B.S., R.C., I.S.S., and S.R. Manuscript was written by O.B.S. and S.R. Final version of the manuscript was reviewed and approved by all the authors.

Supplemental Information

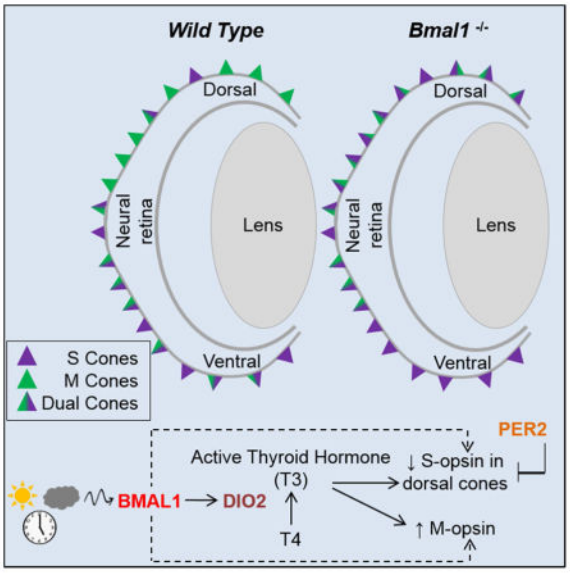
Supplemental Information includes seven figures, a primer list table, experimental procedures and data files related to ChIP-Seq data analysis.

Data File S1, **related to** Figure 7 and S7. Target genes
Data File S2, **Related to** Figure 7 and S7. Trusted Targets
Data File S3, **Related to** Figure 7 and S7. Biological Process
Data File S4, **Related to** Figure 7 and S7. Pathway

functional integrity. Overall, our results suggest a mechanism by which circadian proteins can locally regulate the availability of thyroid hormone and influence tissue development and function.

In Brief

Short and medium wavelength opsins in cone photoreceptors are required for color vision. Sawant et al. show that core circadian clock genes are required for cone opsin expression, gradient maintenance, and cone function and that these effects are mediated by regulation of thyroid hormone signaling.



INTRODUCTION

Vertebrate retinal photoreceptors are specialized light-sensing neurons comprised of rods and cones. Rods are mainly active under dim illumination (photopic) conditions, while cones mediate high acuity vision under bright daylight (scotopic) conditions and are essential for color vision. Functional differences between rods and cones are due to the presence of different light-sensing opsins expressed in respective photoreceptors. Unlike rods, which express just one type of opsin (rhodopsin), cone opsins are widely divergent and classified based on their spectral sensitivity. Humans and closely related primates with trichromatic color vision have three types of cone opsins - short (S), medium (M) and long (L) wavelength-sensitive, while most mammals with dichromatic color vision have only S and M (Applebury et al., 2000; Haverkamp et al., 2005; Nathans, 1999). Despite cone diversity in number and type between species, both gene regulatory networks and developmental patterning in cones exhibit similarities (Viets et al., 2016). In mice, retinal cones are arranged in a dorso-ventral gradient, with M cones predominantly in the dorsal region and S cones enriched in the ventral region (Applebury et al., 2000).

Thyroid hormone (TH) signaling plays an essential role in formation of the dorso-ventral cone gradient. Animals lacking the thyroid hormone receptor TRβ2 lack M opsin expression

and show S opsin expression throughout the retina (Ng et al., 2001). Similarly, loss of RXRγ, which heterodimerizes with TRβ2, results in S opsin expression in the dorsal retina, but M opsin expression remains unaffected (Roberts et al., 2005). TH is predominantly secreted into the circulation as an inactive prohormone thyroxin (T4). In the target tissue, most cytoplasmic T4 is converted into active triiodothyronine (T3) by the activity of enzyme type 2 iodothyronine deiodinase (DIO2), while the related protein DIO3 inactivates T4 and T3 by converting them into reverse T3 and T2, respectively (Gereben et al., 2008). T3 generated by DIO2 contributes to the nuclear pool of T3 and serves as ligand for the thyroid hormone receptors such as TRβ2 (Wu and Koenig, 2000). A number of studies have documented circadian and seasonal dependent oscillations in circulating levels of T3 and intracellular levels of *Dio2* and *Dio3* (Barrett et al., 2007; Bedolla and Torre, 2011; Ono et al., 2008; Peliciari-Garcia et al., 2016; Watanabe et al., 2007). These observations raise the possibility that T3 availability within the tissues could be under a circadian regulation, however molecular mechanisms underlying this regulation are unknown.

Circadian rhythms are generated through the activity of a set of proteins that form an autoregulatory transcriptional and translational feedback loop. The positive transcriptional arm of the loop is mediated by activity of the heterodimeric transcription factors BMAL1 and CLOCK that bind to E-Box motifs of the Period (*Per*) and Cryptochrome (*Cry*) genes and other clock-controlled genes to activate transcription. PER and CRY proteins also form heterodimers and repress their own transcription by blocking BMAL1/CLOCK mediated activation. BMAL1 and CLOCK also activate transcription of the nuclear receptor genes *Rev-Erbα* and *Rorα*. REVERBα inhibits *Bmal1* transcription, whereas RORα enhances it (McMahon et al., 2014; Tosini et al., 2008). Transcripts and proteins encoded by clock genes have been detected in the cells of the inner nuclear layer (INL), ganglion cell layer (GCL), retinal pigmented epithelium (RPE), in cones and at very low levels in rods (Hiragaki et al., 2014; Liu et al., 2012; Mustafi et al., 2013; Ruan et al., 2006). Nonetheless, their role in photoreceptor development and function has not been determined.

Here we find that *Bmal1* loss from mouse cones results in ectopic expression of S opsin in the dorsal retina, while conversely, *Per2* loss results in decreased S opsin expression in the dorsal retina. BMAL1 mediates its action in part by regulating *Dio2* as we report that *Dio2* loss-of-function mutants and *Dio2*/*Bmal1* double heterozygotes exhibit similar S opsin phenotypes. In addition, we use ChIP sequencing to show that *Dio2* is a BMAL1 transcriptional target and thus retinal *Dio2* expression could be under circadian control. Morever, we show that T3 can partially rescue the phenotypes caused by *Bmal1* loss. Finally, our studies indicate that *Bmal1* and *Dio2* are required for cone receptor maintenance, as loss of either promotes progressive defects in photoreceptor function. These studies suggest a function of the circadian clock in cone photoreceptor activities. Our findings have implication for cone degenerative diseases, as a cone clock or its output signals could exert its effects on retinal health by regulating gene expression, signaling and metabolism.

RESULTS

Bmal1 is required for maintenance of S opsin gradient

The dorso-ventral gradient of cone opsins is known to be regulated by TH and its receptors (Glaschke et al., 2011; Ng et al., 2001; Pessoa et al., 2008; Roberts et al., 2006). We have previously shown that light can regulate TH levels in the murine eye and can affect rod photoreceptor development (Sawant et al., 2015). Light is a strong entrainment signal for the circadian clock and TH is an important regulator of metabolic processes. Hence we hypothesized that the effects of light may be due to desynchronization between the retinal circadian clock and TH signaling during photoreceptor development. To determine if disruption of the circadian clock could affect photoreceptor development, we used the photoreceptor specific *Crx-Cre* transgene to delete *Bmal1* from all photoreceptors. *Bmal1* deletion was confirmed by qPCR and western blot analysis on the entire retina (Figure S1). Though the transcript was significantly reduced at P17, we only detected a significant reduction in BMAL1 protein at P24 (Figure S1 AC). Despite the loss of *Bmal1* from all photoreceptors, rod development or function was not affected as assessed by dark adapted ERG a-wave amplitude (Figure S1 D) and rhodopsin western blots (Figure S1 F). However, we did observe an ERG response that was indicative of cone photoreceptors being functionally compromised. (Figure S1 E). This led us to further investigate if the cone photoreceptors were affected by the loss of *Bmal1*.

We coimmunolabeled the retinae with antibodies to S and M opsin to visualize the cones. In the litter mate control retina, the S opsin expressing cones were concentrated in the ventral retina with very few S opsin positive cones in the dorsal retina (Figure 1B, b and b′). By contrast, in *Bmal1* conditional mutants (CKO) there is a drastic disruption in the regular patterning and distribution of S opsin expressing cones with all the cones in the dorsal retina now expressing S opsin. (Figure 1D, d and d′). In the *Crx-Cre* transgene, the Cre expression occurs as early as embryonic day 12 (E12), a developmental time when progenitors are committed to becoming photoreceptors. Early loss of *Bmal1* could result in a cone/rod fate switch but the total number of cones were unaffected suggesting that *Bmal1* alters opsin expression but not cone/rod fate (Figure 1G). To conclusively determine the effects of *Bmal1* deletion on cone photoreceptors, we used the cone specific *HRGP-Cre* transgene to delete *Bmal1* (Le et al., 2004, 2006). Similar to the phenotype detected in the *Crx-Cre; Bmal1*$^{Fl/Fl}$ retina, cone specific deletion of *Bmal1* resulted in S opsin expression in the dorsal retina (Figure 1F, f and f′). We quantified the density of cones expressing either only S opsin (Figure 1I), only M opsin (Figure 1H), both S and M opsin (Figure 1J) and the total number of cones (Figure 1G) in the various regions of the retina as shown in the schematic (Figure S2).

We observed a significant reduction (>85%) in the number of M opsin expressing cones (Figure 1H) and a concurrent increase in the density of dual-cones (Figure 1J). Overall there is a decrease in M opsin expressing cones throughout the retina and this decrease was more evident in the far ventral region of the retina, in the *HRGP-Cre; Bmal1*$^{Fl/Fl}$ animals (Figure S3 D). Immunofluorescence data was validated using western blot analysis (Figure S3 C) and real time qPCR (Figure S3 A–B). In agreement with our observation, both the M opsin

transcript and protein was significantly lower in the *CKO* retina. Thus, these results demonstrate that core circadian clock gene *Bmal1* is required for the maintenance of S opsin gradient and regulates the expression of M opsin in the mouse retina. Interestingly, we observe a similar phenotype in the retinas from genetically deleted *Clock* animals (Figure S4). BMAL1/CLOCK heterodimer regulates the transcription of target genes which include other clock genes like Period and Cryptochrome. Thus, the loss of either *Bmal1* or *Clock* resulting in the same phenotype strengthens our hypothesis that core clock genes are important for cone photoreceptor development.

Per2 is required for overall S opsin levels but not for maintaining the S opsin gradient

Bmal1 and *Period* are known to have opposite effects on cellular processes and opposite phase for their rhythmic expression pattern (Bouchard-Cannon et al., 2013; Jensen et al., 2012). It has been demonstrated that in mice lacking both *Per1* and *Per2*, S opsin levels are reduced (Ait-Hmyed et al., 2013) and this phenotype is exactly opposite to our observations with *Bmal1* deletion. Based on previous analysis (Hiragaki et al., 2014; Liu et al., 2012; Ruan et al., 2006), *Per1* appears to be largely restricted to the INL while *Per2* seems to be more cone specific, suggesting a distinct function for the different *Per* genes. To determine if photoreceptor specific loss of *Per2* would have the opposite effects to the loss of *Bmal1,* we used a floxed allele for *Per2* (Figure S5 A) and deleted *Per2* using the *Crx-Cre* transgene (Figure S5 C). We confirmed loss of *Per2* by qRT-PCR analysis. Loss of *Per2* resulted in the reduction of S opsin transcript but *Per1* and *Per3* were unchanged (Figure 2A). Although the overall dorso-ventral gradient of cone opsins was not altered in *Per2 CKO* retinas (Figure 2F), there was a significant decrease in the number of S opsin positive cones in the dorsal region compared to the control group (Figure 2C, g–h). Thus our data suggest that *Per2* is expressed in the cones (Figure S5 A) and loss of *Per2* results in reduced S opsin levels but S opsin gradient was unaffected. It will be interesting to determine if the loss of Cryptochromes, would result in similar phenotypes as loss of Period. Nevertheless, our findings suggest that *Bmal1* and *Per2* have an opposing effect on S opsin expression.

Dio2 is expressed in the cone photoreceptors

A number of genes involved in thyroid hormone signaling pathway such as *Trβ2*, *Pax8* and *Dio3* are known to play a vital role in establishing the cone opsin gradient (Applebury et al., 2007; Glaschke et al., 2010; Ng et al., 2001; Ng et al., 2010; Pessoa et al., 2008). To determine if any of these genes were transcriptionally altered by loss of *Bmal1*, we analyzed their transcript levels using qRT-PCR at P17 and P24, the ages when we can detect lower levels of *Bmal1* transcript in the *Bmal1 CKO* retina (Figure S1). We only tested for *Dio1, Dio2, Dio3, RXRγ, Trβ2* and *Pax8* transcripts as these could play a role in cone opsin gradient formation. Except for *RXRγ* which was significantly reduced in the *Bmal1 CKO* retina, none of the other transcripts were affected at P17. *RXRγ* is a cone marker and is expressed in the developing cones (Roberts et al., 2005). Thus the observed reduction in the *RXRγ* transcript, could be due to a delay in cone photoreceptor development in the *Bmal1 CKO*. Accordingly at P24 *RXRγ* levels were not different between the control and *CKO* retina (Figure 3B). Interestingly all the deiodinases were altered by the loss of *Bmal1*. However, neither *Dio1* or *Dio3* is known to be expressed at any significant levels in the post natal retina compared to *Dio2*, thus making it less likely that these deiodinases have any role

to play in regulating S opsin expression in the post natal retina. (Figure 3C) (Ng et al., 2017). Moreover, loss of *Dio3* results in apoptosis of cone photoreceptors, whereas in the *Bmal1 CKO* retina, cone numbers are not significantly altered (Figure 1G). On the other hand, in the postnatal retina, the relative expression profile for the *Dio2* transcript is very similar to that of *M* opsin expression (Figure 3E–F) and coincides with the peak period of photoreceptor outer segment extension (LaVail, 1973). Similarly, *Bmal1* transcript levels also increase postnatally but the increase is not as exponential as seen for the *Dio2* or *M* opsin transcripts. The transcript analysis was performed using the entire retina and these transcriptional changes reflect global changes within the retina. To determine if *Dio2* was specifically expressed in the cone photoreceptors, we performed qPCR analysis on isolated cones and rods at P24. Despite the presence of some contaminating rods in our cone enriched population, *Dio2* transcripts could only be detected in the cone enriched population (Figure 3G). Importantly, there is a significant increase in *Dio2* transcript between P11 and P24 in the sorted cones (Figure 3H). Similar to the results acquired from the RNA analysis of the whole retina, *Dio2* transcript levels are significantly lower in the isolated cones from the *Bmal CKO* (Figure 3H) at P11 and P24. Taken together these results indicate that *Dio2* is expressed in the cone photoreceptors and *Dio2* levels are reduced in *Bmal1 CKO* retinas.

Loss of *Dio2* results in ectopic S opsin expression in the dorsal retina

Next, we wanted to test if the ectopic S opsin expression in the *Bmal1* CKO retina is due to alterations in *Dio2* expression. We analyzed the retinas from animals that lack *Dio2* due to the insertion of a null mutation in the *Dio2* gene. We observed an increase in the number of S opsin expressing cones in the dorsal retina and disruption of dorso-ventral S opsin gradient in $Dio2^{-/-}$animals (Figure 4C, d′, d′-2, K-2 and L) compared to the controls (Figure 4A, b′, b′-2, I-2 and J). Similar to *Bmal1 CKO* retina, *Dio2* deletion did not affect total number of cones, but density of dual cones is increased with a concurrent decrease in genuine M cone density (Figure 4E–H). The phenotype is more pronounced in the far dorsal and mid dorsal region, but the cones in the extreme periphery region (FFD-far far dorsal) are unaffected (Figure 4E–H). However, this phenotype is persistent up to 6 weeks (Figure 4K-2). The phenotype of $Dio2^{-/-}$ animals though mild is similar to that of the *Bmal1 CKO* animals suggesting that both *Bmal1* and *Dio2* are required for maintaining the S opsin gradient in the mouse retina.

BMAL1 regulates *Dio2* expression

Bmal1 and *Dio2* mutants have similar phenotype suggesting that the two signaling pathways could cooperate in regulating S opsin expression. BMAL1 is a transcription factor, therefore, one possibility is that BMAL1 directly regulates *Dio2* expression. If the two pathways are interacting, then partially compromising the function of both pathways should result in an additive effect. To test this hypothesis, we quantitated the S cone density in the *Bmal1 CKO;* $Dio2^{+/-}$ double heterozygous retinas and compared it to single heterozygotes of *Bmal1 CKO* and *Dio2*. The S opsin gradient was disrupted in the double heterozygous mutant animals and the number of S opsin positive cones were significantly higher (Figure 5C, C′, D) compared to single heterozygous animals (Figure 5A′, B′, D). We detected the maximal changes in the dorsal retina and hence quantitated the cone densities in the dorsal region.

These findings suggest that there is a genetic interaction between *Bmal1* and *Dio2* and these pathways are important regulators of S opsin patterning in the dorsal retina.

Next we wanted to test if we could detect any oscillation in the *Bmal1* and *Dio2* transcripts within the retina over a circadian time period. We observed rhythmicity in *Bmal1* expression, with peak expression at ZT0 (beginning of the light phase) and lowest expression at ZT12 (beginning of dark phase) (Figure 5E). In the *Bmal1 CKO*, as expected, the overall levels of *Bmal1* transcript were significantly lower ($P<0.001$) but *Bmal1* expression levels were not significantly different among different ZTs (Figure 5E). This is surprising given that only 50% of the transcript is reduced by the loss of *Bmal1* from the photoreceptors (Figure S1). This data suggests that the circadian oscillations of *Bmal1* in the entire retina could be compromised due to deletion of *Bmal1* from the photoreceptors. *Dio2* transcript also exhibited cyclic oscillations fairly similar to that of *Bmal1* except for ZT 20 (Figure 5F). The peak expression for retinal *Dio2* could be detected at ZT 0 and was significantly higher compared to its lowest expression observed just before and during the dark phase (Figure 5F). Overall oscillations of the *Dio2* transcript was eliminated in *Bmal1 CKO* retinas as none of the time points were significantly different. To confirm that *Bmal1* deletion would result in loss of *Dio2*, we performed qPCR analysis on sorted cones for *Bmal1* and *Dio2* transcripts. There is more than 50% reduction in the *Bmal1* and *Dio2* transcripts at P11 and at P24 (Figure 5K and L). The residual expression for *Bmal1* and *Dio2* is due to the activity of the transgene as Cre driven conversion of a reporter transgene was used to isolate the cones (see experimental procedures for details). This data suggest that BMAL1 could be directly regulating *Dio2* expression within the cones.

We then wanted to determine if BMAL1 controls *Dio2* expression. First we performed *in-silico* analysis of the *Dio2* promoter to establish if any of the known canonical (5′-CANNTG-3′) BMAL1 binding E-box elements were present. We identified 6 E-box elements (Jensen et al., 2012; Rey et al., 2011) within 5 kb of the annotated transcription start site of the *Dio2* gene (Figure 5H). Of these 6, there is one highly conserved E-box in the region that is accompanied by another BMAL1 E-box (CATGTG) separated by 7bp (Figure 5H right panel). Interestingly, there is also a known CLOCK binding E-box (CATATG) site right next to the BMAL1 E-box (Yoshitane et al., 2014) separated by 6 bases. This *in-silico* analysis strongly suggested that *Dio2* might be a target of BMAL1. To validate our *in-silico* analysis, we performed chromatin immunoprecipitation (ChIP) assay on the retinal tissues using a BMAL1 antibody and an IgG control antibody. We could specifically amplify the amplicons from the BMAL1 ChIPed DNA samples but not the IgG samples, with primers that span across the 6 E-boxes (Figure 5I). We also performed qPCR analysis on the input and ChIPed DNA with the same primer sets and one primer (P4) that was unrelated to the BMAL1 E-boxes. This analysis indicated a decrease in ChIP efficiency (Figure 5J) for these sites in *Bmal1* CKO, further suggesting that BMAL1 can bind to the *Dio2* promoter. It should be noted that the ChIP efficiency for the primers is variable and this is due to the entire retina being used for the ChIP-Seq. Again, in the ChIPed DNA samples obtained with IgG, we did not detect *Dio2* (Figure 5J). We also validated the ChIP efficiency using primers designed against known BMAL1 E-boxes around upstream region of *Per2* gene (Figure S6 A). ChIP-Seq coverage plots for the *Dio2* gene and 5′ upstream region further confirmed our observations that *Dio2* is a target of BMAL1 (Figure 5G). Thus

our data demonstrate that *Dio2* expression significantly varies over the circadian day and regulation of *Dio2* by BMAL1 is important for spatial distribution of the S opsin expressing cones.

Partial recovery of opsin expression after T3 administration

The phenotype in *Bmal1* CKO animals is very similar to that observed in adult rodents with induced hypothyroidism, where terminally differentiated M opsin cones in the dorsal retina convert to S cones (Glaschke et al., 2011; Pessoa et al., 2008; Roberts et al., 2006). Our data raises the possibility that in the *Bmal1 CKO* retina, due to the loss of *Dio2,* a local reduction in T3 levels could result in an hypothyroid-like cone opsin phenotype. If our hypothesis is correct then we would predict that we should rescue the phenotype in the *Bmal1 CKO* by providing exogenous T3 to the animals. To achieve this, we provided T3 via drinking water to $Bmal1^{Fl/Fl}$ and Hrgp-Cre; $Bmal1^{Fl/Fl}$ littermates from P20–P50 and analyzed their retina for S and M opsin expression. As predicted, T3 supplementation resulted in a decrease in S opsin expression in the dual cones and they begin to appear as genuine M opsin expressing cones (Figure 6A–B, dotted ovals). The far and mid dorsal region were most affected by T3 supplementation (Figure 6O). We validated the immunofluorescence data with transcript analysis. T3 treatment results in an overall reduction in S opsin transcript (Figure 6P). It is interesting to note that at P50, S opsin transcript levels were similar in the vehicle control WT group and T3 treated CKO group (Figure 6P). Though T3 treatment did not result in complete reversal of S opsin gradient as assessed by immunofluorescence staining, we could detect a noticeable rescue in the S opsin transcript levels. It would be interesting to maintain the animals on T3 for longer periods to determine if longer treatment can result in complete reversal of S opsin gradient. However, high levels of T3 can also affect cone survival and overall affect the health of the animals (Ma et al., 2014). Nonetheless, our data demonstrates that T3 supplementation can partially reverse the cone opsin phenotype resulting from *Bmal1* deletion.

Bmal1 and *Dio2* are required for adult cone function and maintenance

Next, we wanted to test if the dorsal increase in the S opsin cones results in functional changes and if *Bmal1 CKO* cones exhibit stronger responses towards short wavelength of light. We performed wavelength specific ERG testing in mice at 6 weeks of age to assess if the observed changes in opsin expression result in impaired cone function. In *HRGP-Cre;* $Bmal1^{Fl/Fl}$ mice, UV light flashes (365nm) that excites S opsin cones elicited a statistically significant increased response at various flash stimuli compared to the control group (Figure 7C). Other wavelengths of light which include blue (445nm), green (520nm) and amber (590nm) light sources showed significantly lower b-wave amplitudes compared to the control group (Figure 7D–F). Cone b-wave ERG responses in *Crx-Cre;* $Bmal1^{Fl/Fl}$ mice were also significantly decreased for all wavelengths tested compared to the control group. Although cone b-wave response for UV light was significantly lower in *Crx-Cre;* $Bmal1^{Fl/Fl}$ mice (Figure 7A), the degree of reduction is less severe than the degree of reduction for other wavelengths (Figure 7C–F) (P=0.024 V/S P<0.001). These results indicate that cone specific deletion of *Bmal1* results in an increase in S opsin cone responses and a decrease in M opsin cone responses.

We performed standard ERGs on *Dio2*$^{-/-}$ animals at 6 weeks of age. *Dio2*$^{-/-}$ mice exhibited approximately 40% reduction in light-adapted cone b-wave response to increasing intensity of achromatic flashes that excite both S and M opsins as compared to control mice (Figure 7G–H). As expected, dark-adapted a-wave was not drastically affected in *Dio2*$^{-/-}$ mice compared to their respective littermate controls (Figure S6 B) since we could not find detectable levels of *Dio2* in the rods (Figure 3G). We also observed a significant reduction in dark-adapted b-wave responses in *Bmal1* CKOs (Figure 7A) as well as in the *Dio2*$^{-/-}$ mice (Figure 7B). As this response is known to reflect the activity of the bipolar cells, this reduction could be due to the loss of *Bmal1* in bipolar cells. This certainly could be the case as we can detect very weak levels of Cre protein in cells of INL besides the photoreceptor (data not shown) and the *Crx-Cre CKO* animals exhibit much stronger phenotype compared to the *HRGP-Cre CKO* animals. However, the reduced dark adapted b-wave amplitude in the *HRGP-Cre CKO* animals also suggest that the Scotopic b-wave response is in part due to compromised cone function. In summary, these results indicate that *Bmal1* and *Dio2* are required for cone photoreceptor function.

Our ChIP-Seq analysis with the BMAL1 antibody identified 752 target genes and none of these appeared in the *Bmal1 CKO* group (Figure S7 and Data File S1, S2). Many of these targets were involved in pathways that regulate cellular homeostasis (Data File S3, S4). This prompted us to investigate if the loss of *Bmal1* would lead to long term changes in the cones. We assessed the retina from 3 month-old animals for cone outer segment (OS) defects. The majority of the cones in *Bmal1* CKO animals appear to have shorter OS compared to the controls (Figure 7K–N). To further evaluate this phenotype, we measured cone OS length compared to the combined OS and IS (inner segment) length, using cone arrestin and S opsin to mark the cones (Zhu et al., 2002). In 3 month-old animals, loss of *Bmal1* resulted in a significant decrease in cone OS length across the entire retina (Figure 7I,O). In the *HRGP-Cre CKO* animals, the OS length of the cones in the far dorsal region was slightly shorter in comparison to the cones from the control animals, but not stastically significant. This could be due to the late onset of *Hrgp-Cre* and we speculate that this phenotype will probably deteriorate over time. We also observed a small (~21%) but statistically significant reduction in cone arrestin transcript levels in *Bmal1 CKO* retinas at 5 months of age (Figure S6 C). This data suggest that *Bmal1* is also required for cone function and maintenance of the cone outer segment.

DISCUSSION

Cells in the rodent retina exhibit circadian activity in processes such as melatonin synthesis (Tosini and Menaker, 1998), disk shedding (LaVail, 1980), and ion channel function (Huang et al., 2015). Nonetheless, whether a circadian clock regulates photoreceptor activity in the mammalian retina has remained unclear, due in part to difficulty in analyzing low levels of expression of various clock genes in those tissues. Here we show that the circadian clock genes *Bmal1* and *Per2* are required to maintain the spectral identity of the cone photoreceptors and *Bmal1* mediates its effect by regulating thyroid hormone signaling within the cone photoreceptors.

Circadian rhythmicity in visual responses

ERG responses from *Bmal1* CKO animals have been reported (Storch et al., 2007), but assessment of cone subtypes and distribution in mutant mice has not been analyzed. Importantly, Storch et al, reported that light-adapted b-wave ERG responses in wild-type mice exhibit circadian rhythmicity, a response abolished in animals lacking retinal *Bmal1*. Based on their observation, the authors concluded that *Bmal1* is required for generation of circadian rhythms in the inner retinal neurons. Here, we observed similar reductions in light-adapted b-wave responses in photoreceptor-specific and cone-specific *Bmal1* knockout mice. Although we did not test mutant mice at different circadian times, our data suggests that differences in light-driven responses observed by Storch et al., could be due to the presence of a functional circadian clock within the cones. Similar circadian changes in cone function have been reported for mice deficient in *Rev-Erbα*$^{-/-}$, which show an increase in dark-adapted b-wave amplitude, a modest increase in light-adapted b-wave amplitude, and unaltered dark-adapted a-wave amplitude (Ait-Hmyed Hakkari et al., 2016). Our data provides further evidence for the existence of a cone specific clock that is required for both developmental and mature functional responses. However, further studies are needed to determine if the cone clock drives rhythmic expression of other retinal factors.

Thyroid hormone and other transcription factors controlling developmental cone opsin gradient

Multiple transcription factors and external signals such as TH control temporal and spatial expression of cone opsins. In mice, peak levels of TH (Alonso et al., 2007; Sawant et al., 2015), TH transporters (Henning and Szafranski, 2016) and *Dio2* (Campos-Barros et al., 2000) are detected in the retina around P12–P18, during the period of photoreceptor development. Based on the expression pattern for S and M opsin, it has been proposed that initially all cones express S opsin by default and then active suppression of the S opsin occurs in the dorsal retina, followed by M opsin expression (Mears et al., 2001; Ng et al., 2001). S opsin suppression in the dorsal retina requires protein-protein interaction between thyroid hormone-bound TRβ2 and RXRγ (Roberts et al., 2005). *Bmal1* deletion by *Crx-Cre* did not disrupt the S opsin gradient at P6 (Figure S6 D), despite the fact that *Crx-Cre* is expressed as early as E12. This observation suggests that either *Bmal1* is not expressed in cone photoreceptors before P6, or *Bmal1* is essential only after P6. We have not confirmed if *Bmal1* is expressed in the cones before P6, but *Bmal1* deletion using the late onset *HRGP-Cre* results in a phenotype that is similar to that observed in the *Crx-Cre CKO* and strongly suggests that *Bmal1* is probably expressed after P6. Despite the important role played by TRβ2 in regulating M opsin expression, there is a considerable delay between detection of high levels of TRβ2 expression and M opsin expression (Applebury et al., 2007; Ng et al., 2009), suggesting that additional factors cooperate with TRβ2 and RXRγ in this activity. One such candidate RORα, when genetically deleted results in decreased expression of S opsin, M opsin and cone arrestin, indicating that RORα is required for overall cone development (Fujieda et al., 2009). In the *Bmal1 CKO*, RORα levels remain unchanged both at P17 or at P24 (data not shown). Other transcription factors such as GTF2IRD1 and chicken ovalbumin upstream promoter-transcription factor (COUP-TF) nuclear receptors are implicated in S opsin suppression (Masuda et al., 2014; Satoh et al., 2009), and *Coup-tfI* (*Nr2f1*) was identified as a BMAL1 target in our ChIP-Seq analysis (data not shown). Our

results raise the possibility that BMAL1 is a master regulator that is required for synchronized and coordinated expression of multiple target genes that can regulate S opsin suppression within the dorsal retina, although further investigations are needed to support this hypothesis.

The role of the circadian clock and thyroid hormone in cone maintenance

It is well documented that TH is required for cone survival and elevated TH levels in adult mice cause cone death (Ma et al., 2014). Similar results have been observed using a zebrafish model in which morpholino-mediated transient knockdown of all three deiodinases promoted photoreceptor loss (Houbrechts et al., 2016). Reduction in levels of thyroid hormone also has a protective effect on cone survival in animal models of retinal degeneration (Ma et al., 2014). Our data suggests that *Bmal1* and *Dio2* are required for cone maintenance in adults. Accordingly, we can detect impairment in cone function at P90 in the photoreceptor specific and cone specific *Bmal1 CKO* and in *Dio2* mutants. Compromised cone function could be the result of the shorter cone outer segments in the *Bmal1 CKO* animals compared to their WT cohorts of the same age. These results suggest a role for TH signaling in adult tissue maintenance. Though TH signaling plays an important role in developing tissues and is a key mediator of growth, role of TH signaling in adult tissue maintenance is still unexplored (For a review, see (Mendoza and Hollenberg, 2017)). It is also important to note that both *Bmal1* and *Dio2* transcripts are rhythmically expressed in the retina, although the functional significance of this pattern needs to be determined. Our findings in $Dio2^{-/-}$ mice as well as the genetic interaction between *Bmal1* and *Dio2* reported here, suggest that cone maintenance is dependent on BMAL1-mediated Dio2 signaling. Moreover, by providing T3 to the *Bmal1 CKO* animals we can bypass the requirement for *Dio2* and partially revert some of the dual cones to M only cones. Based on this analysis we also cannot exclude the possibility that besides *Dio2* and thyroid hormone there are additional signaling pathways that are regulated by *Bmal1* and are important in the patterning of S and M opsin expressing cones in the mouse retina. Nevertheless the partial rescue results suggest that it is possible to revert opsin expression in adult cones and that opsin expression could be manipulated for therapeutic purposes.

In summary, we show that loss of the circadian clock gene *Bmal1* disrupts the dorso-ventral gradient of S and M opsins and increases S opsin expression in the entire retina, whereas loss of *Per2,* reduces S opsin expression. We also show that the levels of the transcript encoding the thyroid-activating enzyme *Dio2* oscillate within the retina during the circadian day and the circadian clock protein BMAL1 binds to the *Dio2* promoter. Our findings suggest a functional role for the circadian clock in the mammalian cone photoreceptor development and provides evidence for a continual role for thyroid hormone signaling in cone photoreceptor maintenance. Our findings maybe of relevance in other tissues, where the circadian clock could alter tissue function by directly regulating *Dio2* expression and thus TH signaling.

EXPERIMENTAL PROCEDURES

Mouse Strains

Transgenic mice carrying the *Dio2* mutation (Stock No: 018985), (Schneider et al., 2001), *Bmal1*$^{flox/flox}$ (Stock No: 018985), (Storch et al., 2007), tdTomato (Stock No: 007914) were purchased from The Jackson Laboratory, Bar Harbor, ME. Other mice used in this study are *HRGP-Cre* (Le et al., 2004, 2006), and *Crx-Cre* (Nishida et al., 2003). Details about mice breeding are included in supplemental experimental procedures. Animal use protocol was approved by the Institutional Animal Care and Use Committees at Cleveland Clinic, and all procedures adhered to the ARVO guidelines for the Use of Animals in Vision Research.

Quantitative Real-Time PCR

Relative fold changes in mRNA expression were determined using the comparative Ct method ($2^{-\Delta\Delta Ct}$ method) and details of RNA isolation, cDNA synthesis and qRT-PCR are mentioned in supplemental experimental procedures.

Immunohistochemistry

Dissected retinas were fixed in 4% PFA for 90 min at room temperature (RT), washed in PBS several times, permeabilized for 30 min using 1% Triton X-100 and blocked for 1 hr at RT with PBS containing 3% BSA and 0.03% Triton X-100. Retinas for flatmount preparation were incubated with primary antibodies [(Goat anti S Opsin, sc-14363, Santa Cruz Biotechnology, Dallas, TX, USA) and (rabbit anti M opsin, AB5405, Millipore, Billerica, MA, USA)] (1:100) for 4 days at 4°C. For cryosectioing, dorso-ventral orientation of mouse eye was determined using guidelines described previously (Wagner et al., 2000). Details about antibodies and immunohistochemistry procedure is described in supplemental experimental procedures.

Cell Isolation by FACS

Retinal dissociation was done using enzyme liberase (Roche Diagnostics, Indianapolis, IN, USA). Cell isolations using fluroscence-activated cell sorting (FACS) were performed on a FACSAriaII (BD Biosciences, San Jose, CA, USA) at the Cleveland Clinic flow cytometry core facility. More information about FACS protocol is described in supplemental experimental procedures.

T3 Supplementation

Oral T3 supplementation (3 μg/mL) via drinking water was done as described previously (Hamidi et al., 2010). T3 supplementation was started either on the day of weaning (P20) (for Figure 6A–O) or on P10 (for Figure 6P–Q) and terminated on P50. Animals were sacrificed on P50. Due to increased lethality associated with T3 supplementation before P10, all rescue experiments were done after P10.

Chromatin Immunoprecipitation (ChIP) Assay

P24 mouse retinas (14–28) and P3 retinas (28–32) were used for the chromatin immunoprecipitation (ChIP) assay using a rabbit anti-BMAL1 antibody (2 μg/sample,

ab3350, Abcam, Cambridge, UK) and a non-immune rabbit IgG (2 μg/sample, 12-370, Abcam, Millipore, Billerica, MA, USA). ChIP protocol used in this study was adapted from a protocol recommended for mouse retinal tissues previously (Peng and Chen, 2013). Ultrasonicator (Model S2, Covaris, Inc. Woburn, MA, USA) was used for fragmentation of chromatin DNA from mouse retinas. ChIP DNA was used for PCR analysis. See supplemental information (Table S1) for a list of primers used. Sequencing on ChIP samples was performed on either single read HiSeq 2500 or HiSeq 4000 (Illumina, San Diego, CA, USA). Sequences from ChIP samples and input controls were aligned to the GRCm38 mouse reference genome (mm10) with bowtie2 (Langmead and Salzberg, 2012). Peak calling was done using HOMER peak caller. See supplemental experimental procedures for more details about ChIP-Seq and data analysis.

Electroretinogram Recordings

P24 or adult 6 week old mice were dark-adapted overnight and then anesthetized using ketamine (40mg/kg) and xylazine (8mg/kg). Pupils were dilated using 1% tropicamide (Bausch and Lomb, Tampa, FL, USA), 2.5% phenylephrine hydrochloride (Akorn Inc., Lake Forest, IL, USA), and 1% cyclopentolate (Bausch and Lomb). Eyes were anesthetized with 0.5% proparacaine hydrochloride (Akorn, Inc.). Mice were placed on a temperature regulated heating pad and ERGs were recorded using an Espion E3 ColorDome Full field Ganzfeld (Diagnosys, LLC, Lowell, MA, USA) as described previously (Ng et al., 2010). Details about rod (scotopic) and wavelength specific photopic responses are mentioned in supplemental experimental procedures.

Statistical Analysis

Statistical significance was determined using SigmaPlot 10.0 software (Systat Software, San Jose, CA, USA). Results comparing two groups were analyzed using Student’s t test. Results in which more than two groups are involved were analyzed using either one-way or two-way ANOVA followed by Tukey post-hoc test or in some case Fisher LSD post-hoc test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was funded by R01 EY027077-01 (S.R.), RPB1503 (S.R.), E.Matilda Ziegler Foundation - MZF1408 (S.R.), Knights Templar Eye Foundation award (S.R.), BrightFocus Foundation award (V.L.B.), California Table Grape Commission research grant (V.L.B), Genentech grant (V.L.B.), VA Merit Award i01-BX002754 (I.S.S.), a National Eye Institute P30-EY025585 core grant and Cleveland Clinic Foundation startup funds (S.R.). We are grateful to Rebecca Fuller, Leigh Perkins and Joey Tate for their assistance in the laboratory, Biological Resources Unit and Veterinary Services at Cleveland Clinic for their assistance with mouse colony management, Genomics Core at the Case Western Reserve University, University of Chicago and Cleveland Clinic for their assistance with ChIP-Seq.

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Highlights

- Circadian clock genes *Bmal1* and *Per2* are required for spatial patterning of cone opsins
- BMAL1 controls expression of the thyroid hormone activating gene *Dio2*
- Regulation of *Dio2* by BMAL1 is required for cone spectral identity
- *Bmal1* and *Dio2* are required for visual function and cone integrity

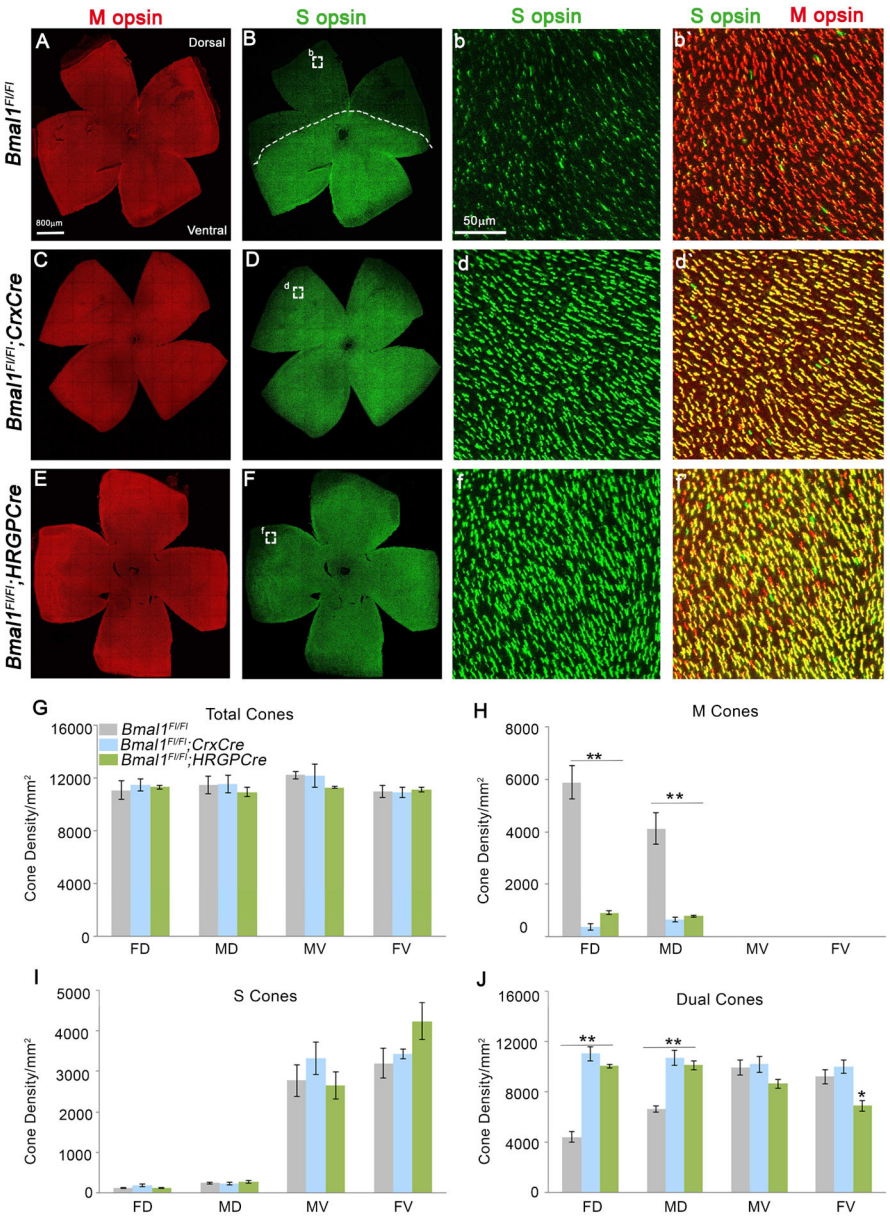

Figure 1. *Bmal1* is required for maintenance of S opsin gradient

(A–F) P24 retinal flat mount preparations coimmunolabeled with S opsin (green) and M opsin (red) from control (A and B) and *Bmal1* conditional mutants (C–F). (B) Dotted line separating low S opsin expressing dorsal (superior) region from S opsin enriched ventral (inferior) region in control retina. (D and F) Loss of dorso-ventral S opsin gradient in *Bmal1* conditional mutants. (b–f′) Representative high magnification images of the region within the square from control (b and b′) and *Bmal1* conditional mutants (d, d′, f and f′). (G–J) Quantification of total cones (G), genuine M opsin cones (H), genuine S opsin cones (I), and dual S+M opsins cones (J) in 20x fields across far dorsal (FD), mid dorsal (MD), mid ventral (MV) and far ventral (FV) regions of the retina indicating that deletion of *Bmal1* results in ectopic S opsin expression in the dorsal retina. Error bars are ± SEM and n=5–11.

Results were analyzed using one-way ANOVA followed by Tukey post test. * indicates $P<0.05$ and ** indicates $P<0.01$.

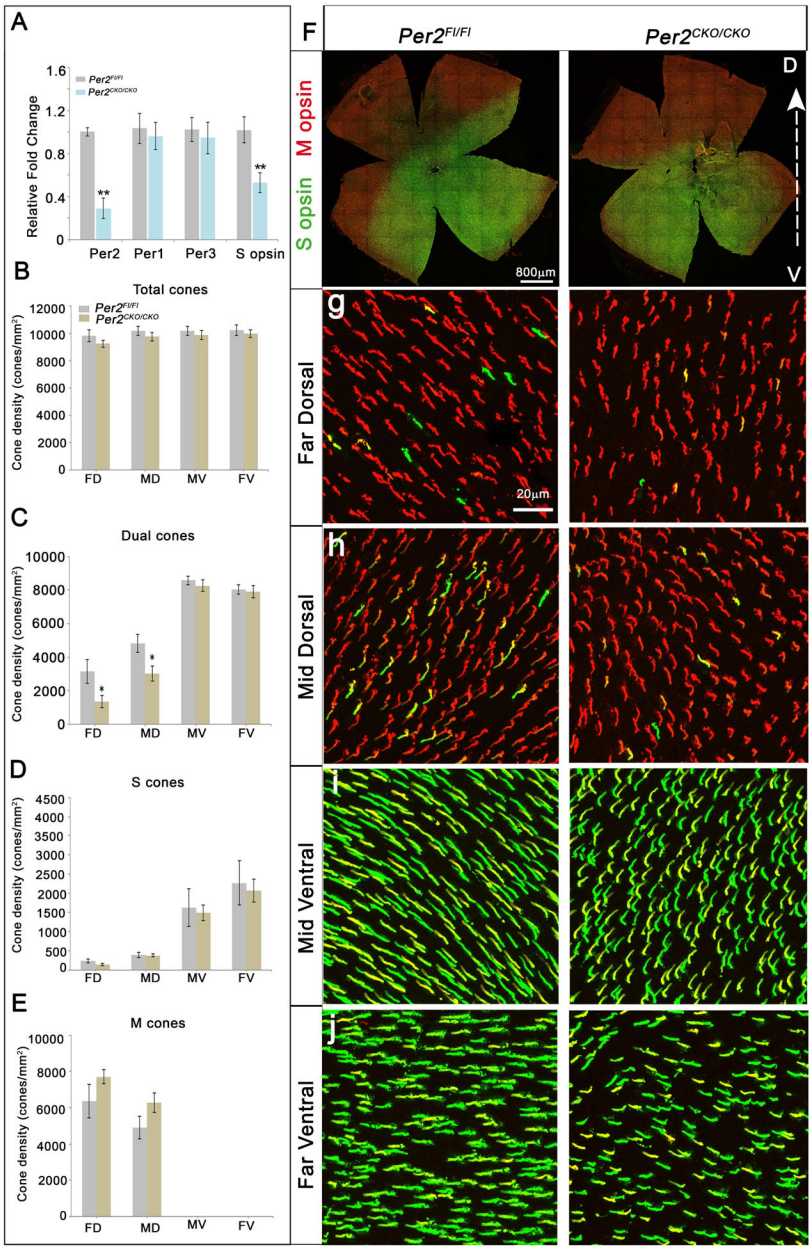

Figure 2. *Per2* is required for overall S opsin levels but not for maintaining S opsin gradient

(A) Relative expression of *Per2*, *Per1*, *Per3* and S opsin transcripts in P24 control and *Crx-Cre; Per2*$^{Fl/Fl}$ retinas using qRT-PCR. Fold change is relative to expression in the control group. Error bars are ± SEM and n=4. ** indicates P<0.01.

(F–j) P42 retinal flat mount preparations coimmunolabeled with S opsin (green) and M opsin (red) for control (F-left panel) and *Per2* conditional mutants (F-right panel). The S opsin gradient is not perturbed in the *Crx-Cre; Per2*$^{Fl/Fl}$ animals, but decrease in S opsin expression was observed at high magnification, (g–j right panels) compared to the control (g–j left panels). D-dorsal and V-ventral.

(B–E) Quantification of total cones (B), dual S+M opsins cones (C), genuine S opsin cones (D), and genuine M opsin cones (E) in 20x fields across far dorsal (FD), mid dorsal (MD),

mid ventral (MV) and far ventral (FV) regions of the retina. Error bars are ± SEM and n=5–6. Results were analyzed using Student's t-test. * indicates $P<0.05$.

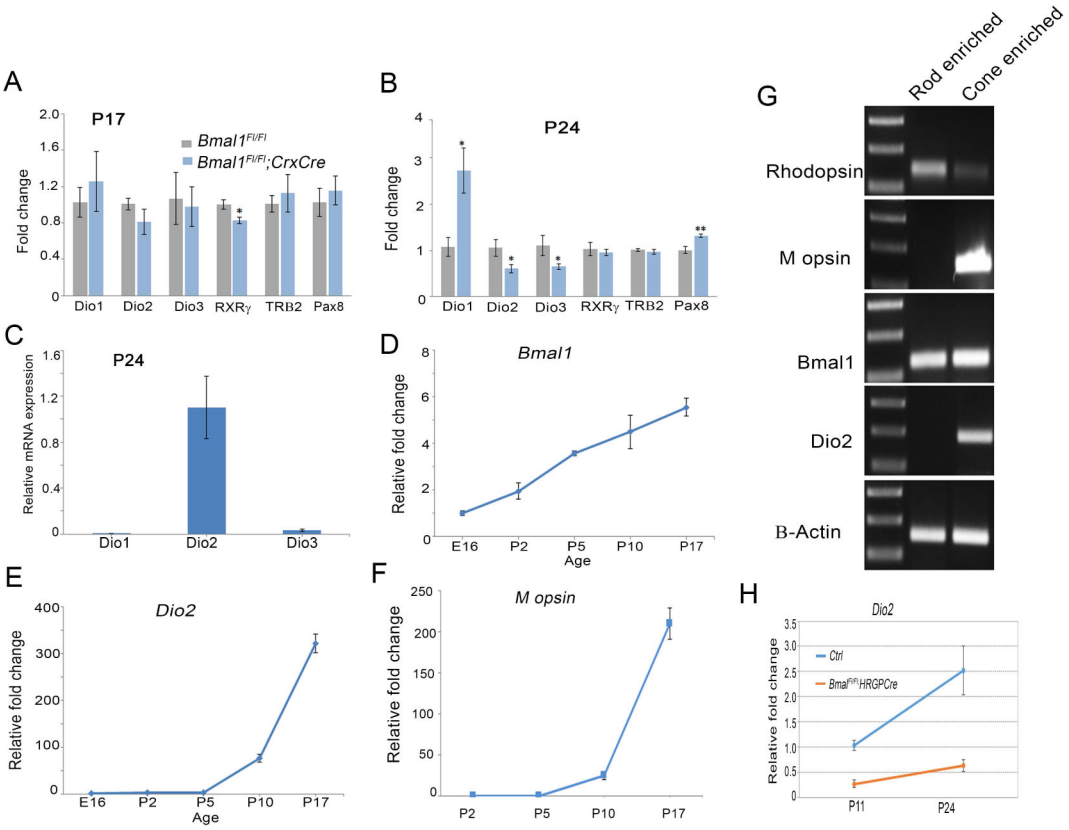

Figure 3. *Dio2* levels are altered in *Bmal1* conditional mutants and *Dio2* is expressed in the cone photoreceptors

(A–B) Relative mRNA expression of *Dio1, Dio2, Dio3, Rxrγ, Trβ2* and *Pax8* in control and *Bmal1* conditional mutant retinas using qRT-PCR. Fold change is relative to expression in the control group.

(C) Relative retinal mRNA expression of *Dio1* and *Dio3* compared to that of *Dio2* at P24 indicating that retinal *Dio2* expression is much higher than that of *Dio1* and *Dio3*.

(D–F) Relative mRNA expression of retinal *Bmal1, Dio2 and M opsin* over the developmental ages indicated.

(G) PCR products of rhodopsin, M opsin, *Bmal1*, *Dio2* and β-actin from FAC sorted cone and rod enriched populations.

(H) Relative *Dio2* mRNA expression in cone-enriched population from control (*tdTomato; Bmal1* $^{Fl/+}$*; Hrgp-Cre*) and *Bmal1* conditional mutant (*tdTomato; Bmal1* $^{Fl/Fl}$*; Hrgp-Cre*) animals at P11 and P24. *Dio2* expression in the control group was significantly higher at P24 compared to P11.

Error bars are ± SEM and n=3–4. Results were analyzed using Student's t-test.* indicates $P<0.05$ and ** indicates $P<0.01$.

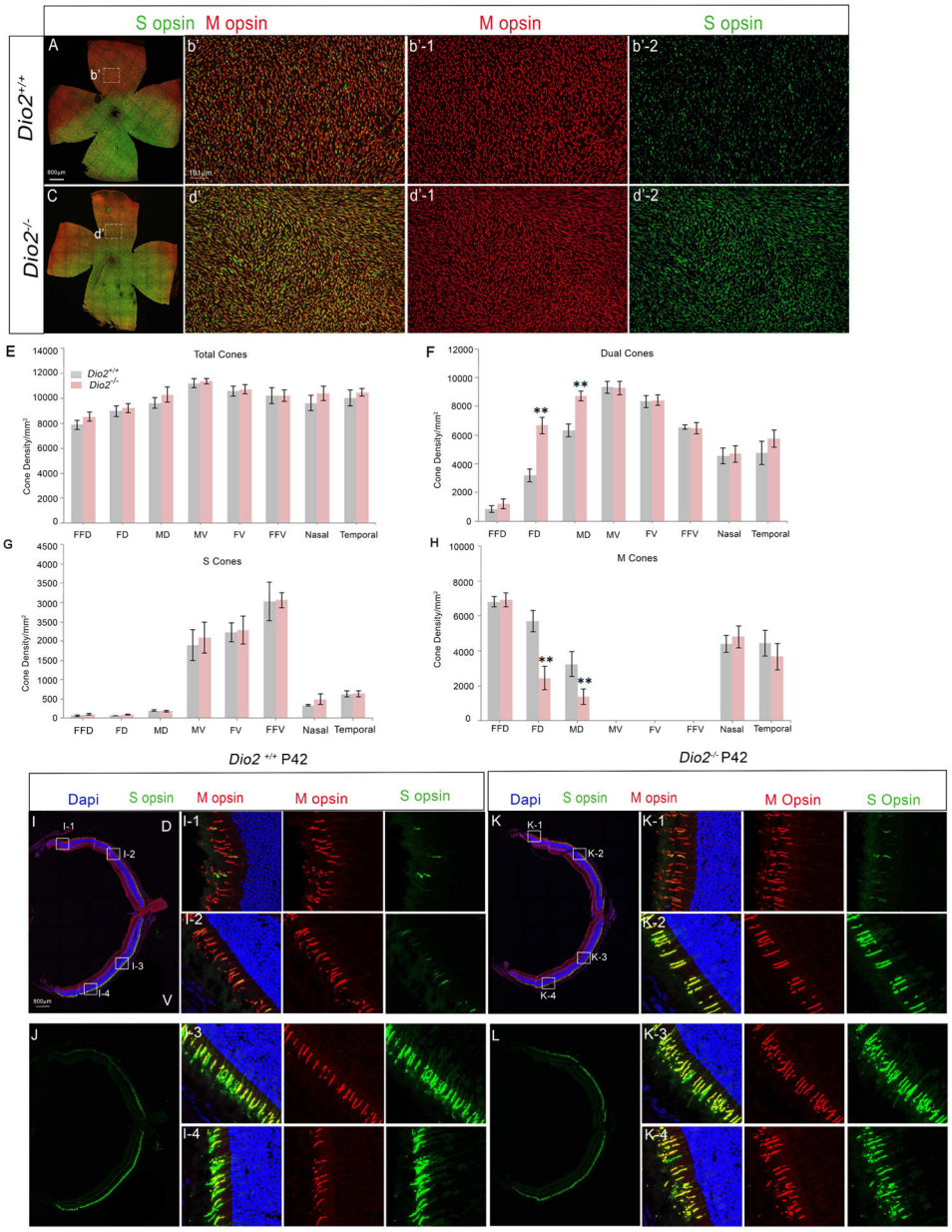

Figure 4. Ablation of *Dio2* ectopic S opsin expressing cones in the dorsal retina

(A–d) P24 retinal flat mount preparations coimmunolabeled with antibodies to S opsin (green) and M opsin (red) from control (A) and *Dio2* mutant (C). Representative high magnification images of the dorsal retina from control (b′) and *Dio2* mutant (d′) showing increased density of dual cones in *Dio2*$^{-/-}$ retina.

(E–H) Quantification of total cones (E), dual S+M opsins cones (F), genuine S opsin cones (G), and genuine M opsin cones (G) in 20x fields across far far dorsal (FFD), far dorsal (FD), mid dorsal (MD), mid ventral (MV), far ventral (FV) and far far ventral (FFV) regions of the retina. Error bars are ± SEM and n=6. Results were analyzed using Student's t-test. * indicates $P<0.05$ and ** indicates $P<0.01$.

(I–L) P42 retinal sections coimmunolabeled with S opsin (green) and M opsin (red) for control (I and J) and *Dio2* mutant (K and L) indicating that in the *Dio2*$^{-/-}$ mutant the phenotype persists in the adults. (l1–4, K1–4) High magnification images of the boxed regions.

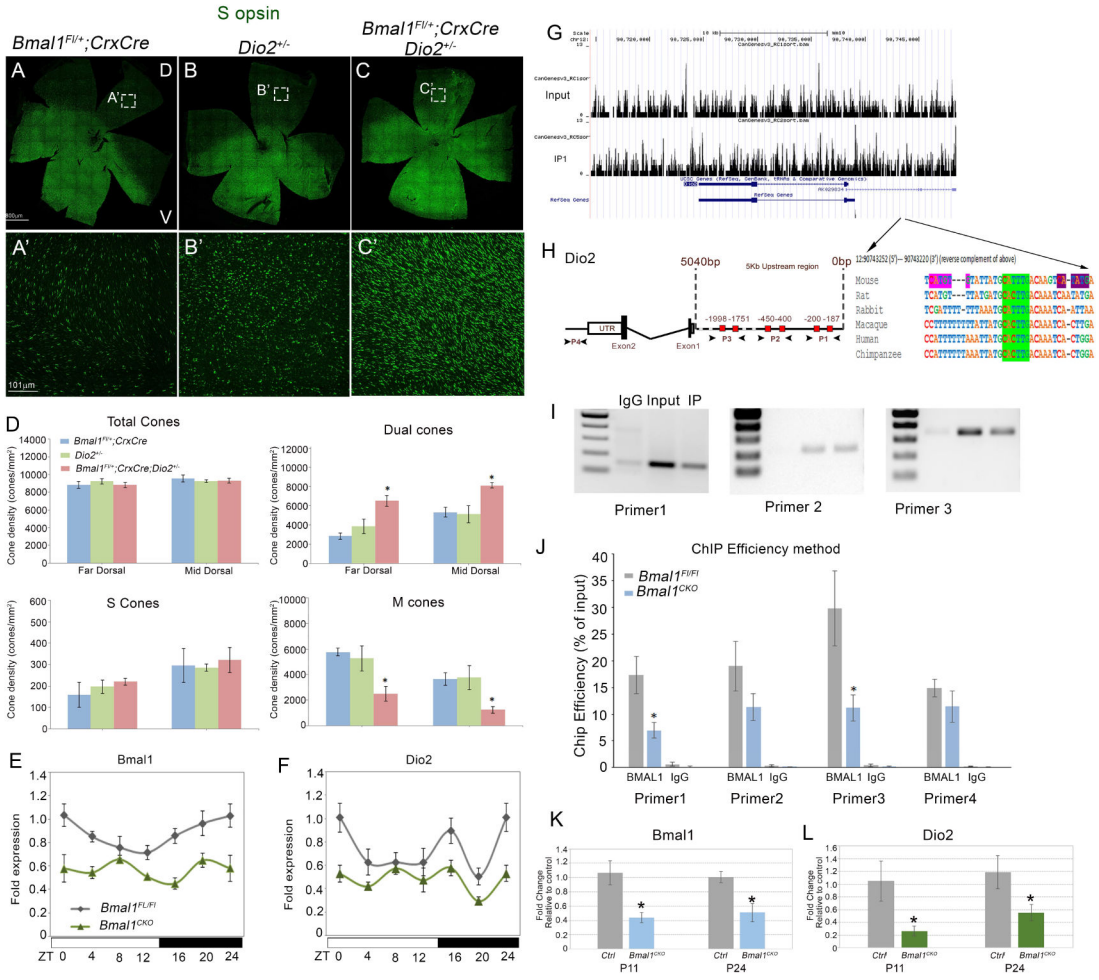

Figure 5. *Bmal1* regulates *Dio2* expression

(A–C) retinal flat mount images and (A′–C′) high magnification images from dorsal region of the retina indicating increase in S opsin (green) positive cones in double heterozygous retina (C′) compared to single heterozygous of *Bmal1* (A′) and *Dio2* (B′).

(D) Quantification of total cones, dual S+M opsins cones, genuine S opsin cones, and genuine M opsin cones in 20x fields across far dorsal (FD) and mid dorsal (MD) regions of the retina. Error bars are ± SEM and n=4–5. Results were analyzed using Student's t-test. * indicates $P<0.05$.

(E–F) Circadian oscillations in retinal *Bmal1* (E) and *Dio2* (F) transcripts from P21 animals of the indicated genotypes. Solid white and black bars on the X-axis indicate light and dark phase, respectively. *Bmal1* and *Dio2* levels were significantly lower in the *Bmal1 CKO* group compared to the control group ($F_{1, 47}=18.83$; $P<0.001$ and $F_{1, 47}=13.50$; $P<0.001$, respectively). Error bars are ± SEM and n=4. Results were analyzed using two-way ANOVA followed by Fisher LSD post-hoc test with genotype and ZT as two independent factors.

(H) Sequence analysis of mouse *Dio2* upstream region that contain known E-boxes (red boxes) that bind to BMAL1. P1, P2 and P3 indicate the primer sets designed to amplify the DNA sequence (I) from the Input, BMAl1 IP (Immunoprecipitated) and IgG IP samples. (J) ChIP-qPCR indicating decrease in ChIP efficiency in P24 *Bmal1 CKO* compared to control.

IgG was used a a non specific antibody control for the ChIP experiments. Error bars are ± SEM and n=3. Results were analyzed using Student's t-test. * indicates $P<0.05$.

(G) Coverage plots of genomic sequence surrounding *Dio2* gene in input and BMAL1 IP retinal samples (P3). Arrows indicate the region in the coverage plot with multiple peaks is a region within the *Dio2* promoter [Chr12: 90743252 (5′) --- 90743220 (3′)] that is highly conserved among different species and contains a conserved E box (green highlight). – indicates spaces in alignment. In mouse, the conserved E-box is accompanied by another BMAL1 E-box (CATGTG) (pink) separated by 7 bases and a known CLOCK binding E-box (CATATG) (violet) separated by 6 bases.

(K–L) Relative expression of *Bmal1* (K) and *Dio2* (L) transcripts in cone-enriched population from control (*tdTomato; Bmal1* $^{Fl/+}$*; Hrgp-Cre*) and *Bmal1* conditional mutant (*tdTomato; Bmal1* $^{Fl/Fl}$*; Hrgp-Cre*) animals at P11 and P24. Error bars are ± SEM and n=3. Results were analyzed using Student's t-test. * indicates $P<0.05$.

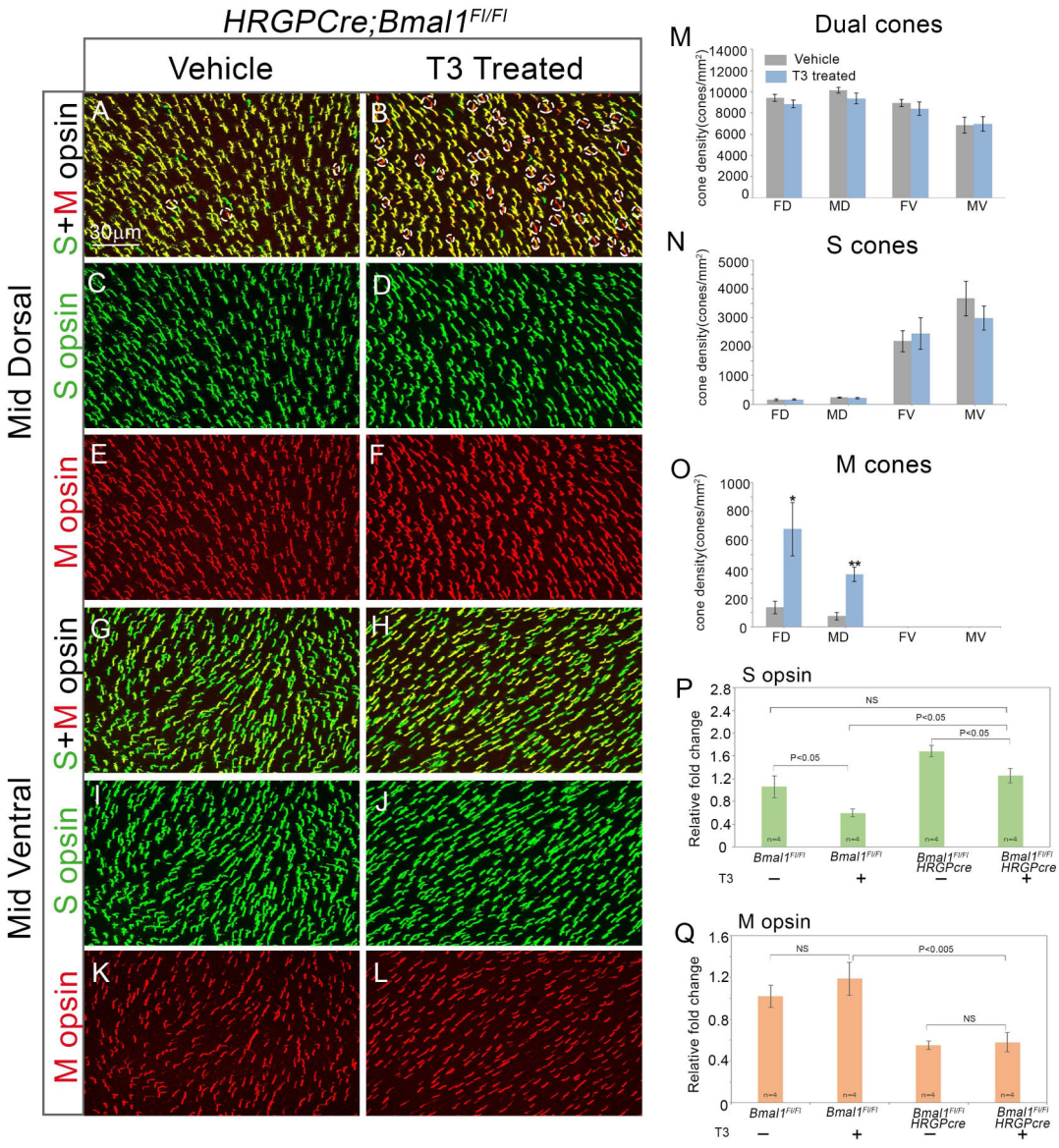

Figure 6. Partial rescue of *Bmal1* conditional mutants after T3 administration

(A–F) High magnification images from dorsal region of the retina indicating a concurrent decrease in S opsin expression and increase in M opsin expression resulting in the appearance of more M opsin cones (dotted ovals) in T3 treated *Bmal1* conditional mutants (B) compared to the untreated vehicle group (A). (G–L) High magnification images from ventral region of the retina. (M–O) Quantification of dual S+M opsins cones (M), genuine S opsin cones (N), and genuine M opsin cones (O) in 20x fields across far dorsal (FD), mid dorsal (MD), mid ventral (MV) and far ventral (FV) regions of the retina. Error bars are ± SEM and n=5. Results were analyzed using Student's t-test. * indicates $P<0.05$ and ** indicates P<0.01.

(P–Q) Relative retinal expression of S opsin (P) and M opsin (Q) transcript in P50 *Bmal1*$^{Fl/Fl}$ and *Hrgp-Cre; Bmal1*$^{Fl/Fl}$ animals from T3 supplemented and vehicle control groups using qRT-PCR. Fold change is relative to the *Bmal1*$^{Fl/Fl}$ vehicle control group. Error

bars are ± SEM and n=4. Results were analyzed using two-way ANOVA followed by Tukey post-hoc test with genotype and treatment (T3 V/S Vehicle) as two independent factors.

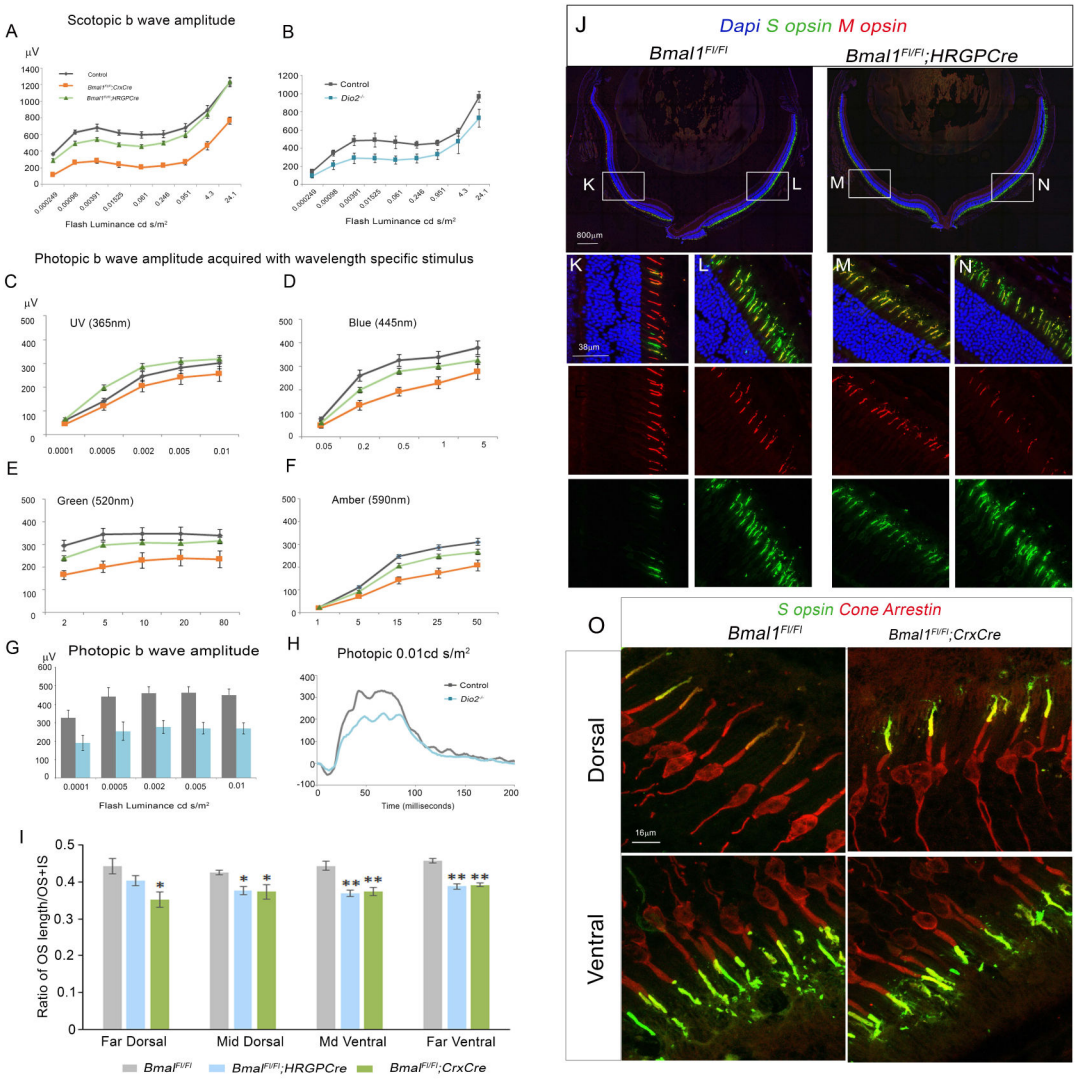

Figure 7. *Bmal1* and *Dio2* are required for adult cone function and maintenance

(A–B) Dark-adapted b-wave responses were decreased in 6 weeks old *Bmal1 CKO* (A) and *Dio2*$^{-/-}$mutants (B) compared to the reponses recorded from the respective littermate controls.

(C–F) Light adapted ERG b-wave responses in 6 weeks old *Bmal1 CKO* and littermate control mice using 365 nm (C), 445 nm (D), 520 nm (E) and 590 nm (F) of light sources. (C) b-wave responses at 365 nm were significantly higher in the *HRGP-Cre; Bmal1*$^{Fl/Fl}$ group ($F_{2, 65}$=18.74; P<0.001). (D–F) b-wave responses at 445 nm ($F_{2, 65}$=35.25; P<0.001), 520 nm ($F_{2, 65}$=41.08; P<0.001) and 590 nm ($F_{2, 65}$=40.36; P<0.001) were significantly lower in both *Bmal1* conditional mutant groups compared to the control group.

(G–H) Light-adapted b-wave responses were significantly decreased in *Dio2* mutants at 6 weeks of age. Error bars are ± SEM and n=4–8. Results were analyzed using two-way ANOVA followed by Tukey post-hoc test with genotype and flash intensity as two independent factors.

(J–N) Dorsal and ventral retinal sections from 3 month old control animal (K and L,) and *Bmal1 CKO* (M and N,) labeled with S opsin (green) and M opsin (red). (O) Dorsal and ventral retinal sections from 3 month old control animal and *Bmal1 CKO* labeled with S opsin (green) and Cone arrestin (red). (I) Quantification of cone outer segment length (OS). The cones from *Bmal1 CKO* retina have shorter OS compared to littermate controls suggesting a role for *Bmal1* in maintenance of the photoreceptors. Results were analyzed using one-way ANOVA followed by Tukey post-hoc test.* indicates $P<0.05$ and ** indicates $P<0.01$.