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Circadian clock protein BMAL1 regulates melanogenesis through *MITF* in melanoma cells

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Abstract

Solar ultraviolet B radiation (UVB) is one of the leading causes of various skin conditions, including photoaging, sunburn erythema and melanoma. As a protective response, the skin has inbuilt defense mechanisms, including DNA repair, cell cycle checkpoint, apoptosis, and melanin synthesis, of which DNA repair, cell cycle and apoptosis have been shown to be under control of the circadian clock. However, the mechanism by which the circadian clock regulates melanin synthesis is not well understood. Using human melanocytes and melanoma cells under circadian synchronized conditions, we report that the microphthalmia-associated transcription factor (MITF), a rate-limiting protein in melanin synthesis, is expressed rhythmically with 24-hour periodicity in the presence of BMAL1, a canonical clock protein. We further demonstrate that BMAL1 binds to the promoter region of *MITF* and transcriptionally regulates its expression, which positively influences melanin synthesis. Finally, we report that an increase in melanin levels due to BMAL1 overexpression protects human melanoma cells from UVB. In conclusion, our studies provide novel insights to the mechanistic role of the circadian clock in melanin synthesis and protection against UVB mediated DNA damage and genomic instability.

SIGNIFICANCE—Melanin synthesis in melanocytes is an endogenous protective mechanism of the skin against solar ultraviolet B radiation (UVB). The present study shows that *MITF*, a rate-limiting protein in the melanin synthesis pathway, is transcriptionally regulated by BMAL1, a canonical clock protein, and positively influences melanin synthesis which potentially protects

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest with the contents of this article.

the skin cells against UVB. Therefore, the mechanism of *MITF* regulation by the circadian clock protein BMAL1 is one of the additional regulatory mechanisms may contribute to the defensive arsenal of the skin against solar UVB.

Keywords

Melanogenesis; Melanin; *MITF*; BMAL1; Ultraviolet radiation

INTRODUCTION

The human body responds to various environmental cues including the light from the Sun. However, along with visible light, solar energy comprises harmful ultraviolet radiation (UVR), which is known to cause various diseases in humans (Armstrong & Kricker, 1993; Linos, Swetter, Cockburn, Colditz, & Clarke, 2009). The skin is the largest organ of the human body affected by solar ultraviolet B radiation (UVB), which is a primary cause of up to 90% of non-melanoma and about 65- 90% of melanoma skin cancer cases (Arnold et al., 2018; D'Orazio, Jarrett, Amaro-Ortiz, & Scott, 2013; Parkin, Mesher, & Sasieni, 2011; Pleasance et al., 2010). UVB primarily affects the skin and activates DNA damage response (DDR) mechanisms like melanin synthesis and nucleotide excision repair (NER) in skin cells. These mechanisms protect and maintain genomic integrity of skin cells (Brenner & Hearing, 2008; DiGiovanna & Kraemer, 2012; Scott et al., 2012).

Melanin is synthesized by melanocytes upon paracrine signaling from keratinocytes and is a potent UVB absorbent which protects keratinocyte DNA from UVB-induced damage. Similarly, the NER system removes UV-induced DNA photolesions, including cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts [(6-4) PPs] (Sancar et al., 2015). Previously, it was reported that Xeroderma pigmentosum complementation group A (XPA), an essential rate limiting NER protein, is transcriptionally regulated by the canonical clock protein BMAL1making the NER activity vary across 24 hours in a circadian manner (Dakup et al., 2018; Kang, Reardon, Kemp, & Sancar, 2009; Kang, Reardon, & Sancar, 2011). As a result, the efficiency of NER protection against UVB is high at a certain time period of the day (Gaddameedhi, Selby, Kaufmann, Smart, & Sancar, 2011). However, unlike NER, not much is known regarding the regulation of melanin synthesis by the circadian clock.

The circadian clock is a self-sustained endogenous timekeeping mechanism that is present in almost every cell of the human body. Environmental cues, including light-dark cycles, temperature, and food intake, act as zeitgebers (time-giver) and synchronize the circadian clock to the external environment. The central clock is located in the suprachiasmatic nucleus of our brain and regulates other peripheral clocks in different tissues such as the liver, heart, and skin via hormonal/neuronal signals. Synchronization of central and peripheral clocks generates physiological and behavioral rhythms (Hastings, Reddy, & Maywood, 2003; Reppert & Weaver, 2002). At the molecular level, circadian rhythms are generated through a transcriptional-translational feedback loop (TTFL). BMAL1 (Brain and muscle ARNT-like protein-1) and CLOCK (Circadian locomotor output cycles kaput) of the canonical clock transcriptionally regulates several clock-controlled genes (CCGs) including core clock genes C*RYs* (Cryptochrome 1, 2) and *PERs* (Period 1, 2, 3). Upon translation,

CRY and PER proteins dimerize and translocate into the nucleus, and bind to the CLOCK/BMAL1 heterodimer and inhibit their transcriptional activity (Reppert & Weaver, 2001; Vitaterna et al., 1999). It takes ~24 hours to complete the TTFL for generating circadian rhythms.

It is well known that the skin has a robust circadian clock, and in recent years, the skin clock has been shown to regulate various biological functions including oxidative stress, metabolism, NER, cell cycle checkpoints, and apoptosis (Gaddameedhi et al., 2011; Gaddameedhi, Selby, Kemp, Ye, & Sancar, 2015; Geyfman et al., 2012; Lin et al., 2009; Wang et al., 2017). The circadian clock helps to maintain cellular homeostasis and protects from environmental stressors, including UVR. However, dysregulation of these cellular processes due to clock disruption caused by shift work, and unscheduled eating, has been positively associated with various skin pathologies, including increased risk of melanoma (Wang et al., 2017; Yousef, Mitwally, Noufal, & Tahir, 2020). Moreover, recent transcriptome studies demonstrated that up to 43% (mouse) and 50% (humans) of all protein-coding genes show circadian rhythm throughout the body as a whole, however this percentage tends to vary depending on the tissue type (Ruben et al., 2018; Zhang, Lahens, Ballance, Hughes, & Hogenesch, 2014).

The circadian clock and melanin synthesis each play a vital role in protection against solar UVB-induced DNA damage. Previous study using human hair follicles and epidermal melanocyte cultures demonstrated that perturbation of circadian core clock genes, *BMAL1* and *PER1* positively influences melanin synthesis along with tyrosine gene expression and MITF phosphorylation levels (Hardman et al., 2015). However, a mechanistic explanation between the molecular clock and melanin synthesis remains to be explored. Therefore, in the current study, we investigated the role of the molecular clock in melanin pigmentation and its protection against UVB by demonstrating transcriptional regulation of *MITF* by a circadian clock protein BMAL1. Using circadian synchronized human melanocytes and melanoma cells, we also reported that *MITF* expression is rhythmic *in vitro* in a circadian manner. Further, our findings suggest that the increase of melanin synthesis through the canonical clock protein, BMAL1, has a protective role against solar UVB exposure.

MATERIALS AND METHODS

Cell culture and synchronization

Information about the sources and culture methods for melanoma lines are provided in Table S2. Secondary cultures of normal human melanocytes (NHM4) were a gift from Dr. William K. Kaufmann at UNC Chapel Hill (Kaufmann et al., 2008) and cultured using the DermaLife Ma Melanocyte complete kit (Lifeline Cell Technology, Cat # LL-0039). Cells were maintained in a cell culture incubator at 37°C with 5% CO_2. These cells were synchronized using 200 nM Dexamethasone (Dex.) (Sigma, Cat # D4902-100MG) for 2 hours (Kiessling et al., 2017; Papp et al., 2015). Once cells reached 85% to 100% confluency, the complete media was removed, and the cells were washed with sterile PBS, which was replaced by serum-free (SF) DMEM (Sigma, Cat # D5796-500ML) medium containing 200 nM Dex. The cells were kept in Dex containing SF media for ~2 hours,

at which point they were washed once with PBS and cultured in fresh complete media according to the cell line requirement (Kiessling et al., 2017).

Mouse skin collection

Skin samples were collected from 8 to 12-week-old C57BL/6J mice with wild-type (WT) genetic background. These mice were maintained under a 12-hour light/12-hour dark (LD 12:12) cycles for at least 4 weeks before and through the duration of the study. Following CO_2 euthanasia, the skin samples were collected after hair depilation and affixed to a section of an index card, covered with aluminum foil, snap-frozen on dry ice, and stored at −80°C until further use (Gaddameedhi et al., 2011) for ChIP assay as described in supplementary methods.

UVB treatment

Cells were treated using a UVP Black-Ray UVB Lamp (VWR, Cat # 36575-052), which emits light between 290-350 nm with peak emission at 312 nm. The UVB dose was measured using a UVX Radiometer (VWR, Cat # 97-0015-02) attached to appropriate sensors (UVB - UVX-31 Sensor 310 nm), as previously described (Gaddameedhi et al., 2011). Doses of 72 and 144 J/m^2 of UVB were selected to treat melanoma cells, as mentioned previously (Abdel-Naser, Krasagakis, Garbe, & Eberle, 2003). Before treatment, the growth medium from the cells was removed, and the cells were washed with sterile PBS. Post-treatment cells were cultured in fresh complete media.

Quantitative real time-PCR (qRT-PCR)

qRT-PCR was performed as described previously (Gaddameedhi, Reardon, Ye, Ozturk, & Sancar, 2012). In brief, SK-MEL27, PMWK, and NHM4 cells transfected with and without siBMAL1 were synchronized as described above, following which the cells were harvested every 3-hours for 24-hours using an appropriate amount of Trizol reagent (Life Technology, Cat # 15596026). RNA was isolated as described previously, and the quantity of RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) (Gaddameedhi et al., 2012). 1 μg of RNA was used to synthesize cDNA using iScript RT Supermix (Bio-Rad, Cat # 1708841). qRT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Cat # 1725124) or PERFECTA SYBR supermix-rox (VWR, Cat # 101414-160) and StepOnePlus Real-Time-PCR System (Fisher Scientific, Cat # 43-765-99) following the manufacturer's protocol. Amplification primers targeted towards *BMAL1*, *MITF*, and *ACTIN*, were ordered from IDT, where Actin was used as an internal control (Table S4). Data was analyzed as per the method previously described (Livak & Schmittgen, 2001). Data reported is the foldchange calculated using $2^{-\Delta\Delta CT}$. Circwave v1.4 was used to evaluate circadian rhythmicity of 24 hour mRNA expression (Oster, Damerow, Hut, & Eichele, 2006).

Statistical analysis

All statistics were done using Prism version 6.01 (GraphPad software). Statistical significance was measured using unpaired two-tailed Student’s t-test, One-way ANOVA, Two-way ANOVA, with Bonferroni and Tukey’s posthoc comparisons, where p-value <

0.05 was considered statistically significant. Circwave v1.4 was used for plotting cosinor analysis, and one-way ANOVA was done to determine statistical significance (Oster et al., 2006).

Additional methods, supplementary tables and figures can be found in the supplementary file.

RESULTS

Loss of *period* gene increases melanin level

To understand the mechanism associated with the circadian clock and melanin synthesis, we measured melanin levels in the absence of a canonical clock gene, *PER2*. Our observation showed significantly increased melanin levels in *PER2* knockout ($PER2^{-/-}$) compared to wild-type (WT) SK-MEL27 human melanoma cells (Fig. 1A). Next, we wanted to understand whether an increase of melanin from the loss of *PER2* gene expression was physiologically relevant. We treated WT and $PER2^{-/-}$ SK-MEL27 cells with UVB and measured UV-induced photoproduct formation by immuno-slot-blot assay. We measured significantly reduced formation of CPDs and (6-4) PPs in $PER2^{-/-}$ compared to WT cells (Fig. 1B & C). We further observed increased mRNA and total protein (all three bands taken together) expression of MITF, a known master regulator of melanin synthesis, in $PER2^{-/-}$ cells compared to WT (Figs. 1D & S1C). As PER2 is a negative regulator of BMAL1, we speculated that the melanin increase in $PER2^{-/-}$ cells was associated with an increase in BMAL1 levels. Interestingly, we observed elevated mRNA and protein levels of BMAL1 in $PER2^{-/-}$ cells compared to WT (Figs. 1D & S1B). As a control, we confirmed the knockout of PER2 expression at the mRNA and protein levels in $PER2^{-/-}$ cells (Figs. 1D & S1A). Since BMAL1 is a canonical clock transcription factor in the TTFL, we hypothesized that the increase in melanin level in $PER2^{-/-}$ cells is due to increased MITF levels through transcriptional activation by BMAL1.

BMAL1 transcriptionally regulates *MITF* and is responsible for its rhythmicity

To test our hypothesis, we performed a BMAL1-targeted chromatin immunoprecipitation (ChIP) assay using wild type mouse skin and SK-MEL27 cells. We used the *PER2* promoter region as a positive control and observed strong enrichment of BMAL1, confirming efficiency of the ChIP assay (Fig. S2A). Next, we observed relative BMAL1 enrichment of ~8-fold at *MITF* promoter region 1 (−1 to −1500 bp) and ~4-fold at region 2 (−1500 to −3000 bp) in mouse skin, as well as ~2-fold at both regions in SK-MEL27 cells (Fig. 2A). Our ChIP results show that BMAL1 binds to the *MITF* promoter region, suggesting the possible transcriptional regulation of *MITF* expression by BMAL1. We confirmed our results with existing BMAL1 ChIP-Seq data of mouse liver (data accessible at NCBI GEO database, accession GSE39860) from Takahashi's group, and found BMAL1 enrichment at the promoter region of *Mitf* (Fig. S2B).

To demonstrate transcriptional regulation of *MITF* by BMAL1, we performed a luciferase reporter assay in BMAL1 knockdown HEK293T cells (Figs. 2B & S2C). We overexpressed BMAL1 protein by a recombinant *BMAL1* MYC-DDK plasmid (BMAL1-OE) and co-

transfected *MITF* promoter-driven luciferase constructs containing an E'-Box sequence (CACGTT), a known DNA binding sequence of BMAL1, in *BMAL1* knockdown HEK293T cells. As a control, we co-transfected *MITF* promoter-driven luciferase constructs with an empty vector plasmid. Upon measuring the luminescence of the different groups mentioned above, we observed that *MITF* promoter constructs without an E'-Box (177/+136 Luc construct) had a weak luciferase signal compared to *MITF* promoter constructs containing an E'-Box (−435/+136 Luc construct), indicating that the E'-box region is necessary for the BMAL1-driven promoter activity. When comparing within the group transfected with the *MITF* promoter constructs with an E'-Box sequence, the presence of BMAL1 produced a stronger luciferase signal compared to the empty vector-transfected control group. The above result confirms the transcriptional regulation of the *MITF* gene by BMAL1, possibly through binding to the E'-Box sequence in the *MITF* promoter.

To further elucidate the transcriptional activation of *MITF* by BMAL1, we performed transient *BMAL1* overexpression (BMAL1-OE), as mentioned earlier, in human melanoma cell lines and confirmed mRNA and protein levels of MITF. Upon transient transfection of BMAL1-OE plasmid in SK-MEL27 cells, we observed a significant increase of *MITF* mRNA compared to WT (Figs. 2C & S3A). As a proof of concept, we performed a series of transductions (4) with BMAL1-OE viral particles to PMWK cells and observed protein levels of BMAL1 and MITF using western blot. We observed a corresponding increase in protein levels of MITF and recombinant BMAL1, such that the increase was directly proportional to the number of times the cells were transduced (Fig. S3B). Furthermore, we confirmed that BMAL1-OE significantly increases melanin levels compared to the control SK-MEL27 cells (Fig. 2D). These observations suggest that BMAL1 positively regulates MITF expression and melanin synthesis.

To investigate the circadian expression of *MITF* in the presence or absence of BMAL1 protein, we collected synchronized human melanoma cells (PMWK & SK-MEL27) and normal human melanocytes (NHM4) at 3-hour intervals over a period of 24 hours under siRNA control or *BMAL1* knockdown conditions. For us to determine whether a gene expression is circadian or not, we utilized two different statistical analyses, Cosinor and ANOVA. Cosinor allows us to fit a cosine wave through the data and gives us an r^2 value which is used to calculate statistical significance using F test, whereas ANOVA allows us to determine the degree of variance among the data points. Significant cosinor analysis means 24-hour circadian oscillation whereas significant ANOVA gives us information regarding tightness of the data (Oster et al., 2006; Refinetti, Lissen, & Halberg, 2007). Therefore, statistical significance of both these analyses are important for determining a circadian rhythm. Upon utilizing the above-mentioned analysis, we report statistically significant rhythmic expression of *MITF* mRNA under non-target siRNA control with the highest and lowest gene expression at ~15 hrs. and ~6 hrs. post-synchronization, respectively (Table S1). Also, the circadian expression of MITF was in-phase with BMAL1 cosine wave in all cell lines (Figs. 3A, C & E). However, a high degree of variation among biological replicates rendered the ANOVA test for NHMs non-significant. To understand whether circadian expression of *MITF* was BMAL1-dependent, we measured *MITF* mRNA levels in *BMAL1* knockdown (siBMAL1)-conditioned cells over the period of 24 hours and reported loss of *MITF* circadian rhythmicity in these cells (Figs. 3B, D & F). Together, these results from

three independent cell lines suggest circadian rhythmic expression of *MITF* in the presence of canonical clock protein BMAL1.

UVB protective effect of BMAL1 regulated melanin synthesis

To determine the physiological relevance of BMAL1-regulated melanin synthesis in UVB exposed cells, we measured the survival of cells post-UVB exposure normalized to non UVB-treated condition (Figs. S3C-E). Based on our earlier observations, increased melanin levels with the *PER2*$^{-/-}$ genetic background protected SK-MEL27 cells from UVB-induced DNA damage. Therefore, we wanted to understand whether an increase in melanin due to *PER2* loss translates to better cell survival upon UVB exposure. Interestingly, we observed significantly increased survival in *PER2*$^{-/-}$ cells compared to WT (Fig. 4A). To tie our observations back to our BMAL1-dependent mechanism, we investigated whether increased melanin due to BMAL1-OE protected cells from UVB-induced cell death. We measured survival upon UVB exposure in synchronized SK-MEL27 (Melanogenesis active) and PMWK (Melanogenesis inactive) cells with siBMAL1 and BMAL1-OE conditions using MTT and clonogenic survival assays in comparision to isogenic control (non-target siRNA). We observed significantly reduced survival in siBMAL1 PMWK cells, whereas no effect on survival was seen in SK-MEL27 cells compared to isogenic control after UVB treatment (Figs. S4A-C). In contrast, significantly increased survival was observed in the SK-MEL27 BMAL1-OE condition compared to isogenic control cells with both MTT and clonogenic assays (Figs. 4B & C). However, the PMWK BMAL1-OE cells did not show increased survival compared to control (Fig. 4D). In conclusion, the survival data suggest that melanin increase with BMAL1-OE in SK-MEL27 cells can potentially enhance protection against UVB irradiation.

DISCUSSION

Our findings are summarized as follows in Fig. 5. First, using human melanoma cell lines and mouse skin, our study suggests that BMAL1 is a novel transcriptional regulator of *MITF* and regulates *MITF* expression in a circadian manner in synchronized human melanoma and melanocyte cell lines. Secondly, we showed that BMAL1 overexpression has the capability to increase melanin level by transcriptionally upregulating MITF levels. Finally, we demonstrate that melanin increase due to positive regulation of *MITF* by BMAL1 can potentially protect against UVB-induced DNA damage and cell death.

Clock and melanin synthesis

The importance of the circadian clock in melanin synthesis has yet to be explored. In the current study, we report rhythmic expression of *MITF*, an essential melanogenesis gene, in human melanoma cells (Fig. 3) which is in accordance with previous studies in mouse liver tissue and zebrafish larva (Li, Li, Wang, Du, & Yan, 2013; Westermark & Herzel, 2013). Another study showed that knock-down of *PER1* in human hair follicles increased melanin levels relative to the control (Hardman et al., 2015), which agrees with our in vitro observation showing increased melanin in *PER2*$^{-/-}$ cells (Fig. 1A). We further confirmed the physiological relevance of increased melanin in *PER2*$^{-/-}$ cells by measuring decreased

levels of UVB-induced DNA photoproducts, which potentially resulted in increased survival against UVB exposure (Figs. 1B, C & 4A).

In addition to observing the clock influence on melanin levels in human hair follicles, Hardman et al. reported that decreased levels of *BMAL1* and *PER1* genes (siRNA approach) led to posttranslational modification, mostly phosphorylation, of MITF possibly through MAPK activation and is responsible for increased melanin levels. They also reported no difference in mRNA level of *MITF* and *BMAL1* gene in si*PER1* group (Hardman et al., 2015). Conversely, our data showed an increase in mRNA and protein levels of BMAL1 and MITF (all MITF bands) in *PER2*$^{-/-}$ cells compared to WT control, which led us to explore transcriptional regulation of *MITF* through BMAL1 (Figs. 1D & S1B-D). However, the difference in observations could be due to the robust and multifaceted nature of the molecular clock, which is attributed to the redundancy of the core clock gene paralogs. Previously, PER1 and PER2 have been shown to have different functions, including clock regulation in the cells, which could be a possible explanation for the difference in observations between the two studies (Zheng et al., 2001). Furthermore, our study does not undermine the possibility of clock-mediated post-translational regulation of MITF and supports the fact that it could be an additional regulatory pathway, as we also observed multiple MITF bands in the PER2$^{-/-}$ group but at present, it is beyond the scope of this manuscript.

MITF: A clock-controlled gene

BMAL1 is considered a rate-limiting subunit of the BMAL1/CLOCK heterodimer complex, which transcriptionally drives the expression of numerous CCGs such as *XPA*, a core NER factor (Dakup et al., 2018; Kang et al., 2009; Lowrey & Takahashi, 2011). It is well established that the BMAL1/CLOCK protein complex binds preferably to a specific *cis*-acting regulatory element, known as the E-Box sequence, at the promoter region of its target genes, which are broadly classified as canonical (E) and non-canonical (E') sequences (Munoz & Baler, 2003; Yoo et al., 2005; Yoshitane et al., 2014). To determine whether the human *MITF* promoter region contains any E-Box sequences, we searched for the presence of well-known E/E'-box sequences within the 3000 base pairs upstream of the transcription start site (TSS) of *MITF* using the eukaryotic promoter database (EPD) site. We found two E'-Box sequences (CACGTT) at positions −188 bp and −1689 bp above the TSS of *MITF* (data not shown) (Dreos, Ambrosini, Groux, Cavin Perier, & Bucher, 2017; Dreos, Ambrosini, Perier, & Bucher, 2015). In line with this, we reported enrichment of BMAL1 protein at the promoter region of mouse and human *MITF* gene through our ChIP data (Figs. 2A & S2A) and ChIP seq data in mouse liver (Fig. S2B). This indicates that there is a strong interaction between BMAL1 protein and the *MITF* promoter region regardless of tissue specificity. However, determining the transcriptional activity of such interaction is of utmost importance.

Since we observed more enrichment of BMAL1 at *MITF* promoter region 1 (−1 to −1500 bp) compared to region 2 (−1501 to −3000 bp) in human melanoma cells, we used a partial sequence of *MITF* promoter region 1 with or without the E'-Box to establish transcriptional regulation of *MITF* by BMAL1 using a reporter assay. We demonstrated that the presence of

an E'-Box sequence in the *MITF* promoter was essential for its transcriptional regulation by BMAL1 (Figs. 2B & S2B). However, we observed high background noise in the BMAL1 knockdown group with the *MITF* promoter region with an E'-Box, which could be due to binding of other transcriptional regulators of *MITF* including CREB, WNT3A, ONECUT2, and PAX3 (Kawakami & Fisher, 2017; Pierrat, Marsaud, Mauviel, & Javelaud, 2012). This observation of increased background signal indicates that BMAL1 is one of the many regulators of *MITF* and could possess a more homeostatic role in maintaining MITF levels the skin. Finally, increased MITF mRNA and melanin levels in transient BMAL1 overexpressed SK-MEL27 cells compared to the control group ties in this mechanism to the phenotypic characteristic seen in $PER2^{-/-}$ cells (Fig. 2C & D). Notably, the level of melanin increase seen in BMAL1-OE and $PER2^{-/-}$ groups are different, which is mainly due to the difference between transient vs. stable clock gene manipulation, combined with the long half-life of melanin. These results provide convincing evidence of the transcriptional regulation of *MITF* by BMAL1 can positively influence melanin synthesis.

However, to establish that *MITF* expression changes according to time of the day over 24 hours in a circadian manner, checking its expression at regular intervals for 24 hours in an entrained in vitro model is essential. At a molecular level, the circadian clock is deemed self-sustained; however, it has the property to get entrained by external environmental stimuli such as light. As cells are grown in a constant cell culture environment, they are not exposed to external stimuli like light for synchronization. Therefore, physical and chemical agents like temperature cycles, serum shock, forskolin, or dexamethasone are commonly used to entrain cells in a tissue culture setting (Balsalobre, Damiola, & Schibler, 1998; Buhr, Yoo, & Takahashi, 2010; Kiessling et al., 2017). In this study, we synchronized SK-MEL27 cells using dexamethasone and report a modest and statistically significant circadian expression of *MITF* in a BMAL1 dependent manner (Fig. 3A). A similar *MITF* rhythm was observed with PMWK (human melanoma cell line) cells and normal human melanocytes (NHM4) as well (Fig. 3C & E). We also report that *MITF* expression in these cells is in phase to *BMAL1* upon comparing the cosine wave statistics; however, the ANOVA result for *BMAL1* was found to be not significant in NHM4 cells, indicating a variability among different data points (Table S1). One of the reasons for this variability could be that NHM4 are slow dividing cells and reach confluency at different rates, which causes a lack of uniform synchronization throughout the plate. Nevertheless, the *MITF* rhythm was lost in all cell lines upon BMAL1 knockdown, bolstering the hypothesis of transcriptional regulation of *MITF* by BMAL1 (Figs. 3B, D & F).

Previously, our lab and others have shown that *XPA*, a rate limiting nucleotide excision repair (NER) protein, is transcriptionally regulated by BMAL1 in mouse skin with high NER activity in the evening compared to the morning (Dakup et al., 2018; Gaddameedhi et al., 2011; Gaddameedhi et al., 2015; Kang et al., 2009). In support, an independent group reported that the NER activity and XPA expression in human skin is opposite to that in mouse (Nikkola et al., 2018; Sarkar & Gaddameedhi, 2018). Our extensive in vitro data and in vivo mouse skin ChIP data in this study shows that BMAL1 binds the promoter region and transcriptionally regulates *MITF*, similar to *XPA*. We hypothesize that the MITF expression in human skin melanocytes will follow our results, being high during the day and low during the evening time, exhibiting a diurnal rhythm. This hypothesis reflects our

everyday living conditions as well, where MITF during the daytime will ensure efficient melanin synthesis when exposed to solar UVR. However, due to the longer half-life of melanin and the complex heterogeneity of human skin, it may be difficult to observe an oscillation in melanin levels on a daily basis but we expect that the skin's ability to produce melanin will vary according to the time of the day (Mujahid et al., 2017; Plikus et al., 2015). In addition, our reporter assay shows that BMAL1 and other transcriptional regulators of *MITF* could work synergistically to regulate MITF levels, making it a robust process to protect against UVR.

Clock and melanin: A protective partnership

The circadian clock has been shown to play a vital role in cell survival by regulating pro-survival genes, including *p53*, *NFκB*, and *BCL-2* (Granda et al., 2005; Lee & Sancar, 2011; Zhanfeng et al., 2016). Similarly, it has been shown that MITF is essential in the survival of melanocytes as well (McGill et al., 2002; Wellbrock et al., 2008). While there have been several studies aiming to understand the role of the clock or MITF independently in regulating cell survival, there are not many studies investigating the partnership or synergistic benefit of both mechanisms, and our study addresses this gap.

The negative (CRY & PER) and positive (BMAL1 & CLOCK) arms of the molecular clock have different effects on clock-controlled genes. It is well accepted that loss of *Period* gene causes a circadian defect in cells (Ko & Takahashi, 2006). In our study knocking out the *PER2* gene seems to be beneficial specifically for cell survival upon UVB exposure in melanin producing cells (Fig. 4A), which can only be due to MITF-mediated melanin increase. However, our study does not distinguish between the type of melanin produced upon $PER2^{-/-}$, nevertheless, the protective nature of the melanin against UVB-mediated photolesion formation, was further emphasized through our immuno-slot-blot data. Together showing the importance of melanin in protecting the cells against UVB and the role of circadian clock in regulating the melanogenesis.

In line with the above observation, we mechanisticaly demonstrated a protective aspect of melanin against UVB exposure due to the BMAL1-MITF transcriptional axis using both SK-MEL27 (melanogenesis active cells) and PMWK (amelanotic) cells. We have shown that upon BMAL1-OE, SK-MEL27 cells were significantly protected against UVB exposure compared to control, potentially due to their ability to produce melanin (Figs. 4B & C). Conversely, the increased survival was not seen in PMWK BMAL1-OE condition (Fig. 4D) rather, the cells were negatively affected. This is probably because the PMWK cells are amelanotic due to the inability of MITF to induce expression of enzymes required for melanin synthesis (Shields et al., 2007). Overall, this observation shows the contribution of BMAL1 and BMAL1-mediated melanin increase to protecting the cells against UVB exposure.

Further, we observed that knockdown of *BMAL1* causes PMWK cells to be statistically more vulnerable to UVB exposure compared to SK-MEL27 Figs. S4A-C. Previously, it has been reported that loss of BMAL1 and UVB exposure are independently responsible for increased reactive oxygen species (ROS) levels in skin (Geyfman et al., 2012; Heck, Vetrano, Mariano, & Laskin, 2003; Premi et al., 2015; Young, 1997). Combined with the

fact that WT melanoma cells used in our study have very low levels of melanin, such that BMAL1 knockdown did not cause detectable reduction in melanin content (data not shown), knocking down BMAL1 made the cells extremely vulnerable to UVB exposure. This phenomenon could be due to one or a combination of the following reasons: 1) increase in ROS level due to UVB exposure and BMAL1 knockdown and/or 2) disruption of clock-mediated DNA repair or oxidative stress response mechanisms.

In summary, we demonstrated a mechanistic relationship between the clock and melanin synthesis, by understanding transcriptional regulation of *MITF*, a melanin synthesis associated gene, by the core clock protein BMAL1. We further demonstrated the circadian regulation of *MITF* at the mRNA level in the presence of BMAL1. Our study supports the idea that the presence of robust circadian clock and melanin synthesis mechanisms helps in better protection against solar UVB radiation. Though our results and conlusion indicate a normal biological mechanism, we believe further studies using normal human melanocytes and in vivo skin models are required to understand the physiological relavance of our findings. As our survival data is mainly done using melanoma cell lines, it would be interesting to decipher the BMAL1-MITF signaling axis contribution in protecting heterogenous skin tissue from UVR. In addition, using human hair follicle tissue we could further extrapolate our mouse BMAL1 ChIP data and MITF rhythmic data in melanocytes to human skin tissue. The above-mentioned future directions would eventually enable us to utilize the BMAL1-MITF axis as a possible therapeutic target to enhance melanin synthesis in future sunscreen development strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

UVR	ultraviolet radiation
UVB	ultraviolet B radiation
Dex	Dexamethasone
NER	nucleotide excision repair
CCG	clock-controlled gene

REFERENCES

Abdel-Naser MB, Krasagakis K, Garbe C, & Eberle J (2003). Direct effects on proliferation, antigen expression and melanin synthesis of cultured normal human melanocytes in response to UVB and UVA light. Photodermatol Photoimmunol Photomed, 19(3), 122–127. [PubMed: 12914596]

Armstrong BK, & Kricker A (1993). How much melanoma is caused by sun exposure? Melanoma Res, 3(6), 395–401. [PubMed: 8161879]

Arnold M, de Vries E, Whiteman DC, Jemal A, Bray F, Parkin DM, & Soerjomataram I (2018). Global burden of cutaneous melanoma attributable to ultraviolet radiation in 2012. Int J Cancer, 143(6), 1305–1314. doi:10.1002/ijc.31527 [PubMed: 29659012]

Balsalobre A, Damiola F, & Schibler U (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell, 93(6), 929–937. [PubMed: 9635423]

Brenner M, & Hearing VJ (2008). The protective role of melanin against UV damage in human skin. Photochem Photobiol, 84(3), 539–549. doi:10.1111/j.1751-1097.2007.00226.x [PubMed: 18435612]

Buhr ED, Yoo SH, & Takahashi JS (2010). Temperature as a universal resetting cue for mammalian circadian oscillators. Science, 330(6002), 379–385. doi:10.1126/science.1195262 [PubMed: 20947768]

D'Orazio J, Jarrett S, Amaro-Ortiz A, & Scott T (2013). UV radiation and the skin. Int J Mol Sci, 14(6), 12222–12248. doi:10.3390/ijms140612222 [PubMed: 23749111]

Dakup PP, Porter KI, Little AA, Gajula RP, Zhang H, Skornyakov E, … Gaddameedhi S (2018). The circadian clock regulates cisplatin-induced toxicity and tumor regression in melanoma mouse and human models. Oncotarget, 9(18), 14524–14538. doi:10.18632/oncotarget.24539 [PubMed: 29581861]

DiGiovanna JJ, & Kraemer KH (2012). Shining a light on xeroderma pigmentosum. J Invest Dermatol, 132(3 Pt 2), 785–796. doi:10.1038/jid.2011.426 [PubMed: 22217736]

Dreos R, Ambrosini G, Groux R, Cavin Perier R, & Bucher P (2017). The eukaryotic promoter database in its 30th year: focus on non-vertebrate organisms. Nucleic Acids Res, 45(D1), D51–D55. doi:10.1093/nar/gkw1069 [PubMed: 27899657]

Dreos R, Ambrosini G, Perier RC, & Bucher P (2015). The Eukaryotic Promoter Database: expansion of EPDnew and new promoter analysis tools. Nucleic Acids Res, 43(Database issue), D92–96. doi:10.1093/nar/gku1111 [PubMed: 25378343]

Gaddameedhi S, Reardon JT, Ye R, Ozturk N, & Sancar A (2012). Effect of circadian clock mutations on DNA damage response in mammalian cells. Cell Cycle, 11(18), 3481–3491. doi:10.4161/cc.21771 [PubMed: 22918252]

Gaddameedhi S, Selby CP, Kaufmann WK, Smart RC, & Sancar A (2011). Control of skin cancer by the circadian rhythm. Proc Natl Acad Sci U S A, 108(46), 18790–18795. doi:10.1073/pnas.1115249108 [PubMed: 22025708]

Gaddameedhi S, Selby CP, Kemp MG, Ye R, & Sancar A (2015). The circadian clock controls sunburn apoptosis and erythema in mouse skin. J Invest Dermatol, 135(4), 1119–1127. doi:10.1038/jid.2014.508 [PubMed: 25431853]

Geyfman M, Kumar V, Liu Q, Ruiz R, Gordon W, Espitia F, … Andersen B (2012). Brain and muscle Arnt-like protein-1 (BMAL1) controls circadian cell proliferation and susceptibility to UVB-induced DNA damage in the epidermis. Proc Natl Acad Sci U S A, 109(29), 11758–11763. doi:10.1073/pnas.1209592109 [PubMed: 22753467]

Granda TG, Liu XH, Smaaland R, Cermakian N, Filipski E, Sassone-Corsi P, & Levi F (2005). Circadian regulation of cell cycle and apoptosis proteins in mouse bone marrow and tumor. FASEB J, 19(2), 304–306. doi:10.1096/fj.04-2665fje [PubMed: 15545298]

Hardman JA, Tobin DJ, Haslam IS, Farjo N, Farjo B, Al-Nuaimi Y, … Paus R (2015). The peripheral clock regulates human pigmentation. J Invest Dermatol, 135(4), 1053–1064. doi:10.1038/jid.2014.442 [PubMed: 25310406]

Hastings MH, Reddy AB, & Maywood ES (2003). A clockwork web: circadian timing in brain and periphery, in health and disease. Nat Rev Neurosci, 4(8), 649–661. doi:10.1038/nrn1177 [PubMed: 12894240]

Heck DE, Vetrano AM, Mariano TM, & Laskin JD (2003). UVB light stimulates production of reactive oxygen species: unexpected role for catalase. J Biol Chem, 278(25), 22432–22436. doi:10.1074/jbc.C300048200 [PubMed: 12730222]

Kang TH, Reardon JT, Kemp M, & Sancar A (2009). Circadian oscillation of nucleotide excision repair in mammalian brain. Proc Natl Acad Sci U S A, 106(8), 2864–2867. doi:10.1073/pnas.0812638106 [PubMed: 19164551]

Kang TH, Reardon JT, & Sancar A (2011). Regulation of nucleotide excision repair activity by transcriptional and post-transcriptional control of the XPA protein. Nucleic Acids Res, 39(8), 3176–3187. doi:10.1093/nar/gkq1318 [PubMed: 21193487]

Kawakami A, & Fisher DE (2017). The master role of microphthalmia-associated transcription factor in melanocyte and melanoma biology. Lab Invest, 97(6), 649–656. doi:10.1038/labinvest.2017.9 [PubMed: 28263292]

Kiessling S, Beaulieu-Laroche L, Blum ID, Landgraf D, Welsh DK, Storch KF, … Cermakian N (2017). Enhancing circadian clock function in cancer cells inhibits tumor growth. BMC Biol, 15(1), 13. doi:10.1186/s12915-017-0349-7 [PubMed: 28196531]

Ko CH, & Takahashi JS (2006). Molecular components of the mammalian circadian clock. Hum Mol Genet, 15 Spec No 2, R271–277. doi:10.1093/hmg/ddl207 [PubMed: 16987893]

Lee JH, & Sancar A (2011). Regulation of apoptosis by the circadian clock through NF-kappaB signaling. Proc Natl Acad Sci U S A, 108(29), 12036–12041. doi:10.1073/pnas.1108125108 [PubMed: 21690409]

Li Y, Li G, Wang H, Du J, & Yan J (2013). Analysis of a gene regulatory cascade mediating circadian rhythm in zebrafish. PLoS Comput Biol, 9(2), e1002940. doi:10.1371/journal.pcbi.1002940 [PubMed: 23468616]

Lin KK, Kumar V, Geyfman M, Chudova D, Ihler AT, Smyth P, … Andersen B (2009). Circadian clock genes contribute to the regulation of hair follicle cycling. PLoS Genet, 5(7), e1000573. doi:10.1371/journal.pgen.1000573 [PubMed: 19629164]

Linos E, Swetter SM, Cockburn MG, Colditz GA, & Clarke CA (2009). Increasing burden of melanoma in the United States. J Invest Dermatol, 129(7), 1666–1674. doi:10.1038/jid.2008.423 [PubMed: 19131946]

Livak KJ, & Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25(4), 402–408. doi:10.1006/meth.2001.1262 [PubMed: 11846609]

Lowrey PL, & Takahashi JS (2011). Genetics of circadian rhythms in Mammalian model organisms. Adv Genet, 74, 175–230. doi:10.1016/B978-0-12-387690-4.00006-4 [PubMed: 21924978]

McGill GG, Horstmann M, Widlund HR, Du J, Motyckova G, Nishimura EK, … Fisher DE (2002). Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell, 109(6), 707–718. [PubMed: 12086670]

Mujahid N, Liang Y, Murakami R, Choi HG, Dobry AS, Wang J, … Fisher DE (2017). A UV-Independent Topical Small-Molecule Approach for Melanin Production in Human Skin. Cell Rep, 19(11), 2177–2184. doi:10.1016/j.celrep.2017.05.042 [PubMed: 28614705]

Munoz E, & Baler R (2003). The circadian E-box: when perfect is not good enough. Chronobiol Int, 20(3), 371–388. [PubMed: 12868535]

Nikkola V, Gronroos M, Huotari-Orava R, Kautiainen H, Ylianttila L, Karppinen T, … Snellman E (2018). Circadian Time Effects on NB-UVB-Induced Erythema in Human Skin In Vivo. J Invest Dermatol, 138(2), 464–467. doi:10.1016/j.jid.2017.08.016 [PubMed: 28844942]

Oster H, Damerow S, Hut RA, & Eichele G (2006). Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleosome assembly genes. J Biol Rhythms, 21(5), 350–361. doi:10.1177/0748730406293053 [PubMed: 16998155]

Papp SJ, Huber AL, Jordan SD, Kriebs A, Nguyen M, Moresco JJ, … Lamia KA (2015). DNA damage shifts circadian clock time via Hausp-dependent Cry1 stabilization. Elife, 4. doi:10.7554/eLife.04883

Parkin DM, Mesher D, & Sasieni P (2011). 13. Cancers attributable to solar (ultraviolet) radiation exposure in the UK in 2010. Br J Cancer, 105 Suppl 2, S66–69. doi:10.1038/bjc.2011.486 [PubMed: 22158324]

Pierrat MJ, Marsaud V, Mauviel A, & Javelaud D (2012). Expression of microphthalmia-associated transcription factor (MITF), which is critical for melanoma progression, is inhibited by both transcription factor GLI2 and transforming growth factor-beta. J Biol Chem, 287(22), 17996–18004. doi:10.1074/jbc.M112.358341 [PubMed: 22496449]

Pleasance ED, Cheetham RK, Stephens PJ, McBride DJ, Humphray SJ, Greenman CD, … Stratton MR (2010). A comprehensive catalogue of somatic mutations from a human cancer genome. Nature, 463(7278), 191–196. doi:10.1038/nature08658 [PubMed: 20016485]

Plikus MV, Van Spyk EN, Pham K, Geyfman M, Kumar V, Takahashi JS, & Andersen B (2015). The circadian clock in skin: implications for adult stem cells, tissue regeneration, cancer, aging, and immunity. J Biol Rhythms, 30(3), 163–182. doi:10.1177/0748730414563537 [PubMed: 25589491]

Premi S, Wallisch S, Mano CM, Weiner AB, Bacchiocchi A, Wakamatsu K, … Brash DE (2015). Photochemistry. Chemiexcitation of melanin derivatives induces DNA photoproducts long after UV exposure. Science, 347(6224), 842–847. doi:10.1126/science.1256022 [PubMed: 25700512]

Refinetti R, Lissen GC, & Halberg F (2007). Procedures for numerical analysis of circadian rhythms. Biol Rhythm Res, 38(4), 275–325. doi:10.1080/09291010600903692 [PubMed: 23710111]

Reppert SM, & Weaver DR (2001). Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol, 63, 647–676. doi:10.1146/annurev.physiol.63.1.647 [PubMed: 11181971]

Reppert SM, & Weaver DR (2002). Coordination of circadian timing in mammals. Nature, 418(6901), 935–941. doi:10.1038/nature00965 [PubMed: 12198538]

Ruben MD, Wu G, Smith DF, Schmidt RE, Francey LJ, Lee YY, … Hogenesch JB (2018). A database of tissue-specific rhythmically expressed human genes has potential applications in circadian medicine. Sci Transl Med, 10(458). doi:10.1126/scitranslmed.aat8806

Sancar A, Lindsey-Boltz LA, Gaddameedhi S, Selby CP, Ye R, Chiou YY, … Ozturk N (2015). Circadian clock, cancer, and chemotherapy. Biochemistry, 54(2), 110–123. doi:10.1021/bi5007354 [PubMed: 25302769]

Sarkar S, & Gaddameedhi S (2018). UV-B-Induced Erythema in Human Skin: The Circadian Clock Is Ticking. J Invest Dermatol, 138(2), 248–251. doi:10.1016/j.jid.2017.09.002 [PubMed: 29389325]

Scott TL, Christian PA, Kesler MV, Donohue KM, Shelton B, Wakamatsu K, … D'Orazio J (2012). Pigment-independent cAMP-mediated epidermal thickening protects against cutaneous UV injury by keratinocyte proliferation. Exp Dermatol, 21(10), 771–777. doi:10.1111/exd.12012 [PubMed: 23078399]

Shields JM, Thomas NE, Cregger M, Berger AJ, Leslie M, Torrice C, … Sharpless NE (2007). Lack of extracellular signal-regulated kinase mitogen-activated protein kinase signaling shows a new type of melanoma. Cancer Res, 67(4), 1502–1512. doi:10.1158/0008-5472.CAN-06-3311 [PubMed: 17308088]

Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, Fruechte EM, … Sancar A (1999). Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. Proc Natl Acad Sci U S A, 96(21), 12114–12119. [PubMed: 10518585]

Wang H, van Spyk E, Liu Q, Geyfman M, Salmans ML, Kumar V, … Andersen B (2017). Time-Restricted Feeding Shifts the Skin Circadian Clock and Alters UVB-Induced DNA Damage. Cell Rep, 20(5), 1061–1072. doi:10.1016/j.celrep.2017.07.022 [PubMed: 28768192]

Wellbrock C, Rana S, Paterson H, Pickersgill H, Brummelkamp T, & Marais R (2008). Oncogenic BRAF regulates melanoma proliferation through the lineage specific factor MITF. PLoS One, 3(7), e2734. doi:10.1371/journal.pone.0002734 [PubMed: 18628967]

Westermark PO, & Herzel H (2013). Mechanism for 12 hr rhythm generation by the circadian clock. Cell Rep, 3(4), 1228–1238. doi:10.1016/j.celrep.2013.03.013 [PubMed: 23583178]

Yoo SH, Ko CH, Lowrey PL, Buhr ED, Song EJ, Chang S, … Takahashi JS (2005). A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. Proc Natl Acad Sci U S A, 102(7), 2608–2613. doi:10.1073/pnas.0409763102 [PubMed: 15699353]

Yoshitane H, Ozaki H, Terajima H, Du NH, Suzuki Y, Fujimori T, … Fukada Y (2014). CLOCK-controlled polyphonic regulation of circadian rhythms through canonical and noncanonical E-boxes. Mol Cell Biol, 34(10), 1776–1787. doi:10.1128/MCB.01465-13 [PubMed: 24591654]

Young AR (1997). Chromophores in human skin. Phys Med Biol, 42(5), 789–802. doi:10.1088/0031-9155/42/5/004 [PubMed: 9172259]

Yousef E, Mitwally N, Noufal N, & Tahir MR (2020). Shift work and risk of skin cancer: A systematic review and meta-analysis. Sci Rep, 10(1), 2012. doi:10.1038/s41598-020-59035-x [PubMed: 32029836]

Zhanfeng N, Chengquan W, Hechun X, Jun W, Lijian Z, Dede M, … Lei Y (2016). Period2 downregulation inhibits glioma cell apoptosis by activating the MDM2-TP53 pathway. Oncotarget, 7(19), 27350–27362. doi:10.18632/oncotarget.8439 [PubMed: 27036047]

Zhang R, Lahens NF, Ballance HI, Hughes ME, & Hogenesch JB (2014). A circadian gene expression atlas in mammals: implications for biology and medicine. Proc Natl Acad Sci U S A, 111(45), 16219–16224. doi:10.1073/pnas.1408886111 [PubMed: 25349387]

Zheng B, Albrecht U, Kaasik K, Sage M, Lu W, Vaishnav S, … Lee CC (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell, 105(5), 683–694. [PubMed: 11389837]

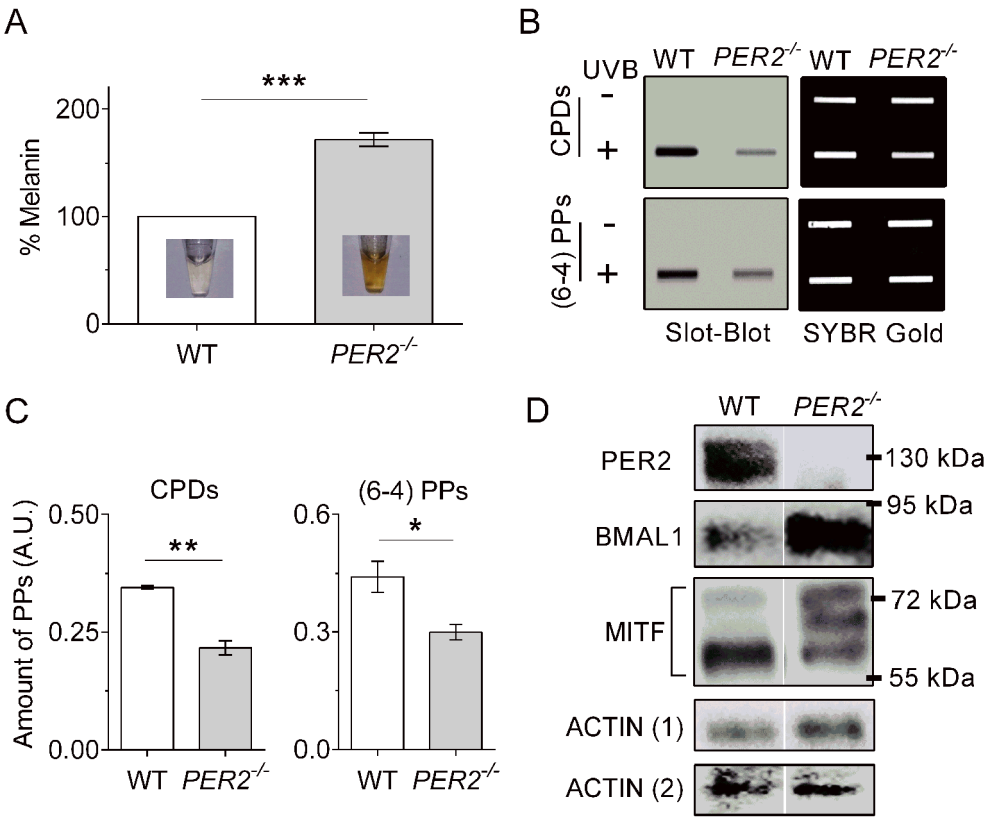

Figure 1. Loss of period gene increases cellular protection against UVB through melanin.

(A) Percentage of melanin measured in Wild-type (WT) and PER2 knockout (*PER2*$^{-/-}$) human melanoma cells (SK-MEL27). Bar graph represents mean ± SEM values of % normalized to WT from 3 biological replicates. Statistical difference was determined using the student's t test (***p<0.001). **(B)** Representative image of slot-blot assay performed using WT and *PER2*$^{-/-}$ SK-MEL27 cells to measure the amount of CPDs and (6-4) PPs formed immediately after UVB exposure (144 J/m^2). SYBR Gold was used as a loading control. **(C)** Quantitation of CPD and (6-4) PP immuno-slot-blot signals from the slot blot assay of WT and *PER*$^{-/-}$ SK-MEL27 cells. Each bar represents the mean ± SEM from 3 biological replicates; statistical analysis was done by using the student's t test with *p<0.05, and **p<0.01 was considered significant. **(D)** Western blot image showing the levels of PER2, BMAL1, and MITF proteins in WT and *PER2*$^{-/-}$ SK-MEL27 cells. Both groups were run on the same gel, but not adjacent to each other, ACTIN (1) is the loading control for PER2 & MITF bands, whereas ACTIN (2) is a loading control of BMAL1 band which was run on a different gel.

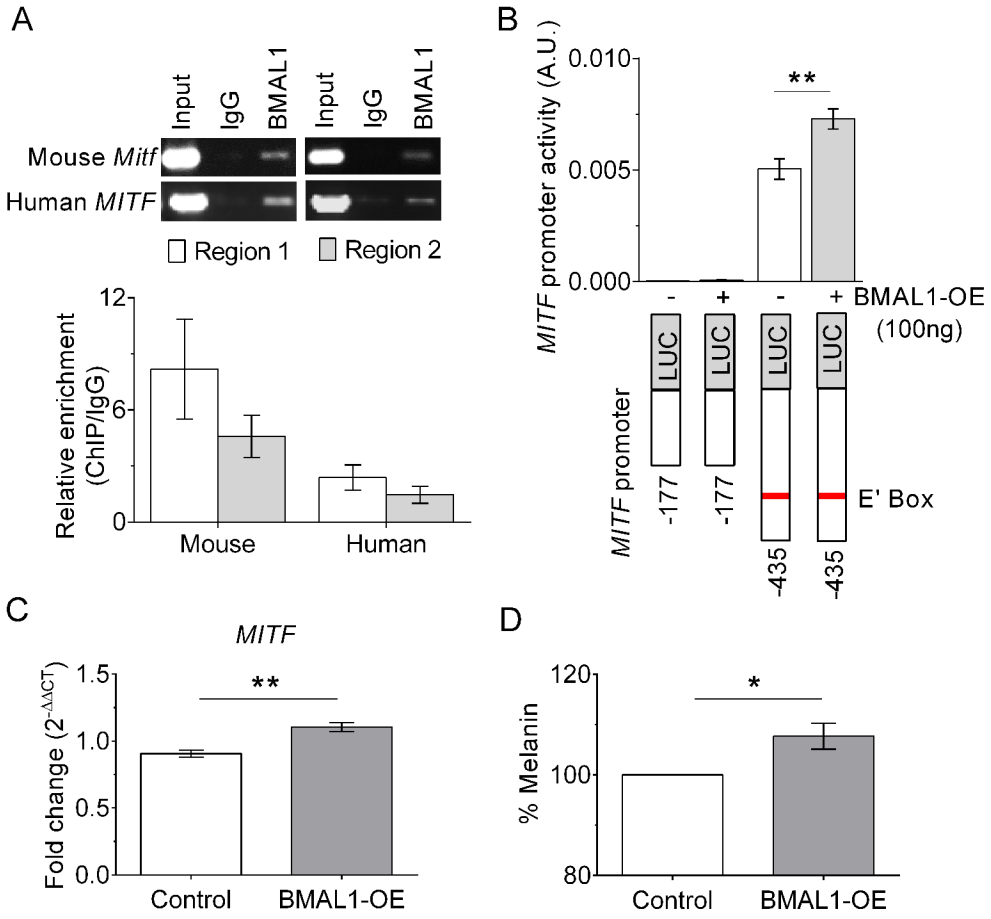

Figure 2. BMAL1 transcriptionally regulates *MITF* by binding to its promoter region.

(A) Upper panel is a representative ChIP-PCR image showing PCR product of *MITF* promoter region 1 (−1 to −1500 bp) and region 2 (−1501 to −3000) from WT mouse skin (*Mitf*) and human melanoma cells (SK-MEL27) (*MITF*). Input is the loading control, and IgG is the negative control. Lower panel is the quantitative bar graph (Mean ± SEM, n=3) representing the relative enrichment of BMAL1 at the *MITF* promoter to IgG. **(B)** Represents the *MITF* promoter activity in *BMAL1* knockdown HEK293T cells co-transfected with MITF partial promoter-driven luciferase plasmids, −177/+136Luc and −435/+136Luc (as shown in the figure), and BMAL1-OE using *BMAL1* MYC-DDK plasmid (100 ng). Luciferase signal was normalized to the *Renilla* signal. (Mean ± SEM, n=3, student's t test, **p<0.01) (**C**) *MITF* mRNA level in SK-MEL27 control and BMAL1-OE conditions, reported as $2^{-\Delta\Delta CT}$ relative to *ACTIN* mRNA (Fold change). **(D)** Percent melanin present in control vs. BMAL1-OE condition in SK-MEL27 human melanoma cells was measured using a synthetic melanin standard curve. Each bar in C & D represent mean ± SEM (n = 4 & 3 resp.) and statistical significance were calculated by student's t- test (*p<0.05).

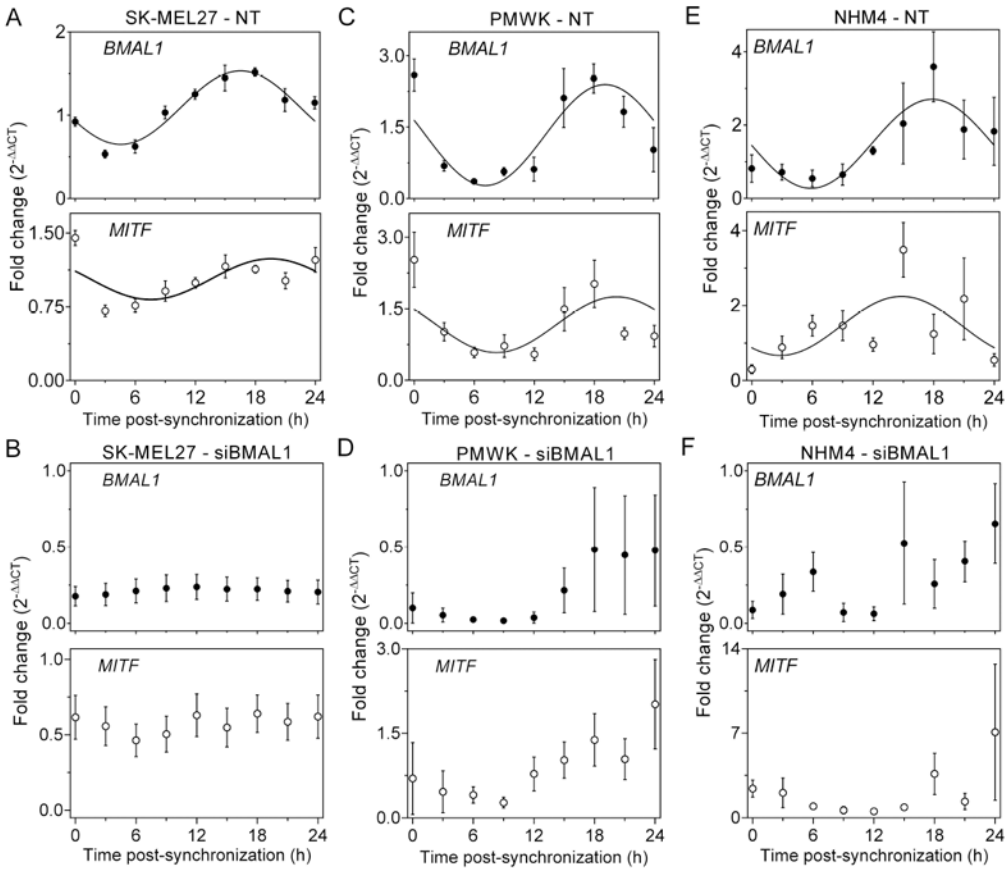

Figure 3. Rhythmic expression of MITF.

(A, C & E) Expression level of *BMAL1* & *MITF* mRNA, reported as $2^{-\Delta\Delta CT}$ relative to *ACTIN* (Fold change), in dexamethasone synchronized human melanoma cells SK-MEL27 (n=6), PMWK (n=3) and NHM4 (n=3) treated with Non-target (NT) siRNA. (**B, D & F**) mRNA level of *BMAL1* and *MITF* in siBMAL1 treated human melanoma and melanocyte cells relative to NT. Each data point represents the mean ± SEM data per time point collected every 3 hours for 24 hours. All the cosine wave fitted in this figure was generated using Circwave v1.4, and statistical significance was measured using F test and ANOVA, where p-value < 0.05 was considered significant (Table S1).

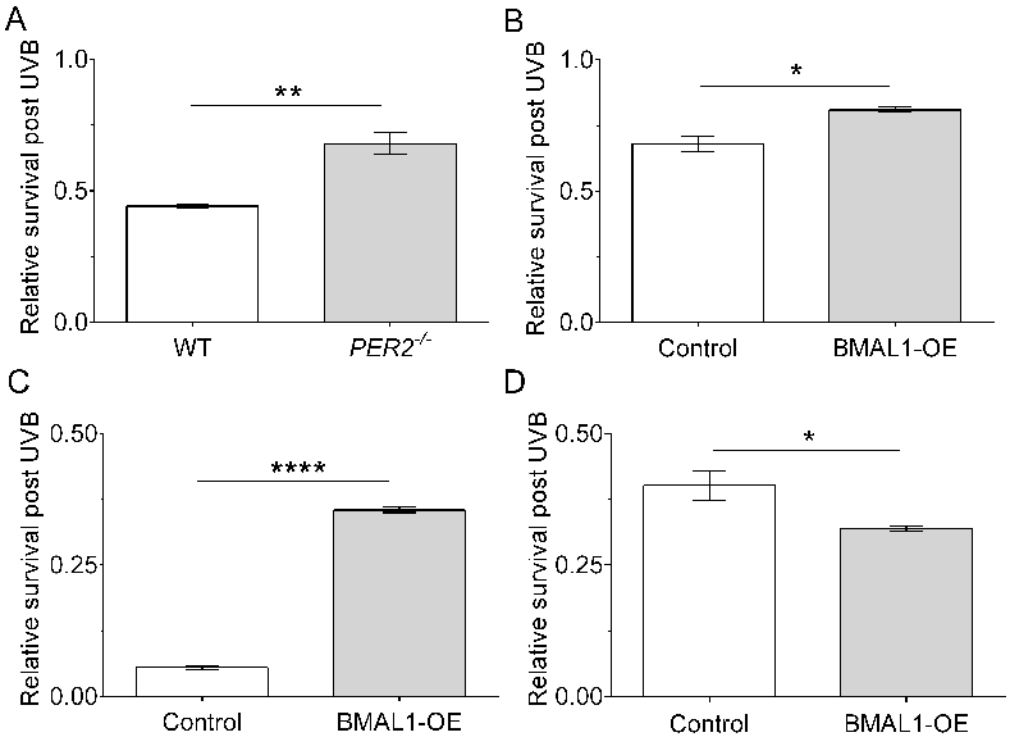

Figure 4. BMAL1 overexpression critical for survival outcomes upon UVB exposure.

(A) Relative survival of Dex. synchronized SK-MEL27 WT vs. *PER2*$^{-/-}$ cells upon UVB exposure (144 J/m^2) measured by MTT assay. (**B & C**) Relative survival of Control and BMAL1-OE SK-MEL27 cells upon UVB (144 J/m^2) exposure determined using MTT (B) and clonogenic assays (C). (**D**) Survival of control and BMAL1-OE conditioned PMWK cells upon UVB (144 J/m^2) exposure was measured by performing the clonogenic assay. For all the above figures, the relative survival was calculated by normalizing the UVB treated group to its non-UVB treated group and is represented as mean ± SEM from 3 biological replicates. Statistical significance was measured using either student's test, where *p<0.05, **p<0.01 & ****p<0.0001 was considered significant.

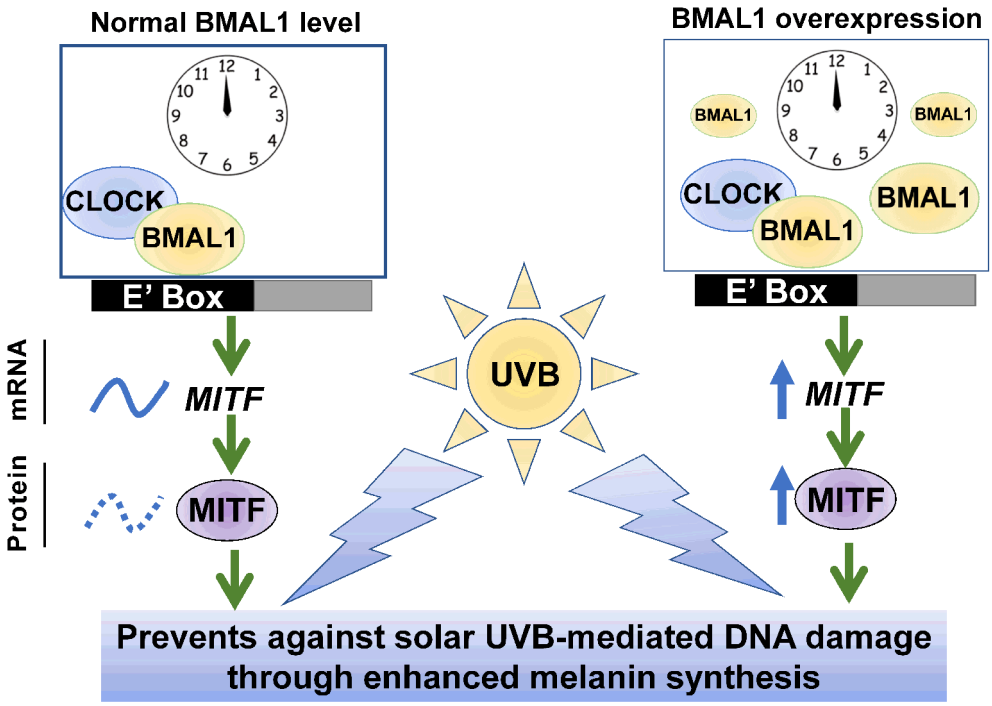

Figure 5. BMAL1 influences melanin synthesis in the skin through MITF.

Schematic representation of a molecular mechanism by which the circadian clock protects the skin against solar UVB. Our study shows that *MITF*, the master regulator of melanin synthesis, is a clock-controlled gene transcriptionally regulated by BMAL1. This clock regulatory mechanism positively influences melanin synthesis and helps better protect skin against UVB-mediated genomic instability.