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Divergence of cAMP signalling pathways mediating augmented nucleotide excision repair and pigment induction in melanocytes

Erin M. Wolf Horrell[1,3] | Stuart G. Jarrett[2,3] | Katharine M. Carter[3] | John A. D'Orazio[1,2,3,4,5]

[1]Department of Physiology, University of Kentucky College of Medicine, Lexington, KY, USA

[2]Department of Toxicology and Cancer Biology, University of Kentucky College of Medicine, Lexington, KY, USA

[3]Markey Cancer Center, University of Kentucky College of Medicine, Lexington, KY, USA

[4]Department of Pharmacology and Nutritional Sciences, University of Kentucky College of Medicine, Lexington, KY, USA

[5]Department of Pediatrics, University of Kentucky College of Medicine, Lexington, KY, USA

Correspondence
John D'Orazio, MD, PhD, Associate Professor of Pediatrics, University of Kentucky College of Medicine, Markey Cancer Center, Combs Research, Building Room 204, 800 Rose Street, Lexington, KY, 40536, USA.
Email: jdorazio@uky.edu

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Abstract

Loss-of-function melanocortin 1 receptor (MC1R) polymorphisms are common in UV-sensitive fair-skinned individuals and are associated with blunted cAMP second messenger signalling and higher lifetime risk of melanoma because of diminished ability of melanocytes to cope with UV damage. cAMP signalling positions melanocytes to resist UV injury by upregulating synthesis of UV-blocking eumelanin pigment and by enhancing the repair of UV-induced DNA damage. cAMP enhances melanocyte nucleotide excision repair (NER), the genome maintenance pathway responsible for the removal of mutagenic UV photolesions, through cAMP-activated protein kinase (protein kinase A)-mediated phosphorylation of the ataxia telangiectasia-mutated and Rad3-related (ATR) protein on the S435 residue. We investigated the interdependence of cAMP-mediated melanin upregulation and cAMP-enhanced DNA repair in primary human melanocytes and a melanoma cell line. We observed that the ATR-dependent molecular pathway linking cAMP signalling to the NER pathway is independent of MITF activation. Similarly, cAMP-mediated upregulation of pigment synthesis is independent of ATR, suggesting that the key molecular events driving MC1R-mediated enhancement of genome maintenance (eg PKA-mediated phosphorylation of ATR) and MC1R-induced pigment induction (eg MITF activation) are distinct.

KEYWORDS
ATR, DNA repair, melanin, melanocortin 1 receptor, microphthalmia, UV

1 | INTRODUCTION

Loss-of-function polymorphisms in the melanocortin 1 receptor (MC1R) signalling axis represent a major inherited risk factor for melanoma,[1-3] a disease which currently affects nearly 1 in 50 people in the United States and accounts for more than 80% of skin cancer deaths.[4] Ultraviolet radiation (UV) is a major environmental driver of melanoma, as evidenced by the abundance of "UV-signature" pyrimidine transitional mutations in many melanomas.[5,6] UV-signature mutations result from the formation of UV photoproducts including cyclobutane pyrimidine dimers (CPDs), which if not repaired in a timely manner, can result in mutations and promote carcinogenesis and tumor progression. [7]

MC1R, a G_s protein-coupled receptor located on the melanocyte cell membrane, is integral to physiologic melanocytic UV responses. MC1R signalling results in the accumulation of cAMP and activation of cAMP-dependent protein kinase (protein kinase A; PKA).[8-11] cAMP stimulation increases synthesis of the brown/black UV-blocking pigment eumelanin[12,13] which accumulates in the epidermis to protect the skin against UV injury.[14,15] Induction of eumelanin synthesis is largely dependent upon PKA-mediated phosphorylation of the cAMP responsive binding element (CREB) transcription factor at Ser133 and subsequent

induction of the microphthalmia-associated transcription factor (MITF) protein.[16,17] In turn, MITF promotes the transcription of melanogenic enzymes including tyrosinase and dopachrome tautomerase.[18,19]

In addition to regulating melanin production, we and others have documented that MC1R signalling protects melanocytes from UV damage by pigment-independent pathways, specifically by protecting the cell against UV mutagenesis and enhancing genomic stability.[11,20-24] MC1R signalling enhances nucleotide excision repair (NER), the genome maintenance pathway responsible for identifying and repairing UV-induced DNA photolesions. Recently, we reported that the critical molecular event linking MC1R signalling to NER is a PKA-mediated post-translational modification of the ataxia telangiectasia-mutated and Rad3-related (ATR) protein. ATR is a global damage response protein that mediates checkpoint blockade of the cell cycle and activates damage repair pathways. When a cell is damaged, ATR promotes G_2/M checkpoint blockade by phosphorylating Chk1 kinase.[25,26] We identified a repair-specific function of ATR downstream of MC1R/cAMP signalling. When ATR is phosphorylated by PKA on its S435 residue, it facilitates NER by accelerating association of the rate-limiting NER factor xeroderma pigmentosum complementation group A protein (XPA) with nuclear UV photolesions.[11] Thus, in melanocytes, PKA-mediated phosphorylation of ATR on the S435 residue changes ATR function away from checkpoint signalling to a more DNA repair-specific physiology.

MC1R signalling positions melanocytes to better cope with UV injury by increasing eumelanin production and by enhancing genomic stability; however, it is unclear whether there may be crosstalk between these cAMP-dependent downstream events. MITF is a cAMP-induced transcription that is critical to melanin induction[18,19] and increases expression of genes associated with NER including *RPA2* and *POLE2* among others.[27] More recently, MITF was reported to promote Wnt signalling in melanocytes,[28] raising the possibility that MITF may influence signalling events. We therefore sought to determine whether MITF is required for MC1R enhancement of NER via PKA-mediated generation of ATR-p435S. Similarly, as ATR is a Ser/Thr kinase with a host of potential targets,[29] we explored whether ATR kinase function may impact MC1R-induced pigment enzyme induction and eumelanin synthesis. We found that basal and cAMP-accelerated NER kinetics are MITF-independent and that MC1R-mediated upregulation of tyrosinase and melanin synthesis is independent of ATR kinase function. Our findings suggest that cAMP-mediated induction of eumelanin synthesis and acceleration of NER diverge downstream of MC1R signalling and offer the opportunity of exploiting DNA repair benefit for increasing resistance of melanocytes to carcinogenic degeneration independent of melanin induction.

2 | METHODS

2.1 | Cell lines and pharmacologic drugs

Transformed melanoma SK-MEL-2 (ATCC) cells and primary human melanocytes (Coriell Institute for Medical Research, Cambden, NJ) were cultured in Roswell Park Memorial Institute (RMPI) media (Life Technologies, Carlsbad, CA) with 10% foetal bovine serum and Cascade Biologics Medium 254 (Life Technologies, Carlsbad, CA), respectively. Cells were not deprived of serum before use in experiments. Forskolin (LC Laboratories, Woburn, MA) and VE-821 (Selleckchem, Houston, TX) were utilized as indicated. siRNA targeted to ATR (Dharmacon, Lafayette, CO) and MITF (Dharmacon, Lafayette, CO) were performed according to the manufacturer's instructions.

2.2 | UV exposure

Cells were treated with 0.1% ethanol vehicle, 10 μmol/L forskolin, 10 μmol/L VE-821 or a combination of 10 μmol/L forskolin and 10 μmol/L VE-821 as indicated for 1 hour prior to UV exposure. Cells were exposed to a double bank of UVB lamps (UV Products, Upland, CA). UV emittance was measured with a Model IL1400A handheld flash measurement photometer (International Light, Newburyport, MA) equipped with separate UVB (measuring wavelengths from 265-332 nm; peak response at 290 nm) and UVA (measuring wavelengths from 315-390 nm; peak response at 355 nm) corresponding to International Light product numbers TD# 26532 and TD# 27108, respectively. Spectral output of the lamps was determined to be roughly 75% UV-B and 25% UV-A. Media were removed from the cells prior to exposure, and cells were exposed to a dose of 10 J/m^2 UVB (unless otherwise stated).

2.3 | Antibodies

Antibodies directed against pSer435-ATR were generated against the peptide CPKRRR(pS)SSLNPS (Amsbio, Cambridge, MA) as previously reported.[11] Commercially available antibodies included anti-CPD (Kamiya Biomedical, Seattle, WA), anti-CREB (Cell Signaling, Danvers, MA), MITF (Cell Signaling, Danvers, MA), anti-pSer133-CREB (Cell Signaling, Danvers, MA), anti-Chk1 (Cell Signaling, Danvers, MA), anti-pSer317-Chk1 (Cell Signaling, Danvers, MA) and antitryosinase (Santa Cruz, Dallas, TX).

2.4 | pSer435-ATR detection

pSer435-ATR kinase assays were performed as described[24] using the biotinylated ATR peptide substrates CPKRRRLSSSLNPS (Genscript, Piscataway, NJ). Cells were plated in a 6-well plate and treated with ethanol vehicle or 10 μmol/L forskolin for 1 hour prior to harvesting. 100 μmol/L biotinylated ATR peptide substrate was added to a streptavidin-coated 96-well plate. 20 μg of whole cell lysate was added to the wells in 40 mmol/L Tris-HCL (pH 7.5), 10 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol (DTT), 100 μg/mL bovine serum albumin and 10 μmol/L ATP. The kinase reaction was performed at 30°C. The reaction was terminated via the addition of 10 μL of 100 mmol/L EDTA at 1 hour. PKA phosphorylation was measuring utilizing the anti-pSer435-ATR primary antibody. The primary antibody was conjugated to an HRP-conjugated anti-rabbit secondary antibody (Abcam, Cambridge, MA) for 1 hour followed by the addition of QuantaBLu (Pierce, Rockford, IL). Fluorescence was detected by plate reader via excitation at 315 nmol/L and emission at 400 nmol/L.

2.5 | L-DOPA staining and pigment quantification

Cells were plated at a density of 3×10^5 per well in a 6-well plate, treated with vehicle or forskolin (as described elsewhere), washed in PBS and then fixed in 4% paraformaldehyde for 10 minutes. After three washes in PBS, L-DOPA (10 mmol/L) was added to the cells for 2 hour at 37°C, followed by a final fixation step in 4% paraformaldehyde for 10 minutes. Photographs were then taken of cell pellets before cells were dissolved in Soluene-350 (Perkin Elmer): water (9:1;v/v) for 10 minutes and heated at 95°C for 30 minutes. Soluene-350 supernatants were analysed at 492 nm on a Multiskan MCC/340 (Thermo Labsystems, Philadelphia, PA) plate reader.

2.6 | SiRNA

Cells were treated with pooled siRNA directed to ATR (ON-TARGET plus SMARTpool; Dharmacon, Lafayette, CO) and MITF (ON-TARGET plus SMARTpool, Dharmacon, Lafayette, CO). siRNA was transfected following the manufacturer's protocol. The siRNA was diluted in serum-free medium such that the final concentration was 25 nmol/L. The DharmaFECT solution was diluted such that 5 uL was added per well (6-well plate). SK-MEL-2 cells were treated with siRNA directed to ATR for 96 hours prior to treatment with vehicle or forskolin. SK-MEL-2 cells and primary human melanocytes were treated with siRNA directed to MITF for 72 hours prior to treatment with vehicle or forskolin as indicated. Duration of vehicle or forskolin treatment is indicated in each experiment. siRNA knockdown was confirmed at the time point indicated for each experiment.

2.7 | Nucleotide excision repair kinetics

Immuno slot blots were performed on whole cell lysates with 6,4 and CPD antibodies via standard methods.[11] Genomic stability was isolated using the DNEasy Qiagen Kit per manufacturer's instructions. DNA concentration was determined by Nanovue nanodrop (GE Healthcare, Pittsburgh, PA) reading. Equal loading of DNA was confirmed by 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, 1 mg/mL; Thermo Fisher, Waltham, MA) staining. DNA was denatured at 95°C for 10 minutes, and 0.1 μg was loaded per well on a slot blot apparatus (Bio-Rad) on a nitrocellulose membrane (Bio-Rad, Hercules, CA). Wells were washed with TE and suction was applied until dry. Membranes were baked (80°C, 1 hour), blocked in 5% milk (20°C, 1 hour, TBS buffer, 0.5% Tween), incubated with anti-CPD (Cosmo Biosciences, San Diego, CA, 1:1000 dilution, 4°C, overnight), washed, incubated in secondary antibody (HRP-anti-mouse, Abcam, Cambridge, MA, 1:10 000, 20°C, 1 hour) prior to visualization by ECL using the STORM system.

2.8 | Immunoblotting

Immunoblots were performed on whole cell lysates in radioimmunoprecipitation assay (RIPA) buffer. Cells were lysed in RIPA buffer for 20 minutes at 4°C. Protein concentration was determined via Bradford protein assay (Life Technologies, Waltham, MA). 10-30 μg of protein was loaded onto a 4-20% gradient SDS-polyacrylamide gel (SDS-PAGE) (Bio-Rad, Hercules, CA). Immuoblotting was either performed using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE) or STORM imaging system (GE Healthcare, Pittsburgh, PA) and analysed using IMAGEJ.

2.9 | Statistics and data analysis

One-way ANOVA with Tukey post hoc test and two-way paired ANOVA analysis were performed using GRAPHPAD PRISM 5.0 (GraphPad Software, San Diego, CA, USA). Data were considered statistically significant if P values were less than 0.05 from multiple independent experiments.

3 | RESULTS

3.1 | MITF depletion does not affect NER kinetics

SK-MEL-2 melanoma cells or primary human melanocytes (PHMs) were pretreated with either scrambled or siRNA directed to MITF prior to incubation with forskolin, an activator of adenylyl cyclase. Accumulation of pSer435-ATR was measured by kinase assay as previously described.[11] Forskolin promoted 6.0-fold and 4.0-fold inductions of ATR phosphorylation of Ser435 in SK-MEL-2 or PHMs, respectively, and levels of pSer435-ATR were not influenced by siRNA-mediated MITF depletion in either cell type (Figure 1A, B). Therefore, we conclude that PKA-mediated generation of ATR-pS435 is MITF-independent. To directly determine whether MITF is required for MC1R-enhanced NER, we measured the effect of MITF depletion on basal and cAMP-enhanced repair of UV-induced photolesions. SK-MEL-2 cells or PHMs were treated with vehicle control or 10 μmol/L forskolin for 30 minutes prior to exposure to a sublethal dose (10 J/m^2) of UVB radiation, and repair of CPDs was assessed at 0, 24, 48 and 72 hours. Treatment with forskolin significantly accelerated NER in either SK-MEL-2 cells or PHMs, and neither basal repair nor forskolin-enhanced repair were affected by siRNA-mediated MITF depletion in either cell type (Figure 1C, D). These data suggest that MC1R-enhanced NER is MITF-independent.

3.2 | ATR is not required for PKA phosphorylation of CREB

While our results suggested to us that MC1R-enhanced NER is independent of MITF, they do not exclude the possibility of signalling pathway crosstalk in which ATR influences cAMP-directed melanocyte responses. An early molecular event following cAMP stimulation is PKA-mediated phosphorylation of the CREB transcription factor, induction of MITF and a variety of downstream pro-differentiation events that follow.[16,17] To understand whether ATR kinase function is required for cAMP-mediated phosphorylation of CREB, we quantified levels of PKA-phosphorylated CREB in SK-MEL-2 cells or PHMs treated with 10 μmol/L forskolin. cAMP stimulation led to increased

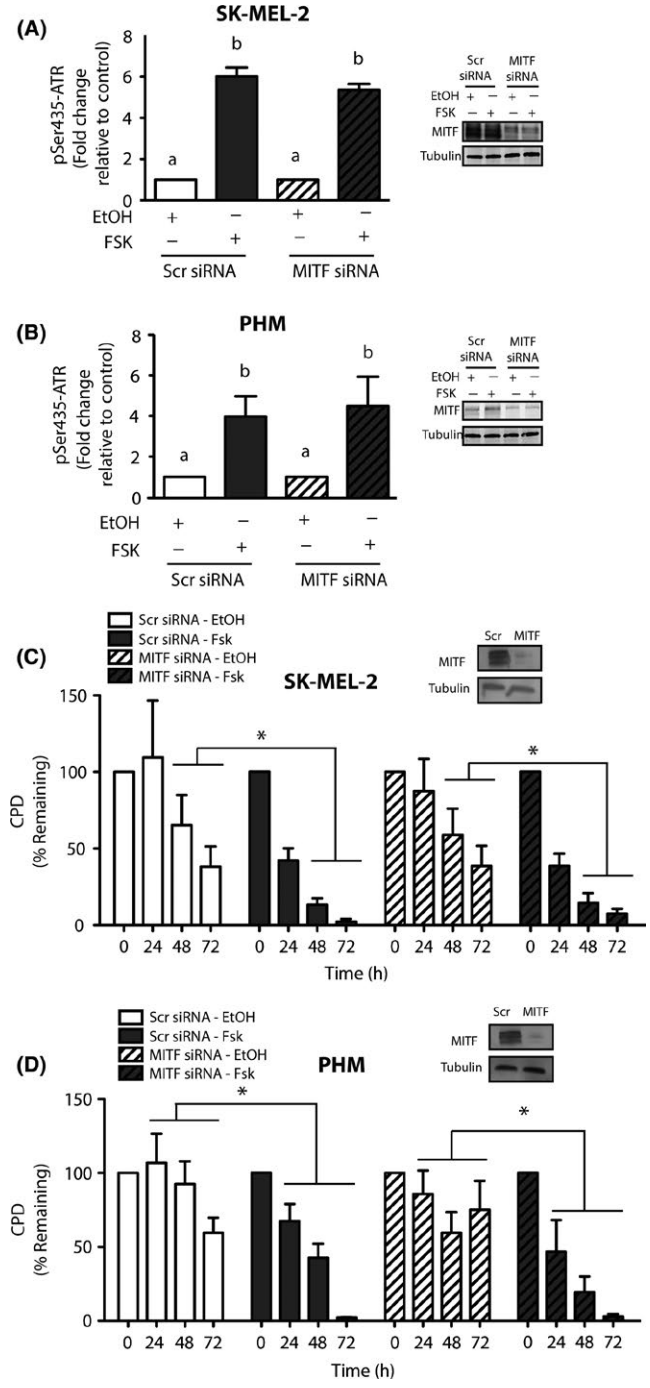

FIGURE 1 MITF inhibition does not affect PKA-mediated generation of pS435-ATR and has no functional impact on melanocyte nucleotide excision repair (NER). (A,B) SK-MEL-2 melanoma cells (n=3) (A,C) and primary human melanocytes (PHMs; n=4) (B,D) were treated with scrambled siRNA or siRNA directed to MITF prior to treatment with 10 μmol/L forskolin. Whole cell lysates were collected at 1 hour, and pSer435-ATR levels were determined by kinase assay (A,B). Values not sharing a common letter are significantly different as determined by one-way ANOVA and Tukey post hoc test ($P<0.05$). Data are expressed as mean-fold change over control±SEM. C,D) Cells were treated with scrambled siRNA or siRNA directed to MITF. Cells were treated with 10 μmol/L forskolin for 30 minutes prior to treatment with 10 J/m2 UVB radiation. MITF knockdown following treatment with siRNA directed to MITF is shown in inset. Significance between control and forskolin treatment as determined by two-way paired ANOVA ($P<0.05$) is denoted by an asterisk (*). Data are expressed as mean CPD remaining±SEM. Insets show degree of MITF knockdown by Western blotting

levels of CREB-pS133 at 1 hour (2.4-fold induction for SK-MEL-2 cells; 2.6-fold induction for PHM), and addition of the ATR kinase inhibitor VE-821 did not affect CREB-pS133 levels (Figure 2A-D). As a control to ensure VE-821 inhibited ATR, we evaluated total and ATR-phosphorylated forms of the checkpoint kinase Chk1. At the dose used, VE-821 inhibited Chk1-pS317 levels without affecting total Chk1 levels (insets, Figure 2A, C), indicating that the drug blocked ATR kinase function. Similar results were noted with siRNA-mediated ATR depletion in SK-MEL-2 cells (Figure S1). Together, these results suggest that ATR is not required for PKA-mediated CREB phosphorylation downstream of cAMP induction.

3.3 | ATR does not impact cAMP-induced MITF activation

To determine whether cAMP-induced MITF activation requires ATR, SK-MEL-2 cells or PHMs were incubated with forskolin and MITF levels were quantified through 6 hour in the presence or absence of the ATR kinase inhibitor VE-821. We noted increased MITF expression by forskolin was not impacted by the ATR kinase inhibitor VE-821 (Figure 3A-D). These findings suggest that ATR is dispensable for cAMP-mediated MITF induction.

3.4 | ATR inhibition does not interfere with cAMP-mediated pigment induction

To directly determine whether ATR was required for MC1R dependent pigmentation, we measured the effect of ATR inhibition on cAMP-enhanced pigmentation.[30] SK-MEL-2 cells or PHMs were incubated (2 hour) in the presence or absence of forskolin (10 μmol/L) and VE- 821 (10 μmol/L). To assess the impact of ATR inhibition on cAMP-mediated melanin induction, we measured pigmentation of SK-MEL-2 cells or PHMs after DOPA staining. [31] Forskolin promoted pigment induction in either cell type, as assessed visually (Figure 4A, B) or spectrophotometrically (Figure 4C, D). These data suggest that cAMP-induced pigmentation is unaffected by ATR kinase function.

4 | DISCUSSION

Inherited loss-of-function MC1R polymorphisms affect millions of individuals and place them at greater risk of melanoma.[1,3] Indeed, the loss of even one functional copy of the MC1R gene results in an accumulation of UV mutations in melanoma equivalent to expected extra UV exposure of more than two decades of life,[32] supporting a link between MC1R signalling and melanocyte genomic stability. Defective pigmentary responses caused by MC1R loss yield a fair-skinned and sun-sensitive phenotype[12] that facilitates UV penetration into the

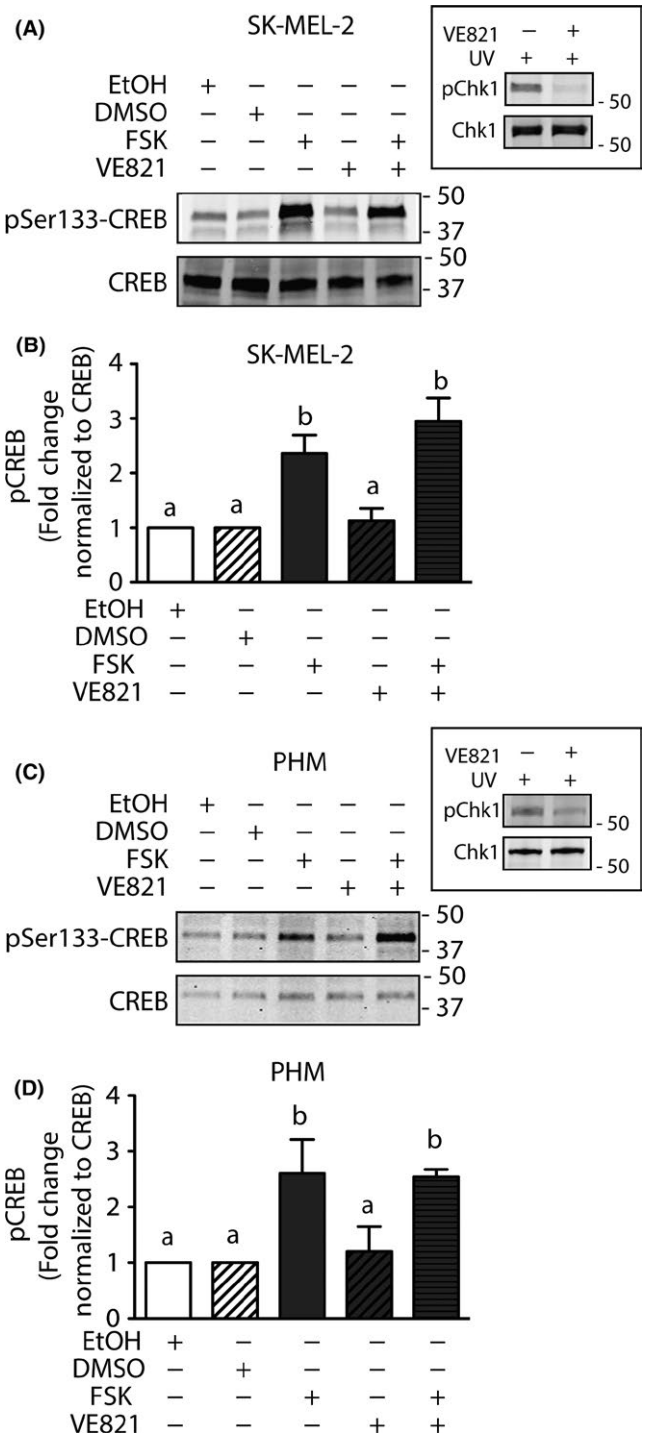

FIGURE 2 ATR inhibition does not affect PKA phosphorylation of CREB. SK-MEL-2 melanoma cells (n=3) (A-B) and PHMs (n=3) (C-D) were treated with 10 μmol/L forskolin, VE821, or a combination of 10 μmol/L forskolin and 10 μmol/L VE-821. Whole cell lysates were collected 1 hour following treatment and immunoblotted for pSer133-CREB. B,D) Composite densitometry quantification of immunoblots (n=3 per condition) in A,C. Values not sharing a common letter are significantly different as determined by one-way ANOVA and Tukey post hoc test ($P<0.05$). Data are expressed as mean-fold change over control±SEM. Panel insets show effect of VE-821 on UV-mediated Chk1 phosphorylation (pChk1 S313)

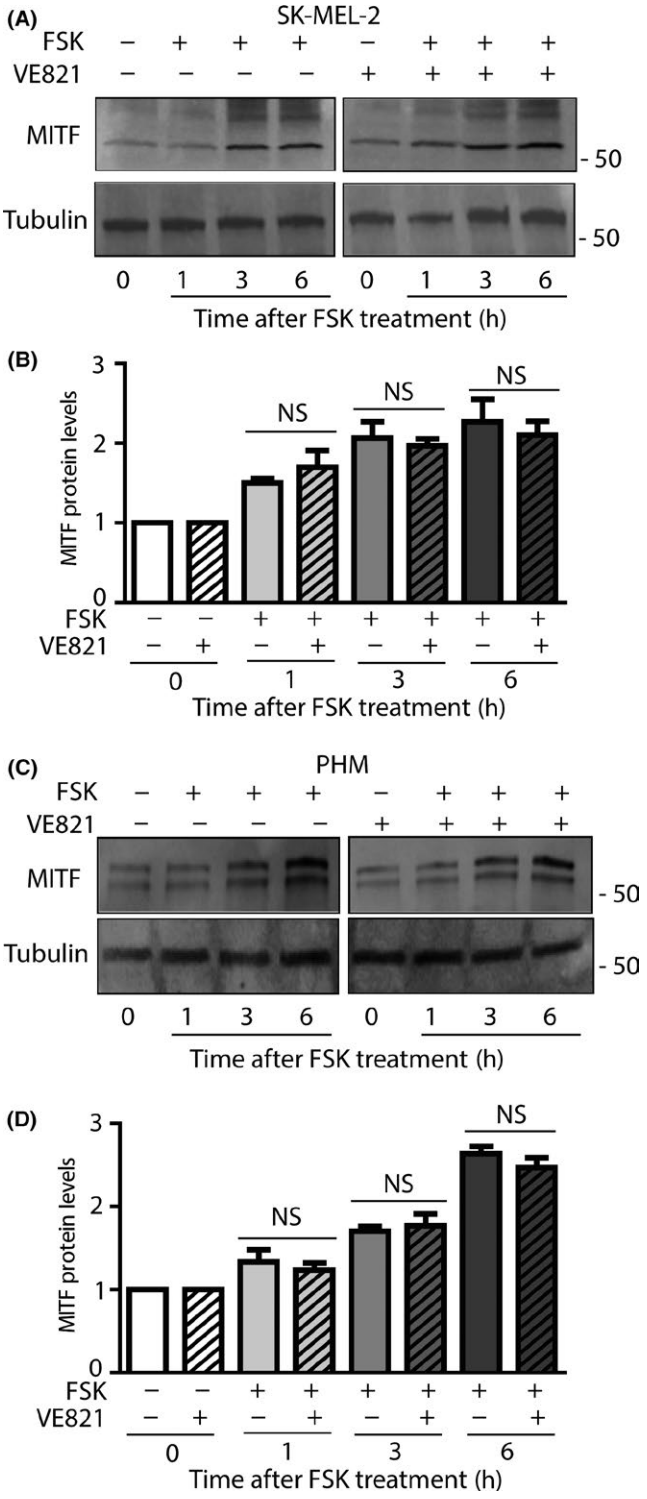

FIGURE 3 Pharmacologic ATR inhibition does not interfere with cAMP-induced Mitf activation. SK-MEL-2 melanoma cells (n=3) (A-B) and PHMs (n=3) (C-D) were treated with 10 μmol/L forskolin, VE821 or a combination of 10 μmol/L forskolin and 10 μmol/L VE-821. Whole cell lysates were collected 1 hour following treatment and immunoblotted for Mitf. B,D) Composite densitometry quantification of immunoblots (n=3 per condition) in A,C. NS denotes that no significant difference was observed between experimental groups as determined by one-way ANOVA and Tukey post hoc test. Data are expressed as mean-fold change over control±SEM

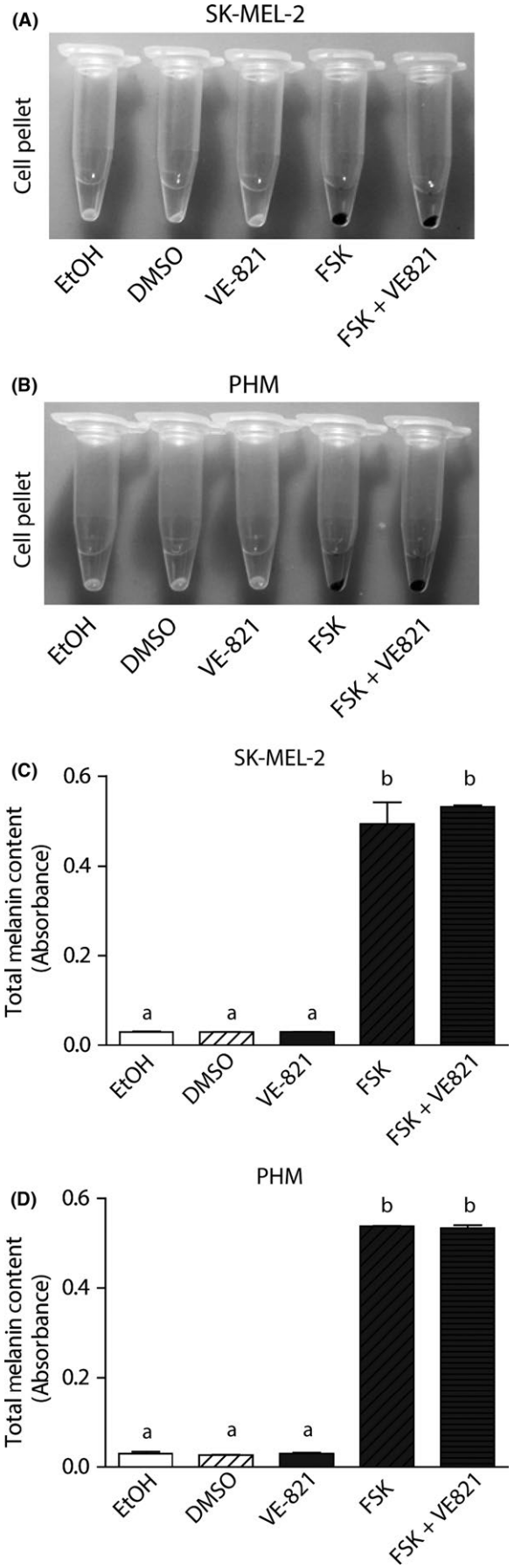

FIGURE 4 ATR inhibition does not interfere with cAMP-induced pigment induction. SK-MEL-2 melanoma cells (n=3) (A,C) and primary human melanocytes (PHMs; n=4) (B,D) were treated with vehicle controls 10 μmol/L VE-821 or 10 μmol/L forskolin. Cells were collected, incubated with L-DOPA (see Methods) and pelleted after 2 hours. (A,B) photographs of microcentrifuge tubes showing cell pellets of indicated conditions. (C,D) Cells were lysed in Soluene-350 solution and spectrophotometry of supernatants was quantified at 492 nm. Values not sharing a common letter are significantly different as determined by one-way ANOVA and Tukey post hoc test ($P<0.05$). Data are expressed as mean-fold change over control±SEM.

skin and a tendency for episodic UV-induced inflammatory episodes (sunburns),[14] both of which favour melanoma development.[33-35] We and others have documented that MC1R loss also results in defects in the ability of melanocytes to repair UV photodamage in nuclear DNA.[21,22,24,36,37] This damage, if left unrepaired, causes transitional pyrimidine "UV-signature" mutations known to cause and promote melanoma development.[5-7] Thus, MC1R signalling, mediated by generation of the second messenger cAMP,[38] is a global regulator of melanocyte UV physiology and UV resistance.

Herein, we report that MITF does not appear to be required for cAMP-enhanced NER. Although MITF functions as a transcription factor inducing specific genes involved in NER,[27] it has also been reported to regulate cell signalling pathways such as Wnt signalling in a positive feedback-mediated manner and Wnt-mediated increases in vesicular structures and endosomal proteins.[28] Our data suggest that MITF is not necessary for PKA phosphorylation of ATR at Ser435, which occurs and exerts its effects within minutes following stimulation of melanocytes by cAMP induction.[11] Furthermore, MITF loss has little impact on basal NER kinetics or cAMP-enhanced repair. While Strub and co-workers reported that MITF induces expression of *RPA*, *XRCC3* and *POLE*,[27] XPA, which is considered to be the rate-limiting factor in NER[39,40] and the key mediator of MC1R-enhanced NER,[11] was not induced by MITF.[27] Our data, however, do not exclude the possibility that MITF influences melanocyte genomic stability in other ways beside the cAMP-ATR-XPA repair axis. Indeed, one possibility is cAMP protective effects on cellular antioxidant levels[10,41] as lower amounts of UV-induced free radicals and oxidative species would be expected to preserve repair enzyme function by preventing their inactivation. Lastly, Mitf levels were documented at the end of the experimental time frame; although unlikely, it may be possible that Mitf expression was less inhibited at earlier time points.

Data presented here also suggest that non-DNA repair physiologic events downstream of MC1R are independent of ATR. Specifically, inhibiting ATR either pharmacologically by the kinase inhibitor VE-821 or by siRNA-directed knockdown had no discernable effect on phosphorylation of CREB by PKA or basal levels or cAMP-induced activation of MITF protein. Moreover, our findings suggest that ATR is not required for cAMP-mediated upregulation of pigmentation; however, our data do not rule out the possibility that ATR function may impact other pigment enzyme expression or function downstream of cAMP signalling. The cell lines used in these studies did not appear to express

pigment in culture either basally or within the time course of our experiments. However, it is possible that low levels of melanin could have contributed to the formation of "dark photoproducts" through oxidative induction of melanin triplet energy state with subsequent formation of CPDs as recently described.[42] Our data did not directly address this as a possibility.

In summary, we have determined that cAMP-mediated augmentation of the melanocyte adaptive pigmentation pathway and acceleration of NER appear to diverge at the level of PKA as would lie downstream of the melanocortin signalling cascade in melanocytes. There is much interest in developing UV-protective and melanoma-preventive strategies based on pharmacologic induction of melanocortin/cAMP signalling in the skin[43-45]; however, to date, there is no way to uncouple the benefits of enhanced DNA repair and genomic stability with pigment enhancement, or possibly other cAMP-directed responses such as melanocyte proliferation. Our findings offer the possibility of developing approaches to enhance melanocyte genomic stability independent of melanin induction.

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All authors substantially contributed to experimental design, acquisition and/or interpretation of the data. EMWH and JAD designed the study. EMWH and SGJ performed the research and analysed the data. EMWH and JAD wrote drafts and critical revisions of the manuscript. Each author approved the submitted and final versions of the manuscript. We are grateful for support from the National Cancer Institute (R01 CA131075), the Melanoma Research Alliance (MRA) and the Regina Drury Endowment for Pediatric Research. We also acknowledge support by the NCI Cancer Center Support Grant (P30 CA177558) and Cancer Training Grant (T32 CA165990).

CONFLICT OF INTERESTS

None of the authors declares any financial or personal relationships that could be viewed as presenting a potential conflict of interest with the design or conclusions of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1 ATR knockdown does not affect PKA-mediated CREB phosphorylation.

Figure S2 MITF knockdown does not affect cAMP-mediated DNA repair of CPDs.

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