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PKA-mediated Phosphorylation of ATR Promotes Recruitment of XPA to UV-induced DNA Damage

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Running Title: cAMP signaling links ATR with DNA repair

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The melanocortin 1 receptor (MC1R), which signals through cAMP, is a melanocytic transmembrane receptor involved in pigmentation, adaptive tanning and melanoma resistance. We report MC1R-mediated or pharmacologically-induced cAMP signaling promotes nucleotide excision repair (NER) in a cAMP-dependent protein kinase A (PKA)-dependent manner. PKA directly phosphorylates ataxia telangiectasia and Rad3-related protein (ATR) at Ser435 which actively recruits the key NER protein xeroderma pigmentosum complementation group A (XPA) to sites of nuclear UV photodamage, accelerating clearance of UV-induced photolesions and reducing mutagenesis. Loss of Ser435 within ATR prevents PKA-mediated ATR phosphorylation, disrupts ATR-XPA binding, delays recruitment of XPA to UV-damaged DNA and elevates UV-induced mutagenesis. This study mechanistically links cAMP-PKA signaling to NER and illustrates potential benefits of cAMP pharmacological rescue to reduce UV mutagenesis in MC1R-defective, melanoma-susceptible individuals.
INTRODUCTION

Cyclic adenosine 3′,5′-monophosphate (cAMP) is a critical intracellular signaling molecule that, in melanocytes, is robustly generated following interactions between the melanocortin 1 receptor (MC1R), a Gs-coupled cell surface receptor, and alpha melanocyte stimulating hormone (MSH) (Beaumont et al., 2011; Palmer et al., 2000). One of the most important inherited risk factors for UV skin sensitivity and development of melanoma is blunted MC1R-mediated cAMP signaling. Persons with loss-of-signaling MC1R polymorphisms (e.g. R151C, R160W and D294H) exhibit a fair-skinned sun-sensitive phenotype and have up to a four-fold increased lifetime risk of melanoma (Valverde et al., 1995). A major route for channeling cAMP signaling is via cAMP-dependent protein kinase (PKA), an enzyme that influences a number of differentiation and survival pathways including biosynthesis of UV-protective melanin pigment (Lin and Fisher, 2007). Though MC1R dysfunction is clearly linked with defective tanning (D'Orazio et al., 2006), MC1R signaling also protects melanocytes against carcinogenesis independent of pigmentation (Abdel-Malek et al., 2009; Bohm et al., 2005; Hauser et al., 2006; Kadekaro et al., 2012). The mechanisms behind pigment-independent MC1R-mediated genome maintenance, however, remain unclear.

UV generates mutagenic DNA photolesions which if unrepaired lead to characteristic “UV-signature mutations” which are causative for melanoma (Hodis et al., 2012). Photolesions engage the nucleotide excision repair (NER) pathway, which corrects UV-induced DNA damage in a multistep process involving numerous repair factors including XPA, ERCC1, ERCC3 (XP-B), XPC, ERCC2 (XP-D), DDB2 (XP-E), ERCC4 (XP-F) and ERCC5 (XP-G) (DiGiovanna and Kraemer, 2012; Lehmann et al., 2011). The importance of NER in resistance to UV-induced cancers is clearly demonstrated by observing the natural history of xeroderma pigmentosum (XP) patients, who through homozygous loss of one of the enzymes that carry out NER, are profoundly predisposed to melanoma and other UV-induced skin cancers (DiGiovanna and Kraemer, 2012). Xeroderma pigmentosum complement group A (XPA), a gene frequently mutated in XP patients, is part of the core incision complex of NER and interacts with DNA as well as many other NER and damage response proteins (Bomgarden et al., 2006; Kang et al., 2011;
Reardon and Sancar, 2005; Svetlova et al., 1999). ATR (ATM and Rad3-related) is critical to UV DNA damage signaling (Ciccia and Elledge, 2010) and is intimately linked with NER (Bomgarden et al., 2006; Lindsey-Boltz et al., 2014).

Herein, we report that a novel cAMP-dependent post-translational modification of ATR promotes its DNA-repair function, thus explaining how MC1R signaling is linked with NER. Specifically, PKA phosphorylates ATR at the Serine 435 (Ser435) position, causing enhanced physical interaction with XPA and accelerated binding to sites of DNA photodamage. PKA-mediated ATR phosphorylation reduces UV-induced mutagenesis, which is likely critical to how MC1R function protects melanocytes against malignant degeneration. Taken together, we report the molecular mechanism by which the MC1R-cAMP-PKA signaling axis enhances NER and reduces UV mutagenesis in melanocytes. Our findings highlight potential anti-mutagenic benefits of pharmacological cAMP stimulation in the skin of MC1R-deficient and melanoma-susceptible individuals.

RESULTS

MC1R Signaling Enhances Repair of UV-Induced Photolesions in vivo and in vitro

In order to determine the role of MC1R function and cAMP signaling in NER, we made use of a murine model of “humanized skin” in which epidermal melanocytes are retained in the skin (D’Orazio et al., 2006; Spry et al., 2009). K14-Scf transgenic animals congenic except for function at the Mc1r or tyrosinase (tyr) loci (Figure 1A) were irradiated with UV to ascertain pigment-independent effects of Mc1r on DNA repair (D’Orazio et al., 2006; Vanover et al., 2009). Clearance of UV-induced cyclobutane pyrimidine dimers (CPD) was impaired in animals expressing inactive Mc1r (Mc1r<sup>e/e</sup>) when compared to those with wild type Mc1r, as measured by time taken to repair 50% of UV damage (repair <i>t</i><sub>1/2</sub> for CPD removal 42 ± 6.3 h vs. 24 ± 0.7 h respectively; <i>p</i> ≤ 0.05) (Figure 1B). We reasoned that since Mc1r<sup>e/e</sup> mice lacked the ability to generate cAMP in response to MSH, cutaneous application of forskolin, a skin-permeable adenyllyl cyclase activator would restore DNA repair of UV photodamage. Topically-applied
forskolin robustly enhanced clearance of UV photodamage in the skin, essentially to \textit{Mc1r} wild type levels (repair $t_{1/2} 23 \pm 2.7$ h vs. $44 \pm 3.3$ h in treated vs. untreated animals respectively; $p \leq 0.05$; Figure 1C). Importantly, neither \textit{Mc1r} status nor forskolin application influenced initial amount of UV-induced DNA damage (Figures S1A and S1B). Since measuring repair in murine whole skin represents the combined influence of numerous cell types, we repeated photolesion clearance studies in B16 immortalized mouse melanocytes. Pre-treatment of B16 cells (\textit{Mc1r}^{\text{EE}}) with either MSH (MC1R agonist), forskolin or the phosphodiesterase inhibitor rolipram enhanced removal of CPDs compared to vehicle-treated cells (Figure 1D), again without affecting amount of initial UV damage (Figures S1C and S1D). Thus, \textit{Mc1r} signaling or pharmacologic stimulation of cAMP optimized melanocytic NER in murine whole skin and in a melanocyte cell line.

**cAMP Signaling Enhances DNA Repair in Human Cells**

To determine whether cAMP-mediated NER enhancement extended to human cells, we measured the effect of forskolin or MSH on clearance of 6-4 photoproducts ([6-4]-PP) and CPDs in a variety of human cell lines. Treatment of MC1R wild-type melanocytes with forskolin or MSH significantly increased repair efficiency (Figures 1E, 1F and Supplemental Table 1). Addition of agouti signaling protein (ASIP), a potent MC1R antagonist (Lu et al., 1994), abrogated any MSH-mediated repair benefit (Supplemental Table 1). The MC1R-defective melanoma cell line A375 exhibited slower repair of UV damage compared to MC1R-wild type cells and had a markedly blunted response to MSH (Supplemental Table 1). However, forskolin enhanced clearance of both [6-4]-PP and CPD in all melanocyte cells tested regardless of MC1R status (Figures 1E and 1F) with no apparent effect on cell proliferation (Figure S2) indicating photolesion clearance was not influenced by proliferative differences. Furthermore, UV damage alone did not promote production of cAMP (Figure S2E), suggesting that in the skin UV-associated melanocytic cAMP increases depend on an intact MC1R signaling pathway initiated by MSH. Interestingly, cAMP-enhanced repair was not limited to melanocytic lines, as a repair benefit was also observed in ARPE-19 and MCF-7 cells (Figures 1E and 1F).
To confirm the specificity and importance of MC1R in melanocytic NER, complementation studies were performed in MC1R-mutant (R151C) A375 melanoma cells. MSH pre-treatment enhanced repair of [6-4]-PP and CPDs when cells were transfected with wild-type MC1R but not when cells were transfected with MC1R mutants (either R151C, R160W or D294H) (Figure 1G and H). Together, these data strongly support an integral role for MC1R/cAMP signaling in UV repair responses and support the hypothesis that pharmacologic induction of cAMP signaling enhances NER.

**Recruitment of XPA to UV-Induced Photolesions is Enhanced by cAMP Signaling**

cAMP stimulation failed to increase total protein levels of core NER factors (Figure S3A), however we explored whether distribution of key NER proteins to sites of UV-induced DNA damage might be affected. We found that cAMP signaling influenced kinetics of XPA nuclear accumulation and recruitment to chromatin after UV-induced DNA damage (Figures 2A, 2B and 2C). Forskolin pre-treatment enhanced the percentage of XPA associated with chromatin, with the greatest chromatin-XPA interaction observed with forskolin and UV together (Figures 2A, 2B and 2C). Immunolocalization studies confirmed XPA accumulation at UV-induced photodamage in the nucleus (Figures 2D and E and Figure S3B). In A375 cells, neither wild-type XPA nor FLAG-tagged XPA efficiently co-localized with [6-4]-PP 30 min after UV exposure. However, pre-treatment with forskolin resulted in a high degree (~2.5-fold increase; \( p \leq 0.05 \)) of XPA/[6-4]-PP co-localization following UV (Figures 2D and 2E). Co-immunoprecipitation experiments confirmed that the XPA/[6-4]-PP interaction on chromatin was enhanced by forskolin treatment (Figure S3C). Furthermore, treatment of MC1R wild-type melanocytes with forskolin or MSH significantly increased chromatin XPA levels and [6-4]-PP repair (Figures S3D and S3E), whereas addition of ASIP, a potent MC1R antagonist that down-regulates cAMP signaling, abrogated MSH-mediated benefit, confirming the importance of XPA and MC1R in the repair of UV-induced DNA damage. Interestingly, DNA-bound XPA was enhanced by forskolin even in the absence of UV, suggesting that cAMP stimulation might somehow enhance XPA-chromatin interactions before
UV damage occurs. We conclude that cAMP signaling enhances and directs accumulation of XPA to chromatin and sites of UV damage and that pharmacologic induction of cAMP “rescues” NER in MC1R-mutant melanocytes otherwise incapable of responding to MSH.

**cAMP-Mediated Signaling Facilitates XPA-ATR Interaction**

To gain further insight into how MC1R signaling affects XPA-chromatin association and NER enhancement, we tested whether cAMP signaling enhanced the ability of XPA to bind DNA repair-relevant proteins in a UV-dependent manner. A proteomic screen of XPA-immunoprecipitated lysates revealed that cAMP-mediated signaling enriched XPA-associated ATR by at least two-fold. Since prior work by others suggested that ATR influenced NER in other cell types (Shell et al., 2009; Wu et al., 2007; Wu et al., 2006), we hypothesized that cAMP enhances NER in melanocytes by promoting biochemical interaction between ATR and XPA. Co-immunoprecipitation experiments confirmed that cAMP stimulation promoted a robust and accelerated physical interaction between ATR and XPA in UV-radiated A375 melanoma cells, as detected either in whole cell lysates (Figure 3A) or with chromatin (Figure 3B).

To confirm the importance of the cAMP-dependent ATR-XPA interaction to NER in melanocytes, A375 cells were transfected with small interfering RNA (siRNA) targeting ATR. ATR knockdown greatly reduced baseline and forskolin-mediated recruitment of XPA to UV-damaged chromatin (~2.0-fold decrease; \( p \leq 0.05 \)) (Figure 3C), blocked forskolin-mediated NER enhancement (Figure 3D) and blunted repair of UV-induced [6-4]-PP (repair \( t_\text{1/2} \), 4.5 ± 0.4 h vs. 2.9 h ± 0.2 h in scrambled siRNA-transfected cells; \( p \leq 0.05 \)) (Figure 3D). These data suggest that cAMP-mediated NER enhancement results from accelerated and increased association of an ATR-XPA complex with UV-damaged DNA and that ATR is critical in mediating MC1R optimization of melanocytic NER.

ATR signals through multiple pathways to promote genome maintenance. Most typically, cell damage leads to ATR phosphorylation and activation of the ATR-Chk1 pathway which promotes G2/M
cell cycle arrest (Cortez et al., 2001; Liu et al., 2000). We tested whether cAMP-mediated ATR phosphorylation on Ser435 promoted Chk1 phosphorylation and cell cycle arrest in melanocytes. Whereas UV exposure led to robust Chk1 phosphorylation, forskolin treatment had no effect on total Chk1 levels or phospho-Chk1 levels (Figure 3E). Similarly, exposing A375 melanocytes to UV promoted a G2/M cell cycle arrest, whereas forskolin treatment had no effect on the cell cycle (Figure 3F), suggesting that cAMP-induced ATR phosphorylation is a distinct form of ATR signaling independent of Chk1 phosphorylation and cell cycle arrest.

**MC1R/cAMP Signaling Suppresses UV-Induced Mutagenesis in an XPA/ATR-Dependent Process**

Since cAMP signaling enhanced clearance of UV-dependent photolesions, we reasoned that cAMP signaling would protect melanocytes against UV-induced mutagenesis. To test this, we performed hypoxanthine phosphoribosyltransferase (*hprt*) mutagenesis screens on a variety of melanocyte cell lines with or without cAMP stimulation. UV exposure resulted in mutant colony formation in all cell lines (Figure 4A), with similar mutation frequencies to other *hprt* mutagenesis studies (Wigan et al., 2012). MC1R mutant cell lines exhibited higher mutation rates when compared to MC1R wild-type cells. Pre-treatment of cells with MSH reduced UV-induced mutagenesis in MC1R wild type cells but failed to affect mutation rates in MC1R mutant lines (Figure 4A). In contrast, forskolin pre-treatment suppressed UV-induced mutagenesis in every cell line tested, irrespective of MC1R status. In the absence of UV, no mutant colonies were observed regardless of MC1R status. We concluded from these observations that cAMP-signaling results in an increased ability of melanocytes to resist UV-induced mutagenesis.

We next explored the need for a physical ATR-XPA interaction on cAMP-mediated protection against UV-induced mutagenesis. To do so, we compared UV responses of XPA-null fibroblasts expressing either mutant XPA (K188A) lacking the ability to bind ATR (Shell et al., 2009) or wild-type XPA (XPA-WT). As was the case in cells of melanocytic lineage, forskolin greatly enhanced chromatin-associated ATR-XPA interaction in the presence of XPA-WT ($p \leq 0.05$) (Figure 4B). However, when
cells were transfected with the XPA construct incapable of binding to ATR (XPA-K188A), no XPA was found on chromatin regardless of cAMP stimulation (Figures 4B). Forskolin pre-treatment suppressed UV-induced mutant colony formation in the XPA-WT expressing fibroblasts ($p \leq 0.05$) but not in cells expressing XPA-K188A (Figure 4C), indicating that cAMP-mediated XPA translocation to chromatin and resultant protection from UV mutagenesis is dependent on a physical ATR-XPA interaction.

**PKA promotes ATR phosphorylation and its interaction with XPA**

Computer motif analysis predicted that ATR possesses a single PKA phosphorylation consensus sequence on Serine 435 (Ser435; RX(p)SX). Phosphorylation of Ser435 on ATR has been previously reported (Liu et al., 2011), however, the kinase responsible for phosphorylating this residue as well as its functional relevance is unknown. We reasoned that cAMP-mediated recruitment of XPA to sites of UV-induced DNA damage would be mediated by PKA-mediated phosphorylation of ATR. Co-immunoprecipitation experiments using a PKA phosphorylation-specific substrate antibody (p-PKA), revealed forskolin treatment increased ATR phosphorylation, and that this phosphorylation was ablated in the presence of the specific PKA inhibitor, H-89 (Figure 5A). We posited that cAMP directs PKA-mediated phosphorylation of Ser435 on ATR to promote interaction with XPA and accumulation at sites of DNA damage. To test this, a site-specific mutant of ATR was engineered containing an alanine substitution at the 435 position (S435A), which completely abolished forskolin-mediated PKA phosphorylation of ATR (Figure 5B). Furthermore, an ATR Ser435 phosphopeptide-specific antibody (p-S435) was generated that recognizes ATR only when phosphorylated specifically at Ser435 (Figure S4A, S4B and S4C). Forskolin pre-treatment increased phosphorylation of Ser435 on ATR following UV exposure (~2-fold induction compared to vehicle treated cells; $p \leq 0.05$) and phosphorylation at this site was ablated in the presence of the specific PKA inhibitor, H-89 (Figure 5C). In addition, forskolin pre-treatment increased the interaction between phosphorylated Ser435 ATR and XPA following UV damage in whole cell lysates (Figures 5D), suggesting that PKA-mediated Ser435 phosphorylation facilitates
ATR’s physical interaction with XPA. Intriguingly, Ser435 in the context of a consensus PKA phosphorylation target sequence (RRX(p)S) appears to be conserved among vertebrates suggesting widespread physiological relevance of the PKA-ATR interaction (Figure 5E).

**PKA Directly Phosphorylates ATR at Serine 435**

To further characterize PKA-mediated phosphorylation of ATR, *in vitro* phosphorylation experiments were performed. Nuclear extracts isolated from ATR hypomorphic Seckel cell-lymphocytes were transfected with either wild-type or Ser435-mutant ATR (S435A) and incubated with recombinant catalytically active PKA. Phosphorylation of wild-type but not S435A-mutant ATR was identified and the reaction was blocked by the specific PKA inhibitor H-89 (Figure 6A). To address whether ATR is a direct substrate for PKA, we tested PKA’s ability to phosphorylate a short ATR peptide containing Ser435 and surrounding residues in a cell-free system. Whereas PKA promoted robust phosphorylation of the wild type ATR peptide containing S435, there was no phosphorylation of the S435A mutant form (Figure 6B). Enzyme kinetic studies of PKA activity revealed similar $K_m$ values for wild-type ATR compared with other known PKA substrates (Figure 6C). Notably, ATR S435A was not phosphorylated by recombinant catalytically active PKA (Figure 6C). The specificity and importance of MC1R in PKA-mediated ATR phosphorylation was confirmed in human embryonic kidney (HEK) cells. Thus, MSH pre-treatment enhanced ATR phosphorylation on Ser435 in HEK 293 cells transfected with wild-type MC1R but had no effect when cells were instead transfected with loss-of-function MC1R mutants (Figure 6D).

We tested the functional consequences of PKA-mediated ATR phosphorylation on NER by measuring clearance of UV photodamage. Recombinant catalytically active PKA enhanced NER in ATR-hypomorphic Seckel lymphocytes transfected with wild type (S435) but not mutant (S435A) ATR (Figure 6E). Further, PKA enhanced NER in a variety of cell types (Figure 6F), suggesting that cAMP-directed PKA activation may also influence NER in non-melanocytic cells. Despite promoting NER, it appears that S435 ATR phosphorylation has little impact on global UV sensitivity (Figure S4D). Together, these
data identify that the Ser435 residue is a direct target of PKA and that its phosphorylation specifically enhances repair of UV-induced DNA damage.

**PKA Phosphorylation of ATR at Serine 435 Suppresses UV-Induced Mutagenesis by Mediating XPA Recruitment to Sites of UV Damage**

Next, we determined the effects of cAMP-induced PKA signaling on cellular localization of ATR and XPA by immunofluorescence confocal microscopy. Whereas vehicle-treated A375 cells demonstrated XPA and ATR in both the cytoplasm and the nucleus, forskolin treatment led to nuclear accumulations of total ATR, phospho-Ser435 ATR and XPA (~2-fold nuclear enrichment compared to vehicle treated cells; \( p \leq 0.05 \)) (Figure S5). To confirm that cAMP signaling promoted a close physical interaction between XPA and ATR in the nucleus, we utilized a proximity ligation assay (PLA) approach (Figure 7A). No XPA-ATR interaction was observed in the absence of UV with or without cAMP stimulation by forskolin. Pre-treatment of cells with forskolin, however, promoted a dramatic UV-dependent increase in nuclear XPA-ATR interaction, suggesting that the association of XPA and ATR in the nucleus is influenced both by cellular damage and by cAMP stimulation. Further, forskolin pre-treatment specifically increased the interaction between phosphorylated Ser435 ATR and XPA on chromatin following UV damage (Figure 7B and 7C).

Co-immunoprecipitation experiments confirmed that recruitment of ATR-XPA to UV-damaged chromatin depends on PKA-mediated phosphorylation of Ser435 on ATR (Figure S6A). In support of this, phosphomimetic mutation of ATR-phospho-S435 (S435D) promoted efficient recruitment of XPA to chromatin even in the absence of UV (Figures 7D and 7E), suggesting that ATR mediates XPA recruitment to photolesions when it has been phosphorylated by PKA on Ser435. Together, these results demonstrate the importance of PKA-directed phosphorylation of Ser435 on ATR for chromatin enrichment of the ATR-XPA complex.
A knock-down and rescue approach was employed to further characterize the physiological importance of PKA-mediated Ser435 ATR phosphorylation in melanocytes. Native ATR was effectively suppressed and cells were transfected with siRNA-resistant S435 wild type, S435A or S435D ATR constructs (Figure S6B). Rescue with either the wild type S435 or the phosphomimetic S435D forms of ATR (but not with the S435A mutant form of ATR) was associated with enhanced repair and reduced mutagenesis (Figures 7F and 7G). Together, these data indicate that PKA-phosphorylation of ATR at Ser435 optimizes repair of UV-induced DNA damage.

DISCUSSION

One of the most important inherited risk factors for development of melanoma is loss of signaling of MC1R, a $G_s$-coupled MSH-activated cell surface receptor on melanocytes. Individuals with loss-of-signaling MC1R polymorphisms exhibit a fair-skinned sun-sensitive phenotype and have up to a four-fold increased lifetime risk of melanoma. Though MC1R dysfunction is clearly linked with defective tanning (D’Orazio et al., 2006) and altered AKT regulation (Cao et al., 2013), MC1R signaling also protects melanocytes against carcinogenesis independent of pigmentation (Hauser et al., 2006). Mediated by robust cAMP second messenger generation, MC1R signaling enhances the rate of clearance of UV-induced DNA photodamage (Abdel-Malek et al., 2006; Dong et al., 2010; Kadekaro et al., 2012). Herein, we report that MC1R signaling enhances NER by a novel and direct PKA-mediated phosphorylation of ATR on Ser435. This is the first report of a post-translational modification of ATR promoting a DNA-repair specific function and the first to mechanistically link PKA to MC1R signaling and the genomic maintenance pathway responsible for clearance of UV-induced photolesions known to be causal for melanoma. Ser435 phosphorylation by PKA promotes ATR’s physical association with XPA and directs XPA to sites of UV damage in the nucleus to accelerate repair of photodamage and protect against UV-induced mutagenesis. Although studies in other systems demonstrate UV-dependent ATR-XPA interactions (Dong et al., 2010; Shell et al., 2009; Wu et al., 2007), our data mechanistically link ATR and
XPA with the MSH-MC1R-cAMP signaling axis, and experiments using the phosphomimetic S435D ATR mutant suggest that Ser435 phosphorylation is both necessary and sufficient for ATR to mediate MC1R-enhanced NER.

PKA-induced phosphorylation of ATR is distinct from the ATR-Chk1 pathway (Cortez et al., 2001; Liu et al., 2000) since phosphorylation of Ser435 does not promote Chk1 phosphorylation or cell cycle arrest, as was described for other ATR phosphorylation events (e.g. Ser435, Ser428, Ser436 and Ser437) (Daub et al., 2008; Dephoure et al., 2008; Liu et al., 2011; Nam et al., 2011). Instead, phosphorylation of Ser435 promotes NER independent of traditional damage signaling coordinated by ATR. PKA-mediated phosphorylation of other genome maintenance proteins have recently been reported (Marazita et al., 2012; Rahmeh et al., 2012), suggesting cAMP signaling may be an important event acting to globally prime DNA repair.

Ser435 is part of a PKA target sequence (RRXS*) within ATR’s predicted nuclear localization sequence (425-DGISPKRRLSSSLNPSKRAP), suggesting that its phosphorylation might impact ATR’s nuclear localization, possibly through interactions with nuclear importins (Li et al., 2013). Indeed, co-localization studies of phosphoSer435 ATR and XPA suggest that PKA modification promotes nuclear entry of ATR-XPA to prime NER for DNA damage. PKA-directed nuclear localization of DNA-PK, another PIKK family member, has also been reported (Huston et al., 2008). Alternatively, PKA-mediated phosphorylation of ATR at Ser435 may optimize NER through enhanced intra-nuclear interactions with XPA to aid transport and/or assembly at sites of UV damage using either enzymatic and/or non-enzymatic mechanism(s). It is possible that the phospho-Ser435 ATR-XPA complex recognizes UV damaged sites through RPA binding (Zou and Elledge, 2003) or perhaps via the excision gap generated by NER (Lindsey-Boltz et al., 2014). In any case, our findings suggest that PKA-mediated phosphorylation of ATR at Ser435 is an important event that dynamically regulates early recruitment/assembly of XPA and possibly other DNA repair proteins to sites of UV damage to optimize NER.
Besides defective pigmentary responses (D’Orazio et al., 2006; Spry et al., 2009) and dysregulated AKT signaling (Cao et al., 2013), our data support the concept that MC1R-signaling defective individuals are melanoma-prone because of blunted cAMP signaling and inadequate genome maintenance (Abdel-Malek et al., 2006; Dong et al., 2010; Kadekaro et al., 2012). Our findings have clear translational potential since pharmacologic targeting of the cAMP signaling by topical application of forskolin clearly enhanced NER in the skin. Thus, it may be possible for fair-skinned, MC1R-defective individuals to greatly reduce melanoma risk by pharmacologically enhancing NER in a cAMP-dependent manner.

EXPERIMENTAL PROCEDURES

Mouse Pigmentation Phenotypes and UV Exposure

Protocols for murine experiments were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. C57BL/6J mice of varying pigment phenotype were crossed with K14-Scf transgenic animals (D’Orazio et al., 2006). Pigmentation phenotypes were: C57BL/6J Mc1r<sup>E/E</sup> Tyr<sup>c2j/c2j</sup> (albino wild, non-pigmented; functional Mc1r) and C57BL/J Mc1r<sup>e/e</sup> Tyr<sup>c2j/c2j</sup> (albino extension, non-pigmented; non-functional Mc1r). Mice were exposed to a single dose of UV irradiation (7,500 J/m²) with lamps emitting a spectral output in the 290 to 400 nm range (UV; UVP).

Cell Lines, Plasmids and UV Exposure

Murine and human melanoma cell lines B16, SK-MEL-2, A375, SBCL2, HEK 293, ARPE-19 and MCF-7 were purchased from ATCC, lightly and heavily pigmented primary melanocytes (Invitrogen), XP-A fibroblasts (Coriell Institute) and Seckel cell- lymphocytes (Coriell Institute) were cultured using standard methods. pcDNA4a containing FLAG-tagged XPA (Unsal-Kacmaz et al., 2007) (Addgene, MA),
pcDNA3.1 vectors containing either wild-type or mutated XPA (K188A) (Shell et al., 2009; Wu et al., 2007; Wu et al., 2006), wild-type ATR (Jiang and Sancar, 2006) or mutated ATR (S435A and S435D; constructed using the QuikChange site-directed mutagenesis kit (Stratagene, CA)), pDONR221 vector containing either wild-type MC1R (DNASU Plasmid Repository) or mutated MC1R (R160W and D294H; constructed using the QuikChange site-directed mutagenesis kit (Stratagene, CA)) were cultivated using standard procedures. siRNA targeted to ATR (Dharmacon) was performed using manufacturer’s instructions. UV at a dose of 10 J/m² (unless otherwise indicated) was delivered to cell cultures with lamps emitting either in UVB or UVC range.

**Antibodies**

The phospho-specific antibody (p-S435) was generated by Amsbio against the peptide CPKRRR(pS)SSLNPS. Commercially available antibodies used, anti-[6-4]-PP (Cosmo. Bio.), anti-CPDs (Kamiya), anti-XPA (Kamiya), anti-FLAG (Sigma), anti-ATR (Cell Signaling), anti-ATR (p-S428) (Cell Signaling), anti-PKA (Cell Signaling) and anti-Chk1 (Cell Signaling).

**DNA Repair Kinetics and Mutagenesis**

Immuno-slot-blots were performed on whole cells and nuclear extracts using either [6-4]-PP or CPD antibodies using standard methods (Mellon et al., 2002). All doses of UV for repair studies were 10 J/cm² unless stated otherwise. Acquired resistance of cells to 6-thioguanine (6-TG) is conferred primarily by mutations within the *hprt* locus and quantified as mutation frequency after selection. Frequencies of UV-induced *hprt* mutations were measured as previously described (Glaab and Tindall, 1997).
**Chromatin Isolation, Immunoprecipitation and Immunoblotting**

Chromatin isolation was performed essentially as previously described (Zou et al., 2002). Briefly, $5 \times 10^6$ cells were washed with PBS and resuspended in Solution A (10 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM MgCl$_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, with protease and phosphatase inhibitors freshly added (Thermo Scientific)). Cells were lysed with 0.5% Triton X-100 incubated for 5 min at 4°C, following which, nuclear proteins were pelleted by centrifugation at 1300 g for 5 min pelleted and washed with solution A and lysed in 400 μL of solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT) for 10 min at 4°C and centrifuged at 15000 g for 3 min. The chromatin was sheared by sonication and digested with micrococcal nuclease (50 units) and proteins resuspended in solution A. Immunoprecipitations were performed overnight at 4°C using 5 μg of antibody and protein G agarose, washed 5 times with RIPA buffer and separated by SDS-PAGE prior immunoblotting. All immunoblotting was performed using ECL and quantified using the STORM system.

**In Vitro Kinase Assays**

PKA kinase assays were carried out in a similar fashion to previous reports (Bondzi et al., 2000). Briefly, 30 μM of peptide (either custom ATR peptides; CPKRRRLSSSLNPS or CPKRRRLASSLNPS or commercially available PKA-substrate peptides; Kemptide, CREBtide, BCL-2 and H1-7 (Santa-Cruz) were used as substrates together with 40 mM Tris-Cl (pH 7.5), 10 mM MgCl$_2$, 1 mM DTT, 100 μg/mL BSA and 10 μM ATP. Reactions were either initiated by the addition of 10 nM recombinant catalytic subunit of PKA enzyme (Invitrogen) or whole cell lysate, carried out at 30°C and stopped by the addition of 10 μL of 100 mM EDTA. The extent of PKA phosphorylation was measured by immune-slot-blots using either anti-pS435 or anti-PKA substrate antibody (Cell Signalling). Detection was accomplished using ECL and quantified using the STORM system. Equal loading of PKA and substrate peptides was
confirmed by SYPRO Ruby staining (Invitrogen). The kinetic parameters of the phosphorylation reaction were calculated by non-linear regression analysis using Graphpad Prism.

**Immunofluorescence and Proximity Ligand Assay (PLA)**

To achieve localized UV irradiation, melanoma cells were grown on plastic chamber slides (Lab-Tek), media was aspirated, and UVC (50 J/m²) applied through sterile UV-absorbing polycarbonate with 3-μm pores (Millipore). The membrane was removed and cells were either processed immediately, or medium was replaced and DNA repair 30 minutes. Cell extraction was carried out *in situ* by 2 washes of 0.05% Nonidet P-40 followed by fixation in 4% paraformaldehyde. Antibodies directed to XPA (Abcam), [6–4] PP (Cosmo. Bio.) and secondary antibodies conjugated to DyLight Fluors (DyLight 549 and 488; Jackson ImmunoResearch) were used for immunodetection. PLA assay (DuoLink) was performed on 16 well chamber slides (Lab-Tek) and exposed to UV (10 J/m²). After antibodies were incubated overnight, fluorescence probes were used to visualize close proximity protein interactions as previously described (Soderberg et al., 2006). All fluorescence images were obtained using a Leica SP5 inverted confocal laser scanning microscope.

**BrdU/PI staining for cell cycle**

Sub-confluent A375 cells were treated with 5-bromo-2'-deoxyuridine (BrdU)/thymidine at final concentrations of 20 μM using standard procedures for anti-BrdU-fluorescence-based flow cytometry.

**Statistical Analysis**
All statistical assays, Student’s t test, and One Way ANOVA were performed using GraphPad Prism 5.0 (GraphPad Software, CA). Data were considered statistically significant if $p$ values were less than 0.05 from three independent experiments.

**AUTHOR CONTRIBUTIONS**

Conceived and designed the experiments: SGJ, JAD. Performed the experiments: SGJ, EMWH, PAC, JCV, MCB. Provided reagents and insight: YZ. Analyzed data: SGJ, EMWH, PAC, JCV. Wrote the paper: SGJ, JAD.

**ACKNOWLEDGMENTS**

We are indebted to David Fisher, Daret St.Clair and David Cortez and for helpful comments and insight. We thank Carol Beach and Haining Zhu of the University of Kentucky Proteomics Core Facility. This work was supported by the following NIH grants: R01 CA131075, UL1TR000117 and ES07266, T32 ES007266, T32CA165990. We also thank the Drury Pediatric Research Endowed Chair Fund, Wendy Will Case Cancer Research Fund, the Markey Cancer Foundation, the Children’s Miracle Network and the Jennifer and David Dickens Melanoma Research Foundation.
FIGURE LEGENDS

Figure 1. Mc1r Signaling Enhances Repair of UV-Induced Photolesions in vivo and in vitro.

(A) Images of congenic K14-Scf transgenic animal system defective at either the melanocortin 1 receptor (Mc1r) and/or tyrosinase (tyr) loci. Note that Mc1r loss results in a profound pigmentary phenotype in tyrosinase-intact animals but not their albino counterparts.

(B) Kinetics of UV-induced CPD repair in Mc1r Wild-type/albino (Mc1r<sup>E/E</sup>, Tyr<sup>c2j/c2j</sup>) vs. Mc1r Mutant/albino (Mc1r<sup>e/e</sup>, Tyr<sup>c2j/c2j</sup>) mice, *p ≤ 0.05.

(C) Kinetics of UV-induced CPD repair in Mc1r Mutant/albino (Mc1r<sup>e/e</sup>, Tyr<sup>c2j/c2j</sup>) mice treated topically either with vehicle or forskolin (80 mM), *p ≤ 0.05.

(D) Effect of cAMP agonists, MSH (100 nM), forskolin (20 μM) or rolipram (50 μM) on UV-induced CPD repair in B16 mouse melanoma cells. *signifies significant differences in % repair between vehicle and cAMP agonist treated cells, p ≤ 0.05.

(E and F) Forskolin enhances repair of [6-4]-PP and CPD in human cells. Cells were pre-treated with forskolin (20 μM) 30 min before UV exposure. The time taken to repair 50% of initial damage (repair t<sub>½</sub> (h)) was calculated and expressed as mean ± SEM, *signifies significantly different repair t<sub>½</sub> between vehicle and forskolin treated cells, p ≤ 0.05.

(G and H) Kinetics of UV-induced DNA repair ([6-4]-PP and CPD) in A375 cells either transfected with wild-type MC1R or MC1R mutants (R151C, R160W or D294H) and pre-treated with MSH for 30 min
and UV-irradiated. *signifies significantly different % repair between MC1R-WT and MC1R-mutant-expressing forskolin treated cells, \( p \leq 0.05 \).

**Figure 2. cAMP Signaling Enhances UV-Induced Chromatin Associated XPA.**

(A, B and C) Primary human melanocytes, SK-MEL-2 and A375 human melanoma cells were pre-treated with either vehicle control or forskolin (20 \( \mu \)M) for 1 h and UV-irradiated (10 J/m\(^2\)). Whole cell lysates or nuclear fractions containing soluble or chromatin fractions were collected at indicated times and immunoblotted for XPA. Percentages of chromatin-bound XPA from total cellular XPA were determined from 3 experiments. Histone 2B levels are indicated as a loading control.

(D) Double immunofluorescence and confocal microscopy was carried out to confirm the extent of co-localization of XPA (red) and [6-4]-PP (green) in A375 cells 30 minutes after filtered UVC exposure (50 J/m\(^2\); 3 micron pores) pre-treated (1 h) with vehicle control or forskolin (20 \( \mu \)M). Bars represent 5 \( \mu \)m.

(E) Quantification of XPA co-localization with [6-4] PP in cell lines. In each experiment, at least 50 cells were counted and the number of co-localized XPA/[6-4]-PP foci per nucleus quantified, \( *p \leq 0.05 \).

**Figure 3. cAMP-Mediated Signaling Promotes the Interaction Between XPA and ATR.**

(A) Co-immunoprecipitation experiments using whole cell extracts with either an anti-XPA or anti-ATR antibody and immunoblotted reciprocally after vehicle or forskolin (20 \( \mu \)M) pre-treatment (1 h) and UV exposure (10 J/m\(^2\)). Input 10% of total cellular lysate.
(B) Co-immunoprecipitation experiments using chromatin-bound proteins with either an anti-XPA or anti-ATR antibody and immunoblotted reciprocally after vehicle or forskolin (20 μM) pre-treatment (1 h) and UV exposure (10 J/m²). Input 10% of total nuclear extract.

(C) A375 cells were transfected with either scrambled siRNA (Ctrl) or siRNA targeted to ATR. Chromatin-bound proteins were immunoblotted with an anti-XPA antibody after vehicle or forskolin (20 μM) pre-treatment (1 h) and UV exposure (10 J/m²). Input 10% of total nuclear extract.

(D) Kinetics of UV-induced [6-4]-PP repair in A375 cells transfected with either scrambled siRNA (Ctrl) or siRNA targeted in ATR. Shown in the inset is ATR protein levels in scrambled vs. ATR-siRNA-treated cells. Values not sharing a common letter are significantly different as determined by One Way ANOVA, \( p \leq 0.05 \).

(E) Total Chk1 and phosphorylated Chk1 protein levels in A375 cells pre-treated with vehicle control or forskolin (20 μM) for 1 h and mock or UV-irradiated (10 J/m²).

(F) Anti-BrdU-fluorescence-based flow cytometry in A375 cells pre-treated with vehicle, forskolin (20 μM) or UV-irradiated (10 J/m²).

Figure 4. The cAMP-Mediated XPA and ATR Interaction is Required for Suppression of UV-Induced Mutagenesis.

(A) SK-MEL-2, A375 and SBCL2 melanoma cell lines were pre-treated with either vehicle control, MSH (100 nM) or forskolin (20 μM) for 1 h and either mock-treated or exposed to UV (10 J/m²), with colony-forming efficiency determined at 21 days after-6-TG treatment, \(* p \leq 0.05\).

(B) XP-A-null fibroblasts expressing either XPA-WT or XPA-K188A were exposed to UV (10 J/m²) and either processed immediately or allowed to repair for the indicated times. Immunoprecipitations were
performed on chromatin-bound proteins with either anti-XPA or anti-ATR antibody and immunoblotted reciprocally. Input 10% of total nuclear lysate.

(C) XP-A-null fibroblasts expressing either XPA-WT or XPA-K188A were pre-treated with either vehicle control or forskolin (20 μM) for 1 h and either mock-treated or exposed to UV (10 J/m²), with colony-forming efficiency determined at 21 days post-6-TG treatment. Values not sharing a common letter are significantly different as determined by One Way ANOVA ($p \leq 0.05$).

**Figure 5. PKA promotes ATR phosphorylation and its interaction with XPA**

(A) Co-immunoprecipitation experiments using A375 whole cell extracts with either an phospho-PKA substrate antibody that detects proteins phosphorylated at the PKA consensus sequence (RXS*X) (p-PKA) or anti-ATR and immunoblotted reciprocally after vehicle or forskolin (20 μM) pre-treatment. Cells were either mock-treated or exposed to UV (10 J/m²). Input 10% of total lysate. H-89 treatment was included to confirm PKA involvement.

(B) Co-immunoprecipitation experiments using A375 cells that were either transfected with wild-type FLAG expressing ATR (ATR-WT) or FLAG expressing mutant ATR with the PKA consensus motif modified via site-directed mutagenesis (S435A). Whole cell extracts were incubated with either a phospho-PKA substrate antibody (p-PKA) or anti-FLAG and immunoblotted reciprocally after vehicle or forskolin (20 μM) pre-treatment. Cells were either mock-treated or exposed to UV (10 J/m²). Input 10% of total lysate.

(C) A375 cells were pre-treated with forskolin (20 μM), exposed to UV (10 J/m²) and total levels of phosphorylated Ser435 were determined at 0, 0.5 and 2 h post-UV exposure in whole cell extracts using an antibody specific to phosphorylated S435 (p-S435).
(D) Co-immunoprecipitation experiments using A375 whole cell extracts with either an anti-phospho-Ser435 (p-S435) or anti-XPA, immunoblotted reciprocally after vehicle or forskolin (20 μM) pre-treatment. Cells were either mock-treated or exposed to UV (10 J/m²). Input 10% of total lysate.

(E) Sequence alignment of ATR orthologous at Serine 435 and surrounding residues among vertebrates. Red-shaded boxes show the conserved Serine, black-shaded boxes indicate conserved residues and yellow boxes denote residues with similar characteristics.

Figure 6. PKA Directly Phosphorylates ATR at Serine 435

(A) Seckel cell (ATR hypomorph)-lymphocytes were either transfected with wild-type FLAG expressing ATR (ATR-WT) or FLAG expressing mutant ATR (S435A). Whole cell lysates were immunoprecipitated with anti-FLAG and incubated with purified recombinant PKA (10 nM). Phosphorylation was determined by immunoblotting with anti-pS435. H-89 treatment was included to confirm PKA involvement.

(B) Direct PKA kinase assays were carried out using 30 μM peptide (either CPKRRRLSSSLNPS (wild type) or CPKRRRLASSLNPS (S435A mutant)) as substrates together with recombinant PKA (10 nM) and ATP (20 μM) for 10 min. The extent of phosphorylation at Ser435 was measured by immune-slot-blot using anti-pS435. Equal loading of PKA and substrate peptides was confirmed by SYPRO Ruby staining.

(C) Direct PKA kinase assays were carried out using 30 μM of indicated peptide (all containing a PKA phosphorylation site) as substrates together with recombinant PKA (10 nM) and ATP (20 μM) for 10 min. The extent of phosphorylation at Ser435 was measured by immune-slot-blot using anti-PKA-substrate antibody. The kinetic parameters of the phosphorylation reaction were calculated by non-linear regression analysis.
(D) HEK293 cells (MC1R null) were transfected with either wild-type MC1R or mutant MC1R (R160W or D294H) and pre-treated with MSH for 30 min. Whole cell lysates were incubated with 30 μM peptide (CPKRRRLSSSLNPS) as a substrate for 10 min. The extent of phosphorylation at Ser435 was measured by immune-slot-blot using anti-pS435. The kinetic parameters of the phosphorylation reaction were calculated by non-linear regression analysis.

(E) Seckel cell-lymphocytes were either transfected with wild-type FLAG expressing ATR (ATR-WT) or FLAG expressing mutant ATR (S435A) and exposed to UV (10 J/m²). Recombinant PKA was incubated with nuclear extracts for either 0, 1 or 3 h post UV exposure and [6-4]-PP removal was determined using immune-slot-blot using anti-pS435.

(F) Human cell lines (A375, MCF-7 and ARPE-19) were exposed to UV (10 J/m²) and recombinant PKA (either 1, 100 or 500 nM) incubated with nuclear extracts and DNA repair measured using anti-[6-4]-PP immunoblotting at either 0, 1 or 3 h post UV exposure. Graph shows linear regression analysis of time taken to repair 50% of initial damage (repair t½ (h)) correlated with the amount of recombinant PKA added to nuclear extract (i.e. 1, 100 or 500 nM).

Figure 7. PKA Phosphorylation of ATR Suppresses UV-Induced Mutagenesis by Mediating XPA Recruitment to Sites of UV Damage.

(A) Proximity ligation assay (PLA) was carried out to confirm a physical association between XPA and FLAG expressing ATR. Green detection events signify juxtaposition between anti-XPA and anti-FLAG (ATR) antibodies. Nucleus and actin were stained with DAPI (blue) and phalloidin (pink), respectively.

(B) Co-immunoprecipitation experiments using chromatin-bound proteins isolated from A375 cells with either anti-phospho-Ser435 (p-S435) or anti-XPA, and immunoblotted reciprocally after vehicle or forskolin (20 μM) pre-treatment and mock-treated or exposed to UV (10 J/m²). Input 10% of total lysate.
(C) Proximity ligation assay (PLA) between XPA and ATR phosphorylated on Ser435 on chromatin. A375 human melanoma cells were pre-treated with either vehicle control or forskolin (20 μM) and either mock-treated or UV-irradiated (10 J/m²). *In situ* detergent extraction was used to remove all soluble proteins but retain chromatin binding proteins. Green detection events signify a close interaction between anti-XPA and anti-pS435 (ATR) antibodies. Nuclei were stained with DAPI (blue). In each experiment, at least 30 cells were analyzed.

(D and E) A375 cells were transfected with an either empty vector, ATR-WT, ATR-S435A or S435D and either mock treated or exposed to UV (10 J/m²). Immunoblots were performed on chromatin-bound proteins with anti-XPA.

(F) Repair of [6-4]-PP. A375 cells were treated with siRNA directed to ATR, and then transfected with either FLAG-ATR-WT, FLAG-ATR-S435A or FLAG-ATR-S435D. Cells were pre-treated with forskolin (20 μM) and either mock-treated or exposed to UV (10 J/m²). Repair was measured at 3 and 9 h post-damage using an antibody specific for [6-4]-PP.

(G) A375 cells were treated with i) scrambled siRNA, ii) siRNA directed to ATR, iii) siRNA directed to ATR and transfected with FLAG-ATR-WT, iv) siRNA directed to ATR and transfected with FLAG-ATR-S435A, (v) siRNA directed to ATR and transfected with FLAG-ATR-S435D or (vi) the specific ATR inhibitor, VE-821. Endogenously expressed ATR knockdown and transfected ATR expression was confirmed by Immunoblot. Cells were pre-treated with forskolin (20 μM) or vehicle and exposed to UV (10 J/m²), with colony-forming efficiency determined at 21 days post-6-TG treatment. Note no colonies were observed in the absence of UV. Values not sharing a common letter are significantly different as determined by One Way ANOVA ($p \leq 0.05$).

REFERENCES

induced DNA damage and cytotoxicity. FASEB journal: official publication of the Federation of American Societies for Experimental Biology 20, 1561-1563.


Primary human melanocytes (MC1R-WT)

Fraction    | I.B | Vehicle   | Forskolin
-----------|-----|-----------|-----------
Soluble nuclear | XPA |           |           
Chromatin     | XPA |           |           
Whole cell    | XPA |           |           

% Chromatin-bound XPA

- UV | 0.5 | 2   | 4   
Time after UV (h)

H2B

SK-MEL-2 (MC1R-WT)

Fraction    | I.B | Vehicle   | Forskolin
-----------|-----|-----------|-----------
Soluble nuclear | XPA |           |           
Chromatin     | XPA |           |           
Whole cell    | XPA |           |           

% Chromatin-bound XPA

- UV | 0.5 | 2   | 4   
Time after UV (h)

H2B

A375 (MC1R-R151C)

Fraction    | I.B | Vehicle   | Forskolin
-----------|-----|-----------|-----------
Soluble nuclear | XPA |           |           
Chromatin     | XPA |           |           
Whole cell    | XPA |           |           

% Chromatin-bound XPA

- UV | 0.5 | 2   | 4   
Time after UV (h)

H2B

A375 (MC1R-R151C)

- UV

Vehicle

+UV

Forskolin

+UV

6-4 PP | XPA | Merge | DAPI

No. merged signals/nucleus

A

B

C

D

E

Figure
Figure 4

A

Mutation Frequency ($10^{-4}$)

- UV  Vehicle  MSH  Forskolin

Sk-Mel-2 (MC1R-WT)

A375 (MC1R-R151C)

SBCL2 (MC1R-V60L)

B

XPA-WT - Chromatin

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Input

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Time after UV (h)

XPA-K188A - Chromatin

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Input

- UV  0.5  2

Time after UV (h)

C

Mutation Frequency ($10^{-4}$)

- UV  Vehicle  Forskolin

XPA-WT  XPA-K188A

Vehicle

Forskolin

Jarrett et al, Figure 4
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- **p-S435/XPA:** Proportions of p-S435/XPA
- **p-PKA/ATR:** Proportions of p-PKA/ATR
- **I.B:** Immunoblot

**Figure:**

- **A:** Whole cell lysate
- **B:** Whole cell lysate
- **C:** Whole cell lysate
- **D:** Whole cell lysate
- **E:** Ser435

**Species:**

- Homo sapiens
- Pan troglodytes
- Equus ferus caballus
- Bos primigenius
- Ovis aries
- Rattus norvegicus
- Mus musculus
- Heterocephalus glaber
- Ceanothus griseus
- Anas platyrhynchos
- Ceanothus mydas
- Alligator mississippiensis
- Xenopus laevis
- Danio rerio
A. Whole cell lysate
   - Recomb. PKA
   - I.P FLAG
   - I.B p-S435
   - Input
   - ATR

B. Peptide
   - PKA
   - H-89
   - anti-pS435
   - SyproStain (10% Input)

C. PKA Phosphorylation (A.U)
   - Kemptide = 4 μM
   - CREBtide = 10 μM
   - ATR S435 = 19 μM
   - BCL-2 = 54 μM
   - Histone-1 = 60 μM
   - ATR S435A = N/A

D. S435 Phosphorylation (A.U)
   - MC1R wt = 15 μM
   - MC1R D294H = 51 μM
   - MC1R null = 61 μM
   - MC1R R160W = 45 μM

E. Nuclear extract
   - Recomb. PKA
   - I.B 6-4 PP
   - ATR-S435A
   - ATR-WT

F. (6-4)-PP Repair ½ (h)
   - A375 - $r^2 = 0.81$
   - MCF-7 - $r^2 = 0.61$
   - ARPE-19 - $r^2 = 0.72$
Figure 7

A:

PLA: ATR-XPA

Control
Forskolin
UV
UV + Forskolin

DAPI

B:

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<thead>
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<th>+ UV</th>
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Chromatin

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Whole cell lysate

Input

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<td>ATR</td>
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C:

- Forskolin
+ Forskolin

D:

-UV

Chromatin

Input

- vector
ATR-WT
S435A
S435D

I.B

E:

+UV

Chromatin

Input

- vector
ATR-WT
S435A
S435D

I.B

F:

% [6-4] PP Repair

Time-post UV (h)

Vehicle
Forskolin

WT
S435A
S435D

G:

Mutation Frequency (10^-4)

Vehicle
Forskolin

Ctrl
sIRNA
ATR
ATR-S435A
ATR-S435D
VE-821

Chl
sIRNA
ATR
ATR-S435A
ATR-VE-821

No. of pS435-XPA Signals /Nucleus

-UV
+UV

Forskolin

0 1 2 3 4 5

-    -  +  + Forskolin
UV -- ++

- Forskolin
+ Forskolin

0 10 20 30 40 50

1.0    1.0      10    3.5

+UV

-UV

1.0    1.0      10    3.5

+UV

-UV
Inventory of all Supplemental Items

Figure S1. Mc1r status does not influence the initial amount of UV-induced DNA damage in vivo or in vitro. These data show that Mc1r specifically enhances repair of photoproducts without affecting the initial amount of UV-induced DNA damage.

Figure S2. cAMP Does Not Influence Cell Proliferation in Melanocytes. This figure demonstrates that none of the treatments used in this study impact cell proliferation, thus supporting our conclusion that MC1R activity enhances DNA repair and that clearance of UV photolesions. Further, we show that UV damage alone does not cause an intracellular cAMP increase.

Figure S3. cAMP Signaling Mediates Recruitment of XPA to UV-induced Photolesions. This figure shows that cAMP does not impact protein levels of core NER factors but does enhance recruitment of XPA to photolesions.

Figure S4. The Anti-pS435 Antibody is Specific For Phosphorylation of Serine 435 on ATR. These data confirm that the newly generated anti-phospho Ser435 antibody is specific to this residue.

Figure S5. cAMP Promotes Nuclear Enrichment of ATR, ATR-pS435 and XPA. This figure highlights that cAMP treatment enhances nuclear levels of ATR phosphorylated at Ser435.

Figure S6. Recruitment of ATR-XPA is Dependent upon Phosphorylation of Ser435. These data show that recruitment of ATR-XPA to UV-damaged chromatin depends on PKA-mediated phosphorylation of Ser435 on ATR.

Supplemental Table 1. This table shows that cAMP-mediated signaling enhances the repair of UV-induced DNA damage in a variety of human melanocyte and non-melanocyte cell lines.
Figure S1. Mc1r Status Does Not Influence the Initial Amount of UV-Induced DNA Damage in vivo or in vitro. The relative abundance of UV-induced CPD in (A and B) Albino (C57BL/6J Tyr<sup>c2j/c2j</sup>, Mc1<sup>e/E</sup>) and albino extension (C57BL/6J Tyr<sup>c2j/c2j</sup>, Mc1<sup>e/e</sup>) mice and (C and D) B16 mouse melanoma cells either treated with vehicle, MSH (100 nM), forskolin (20 μM) or rolipram (50 μM).
Figure S2. cAMP Does Not Influence Cell Proliferation in Melanocytes. (A, B, C and D) Cells were treated with either vehicle control, α-MSH (100 nM), α-MSH (100 nM) + ASIP (100 nM), or forskolin (20 μM). Cells were counted in duplicate wells at 24, 48 and 72 h after indicated treatment. (E) A375 cells were pre-treated with either vehicle or forskolin and mock-treated or exposed to UV. cAMP levels were measured at the indicated times using an cAMP ELISA kit.
**Figure S3. cAMP Signaling Mediates Recruitment of XPA to UV-Induced Photolesions.** (A) Protein levels of NER core factors were determined following forskolin treatment (B) FLAG-tagged XPA-expressing A375 melanoma cells pre-treated with either forskolin (20 μM) or vehicle control for 1 hr and UV-irradiated through a polycarbonate UV-absorbing filter containing 3-μm pores (50 J/m²; UVC) and quantification of XPA co-localization with [6-4] PP. In each experiment, at least 50 cells are counted to determine the % of cells containing co-localized foci. (C) Co-immunoprecipitation experiments using chromatin extracted from A375 cells with either anti-[6-4]-PP or anti-XPA antibodies, and immunoblotted reciprocally after vehicle or forskolin (20 μM) pre-treatment and mock-treated or UV exposed (input 10% of total lysate). (D) MC1R wild-type melanocytes were either treated with forskolin, MSH, MSH + agouti signaling protein or forskolin + agouti signaling protein before UV exposure and chromatin XPA levels were determined via immunoblotting. (E) The same treatment groups were also used to analyze the effect on the repair of [6-4]-PP.
Figure S4. The Anti-pS435 Antibody is Specific for Phosphorylation of Serine 435 on ATR and S435 does not Influence Cell Sensitivity to UV. (A) Affinity purified antibody (anti-pS435 and anti-S435) was added to 5 ng of peptide to determine specificity. (B) A375 cells were transfected with either ATR-WT (FLAG) or ATR-S435A (FLAG) and pre-treated with forskolin (20 μM) for 1 hr followed by UV exposure. Cells were immunoprecipitated with anti-FLAG and immunobotted with either anti-pS435 or anti-p428 (commercially available; Cell Signaling). Note the addition of phosphatase removed p-S435 and p-428 antibody recognition. (C) A375 cells were transfected with either ATR-WT (FLAG) or ATR-S435A (FLAG) and pre-treated with vehicle or forskolin (20 μM) for 1 hr and immunoprecipitated with anti-FLAG and immunobotted with anti-pS435. (D) A375 cells were treated with i) scrambled siRNA, ii) siRNA directed to ATR, iii) siRNA directed to ATR and transfected with FLAG-ATR-WT, iv) siRNA directed to ATR and transfected with FLAG-ATR-S435A; (v) ATR-kinase-dead or (vi) the specific ATR inhibitor VE-821. Extent of endogenously expressed ATR knockdown and transfected ATR expression was determined by Western Blot. Cells were exposed to varying doses of UV. Cell viability was determined 24 hrs following UV exposure by MTT.
Figure S5. cAMP Promotes Nuclear Enrichment of ATR, ATR-pS435 and XPA. Immunofluorescence and confocal microscopy were carried out to determine the effect of cAMP on cytoplasmic/nuclear distribution of ATR, ATR-pS435 and XPA. Cells were pre-treated with either forskolin (20 μM) or vehicle and the cytoplasmic/nuclear ratio of each protein was determined. All fluorescence images were obtained using a Leica SP5 inverted confocal laser scanning microscope and analyzed as inverted grey scale images. Data were considered statistically significant if p values were less than 0.05.
Figure S6. Recruitment of ATR-XPA is Dependent upon Phosphorylation of Ser435. (A) Co-immunoprecipitation experiments using chromatin extracted from mock-treated or UV exposed A375 cells ± H-89 or A375 cells expressing wild-type FLAG-ATR (ATR-WT) or FLAG-mutant ATR (ATR-S435A) following either pre-treatment with vehicle or forskolin (20 μM). (B) Representative Western Blot confirming that endogenous ATR expression in A375 cells was reduced following siRNA-directed knockdown and that cells transfected with FLAG-ATR constructs expressed FLAG.
Supplemental Table 1. cAMP-mediated signaling enhances the repair of UV-induced DNA damage in a variety of human melanocyte and other cell lines.

<table>
<thead>
<tr>
<th>Cell type and treatment</th>
<th>[6-4]-PP repair t½ (h)</th>
<th>CPD repair t½ (h)</th>
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<tr>
<td>Primary human melanocytes (MC1R-WT) (Lightly pigmented)</td>
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<tr>
<td>Vehicle Control</td>
<td>2.2 ± 0.4</td>
<td>49.2 ± 2.0</td>
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<tr>
<td>α-MSH</td>
<td>1.2 ± 0.3</td>
<td>27.6 ± 6.7</td>
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<td>α-MSH + ASIP</td>
<td>3.1 ± 0.5</td>
<td>67.3 ± 5.7</td>
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<td>Forskolin</td>
<td>0.9 ± 0.2</td>
<td>22.4 ± 3.1</td>
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<tr>
<td>Primary human melanocytes (MC1R-WT) (Heavily pigmented)</td>
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<td>Vehicle Control</td>
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<td>52.2 ± 3.1</td>
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<tr>
<td>α-MSH</td>
<td>1.2 ± 0.4</td>
<td>30.1 ± 2.4</td>
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<td>α-MSH + ASIP</td>
<td>2.8 ± 0.4</td>
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<tr>
<td>Forskolin</td>
<td>0.9 ± 0.3</td>
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<td>Sk-Mel-2 (MC1R-WT)</td>
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<tr>
<td>α-MSH</td>
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<td>α-MSH + ASIP</td>
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<td>A375 (MC1R-R151C)</td>
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<td>Vehicle Control</td>
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<tr>
<td>Forskolin</td>
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<td>21.2 ± 3.3</td>
</tr>
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</table>

Repair t½ (h) expressed as time taken for [6-4] PP or CPDs to repair 50% of initial damage and expressed as mean ± SEM. Cells were pre-treated with α-MSH (100 nM), agouti signaling protein (ASIP; 100 nM) or forskolin (20 μM) before UV exposure (10 J/cm²), and abundance of either [6-4]-PP or CPD was calculated. *signifies repair t½ significantly different photoproduct repair compared to vehicle control, as determined by two-way ANOVA and mean separation.