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AKAP12 mediates PKA-induced phosphorylation of ATR to enhance nucleotide excision repair

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ABSTRACT

Loss-of-function in melanocortin 1 receptor (MC1R), a \(G_\text{S}\) protein-coupled receptor that regulates signal transduction through cAMP and protein kinase A (PKA) in melanocytes, is a major inherited melanoma risk factor. Herein, we report a novel cAMP-mediated response for sensing and responding to UV-induced DNA damage regulated by A-kinase-anchoring protein 12 (AKAP12). AKAP12 is identified as a necessary participant in PKA-mediated phosphorylation of ataxia telangiectasia mutated and Rad3-related (ATR) at S435, a post-translational event required for cAMP-enhanced nucleotide excision repair (NER). Moreover, UV exposure promotes ATR-directed phosphorylation of AKAP12 at S732, which promotes nuclear translocation of AKAP12–ATR–pS435. This complex subsequently recruits XPA to UV DNA damage and enhances 5′ strand incision. Preventing AKAP12’s interaction with PKA or with ATR abrogates ATR-pS435 accumulation, delays recruitment of XPA to UV-damaged DNA, impairs NER and increases UV-induced mutagenesis. Our results define a critical role for AKAP12 as an UV-inducible scaffold for PKA-mediated ATR phosphorylation, and identify a repair complex consisting of AKAP12–ATR–pS435–XPA at photodamage, which is essential for cAMP-enhanced NER.

INTRODUCTION

Ultraviolet (UV) radiation is among the most important causative risk factors for cutaneous melanoma, an aggressive malignancy whose incidence has risen sharply over the past several decades (1). A critical inherited risk factor for UV skin sensitivity and melanoma is loss of signaling of the melanocortin 1 receptor (MC1R), a \(G_\text{s}\) protein-coupled cell surface receptor on melanocytes activated by melanocyte stimulating hormone (MSH). MC1R function, mediated by cyclic adenosine 3,5-monophosphate (cAMP)-dependent signaling, is central to UV resistance by promoting melanin synthesis (2) and enhancing DNA repair of mutagenic UV photodamage (3–6).

DNA repair is essential for maintaining the integrity of the genome, which when faulty contributes to mutagenesis, genetic instability and carcinogenesis. The nucleotide excision repair (NER) pathway is the primary system for removing UV-induced mutagenic photolesions such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts ([6-4]-PPs). The xeroderma pigmentosum complementation group proteins (XPs), which include XPA through XPG, play critical roles in coordinating and promoting NER (7). NER corrects UV-induced DNA damage in a multistep process involving recognition of helical distorting lesions by XPC–RAD23B (8), and in some cases UV–DDB (9). Recruitment of transcription factor II H (containing XPB and XPD) leads to strand separation, enabling other NER factors to bind, including XPA, replication protein A (RPA), XPG, and excision repair cross-complementation group 1 (ERCC1)-XP-F (10,11). Once ERCC1-XP-F is correctly positioned on DNA via its interaction with XPA, it incises the damaged strand 5′ to the lesion (12), followed by XPG performing the 3′ incision (13). DNA is restored to its original form by the action of replicative DNA polymerases and associated factors using the undamaged complementary strand as a template (14–16). Ataxia telangiectasia mutated and Rad3-related (ATR) is critical to UV DNA damage signaling (17,18), cell survival (19–22) and is linked with NER (23–25). We recently described a molecular pathway linking MC1R signaling with NER through a protein kinase A (PKA)-mediated phosphorylation event on ATR at S435, which accelerates XPA recruitment to sites of UV-induced DNA damage (5).

PKA is composed of catalytic (C) and regulatory (R) subunits arranged as a tetrameric \(R_2C_2\) inactive holoenzyme...
(26). When cAMP levels are low, the PKA holoenzyme is maintained in an inactive state; however, upon binding of cAMP to R subunits, the C subunits are released as active monomers. A-kinase anchoring proteins (AKAPs) are scaffolding proteins that regulate cellular cAMP responses by spatiotemporally coordinating PKA with target proteins specific to individual activation stimuli (27,28). AKAP12 (also called Gravin and SSeCKS) has been implicated in a wide range of cell functions, including tumor suppression (29–31), cytoskeletal architecture (32,33), β2-adrenergic receptor desensitization/resensitization (34,35) and cell cycle regulation (36–38). AKAP12 activities have been described at the plasma membrane, the cell periphery and at perinuclear regions of the cytoplasm (28). Although AKAP12 possesses multiple nuclear localization sequences (39), the molecular dynamics that control nuclear translocation remain poorly understood. In support of a nuclear function, AKAP12 localizes to centrosomes and mitotic spindles in dividing cells and interacts with Polo-like kinase 1, an important regulator of mitotic progression and genomic stability (37). AKAP12 has also been reported at sites of stalled replication forks following nucleotide depletion (40), however to date, AKAP12 has not been implicated in DNA repair.

Here, we identify a novel cAMP-directed pathway for sensing and repairing UV-induced DNA damage. Mechanistically, AKAP12 regulates PKA-mediated phosphorylation of ATR-pS435 downstream of MC1R/cAMP signaling within the cytoplasm. With UV damage, ATR phosphorylates AKAP12 at S732 which stimulates nuclear translocation of an AKAP12–ATR–pS435 complex that results in enhanced 5′ strand incision of NER. Disruption of the AKAP12–ATR axis abolishes ATR phosphorylation at Serine 435, reduces recruitment of XPA to photoproducts, blunts NER and elevates UV-induced mutagenesis. This study defines a nuclear role for AKAP12 as a critical regulator of NER and uncovers a novel pathway of NER regulation in melanocytes.

MATERIALS AND METHODS

Cell lines, plasmids, gene silencing, pharmaceutical inhibitors, antibodies and UV exposure

HEK293, A375, SK-Mel-2 and WM3211 cell lines (ATCC), XP-A fibroblasts (Coriell Institute) and primary melanocytes (Coriell Institute) were cultured using standard methods. pcDNA3.1 vectors containing wild-type XPA (41), wild-type ATR (42), ATR-S435A and ATR-S435D (5), HA-tagged AKAP12 or truncated mutants AKAP12Δ353-1537 (43,44) and AKAP12Δ353-1537-1783, pDONR221 vector containing either wild-type MC1R (DNASU Plasmid Repository) or MC1R-R160W and MC1R-D294H (5), were all cultivated using standard procedures. Mutants of HA-tagged AKAP12 were engineered to abolish PKA binding (AKAP12Δ353-1537; AKAP12ΔPKA), prevent ATR binding (AKAP12Δ353-580; AKAP12ΔATR), impair transport of AKAP12 to the nucleus (AKAP12Δ353-1783; AKAP12ΔNLS) and non-phosphorylatable mutants of AKAP12-S337A, -S505A, -S732A and -S887A (Aligent QuickChange II XL mutagenesis kit). CRISPR targeted to AKAP12 (Santa Cruz) and siRNA targeted to ATR, AKAP12, XPG, SLX4 and PKA (Dharmacon) was performed using manufacturer’s instructions. Inhibitors for ATR kinase activity (VE-821), ataxia telangiectasia mutated (ATM) kinase activity (KU-55933) and AKAP-R binding (a stearated competitive peptide; st-Ht31) were all used at a concentration of 10 μM. Antibodies used were ATR-pS435 and ATR-WT (5), [6-4]-PP (Cosmo. Bio., Cat# CAC-NM-DND-001), CPD (Kamiya, Cat # MC-068), AKAP12 (Thermo Fisher Scientific, Cat # PA5-21759), XPA (Cell Signaling, Cat # 14607S), XPC (Cell Signaling, Cat # 14768), ATR (Cell Signaling, Cat # 13934), phospho-ATM/ATR substrate (Cell Signaling, Cat # 2851), phospho-PKC substrate (Cell Signaling, Cat # 2261), RII subunit-PKA (Abcam, Cat # 38949), catalytic subunit-PKA (Cell Signaling, Cat # 4782), HA (Cell Signaling, Cat # 3724), H2A (Cell Signaling, Cat # 12349), Aly (Santa Cruz, Cat # 32311) and FLAG (Cell Signaling, Cat # 8146). UV radiation was measured via a Model IL1400A handheld flash measurement photometer (International Light) with UV lamps emitting a spectral output in the 290–400 nm range (72% UVB, 27% UVA, <0.01% UVC) (UVP, Upland, CA, USA). UV exposure was performed when media was removed from the cells. A dose of 10 J/m2 of UVB was delivered to cell cultures except for DNA repair/mutagenesis studies that used 20 J/m2 of UVB and photomutation confocal imaging studies which used 10 J/m2 of UVC.

Sub-cellular fractionation, immunoprecipitation and immunoblotting

Sub-cellular fractionation was performed with ~2 × 106 cells, which were washed with phosphate buffered saline and resuspended in 200 μl of solution A (10 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol (DTT), 10 mM NaF, 1 mM Na2VO3 and protease inhibitors (Halt Protease and Phosphatase inhibitors; Thermo Scientific). Cells were lysed with the addition of Triton X-100 (0.05%), followed by incubation at 4°C for 10 min. Cytoplasmic proteins were separated from nuclei by centrifugation at 1000 g for 5 min. Isolated nuclei were washed with solution A and lysed in 200 μl of solution B (3 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM DTT). The soluble nuclear proteins were separated from chromatin by centrifugation at 2000 g for 5 min. To release the proteins associated with DNA for oligonucleotide retrieval assay-immunoprecipitation (ORiP) assays, the pellet was treated with DNase I (50 U) for 30 min at 37°C in 60 mM Tris–HCl (pH 7.5), 2.5 mM MgCl2 and 11 mM CaCl2). The reaction was stopped by the addition of 1 mM EGTA and the reaction centrifuged at 2000 g for 5 min at 4°C to obtain the supernatant. The isolated fractions were analyzed by western blotting to determine purity using anti-Tubulin, anti-Aly and anti-H2A antibodies, with only confirmed pure samples used in assays. To obtain chromatin containing proteins for western blotting, the chromatin pellet was washed once with solution B and centrifuged at 10 000 g for 1 min. Isolated chromatin was resuspended in 20 mM Tris–HCl, pH 7.9, 100 mM NaCl, 20% glycerol, 0.1 Nonidet P-40 and...
sheared by sonication. Chromatin-bound proteins were obtained after centrifugation at 10 000 g for 10 min. Samples were resuspended in 100 μl of sodium dodecyl sulphate (SDS) sample buffer and heated at 95°C for 10 min before western analysis. When Co-IP analysis was performed, 10% of lysates were set aside for input loading lysates. Either 5 μg of antibody or non-immune IgG was incubated with samples overnight at 4°C. Following 2 h incubation with Protein A agarose beads (GE Healthcare), samples were resolved on Tris-SDS gels prior to immunoblotting and visualization by ECL using the STORM system.

Oligonucleotide retrieval-immunoprecipitation (ORiP)

Synthetic oligonucleotides (Molecular Beacons) were assembled to form 5′-biotinylated duplex DNA fragments that acts as substrates for NER proteins. A 30-nt oligonucleotide, 5'-CTCGTACGATTTTTTTTTTTTTTTTTTTTTTTTTAAGCTTTTATGTCC-3', was exposed to 10 J/m² of UVC, annealed and ligated with two oligonucleotides as previously described (43,44) for AKAP12 binding studies. For XPA and ERCC1-XPF binding a 5′-biotinylated stem-loop substrate (GCCAGCGCTCGG(T)22CCGAGCGCTGGC) was utilized (45). After indicated treatments, chromatin extracts (50 μg) were incubated with the biotinylated oligonucleotide in streptavidin-coated 96 well plates (Thermo-Scientific) (0.01 nM per well) for indicated times at 30°C. Wells were washed with 40 mM Tris–HCl (pH 7.5) containing 0.01% bovine serum albumin (BSA) (wash buffer) followed by fixation in 4% paraformaldehyde. After three washes, 2 μg of either anti-XPA, anti-ERCC1, anti-XPF or anti-AKAP12 was added for 1 h. Detection was accomplished using an HRP-conjugated anti-rabbit secondary antibody (Abcam) for 1 h followed by the addition of 1-Step Ultra TMB ELISA Substrate (Pierce) to each well and absorbance measured at 400 nm.

PKA and PKC kinase assays

PKA kinase assays were performed using biotinylated ATR peptide substrates containing S435 and its surrounding residues, CPKRRRSLSSLNPS or CPKRRRLASSLNPS (Genscript). protein kinase C (PKC) kinase assays were performed using a biotinylated CREB peptide substrate, CKRREILSRPRPYSRK (Genscript). Reactions containing peptide substrate (10 μM) were performed in streptavidin-coated 96-well plates by the addition of nuclear lysate (500 μg) in 40 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 100 μg/ml BSA and 10 μM ATP. Kinase reactions were carried out at 30°C with gentle agitation and terminated by the addition of 10 μl of 100 mM EDTA. PKA and PKC phosphorylation was measured with either an anti-ATR-pS435 or PKC phosphorylation substrate antibody, respectively. Detection was accomplished using an HRP-conjugated anti-rabbit secondary antibody (Abcam) for 1 h followed by the addition of 1-Step Ultra TMB ELISA Substrate (Pierce) to each well and absorbance measured at 400 nm.

Immunofluorescence, in situ detergent extraction and proximity ligation assay

Following damage, cells were either processed immediately or medium was replaced and DNA repair allowed for indicated periods. Cell extraction was carried out in situ by washes of 0.1% Nonidet P-40 for 10 min on ice to remove all soluble proteins. Following fixation in 4% paraformaldehyde and cell permeabilization with 0.3% Triton X-100, cells were blocked overnight in 10% donkey serum at 4°C. After incubation with indicated primary and secondary antibodies, cells were mounted with Prolong Gold antifade. Proximity ligation assay (Duolink, Sigma) was performed using the manufacturer’s instructions and as previously described (5). All fluorescence images were obtained using a Leica DMI 6000 confocal microscope using ×100 objective (1.4 numerical aperture) with LAS AF 2.7.2.9586 software (Leica Application Suite Advanced Fluorescence). Maximum intensity images from focal plane z-stacks (spaced 0.2 μm apart) were acquired and deconvoluted. Quantification of fluorescent signals was performed using Image J software.

DNA repair kinetics and mutagenesis

Cells were exposed to 20 J/m² of UVB and immuno-slot blots were performed with either [6-4]-PP or CPD antibodies. The frequencies of UV-induced hprt mutations were measured as previously described (46).

Endonuclease assays

Stem-loop oligonucleotide (GCCAGCGCTCGG(T)22CCGAGCGCTGGC) labeled with fluorescent dye 6-FAM at the 5′-end (IDT) and as described previously (45) and a bubble substrate 5′CCAGTGATCACA TACGCTTTGCTA GGACATCCCCCCCCCCCCCCCCCCCCCCCCCCCC CCCAGTGGCCACGTGTGA TGCCCACTTTGACGCCG3′ labeled with fluorescent dye 6-FAM at the 5′-end (IDT) as previously described (47) were annealed in annealing buffer (10 mM Tris pH 8.0, 1 mM EDTA and 100 mM NaCl) by heating at 90°C for 5 min along with an equal amount of unlabeled bubble-down oligonucleotides 5′CGGTCAACGTGGGCTATACACGTGGCGCTGTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT CAGAAGCTTGACGTTGA TCGCAAGCTTGAGGTTACGTT5′. Chromatin fractions (50 μg; in 25 mM Tris–HCl pH 8.0, 40 mM NaCl, 1.0% NP-40, 0.4M MnCl₂) were incubated with 100 nM of the substrates in a volume of 50 μl for indicated times at 37°C with reactions quenched by the addition of 10 μl of 80% formamide/10 mM EDTA. After heating at 95°C for 5 min, 10 μl of each sample were analyzed on 4–20% polyacrylamide gels and a STORM phosphoimager using the blue channel (Molecular Dynamics).

RESULTS

ATR interacts with AKAP12

MC1R signaling is critical for genome stability and UV resistance in melanocytes and enhances NER through cAMP-dependent PKA-mediated phosphorylation of ATR (5). To understand how PKA-mediated ATR phosphorylation...
is regulated, we screened binding partners of ATR–pS435 using a phospho-specific antibody. A proteomic study of UVB-exposed melanocytes identified an association between ATR–pS435 and AKAP12. Co-immunoprecipitation (Co-IP) experiments confirmed that UV exposure enhanced the physical interaction between ATR and AKAP12 in primary and transformed melanocytes as well as in HEK293 cells (Figure 1A). In order to validate our results, Co-IP was performed in HEK293 cells transfected with plasmids encoding either full-length or truncated forms of AKAP12. The AKAP12 mutant that retained the N terminus (AKAP12\textsuperscript{NLS-1783}) interacted with ATR as efficiently as the full length AKAP12 (Figure 1B), however the AKAP12 mutant that retained the C terminus (AKAP12\textsuperscript{51-839}) failed to associate with ATR, suggesting that AKAP12 binds ATR through its N-terminal region.

We investigated the sub-cellular distribution of UV-induced ATR–AKAP12 interactions by proximity ligation, a sensitive method that detects protein–protein interactions (48). In the absence of UV-induced damage no association was observed between ATR and AKAP12 (Figure 1C). Following UV however, a robust ATR–AKAP12 interaction was evident in both the cytoplasmic and nuclear compartments (Figure 1C). Since AKAPs are known to scaffold PKA-mediated phosphorylation, we reasoned that the UV-induced nuclear translocation of AKAP12–ATR might be dependent on PKA function. To investigate, we generated AKAP12 mutants lacking amino acids 1537–1560 (AKAP12\textsuperscript{APKA}), corresponding to the regulatory subunit (R) binding domain of PKA (49), AKAP12 lacking amino acids 5–500 which prevents ATR binding (AKAP12\textsuperscript{ΔATR}) and AKAP12 lacking amino acids 501–767 with a deleted nuclear localization domain (AKAP12\textsuperscript{ΔNLS}) (39) (Figure 1D and Supplementary Figure S1A). A CRISPR/Cas9-edited AKAP12 null background in HEK293 cells was utilized for AKAP12 transfection studies (Supplementary Figure S1B). We observed equal expression of wild-type AKAP12 and deletion variants (AKAP12\textsuperscript{APKA}, AKAP12\textsuperscript{ΔNLS} and AKAP12\textsuperscript{ΔATR}), validated mutation-specific defects (i.e. impaired R subunit binding, lack of ability to localize to the nucleus and defective ATR binding, respectively) and confirmed that UV did not influence total cellular AKAP12 mRNA (Supplementary Figure S1A–J). Following UV exposure, ATR–AKAP12-WT and ATR–AKAP12\textsuperscript{ΔPKA} interactions were detected in purified cytoplasmic, nuclear and chromatin fractions (Figure 1E and Supplementary Figure S1J). In contrast, AKAP12\textsuperscript{ΔATR} failed to bind ATR and ATR–AKAP12\textsuperscript{ΔNLS} interactions were only present in the cytoplasm (Figure 1E). Together, these data suggest that UV induced AKAP12–ATR binding occurs in the cytoplasm, in the nucleus and at chromatin, that nuclear or chromatin localization of the complex requires AKAP12's nuclear localization domain and that PKA binding is not required for the UV-induced ATR–AKAP12 interaction.

Since AKAPs provide the critical cellular function of directing PKA to subcellular compartments (50), we tested whether the PKA holoenzyme co-distributed with AKAP12 in the nucleus (Figure 1F). HA-tagged AKAP12 plasmids encoding wild-type AKAP12, AKAP12\textsuperscript{ΔPKA}, AKAP12\textsuperscript{ΔNLS} or AKAP12\textsuperscript{ΔATR} were used to perform pull-downs from cytoplasmic, nuclear and chromatin lysates using an anti-HA antibody. Immunoprecipitation of AKAP12 followed by immunoblotting with the catalytic (C) or the regulatory (R) subunit of PKA clearly showed that PKA-AKAP12 interactions only occurred in the cytoplasm.

### ATR phosphorylates AKAP12

The observation that UV damage promoted the nuclear translocation of an ATR–AKAP12 complex raised the possibility that ATR's kinase activity might regulate nuclear translocation of AKAP12. To test this, we investigated AKAP12 localization by confocal microscopy in ATR siRNA-silenced HEK293 cells transfected with siRNA-resistant wild-type or kinase-dead-ATR (ATR-KD) plasmids. Consistent with previous reports (51), AKAP12 was found predominantly in the cytoplasm under basal conditions, however UV exposure resulted in translocation of AKAP12 into the nucleus (Figure 2A). Immunofluorescence experiments revealed that UV-induced translocation of AKAP12 was dependent on ATR's kinase activity. The expression of either ATR-KD or the addition of the ATR kinase inhibitor VE-821 to ATR-WT expressing cells caused AKAP12 to be retained within the cytoplasm (Figure 2B and C). In contrast, pharmacological inhibition of ATM kinase activity did not influence UV-induced AKAP12 nuclear translocation (Figure 2D). Additionally, AKAP12 was detected in purified nuclear and chromatin fractions only when ATR-WT was expressed but not with ATR-KD or with treatment of an inhibitor of ATR (VE-821) (Figure 2E and Supplementary Figure S2A). In support of AKAP12's UV-induced nuclear translocation being dependent on its phosphorylation by ATR, we performed Co-IP experiments pulling down AKAP12. Immunoblot analysis after UV exposure using an ATR/ATM phosphorylation-specific antibody, demonstrated that SQ/TQ phosphorylation within AKAP12 was initially observed in cytoplasmic fractions, which decreased proportionally over time concomitantly with its nuclear accumulation (Figure 2F and Supplementary Figure S2B and C).

A large-scale proteomic analysis of proteins phosphorylated in response to DNA damage by ATM and ATR previously identified four serine residues of AKAP12: S388, S505, S732 and S887 (52). To determine whether these phosphorylation sites are functionally important for UV-induced nuclear translocation of AKAP12, we constructed non-phosphorylatable serine-to-alanine mutations in these residues. Mutations of S338, S505 and S887 did not alter the ability of AKAP12 to localize to the nucleus in HEK293 cells and forskolin pre-treatment, which mimics MC1R signaling by activating adenylyl cyclase to raise cytoplasmic cAMP levels, elevated nuclear levels of each of these AKAP12 variants. In stark contrast, mutation of S732 resulted in AKAP12 nuclear levels being almost negligible in the presence or absence of forskolin (Figure 2G), suggesting that ATR-mediated phosphorylation of AKAP12 at the S732 position is required for UV-dependent AKAP12 nuclear localization.
Figure 1. AKAP12 interacts with ATR. (A) Cells were either mock-treated or exposed to UVB (10 J/m²). After 30 min, whole cell lysates were sampled and reciprocal Co-IP and immunoblotting of ATR and AKAP12 were performed. Input represents 10% of total cellular lysate. (B) Full-length HA-tagged AKAP12 and truncated HA-tagged AKAP12 mutants were transfected in HEK293 cells expressing FLAG-tagged ATR and exposed to UVB (10 J/m²). Co-IP with anti-HA followed by immunoblotting with either anti-ATR or anti-FLAG at 30 min post-UVB. Input represents 10% of total cellular lysate. (C) Proximity ligation confirming sub-cellular localization of the ATR–AKAP12 interaction in HEK293 cells at 30 min after UVB (10 J/m²) or mock treatment. PLA was performed with anti-AKAP12 and anti-ATR antibodies. Green detection events signify juxtaposition between AKAP12 and ATR in maximum intensity projection images. Nuclei were stained with DAPI (blue). Bar represents 50 μm. (D) Schematic diagram of wild-type AKAP12 and mutant variants that were engineered to either prevent the AKAP12–ATR interaction (AKAP12ΔATR), prevent nuclear localization (AKAP12ΔNLS) or prevent PKA-RII-AKAP12 binding (AKAP12ΔPKA). (E) Co-IP of ATR and immunoblotting of HA-tagged wild-type or HA-tagged mutant AKAP12 (AKAP12ΔPKA, AKAP12ΔATR and AKAP12ΔNLS). Cells were either mock-treated or exposed to UVB (10 J/m²) and cytoplasm, nucleus and chromatin fractions extracted (30 min). Input represents 10% of total cell lysate. (F) Co-IP of HA-tagged wild-type or HA-tagged mutant AKAP12 with anti-HA and immunoblotting of RII and C subunits of PKA. HEK293 cells were either mock-treated or exposed to UVB (10 J/m²) and cytoplasmic, nuclear and chromatin fractions obtained. Input represents 10% of total cellular levels of the respective fractions. Data in all panels are representative from three independent experiments.
Figure 2. ATR phosphorylates AKAP12. Confocal imaging of AKAP12 in HEK293 treated with siRNA-targeted to ATR and transfected with siRNA-resistant (A) wild-type ATR (ATR-WT) or (B) kinase-dead ATR (ATR-KD). Confocal imaging of AKAP12 in HEK293 cells expressing ATR-WT and treated with either an inhibitor against (C) ATR (VE-821; 10 μM) or (D) ATM (KU-55933; 10 μM). Cells were either mock-treated or exposed to UVB (10 J/m²) and AKAP12 staining was determined 30 min post-damage. Green staining signifies AKAP12 maximum intensity projection images. Nuclei were stained with DAPI (blue). Bar represents 50 μm. (E) AKAP12 levels in cell fractions of ATR-siRNA silenced HEK293 cells transfected with siRNA-resistant wild-type (ATR-WT) or kinase-dead ATR (ATR-KD) and ATR-WT expressing HEK293 cells treated with ATR kinase inhibitor (VE-821; 10 μM). Cells were either mock-treated or exposed to UVB (10 J/m²) and cytoplasmic, nuclear and chromatin fractions extracted. Input represents 10% of total cellular fraction. (F) Immunoprecipitation of AKAP12 in HEK293 cells expressing HA-tagged wild-type AKAP12 with an anti-HA antibody and immunoblotting with an ATR/ATM phosphorylation-specific antibody that detects phosphorylated SQ/TQ motifs in ATR-WT-transfected HEK293 cells at 15 and 30 min post-UVB (10 J/m²). Percentages of ATR-phosphorylated-AKAP12 (ATR-pAKAP12) interactions from total ATR–AKAP12 interactions were determined from three experiments. Input represents 10% of respective fraction; AKAP12-bound ATR is also shown. (G) AKAP12 CRISPR-deleted HEK293 cells were transfected with HA-tagged wild-type AKAP12 or HA-tagged AKAP12 mutants (AKAP12ΔPKA, AKAP12ΔATR or AKAP12ΔNLS) and were treated with either vehicle or forskolin (10 μM) for 30 min and exposed to UVB (10 J/m²). Cytoplasmic and nuclear fractions were probed with an anti-HA antibody. Input represents 10% of respective fraction. Data in all panels are representative from three independent experiments.
To further interrogate the role of ATR’s kinase activity for AKAP12's ability to associate with UV-damaged DNA, we utilized a technique termed ‘ORiP’ to measure protein–DNA interactions (53) using chromatin fractions isolated from HEK293 cells expressing either wild-type ATR or a kinase dead variant (ATR-KD). The ORiP assay relies on UV exposure or mock treatment of cells, isolation of chromatin fractions, incubation with a biotinylated oligonucleotide construct containing either a non-damaged or UV-damaged fragment, retrieval of the oligonucleotide by streptavidin and identification of bound proteins. After UV treatment, no AKAP12 binding was observed on a non-damaged substrate (Figure 3A). However, chromatin fractions isolated from UV-damaged cells expressing ATR-WT demonstrated robust binding of AKAP12 to photoprod-uct containing-substrate, while no binding of AKAP12 was detected in cells expressing ATR-KD (Figure 3B). Further, addition of the ATR inhibitor, but not an ATM inhibitor abolished AKAP12-photodamage interaction in wild-type ATR-expressing cells (Figure 3B). Using the ORiP substrate, AKAP12 was immunoprecipitated and immunoblotted with an ATR/ATM phosphorylation-specific antibody. We confirmed an ATR-phosphorylated AKAP12 association only occurred with UV-damaged DNA-substrate (Figure 3C and D) and introduction of lambda phosphatase 15 minutes into the ORiP reaction caused a dramatic loss of ATR-phosphorylated AKAP12-DNA binding (Figure 3D). Together, these results identify AKAP12 as a UV-inducible binding partner of ATR and suggest that ATR phosphorylates AKAP12 to facilitate AKAP12's accumulation on UV-damaged chromatin.

**AKAP12 is critical for PKA-mediated ATR phosphorylation at S435**

Because a major function of AKAP proteins are to support PKA phosphorylation of target proteins, we reasoned that AKAP12 might be required for MC1R/cAMP-induced phosphorylation of ATR at S435. To test this, *in vitro* kinase assays using an ATR peptide containing S435 and an ATR-s435 phosphospecific antibody were performed (53). We tested the ability of HEK293 cells expressing wild-type AKAP12 or AKAP12 ΔPKA to support S435 phosphorylation of ATR. HEK293 cells transfected with wild-type MC1R demonstrated cAMP-enhanced phosphorylation of S435 after treatment with either MC1R's agonist MSH or with forskolin (Figure 4A and Supplementary Figure S3A). In contrast, we observed no MSH-induced ATR-s435-specific kinase activity in HEK cells expressing mutant MC1R (MC1R-R151C or MC1R-D294H) (Figure 4B and C) or in wild-type MC1R-expressing HEK293 cells treated with MSH concomitantly with MC1R antagonists human β-defensin 3 (HBD3) or agouti signaling protein (ASIP) (Supplementary Figure S3B and C). Importantly, rescue of AKAP12 expression with AKAP12 ΔPKA failed to support PKA-mediated phosphorylation of the ATR S435 peptide (Figure 4D–F).

We also measured the ability of cytoplasmic fractions of AKAP-null HEK293 cells transfected with wild-type AKAP12, AKAP12 ΔPKA, AKAP12 ΔNLS or AKAP12 ΔATR constructs to support PKA-mediated phosphorylation of an S435 ATR-containing peptide by ELISA. A robust forskolin-induced PKA-mediated phosphorylation of the peptide in cells transfected with wild-type AKAP12 or AKAP12 ΔNLS-transfected constructs occurred, but not in cells harboring AKAP12 ΔPKA or AKAP12 ΔATR (Figure 4G). Immunoblot analysis confirmed UV-induced and cAMP-enhanced cytoplasmic generation of ATR-pS435 in cells expressing AKAP12s capable of binding PKA (Figure 4H). Together, these data indicate that PKA-mediated phosphorylation of ATR on S435 is dependent on AKAP12, that this event occurs in the cytoplasm, that AKAP12 binding to PKA and ATR (but not nuclear localization) is required and that cellular UV damage promotes ATR-pS435 accumulation.

As we showed that AKAP12 is a substrate for ATR’s kinase activity (Figures 2 and 3), we explored whether ATR-pS435 is a prerequisite for ATR-mediated AKAP12 phosphorylation. Immunoprecipitation experiments in ATR-hypomorphic Seckel cells transfected with wild-type ATR confirmed that ATR’s phosphorylation of AKAP12 is a UV-mediated event and that it occurs independently of cAMP-stimulation (Supplementary Figure S4A and B). We also observed ATR-mediated AKAP12 phosphorylation in Seckel cells transfected with S435A ATR incapable of being phosphorylated by PKA (Supplementary Figure S4C and D). These data suggest that ATR’s phosphorylation of AKAP12 occurs after UV radiation independently of its phosphorylation by PKA.

Since AKAP12 interacts not only with PKA but also with PKC (54,55), we determined whether PKC inhibition influenced AKAP12-mediated generation of ATR-pS435. After confirming PKC inhibition by BIM I and PKA inhibition by H-89 (Supplementary Figure S5A), loss of AKAP12-dependent, forskolin-induced ATR-pS435 accumulation by H-89 but not by BIM I occurred (Supplementary Figure S5B). *In vitro* PKA kinase assay confirmed forskolin-induced phosphorylation of a peptide containing S435 and surrounding residues inhibited by H-89 but not BIM I and no induction by the PKC-activating phorbol ester PMA (Supplementary Figure S5C–E). Together, these data suggest that PKA-mediated phosphorylation of ATR on S435 is scaffolded by AKAP12 and is not dependent on PKC.

**AKAP12 enhances XPA localization to UV-induced DNA damage**

Since phosphorylation of ATR at S435 promotes NER by recruiting XPA to photolesions, we reasoned that AKAP12 is necessary for efficient transport of XPA to UV damage. UV exposure promoted XPA's association with chromatin and levels were enhanced 2.1-fold with forskolin pre-treatment in AKAP12-null HEK293 cells rescued with wild-type AKAP12 (compared to UV treatment alone). In contrast, AKAP12 ΔPKA expressing cells did not demonstrate any forskolin-mediated enhanced of chromatin-XPA levels (Figure 5A and Supplementary Figure S6A). Proximity ligation in AKAP12-null HEK293 cells transfected with either wild-type AKAP12 or AKAP12 ΔPKA confirmed that co-localization of XPA with [6-4]-PP was enhanced with forskolin pre-treatment and dependent on PKA–AKAP12 interactions (Figure 5B and C). Similarly,
siRNA-knockdown of AKAP12 in A375 melanoma cells prevented forskolin enhancement of XPA interaction with photodamage (Supplementary Figure S6B) or chromatin (Supplementary Figure S6C). Introduction of siRNA-resistant wild-type ATR rescued forskolin-enhanced chromatin interactions whereas ATR-S435A did not and the phosphomimetic ATR-S435D resulted in robust forskolin-independent XPA-chromatin interactions (Supplementary Figure S6D and E).

Next, we explored which components of the AKAP12–ATR axis are necessary for promoting XPA recruitment to chromatin. The siRNA-targeted knockdown of AKAP12, ATR or PKA each prevented XPA accumulation on chromatin after UV (Supplementary Figure S6F), suggesting that AKAP12, ATR and PKA are all necessary for cAMP-mediated enhancement of XPA binding to photodamage. Similarly, in AKAP12-null HEK293 cells with ATR expression inhibited by siRNA (Supplementary Figure S6G), forskolin-enhanced binding of XPA to UV-damaged chromatin in AKAP12-WT but not AKAP12ΔPKA-expressing cells when siRNA-resistant ATR was expressed (Figure 5D). In contrast, there was no forskolin enhancement of XPA-chromatin interaction in S435A ATR-expressing cells (Figure 5E) and increased basal XPA-chromatin interactions in S435D ATR-expressing cells (Figure 5F). In addition, forskolin pre-treatment enhanced the binding kinetics of XPA to a UV-damaged substrate as measured by ORIP (Supplementary Figure S6H), however no binding of the substrate was observed in cells expressing AKAP12ΔPKA (Supplementary Figure S6I) or not exposed to UV (Supplementary Figure S6J). Altogether, these data suggest that each component of the AKAP12–ATR–PKA axis is necessary for MC1R/cAMP-enhanced XPA localization to UV-damaged chromatin.

AKAP12 associates with UV-induced photoproducts

Proximity ligation analyses revealed co-localization of wild-type AKAP12 but not AKAP12ΔPKA with [6-4]PP and CPDs, both of which were enhanced by forskolin
Figure 4. MC1R-cAMP signaling enhances the kinetics of ATR-pS435 generation. (A–C) HEK293 cells expressing either wild-type MC1R or a defective MC1R variant (R151C and R294H) were transfected with wild-type AKAP12. Cells were treated with either MC1R agonist, α-MSH (100 nM), cAMP enhancer (independent of MC1R), forskolin (10 μM) or vehicle for 30 min. Whole-cell lysates were incubated with 10 μM peptide (CPKRRRLSSLSNPS) as a phosphorylatable substrate for 3 min. Kinetic parameters of the phosphorylation reaction were calculated by non-linear regression analysis for the peptide substrate using anti-ATR-pS435 antibody coupled with fluorescence detection. (D–F) HEK293 cells expressing either wild-type MC1R or a defective MC1R variant (R151C and R294H) were transfected with AKAP12 ΔPKA. Cells were treated with either MC1R agonist, α-MSH (100 nM), cAMP enhancer (independent of MC1R), forskolin (10 μM) or vehicle for 30 min. Whole-cell lysates were incubated with 10 μM peptide (CPKRRRLSSLSNPS) as a phosphorylatable substrate for 3 min. Kinetic parameters of the phosphorylation reaction were calculated by non-linear regression analysis for the peptide substrate using anti-ATR-pS435 antibody coupled with fluorescence detection. (G) CRISPR-deleted AKAP12 HEK293 cells were transfected with either wild-type AKAP12 or AKAP12 ΔPKA, AKAP12 ΔATR or AKAP12 ΔNLS mutants. Cytoplasmic fractions were treated with the cAMP enhancer, forskolin (50 μM) for 10 min and the ability of fractions to support PKA-mediated phosphorylation of an S435-containing ATR peptide were measured by ELISA; A.U., arbitrary units. Treatments significantly different from vehicle were determined by one-way ANOVA; *P < 0.05. (H) CRISPR-deleted AKAP12 HEK293 cells expressing either AKAP12 or an AKAP12 mutant (AKAP12 ΔPKA, AKAP12 ΔATR and AKAP12 ΔNLS) were pre-treated with vehicle or forskolin (10 μM) for 30 min and mock or UVB irradiated (10 J/m²). Immunoblots of cytoplasmic ATR-pS435 generation was determined. Data in all panels are representative from three independent experiments and error bars are standard deviation.
Figure 5. AKAP12 enhances XPA localization to UV-induced DNA damage. (A) CRISPR-deleted AKAP12 cells expressing HA-tagged AKAP12-WT or HA-tagged AKAP12ΔPKA were pre-treated with vehicle or forskolin (10 μM) for 30 min and mock treated or UVB irradiated (10 J/m²). Immunoblots were performed on chromatin-fractions with anti-XPA. Inputs represent total chromatin H2A levels and 10% of whole cell lysate levels of XPA. (B) XPA-[6-4]-PP interactions were determined by proximity ligation using anti-XPA and anti-[6-4]-PP antibodies. CRISPR-deleted AKAP12 HEK293 cells were transfected with either wild-type AKAP12 or AKAP12ΔPKA and treated with either vehicle or forskolin (10 μM) and exposed to UVC (10 J/m²). Green detection events signify juxtaposition between AKAP12 and XPA in maximum intensity projection images 30 min after UV exposure. Nuclei were stained with DAPI (blue). Bar represents 50 μm. (C) Quantification of the XPA-[6-4]-PP colocalization shown in panel C. At least 100 cells were counted from representative fields from two separate experiments and extent of interaction is expressed as fold-change compared to the damage-null cells obtained from maximum intensity images from focal plane z-stacks. Values not sharing a common letter were significantly different as determined by one-way ANOVA; *P ≤ 0.05. Data are expressed as mean ± SEM. (D-F) Chromatin-associated XPA levels in CRISPR-deleted AKAP12 HEK293 cells expressing either wild-type AKAP12 or AKAP12ΔPKA and with ATR expression inhibited by siRNA. Cells were transfected with siRNA-resistant ATR-WT, ATR-S435A or ATR-S435D and treated with vehicle or forskolin (10 μM) and exposed to UVB (10 J/m²) or mock-treated. Data are expressed as the percent of chromatin-bound XPA to total cellular XPA. Treatments significantly different from vehicle were determined by one-way ANOVA; *P ≤ 0.05. Data in all panels are representative from three independent experiments and error bars are standard deviation.
pre-treatment in HEK293 cells (Figure 6A–F). Immunocolocalization studies were conducted to verify that the protein complex of AKAP12, XPA and ATR-pS435 was recruited directly to UV-irradiated DNA. HEK293 cells were UV-irradiated and after removing soluble nuclear proteins by in situ detergent extraction, application of anti-AKAP12 (pink foci), anti-XPA (green foci) and anti-ATR-pS435 (red foci) revealed co-localization events (white foci). Whereas no co-localization was observed in the absence of DNA damage, UV recruited XPA, AKAP12 and ATR-pS435 to photodamage, especially with forskolin pre-treatment (Figure 6G). Cells expressing AKAP12ΔPKA (Figure 6H) or deleted for AKAP12 (Supplementary Figure S7A), failed to exhibit forskolin-induced co-localization events. In addition, mutation of AKAP12 at S732 to a non-phosphorylatable alanine prevented nuclear accumulation of ATR-pS435 which is key molecular mediator of cAMP-enhanced repair (Supplementary Figure S7B).

Taken together, these data strongly implicate localization of AKAP12 by an ATR-dependent mechanism at sites of nuclear photodamage.

AKAP12 enhances NER

XPA is responsible for recruiting the ERCC1-XPF endonuclease in NER which catalyze the 5′ incision step of the damaged DNA strand (12,56). We reasoned, therefore, that cAMP signaling and AKAP12 might enhance the protein levels of ERCC1. To test this we measured biochemical interactions between XPA and ERCC1-XPF basally and with cAMP stimulation. Immunoprecipitation experiments using chromatin fractions isolated from CRISPR-AKAP12 deleted HEK293 cells expressing either AKAP12-WT or deletion variants (AKAP12ΔPKA, AKAP12ΔNLS, AKAP12ΔATR or AKAP12-S732A) demonstrated that cAMP enhanced interactions between XPA and ERCC1 following UV exposure with AKAP12-WT (2.1-fold increase compared to UV treatment alone) but not with any of the AKAP12 mutants tested (Figure 7A). To further explore cAMP-enhanced XPA-ERCC1-XPF interactions, we used a biotinylated stem-loop DNA substrate known to be a substrate for ERCC1-XPF and measured interactions by ORIP in HEK chromatin fractions expressing either AKAP12-WT or deletion variants (AKAP12ΔPKA, AKAP12ΔNLS, AKAP12ΔATR or AKAP12-S732A) (Figure 7B). Without UV exposure of the cells, we observed no binding of either XPA or ERCC1-XPF to the stem loop structure (Supplementary Figure S8), however, UV-damaged cellular lysates demonstrated binding of either XPA or ERCC1-XPF to the stem loop structure. cAMP-pre-treatment of cells enhanced binding between the stem loop substrate and either XPA or ERCC1-XPF in the presence of AKAP12-WT but not the AKAP12ΔPKA, AKAP12ΔNLS, AKAP12ΔATR or AKAP12-S732A mutants (Figure 7B). Furthermore, chromatin fractions isolated from UV-damaged cells expressing AKAP12-WT demonstrated enhanced ERCC1 levels when pre-treated with forskolin, but not the AKAP12ΔPKA, AKAP12ΔNLS, AKAP12ΔATR or AKAP12-S732A mutants (Supplementary Figure S9).

Since AKAP12 facilitated cAMP-dependent XPA recruitment to damaged DNA, we reasoned AKAP12 may be functionally necessary for cAMP-enhancement of ERCC1-XPF incision. An ERCC1-XPF-mediated incision assay was studied using a fluorescently labeled stem-loop substrate incubated with chromatin fractions isolated from HEK293 cells expressing either AKAP12-WT or AKAP12 deletion variants (Figure 7C and D). Initially, we confirmed that the observed stem-loop incision was due to ERCC1-XPF (Supplementary Figure S10A and B). In the absence of cellular UV damage, no incision of the stem-loop DNA substrate was observed. In contrast, UV exposure resulted in cleavage of stem-loop substrate, with a 2-fold increase (P ≤ 0.05) in incision after cAMP-pre-treatment compared to UV treatment alone, but only with expression of AKAP12-WT (Figure 7C). No significant incision enhancement was observed with any of the AKAP12 mutants (Figure 7D). Furthermore, neither AKAP12 nor cAMP impacted levels of NER factors, XBP, XPC, XPD and XPG on UV-damaged chromatin (Supplementary Figure S11) nor the endonuclease activity of XPG (Supplementary Figure S12). To confirm that incision was enhanced downstream of MC1R, stimulation of MC1R-intact cells by MSH promoted ERCC1-XPF incision by 2-fold (P ≤ 0.05) compared to UV treatment alone. However, incubation of cells with MC1R antagonists ASIP or human β-defensin three blocked MSH-mediated enhancement of incision (P ≤ 0.05) (Supplementary Figure S13).

AKAP12 promotes repair of UV-induced DNA damage and suppresses mutagenesis

Since AKAP12 enhanced cAMP-induced PKA-mediated ATR phosphorylation at S435, XPA localization to DNA damage and ERCC1-XPF incision, we reasoned that AKAP12 protects melanocytes against UV-induced mutagenesis. In order to determine the extent to which AKAP12 is required for repair of UV-induced DNA damage, a panel of cell lines expressing wild-type AKAP12 or defective variants (AKAP12ΔPKA, AKAP12ΔATR or AKAP12ΔNLS) was exposed to UV and repair of [6-4]-PP was measured. In all cell types, wild-type AKAP12 expression resulted in a forskolin-induced enhancement of [6-4]-PP repair (Table 1). However, AKAP12-null or either AKAP12ΔATR or AKAP12ΔNLS variants did not display any forskolin-mediated DNA repair benefit (Table 1). Reasoning that photodamage repair efficiency would affect UV mutagenesis risk, we studied the influence of AKAP12 on UV-induced mutagenesis. Basal and UV-induced mutagenesis screens of hprt were performed with or without cAMP stimulation. Without UV, no mutant colonies were observed regardless of AKAP12 status (Table 1). In wild-type AKAP12 cells, forskolin pre-treatment significantly reduced UV-induced mutagenesis in all cell types, however expression of either AKAP12ΔPKA, AKAP12ΔATR or AKAP12ΔNLS abrogated any benefit of cAMP stimulation on reduction of UV-induced mutagenesis (Table 1). Taken together, these data indicate that AKAP12 serves as a UV-inducible scaffold needed for phosphorylation of ATR at S435, a post-translational modification that reduces genomic UV damage and mutagenesis downstream of MC1R signaling in melanocytes.
Figure 6. AKAP12 interacts with UV-induced photoproducts. (A and B) AKAP12 CRISPR-deleted HEK293 cells were transfected with wild-type AKAP12 or AKAP12ΔPKA were treated with either vehicle or forskolin (10 μM) and exposed to UVC (10 J/m²). The interaction between AKAP12 and CPDs at 30 min was determined by proximity ligation using anti-AKAP12 and anti-CPD antibodies. Green detection events signify juxtaposition between AKAP12 and [6-4]-PP in maximum intensity projection images. Nuclei were stained with DAPI (blue). Bar represents 50 μm. (C) Quantification of the AKAP12-CPD localization shown in panel A and B. At least 100 cells were counted from representative fields from two separate experiments. Values not sharing a common letter were significantly different as determined by one-way ANOVA; P ≤ 0.05. (D and E) CRISPR-deleted AKAP12 HEK293 cells treated as described in (A). Interaction between AKAP12 and [6-4]-PP was determined by proximity ligation using anti-AKAP12 and anti-[6-4]-PP antibodies at 30 min after UV. Green detection events signify juxtaposition between AKAP12 and [6-4]-PP in maximum intensity projection images. Nuclei were stained with DAPI (blue). Bar represents 50 μm. (F) Quantification of the AKAP12-[6-4]-PP localization shown in panels d and e as described in (C). (G and H) Confocal imaging of AKAP12, XPA and ATR-pS435 in HEK293 cells transfected with wild-type AKAP12 or AKAP12ΔPKA. Cells were exposed to 10 J/m² of UVC followed by in situ detergent extraction. Co-localization of AKAP12 (pink), XPA (green) and ATR-pS435 (red) is observed as white nuclear foci. Bar represents 50 μm. Data in all panels are representative from three independent experiments and error bars are standard deviation.
Figure 7. cAMP enhances ERCC1-XPF-mediated DNA incision. (A) AKAP12 CRISPR-deleted HEK293 cells were transfected with wild-type AKAP12, AKAP12ΔPKA, AKAP12ΔATR, AKAP12ΔNLS or AKAP12-S732A and were treated with either vehicle or forskolin (10 μM) and exposed to UVB (10 J/m²). After 5 min, nuclear fractions were immunoprecipitated with anti-XPA and immunoblotted with anti-ERCC1. Input represents levels of XPA and ERCC1 in 10% of total whole cellular lysate. (B) AKAP12 CRISPR-deleted HEK293 cells were transfected with wild-type AKAP12, AKAP12ΔPKA, AKAP12ΔATR, AKAP12ΔNLS or AKAP12-S732A and were treated with either vehicle or forskolin (10 μM) and exposed to UVB (10 J/m²). After 30 min, chromatin fractions were incubated with biotinylated stem-loop substrate for indicated times. Levels of XPA, ERCC1 and XPF bound to the DNA fragment were quantified by ORiP as described in ‘Materials and Methods’ section. Treatments significantly different from vehicle were determined by one-way ANOVA; *P ≤ 0.05. AKAP12 CRISPR-deleted HEK293 cells were transfected with (C) wild-type AKAP12 or (D) AKAP12ΔPKA, AKAP12ΔATR, AKAP12ΔNLS or AKAP12-S732A and were treated with either vehicle or forskolin (10 μM) and exposed to UVB (10 J/m²). After 30 min, chromatin fractions were incubated with FAM-labeled stem-loop substrate at 37°C and products visualized on 4–20% polyacrylamide gels. Data in all panels are representative from three independent experiments and error bars are standard deviation. Treatments significantly different from each were determined by one-way ANOVA.
DISCUSSION

Inherited dysfunction of the MC1R, a Gs protein-coupled receptor that signals through cAMP and PKA, is a bona fide melanoma risk factor. We previously reported that MC1R promotes melanocytic genomic stability by stimulating phosphorylation of ATR at S435 by PKA which promotes ATR–XPA interactions, enhances binding to nuclear photodamage and accelerates NER (5). Here we describe a central role for AKAP12 as a critical regulator of this event, necessary for PKA-mediated ATR phosphorylation and enhancement of cellular responses to UV-induced DNA damage. For the first time, these data place AKAP12 directly at sites of nuclear photodamage in complex with ATR and XPA.

We found that UV promotes cytoplasmic ATR-mediated phosphorylation of AKAP12 and, in the context of cAMP signaling, phosphorylation of ATR at S435 by PKA. ATR’s phosphorylation of AKAP12 is required for nuclear localization of the AKAP12–ATR–pS435 complex, however, neither PKA C nor R subunits accompany AKAP12–ATR–pS435 to the nucleus. We further identified that S732 on AKAP12 is a UV-induced phosphorylation site important for nuclear translocation. Indeed, S732 is part of an ATR–pS435 to the nucleus. We further identified that S732 phosphorylation of AKAP12 is required for nuclear localization. For the first time, these data place AKAP12 directly at sites of nuclear photodamage in complex with ATR and XPA.

Our findings implicate AKAP12 as a DNA damage-induced scaffold that coordinates cytoplasmic and nuclear events critical to NER. Specifically, AKAP12 is necessary for PKA-mediated ATR phosphorylation and accompanies ATR–pS435 and XPA to nuclear photodamage. Given the central role of XPA in NER (57,58), the discovery of AKAP12 as a scaffold protein that facilitates XPA’s transport to photodamage introduces a novel regulatory mechanism for NER. We hypothesize that AKAP12 mediates a scaffold for the docking and integration of damage sensing and repair proteins, particularly in the context of MC1R/cAMP signaling. In so doing, AKAP12 provides an appropriate ‘microenvironment’ to enable interactions between ATR and XPA and optimize assembly of DNA repair complexes. Indeed, the ability of AKAP12 to localize to [6-4]-PP and CPDs and to enhance XPA recruitment of ERCC1-XPF supports such a scaffolding role. It is uncertain how AKAP12 enhances NER; possibilities include the higher abundance of the nuclease ERCC1-XPF that may promote more strand incision events or the scaffolding ability of AKAP12 to contribute to assembly of pre-incision NER complexes. The recent identification of AKAP12 interacting with ATR at collapsed replication forks (40), suggests that non-UV damage signals may promote AKAP12 functioning with ATR and raises the possibility of functional relevance for AKAP12 in genomic maintenance pathways beyond NER.

Interactions between XPA and ERCC1 have been reported independently of DNA damage (56,59–61), however our data suggest a potential UV-inducible component to their association. This is supported by a previous study showing enhancement of XPA–ERCC1 interactions after UV-irradiation (62). Mechanisms by which UV damage responses impact XPA–ERCC1 interactions are unclear, however others have reported that XPA–ERCC1 interactions are strengthened in the presence of the mitotic regulator Nlp, which putatively acts as a scaffold to enhance interactions. Indeed, S732 thwarts nuclear accumulation of AKAP12–ATR–pS435 and prevents cAMP enhancement of NER.

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either associated with gene deletion, promoter hypermethylation or changes in chromatin modeling (64). The fate of AKAP12 expression, however, is also dependent upon the tumor microenvironment as hypoxxia induces an AKAP12 variant, AKAP12v2 which alters PKA signaling (31). Our studies suggest that AKAP12 may protect against early mutagenic events in melanocyte carcinogenesis by promoting genomic stability through enhanced DNA repair and resistance to UV-induced mutagenesis. Thus dysregulation of AKAP12 expression/function may represent a ‘double-hit’ by which cells not only are prone to UV-induced mutations but also more genetically unstable and susceptible to changes in cell survival, migration, and invasion. Our data introduce a previously unknown nuclear function for AKAP12 in NER and further our understanding of how NER may be regulated in melanocytes. By linking its newly identified role in DNA repair with previously-described nuclear functions, AKAP12 is emerging as an important regulator of cell cycle, DNA replication and DNA repair.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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