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ATR Kinase Activity Limits Mutagenesis and Promotes the Clonogenic Survival of Quiescent Human Keratinocytes Exposed to UVB Radiation

Kavya Shaj, Rebekah J. Hutcherson, Michael G. Kemp*

Department of Pharmacology and Toxicology, Wright State University Boonshoft School of Medicine, Dayton, Ohio 45435

Abstract

The ATR protein kinase has well-described roles in maintaining genomic integrity during the DNA synthesis phase of the cell cycle. However, ATR function in cells that are not actively replicating DNA remains largely unexplored. Using HaCaT and telomerase-immortalized human keratinocytes maintained in a confluent, non-replicating state in vitro, ATR was found to be robustly activated in response to UVB radiation in a manner dependent on the nucleotide excision repair factor and DNA translocase XPB. Inhibition of ATR kinase activity under these conditions negatively impacted acute cell survival and cytotoxicity and severely inhibited the ability of UVB-irradiated HaCaT keratinocytes to proliferate upon stimulation with growth factors. Furthermore, ATR kinase inhibition in quiescent HaCaT keratinocytes potentiated UVB mutagenesis at the hypoxanthine phosphoribosyltransferase locus. Though ATR inhibition did not impact the rate of removal of cyclobutane pyrimidine dimers from genomic DNA, elevated levels of PCNA mono-ubiquitination and chromatin-associated PCNA and RPA indicate that excision gap filling synthesis was altered in the absence of ATR signaling. These results indicate that the ATR kinase plays important roles in preventing mutagenesis and in promoting the proliferative potential of quiescent keratinocytes exposed to UVB radiation.

Graphical Abstract

ATR is activated following the removal of UV photoproducts (T$\hookrightarrow$T) from the genome in quiescent keratinocytes. In the absence of ATR kinase signaling, the gap filling process is abrogated and leads to increased PCNA mono-ubiquitination and RPA-coated ssDNA. These events are then associated with increased mutagenesis and reduced survival.

INTRODUCTION

During the DNA synthesis phase of the cell cycle, DNA lesions induced by ultraviolet (UV) wavelengths of light and many other environmental and chemotherapeutic genotoxins impair the progression of the replicative DNA polymerases and activate the ATR (ataxia

*Corresponding author: mike.kemp@wright.edu (Michael G. Kemp).

SUPPORTING INFORMATION

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telangiectasia-mutated and rad3-related) protein kinase. In this context, ATR has generally been shown to promote genomic stability through several mechanisms (1), including the stabilization of stalled replication forks (2), the inhibition of replication origin firing (3), the promotion of translesion synthesis (4–6), and by delaying entry into the G2/M phase of the cell cycle (7,8). ATR also enables the efficient removal of UV photoproducts from genomic DNA during S phase (9,10). Loss or inhibition of ATR therefore sensitizes proliferating cells to the lethal effects of UV radiation and other DNA damaging agents (6,7,11–13).

The function of ATR in cells that are not actively replicating DNA or progressing through the cell cycle is much less understood. Though several studies have shown that common ATR substrates become phosphorylated in quiescent, non-replicating cells after UV exposure (14–21), the actual functions of ATR under these circumstances have not been characterized. Given that the overwhelming majority of cells in the body are in a quiescent or differentiated state, including skin epidermal keratinocytes that are routinely exposed to UV wavelengths of sunlight, understanding the replication- and cell cycle-independent functions of ATR may uncover novel roles for this protein kinase in the DNA damage response in contexts that may be relevant to mutagenesis and carcinogenesis of quiescent skin stem cell populations. Moreover, the development of highly specific small molecule inhibitors of ATR for use in cancer chemotherapy regimens (22–24) makes a full characterization of ATR kinase function important for understanding potential side effects of ATR kinase inhibitor-based therapies in human subjects.

Thus, in this report, the function of the ATR protein kinase was examined in human keratinocytes maintained in a quiescent state *in vitro*. ATR signaling was found to be activated following UVB exposure in a manner dependent on the nucleotide excision repair (NER) protein XPB. Moreover, the loss of ATR kinase signaling negatively impacted several cellular UVB responses, including acute toxicity, proliferative capacity, and mutagenesis. These negative outcomes were correlated with increased levels of chromatin-bound RPA (Replication Protein A) and PCNA and with elevated mono-ubiquitination of PCNA. These results imply that in the absence of ATR kinase signaling, UVB-irradiated quiescent keratinocytes are more reliant on the translesion synthesis pathway of DNA synthesis to maintain genomic integrity. These results therefore demonstrate that ATR plays important roles in the UVB DNA damage response in both proliferating cells and quiescent, non-replicating cells.

MATERIALS AND METHODS

Cell culture.

HaCaT keratinocytes were cultured in DMEM supplemented with 10% Fetal Clone III (Hyclone), 6 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Telomerase-immortalized normal human foreskin keratinocytes (N-TERTs) (25) were cultured in EpiLife medium containing human keratinocyte growth supplement (Thermo Fisher Scientific) and penicillin/streptomycin. All cells were maintained in a 5% CO₂ humidified incubator at 37°C and monitored periodically for mycoplasma contamination (Sigma Venor GeM Kit). To generate non-replicating, quiescent cells, HaCaT cells were plated so that they would reach confluence in 2–3 days. Cells were then maintained for an

additional 2 days in DMEM supplemented with 0.5% Fetal Clone III and penicillin/streptomycin. Quiescent N-TERTs were generated by growing the cells to confluence and then incubating for 2 days in basal EpiLife medium without any additional growth factors. Cells were treated with DMSO, the ATR inhibitor (ATRi) VE-821 (Selleckchem or APExBio; 10 μ M), triptolide (Sigma; 1 μ M), spironolactone (APExBio; 10 μ M), or THZ1 (APExBio; 1 μ M) for 30 min (or for 2 hr for spironolactone) prior to UVB exposure using a Philips F20T12 broadband UVB bulb at a dose rate of 5 J/m²/sec. With the exception of cell growth, colony formation, and mutagenesis assays, cells were incubated with the indicated compound throughout the experiments.

Protein immunoblotting.

Cells were scraped from the plate into cold PBS, pelleted, and lysed on ice in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% Triton X-100, and then soluble lysates were prepared by centrifugation at maximum speed in a microcentrifuge for 10–15 min. Chromatin-associated proteins were enriched from cells following three extractions with a modified cytoskeletal buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 1 mM Na₃VO₄, 10 mM NaF, and 0.1% Triton X-100). Soluble and chromatin lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and then stained briefly with Ponceau S. Following washing with TBST (Tris-buffered saline containing 0.1% Tween-20) and blocking in 5% milk in TBST, blots were probed with 1:2000 dilutions of antibodies against phospho-KAP1 (Ser824; Bethyl A300–676A-M), phospho-p53 (Ser15; Cell Signaling 8284), phospho-CHK2 (Thr68; Cell Signaling 2621), phospho-DNA-PK (Ser2056; Cell Signaling 4215), phospho-ATR (Thr1989; GeneTex 128145), PCNA (Santa Cruz sc-10), or actin (Santa Cruz I-19) overnight in TBST. Chemiluminescence was visualized with either Clarity Western ECL substrate (Bio-Rad) or SuperSignal West Femto substrate (Thermo Scientific) using a Molecular Imager Chemi-Doc XRS+ imaging system. Signals in the linear range of detection were quantified using Image Lab (Bio-Rad) and normalized as previously described (19,26).

DNA immunoblotting.

Bromodeoxyuridine (BrdU) was added to a final concentration of 10 μ g/ml to the culture medium of proliferating or quiescent cells for either 15 min (HaCaT) or 30 min (N-TERT). Genomic DNA was purified using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) and quantified using PicoGreen fluorescence (Invitrogen) on a Synergy H1 Hybrid Multi-Mode microplate reader (BioTek). DNA was immobilized on nitrocellulose, dried, and immunoblotted for BrdU or cyclobutane pyrimidine dimers (CPDs) as previously described (26,27). Blots were stripped and re-probed with an anti-ssDNA antibody (Millipore MAB3034; 1:10,000 dilution) to detect total DNA and ensure equal loading. Chemiluminescent signals were quantified by densitometry and normalized to either the proliferating or non-irradiated control, which was set to an arbitrary value of 100.

Cell survival.

Acute cell survival was determined using crystal violet staining as previously described (19) or with an MTT assay, in which methylthiazolyldiphenyl-tetrazolium bromide (MTT) was added to cell culture medium for 30 min before solubilization with DMSO and

spectrophotometric detection at 550 nm. Cytotoxicity was measured with the Pierce LDH Cytotoxicity Assay Kit (ThermoScientific 88953). The proliferative capacity of quiescent HaCaT cells was determined by two different approaches. Using a classical colony formation assay, 100–200 cells were plated per 100 mm plate ($1.3\text{--}2.6\text{ cells/cm}^2$) 5 days after treatment. The culture medium contained 0.5% Fetal Clone III throughout the 5 days following irradiation, but DMSO/ATRi-containing medium was replaced with low serum medium 2 days after UVB exposure. Upon re-plating, the cells were plated in normal medium containing 10% Fetal Clone III. Cells were allowed to grow and form colonies for 10–14 days, after which cells were stained with crystal violet and counted. In a second cell proliferation assay to monitor cell growth, 150,000 quiescent cells were plated per well of a 6-well plate (approximately $15,600\text{ cells/cm}^2$) in normal growth medium 5 days after UVB exposure. Cells were stained with crystal violet 1, 3, 5, 7, 9, and 11 days later. The dye was solubilized and quantified with a spectrophotometer as described above to measure cell proliferation (19)

HPRT mutagenesis assay.

HPRT mutagenesis assays were performed essentially as described (27), except that cells were quiescent at the time of treatment and were incubated for 5 days prior to plating and selection with 6-thioguanine.

RESULTS

ATR is activated in UVB-irradiated quiescent keratinocytes in an XPB-dependent manner

To ensure that a quiescent population of keratinocytes containing few replicating cells could be generated in culture in vitro, HaCaT keratinocytes maintained in either a sub-confluent proliferating state or in a confluent state deprived of growth factors were incubated briefly with BrdU, and then genomic DNA was purified for DNA immunoblot analysis. As shown in Figure 1a, the quiescent HaCaT cells incorporated very little BrdU into their genomic DNA, thus ensuring that the cells have exited the cell cycle and are largely in a non-replicating state.

Quiescent HaCaT cells were next exposed to 200 J/m^2 of UVB and then harvested 20 min later for immunoblot analysis to determine whether UVB activates ATR kinase signaling in non-replicating cells. Figure 1b shows that the phosphorylation of several DNA damage substrate proteins was stimulated by UVB exposure, including the heterochromatin protein KAP1, the cell cycle checkpoint kinase CHK2, and the tumor suppressor protein p53. Moreover, UVB also induced an increase in ATR autophosphorylation on Thr1989 (28,29). With the exception of CHK2, which is primarily a target of the related ATM kinase, all of these UVB-dependent phosphorylation events were severely abrogated by treatment of the cells with the small molecule ATR kinase inhibitor VE-821 (Figure 1b, c).

HaCaT keratinocytes contain mutations in both alleles of p53 and have an abnormal chromosome number (30,31), which could influence the cellular response to UVB radiation. Thus, to determine whether UVB activates ATR kinase signaling in normal keratinocytes, telomerase-immortalized neonatal foreskin keratinocytes (N-TERTs) (25) maintained in a

non-replicating state via confluence arrest and growth factor withdrawal were next examined for UVB-dependent ATR kinase activation. DNA immunoblotting results in Figure 2a demonstrated that the level of DNA synthesis in the quiescent N-TERTs was reduced by more than 80% in comparison to sub-confluent, proliferating N-TERTs. As shown in Figure 2b and c, UVB-induced KAP1 and p53 phosphorylation was blocked by ATR kinase inhibition in the quiescent N-TERT keratinocytes. These results demonstrate that ATR becomes rapidly activated in non-replicating quiescent keratinocytes in response to UVB radiation.

A recent study demonstrated that the DNA translocase and TFIIH (transcription factor II-H) component XPB plays an important role in the activation of ATR kinase signaling in non-replicating cells exposed to *N*-acetoxy-2-acetylaminofluorene (NA-AAF) (26), a metabolic derivative of fluorene that generates bulky adducts on guanine residues in DNA. As a component of TFIIH, XPB plays important roles in both NER and transcription (32). XPB can be depleted from keratinocytes via proteosomal degradation with the pharmacological agent spironolactone (SP) (27,33), and its ATPase/DNA translocase activities can be inhibited with the natural product triptolide (TPL) (34,35). To determine whether XPB is required for ATR kinase signaling in UVB-irradiated quiescent cells, HaCaT keratinocytes were pre-treated cells with DMSO, TPL, SP, or with THZ1, which inhibits the Cdk7 kinase activity of TFIIH (36), before exposure to UVB radiation. As shown in Figure 3a and quantified in Figure 3b, both XPB-targeting small molecules significantly abrogated the ability of UVB radiation to induce KAP1 and p53 phosphorylation. In contrast, THZ1 which inhibits transcription but not NER, did not impact ATR signaling. These results indicate that XPB presence and activity during NER are required for the proper activation of ATR kinase signaling in non-replicating, quiescent HaCaT cells exposed to UVB radiation.

ATR kinase inhibition is modestly cytotoxic in UVB-irradiated quiescent keratinocytes

Cell viability following UVB exposure was next examined to determine the functional consequences of ATR kinase inhibition in quiescent keratinocytes. Figure 4a demonstrates that the ATR kinase inhibitor VE-821 partially sensitized HaCaT cells to UVB radiation. Similar results were obtained with the ATR inhibitor AZD6738 (data not shown). The ATRi-dependent loss of viability is associated with increased cytotoxicity, as determined by measuring the activity of lactate dehydrogenase in the culture medium 1 day following UVB exposure (Figure 4b). Similarly, quiescent N-TERTs are similarly modestly sensitized by ATR kinase inhibition (Figure 4c) and display elevated cytotoxicity relative to vehicle-treated control cells (Figure 4d). These results indicate that ATR kinase activity helps to maintain the acute viability of non-replicating keratinocytes exposed to UVB radiation.

Inhibition of ATR kinase activity in quiescent keratinocytes exposed to UVB radiation negatively impacts colony forming ability and induces mutagenesis

Though many cells in the body exist in a non-replicating quiescent state, cells are frequently induced to proliferate in response to stress and growth factor stimulation. It is therefore important to determine how the inhibition of ATR kinase activity in UV-irradiated quiescent keratinocytes affects the proliferative potential of the cells. A schematic of this experimental approach is outlined in Figure 5a and involved incubating UVB-irradiated quiescent HaCaT

cells for several days prior to re-plating and stimulation with growth factors. Thus, in these experiments, ATR was only transiently inhibited while cells are in the quiescent state and not during the period of growth factor stimulation. Importantly, UVB exposure did not induce any proliferation during this 5 day incubation period prior to re-plating (Figure S1). As shown using a clonogenic survival assay (Figure 5b), the inhibition of ATR in quiescent HaCaT cells was associated with a 3- to 6-fold reduction in colony forming ability. Additional experiments in which quiescent cells were re-plated at a higher density than in the clonogenic survival assays following treatment to monitor short-term population growth confirmed that ATR inhibition in the quiescent state slowed the recovery of the quiescent cells upon stimulation with growth factors (Figure S2).

UVB radiation has the capacity to induce DNA mutations that can alter cell proliferation, viability, and carcinogenesis. To determine whether ATR kinase inhibition impacts mutagenesis in UVB-irradiated quiescent keratinocytes, HPRT mutagenesis assays were performed by quantifying the number of cell clones that develop mutations at the HPRT locus and subsequently become resistant to the nucleotide analog 6-thioguanine. As shown in Figure 5c, DMSO-treated cells exposed to 100 J/m² UVB exhibited a mutant frequency of approximately 17 per million cells. In striking contrast, the mutant frequency was approximately 10-fold higher (171 mutants per million cells) in the quiescent HaCaT cells treated with the ATR inhibitor prior to UVB exposure. Control experiments that involved labeling genomic DNA with BrdU for 24 hr at various time points following UVB exposure failed to show any evidence that either UVB radiation or ATR kinase inhibition resulted in more cell proliferation or DNA synthesis under these cell culture conditions (Figures S1, S2). These results indicate that ATR kinase function is important to prevent UVB mutagenesis in keratinocytes maintained in a quiescent, non-proliferating state.

Loss of ATR kinase activity results in increased PCNA monoubiquitination in UVB-irradiated quiescent keratinocytes

Nucleotide excision repair (NER) plays a critical role in preventing mutagenesis through the removal of mutagenic cyclobutane pyrimidine dimers (CPDs) and other UV photoproducts from genomic DNA (37,38). Immunoblot analyses of genomic DNA were therefore next performed to determine whether the loss of ATR kinase activity affected the rate UVB photoproduct removal in quiescent keratinocytes. However, ATR kinase inhibition did not significantly impact the rate of CPD removal from the genomic DNA of UVB-irradiated HaCaT keratinocytes (Figure 6a). Thus, the diminished proliferative capacity and increased mutagenesis of quiescent keratinocytes with inactive ATR is likely not due to defects in nucleotide excision repair. The excision of UV photoproducts leaves single-stranded DNA (ssDNA) gaps in genomic DNA that must be filled in by the action of DNA polymerases to complete the repair process (37). In the absence of gap filling, it is possible that the excision gaps may be prone to breakage and result in the formation of double-strand breaks (DSBs). Indeed, a previous study demonstrated that quiescent cells exposed to UV radiation resulted in a transient NER-dependent generation of DSBs and is associated ATM-CHK2 kinase signaling (15). The loss of ATR kinase signaling could exacerbate this DSB formation and enhance DSB response signaling, which could contribute to cell lethality and mutagenesis. However, immunoblot analyses of CHK2 and DNA-PK phosphorylation as markers of DSB

signaling were not enhanced by ATR inhibition (Figure 6b, c), suggesting that the loss of ATR signaling does not result in the collapse of excision gaps into DSBs.

It was also possible that the gap filling process occurs abnormally in the absence of ATR kinase signaling. The single-stranded DNA binding protein RPA (Replication Protein A) and the polymerase clamp protein PCNA (proliferating cell nuclear antigen) are known to associate with the excision gaps, and thus UVB-irradiated quiescent HaCaT cells were fractionated to enrich for chromatin-associated proteins. As shown in Figure 7a, both proteins were found to be enriched on chromatin following UVB exposure, and this response was abrogated by treatment of cells with the NER inhibitors SP and TPL (Figure 7b). Interestingly, the levels of chromatin-associated RPA and PCNA were both transiently elevated when ATR was inhibited (Figure 7c, d). Moreover, a characteristic mono-ubiquitinated form of PCNA was also observed to be present at increased levels when ATR was inhibited. PCNA mono-ubiquitination is frequently considered to be a biochemical signal for the recruitment of specialized, potentially mutagenic translesion synthesis (TLS) polymerases to sites of DNA damage and replication stress (42), and previous data had suggested that TLS polymerases (and particularly pol kappa) contribute to gap filling synthesis under such circumstances in quiescent cells (39–41). Thus, though TLS polymerases may promote the survival of quiescent cells following UV exposure, their decreased fidelity may increase the risk of mutagenesis. Together, these results indicate that the gap filling DNA synthesis that occurs in UVB-irradiated quiescent cells in the absence of ATR signaling may involve abnormal gap filling synthesis and be more dependent on TLS polymerases that help to promote survival but at the risk of increased mutagenesis (Figure 8).

DISCUSSION

Much of the understanding of ATR kinase function in the cellular response to DNA damage induced by UV radiation and other genotoxins is based on model systems and approaches in which DNA synthesis and cell cycle progression are major components (1). However, given that most cells in the body, including keratinocytes in the epidermal layer of the skin, are not actively synthesizing DNA at the time of UVB exposure, there are likely new insights to be gained on the role of ATR in the cellular DNA damage response by employing experimental approaches in which DNA replication and cell cycle progression are avoided.

Quiescent, non-replicating keratinocytes were used here in vitro to begin to understand these novel functions of ATR in the response to UVB radiation. Consistent with previous studies using UVC and other DNA damaging agents (19,26), ATR activation occurs rapidly in non-replicating keratinocytes following UVB irradiation and requires the function of the XPB DNA translocase (Figure 1–3), which has roles in both NER and transcription (32). Though both DNA repair intermediates (16,17,20,21,43) and transcription stalling (19,26,44) have been implicated in ATR activation in non-replicating cells, the greater accumulation of RPA and PCNA on chromatin in the absence of ATR signaling (Figure 6) may indicate that unfilled excision gaps are the major signaling that activates ATR in response to UVB radiation.

The loss of ATR kinase signaling in quiescent UVB-irradiated keratinocytes has a modest negative effect on acute survival and cytotoxicity (Figure 4) and more dramatically disrupts proliferative capacity upon growth factor stimulation (Figure 5). These results indicate that the loss of ATR kinase function during the acute UVB response has long-term effects, including through a reduced clonogenic growth potential and increased mutagenesis (Figure 5). In addition to a transient elevation in RPA and PCNA association with chromatin in quiescent UVB-irradiated keratinocytes lacking ATR kinase signaling (Figure 7), the increased PCNA mono-ubiquitination that was observed indicates that such cells are at a greater reliance on potentially mutagenic TLS polymerases to process and fill the excision gaps that are generated during NER. This dependence on the TLS pathway at early time points following UVB exposure may provide a mechanism for the increased mutagenesis that is seen in cells lacking ATR kinase function (Figure 5c). The precise mechanism by which ATR kinase signaling impacts gap filling processes and the potential influence on the TLS pathway in quiescent cells (Figure 7) is not fully clear, and the relevant protein substrates of ATR in this context remains to be characterized. Moreover, given that ATR has been shown to directly phosphorylate pol eta (4,5) and to be required for the transcriptional induction of TLS polymerases in replicating cells (45), it will be important to determine how these regulatory events influence UVB responses in quiescent cells as well. Nonetheless, as ATR kinase inhibitors have entered clinical trials for the treatment of various cancers (23,24), it may be important to examine whether such regimens elevate skin cancer risk in susceptible individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. UVB radiation activates ATR kinase signaling in quiescent human HaCaT keratinocytes.

(a) DNA synthesis was quantified by genomic DNA immunoblotting in proliferating and quiescent HaCaT cells pulsed with BrdU. (b) Quiescent HaCaT cells treated with vehicle (DMSO) or the ATR inhibitor (ATRi) VE-821 were exposed to 200 J/m² of UVB radiation and then harvested 20 min later (or 4 hr, for ATR autophosphorylation) later for immunoblotting with the indicated antibodies. (c) Quantitation (average and SEM) of three independent experiments performed as in (b) is shown.

Figure 2. ATR kinase signaling is activated in UVB-irradiated, telomerase-immortalized normal human keratinocytes.

(a) DNA synthesis was examined in proliferating N-TERT keratinocytes and N-TERTs maintained at confluence without growth factors for 2 days as in Figure 1. (b) Activation of ATR kinase signaling was analyzed and quantified (c) in N-TERTs as described for HaCaT cells in Figure 1. The asterisks indicate that the level of DNA synthesis or protein phosphorylation is significantly different between the treatment groups ($p < 0.05$).

Figure 3. The DNA translocase and NER protein XPB is required for ATR kinase activation in quiescent keratinocytes exposed to UVB radiation.

(a) Quiescent HaCaT cells were treated with vehicle (DMSO), triptolide (TPL), spironolactone (SP), or the Cdk7 inhibitor THZ1 before exposure to 200 J/m² of UVB. Cells were harvested 20 min later and analyzed by immunoblotting. (b) Quantitation (average and SEM) of three independent experiments performed as in (a). The asterisks indicate that the level of protein phosphorylation is significantly different between the treatment groups (p<0.05).

Figure 4. ATR kinase inhibition in UVB-irradiated quiescent keratinocytes results in reduced acute survival and increased cytotoxicity.

(a) HaCaT cells treated with DMSO or ATRi were exposed to the indicated fluence of UVB radiation, and surviving cells were stained with crystal violet 3 days later. (b) Cytotoxicity was assessed in quiescent HaCaT cells as in (A) 1 day following UVB exposure using an LDH assay. (c) Cell survival and (d) cytotoxicity were measured 1 day after UVB exposure in N-TERT keratinocytes with MTT and LDH assays, respectively.

Figure 5. ATR kinase inhibition impedes the proliferative potential of quiescent keratinocytes and enhances mutagenesis.

(a) Design of experiments to measure cell proliferation and mutagenesis upon stimulation of quiescent keratinocytes with growth factors. (b) Clonogenic survival assays were performed 5 days following the exposure of quiescent HaCaT cells to the indicated doses of UVB radiation in the absence or presence of ATRi. (c) Quiescent HaCaT cells treated with DMSO or ATRi and exposed to 100 J/m² UVB were selected with 6-thioguanine to quantify the number of cell clones with mutations at the HPRT locus. The asterisks indicate significant differences between the DMSO and ATRi treatment groups ($p < 0.05$).

Figure 6. ATR kinase inhibition in quiescent HaCaT keratinocytes does not impact UVB photoproduct removal or DSB signaling events.

(a) Removal of cyclobutane pyrimidine dimers (CPDs) from genomic DNA of quiescent HaCaT cells exposed to 100 J/m² UVB was measured by DNA immunoblotting. (b) Quiescent HaCaT cells treated with DMSO or VE-821 (10 μM) and exposed to 100 J/m² UVB were harvested at the indicated time points for analysis of DSB response signaling.

Figure 7. ATR kinase inhibition in quiescent HaCaT keratinocytes leads to increased chromatin-associated RPA and PCNA and to elevated PCNA mono-ubiquitination.

(a) Chromatin-associated proteins were enriched from cells 1 hr after exposure to 200 J/m² of UVB in the absence or presence of the indicated NER inhibitor. (b) Quantitation of chromatin-associated RPA and PCNA from three independent experiments. (c) Chromatin fractions of cells were isolated at the given time points following UVB exposure and analyzed by immunoblotting. Note that longer exposures using a stronger chemiluminescent substrate were used to detect PCNA mono-ubiquitination. (c) The levels of chromatin-bound RPA, PCNA, and mono-ubiquitinated PCNA were quantified from 3–5 independent experiments. Asterisks indicate significant differences ($p < 0.05$) between the DMSO and ATRi treatment groups.

Figure 8.

Model summarizing the effects of ATR kinase inhibition in UVB-irradiated quiescent cells. ATR is activated following the removal of UV photoproducts (T$\diamond$T) from the genome. In the absence of ATR kinase signaling, the gap filling process is abrogated and leads to increased PCNA mono-ubiquitination and RPA-coated ssDNA. These events are then associated with increased mutagenesis and reduced survival.