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Inhibition of mTORC2 enhances UVB-induced apoptosis in keratinocytes through a mechanism dependent on the FOXO3a transcriptional target NOXA but independent of TRAIL

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Abstract

The primary cause of non-melanoma skin cancer (NMSC) is ultraviolet B (UVB) radiation. We have shown previously that mTORC2 inhibition sensitizes keratinocytes to UVB-induced apoptosis mediated by the transcription factor FOXO3a. FOXO3a is a key regulator of apoptosis and a tumor suppressor in several cancer types. Activation of FOXO3a promotes apoptosis through the coordinated expression of a variety of target genes, including TRAIL and NOXA. We hypothesized that in the setting of mTORC2 inhibition, the UVB-induced expression of these factors would lead to apoptosis in a FOXO3a-dependent manner. Using spontaneously immortalized human keratinocytes (HaCaT cells), we observed that both TRAIL and NOXA expression increased in cells exposed to UVB and the TOR kinase inhibitor Torin-2. Similar to knockdown of FOXO3a, NOXA knockdown reversed the sensitization to UVB-induced apoptosis caused by mTORC2 inhibition. In contrast, loss of TRAIL by either knockdown or knockout actually enhanced expression of nuclear FOXO3a, which maintained apoptosis. These surprising results are not due to faulty death receptor signaling in HaCaT cells, as we found that the cells undergo extrinsic apoptosis in response to treatment with recombinant TRAIL. Even more striking, TRAIL knockout cells were sensitized to recombinant TRAIL-induced apoptosis compared to wild-type HaCaT cells, with the largest increase occurring in the presence of mTORC2 inhibition. Taken together, these studies provide strong evidence that mTORC2 controls UVB-induced apoptosis by regulating NOXA expression downstream of FOXO3a. Moreover, FOXO3a transcriptional activation by mTORC2 inhibitors may be a valuable target for prevention or therapy of NMSC, especially in cases with low endogenous TRAIL.

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Author Contributions

LMS and RPF designed the study and wrote the paper. AMN helped with RT-qPCR and exogenous TRAIL experiments as well as provided insight and help with designing experiments to study death receptor-induced keratinocyte apoptosis. LMS, AMN, and RPF all analyzed the results and approved the final version of the manuscript.

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Conflict of Interest

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

Keywords

UVB; TRAIL; NOXA; mTOR; non-melanoma skin cancer; apoptosis

1. Introduction

The incidence of non-melanoma skin cancer (NMSC), although not required to be reported to cancer registries, far exceeds any other form of cancer in the United States (US). Currently, an estimated 3.3 million people suffer from NMSC in the US, representing a striking 300% increase in cases since 1994 [1]. Ultraviolet (UV) radiation, specifically UVB (280–320nm) radiation, is the primary cause of NMSC [2]. While NMSC is treatable if detected early, a minority of cutaneous squamous cell carcinomas (cSCCs) can recur or metastasize, accounting for approximately 25% of deaths attributed to skin cancer each year in the US [3]. Patients who have a history of NMSC are at an increased risk to acquire additional NMSCs as well as melanoma, and are at a 2-fold increase risk for developing other malignancies [4]. If the injured keratinocyte cannot repair the UVB-induced DNA damage, it will either undergo cell cycle arrest or initiate apoptosis as a regulatory defense mechanism [5]. However, UVB irradiation also induces pro-survival pathways in keratinocytes that oppose and counter apoptosis [6–11]. Identifying how these pathways are regulated and understanding the subsequent balance between apoptosis and cell survival in keratinocytes will aid in the development of novel targeted clinical therapies against NMSC.

Both the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) and mTORC2 are crucial in NMSC development, and mTOR may be a potential target for chemoprevention in patients at high risk for developing NMSC. Specifically, kidney transplant patients prescribed the mTORC1 inhibitor rapamycin for immunosuppression have significantly lower incidence of NMSC compared with patients receiving cyclosporine A [12]. We and others have demonstrated that UVB activates mTORC1 downstream of phosphoinositide 3-kinase (PI3K), which enhances cell growth and proliferation during cSCC promotion [10, 13–17]. Our work also demonstrates that mTORC2 activation supports UVB-induced cSCC initiation by allowing the survival of damaged cells via AKT-dependent phosphorylation and cytoplasmic sequestration of the pro-apoptotic transcription factor FOXO3a [18, 19]. Conversely, inhibition of mTORC2 by either pharmacologic or genetic means attenuates AKT activity and allows retention of active FOXO3a in the nucleus, thus triggering increased apoptosis in irradiated keratinocytes [18].

Apoptosis of damaged keratinocytes limits survival of initiated cells and reduces subsequent tumorigenesis. Therefore, this work focuses on understanding the downstream transcriptional targets of FOXO3a that regulate cell death in response to UVB. Specifically, FOXO3a can activate the extrinsic apoptotic pathway through increased TRAIL (TNF-related apoptosis-inducing ligand) expression, as well as initiate NOXA-dependent intrinsic cell death in multiple cell types [20]. TRAIL is a cytokine ligand that belongs to the tumor necrosis factor superfamily [21]. TRAIL binds two main death receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), on the cell surface, which initiate downstream caspase-8 dependent apoptosis [22, 23]. Because TRAIL is an extracellular ligand, the application of TRAIL-

induced apoptosis in cancer therapy has been studied extensively [24–26]. It has been shown that exploitation of the TRAIL-death receptor pathway is an effective strategy to stimulate apoptosis in head and neck cancers [27, 28], melanoma [29] and other skin diseases [30, 31]. Based on these successes, understanding how to manipulate and maximize the responsiveness of the TRAIL pathway in keratinocytes can help develop novel therapies to prevent and treat NMSC.

The FOXO3a target gene NOXA is characterized as a BH3-only protein and part of the caspase-dependent intrinsic apoptotic pathway [11]. Upon induction, NOXA activates the proteins BAX (Bcl-2 homologous antagonist) and BAK (BAX/killer), which are inhibited by Bcl-2 (B-cell lymphoma 2)-like proteins [32], resulting in depolarization of the mitochondria [33]. NOXA accomplishes this task in two ways: indirectly by inhibiting the Bcl-2-like protein Myeloid Cell Leukemia Sequence 1 (Mcl-1) and by directly binding to and activating BAX and BAK [34–36]. NOXA, like TRAIL, plays an important role in multiple forms of skin cancer, including NMSC [37], but the factors that mediate mTORC2/FOXO3a-dependent apoptosis in keratinocytes have not been identified. These studies demonstrate that UVB generates a unique FOXO3a-dependent cell death response when mTORC2 is inhibited, leading to increased caspase-dependent apoptosis that is regulated at least in part by NOXA-mediated intrinsic apoptosis but not by TRAIL-dependent extrinsic apoptosis. Further, we identified a novel TRAIL-dependent negative regulation of FOXO3a that may be employed in cSCC therapy.

2. Methods and Materials

2.1. Cell Culture

The spontaneously-immortalized, human keratinocyte HaCaT cells were obtained from the German Cancer Research Center and were used at passage <25. A431 cells were a gift from Dr. Ryan Hobbs (Pennsylvania State College of Medicine, Hershey, PA). Both cell lines were grown in DMEM containing 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin and 1% Glutamax (ThermoFisher Scientific, Waltham MA). Cells were monitored for consistent growth using growth curve analyses. Cells were frozen and stored in liquid nitrogen and experiments were conducted using cells within 5 passages of each other. All cell lines are reported to be UVB sensitive [18, 19, 38] and the UVB and drug treatment experiments (Section 2.4) were conducted using cells that had reached 70–80% confluence.

2.2. Lentiviral-mediated production, transduction, and single colony selection.

ViraPower™ Lentiviral Expression Systems (ThermoFisher Scientific) was used according to manufacturer's protocols to generate pLKO.1 constructs for sh-mSIN1 (mSin1 shRNA #1, a gift from Dr. David Sabatini, Cambridge MA; Addgene #13483), sh-FOXO3a (Sigma-Aldrich, St. Louis MO - TRCN0000040102), sh-TRAIL (a gift from Dr. Wafik El-Deiry, Fox Chase Cancer Center, Philadelphia PA), sh-NOXA (Sigma-Aldrich – TCRN0000338867), and a U6 scrambled construct (Scramble, a gift from Dr. David Sabatini; Addgene #1864). Production of CRISPR/Cas9 viral production was performed using the same protocol as above but using pLentiCRISPR v2 constructs containing guide

RNA for either mSIN1 [GenScript, Piscataway NJ; gRNA sequence (TGCTGGCAGTATACAAGCGA)], or TRAIL [GenScript; gRNA sequence (ACTCCGTCAGCTCGTTAGAA)]. See Figures S1 and S2 for PCR and sequence validation of CRISPR knockout cells. HaCaT and A431 cells were incubated with viral particles for 24–48 h and selected with puromycin (1.0–1.5 $\mu\text{g/mL}$) in complete medium for 72 h following transduction. Single clones for the knockout cell lines were isolated, amplified, and re-selected twice with puromycin. Double knockdown of FOXO3a and mSIN1 was accomplished through initial knockdown of FOXO3a as described above. The sh-FOXO3a cells were then subjected to a second transduction with sh-mSIN1 48 h prior to UVB exposure. Double knockout of mSIN1 and TRAIL was accomplished with initial knockout of mSIN1 as described above. The mSIN1-KO cells were then subjected to a second transduction with TRAIL-CRISPR viral particles for an additional 48 h in order to knock out TRAIL. Single colony selection was then repeated. Knockdown of FOXO3a in the TRAIL-KO cells was accomplished with initial knockout of TRAIL as described above. The TRAIL-KO cells were then subjected to a second transduction with sh-FOXO3a 48 h prior to UVB exposure. Western blot analysis of mSIN1, FOXO3a, and TRAIL, protein levels were performed to assess both knockout and levels of knockdown (Figure S1b–d).

2.3. Genomic DNA Extraction and PCR Purification.

Genomic DNA of the HaCaT CRISPR cell lines were isolated using the DNeasy Blood & Tissue Kit (Qiagen, Valencia CA) as per the manufacturer's instructions. Primers flanking the guide RNA region of the respective CRISPR plasmids were used to generate PCR products from genomic DNA in each CRISPR KO cell line (TRAIL Forward Primer: TCTCAACCCAGATGCAGGAC; TRAIL Reverse Primer: AGGAATTCTTCTGGCCTTCTC; mSIN1 Forward Primer: GTCTACCTCCCTCTGCACTC; mSIN1 Reverse Primer: GTGCAGAAGTGTGGTTCCAG; Figures S1a and S2). Purification of the PCR products were done using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison WI) as per the manufacturer's instructions prior to sequencing.

2.4. UVB and Drug Treatment.

For UVB exposure, cells were washed twice with PBS, then in a minimal volume of PBS, the monolayer was exposed to UVB (FS20 UVB bulbs, National Biological, Cleveland OH) at a physiological dose of 35 mJ/cm^2 . Bulb intensity was measured at the beginning of each experiment using a UVB 500C meter (National Biological). After irradiation, PBS was removed and conditioned medium with drug treatments was added back. Torin 2 (Tocris Bioscience, Bristol UK), rapamycin (Developmental Therapeutics Program, National Cancer Institute, Bethesda MD), or DMSO vehicle was added to the tissue culture medium at a 1:1000 dilution (50 μM Stock) 1 h prior to UVB treatment. The range of incubation times following UVB exposure were based on our previously reported data for optimal results for both Western and FACS analysis [18, 19].

2.5. Western blotting analysis.

Cells were washed twice with ice-cold PBS, harvested directly in 1 $\times$ SDS sample buffer, and boiled for 10 min. Whole cell extracts were stored at -20°C until use. Western blotting was

performed as described previously [10] using antibodies against mSIN1 [CS #12860; Molecular Weight (MW) – 74, 78 kDa], AKT (CS #4691; MW – 60 kDa), p-AKT^{S473} (CS #4060; MW – 60 kDa), FOXO3a (CS #2497; MW – 82 kDa), Caspase-3 (CS #9662; MW – cleaved 17 & 19 kDa / full length 35 kDa), PARP (CS #9542; MW – cleaved 89 kDa / full length 116 kDa), BIM (CS #2819; MW – 23 kDa), Caspase-8 (CS #9746; MW – cleaved 18 & 43 kDa / full length 57 kDa) (all 1:1000 - Cell Signaling, Beverly MA), TRAIL (CS #3219; MW – 28–30 kDa), DcR2 (CS #8049; MW – 45–60 kDa), FasL (CS #4273; MW – 40 kDa), CD95/FasR (CS #4233; MW – 45–50 kDa), SMC1 (CS #6892; MW – 145 kDa), NOXA (CS #14766; MW – 10 kDa), BID (CS #2002; MW – truncated 15 kDa / full length 22 kDa), Bcl-2 (CS #2872; MW – 26 kDa), P-S6K1^{T389} (CS #9205; MW 70 kDa), p70 S6K1 (CS #9202; MW 70 kDa), P-p38^{T108/Y182} (CS #9211; MW – 43 kDa), p38 MAP Kinase (CS #9212; MW – 43 kDa), p-ERK1/2^{T202/Y204} (CS# 9101; MW – 42 & 44 kDa), p44/42 MAPK (ERK1/2; CS #9102; MW – 42 & 44 kDa) (all 1:500 - Cell Signaling), DR4/TRAIL-R1 (Ab #13890; MW – 52 kDa) (1:250 - Abcam, Cambridge, UK), and GAPDH (#10494-1-AP; MW – 37 kDa) (1:2000 – Proteintech, Rosemont IL). All antibodies were authenticated (see Section 2.12). Comparisons of protein levels were normalized within the same gel and not between gels to allow for direct comparison of protein levels. Each blot is representative of at least 2 experiments with similar results. Pierce™ Reversible Protein Stain Kit (MemCode, Thermo Scientific, Rockford IL) was used where indicated to validate equal loading.

2.6. Quantitative real-time PCR (qRT-PCR).

RNA from HaCaT cells was isolated following UVB irradiation using the RNeasy Mini Kit (Qiagen) with DNase I digestion and converted to cDNA using the High-Capacity RNA-to-cDNA conversion kit (ThermoFisher Scientific) following the manufacturer's protocol. qRT-PCR was performed for *Trail* (TNFSF10 #Hs00921974_m1 - Applied Biosystems™, ThermoFisher Scientific) along with 18S or ribosomal protein, large P0 (RPLP0) (housekeeping gene – ThermoFisher Scientific) using inventoried TaqMan reagents measured on the LightCycler 96 instrument (Roche Life Sciences, Madison WI). Differences in gene expression were assessed by comparative CT values with fold change calculations. Biological replicates (n = 3–6) were examined for each time point.

2.7. Fluorescence-activated cell sorting (FACS) Analysis.

Apoptosis was assessed by flow cytometry using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes NJ) according to the manufacturer's instructions. The percentage of early apoptotic [Annexin V positive / 7-Aminoactinomycin D (7-AAD) negative] and late apoptotic/necrotic (Annexin V / 7-AAD positive) cells was determined using a FACSCalibur cytometer (Beckman Coulter, Jersey City NJ) and analyzed with Modfit LT software (Verity Software). Total cell death was measured as the percent of cells undergoing both early and late apoptosis/necrosis (Annexin V positive cells). All UVB-treated groups were significantly different ($P < 0.001$) from their No-UVB counterparts. See Figure S8 for representative dot plots of the flow cytometry.

2.8. Cellular fractionation.

Cellular fractionation of HaCaT cells was performed as described previously [39]. Briefly, the cells were pelleted, re-suspended in nuclear isolation buffer (10mM KCl, 10mM Tris, pH 7.5, 0.625% NP-40), incubated on ice for 20 min, and centrifuged at $1000 \times g$ at 4°C for 10 min. The supernatant was saved as the cytoplasmic fraction and further clarified by centrifugation at $16,000 \times g$ for 10 min, which was then combined with 4 $\times$ Laemmli Sample Buffer (4 $\times$ LDS, Biorad, Philadelphia PA). The nuclear pellet was washed three times with ice-cold 1 $\times$ PBS, re-suspended in 4 $\times$ LDS and run through a QIAshredder (Qiagen) at $16,000 \times g$ for 2 min at room temperature. Both the cytoplasmic and nuclear samples were boiled for 10 min and then analyzed by Western blotting.

2.9. Forkhead Response Element (FHRE) Luciferase Assay.

The FHRE was cloned into the pGL3-Basic vector (Promega), which contains the Firefly luciferase gene directly downstream of the FHRE (pGL3-FHRE – a gift from Dr. Michael Greenburg, Boston MA; Addgene #1789). HaCaT cells were grown to ~60% confluency in 3.5 cm plates and transfected with either the FHRE vector or the pGL3-control firefly vector containing the SV-promoter (Promega) as described above. In order to control for transfection efficiency, cells were co-transfected of the pRL-SV40 vector containing the Renilla luciferase reporter gene (Promega). Cells were subjected to UVB irradiation 48 h after transfection. Cells were lysed in 1 $\times$ Passive Lysis Buffer (Promega) at specific timepoints before and after UVB-exposure and then assayed using the Dual-Luciferase Kit as per the manufacturer's instructions (Promega). Values listed are fold change in luciferase luminescence at the 1 h and 3 h post-UVB compared to their respective No-UVB control group. The data were normalized to a Renilla-driven luciferase plasmid.

2.10. Cellular transfection.

HaCaT cells were grown to ~60% confluency in 3.5 cm plates and transfected with the indicated plasmids and concentrations using Lipofectamine 2000 as per the manufacturer's instructions (ThermoFisher). This includes 1 μg of either the pGL3-control vector (Promega) or the pGL3-FHRE Firefly luciferase reporters co-transfected with 0.1 μg of the pRL-SV40 Renilla luciferase reporter (Promega) for the luciferase assay. UVB treatment was performed 48 h following transfection.

2.11. Recombinant TRAIL Treatment.

HaCaT cells were plated in triplicate and allowed to grow in culture medium with FBS to ~80% confluency. Torin 2 or DMSO vehicle was added to the tissue culture medium at a 1:1000 dilution (50 μM Stock) for 1 h prior to addition of TRAIL. Following incubation with drug, either recombinant TRAIL (ThermoFisher) or 1% BSA vehicle was added directly to the medium to achieve a final concentration of 300ng/mL. Cells were placed back in the incubator for either 3 or 24 h hours and then harvested in 1 $\times$ Sample Buffer.

2.12. Authentication.

The following approaches have been used in these studies to properly validate key reagents used to ensure rigor, transparency and reproducibility of data:

All antibodies used for Western blotting are validated for each corresponding application. Our validation involves the use of any given antibody in both control wild type cells, in cells where the function of the gene of interest is either silenced or knocked-out and where possible in cells known to overexpress the target protein. For all antibodies used, company names are provided and raw data are available for all gels with information on the MW at which bands run. All assays use both positive and negative controls for key biological resources and rigor of data is established through replications in several independent experiments performed on independent days in a blinded manner combined with statistics.

All constructs are fully sequenced, catalogued and glycerol stocks are generated for longevity. All cell lines are used for less than 20–25 passages after removing from freeze. Cells are also monitored by observation of morphology and growth curve analysis. All cells undergo mycoplasma contamination checks, and the HaCaT cells used in the experiments described were found to be mycoplasma-free.

2.13 Statistical Analysis.

Data directly comparing two groups (i.e. No-UVB vs UVB) were analyzed using the two-tailed Student's *t*-test. A two-way analysis of variance (ANOVA) was used when comparing interaction of the above values between groups (i.e. wild-type vs mTORC2-disrupted). Data were displayed as mean or percent change/fold difference $\pm$ SE. Differences between values were considered significant when $p < 0.05$. Experiments requiring *t*-test or ANOVA analysis include at least 3 biological replicates per experimental group. Bonferroni post-hoc test was used for ANOVA.

3. Results

3.1. Inhibition of mTORC2 increases TRAIL mRNA and protein in a FOXO3a-dependent manner

We examined the relative mRNA expression of TRAIL following inhibition of mTORC2 in mock-irradiated control HaCaT cells and in cells 6 h after UVB irradiation. Overnight treatment with the TOR kinase inhibitor Torin 2 increased TRAIL expression 6-fold compared to control, while cells treated with rapamycin did not show increased TRAIL mRNA (Figure 1a). Expression of TRAIL remained significantly elevated in the Torin 2-treated cells in response to UVB, and this was reversed by simultaneously knocking down FOXO3a (sh-FOXO3a; Figure 1a). The protein levels of TRAIL also increased under similar conditions following knockdown of the essential mTORC2 component, mSIN1 (sh-mSIN1; Figure 1b). Loss of mSIN1 disrupts formation of the mTORC2 complex without impacting mTORC1 activity [18, 40], thus inhibiting downstream pathways uniquely dependent on mTORC2. Knockdown of FOXO3a likewise rescued the observed increase in TRAIL (Figure 1b), suggesting that induction of TRAIL protein expression is dependent on FOXO3a. Together, these results demonstrate that both TRAIL transcription and protein levels are increased in a FOXO3a-dependent manner when mTORC2-dependent signaling is disrupted. The FOXO3a-dependent increase in TRAIL mRNA and protein expression in the “No-UVB” samples is consistent with our previous work, which described that inhibition of mTORC2 prior to UVB-exposure transiently increases FOXO3a localization and abundance

in the nucleus, where it is transcriptionally active [18]. Importantly, despite this increase in both nuclear FOXO3a and TRAIL, these cells still require UVB exposure to undergo apoptosis.

We employed both CRISPR/Cas9 targeting of the TRAIL gene and shRNA targeting of TRAIL mRNA to generate HaCaT cells with either stable knockout (TRAIL-KO) or knockdown (sh-TRAIL) of endogenous TRAIL, respectively. We also used CRISPR/Cas9 technology to generate a dual knockout of mSIN1 and TRAIL (mSIN1/TRAIL-KO; Figures S1, S2). We assessed levels of apoptosis using FACS for Annexin V and 7-AAD. The levels of early apoptosis and total cell death were increased 10% to 20% with either knockout of mSIN1 or treatment with Torin 2, respectively, following UVB exposure (Figure 2a). Since this effect is rescued by simultaneous knockdown of FOXO3a [18], we expected loss of TRAIL to phenocopy this result. Surprisingly however, neither knockout nor knockdown of TRAIL had any effect on the increased apoptotic sensitivity that resulted from mTORC2 inhibition (Figure 2a). These results were confirmed both by immunoblot analysis of caspase-3 and PARP cleavage in HaCaT cells with knockdown of TRAIL (sh-TRAIL; Figure 2b) as well as visual inspection of the TRAIL-KO cells in culture (Figure 3a). Interestingly, loss of TRAIL unexpectedly increased FOXO3a protein expression compared to cells with intact endogenous TRAIL, and this effect was even further enhanced in TRAIL-disrupted cells when mTORC2 was inhibited (Figure 2b). This suggests TRAIL and mTORC2 may cooperate to negatively regulate FOXO3a activity.

We used A431 cells [41] to confirm in a second keratinocyte line that loss of TRAIL did not reverse the increased sensitivity to apoptosis observed with mTORC2 inhibition (Figure 3b). During a UVB time course, both the HaCaT and A431 cells show similar cleavage of caspase-3 and PARP in the absence of mTORC2 activity (Torin 2 treatment) whether or not TRAIL was knocked down (Figure 2b, 3b). Thus, under conditions where mTORC2 signaling is disrupted, blocking TRAIL is not sufficient to reverse the increased sensitivity to UVB-induced apoptosis in keratinocytes despite the observed increase in TRAIL mRNA and protein expression.

3.2. Loss of TRAIL enhances expression of nuclear FOXO3a and FOXO3a-dependent apoptosis in mTORC2-inhibited keratinocytes exposed to UVB

While the TRAIL pathway is classically identified as an inducer of apoptosis, recent studies have described and illuminated a non-canonical role of the TRAIL death receptor pathway in promoting cell survival in certain TRAIL-resistant cancer and non-transformed cell lines [42], which could explain our results. Therefore, to study this TRAIL-dependent mechanism, we examined UVB-inducible signaling pathways known to be involved in NMSC [11] to determine if the knockout of endogenous TRAIL altered their induction. These pathways include mTORC1 (P-S6K^{T389}), mTORC2 (P-ATK^{S473}), MEK/ERK (P-ERK1/2^{T202/Y204}), p38 MAPK (P-p38^{T180/Y182}), and NF- κ B (P-IKK α/β ^{S176/180}). We found that only mTOR-dependent pathways were appreciably increased following TRAIL-KO in response to UVB (P-ATK^{S473} and P-S6K^{T389}) (Figure S3). In contrast, ERK and p38 phosphorylation were relatively unaffected (Figure S3). Total IKK α/β protein levels

remained unchanged during the UVB time course, while phosphorylation of IKK α/β was below the level of detection in all samples (data not shown).

We next investigated the surprising finding that FOXO3a protein levels were increased in the absence of TRAIL. To our knowledge, a regulatory loop between TRAIL and FOXO3a has not been described. We hypothesized that increased FOXO3a-dependent apoptosis in HaCaT cells with reduced endogenous TRAIL could account for the observation that neither TRAIL knockout nor knockdown reversed the effect of mTORC2 inhibition (Figure 2). In order to test this hypothesis, it was first necessary to determine the fraction of FOXO3a present in the nucleus, where it would be transcriptionally active. We performed a nuclear/cytoplasmic fractionation of the TRAIL-disrupted HaCaT cells with and without mTORC2 inhibition to assess the overall levels of nuclear FOXO3a in response to UVB. FOXO3a protein expression in both whole cell lysates and the cytoplasmic fractions was greater in control cells compared with mTORC2-inhibited cells following UVB exposure (Figure 4a and b). [Note that phosphorylation of AKT at Ser 473 in the whole cell and cytoplasmic fractions is used as a marker for mTORC2 activity (Figure 4a and b)]. Conversely, FOXO3a nuclear expression was enhanced in both TRAIL knockout and control cells following inhibition of mTORC2 (Figure 4c). Importantly, the greatest increase in nuclear FOXO3a was observed under conditions where both TRAIL and mTORC2 are disrupted (Figure 4c). To confirm that increased nuclear FOXO3a correlates with higher FOXO3a activity, we measured FOXO3a-dependent transcription using luciferase analysis of the forkhead responsive element (FHRE) under the same experimental conditions. As expected from the cell fractionation results, knockout of TRAIL further increased FOXO3a transcriptional activity in mTORC2-inhibited cells in response to an apoptotic dose of UVB (Figure 4d).

The above results suggest that increased FOXO3a nuclear localization and activity is responsible for the inability of TRAIL knockout to reverse the effects of mTORC2 inhibition. To address this, we examined cell death 6 h and 9 h after UVB in Torin 2-treated cells under the following conditions: sh-FOXO3a, TRAIL-KO, and TRAIL-KO/shFOXO3a dual inhibition. FOXO3a knockdown alone reversed the effects of mTOR inhibition, while cleavage of caspase-3 and PARP were similar following treatment with Torin 2 at both time points in TRAIL-KO cells compared to wild-type cells. Importantly, this Torin 2-dependent increase was completely reversed in TRAIL-KO/sh-FOXO3a cells (Figure 5a). These results were confirmed using FACS analysis to examine both early apoptosis and total cell death (Figure 5b), as well as microscopic visual inspection of the cells (Figure 5c), supporting the conclusion that in the absence of TRAIL, UVB-induced apoptosis following inhibition of mTOR signaling continues to be dependent on FOXO3a.

3.3. The death receptor pathway is intact in HaCaT cells and is regulated by mTORC2/FOXO3a signaling as well as endogenous levels of TRAIL

To determine if the results above were due to faulty TRAIL death receptor signaling in HaCaT cells, we investigated whether recombinant TRAIL (300ng/mL) could induce apoptosis in the absence of UVB. As expected, treatment with TRAIL increased activation of the direct TRAIL death receptor target caspase-8, as well as caspase-3, resulting in cleavage of PARP and indicating that the TRAIL apoptotic pathway is intact and responsive

to TRAIL (Figure 6). Surprisingly, inhibition of mTORC2 sensitized HaCaT cells to TRAIL-induced death receptor pathway activation, and this was completely rescued by simultaneously knocking down FOXO3a, indicating the process was dependent on FOXO3a (Figure 6). These results mirror the regulation noted in UVB-induced apoptosis (Figure 5a). Even more striking was the observation that TRAIL-KO cells were further sensitized to recombinant TRAIL-induced apoptosis compared to wild-type HaCaT cells, with the largest increase occurring in the presence of mTORC2 inhibition (Figure 6). Immunoblot examination of TRAIL-R1 (DR4), the primary TRAIL receptor responsible for apoptosis in HaCaT cells [43], shows no difference in receptor expression regardless of mTORC2 activity or the presence of either FOXO3a or TRAIL as well as in TRAIL-deficient cells following UVB-exposure (Figure S4). TRAIL-R2 (DR5) was below the limit of detection (data not shown). Taken together, these results suggest both mTORC2 signaling and levels of endogenous TRAIL regulate TRAIL-specific, caspase-8 mediated cell death.

3.4. The FOXO3a transcriptional target NOXA mediates mTORC2 regulation of UVB-induced apoptosis

Our results suggest a FOXO3a transcriptional target other than TRAIL is responsible for sensitizing cells to UVB-induced apoptosis when mTORC2 is inhibited. Thus, we investigated mTORC2-dependent expression of a number of intrinsic and extrinsic apoptotic proteins that are known direct FOXO3a targets [11], including FasL, FasR/CD95, TRAIL, DR4, Dcr2, Bcl-2, BIM, BID, and NOXA, 6 h following UVB exposure in sh-control, sh-FOXO3a, and TRAIL-KO HaCaT cells treated with Torin 2 (Figures S5, S6). Of these candidates, the BH3-only protein NOXA was the only apoptotic protein that was increased by mTORC2 inhibition in both control and TRAIL-KO cells exposed to UVB. Further, the increased expression of NOXA was blocked with knockdown of FOXO3a, consistent with a regulatory role in UVB-induced FOXO3a-dependent apoptosis (Figures 7a and S5). Interestingly, NOXA protein expression was highest in the Torin 2-treated TRAIL-KO cells (Figure 6a), suggesting a compensatory FOXO3a-dependent increase in NOXA when mTORC2 is inhibited in the absence of endogenous TRAIL.

To determine whether NOXA mediates the effect of mTORC2 disruption in cells exposed to UVB, we knocked down NOXA expression by shRNA (sh-NOXA; Figure 7b) and examined apoptosis. Both FACS analysis and visual inspection of the cells show that knockdown of NOXA reversed the increased sensitivity to UVB-induced apoptosis brought about by treatment with Torin 2 (Figure 7c, 7d). These data suggest that inhibition of mTORC2 activates a FOXO3a-dependent apoptotic response to UVB that is mediated at least in part by NOXA (see Figure S7 for model).

4. Discussion

Understanding the mechanisms of UVB-induced apoptosis and cell survival in keratinocytes is vital, as greater insight into how these processes are activated, regulated, and potentially exploited is essential for developing novel therapies for NMSC. We show that the BH3-only protein, NOXA, is an important contributor to the increased sensitivity to UVB-induced apoptosis observed when mTORC2 signaling is inhibited. Moreover, NOXA protein

expression is dependent on FOXO3a, suggesting NOXA is likely the primary downstream transcriptional target of FOXO3a under these conditions. Surprisingly, we also identified a novel TRAIL-dependent negative regulation of FOXO3a protein expression and activity in keratinocytes. In doing so, we demonstrated that inhibition of mTORC2 in TRAIL-KO cells increases the sensitivity to apoptosis caused by the addition of recombinant TRAIL. Altogether, our data suggest that under conditions of mTORC2 inhibition, both the intrinsic (*via* NOXA) and the extrinsic (*via* TRAIL) FOXO3a-dependent apoptotic pathways may be exploited for the potential chemoprevention and treatment of NMSC.

Regulation of FOXO3a and the development of strategies to maximize its pro-apoptotic activity has been extensively investigated in many disease pathologies [24–26, 44, 45]. FOXO3a-regulated apoptotic proteins target caspase-dependent apoptosis through either extrinsic death receptors (e.g. TRAIL), or the intrinsic cellular stress pathway (e.g. NOXA) [20, 46–49]. TRAIL is an central regulator of apoptosis in both normal skin [31, 43] and skin pathologies, including skin cancer [50–52] [53]. In contrast, some studies indicate UVB exposure downregulates both endogenous TRAIL and its receptor in normal skin, actinic keratosis, and cSCC [54], implying TRAIL has a lesser role in sun-damaged skin. We identified an increase in TRAIL mRNA and protein in mTORC2-inhibited keratinocytes following UVB exposure, suggesting that FOXO3a-dependent induction of endogenous TRAIL is responsible for the increased apoptosis when mTORC2-signaling is disrupted. However, this was not the case as both knockdown and knockout of TRAIL in our system did not mitigate the effect of mTORC2 inhibition.

Recent studies have shown that activation of the TRAIL death receptor pathway in certain TRAIL-resistant cancers and non-transformed cell lines can counter-intuitively activate cell survival pathways [42]. Based on these findings, reducing endogenous levels of TRAIL in our system may induce two competing responses. Specifically, a reduction in TRAIL would prevent direct TRAIL-dependent caspase-3 activation. However, diminished TRAIL-DR activation may indirectly alter other cell survival pathways, leading to increased apoptosis. To determine whether this potential attenuation of cell survival may be the reason levels of UVB-induced apoptosis in scrambled and TRAIL-deficient keratinocytes are not different, we examined UVB-inducible pathways that have been shown to be regulated by TRAIL [42] and are also important in NMSC (PI3K/mTOR, MEK/ERK, p38 MAPK, and NFκB) [11]. Interestingly, the only cell survival pathway that was markedly altered in the TRAIL-KO cells lines following UVB exposure was the PI3K/mTOR pathway. However, we found that TRAIL knockout in fact leads to activation of this pathway, which should theoretically decrease the apoptotic response.

In further examination of these novel results, we found FOXO3a levels were significantly higher in cells that lack endogenous TRAIL. As stated above, we identified increased P-AKT^{S473} in both untreated and UVB-treated TRAIL-KO cells (Figure 3a, Figure S3) suggesting an induction in mTORC2 activity when TRAIL is lost, since mTORC2 phosphorylates AKT at this site. This activation of AKT could potentially explain the increased FOXO3a seen in cells that lack endogenous TRAIL, since FOXO3a is phosphorylated and stabilized by AKT [18]. However, following inhibition of mTORC2, both total and nuclear FOXO3a protein expression in the TRAIL knockout and knockdown

cells is substantially increased, as is FOXO3a transcriptional activity, indicating the mechanisms behind the TRAIL/FOXO3a negative regulation do not completely depend on mTORC2. This suggests that an additional TRAIL-responsive pathway other than the ones examined above may work in combination with mTORC2 to cause this novel FOXO3a regulation. Importantly, knocking down FOXO3a in our TRAIL-KO cell lines rescued the increased sensitivity to UVB-induced apoptosis following mTORC2-inhibition in a similar manner to cells with intact TRAIL, indicating the apoptotic mechanism in these TRAIL-deficient cells is still dependent on FOXO3a.

Numerous cancer therapies and trials use both recombinant TRAIL and agonistic anti-TRAIL receptor antibodies to stimulate apoptosis in cancer cells [45]. However, tumors are commonly resistant to TRAIL-induced treatments, leading to the rise in research aimed at increasing the sensitivity of cancer cells to recombinant TRAIL [44]. In many instances, the resistance to TRAIL is caused by the PI3K/mTOR/AKT pathway, as chemical inhibition of this pathway in combination with recombinant TRAIL therapy has successfully enabled TRAIL-resistant cancer cells to undergo apoptosis [55–58]. Most NMSCs maintain substantially increased PI3K/mTOR activity [59], and our results indicate that this characteristic will likely make them resistant to TRAIL-induced apoptosis. Therefore, our results strongly suggest that inhibiting the PI3K/mTOR/AKT pathway will sensitize keratinocytes to recombinant TRAIL-induced apoptosis in a FOXO3a-dependent manner and that lesions with low levels of endogenous TRAIL may be especially sensitive to treatment with recombinant TRAIL.

In examining other apoptotic transcriptional targets of FOXO3a in both our scrambled and TRAIL-KO cell lines, we identified NOXA as a key contributor to the increased sensitivity to apoptosis observed in HaCaT cells following mTORC2 inhibition. Interestingly, NOXA has been shown previously to be important in UVB-induced cell death in keratinocytes; however, those studies demonstrate that NOXA-induced apoptosis was dependent on activation of either p53 [60] or p38/HIF-1 α [61]. Conversely, our data show that NOXA-mediated UVB-induced keratinocyte cell death can occur in a FOXO3a-dependent but p53-independent manner, as HaCaT cells have an inactivating mutation in both alleles of p53, which is common in sun damaged skin [62].

5. Conclusion

In summary, these data demonstrate a novel mTORC2/FOXO3a-dependent apoptosis regulatory mechanism in response to UVB. Following inhibition of mTORC2, which sensitizes keratinocytes to UVB-induced cell death, we observe an accumulation of nuclear FOXO3a that corresponds to increased FOXO3a-dependent transcription of both TRAIL and NOXA and subsequent apoptosis *via* the NOXA-induced intrinsic pathway. Interestingly, unlike knockdown of FOXO3a, NOXA knockdown did not completely rescue the increased sensitivity to UVB-induced apoptosis caused by mTORC2 inhibition. This could be due to other FOXO3a-dependent apoptotic pathways that are active in the shNOXA cells, including TRAIL-induced apoptosis. Likewise, the fact that levels of FOXO3a-dependent apoptosis are unchanged by loss of TRAIL can be explained by a compensatory increase in FOXO3a-dependent NOXA expression. Taken together, these data suggest that mTORC2 inhibition

may activate both NOXA- and TRAIL-dependent apoptosis in response to UVB (see Figure S7 for model). Thus, our results highlight key early changes in keratinocyte proteins and pathways that take place upon UVB exposure. From a clinical perspective, characterizing the complex mechanisms behind increased survival of UVB-damaged cells will be critical for the advancement of our knowledge of skin cancer development and essential for both developing new cost-effective therapies and establishing better prevention strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

7-AAD	7-Aminoactinomycin D
FACS	fluorescence-activated cell sorting
FHRE	forkhead response element
FOX	forkhead box
mTOR	mechanistic (mammalian) target of rapamycin
mTORC2	mTOR complex 2
NMSC	non-melanoma skin cancer
PI3K	phosphoinositide 3-kinase
(c)SCC	(cutaneous) squamous cell carcinoma
TRAIL	TNF-related apoptosis-inducing ligand
UVB	ultraviolet-B
WT	wild-type

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Highlights:

- Regulation of TRAIL and NOXA is dependent on mTOR/FOXO3a signaling following UVB
- TRAIL loss had no effect on UVB-induced apoptosis in cells with mTORC2 inhibition
- Knockout of TRAIL sensitizes cells to recombinant TRAIL-induced cell death
- Increased NOXA drives UVB-induced apoptosis following inhibition of mTORC2

Figure 1. Disruption of mTORC2 increases FOXO3a-dependent transcription of TRAIL. (a) Relative fold change in *Trail* mRNA expression in HaCaT cells treated with either rapamycin or Torin 2 following UVB irradiation (35mJ/cm²). Data are normalized to RPLP0. Graph results are represented as the mean $\pm$ SEM of 3–6 independent experiments; paired t-tests with $p < 0.05$. UVB-treated experimental groups within each timepoint with means that are statistically different from one another ($p < 0.05$) are indicated with different letters above the bars. (b) Time-course immunoblot analysis of TRAIL protein expression 6 h post-UVB exposure. The relative density for TRAIL was normalized to GAPDH. Graph results are the mean of two independent experiments.

Figure 2. Loss of TRAIL does not rescue mTORC2-dependent apoptosis in response to UVB. (a) FACS analysis was performed to measure early apoptosis and total cell death 18 h post UVB for the indicated groups (n=3–6 biological replicates / group). Graph results are represented as the mean $\pm$ SEM. UVB-treated experimental groups with means that are statistically different from one another ($P < 0.05$) are indicated with different letters above the bars. (b) Immunoblot analysis of caspase-3 and PARP following UVB irradiation. The relative protein densities for cleaved caspase-3 and cleaved PARP were normalized to GAPDH. Bar graphs represent the mean $\pm$ SEM of three independent experiments.

Figure 3. Levels of UVB-induced cleavage of caspase-3 and PARP are not reduced following knockdown of TRAIL in keratinocytes.

(a) Camera image of HaCaT cells 18 h post 35 mJ/cm² UVB after the indicated treatments.

(b) Verification of HaCaT data using A431 cells showing immunoblot analysis (whole-cell extracts) of caspase-3 and PARP following treatment of 50 nM Torin 2 in Scrambled and sh(TRAIL) A431 cells prior to and after UVB irradiation (35 mJ/cm²). The relative protein densities for cleaved caspase-3 and cleaved PARP were normalized to GAPDH. Knockout or knockdown of TRAIL in both cell lines was achieved as described in the Materials and Methods. Bar graphs represent the mean $\pm$ SEM of three independent experiments.

Figure 4. Inhibition of mTORC2 combined with TRAIL knockout increases relative UVB-induced nuclear FOXO3a abundance and FHRE activity compared to cells with disruption of mTORC2 alone.

(a-c) Time-course analysis of cells following UVB exposure. Immunoblot analysis of (a) whole-cell, (b) cytoplasmic, and (c) nuclear extracts of the indicated HaCaT cells. The relative densities of whole-cell and cytoplasmic FOXO3a were normalized to GAPDH while nuclear FOXO3a was normalized to SMC1. Bar graphs represent the mean $\pm$ SEM of three independent experiments. (d) FHRE luciferase activity in response to UVB in the indicated HaCaT cells. Graph of results represents the mean $\pm$ SEM of three independent experiments.

Figure 5. Increased sensitization to UVB-induced apoptosis in TRAIL-KO cells with disrupted mTORC2 signaling is rescued by knockdown of FOXO3a.

(a) Immunoblot analysis of caspase-3 and PARP in the indicated experimental groups following UVB. The relative protein densities were normalized to GAPDH. Bar graphs represent the mean $\pm$ SEM of three independent experiments. Protein loading (Protein S.) was assessed by MemCode reversible protein staining. (b) FACS analysis was performed 18 h post UVB ($n=3-6$ / group). Graph results are represented as the mean $\pm$ SEM. UVB-treated experimental groups with means that are statistically different from each other ($P < 0.05$) are indicated with different letters above the bars. (c) Camera image of cells 18 h post UVB for the indicated HaCaT cell groups.

Figure 6. Inhibition of mTORC2 sensitizes HaCaT cells to recombinant TRAIL-induced caspase-8 activation.

Immunoblot analysis (whole-cell extracts) of caspase-8, caspase-3, and PARP in the indicated HaCaT experimental groups 3 h and 24 h after treatment of 300ng/mL recombinant TRAIL. The relative protein densities for cleaved caspase-8 (18 kDa fragment), cleaved caspase-3, and cleaved PARP were normalized to GAPDH. Graph results are the mean $\pm$ SEM of three independent experiments (N = 3). Experimental groups with means that are statistically different from each other ($P < 0.05$) are indicated with different letters above the bars. Protein loading (Protein S.) was assessed by MemCode reversible protein staining.

Figure 7. Knockdown of NOXA rescues the mTORC2-dependent sensitization to apoptosis in response to UVB exposure.

(a) Immunoblot analysis of NOXA in the HaCaTs following UVB irradiation. The relative protein densities were normalized to GAPDH. Graph results are the mean $\pm$ SEM of three independent experiments (N = 3). Protein loading (Protein S.) was assessed by MemCode reversible protein staining. Note: The continuous bottom band (<10 kDa) in the NOXA image is a non-specific staining artifact. (b) Immunoblot analysis of NOXA protein expression in HaCaT cells. (c) FACS analysis was performed 18 h post UVB (n=3–6 / group). Graph results are represented as the mean $\pm$ SEM. UVB-treated experimental groups with means that are statistically different from each other ($P < 0.05$) are indicated with different letters above the bars. (d) Camera image of HaCaT cells 18 h post UVB.