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Supplemental Information

MC1R Is a Potent Regulator of PTEN after UV Exposure in Melanocytes

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SUPPLEMENTAL INVENTORY

Supplemental Experimental Procedures

Subjects, Treatment Regimen and ssUVR Exposure

Eligible volunteers were Caucasian with Anglo-Celtic ancestry and >18 years (with Fitzpatrick skin type Classification I-III). Volunteers in this study had sites on their lower back undergo solar simulated ultraviolet radiation (ssUVR) treatment. The non-exposed site (Non-UVR) was collected prior to undertaking the UVR treatment. A solar simulator Model 601 fitted with a 300 watt xenon arc lamp with UV filters was used to administer the ssUVR dose (Solar Light Co, Philadelphia, PA). The treatment regime dose of (2 MED) ranging from 44-130 mJ/cm^2 was applied via a fiber optic cable to the sun exposed sites. Skin biopsy samples were collected at 24 hr and 14 days post ssUVR and processed for histology. Approval to perform the study was given by the Human Research Ethics Committee of the Queensland Institute of Medical Research and the Queensland University of Technology. All participants gave their written informed consent to take part.

Genotyping

Saliva samples were collected and DNA was extracted using Oragene saliva kits (DNA genotek, Ottawa, ON, Canada) following the manufacturer's instructions. Genotyping was performed using the MassArray platform (Sequenom Inc, San Diego, CA, USA). An optimized multiplex assay of all nine common variants of *MC1R* (I155T, R142H, D84E, R160W, D294H, V92M, R163Q, V60L, R151C) were used as previously described (Duffy et al., 2004).

Plasmids and shRNA Constructs

Flag-Smurf1, Flag-Smurf2, Flag-WWP1, Flag-WWP2, Flag-NEDD4, Flag-NEDD4L, Flag-Itch, pLKO-shp16, pSG5L-HA-PTEN WT, 4A, 4E and C124S were purchased from Addgene. Different lengths of GFP-PTEN constructs were cloned into pEGFP-C2 vector at the *Eco*RI site as described previously (Leslie et al., 2000). pcDNA3-Flag-MC1R WT and RHC variants R151C, R160W and

D294H were generated as previously described (Sanchez-Laorden et al., 2006). Non-RHC MC1R variants F76Y, V174I and P230L mutants were generated by using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The Flag-MC1R truncation and deletion mutants were generated with site directed mutagenesis kit (Agilent Technologies). To generate the MC1R expression plasmids for retroviral infection, the cDNAs of the WT and RHC variants were subcloned into pQCXIP (Clonetech) at the *Not*I/*Eco*RI sites, or pLenti-CMV-Blast at the *Bam*HI/*Sal*I sites, respectively. To make GST-MC1R fusion protein, WT MC1R and RHC variants R151C, R160W and D294 H, cDNAs were amplified by PCR and inserted into pGEX-6P-1 at the *Eco*RI/*Xho*I sites. shRNA constructs targeting human MC1R (Cat. No. RHS4533-EG4157), mouse MC1R (Cat. No. RMM4534-EG17199) were purchased from OpenBiosystems. The most efficient knockdown cell lines with shmMC1R-A (target sequence: 5'- AATGGAGATCAGGAAGGGATG-3') or shMC1R-1 (target sequence: 5'- AAATGTCTCTTTAGGAGCCTG-3') were used in most assays. The target sequence for mouse shPTEN: shPTEN-1 5'-ATATCTTCACCTTTAGCTGGC-3'; and shPTEN-2 5'-AAGGGTTTGATAAGTTCTAGC-3'. The target sequence for human shPTEN: 5'-TTCCTTGTCATTATCTGCACG-3'.

Transfection, Lentiviral and Retroviral Infections

To generate stable knockdown of MC1R in B16 and human primary melanocytes, mouse or human specific short hairpin RNAs and scrambled shRNA controls were purchased from OpenBiosystems. The shRNA plasmids were co-transfected with psPAX2 (Addgene #12260) and pMD2.G (Addgene #12259) in 293-FT (ATCC) using Lipofectamine 2000 (Invitrogen). Lentiviruses were collected after 48 hr and cells were then infected for 24 h in the presence of polybrene (8 μg/ml) and selected with puromycin (2 μg/ml) for 3 days.

To generate cells with stable expression of WT MC1R and MC1R variants R151C, R160W and D294H, 293T cells were co-transfected with MC1R constructs in pQCXIP, VSV-G and pUMVC (Addgene #8449) plasmids using Lipofectamine 2000. Retroviruses were harvested after 48 hr and

B16 or human primary melanocytes shMC1R cells were infected with retroviruses in the presence of polybrene (8 μg/ml). After 24 hr, cells were selected with puromycin (2 μg/ml) for 3 days. To generate cells with stable expression of WT MC1R and MC1R variants R151C, R160W and D294H in MC1R-depleted hTERT/CDK4(R24C)/p53DD melanocytes, Blasticidin (5 μg/ml) was used for the selection of infected cells for 5 days after lentiviral infection.

Clonogenic Survival and Soft Agar Assays

For clonogenic survival assays, cells were exposed to different doses at 0, 50, 100, 200 and 300 J/m^2 of UVB as described above. After UV irradiation, cells were trypsinized and plated in triplicate at different cell concentrations to obtain enough colonies. After 10 days, colonies larger than 50 cells were stained and quantified as described (Li et al., 2010). Percent of survival was calculated from colonies formed against untreated controls. For cell survival at time points of 3 hr and 24 hr after 100 J/m^2 of UVB exposure, the relative number of living cells was measured with MTT assay kit (Roche).

The clonogenic survival and soft agar assays for hTERT/p53DD/CDK4(R24C) melanocytes were performed as described previously (Garraway et al., 2005). Briefly, for the melanocyte growth promoting withdrawal experiments, hTERT/p53DD/CDK4(R24C) melanocytes with or without MC1R depletion were untreated or treated with 20 J/m^2 UVB in the presence of 1 μM α-MSH before plating into 6-well plate at 1,000 cells per well. DMEM media was used with 10% FBS and penicillin/streptomycin/glutamine. For the MC1R rescue experiments, the MC1R depleted hTERT/p53DD/CDK4(R24C) melanocytes were further infected with lenti-viruses encoding EV, WT-MC1R or RHC variants R151C, R160W and D294 H. The resulting cells were plated into 6-well plates as described above.

For soft agar assays, cells (10,000 per well) were seeded in 0.5% low-melting-point agarose in DMEM with 10% FBS, layered onto 0.8% agarose in DMEM/10% FBS. The plates were kept in the cell culture incubator for 30 days after which the colonies >50 μm were counted under a light microscope.

Immunoblot Analysis

Anti-PTEN (9188), anti-phospho-PTEN (Ser380/Thr382/383) (9554), anti-AKT (pan) (2920), anti-phospho-AKT (Ser473) (3787), anti-R-Ras (8446), anti-PDK1 (5662), anti-phospho-PDK1 (Ser241) (3438), and anti-phospho-AKT (Thr308) (4056) antibodies were purchased from Cell Signaling. Anti-MC1R (N-19), anti-p16 (sc-467) and anti-p21 (sc-469) antibodies were purchased from Santa Cruz. Anti-WWP2 (A302-935A) and anti-NEDD4 (A303-253A) antibodies were purchased from Bethyl Laboratories. Polyclonal anti-Flag antibody (F-2425), monoclonal anti-Flag antibody (F-3165), anti-Flag agarose beads (A-2220), anti-HA agarose beads (A-2095), peroxidase-conjugated anti-mouse secondary antibody (A-4416) and peroxidase-conjugated anti-rabbit secondary antibody (A-4914) were purchased from Sigma. The oxidized PTEN was separated and detected as described previously (Cao et al., 2009).

FACS Analysis for Cell Cycle Phases Distribution and Apoptosis

Melanoma or human primary melanocyte cells were treated with or without UV, harvested 24 h post UV irradiation by trypsinization, and fixed with cold 70% ethanol. For cell cycle analysis, after two washes in PBS, the cells were resuspended in PBS containing 50 μg/ml propidium iodide (PI) and 100 μg/ml ribonuclease. After incubation at 37ºC for 30 min, the cells were analyzed by FACScan flow cytometer (Becton Dickinson).

Apoptosis analysis was performed with Annexin V binding assay following the manufacturer's instructions (BD Biosciences). Briefly, 24 h after UV irradiation, cells were harvested, washed with PBS and counted. A portion of 10^5 cells were co-stained with PE-Annexin V and 7-AAD and analyzed by FACScan flow cytometer. The cells stained positively for Annexin V were taken as apoptotic cells.

Immunohistochemistry

Mouse skins or foreskins were fixed in 10% formalin solution at 4°C overnight, paraffin-embedded and then cut in 5 μm-thick sections (Dermpath core facility, Boston University). Sections were then deparaffinized, rehydrated, and stained with phospho-Akt (Ser473) (1:100), AKT (1:200), phopho-PTEN (Ser380/Thr382/383) (1:200), PTEN (1:200) and counterstained with hematoxylin. Briefly, for antigen retrieval, sections were heated in a boiling water bath in 10 mM sodium citrate buffer (PH 6.0) for 20 min prior to immunostaining. Nonspecific staining was blocked by pre-incubation with Tris Buffered Saline (TBS)/0.1% Tween-20/5% normal goat serum (Jackson ImmunoResearch) for 1 hour at room temperature.

Tissue sections were incubated with the primary antibody at 4°C overnight, and were subsequently incubated with secondary antibodies and detected with DAB substrate (Dako EnVision + System HRP, Dako) following manufacturer's instructions. Coverslips were mounted onto glass slides with permanent mounting medium (VectaMount). All images were taken with Olympus Inverted microscope (Cellular imaging core, Boston University).

Immunofluorescence

The PTEN protein was detected using the rabbit anti-PTEN antibody (Cell Signaling) diluted 1/50 and melanocytes were identified using anti-tyrosinase (Dako, Denmark) diluted 1/50. For dual staining of PTEN and tyrosinase, PTEN was detected using the anti-rabbit Alexa-Fluor 488 (Invitrogen) and tyrosinase was detected using the biotinylated donkey anti-mouse (Jackson Laboratory, ME, USA) secondary antibody with streptavidin linked Alexa-Fluor 555 (Invitrogen, Carlsbad, CA, USA). Negative controls were processed in parallel using an identical protocol, but with the omission of the primary antibody. All slides were cover-slipped using Vectashield-DAPI mounting media (Vector laboratories, Burlingame, CA, USA). Three images per sample were captured using the DeltaVision Microscope system (Applied Precision, Issaquah, WA, USA) and each image contained on average a 300 μm length of epidermis, all cells in the epidermis were scored. The DeltaVision tracking software

and stage technology allowed non-overlapping images to be collected along each skin section. Sections were counted using volocity image software (PerkinElmer, Waltham, MA, USA) and cells positively stained for PTEN or tyrosinase were counted only if a visible DAPI staining nuclei was observed. All staining and quantification procedures were performed blind to the samples ID.

Co-immunoprecipitation

Co-immunoprecipitation of MC1R with PTEN was performed as described previously (Cao et al., 2009) with minor modifications. Cells were washed twice with ice-cold phosphate-buffered saline and lysed in lysis buffer containing 50 mM Tris pH 7.4; 1% Triton X-100; 0.5 mM EDTA; 0.5 mM EGTA; 150 mM NaCl; 10% Glycerol; 1 mM phenylmethylsulfonyl fluoride, complete protease inhibitor cocktail (Roche) on ice for 30 min. The supernatant was collected after centrifugation at 15,000 × g for 15 min at 4 °C, and 500 µg of total cell lysate was incubated with 20 µl of either HA-conjugated agarose beads (Sigma) or anti-Flag M2 affinity gel (Sigma) in a total volume of 500 µl lysis buffer for 16 hr with rotation at 4 °C. After three washes with 1 ml of lysis buffer, the bound proteins were released by boiling in 30 µl of SDS loading buffer and detected as described above.

GST-Pull Down Assays

GST-MC1R protein was expressed and purified as described (Fine et al., 2009) with minor modification. Briefly, GST-MC1R was cloned into pGEX-6P-1 empty vector, and then transformed into BL21(DE3)pLysD competent cells (New England Biolabs, MA, USA). Protein expression was induced at OD=0.4 with 0.2 mM IPTG for 4 hr at 37°C. Bacterial cells were then pelleted and sonicated in lysis buffer containing 400 mM NaCl, 50 mM Tris pH 7.2, 1% Triton X-100,1mM EDTA, 1 mM DTT, 50 µM PMSF, 5 mM benzamidine hydrochloride hydrate and 3 µM aprotinin. Lysates were centrifuged at 20,000 × g for 45 minutes at 4ºC and the supernatant was further cleared by passing through a 0.45 micron filter. The filtered supernatant was incubated overnight at 4°C with glutathione affinity matrix. The matrix was washed with buffer containing 50 mM Tris pH 7.6, 50 mM

NaCl and 5 mM $MgCl_2$, and the GST-tagged fusion protein was eluted from the matrix by incubation with 30 mM reduced glutathione.

Recombinant GST-MC1R WT, MC1R variants R151C, R160W and D294H, and His-PTEN (Sigma) interaction was performed as described before (Cao et al., 2009). Briefly, equal molars of GST-MC1R or GST-EV and His-PTEN were incubated in 500 µl reaction buffer containing 20 mM Hepes (pH 8.0), 150 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 50 µM PMSF, 5 mM benzamidine hydrochloride, 3 µM aprotinin and 1% Triton X-100 overnight at 4°C. After the incubation, pre-washed glutathione agarose beads were added and further incubated 4 h at 4°C. The GST beads were washed extensively with reaction buffer and the proteins were eluted with SDS-PAGE sample buffer and analyzed on 10% SDS-PAGE.

PTEN Inositol Phosphatase Activity Measurement

Cells were lysed in phosphatase inhibitors free lysis buffer. PTEN was immunoprecipitated with HA-conjugated agarose beads (Sigma) overnight at 4ºC. The immunoprecipitates were thoroughly washed three times with 1000 µL PTEN reaction buffer (25 mM Tris, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 10 mM DTT). The phosphatase activity was detected with Malachite Green assay kit (K-1500, Echelon Biosciences) following manufacturer's instructions. Briefly, the immunoprecipitated PTEN proteins were incubated with 200 µM $PI(3,4,5)P_3$ in PTEN reaction buffer at 37°C for 20 min, the reactions were then transferred in triplicate to 96-well plate, the enzyme reactions were stopped with Malachite green solution and further incubated at room temperature for 20 min with gentle shaking. Absorbance was measured at 620 nm on microplate reader, and free phosphate released was calculated from a standard curve.

Gel Filtration Chromatography

The gel filtration experiment was performed as described previously (Wan et al., 2011). More specifically, to prepare the cell lysates for gel filtration analysis, cells were washed with phosphate-buffered saline, then lysed in EBC buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40), and filtered through a 0.45 μm syringe filter. 500 μl of lysate (4 mg/ml) was loaded onto a Superdex 200 10/300 GL column (GE Lifesciences, Cat. No.: 17-5175-01). Chromatography was performed using an AKTA FPLC (GE Lifesciences, Cat. No.: 18-1900-26), and protein complexes were resolved by eluting with the EBC buffer at 0.5 ml/min. Eluent was collected in 500 μl fractions. 40 μl aliquots of these fractions were separated by SDS-PAGE and Western blot analysis was performed with the indicated antibodies to probe the fractionated protein complexes. Prior to running cell lysates, the molecular weight resolution of the columns was first estimated by running Gel Filtration Calibration Kit (GE Lifesciences, Cat. No.: 28-4038-42) to detect their retention times on Coomassie-stained SDS-PAGE protein gels.

Chemical Cross-linking

Cells were washed with phosphate-buffered saline (PBS), and lysed in ice-cold PBS buffer containing 0.5% NP-40. 1000 μg of the cell lysates were immunoprecipitated with MC1R antibody in a total volume of 500 μl in PBS supplemented with protease inhibitors overnight at 4ºC, the immunoprecipitated proteins were then eluted and crosslinked with 2 mM DSP (Pierce/Thermo fisher, Catalog #22585) on ice for 2 hr before analysis by SDS-PAGE.

***In Vivo* Tumorigenesis Assay**

In vivo tumorigenesis assay of hTERT/p53DD/CDK4(R24C)/BRAF^{V600E} melanocytes was performed as described previously (Garraway et al., 2005), Briefly, 3×10^6 control or MC1R-depleted hTERT/p53DD/CDK4(R24C)/BRAF^{V600E} melanocytes were mixed with matrigel (1:1) and injected subcutaneously into the flanks of male nude mice. Tumor size was measured every 3 days with a

caliper, and the tumor volume was determined as mentioned above. Three weeks after inoculation, the tumors were dissected to measure their weights. All mice experiments were approved by the Harvard University and Beth Israel Deaconess Medical Center Animal Care and Use Committee, and the experiments were performed according to the relevant regulatory standards.

Detection of PTEN Aberrations in Human Melanomas

PTEN mutations were assayed by PCR amplification and sequencing of the entire *PTEN* coding region and flanking intronic sequences (Nassif et al., 2004). Allelic loss at the *PTEN* locus was determined using whole-genome SNP arrays (Stark and Hayward, 2007).

Statistical Analyses

All quantitative data were presented as the mean ± SEM of at least three independent experiments by Student's *t* test for between group differences and analysis of variance for comparisons among three or more groups. The association between MC1R variants and PTEN dysfunction was explored using Pearson's square Chi test. The $\boldsymbol{p} < 0.05$ was considered as statistically significant.

Fig. S1, related to Figure 1

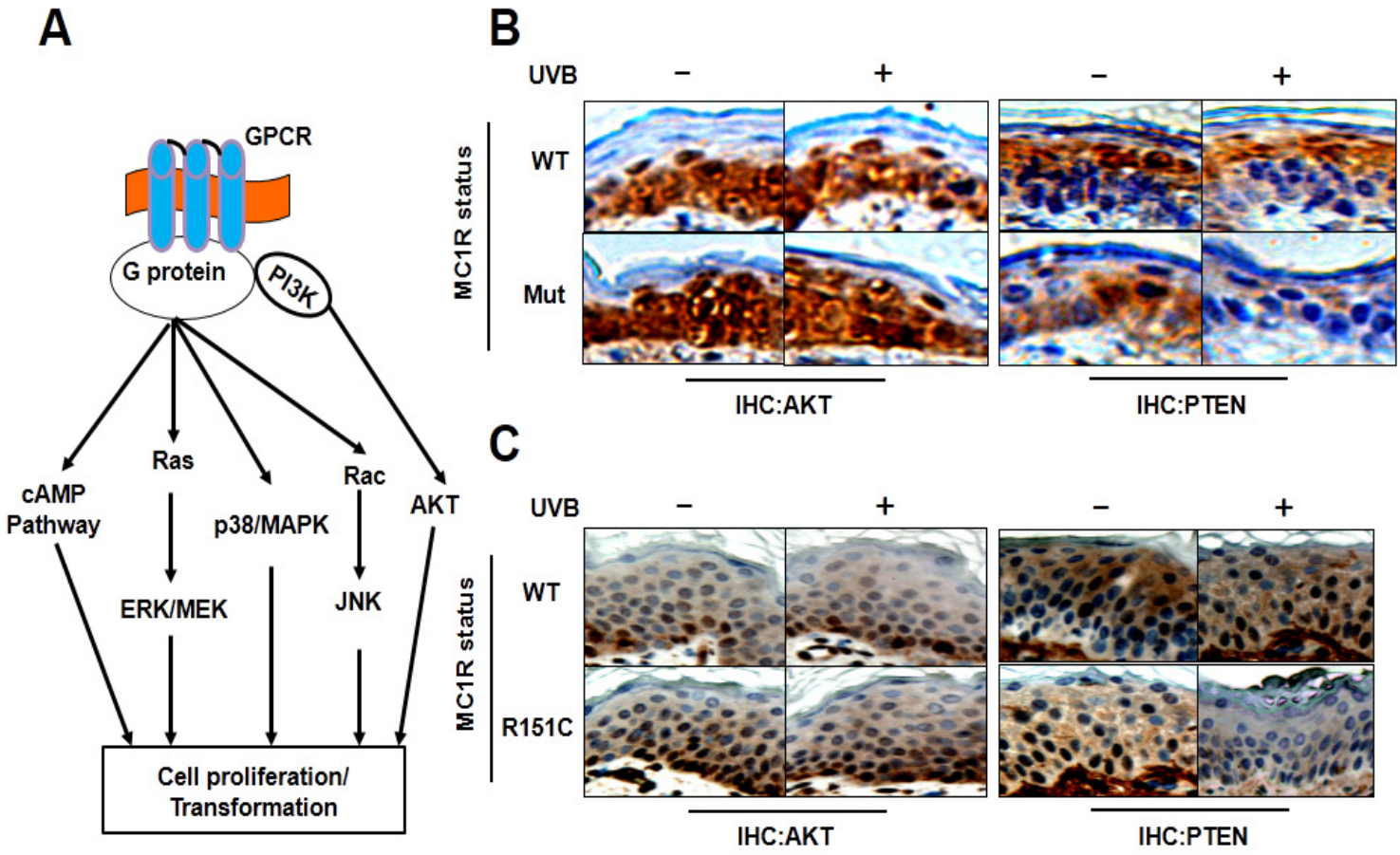

Figure S1. Loss-of-Function MC1R Variant Augments UV-Induced AKT Phosphorylation and PTEN Phosphorylation In Vivo, Related to Figure 1

A. Schematic illustration of the G protein-coupled receptor signaling pathways.

B. Immunohistochemistry (IHC) staining of total PTEN and total AKT in mouse skin with or without UVB irradiation. Albino mice with wild-type (WT) MC1R or loss-of-function MC1R frameshift mutation (Mut) were irradiated with 100 J/m^2 UVB and subjected to IHC at 3 hr after UVB irradiation.

C. Immunohistochemical staining (IHC) of total PTEN and total AKT in human foreskins before and after UVB irradiation. Human foreskins collected from Caucasian with wild-type MC1R (WT) or MC1R (R151C) were exposed to 100 J/m^2 UVB and subjected to IHC 3 hr post UVB irradiation.

Fig. S2, related to Figure 2

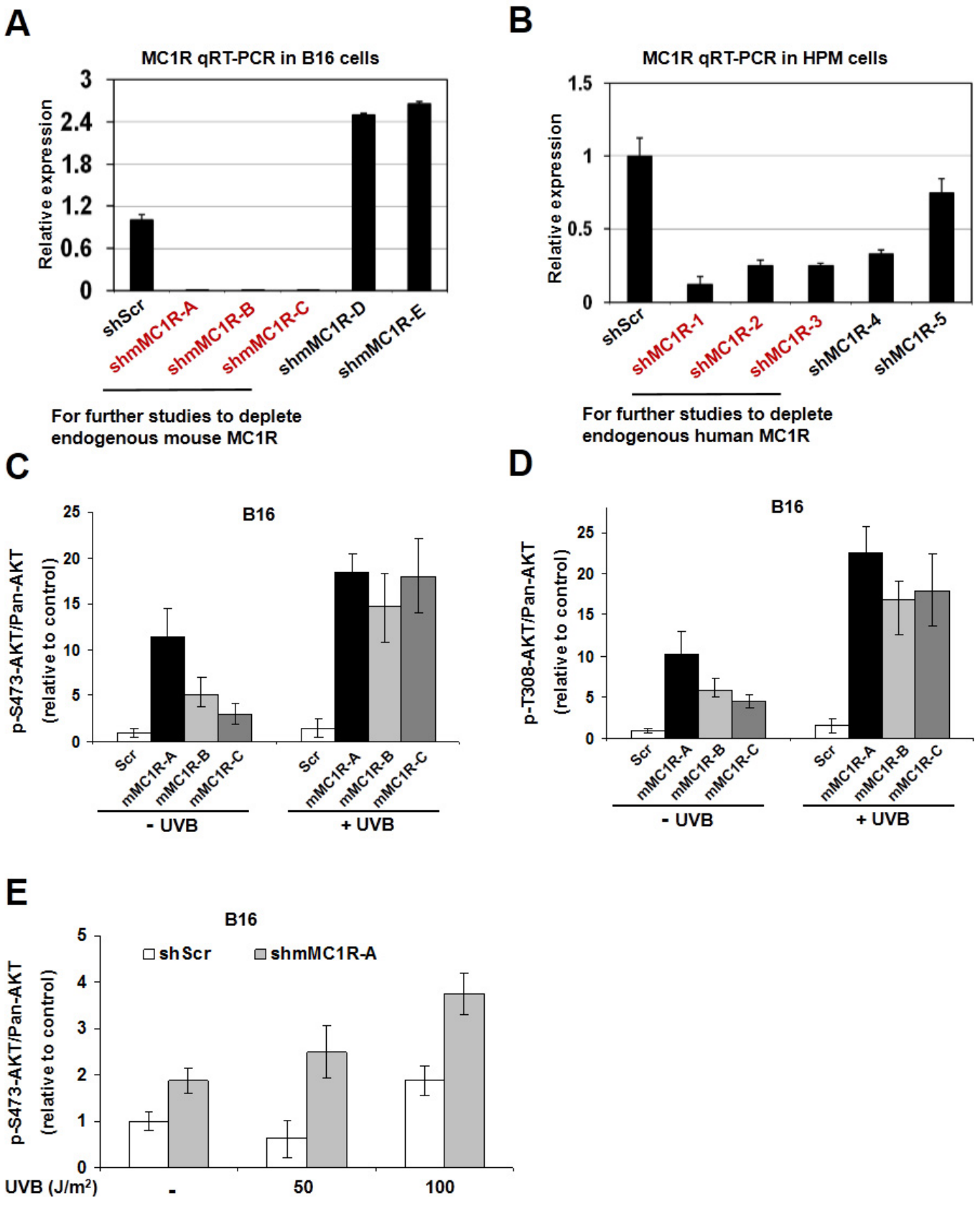

Figure S2. Depletion of MC1R Leads to Elevated AKT Phosphorylation and PTEN Inactivation in Response to UVB Exposure, **Related to Figure 2**

A-B. Quantitative real time RT-PCR analysis was performed to demonstrate the efficient depletion of MC1R mRNA in B16 cells (**A**) and in human primary melanocytes (HPMs) (**B**). Five independent shMC1R lentiviral constructs specific to the mouse or human MC1R gene were used for this analysis. Data are represented as mean ± SEM, n=3.

C-D. Quantification of the pSer473-AKT (**C**) or pThr308 (**D**) band intensities in **Figure 2A** pSer473 or pThr308-AKT bands were normalized to total AKT, then normalized to shScr control without UVB treatment. Data are represented as mean ± SEM, n=3.

E. Quantification of the pSer473-AKT band intensities in **Figure 2C**. pSer473-AKT bands were normalized to total AKT, then normalized to shScr control without UVB treatment. Data are represented as mean ± SEM, n=3.

Fig. S3, related to Figure 3

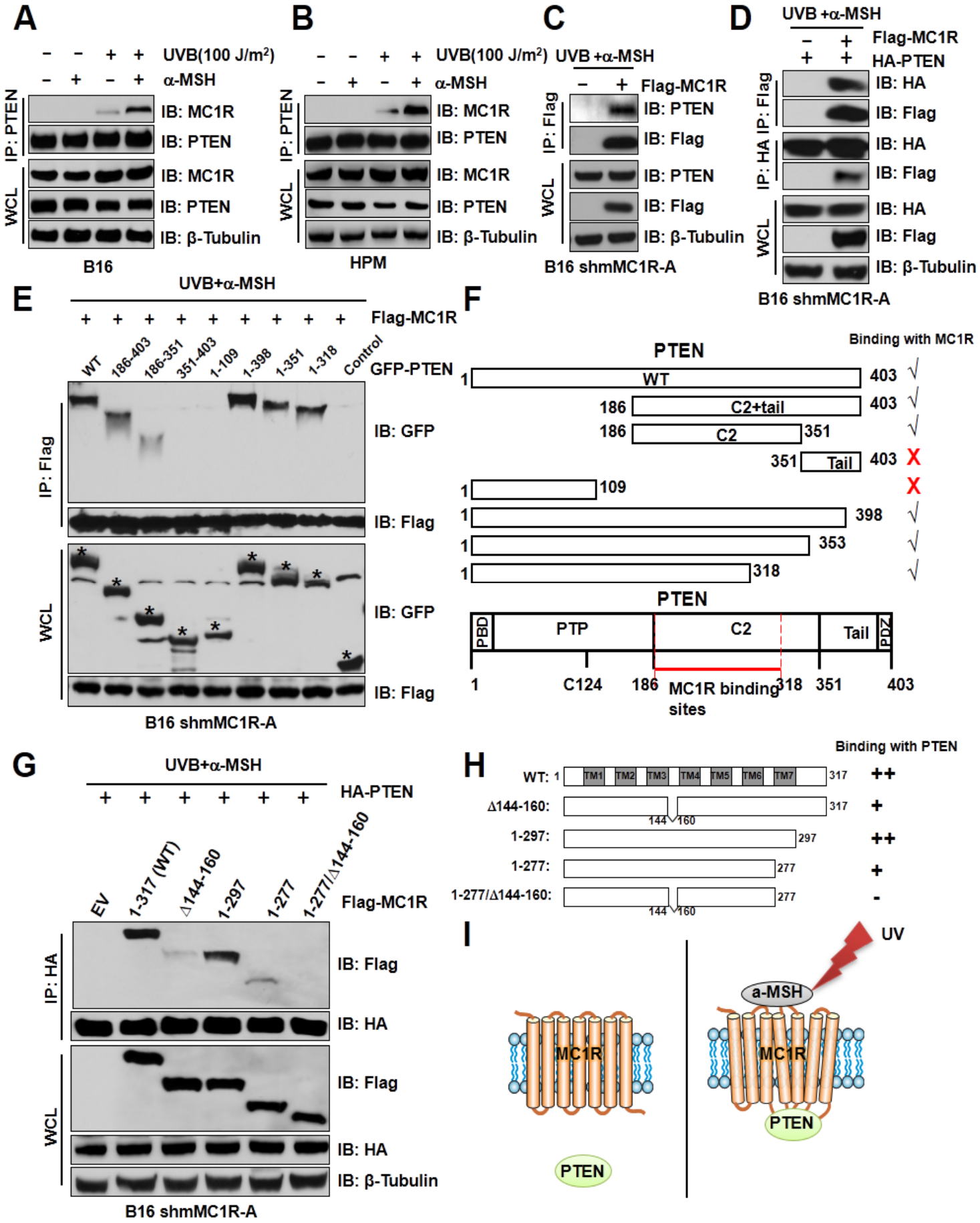

Figure S3. UVB Irradiation Induces MC1R Interaction with PTEN in Melanocytes, Related to Figure 3

A-B. α-MSH is required for MC1R/PTEN interaction upon UVB exposure. B16 cells (**A**) and human primary melanocytes (HPMs) (**B**) were treated with 1 μM α-MSH for 30 min followed by irradiation with 100 J/m^2 UVB before harvesting for immunoprecipitation (IP) and immunoblot (IB) analysis. The 26S proteasomal inhibitor MG132 (25 μM) was used to inhibit protein degradation.

C-D. B16 cells were transfected with the indicated plasmids. 48 hr later, the cells were treated with 1 μM α-MSH for 30 min followed by irradiation with 100 J/m^2 UVB before harvesting for immunoprecipitation (IP) and immunoblot (IB) analysis. The 26S proteasomal inhibitor MG132 (25 μM) was used to inhibit protein degradation.

E-F. (**E**) B16 cells were transfected with various GFP-PTEN truncation constructs together with Flag-WT-MC1R.After 48 hr, the cells were treated with 1 μM α-MSH for 30 min followed by irradiation with 100 J/m^2 UVB before harvesting for immunoprecipitation (IP) and immunoblot (IB) analysis. The 26S proteasomal inhibitor MG132 (25 μM) was used to inhibit protein degradation. (**F**) Schematic illustration of the interaction of various GFP-tagged PTEN fragments with MC1R.

G-H. (**G**) B16 cells were transfected with various Flag-MC1R constructs together with HA-PTEN. After 48 hr, the cells were treated with 1 μM α-MSH for 30 min followed by irradiation with 100 J/m^2 UVB before harvesting for immunoprecipitation (IP) and immunoblot (IB) analysis. The 26S proteasomal inhibitor MG132 (25 μM) was used to inhibit protein degradation. (**H**) Schematic illustration of the interaction of various MC1R fragments with PTEN.

I. Schematic diagram of the proposed mechanism through which α-MSH binds to MC1R and subsequently induces MC1R conformational changes to facilitate the MC1R-PTEN interaction upon UVB exposure.

Fig. S4, related to Figure 4

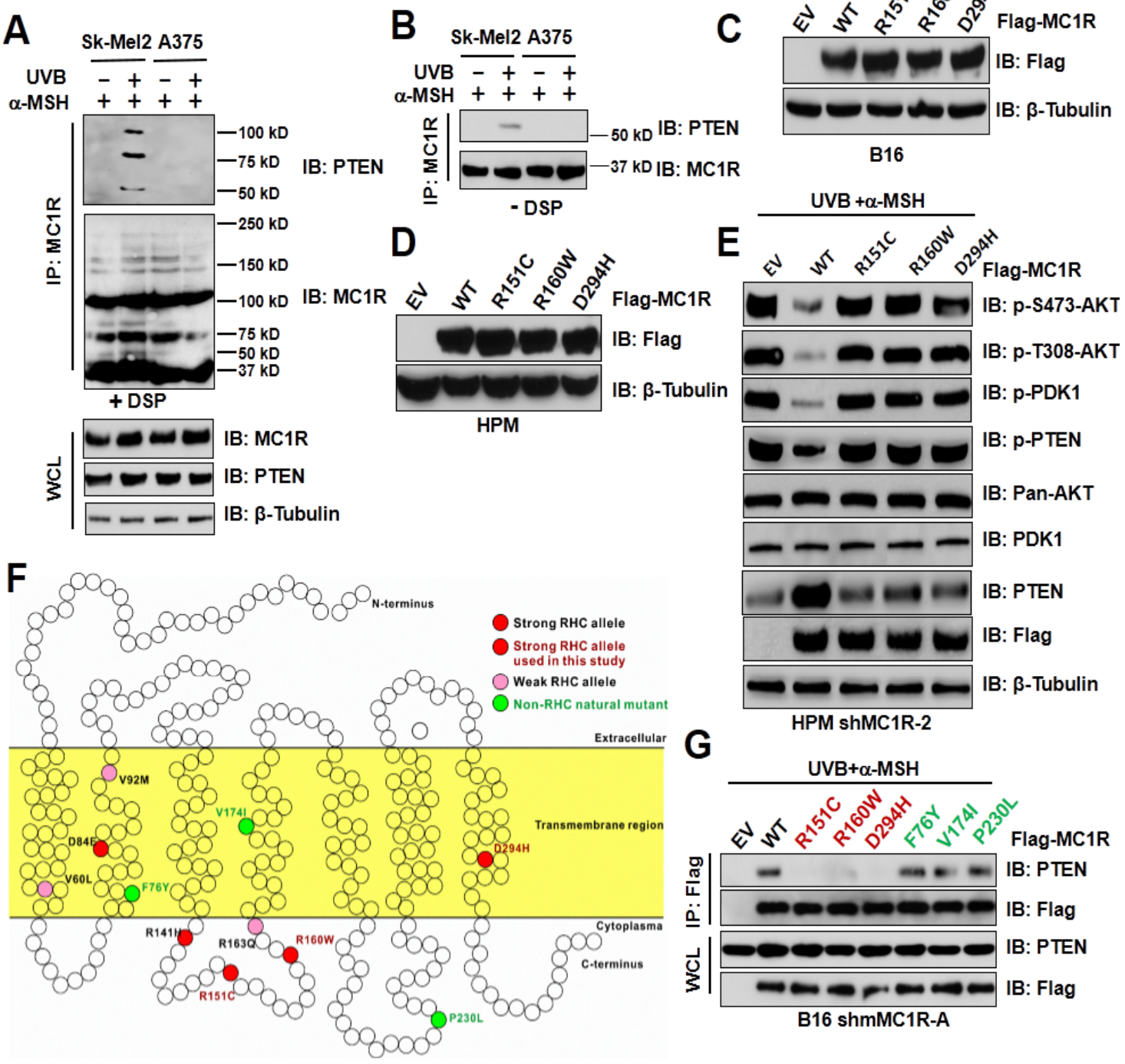

Figure S4. MC1R Variants are Defective in Interacting with PTEN, Related to Figure 4

A-B. IP and IB analysis of the WCL derived from SK-Mel2 (WT-MC1R) or A375 (R151C-MC1R) cells with or without UVB exposure (100 J/m^2). Cells were pretreated with the MG132 (25 μM) for 3 hr and with 1 μM α-MSH for 30 min before UV exposure and harvested at 3 hr after UVB exposure. Immunoprecipitated MC1R with its associated proteins were further crosslinked with DSP (**A**) or untreated as a control (**B**) before analysis by SDS-PAGE.

C-D. B16 cells (**C**) or human primary melanocytes (HPMs) (**D**) were infected with retro-viral constructs encoding Flag-MC1R WT or variants (R151C, R160W or D294H). The expression of MC1R was detected by IB analysis.

E. IP and IB analysis of the whole cell lysates derived from shMC1R-HPM cells infected with the indicated Flag-MC1R retro-viral constructs. Cells were treated with 1 μM α-MSH for 30 min before UVB exposure (100 J/m^2).

F. Membrane topology of MC1R with indicated RHC-associated or non-RHC mutants marked as red and green, respectively. Yellow color is used to indicate the plasma membrane region.

G. IP and IB analysis of WCL derived from shMC1R-B16 cells transfected with the indicated Flag-MC1R plasmids. Cells were pretreated with the MG132 (25 μM) for 3 hr and with 1 μM α-MSH for 30 min before UVB exposure (100 J/m^2).

Fig. S5, related to Figure 5

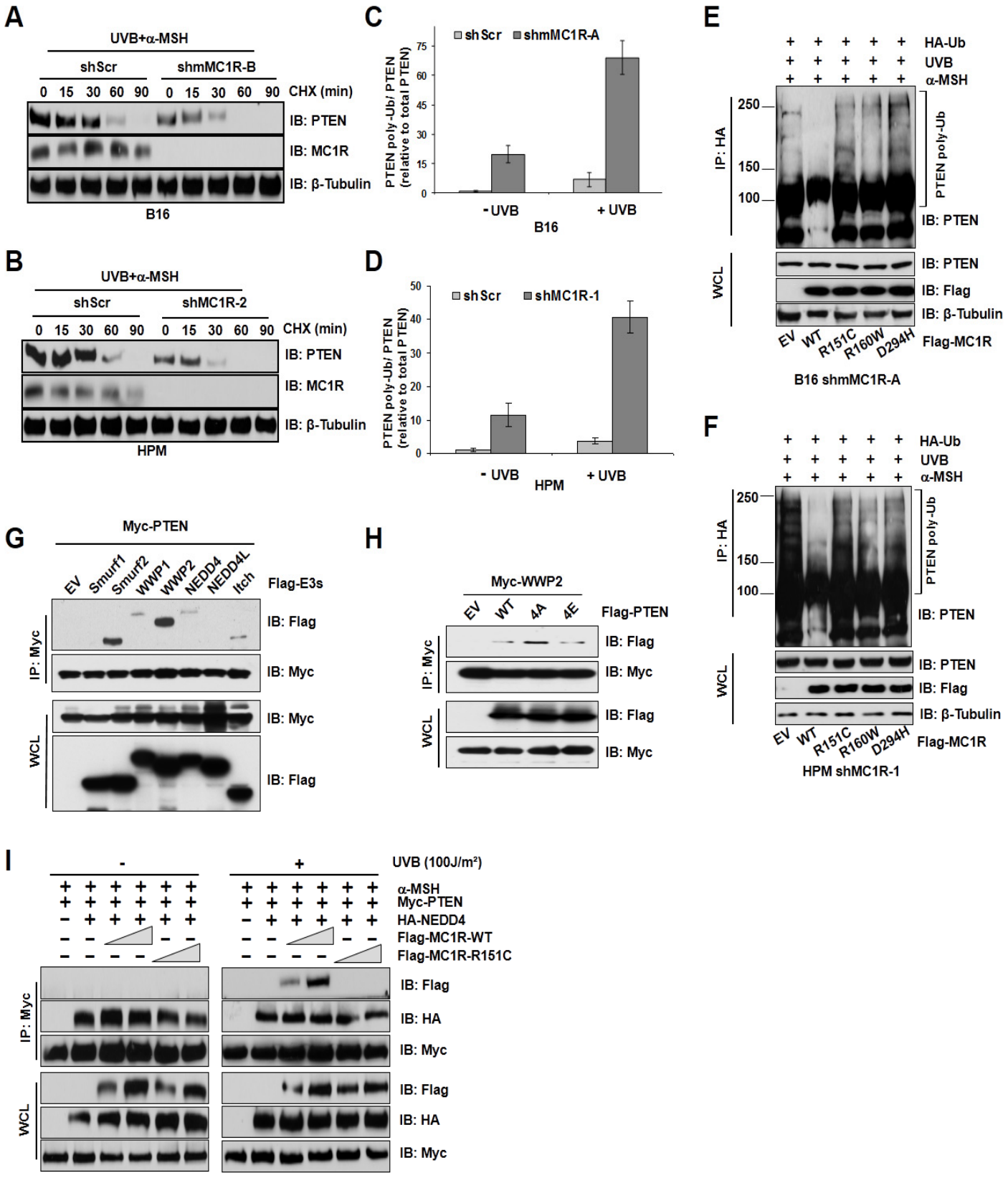

Figure S5. PTEN Binds to a Subset of NEDD4 Family Ubiquitin E3 Ligases, Related to Figure 5

A-B. B16 cells (**A**) or human primary melanocytes (HPMs) (**B**) were infected with shMC1R (with shScr as a negative control) to deplete endogenous MC1R. The resulting cells were pretreated with 1 μM α-MSH for 30 min followed by treatment with 50 μg/ml cycloheximide (CHX) before UVB irradiation (100J/m^2), and then were cultured in media containing CHX. The cells were harvested at different time points for immunoblot (IB) analysis.

C-D. Quantification of the ubiquitinated PTEN band intensities in **Figure 5G** and **5H,** respectively. Intensities of ubiquitinated PTEN lanes were normalized to total PTEN, then normalized to shScr control without UVB treatment. Data are represented as mean ± SEM, n=3.

E-F. B16 cells (**E**) or HPMs (**F**) were infected with the shMC1R lentiviral construct (with shScr as a negative control) to deplete endogenous MC1R. The resulting cells were transfected with HA-ubiquitin and the indicated Flag-MC1R constructs, 40 hr post-transfection, cells were pretreated with 25μM MG132 for 6 hr and 1 μM α-MSH for 30 min prior to UVB irradiation (100J/m^2). The cells were harvested 3 hr after UVR and then immunoprecipitated with HA-conjugated agarose beads. Ubiquitin bound PTEN was analyzed by SDS-PAGE and IB analysis using anti-PTEN antibody.

G. Immunoprecipitation (IP) and immunoblot (IB) analysis of the whole cell lysates derived from 293T cells transfected with the Myc-PTEN plasmid and the indicated Flag-NEDD4 family E3 ligase plasmids. Cells were treated with 10 μM MG132 for 12 hr before harvesting for the assays.

H. Immunoprecipitation (IP) and immunoblot (IB) analysis of the whole cell lysates derived from 293T cells transfected with Myc-WWP2 and the indicated Flag-PTEN plasmids. Cells were treated with 10 μM MG132 for 12 hr before harvesting for the assays.

I. Immunoprecipitation (IP) and immunoblot (IB) analysis of the whole cell lysates derived from B16 cells transfected with Myc-PTEN, HA-NEDD4 and the indicated Flag-MC1R plasmids. Cells were pre-incubated with 1 μM α-MSH for 30 min before UVB exposure (100 J/m^2) and harvested at 3 hr after UVB exposure.

Fig. S6, related to Figure 6

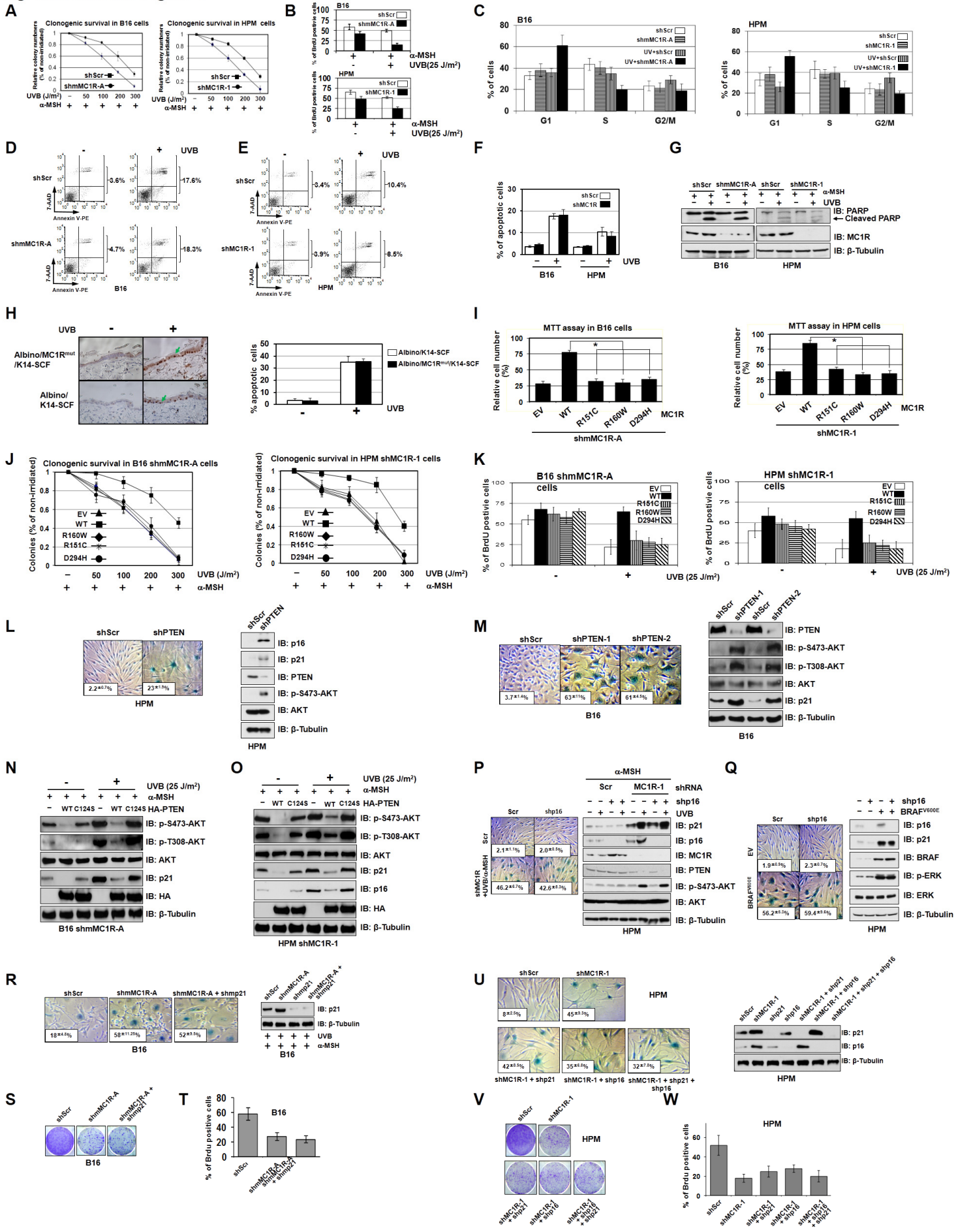

Figure S6. MC1R Protects Melanocytes from UVB-Induced Growth Inhibition, Related to Figure 6

A-C. B16 cells (**A**) or human primary melanocytes (HPMs) (**B**) were infected with shMC1R (with shScr as a negative control) to deplete endogenous MC1R. The resulting cells were treated with 1μM α-MSH for 30 min, and were then irradiated with different doses of UVB as indicated. The cells were seeded at equal density for clonogenic survival assay for 10 days after UVR before staining with crystal violet for formed colonies (**A**); plated for BrdU incorporation assay (**B**); or analyzed for cell-cycle distribution by flow cytometry (**C**). Data are represented as mean ± SEM, n=3.

D-F. Annexin V/7-AAD double staining analysis to detect apoptotic cell population of control and MC1R-depleted B16 cells (**D**) or human primary melanocytes (HPMs) (**E**). Specifically, cells were treated with 1 μM α-MSH for 30 min, and were then irradiated with 100 J/m^2 UVB before being subjected to FACS analysis 3 days after UVR. The results shown in **F** were obtained from three independent experiments and the data are represented as mean ± SEM.

G. The cells generated from **D-E** were harvested and the lysates were subjected to immunoblot (IB) analysis.

H. The MC1R variant minimally affects UVB-induced apoptosis in epidermis *in vivo*. Black skin sections were obtained from albino/MC1R^{mut}/K14-SCF (n=4) and albino/K14-SCF mice (n=4) at baseline and 12 hr after UVB (500 J/m^2) irradiation and were subjected to TUNEL-peroxidase assay, followed by counterstaining with hematoxylin. At least 2 skin sections from each time point were analyzed from every mouse. Green arrows indicate the positive cells. Results were quantified by counting TUNEL-positive cells / total cells in epidermis. Data are represented as mean ± SEM (*p< 0.05), n=3.

I-J. MC1R-depleted B16 cells or human primary melanocytes (HPMs) were infected with Flag-MC1R WT and variants encoding retro-viruses as indicated. The resulting cells were treated with 1 μM α-MSH for 30 min followed by irradiation with or without UVB as indicated. (**I**) The cell viability at 24 hr after UVB exposure (100 J/m^2) was determined by MTT assays in B16 cells and in HPM cells. **(J)** B16 cells and HPM cells were seeded at equal densities for clonogenic survival assays before staining with crystal violet 10 days after UVR. Data are represented as mean ± SEM n=3.

K. MC1R-depleted B16 cells or human primary melanocytes (HPMs) were infected with Flag-MC1R WT and variants encoding retro-viruses as indicated. The resulting cells were treated with 1 μM α-MSH for 30 min followed by irradiation with or without UVB (25 J/m^2). The cells were analyzed for BrdU incorporation 3 days after UVB exposure. Data are represented as mean ± SEM, n=3.

L-M. PTEN depletion induces premature senescence in melanocytes. Human primary melanocytes (HPMs) or B16 cells were infected with the shPTEN lentiviral vector (with shScr as a negative control). The resulting cells were analyzed for SA-β-gal activity 10 days after selection (**L**); or harvested for immunoblot (IB) analysis (**M**). One representative image out of three independent experiments was shown. Data are represented as mean ± SEM, n=3.

N-O. MC1R-depleted B16 cells (**N**) or human primary melanocytes (HPMs) (**O**) were infected with retro-viruses encoding HA-PTEN WT and C124S. The resulting cells were then treated with 1 μM α-MSH for 30 min followed by irradiation with or without UVB (25 J/m^2) before harvesting for immunoblot (IB) analysis.

P. Normal or MC1R-depleted human primary melanocytes (HPMs) were further infected with the shp16 lentiviral construct (or shScr as a negative control) to deplete endogenous p16 (either in WT-MC1R or MC1R-depleted background as indicated). The resulting cells were treated with 1 μM α-MSH for 30 min and were then irradiated with or without UVB (25 J/m^2). Cells were analyzed for SA-β-gal activity 10 days after UVB exposure and subjected to immunoblot (IB) analysis. One representative image out of three independent experiments was shown. Data are represented as mean ± SEM, n=3.

Q. Human primary melanocytes (HPMs) control cells or cells stably expressing $BRAF^{V600E}$ were infected with the shp16 lentiviral vector (or shScr as a negative control) to deplete endogenous p16. The resulting cells were analyzed for SA-β-gal activity 10 days after selection and subjected to immunoblot (IB) analysis. One representative image out of three independent experiments was shown. Data are represented as mean ± SEM, n=3.

R-T. Normal or MC1R-depleted B16 cells were further infected with the shp21 lentiviral construct (or shScr as a negative control) to deplete endogenous p21. The resulting cells were treated with 1 μM α-MSH for 30 min and were then irradiated with or without UVB (25 J/m^2). Cells were analyzed for SA-β-gal activity 10 days after UVB exposure and subjected to immunoblot (IB) analysis in **R**..One representative image out of three independent experiments was shown in **R**. Data are represented as mean ± SEM, n=3. Additionally, these cells were used for clonogenic survival assays before staining with crystal violet 10 days after UVR in **S**, or for BrdU incorporation 3 days after UVB exposure in **T**. Data are represented as mean ± SEM, n=3 in **T**.

U-W. Normal or MC1R-depleted HPM cells were further infected with the shp16 or/and shp21 lentiviral construct (or with shScr as a negative control) to deplete endogenous p16 or/and p21. The resulting cells were treated with 1 μM α-MSH for 30 min and were then irradiated with or without UVB (25 J/m^2). Cells were analyzed for SA-β-gal activity 10 days after UVB exposure and subjected to immunoblot (IB) analysis in **U**. One representative image out of three independent experiments was shown in **U**. Data are represented as mean ± SEM, n=3. Additionally, these cells were used for clonogenic survival assays before staining with crystal violet 10 days after UVR in **V**, or for BrdU incorporation 3 days after UVB exposure in **W**. Data are represented as mean ± SEM, n=3 in **W**.

Fig. S7, related to Figure 7

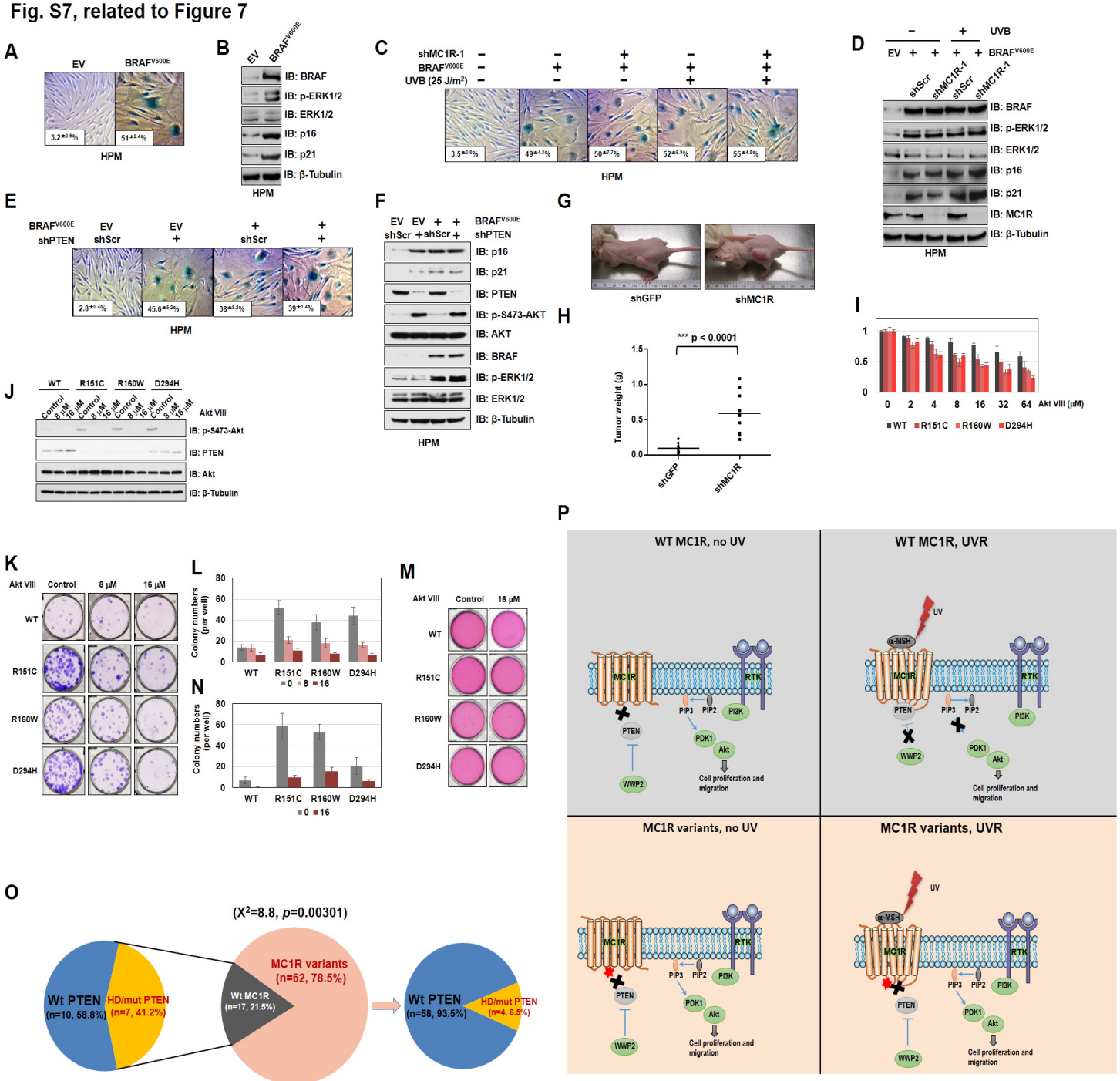

Figure S7. MC1R Deficiency Cooperates with $BRAF^{V600E}$ to Induce Melanomagenesis, Related to Figure 7

A-B. Human primary melanocytes (HPMs) were infected with the EV control or $BRAF^{V600E}$ encoding retroviruses. The resulting cells were analyzed for SA-β-gal activity (**A**) and subjected to immunoblot (IB) analysis (**B**) 10 days after selection in 50 μg/ml Zeocin. One representative image out of three independent experiments was shown in **A**. Data are represented as mean ± SEM, n=3.

C-D. Human primary melanocytes (HPMs) stably expressing the EV control or $BRAF^{V600E}$ were infected with the shMC1R lentiviral construct (with shScr as a negative control) to deplete endogenous MC1R. The resulting cells were treated with 1 μM α-MSH for 30 min followed by irradiation with or without UVB (25 J/m^2). Cells were analyzed for SA-β-gal activity 10 days after UVB exposure (**C**) and subjected to immunoblot (IB) analysis (**D**). One representative image out of three independent experiments was shown in **C**. Data are represented as mean ± SEM, n=3.

E-F. Human primary melanocytes (HPMs) stably expressing EV control or $BRAF^{V600E}$ were infected with the shPTEN lentiviral construct (with shScr as a negative control) to deplete endogenous PTEN. Cells were analyzed for SA-β-gal activity 10 days after selection (**E**), and subjected to immunoblot (IB) analysis (**F**). One representative image out of three independent experiments was shown in **E**. Data are represented as mean ± SEM, n=3.

G. Representative pictures of formed xenografts in **Figure 7J**. Images were taken 3 weeks after injection.

H. Mass of dissected tumors (**Figure 7K**) was measured and presented to illustrate that depletion of MC1R promoted the *in vivo* tumorigenesis of hTERT/p53DD/CDK4(R24C)/$BRAF^{V600E}$ melanocytes. *p* value was calculated with the Student's *t* test. Data are represented as mean ± SEM, n=10.

I-N. Cell viability assay (**I**), IB analysis (**J**), colony formation assay (**K-L**) and soft agar assay (**M-N**) using cells generated in **Figure 7L** untreated or treated with indicated concentrations of AKT inhibitor. Error bars represent mean ± SEM, n=3 in **I**, **L** and **N**.

O. The correlation between PTEN and MC1R status in 39 melanoma cell lines derived from patients ($p < 0.05$ by Pearson's Chi-squared test).

P. Schematic diagram to illustrate that compared with humans with WT-MC1R, humans with red hair and fair skin that harbor RHC type of MC1R variants, could not protect PTEN from UVR-induced ubiquitination and destruction, thereby leading to elevated AKT signaling. Our work further showed that elevated AKT signaling in humans with RHC type of MC1R variants synergizes with the $BRAF^{V600E}$ oncogenic mutation to promote melanomagenesis. This MC1R-mediated regulation of the PTEN/AKT signaling axis provides a molecular basis for the observation that red-haired individuals are much more prone to developing nevi and melanoma.

Table S1. The *MC1R* Genotypes of Human Skin Samples Used in Figure 1, Related to Figure 1

subject ID	slide label	MC1R Genotype (r=1,rr=11,R=R,W=wildtype)	MC1R Genotype codon change		
UV-003	PTEN4	R1	R151C-/+	V92M-/+	
UV-015	PTEN10	R1	R151C-/+	R163Q-/+	
UV-020	PTEN16	R1	V60L-/+	R151C-/+	
UV-040	PTEN34	R1	R160W-/+	V92M-/+	
UV-064	PTEN60	R1	D84E-/+	V60L-/+	
UV-045	PTEN43	R11	V60L-/+	R151C-/+	R163Q-/+
UV-057	PTEN52	R11	V60L-/+	R151C-/+	V92M-/+
UV-033	PTEN25	RR	R151C-/+	R160W-/+	
UV-048	PTEN46	RR	R151C-/+	R160W-/+	
UV-036	PTEN28	RR1	R151C+/-	R160W-/+	R163Q-/+
PUV-00333	PTEN1	W			
UV-013	PTEN7	W			
UV-016	PTEN13	W			
UV-028	PTEN19	W			
UV-030	PTEN22	W			
UV-037	PTEN31	W			
UV-043	PTEN37	W			
UV-044	PTEN40	W			
UV-049	PTEN49	W			
UV-059	PTEN57	W			

Table S2. PTEN Expression In Vivo 24 Hr and 14 Days following ssUVR Exposure, Related to Figure 1

% PTEN Staining		Non-UVR	ssUVR 24 hr	ssUVR 14 days	Δ ssUVR 24 hr and Non-UVR	Δ ssUVR 14 days and Non-UVR
Epidermal Melanocytes n=20	Mean	90.0	72.7	80.5	-17.3	9.5
	SD	10.9	22.2	10.6		
	P value				<0.01	<0.005

Table S3. The Association between *MC1R* and PTEN Expression in Melanocytes 24 Hr and 14 Days following ssUVR Exposure, Related to Figure 1

Genotype	N	Baseline Mean	ssUVR / 24 hr	*p*-value*
Variants	10	90.7 (11.9)	68.3 (19.6)	0.008
WT	10	89.2 (10.2)	77.1 (24.7)	0.18
Genotype	**N**	**Baseline Mean**	**ssUVR / 14 day**	***p*-value***
Variants	10	90.7 (11.9)	78.5 (11.6)	0.03
WT	10	89.2 (10.2)	82.6 (9.7)	0.17

( ) = std dev.

**p* value = paired t-test change between ssUVR and Non-UVR

Baseline mean represents average percent of melanocytes expressing PTEN at the Non-UVR site prior to any treatment.

Table S4. The Calculated p Values for the Detected PTEN Activity Changes after UVB Exposure, Related to Figure 4

	B16 (related to Figure 4I)				HPM (related to Figure 4J)			
UVB	WT	R151C	R160W	D294H	WT	R151C	R160W	D294H
0 vs 5 min	N.S.	N.S.	0.045	0.048	N.S.	N.S.	N.S.	N.S.
0 vs 3 h	N.S.	0.012	0.018	0.0095	N.S.	0.0055	0.0042	0.0038

p value = paired t-test change between the determined PTEN activity of untreated and treated cells for 5 min or 3 h.
N.S. = not significant

Table S5. Mutation Status of PTEN, MC1R, and Selected Other Genes in Melanoma Cell Lines, Related to Figure 7

Cell Lines	MC1R	PTEN	NRAS	BRAF	p16	p14ARF	TP53
A02-JLO	R151C+/-, 163Q+/-	wt	wt	V600E	wt	wt	Q192Stop
A06-MLC	wt		wt	V600E	HD	HD	wt
A07-RJM	wt	wt	G12S	wt	E2K	wt	Q104Stop
A11-IC	V60L+/-	wt	wt	wt	HD	(HD)	wt
A15-BEP	V60L-/-	wt	wt	V600K	WT(methylated)	wt	wt
D01-JEB	R160W+/-, 163Q+/-	wt	Q61K	wt	HD	HD	wt
D04	R163Q+/-	wt	Q61L	wt	HD	HD	wt
D05	R151C +/-	wt	wt	V600E	8bp del, FS codon11	wt	wt
D08	R160W-/-	wt	Q61K	wt	WT(methylated)	wt	wt
D10	R160W-/-	wt	wt	wt	wt	wt	R248Q
D11	D294H-/-	wt	Q61L	wt	P114L	wt	wt
D14	wt	HD	wt	V600E	P114L	wt	G266E
D17	R151C +/-, R160W+/-	wt	wt	V600E	WT(methylated)	wt	wt
D20	wt	HD	wt	V600E	wt	wt	R248Q
D22	D294H-/-	wt	wt	wt	wt	wt	E287K
D24	F134F-/-	wt	wt	wt	HD	HD	wt
D25	V60L-/-	wt	wt	V600E	wt	wt	D281N
D28	T95M+/-	wt	wt	V600K	P114L	wt	wt
D29	V60L+/-, D294H+/-	wt	wt	V600E	HD	HD	wt
D32	R160W-/-	Del 1 bp at codon 101	wt	I462I ,V600E	HD	HD	wt
D35	D294H+/-	wt	wt	wt	IVS1-1G<A	wt	wt
D36	V60L-/-	HD	wt	V600E	R80Stop	P94L	n/a
D38	R160W+/-	wt	wt	wt	wt (methylated)	wt	wt
D40	R151C +/-	wt	wt	V600E	HD	HD	wt
D41	R160W+/-	wt	wt	V600E	wt	wt	R213Stop
JA	R160W +/-, S315S +/-	wt	wt	V600E	wt	wt	S127F
MM383	wt	wt	wt	V600E	WT (methylated)	wt	wt
MM485	R151C +/-, R160W +/-	wt	Q61R	wt	W110Stop	G125R	R196Stop
MM488	R160W+/-, R163Q+/-	HD	wt	V600E	HD	HD	wt
MM537	R151C-/-	wt	wt	V600E	HD	HD	wt
MM540	V60L +/-, R151C +/-	wt	wt	V600E	WT(methylated)	wt	S366P
MM548	I155T+/-	wt	wt	V600E	R99_Del3bp	PG113-114R	wt
MM576	wt	wt	wt	V600E	wt	wt	L350P
MM595	D84E +/-	wt	wt	V600E	HD	HD	wt
MM608	wt	wt	wt	V600E	HD	HD	wt
MM622	R163Q-/-	L139Stop	wt	V600E	InsT_FrameS to ARF	Ins. FS at codon 81	wt
MM628	V60L+/-,R306H +/-	wt	G13R	wt	P114L	wt	wt
MM647	V60L+/-, R151C+/-	wt	wt	wt	Aberrant transcript	wt	wt
MM648	R160W +/-	wt	wt	V600E	HD	HD	wt

Cell Lines	MC1R	PTEN	NRAS	BRAF	p16	p14ARF	TP53
C001	R151C +/-	wt	Q61K	wt	wt	wt	E258_S261del (EDSS), 771_782delGGAAGACTCCAG
C002	V60L -/-	wt	Q61K	wt	HD	HD	wt
C006	wt	wt	Q61L	wt	wt	wt	wt
C011	R160W -/-	wt	wt	V600E	wt	wt	wt
C012	R151C -/-	wt	wt	V600E	n/a	n/a	n/a
C013	R151C +/-, D294H +/-	wt	Q61L	wt	wt	wt	wt
C017	R160W +/-	wt	wt	V600E	wt	wt	wt
C021	V92M +/-, I155T +/-, T314T -/-	wt	wt	wt	wt	wt	N247K, R248W- 741CC>AT
C022	R160W -/-, D294H +/-	wt	wt	wt	wt	wt	n/a
C025	V60L -/-, P230L -/-	wt	wt	wt	n/a	n/a	n/a
C027	V60L +/-	wt	Q61K	wt	wt	wt	wt
C028	wt	HD	wt	V600E	HD	HD	n/a
C037	wt	wt	wt	wt	wt	wt	wt
C042	V60L -/-	HD	wt	V600E	HD	HD	wt
C044	R151C -/-	HD	wt	V600E	wt	wt	n/a
C045	R163Q +/-, D294H +/-	wt	wt	V600E	wt	wt	wt
C052	V92M+/-, T314T +/-	wt	wt	wt	wt	wt	wt
C054	V92M -/-, T314T -/-	wt	Q61K	wt	HD	wt	wt
C057	wt	HD	wt	V600E	wt	wt	wt
C058	V92M -/-, T314T -/-	wt	wt	L597S	HD	HD	wt
C060	V60L +/-, I264I +/-	wt	wt	V600E	wt	wt	wt
C062	wt	HD	wt	V600E	HD	HD	wt
C065	V92M +/-, R160W -/-, T314T +/-	wt	wt	V600E	wt	wt	N247K, 741CC>AA
C067-L	wt	wt	wt	wt	wt	wt	wt
C071	V60L +/-, D84E +/-	HD	wt	V600E	HD	HD	K120X, 357_358insT
C074	wt	HD	wt	V600E	n/a	n/a	n/a
C077	V60L +/-, R151C +/-	wt	wt	wt	wt	wt	Q100X, 297CC>TT
C078	V60L -/-	wt	wt	V600E	LOH	LOH	wt
C081	wt	wt	wt	V600K	HD	HD	n/a
C083	wt	wt	Q61L	wt	wt	wt	n/a
C084	V92M +/-, R151C -/-, T314T+/-	wt	wt	wt	wt	wt	wt
C086	D294H +/-	wt	wt	wt	wt	wt	E286X, G856T
C088	V60L+/-	wt	wt	V600K	wt	wt	wt
C089	V92M +/-, T314T +/-	wt	wt	V600E	HD	HD	wt
C094	V38M +/-, R163Q +/-	wt	wt	V600E	n/a	n/a	n/a
C096	D294H -/-	wt	Q61R	wt	HD	wt	wt
C097	R151C -/-, D294H +/-	wt	wt	V600E	HD	HD	wt
C100	R163Q +/-	wt	wt	G469R	wt	wt	R342X, C1025T
C106	V92M -/-, T314T -/-	wt	Q61L	wt	wt	wt	wt
C108	wt	wt	wt	V600K	wt	wt	wt

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