Activation of Nrf2 reduces UVA-mediated MMP-1 upregulation via MAPK/AP-1 signaling cascades: the photoprotective effects of sulforaphane and hispidulin

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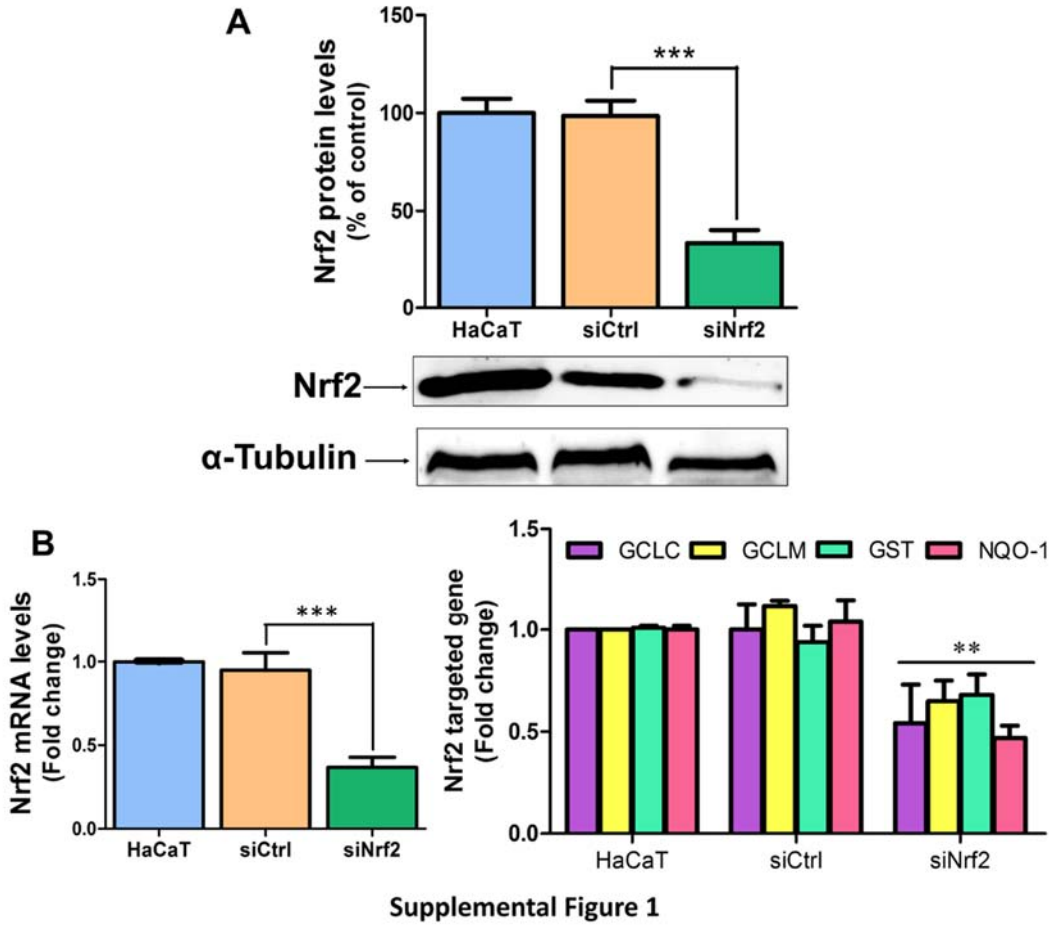
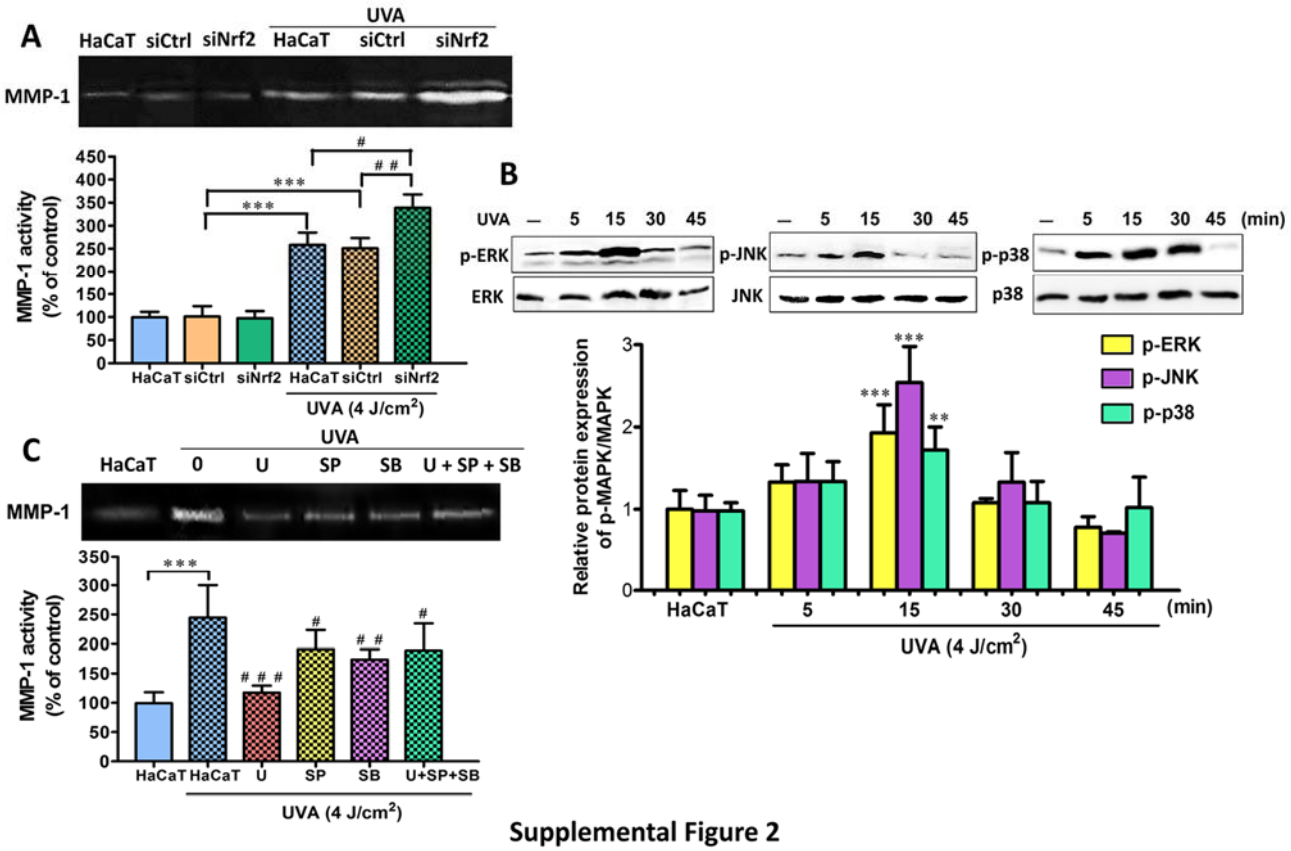


Supplemental Figure 1. The knockdown efficiency of small-interfering RNA-mediated silencing of Nrf2 in HaCaT cells.

Nrf2 protein expression (A) and mRNA expression of Nrf2 and its target genes (GCLC, GCLM, GST and NQO-1) (B) in HaCaT cells transfected with 5 nM of siNrf2 or siCtrl for 48 h. Nrf2 protein levels were detected by Western blot analysis. Data was normalized using α-Tubulin as an internal standard. Nrf2 protein was detected at 68 kDa and α-Tubulin, the loading control, at 50 kDa. mRNA levels were evaluated by real-time RT-PCR. Data was shown as mean ± SD. The statistical significance of differences was evaluated by one-way ANOVA followed by Dunnett's test. ** $P < 0.01$; *** $P < 0.001$ versus untransfected control cells.

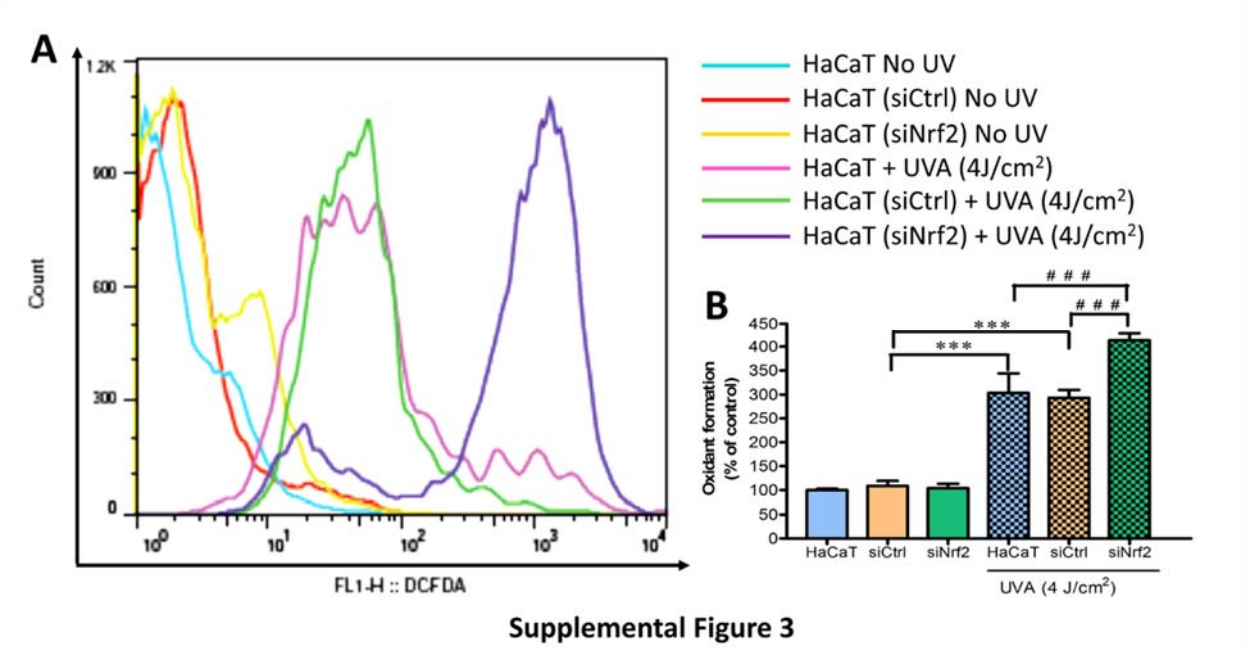


Supplemental Figure 2

Supplemental Figure 2. Nrf2 depletion enhanced UVA-induced MMP-1 activity in association with phosphorylation of ERK, JNK and p38 MAPK in keratinocyte HaCaT cells.

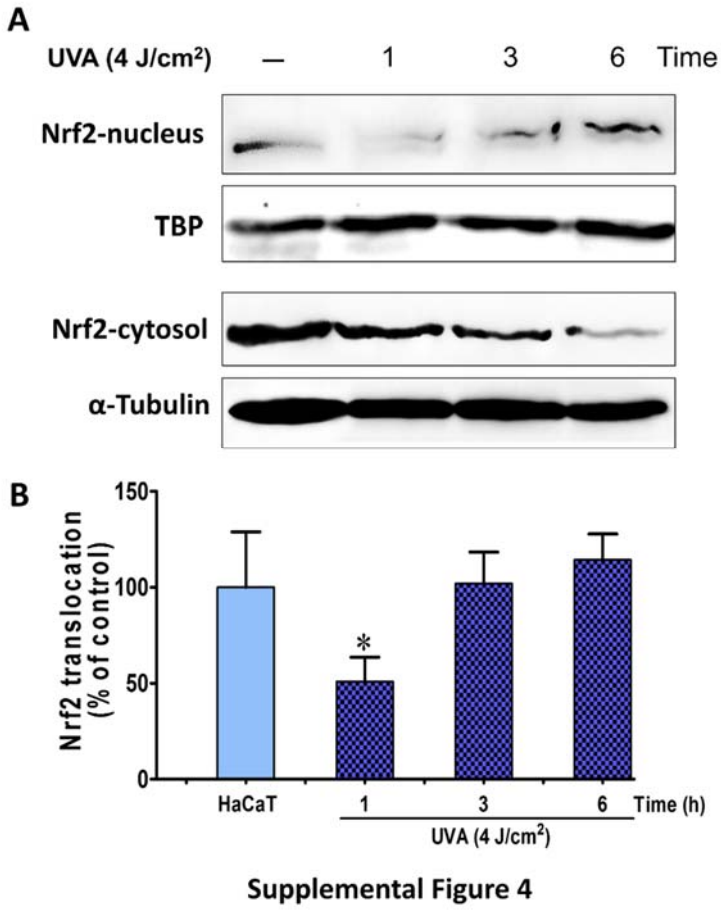
(A) MMP-1 activity in HaCaT cells transfected with siNrf2 or siCtrl for 48 h prior to UVA (4 J/cm^2) irradiation. The cells were harvested at 24 h post-irradiation for detection of MMP-1 activity by zymography. Data was expressed as mean ± SD. The statistical significance of differences was evaluated by one-way ANOVA followed by Dunnett's test. *** $P < 0.001$ versus siCtrl-transfected cells without UVA irradiation. # $P < 0.05$; ## $P < 0.01$ versus siNrf2-transfected cells irradiated with UVA. (B) Time-dependent effects of UVA (4 J/cm^2) irradiation on phosphorylation of MAPKs in HaCaT cells. Phosphorylated-MAPK protein levels were measured at 5, 15, 30, 45 min following irradiation. The statistical significance of differences between non-irradiated controls and the UVA-irradiated groups was evaluated by one-way

ANOVA followed by Dunnett's test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus unirradiated control cells. (C) MMP-1 activity in HaCaT cells pre-treated with 1 μM of U0126, SP600125 and SB203580 for 1 h prior to UVA (4 J/cm^2) exposure. The statistical significance of differences between non-irradiated controls and the UVA-irradiated groups was evaluated by independent Student's *t*-test. *** $P < 0.001$ versus unirradiated control cells. The statistical significance of differences between non-irradiated groups pre-treated with compounds and the irradiated control groups was evaluated by one-way ANOVA followed by Dunnett's test. # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ versus UVA-irradiated cells.



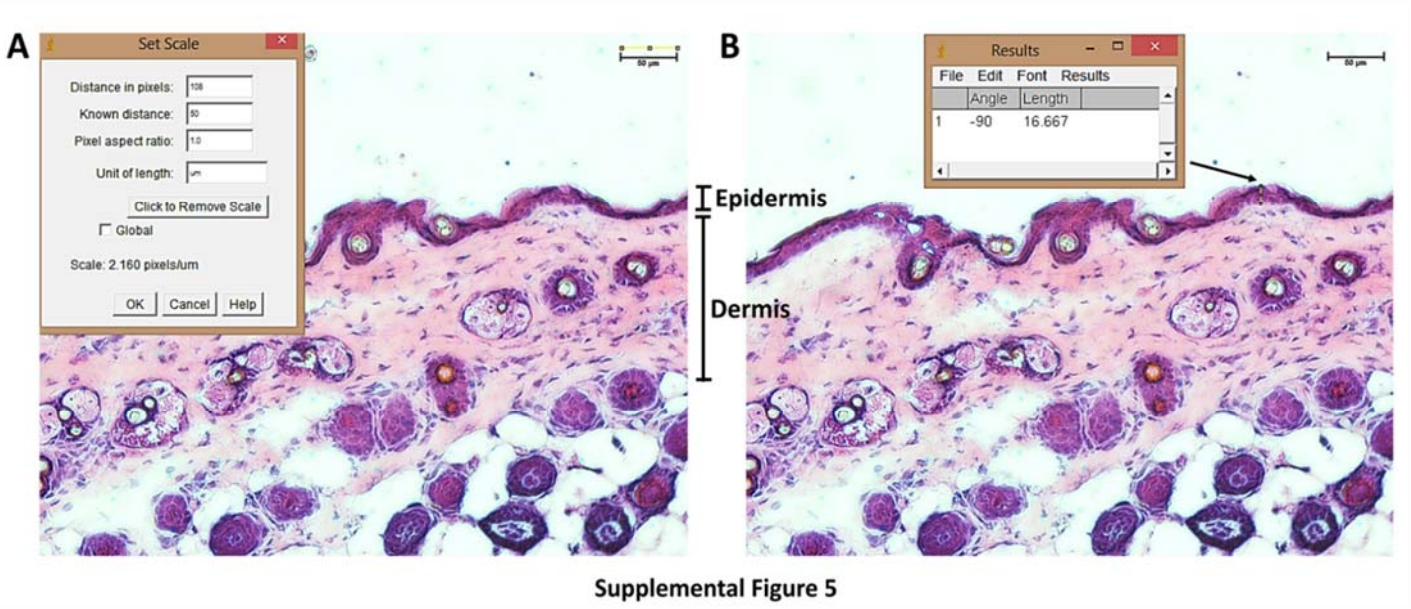
Supplemental Figure 3. Depletion of Nrf2 increased ROS levels in UVA-irradiated HaCaT cells.

(A) ROS levels were detected in HaCaT cells transfected with siNrf2 or siCtrl at 1 h following UVA (4 J/cm^2) irradiation. (B) Quantitative analysis of oxidant formation. Determination of DCFDA was performed by flow cytometry and data was expressed as a percentage of control (100%, non-irradiated and untransfected cells). The statistical significance of differences was evaluated by one-way ANOVA followed by Dunnett's test. *** $P < 0.001$ versus siCtrl-transfected cells without UVA irradiation. ### $P < 0.001$ versus siNrf2-transfected cells irradiated with UVA.



Supplemental Figure 4. The time-dependent changes in Nrf2 nuclear accumulation in HaCaT cells exposed to UVA irradiation.

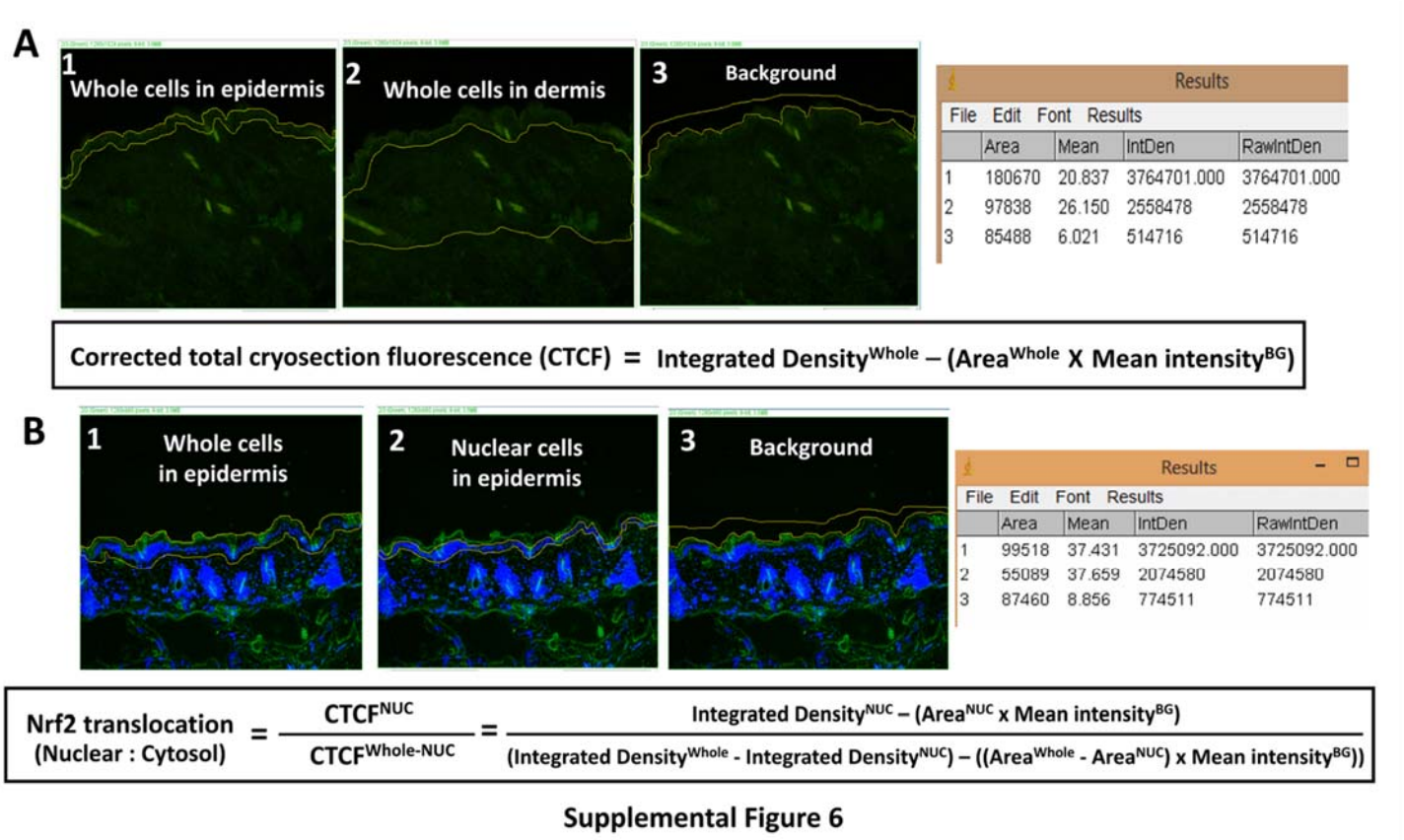
(A) Time-dependent effects of UVA (4 J/cm^2) on Nrf2 nuclear translocation in HaCaT cells harvested at 1, 3 and 6 h after UVA exposure. Western blot was performed to determine Nrf2 nuclear translocation. Nrf2 was detected at 68 kDa, TATA-binding protein (TBP), the loading control for nuclear protein, at 37 kDa and and α-Tubulin, the loading control for cytosol protein, at 50 kDa. (B) Quantitative analysis of Nrf2 nuclear translocation (% of control) expressed as mean ± SD. The statistical significance of differences was evaluated by one-way ANOVA followed by Dunnett's test. * $P < 0.05$ versus unirradiated control cells.



Supplemental Figure 5

Supplemental Figure 5. Epidermal thickness analysis of images by image J software.

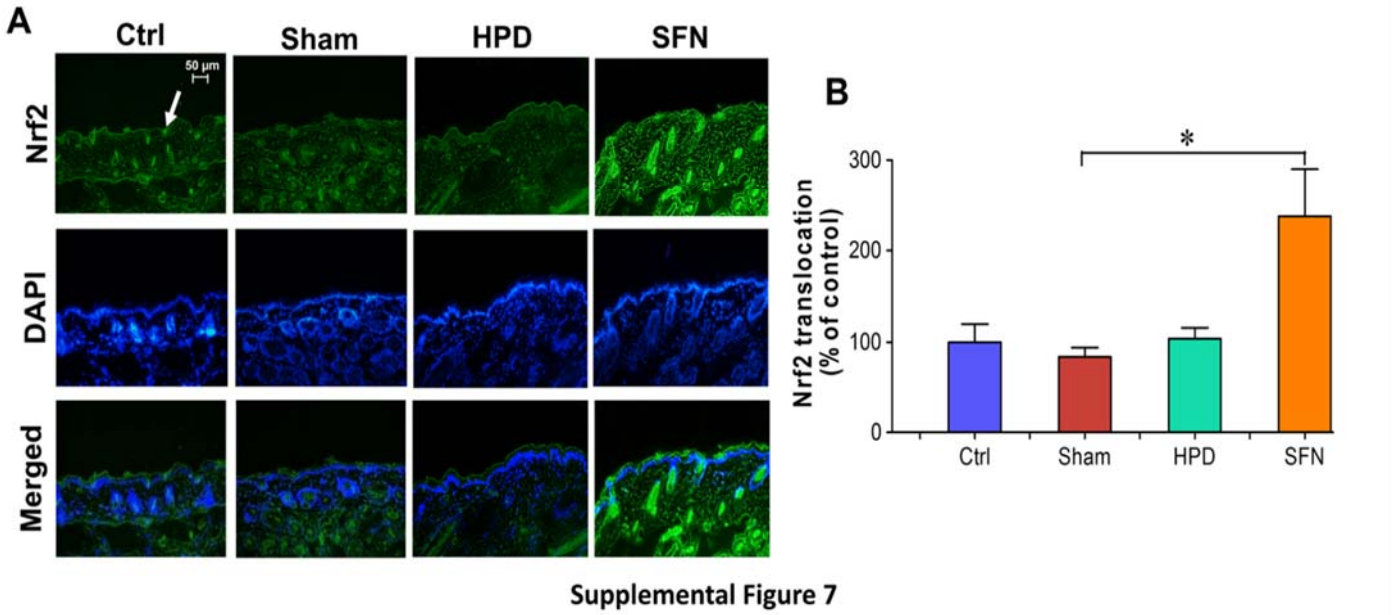
(A) The distance in pixels (108 pixels) were defined from scale bar (50 μm) indicated by inverted fluorescent microscope with camera (Ti-S Intensilight Ri1 NIS-D) as shown in yellow line. (B) Drawing the yellow line in epidermis and measured by ImageJ. The data were generated length in μm unit.



Supplemental Figure 6

Supplemental Figure 6. Fluorescence intensity analysis of images by image J software. (A) To determined protein markers, images of FITC staining were split into green and blue channels. Whole cells in epidermis (1) and whole cells in dermis (2) and background (3) were segmented as shown in yellow lines. Images from 3 rounds of FITC staining were quantified in pixel intensity at green channel. The intensity data measured by ImageJ were generated area, mean intensity and integrated density of yellow line selection. The corrected total cryosection fluorescence (CTCF) = integrated density – (area of selected cell × mean fluorescence of background readings) was calculated as shown in equations. (B) To determined Nrf2 translocation, images of FITC/DAPI staining were split into green and blue channels. Whole cells (1) and nuclear cells (2) in epidermis and background (3) were segmented as shown in yellow lines. Nuclear masks were generated from DAPI staining. Epidermis area was located and confirmed by the pathologist followed by morphological image (Supplementary Fig. S5). Images from 3 rounds of FITC staining were quantified in pixel intensity at green

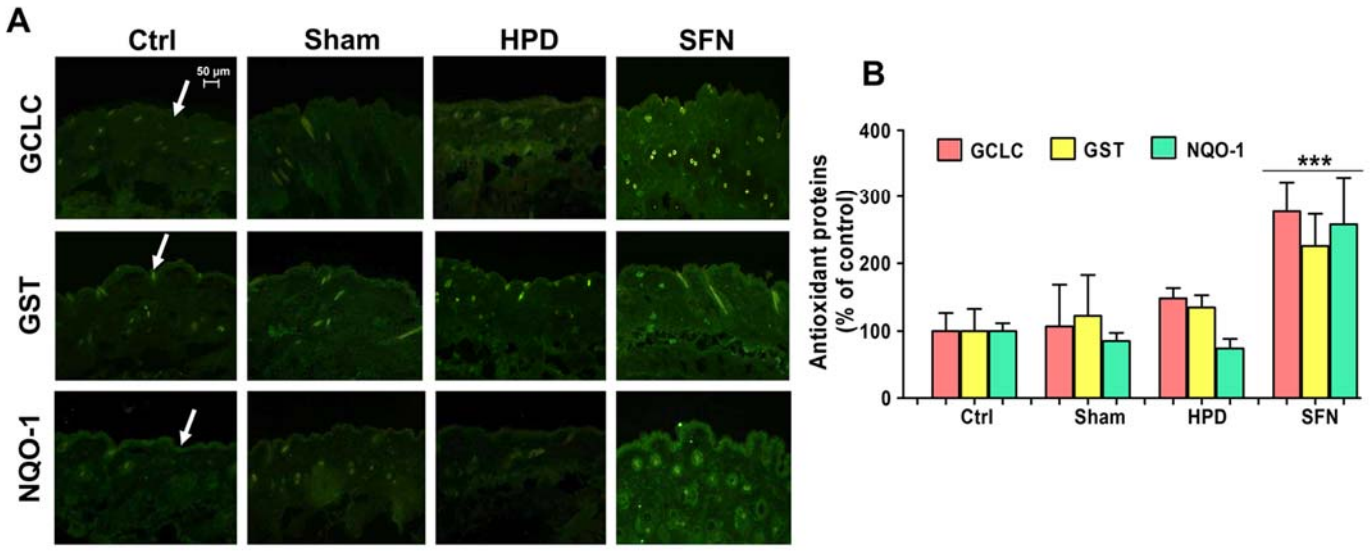
channel. The intensity data measured by ImageJ were generated area and integrated density of yellow line selection. The ratio of nuclear and cytosolic Nrf2 was calculated as shown in equations.



Supplemental Figure 7

Supplemental Figure 7. The effect of hispidulin (HPD) and sulforaphane (SFN) on Nrf2 nuclear localization in unirradiated control skin.

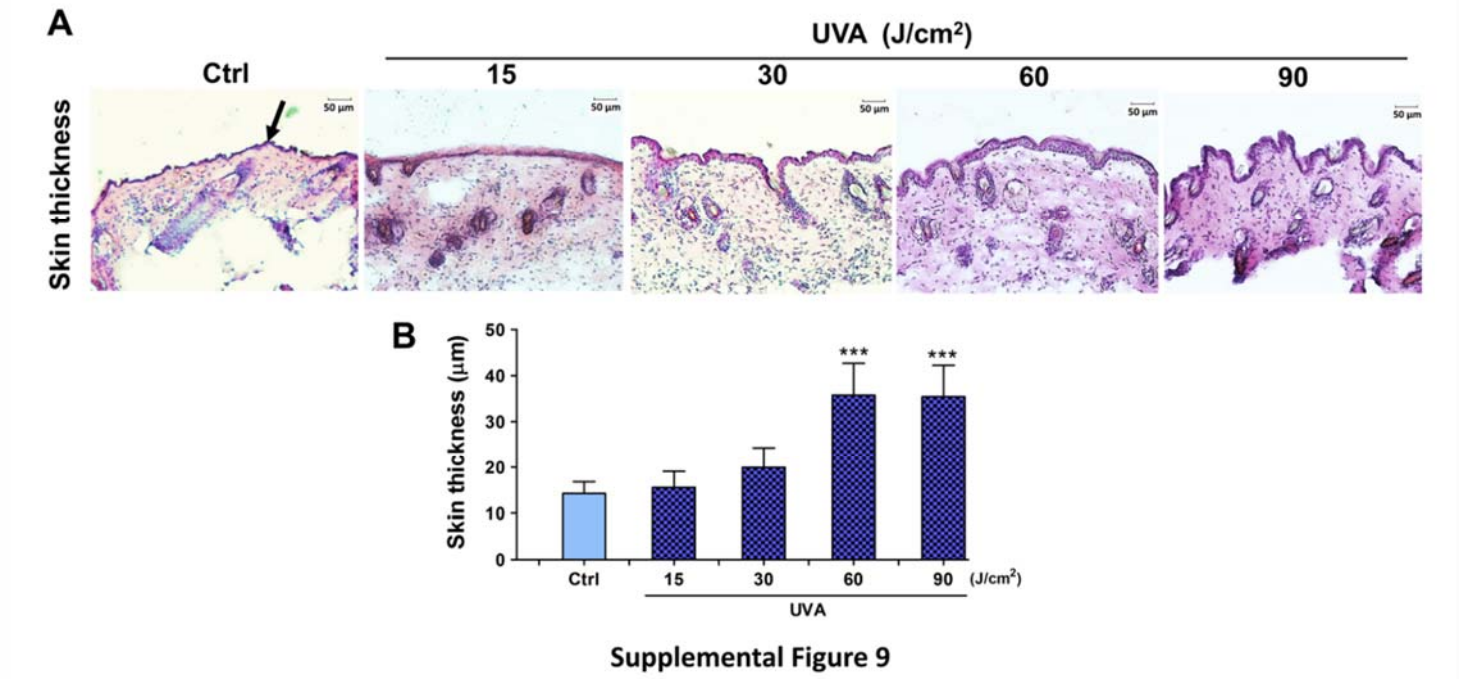
(A) Mice were topically treated over the dorsal skin with 20 µl of vehicle [ethanol:acetone (1:1, v/v)] as sham group, HPD at the highest dose used in this study (200 μM/cm^2) and SFN (0.6 μM /cm^2) for 1 h (3 times a week for 2 weeks) without UVA exposure. The skin was collected at 1 h following the last treatment. Immunofluorescence staining using anti-Nrf2 antibody and FITC-conjugated secondary antibody was performed to determine Nrf2 nuclear localization indicating Nrf2 activation in mouse epidermis. As shown with the white arrow, FITC-conjugated secondary antibody staining indicated location of Nrf2 (green) by anti-Nrf2 antibody. DAPI staining indicated the location of the nucleus (blue) and the merged image indicated the nuclear localization of Nrf2. (B) Levels of nuclear/cytosolic Nrf2 in mouse epidermis topically treated with HPD or SFN alone were quantified by ImageJ software and were expressed as mean ± SD, $n = 9$. The statistical significance of differences between sham control groups and the compound-treated groups was evaluated by one-way ANOVA followed by Dunnett's test. * $P < 0.05$ versus untreated control skin.



Supplemental Figure 8

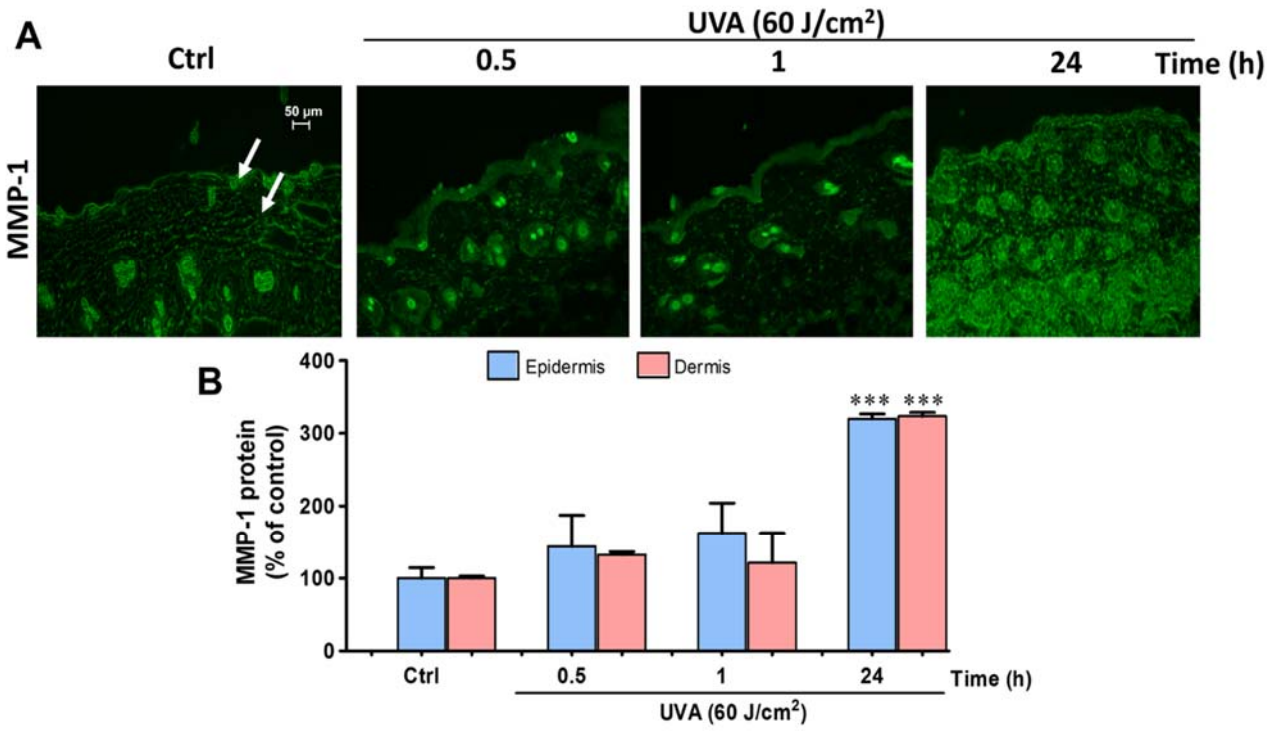
Supplemental Figure 8. The effect of hispidulin (HPD) and sulforaphane (SFN) on Nrf2 targeted proteins in unirradiated control skin.

(A) Mice were topically treated over the dorsal skin with 20 µl of vehicle [ethanol:acetone (1:1, v/v)] as sham group, HPD at the highest dose used in this study (200 μM/cm^2) and SFN (0.6 μM /cm^2) for 1 h (3 times a week for 2 weeks) without UVA exposure. The skin was collected at 6 h following the last treatment. Immunofluorescence staining was performed to determine Nrf2 targeted proteins (GCLC, GST and NQO-1) indicated with the white arrow. (B) Protein levels of GCLC, GST and NQO-1 in mouse skin topically treated with HPD or SFN alone were quantified by ImageJ software. Data was shown as mean ± SD, n = 9. The statistical significance of differences between sham control groups and the compound-treated groups was evaluated by one-way ANOVA followed by Dunnett's test. *** $P < 0.001$ versus sham control skin.



Supplemental Figure 9. Dose-dependent effects of UVA irradiation on the epidermal thickness of mouse skin.

(A) Images of H&E staining for epidermal thickness as indicated by the black arrow. Mouse dorsal skin was irradiated with different doses of UVA irradiation (15, 30, 60 and 90 J/cm^2) and was collected at 1 h after the last irradiation. (B) The epidermal thickness of mouse skin were quantified by ImageJ software. Data was shown as mean ± SD, n = 9. The statistical significance of differences between unirradiated control groups and the UVA-irradiated groups was evaluated by one-way ANOVA followed by Dunnett's test. *** $P < 0.001$ versus unirradiated control skin.



Supplemental Figure 10

Supplemental Figure 10. The effects of UVA (60 J/cm^2) irradiation on MMP-1 expression in mouse skin.

(A) Images of immunofluorescence staining for MMP-1 protein in mouse epidermis and dermis. Mouse skin was collected at 0.5, 1 and 24 h after the final irradiation with UVA (10 J/cm^2/session 3 times a week for 2 weeks; a total dose of 60 J/cm^2). (B) MMP-1 protein expression in mouse epidermis and dermis was quantified by ImageJ software. Data was shown as mean ± SD, $n = 9$. The statistical significance of differences between unirradiated control groups and the UVA-irradiated groups was evaluated by one-way ANOVA followed by Dunnett's test. *** $P < 0.001$ versus unirradiated control skin.