Supplementary Materials

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"Differential modulation of keratin expression by sulforaphane occurs via Nrf2-dependent and independent pathways in skin epithelia"

List of Elements

Supplemental Figure S1.
Examining keratins as targets of the Keap1-Nrf2-ARE pathway in mouse epidermis.
Keratin expression was evaluated, using RT-PCR (A) and indirect immunofluorescence (B), in hypomorphic Keap1 ($Keap1^{fl/fl}$) mice, in which expression of Keap1 is significantly reduced and Nrf2 signaling activity is increased. A) RT-PCR amplification of the Keap1, K6, K14, K16, and K17 transcripts in $Keap1^{fl/fl}$ mouse skin compared to wildtype littermates. Data in histogram (left) reported as mean ± SEM, combining 3 independent experiments. The agarose gel at right (ethidium-bromide stained PCR products) shows representative RT-PCR results. HPRT, hypoxanthine-guanine phosphorybosyltransferase. B) Indirect immunofluorescence staining for K16, K6 and K17 (see labels at left) in wildtype (left column) and Keap1fl/fl littermates (center and right columns), further illustrating the upregulation of K6 and K16 but not K17.
Epi, epidermis; hf, hair follicle. Bar = 100 micrometers.

Supplemental Figure S2.
Quantitation of indirect immunofluorescence signal for specific keratin antigens in mouse epidermis following treatment with SF and GME or BSO.
Three areas of epidermis (25 μm^2 each) were randomly selected in one representative section of mouse skin tissue immunostained for the antigen of interest. The pixel density of immuno-fluorescence signal in epidermis following treatment with SF alone, or in combination with BSO (A) or GME (B), was measured (see Methods). Fold change (mean ± SEM) is expressed relative to the SF treatment alone (control), and is the aggregate of three independent experiments. Student's t-test: *P<0.05, **P<0.01, ***P<0.001.

Supplemental Figure S3.
Role of MAP kinases in sulforaphane-mediated keratin induction in mouse epidermis.
The activity status of MAP kinases in skin tissue extracts following treatment with SF alone, or in combination with inhibitors to p38 (SF + SB), JNK (SF + SP), or ERK (SF + UO) kinases, was measured. Activity of p38, JNK, ERK, and Jun was indirectly assessed by measuring their phosphorylation status using commercially available antibodies that recognize well-defined phosphoepitopes (see Materials and Methods). Fold-activation (mean ± SEM) is expressed relative to the SF treatment alone (control), and is the aggregate of three independent experiments. Student's t-test: *P<0.05, **P<0.01, ***P<0.001.

Supplemental Figure S4.
Quantitation of indirect immunofluorescence of keratins in mouse epidermis following treatment with SF and MAP kinase inhibitors.
Three areas of epidermis (25 μm^2 each) were randomly selected in one representative section of mouse skin tissue immunostained for the antigen of interest (K16 or K17). The pixel density of immunofluorescence signal in epidermis following treatment with SF alone, or in combination with inhibitors to p38 (SF + SB), JNK (SF + SP), or ERK (SF + UO) kinase, was measured (see Materials and Methods). A and B present the results of treatment of SKH-1 hairless adult mice, whereas C shows data from treatment of $Nrf2^{-/-}$ mice. Fold change (mean ± SEM) is expressed

relative to the SF treatment alone (control), and is the aggregate of three independent experiments. Student's t-test: *P<0.05, **P<0.01, ***P<0.001.

Supplemental Table S1.
Quantitation of indirect immunofluorescence for select keratin antigens in epidermis from immunostained sections of mouse skin subjected to various treatments.
Three areas of epidermis (25 μm^2 each) were randomly selected in one representative section of mouse skin tissue immunostained for the antigen of interest (K16 or K17). The pixel density of immunofluorescence signal was obtained as described under "Materials and Methods" in the main text. Data is here reported in as mean pixel density per 25μm^2 area of epidermis $\pm$ SEM. The Student's t-test was used to analyze the data; P<0.05, statistically significant difference.

Supplemental Table S2.
Measurement of total glutathione levels in full-thickness skin after various treatments.
Data reported in two different ways: per unit volume of sample, and per milligram wet weight of skin tissue. See Materials and Methods in the main text for additional details. Note: The measurements obtained for full-thickness skin samples in female SKH-1 hairless mice are less than those measured for epidermis alone in otherwise similar conditions, as reported by others (Lu *et al.*, 1999). That glutathione levels are 2-3 fold higher in epidermal keratinocytes than in fibroblasts cultured from the same foreskin biopsy (Tyrrell and Pidoux, 1988), such that this likely accounts for the difference. The Student's t-test was used to analyze the data; P<0.05, statistically significant difference.

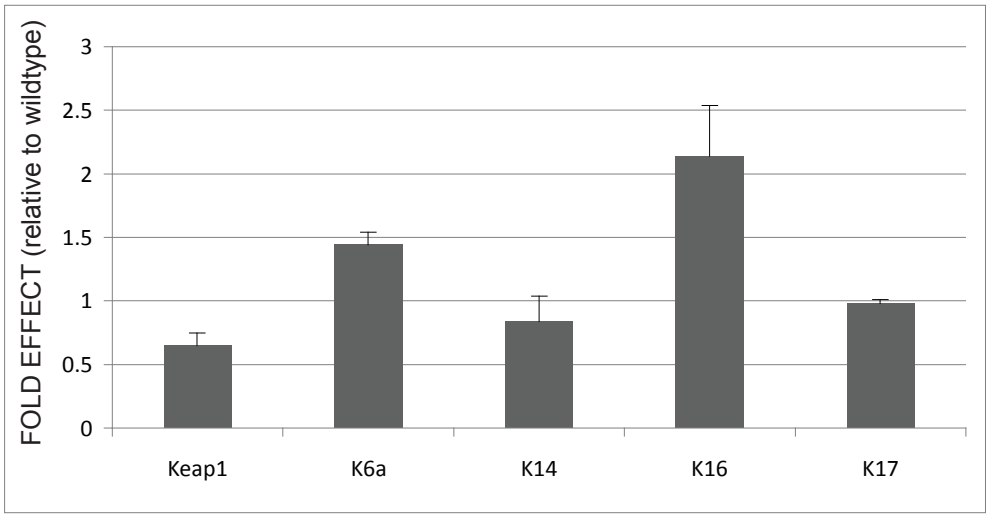
Supplemental Table S3.
Measurement of MAP kinase activity in full-thickness skin extracts after various treatments.
Data was obtained using a using a Sandwich ELISA assay and is reported here as mean $absorbance_{450nm} \pm$ SEM. See Materials and Methods in main text for additional details. The Student's t-test was used to analyze the data; P<0.05, statistically significant difference.

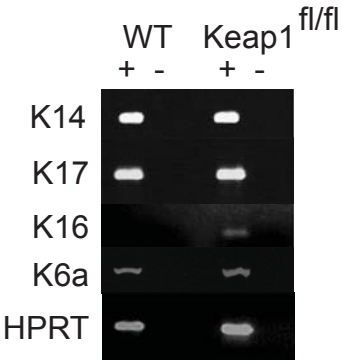
References

Lu SC, Huang ZZ, Yang H, Tsukamoto H (1999) Effect of thioacetamide on hepatic expression of gamma-glutamylcysteine synthetase subunits in the Rat. *Toxicol Appl Pharmacol* 159:161-168.

Tyrrell RM, Pidoux M (1988) Correlation between endogenous glutathione content and sensitivity of cultured human skin cells to radiation at defined wavelengths in the solar ultraviolet range. *Photochem Photobiol* 47:405-412.

A





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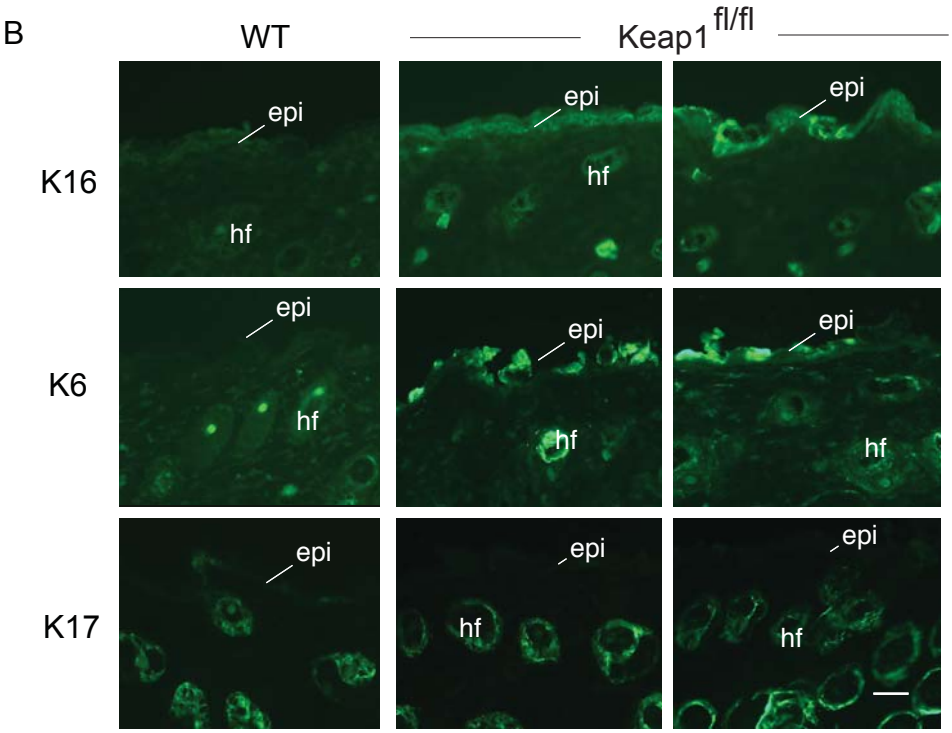
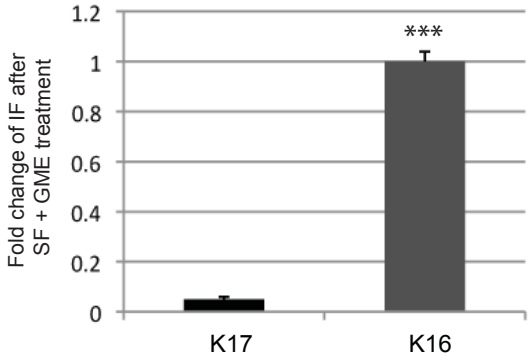


Kerns et al., Supplemental Figure 1

A



See Figure 3C and Supplementary Table S1d

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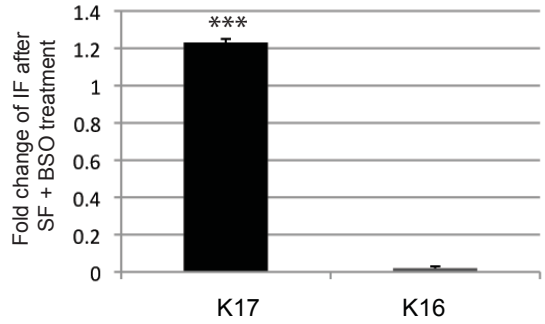
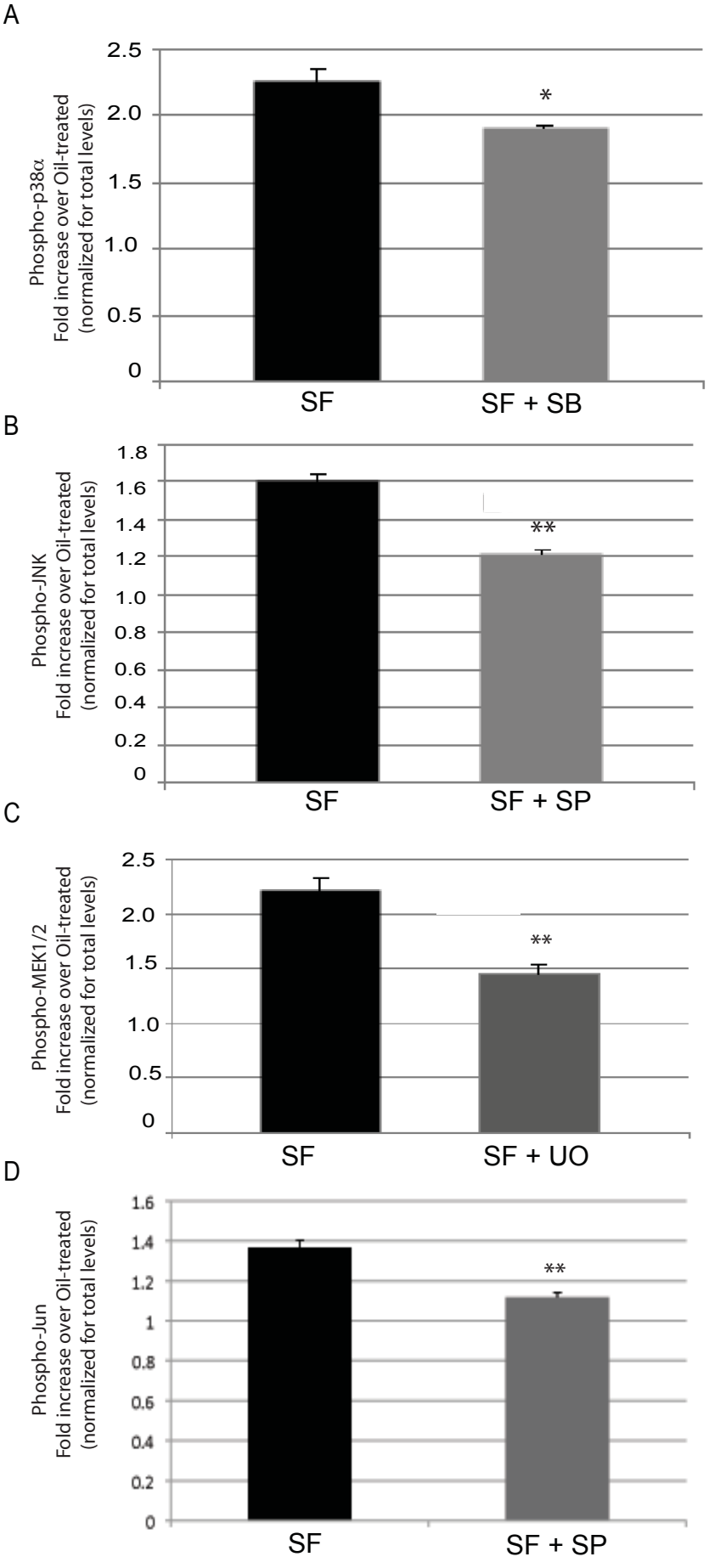


See Figure 3D and Supplementary Table S1e

Kerns et al., Supplemental Figure 2



See Supplementary Table S3c

Kerns et al., Supplemental Figure 3

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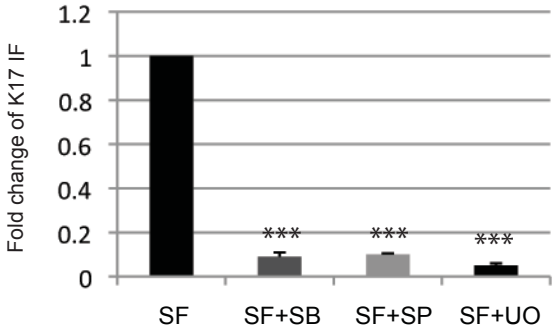

See Supplementary Table S1f

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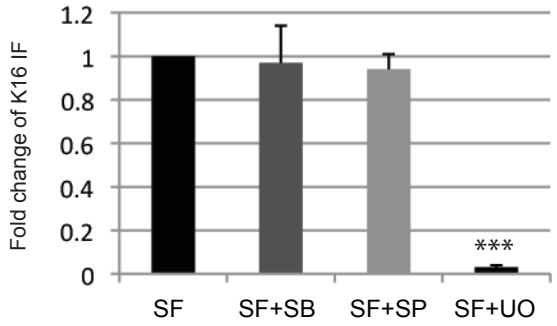

See Supplementary Table S1f

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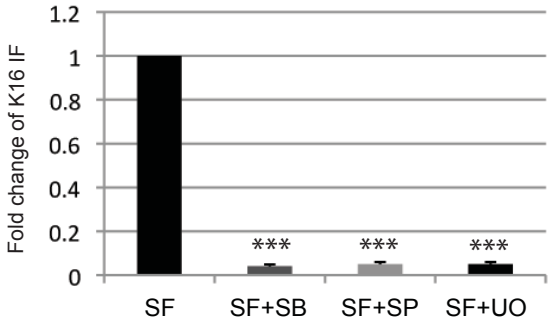

See Supplementary Table S1g

Kerns et al., Supplemental Figure 4

Supplemental Table S1. Quantitation of Immunofluorescence Signal in Mouse Skin Epidermis.

a. Epidermal immunofluorescence (IF) following topical SF treatment of Nrf2 null females in SKH-1 hairless background (n = 3; mean pixel density per 25 μm^2 area of epidermis ± SEM). Complement to Figure 1B.

	K17	P value	K16	P value	K6	P value
Oil (topical)	5.21 ± 1.19		40.92 ± 1.09		21.43 ± 1.80	
SF (topical)	53.86 ± 3.60	**0.0002**	115.98 ± 4.50	**0.0001**	21.82 ± 2.13	0.8955

b. Epidermal immunofluorescence (IF) following topical SF treatment of Nrf2 null 8 day old mice in C57Bl/6 background (n=3, mean pixel density per 25 μm^2 area of epidermis ± SEM). Complement to Figure 1C.

	K17	P value	K16	P value
Oil (topical)	13.10 ± 1.41		16.93 ± 2.15	
SF (topical)	73.10 ± 6.36	**0.0008**	110.53 ± 1.76	**0.0001**

c. Epidermal immunofluorescence (IF) following systemic BSO or GME treatment of SKH-1 hairless females (n=3, mean pixel density per 25 μm^2 area of epidermis ± SEM). Complement to Figure 1, frames E & F.

	K17	P value	K16	P value
PBS (ip)	7.66 ± 0.48		12.44 ± 0.93	
BSO (ip)	101.20 ± 2.37	**0.0001**	21.36 ± 0.88	**0.0022**
GME (ip)	11.02 ± 1.15	0.0543	81.09 ± 8.52	**0.0013**

d. Epidermal immunofluorescence (IF) following topical SF treatment and systemic GME treatment of SKH-1 hairless females (n=3, mean pixel density per 25 μm^2 area of epidermis ± SEM). Complement to Figure 3C and Supplementary Figure S2A.

	K17	P value	K16	P value
SF (topical) + PBS (ip)	163.83 ± 1.11		145.21 ± 3.31	
SF (topical) + GME (ip)	8.21 ± 0.74	**0.0001**	144.95 ± 5.57	0.2600

e. Epidermal immunofluorescence (IF) following topical SF treatment and systemic BSO treatment of Nrf2 null females in SKH-1 hairless background (n=3, mean pixel density per 25 µm^2 area of epidermis ± SEM). Complement to Figure 3D and Supplementary Figure S2B.

	K17	P value	K16	P value
SF (topical) + PBS (ip)	124.45 ± 1.81		169.67 ± 2.08	
SF (topical) + BSO (ip)	151.38 ± 2.78	**0.0013**	3.62 ± 2.19	**0.0001**

f. Epidermal immunofluorescence (IF) following topical SF treatment and topical MAPK inhibitor treatment of SKH-1 hairless females (n=3, mean pixel density per 25 µm^2 area of epidermis ± SEM). Complement to Supplementary Figure S4, frames A (K17) and B (K16).

	K17	P value	K16	P value
SF (topical)	88.22 ± 3.56		88.39 ± 8.59	
SF (topical) + SB (topical)	8.12 ± 1.41	**0.0001**	85.35 ± 14.64	0.8666
SF (topical) + SP (topical)	8.53 ± 0.53	**0.0001**	74.62 ± 6.29	0.2655
SF (topical) + UO (topical)	4.52 ±0.33	**0.0001**	2.45 ± 0.42	**0.0006**

g. Epidermal immunofluorescence (IF) following topical SF treatment and topical MAPK inhibitor treatment of Nrf2 null females in SKH-1 hairless background (n=3, mean pixel density per 25 µm^2 area of epidermis ± SEM). Complement to Supplementary Figure S4C.

	K16	P value
SF (topical)	146.05 ± 3.06	
SF (topical) + SB (topical)	5.70 ± 0.63	**0.0001**
SF (topical) + SP (topical)	7.52 ± 0.54	**0.0001**
SF (topical) + UO (topical)	7.60 ± 1.41	**0.0001**

Supplemental Table S2. Measurement of Total Glutathione Levels in Mouse Skin Tissue

a. Total glutathione levels following topical treatment. Complement to Figure 2A.

Timepoint	Treatment	nmole of glutathione/ ml of sample (mean ± SE, 3 mice per group)	µg of glutathione /mg of wet weight of whole skin (mean ± SE, 3 mice per group)	P value
1hr 30min	Oil (topical)	8.89 ± 0.28	0.027 ± 0.001	
1hr 30min	SF (topical)	8.92 ± 0.30	0.027 ± 0.001	0.9452
2hrs	Oil (topical)	9.91 ± 1.81	0.030 ± 0.005	
2hrs	SF (topical)	4.98 ±2.17	0.015 ± 0.006	0.1560
5hrs 30min	Oil (topical)	6.74 ± 1.14	0.020 ± 0.003	
5hrs 30min	SF (topical)	7.94 ± 1.04	0.024 ± 0.003	0.4802
24hrs	Oil (topical)	9.73 ± 0.23	0.029 ± 0.001	
24hrs	SF (topical)	11.77 ± 0.05	0.035 ± 0.0001	**0.001**

b. Total glutathione levels following systemic BSO treatment. Complement to Figure 2B.

Treatment	nmole of glutathione / ml of sample (mean ± SE, 3 mice per group)	µg of glutathione /mg of wet weight of whole skin (mean ± SE, 3 mice per group)	P value
PBS (ip)	8.36 ± 2.06	0.025 ± 0.006	
BSO (ip)	4.57 ± 0.54	0.014 ± 0.002	0.1497

c. Total glutathione following systemic GME treatment. Complement to Figure 2C.

Treatment	nmole of glutathione / ml of sample (mean ± SE, 3 mice per group)	µg of glutathione /mg of wet weight of whole skin (mean ± SE, 3 mice per group)	P value
PBS (ip)	6.19 ± 0.30	0.019 ± 0.001	
GME (ip)	10.33 ± 0.59	0.031 ± 0.002	**0.003**

d. Total glutathione levels following topical SF and systemic GME treatment. Complement to Figure 3A.

Treatment	nmole of glutathione / ml of sample (mean ± SE, 3 mice per group)	µg of glutathione /mg of wet weight of whole skin (mean ± SE, 3 mice per group)	P value
PBS (ip) + Oil (topical)	9.91 ± 1.81	0.030 ± 0.005	
PBS (ip) + SF (topical)	4.98 ± 2.17	0.015 ± 0.007	0.1560
GME (ip) + SF(topical)	8.16 ± 0.53	0.024 ± 0.002	0.4060

e. Total glutathione levels following topical SF and systemic BSO treatment. Complement to Figure 3B.

Treatment	nmole of glutathione / ml of sample (mean ± SE, 3 mice per group)	µg of glutathione /mg of wet weight of whole skin (mean ± SE, 3 mice per group)	P value
PBS (ip) + Oil (topical)	9.73 ± 0.23	0.029 ± 0.001	
PBS (ip) + SF (topical)	11.77 ± 0.05	0.035 ± 0.0001	**0.001**
BSO (ip) + Sf (topical)	7.67 ± 0.09	0.023 ± 0.0002	**0.001**

Supplemental Table S3. Measurements of MAP Kinase Activity in Skin Tissue Extracts.

a. MAPK levels 2 hours after topical SF and systemic GME treatment (mean absorbance$_{450nm}$ ± SEM, n=3). Complement to Figure 4A.

	P-p38	P value*	P-SAP/JNK	P value*	P-MEK1/2	P value*	pERK1/2	P value*
Oil (topical)	0.18 ± 0.01		0.07 ± 0.09		0.08 ± 0.01		0.24 ± 0.02	
SF (topical)	0.22 ± 0.003	**0.0162**	0.13± 0.004	**0.5418**	0.18 ± 0.008	**0.0015**	0.28 ± 0.003	0.1191
SF (topical) + GME (ip)	0.12 ± 0.009	**0.0112**	0.10 ± 0.030	0.7676	0.08 ± 0.001	1.0000	0.19 ± 0.008	0.0810

b. MAPK levels 24 hours after topical SF and systemic BSO treatment (mean absorbance$_{450nm}$ ± SEM, n=3). Complement to Figure 4B.

	P-p38	P value*	P-SAP/JNK	P value*	P-MEK1/2	P value*	pERK1/2	P value*
Oil (topical)	2.94 ± 0.02		0.09 ± 0.01		0.10 ± 0.03		1.81 ± 0.03	
SF (topical)	2.76 ± 0.01	**0.0013**	0.09 ± 0.002	1.0000	0.12 ± 0.002	0.5423	1.65 ± 0.06	0.0756

c. MAPK levels after topical SF and inhibitor treatment (mean absorbance$_{450nm}$ ± SEM, n=3). Complement to Supplementary Figure 3A-D.

		Fold increase over oil-treated	P value
SF (topical)	P-MEK1/2	2.22 ± 0.10	
SF (topical) + UO (topical	P-MEK1/2	1.45 ± 0.07	**0.0032**
SF (topical)	P-p38α	2.26 ± 0.09	
SF (topical) + SB (topical)	P- p38α	1.91 ± 0.02	**0.0192**
SF (topical)	P-JNK	1.60 ± 0.04	
SF (topical) + SP (topical)	P-JNK	1.21 ± 0.03	**0.0015**
SF (topical)	P-Jun	1.35 ± 0.04	
SF (topical) + SP (topical)	P-Jun	1.12 ± 0.03	**0.0100**