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Supplemental Information

A UV-Independent Topical Small-Molecule Approach for Melanin Production in Human Skin

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Supplemental Figure Legends

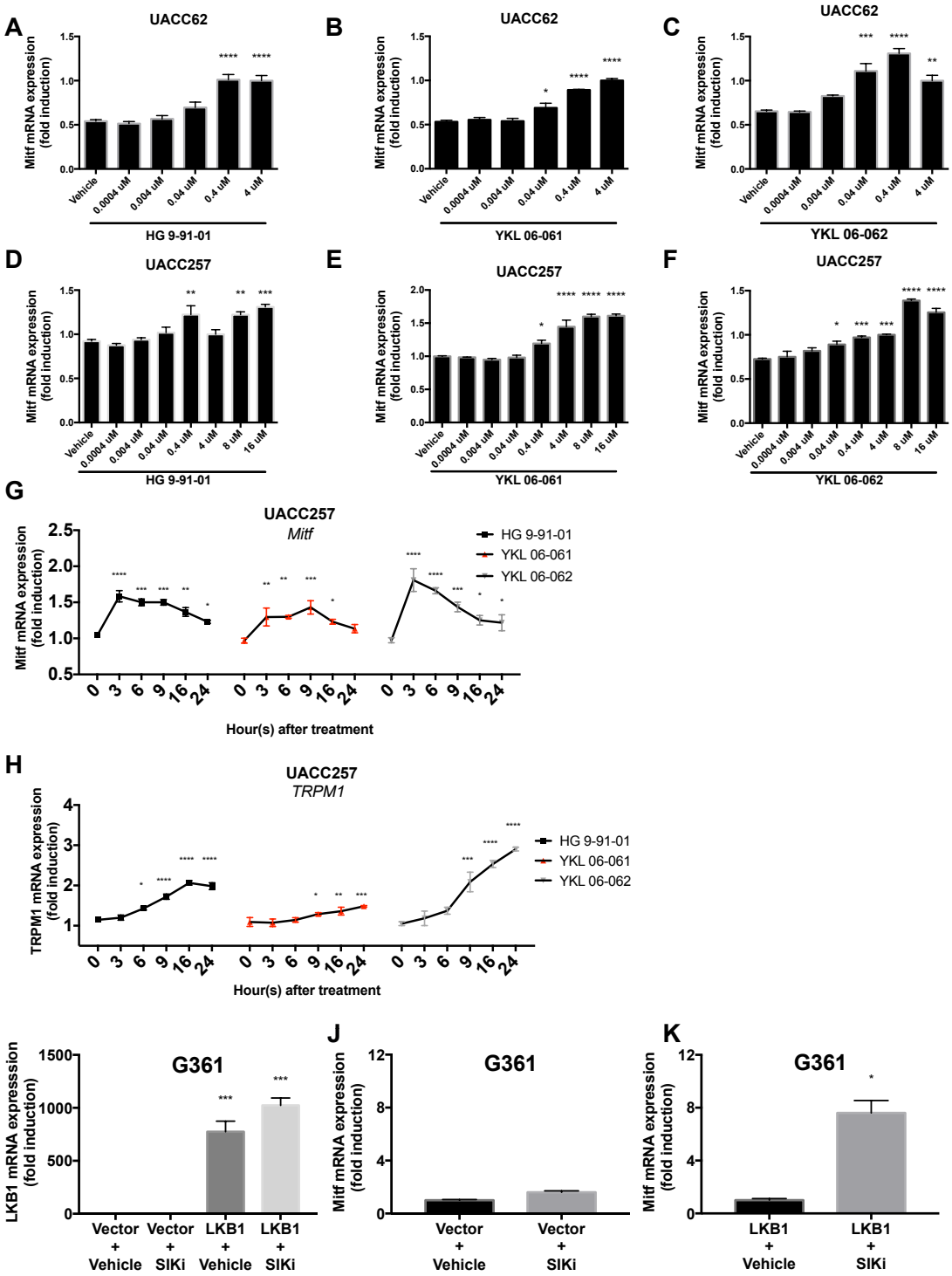


Figure S1: Inhibition of SIK by HG 9-91-01, YKL 06-061, and YKL 06-062 in UACC62 and UACC257 melanoma cells induces *MITF* expression. Related to Figure 1 and Figure 3. mRNA expression of *MITF* relative to *RPL11* mRNA and vehicle control quantified by qRT-PCR in human melanocytes, UACC62 melanoma cells **(A)**, **(B)**, **(C)** and UACC257 melanoma cells **(D)**, **(E)**, **(F)** treated with HG 9-91-01 **(A)**, **(D)**, YKL 06-061 **(B)**, **(E)**, or YKL 06-062 **(C)**, **(F)** (n=3, mean ± SEM). mRNA expression of *MITF* **(G)** and *MITF*-dependent gene *TRPM1* **(H)**, relative to *RPL11* and vehicle control (70% ethanol; 30% propylene glycol) at each time point, in normal human melanocytes treated with 4 μM of SIK inhibitor, quantified by qRT-PCR (n=3, mean ± SEM). **(I)** *LKB1* mRNA expression in G361 melanoma cells transduced with control vector or wild-type LKB1 overexpression vector (n=3, mean ± SEM). **(J)** *MITF* mRNA expression in control vector transduced G361 melanoma cells as described in **I** treated with 1 uM salt-inducible kinase inhibitor (SIKi) HG 9-91-01 or vehicle (dimethyl sulfoxide) (n=3, mean ± SEM). **(K)** *MITF* mRNA expression in LKB1 transduced G361 melanoma cells as described in **I** treated with 1 uM salt-inducible kinase inhibitor (SIKi) HG 9-91-01 or vehicle (dimethyl sulfoxide) (n=3, mean ± SEM). For graphs in **A**-**F**, statistical significance is reported as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVA with Dunnett's multiple comparisons test comparing treatment dose to vehicle control. For graphs in **G** and **H**, statistical significance is reported as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, repeated measures one-way ANOVA with Dunnett's multiple comparisons test comparing each time point to time point zero. For graph in **I**, statistical significance is reported as follows: ***P < 0.001, one-way ANOVA with Dunnett's multiple comparisons test comparing all experimental groups to vector + vehicle experimental group. For graphs in **J** and **K**, statistical significance is reported as

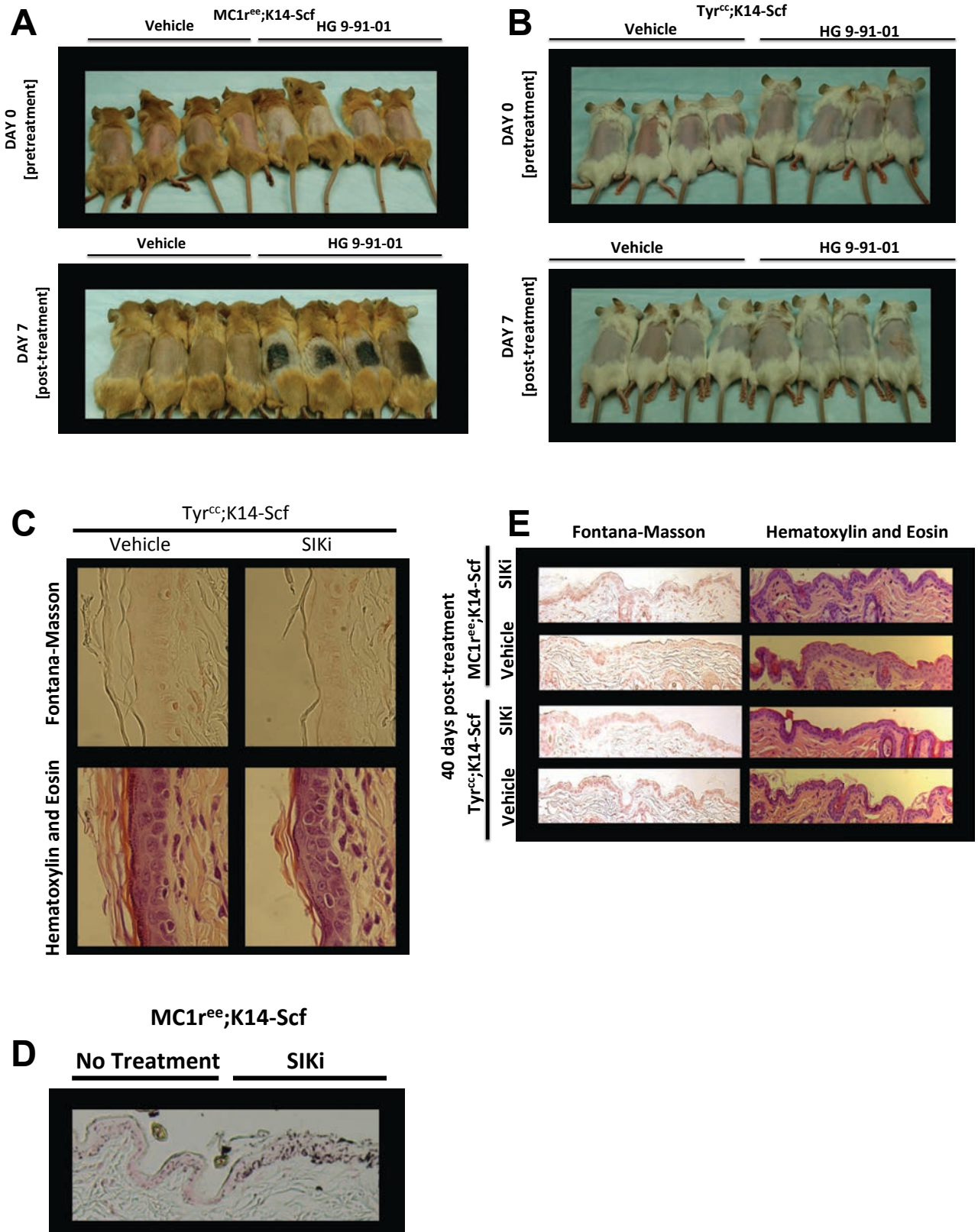
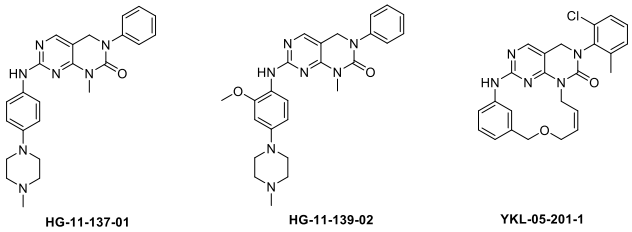
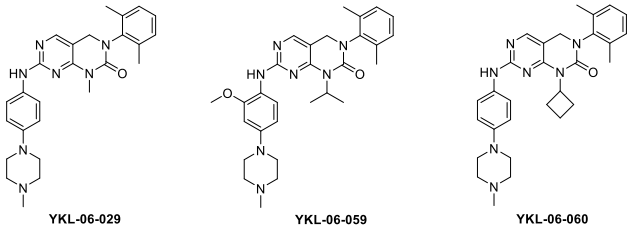


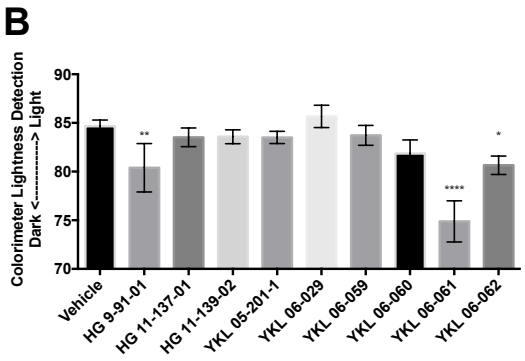
Figure S2: **Expansion of Figure 2**. *Mc1r*$^{e/e}$;K14-SCF mice **(A)** and *Tyr*$^{c/c}$;K14-SCF mice **(B)** before treatment (Day 0) and after 7 days of treatment (Day 7) with 30 µL of vehicle control (70% ethanol; 30% propylene glycol) or 37.5 mM HG 9-91-01 (image of all replicates of mice represented in **Figure 2A**). **(C)** Fontana-Masson (eumelanin) [top panel] and hematoxylin and eosin stains [bottom panel] of *Tyr*$^{c/c}$;K14-SCF mice as described in **Figure 2A** (x630 magnification) (image is representative of n=4 experiments). Fontana-Masson (eumelanin) stained skin sections of *Mc1r*$^{e/e}$;K14-SCF mice treated with 37.5 mM HG 9-91-01 for 7 days as described in **Figure 2A**. **(D)** Image is at the margin of treated and untreated area (x100 magnification). **(E)** Fontana-Masson (eumelanin) [left panels] and hematoxylin and eosin stains [right panels] of *Mc1R*$^{e/e}$;K14-SCF mice and *Tyr*$^{c/c}$;K14-SCF mice with vehicle or 37.5 mM HG 9-91-01 as described **Figure 2E** (x200 magnification)**.**

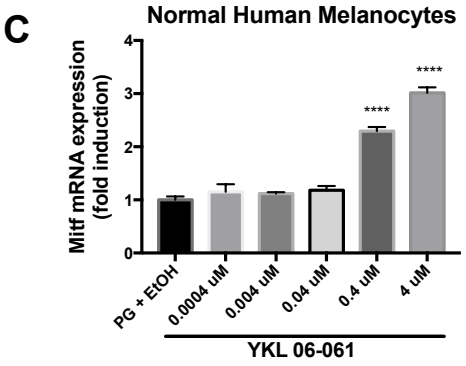
A

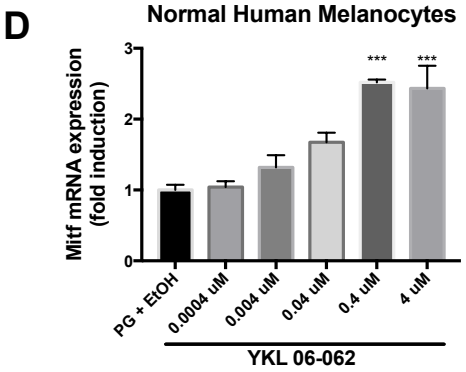
Molecule Name	Molecular weight (g/mol)	CLogP	H-bond donors	H-bond acceptors
HG-9-91-01	567.681	6.01	2	8
HG-11-137-01	429.518	3.34	1	6
HG-11-139-02	459.544	3.19	1	7
YKL-05-201-1	447.917	4.74	1	5
YKL-06-029	457.571	4.37	1	6
YKL-06-059	515.65	4.99	1	7
YKL-06-060	497.634	5.28	1	6
YKL-06-061	527.66	5.12	1	7
YKL-06-062	525.688	6.17	1	6

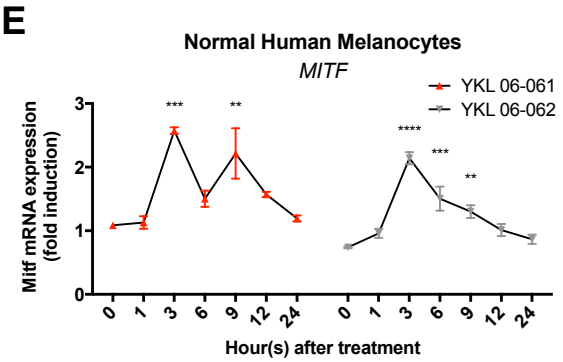












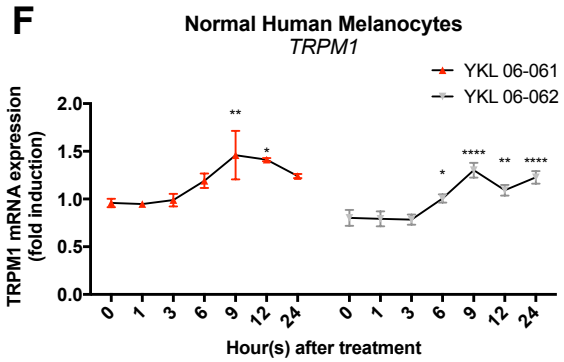


Figure S3: Second generation SIK inhibitors are as efficacious in inducing the pigmentation pathway as HG 9-91-01. Related to Figure 3. **(A)** Physiochemical properties of SIK inhibitors and structures of SIK inhibitors HG 11-137-01, HG 11-139-02, YKL 05-201-1, YKL 06-029, YKL 06-059, YKL 06-060 **(B)** Reflective colorimetry measurements (L* white-black color axis) of human breast skin explants treated with passive application of vehicle control (70% ethanol; 30% propylene glycol) or 37.5 mM of SIK inhibitors HG 9-91-01, HG 11-137-01, HG 11-139-02, YKL 05-201-1, YKL 06-029, YKL 06-059, YKL 06-060, YKL 06-061, or YKL 06-062 for 8 days (30 uL; 2x/day). Colorimeter analysis 1 day after end of treatment (n=3, mean ± SEM). mRNA expression of *MITF* relative to *RPL11* mRNA and vehicle control in normal human melanocytes, treated with YKL 06-061 **(C),** YKL 06-062 **(D)**, or vehicle control (70% ethanol; 30% propylene glycol) **(C,D)**, quantified by qRT-PCR (n=3, mean ± SEM). mRNA expression of *MITF* **(E)** and *MITF*-dependent gene *TRPM1* **(F)** relative to *RPL11* and vehicle control at each time point, in normal human melanocytes over 24 hours after 4 μM YKL 06-061, YKL 06-062, or vehicle control treatment, quantified by qRT-PCR (n=3, mean ± SEM). For graph in **B**, statistical significance is reported as follows: *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA with Dunnett's multiple comparisons test comparing each treatment group to vehicle control. For graphs in **C** and **D**, statistical significance is reported as follows: ***P < 0.001, ****P < 0.0001, one-way ANOVA with Dunnett's multiple comparisons test comparing treatment dose to vehicle control. For graphs in **E** and **F**, statistical significance is reported as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, repeated measures one-way ANOVA with Dunnett's multiple comparisons test comparing each time point to

Supplemental Experimental Procedures

Quantitative real time polymerase chain reaction (qRT-PCR). mRNA was extracted from cells using the RNeasy Mini Kit (Qiagen). KAPA SYBR ® FAST Universal One-Step qRT-PCR Kit (KAPA BIOSYSTEMS) was used to prepare mRNA samples for qRT-PCR and samples were analyzed using the 7500 Fast Real Time PCR System (Applied Biosystems). The relative expression of each gene was calculated by 7500 Fast System Software, which utilizes *Ct* normalized to mRNA levels of *RPL11* to calculate relative expression. Results are reported relative to control cells. Primer sequences used: human M-specific *MITF* forward 5'-CATTGTTATGCTGGAAATGCTAGAA-3', human M-specific *MITF* reverse 5'-GGCTTGCTGTATGTGGTACTTGG-3', *TRPM1* forward 5'-ATGCCTTGAAAGACCACTCCTCCA-3', *TRPM1* reverse 5'-TGTGGGAGTTGTTGAGCACAGAGA-3', *RPL11* forward 5'- GTTGGGGAGAGTGGAGACAG-3', *RPL11* reverse 5'-TGCCAAAGGATCTGACAGTG-3'.

Kinase activity *in vitro* assay. The biochemical activities against SIK2 were measured by Caliper-based mobility shift assay (PerkinElmer). For these experiments, full length His6-MBP-tagged hSIK2 (4 nM) was incubated with HG-9-91-01 derivatives in buffer containing 100 mM HEPES 7.5, 10 mM $MgCl_2$, 2.5 mM DTT, 0.004% Tween 20, 0.003% Brij-35, 30 μM ATP, and 1.5 μM ProfilerPro FL-Peptide 10 (5-FAMKKKVSRSGLYRSPSMPENLNRPR-COOH, PerkinElmer, Catalog No. 760354) at room temperature. Reactions were quenched by adding 20 mM EDTA (pH 8) after 1 hour, and percentage of substrate conversion was measured by LabChip EZ Reader II (PerkinElmer). IC50s for SIK2 inhibition were calculated using SmartFit nonlinear regression in Genedata Screener software suite (Genedata).
All other *in vitro* kinase assay were conducted using the SelectScreen Kinase Profiling Service at Thermo Fisher Scientific (Madison, WI). The protocols are available from the Thermo Fisher Scientific website.

Cell Culture. UACC257 human melanoma cells and UACC62 human melanoma cells were grown in RPM1 medium + 1% penicillin/streptomycin/glutamine + 5% fetal bovine serum. Normal human melanocytes were grown in TIVA medium (HAM's F-12, 1% penicillin/streptomycin/glutamine, 10% fetal bovine serum, 50ng/mL 12-O-tetradecanoyl phorbol-13-acetate, 1 x 10^{-4} M 3-isobutyl-1-methyl xanthine, 1 μM Na_3VO_4, 1 x 10^{-3} M N^6,2'-O-dibutyryladenosine 3:5-cyclic monophosphate), and were starved for 24 hours in HAM's F-12 + 1% penicillin/streptomycin/glutamine before all molecular experiments.

Lentiviral Transduction. Lentivirus was produced by cotransfection of 293T cells with LKB1-expressing lentivirus vector, packaging system VSV-G (Cell Biolabs Inc), and psPAX2 (Addgene). 1 x 10^6 G361 melanoma cells were plated in each well of a 6-well plate. Cells were infected with virus in the presence of 8 ug/mL of polybrene infection reagent (Sigma-Aldrich). Plates were spun at room temperature for 1.5 hours at 1,000 RPM. Media was changed to viral-free media 24 hours after infection. 48 hours after infection cells were treated in 1 uM of SIKi or vehicle control (DMSO) and mRNA samples were collected after 3 hours.

Photos. Photos were taken using a Nikon D50 DSLR camera with a Nikon Nikkor 40 mm f/2.8 DX G AF-S lens. Shutter speed ranged from 1/40 to 1/250 and aperture ranged from F3-F8. Ott-Lite Model L139AB lamps were used to create uniform lighting for photos.

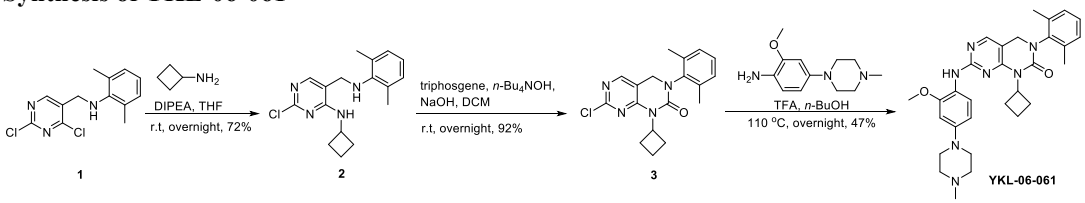
Cell pelleting experiments. 1x10^5 UACC257 cells were plated per well in a 6-well plate. Cells were treated daily (1x/day) with 4 μM of SIK inhibitor HG 9-91-01 or vehicle control (30% propylene glycol + 70% ethanol). After 3 days, cells were detached with 0.25% trypsin, resuspended in RPMI media, and centrifuged. RPMI media was removed and pellets were washed 1x with PBS and allowed to dry before imaging.

Mouse pigmentation experiments. For *in vivo* darkening experiment, male and female mice ranging from 1.5-3 months old were used. For *in vivo* time course experiment, male and female mice ranging from 2.5-6 months were used. Animals were waxed and treated with vehicle control (30% propylene glycol + 70% ethanol) or 37.5 mM HG 9-9-01 until uniform gross darkening was visible, as stated in figure legends. Daily differences in darkening of the skin were measured by reflective colorimetry (when hair was re-growing, no measurement was taken until hair was long enough to be waxed). Skin was harvested, fixed, and processed for paraffin embedding. Sections were cut from paraffin blocks, and sections were stained utilizing hematoxylin and eosin (morphology) and Fontana-Masson (melanin). Melanin dissolution was conducted utilizing the NaOH lysis method as described previously by Wakamatsu and Ito. In the time course experiment, one vehicle-treated *Tyr*$^{c/c}$;K14-SCF mouse (Day 11) and two vehicle-treated *Mc1r*$^{e/e}$;K14-SCF mice (Day 23 and Day 34) died due to technical reasons.

Human pigmentation experiments. Full thickness human breast skin explants were cultured in petri dishes with a solid phase and liquid phase phenol red free DMEM medium with 20% penicillin/streptomycin/glutamine, 5% fungizone (Gibco), and 10% fetal bovine serum. Explants were treated daily with vehicle or SIK inhibitor as indicated in figure legends. Passive application refers to simply applying the treatment to skin without further rubbing or manipulation. Mechanical application refers to application of agents to skin with further rubbing of treatment with 10 clockwise turns of a gloved cotton swab applicator. Skin was harvested, fixed, and processed for paraffin embedding. Sections were cut from paraffin blocks, and sections were stained utilizing hematoxylin and eosin (morphology) and Fontana-Masson (melanin).

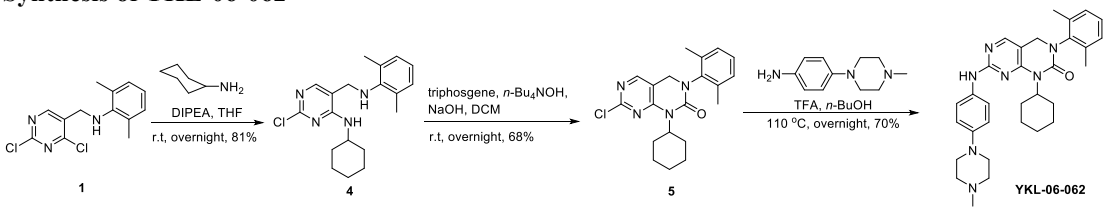
Synthetic procedure of small molecule SIK inhibitors:
Unless otherwise noted, reagents and solvents were used as received from commercial suppliers. Proton nuclear magnetic resonance spectra were obtained on Bruker AVANCE spectrometer at 400 MHz for proton. Spectra are given in ppm (δ) and coupling constants, J, are reported in Hertz. The solvent peak was used as the reference peak for proton spectra. LC-MS spectra were obtained on Agilent 1100 HPLC LC-MS ion trap electrospray ionization (ESI) mass spectrometer.

Synthesis of YKL-06-061



2-chloro-N-cyclobutyl-5-((2,6-dimethylphenylamino)methyl)pyrimidin-4-amine (2)
A mixture of N-((2,4-dichloropyrimidin-5-yl)methyl)-2,6-dimethylaniline **(1)** (1.10 g, 3.91 mmol), cyclobutanamine (1.67 g, 23.49 mmol) and DIPEA (6 mL) in THF (100 mL) was stirred at room temperature overnight. Then the mixture was concentrated, the residue was purified by flash column (eluting with ethyl acetate /PE = 0-15%) to give 2-chloro-N-cyclobutyl-5-((2,6-dimethylphenylamino)methyl)pyrimidin-4-amine **(2)** as white solid (0.90 g, yield 72%). LCMS (m/z): 317 $[M + H]^+$.
7-chloro-1-cyclobutyl-3-(2,6-dimethylphenyl)-3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one (3)
A mixture of 2-chloro-N-cyclobutyl-5-((2,6-dimethylphenylamino)methyl)pyrimidin-4-amine **(2)** (0.75 g, 2.37 mmol) and triphosgene (1.05 g, 3.56 mmol) in DCM (100 mL) was stirred at room temperature for 1 hour, the a solution of NaOH (1.90 g, 47.5 mmol) and *n*-Bu_4NOH (78 mg, 0.301 mmol) in H_2O (24 mL) was added, the resulting mixture was stirred at room temperature overnight. The organic layer was washed with H_2O (50 mL × 2), dried with Na_2SO_4, filtered and concentrated, the residue was purified by flash column (eluting with ethyl acetate /PE = 0-15%) to give 7-chloro-1-cyclobutyl-3-(2,6-dimethylphenyl)-3,4-dihydropyrimido[4,5-d] pyrimidin-2(1H)-one **(3)** as white solid (750 mg, yield 92%). LCMS (m/z): 343 $[M + H]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 8.11 (s, 1H), 7.11-7.18 (m, 3H), 4.83-4.92 (m, 1H), 4.47 (d, *J* = 0.8Hz, 2H), 2.52-2.59 (m, 4H), 2.22 (s, 6H), 1.74-1.92 (m, 2H).
1-cyclobutyl-3-(2,6-dimethylphenyl)-7-(2-methoxy-4-(4-methylpiperazin-1-yl)phenylamino)-3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one (YKL-06-061)
A mixture of 7-chloro-1-cyclobutyl-3-(2,6-dimethylphenyl)-3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one **(3)** (70 mg, 0.204 mmol), 2-methoxy-4-(4-methylpiperazin-1-yl)aniline (66 mg, 0.298 mmol) and TFA (0.5 mL) in *n*-BuOH (5 mL) was stirred at 110°C overnight, the mixture was concentrated, the residue was purified by prep-HPLC (0.05%NH_4HCO_3 in CH_3CN-H_2O) to give **YKL-06-061** as white solid (50 mg, yield 47%). LCMS (m/z): 528 $[M + H]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 8.22 (d, *J* = 8.4 Hz, 1H), 7.95 (s, 1H), 7.36 (s, 1H), 7.09 - 7.16 (m, 3H), 6.56 - 6.60 (m, 2H), 4.90 - 4.98 (m, 1H), 4.37 (s, 2H), 3.90 (s, 3H), 3.20 (t, *J* = 5.2 Hz, 4H), 2.58 - 2.68 (m, 6H), 2.46-2.54 (m, 2H), 2.38 (s, 3H), 2.23 (s, 6H), 1.74-1.89 (m, 2H).

Synthesis of YKL-06-062



2-chloro-N-cyclohexyl-5-((2,6-dimethylphenylamino)methyl)pyrimidin-4-amine (4)
A mixture of A mixture of N-((2,4-dichloropyrimidin-5-yl)methyl)-2,6-dimethylaniline **(1)** (1.5g, 5.34 mmol), cyclohexanamine (3.17 g, 32.0 mmol) and DIPEA (6 mL) was stirred at room temperature overnight. Then the mixture was concentrated, the residue was purified by flash column (eluting with ethyl acetate /PE = 0-12%) to give 2-chloro-N-cyclohexyl-5-((2,6-dimethylphenylamino)methyl)pyrimidin-4-amine **(4)** as white solid (1.5 g, yield 81%). LCMS (m/z): 345 $[M + H]^+$.
7-chloro-1-cyclohexyl-3-(2,6-dimethylphenyl)-3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one (5)
A mixture of 2-chloro-N-cyclohexyl-5-((2,6-dimethylphenylamino)methyl)pyrimidin-4-amine **(4)** (1.50 g, 4.36 mmol) and triphosgene (1.94 g, 6.54 mmol) in DCM (100 mL) was stirred at room temperature for 30 minutes, then a solution of NaOH (3.49 g, 87.3 mmol) and n-Bu_4NOH (78 mg, 0.301 mmol) in H_2O (43 mL) was added, the resulting mixture was stirred at room temperature overnight. The organic layer was washed with H_2O (50 mL × 2), dried with Na_2SO_4, filtered and concentrated to give crude product, then purified by flash column (eluting with ethyl acetate /PE = 0-20%) to give 7-chloro-1-cyclohexyl-3-(2,6-dimethylphenyl)-3,4-dihydro-
pyrimido[4,5-d]pyrimidin-2(1H)-one **(5)** as white solid (1.1 g, yield 68%). LCMS (m/z): 371 $[M + H]^+$.
^{1}H-NMR ($CDCl_3$, 400 MHz): δ 8.08 (s, 1H), 7.11-7.18 (m, 3H), 4.63-4.71 (m, 1H), 4.49 (d, *J* = 0.8Hz, 2H), 2.45-2.56 (m, 2H), 2.22 (s, 6H), 1.65-1.86 (m, 5H), 1.34-1.43 (m, 2H), 1.17-1.28 (m, 1H).
1-cyclohexyl-3-(2,6-dimethylphenyl)-7-(4-(4-methylpiperazin-1-yl)phenylamino)-3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one (YKL-06-062)

A mixture of 7-chloro-1-cyclohexyl-3-(2,6-dimethylphenyl)-3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one **(5)** (94 mg, 0.254 mmol), 4-(4-methylpiperazin-1-yl)aniline (97 mg, 0.508 mmol) and TFA (0.5 mL) in *n*-BuOH (8 mL) was stirred at 110°C overnight. The mixture was concentrated, the residue was purified by prep-HPLC (0.05%NH_4HCO_3 in CH_3CN-H_2O) to give **YKL-06-062** as light yellow solid (94 mg, yield 70%). LCMS (m/z): 526 $[M + H]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.92 (s, 1H), 7.46 - 7.50 (m, 2H), 7.09 - 7.15 (m, 3H), 7.02 (s, 1H), 6.93 - 6.96 (m, 2H), 4.61 - 4.69 (m, 1H), 4.38 (s, 2H), 3.19 (t, *J* = 5.2 Hz, 4H), 2.61 (t, *J* = 5.2 Hz, 4H), 2.43-2.53 (m, 2H), 2.36 (s, 3H), 2.23 (s, 6H), 1.76 - 1.86 (m, 4H), 1.66 (d, *J* = 12.4 Hz, 1H), 1.32 - 1.43 (m, 2H), 1.14-1.23 (m, 1H).