Supplementary Material

Nuclear entry of CRTC1 as druggable target of acquired pigmentary disorder

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Table S1. Nucleotide sequence of RT-PCR primer

Target	Nucleotide sequence (amplicon)	
β-Catenin (B16-F0)	Forward	5’-TGCAGATCTTGGACTGGAC-3’
	Reverse	5’-CATGCTCCATCATAGGGTCCA-3’ (133 bp)
CRTC1 (B16-F0)	Forward	5’-TCCCCAACATCATCCTCAC-3’
	Reverse	5’-GGTCAATCTTCAGCTCGTC-3’ (138 bp)
KPNA1 (MNT1)	Forward	5'-GTGATCTCCTCACGGTCATG-3'
	Reverse	5'-CATAGGAGCCTCACACTG-3' (314 bp)
KPNA2 (MNT1)	Forward	5’-TCTGCTTGGGCACTCACT-3’
	Reverse	5’-TGCAGGAGCCGAACTAAG-3’ (365 bp)
KPNA4 (MNT1)	Forward	5’-ATGCTTCAAGTGATAACCAAGG-3’
	Reverse	5’ CAAGACAATGGACTAAAATGG-3’ (135 bp)
MITF-M (B16-F0)	Forward	5’-CTAGAATACAGTCACTACCAG-3’
	Reverse	5’-CCATCAAGCCCAAAATTTCTT-3’ (404 bp)
MITF-M (guinea pig)	Forward	5’-GGTGCCGATGGAAGTCCTTA-3’
	Reverse	5’-CCATCAAGCCCAGAATTTCTT-3’ (384 bp)
MITF-M (HEM, MNT1)	Forward	5'-TCTACCGTCTCTCACTGGATTGG-3'
	Reverse	5'-GCTTTACCTGCTGCCGTTGG-3’ (142 bp)
SOX10 (B16-F0)	Forward	5’-CAGACTGGAGGAGAGGTCGG-3’
	Reverse	5’-GGTCTTGTTCCTCGGCCATG-3’ (122 bp)
SOX10 (MNT1)	Forward	5'- AGTACCCGCACCTGCACA-3'
	Reverse	5'- GAAGGGGCGCTTGTCACT-3’ (86 bp)
TYR (B16-F0)	Forward	5’-CATTTTTGATTTGAGTGTCT-3’
	Reverse	5’-TGTGGTAGTCGTCTTTGTCC-3’ (1,211 bp)
TYR (guinea pig)	Forward	5’-CATCTTTGATTTGAGTGTC-3’
	Reverse	5’-CTTTTACAAATGGCTTTGAT-3’ (1,231 bp)
β-Actin (B16-F0)	Forward	5’-TGGAATCCTGTGGCATCCATGAAAC-3’
	Reverse	5’-TAAAACGCAGCTCAGTAACAGTCCG-3’ (349 bp)

β-Actin (B16-F0)	Forward	5’-TGCAGATCTTGGACTGGAC-3’
	Reverse	5’-CATGCTCCATCATAGGGTCCA-3’ (134 bp)
β-Actin (HEM, MNT1)	Forward	5'-GAGACCTTCAACACCCCAGCC-3'
	Reverse	5'-GGCCATCTCTTGCTCGAAGTC-3‘ (312 bp)

Table S2. Nucleotide sequence of PCR primer in ChIP assay

Target	Nucleotide sequence (amplicon)		ChIP
CREB-responsive Element (CRE)	Forward	5'-TGGGGACTTGGCCTTGATCT-3'	Anti-CRTC1
	Reverse	5'-ATATCAGTTTCCCTGCTGGCT-3' (114 bp)	
LEF1-responsive element	Forward	5'-TGGGGACTTGGCCTTGATCT-3'	Anti-β-catenin
	Reverse	5'-ATATCAGTTTCCCTGCTGGCT-3' (114 bp)	
SOX10-responsive element	Forward	5'-TCTGAAGAGGGCATCCAGCT-3'	Anti-SOX10
	Reverse	5'-AGATCAAGGCCAAGTCCCCA-3' (102 bp)	

Quantitative PCR was designed to encompass CREB-, LEF- or SOX10-responsive *cis*-acting element at the MITF-M promoter in mice. CRTC1 and β-catenin assemble to CREB and LEF1 on the DNA motifs, respectively.

Table S3. Nucleotide sequence of siRNA

Target	Nucleotide sequence
CRTC1 (B16-F0)	5’-UGGACAGAGUAUAUCGUGA-3’
KPNA1 (MNT1)	5’-AAUGUGCUUUCCUGGUUGCUG-3’
KPNA2 (MNT1)	5’-CAGUGUUCCGAGACUUGGUUA-3’
KPNA4 (MNT1)	5’-CAACUUAUGUCGCCACAAA-3’

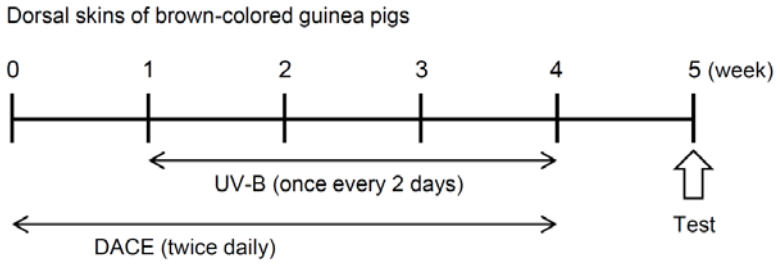


Figure S1. Experimental protocol of UV-B-induced skin hyperpigmentation. Dorsal skins of guinea pigs were topically treated with DACE (0.03-0.1%) in a twice-daily regimen for four weeks, and irradiated with UV-B (350 mJ/cm^2) once every two days for three weeks. Skin hyperpigmentation was measured at one week after the end of the UV-B radiation.

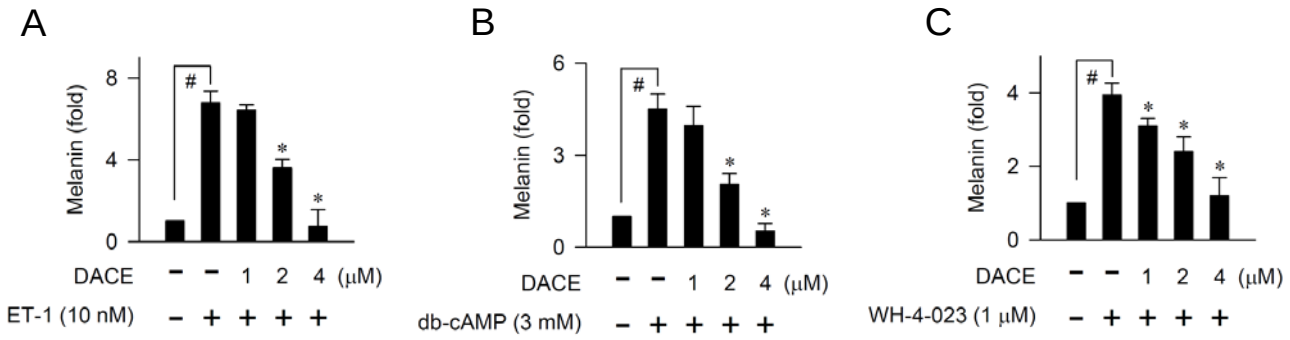


Figure S2. Effect of DACE on ET-, db-cAMP- or WH-4-023-induced melanin production. B16-F0 cells were stimulated with ET-1 (A), db-cAMP (B) or WH-4-023 (C) for 72 h in the presence of DACE. Melanin pigments were quantified by measuring absorbance values at 405 nm, and are represented as relative fold change. Data are mean ± SEM (n = 5). $^{\#}P < 0.05$ vs. medium alone. $^{*}P < 0.05$ vs. ET-1, db-cAMP or WH-4-023 alone.

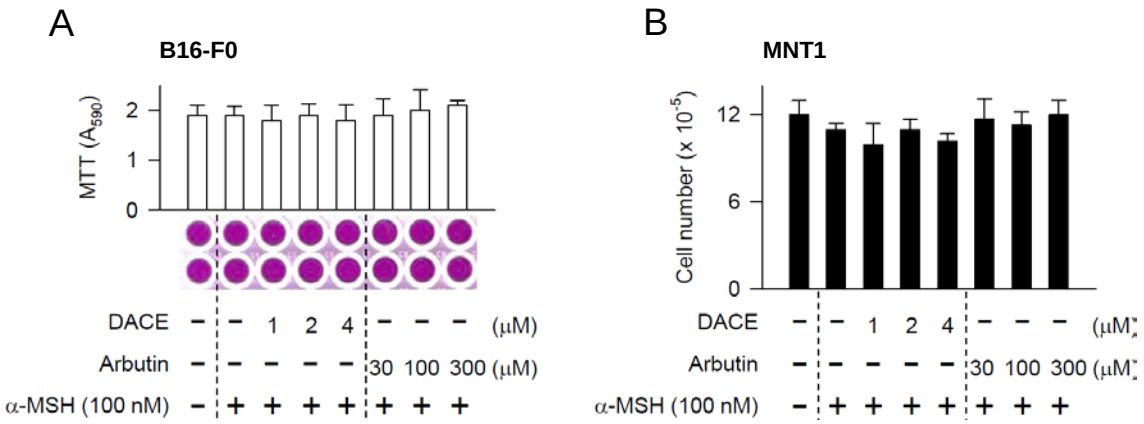


Figure S3. Effect of DACE on cell viability. (A) B16-F0 cells were incubated with DACE for 72 h in the presence of α-MSH. The cells were reacted with 0.5 mg/ml MTT for 1 h. Formazan crystals were dissolved in 99% DMSO, and measured absorbance values at 590 nm (A_{590}). (B) MNT1 cells were incubated with DACE for 72 h in the presence of α-MSH. Cell numbers were counted after exclusion with trypan blue dye. Data are mean ± SEM (n = 3)

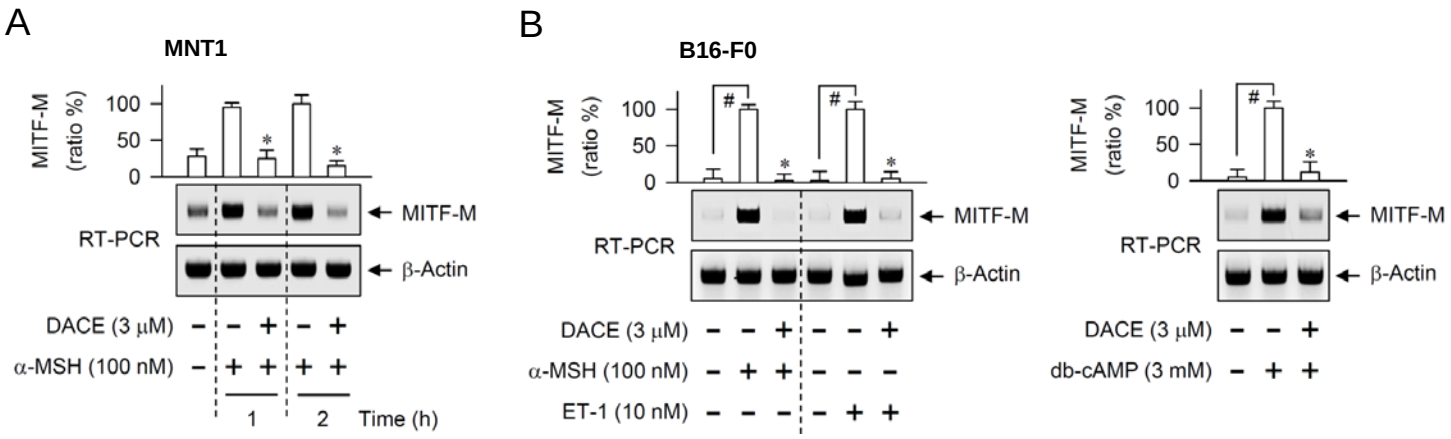


Figure S4. Effect of DACE on α-MSH-, ET-1- or db-cAMP-induced MITF-M transcription. (A) MNT1 cells were pretreated with DACE for 2 h and stimulated with α-MSH for indicated time points in the presence of DACE. (B) B16-F0 cells were pretreated with DACE for 2 h and stimulated with α-MSH, ET-1 or db-cAMP for another 2 h in the presence of DACE. Total RNAs were subjected to RT-PCR analysis of MITF-M with the internal control β-actin. Data are mean ± SEM (n = 3). $^{\#}P < 0.05$ vs. medium alone. $^{*}P < 0.05$ vs. α-MSH, ET-1 or db-cAMP alone.

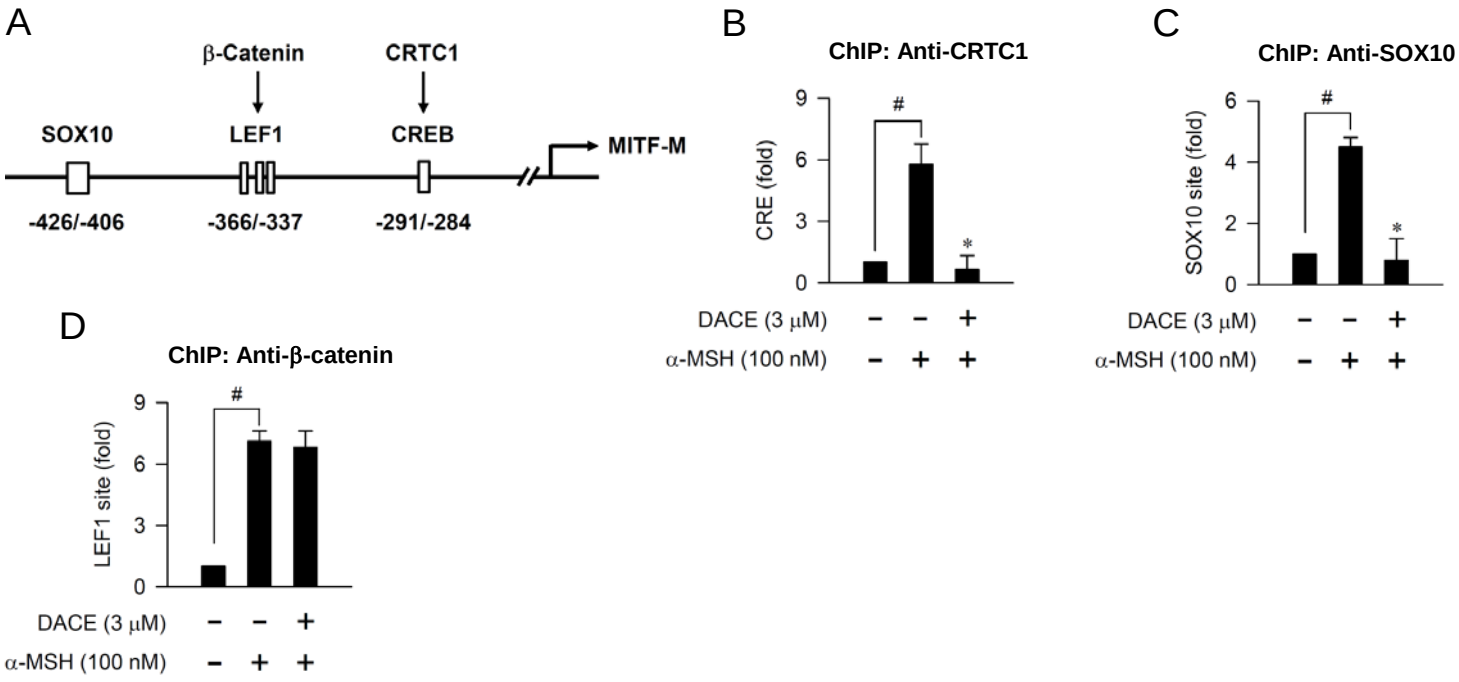


Figure S5. Effect of DACE on α-MSH-induced transcriptional ability of CREB/CRTC1 heterodimer, SOX10 or LEF1/β-catenin heterodimer. Schematic representation of CREB-, LEF1- or SOX10-responsive *cis*-acting elements at the MITF-M promoter in mice. CRTC1 or β-catenin assembles to CREB or LEF1 on the DNA motif. B16-F0 cells were pretreated with DACE for 2 h, stimulated with α-MSH for 20 min (B, D) or 1 h (C) in the presence of DACE, and subjected to chromatin immunoprecipitation (ChIP) analysis. After cross-linked between DNA and proteins, chromatin fragments were precipitated with anti-CRTC1 (B), anti-SOX10 (C) or anti-β-catenin antibody (D). Input and precipitated DNAs were subjected to quantitative PCR encompassing CREB-responsive CRE motif (B), SOX10- (C) or LEF1-responsive *cis*-acting element (D) at the MITF-M promoter. Data are mean ± SEM (n = 3). $^{\#}P < 0.05$ vs. medium alone. $^{*}P < 0.05$ vs. α-MSH alone.

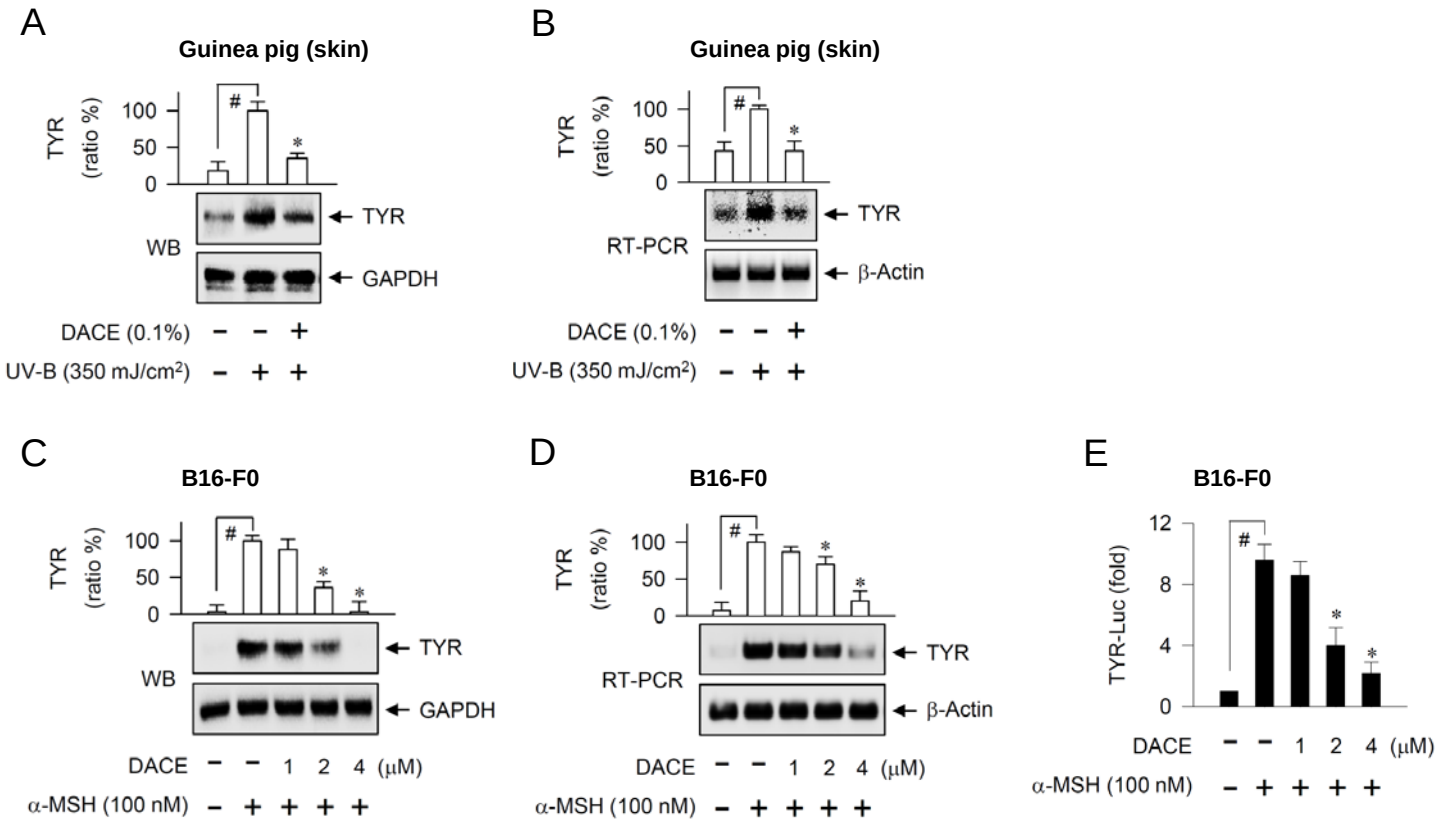


Figure S6. Effect of DACE on TYR expression. (A, B) Dorsal skins of guinea pigs were exposed to UV-B radiation (350 mJ/cm^2) and treated topically with DACE according to the protocol in Figure S1. Skin tissues were biopsied. B16-F0 cells were stimulated with α-MSH for 48 h (C) or 18 h (D) in the presence of DACE. (A, C) Protein extracts were subjected to Western blot analysis (WB) with anti-TYR or anti-GAPDH antibody. (B, D) Total RNAs were subjected to RT-PCR analysis of TYR with the internal control β-actin. (E) B16-F0 cells were transfected with TYR-Luc reporter construct in combination with *Renilla* control vector. The transfected cells were pretreated with DACE for 2 h and stimulated

with α-MSH for 20 h in the presence of DACE. Firefly luciferase activity, reporting promoter activity of TYR gene, is represented as relative fold change after normalizing to *Renilla* activity as a reference of transfection efficiency. Data are mean ± SEM (n = 3). $^{\#}P < 0.05$ vs. normal skin (A, B) or medium alone (C-E). $*P < 0.05$ vs. UV-B alone (A, B) or α-MSH alone (C-E).

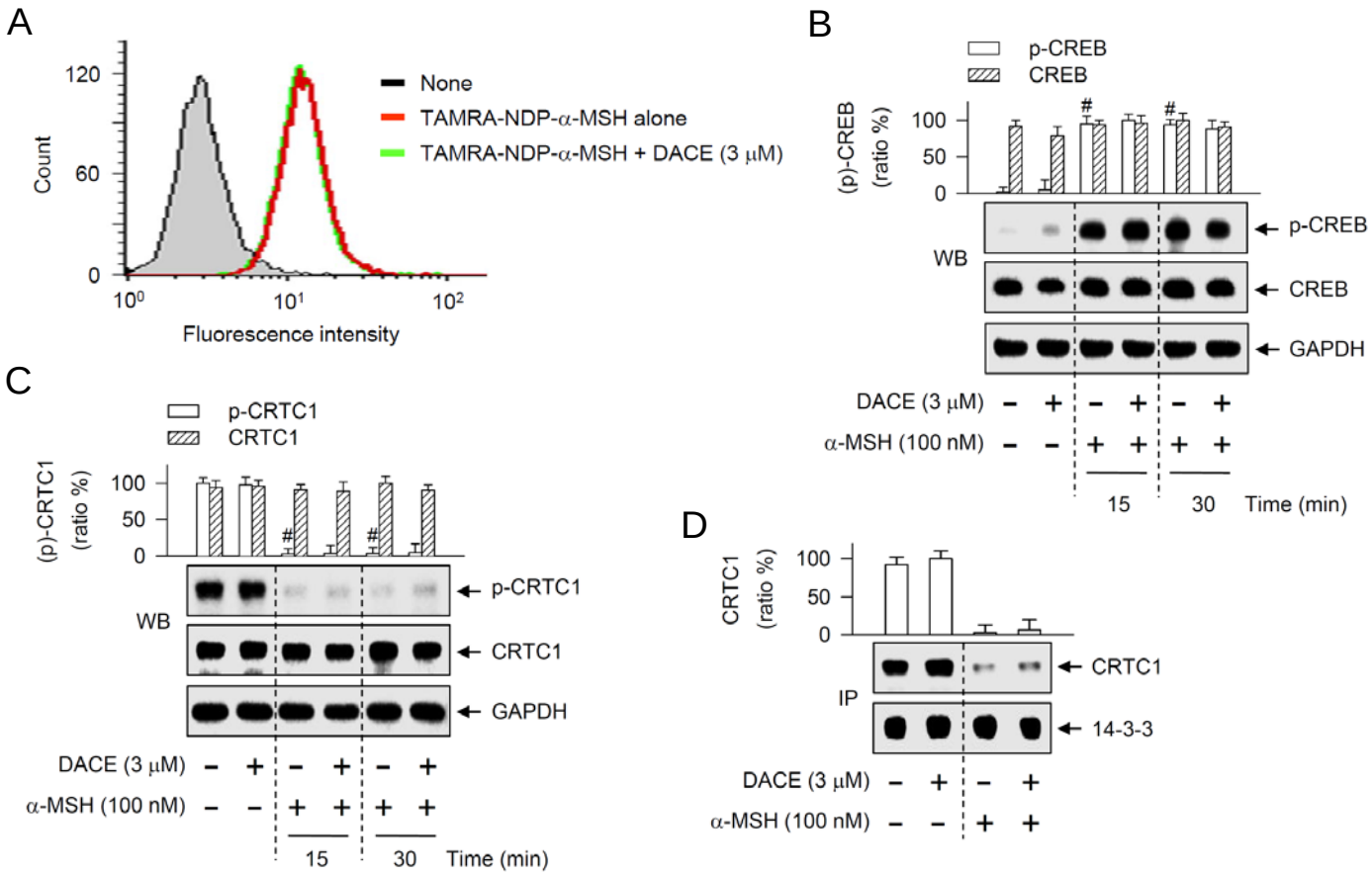


Figure S7. Effect of DACE on the α-MSH-binding to receptor, the phosphorylation of CREB, the dephosphorylation of CRTC1 or the tethering of CRTC1 by 14-3-3 proteins. (A) B16-F0 cells were incubated with 500 nM TAMRA-NDP-α-MSH, a fluorescent α-MSH probe, for 30 min in the presence of DACE, and subjected to flow cytometric analysis. B16-F0 cells were pretreated with DACE for 2 h and stimulated with α-MSH for indicated time points (B, C) or 15 min (D) in the presence of DACE. Cell extracts were subjected to Western blot analysis (WB) with

anti-p-CREB, anti-CREB or anti-GAPDH antibody (B) and with anti-p-CRTC1, anti-CRTC1 or anti-GAPDH antibody (C). (D) For immunoprecipitation (IP), cell extracts were precipitated with the pan antibody against 14-3-3 proteins, and probed with anti-CRTC1 or anti-14-3-3 antibody to detect the co-precipitates. Data are mean ± SEM (n =3). $^{\#}P < 0.05$ vs. medium alone.

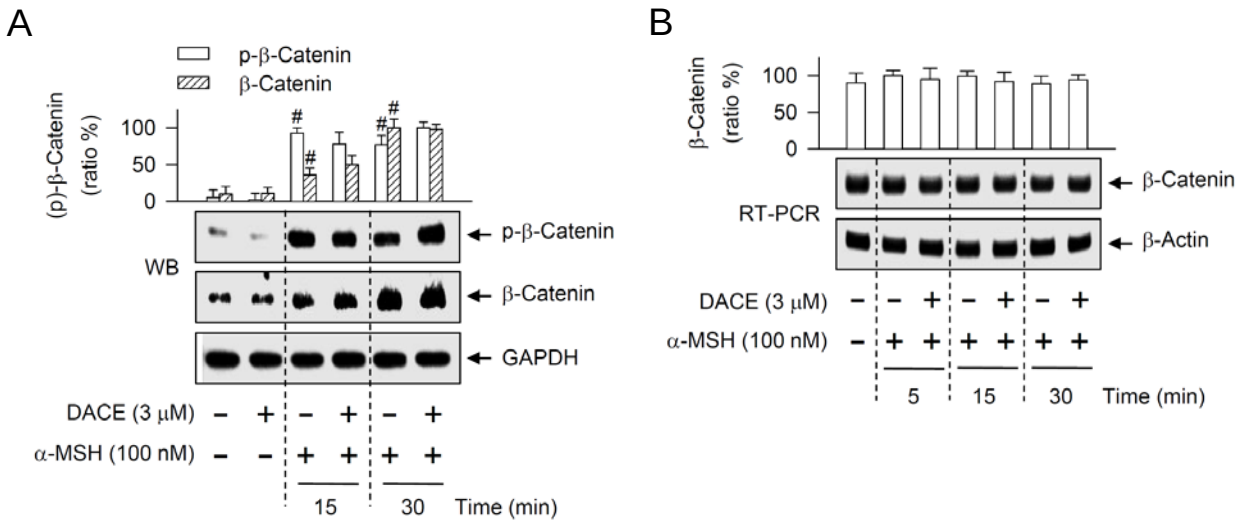


Figure S8. Effect of DACE on the phosphorylation or expression of β-catenin. B16-F0 cells were pretreated with DACE for 2 h and stimulated with α-MSH for indicated time points in the presence of DACE. (A) Cell extracts were subjected to Western blot analysis (WB) with anti-p-β-catenin, anti-β-catenin or anti-GAPDH antibody. (B) Total RNAs were subjected to RT-PCR analysis of β-catenin with the internal control β-actin, Data are mean ± SEM (n = 3). $^{\#}P < 0.05$ vs. medium alone.

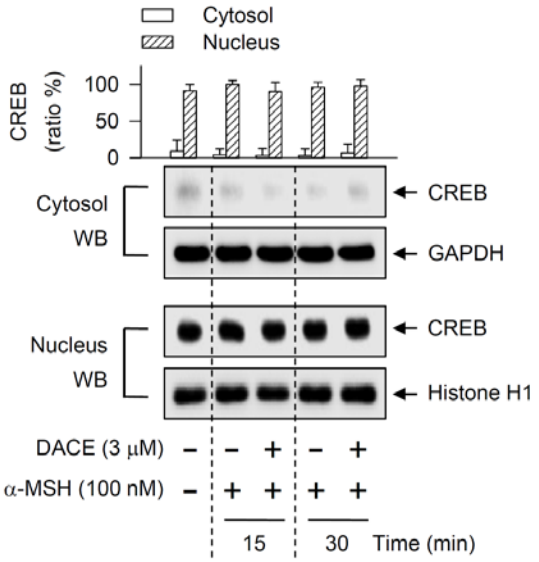


Figure S9. Effect of DACE on the nuclear retention of CREB. B16-F0 cells were pretreated with DACE for 2 h and stimulated with α-MSH for indicated time points in the presence of DACE. Cell extracts were partitioned between the cytosol and the nucleus, and subjected to Western blot analysis (WB) with anti-CREB, anti-GAPDH or anti-histone H1 antibody. Data are mean ± SEM (n = 3).

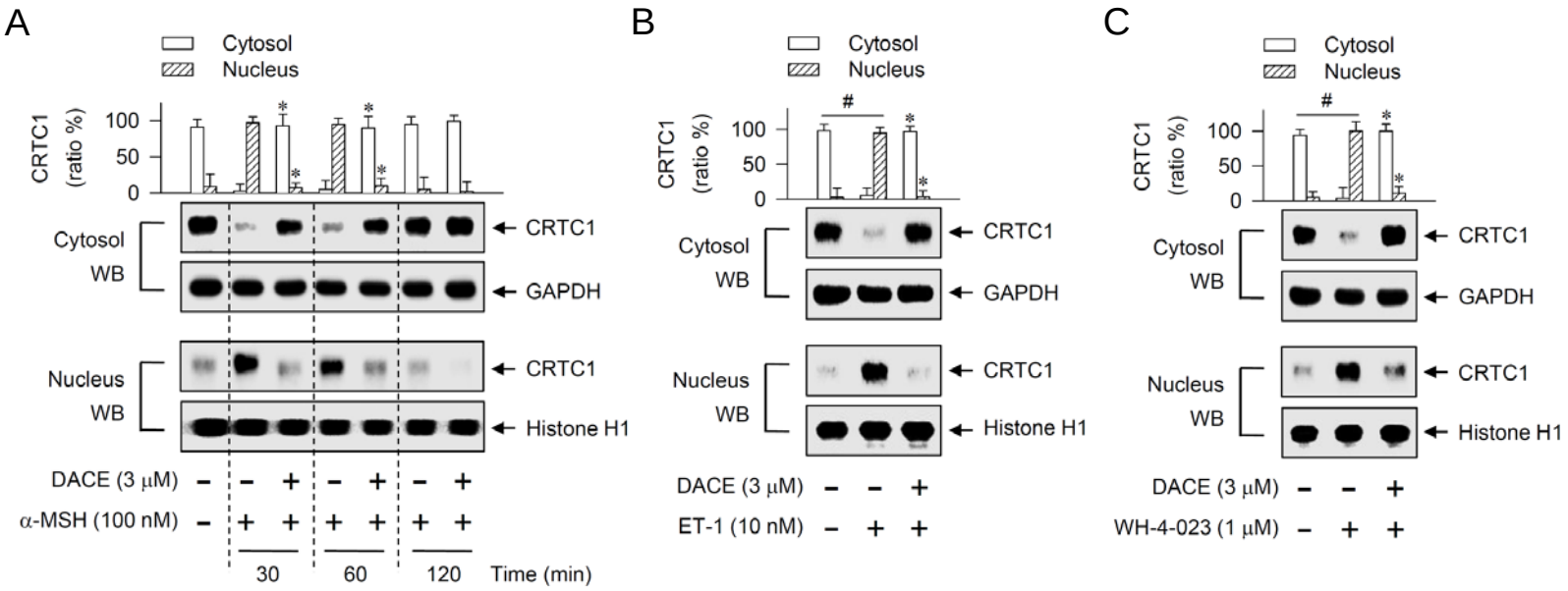
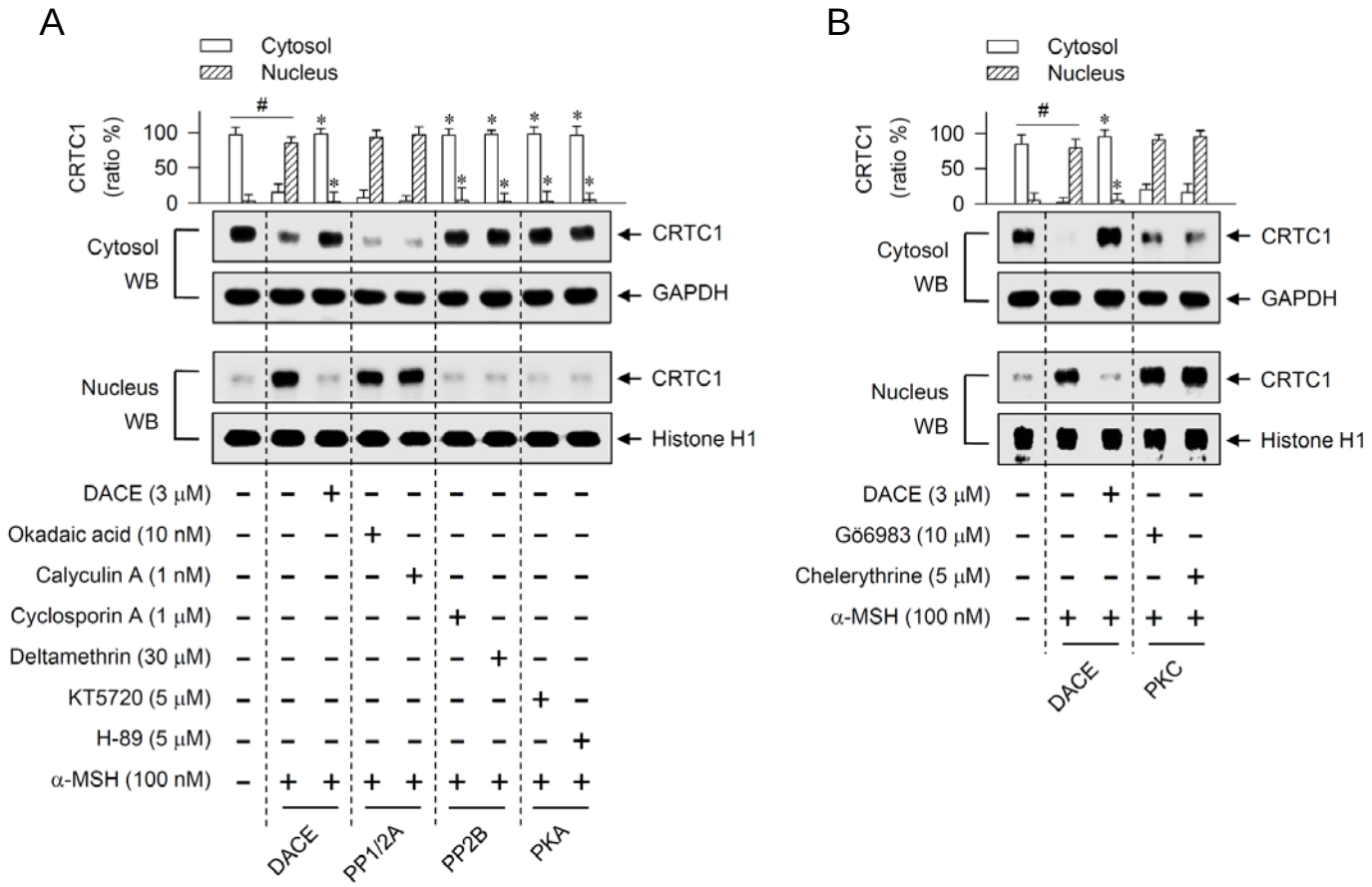


Figure S10. Effect of DACE on α-MSH-, ET-1- or WH-4-023-induced nuclear import of CRTC1. (A) B16-F0 cells were pretreated with DACE for 2 h and stimulated with α-MSH for indicated time points (A), ET-1 for 20 min (B) or WH-4-023 for 20 min (C) in the presence of DACE. Cell extracts were partitioned between the cytosol and the nucleus, and subjected to Western blot analysis (WB) with anti-CRTC1, anti-GAPDH or anti-histone H1 antibody. Data are mean ± SEM (n = 3). **P* < 0.05 vs. α-MSH, ET-1 or WH-4-023 alone. $^{\#}P$ < 0.05 vs. medium alone.

(Figure S11)


A
Cytosol
Nucleus
CRTC1 (ratio %)
100
50
0
#
*
Cytosol WB
CRTC1
GAPDH
Nucleus WB
CRTC1
Histone H1
DACE (3 μM)
Okadaic acid (10 nM)
Calyculin A (1 nM)
Cyclosporin A (1 μM)
Deltamethrin (30 μM)
KT5720 (5 μM)
H-89 (5 μM)
α-MSH (100 nM)
DACE
PP1/2A
PP2B
PKA
B
Cytosol
Nucleus
CRTC1 (ratio %)
100
50
0
#
*
Cytosol WB
CRTC1
GAPDH
Nucleus WB
CRTC1
Histone H1
DACE (3 μM)
Gö6983 (10 μM)
Chelerythrine (5 μM)
α-MSH (100 nM)
DACE
PKC

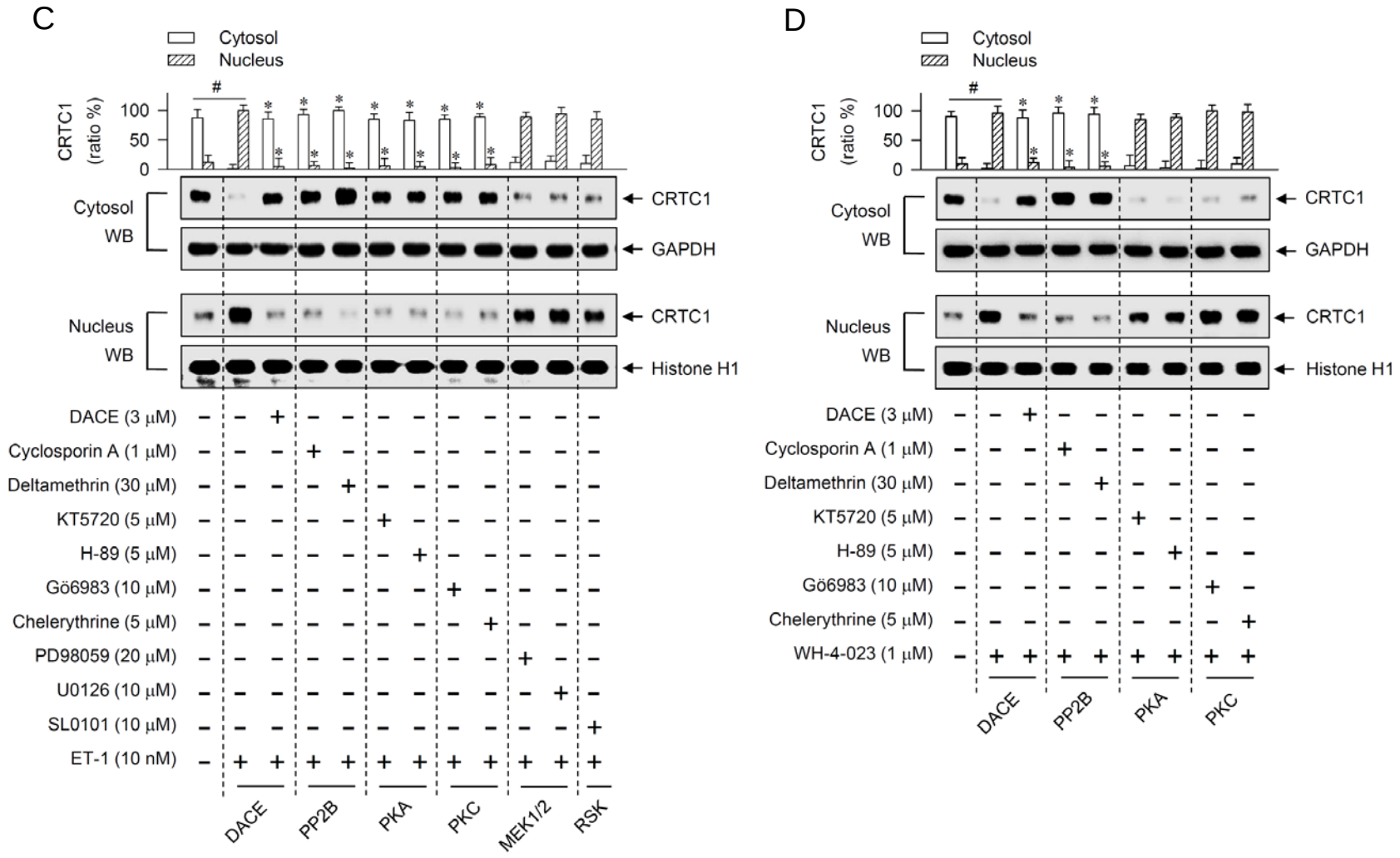


Figure S11. Effects of phosphatase inhibitor and kinase inhibitor on the nuclear import of CRTC1. B16-F0 cells were pretreated with each inhibitor for 2 h and stimulated with α-MSH (A, B), ET-1 (C) or WH-4-023 (D) for 20 min in the presence of each inhibitor. Cell extracts were

partitioned between the cytosol and the nucleus, and subjected to Western blot analysis (WB) with anti-CRTC1, anti-GAPDH or anti-histone H1 antibody. Data are mean ± SEM (n = 3). $^{\#}P < 0.05$ vs. medium alone. $*P < 0.05$ vs. α-MSH, ET-1 or WH-4-023 alone.

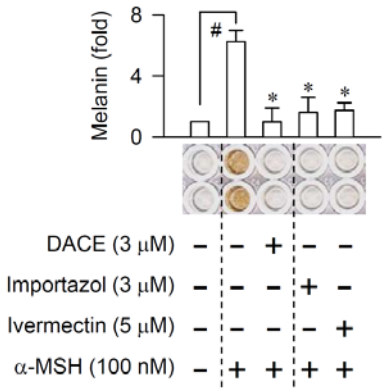


Figure S12. Effects of importazol and ivermectin on melanin production. B16-F0 cells were stimulated with α-MSH for 72 h in the presence of importazol or ivermectin. Melanin pigments were quantified by measuring absorbance values at 405 nm, and are represented as relative fold change. Data are mean ± SEM (n = 3). $^{\#}P < 0.05$ vs. medium alone. $^{*}P < 0.05$ vs. α-MSH alone.