Doctor of Philosophy

CRTC3 depletion prevents melanocyte differentiation in epidermis and hair follicle

표피와 모낭에서 CRTC3 결핍 시
멜라닌 세포 분화 억제에 관한 연구

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CRTC3 depletion prevents melanocyte differentiation in epidermis and hair follicle

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CRTC3 depletion prevents melanocyte differentiation in epidermis and hair follicle

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Abstract

The mechanisms that lead to variation in human skin and hair color are not fully understood and melanocyte system is the most important strategy against environmental hazards. To better understand the molecular control of skin and hair color variation and tanning response (as a tool of environmental prevention response) to cAMP stimuli (UVR), we knocked out modulation of the expression of CRTC3 (CREB-regulated transcriptional co-activator 3), CREB coactivator in mice. Depletion of CRTC3 was sufficient to lighten the color of the mouse hair and tail (ear) skin in Knock-out (K/O). Ultra-structural studies revealed that depletion of CRTC3 inhibited the accumulation of both early and mature melanosomes while melanin contents and early and late melanogenesis-associated genes were much reduced in hairy dorsal skin and tail skin. Knock-down (K/D) of CRTC3 in mel-ab cells and primary cultured cells from the mice showed consistent results with those of in vivo and cAMP tended to recover the downregulation of melanogenesis genes. These results indicated that CRTC3 is a determinant of color of skin and hair both ways of facultative (basal) and stimulated state of adapted melanogenesis. CRTC3 contributes to melanogenesis by modulating CRTC3/MITF.

Key words: skin, melanocyte, melanin, CRTC3, CREB

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List of Abbreviation

CRTC3 (CREB-regulated transcriptional co-activator 3)

CREB: cAMP response element binding protein

cAMP: cyclic adenosine monophosphate

DOPA: 3,4-dihydroxy-L-phenyl-alanine

DHICA: 5,6-dihydroxyindole-2-carboxylic acid

DHI: 5,6-dihydroxyindole

Tyrp1: Tyrosinase related protein-1

DCT (Tyrp2): Tyrosinase related protein-2

MITF: Microphthalmia-associated transcription factor

PKA: protein kinase A

MAPK: Mitogen-activated protein kinase

ERK: Extracellular signal-regulated kinase

Introduction

The skin is the largest organ of the body [1], and acts as most important barriers from biological, physical and chemical attacks, such as bacteria, ultraviolet (UV) irradiation and allergens. It also prevents water evaporation from the body and keeps the body temperature [2,3]. Anatomically, the skin is divided into three main layers; epidermis, dermis (papillary and reticularis) and hypodermis (subcutaneous fat layer) (figure 1). The epidermis, the outermost layer of the skin, is composed of three types of cells; keratinocytes, melanocytes and Langerhans cells. Firstly, the keratinocytes are the predominant cells in the epidermis and hair follicle which produce and store the keratin that gives hair, nails, and skin their hardness, strength, and water-resistant properties. Namely, the keratin is an intracellular fibrous protein that forms a skin barrier for protection from environmental damage. Secondly, Melanocytes are specialized in the synthesis of melanosome and pigment-containing organelles. And melanocytes are mainly present in the basal layer of epidermis and hair follicles at the frequency of one in every five to ten basal keratinocytes. Thirdly, the Langerhans cells play a significant role in skin immune system and serve as dendritic antigen-presenting cells in the basal layers of the epidermis. Both keratinocytes and melanocytes are anatomically related by forming the 'epidermal-melanin unit'. The interaction between keratinocytes and melanocytes is responsible for the synthesis, transfer, transport, and the deposition of melanosomes in the skin. This complete process is known as melanin production. The melanocytes have several

steps of life cycles including embryonic neural crest cells (melanoblasts), migration and proliferation of melanoblasts, differentiation of melanoblasts into melanocytes, maturation of melanocytes, transport of mature melanosomes to keratinocytes and eventual cell death [4, 5]. For the melanocyte development, stem cell factor (SCF) and its receptor (SCFR) on the melanocytes have crucial role [6].

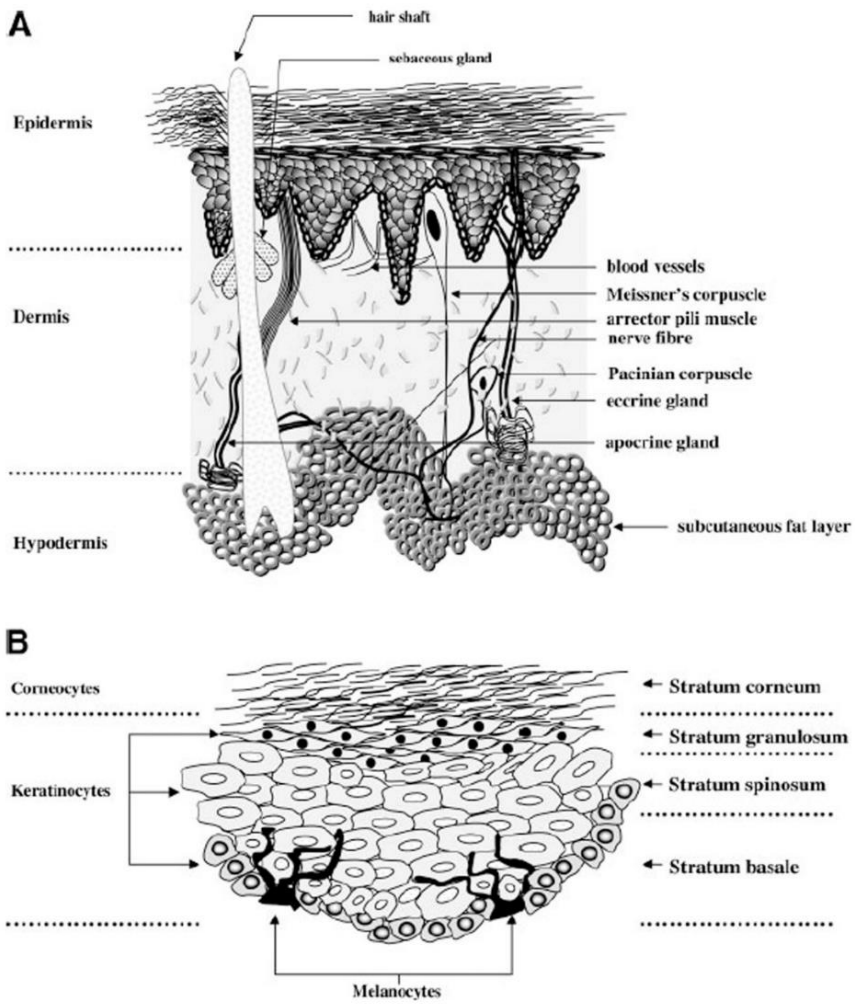

Figure 1. Structure of the skin [7].

A) The different layers and components. B) Layers of the epidermis.

Most animal species have developed unique skin pigmentation patterns. Such pigmentations are utilized to communicate between individual of a species, to appeal sexual attractiveness to a potential mate, and most importantly to defend their species and maintenance of the order of ecosystem. The skin pigmentation pattern of lower vertebrates can change rapidly through the process of aggregation and dispersion, which matches skin pattern with the environmental background and protects themselves from predators [8, 9, 10]. Melanin is a chemically inert yet stable pigment that gives skin and hair its color. In human, as skin cancer is increasing rapidly, in one side, melanin synthesis is essentially beneficial for the maintenance of the skin tissue homeostasis and has an important role against skin cancer in response to environmental hazards such as UVR. In the other side, vitamin D_3 synthesis requires UVR irradiation thus excessive melanin disturbs vitamin D_3 synthesis. Therefore, the melanogenesis process of melanocyte system of human skin must be strictly and reversibly controlled on demand [11,12]. Further, understanding the molecular mechanisms of skin color regulation could lead to the development of new strategies to prevent skin cancer in lighter skin types that are more susceptible to UV-induced damage and to treat hyper-or hypo-pigmentation skin disorders.

Indeed, melanin deposition in human skin tissue is tightly regulated by two major steps, the expression of melanin synthesis genes and ensuing enzymatic reaction of melanin

biosynthesis by these genes. MITF, the master regulator of melanogenesis, integrates various upstream signals and controls the first step of melanogenesis by regulating the expression of downstream target genes, tyrosinase (TYR), and two tyrosinase-related enzymes, tyrosinase related protein-1 (TRP-1) and dopachrome tautomerase (DCT) [13].

Melanins are synthesized from tyrosine via an enzymatic reaction catalyzed by TYR while TRP-1 and DCT are also required to generate the final melanin product. TYR is a membrane glycoprotein controlling the rate of melanin biosynthesis, which catalyzes hydroxylation of L-tyrosine to L-dihydroxydroxyphenylalnine (L-DOPA) as well as the oxidation of DOPA to L-DOPA-quinone and L-DOPA-quinone intermediate is further acted upon by TRP-1 and DCT to form eumelanin. In a condition of low or absence of DCT and TRP-1 expression with high cysteine supply, L-DOPA-quinone intermediate reacts with cysteine and turns into pheomelanin (Figure 2).

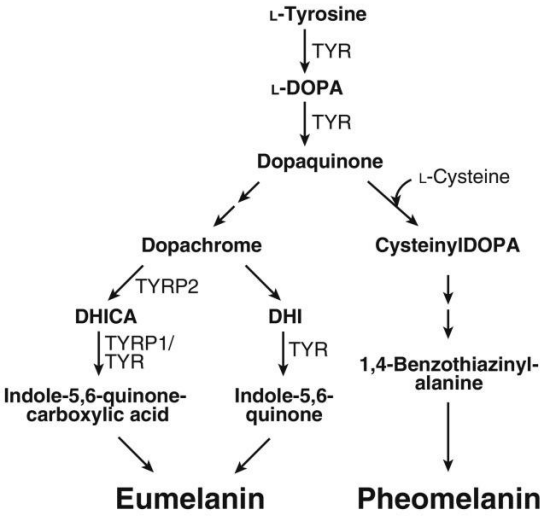

Figure 2. Pathway of melanogenesis [14].

Melanin synthesis occurs within the melanosome, a specific lysosome-related organelle that matures through four morphologic stages (I-IV). Stage I melanosomes are spherical vacuoles that lack TYR activity and melanin. They contain intralumenal fibrils that are comprised mainly of luminal fragments of PMEL17/gp100, an integral membrane protein specifically expressed in pigment-producing cells. In the stage II melanosomes of the elongated, fibrillar organelles, TYR is transported to them and initiates melanin synthesis, and deposits pigment onto internal fibrils that are characteristic of stage III melanosome. Mature stage IV melanosomes are either elliptical or ellipsoidal in shape and demonstrate complete melanization with little TYR enzymatic activity without visible amyloid fibrils. Mature stage IV melanosomes are transferred from melanocytes to adjacent keratinocytes where they accumulate as melanin caps above the keratinocyte nuclei and absorb hazardous UVR which can damage the DNA of skin. The proteins which are involved melanosome formation can be expressed as 'early differentiation' which melanization processes are called late differentiation (table 1).

Early melanosome (stages I and II) **- acidic pH**	PMEL (gp100), Mlan-A (MART-1)
Late melanosome (stages III and IV) **- neutral pH**	Tyrp1, DCT, tyrosinase
Melanosome transporter	ATP7A, OCA2, SLC24A5, SLC45A2, TPC2

Table 1. Melanosomal proteins

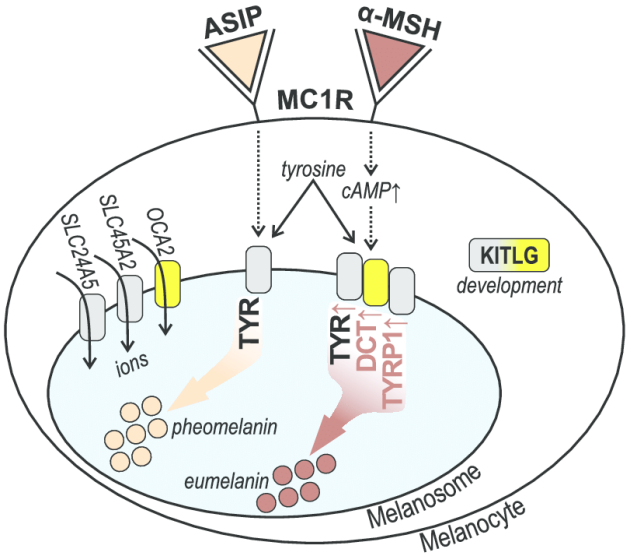

Figure 3. Melanosome transporter [15]

The process of melanogenesis is under hormonal control such as melanocyte stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH), derived from pro-opiomelanocortin (POMC). The melanocortin 1 receptor (MC1R), a seven transmembrane G protein coupled receptor, plays a key role in determining the type of melanin (eumelanin or pheomelanin). Upon being stimulated, MC1R increases the levels of intracellular cAMP, which induces melanogenesis.

Regulation of MITF is the most crucial way of melanocyte biology as MITF is the central master of interconnecting upstream signals and downstream effector signals in melanocytes. Among various upstream signals, although multiple signaling pathways and factors have been shown to be involved in the regulation of MITF expression, cAMP and CREB appears to be the principal mediators in UVR-induced MITF expression and adaptive

melanogenesis. CREB is a ubiquitously expressed basic leucine zipper (bZip) transcription factor that present in the nucleus which binds cAMP-responsive element (CRE) motif of the regulatory region of target genes and its transcriptional activity is reversibly regulated by the phosphorylation status of CREB at Ser133 [17,18]. UVR exposure stabilizes transcription factor p53 which, through binding directly to the pro-opiomelanocortin (POMC) promoter, induces POMC transcription and alpha melanocyte-stimulating hormone (αMSH) and adrenocorticotropic hormone (ACTH) secretion in keratinocytes [19]. αMSH/ACTH released from keratinocytes by triggering melanocortin receptor 1 (MC1R) and Gαs increases intracellular cAMP levels and protein kinas A (PKA) activity in melanocytes. PKA then by phosphorylating CREB at Ser133 site, recruits CBP/p300 recruitment and stimulates its target gene expression, such as MITF [20].

However, despite of numerous studies suggesting the importance of CREB in UVR-induced melanogenesis [21], yet there is no compelling *in vivo* genetic evidence confirming the key role of CREB in this pathway [22] of skin. Recently, three isoforms of CREB-regulated transcription coactivators (CRTCs) were identified to be expressed exclusively and/or redundantly in various organs. The CRTCs have been found that along with CREB phosphorylation, the recruitment of CRTCs to CREB complex is additionally required for full activation of CREB-mediated transcription [23, 24, 25]. Moreover, unlike CREB knockout

(K/O) mice that died after birth, individual CRTC knockout mice survived and exhibited the CREB signaling related traits of appetite, reproduction, glucose, and energy metabolic phonotypes [26, 27, 28].

Based on these backgrounds, we hypothesized K/O of CREB coactivators may be involved in development, differentiation or terminal maturation of melanocytes. To address this question in vivo, we genetically engineered CRTC3 mutant mice. These mice and corresponding mouse cells experiment with cAMP (forskolin) stimulation may offer a good incentive to understand the physiological role of CREB in skin melanocyte system in relation to CRTC3/CREB in facultative and UVR/cAMP-stimulated melanogenesis and pathologic skin hyperpigmentation.

Materials and Methods

1. Animals

C57BL6/J CRTC3 WT (CRTC3+/+), hetero (CRTC3+/-), and CRTC3-null mice were reared under temperature-controlled specific pathogen-free conditions with a 12 h light/dark cycle, free access to water, and a normal chow diet (Purina, Pyeongtaek, Republic of Korea). Age-matched experimental animals of both genders, which were 2-months-old adults and post-natal day 1 pups, were used. C57BL6/J and KRT14-SCF transgene-harbored C57BL6/J mice (KRT14-SCF) were purchased from Jackson Laboratories. Knockouts of CRTC3 were made as previously described [29]. C57BL6/J WT or knockouts of CRTC3 were crossed with KRT14-SCF mice, producing KRT14-SCF; C57BL6/J or KRT14-SCF; CRTC3-null, where both mice had the same genetic background as C57BL6/J.

2. Cell Culture

Mel-ab mouse melanocytes were cultured in Dulbecco's Modified Eagle's medium (DMEM, WELGENE) supplemented with 10% fetal bovine serum 100 nM 12-O-tetradecanoylphorbol-13-acetate (Sigma-Aldrich, St. Louis, MO, USA), 1 nM cholera toxin (Cayman Chemicals, Ann Arbor, MI, USA), and 1% Antibiotic-Antimycotic solution (A.A, Thermo Scientific, Rockford, IL, USA). All cells were maintained in a humid environment at 5% CO_2. B16F10

murine melanoma cells were maintained in DMEM containing 10% fetal bovine serum and 1% A.A.

Normal human epidermal melanocytes (NHMs) (Invitrogen, Carlsbad, CA, USA) at passage 3–5 were cultured in medium 254 supplemented with human melanocyte growth supplement (Invitrogen, Carlsbad, CA, USA). Coculture of NHMs and human keratinocytes HaCaT was generated in M254 and DMEM mixed medium, at a seeding ratio of 2:1 (for melanin assays, Western blotting and qRT-PCR). NHMs were seeded into a 6-well plate at a density of 6×10^5 cells per well. On the next day, HaCaT cells were added to each well at a density of 3×10^5 cells for the coculture.

3. Subcellular localization of CRTC3 and CRTC3 shRNA transfection

Plasmid constructs encoding the CRTC3-EGFP fusion gene were transfected into B16F10 cells using PEI reagents. After 24 h of transfection, CRTC3-EGFP-transfected B16F10 cells were treated with FSK or TPA as indicated by subcellular localization of CRTC3 was monitored by fluorescence microscopy (Observer.Z1, Carl Zeiss, Oberkochen, Germany). To knockdown CRTC3 in Mel-Ab and HaCaT cells, specific short hairpin RNA for CRTC3 was designed and cloned to a pLKO.1 vector. Lentivirus

containing shCRTC3i was infected into Mel-Ab cells followed by selection of puromycin-resistant cells. A shunscrambled RNAi (shUSi)was used as a control. All cloning procedures into AgeI and EcoRI sites were performed according to the common digestion-ligation protocol. For overexpression of CRTC3 in Mel-Ab cells, the gene was cloned in pCDH-CMV-MCS-EF1-puro vector, which has resistance to puromycin for selecting stably transduced cells. Cloning procedures into EcoRI and NotI sites were performed according to the common digestion-ligation protocol.

4. Antibodies

In this experiment, following antibodies were used: tyrosinase (ab180753 and sc-73244; Santa Cruz, Dallas, TX, USA), TRP-1 (ab178676), DCT (ab74073), MITF (C5, ab12039), SOX10 (ab227680), and pmel (ab137078) were obtained from Abcam (Cambridge, UK). total extracellular signal-regulated kinase (ERK, #9102), phospho-ERK (pERK, #3192) and total CREB (#4820), pCREB (ser133, #14001) were purchased from Cell Signaling Technology (Danvers, MA, USA). α-tubulin (Gentex, Holland, MI, USA) and HSP90 (sc-13119, Santa Cruz) were used as an internal loading control. Secondary antibodies used for western blotting

were as below: goat anti-rabbit IgG- horseradish peroxidase (HRP) (1:5,000), goat anti-mouse IgG-HRP (1:5,000), mouse anti-goat IgG-HRP (1:5,000)

5. Western blot analysis

Total protein was extracted from Mel-ab cells washed once with cold PBS and lysed in protein lysis buffer (1% SDS in 100 mM Tris and 5 mM EDTA, pH 7.4), followed by incubation at 100 °C for 10 min. Protein concentrations were determined using a Bradford assay kit (Biorad, Hercules, CA, USA). Next, 20 μg of protein samples per lane were separated by 5.5% or 8% SDS-polyacrylamide gel electrophoresis and transferred onto 0.45μm nitrocellulose membranes (GE healthcare, Chicago, IL, USA). Following block with Tris-buffered saline containing 0.5% Tween 20 and 5% BSA, blots were incubated with the appropriate primary antibodies at a dilution of 1:1000 and then further incubated with horseradish peroxidase-conjugated secondary antibodies. Bound antibodies were detected using an Enhanced Chemiluminescence Kit (Thermo Fisher Scientific, Cheshire, UK). Image analysis was performed using Image J 1.52a software (National Institute of Health, Bethesda, MA, USA) to determine the relative band densities. Membranes were washed with Tris-buffered saline containing 0.1% Tween 20, incubated with secondary antibodies, and signals were detected using ECL Western Blotting Detection Reagents (Thermo Fisher Scientific).

6. Cell viability assay

Viability of cultured cells and cytotoxicity of samples were evaluated using WST assay (Ez-Cytox Cell Viability Assay Kit, Dogen-Bio Co., Ltd., Seoul, Korea). Mel-ab cells were seeded into 24-well culture plate at a density of 6×10^4 cells/well with DMEM. After incubation at 37°C for 24 h, the medium was replaced with fresh medium and cells were treated 10 μM FSK for indicated time period. After treatment, the Ex-Cytox reagent added in medium each well (1/10 volume of the culture medium in a well). After 1 h incubation, the absorbance of each well was measured at 450 nm by microplate reader.

7. Melanin content and intracellular tyrosinase activity assay

Mel-Ab cells were plated onto 6-well culture plates at a density of 3×10^5 in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic solution (without 12-O-tetradecanoylphorbol-13-acetate and cholera toxin) were treated with 10 μM FSK, as indicated in the figures. After treating for 72 hours, the cells were dissolved in 550 μL of 1 N NaOH at 100 °C for 30 min, and the amount of melanin in the supernatant was measured using a microplate reader at an optical density of 405 nm, normalized to the amount of protein used, and reported as the percent change relative to that in the untreated controls.

The tyrosinase activity was evaluated by measuring the rate of dopachrome formation of L-DOPA. After 24 hours incubation, the cells were treated with 10 μM FSK for 3 days, and then the cells were washed by cold PBS and lysed in phosphate buffer (pH 6.8) containing 1% Triton X-100 with repeated freeze/thaw cycles. The lysates were clarified by centrifugation at 15,000 rpm at 4 °C for 10 min. After quantifying the protein levels of the lysate and adjusting the protein concentrations with lysis buffer, 90 μL of supernatant mixed with 10 μL of 10 mM L-DOPA in tyrosinase lysis buffer was incubated at 37 °C. Cellular tyrosinase activity was measured by reading the absorbance at 475 nm using a microplate reader every 10 min for at least 1 h.

8. Isolation of mouse primary melanocytes

The primary melanocytes from newborn control (C57BL6) and CRTC3 KO mice were euthanized by decapitation. The mice were washed by immersion in 70% ethanol for 10 min, then washed by sterile PBS. Superficial longitudinal dorsal incision with the scalpel throughout the whole skin after removing extremities from the mice using surgical scissors. To separate epidermis from dermis, whole skins of mice were incubated in 5 mg/ml Dispase II (Sigma-Aldrich, St. Louis, MO, USA) diluted in PBS at 37℃ for 1.5 hour, allowing the fully spread tissues to float. Epidermis was gently separated from the dermis and chopped into

pieced of about 2 mm X 2 mm fragments. And then, the tissues were incubated for 10 min in TrypLE (GIBCO, Grand Island, NY, USA) at 37°C with gently shaking. The dissociated cells were collected by 100uM pore cell strainer and centrifugation (200 g, 10 min). After removing supernatants, the pellets were resuspended mechanically by repeated pipetting up and down with MBM4 medium (Lonza, Basel, Switzerland). The epidermal cell mixtures in pellets were counted and plated on a culture dishes at a density of $1X10^5$ cells/cm^2. The culture medium was changed after 24 h and kept for another 3 days.

9. Immunohistochemical analysis

Mouse skin tissues were fixed overnight in 4% paraformaldehyde in PBS at 4°C and paraffin embedded. Paraffin-embedded tissues were cut into multiple sections (6-μm-thick) and stained with indicated antibodies and Hematoxylin and Eosin (Vector Laboratories, Burlingame, California, USA) for morphological observation. The melanin index was determined by measuring the percentage of stained area to the total tissue area using ImageJ software (National Institutes of Health). For proceeding with the immunohistochemistry (IHC) staining, the sections were deparaffinized, rehydrated and processed with antigen retrieval solution (Vector). Slides were incubated with indicated antibodies. Next day, after washing 3 times with PBS, add biotinylated secondary antibodies were added and incubated at room

temperature for 30 min. After washing 3 times with PBS, they were incubated with avidin-peroxidase (ABC kit; Vector Laboratories) at room temperature for 1h. The slides were developed using NovaRed substrate kit (Vector Laboratories). Thereafter, dehydrate slides and mounting with coverslips.

Melanin pigment was visualized using Fontana–Masson stain, and multiple areas were randomly photographed using a phase-contrast microscope (BX53, Olympus, Tokyo, Japan). The melanin index was determined by measuring the stained area normalized to total epidermal area using Image J and expressed as percent change relative to controls.

10. Transmission electron microscopy (TEM)

Mel-Ab cells were treated with FSK for 72 h and prepared for electron microscopic analysis. Briefly, cultured cells were detached and fixed in a mixture of 4% paraformaldehyde and 2% glutaraldehyde, followed by osmium tetroxide for 2 h. Two-month-old CTRL (C57BL6/J) and CRTC3-null mice tail tissues were also fixed in the same manner. Then, the fixed samples were dehydrated with ethanol and embedded in epon-araldite resin. Ultrathin sections were stained with 2% uranyl acetate and lead citrate, and then examined by Tecnai 10 TEM (Fei, The Netherlands).

11. Real time quantitative PCR

Total cellular RNA was extracted from the cells using a FavorPrep™ Total RNA Purification Mini Kit according to the manufacturer's instructions (Favorgen, Ping-Tung, Taiwan). Following isolation, the quantity and quality of the RNA were determined using a NanoDrop® ND-1000 Spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). Single-stranded cDNA was synthesized from 1 μg of total RNA using a Revert Aid First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, USA). qRT-PCR was performed using a LightCycler® 480II machine coupled with SYBR Green chemistry (Roche Applied Science, Penzberg, Germany). Initial denaturation was performed at 95 °C for 5 min, followed by amplification at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s for 45 cycles. The cDNA obtained was amplified with the primers listed in **Table 1.**

Table 1. List of primers used for qRT-PCR (F: Forward, R: Reverse)

Name	Forward (3' → 5')	Reverse (5' → 3')
mL32	TCTGGTGAAGCCCAAGATCG	CCTCTGGGTTTCCGCCAGTT
mCRTC3	GAAGTTCAGTGAGAAGATCGC	CCCCGTGGTACTGGGTAAG
mNR4A2	AGTCTGATCAGTGCCCTCGT	GATCTCCATAGAGCCGGTCA
mCREB	CCAGTCTCCACAAGTCCAAACA	GGCACTGTTAGAGTGGTGGTATG
mERK1	CCTGCTGGACCGGATGTTA	TGAGCCAGCCTTCCTCTAC

mERK2	GGAGCAGTATTATGACCCAAGTGA	TCGTCCACTCCATGTCAAACT
mMLANA	GACGAAGTGGATACAGAACCTTG	CTCTTGAGAAGACAGTCGGCTG
mMITF-M	GGGATGCCTTGTTTATGGTG	CACCGCAGACCACTTAGTCC
mTyrp-1	CCCCTAGCCTATATCTCCCT	TACCATCGTGGGGATAATGG
mDCT-	CTTTGCAACCGGGAAGAACG	CCGACTAATCAGCGTTGGGT
mTyr	TTATGCGATGGAACACCTGA	GAGCGGTATGAAAGGAACCA
mPmel	CAAGTTCCCCTGGACTGTGT	GTGCTACCATGTGGCATTTG
mSOX10	CGGACGATGACAAGTTCCCC	GTGAGGGTACTGGTCGGCT
mSLC24A5	AGAGCACGGATGGAGGTATCGT	GCAACATCCTGCGACAGTCCAA
mSLC45A2	ACACAGAGCAGCCAGTACAGGA	CAATCAGGTGGCTGACGCAAAG
mOCA2	ATAGTGAGCAGGGAGGCTGT	ACTGATGGGCCAGCAAAAGA
mSCF	TCCGAAGAGGCCAGAAACTA	TCCCTTTCTCGGGACCTAAT
mET1	ACTTCTGCCACCTGGACATC	GGTGAGCGCACTGACATCTA
mBFGF	AAGCGGCTCTACTGCAAGAACG	CCTTGATAGACACAACTCCTCTC
mPOMC	AAGTGGAGATTCAACACCATTCTTAA	GTCCAGAGCTGAGACACCCTTAC
hGAPDH	CCCATCACCATCTTCCAGGAG	GTTGTCATGGATGACCTTGGC
hCRTC3	GCACCAGCCTGTTCAAAGAC	TCTGCAGCTCCTCTTCCAGT
hMITF-M	TCTACCGTCTCTCACTGGATTGG	GCTTTACCTGCTGCCGTTGG
hTyrp-1	CCCCTAGCCTATATCTCCCTTTT	TACCATCGTGGGGATAATGGC
hDCT	TGTGCAAGATTGCCTGTCTC	GTTGCTCTGCGGTTAGGAAG
hTyr	TCAGCACCCCACAAATCCTAA	AATCGGCTACAGACAATCTGC
hPmel	GAAGACCTGGGGGCCAATACT	TGAAGGCTGAGCTGGAATGA
hSOX10	ATGAACGCCTTCATGGTGTGGG	CGCTTGTCACTTTCGTTCAGCAG
hSLC24A5	AGCGCAGAGATGGAGGCATCAT	TGTGCCTGCAACATCCTGAGAC

hSLC45A2	CTTTGCATCAGCCACCTCATTGG	TCCAACCTCGACTCCTCTTTCG
hOCA2	AGGAGAAGCGAGCACTCAGTGA	CACCTGGGTTTCTACACTTCCG
hSCF	TGGTGGCAAATCTTCCCAAAAAG	CAATGACTTGGCAAAACATCCA
hET1	AAGGCAACAGACGCTGAAAAT	CGACCTGGTTTGTCTTAGGTG
hBFGF	AGCGGCTGTACTGCAAAAACGG	CCTTTGATAGACACAACTCCTCTC
hPOMC	CTGGAGAGCAGCCAGTGTCAG	AGAGGCTGCTCGTCGCCATTTC

12. Statistical analysis

Data are represented as mean ± SEM, and statistical significance was determined by an unpaired student t-test using the GraphPad Prism program. In this study, $P < 0.05$, $P < 0.01$, and $P < 0.001$ are represented as *, **, and ***, respectively, and were considered statistically significant.

Results

1. CRTC3 whole body K/O mice skin showed less melanin accumulation.

The fur and skin pigmentation on the ear and tail in CRTC3 null and heterozygote (HT) mice compared to C57BL/6 wild type (WT) mice (Fig 1. A). Genotype was analyzed by PCR analysis (Fig 1. B). When visualizing WT, HT and null fur pigment and structure by phase-contrast microscopy, null mice had lighter fur color than WT and HT, but all types had same structure and thickness (Fig 1. C). When we analyzed hair melanin content in CRTC3 WT, HT and null mice, the fur melanin was significantly decreased in null mouse (63.7%), and HT mouse (86.5%) was also decreased, although not significantly compared to WT (100% pigmentation) mouse (Fig 1. D). We also adopted K14-SCF (stem cell factor) mouse and produced K14-SCF-CRTC3 null mice in order to observe change of epidermal melanocyte biology. In the K14-SCF transgenic mouse model system, constitutive expression of SCF by epidermal keratinocytes results in retention of melanocytes in the interfollicular basal layer and pigmentation of the epidermis itself [30, 31, 32]. The K14-SCF WT mice had a more intensely eumelanotic phenotype with black skin and fur due to robust accumulation of epidermal eumelanin than control C57BL/6 WT mice. K14-SCF-CRTC3 null mice had lighter skin and fur color than that of K14-SCF control mouse (Fig 1. E).

On visualization by phase-contrast microscopy, the color of fur in CRTC3 Null mice

was much lighter than that of WT mouse showing less melanized melanosomes in follicular epithelium and hair shaft (Fig 2. A). While melanocytes are primarily found in the skin and hair follicles in human, they are largely restricted to the hair follicles in adult mice. In adult mice, epidermal melanocytes are only conserved on ear, tail and feet. Therefore, tail skin was stained by Fontana-masson solution. The melanin accumulation was much down-regulated in CRTC3 null 2-month-old adult mouse tail skin (Fig 2. B, C). Subsequently, we estimated whether the reduction of melanin is associated with altered melanogenic gene expression, assessed by RNA sequencing analysis (Table 1) and Western blotting in 2-month-old CRTC3 WT (n=3) and null (n=3) mice tail tissue (Fig 2. D). As expected, the level of MITF, tyrosinase, Tyrp1 and DCT was significantly down-regulated in CRTC3 null mice tail skin tissue (Fig 2. E, F), indicating CRTC3 ablation resulted in light coat color and has down-regulated in melanin accumulation.

Next, we wondered whether thus number of surviving melanocytes would reduce in CRTC3 null mice, Hematoxylin-eosin (H&E) staining was performed for counting the number of epidermal melanocytes and hair follicle. There was no difference of epidermal and hair follicular melanocytes identified as cells surrounded by clear halos at the dermal-epidermal junction between CRTC3 null and WT mouse (Fig 3. A, B). Indicating that, melanin accumulation is reduced in the tail skin of CRTC3 Null mice without significant reduction of

melanocytes in epidermis and hair follicle (Fig 3. B). By electron microscopy, rather melanosomes of hair unit are markedly decreased in Null mice on tail skin and those of tail epidermis are almost completely invisible in Null mice (Fig 3. C, D, E). When observing ocular structure in CRTC3 Null mice, microphthalmia or were not observed in decoloration (Fig 3. F, G).

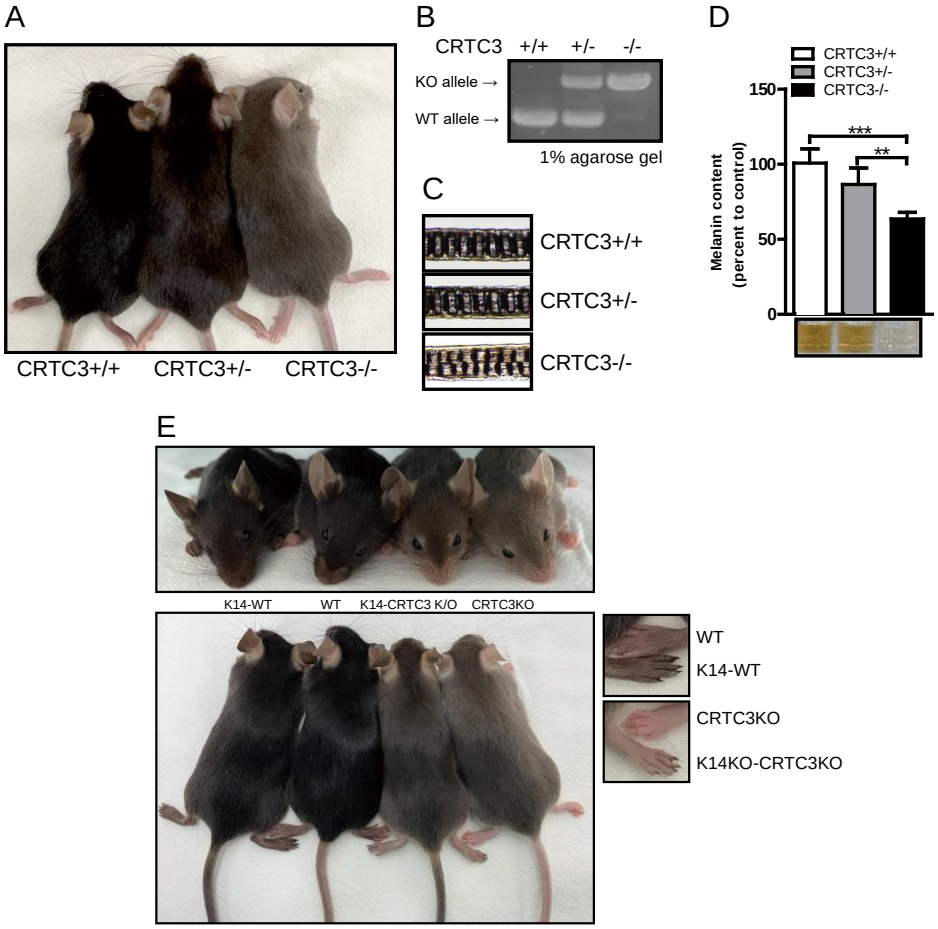

Figure 1. Comparison of hair and fur pigmentation between CRTC3 WT, K/O and K14-SCF WT and K14-SCF CRTC3 K/O mice skin.

(A, C) Detailed comparison of hair and fur pigmentation between 2-month-old CRTC3 WT, HT and Null mice. (B) Genotypes of WT, heterozygous and CRTC3 mutant mice genomic DNA by PCR analysis. WT and K/O shows about 650bp and 976bp, respectively. (D) The hair melanin content of CRTC3 WT, HT and K/O (n=4, 3, 5 per group) mouse. (E) Detailed comparison of hair and fur pigmentation between 3-month-old K14 WT, CRTC3 WT, K14-CRTC3 null and CRTC3 Null mice. Data are expressed as means ± SD of three independent experiments. ** $p < 0.01$ and *** $p < 0.001$ vs controls

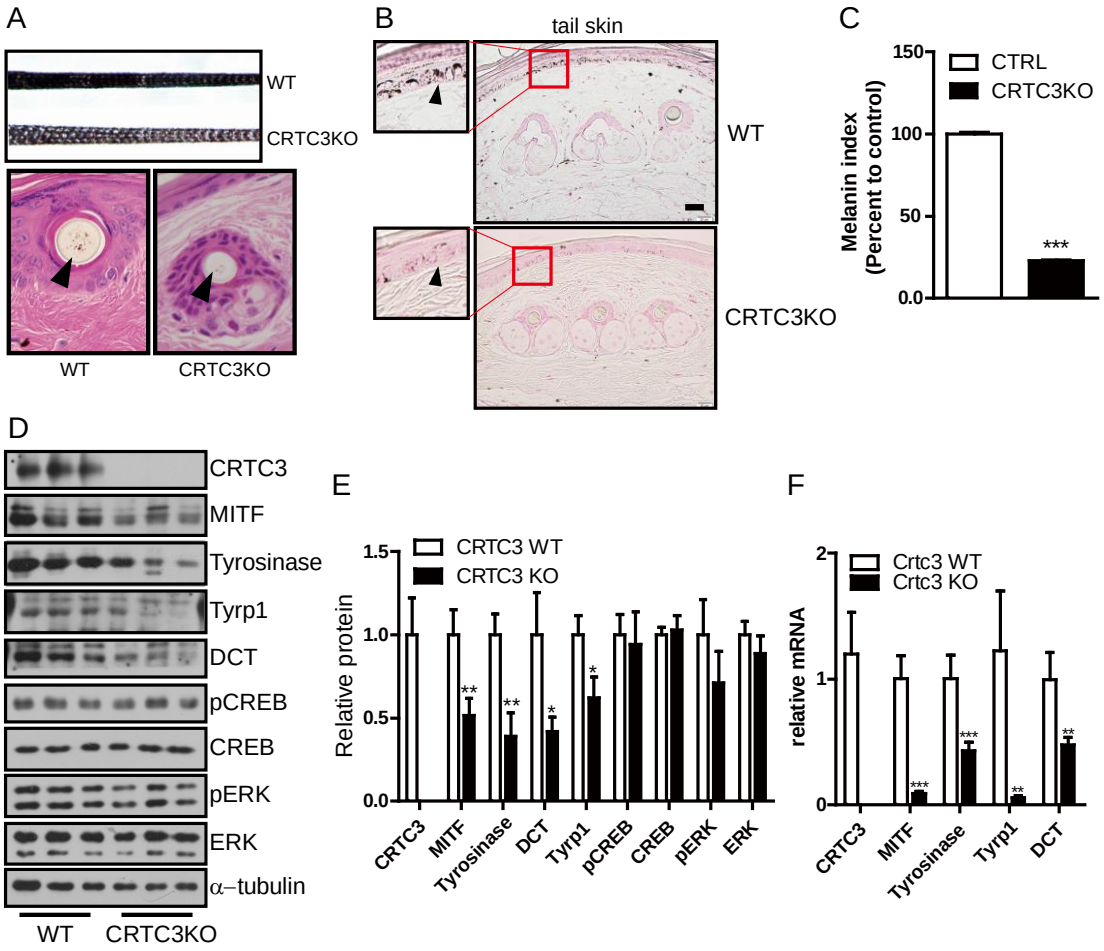

Figure 2. CRTC3 whole body K/O mice skin showed less melanin accumulation.

(A) Representative phase-contrast microscopic images and H&E staining of CRTC3 WT and Null mice hair. (B, C) Melanin accumulation was visualized quantified using Fontana-masson staining and the melanin index. (D) Protein expression of melanogenic genes (tyrosinase, MITF, Tyrp1 and DCT) in mouse tail tissue as assessed by Western blotting. α-tubulin was used as an internal loading control. (E) Quantification relative protein amount was represented by graph. (F) Relative mRNA expression of melanogenic genes (tyrosinase, MITF, Tyrp1 and DCT) in mouse tail tissue as assessed by qRT-PCR. Data are expressed as means ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs controls

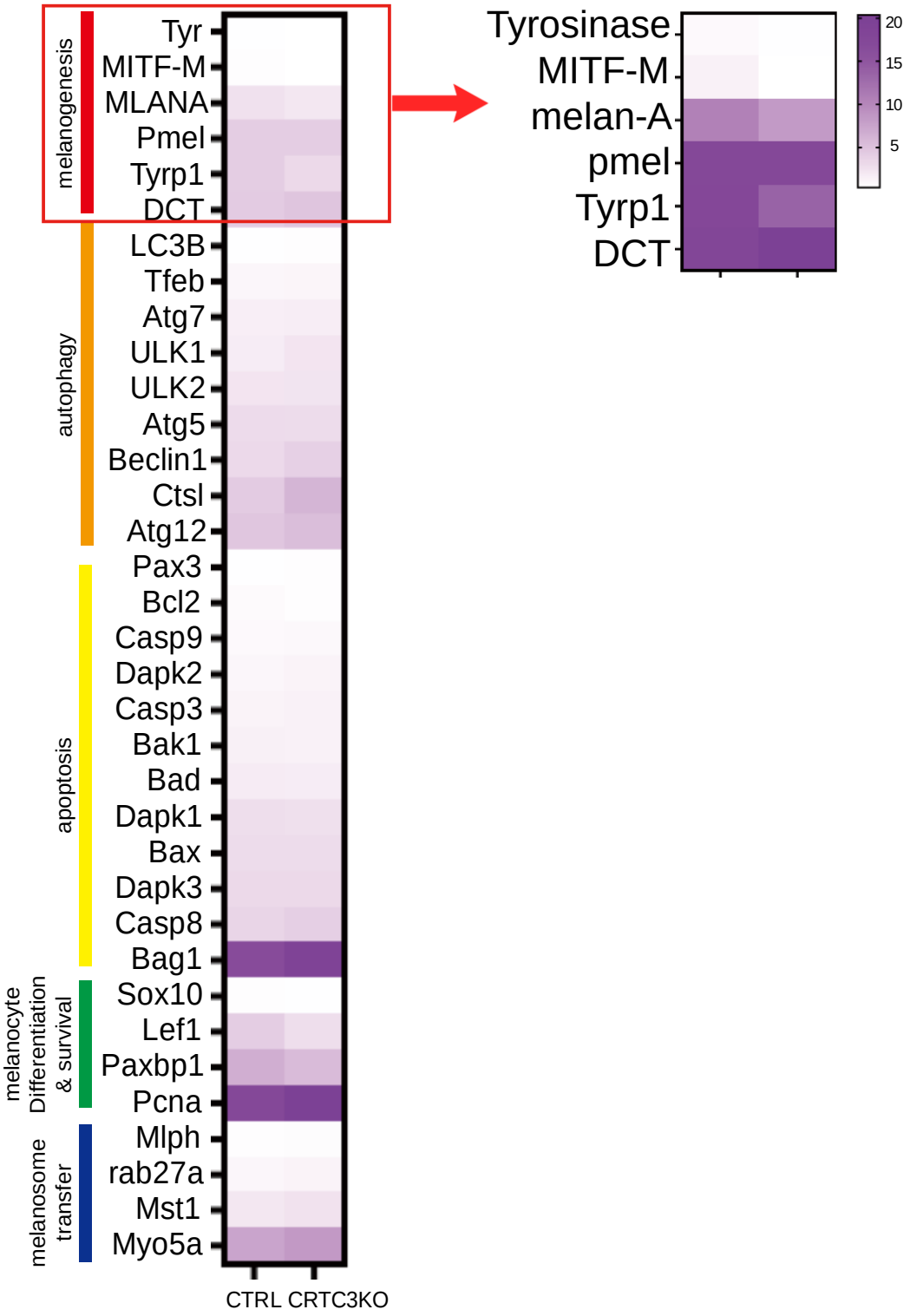

Table 1. RNA sequencing analysis of WT vs CRTC3 K/O mice tail skin.

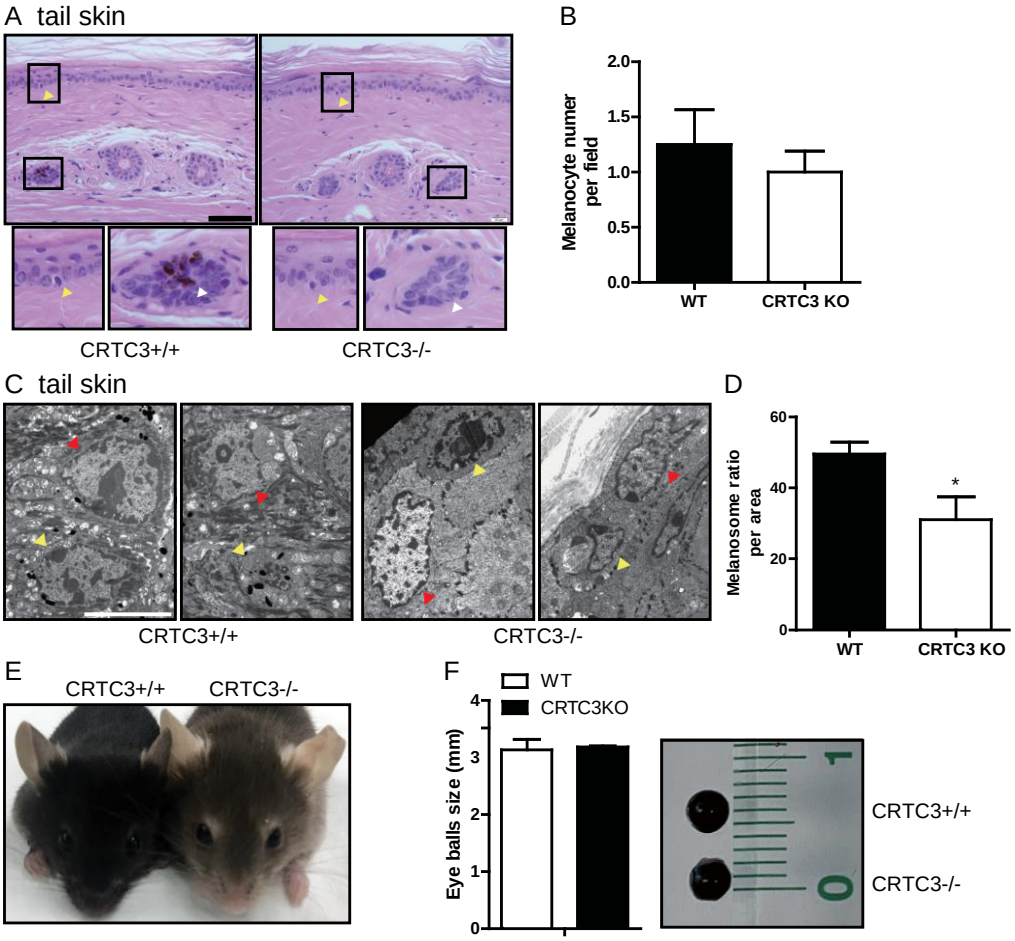

Figure 3. Melanocyte number was not different between WT and CRTC3 null mice skin.

(A), (B) Hematoxylin-eosin (H&E) staining of CRTC3 WT and null 2-month-old mouse tail tissue. (Bar = 50 μm) The number of melanocytes in epidermis of CRTC3 WT and Null mice tail tissue was calculated. (C) Electron microscopic (EM) images of epidermal cells and melanosomes in CRTC3 WT and Null mice tail tissue. The yellow and red arrowheads indicate the melanocyte and keratinocyte, respectively. (Bar = 5 μm) (D) The ratio of melanosome in CRTC3 WT and Null mice tail tissue based on EM images analyzed by ImageJ program. (E, F) The picture of Crtc3 WT and Null mice eyeball size.

2. cAMP/PKA signals activate CREB target genes.

Among multiple signaling pathways implicated in melanogenesis, only cyclic AMP (cAMP)-protein kinase A (PKA) and protein kinase C (PKC) is known a positively correlation with both melanin synthesis and phosphorylation of CREB at Ser 133 site [33, 34], but unlike PKA, PKC does not promote CBP/p300 recruitment to CREB or CREB target gene expression [35, 36] To test whether this signal discrimination is associated with function of CRTC3, FSK (forskolin) (adenyly cyclase activator) or phorbol ester TPA (12-O-Tetradecanoylphorbol-13-acetate) (PKC activator) were treated on mouse melanocytes Mel-Ab cells, a spontaneously immortalized melanocyte line that produces large quantities of melanin [37]. When exposed to FSK, the melanogenesis-associated protein levels of MITF, tyrosinase, Tyrp1 and DCT increased, but TPA did not (Fig 4. A). In consistent with this result, melanin content was increased by FSK but, it was decreased by TPA in Mel-Ab cells (Fig 4. B).

It is well-known that, protein phosphorylation is an important reversible mechanism for cellular signal transduction [38]. As such, CRTC nuclear shuttling is regulated by phosphorylation and dephosphorylation of CRTC leads to state which make CRTC enter into nucleus. FSK treatment induced nuclear migration of CRTC3, but TPA led to cytoplasmic localization of CRTC3. (Fig 4. C, D). The mRNA expression levels of NR4A2 and MITF rapidly rose and declined, consistent with the well-known burst-attenuation kinetics of CREB

transcriptional target gene expression (Fig 4. E). Treatment of FSK or TPA increased phospho-CREB to the same extent (Fig 4. C, F) but the CREB target genes increased only by FSK (Fig 4. E, F).

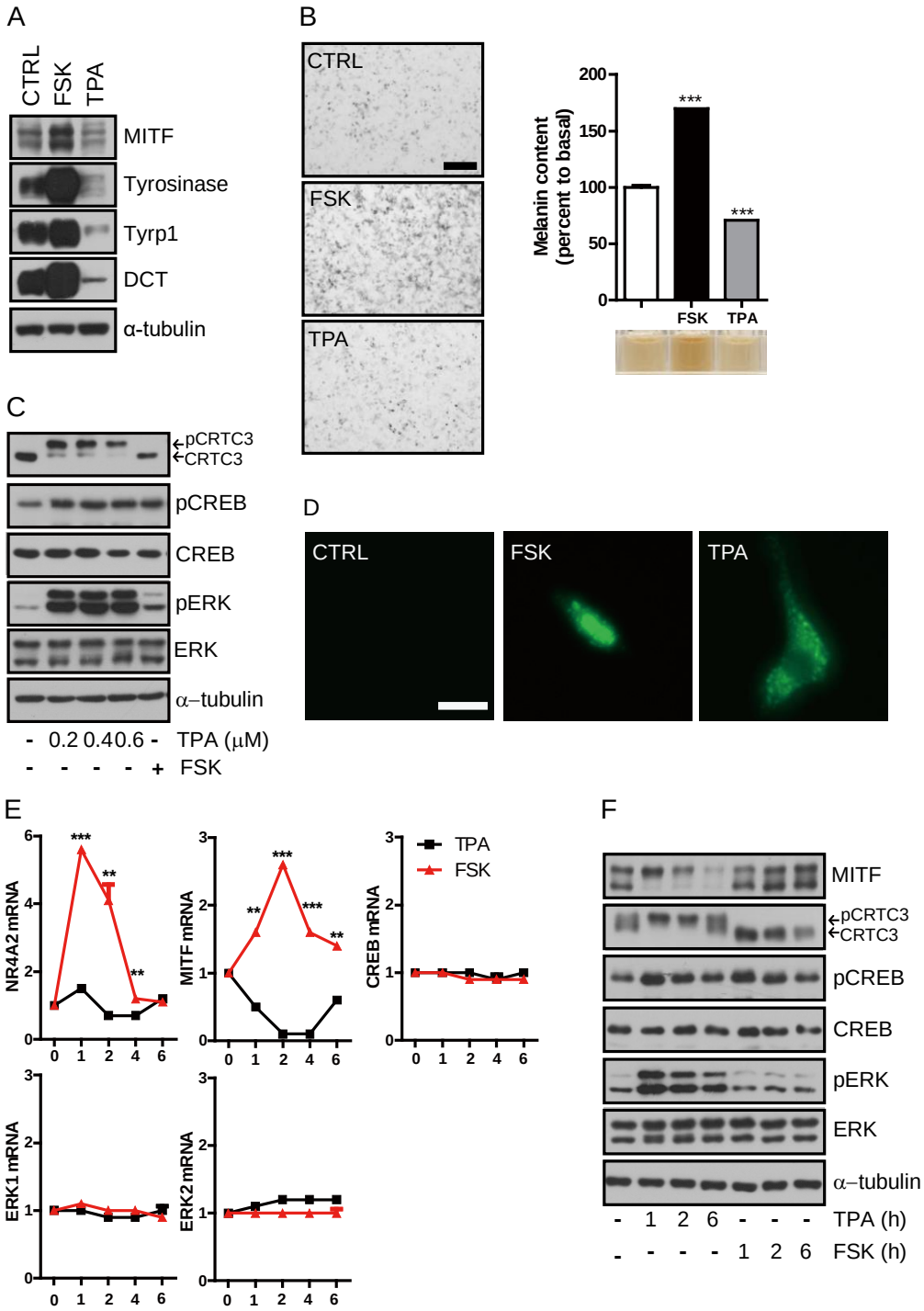

Figure 4. cAMP/PKA signals activate CREB target genes.

(A) Mel-Ab cells were treated with FSK and TPA for 72 h, MITF, tyrosinase, Tyrp1, and DCT levels were analyzed using Western blotting. (B) Melanin content in Mel-Ab cells by phase-contrast microscopy following treatment with FSK or TPA. Melanin content is presented as the percent change

relative to that in the vehicle-treated controls. (D) In B16F10 cells transfected with a plasmid containing CRTC3-EGFP were treated with FSK and TPA subcellular localization of CRTC3 was examined. (E) Short-term effects of FSK and TPA (1-6 h) on expression levels of CREB target gene (NR4A2, MITF), CREB, ERK1 and 2 mRNAs. (F) Relative effect of FSK and TPA for 72h on amounts of MITF, phospho (Ser133) CREB (P-CREB) and phospho-extracellular signal-related kinase (P-ERK). A-tubulin was used as an internal loading control. FSK; 10 μM, TPA; 200 nM. Data are expressed as means ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs controls

2. The level of melanogenic genes in CRTC3 O/E and K/D Mel-Ab cell.

We made CRTC3 knock-down (K/D) and CRTC3 overexpression (O/E) Mel-Ab cell lines by lentiviral infection (Fig 5. A, B). There was no significant difference of cell viability in CRTC3 K/D, O/E Mel-Ab cells and control cells at both basal and FSK-induced state (Fig 5. C, D). In CRTC3 O/E Mel-Ab cells, FSK-induced increase in melanin content was observed in a duration-dependent manner (Fig 6. A, B). In accordance with increment of melanin content significant increase of tyrosinase activity was demonstrated in CRTC3 O/E cells (Fig 6. C). The melanin content of Mel-Ab cells was much less in the CRTC3 K/D cells, although it is reactively increased by FSK treatment in a time-dependent manner (Fig 6. D, E). In keeping with this result, K/D of CRTC3 inhibited cellular tyrosinase activity in Mel-Ab cells (Fig 6. F).

To investigate the expression of melanogenesis related mRNA and proteins levels, western blotting and RT-PCR analysis were performed. When overexpressing CRTC3 in Mel-Ab cells, the mRNA and protein levels of MITF, Tyrp1, DCT, and tyrosinase were increased (Fig 7. A, B), suggesting that this could be resulted from an upregulation of MITF transcription. Indeed, we found that CRTC3 O/E in Mel-Ab cells enhanced melanogenesis-associated genes. When we also observed protein and mRNA levels in a short-term (1-6 h) or long-term (24-72 h) effect of FSK treatment in CRTC3 K/D Mel-Ab cells. Levels of Tyrp1, DCT and tyrosinase were decreased in CRTC3 K/D cells at 24 to 72h. FSK induced increase of these genes, but in

CRTC3 K/D cells mitigated the increment of them by FSK (Fig 7-C, D). MITF transcription showed a peak at 2 h of cAMP production by FSK, but it was again attenuated by CRTC3 K/D. On the other hand, K/D of CRTC3 caused a delay and impairment of upregulation of the melanocyte master transcriptional regulator, MITF, after FSK induced elevation of cAMP levels (Fig 7. F). CREB phosphorylation is also required for CREB activation. Reflecting the well-known burst-attenuation kinetics of CRTC/CREB target gene expression, the mRNA levels of tyrosinase, Tyrp1 and DCT were gradually increased. Accordingly, the protein expression of tyrosinase and Tyrp1 did not show alteration up to 6h (Fig 7. E, F). Taken together, these results demonstrate that CRTC3 activation is prerequisite for melanogenesis in melanocytes.

mRNA levels of CREB were not significantly altered during the time of FSK treatment (Fig 8. A, B). In addition, to determine whether the PKC signaling pathway is not related CRTC3, we analyzed PKC activity in CRTC3 O/E and K/D Mel-Ab cells by assessing protein level of phospho-PKC substrate (Fig 8. C, D).

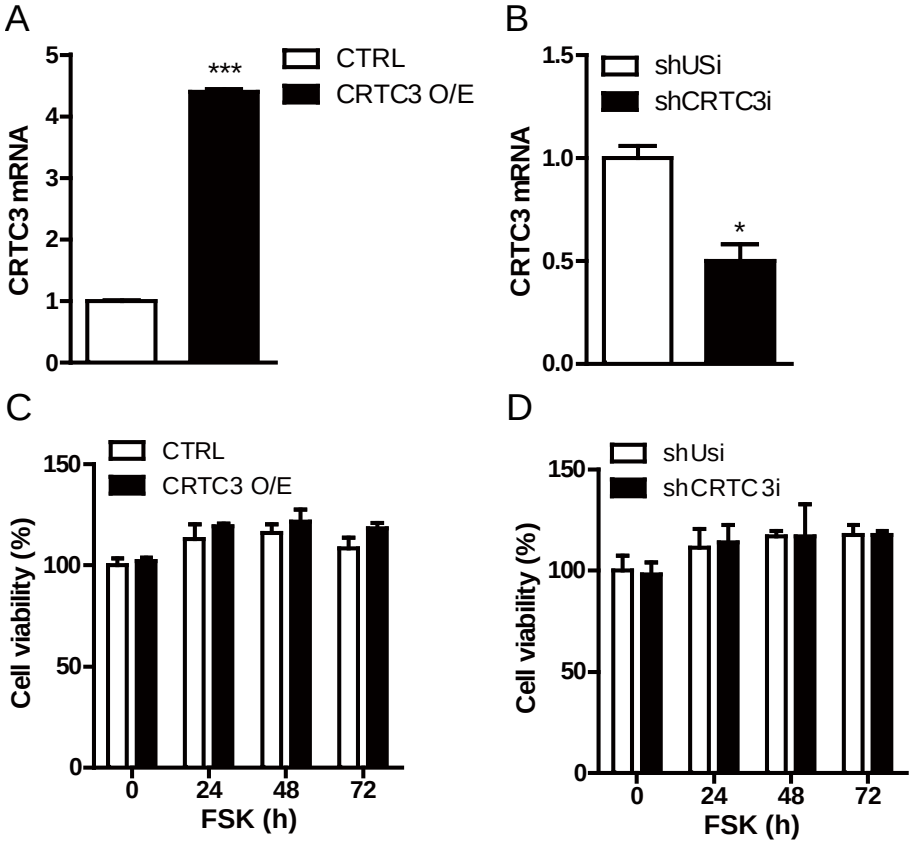

Figure 5. Effect on cell viability in CRTC3 K/D and O/E Mel-Ab cells.

After selecting puromycin resistant Mel-Ab cells, CRTC3 overexpression (O/E) (A) and knock down (K/D) (B) efficiency was assessed by qRT-PCR. (C, D) The effect of cell viability in CRTC3 O/E and CRTC3 K/D Mel-Ab cells. FSK; 10 μM, Data are expressed as means ± SD of three independent experiments. * $p < 0.05$ and *** $p < 0.001$ vs controls

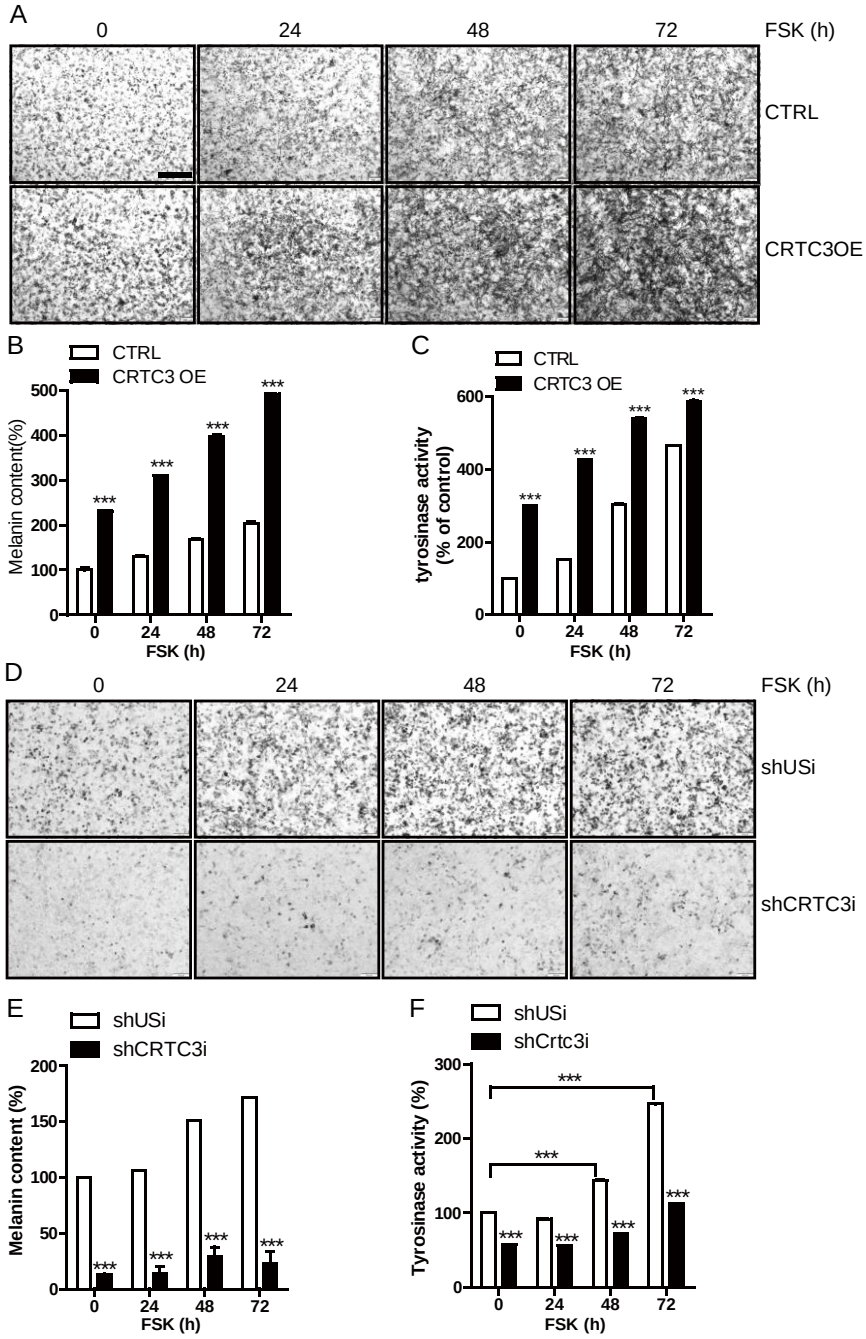

Figure 6. FSK-induced melanin content and tyrosinase activity in CRTC3 O/E and K/D cells

Microscopic images in the CRTC3 O/E (A) and K/D (D) Mel-Ab cells after 24-, 48- and 72h treatment of FSK. Melanin content (B) and tyrosinase activity (C) of CRTC3 O/E and K/D Mel-Ab cells with or

without the FSK was examined (expressed as percent change in control). FSK; 10 μM, Data are expressed as means ± SD of three independent experiments. *** $p < 0.001$ vs controls

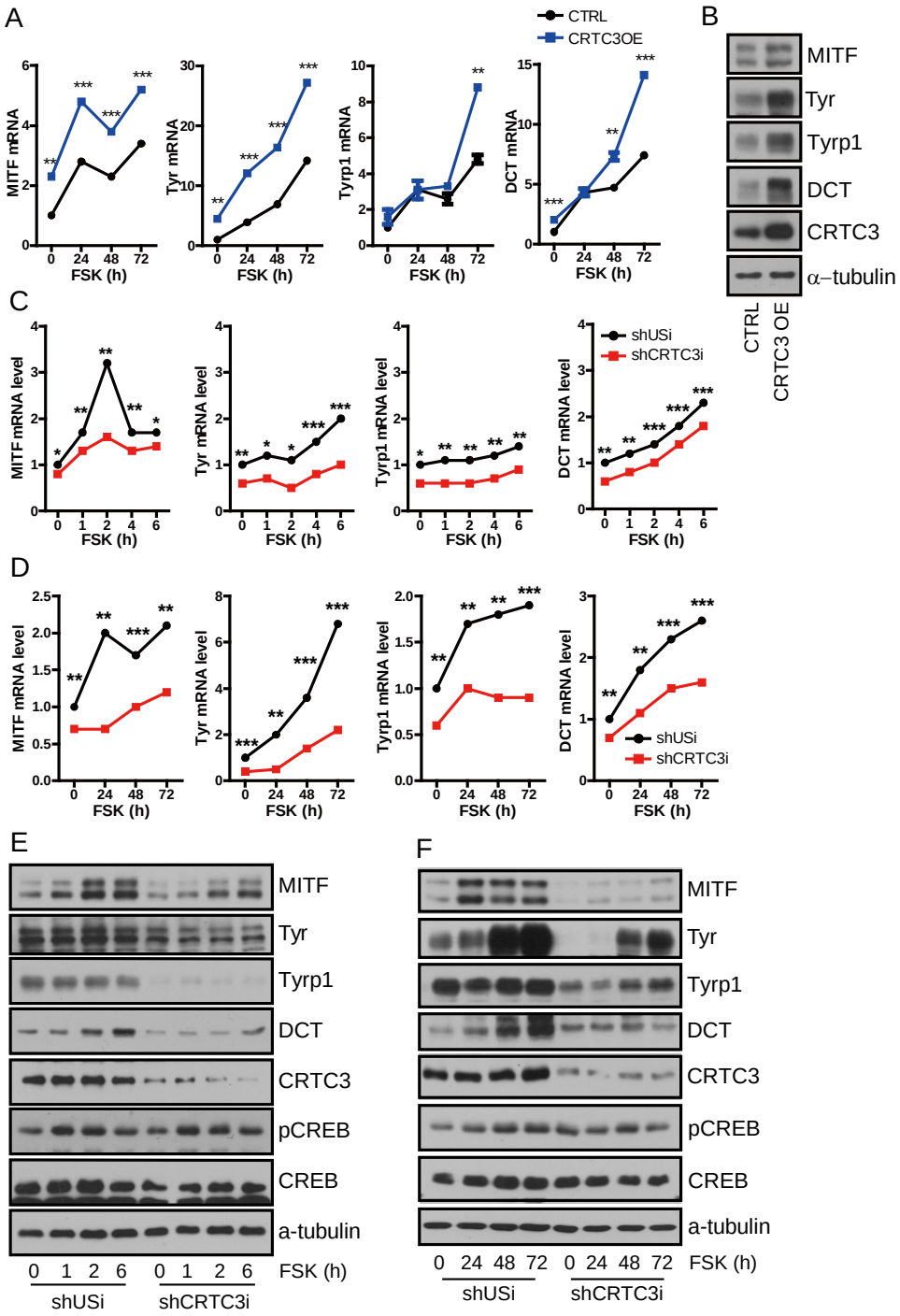

Figure 7. The level of melanogenic genes in CRTC3 O/E and K/D Mel-Ab cell.

(A) The relative mRNA levels of MITF, tyrosinase, Tyrp1 and DCT mRNA in the CRTC3 O/E Mel-Ab cells for up to 72h. (B) The protein levels of MITF, Tyrp1, DCT, tyrosinase and CRTC3 in CRTC3 O/E Mel-Ab cells were assessed by Western blotting. (C, D) Short-term (1-6 h) and long-term (24-72h) effects of FSK on expression levels of melanogenic gene mRNAs. (E, F) Short-term (1-6 h) and long-term (24-72h) effects of FSK on expression levels of melanogenic gene by Western blotting. The protein levels of pCREB and CREB in CRTC3 K/D Mel-Ab cells for 72hr effect of FSK treatment by Western blotting were not changed. FSK; 10μM, Data are expressed as means ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs controls

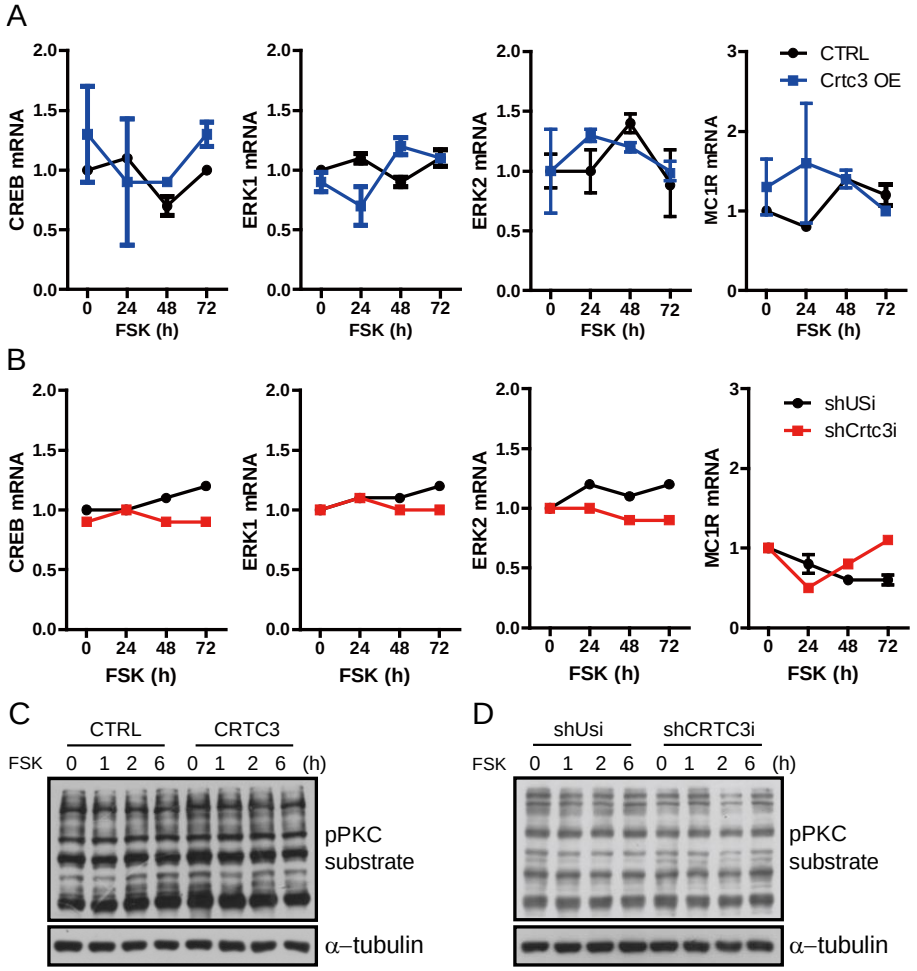

Figure 8. The level of melanogenesis-associated molecules in CRTC3 O/E and K/D Mel-ab cell.

(A, B) The relative mRNA level of CREB, ERK1, 2 and MC1R in CRTC3 O/E and CRTC3 K/D Mel-Ab cells by qRT-PCR were not significantly different. (C, D) The effect of CRTC3 O/E and K/D on protein kinase C (PKC) activity in Mel-Ab cells was assessed by Western blotting with phospho-PKC substrate antibody.

3. Melanosome maturation markedly reduced in CRTC3 K/D Mel-Ab cells.

Melanocytes synthesize melanin inside a special membrane bound organelle, melanosomes. We observed melanosome accumulation by electron microscopy (EM) analysis in control and CRTC3 K/D Mel-Ab cells. In control cells, there were randomly scattered immature pre-melanosomes to highly matured melanosomes in the cytoplasm (Fig 9. A). As melanosome stages were well defined by morphology [39, 40], we analyzed degrees of melanization from stage I to stage IV melanosomes. The number of both early and mature melanosomes in CRTC3 K/D Mel-Ab cells were decreased in both basal- and cAMP-stimulated levels (Fig 9. B). Thus, we investigated expression level of melanosome-related transcription in basal and FSK-induced CRTC3K/D Mel-Ab cells compared to control cells. The relative mRNA levels of melanoblast marker PMEL, which is highly enriched in stage II, were significantly downregulated in both basal and FSK stimulated CRTC3 K/D Mel-ab cells (Fig 9. C). Late melanogenesis marker, tyrosinase, Tyrp1 and DCT were downregulated, while SOX10 and LEF1which is not downstream of MITF are well maintained in CRTC3 K/D Mel-Ab cells. Taken together, these observations support a requirement for CRTC3 in the maturation of premelanosome and all step of melanogenesis.

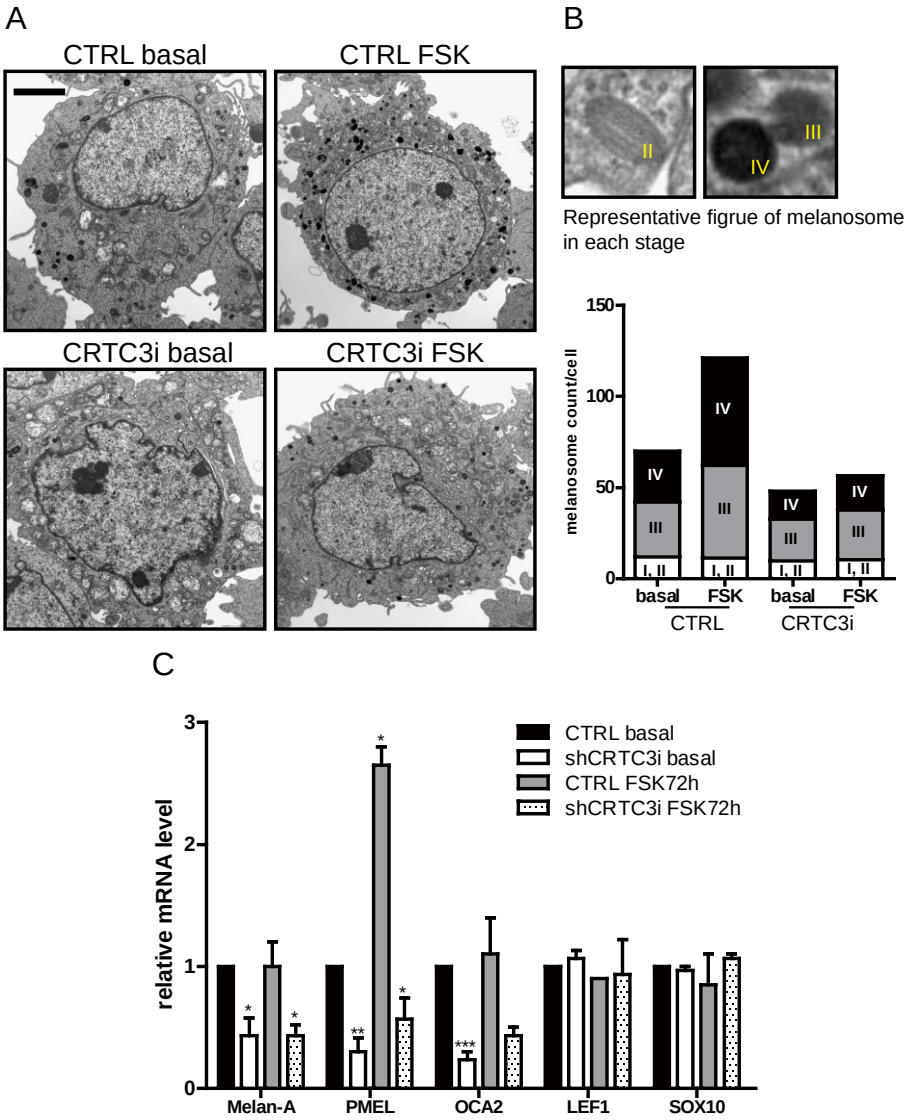

Figure 9. The melanosome maturation markedly reduced in CRTC3 K/D Mel-Ab cells.

(A) Electron microscopy images of FSK-induced melanosome maturation in CRTC3 K/D Mel-Ab cells compared to control Mel-Ab cells. (Bar = 2 μm) (B) Quantification of stage I-IV melanosomes in control and FSK-treated (72hr) cells. Right panel indicates classic stage I-IV melanosomes. (C) mRNA expression level of MALENA, PMEL, SLC24A5, SLC45A2, OCA2, LEF1 and SOX10 in CRTC3 K/D Mel-Ab cells treated with FSK for 72h were calculated by qRT-PCR.

6. Newborn mice skin tissue of CRTC3 null showed downregulation of epidermal melanogenesis-associated gene expression.

In C57BL/6 mice, melanocytes are known to be largely restricted to the hair follicles [41]. However, up to 3-day-old mice have still substantial numbers of melanocytes in the interfollicular epidermis. We first examined the neonatal mice (postnatal day 0 to 6) dorsal skin color (Fig 10. A). We noticed that 2-month-old CRTC3 K.O mice showed lighter fur color on dorsal area, we also investigated the dorsal fur color of newborn mice in order to identify any melanocyte development change. CRTC3 depletion causes downregulation of melanogenesis-associated signal pathways (Fig 10. B, C). In results, neonatal (postnatal day 0) C57 control and Null mice lack both epidermal melanocytes and melanin pigment in the dorsal skin, CRTC3 Null mice shows no difference of epidermal thickness, skin structure configuration and dermal collagen (Fig 11. A). The melanocyte lineage is derived from neural crest cells via a SOX10 (sex-determining region Y (SRY)-box 10)-positive melanoblast/glial bipotent progenitor. These progenitor cells are then committed into melanoblasts with the expression of DCT, tyrosine protein kinase KIT (KIT) and MITF. MITF targets a number of genes which are involved in cell survival (BCL2, PAX3, SOX10 and LEF1), cell proliferation (Ki67, PCNA, p21, p16, CDK2, and TBX2), and differentiation (tyrosinase, Tyrp1, DCT, PMEL, Melan-A and MC1R) [Ling Hou, 2008]. Immunohistochemically, CRTC3 was not seen

in the epidermis in CRTC3 Null mice compared to discernible positivity of CRTC3 on epidermis of WT control. Instead, back skin section showed that melanosomes (assessed by PMEL) of hair unit are markedly decreased in CRTC3 Null mice but the number of melanocytes which was identified by SOX10 staining, was not diminished in hair bulb follicular epithelium. Significantly Ki67 levels were not different between WT and CRTC3 null mice and it was expressed on keratinocytes (Fig 11. C, D). These results revealed that there is no significant difference in the number of melanocytes in CRTC3 K/D cell, indicating that CRTC3 null may not affect melanocyte survival or proliferation profoundly.

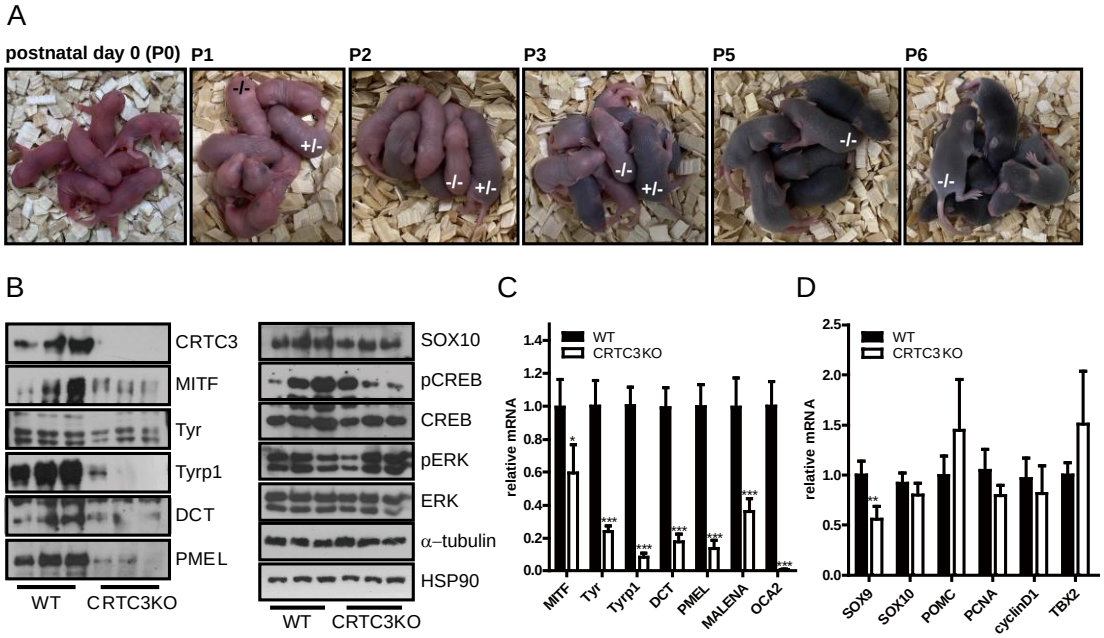

Figure 10. Newborn mice skin tissue of CRTC3 null showed downregulation of epidermal melanogenesis-associated gene expression.

(A) CRTC3 null mice appearance of pups by age. Western blot (B) and qRT-PCR (n=4 per group) (C-D) analysis for melanocyte biology associated genes of CRTC3 WT and null newborn mice dorsal skin tissue. Data are expressed as means ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. controls.

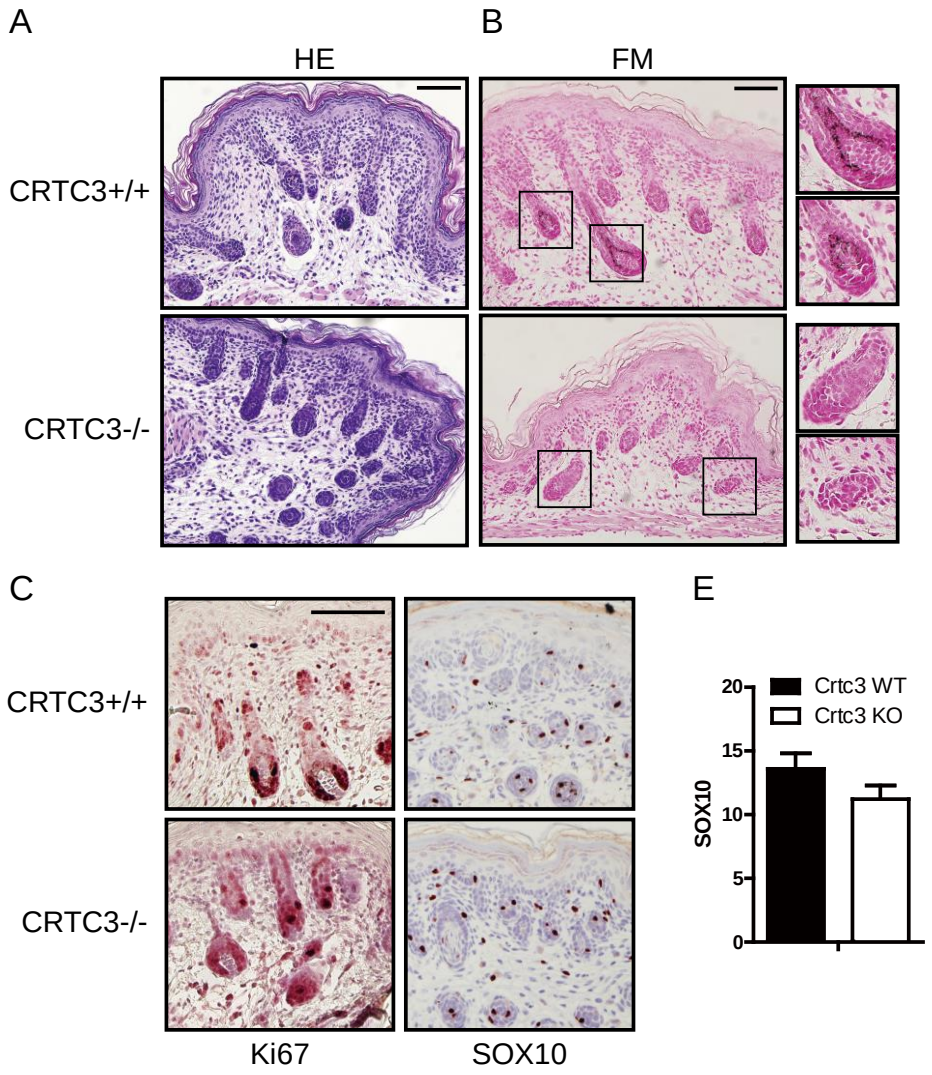

Figure 11. Immunohistochemical analysis of neonatal (P1) CRTC3 WT and null mice.

(A) Hematoxylin-eosin (H&E) staining of dorsal sections prepared from CRTC3 WT and null mice. (B) Representative microscopic images of Fontana–Masson-stained paraffin-embedded skin sections. (C, D) Microscopic images of paraffin-embedded tissue sections were immunostained with anti-Ki67, and SOX10. Immunostaining was visualized with the Vectastain ABC kit, using Novared as substrate (red color). (E) Quantified number of SOX10 in epidermis of CRTC3 WT and null P1 mice dorsal skin tissue based on multiple IHC images calculation (Bar = 50 μm). Data are expressed as means ± SD of three independent experiments. * $p < 0.05$ vs. controls

7. In coculture with human melanocyte and CRTC3 K/D HaCaT cells did not affect melanogenesis.

Similar to the results in mouse melanocytes Mel-ab cells (shown in Fig1-C, F), treatment to FSK or TPA increased phosphorylation of CREB in human melanocytes and keratinocytes (Fig 12. A). CRTC3 K/D of HaCaT cells also showed reduction of CREB target genes (NR4A2, ATF3) expression (Fig 12. B). To elucidate any role of CRTC3 K/D of keratinocytes on melanogenesis, the effect of FSK treatment in a coculture of normal human melanocytes and CRTC3 K/D HaCaT cells was assessed. CRTC3 K/D of HaCaT cells did not affect cellular proliferation. In coculture with human melanocytes transcription and translation level of melanogenesis-associated gene was not altered significantly (Fig 12. C, D, E, F).

And there was no difference of stem cell factor (SCF), endothelin (ET)-1 (Fig 12. G) and POMC (Fig 12. H) mRNA levels in control and CRTC3 K/D HaCaT. Moreover, CRTC3 whole body Null mice was not accompanied by alteration of serum POMC. There was no significant difference in serum POMC level between WT and CRTC3 null mouse measured by ELISA (Fig 12. I).

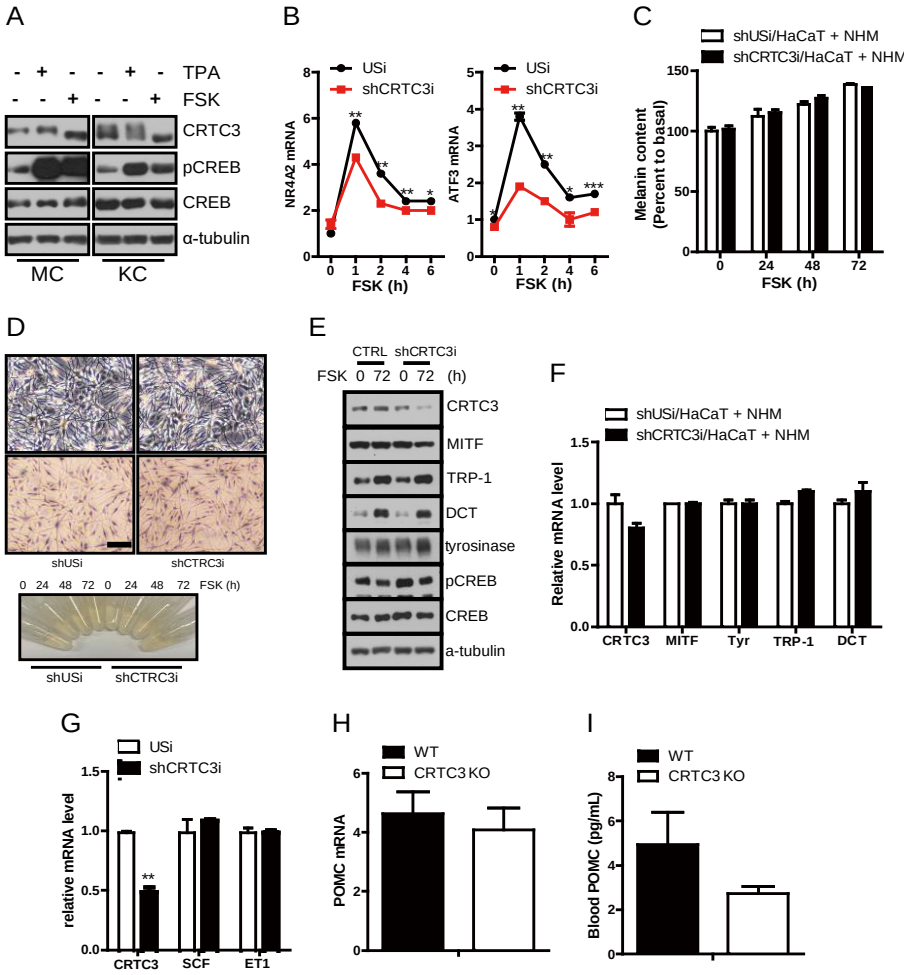

Figure 12. In coculture with human melanocyte and CRTC3 K/D HaCaT cells did not affect melanogenesis.

(A) Normal human melanocytes (NHM) and HaCaT cells (keratinocytes) were treated with FSK or TPA in order to examine expression of CRTC3, phospho- and total- CREB analyzed by Western blotting. α-tubulin was used as a loading control. (B) The level of CREB target genes (NR4A2, ATF3) expression. (C) FSK-induced melanin content in coculture with NHM and CRTC3 K/D HaCaT cells. (D, E) Microscopic images and protein levels (MITF, Tyrp1, DCT, tyrosinase, phospho- and total CREB) in co-culture with NHM and CRTC3 K/D HaCaT. (F) Relative mRNA levels of melanogenesis-related genes in coculture with NHM and CRTC3 K/D HaCaT. Relative mRNA levels of SCF, ET-1 (G) and

POMC (H) in control and CRTC3 K/D HaCaT. (I) POMC level of CRTC3 WT and Null mice blood serum was assessed by ELISA. FSK; 10 μM, TPA; 200 nM.

8. Mouse primary melanocyte culture in WT, CRTC3 HT and K/O mice.

Compared to WT and HT mice, primary cultured melanocytes from CRTC3 null mice showed significant less melanin accumulation (Fig 12. A) and expression of MITF, tyrosinase, Tyrp1, DCT and PMEL were downregulated. And there was no difference in SOX10 level (Fig 12. B). In microscopic images, melanocytes cultured in CRTC3 null mice were less differentiated (Fig 12. C, D).

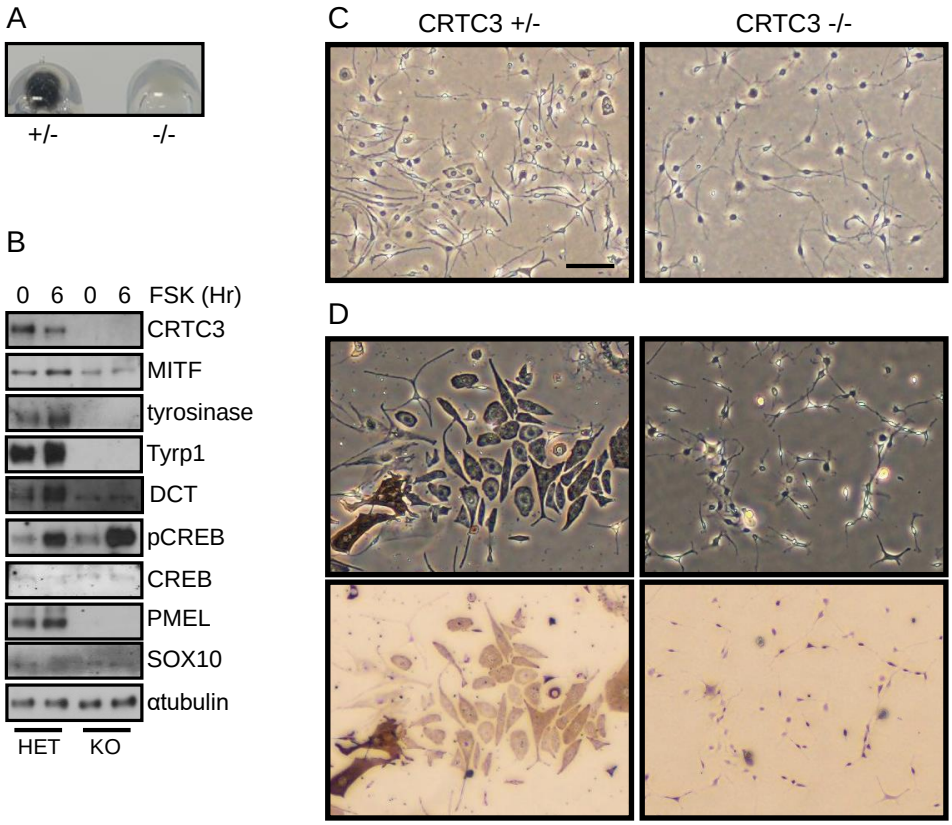

Figure 13. The level of melanogenic genes in mouse primary melanocyte culture

(A) Pellets of mouse primary melanocyte with CRTC3 HT (left vial) and KO (right vial). (B) Protein level of melanogenesis-related genes in CRTC3 HT and KO primary melanocyte. (C, D) Microscopic images of CRTC3 HT and KO primary melanocyte. Lower figures show melanosomal distribution in bright-field image (Bar = 100 μm).

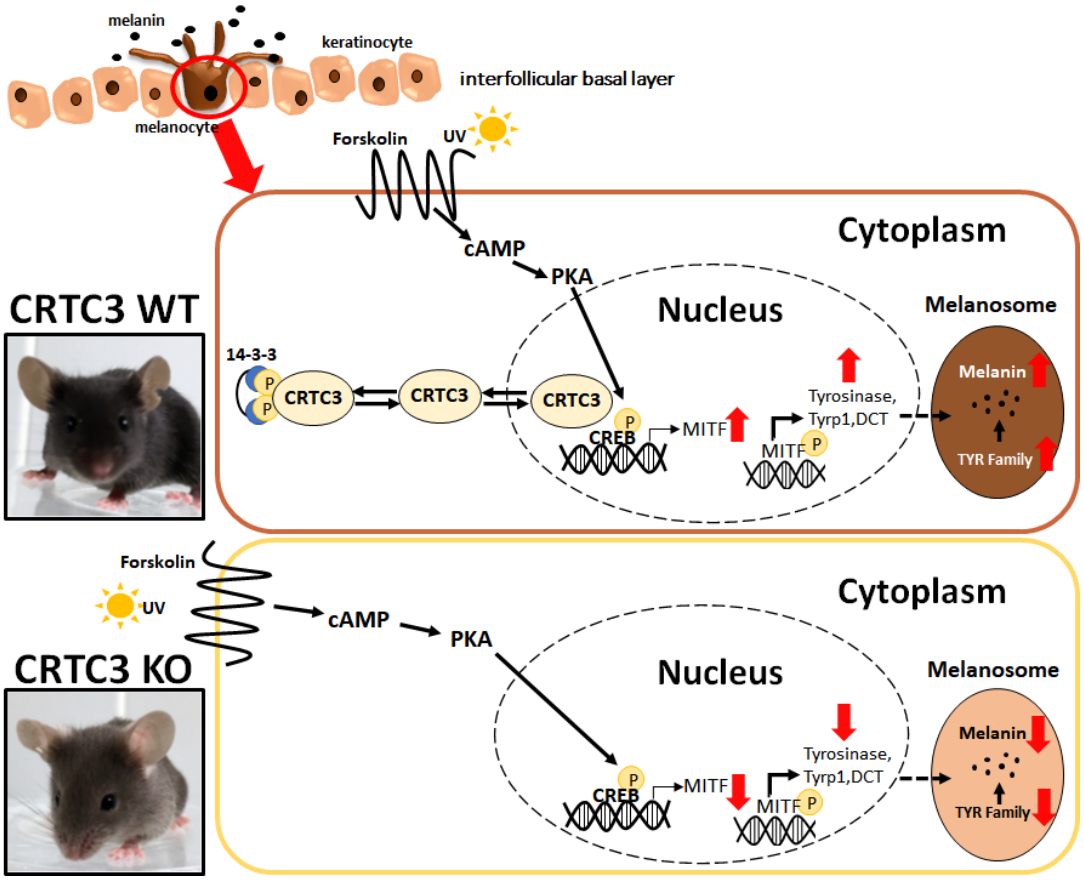

Figure 14. Schematic model of melanogenic modulation by CRTC3/CREB/MITF signaling

Discussion

Melanogenesis is a pivotal process of melanin production and is important to protect skin from the ultraviolet radiation. However, an abnormal accumulation of melanin can cause hyper- or hypopigmentary disorders such as melasma, lentigo, cafe au lait spot and vitiligo. The cAMP response element binding protein (CREB) signaling pathway is involved in regulating microphthalmia-associated transcription factor (MITF), a key modulator in the transcription of genes related to melanin synthesis. Recently, we reported that CREB-regulated transcription co-activators (CRTCs) are required for transcription of CREB. In this study, we characterized CRTC whole body null mouse related to mouse coat color.

The CRTC family members include CRTC1, CRTC2, and CRTC3. CRTC1 is mainly expressed in certain regions of the brain (prefrontal cortex, cerebellum, and hypothalamus) [42, 43]. CRTC1 K/O mice are obese, hyperphagic, and leptin resistant, and thus CRTC1 appears to have a role in appetite suppression. The Creb1 coactivator Crtc1 is required for energy balance and fertility [44]. CRTC2 is ubiquitously expressed throughout the body, but has primarily been identified with roles of gluconeogenesis and hepatic insulin sensitivity [45]. Insulin modulates gluconeogenesis by inhibition of the coactivator TORC2 [45- 48]. Similarly, CRTC3 is expressed throughout the body, but has primarily been identified with increasing insulin resistance in adipose tissue and energy balance through catecholamine signaling [29]. Interestingly, despite the fact that the expression of CRTC1 and CRTC2 was observed in

melanocytes and/or melanoma cell lines [48, 49], CRTC1 and CRTC2-null mice did not present any skin and coat color changes (data not shown). Previously, we and other researchers observed overlapping expression of CRTC2 and CRTC3, but independent phenotypes occurred in the liver and adipose tissue of CRTC2 and CRTC3-null mice [28, 50].

We used lentivirus for knock down of CRTC3 in Mel-Ab cells and explored melanogenesis. Knock-down (K/D) of CRTC3 significantly reduced melanin content and tyrosinase activity, and also downregulated the level of melanogenesis-associated genes and proteins, including MITF, tyrosinase, tyrosinase related protein 1 (Tyrp1) and dopachrome tautomerase (DCT) in both cell and tissue level. Tyrosinase, Tyrp1, and DCT play a role in the biosynthetic pathway of melanin in melanocytes. Tyrp1 and DCT are involved in stabilizing tyrosinase and modulating its catalytic activity. MITF is a major transcription factor in the regulation of tyrosinase activity and expression of Tyrp1 and DCT genes. An upregulation of MITF expression resulted from the increase of intracellular cAMP concentration. cAMP has been evidenced to play a key messenger in the regulation of skin pigmentation as increase of intracellular cAMP upregulates MITF expression [50]. Although biochemical rate of melanin biosynthesis is determined by tyrosinase activity of melanocyte, differentiation process including the levels of tyrosinase, DCT, and Tyrp1, melanosome biogenesis, melanosome transporters in association with ion channels (pH), finally and eumelanin/pheomelanin ratio,

differentiation processes as well as proliferation and survival of melanocytes are centrally regulated by MITF [51]. Mutations in MITF cause hypopigmentation and increase the risk for familial melanoma in humans. There are multiple different isoforms of MITF, and it is currently unclear how these different isoforms contribute to pigment cell development in the eye, hair, and skin. Melanocytes express two isoforms of MITF, MITF-A and MITF-M. MITF-A knockout mice have a subtle loss of pigmentation in the hair, while MITF-M knockout mice lack melanocytes in the epidermis, hair follicle, iris, and choroid [52]. The synthesis of the melanin pigment is ascertained by tyrosinase, the key enzyme in pigment synthesis, and two related enzymes, tyrosinase-related protein 1 (Tyrp1) and dopachrome tautomerase (DCT), which are strongly implicated in eumelanin synthesis [53]. As tyrosinase (TYR) is a key enzyme in melanin synthesis, depletion of TYR in mice results in mosaic pigmentation or total albinism. Conditional deletion of allele of the transmembrane domain KIT showed white spotting phenotype indicating that melanocyte-selective impairment of KIT interfered with normal melanocyte development and overexpression of KITL, endothelin-3 or hepatocyte growth factor (HGF) was unable to rescue melanocytes in these mice [54]. TYR depletion did not significantly inhibit total melanin accumulation but reduced mature melanosomes [55].

Melanosomes are synthesized and stored melanins through four morphologically distinct stages. The first two early stages called pre-melanosome lack pigment. In stage I, a

vesicle builds inside a fibrillar matrix formed by glycoproteins (PMEL, Melan-A). As a next step, pre-melanosomal vesicles get tyrosinase and other enzymes of melanogenesis in stage II. Melanin is polymerized and settled on the internal fibrils in stage III by melanosomes. Finally in the last stage IV melanosome, melanosome maturation is fulfilled melanin [56]. Melanosomal pH plays a critical role in melanogenesis. Pre-melanosomes maintain acidic pH while stages III–IV melanosomes exhibit neutral pH, which may be a result of combinational function of the newly deposited ion transport proteins. OCA2 (Oculocutaneous Albinism II) and SLC45A2 (MATP/AIM1) proteins are reported to be positive regulators for pH neutralization. On the other hand, two-pore channel 2 was found to be a negative regulator for pH neutralization. Under neutral pH, tyrosinase is functional and melanin is synthesized [57, 58]. SLC45A2 is a transporter protein that mediates melanin synthesis. It may regulate the pH of the melanosome, affecting tyrosinase activity. SLC45A2 is also a melanocyte differentiation antigen that is expressed in a high percentage of melanoma cell lines [59]. In melanocytic cell types, the SLC45A2 gene is regulated by MITF [60, 61].

Melanocortin system is unique in that its activity is regulated by endogenous agonist and antagonist. The critical role of CREB in linking UV stimuli to melanogenesis has been well documented. CREB is a ubiquitously expressed basic leucine zipper (bZip) transcription factor that binds cAMP-responsive element (CRE) motif of the regulatory region of genes.

CREB present in the nucleus occupying CRE motif of target genes and its transcriptional activity is reversibly regulated by the phosphorylation status of CREB at Ser133 [18]. If non-cAMP signals not enoughly exposed, CREB-CBP complex formation is blocked. The levels of CREB Ser133 phosphorylation in response to non-cAMP signals may not be high enough to promote the CREB–CBP interaction. Unexpectedly, systemic CREB K/O mice targeting exon 2 did not show any or only mild phenotype: ATF1 level is similar but CREBβ and CREM appears to be upregulated [27]. Although Ser133 phosphorylation of CREB has long been accepted as a whole marker of transcriptional activity of CREB [16, 17, 33], systemic deletion of bZip domain CREB knock out mice died perinatally [25] and conditional K/O mice did not show.

Since the discovery that transcriptional activity of CREB is regulated by reversible phosphorylation through PKA, phosphorylation of CREB at ser133 has been accepted as a whole marker of transcriptional activity of CREB [16, 17, 33]. cAMP regulated gene transcription appears to be mainly mediated by three combinations of hetero or homo dimeric CREB family genes, CREB1, CREM, and ATF1. In systemic CREB K/O mice, although ATF1 level is similar but CREBβ and [26] CREM is upregulated [26].

Patients with acrodysostosis who carries mutations in PRKAR1A (gain of function, regulatory subunit of PKA, presented pigmented skin leisions [28]. Pigmented schwannomas

was observed in CNC [42]. In addition, mutations in the PRKAR1A gene are also associated with pigmented skin lesions in acrodysostosis patients [21]. This gain of function mutation in PRKAR1A leads to constitutive inhibition of PKA activity (catalytic subunits) by the inability of the regulatory domain to dissociate from the catalytic domain of PKA. Taken together, these observations show other mechanisms for cAMP activity in pigmentation due to PKA inactivation in these patients. Alternatively, other signaling pathways might be compensating for this deficiency by activating PKA effector, CREB.

CRTC K/D of HaCaT cells also reduced CREB target genes and did not affect cellular proliferation. And there was no significant difference in serum POMC level between CRTC3 K/O and WT mouse measured by ELISA. The protein level of SOX10 in CRTC3 K/D and WT Mel-Ab cell was same. This result implies that there is no difference in the number of melanocytes in CRTC3 K/D cell. And, upstreams of MITF transcription factors are not reduced in CRTC 3 K/O mice and are maintained, with AXIS other than CREB/MITF remaining and melanocyte develop and survival guaranteed. CRTC3 is a target that reduces melanin, but does not reduce the number of cells and does not have the risk of causing vitiligo. These data suggest that regulation of CRTC3 activity may provide novel therapeutic approaches for pigmentation disorders and CRTC3 may work on the melanocyte specifically, not on the keratinocyte or systemic alteration even in whole body K/O. Understanding the detailed molecular

mechanisms of precise skin color regulation by CRTC3 modulation could lead to the development of new strategies to maintain skin health and treat hyper- or hypo-pigmentation skin disorders.

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국문 요약

인간의 피부와 머리카락 색 변화를 일으키는 메커니즘관련 연구는 아직 완벽하게 밝혀진 바 없다. 피부색 또는 마우스 털색을 결정 짓는 데는 매우 다양한 신호전달 경로들과 인자(factor)들이 필요하다. 그중 MITF를 조절하는 CREB은 멜라닌 합성관련 유전자 전사과정의 핵심 조절자에 해당한다. 이러한 CREB 활성기능을 알기 위해 CREB 의 공활성인자(co-activator)인 CRTC3 의 녹아웃(Knock out) 마우스에 관한 연구가 필요하기 때문에 CRTC3 의 역할에 초점을 두고 연구하였다. CRTC3 KO 마우스의 털색은 야생형 마우스에 비해 흐린 회색빛을 띤다. 우선 CRTC3 KO 마우스 꼬리 조직에서 폰타나 매쓴 염색을 통해 멜라닌 축적 정도가 KO 마우스에서 감소한 것을 관찰하였다. 멜라닌관련 유전자의 핵심 조절인자인 MITF 유전자와 단백질수준이 감소해 있었고 그 하위에 존재하는 대표적인 멜라닌 생성관련 단백질인 tyrosinase, Tyrp1, DCT 또한 모두 감소해 있었다.

인비트로 수준에서는 마우스 멜라노사이트인 Mel-Ab 세포에 CRTC3 낙다운 혹은 과발현을 유도하였을 때 낙다운시에는 멜라닌관련 유전자 혹은 단백질이 감소, 과발현시에는 증가함을 확인하였다. 추가적으로 마우스 일차 멜라노사이트 배양을 통해 CRTC3 KO 마우스 세포에서 멜라닌 생성관련 유전자가 감소되어 있음을 확인하였다. 멜라노사이트의 생존마커인 SOX10 의 단백질,

mRNA 수준과 조직염색에서의 차이는 없었기 때문에 멜라노사이트 수의 차이는 없음을 알 수 있었다. 또한 케라티노사이트에 CRTC3 를 낙다운 시킨후 멜라노사이트와 공배양을 한 결과 생성되는 멜라닌의 차이는 없음을 확인함으로써 CRTC3 K/O 마우스의 털색 차이는 케라티노사이트가 아닌 멜라노사이트로부터 유래함을 알 수 있었다.

선행 연구결과에서는 CREB 의 인산화만이 CREB 활성의 마커로써 여겨져 왔으나 본 연구를 통해 CRTC3 의 활성화가 CREB 의 전사활성을 위해서 꼭 필요한 인자인 것으로 알 수 있었다. 즉, CRTC3 는 멜라닌 형성 조절에 새롭고 중요한 물질이 될 수 있을 것으로 보인다. 더 나아가 유병률이 높고 난치성인 색소성질환의 약물개발에 새로운 핵심물질이 될 수 있을 것으로 기대된다

<핵심 단어> CRTC3, CREB, MITF, 멜라닌형성