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# Epistatic connections between microphthalmia-associated transcription factor and endothelin signaling in Waardenburg syndrome and other pigmentary disorders

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ABSTRACT **Waardenburg syndrome (WS) is an inherited sensorineural deafness condition in humans caused by melanocyte deficiencies in the inner ear and forelock. Mutation of microphthalmia-associated transcription factor (MITF) is known to produce WS type IIA whereas mutations of either endothelin (EDN) or its receptor endothelin receptor B (EDNRB) produce WS type IV. However, a link between MITF haploinsufficiency and EDN signaling has not yet been established. Here we demonstrate mechanistic connections between EDN and MITF and their functional importance in melanocytes. Addition of EDN to cultured human melanocytes stimulated the phosphorylation of MITF in an EDNRB-dependent manner, which was completely abolished by mitogen-activated protein kinase kinase inhibition. The expression of melanocyte-specific MITF mRNA transcripts was markedly augmented after incubation with EDN1 and was followed by increased expression of MITF protein. Up-regulated expression of MITF was found to be mediated *via* both the mitogen-activated protein kinase-p90 ribosomal S6 kinase-cAMP response element-binding protein (CREB) and cAMP-protein kinase A-CREB pathways. In addition, EDNRB expression itself was seen to be dependent on MITF. The functional importance of these connections is illustrated by the ability of EDN to stimulate expression of melanocytic pigmentation and proliferation markers in an MITF-dependent fashion. Collectively these data provide mechanistic and epistatic links between MITF and EDN/EDNRB, critical melanocytic survival factors and WS genes.—Sato-Jin, K., Nishimura, E. K., Akasaka, E., Huber, W., Nakano, H., Miller, A., Du, J., Wu, M., Hanada, K., Sawamura, D., Fisher, D. E., Imokawa, G. Epistatic connections between microphthalmia-associated transcription factor and endothelin signaling in Waardenburg syndrome and other pigmentary disorders. *FASEB J.* 22, 1155–1168 (2008)**

*Key Words: cAMP response element-binding protein • CREB • cyclin-dependent kinase 2 • melanocyte • endothelin B receptor*

Highly organized but complex interactions between environmental cues, signal transduction pathways, and transcription factors underlie the development of a cell lineage. Mammalian pigmentation genes provide an attractive system in which to analyze such interactions *in vivo* because melanocyte distribution and pigmentation are visually detectable. These properties have allowed for the identification of numerous coat color mutations in mice, including a smaller number of white spotting genes, which are essential for melanocyte development or survival (as opposed to pigmentation *per se*). In human skin, mutations or hyperexpression of several pigmentation genes may be associated with hypo- or hyperpigmentary disorders. Melanocyte progenitors exit from the dorsal neural tube and migrate toward the ventral midline through the dorsolateral pathway to colonize the dermis, epidermis, and hair follicles of the skin. They undergo proliferation and differentiation dependent on environmental cues during the colonization process. Once in the skin, melanocytes may respond to exogenous signals to stimulate their growth and melanogenesis, thereby altering skin pigmentation levels. The molecular mechanisms whereby these cues are translated into altered melanocyte cell fate, proliferation, or differentiation are not well understood.

Mutations of the white spotting genes have been similarly found in human hereditary deafness/depig-

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mentation syndromes. Recent studies have shown that the transcription factor PAX3, which is responsible for Waardenburg syndrome (WS) type I or III depending on the precise mutation, modulates expression of the central melanocytic transcriptional regulator microphthalmia-associated transcription factor (MITF) (1–3). Similarly, the SOX10 transcription factor modulates MITF expression and is mutated in WS type IV (1, 2, 4, 5). Additional studies have shown that KIT, which is mutated in the piebaldism plus deafness condition (Woolf's syndrome), triggers a signaling pathway that leads to MITF modulation *via* phosphorylation (6, 7).

Mutation of endothelin3 (EDN3) or its receptor endothelin receptor B (EDNRB) causes loss of melanocytes as well as enteric ganglion cells (which are also neural crest derivatives) both in humans with WS type IV and in mutant mice (8–10). EDNRB is required between embryonic day 10 and 12.5 when melanoblasts, which have just exited from the dorsal neural tube, are proliferating and dispersing along the dorsolateral pathway (11). The significant increase in melanoblasts seen in wild-type embryos during this time window is absent in $Ednrb^{s-l/s-l}$ and $Edn3^{ls/ls}$ mutant mice (12, 13), indicating that EDN3/EDNRB signaling is critical for melanoblast proliferation (and subsequent survival) during development.

EDN peptide derived from endothelial cells is a potent vasoconstrictor of vascular smooth muscle (14). We demonstrated previously that human epidermal keratinocytes secrete EDN1 and EDN2 in response to ultraviolet (UV) irradiation (15, 16). Those EDNs can act on melanocytes as melanogens in conjunction with their mitogenic properties, playing an essential role in stimulating epidermal pigmentation in several hyperpigmentary disorders including UVB melanosis (17). A number of *in vitro* experiments have also demonstrated that EDN1 as well as the related factor EDN3 (which binds and activates the same receptor) promote melanocyte proliferation and differentiation in cell culture experiments (18). Characterization of the signaling pathways involved in those responses demonstrated that the EDN-induced signal transduction pathway dominates the activation of protein kinase C (PKC) through EDNRB, resulting in activation of the mitogen-activated protein kinase (MAPK) pathway *via* a convergent point of Raf-1 (19–21). EDN signaling is also thought to target activation of p90 ribosomal S6 kinase (RSK) family kinases downstream of MAPK (22). In contrast to the action of EDN1, ligands such as basic fibroblast growth factor (bFGF) and stem cell factor (SCF), which are associated with tyrosine kinases, stimulate melanization of human melanocytes only weakly despite their distinct actions as mitogens (23–25). Therefore, the signaling mechanisms involved in the dual and potent biological effects of EDN1 on proliferation and differentiation of human melanocytes remains unclear, and molecular targets of EDNs downstream of MAPK that underlie EDN-induced melanoblast or melanocyte proliferation/differentiation are not well understood.

*MITF*, one of the WS genes, encodes a tissue-restricted transcription factor of the basic helix-loop-helix leucine-zipper type (26), which recognizes E-box-containing promoter/enhancer elements (27, 28). It regulates melanocytic pigmentation as a transcription factor regulating the key melanogenic enzymes tyrosinase, TRP1 and TRP2 (29–32), and is also associated with melanocyte survival (26, 28). Mutation of *MITF* results in WS type IIA in humans (33–35), causing hypopigmentation of the skin and hair and corresponding white coat color and deafness in mice. Interestingly, germline mutations at loci encoding MITF, EDNRB, or its ligand EDN3, lead to strikingly similar defects in melanocytes (10, 36, 37). The phenotypic overlap between *MITF* and *EDN/EDNRB* mutations in humans and mouse suggests the existence of a common functionally important pathway for melanocyte development and its cellular responses to environmental stimuli.

Recently, new roles for MITF in melanocytes have been proposed, such as regulating the apoptotic inhibitor *Bcl*II (38), the cyclin-dependent kinase inhibitor gene p21 (Cip1) (39), p16/Ink4a (40), and/or cyclin-dependent kinase 2 (CDK2) (41) in addition to regulating transcription of melanogenic enzymes. Thus, characterizing the dynamics of MITF and its modulation within melanocytes during EDN stimulation, which reflects *in vivo* abnormal pigmentary events, would provide insights into the regulatory roles of melanocyte-specific MITF (MITF-M) in melanocytes. Here, we used EDN stimulation of intracellular signaling to examine the dynamics of MITF-M in normal human melanocytes. We demonstrate that EDN signals modulate the MITF *via* three kinetically distinct mechanisms, thereby connecting an essential growth factor/receptor to a master transcription factor MITF, which is pivotal to melanocyte fate.

## MATERIALS AND METHODS

### Cell culture and treatment

Primary human melanocytes from neonatal foreskins (provided by Dr. Ruth Halaban, Yale University, New Haven, CT, USA) were maintained between passages 1 and 3 in F10 medium (GIBCO-Life Technologies, Inc., Rockville, MD, USA) supplemented with 7% fetal bovine serum (FBS), penicillin/streptomycin/glutamine (GIBCO-Life Technologies, Inc.), $1 \times 10^{-4}$ M 3-isobutyl-1-methylxanthine (Sigma-Aldrich Corp., St. Louis, MO, USA), 50 ng/ml 12-*O*-tetradecanoyl phorbol-13-acetate (Sigma-Aldrich Corp.), 1 μM $Na_3VO_4$, and $1 \times 10^{-3}$ M $N^6$,2′-*O*-dibutyryladenosine 3:5-cyclic monophosphate (Sigma-Aldrich Corp.). Primary human melanocytes from another source (Cascade Biologics, Portland, OR, USA) were maintained in medium 254 (Cascade Biologics) supplemented with 3 ng/ml recombinant bFGF, 5 μg/ml insulin, 0.18 μg/ml hydrocortisone, 5 μg/ml transferrin, 3 μg/ml heparin, 10 ng/ml phorbol-12-myristate-13-acetate (PMA), 0.2% (v/v) bovine pituitary extract (BPE), and 0.5% (v/v) FBS (Cascade Biologics). The human melanoma cell line 501 MEL (gift of Dr. Ruth Halaban) was grown in F10 medium with 10% FBS plus penicillin/streptomycin/glutamine. In evaluation of signaling changes, human pri-

mary melanocytes were seeded in medium 254 or F10 medium and at 24 h before the addition of ligands, the medium was replaced with medium 254 depleted of FBS, PMA, bFGF, and BPE or F10 medium supplemented only with penicillin/streptomycin/glutamine. Human primary melanocytes were stimulated with 10 nM EDN1 (Calbiochem, San Diego, CA, USA or Sigma-Aldrich Corp.)/EDN3 (Calbiochem), 20 ng/ml recombinant human SCF (R&D Systems, Minneapolis, MN, USA), and forskolin (FSK) (20 μM) with or without 20 μg/ml cycloheximide, 20 or 40 μM mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 (New England BioLabs, Ipswich, MA, USA or Sigma-Aldrich Corp.), 1 μM PKC inhibitor Gö6983 (Calbiochem), 5 or 10 μM protein kinase A (PKA) inhibitor H89 (D. Western Therapeutics Institute, Inc, Nagoya, Japan), 10 μM p38 MAPK inhibitor SB203580 (Calbiochem), 100 nM phosphoinositol 3-kinase (PI3 kinase) inhibitor wortmannin (Calbiochem), 1 μM Akt inhibitor SH-6 (Calbiochem), or 25 μM proteasome inhibitor MG132 (Calbiochem) for the indicated times after 24 h of starvation as described above. The 501 MEL human melanoma cells were stimulated by EDN1/3 and FSK with or without the MEK inhibitor PD98059 in F10 medium supplemented only with penicillin/streptomycin/glutamine.

**Adenovirus infection**

Adenoviruses were used as described previously (42) and were engineered to overexpress either wild-type human *MITF*, R215del (dominant-negative *MITF*), or a green fluorescence protein (GFP)/wee1-truncation hybrid (which targets GFP to the nucleus as vector control), all under the control of the elongation factor α promoter (42). Subconfluent primary human melanocytes were incubated with concentrated adenoviruses in serum-free F10 medium supplemented with 10 mM $MgCl_2$ for 30 min at a multiplicity of infection of 200 for each virus. After infection the medium was replaced by fresh medium and cultured for indicated times until stimulation.

**Quantitative reverse transcriptase (RT) -polymerase chain reaction (PCR)/TaqMan**

RNA was isolated using the Ambion RNAqueous kit (Ambion, Austin, TX, USA) and quantitated by spectrophotometry (Beckman, Miami, FL, USA). TaqMan One-Step RT-PCR Master Mix Reagents as well as GAPDH Control Reagents (Applied Biosystems, Foster City, CA, USA) were used for quantitative RT-PCR reactions, each containing 100 ng of total sample RNA. Reactions were run for 40 cycles under the following conditions: stage 1: 48°C, 30 min; stage 2: 95°C, 10 min; stage 3: 94°C, 20 s; and stage 4: 62°C, 1 min. The message of human MITF, CDK2, and silver homolog (SILV) was detected using the following primers (Integrated DNA Technologies, Coralville, IA, USA) and TaqMan probe (Applied Biosystems): CDK2: forward 5′-ATG GAG AAC TTC CAA AAG GTG GA-3′, reverse 5′-CAG GCG GAT TTT CTT AAG CG-3′ primers and CDK2 probe 5′-6-FAM-ATC GGA GAG GGC ACG TAC GGA GTT GT-TAMRA-3′; SILV: forward 5′-TCT GGG CTG AGC ATT GGG-3′, reverse 5′-AGA CAG TCA CTT CCA TGG TGT GTG-3′ primers and SILV probe 5′-6-FAM-CAG GCA GGG CAA TGC TGG GC-TAMRA-3′; and EDNRB: forward 5′-TGA GTC TAT GTG CTC TGA GTA TTG ACA-3′, reverse 5′-ACC TAT GGC TTC AGG GAC AGC-3′ primers and EDNRB probe 5′-6-FAM-TGT TTT GAT TTG GGT GGT CTC TGT GGT TCT-TAMRA-3′. All reactions were run in triplicate on an ABI-PRISM 7700 instrument (Applied Biosystems), and gene message levels were normalized to GAPDH expression.

**Quantitative RT-PCR/SYBR Green**

RNA was isolated using an RNeasy mini-protocol kit (Qiagen, Valencia, CA, USA) and quantitated by spectrophotometry (Beckman). The first-strand cDNA was synthesized from total RNA using Rever Tra Ace (TOYOBO, Nagoya, Japan). Reactions were run under the following conditions: stage 1 30°C, 10 min; stage 2: 42°C, 20 min; and stage 3: 99°C, 5 min. iQ SYBR Green Supermix Reagents (Bio-Rad Laboratories, Hercules, CA, USA) were used for quantitative RT-PCR reactions, each containing 10 ng of total sample RNA. Reactions were run for 40 cycles under the following conditions: stage 1: 95°C, 15 s; stage 2: 61°C for 30 s; and stage 3: 72°C, 30 s. The message of human MITF-M and GAPDH was detected using the following primers (FASMAC, Atsugi, Japan). MITF-M: forward 5′-TCC GTC TCT CAC TGG ATT GGT G-3′, reverse 5′-CGT GAA TGT GTG TTC ATG CCT GG-3′; and GAPDH: forward 5′-GCC ATC AAT GAC CCC TTC ATT-3′, reverse 5′-TTG ACG GTG CCA TGG AAT TT-3′. All reactions were run in triplicate on a DNA Engine Opticon 2 Real-Time PCR Detection System (Bio-Rad Laboratories), and gene message levels were normalized to GAPDH expression.

**Gel electrophoresis, immunoblotting, and immunoprecipitation**

For immunoblot analysis, cells were lysed in lysis buffer [50 mM Tris (pH 7.6), 150 mM NaCl, and 1% Triton X-100] plus protease inhibitors (Complete mini-tablets; Boehringer Mannheim, Mannheim, Germany) and phosphatase inhibitors (20 mM NaPP, 10 mM NaF, and 1 mM $Na_3VO_4$, and 1 mM $Na_3VO_4$) and centrifuged at 13,000 rpm for 15 min. The supernatant was harvested and lysed in 2× loading buffer [125 mM Tris (pH 6.8), 4.6% sodium dodecyl sulfate (SDS), 20% glycerol, and 0.04% pyronin Y]. The mixture was then boiled for 5 min. Samples were solubilized in SDS sample buffer plus 50 mM dithiothreitol and boiled for 5 min. Total protein from cell cultures of human melanocytes was subjected to Western blotting with anti-MITF (C5; NeoMarkers, Fremont, CA, USA), anti-phospho-extracellular signal-regulated kinase (ERK) 1/2 (Cell Signaling Technologies, Danvers, MA, USA or Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-cAMP response element-binding protein (CREB) (Cell Signaling Technologies), anti-phospho-MEK1/2 (Cell Signaling Technologies), anti-CREB (Cell Signaling Technologies), anti-ERK (Santa Cruz Biotechnologies), anti-MEK (Santa Cruz Biotechnologies), anti-β-actin (Sigma-Aldrich Corp.), and anti-α-tubulin (Sigma-Aldrich Corp.) antibody. Samples were run on SDS-PAGE gels, transferred onto nitrocellulose, blocked with 5% nonfat dry milk in Tris-buffered saline (TBST) and probed with the respective antibodies in TBST overnight at 4°C. Membranes were washed 3 times for 15 min with TBST, probed with peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ, USA or ICN Biomedicals Inc, Solon, OH, USA) washed three times for 30 min in TBST and developed by ECL (Amersham).

For immunoprecipitation, cells were lysed with lysis buffer as above plus protease inhibitors. The soluble fraction was incubated overnight at 4°C with anti-MITF antibody (D5) or anti-phospho-S73 MITF (43), and subsequently protein G agarose beads (GIBCO-Life Technologies, Inc.) were added and the solution was incubated for an additional 1 h at 4°C. Beads were washed three times with cold PBS, resuspended in SDS sample buffer, and boiled for 5 min. The eluted proteins were resolved on SDS-PAGE and immunoblotted with anti-MITF antibody (C5).

### Transient *in vitro* reporter assay

For reporter assays, 501 MEL cells were plated in a 96-well black and white tissue culture plate (Wallac, Waltham, MA, USA) to a density of $1 \times 10^4$ cells/well. The following day, cells were transfected with 40 ng of promoter [pGL2.basic, pGL2.MITF cAMP response element (CRE) wild-type (CRE wt), or pGL2.MITF (ΔCRE) (CRE mut)] and 20 ng of pRL/null (Promega, Southampton, UK) using FuGene6 transfection reagent (Roche Molecular Biochemicals, Basel, Switzerland) as described previously (44). Cells were allowed to incubate with the transfection mixture for 20 h. The cells were then incubated with 20 μM FSK, 10 nM EDN1/3, or MEK inhibitor PD98059 for an additional 6 h. Cells were washed once with PBS and lysed with 20 μl of 1× passive lysis buffer (Promega). The assay samples were then analyzed on a 96-well plate luminometer (EG&G Berthold, Bad Wildbad, Germany) using a Dual-Luciferase kit (Promega). Luciferase signals were normalized to corresponding *Renilla* signals. Results are expressed as fold activation over unstimulated vector control transfection for each individual promoter set and are plated as the mean ± SE from at least three independent data points.

## RESULTS

### EDN signaling elicits phosphorylation of MITF

We first examined posttranslational modulation of MITF *via* EDN signaling to assess whether treatment with EDN1 stimulates the phosphorylation of MITF in cultured human melanocytes. Western blotting of human melanocytes using antibody to MITF revealed that there are two MITF species with relative mobilities corresponding to molecular masses of 62 and 66 kDa (**Fig. 1*A***). The identities of the two MITF species with different mobilities were suggested to be unphosphorylated and phosphorylated MITFs because of their changes with rapid kinetics and prior analyses by phosphotryptic mapping (7). Activation of signaling by EDN1 completely shifted the lower unphosphorylated MITF band to the upper phosphorylated MITF band with a peak at 10 min postincubation. This shift occurred rapidly but transiently and the lower unphosphorylated band reappeared within 40 min. The mobility shift was abrogated by BQ788, a selective EDNRB

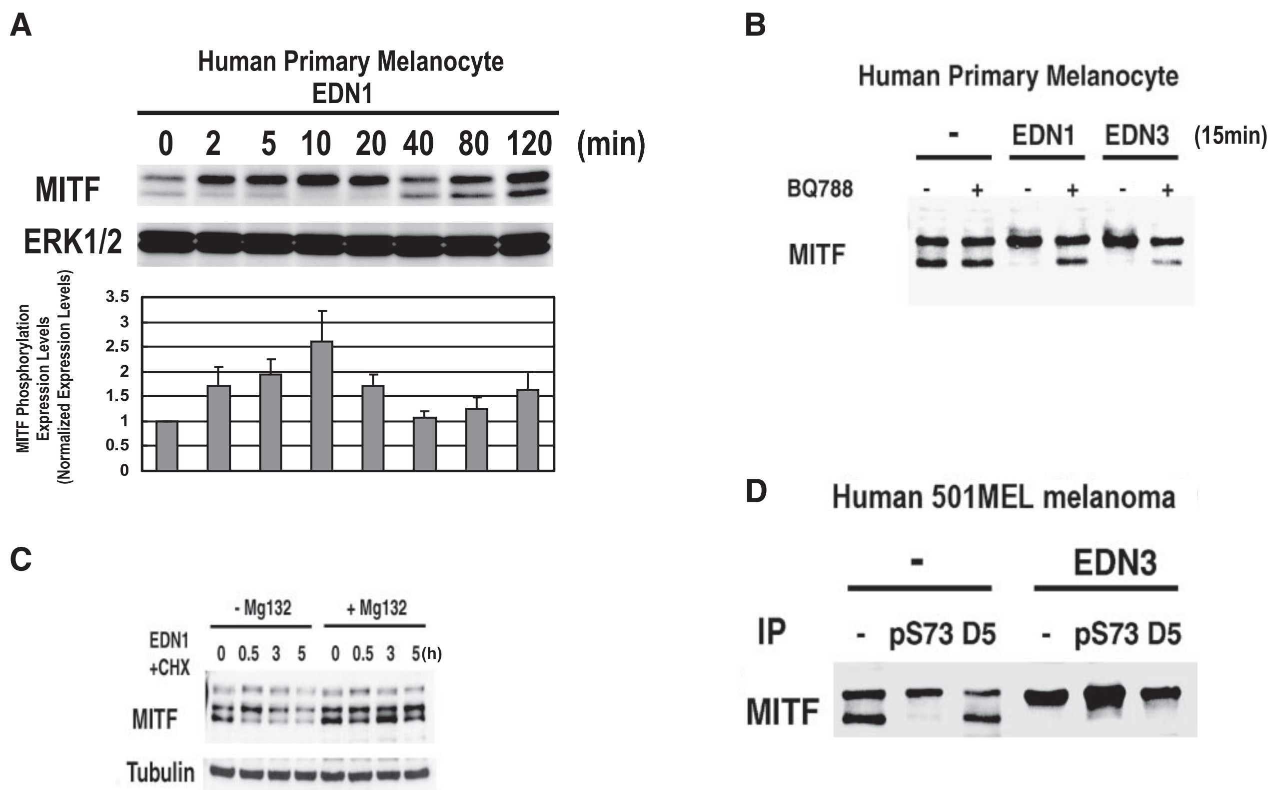



**Figure 1.** *A*) EDN1 elicits a marked phosphorylation of MITF. Human primary melanocytes were stimulated with EDN1 (10 nM). Lysates were harvested at the indicated times. EDN stimulation produced a mobility shift of MITF. Total protein blots were probed for MITF and ERK1/2. Western blotting shows representative data. Densitometric data represent SEM from three independent experiments using melanocytes from the same donors. *B*) The EDNRB antagonist BQ788 abolishes the EDN1/3-induced phosphorylation of ERK. Human primary melanocytes were treated with 10 nM EDN1/3 in the presence or absence of BQ788 (10 μM). Total protein blots were probed for MITF. *C*) MG132 prevents MITF degradation mediated through proteasomal degradation. Human primary neonatal melanocytes were stimulated with EDN1 (10 nM) and cycloheximide (CHX) (20 μg/ml) in the presence or absence of MG132 protease inhibitor (25 μM). Lysates were harvested at the indicated times and immunoblotted with MITF and α-tubulin antibodies. *D*) S73 of MITF is phosphorylated by EDN3 stimulation. Human 501 MEL melanoma cells were stimulated with EDN3 (10 nM). Lysates were immunoprecipitated (IP) with anti-phospho-S73 (pS73) MITF antibodies or anti-MITF antibody (D5) followed by Western blotting for MITF (C5).

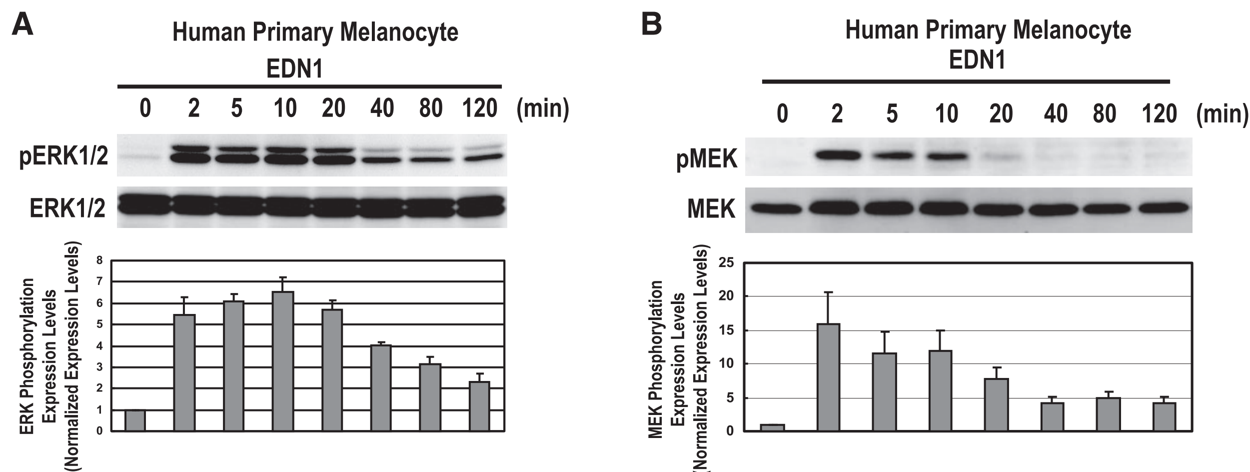



**Figure 2.** A) EDN1 elicits a marked phosphorylation of ERK1/2 followed by the phosphorylation of MITF. Human primary melanocytes were stimulated with EDN1 (10 nM). Lysates were harvested at indicated times. Total protein blots were probed for phospho-ERK1/2 (pERK1/2) and ERK1/2. Western blotting shows representative data. Densitometric data represent SEM from three independent experiments using melanocytes from the same donors. *B*) EDN1 elicits a marked phosphorylation of MEK followed by phosphorylation of MITF or ERK1/2. Human primary melanocytes were stimulated with EDN1 (10 nM). Lysates were harvested at indicated times. Total protein blots were probed for phospho-MEK (pMEK) and MEK. Western blotting shows representative data. Densitometric data represent SEM from three independent experiments using melanocytes from the same donors.

receptor antagonist (Fig. 1*B*). Proteasome inhibition using MG132 (Fig. 1*C*) stabilized MITF in the upper phosphorylated form induced by EDN1 treatment, suggesting prevention of MITF degradation mediated through proteasomal degradation. Immunoprecipitation with anti-phospho-S73 MITF antibody showed that serine 73 of MITF is phosphorylated by EDN stimulation (Fig. 1*D*).

### EDN signaling elicits the phosphorylation of ERK1/2 and MEK

EDN1 stimulation also increased the phosphorylation of ERK1/2 with a peak at 10 min postincubation (**Fig. 2*A***), which correlated temporally with the shift in MITF protein. The increased phosphorylation occurred rapidly but transiently from 2 to 20 min after incubation with EDN1; the phosphorylation of ERK1/2 was markedly diminished within 40 min postincubation. Further, the increased phosphorylation of ERK1/2 was accompanied by increased phosphorylation of MEK during a comparable time; the increased phosphorylation occurred rapidly but transiently from 2 to 20 min postincubation and almost disappeared within 40 min (Fig. 2*B*).

### Phosphorylation of MITF is specifically abolished by inhibitors of MEK and PKC

To elucidate the signaling mechanisms involved in phosphorylation of MITF, we compared time courses of phosphorylation of MITF and ERK1/2 and used several specific signaling inhibitors at 10 min postincubation when the phosphorylation of MITF reaches a maximum. Western blot analysis revealed that phosphorylation of ERK1/2 occurred at a time course similar to the mobility shift of MITF with a peak at 10–20 min postincubation with EDN3 in human melanocytes and human melanoma cells (501 MEL) (**Fig. 3**). Treatment with the MEK inhibitor PD98059 (20 μM), 60 min before addition of EDN1, diminished the increased phosphorylation of MITF in the presence or absence of cycloheximide, a translation inhibitor (which blocks *de novo* protein synthesis) (**Fig. 4*A, B***). Three independent experiments showed that MEK inhibition elicits a significant decrease in EDN-induced MITF phosphorylation (**Fig. 5*A***). The MEK inhibitor PD98059 also significantly decreased the EDN-induced phosphorylation of

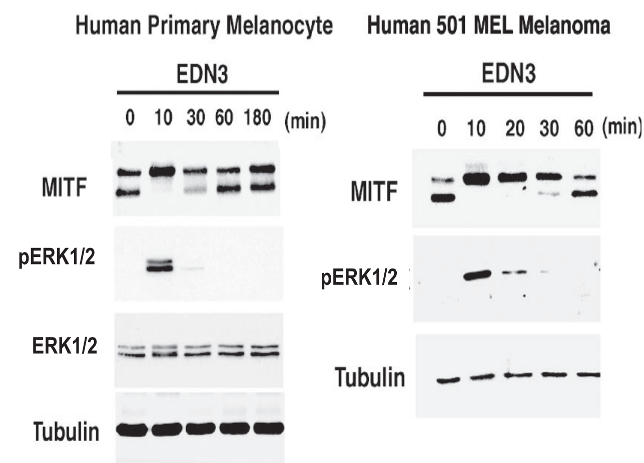



**Figure 3.** EDN3 stimulation produced a mobility shift of MITF accompanied by ERK phosphorylation. Human primary melanocytes and human 501 MEL melanoma cells were stimulated with EDN3 (10 nM) for the indicated times, and total protein blots were probed for MITF (top), phospho-ERK1/2 (pERK1/2) and ERK1/2 (center), and α-tubulin (bottom).

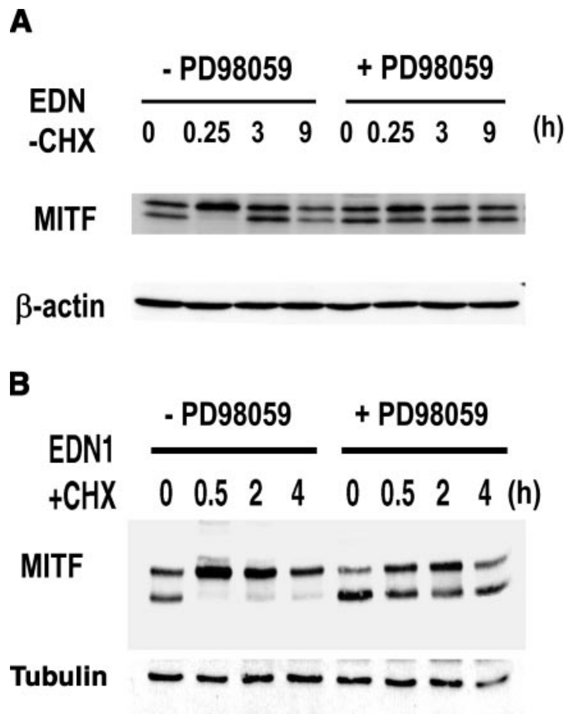



**Figure 4.** PD98059 abolishes EDN1-induced phosphorylation of MITF. Human primary melanocytes were stimulated with EDN1 (10 nM) and without (*A*) or with (*B*) cycloheximide (CHX) (20 μg/ml) in the presence or absence of MEK inhibitor PD98059 (20 μM). Lysates were harvested at indicated times and immunoblotted with MITF and α-tubulin or β-actin antibodies.

ERK1/2 (Fig. 5*B*). Although MEK inhibition was incomplete, it also suppressed a corresponding degree of MITF phosphorylation.

Similarly, treatment with the PKC inhibitor Gö6983 (1 μM), 60 min before addition of EDN1, significantly abolished the increased phosphorylation of MITF (**Fig. 6*A***), which was accompanied by marked inhibition in the phosphorylation of ERK1/2 (Fig. 6*B*), consistent with the possibility that suppression of MITF phosphorylation by the PKC inhibitor was mediated *via* the inhibition of ERK1/2 phosphorylation. In contrast, inhibitors of other kinases such as PKA (H89, 5 μM), p38 MAPK (SB203580, 10 μM), PI3 kinase (wortmannin, 100 nM), and Akt (SH-6, 1 μM) did not significantly inhibit the phosphorylation of MITF (**Fig. 7*A–D***).

### EDN signaling stimulates the expression of MITF

Because evidence suggests that MITF expression is regulated by a signal transduction pathway using cAMP as a second messenger (45, 46) and because we have already shown that EDN signaling exerts a rapid and marked increase in the level of cAMP in human melanocytes (19), we determined whether EDN1 alters MITF expression using quantitative RT-PCR analysis and Western blotting. Within 40 min after EDN1 stimulation there was an increase in the intensity of MITF-M mRNA transcripts, relative to control GAPDH levels (**Fig. 8*A***). This increase occurred transiently with a peak at 40 to 80 min postincubation and returned to the control level within 3 h. Western blotting analysis during 24 h after incubation with EDN1 revealed that within 2 h of treatment there was an increase in the intensity of MITF protein (upper + lower bands), relative to control β-actin levels (Fig. 8*B*). This increase occurred transiently with a peak at 2 to 3 h postincubation and returned to the control level within 24 h. The increased production of MITF protein was significantly diminished by the PKA inhibitor H89 (Fig. 8*C*), suggesting that the stimulation was dependent on the cAMP-PKA pathway.

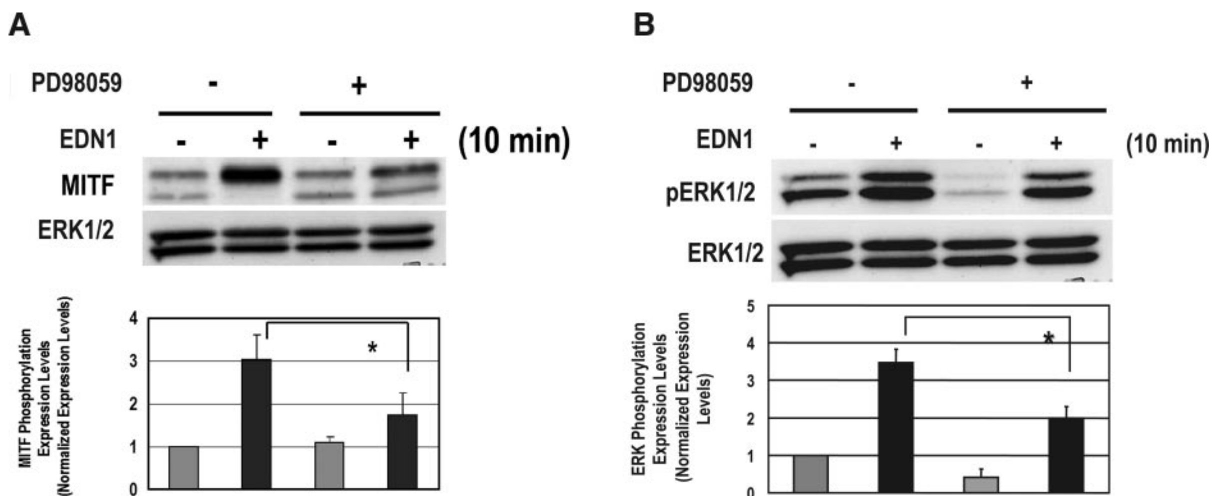



**Figure 5.** PD98059 abolishes the EDN1-induced phosphorylation of MITF and ERK1/2. Human primary melanocytes were stimulated with or without EDN1 (10 nM) in the presence or absence of PD98059 (20 μM). Lysates were harvested at 10 min and immunoblotted with MITF and ERK1/2 (*A*) or with phospho-ERK1/2 (pERK1/2) and ERK1/2 (*B*). Western blotting shows representative data. Densitometric data represent mean ± SD from three independent experiments using melanocytes from the same donors. $^{*}P < 0.05$.

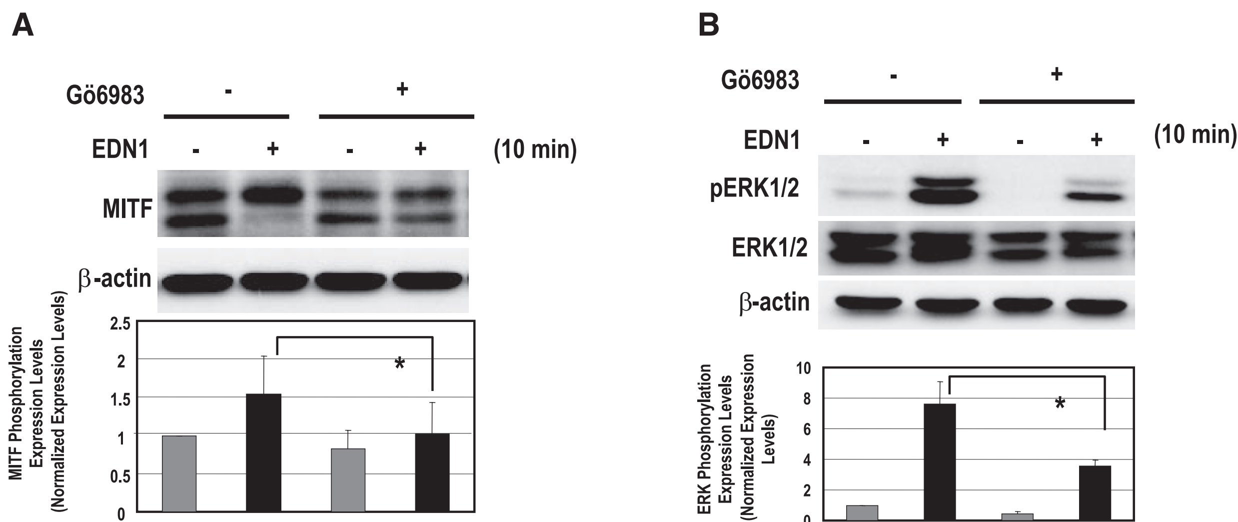



**Figure 6.** Gö6983 diminishes the EDN1-induced phosphorylation of MITF and ERK1/2. Human primary neonatal melanocytes were stimulated with or without EDN1 (10 nM) in the presence or absence of PKC inhibitor Gö6983 (1 μM). Lysates were harvested at 10 min and immunoblotted with MITF and ERK1/2 (*A*) or with phospho-ERK1/2 (pERK1/2) and ERK1/2 (*B*). Western blotting shows representative data. Densitometric data represent mean ± SD from three independent experiments using melanocytes from the same donors. $^*P < 0.05$.

### EDN signaling activates CREB factors

To examine whether the transcription of the *MITF* gene is dependent on binding of CREB to the MITF promoter, luciferase reporter assays were performed using plasmids containing the melanocyte-restricted MITF promoter (46) driving luciferase in 501 MEL melanoma cells (47). These assays showed that EDN (as well as SCF) significantly induces MITF promoter activity (**Fig. 9** and data not shown). The melanocytic MITF promoter has a CRE consensus sequence where CREB/ATF family members bind and transactivate (48). It has been shown that whereas cAMP signaling stimulates the MITF promoter *via* this conserved CRE (45, 46), ERK/MAPK activation in melanocytes stimulates RSK, which is known to phosphorylate and activate CREB factors in other lineages (49, 50). We therefore examined the possibility that activation of the MITF promoter is mediated by the CRE site. As shown in Fig. 9 (black bars), EDN-induced MITF promoter activation was abrogated when the CRE site was mutated. Furthermore, the EDN responsiveness was also lost with MEK inhibitor PD98059 treatment before EDN stimulation. These data are consistent with the possibility that EDN signaling induces *MITF* gene transcription using CREB phosphorylation/activation significantly *via* the MAPK-p90RSK pathway. Indeed, as seen in **Fig. 10*A***, Western blot analysis of EDN1/3 or SCF-treated melanocytes using anti-phospho-ERK and anti-phospho-CREB showed that both EDN and SCF stimulations induce

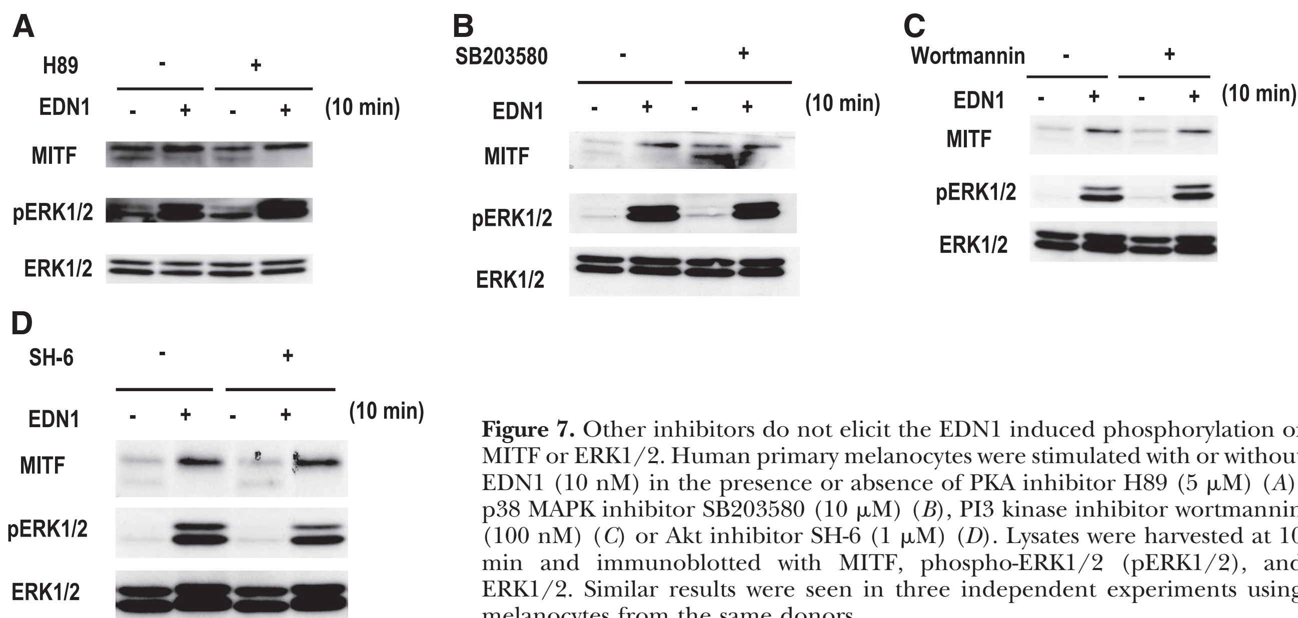
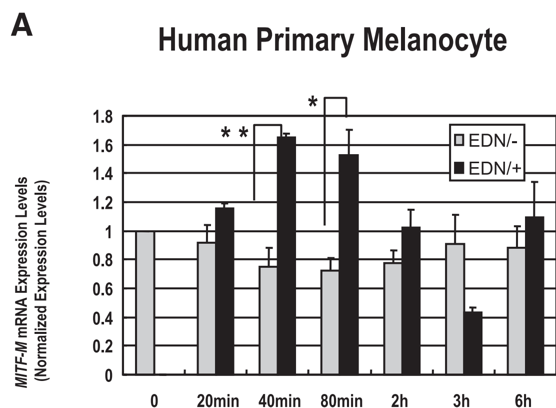
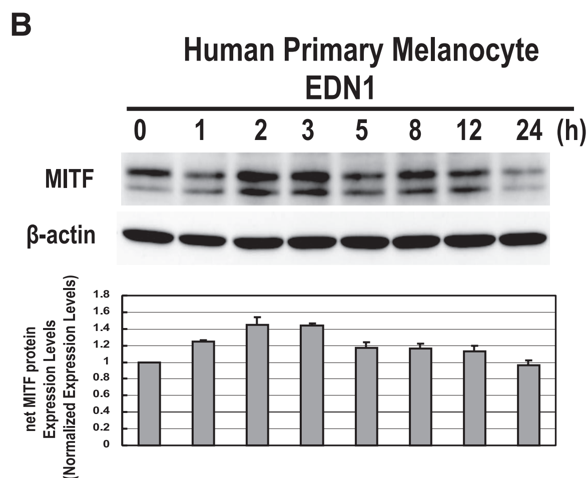



**Figure 7.** Other inhibitors do not elicit the EDN1 induced phosphorylation of MITF or ERK1/2. Human primary melanocytes were stimulated with or without EDN1 (10 nM) in the presence or absence of PKA inhibitor H89 (5 μM) (*A*), p38 MAPK inhibitor SB203580 (10 μM) (*B*), PI3 kinase inhibitor wortmannin (100 nM) (*C*) or Akt inhibitor SH-6 (1 μM) (*D*). Lysates were harvested at 10 min and immunoblotted with MITF, phospho-ERK1/2 (pERK1/2), and ERK1/2. Similar results were seen in three independent experiments using melanocytes from the same donors.



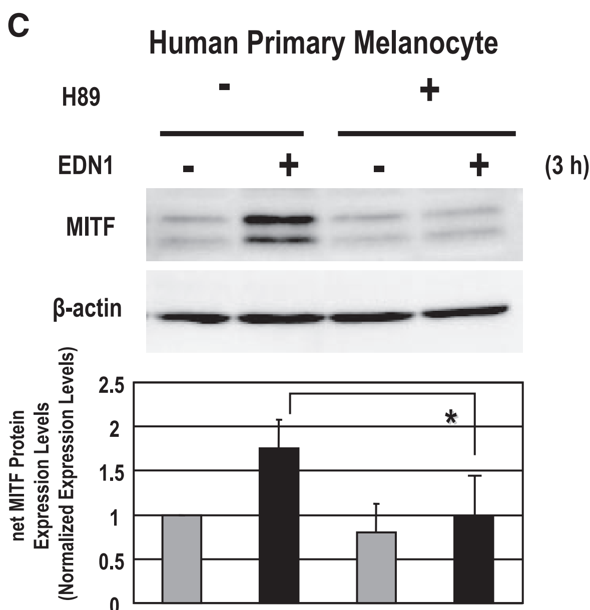




**Figure 8.** *A*) EDN1 increases transcription of *MITF-M*. Human primary melanocytes were treated with 10 nM EDN1 and were harvested and solubilized after the incubation times. Total RNA extracts from treated or control human melanocytes were reverse-transcribed and the cDNAs were PCR amplified with specific primer sets. $^{**}P < 0.01$; $^{*}P < 0.05$. *B*) EDN1 stimulates production of MITF protein. Human primary melanocytes were stimulated with EDN1 (10 nM). Lysates were harvested at the indicated times. Total protein blots were probed for MITF and β-actin. Western blotting shows representative data. Densitometric data represent SEM from three independent experiments using melanocytes from the same donors. *C*) Human primary melanocytes were stimulated with or without EDN1 (10 nM) in the presence or absence of H89 (5 μM). Lysates were harvested at 3 h and immunoblotted with MITF and β-actin. Western blotting shows representative data. Densitometric data represent mean ± SD from three independent experiments using melanocytes from the same donors. $^{*}P < 0.05$.

significant MAPK and CREB phosphorylation, whereas FSK treatment did not induce comparable ERK/MAPK phosphorylation, although it did trigger strong CREB phosphorylation that was not abrogated by MEK inhibitor (Fig. 10*B*).

The time course study of the phosphorylation of CREB revealed that CREB was markedly phosphorylated by EDN stimulation with a broad peak from 3 through 30 min postincubation (**Fig. 11*A***). The CREB phosphorylation induced by EDN was abrogated to a different extent depending on postincubation times by the PKA inhibitor (H89) or the MEK inhibitor (PD98059) (Fig. 11*B–E*). Although CREB phosphorylation was significantly abolished at 5 min postincubation by the MEK inhibitor but not by the PKA inhibitor (Fig. 11*B, C*), the induced phosphorylations at 15 and 30 min postincubation were significantly abrogated by the PKA inhibitor but not by the MEK inhibitor (Fig. 11*B, D, E*), suggesting a biphasic activation of CREB due to the differential time course of signaling in the MAPK-RSK and cAMP-PKA pathways. These findings, taken together, suggest that EDN signaling up-regulates MITF expression significantly through both MAPK-RSK and cAMP-

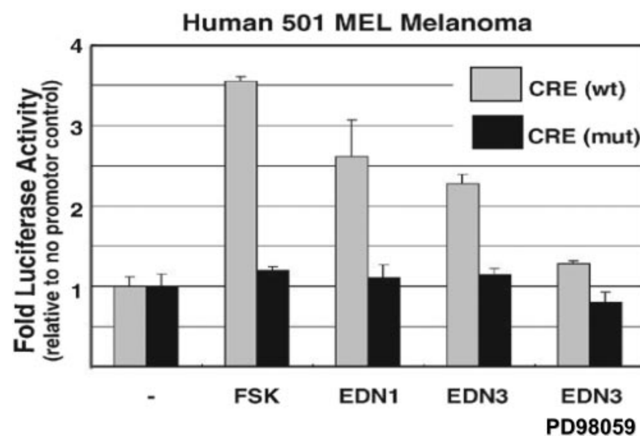



**Figure 9.** EDN1/3 Significantly induces MITF promoter activity. 501 MEL melanoma cells were transfected with the human MITF promoter (−387 to +97) or CREB binding site (CRE) mutant promoter driving luciferase. Transfected cells were treated with FSK or EDN1/3. Further, in one case, EDN3 was added after PD98059 (20 μM) treatment. Luciferase activity was corrected for transfection efficiency using constitutive *Renilla* (sea pansy) luciferase activity. Luciferase activities were normalized with no promoter control (pGL2.basic). The value of no stimulation control was normalized to 1. Data represent SEM from three independent experiments using melanocytes from the same donors.

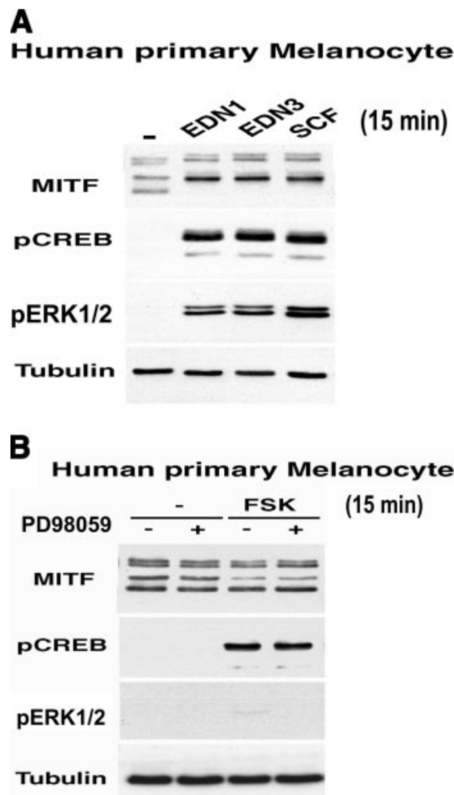



**Figure 10.** *A*) EDN1/3 and SCF elicit a marked phosphorylation of CREB and ERK1/2. Human primary melanocytes were stimulated with EDN1 (10 nM), EDN3 (10 nM), and SCF (20 ng/ml). Total protein blots were probed for MITF, phospho-CREB (pCREB), phospho-ERK1/2 (pERK1/2), and α-tubulin. *B*) FSK induces a marked CREB phosphorylation that is not abrogated by PD98059. Human primary melanocytes were stimulated with FSK (20 μM) in the presence or absence of 20 μM PD98059 for 15 min. Total protein blots were probed for MITF, phospho-CREB, phospho-ERK1/2, and α-tubulin.

PKA pathways, which converge on CREB and exhibit differential (complementary) kinetics.

### EDN induces MITF-dependent transactivation of the cell cycle regulator CDK2 and the melanosomal protein SILV

The above results place MITF downstream of EDN signals and suggest that significant aspects of EDN-induced melanocyte proliferation or differentiation may be mediated by MITF. We have recently found that *SILV* and *CDK2* are both MITF target genes (41, 51). Quantitative RT-PCR revealed that EDN stimulation significantly increased the expression levels of SILV and CDK2 in primary melanocytes (**Fig. 12**). We therefore asked whether up-regulation of these targets by EDN was mediated by MITF. To test this hypothesis, EDN3 stimulation was repeated in the presence of control or dominant-negative MITF adenoviruses (52) in primary melanocytes (Fig. 12). The increased expression levels of *SILV* and *CDK2* induced by EDN administration were both selectively blocked by dominant-negative MITF. The dominant-negative MITF virus itself did not affect MAPK activation (data not shown). These results demonstrate that EDN stimulates expression of *SILV* and *CDK2*, two genes that are involved in melanocyte differentiation and proliferation, respectively, and this stimulated expression is dependent on endogenous MITF.

### Expression of EDN receptor EDNRB is dependent on MITF

Analysis of MITF expression in human melanocytes stimulated with EDN1/3 suggested that the EDN-MITF signaling pathways are regulated by multiple feedback pathways. Our previous microarray analysis suggested that expression of EDNRB is regulated *via* MITF in human melanocytes (38). Thus, we examined EDNRB expression by human melanocytes infected with adenovirus expressing the wild-type and dominant-negative form of MITF. As shown in **Fig. 13**, EDNRB mRNA expression was significantly induced with wild-type MITF and inhibited by dominant-negative MITF, indicating that EDNRB expression is dependent on MITF.

## DISCUSSION

Despite increasing knowledge of the functions of MITF in melanoblasts/melanocytes during their development and of the roles of EDNs in several pigmentary disorders (10, 33–37), the effects of EDN-activated intracellular signaling on MITF dynamics, including its phosphorylation, have received little attention. Thus, it remains to be clarified as to how the activation of the PKC, MAPK, and cAMP pathways initiated by EDN receptor binding (19–21) are linked to survival as well as to the stimulation of mitogenesis and melanogenesis of melanocytes in association with the dynamics of MITF. Although MAPK is known to link MITF to KIT signaling in melanocytes *via* MITF phosphorylation (7, 53), it had not been established whether stimulation of human melanocytes with EDN affects the phosphorylation of MITF due to the activation of the MAPK pathway. Here we demonstrate that EDN1 signaling elicits a marked phosphorylation of MITF with a peak at 10 min postincubation with EDN1. This phosphorylation of MITF occurs at $Ser^{73}$ in an EDNRB-sensitive manner and is accompanied by or preceded by the phosphorylation of ERK1/2 and MEK, which indicates activation of the MAPK pathway. Treatment with a MEK inhibitor markedly suppresses the phosphorylation of MITF. The inhibition of MITF phosphorylation is accompanied by a significant suppression of the phosphorylation of ERK1/2 at a comparable time. Because ERK2 is the kinase responsible for this MITF phosphorylation (7) and because the increased phosphorylation of ERK1 and 2 occurs with response kinetics similar to those of EDN1, these findings strongly suggest that activation of the MAPK pathway by EDN1 directly

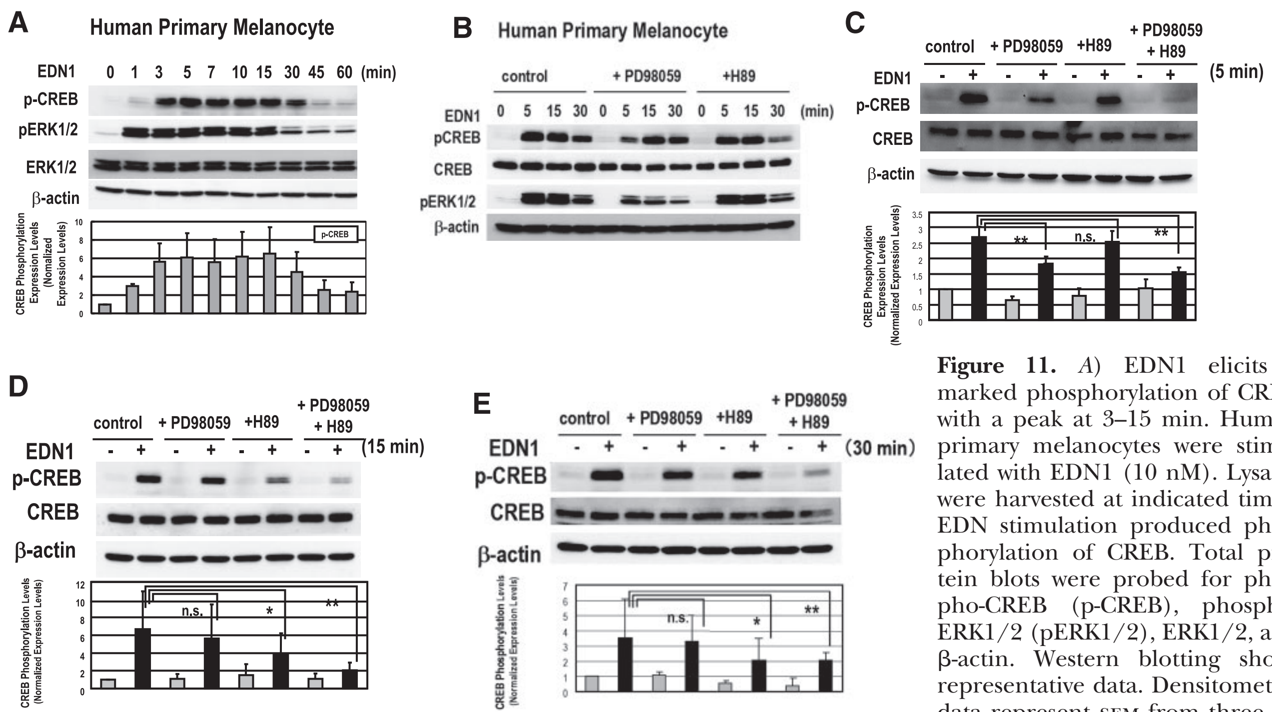



**Figure 11.** *A*) EDN1 elicits a marked phosphorylation of CREB with a peak at 3–15 min. Human primary melanocytes were stimulated with EDN1 (10 nM). Lysates were harvested at indicated times. EDN stimulation produced phosphorylation of CREB. Total protein blots were probed for phospho-CREB (p-CREB), phospho-ERK1/2 (pERK1/2), ERK1/2, and β-actin. Western blotting shows representative data. Densitometric data represent SEM from three independent experiments using melanocytes from the same donors. *B*) PD98059 or H89 diminishes the EDN1-induced phosphorylation of CREB. Human primary neonatal melanocytes were stimulated with EDN1 (10 nM) in the presence or absence of PD98059 (40 μM) and H89 (10 μM). Lysates were harvested at indicated times and immunoblotted with phospho-CREB, CREB, phospho-ERK1/2, and β-actin. *C–E*) PD98059 and H89 diminishes the EDN1-induced phosphorylation of CREB with a different time course. Human primary neonatal melanocytes were stimulated with or without EDN1 (10 nM) in the presence or absence of PD98059 (40 μM) and H89 (10 μM). Lysates were harvested at 5 min (*C*), 15 min (*D*), or 30 min (*E*) and immunoblotted with phospho-CREB, CREB, and β-actin. Western blotting shows representative data. Densitometric data represent mean ± SD from three independent experiments using melanocytes from the same donors. $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., not significant.

triggers MITF phosphorylation in human melanocytes similar to the stimulation by SCF.

In the signaling pathway initiated by EDN1 activation of the EDNRB in human melanocytes, we found that the activation of PKC is linked probably at a convergent point of Raf-1 to the phosphorylation of ERK1/2, which indicates activation of the MAPK pathway (21). In addition, we demonstrated that EDN1 signaling elicits a marked increase in intracellular cAMP levels in a PKC-dependent manner, indicating cross-talk between PKC and the cAMP pathway (19). Based on the above observations regarding EDN signaling, we used several specific signaling inhibitors in this study to show that MITF phosphorylation is significantly abolished by a

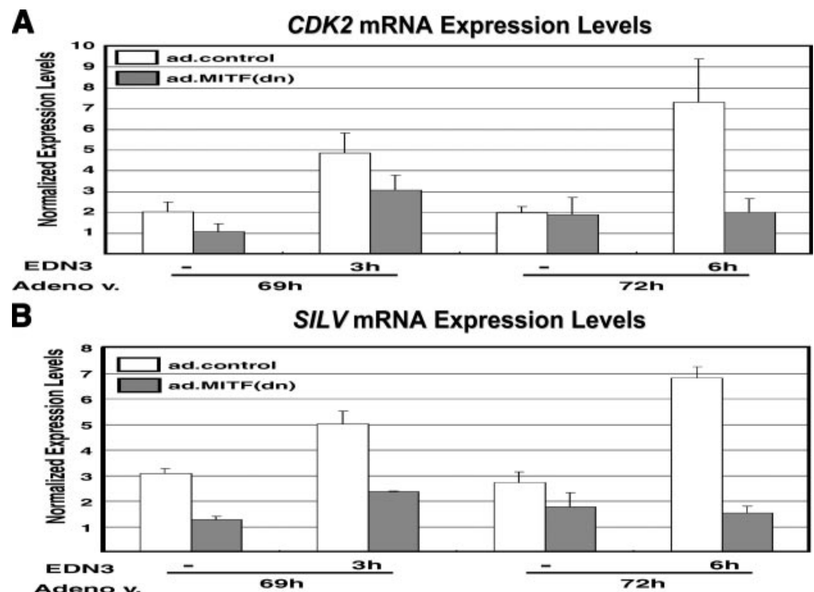



**Figure 12.** EDN induces MITF-dependent transactivation of cell cycle regulator CDK2 and melanosomal protein SILV. CDK2 (*A*) and SILV (*B*) mRNA expressions were examined in human primary melanocytes infected with adenovirus (ad.) expressing the dominant-negative (dn) form of MITF and stimulated with EDN3. Data represent SEM from three experiments using melanocytes from the same donors.

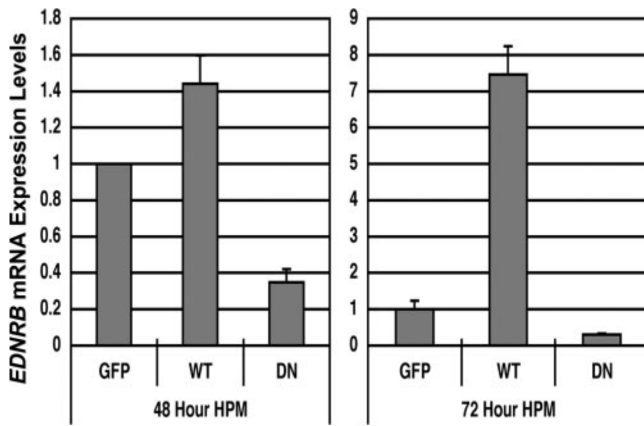



**Figure 13.** EDNRB expression is dependent on MITF function. EDNRB mRNA expression were examined in human primary melanocytes infected with adenovirus expressing the wild-type (WT) or dominant-negative (DN) form of MITF. Data represent SEM from three experiments using melanocytes from the same donors.

specific PKC inhibitor, but not by p38 MAPK, PI3 kinase, Akt, or PKA inhibitors. The fact that the inhibition of MITF phosphorylation by the PKC inhibitor is accompanied by a significant suppression of ERK1/2 phosphorylation suggests that the MITF phosphorylation induced by EDN signaling is not directly triggered by the activation of PKC but is evoked by the activation of ERK1/2, which occurs subsequently as a result of the activation of PKC.

In contrast to MITF Ser$^{73}$ phosphorylation, which is associated with the signal transduction pathway involving MAPK, MITF expression is probably regulated by a signal transduction pathway using cAMP as a second messenger as the MITF promoter contains a CRE (48). In fact, elevated levels of intracellular cAMP, triggered either by α-melanocyte-stimulating hormone (α-MSH) or by FSK, lead to rapid and potent induction of the MITF promoter (45, 46). Because EDN1 signaling results in a marked increase in cAMP levels in human melanocytes in a PKC-dependent manner (19), we asked whether EDN1 stimulation alters MITF expression *via* CREB phosphorylation. In this connection, our study demonstrates that EDN1 treatment elicits a rapid increase in MITF-M mRNA transcripts with a peak at 40–80 min postincubation. This is followed by a rapid and transient increase in MITF protein with a peak at 2–3 h after EDN1 stimulation. As for signaling mechanisms underlying stimulated MITF expression, we found that *1*) the increased production of MITF protein was significantly diminished by the PKA inhibitor H89, *2*) EDN-induced MITF promoter activation was abrogated when the CRE site was mutated, and *3*) EDN stimulation induces a significant CREB phosphorylation in concert with MAPK phosphorylation. These findings strongly suggest that the increased expression of MITF after EDN stimulation is mediated *via* CREB activation. As for the signaling pathway leading to CREB activation after EDN stimulation, we demonstrated that both the potent induction of the MITF promoter and CREB phosphorylation by EDN stimulation are abrogated by the MEK inhibitor (PD98059). Because CREB phosphorylation after FSK stimulation was not significantly affected by the MEK inhibitor, our results suggest the possibility that the MAPK-RSK pathway is also implicated in EDN-induced *MITF* gene expression through CREB phosphorylation/activation. This finding is consistent with our results (see Fig. 10*A*) that SCF stimulation induces CREB phosphorylation concomitant with ERK phosphorylation despite the fact that SCF signaling does not up-regulate intracellular cAMP levels (19). It is known that CREB is activated by cAMP-dependent protein kinase as well as by all three members of the RSK family (RSK1–3) in cells stimulated by activators of the Ras-MEK-ERK1/2 cascade (49). Therefore, as depicted in **Fig. 14**, it is likely that EDN signaling triggers MITF expression *via* CREB phosphorylation/activation due to both cAMP-PKA and MAPK-p90RSK cascades, which occur with different (biphasic) kinetics, PKA being much later than tMAPK.

α-MSH, KIT, and EDN signaling pathways are implicated in the modulation and the expression of MITF, but they do so in very different ways. α-MSH stimulation of melanocytes up-regulates cAMP, which increases the transcription of *MITF* through a CRE in the MITF promoter (45, 46). In contrast, KIT stimulation elicits a very rapid MAPK-mediated phosphorylation of MITF, which induces the enhanced recruitment of p300/CREB-binding protein (54), the coactivator family that interacts with and modulates the transcriptional activity of *MITF*. All of that occurs over the course of minutes. On the other hand, EDN stimulation not only elicits very rapid MAPK-mediated phosphorylation of MITF within minutes but also profoundly increases MITF protein expression within hours. These dual actions

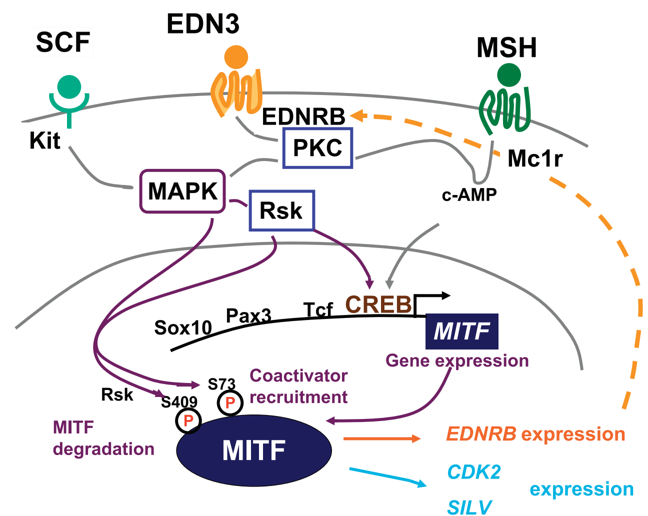



**Figure 14.** Schematic diagram of the EDN-MITF signaling pathway. EDN signals profoundly regulate the central melanocyte transcription factor MITF in two ways: *1*) up-regulation of the *MITF* gene and *2*) direct MITF phosphorylation through MAPK. This pathway forms a feedback loop with MITF-dependent up-regulation of *EDNRB* gene expression.

triggered by EDN stimulation are likely to be associated with the dual potent effects of EDN on both the proliferation and the differentiation of human melanocytes (15).

It is well known that KIT-mediated MAPK phosphorylation triggers a short-lived MITF activation as well as a net degradation of MITF mediated by proteasomes (7, 53). Our data also showed that a rapid disappearance of phosphorylated MITF during EDN stimulation occurs *via* its rapid degradation through proteasomes. EDN1 signaling elicits the increased production of MITF protein and sustains MITF protein at more than control levels over 24 h after the addition of EDN1. The increased production of MITF protein is significantly diminished by the PKA inhibitor H89, suggesting that the stimulation occurs at least *via* the cAMP pathway. Thus, it is likely that the cAMP-mediated new production of MITF protein may compensate to some extent for MITF degradation, which is stimulated by MAPK-mediated MITF phosphorylation and subsequent ubiquitination.

In this study, we demonstrate a molecular link between *MITF* and *EDN/EDNRB*, mutations, which produce WS type II and IV, respectively. Our findings show that EDN signaling modulates the MITF by up-regulation of *MITF* gene expression and MITF phosphorylation, which is associated with altered half-life. These effects are both mediated by the ERK/MAPK pathway but follow distinct kinetics. Furthermore, our transfection studies using dominant-negative MITF demonstrate that expression of EDNRB is dependent on MITF function, suggesting that EDNRB is a downstream target of MITF. These findings connect several central regulators of melanocyte development/proliferation/differentiation into a signaling pathway that is disrupted in WS types II and IV. The deafness and white forelock (spotting) seen in patients with WS types I–IV and Woolf syndrome thus probably results from MITF dosage insufficiency due to disruption of *1*) signaling pathways that modulate MITF expression (EDN3, EDNRB, and KIT), *2*) transcription factors that regulate MITF expression (PAX3 and SOX10), or *3*) mutations within *MITF* itself. The finding of WS type II *MITF* null mutant alleles (34) is consistent with the conclusion that MITF dosage (haploinsufficiency) is the critical determinant of this autosomal dominant (heterozygous) clinical syndrome. In addition, the discovery of signaling pathways that modulate MITF promoter activity might theoretically permit therapeutic up-regulation of the remaining wild-type *MITF* allele in these patients. This possibility may also exist through the MSH pathway, which signals *via* cAMP to CREB and similarly stimulates MITF expression in melanocytes (45, 46).

As for signaling mechanisms underlying EDN-induced proliferation and differentiation (melanin synthesis) in association with MITF function, the present study demonstrated that EDN induces MITF-dependent transactivation of the *CDK2* and *SILV* genes. CDK2 is a major cell cycle regulator important for S phase progression (54). Therefore, a direct link of MITF with *CDK2* gene transactivation during EDN signaling may contribute to the EDN-induced stimulation of melanocyte proliferation (21). SILV (PMEL17) is a melanosomal protein (55) whose mutation in mice (*si/si*) results in reduced melanosome number and the silver pigment color defect (56). Details of the transcriptional regulation of these genes by MITF have been described previously (41, 51). The induction of *SILV* gene transactivation during EDN signaling is also consistent with EDN-induced stimulation of melanin synthesis (including melanosome formation) in human melanocytes (21) as MITF can act as a transcription factor for multiple components in the pigmentation pathway (29–32).

In conclusion, as depicted in Fig. 14, EDN signals profoundly regulate the central melanocyte transcription factor MITF in two ways: *1*) direct MITF phosphorylation through MAPK and *2*) up-regulation of *MITF* gene expression. This pathway is also definitively associated with up-regulation of EDNRB gene expression. Thus, our findings associate EDN/EDNR signaling with MITF modulation as a central regulator of melanocyte fate, leading to the formation of a feedback loop with strong epistatic connections. The dynamics of MITF phosphorylation and production initiated by the activation of EDN/EDNR provides a deep insight into the pathogenesis of WS types II and IV as well as the biological mechanisms underlying other EDN-related hyper- or hypopigmentary disorders. FJ

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