CtBP2 Downregulation during Neural Crest Specification Induces Expression of Mitf and REST, Resulting in Melanocyte Differentiation and Sympathoadrenal Lineage Suppression

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Trunk neural crest (NC) cells differentiate to neurons, melanocytes, and glia. In NC cultures, cyclic AMP (cAMP) induces melanocyte differentiation while suppressing the neuronal sympathoadrenal lineage, depending on the signal intensity. Melanocyte differentiation requires activation of CREB and cAMP-dependent protein kinase A (PKA), but the role of PKA is not understood. We have demonstrated, in NC cultures, cAMP-induced transcription of the microphthalmia-associated transcription factor gene (Mitf) and the RE-1 silencing transcription factor gene (REST), both Wnt-regulated genes. In NC cultures and zebrafish, knockdown of the corepressor of Wnt-mediated transcription C-terminal binding protein 2 (CtBP2) but not CtBP1 derepressed Mitf and REST expression and enhanced melanocyte differentiation. cAMP in NC and B16 melanoma cells decreased CtBP2 protein levels, while inhibition of PKA or proteasome rescued CtBP2 degradation. Interestingly, knockdown of homeodomain-interacting kinase 2 (HIPK2), a CtBP stability modulator, increased CtBP2 levels, suppressed expression of Mitf, REST, and melanocyte differentiation, and increased neuronal gene expression and sympathoadrenal lineage differentiation. We conclude that cAMP/PKA via HIPK2 promotes CtBP2 degradation, leading to Mitf and REST expression. Mitf induces melanocyte specification, and REST suppresses neuron-specific gene expression and the sympathoadrenal lineage. Our studies identify a novel role for REST in NC cell differentiation and suggest cross talk between cAMP and Wnt signaling in NC lineage specification.

The neural crest (NC) is a transient, embryonic cell population of migratory and pluripotent cells that differentiate into diverse cell types. Trunk NC cells give rise to the neuronal cell types of sympathoadrenal (SA) and sensory neurons and the nonneuronal cell types of melanocytes and glia (44, 45). Lineage segregation of NC cells is instructed by the interplay of microenvironmental signals (61, 64). Cyclic AMP (cAMP) is an intracellular signaling molecule that transmits extracellular signals to modulate NC cell fate (7, 14, 35, 53, 59). Our earlier findings have demonstrated dual inputs of cAMP signaling in NC cell differentiation depending on the signal intensity (9). Specifically, moderate activation of cAMP signaling, in combination with BMP-2, exerts a synergistic induction in SA lineage specification; by contrast, robust activation of cAMP signaling promotes melanocyte differentiation while suppressing SA cell development. Activation of the cAMP response element-binding protein (CREB) by cAMP signaling is necessary for melanocyte differentiation. However, expression of the constitutively active CREB is insufficient to induce melanocyte differentiation in the absence of activated protein kinase A (PKA), suggesting additional roles for cAMP signaling in specifying the melanocytic lineage (37).

Wnt is a well-established extracellular signaling molecule involved in NC induction and lineage specification (24, 30, 36, 47). Specifically, Wnt signaling promotes melanocyte differentiation by inducing transcription of the microphthalmia-associated transcription factor gene (Mitf) via a Lef1/TCF site(s) in the Mitf promoter (25, 38, 70, 82). Mitf is the melanocyte-determining transcription factor, as well as the earliest marker of commitment to the melanocytic lineage (5, 28, 56, 68, 71, 75, 80). In zebrafish, the canonical Wnt pathway acting via Lef1/TCF regulates transcription of nacre, the zebrafish homologue of Mitf, resulting in pigment cell development at the expense of other NC-derived cell lineages (22, 23, 50).

In addition to Mitf (22), another Wnt target gene with a potential role in NC fate determination is the RE-1 silencing transcription factor gene (REST) (55, 81), a transcriptional repressor of neuron-specific genes, expressed in nonneuronal cells and neural progenitors (17, 65). The repressor activity of REST suppresses expression of a wide network of neuronal genes, including those encoding tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) (39, 40), both required for catecholamine synthesis. In addition, REST suppresses expression of Ash-1 (57), a proneural transcription factor required for SA lineage commitment (33, 52).

The transcriptional corepressor C-terminal binding protein (CtBP) (3, 15, 16, 58, 73) is a component of Wnt-regulated transcription. Multiple mechanisms have been indicated for CtBP in Wnt signaling. Specifically, in Xenopus, in human 293 embryonic kidney, and in human colorectal cancer cell lines, CtBP functions as a direct corepressor of Tcf-3 (12) and Tcf-4.
(18, 74). In drosophila, CtBP prevents free nuclear β-catenin from binding to Tcfs by sequestering β-catenin via adenomatous polyposis coli (APC), thereby repressing Tcf-mediated transcription (29). A third mechanism, also in drosophila, is that CtBP binds to Wnt-related enhancers to repress transcription of Wnt target genes independently of Tcf (27).

Two isoforms of CtBP, CtBP1 and CtBP2, exist in vertebrates, exhibiting both overlapping and distinct expression patterns and functions. Specifically, migrating NC cells express CtBP2, whereas dorsal root ganglia express CtBP1 (76). Mice harboring mutations in CtBP1 or CtBP2 display distinct developmental defects. CtBP1 mutant mice are smaller but are mostly viable and fertile. In contrast, mutation in CtBP2 causes embryonic lethality, with phenotypes including axial truncation, delayed neural development, and aberrant heart formation (31).

A CtBP modulator identified by the yeast three-hybrid system (84) is homeodomain interacting protein kinase 2 (HIPK2), a serine/threonine kinase involved in regulation of cell growth, apoptosis, and development (10, 13, 21, 62, 77). In response to UV irradiation or overexpression, HIPK2 phosphorylates CtBP, targeting it for proteasomal degradation (83, 84). Similar to CtBP, HIPK2 is involved in Wnt signaling. HIPK phosphorylates CtBP, stabilizing cytosolic β-catenin via phosphorylation, promoting nuclear accumulation and enhancing Wnt signaling and target gene expression (46).

Using primary cultures of avian NC cells and zebrafish as an animal model, we investigated the roles of cAMP signaling in neural tube development. This pathway implies a convergence of the signaling pathway. cAMP signaling via HIPK2 initiates proteasomal degradation of CtBP2. Downregulation of the CtBP2 protein derepresses expression of the Wnt target genes Mif and REST. On the one hand, Mif promotes melanocyte specification by activating expression of enzymes for melanin synthesis. On the other, REST suppresses the SA lineage by repressing expression of neuronal genes required for neuron development. This pathway implies a convergence of the cAMP and Wnt signaling pathways in the process of NC lineage specification.

**MATERIALS AND METHODS**

**Cell culture.** Primary cultures of trunk neural crest (NC) cells were prepared from Japanese quail (Coturnix coturnix) embryos as described by Bilodeau et al. (9). Briefly, stage 12 to 13 embryos were dissected from quail eggs grown for 47.5 h. Neural tubes from the trunk region, including the last five somites, were isolated by pancreatin digestion and plated in Vitrogen 100 (Collagen Corpora-

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TABLE 1. Primers used in this work

After 42 h, allowing NC cell migration, neural tubes were removed, and the remaining cells were collected in growth medium with trypsin-EDTA and replated at a density of 320 cells/mm² in bovine fibronectin (Sigma)-coated dishes. Medium was replaced 2 h later, and treatments were added at this time point unless otherwise stated. This culture is considered day 0 NC culture. Half the total volume of growth medium was exchanged every other day thereafter. The B16-F10 cell line, obtained from American Type Culture Collection, was cultured in Dulbecco’s modified Eagle’s medium (Gibco) containing 10% fetal bovine serum (Atlanta Biologicals), as previously described (8).

**Growth media and reagents.** Growth medium for NC cultures contained 75 ml of Dulbecco’s modified Eagle’s medium–Ham’s F-12 medium (Mediatech), 15 ml of heat-inactivated horse serum (HyClone), 10 ml of d'ay 12 chicken embryo extract, 10 mg of gentamycin sulfate, 10 mg of kanamycin sulfate, 1 ml of 7.5% sodium bicarbonate, 1 ml of 0.2 M l-glutamine, and 1 ml of vitamin mixture (0.05 mg/ml 6-D-imidazole, 0.8 mg/ml l-ascorbic acid, 0.25 mg/ml oxidized glutathione, pH 6.0). Forskolin (Enzo Life Sciences), MG-132 (Enzo Life Sciences), and H89 (Sigma) were reconstituted in dimethyl sulfoxide.

**Real-time PCR.** Total RNA was isolated with Trizol (Invitrogen) reagent as described by the manufacturer. cDNA was synthesized by reverse transcription-PCR (RT-PCR) using total RNA and the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed on the 7300 real-time PCR system (Applied Biosystems). A 20-µl reaction mixture contained 1X SYBR green PCR master mix (Applied Biosystems), 2 µl of cDNA template, and 1 µM primer pair. PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. Primer sequences are provided in Table 1.

**Immunoblot analysis.** Whole-cell extracts (WCE) were prepared by collecting cells in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with phosphatase inhibitor cocktail (1:100 dilution; Sigma) and protease inhibitor (1:1,000 dilution; Sigma). Cell suspensions were sonicated on ice for 10 s and clarified by centrifugation. Nuclear extracts were prepared by immunoprecipitation with protein G agarose beads (Roche) at 4°C. After 3 washes with TBSN buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 5 mM EGTA, 1.5 mM EDTA, 0.5 mM Na3VO4), protein complexes were recovered by boiling the samples in 2X SDS sample buffer. Supernatants were analyzed by immunoblotting.

**siRNA and DNA transfection of NC cultures.** Short interference RNA (siRNA) duplexes targeting different sites of CtBP2/CtBP1/HIPK2 were designed and synthesized using Stealth RNAi siRNA Technology (Invitrogen). Stealth RNAi siRNA negative control was used as a transfection control (Invitrogen). Three siRNA duplexes targeting the same gene were mixed as a
siRNA pool. Mixture of siRNA (200 pmol) was transfected with 10 μl of Lipofectamine 2000 reagent into a 60-mm dish of NC cells. The final concentration of siRNA in NC cultures was 40 nM. Each NC culture was transfected twice, at 24 h after neural tube isolation, i.e., 24 h before replating, and immediately after replating. Opti-MEM I reduced serum medium (Gibco), without antibiotics, was used to dilute siRNA and Lipofectamine 2000 during transfection. DNA plasmids (2 μg) for pCMV-Flag-ubiquitin, pcDNA3-HIPK2K25R, and pcDNA3 empty vector were transfected into NC cells with FuGENE 6 transfection reagent (Roche) at 24 h after neural tube isolation. Sequences of siRNAs are provided in Table 2.

**RESULTS**

Cyclic AMP signaling induces transcription of Mitf and REST and melanocyte differentiation. To investigate the mechanism by which cAMP signaling induces melanocyte differentiation, we employed quail primary NC cultures as the in vitro cellular model and used forskolin, a potent adenylyl cyclase activator, to elevate intracellular cAMP levels. NC cultures treated with 100 μM forskolin for 1 to 3 days exhibited a progressive increase in melanocytes (pigmented cells) in comparison to untreated cultures (Fig. 1A). In the absence of forskolin, less than 2% of the total cell population was melanocytes; by contrast, in the presence of forskolin, the melanocyte number increased from 4% on day 2 to approximately 12% on day 3 (Fig. 1B). Forskolin-induced melanocyte differentiation was completely suppressed by H89, a PKA inhibitor (Fig. 1A and B), indicating the increased melanocyte number by forskolin treatment was solely mediated by cAMP/PKA signaling.

To understand the effect of cAMP signaling on melanocyte differentiation, we examined expression of two genes, Mitf and REST, in primary NC cultures. Mitf is a known cAMP-regulated gene (4, 60). Intriguingly, constitutively active CREB is insufficient to induce Mitf transcription in primary NC cultures (37), suggesting PKA regulates additional events involved in Mitf transcription. Furthermore, despite the well-established role of REST as a neuron-specific transcriptional repressor (17, 65), the involvement of REST in NC differentiation is unknown. Real-time PCR quantification of RNA isolated from day 1 to day 3 of NC cultures shows that cAMP signaling induced transcription of both Mitf and REST. Specifically, on day 3 of NC culture, cAMP signaling enhanced Mitf and REST mRNA by nearly 3-fold relative to levels for the untreated control (Fig. 1C). To further investigate the regulation of Mitf and REST by cAMP signaling, NC cells were treated with increasing concentrations of forskolin. Immunoblots of the lysates demonstrated the levels of phospho-CREB, Mitf, and REST were all induced by 1 μM forskolin and progressively increased following treatment with increasing forskolin concentrations (Fig. 1D). Quantification of the immunoblots indicates a 4.7-fold induction of phospho-CREB, a 2.3-fold induction of Mitf, and a 3.2-fold induction of REST by treatment with 100 μM forskolin (Fig. 1E). Thus, cAMP signaling promotes melanocyte differentiation via PKA and upregulates Mitf and REST expression in a dose-dependent manner.

**Knockdown of CtBP2 but not CtBP1 enhances Mitf and REST expression and promotes melanocyte differentiation.** Since CtBP interferes with Wnt signaling (12, 27, 29), which regulates Mitf and REST transcription (22, 55), we studied CtBP proteins in the NC cell lineage specification. To examine the role of each CtBP1 and CtBP2, we knocked down their expression using siRNA. The transfection efficiency of siRNAs

![Table 2. Sequences of siRNA duplexes](http://mcb.asm.org/)

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FIG. 1. cAMP signaling in NC cells induces melanocyte differentiation and expression of Mitf and REST. (A to C) Primary NC cultures, day 1 through day 3, were grown without (−) or with (+) 100 μM forskolin or the combination of 100 μM forskolin and 10 μM H89. The concentrations of forskolin and H89 are the same in the subsequent figures unless otherwise indicated. (A) Phase-contrast images. (B) Quantification of melanocytes using the ImageJ software program. Quantification is from at least 3 randomly chosen fields of 3 independent experiments. *, P < 0.05; **, P < 0.01, Student's t test; meanings are the same in the subsequent figures unless otherwise indicated. (C) Real-time PCR quantification of Mitf and REST mRNA levels using total RNA isolated from NC cultures, d, day. (D and E) Primary NC cultures treated for 3 days with 1 to 100 μM forskolin. (D) Immunoblots of REST and Mitf using whole-cell extracts (WCE) isolated from the NC cultures. For Phospho-CREB immunoblot, NC cells were treated with the indicated concentration of forskolin for 1 h. Total CREB and actin serve as internal controls. (E) Quantification of immunoblots for indicated molecules by ImageJ software from at least 3 independent experiments. WB, Western blot.
in NC cells was calculated to be 63.6% ± 1.1%, employing siGLO control siRNA (Fig. 2A). Immunoblots confirmed the effective knockdown of the CtBP2 protein by 65% and the CtBP1 protein by 42% following siRNA transfection (Fig. 2B). NC cultures transfected with control (scrambled) siRNA and treated with 1 to 100 μM forskolin gave rise to an increasing number of melanocytes. Interestingly, significantly more melanocytes were observed in NC cultures transfected with CtBP2 siRNA versus CtBP1 siRNA or scrambled siRNA (Fig. 2C). NC cultures transfected with CtBP2 siRNA and treated with 1 to 100 μM forskolin exhibited 2.5- to 4.1-fold increases in melanocyte number relative to cultures transfected with CtBP1
siRNA or scrambled siRNA. Moreover, the total number of melanocytes exhibited a progressive increase ranging from 10 to 52% in CtBP2 siRNA-transfected cultures treated with 1 to 100 μM forskolin (Fig. 2D). Transfection of CtBP1 or CtBP2 siRNA resulted in an approximately 50% reduction in the mRNA level of each gene. Interestingly, CtBP2 knockdown led to more than a 2-fold induction in both Mitf and REST mRNAs, whereas no such induction was observed with CtBP1 knockdown (Fig. 2E). Similarly, knockdown of CtBP2 but not CtBP1 resulted in increased protein levels of Mitf and REST, detected by immunoblots (Fig. 2F). Differentiation markers were likewise altered by CtBP2 knockdown: the melanocyte-specific enzyme tyrosinase was increased, whereas the neuronal Phox2a and tyrosine hydroxylase (TH) were reduced (Fig. 2F). Since tyrosinase is both a melanocyte marker and a regulatory target of Mitf (5, 6), tyrosinase induction suggests that more cells had adopted the melanocyte fate. Conversely, Phox2a and TH are both transcriptional targets of REST and developmental markers of SA cells (2, 26, 51, 57, 67). Down-regulation of Phox2a and TH suggests fewer cells were committed to the SA lineage.

To determine the effect of CtBP on the number of cells committed to the melanocytic lineage, we examined the appearance of the melanoblast early marker MelEM (54) in NC cultures transfected with CtBP1 or CtBP2 siRNAs (Fig. 3A), and the number of cells exhibiting positive immunostaining was quantified (Fig. 3B). In parallel, we monitored development of the neuronal SA lineage by TH immunostaining (Fig. 3C). Under conditions of CtBP2 but not of CtBP1 knockdown, we observed a nearly 2-fold increase in MelEM-positive cells (Fig. 3B). In contrast, CtBP2 knockdown suppressed development of the SA lineage (Fig. 3C). Since CtBP2 knockdown alleviated the transcriptional repression of Mitf and REST, thereby promoting differentiation of melanocytes at the expense of SA cells, we conclude that CtBP2 has an instructive role in NC lineage determination. In contrast, CtBP1 knockdown exerted no significant change in development of NC cells (Fig. 2 and 3), suggesting CtBP1 does not regulate NC cell differentiation.

CtBP2 knockdown in zebrafish increases Mitf and REST expression and melanocyte (melanophore) differentiation. Zebrafish is a powerful and highly amenable animal model for in vivo study of NC lineage specification due to conserved features of NC cells among vertebral species (45). Moreover, zebrafish has been extensively used to study pigment cell (melanocyte) development because of its rapid ex utero embryonic development and large transparent embryos (19, 20, 22, 23, 50). Accordingly, to explore the function of CtBP2 in melanocyte differentiation in vivo, we designed two morpholinos targeting zebrafish CtBP2. The morpholino CtBP2a was designed to interfere with mRNA translation and CtBP2b with mRNA splicing. The CtBP2a morpholino (0.4 mM) microin-
jected into one-cell-stage embryos resulted in various developmental defects, including severe axial truncation, aberrant brain and ear development, and abnormal heart formation (data not shown). These phenotypes agree with defects documented in CtBP2-null mouse embryos (31). In addition, the zebrafish embryos displayed an aberrant pigmentation pattern with irregular distribution of melanophores (data not shown), the zebrafish counterpart of melanocytes. Similar phenotypes were generated by injection of 0.4 mM CtBP2b morpholino (data not shown). Microinjection of the morpholino CtBP2a or CtBP2b at a concentration of 0.25 mM resulted in less-severe defects and a more-obvious change in pigmentation. Injection of either morpholino increased the number of melanophores on the dorsal aspect, especially in the head region of 3-day-postfertilization (dpf) zebrafish (Fig. 4A). Dorsal melanophores were counted from the anterior end of the head to the posterior end of the yolk sac following epinephrine treatment to contract melanosomes (Fig. 4B). Injection of mixed CtBP2 morpholinos (0.1 mM CtBP2a plus 0.1 mM CtBP2b) induced a statistically significant (P = 0.015) increase in the number of melanophores compared with the control morpholino injection.

To investigate whether diminished levels of CtBP2 regulate Mitf and REST expression, RNA isolated from 24- hpf embryos of wild-type zebrafish was quantified by real-time PCR. Mitf and REST mRNA levels were both increased following injection of either the CtBP2a or CtBP2b morpholino (Fig. 4C), demonstrating in vivo regulation of Mitf and REST by CtBP2.

Mitfa-GFP transgenic zebrafish (19) were also used to examine whether CtBP2 regulates Mitf transcription in vivo. Mitfa is the zebrafish isoform of Mitf, responsible for NC-derived melanophore development (50). The Mitfa promoter contains conserved cis-acting elements, including functional Lef/Tcf binding sites (22). Mitfa-GFP zebrafish carry a hemizygous allele with the GFP reporter gene under the control of the Mitfa promoter. To ensure the same basal GFP level, female transgenic fish were crossbred with male wild-type fish; the resulting embryos were injected at the one-cell stage. At 24 hpf, when GFP is expressed (19), fluorescent embryos were screened to compare expression levels of GFP. In GFP-expressing embryos injected with either of the CtBP2 morpholinos, more GFP-positive cells were present, exhibiting a higher intensity of fluorescence (Fig. 4D), thereby indicating that downregulation of CtBP2 enhances Mitfa promoter activity. Indeed, employing confocal microscopy images of morpholino-injected embryos, we quantified increased GFP fluorescence from the head region upon CtBP2 knockdown (Fig. 4E and F).

Taken together, these in vivo data (Fig. 4) support the results derived from the in vitro-cultured primary NC cells (Fig. 2 and 3) and demonstrate that downregulation of CtBP2 derepresses transcription of both Mitf and REST, allowing pluripotent NC cells to adopt the melanocytic fate.

**Cyclic AMP targets CtBP2 for proteasomal degradation and modulates association of CtBP2 and β-catenin.** The induction of Mitf and REST expression by both cAMP signaling (Fig. 1) and CtBP2 knockdown (Fig. 2) led us to explore the connection between cAMP and CtBP2. As shown in Fig. 5A, in day 2 NC cultures, 100 μM forskolin decreased the protein level of CtBP2 to less than one-third of the control level but had no significant effect on nuclear β-catenin protein levels. To elucidate the mechanism underlying this reduction, in addition to forskolin, cells were also treated with MG-132, an inhibitor of proteasomal degradation. Treatment with MG-132 for 4 h reversed by nearly 80% the forskolin-induced decrease in CtBP2, suggesting that cAMP signaling reduces the CtBP2 protein level via proteasome-mediated degradation. The same observation was made in B16 murine melanoma cells (Fig. 5B). In B16 cultures, levels of CtBP2 protein decreased with treatment with increasing concentrations of forskolin (1 to 100 μM) in a dose-dependent manner. Interestingly, this decrease was rescued by inhibition of the proteasome by addition of MG-132. Also, the forskolin-induced CtBP2 decrease was reversed by H89, an inhibitor of PKA (Fig. 5B). Since cAMP transmits signals by activating PKA, the capability of H89 to inhibit CtBP2 degradation confirms that CtBP2 is degraded in response to cAMP signaling. This conclusion can also be drawn from the immunofluorescence (IF) staining studies in B16 cultures (Fig. 5C). Forskolin treatment significantly reduced the number of CtBP2-positive cells. However, treatment with MG-132 or H89 rescued positively stained cells to a ratio similar to that of the control (Fig. 5D). We interpret these results to mean that cAMP signaling via PKA induces degradation of the CtBP2 protein via the proteasome.

To further investigate cAMP-mediated proteasomal degradation of CtBP2, NC cells were transfected with a pCMV-Flag-ubiquitin-expressing plasmid. The level of ubiquitinated CtBP2 was detected by precipitating protein extracts with CtBP2 antibody and immunoblotting with ubiquitin antibody (Fig. 5E). The highest level of ubiquitinated CtBP2 was detected in NC cells treated with a combination of forskolin and MG-132. Since ubiquitination is necessary for protein degradation via the proteasome pathway, these results support that cAMP signaling downregulates CtBP2 via proteasomal degradation.

A mechanism of CtBP action involves sequestration of β-catenin via APC, thereby repressing Wnt signaling and expression of Wnt-responsive genes (29). Since both Mitf and REST are Wnt-regulated genes, we tested whether CtBP2 degradation by cAMP signaling increases the level of free nuclear β-catenin, thereby inducing transcription of Mitf and REST. Immunoprecipitation (co-IP) assays were performed to study the association between CtBP2 and β-catenin in NC cells (Fig. 5F). CtBP2 was detected by immunoblotting of β-catenin immunoprecipitates and vice versa, showing physical interaction between the two proteins. Importantly, forskolin treatment reduced this interaction to less than half of that of the control in both IP experiments. Upon activation of cAMP signaling by forskolin addition, the amount of β-catenin that coprecipitates with CtBP2 decreased; a similar change was observed in the amount of CtBP2 coprecipitating with β-catenin (Fig. 5F). These data indicate that the association between β-catenin and CtBP2 is negatively regulated by cAMP signaling. Taking this together with our previous finding that cAMP induces CtBP2 degradation, we conclude that cAMP signaling modulates the association of nuclear β-catenin and CtBP2 by targeting CtBP2 for degradation. In turn, the reduction in the protein level of CtBP2 enables free nuclear β-catenin to induce transcription of the Wnt-regulated genes Mitf and REST.

HIPK2 knockdown suppresses melanocyte differentiation by stabilizing CtBP2. Earlier studies have demonstrated that UV
FIG. 4. Knockdown of CtBP2 increases melanocyte differentiation in zebrafish. (A, B, D, and E) Zebrafish embryos injected with 0.25 mM standard control, CtBP2a, CtBP2b, or the combination of CtBP2a and -b (0.1 mM + 0.1 mM) morpholinos. (A and B) Representative phase-contrast images of 3-dpf wild-type larvae injected with the indicated morpholinos. In panel B, the 3-dpf larvae injected with control or mixed CtBP2 morpholinos were treated with 1 μg/ml epinephrine for 10 min to contract melanosomes. Quantification of melanophore numbers is from 10 embryos/group. (C) Real-time PCR quantification of Mitf and REST mRNA levels using total RNA isolated from 24-hpf wild-type embryos. (D) Green fluorescence and phase-contrast images of 24-hpf Mitfa-GFP transgenic embryos. (E) Confocal images of Mitfa-GFP transgenic embryos injected with control or mixed CtBP2 morpholinos. (F) GFP fluorescence in the head region quantified by ImageJ software, using morpholino-injected Mitfa-GFP transgenic embryos as with panel E.
irradiation or overexpression of HIPK2 induces CtBP degradation via the proteasome pathway (83, 84). Therefore, we investigated if HIPK2 is involved in NC cell fate determination by regulating CtBP2. To address this question, HIPK2 was knocked down with a mixture of siRNAs targeting three different sites of the gene. NC cultures transfected with HIPK2 siRNA in the presence of 100 μM forskolin gave rise to few melanocytes from 2 to 6 days of treatment (Fig. 6A). Melanocytes were rare in day 2 NC cultures following HIPK2 knockdown and showed a reduction to approximately one-third of the control level on days 4 and 6. In contrast, melanocytes in NC cultures transfected with scrambled siRNA reached 20% of the cell population on day 6 (Fig. 6B).

Employing real-time PCR quantification, we confirmed that siRNA transfection diminished HIPK2 mRNA levels to less than 40% of control levels. Interestingly, both Mitf and REST mRNAs showed a dramatic decrease, whereas the mRNA of the proneural transcription factor Ash-1 increased in the presence of 100 μM forskolin (Fig. 6C). Decreased Mitf and REST expression was also detected at the protein level by immunoblotting (Fig. 6D). Moreover, HIPK2 knockdown rescued CtBP2 from degradation in the presence of 100 μM forskolin. 

FIG. 5. cAMP signaling induces proteasomal degradation of CtBP2 in NC cells. (A to E) Day 3 primary NC or B16 cultures treated without (−) or with (+) 100 μM forskolin, the combination of 100 μM forskolin and 5 μM MG-132, or the combination of 100 μM forskolin and 10 μM H89, as indicated. In NC cultures, MG-132 and H89 were added 4 h before cell harvesting; in B16 cultures, cells were treated for 2 h before harvesting. (A) Immunoblots of CtBP2 and β-catenin using nuclear extracts isolated from NC cultures. Quantification is relative to CREB, an internal control. (B) Immunoblots of CtBP2 protein using nuclear extracts isolated from B16 cultures. Quantification is relative to CREB. (C) Immunofluorescence (IF) staining of CtBP2 in B16 cultures. Hoechst stains cell nuclei. (D) Quantification of CtBP2-positive cells from panel C was performed by ImageJ software, counting a total of 1,500 cells from 3 independent experiments. (E) Immunoblots of ubiquitin using CtBP2 immunoprecipitates of WCE isolated from NC cultures treated as indicated. NC cultures were transfected 24 h prior to replating with pCMV-Flag-ubiquitin plasmid. (F) Immunoblots of β-catenin or CtBP2 using immunoprecipitates of CtBP2 or β-catenin, respectively, derived from WCE isolated from day 3 primary NC cultures, treated as indicated. A representative assay from at least 3 independent experiments is shown in panels A, B, E, and F.
FIG. 6. Knockdown of HIPK2 suppresses melanocyte differentiation in NC cells. (A and B) Day 2, day 4, and day 6 primary NC cultures transfected with 40 nM siRNA for scrambled control or HIPK2 and treated with 100 μM forskolin. (A) Phase-contrast images. (B) Percentage of melanocytes quantified by ImageJ software. Quantification is from at least 3 randomly chosen fields of 3 independent experiments. (C to G) Day 3 or day 5 (G) primary NC cultures transfected with 40 nM siRNA for scrambled control or HIPK2 and treated with 100 μM forskolin or 1 to 50 μM forskolin (F). (C) Real-time PCR quantification of HIPK2, Mitf, REST, and Ash-1 mRNA levels using total RNA isolated from the cultures. (D) Immunoblots of Mitf, REST, and CtBP2 protein using WCE isolated from indicated NC cultures. A representative assay from at least 3 independent experiments is shown. (E) IF staining of CtBP2 in indicated cultures. (F) Immunoblots of Phox2a and TH using WCE isolated from indicated cultures. (G) IF staining of TH and MelEM in day 5 and day 3 NC cultures, respectively. (H) Quantification of MelEM-positive cells from a total of 1,500 cells.
as shown by both immunoblots (Fig. 6D) and IF staining (6E). We interpret these results to mean that cAMP signaling mediates CtBP2 degradation via HIPK2. HIPK2 knockdown stabilizes CtBP2, which in turn represses Mitf and REST transcription, thereby suppressing melanocyte differentiation. On the other hand, since Ash-1, required for SA cell differentiation (33, 52), is induced by HIPK2 knockdown in the presence of 100 μM forskolin (Fig. 6C), we reason that differentiation of the SA lineage is increased.

In accordance with the upregulation of Ash-1 in the presence of 100 μM forskolin, knockdown of HIPK2 also enhanced expression of Phox2a and TH, two neuron-specific SA markers repressed by REST (Fig. 6F). Specifically, NC cultures treated with increasing concentrations of forskolin (1 to 50 μM) exhibited progressively decreasing protein levels of Phox2a and TH both in the control and in HIPK2 knockdown NC cultures, in agreement with findings in our earlier studies (9, 37). Interestingly, at the same forskolin concentration, knockdown of HIPK2 allowed expression of higher levels of the neuronal proteins Phox2a and TH. Likewise, HIPK2 knockdown enabled NC cells to adopt the neuronal lineage (TH-positive cells) in the presence of 100 μM forskolin (Fig. 6G). Conversely, HIPK2 knockdown decreased by nearly 50% the number of NC cells adopting the melanocytic lineage (MEL-positive cells) (Fig. 6G and H). Together, these findings demonstrate that HIPK2 knockdown promotes SA cell differentiation while opposing the melanocytic fate. We propose that HIPK2 acts as a cAMP sensor to affect a balance between the two cell lineages.

**HIPK2 knockdown in zebrafish represses Mitf and REST expression, interfering with melanophore development.** To confirm in vivo the regulatory effects of HIPK2 on NC cell differentiation (Fig. 6), we investigated in zebrafish the effect of HIPK2 knockdown. Two morpholinos, HIPK2a and HIPK2b, were designed to block zebrafish HIPK2 mRNA splicing at different sites. Electrophoresis of RT-PCR products revealed the change in HIPK2 mRNA expression following morpholino injection. No significant alteration in the level of HIPK2 was induced by injecting 1.0 mM standard control morpholino; by contrast, the mixture of HIPK2 morpholinos (0.5 mM HIPK2a plus 0.5 mM HIPK2b) caused a dramatic reduction in HIPK2 mRNA levels (Fig. 7A). This result shows that HIPK2 expression was effectively blocked by the morpholino mixture, whereas the control morpholino had no detectable effect.

Wild-type zebrafish embryos injected with 1.0 mM HIPK2a morpholino displayed lighter pigmentation of body and eyes than embryos injected with 1.0 mM standard control morpholino (Fig. 7B). A similar phenotype was observed in embryos injected with 1.0 mM HIPK2b morpholino (data not shown). To test if a synergistic effect could be induced on the phenotype, injection of the HIPK2 morpholino mixture generated the most significant decrease in body and eye pigmentation compared with HIPK2a or HIPK2b injection alone (Fig. 7B and data not shown). We conclude that HIPK2 knockdown interferes with melanophore development in zebrafish embryos.

To monitor the in vivo regulation of Mitf promoter activity by HIPK2, Mitfa-GFP female transgenic fish were cross-bred with male wild-type fish; the resulting embryos were injected at the one-cell stage and screened the same way as in the CtBP2 knockdown experiments (Fig. 4D). Injection of 1.0 mM HIPK2a or the mixture of two morpholinos similarly decreased green fluorescence in the embryos (Fig. 7C), suggesting low promoter activity and repressed transcription of Mitf upon HIPK2 knockdown. Real-time PCR quantification of RNA isolated from 24-hpf zebrafish embryos demonstrated that the HIPK2 morpholino mixture reduced the HIPK2 mRNA level to less than 10% of the control and reduced Mitf and REST mRNAs by 60% (Fig. 7D). Using confocal microscopy images of morpholino-injected zebrafish, we quantified significantly decreased GFP fluorescence from the head region in HIPK2 knockdown embryos (Fig. 7E and F), suggesting HIPK2 knockdown reduced the number of cells that are differentiating into melanocytes. These results together with the HIPK2 knockdown studies in primary NC cultures (Fig. 6) demonstrate that HIPK2 knockdown represses expression of Mitf and REST, as well as melanocyte/melanophore development.

**Expression of constitutively active HIPK2 induces CtBP2 degradation and melanocyte differentiation.** The studies of Fig. 6 and 7 demonstrate that HIPK2 activity positively regulates Mitf and REST transcription and promotes commitment of NC cells to the melanocytic fate. HIPK2 is covalently modified at lysine (K) residue 25 by small ubiquitin-related modifier 1 (SUMO-1). Sumoylation at K25 abolishes the effector function of HIPK2 (34, 41). Lysine-to-arginine (K to R) substitution of amino acid residue 25 blocks HIPK2 sumoylation, thereby generating constitutively active HIPK2K25R (34). To further determine the role of HIPK2 in NC lineage specification, we examined cell differentiation and gene expression in NC cultures expressing HIPK2K25R.

Phase-contrast images showed a significant difference in the number of melanocytes between control and HIPK2K25R-expressing NC cultures (Fig. 8A). Transfection of pcDNA3-HIPK2K25R induced a 4.4-fold increase in melanocyte number on day 2 compared with transfection of pcDNA3 empty vector. On day 4, in HIPK2K25R-expressing cultures treated with 1 μM forskolin, melanocytes reached 17% (Fig. 8B), comparable to results with treatment with 100 μM forskolin (Fig. 1B). Results from real-time PCR and immunoblotting indicate that in the presence of HIPK2K25R expression, melanocyte differentiation is associated with higher mRNA and protein levels of Mitf and REST (Fig. 8C and D) and decreased CtBP2 protein levels, detected by immunoblotting and IF staining (Fig. 8D and E). We conclude that HIPK2 induces Mitf and REST expression by downregulating CtBP2, thereby promoting melanocyte differentiation.

**DISCUSSION**

The importance of cAMP signaling in melanocyte differentiation has been well defined by the functional characterization of a classical CRE site in the Mitf promoter; this CRE site, conserved from zebrafish to the human Mitf gene, mediates transcription of the Mitf gene and melanocyte differentiation by cAMP signaling (4, 60). Intriguingly, in primary cultures of NC cells, our earlier findings (37) have demonstrated that constitutively active CREB is insufficient to induce Mitf transcription and melanocyte differentiation without PKA activation. We proposed that cAMP signaling via PKA regulates
additional factors beyond CREB activation, necessary for NC cell specification (37). We have also shown that in primary NC cultures, high-intensity cAMP signaling, e.g., 100 μM forskolin, exclusively promotes melanocyte differentiation by inhibiting the SA lineage, suggesting a link in the mechanism determining these exclusive cell fates (9, 37).

In this study, employing primary NC cultures and zebrafish as the animal model, we have demonstrated the mechanism by which cAMP signaling regulates this exclusive cell fate outcome of either the melanocytic or sympathoadrenal (SA) lineage. We have provided evidence that both melanocyte differentiation and SA lineage suppression are inversely regulated.
by CtBP2. cAMP signaling downregulates CtBP2 through HIPK2, targeting CtBP2 for proteasomal degradation. Down-regulation of CtBP2 by cAMP signaling induces expression of both the melanocyte-determining transcription factor Mitf and the neuron-specific transcriptional repressor REST, thereby mediating melanocyte differentiation and SA lineage suppression, respectively. In contrast to the well-defined regulation of Mitf by cAMP signaling, the regulation of REST by cAMP signaling in NC cells is novel.

Specifically, in NC cells, cAMP signaling induces, in a dose-dependent manner, expression of both Mitf and REST (Fig. 1C and D). Although the extracellular signal(s) that activates the cAMP pathway during NC specification in vivo is unknown, various extracellular signals in vivo can converge to activate the cAMP pathway and generate gradients of activation of cAMP signaling. Furthermore, Wnt is a well-established extracellular signaling molecule involved in NC induction and lineage specification (36, 47). Since CtBP2 interferes with Wnt signaling by sequestering β-catenin and cAMP signaling promotes proteasomal degradation of CtBP2, our findings imply a convergence between the cAMP and Wnt signaling pathways in inducing transcription of Mitf and REST and the resulting specification of the melanocytic lineage.

**CtBP2 mediates cAMP-induced Mitf and REST expression.** CtBP1 and CtBP2 isoforms share similar expression patterns and overlapping roles but also exhibit unique functions. For example, CtBP2 but not CtBP1 is detected in migrating NC cells; depletion of CtBP2 but not CtBP1 is embryonic lethal in mice; CtBP2-null

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**FIG. 8.** Constitutively active HIPK2K25R induces melanocyte differentiation via CtBP2 downregulation. (A to E) Day 2 and day 4 (A and B) or day 3 (C to E) primary NC cultures transfected with pcDNA3 empty vector or pcDNA3-HIPK2K25R plasmid and treated with 1 μM forskolin. (A) Phase-contrast images. (B) Percentage of melanocytes quantified by ImageJ software. Quantification is from at least 3 randomly chosen fields of 3 independent experiments. (C) Real-time PCR quantification of HIPK2, CtBP2, Mitf, and REST mRNA levels using total RNA isolated from indicated cultures. (D) Immunoblots of Mitf, REST, and CtBP2 protein using WCE isolated from indicated cultures. A representative assay from at least 3 independent experiments is shown. (E) IF staining of CtBP2 in indicated cultures.
mice exhibit severe developmental defects in comparison to CtBP1-null mice (31, 76). Our study supports these findings, demonstrating that CtBP2 knockdown promotes melanocyte differentiation by inducing Mitf and REST expression in pluripotent NC cells. In contrast, CtBP1 knockdown has no significant effect on NC cell lineage specification (Fig. 2 and 3). In vivo knockdown of CtBP2 in zebrafish embryos enhances melanocyte differentiation as well as Mitf and REST transcription (Fig. 4). Therefore, we conclude that CtBP1 and CtBP2 play distinct roles in NC cell development; only CtBP2 participates in specification of the melanocytic lineage.

The induction of Mitf and REST expression, both by cAMP signaling (Fig. 1) and CtBP2 knockdown (Fig. 2), led us to establish the link between cAMP signaling and CtBP2. First, we showed that the protein level of CtBP2 is regulated by cAMP signaling in a dose-dependent manner (Fig. 5B), suggesting that the intensity of activation of the cAMP pathway in vivo determines the intracellular level of the CtBP2 protein. Second, inhibition of CtBP2 degradation and the resultant accumulation of ubiquitinated CtBP2 by MG-132 treatment demonstrate that CtBP2 is degraded via the proteasome pathway (Fig. 5A to D). This mechanism coincides with the known regulation of CtBP degradation by HIPK2 (83, 84) and identifies a link between cAMP signaling and HIPK2 activation. Further studies are needed to determine how cAMP signaling activates HIPK2. Interestingly, we have noted two putative PKA phosphorylation sites in the amino acid sequence of HIPK2 that may regulate HIPK2 activation. Third, the interaction of CtBP2 and β-catenin is negatively regulated by cAMP signaling (Fig. 5E), identifying a molecular mechanism by which CtBP2 regulates concurrently both Mitf and REST expression. As proposed by Hamada and Bienz (29), CtBP2 sequesters nuclear β-catenin to prevent it from activating transcription of Wnt target genes. We demonstrate that in the presence of cAMP signaling, degradation of CtBP2 frees nuclear β-catenin, allowing it to activate transcription of the Wnt target genes Mitf and REST.

HIPK2 modulates NC cell fate by regulating CtBP2. Both in vitro and in vivo data show HIPK2 knockdown decreases Mitf and REST expression, suppressing melanocyte differentiation (Fig. 6 and 7). Conversely, expression of constitutively active HIPK2K25R induces Mitf and REST expression and promotes melanocyte differentiation in cultured NC cells (Fig. 8). Since knockdown of HIPK2 stabilizes CtBP2 (Fig. 6D and E) and expression of constitutively active HIPK2K25R downregulates CtBP2 (Fig. 8D and E), we conclude that HIPK2 regulates NC cell development via CtBP2. Moreover, stabilization of CtBP2 by HIPK2 knockdown in the presence of 100 μM forskolin (Fig. 6D and E) reveals that HIPK2 mediates cAMP-induced CtBP2 degradation. These findings place HIPK2 activation and CtBP2 degradation, along with Mitf and REST expression, under regulation of the cAMP signaling pathway, inducing melanocyte differentiation of NC cells (Fig. 9).

In contrast to the reduced expression of Mitf and REST upon HIPK2 knockdown, expression of the Phox2a and TH neuronal genes increased, even in the presence of increasing concentrations of forskolin (Fig. 6F). Notably, the inverse changes in the expression of the SA lineage markers Phox2a and TH after CtBP2 knockdown versus HIPK2 knockdown (Fig. 2E, 6F) suggest that CtBP2 and HIPK2 exert opposite effects in the numbers of cells adopting the SA fate. Indeed, knockdown of CtBP2 increased the number of NC cells committed to the melanocytic lineage, whereas knockdown of HIPK2 increased the number of NC cells committed to the neuronal cell lineage (Fig. 3 and 6). The mechanism by which HIPK2 negatively regulates development of the neuronal lineage is under investigation. Our ongoing studies (M. H. Shin and O. M. Andrisani, unpublished data) suggest that HIPK2 mediates by phosphorylation the inactive state of the transcription factor Phox2a (66). Since Phox2a and TH are REST targets (39, 57) and REST expression is inversely modulated by CtBP2 and HIPK2 function, the concurrent regulation of Mitf and REST by CtBP2 implies a dynamic balance between the melanocytic and neuronal cell fate adopted by pluripotent NC cells. This notion is supported by findings from an earlier zebrafish study. Specifically, in zebrafish embryos, activation of Wnt signaling promotes pigment cell development at the expense of other NC derivatives; by contrast, inhibition of Wnt signaling promotes a neural fate at the expense of pigment cells (23). Since CtBP2 interferes with Wnt signaling and both Mitf and REST are Wnt target genes, our data provide a molecular mechanism underlying the effects caused by activation or inhibition of Wnt signaling in zebrafish NC lineage specification.

Cross talk between cAMP signaling and the canonical Wnt pathway. The best-known example of cross talk between cAMP signaling and the canonical Wnt pathway is inactivation of glycogen synthase kinase 3β (GSK-3β) by PKA-mediated...
phosphorylation, leading to β-catenin stabilization and activation of Wnt-responsive transcription (49, 69). Another study demonstrated that PKA directly phosphorylates β-catenin on Ser675, inhibiting its ubiquitination and thereby stabilizing β-catenin (32).

Herein, we show an additional mechanism of integration between cAMP signaling and the canonical Wnt pathway. In this novel pathway, cAMP signaling via PKA targets CtBP2 for proteasomal degradation by activating HIPK2. The decreased protein level of CtBP2 results in the release of nuclear β-catenin from sequestration by the CtBP2/APC complex; free nuclear β-catenin activates Lef1/Tcf-mediated transcription of Wnt target genes (Fig. 9).

This cross talk between CAMP signaling and the canonical Wnt pathway is especially plausible in NC lineage specification. Both cAMP and Wnt signaling are instructive signals in melanocyte differentiation at the expense of other NC derivatives (23, 37), and significantly, expression of Wnts occurs in the microenvironment of differentiating NC cells in vivo (36, 47). We conclude that in addition to CREB activation (37), the cAMP pathway via PKA induces the proteasomal degradation of CtBP2 signaled by HIPK2 activation, resulting in transcriptional induction of both the Mif and REST genes. The increased expression of REST suppresses neuronal SA lineage differentiation, whereas the increased expression of Mif induces the melanocytic lineage.

In addition to CtBP2 acting as a negative regulator of Mif transcription, shown in the studies herein, recent studies have demonstrated that the forkhead transcription factor Foxd3, a marker of premigratory NC cells (43), is also a negative regulator of Mif transcription (19, 20, 72). Significantly, the expression of Foxd3 and that of Mif are mutually exclusive in melanocytes (19, 20). Interestingly, putative Foxd3 binding sites exist within 1,000 bp from the +1 site of the REST and HIPK2 promoters (http://alagen.isi.upc.es/cgi-bin/promo_v3/promomini. cgi?dirDB = TF.8.3), suggesting concurrent repression of REST, HIPK2, and Mif by Foxd3. Furthermore, the mutually exclusive expression patterns of Foxd3 and Mif suggests that Foxd3 expression may be negatively regulated by cAMP signaling similarly to that of CtBP2. Further studies are needed to investigate the possibility of this intriguing signaling network in embryonic development and in human disease (1, 78).

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