α-Melanocyte-stimulating Hormone Signaling Regulates Expression of microphthalmia, a Gene Deficient in Waardenburg Syndrome*

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The pituitary peptide α-melanocyte-stimulating hormone (α-MSH) stimulates melanocytes to up-regulate cAMP, but the downstream targets of cAMP are not well understood mechanistically. One consequence of α-MSH stimulation is increased melanization attributable to induction of pigment enzyme enzymes, including tyrosinase, which catalyzes a rate-limiting step in melanin synthesis. The tyrosinase promoter is a principle target of the melanocyte transcription factor Microphthalmia (Mi), a factor for which deficiency in humans causes Waardenburg syndrome II. We show here that both α-MSH and forskolin, a drug that increases cAMP, stimulate a rapid increase in Mi mRNA and protein levels in both melanoma cell lines and primary melanocytes. This up-regulation requires a cAMP-responsive element within the Mi promoter, and the pathway leading to Mi stimulation is subject to tight homeostatic regulation. Although cAMP signaling is ubiquitous, the Mi promoter was seen to be cAMP-responsive in melanocytes but not in non-melanocytes. Moreover, dominant negative interference with Mi impeded successful α-MSH stimulation of tyrosinase. The regulation of Mi expression via α-MSH thus provides a direct mechanistic link to pigmentation. In addition, because the human melanocyte and deafness condition Waardenburg syndrome is sometimes caused by haploinsufficiency of Mi, its modulation by α-MSH suggests therapeutic strategies targeted at up-regulating the remaining wild type Mi allele.

Waardenburg syndrome II is a human dominant hereditary pigmentation and deafness condition in which mutations in the Microphthalmia (Mi) transcription factor gene have been identified (1–3). Homozygous microphthalmia mutations in mice result in complete absence of the melanocyte lineage (4), which permitted identification and cloning of Mi (5–7). It is thought that inner ear melanocytes are functionally deficient in Waardenburg patients, suggesting an essential although poorly understood role for pigment cells in sensorineural hearing. Most human mutations in Mi are likely to be functionally null (1–3) based largely on structure-function relationships previously examined in a series of murine Mi mutations (8, 9). Because human Waardenburg Syndrome II often results from haploinsufficiency, it is of interest to understand pathways that might up-regulate Mi expression on the remaining (wild type) Mi allele.

Recent work has demonstrated that Mi protein function is subject to regulation in response to cytokine signaling. Activation of the c-Kit cytokine receptor on pigment cells by Steel factor was found to trigger activation of mitogen-activated protein kinase, which directly phosphorylates Mi at serine 73 (10). The transcriptional activity of Mi is modulated through interactions with the transcriptional coactivator p300/CREB-binding protein (11), the specific recruitment to Mi of which is regulated by mitogen-activated protein kinase phosphorylation (12). c-Kit signaling thus increases histone acetyl transferase activity associated with Mi, significantly enhancing the transcriptional potential of Mi (12) although not its expression level per se. The pathway also correlates with the phenotypic overlap of mi, Steel, and c-kit mutant mice, all of which are devoid of melanocytes (4).

Earlier studies have identified Mi as the major transcriptional regulator of the pigment enzyme genes tyrosinase and tyrosinase-related proteins 1 and 2 (8, 13–16), factors required for pigmentation (for review, see Refs. 17 and 18). Mi thus appears to reside at a very central position relative to regulation of pigmentation in melanocytes. For this reason it is plausible that other pathways associated with pigmentation might also operate through regulation of Mi function or expression.

α-Melanocyte-stimulating hormone (α-MSH) is a pituitary-derived peptide that stimulates melanin production in melanocytes (19). The α-MSH receptor, Mc1r, is a member of the seven-transmembrane receptor superfamily (20). Stimulation of the Mc1r receptor activates adenyl cyclase via G protein signaling, leading to an increase in intracellular cAMP (20). Treatment of melanocytes with agents that increase cAMP such as forskolin leads to induction of tyrosinase and increased melanin production (15). Finally, pigment enzyme activity is induced after α-MSH stimulation. These studies have suggested either transcriptional (16) or post-transcriptional (21) mechanisms for this up-regulation. Although the tyrosinase promoter could in principal be a direct target of cAMP, the tyrosinase promoter is unresponsive to forskolin in nonmelanocytes (15; see below), and the sequence of a large portion of the tyrosinase promoter has not revealed any cAMP-responsive elements (CREs) (for review, see Ref. 22). Another possibility is that the tyrosinase promoter could be modulated by a factor that itself is subject to cAMP-dependent regulation. Interestingly, melanoma cells themselves often down-regulate pigment-
tation by a variety of mechanisms, some involving degradation of tyrosinase (23). Mi binds as a dimer to a subset of “E box” DNA sequences (8) found in target genes. Loss of the Mi binding site significantly disrupts tyrosinase promoter activity within melanocytes as well as α-MSH responsiveness (15), suggesting that Mi activity could link α-MSH signaling to tyrosinase induction. In addition, stimulation by α-MSH enhances tyrosinase activity, and in one study this was found to be accompanied by increased levels of Mi DNA binding activity, although either absence or presence of Mi protein increases have both been described after α-MSH stimulation (15, 16).

The *microphthalmia* gene contains two promoter/exon-1 combinations, which are alternatively spliced onto a common downstream body (9). One of these promoter/exon 1 cassettes appears to be expressed exclusively in melanocytes (9). Recently, a fragment from the melanocytic *microphthalmia* promoter was cloned (24) and found to contain a cAMP-responsive element. This element suggested a mechanistic link between α-MSH signaling and induction of tyrosinase via regulation of Mi expression. We demonstrate here that elevated intracellular cAMP, triggered by either α-MSH or forskolin, does, in fact, lead to rapid and potent induction of the Mi promoter. This induction is dependent on an intact CRE, which importantly was found to be cAMP-inducible only in melanocytes. Moreover, use of a dominant negative Mi mutant suggested that this up-regulation of Mi expression is essential to the mechanism through which α-MSH up-regulates tyrosinase expression.

**EXPERIMENTAL PROCEDURES**

*Cell Culture—*The B16, 501mel (kindly provided by Ruth Halaban; Ref. 25), and BHK cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal calf serum, and 5% CO₂. The BAC1.2F5 cell line (macrophages) was grown in DMEM and 10% fetal calf serum plus 20% L cell conditioned media, and the C57 mast cell line (generously provided by Dr. S. Galli) was grown in DMEM, 10% fetal calf serum, and 0.2 μM β-mercaptoethanol. Primary human neonatal melanocytes were obtained from Clonetics and maintained as recommended by the supplier.

*Western Blot—*Cells were treated with forskolin (20 μM), α-MSH (1 μM), or cycloheximide (20 μg/ml) in DMEM and 10% fetal calf serum for indicated times. At harvest, cells were washed with phosphate-buffered saline, extracted with SDS sample buffer (250 mM tris, 4% SDS, 20% glycerol), and immediately boiled. Protein extracts were resolved using either 8.5% or 10% SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose. Prestained size standards were used (Life Technologies, Inc.). The antibodies used for Western blot analysis include mouse anti-Mi (10), mouse anti-α-tubulin (Sigma, T9026), rabbit anti-phospho-CRE-binding protein (CREB; New England Biolabs, 9191), rabbit anti-CREB (New England Biolabs, 9192), rabbit anti-protein kinase A (PKα,β,γ; Santa Cruz, 903, 904, 905), rabbit anti-protein phosphatase 1 (PP1; Santa Cruz, 443), and horseradish peroxidase goat anti-rabbit and mouse (Cappel, 55550, 55876). Mi, tubulin, phospho-CREB, CREB, and PP1 signals were quantitated by densitometry (MultiAnalysis, Bio-Rad) of enhanced chemiluminescence-exposed films. To determine the linear range of quantitation by this method, standard curves were generated using recombinant Mi protein. Quantitation was found to be linear over the range of 2–38 pg (i.e. 19-fold). Data points in this range matched the linear best fit with r = 0.992. Experimental data points all lay within this linear range, with the exception of two late time points of Mi decay in the presence of cycloheximide (see Fig. 2A) indicated as asterisks (see Fig. 2, D and E).

*Northern Blot—*1.5 μg of poly(A⁺)− selected RNA (Qiagen) from B16 cells was separated on a 1% formaldehyde gel, blotted onto a Hybond A-Plus membrane (Amerham Pharmacia Biotech), and cross-linked by UV irradiation. Mouse Mi cDNA fragment (corresponding to amino acids 122–590), rabbit protein phosphatase 1α (amino acids 121–820) (26), or full length human glyceraldehyde 3'-phosphate dehydrogenase cDNA fragments were 32P random-primed labeled and hybridized to immobilized RNA and washed at high stringency (0.2 × SSC, 0.1% SDS at 55 °C), followed by autoradiography. RNA standards were used (Life Technologies, Inc.).

**RESULTS AND DISCUSSION**

Stimulation of the Mc1R receptor by α-MSH initiates a signal transduction cascade that increases cAMP levels through adenyl cyclase. The recent cloning of the *microphthalmia* promoter revealed a CRE just upstream of the TATA box (24), suggesting that Mi expression could be regulated by a signal transduction pathway using cAMP as second messenger. To test whether α-MSH does alter Mi expression, human or murine melanoma cells were treated with α-MSH, and Mi levels were assayed by Western blot. Mi migrates as a doublet in pigment cells, and the two bands differ by the presence of mitogen-activated protein kinase phosphatase 3 phosphorylation at serine 73, as demonstrated by two-dimensional phosphotryptic mapping (10). Within 1 h after α-MSH stimulation there is an increase in the intensity of the lower migrating band but not the upper band. By 2 h and beyond, both upper and lower bands are substantially enhanced and equal in intensity, relative to control tubulin levels (Fig. 1A, quantitated in Fig. 2, D and E). Mi protein thus appears to be up-regulated by α-MSH treatment, and the reproducible appearance of the lower band induction before that of the upper band is consistent with previous evidence that the upper migrating form represents a phosphorylation of the lower isoform (10).

Another test that up-regulation of Mi was related to cAMP signaling came from stimulation of melanoma cells by the cAMP-inducing compound forskolin (Fig. 1B). Forskolin triggered a nearly identical pattern of Mi up-regulation as seen with α-MSH, including previous appearance of the lower migrating form within the doublet after only 1 h, followed by induction of both doublet bands. Maximal accumulation of Mi protein in both human and mouse melanoma cells occurred 3–4 h after drug treatment. Although B16 cells turn (and remain) deeply pigmented by this treatment (data not shown), Mi levels rise only transiently and after ~4 h begin to decrease, ultimately to undetectable levels far below basal by 72 h (Fig. 1C).

Forskolin treatment also led to induction of Mi protein in primary neonatal melanocytes (Fig. 1D); however it did not elicit Mi expression in nonmelanocytic cell types such as fibroblasts or kidney cells (data not shown). Forskolin also did not enhance Mi expression in the nonmelanocytic cell types that normally express Mi, such as macrophages and mast cells. These lineages use a distinct promoter to express an alternative isoform of Mi. As shown in Fig. 1E, monocytes and mast cells express a larger isoform of Mi (27), the levels of which, unlike in melanocytes, do not significantly change with forskolin.

It is formally possible that the increase in Mi protein could
result from changes in transcription, mRNA stability and processing, translational efficiency, or protein stability. The translational inhibitor cycloheximide (CHX) completely blocked the α-MSH induction of Mi protein (Fig. 2A), which in non-CHX-treated cells produced an ~10-fold induction after 4 h (Fig. 2D). Moreover, CHX treatment suggested that Mi has a very short half-life relative to α-tubulin.

Northern blot analysis indicated that α-MSH treatment leads to a rapid rise in Mi mRNA, peaking at 2 h (Fig. 2B). This mRNA induction is unaffected by CHX and suggests that up-regulation of Mi is pretranslational. After 2 h, Mi mRNA levels return to baseline, likely reflecting homeostatic down-regulation and consistent with the subsequent decrease in Mi protein beginning at 4 h (see Fig. 1, A and B). Interestingly, this fall in Mi mRNA levels is severely retarded by CHX, suggesting that new protein synthesis is required for the homeostatic down-regulation of Mi mRNA levels. Because of the presence of a CAMP-responsive element consensus sequence in the melanocytic Mi promoter (24), it appeared likely that the up-regulation as well as the homeostatic down-regulation could involve a CREB transcription factor family member.

cAMP-dependent transcriptional induction of the somatostatin promoter and its homeostatic down-regulation have been previously described in nonmelanocytes (28). In this case, high levels of cAMP activate PKA, leading to nuclear translocation in which PKA phosphorylates the CREB at serine 133 (29). The consequence of phosphorylation of CREB is recruitment of the transcriptional cofactor CREB-binding protein and transcriptional up-regulation of the CRE-containing promoter (30, 31). Within hours, CREB phosphorylation is down-regulated by the action of PP1 as well as the removal of PKA from the nucleus via binding to protein kinase A inhibitor, a cytoplasmic shuffling factor (33).

To examine activation of CREB in α-MSH-treated melanoma cells, extracts were Western blotted with an antibody that specifically recognizes phosphoserine 133 CREB as well as phospho-ATF-1. As shown in Fig. 2C, both phospho-CREB and phospho-ATF-1 levels rise within 1 h after treatment with α-MSH in both the absence and presence of CHX. The up-regulation of the Mi promoter follows the same approximate kinetics as the somatostatin promoter (32). Phospho-CREB peaks at 1 h, followed by the Mi mRNA peak at 2 h, followed by the Mi protein peak at 3 h (Figs. 1, A and B, and 2, A–D and F).

In α-MSH-treated melanoma cells phospho-CREB and -ATF-1 levels first rise, peak at 1 h, and then fall rapidly. Concurrent CHX treatment does not affect the activation and phosphorylation of CREB and ATF-1 but interferes with subsequent down-regulation, leading to a more gradual decrease in phospho-CREB and -ATF-1 (Fig. 2, C and F). When phospho-CREB levels are quantitated and normalized to overall (non-phospho-specific) CREB levels, CHX is seen to stabilize the phospho-specific form (Fig. 2F). Careful inspection of overall CREB analysis (Fig. 2C) also reveals a mobility shift associated with phosphorylation, and the shifted isoform fails to revert in the presence of CHX. These data suggest an important role for new protein synthesis in regulating phosphorylation of phospho-CREB.

Because one means of modulating phospho-CREB is through phosphatase action of PP1 (32), PP1α expression was assessed at both the mRNA and protein levels. In α-MSH-treated cells, a slight increase of PP1α protein was observed in the absence of CHX. In the presence of CHX, PP1α protein levels declined (Fig. 3A). This was accompanied by a corresponding decline in PP1α mRNA (Fig. 3B). Because the decline in PP1α levels in CHX parallels the maintenance of phospho-CREB and sustained Mi mRNA expression, these results are consistent with the possibility that PP1α may contribute to the homeostatic down-regulation of Mi induction by α-MSH. Importantly, other levels of regulation may also function, such as increased kinase activity. PKA isoform levels do not substantially change under these conditions (data not shown).

Because these data suggest CREB and ATF-1 as transcriptional mediators of α-MSH signaling in melanoma cells, a series of reporter assays was undertaken to analyze the requirement of a functional CREB binding site in the melanocytic Mi promoter for cAMP induction. Transfection of a firefly luciferase reporter driven by 484 bp (~387 to +97) of the human Mi promoter into B16 melanoma cells produced a 20-fold induction...
of luciferase activity relative to the promoterless (control) pGL2.basic (Fig. 4A). After transfection into B16 melanoma cells, forskolin treatment further augmented Mi promoter activity by an additional 2–3-fold (Fig. 4B), similar in magnitude to that seen with other cAMP-responsive elements (see below). Importantly, the Mi promoter was inactive and uninducible in nonmelanocyte (BHK) cells (Fig. 4, A and B), recapitulating the tissue-restricted expression of Mi.

The CRE (TGACGTCA) found within the Mi promoter appears to be important for promoter activity in melanoma cells, because mutation of the canonical CRE sequence rendered the promoter construct largely (although not entirely) inactive (Fig. 4A) as well as forskolin nonresponsive (Fig. 4B). Thus the CRE in the Mi promoter displayed tissue-restricted (melanocytic) cAMP responsiveness. In contrast, a 449-bp fragment from the somatostatin promoter (generously provided by M. Montminy) containing the identical CRE core (TGACGCTCA) produced a 2–3-fold induction in either B16 or BHK cells after forskolin treatment and cAMP up-regulation (Fig. 4B). These data imply the existence of cell type-restricted CRE responsiveness and suggest that the context in which the CRE exists, rather than the CRE sequence itself, may confer tissue specificity. Although the mechanism underlying this restriction is currently unknown, this observation is potentially of importance in explaining how the ubiquitous cAMP signaling pathway regulates an exquisitely tissue-specific factor such as Mi.

A similar example of context-dependent cAMP responsiveness was recently reported in deletional studies of the brain-derived neurotrophic factor promoter, in which an intact CRE was needed for phospho-CREB activity, but sequences upstream of the CRE were also essential (34, 35). These observations suggest that other transcription factors bind to this upstream site and may cooperatively interact with CREB. For the Mi promoter, a melanocyte-specific transcription factor might bind sites flanking the CRE and either contribute to basal activity or even interact with CREB cooperatively. Future studies should reveal how this tissue-restricted CRE response is regulated.

It has been reported that induction of the tyrosinase promoter after forskolin requires an intact E box element (15). This sequence is bound by Mi in vitro (8) and transactivated by Mi in vivo (8, 13, 14). We therefore asked whether α-MSH-stimulates the tyrosinase promoter activity via up-regulation of Mi levels, or more specifically whether Mi function is necessary for α-MSH-mediated tyrosinase stimulation. Luciferase reporter constructs under the control of either the human tyrosinase promoter or the Mi promoter (control) were cotransfected into B16 cells. Treatment with α-MSH induced both the tyrosinase promoter as well as the Mi promoter above the basal levels found in B16 cells (Fig. 4C). Endogenous Mi protein was

| A | B16 Treated with α-MSH (-)CHX (+)CHX |
|---|---|
| **αMSH** | **0** | **1** | **2** | **3** | **4** | **0** | **1** | **2** | **3** | **4** |
| **Mi** | - | - | - | - | - | *64kD* | - | - | - | - |
| **Tubulin** | - | - | - | - | - | **52kD** | - | - | - | - |
| **B** | **Mi mRNA** | - | - | - | - | - | - | - | - | - |
| **GAPDH mRNA** | - | - | - | - | - | - | - | - | - | - |
| **C** | **P-CREB** | - | - | - | - | - | - | - | - | - |
| **P-ATF1** | - | - | - | - | - | - | - | - | - | - |
| **Protein** | - | - | - | - | - | - | - | - | - | - |
| **D** | **Phospho-CREB Fold Induction** | - | - | - | - | - | - | - | - | - |

**Fig. 2. Increased cAMP induces Microphthalmia and phospho-CREB.** B16 cells were treated with α-MSH for 0–4 h as indicated in the absence or presence of CHX. A, cell extracts were Western blotted using anti-Mi and anti-tubulin antibody. B, mRNA extracted from matched plates was Northern blotted and probed with either Mi or glyceraldehyde 3’-phosphate dehydrogenase (GAPDH) as control. C, cell extracts were Western blotted with either anti-phospho-CREB or anti-CREB antibodies. Extracts for the non-phospho-CREB blot were electrophoresed through a 10% polyacrylamide gel to better resolve the phosphorylated isoform (doublet), whereas other Western blots represent 8.5% gels. D, quantitation of Mi induction (as in A, combined upper and lower bands) from three independent experiments was generated by densitometric analysis and normalized to signal from non-drug-treated cells and expressed as the mean and S.D. Asterisks indicate signal intensities that were below the linear range for quantitation (see “Experimental Procedures”). E, intensities of upper (phospho) and lower (non-phospho) Mi bands were analyzed (as in A) and expressed as the mean and S.D. of ratios from three independent experiments. F, quantitation of phospho-CREB induction (as in C) from three independent experiments was generated by densitometric analysis and normalized to non-drug-treated cells and total CREB signal and expressed as the mean and S.D.
functionally interfered with by expression of dominant negative Mi. Mi-dn(R215del) is a naturally occurring (and previously characterized) mutation that contains a codon deletion within the basic domain of Mi. This allele, $M_i^{dn}$, is dominantly inherited in mice and is biochemically dominant negative through preserved dimerization activity coupled to ablated DNA recognition (8). Dominant negative Mi produced a dose-dependent inhibition of both basal and $\alpha$-MSH-stimulated tyrosinase promoter activity (Fig. 4C). As control, dominant negative Mi had no effect on the ability of $\alpha$-MSH to activate the Mi promoter construct (Fig. 4C). These results suggest that Mi is necessary for the $\alpha$-MSH-triggered signaling cascade that induces tyrosinase expression.

Taken together these observations describe a mechanistic connection between $\alpha$-MSH and the transcriptional induction of pigmentation. After binding to the Mc1r receptor, $\alpha$-MSH initiates a signaling cascade beginning with G protein activation of adenyl cyclase that produces an increase in intracellular cAMP. A cAMP-regulated kinase then likely activates a member of the CREB family, which, in a context- and cell type-dependent fashion, activates the CRE within the Mi promoter, presumably via cAMP-binding protein- and p300-regulated co-activation. Accumulated Mi protein then secondarily regulates the tyrosinase promoter (and likely other melanocytic promoters), inducing expression of tyrosinase and driving pigmentation.

These studies also highlight a number of unresolved questions relating to the same pathway. Although Mc1r is known to up-regulate cAMP levels (20), it is unclear precisely which kinase activates the transcription factor responsible for CRE-mediated activation in melanocytes. PKA as well as p90RSK are candidate CREB kinases, although their precise roles in cytokine-mediated melanocyte signaling remain to be fully elucidated (36). In addition, the previous questions regarding pigment enzyme up-regulation at the protein versus mRNA level might possibly relate to this indirect pathway involving Mi and varied RNA or protein stabilities coupled to the apparent ho-

meostatic down-regulation of Mi described above.

Humans heterozygous for a mutation in Mi are afflicted with Waardenburg syndrome type II (1–3). These patients suffer significant sensorineural hearing loss and have white forelocks attributable to melanocyte dysfunction and loss. One other factor associated with Waardenburg syndrome, Pax-3, has recently been implicated as another transcriptional regulator of Mi expression (37). Because Mi mutations in Waardenburg patients are typically null (38), the dominant inheritance likely derives from haploinsufficiency. Therefore, it will be of importance to better understand the state of residual inner ear melanocytes in such patients for the possibility that $\alpha$-MSH might provide a tissue-specific means of rescuing Mi expression in these cells.

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**Fig. 3.** Protein phosphatase levels correlate with phospho-CREB and Mi expression. B16 cells were treated with $\alpha$-MSH for 0–4 h in the absence and presence of CHX (extracts used in Fig. 2). A, Western blot analysis of PP1$\alpha$ expression. Quantitation of PP1$\alpha$ from three independent experiments was generated by densitometric analysis and normalized to signal from non-drug-treated cells. B, Northern blot analysis of PP1$\alpha$ expression. The Northern blot was controlled by hybridization with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. The two RNA isoforms likely represent the previously characterized mutation that contains a codon deletion within the basic domain of Mi. This allele, $M_i^{dn}$, is dominantly inherited in mice and is biochemically dominant negative through preserved dimerization activity coupled to ablated DNA recognition (8). Dominant negative Mi produced a dose-dependent inhibition of both basal and $\alpha$-MSH-stimulated tyrosinase promoter activity (Fig. 4C). As control, dominant negative Mi had no effect on the ability of $\alpha$-MSH to activate the Mi promoter construct (Fig. 4C). These results suggest that Mi is necessary for the $\alpha$-MSH-triggered signaling cascade that induces tyrosinase expression.

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miny for the somatostatin luciferase reporter construct and PP1α plasmid.

REFERENCES

1. Hughes, A. E., Newton, V. E., Liu, X. Z., and Read, A. P. (1994) *Nat. Genet.* 7, 509–512
2. Tassabehji, M., Newton, V. E., and Read, A. P. (1994) *Nat. Genet.* 8, 251–255
3. Tachibana, M. (1997) *Pigment Cell Res.* 10, 25–33
4. Silvers, W. K. (1979) *The Coat Colors of Mice: A Model For Mammalian Gene Action and Interaction*, Springer-Verlag, New York
5. Hodkinson, C. A., Moore, K. J., Nakayama, A., Steingrimsson, E., Copeland, N. G., Jenkins, N. A., and Arnheiter, H. (1993) *Cell* 74, 395–404
6. Hughes, M. J., Lingrel, J. B., Krakowsky, J. M., and Anderson, K. P. (1993) *J. Biol. Chem.* 268, 20687–20690
7. Tachibana, M., Perez-Durado, L. A., Nakayama, A., Hodgkinson, C. A., Li, X., Schneider, M., Miki, T., F, J., Francke, U., and Arnheiter, H. (1994) *Hum. Mol. Genet.* 3, 553–557
8. Hodkinson, C. A., Steingrimsson, E., McGill, G., Hansen, M. J., Vaught, J., Hodgkinson, C. A., Arnheiter, H., Copeland, N. G., Jenkins, N. A., and Fisher, D. E. (1994) *Genes Dev.* 8, 2770–2780
9. Steingrimsson, E., Moore, K. J., Lamoreux, M. I., Ferre, D. A. A. R., Burley, S. K., Zimring, D. C., Skow, L. C., Hodgkinson, C. A., Arnheiter, H., Copeland, N. G., and Jenkins, N. A. (1994) *Nat. Genet.* 8, 256–263
10. Hodenstiel, T. J., Price, E. R., Takemoto, C., and Fisher, D. E. (1996) *Nature* 381, 298–301
11. Sato, S., Roberts, R., Gambino, G., Cook, A., Kouzarides, T., and Goding, C. R. (1997) *Oncogene* 14, 3083–3092
12. Price, E. R., Ding, H.-F., Badalian, T., Bhattacharya, S., Takemoto, C., Yee, T. P., Hodenstiel, T. J., and Fisher, D. E. (1998) *J. Biol. Chem.* 273, 17983–17986
13. Bentley, N. J., Eisen, T., and Goding, C. R. (1994) *Nat. Genet.* 8, 256–263
14. Yasumoto, K., Yokoyama, K., Shibata, K., Tomita, Y., and Shibahara, S. (1994) *Mol. Cell. Biol.* 14, 8058–8070
15. Erdmann, T., Bille, K., Ortonne, J., and Ballestri, R. (1996) *J. Cell Biol.* 134, 747–755
16. Bertolotto, C., Busca, R., Abe, P., Bille, K., Aberdam, E., Ortonne, J. P., and Ballestri, R. (1998) *Hum. Mol. Genet.* 7, 8058–822
17. Urabe, K., Araca, P., and Hearing, V. J. (1993) *Pigment Cell Res.* 6, 186–192
18. Bash, G. S. (1996) *Trends Genet.* 12, 299–305
19. Lerner, A. B. (1993) *Ann. NY Acad. Sci.* 680, 1–12
20. Montminy, M. R., Robbins, L. S., Steingrimsson, E., and Read, A. P. (1994) *Science* 267, 1248–1251
21. Abel, M. Z., Swope, V. B., Suzuki, I., Akcali, C., Harriger, M. D., Boyce, S. T., Urabe, K., and Hearing, V. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 1789–1793
22. Ferguson, C. A., and Kidson, S. H. (1997) *Pigment Cell Res.* 10, 127–138
23. Halaban, R., Cheng, E., Zhang, Y., Moellmann, G., Hanlon, D., Michalak, M., Setaluri, V., and Hebert, D. N. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 6210–6215
24. Fuse, N., Yasumoto, K., Suzuki, H., Takahashi, K., and Shibahara, S. (1998) *Biochem. Biophys. Res. Commun.* 219, 702–707
25. Zaki, R., Perlis, R., Eliyahu, S., Yarden, Y., Givol, D., Lyman, S. D., and Halaban, R. (1993) *Oncogene* 8, 2221–2229
26. Cohen, P. T. (1998) *FEBS Lett.* 437, 17–23
27. Weilbaecher, K. N., Hershey, C. L., Takemoto, C. M., Horstmann, M. A., Hodenstiel, T. J., Tashjian, A. H., and Fisher, D. E. (1998) *J. Biol. Chem.* 273, 20687–20690
28. Montminy, M. (1997) *Ann. Rev. Biochem.* 66, 897–922
29. Hagiwara, M., Brindle, P., Harootunian, A., Armstrong, R., Rivier, J., Vale, W., Tsien, R., and Montminy, M. R. (1995) *Mol. Cell. Biol.* 15, 4852–4859
30. Kwok, R. P., Lundhblad, J. R., Chirivia, J. C., Richards, J. P., Bachinger, H. P., Brennam, R. G., Roberts, S. G., Green, M. K., and Goodman, R. H. (1994) *Nature* 370, 223–226
31. Arias, J., Alberts, A., Brindle, P., Claret, F. X., Smeal, T., Karin, M., Feramisco, J., and Montminy, M. R. (1995) *Nature* 370, 256–263
32. Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., and Montminy, M. (1992) *Cell* 70, 105–113
33. Fantozzi, D. A., Harootunian, A. T., Wen, W., Taylor, S. S., Feramisco, J. R., Tsien, R. Y., and Meinkoth, J. L. (1994) *J. Biol. Chem.* 269, 2676–2686
34. Tao, X., Finkbeiner, S., Arnold, D., Shyamsunder, A., and Greenberg, M. (1998) *Neuron* 20, 797–792
35. Shieh, P., Hu, S., Bobb, K., Timmusk, T., and Ghosh, A. (1998) *Neuron* 20, 727–740
36. Bohn, M., Finkbeiner, S., Meng, E., Alvarez-Franco, M., Wagner, S., Sasse-Corsin, P., and Halaban, R. (1995) *Cell Growth & Differ.* 6, 291–302
37. Watanabe, A., Takeda, K., Fliopis, B., and Takizawa, M. (1998) *Nat. Genet.* 18, 283–286
38. Read, A. P., and Newton, V. E. (1997) *J. Med. Genet.* 34, 656–665
