Chemistry & Biology, Vol. 12, 477–484, April, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.02.014

Dissection of Melanogenesis with Small Molecules Identifies Prohibitin as a Regulator

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Summary

Bioactive compounds can be used to selectively modulate gene function. We utilized a chemical genetic approach to dissect the mammalian pigmentation pathway and identify protein regulators. We screened a tagged library of 1170 small molecules in a cell-based assay and discovered a class of pigment-enhancing chemicals. From this class we characterized the small molecule melanogenin. Using melanogenin bound to an affinity matrix and amino acid sequencing, we identified the mitochondrial protein, prohibitin, as an intracellular binding target. Studies employing siRNA demonstrate that prohibitin is required for melanogenin to exert its propigmentary effects and reveal an unsuspected functional role for this protein in melanin induction. This represents a mechanism by which propigmentary signals are transduced and ultimately provides a potential target for the treatment of pigmentary disorders.

Introduction

Chemical genetics is a large-scale method that facilitates the discovery and identification of novel proteins and their function through the screening of small molecules in cells [1, 2]. Conditional and temporal control of protein function and expression may be achieved through the use of small molecules, and selective modulation of individual functions of multifunctional proteins may also be accomplished [1]. Importantly, small molecules can be used to directly identify protein targets in biological pathways through affinity purification [1–4]. Such target identification may provide new avenues for rational drug design and gene-specific therapies [1–4].

Melanin plays an important role in protection of the skin from the deleterious effects of UV radiation. The highest incidence of skin cancer is found in individuals with low levels of endogenous pigmentation [5]. Agents that promote pigmentation hold the potential to reduce the incidence of UV-induced skin damage and carcinogenesis [5]. Additionally, the mammalian pigmentation response is complex and remains incompletely understood. A complete molecular understanding of the pigmentation pathway will facilitate the treatment of disorders such as oculocutaneous albinism, vitiligo, and melanoma. Studies from the mouse genome suggest that >100 loci are involved in pigmentation, many of which remain unidentified. Systematic, large-scale screens hold promise in the identification of these loci; such studies have not yet been attempted. Cell-based chemical genetics has been successfully utilized in the dissection of other fundamental cellular pathways such as glucose signaling [6], myotube fission [7], mitosis [8], and plant auxin signaling [9], yielding potentially therapeutic small molecules and protein targets. Here we discuss our chemical genetic approach to pigmentation.

Using this approach, we have characterized the small-molecule probe melanogenin and identified a specific intracellular target as the mitochondrial protein prohibitin. Studies employing siRNA reveal a functional role for prohibitin in the pigmentation response, representing what we believe to be a novel mechanism by which melanogenic signals are transduced and providing a potential target for specific therapies.

Results

Screening of a Tagged Triazine Library for Inducers of Pigmentation in Melanocytes Yields the Potent Promoter Melanogenin

We constructed a 1170-member tagged triazine-based chemical library (Figure 1A) using an orthogonal synthetic approach [10] for both screening and subsequent target identification. We screened this tagged trisubstituted triazine library for compounds that promoted pigmentation in cultured melanocytes (Figure 1A). Screening was performed in 24-well plates (Figure 1A) followed by quantitative melanin assay. Vehicle-treated melanocytes, as well as cells treated with the melanin induction agent isobutylmethylxanthine (IBMX), served as negative and positive controls, respectively. We established a minimum increase in melanin formation of 160% over untreated melanocytes as significant for promoters. Using this criterion, we identified a class of pigment-enhancing chemicals from the library, resynthesized and rescreened these compounds, and established a subclass of 23 confirmed promoters, of which melanogenin is a representative member (Figure 1A). All confirmed active chemicals were noncytotoxic at concentrations below 20 μM (data not shown). Melanogenin was a potent inducer of pigmentation with an EC_{50} of 2.5 μM (data not shown).

To create an optimal negative control for future target isolation experiments, we analyzed the initial screening results in order to identify an inactive derivative of melanogenin. We identified E28, a molecule that differs structurally from melanogenin only at the R_1 position (Figure 1B) yet is inactive at multiple concentrations

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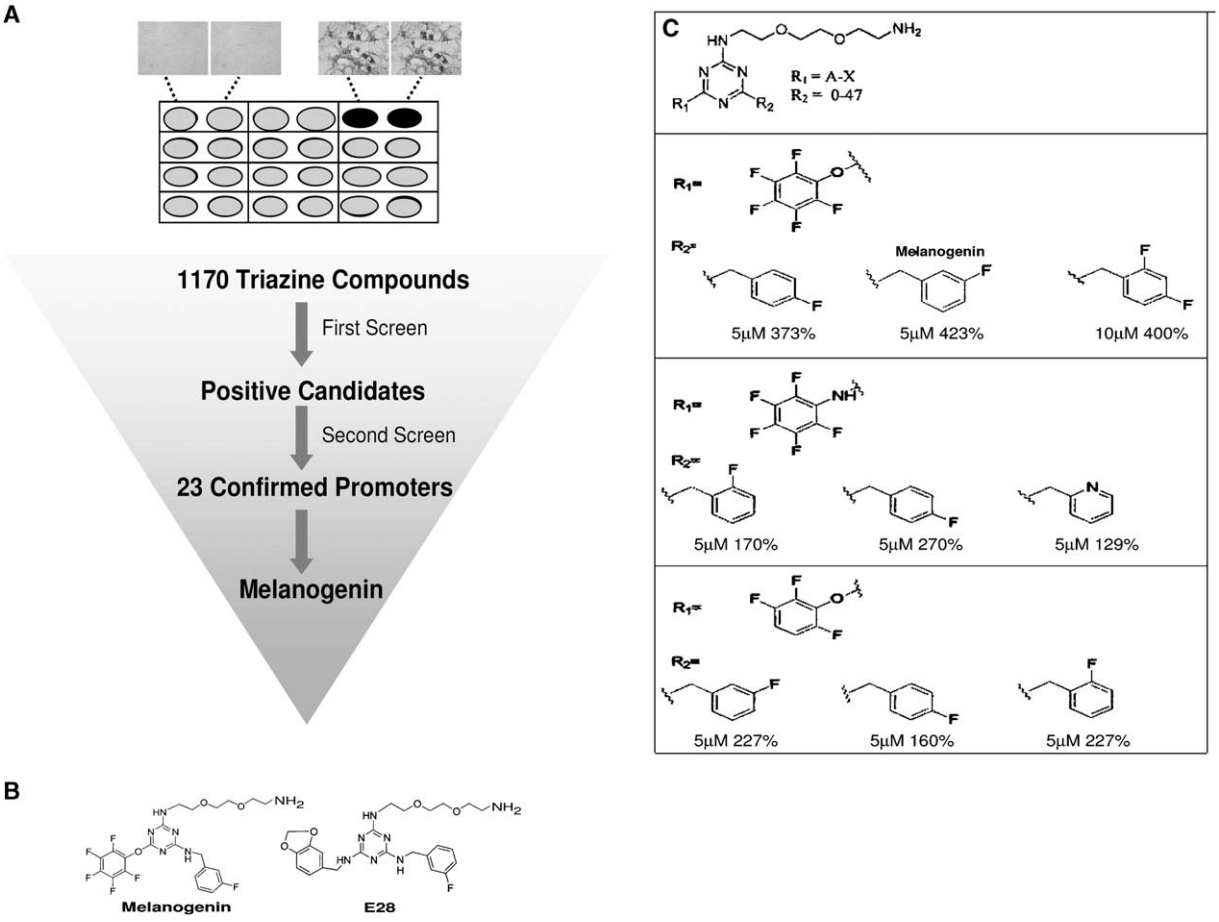

Figure 1. A Chemical Genetic Approach to Pigmentation Yields Melanogenin, Believed to Be a Novel Small-Molecule Inducer of Melanogenesis

(A) In vitro screening of a 1170-member trisubstitued triazine library in cultured wild-type murine melanocytes.

(B) Chemical structures of melanogenin and an inactive derivative E28. Note that E28 differs structurally from melanogenin only at the R_1 position.

(C) Structure-activity relationship analysis of triazine library members. Structural variations were produced by chemically modifying the triazine structure at the R_1 and R_2 positions. Chemical activity is expressed as the percent increase in pigmentation above untreated melanocytes.

(data not shown). Resynthesis and rescreening alone and in combination with melanogenin was performed to ensure that E28 acted neither as a stimulator of pigmentation nor as an antagonist of melanogenin activity.

Multiple structural derivatives of melanogenin were created by varying the functional groups at the R_1 and R_2 positions (Figure 1C). Structure-activity relationship (SAR) analysis of the confirmed promoters revealed high activities correlating with electronegative fluorinated R_1 and R_2 groups, with the most significant induction resulting from melanogenin, in which fluorination exists at the *meta* position (Figure 1C).

Characterization of the Cellular Response to Melanogenin

Melanogenin induced pigmentation and dendricity in melanocytes (Figures 2A and 2B) and melanoma cells (Figure 2F), which was a result not observed in cells treated with DMSO vehicle. Melanogenin induced melanin formation in a dose-dependent manner and was more potent than the known stimulator IBMX (Figure 2C). E28 was entirely inactive (Figure 2C). Western blotting revealed that melanogenin induced an upregulation in levels (Figure 2D) and activity (Figure 2E) of tyrosinase, the rate-limiting enzyme in melanogenesis, whereas E28 had no significant effect (Figure 2D). Untreated melanocytes, as well as albino melan-C melanocytes (expressing an inactive mutant tyrosinase) [12], served as controls in the tyrosinase activity assay. Melanogenin had no effect on levels of a closely related protein involved in pigmentation, tyrosinase-related protein-1 (Trp-1), illustrating the specificity of its effect (Figure 2D). An additive pigmentary effect was observed when murine melanoma cells (Figures 2F and 2G), as well as normal human melanocytes (data not shown), were treated with melanogenin in combination with the pigmentation promoters IBMX, α-MSH, and the MAPK inhibitor PD98059.

Identification of Prohibitin as a Melanogenin Protein Target

Protein target isolation required covalent attachment of melanogenin via its internal linker to an agarose bead affinity matrix (ethanolamine-coated to prevent non-

specific binding). In preparation for affinity matrix synthesis, bulky benzoyl and acetyl groups were substituted at the future site of agarose bead attachment. These functionalized melanogenin molecules retained activity, suggesting that attachment of the affinity matrix would not affect biological function (Figure 3A). Equivalent functionalization of E28 did not alter its lack of activity (data not shown). The synthesis of melanogenin and E28 affinity matrices was accomplished (Figure 3B), and identification of melanogenin binding proteins was performed by incubation of matrix bound melanogenin with total melanocyte protein extract and fractionating retained targets using SDS-PAGE. In multiple experiments, a 32 kDa band was identified that bound specifically to the melanogenin matrix but not to the ethanolamine-coated agarose beads alone nor to the E28 matrix (Figure 3C). This species was cut from the gel, trypsin-digested, and sequenced using mass spectrometry. An NCBI conserved domain search of the peptide fragments yielded 98.9% homology with prohibitin (Figure 3D).

To verify the specificity of melanogenin-prohibitin binding, we incubated melanocyte protein extract with either melanogenin matrix or ethanolamine-coated agarose beads alone. Samples were briefly preincubated with free melanogenin, inactive E28, or vehicle before adding the melanogenin affinity matrix. Following protein resolution and immunoblotting for prohibitin, we found that free melanogenin, but not inactive E28 or DMSO vehicle, effectively competed for the binding of prohibitin to the melanogenin matrix (Figure 3E). We therefore concluded that melanogenin binds prohibitin specifically.

Identification of a Functional Role for Prohibitin in Melanin Induction

Wild-type melanocytes were transfected with negative control (no mammalian homology) siRNA, two distinct prohibitin siRNA sequences, or Lamin A/C (irrelevant mammalian) siRNA. Adequate and specific prohibitin gene silencing was achieved and demonstrated through immunoblotting (Figure 4A). After 24 hr, transfected cells were treated with DMSO vehicle, 5 μM melanogenin, 5 μM E28, or 100 μM IBMX and harvested after 48 hr for melanin assay. Cells transfected with prohibitin siRNA, but not negative control or Lamin A/C siRNA, displayed a significantly attenuated response to melanogenin (Figure 4B). Those cells transfected with either prohibitin siRNA sequence also displayed a modest attenuation in their response to the known promoter IBMX (Figure 4B).

Discussion

In this study we employed the use of a chemical genetic approach to dissect the mammalian pigmentation pathway. Using a tagged triazine library and affinity purification methods, we have identified prohibitin as a target of melanogenin. In addition, through the use of siRNA technology, we report what we believe to be a novel role for prohibitin in melanin induction.

The melan-A cell line utilized in the cell-based screening assay is a spontaneously immortalized, nontumorigenic line of melanocytes obtained from the skins of C57Bl/6J mice [11]. Melan-A cells provide a number of attractive characteristics for the chemical genetics approach we employed. They divide rapidly, but maintain a highly differentiated phenotype with apparently normal and well-studied melanosomal structure and expression of pigmentation-related proteins.

Our chemical library provided several advantages, including the rapid and inexpensive achievement of chemical diversity as well as offering the potential for a high biological "hit" rate. The triazine scaffold has 3-fold symmetry, enabling substantial flexibility in diversity-generating modifications. Due to its ease of manipulation and the low price of the starting material, triazine has elicited considerable interest as a scaffold in combinatorial libraries, resulting in the publication of several triazine-based libraries [10, 16–18]. It is known that a high percentage of proteins interact with purines and pyrimidines (e.g., adenosine, ATP, GTP, cyclic AMP, etc.); the structural similarity between triazines and purines and pyrimidines is hypothesized to increase the chance that library molecules will interact with target proteins.

Despite its power and elegance, the chemical genetic approach is limited by certain methodological difficulties. Traditionally, in order to add a linker to an active molecule without activity loss, a thorough structure-activity relationship study of the chemical is required to find the proper site for linker addition. This procedure is cumbersome and sometimes impossible. To avoid this problem, we designed our library of small molecules to carry an internal tag from the outset [10, 17]. Thus, compounds found to be active in screening could be directly connected via their internal tags to create affinity resins without the need for extensive SAR studies in order to find a linker site that does not disrupt activity.

Several experiments were performed to investigate whether melanogenin could act in combination with other known pigmentation promoters. Our results demonstrated a remarkable additive effect when melanogenin was added in combination with the known stimulators IBMX, α-MSH, and the MAPK inhibitor PD98059 to both melanocytes (data not shown) and melanoma cells (Figures 2F and 2G). These findings suggest a convergence of the pathways modulated by melanogenin with those affected by the other agents.

We cannot as yet distinguish between the possibility that melanogenin's effects are specific to pigmentation pathways in melanocytes and that of it having a role in differentiation. Indeed, pigmentation and differentiation are often viewed as being closely linked, as demonstrated by the role of micropthalmia-associated transcription factor (MITF) in the regulation of melanogenesis [19]. Whereas no gross effects of melanogenin on cell growth were observed, treatment results in a striking increase in dendricity in both melanocytes and melanoma cells. This phenotypic change suggests the distinct possibility that melanogenin's effects are carried out via modulation of melanocyte differentiation.

Further studies employing affinity purification methods identified prohibitin as a melanogenin binding target. Prohibitin is a mitochondrial protein that exists in a dimeric complex with Bap37 on the inner mitochondrial membrane [20, 21], although a nuclear localization has been observed by some authors [22]. Immunofluorescence microscopy demonstrated mitochondrial pro-

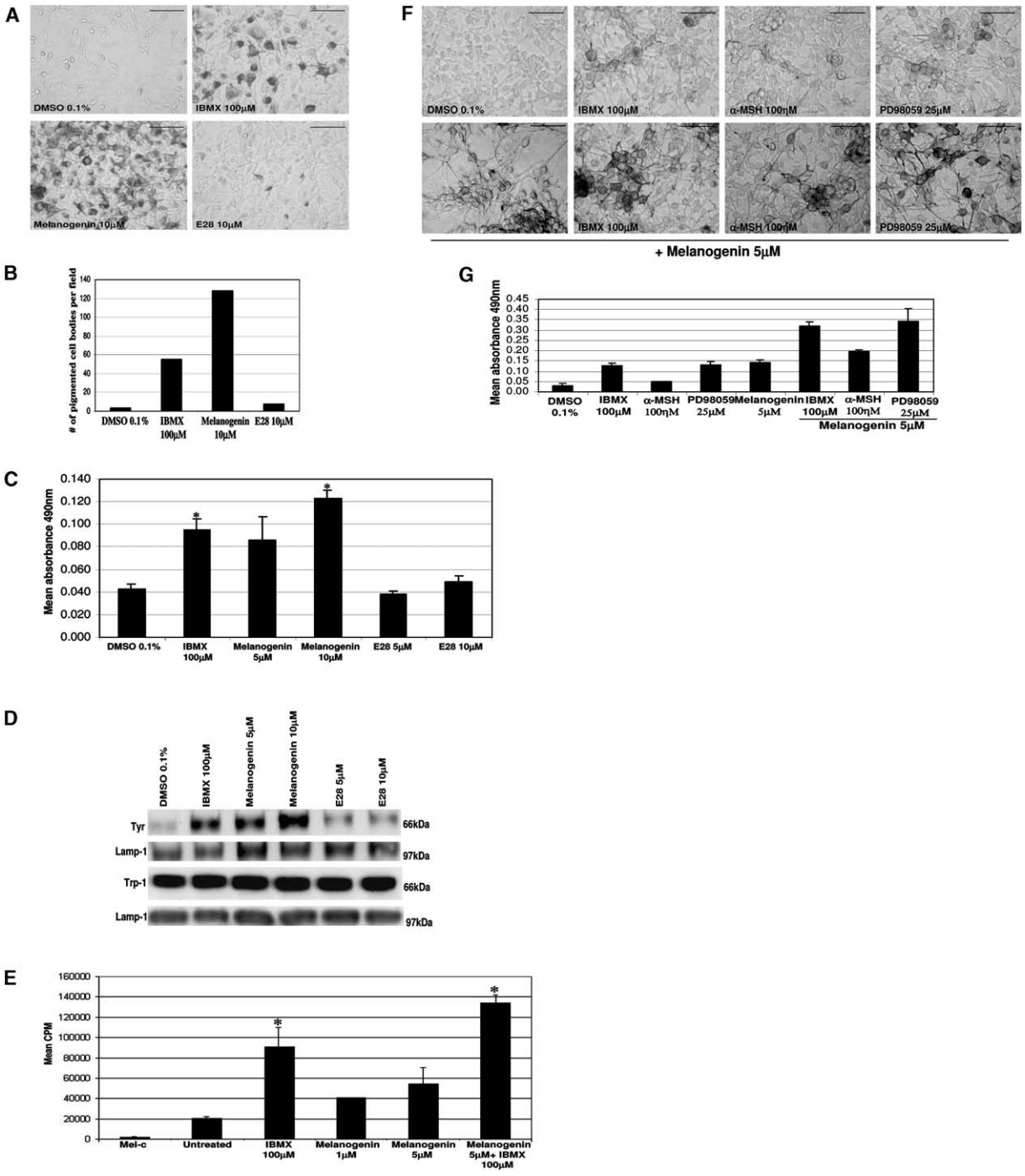

Figure 2. Melanogenin Induces Melanin Formation, Tyrosinase Protein Levels, and Tyrosinase Activity in a Dose-Dependent Fashion in Melanocytes and Melanoma Cells and Acts in Combination with Known Promoters

(A) Phase-contrast images of wild-type cultured murine melanocytes treated with 0.1% dimethylsulfoxide (DMSO) vehicle, positive-control 100 μM isobutylmethylxanthine (IBMX), 10 μM melanogenin, or 10 μM E28 (scale = 250 μm). Note that the melanogenin EC_{50} is 2.5 μM and that significant cytotoxicity does not occur at concentrations below 20 μM.

(B) The number of pigmented cell bodies was quantified from each phase photograph using Scion Image and Adobe Photoshop.

(C) Melanin assay of melanocytes treated with melanogenin and the inactive molecule E28. Data marked with an asterisk represent statistically significant increases in pigmentation above vehicle-treated melanocytes using the Student's t test ($p < 0.01$). Error bars represent ±1 SD. Note that data adjusted for total protein yielded identical results.

(D) Antityrosinase and anti-Trp-1 Western blots from melanocytes treated with melanogenin, IBMX, DMSO vehicle, and E28. Tyrosinase-related protein 1 (Trp-1) is a melanogenic protein unaffected by melanogenin treatment. Lysosomal-associated membrane protein 1 (Lamp-1) is a marker of late endosomes and serves as a loading control. Lamp-1 is represented twice, as the Tyr and Trp-1 results are the product of distinct SDS-PAGE gels. Data shown in (A)–(D) are from the same representative experiment.

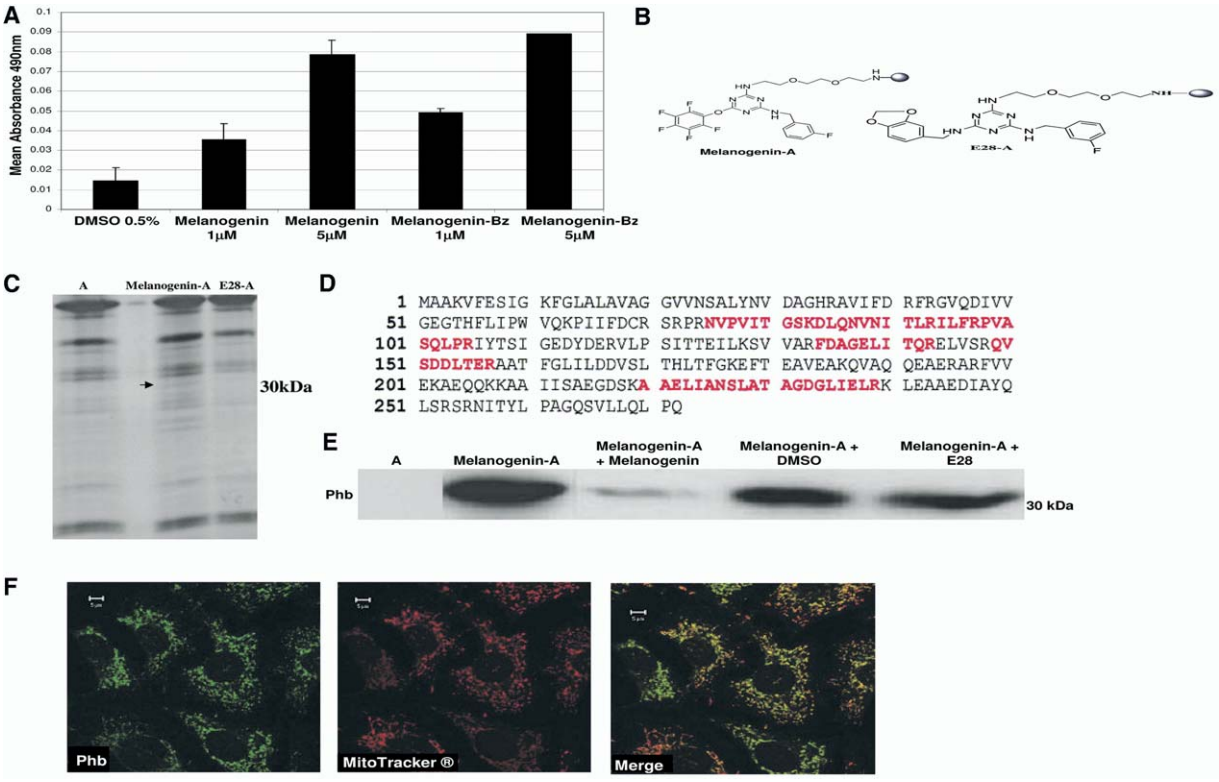

Figure 3. Immobilized Melanogenin Binds the Mitochondrial Protein Prohibitin Specifically

(A) Melanogenin substituted with the benzoyl (Bz) functional group at the future site of affinity matrix attachment retains its in vitro activity. E28 with the identical substitution remained inactive (data not shown). Error bars represent ±1 SD.

(B) Chemical structures of the melanogenin and E28 affinity labels. Melanogenin and E28 are immobilized on activated agarose beads (Affigel 10) coated with ethanolamine to block nonspecific binding sites.

(C) Silver-stained 5%–15% gradient SDS-PAGE gel illustrating melanogenin-specific protein binding. "A" denotes the unconjugated ethanolamine-treated agarose bead matrix. "Melanogenin-A" and "E28-A" represent the corresponding triazine library members linked to agarose bead affinity matrices.

(D) Amino acid sequence results of a 32 kDa melanogenin-specific protein cut from the SDS-PAGE gel. The protein was digested with trypsin, yielding amino acid fragments that were subsequently identified using mass spectrometry. These fragments are illustrated in red and overlap with the murine prohibitin sequence, yielding 98.9% homology by NCBI conserved domain search.

(E) Antiprohibitin Western blot from melanocyte extract incubated with melanogenin-A or agarose beads alone. Melanogenin-prohibitin binding was substantially diminished upon 10 min preincubation with free melanogenin but not with inactive E28 or DMSO vehicle.

(F) Immunofluorescence studies illustrating mitochondrial prohibitin localization in melan-A melanocytes.

hibitin localization in melanocytes (Figure 3F). Treatment with melanogenin did not alter prohibitin cellular location (data not shown). Functional roles for prohibitin in diverse biological processes, such as aging [23], senescence [24, 25], development [25], and tumor suppression [25–29], have been proposed. These functions may be explained by data that suggest that prohibitin may function chiefly as a molecular chaperone [30]. Interestingly, it has recently been shown, using a proteomic approach, that prohibitin is highly expressed in murine epidermis [31].

To determine whether prohibitin has a functional role

(E) Tyrosinase enzyme activity assay on melanocyte extracts in which cells were previously treated with IBMX, melanogenin, IBMX, and melanogenin in combination. Untreated melanocytes and melan-C melanocytes (melanocytes containing mutant, inactive tyrosinase) [15] served as controls in this experiment. The asterisk indicates a statistically significant increase in enzyme activity above untreated melanocytes using Student's t test ($p < 0.05$). Error bars represent ±1 SD. Note that data adjusted for total protein yielded identical results. Tyrosinase activity was also statistically significantly increased in those cells treated with melanogenin in combination with IBMX above either compound tested alone ($p < 0.05$).

(F) Phase photographs of B16F10 murine melanoma cells treated with melanogenin in combination with known promoters α-MSH, IBMX, and the MAPK inhibitor PD98059, illustrating a dramatic increases in melanogenesis and dendricity.

(G) Melanin assay of B16F10 melanoma cells reveals an additive effect in response to treatment with melanogenin in combination with the known promoters IBMX, α-MSH, and PD98059. All chemicals (including combinations) were tested at noncytotoxic concentrations as measured by cell viability assay (data not shown). Data adjusted for total protein yielded identical results. All combination treatments represent a statistically significant increase in melanogenesis above untreated melanoma cells using Student's t test ($p < 0.01$). The increase in melanin production as a result of the melanogenin/promoter combinations was also statistically significant when compared to any of the promoters tested alone ($p < 0.05$). These results are identical in melan-A mouse melanocytes. Error bars represent ±1 SD.

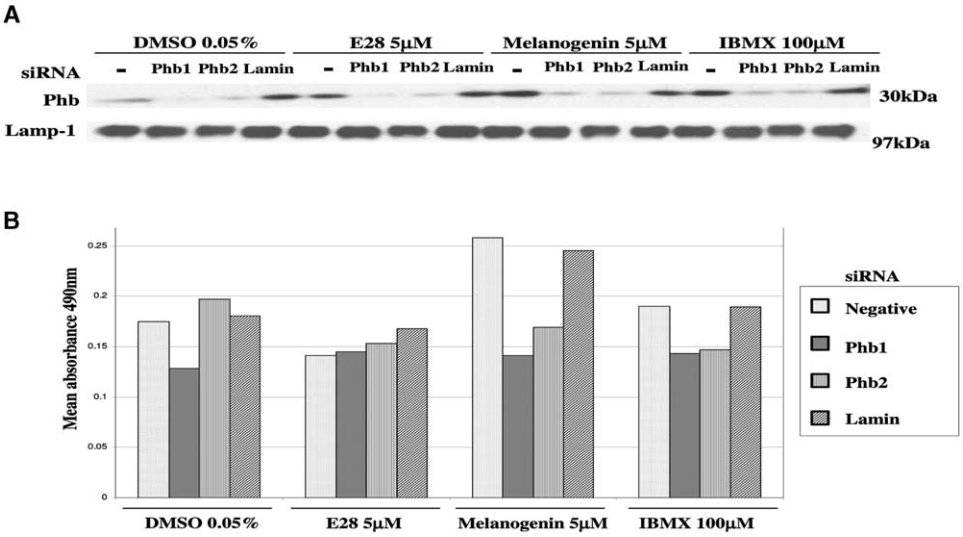

Figure 4. Prohibitin Gene Silencing Effectively Attenuates the Cellular Response to Melanogenin

(A) Antiprohibitin Western blot from melanocytes transfected with two distinct prohibitin siRNA sequences (Phb1 and Phb2), negative control siRNA, or Lamin A/C siRNA followed by 0.05% DMSO, 5 μM melanogenin, 5 μM E28 or 100 μM IBMX treatment. Lamp-1 serves as a loading control.

(B) Melanin assay of melanocytes transfected with two distinct prohibitin siRNA sequences (Phb1 and Phb2), negative control siRNA, or Lamin A/C siRNA followed by 0.05% DMSO, 5 μM melanogenin, 5 μM E28, or 100 μM IBMX treatment. Note that all data in this figure that were adjusted for total protein yielded identical results. All data are the product of the same representative experiment.

in the induction of pigmentation, siRNA technology was utilized to selectively silence prohibitin gene expression (Figure 4A). We demonstrated a significant attenuation in the cellular response to melanogenin following specific prohibitin gene silencing (Figure 4B). These data suggest that prohibitin expression is required for the stimulation of pigmentation by melanogenin, and thus implicate prohibitin in inducible, rather than endogenous, melanogenesis. The intermediary proteins mediating this cellular response, as well as the nature of the interaction between melanogenin and prohibitin, have yet to be further characterized.

Significance

We have demonstrated the use of a chemical genetic approach to the dissection of melanogenesis and identified what we believe to be a novel class of pigment-promoting molecules. We have characterized the small-molecule probe, melanogenin, identified a specific intracellular target as the mitochondrial protein prohibitin, and discovered a functional role for this protein in melanin induction. These results may ultimately aid in the pursuit of therapies for pigmentary disorders by providing a potential target for rational drug design.

Experimental Procedures

Chemical Synthesis and Library Design

The synthetic method for the tagged triazine library has been reported previously [10]. Briefly, a solid-phase method was used to construct a tagged triazine library, where three building blocks were assembled orthogonally yielding 1170 highly pure compounds. Each compound contained a triethyleneglycol (TG) linker at one of the triazine scaffold sites.

Buffers

Buffers used were an extraction buffer (50 mM Tris [pH 7.5]; 2 mM ethylenediamine tetraacetic acid [EDTA] [pH 7.8]; 150 mM NaCl; 1% Triton X-100) containing protease inhibitor cocktail and a bead buffer (50 mM Tris [pH 7.4]; 250 mM NaCl; 5 mM EDTA; 5 mM EGTA; 5 mM NaF; 0.1% Nonidet P-40) containing protease inhibitor cocktail.

Cell Culture and Screening Method

Melan-A melanocytes [11] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM nonessential amino acids 100×, 100,000 U liter^{-1} penicillin, 100 mg liter^{-1} streptomycin, and 200 ηM tetradecanoyl phorbol acetate (TPA-Sigma) at 37°C and 8% CO_2. B16F10 murine melanoma cells were maintained and cultured as above without the addition of TPA. Melan-C melanocytes [12] were maintained as melan-A melanocytes with the addition of 100 ηM β-mercaptoethanol.

Screening in melan-A melanocytes was performed in 24-well plate format with a seeding density of 50,000 cells/cm^2. Library compounds stored in 96-well plates at a stock concentration of 10 mM in dimethylsulfoxide (DMSO) were tested in vitro at a final concentration of 5μM. For each experiment, a DMSO (Sigma) vehicle control and an isobutylmethylxanthine (Sigma) positive control were utilized. Cells were cultured for 72 hr followed by quantitative melanin assay. Cell photographs were obtained after 72 hr using an Axiovert 10 (Carl Zeiss) phase microscope and processed using Adobe Photoshop 6.0 (Adobe systems). Cell viability was assessed using the CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega).

Melanin Assays

Cells were rinsed with phosphate-buffered saline (PBS) and lysed with extraction buffer at 4°C. Cell extracts were then spun at 14,000 rpm for 5 min at 4°C. Supernatants were removed and protein concentration was determined using the Bradford method. The remain-

ing pellet was assayed for melanin by rinsing twice with ethanol-ether (1:1) and dissolving it in 250 μl of 2 N NaOH/20% DMSO at 60°C. A 200 μl aliquot of the resulting solution was measured for absorbance at 490 nm. α-melanocyte-stimulating hormone (MSH; Sigma), PD98059 (Biomol), and IBMX (Sigma) were utilized with melanogenin for synergy experiments.

Western Blotting

Following protein concentration determination using the Bradford method, 10 μg of total protein extract was combined with 2× Laemmli sample buffer containing β-mercaptoethanol. Samples were heat denatured and resolved on 10% SDS-PAGE gels. Following protein transfer to PVDF membrane, samples were probed with primary antibodies to tyrosinase (rabbit polyclonal α-PEP-7), Trp-1 (rabbit polyclonal α-PEP-1), prohibitin (mouse monoclonal-Lab Vision/Neomarkers), and Lamp-1 (rat polyclonal 1D4B-Developmental Studies Hybridoma Bank, Iowa City, IA). The α-PEP-7 and α-PEP-1 antibodies were gifts from Dr. Vincent Hearing, National Cancer Institute. Following wash cycles, membranes were probed with secondary donkey anti-rabbit IgG (Amersham), goat anti-mouse IgG (Accurate Chemical & Scientific Corporation), or goat anti-rat IgG (Accurate Chemical & Scientific Corporation) antibodies. Membranes were washed and films were generated using ECL detection reagent (NEN Life Science Products) and X-OMAT processing.

Tyrosinase Assays

Tyrosinase activity assays were performed on melanocyte cell extracts as previously described [13, 14]. Briefly, aliquots of cellular extract normalized for total protein were incubated with 1.5 μCi ^{3}H tyrosine and 1.5 mg ml^{-1} DOPA for 1 hr at 37°C in 0.1 M $NaPO_4$ and 1% (v/v) Triton X-100. The reaction was terminated through the addition of 10% (w/v) activated charcoal in 0.1 M citric acid and filtered through a cationic exchange resin column. The ^3H_2O reaction product was measured using a Beckman LS 6000SC scintillation counter (Beckman Instruments).

Affinity Chromatography

Before use, 25 μl of packed affinity resin (4 μmol/ml stock concentration stored as a 50% v/v slurry in 2% NaN_3 at 4°C) was washed three times with 1 ml of bead buffer. The beads were resuspended in 400 μl of this buffer and an equal volume, containing 500 μg of total protein extract, was added to each sample. The tubes were subsequently rotated at 37°C for 30 min. After removing the supernatant by centrifugation at 12,000 rpm, the beads were washed extensively with bead buffer. The bound proteins were subsequently recovered with 30 μl of 2× Laemmli sample buffer, containing β-mercaptoethanol, followed by denaturation and resolution using SDS-PAGE.

For competition experiments, free melanogenin, E28, or DMSO was first added in 5-fold excess to each 500 μg protein sample and incubated for 10 min rotating at 37°C. Following this preincubation, 25 μl of packed melanogenin affinity resin or the ethanolamine-coated agarose beads alone were added to the samples and allowed to incubate for an additional 20 min. Proteins were subsequently recovered and resolved, as detailed above, followed by transfer to PVDF membrane and immunoblotting for prohibitin.

Electrophoresis and Protein Identification

Following denaturation, proteins were resolved on a 5%–15% gradient SDS-PAGE gel. Visualization of bound proteins was accomplished using the PlusOne silver staining kit (Amersham Biosciences). The band of interest was excised from the gel under sterile conditions, destained, and digested with trypsin. Amino acid sequencing was performed using a Micromass QTOF mass spectrometer. These data were used to search the NCBI nonredundant protein database using the Mascot search engine.

Immunofluorescence

Melan-A melanocytes were prepared as described previously [15]. MitoTracker (Molecular Probes) was added to cells in DMEM media at a final concentration of 200 ηM and incubated for 20 min at 37°C. The media were removed and the cells were washed with PBS. Following removal of PBS, the cells were fixed with cold methanol for 5 min at –20°C. The cells were washed with PBS followed by permeabilization in 0.5% saponin/PBS for 15 min at room temperature. Following a PBS rinse the cells were incubated with rabbit polyclonal anti-prohibitin antibody (Lab Vision/Neomarkers) at a 1:30 dilution for 1 hr at 37°C. The cells were then washed with PBS and incubated with Alexa 488 conjugated anti-rabbit antibody (Molecular Probes) at a 1:200 dilution for 1 hr at 37°C. Slides were subsequently treated with ProLong Antifade (Molecular Probes) and analyzed using a confocal microscope (LSM510; Carl Zeiss). Data were processed using Adobe Photoshop 6.0 (Adobe Systems).

siRNA Studies

Custom prohibitin siRNA was purchased from Ambion, Inc. Negative control (no mammalian homology) siRNA, Lamin A/C (irrelevant mammalian) siRNA, and RNAiFect transfection reagent were purchased from Qiagen. At 24 hr prior to transfection, melanocytes were seeded in triplicate in 24-well plate format at a density of 50,000 cells/well and incubated in media containing 10% serum, antibiotics, and growth factors. After 24 hr, 1.5 μg of sample siRNA was diluted in low-serum media containing antibiotics (Opti-MEM I-Gibco) to a final volume of 100 μl and vortexed. Complex formation was achieved by adding 9 μl of RNAiFect transfection reagent to the diluted siRNA. Complexes were mixed, incubated, and added drop-wise to cells contained in 300 μl of low-serum media with antibiotics and TPA. Cells were incubated under normal growth conditions for 3 hr, at which point the low-serum media containing siRNA complexes was removed and replaced with media containing 10% serum, antibiotics, and growth factors. Gene silencing was allowed to proceed for 24 hr before the addition of 5 μM melanogenin or 0.05% DMSO vehicle to wells containing negative control, prohibitin, or Lamin A/C siRNA. Samples were harvested following a 48 hr incubation and subjected to quantitative melanin assay. Gene silencing was verified by immunoblotting at the time of harvest. All experimental data are derived from triplicate samples.

Statistical Analysis

Data analyses were performed using the two-tailed Student's t test.

Acknowledgments

The authors are grateful to Dr. Vivekananda Shetty of the New York University Mass Spectrometry Laboratory and Daniela Dodov and Daniel Walsh of the New York University Department of Chemistry. The authors also thank Dr. Willis Huang of the University of Rochester for critical review of this manuscript and Dr. Vince Hearing of the National Cancer Institute for his generous gift of the antityrosinase and anti-Trp-1 antibodies. This work was supported by Public Health Service grant AR41880. The authors declare that they have no conflict of interest in the writing of this article.

Received: August 27, 2004
Revised: January 8, 2005
Accepted: February 16, 2005
Published: April 21, 2005

References

1. Tan, D. (2002). Sweet surrender to chemical genetics. Nat. Biotechnol. *20*, 561–562.
2. Schreiber, S.L. (2000). Target-oriented and diversity-oriented organic synthesis in drug discovery. Science *287*, 1964–1968.
3. Burdine, L., and Kodadek, T. (2004). Target identification in chemical genetics: the (often) missing link. Chem. Biol. *11*, 593–597.
4. Knockaert, M., Gray, N., Damiens, E., Chang, Y.T., Grellier, P., Grant, K., Fergusson, D., Mottram, J., Soete, M., Dubremetz, J.F., et al. (2000). Intracellular targets of cyclin-dependent kinase inhibitors: identification by affinity chromatography using immobilised inhibitors. Chem. Biol. *7*, 411–422.

5. Brown, D.A. (2001). Skin pigmentation enhancers. J. Photochem. Photobiol. B *63*, 148–161.
6. Kuruvilla, F.G., Shamji, A.F., Sternson, S.M., Hergenrother, P.J., and Schreiber, S.L. (2002). Dissecting glucose signaling with diversity-oriented synthesis and small-molecule microarrays. Nature *416*, 653–657.
7. Rosania, G.R., Chang, Y.T., Perez, O., Sutherlin, D., Dong, H., Lockhart, D.J., and Schultz, P.G. (2000). Myoseverin, a microtubule-binding molecule with novel cellular effects. Nat. Biotechnol. *18*, 304–308.
8. Mayer, T.U., Kapoor, T.M., Haggarty, S.J., King, R.W., Schreiber, S.L., and Mitchison, T.J. (1999). Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. Science *286*, 971–974.
9. Zhao, Y., Dai, X., Blackwell, H.E., Schreiber, S.L., and Chory, J. (2003). SIR *1*, an upstream component in auxin signaling identified by chemical genetics. Science *301*, 1107–1110.
10. Khersonsky, S.M., Jung, D.W., Kang, T.W., Walsh, D.P., Moon, H.S., Jo, H., Jacobson, E.M., Shetty, V., Neubert, T.A., and Chang, Y.T. (2003). Facilitated forward chemical genetics using a tagged triazine library and zebrafish embryo screening. J. Am. Chem. Soc. *125*, 11804–11805.
11. Bennett, D.C., and Cooper, P.J. (1987). A line of non-tumorigenic mouse melanocytes, syngenic with the B16 melanoma and requiring a tumor promoter for growth. Int. J. Cancer *39*, 414–418.
12. Bennett, D.C., Cooper, P.J., Dexter, T.J., Devlin, L.M., Heasman, J., and Nester, B. (1989). Cloned mouse melanocyte lines carrying the germline mutations albino and brown: complementation in culture. Development *126*, 379–385.
13. Orlow, S.J., Chakraborty, A.K., Boissy, R.E., and Pawelek, J. (1990). Inhibition of induced melanogenesis in Cloudman melanoma cells by four phenotypic modifiers. Exp. Cell Res. *191*, 209–218.
14. Pomerantz, S. (1996). The tyrosine hydroxylase activity of mammalian tyrosinase. J. Biol. Chem. *241*, 161–168.
15. Hall, A., Krishnamoorthy, L., and Orlow, S.J. (2003). Accumulation of tyrosinase in the endolysosomal compartment is induced by U18666A. Pigment Cell Res. *16*, 149–158.
16. Scharn, D.W.H., Reineke, U., Schneider-Mergener, J., and Germeroth, L. (2000). Spatially addressed synthesis of amino- and amino-oxy-substituted 1,3,5-triazine arrays on polymeric membranes. J. Comb. Chem. *2*, 361–369.
17. Williams, D.R., Jung, D.-W., Khersonsky, S., Heidary, N., Chang, Y.-T., and Orlow, S.J. (2004). Identification of compounds that bind the F_1F_0 mitochondrial ATPase by screening a tagged triazine library for the correction of oculocutaneous albinism type 2. Chem. Biol. *11*, 1251–1259.
18. Stankova, M.L.M. (1996). Library generation through successive substitution of trichlorotriazine. Mol. Divers. *2*, 75–80.
19. Gaggioli, C., Busca, R., Abbe, P., Ortonne, J.P., and Ballotti, R. (2003). Microphthalmia-associated transcription factor (MITF) is required but is not sufficient to induce the expression of melanogenic genes. Pigment Cell Res. *16*, 374–382.
20. Bacher, S., Achatz, G., Schmitz, M.L., and Lamers, M.C. (2002). Prohibitin and prohibitone are contained in high-molecular weight complexes and interact with alpha-actinin and annexin A2. Biochimie *84*, 1207–1220.
21. Darmon, A.J., and Jat, P.S. (2000). BAP37 and prohibitin are specifically recognized by an SV40T antigen antibody. Mol. Cell Biol. Res. Commun. *4*, 219–223.
22. Fusaro, G., Dasgupta, P., Rastogi, S., Joshi, B., and Chellappan, S. (2003). Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. J. Biol. Chem. *278*, 47853–47861.
23. Nijtmans, L.G., Artal, S.M., Grivell, L.A., and Coates, P.J. (2002). The mitochondrial PHB complex: roles in mitochondrial respiratory complex assembly, ageing and degenerative disease. Cell. Mol. Life Sci. *59*, 143–155.
24. Coates, P.J., Nenutil, R., McGregor, A., Picksley, S.M., Crouch, D.H., Hall, P.A., and Wright, E.G. (2001). Mammalian prohibitin proteins respond to mitochondrial stress and decrease during cellular senescence. Exp. Cell Res. *265*, 262–273.
25. McClung, J.K., Jupe, E.R., Liu, X.T., and Dell'Orco, R.T. (1995). Prohibitin: potential role in senescence, development and tumor suppression. Exp. Gerontol. *30*, 99–124.
26. Wang, S., Nath, N., Adlam, M., and Chellappan, S. (1999). Prohibitin, a potential tumor suppressor, interacts with RB and regulates E2F function. Oncogene *18*, 3501–3510.
27. Wang, S., Zhang, B., and Faller, D.V. (2002). Prohibitin requires Brg-1 and Brm for the repression of E2F and cell growth. EMBO J. *21*, 3019–3028.
28. Menssen, A., and Hermeking, H. (2002). Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes. Proc. Natl. Acad. Sci. USA *99*, 6274–6279.
29. Wang, S., Nath, N., Fusaro, G., and Chellappan, S. (1999). Rb and prohibitin target distinct regions of E2F1 for repression and respond to different upstream signals. Mol. Cell. Biol. *19*, 7447–7460.
30. Nijtmans, L.G.J., de Jong, L., Artal Sanz, M., Coates, P.J., Berden, J.A., Back, J.W., Muijsers, A.O., van der Spek, H., and Grivell, L.A. (2000). Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. EMBO J. *19*, 2444–2451.
31. Huang, C.-M., Foster, K.W., DeSilva, T., Zhang, J., Shi, Z., Yusuf, N., Van Kampen, K.R., Elmets, C.A., and Tang, D.C. (2003). Comparative proteomic profiling of murine skin. J. Invest. Dermatol. *121*, 51–64.