Central Role of p53 in the Suntan Response and Pathologic Hyperpigmentation

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SUMMARY

UV-induced pigmentation (suntanning) requires induction of α-melanocyte-stimulating hormone (α-MSH) secretion by keratinocytes. α-MSH and other bioactive peptides are cleavage products of pro-opiomelanocortin (POMC). Here we provide biochemical and genetic evidence demonstrating that UV induction of POMC/MSH in skin is directly controlled by p53. Whereas p53 potently stimulates the POMC promoter in response to UV, the absence of p53, as in knockout mice, is associated with absence of the UV-tanning response. The same pathway produces β-endorphin, another POMC derivative, which potentially contributes to sun-seeking behaviors. Furthermore, several instances of UV-independent pathologic pigmentation are shown to involve p53 "mimicking" the tanning response. p53 thus functions as a sensor/effector for UV pigmentation, which is a nearly constant environmental exposure. Moreover, this pathway is activated in numerous conditions of pathologic pigmentation and thus mimics the tanning response.

INTRODUCTION

Ultraviolet radiation (UV) represents a definitive risk factor for skin cancer, especially when exposure occurs in combination with certain underlying genetic traits such as red hair and fair skin (Fitzpatrick and Sober, 1985; Holick, 2001). Pigmentation of the skin results from the synthesis of melanin in the pigment-producing cells, the melanocytes, followed by distribution and transport of pigment granules to neighboring keratinocytes. It is commonly believed that melanin is crucial for absorption of free radicals that have been generated within the cytoplasm by UV, and it acts as a direct shield from UV and visible light radiation (Pathak and Fanselow, 1983; Riley, 1997; Bykov et al., 2000). Molecular and genetic data indicate that variations in the coding region of the melanocortin-1-receptor (MC1R) play an important role in tanning and pigmentation in humans (Valverde et al., 1995). MC1R is expressed in melanocytes and is activated by its ligand α-melanocyte-stimulating hormone (α-MSH). This propigmentation hormone is produced and secreted following UV by both keratinocytes and melanocytes in the skin (Schauer et al., 1994; Chakraborty et al., 1996). The gene encoding α-MSH is pro-opiomelanocortin (POMC), a multicomponent precursor for α-MSH (melanotropic), ACTH (adrenocorticotropic), and the opioid peptide β-endorphin. Normal synthesis of α-MSH and ACTH is an important determinant of constitutive human pigmentation and the cutaneous response to UV (Lunec et al., 1990; Chakraborty et al., 1996; Kippenberger et al., 1996) since mutations in the POMC gene result in a red-hair phenotype (like that of MC1R alleles) in addition to metabolic abnormalities, such as adrenal insufficiency and obesity (Krude et al., 1998). Several independent reports have demonstrated synthesis of α-MSH and ACTH by epidermal keratinocytes and melanocytes (Iyengar, 1994; Schauer et al., 1994; Schwarz et al., 1995; Gilchrest et al., 1996; Wintzen et al., 1996; Tsatmali et al., 2000; D'Orazio et al., 2006), and the cutaneous α-MSH content showed little change

after hypophysectomy (Eberle, 1998). When taken together with the fact that circulating levels of α-MSH and ACTH are low in humans, these findings suggest that cutaneous activity of these two hormones might involve primarily local paracrine, or perhaps autocrine, effects within the epidermis (Abdel-Malek et al., 2000; Tsatmali et al., 2000). Although expression of POMC/α-MSH is critical for the UV-photopigmentation response, the underlying mechanism of UV-mediated expression of α-MSH is not known.

The tumor-suppressor protein p53 (Lane and Crawford, 1979; Linzer and Levine, 1979) is a transcription factor that plays a pivotal role in the cellular response to genotoxic stressors such as UV and chemically induced DNA damage (Fields and Jang, 1990; Farmer et al., 1992). It has been shown to directly activate transcription of numerous genes such as those that regulate cell-cycle progression, apoptotic cellular pathways, and others (Levine et al., 2006). Loss of function of *p53* leads to aberrant cell growth and survival responses and, as such, p53 dysregulation plays an integral part in the genesis of human cancer. In the skin, p53 function is critical for the retention of tissue integrity following UV irradiation. $p53^{-/-}$ mice exhibited an enormously enhanced propensity to develop tumors following UVB by week 16, while none of the comparably treated $p53^{+/+}$ mice developed skin tumors after 17 weeks (Li et al., 1998). UV can induce "signature" mutations in the p53 gene, which are almost exclusively dipyrimidine C to T substitutions that include CC to TT frameshift mutations; these are rarely seen in noncutaneous tumors (Brash et al., 1991; Ananthaswamy et al., 1997). These mutations were found in the skin of UV-irradiated mice months before tumor development (Ananthaswamy et al., 1997). Conversely, mutations in *p53* are absent from most melanomas (Lubbe et al., 1994). In addition to the above activities, p53 has been shown to be essential for the formation of "sunburn cells," which are a hallmark of sunburns (Ziegler et al., 1994). These apoptotic keratinocytes are absent in $p53^{-/-}$ mice following UV irradiation. This important discovery provided a striking example of *p53*'s pivotal role in regulating keratinocyte apoptosis in the context of a naturally occurring environmental exposure. Collectively, these observations led us to examine the possibility that *p53* may also participate in regulation of the pigmentation response to UV.

RESULTS

UV Treatment Leads to Upregulation of POMC mRNA
Previous data had suggested that the *POMC* gene is upregulated at both protein and mRNA levels following UV irradiation of skin (Iyengar, 1994; Schauer et al., 1994; Schwarz et al., 1995; Gilchrest et al., 1996; Wintzen et al., 1996; Tsatmali et al., 2000; D'Orazio et al., 2006). Although RNA upregulation could occur through a variety of mechanisms, we examined the proximal 1 kb promoter region of the *POMC* gene to search for consensus transcription-factor-binding elements that are conserved between human, rat, and mouse. Among the various consensus elements found, one was particularly noteworthy due to its known regulation by UV: *p53*. We therefore examined primary human keratinocytes and the mouse keratinocyte line PAM212 for both POMC and p53 levels following UV, as shown in Figure 1. A 100 J/m^2 UVB dose was administered in this experiment (see Experimental Procedures). This dose is equivalent to the standard erythema dose (SED; Diffey et al., 1997; CIE Standard, 1998), which is commonly used as a measure of sunlight. As a point of reference, the ambient exposure on a clear summer day in Europe is approximately 30–40 SED. Also, an exposure dose of 4 SED would be expected to produce moderate erythema on unacclimated white skin but minimal or no erythema on previously exposed (tanned) skin. UV markedly induced expression of POMC mRNA and protein by 6 hr, and *p53* induction was already maximal by 3 hr, which is consistent with its known stabilization by UV (Figures 1A and 1B). At 24 hr the levels of POMC protein were lower relative to those found after 6 hr in keratinocytes (human as well as mouse), probably as a result of the proteolytic processing and secretion by keratinocytes (Schauer et al., 1994; Chakraborty et al., 1996). ELISA analysis of the corresponding culture media demonstrated >30-fold induction of α-MSH secretion by keratinocytes after UV (Figure S2).

POMC Is Induced by *p53* Overexpression
To test whether *POMC* is a p53-responsive gene in keratinocytes, we introduced pcDNA-HA-*p53* or empty vector into the PAM212 keratinocyte cell line, and we assessed POMC expression by a real-time quantitative RT-PCR assay and immunoblotting (Figure 1C). POMC expression was significantly induced in response to p53 at both mRNA and protein levels. The rapid induction of POMC following UV radiation of keratinocytes is consistent with the rapid, posttranslational stabilization that is responsible for p53 upregulation following UV (Kastan et al., 1991; Gottifredi et al., 2000; Vogelstein et al., 2000). To test whether POMC induction by p53 correlates with other p53-mediated functions such as apoptosis, the PAM212 keratinocyte cell line was transfected with varying doses of pcDNA-HA-p53 and assessed for apoptotic cells (by flow cytometry) as well as POMC mRNA (by qPCR). p53 overexpression triggered both POMC expression and apoptosis, and there was no obvious difference in threshold for these two endpoints (Figure S3), though differences might exist in other settings, such as within skin or with specific genetic backgrounds. Examination of POMC mRNA stability was also undertaken (±UV), and its decay kinetics were measured in the presence of actinomycin D (Figure S1). No significant changes in POMC mRNA stability were observed with UV.

UV-Mediated Upregulation of POMC Requires p53
To examine whether *p53* is required for UV-mediated induction of POMC, we stably introduced a synthetic
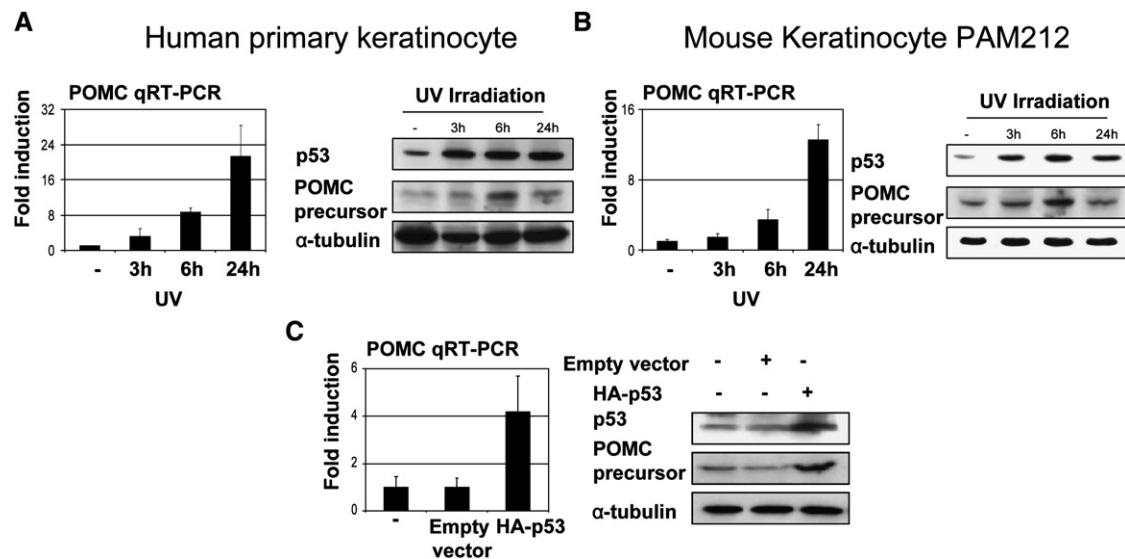
Figure 1. *POMC* Is Induced by UV or *p53* Overexpression

(A and B) Human primary keratinocytes (HPK) and PAM212 cells were irradiated with UV as described in Experimental Procedures. RNA and protein were collected at time 0 and at different time points after irradiation as indicated. The left panels represent POMC RNA levels as measured by quantitative RT-PCR and normalized to GAPDH. Results are expressed as the mean of the experiment done in triplicate ± the standard error of the mean (SEM). Induction is calculated relative to POMC levels in untreated cells. POMC and p53 protein levels, which were analyzed by western blot, are shown on the right along with α-tubulin, which served as loading control.

(C) PAM212 cells were transfected with either empty pcDNA plasmid, HA-*p53* plasmid, or no plasmid. POMC RNA levels were measured by quantitative RT-PCR and normalized to GAPDH. Results are expressed as the mean of the experiment done in triplicate ± the SEM. POMC and p53 protein expression were analyzed by western blot, and α-tubulin was used as a loading control.

dominant-negative *p53* allele (p53DD; Shaulian et al., 1992) into the PAM212 keratinocyte line and into the human primary foreskin keratinocytes (HFK) "PAMDD" and "HFKDD." As shown in Figures 2A and S4A, ectopic expression of p53DD was seen to abrogate induction of both mRNA and protein levels of POMC following UV. We also studied keratinocytes from wild-type and *p53* null mice (littermates). *p53* nullizygous keratinocytes exhibited no measurable POMC mRNA upregulation following UV irradiation (Figure 2B). Of note, western blotting demonstrated that basal POMC expression (prior to UV) was not significantly diminished in the absence of p53, which suggests that p53 is not globally required for POMC expression but is essential for the UV-responsive induction of POMC in keratinocytes. This finding is corroborated by the obvious fact that $p53^{-/-}$ C57Bl6 mice exhibit black fur.

POMC Is a Direct Transcriptional Target of p53

A potential p53-binding site was identified in the *POMC* 5′-flanking region ∼300 bp upstream of the transcription initiation site in humans, and a similar site was identified in the mouse promoter (Bargonetti et al., 1991; Kern et al., 1991). A series of luciferase reporters was tested for UV responsiveness after being transfected into PAM212 keratinocytes. As shown in Figures 3A and 3B, deletion mutants, as well as a site-specific mutation at the p53 consensus element, abrogated UV responsiveness of

the *POMC* promoter. Furthermore, parallel transfections into PAM212 and PAM212/p53DD revealed that suppression of endogenous p53 is sufficient to abrogate the UV-induced reporter activity (Figure 3C). Classical electrophoretic mobility shift assay (EMSA) demonstrated a UV-induced DNA-binding activity that was supershifted by anti-p53 antibody and that had sequence specificity for the p53 consensus probe (but not the point mutant) in keratinocyte nuclear extracts (Figure 3D). To determine whether p53 occupies the endogenous *POMC* promoter in cells, we used chromatin immunoprecipitation (ChIP) from UV-irradiated versus unirradiated mouse keratinocytes (PAM212) or human primary keratinocytes. p53 binding to the *POMC* promoter was detected following UV, whereas no association was detected in unirradiated cells (Figure 3E). Controls included ChIP of the p53 response element in the *p21* promoter, in the actin promoter, and in intronic sequences of the *POMC* gene (negative controls). The human p53 protein was also able to bind to the mouse *POMC* promoter (Figure S4B). These data suggest that p53 directly modulates transcriptional activity of the *POMC* promoter following UV.

UV/p53 Induction of POMC Occurs Preferentially in Keratinocytes

To assess whether the induction of POMC by UV (via p53) occurs in nonkeratinocytes, we exposed melanocytes, fibroblasts, and spleen cells to UV. All three lineages
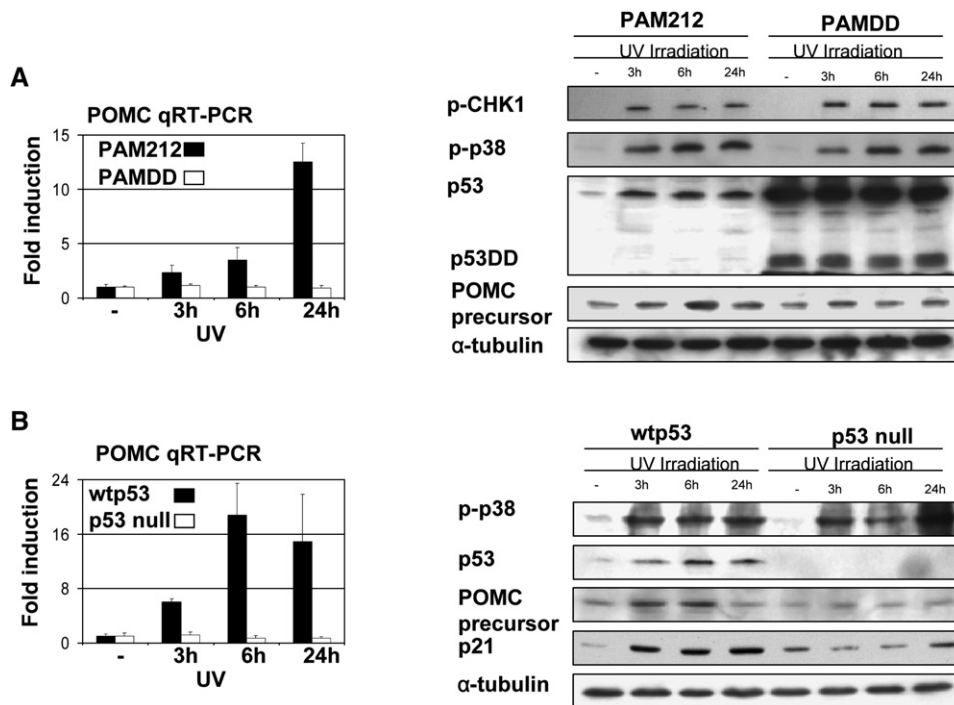
Figure 2. _p53_ Is Required for _POMC_ Transactivation by UV

(A) PAM212 cells were transfected with dominant-negative _p53_ (_p53_DD) plasmid and selected for a stable expression line (PAMDD). UV-irradiated PAM212 or PAMDD cells were processed for RNA and protein isolation. UV induction of POMC RNA and protein is inhibited in the absence of _p53_ activity. Results of RNA levels are expressed as the mean of the experiment done in triplicate ± the SEM.

(B) Mouse primary keratinocytes isolated from wild-type _p53_ (+/+) or _p53_ knockout mice (−/−) were UV irradiated. RNA and protein levels of POMC are shown. Results of RNA levels are expressed as the mean of the experiment done in triplicate ± the SEM. As seen on the western blot, basal POMC levels are not significantly different in the absence of _p53_, whereas induction is _p53_ dependent.

displayed reproducible POMC induction, but the magnitude of the effect was significantly greater in keratinocytes (16- to 25-fold in keratinocytes versus ∼3-fold in nonkeratinocytes; Figures S5A and S6). Using _p53_$^{-/-}$ primary melanocytes, we found that even the modest (∼3-fold) induction of POMC by UV in melanocytes appears to require p53 (Figure S5B). The degree of p53 induction by UV did not predictably correlate with POMC induction in other cell types (e.g., melanocytes or mouse primary spleen cells; Figures S5A and S6), which suggests tissue-specific differences in _POMC_ promoter responsiveness to p53 following UV.

Deficient Tanning Response of _p53_$^{-/-}$ Mice
To test the in vivo requirement of p53 for UV pigmentation, age-matched wild-type and _p53_ null C57Bl6 mice were subjected to UV; this was followed by an evaluation of their ears and tails, which are two locations that contain epidermal melanocytes (furry regions lack epidermal melanocytes; Nordlund et al., 1986). As shown in Figures 4A, S7A, and S7B, visible tanning of ears and tails was observed in wild-type but not in _p53_ null mice. Interestingly, baseline pigmentation was not appreciably different in fur of _p53_ wild-type versus that of null mice but was reproducibly slightly lighter in epidermal tail skin of _p53_ nulls

(Figure S7A). Histologic analyses revealed absence of both POMC and melanin induction in UV-irradiated _p53_$^{-/-}$ skin (Figures 4B and 4C). POMC mRNA induction was also directly measured in skin of the same mice following UV radiation. As shown in Figure 4D, significant POMC mRNA induction was observed following UV but was absent in _p53_$^{-/-}$ mice. Aside from α-MSH, another proteolytic cleavage product of POMC is the opioid receptor ligand β-endorphin, which previously has been suggested to be a mediator of sun-seeking behavior in man (Wintzen et al., 1996, 2001; Kaur et al., 2006). As shown in Figure 4E, expression of β-endorphin, like that of α-MSH, was induced by UV in a p53-dependent manner. These data indicate that p53 is essential for in vivo POMC induction following UV and establish p53 as an integral molecule in the tanning response.

To explore whether similar events occur in the UV response of human skin, discarded normal human skin specimens were exposed to UV and stained over a time course for p53, α-MSH peptide, and the melanocytic transcription factor MITF. Induction of MITF by α-MSH/ MC1R/cAMP indicates activation of the pigmentation pathway (D'Orazio et al., 2006) and serves to identify melanocytes in the basal epidermis. As shown in Figure 5, p53 is rapidly induced in virtually every epidermal
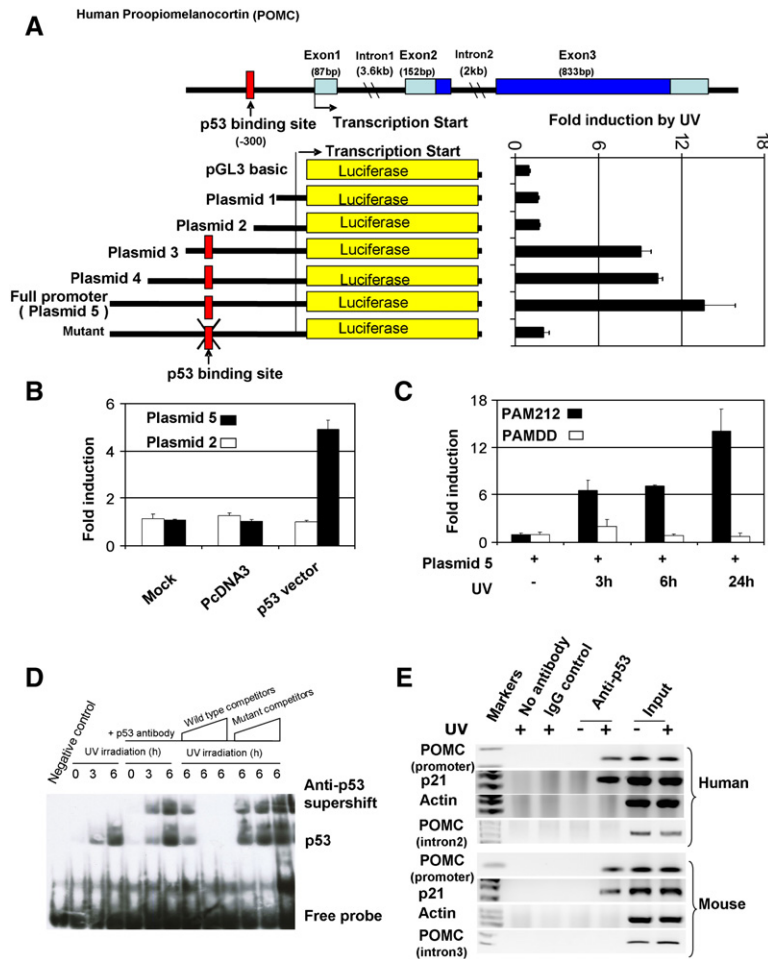
Figure 3. *p53* **Binds and Transactivates the *POMC* Promoter In Vitro**

(A) shows a schematic representation of the *POMC* locus, which indicates location of a *p53*-binding consensus sequence that is highly conserved between the human and mouse promoters. Promoter activity was studied using reporter assays with different promoter portions (including or lacking the *p53*-binding site), which drove expression of firefly luciferase. Relative luciferase activity was normalized to empty pGL3-basic plasmid using the dual-luciferase assay system (Promega). Results are expressed as the mean of the experiment done in triplicate ± the SEM. Analysis of *POMC* promoter transactivation capacity, following UV irradiation of PAM212 cells transiently transfected with *POMC* promoter (plasmid 5), employed a series of deletion mutants or a point mutant ("X") lacking 5 nucleotides at the *p53* consensus site.

(B) *POMC* promoter fragments (plasmids 2 and 5 shown in A) were cotransfected with *p53* plasmid or empty vector into PAM212 cells and used for reporter assays. Results are expressed as the mean of the experiment done in triplicate ± the SEM.

(C) Measurement of *POMC* promoter transactivation capacity following UV irradiation of PAM212 or PAMDD cells is shown. Results are expressed as the mean of the experiment done in triplicate ± the SEM.

(D) shows in vivo association of p53 with the *POMC* promoter by chromatin immunoprecipitation. Protein-DNA complexes extracted from human primary keratinocytes (HPKs) and PAM212 were precipitated with p53 antibody (CM5). PCR was performed with primers specific for the *POMC* promoter sequences, and amplified DNA was resolved by agarose gel electrophoresis.

(E) Nuclear extracts isolated from PAM212 cells at different time points following UV irradiation were incubated with Biotin-labeled p53-binding site oligonucleotide, and probe binding was analyzed by gel shift. The complex formed in UV-irradiated PAM212 cell nuclear extract with radiolabeled probe can be specifically supershifted using antibody against p53 (PAb421). The complex visualized by EMSA is competed away by adding unlabeled wild-type p53 probe but not a mutant probe.

keratinocyte by 1 hr after UV exposure. α-MSH is expressed later (3–6 hr) and is also seen throughout the epidermal keratinocyte population. MITF is strongly induced at 6 hr and localizes to the melanocyte nuclei that are found in the basal epidermal population (Figure 5) as previously reported by King et al. (1999). These studies indicate a similar temporal induction of signaling components following UV irradiation of either mouse or human skin.

Role of p53 in Non-UV Induction of Pigmentation
The role of p53 in the UV-pigment response is notable because p53 protein may be stabilized by various non-UV stressors, which raises the possibility that it may participate in cutaneous pigmentation in a variety of non-UV-associated settings. To test this, PAM212 keratinocytes were treated with the topoisomerase inhibitor etoposide, and induction of p53 and POMC was measured. As shown in Figure 6A, both p53 and POMC were induced. A simple

test of the possibility that p53 may participate in non-UV skin hyperpigmentation is found in the response to topical 5-fluoro-uracil (5-FU), a known inducer of *p53* (Lowe et al., 1994). This drug is used in multiple human dermatologic conditions and has been described to induce hyperpigmentation as a side effect in a fraction of patients (PDR staff, 2005). As shown in Figures 6B and 6C, chronic exposure to topical 5-FU induced hyperpigmentation in *p53* wild-type but not *p53*$^{-/-}$, mouse skin, which suggests that non-UV triggers of p53 may also induce pigmentation.

This result is consistent with previous reports that DNA damage (or its repair) can stimulate tanning (Eller et al., 1994, 1996). Ionizing radiation is also well known for hyperpigmentation induction (as well as p53 induction). Collectively these observations suggest that a common mechanism may involve p53-mediated mimicking of the UV-POMC axis in keratinocytes.
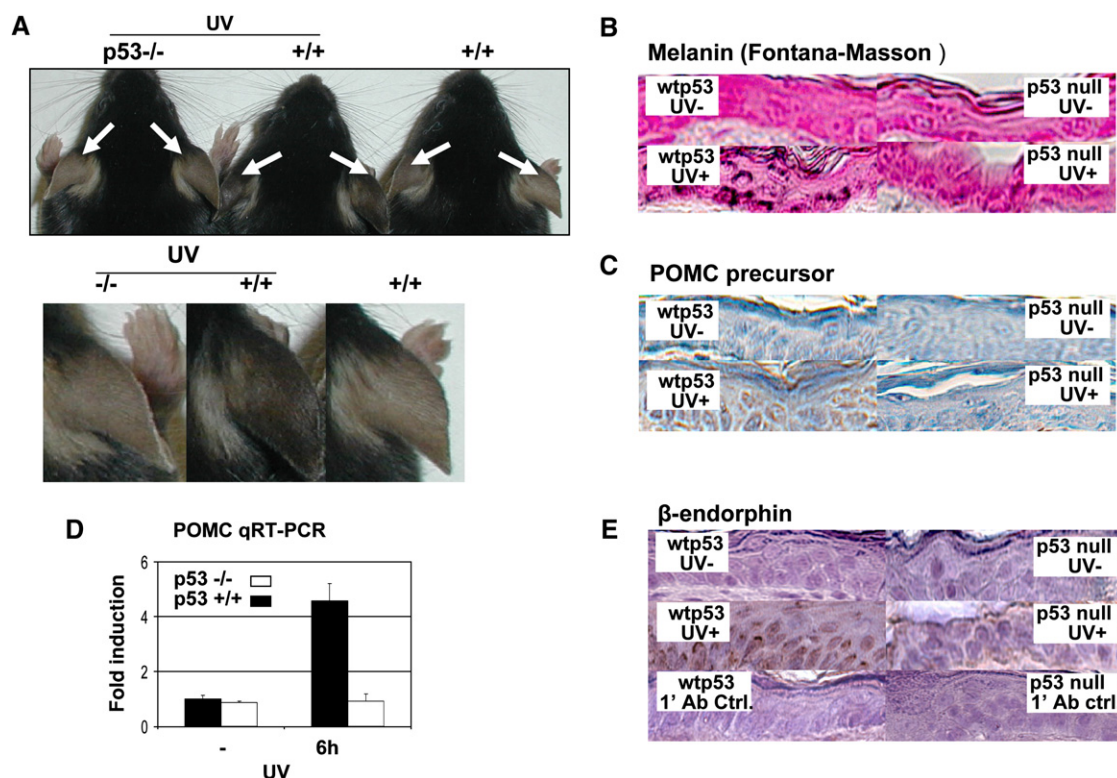
Figure 4. *p53* Promotes Pigment Production by Transcriptional Activation of *POMC*

(A) Three *p53 (+/+)* and three knockout *(−/−)* mice were irradiated with 40 J/m^2 UVB once a day, 5 days per week, for 10 weeks. Arrows indicate pigmentation differences between the ear skin of *p53* wild-type and knockout mice. Ear skin was employed because, unlike trunk/fur-bearing skin, epidermis of the ear contains melanocytes (akin to human skin). $p53^{+/+}$ mice tanned markedly in contrast to *p53* null mice or unirradiated $p53^{+/+}$ mice.

(B) Fontana-Masson staining of ear sections from the mice shown in (A) reveals differences in melanin content.

(C) POMC protein expression in UV-irradiated and unirradiated *p53* wild-type or knockout mice is shown as detected by immunohistochemistry.

(D) Expression of POMC mRNA in epidermis from UV-irradiated and unirradiated *p53* null and wild-type mice was measured by real-time RT-PCR. Results are expressed as the mean of the experiment done in triplicate ± the SEM.

(E) Expression of β-endorphin mRNA in epidermis of *p53* null and wild-type mice as detected by immunohistochemistry is shown.

p53 Mutation and Melanocytic Colonization in Basal Cell Carcinoma

Basal cell carcinoma (BCC) is one of the most common cancers in man and is a cutaneous malignancy that is commonly associated with *p53* mutation (Zhang et al., 2001). A fraction of BCC tumors exhibit pigmentation, even though they are keratinocyte-derived neoplasms, due to melanocytic colonization in the tumor (Bleehen, 1975). Given the above connection between keratinocytic p53, α-MSH, and melanocytic pigmentation, we obtained 23 human BCC specimens and examined both *p53* mutational status and melanocytic colonization as assessed by immunohistochemical staining for MITF (King et al., 1999; Granter et al., 2002). As shown in Table S1, *p53* mutations were identified in 8 of 23 specimens. There was a perfect concordance between *p53* wild-type status and melanocytic colonization (demonstrated by the melanocytic marker *MITF*) as compared to *p53*-mutated tumors that lacked colonizing melanocytes (Figure 7;

arrows indicate *MITF*-positive cells [melanocytes]). Immunohistochemistry for *p53* revealed strong positive staining in *p53*-mutated cases, which was presumably due to the previously described stabilizing effects of many mutations (Levine et al., 2006). These data demonstrate a tight correlation between *p53* mutational status and activation of *MITF* in adjacent melanocytes for human BCC specimens. Activation of *p53* in the setting of oncogenesis is thus likely to represent another example of a non-UV signal that induces the tanning-pigmentation response.

DISCUSSION

We demonstrate that the tumor-suppressor protein p53 promotes cutaneous pigmentation following UV irradiation by direct transcriptional activation of *POMC* in the skin and that p53 absence ablates the tanning response. These data suggest that p53 activation in keratinocytes

UV | - | 1h | 3h | 6h
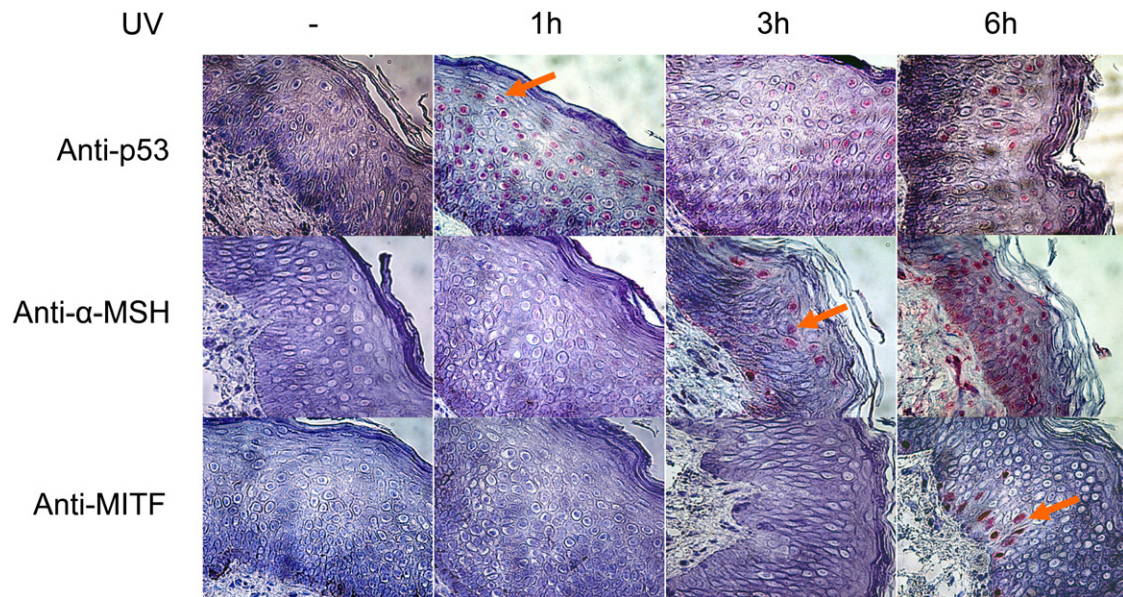
Anti-p53

Anti-α-MSH

Anti-MITF

Figure 5. Immunohistochemical Staining of p53, α-MSH, and MITF in Human Foreskin with or without UV

Sections of human foreskin were taken at different time points for immunohistochemistry. Arrows reveal the first time point when the staining marker is positive.

represents a "UV sensor/effector" for skin pigmentation, and its key mechanistic role is transcriptional activation of *POMC*. The essential role of *POMC*/MSH in the UV-pigment response has been demonstrated by the UV-sensitivity phenotype of humans who harbor mutations in either *POMC*/MSH or its receptor *MC1R* (Kadekaro et al., 2003; Rees, 2003). Thus the identification of p53 as a critical UV-induced transcriptional regulator of *POMC* helps to clarify a key link in the UV pathway that ultimately leads to melanocytic synthesis of melanin.
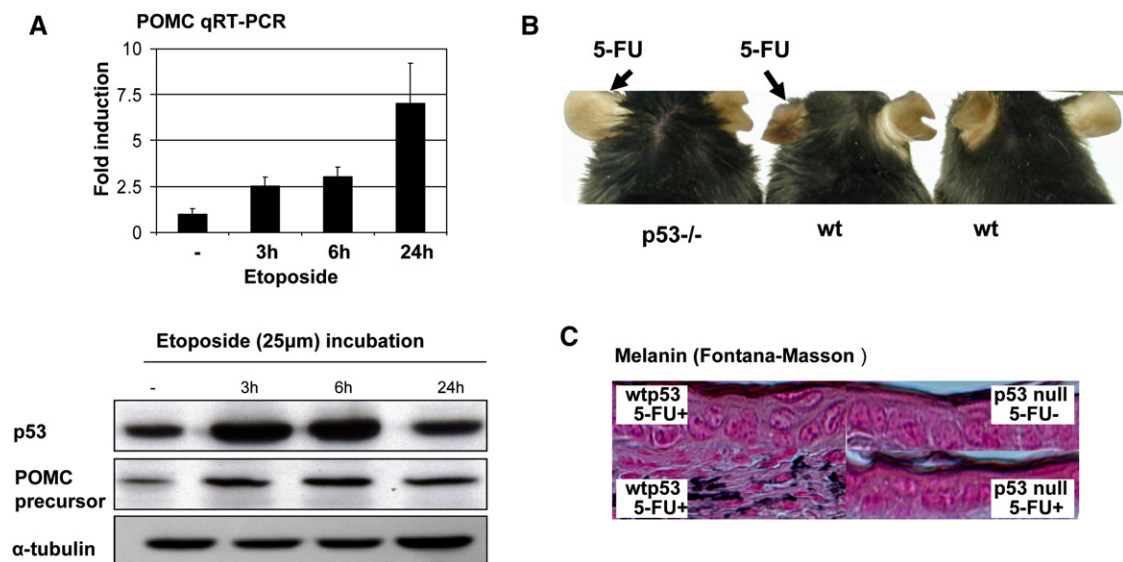

Figure 6. Upregulation of Endogenous POMC Following Chemically Induced DNA Damage

(A) PAM212 cells were treated with 25 μM etoposide for different time points as indicated. POMC RNA (top) and protein (bottom) levels were normalized to GAPDH and α-tubulin, respectively. Results of RNA levels are expressed as the mean of the experiment done in triplicate ± the SEM.
(B) Three *p53 (+/+)* and three knockout *(−/−)* mice were treated with 2% 5-FU once a day, 5 days per week, for 3 weeks. Arrows indicate pigmentation differences between *p53* wild-type and knockout mice in ear skin. *p53^{+/+}* mice tanned markedly in contrast to *p53* null mice or unirradiated *p53^{+/+}* mice.
(C) Fontana-Masson staining of ear sections from the mice reveals differences in melanin content.
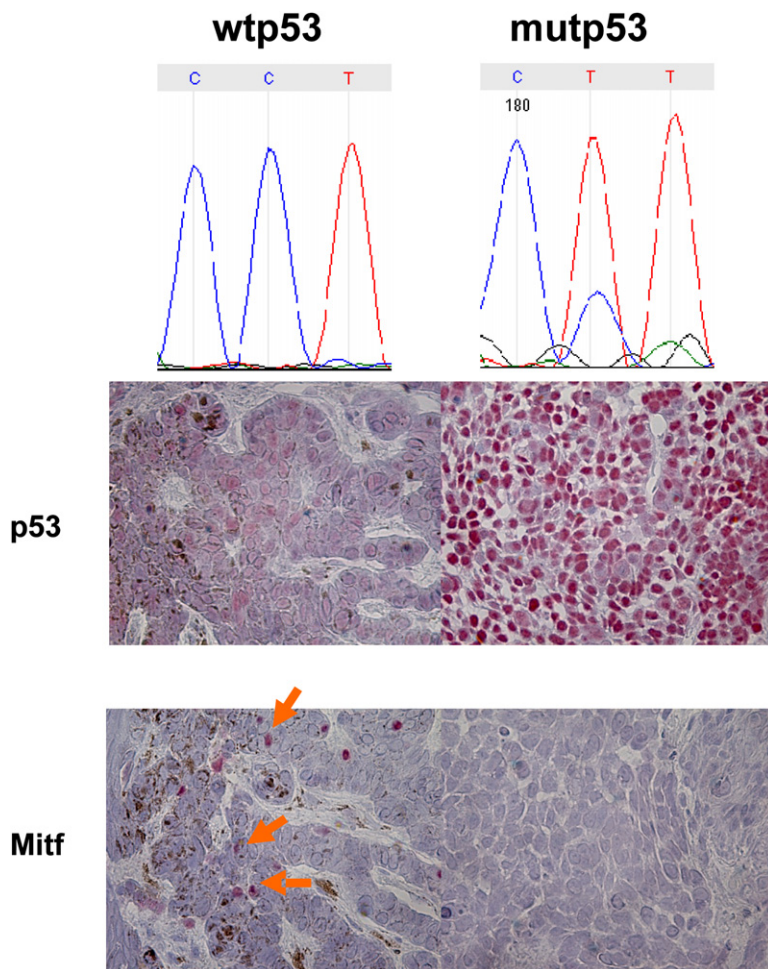
Figure 7. Melanocytic Colonization Correlates with p53 Status in Basal Cell Carcinoma

p53 and MITF protein expression in basal cell carcinoma with wild-type or mutant p53 are shown as detected by immunohistochemistry. A representative *p53* mutant sequence is shown. Mutant p53 is stabilized and produces positive staining in pigmented basal cell carcinomas. Presence of MITF-positive cells (arrows) signifies colonizing melanocytes within the *p53* wild-type keratinocyte tumor.

While pituitary control of *POMC* transcription is quite well delineated, the mechanism(s) of activation in non-pituitary sites have been incompletely understood (Newell-Price, 2003). Tissue-specific positive and negative *POMC* regulation have been described and have been shown to be mediated by several transcription factors, including AP-1 (Boutillier et al., 1991), Nurr77 (Philips et al., 1997), Ptx1 (Lamonerie et al., 1996), glucocorticoid receptor (Therrien and Drouin, 1991), T-box factor Tpit (Lamolet et al., 2001), NF-kappa B (Karalis et al., 2004) in the pituitary gland, and E2F (Picon et al., 1995, 1999) in the small-cell lung cancer cell line DMS-79. Regulation of *POMC* in DMS-79 cells has been reported to differ from that in AtT20 cells (Newell-Price, 2003). The *POMC* promoter is methylated in certain nonexpressing tissues but unmethylated in expressed tissues, including the POMC-expressing DMS-79 small-cell lung cancer cell line (Newell-Price, 2003). One recent report suggested that UV-induced activation of POMC expression was mediated by p38 stress-activated kinase signaling to the transcription factor USF-1 in melanocytes (Corre et al., 2004). The p53-mediated *POMC* regulation reported here is not inconsistent with that finding because the significantly stronger POMC induction observed in keratinocytes as compared to melanocytes (18- to 50-fold versus ~3- to 4-fold; Figure S1) is consistent with having distinct mechanisms of regulation, although based upon $p53^{-/-}$ melanocytes we find that p53 is essential for UV induction of *POMC* in melanocytes as well (Figure S2B). It remains to be determined to what degree melanocytic POMC/MSH contributes to actual pigmentation (something not addressed here), although evidence that is consistent with a major role for paracrine signaling has been reported (Imokawa, 2004; Im et al., 1998; Friedmann and Gilchrest, 1987; Schwarz et al., 1995; Chakraborty et al., 1996; Gilchrest et al., 1996; Wintzen et al., 1996; Sturm, 1998; Tsatmali et al., 2000; D'Orazio et al., 2006). Of note, as the most superficial cells in skin, keratinocytes are likely to perceive greater UV irradiation than melanocytes (situated at the base of the epidermis). Although *POMC*/MSH is rate limiting in the UV-pigmentation response, and small-molecule stimulation of cAMP can rescue *MC1R* deficiency in a "redhead" mouse model (D'Orazio et al., 2006), these data do not imply that other keratinocyte-derived factors are not also important to UV responses in skin. Indeed endothelin-1, β-FGF, NO, SCF, and other

factors have been implicated in pigmentation and the UV-tanning response (Bayerl et al., 1995; Sands et al., 1995; Suzuki et al., 1999; Barsh et al., 2000; Naysmith et al., 2004; Rees, 2004; Cadet et al., 2005), and their precise contributions remain to be determined, as does the question of how their induction is regulated.

Although the pathway through which p53 stimulates pigmentation has not previously been known, a role for p53 in pigmentation has been observed by other investigators. Gilchrest and colleagues have utilized thymidine dinucleotides (pTpT) as mimics of UV-DNA damage to enhance melanogenesis (Goukassian et al., 1999), and this effect requires p53 (Khlgatian et al., 2002). Various DNA-damaging triggers that activate p53 have been seen to stimulate expression of the pigment enzyme tyrosinase and tanning (Kichina et al., 1996; Khlgatian et al., 2002). However, no p53 consensus site was observed in the tyrosinase promoter (Bargonetti et al., 1991; el-Deiry et al., 1992; Khlgatian et al., 2002), which suggests that this regulation, as described here, is indirect (Khlgatian et al., 2002) since the entire pigmentation machinery, including tyrosinase, is thought to be transcriptionally activated by MITF (Chin et al., 2006). The identification of p53's transcriptional regulation of POMC/MSH in response to UV thus likely explains multiple well-described features of the cutaneous pigmentation response.

A central role has been previously established for p53 in modulating UV-induced apoptosis in skin keratinocytes (Ziegler et al., 1994). The current findings suggest that, aside from its control of intracellular growth or survival, p53 modulates a secretory pathway that contributes importantly to the physiologic response to UV in skin. The coordinate induction of β-endorphin together with α-MSH may further illustrate this role with its potential analgesic activity that ameliorates symptoms of local inflammation while possibly contributing to sun-seeking behaviors, as has been suggested (Levins et al., 1983; Zanello et al., 1999; Gambichler et al., 2002; Kaur et al., 2006). Studies are underway to examine the possibility that p53 may regulate expression of additional secreted factors that participate in the cutaneous response to UV damage.

The ability of diverse stresses to trigger stabilization of p53 led to the hypothesis that multiple instances of clinical hyperpigmentation may arise due to such p53-mediated mimicking of the UV-pigmentation pathway. In addition to DNA-damaging agents, nongenotoxic stressors, such as postinflammatory hyperpigmentation, might also induce p53. For this reason it is perhaps not surprising that a large variety of reactive, as well as neoplastic, conditions of human skin may be associated with hyperpigmentation. The tight correlation between p53 mutational status and melanocytic colonization within BCCs that is described here likely represents one such example. The experiments reported here do not address whether MSH plays a chemotactic role for melanocytes, which is an interesting possibility that is under investigation. It is possible that polymorphisms in p53 or p53-related pathways may provide selective advantages at distinct latitudes based upon regulation of the UV-induced pigmentation response (Levine et al., 2006). Numerous benign skin conditions are associated with hyperpigmentation and may thus signify the presence of activated p53 from diverse stresses.

EXPERIMENTAL PROCEDURES

Animals, Cell Lines, and Reagent

p53-deficient (−/−) mice were C57BL/6 TSG-p53 N12 and purchased from Taconic Farms (Hudson, NY, USA). These p53-deficient mice were originally generated by Donehower et al. (1992). B16/F10 mouse melanoma cell line was purchased from ATCC. Primary keratinocytes and melanocytes were isolated and grown from normal human or mouse skin as described (Marcelo et al., 1978; Dunham et al., 1996; Horikawa et al., 1996). Human or mouse primary keratinocytes were cultured in keratinocyte serum-free medium (SFM; Invitrogen Corporation, USA) and were studied in passage 2 after limited in vitro expansion from primary cultures. The mouse keratinocyte cell line PAM212 was generously shared by Dr. Stuart Yuspa (NIH). B16/F10 mouse melanoma cell line was purchased from ATCC.

Primary keratinocytes and melanocytes were isolated and grown from normal human or mouse skin as described (Marcelo et al., 1978; Dunham et al., 1996; Horikawa et al., 1996). Briefly, human or mouse primary keratinocytes were cultured in keratinocyte SFM (Invitrogen Corporation, USA). Cell cultures were studied in passage 2 after limited in vitro expansion from primary cultures. Melanocyte and fibroblast contamination were eliminated by differential trypsinization. Cells were grown in humidified incubators supplemented with 5% CO_2 to 40%–60% confluence prior to their use in irradiation experiments. Etoposide was obtained through Sigma (St. Louis, MO, USA).

UV Exposure

Foreskins were exposed to UV in a Stratalinker UV chamber (Stratagene, Cedar Creek, TX, USA) equipped with 15 W 254 nM UVB bulbs (Germicidal lamp FG15T8, made in Japan) at a dose of 100 J/m^2. After irradiation, foreskins were incubated in DMEM medium in humidified incubators that were supplemented with 5% CO_2 until the time of assay.

Animals were exposed to ultraviolet irradiation in a custom-made lucite chamber (Plastic Design Corporation, MA, USA) that was designed to allow freedom of movement during irradiation. UV was delivered by a double bank of UVB lamps. UVA was filtered by chamber (Plastic Design Corporation, MA, USA), and UV emittance was measured with the use of a UV photometer (UV Products, Upland, CA, USA) that was equipped with a UVB-measuring head. Skin samples were biopsied at indicated time points after UV exposure. In the case of in vitro UV experiments, cells/foreskins were exposed to UV in a Stratalinker UV chamber. Adherent cells were irradiated through a small volume of PBS at a dose of 100 J/m^2. After irradiation, the PBS was aspirated from the wells, and the cells were fed with media for incubation until time of assay.

Histology

Immunohistochemical studies were performed on discarded BCC specimens using formalin-fixed, paraffin-embedded tissue. Primary antibodies included monoclonal antibody DO-7 (p53 antibody, Dako), polyclonal M0939 (α-MSH antibody, Sigma), and monoclonal antibody D5 (MITF antibody). Staining was performed with Dako DAB or AEC Detection Kit (Dako EnVision + System HRP, Dako). Tissues known to express the antigen of interest were used as positive controls, whereas removal of the primary antibodies in the test tissues was used for negative controls. Only MITF-antibody nuclear staining was regarded as positive.

Animals were either sacrificed by CO_2 or anesthetized with isoflurane anesthesia prior to ear sampling. Ear sections were immediately placed in 10% buffered formalin until paraffin embedding and sectioning (done by the rodent histopathology core service at Harvard Medical School). Hematoxylin/eosin staining and Fontana-Masson staining, were performed by the histopathology core. Immunohistochemistry was performed according to standard protocols with the following anti-p53 antibodies: DO-7 (Calbiochem, OPO3L); CM-5 (Vector, VP-P56); anti-POMC (Pro Sci, XW-7447 and Phoenix, H-029-30); and anti-MITF (C5 or D5; Hemesath et al., 1998).

Real-Time RT-PCR, Western Blotting, and Enzyme Immunoassay

For quantitative RT-PCR, total RNA was converted into cDNA using SuperScript III Reverse Transcriptase kit (Invitrogen). cDNA expression was quantified using QuantiTect Probe RT-PCR kits (Qiagen, Valencia, CA, USA) and the ICycler machine (BioRad, Hercules, CA, USA). Gene-specific primer sets are as reported (J.A.D'O.). Taqman PCR reactions were done in triplicate for each sample and normalized to GAPDH.

Western blotting was performed using the following anti-p53 antibodies: DO-7 (Calbiochem, OPO3L); CM-5 (Vector, VP-P56); IC12-MAB (Cell Signaling, 2524); and Pab241 (Oncogene, AB-1); as well as anti-POMC (Pro Sci, XW-7447 and Phoenix, H-029-30). Enzyme immunoassay was performed using the α-MSH EIA kit (Phoenix Pharmaceuticals Inc., EK-043-01).

Luciferase Reporter Assay

A fragment of the human POMC promoter (−680 to +1, relative to the transcription start site) and a series of unidirectional truncations from the 5′ end of POMC (−580/+1, −480/+1, −280/+3, and −101/+1) were generated by PCR and were inserted into the PGL-3 basic vector (Promega) upstream of the luciferase reporter gene in 6-well plates (2 μg DNA/well) using Lipofectamine 2000 (GIBCO BRL) according to the manufacturer's instructions. Promoter constructs were cotransfected with the pRL-TK plasmids (Promega). Twenty-four hours after transfection, the cells were irradiated by UVB (100 J/m^2) and 24 hr later were lysed and assayed using dual-luciferase reagents (Promega). Promoter activity was measured by luciferase levels and normalized to the constitutively expressed *Renilla*.

p53-Binding Assay

EMSAs were done using the LightShift Chemiluminescent EMSA kit (Pierces Biotechnology Inc., Rockford, IL, USA) according to the manufacturer's instructions. For competition experiments, 5-, 15-, or 50-fold excess unlabeled POMC oligo (wild-type 5′- AGGCAA GATGTGCCTTGCGCTC-3 or mutant 5′-CCCGAAGATGTGCCTTGG CAAA-3′ in double-stranded configurations) was incubated with the extract for 10 min before the addition of labeled oligo, and the incubation proceeded for an additional 20 min at room temperature. In supershift experiments, 1 μl of anti-p53 antibody (AB1, Oncogene) was subsequently added and incubated for an additional 15 min at room temperature.

Chromatin immunoprecipitation was performed as described (Flores et al., 2002; Cui et al., 2005) with anti-p53 antibody (Ab1, Oncogene) and control anti-IgG (Santa Cruz Biotechnology, Inc.). DNA released from precipitated complexes was amplified using primers for the p21 and actin promoters and for the POMC promoter region (from −160 to −64 of mouse POMC promoter and from −381 to −260 of human POMC promoter). Primers are shown in Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include seven figures, one table, and Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/128/5/853/DC1/.

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