

Mitf dosage as a primary determinant of melanocyte survival after ultraviolet irradiation

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KEYWORDS apoptosis/melanocyte/melanoma/Mitf/ultraviolet/Fas/mice

PUBLICATION DATA Received 14 January 2006, revised and accepted for publication 23 January 2009

doi: 10.1111/j.1755-148X.2009.00551.x

Summary

Microphthalmia-associated transcription factor (Mitf) is essential for melanocyte development and function and regulates anti-apoptotic *Bcl2* expression. We hypothesized that cellular deficiency of *Mitf* can influence melanocyte survival in response to ultraviolet (UV) radiation. Primary melanocyte cultures were prepared from neonatal wild-type mice and congenic animals heterozygous for *Mitf* mutations *Mitf*^{mi-vga9/+} and *Mitf*^{mi-wh/+} and exposed to UV irradiation. Wild-type melanocytes were more resistant to UV-induced apoptosis than melanocytes partially deficient in Mitf activity, as determined by relative levels of intracellular melanin and relative activation of Mitf target genes *Tyr*, *Tyrp1*, *Dct*, and *Cdk2*. Comparative experiments with wild-type cells and congenic albino melanocytes demonstrated that these differences are not due to differences in melanin content, implicating Mitf as a primary determinant of UV-dependent melanocyte survival. *Mitf* activity correlated directly with resistance to UV-induced apoptosis in melanocytes. Mitf was important not only for regulating the expression of anti-apoptotic Bcl-2 following UV irradiation, but also the expression of the pro-apoptotic BH3-only Bad protein and activation of the extrinsic apoptotic pathway. Hence, Mitf is a multifaceted regulator of UV-induced apoptosis in melanocytes.

Introduction

Although skin cancer is the most common malignancy in the United States, understanding its causes, particularly those of melanoma—the most lethal form of skin

cancer—is limited. Ultraviolet (UV) radiation (280–400 nm) is the major environmental carcinogen for non-melanoma skin cancer and episodes of sunburn from solar or tanning bed UV radiation during childhood to young adulthood significantly increase the risk of

Significance

Ultraviolet (UV)-induced apoptosis is an important mechanism that prevents the accumulation of DNA damage in irradiated skin cells. High intensity UV exposure, for example, leads to the p53-dependent formation of 'sunburn cells', or apoptotic keratinocytes, in human skin. The determinants of UV-induced apoptosis in melanocytes are less well understood. Mitf is a transcription factor critical for melanocyte survival at various stages of development at least in part through its regulation of anti-apoptotic Bcl-2. Our finding that Mitf protects melanocytes against UV-induced apoptosis extends these findings to other apoptotic pathways, and reveals a primary role of Mitf in the protection of the melanocyte against UV assault. Modulation of melanocyte apoptosis in response to UV may be an important mode of cutaneous photoprotection.

developing melanoma (Lindholm et al., 2004; Young, 2004). As a first line of defense against chemical and physical agents, the skin has evolved special mechanisms to protect against damage by UV radiation. Melanocytes comprise approximately 4% of the cells in human epidermis. They protect the skin from photodamage by producing melanin, a pigment with natural sunscreen and antioxidant properties, and transferring melanin to keratinocytes (De Leeuw et al., 2001; Hoogduijn et al., 2004; Smit et al., 2001; Tadokoro et al., 2003, 2005b). Melanocytes have a low capacity for self-renewal. Therefore mechanisms that regulate melanocyte survival and maintenance of genomic stability are important in preventing cell death and malignant transformation.

The microphthalmia-associated transcription factor (MITF/Mitf) is a master regulator of melanocyte differentiation, development and survival (Goding, 2000; Steingrimsson et al., 2004; Widlund and Fisher, 2003). MITF/Mitf is a basic helix-loop-helix leucine zipper (bHLHZip) transcription factor that is critical in the transcription of pigment pathway genes such as tyrosinase (*Tyr*), tyrosinase-related protein-1 (*Tyrp-1*) and dopachrome tautomerase (*Dct*) (Bentley et al., 1994; Du et al., 2003; Jiao et al., 2004; Ludwig et al., 2004; Schwahn et al., 2005; Tomita et al., 1989; Yasumoto et al., 1997). MITF/Mitf binds to the M-box motif in these promoters to increase their expression and production of melanogenic proteins. In humans, mutations in the *MITF* gene lead to Waardenburg Syndrome type IIa which is characterized by skin and hair discoloration and deafness due to a lack of melanocytes in skin, hair, and inner ear (Price and Fisher, 2001; Tassabehji et al., 1994).

Microphthalmia-associated transcription factor/Mitf controls the survival of both melanocytes and melanoma cells through its regulation of the anti-apoptotic molecule Bcl-2 (McGill et al., 2002), which is expressed at high levels in the skin (Plettenberg et al., 1995). MITF is a substrate for caspase cleavage in human melanocytes and melanoma cells (Larribere et al., 2005). The C-terminal fragment of MITF protein generated by caspase cleavage has proapoptotic activity that sensitizes melanoma cells to death signals while an uncleavable form of MITF renders them resistant to apoptosis. Thus, the balance between the cleaved and uncleaved form of MITF influences melanocyte survival. Overexpression of *MITF* enhanced melanocyte survival and contributed to malignant transformation (Garraway et al., 2005). However, overexpression of Bcl-2 in *MITF*-deficient cells only partially rescued them from apoptosis, suggesting that MITF regulates additional survival genes. For example, Busca et al. (2005) showed that MITF binds to the *Hif-1 α* promoter, strongly stimulating *Hif-1 α* gene expression which in turn, reduced caspase 3 cleavage.

Ultraviolet radiation induces the production of nerve growth factor, stem cell factor, α -MSH and a variety of

cytokines by non-melanocytic cells in the skin which can inhibit apoptosis in melanocytes by inducing DNA repair and upregulating Bcl-2 or reducing Bax production (Blume-Jensen et al., 2000; Bohm et al., 2005; Karlsson et al., 2003; Ono and Han, 2000; Rosette and Karin, 1996; Sakata et al., 2003; Zhai et al., 1996). However, the role MITF plays in mediating the effects of UV radiation on melanocyte function and survival is poorly understood. UV radiation can stimulate melanogenesis by directly increasing MITF expression (Kim et al., 2003; Tadokoro et al., 2005a) or *MITF* promoter activity (Lin et al., 2002), and indirectly by triggering Akt, p38 and cAMP pathways which activate the transcription factors cAMP response element binding protein (CREB), NF κ B and *MITF* (Goding, 2000; Kadekaro et al., 2003; Goding, 2000; Kadekaro et al., 2003). Phosphorylation of p38 activates the Usf-1 transcription factor, which is required for UV activation of the tyrosinase promoter, pro-opiomelanocortin (POMC) and MC1R genes that play a crucial role in regulating melanin synthesis (Corre et al., 2004; Galibert et al., 2001).

Because exposure of melanocytes to UV radiation is an important risk factor in the etiology of melanoma, we investigated whether mutations in *Mitf* may influence apoptosis in melanocytes after UV exposure. We used two congenic strains of mice, C57BL/6J-*Mitf*^{Mi-wh/+} and C57BL/6J-*Mitf*^{Mi-vga9/+}, that are heterozygous for mutations in the *Mitf* gene to examine the role of *Mitf* in regulating mitochondrial and receptor-triggered apoptotic pathways in cultures of UV-irradiated primary melanocytes.

Results

Mice with *Mitf* mutations and the primary cultures of melanocytes established from them

We investigated the role of *Mitf* in regulating apoptotic pathways using cells derived directly from mice with *Mitf* mutations with distinct pigmentary and molecular phenotypes. Over two dozen spontaneous and induced mutations in *Mitf* have been described to date (Steingrimsson et al., 2004). We used mice with two distinct *Mitf* mutations, *Mitf*^{Mi-wh} and *Mitf*^{Mi-vga9}, whose mutations can affect coat color either in the homozygous (C57BL/6-*Mitf*^{Mi-wh/Mi-wh} and C57BL/6-*Mitf*^{Mi-vga9/mi-vga9}) or heterozygous (C57BL/6-*Mitf*^{Mi-wh/+} only) states (Figure 1). The *Mitf*^{Mi-wh} mutation is an Ile212Asn substitution that affects the DNA-binding domain of the *Mitf* protein, resulting in severely defective DNA-binding activity and, in the absence of the alternatively-spliced 6 amino acid exon 6A, a dominant-negative effect (Hemesath et al., 1994; Steingrimsson et al., 2004). Mice homozygous for the *Mi-wh* mutation (Figure 1A) have white coats, small, slightly pigmented eyes, inner ear defects and reduced fertility. Heterozygotes have a substantial ventral white spot and a gray-brown hypopigmented coat color on the C57BL/6 background

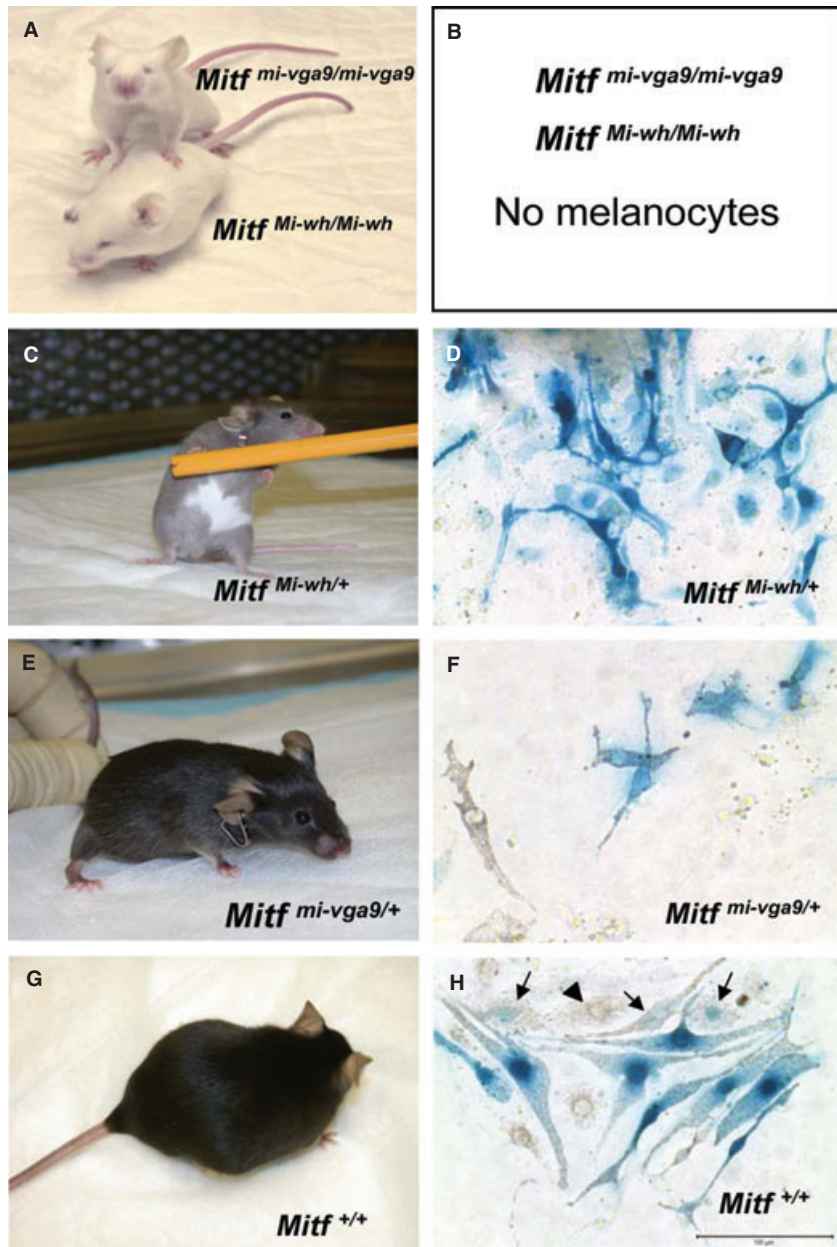


Figure 1. Phenotype of *Mitf* mutant mice and their melanocytes. (A) C57BL/6J-*Mitf*^{Mi-wh/Mi-wh} and C57BL/6J-*Mitf*^{mi-vga9/mi-vga9} homozygous mutant mice. The homozygous mutants lack melanocytes, have microphthalmia (small eyes) and are deaf due to defective melanocyte development. (B) No melanocytes can be isolated from (A). (C) C57BL/6J-*Mitf*^{Mi-wh/+} heterozygous mouse. The *Mi-wh* mutation in *Mitf* has a dominant negative effect in vitro and a semi-dominant phenotype in vivo, resulting in grey coat color and large belly spot. (D) Cultured C57BL/6J-*Mitf*^{Mi-wh/+} melanocytes expressing [Tg]*Dct-lacZ* transgene and stained with X-gal. (E) C57BL/6J *Mitf*^{mi-vga9/+} heterozygous mouse. (F) Cultured C57BL/6J-*Mitf*^{mi-vga9/+} melanocytes expressing [Tg]*Dct-lacZ* transgene and stained with X-gal. (G) C57BL/6 mouse with wild type *Mitf*. (H) Cultured C57BL/6J wild-type melanocytes expressing [Tg]*Dct-lacZ* transgene and stained with X-gal. Arrows indicate melanin-containing cells that are losing their [Tg]*Dct-lacZ* expression. Arrowhead indicates a melanin-containing melanocyte no longer expressing [Tg]*Dct-lacZ*. All melanocyte cultures were stained for β -galactosidase expression with X-gal (blue). Bar = 100 μ m.

(Figure 1C) (Steingrímsson et al., 2004). In contrast, the transgene insertional mutation *mi-vga9* severely reduces the expression of the *Mitf* gene (Hodgkinson et al., 1993; Tachibana et al., 1992). Mice homozygous for this mutation (Figure 1A), like C57BL/6-*Mitf*^{Mi-wh/Mi-wh} mice, lack melanocytes, resulting in a white coat phenotype with small red eyes and hearing defects (Hodgkinson et al., 1993; Steingrímsson et al., 2004). Heterozygotes have a normal pigmented phenotype (Figure 1E), although, unlike wild-type mice, they can exhibit a visible phenotype in the compound heterozygous state with other *Mitf* mutant alleles (Bismuth et al., 2008; Goding, 2000). Hence, C57BL/6 mice and congenic C57BL/6-*Mitf*^{Mi-wh/+} and C57BL/6-*Mitf*^{mi-vga9/+} mice comprise

an allelic series of increasing functional severity on both the phenotypic and molecular level (C57BL/6-*Mitf*^{Mi-wh/+} > C57BL/6-*Mitf*^{mi-vga9/+} > C57BL/6). This provides a convenient framework for investigating the effects of relative *Mitf* dosage in primary cells derived from the mice. In order to examine the effects of these mutations on melanocytes, congenic mice heterozygous for these mutations were created by crossing male C57BL/6-*Mitf*^{Mi-wh/Mi-wh} and C57BL/6-*Mitf*^{mi-vga9/mi-vga9} mice with C57BL/6J-[Tg]*Dct-LacZ* females (Hornyak et al., 2001). Intercross with the C57BL/6J-[Tg]*Dct-LacZ* mice and expression of the *Dct-LacZ* transgene permitted the identification of cells of melanocytic lineage in cultures from these animals.

Primary cultures of dermal cells from newborn heterozygous and wild-type *Mitf* mice initially contained approximately 2–4% melanoblasts and melanocytes, detected by their expression of the *Dct-LacZ* reporter transgene (not shown). Following two rounds of geneticin selection removing rapidly dividing fibroblasts (Halaban and Alfano, 1984), 75–90% of the remaining cells were of the melanocytic lineage, positive for *LacZ* expression. The time from initial isolation of dermal cells from newborn mice to the time of study after two rounds of geneticin treatment was approximately 1 month.

Cultures of dermal cells from C57BL/6-*Mitf*^{Mi-wh/+}[Tg]*Dct-LacZ* mice generally lacked visible melanin but were identifiable as melanoblasts or melanocytes because of their dendritic morphology and the presence of blue stain for β -galactosidase (Figure 1D). Melanocytes from C57BL/6-*Mitf*^{Mi-vga9/+} [Tg]*Dct-LacZ* mice were initially dendritic and unpigmented but began to lose their dendrites and acquired melanin by the third week of culture (Figure 1F). Interestingly, we noted that melanocyte cultures from wild type C57BL/6-*Mitf*^{+/+}[Tg]*Dct-LacZ* mice (Figure 1H, arrows and Figure S1) tended to lose dendricity and evidence of *Dct:LacZ* activity as cytoplasmic melanin granules appeared.

The melanin content of the cultures was determined chemically (Figure 2B). C57BL/6-*Mitf*^{+/+}[Tg]*Dct-LacZ* cells with wild-type *Mitf* produced twice as much melanin as melanocytes from C57BL/6-*Mitf*^{Mi-vga9/+}[Tg]*Dct-LacZ* mice even though these animals are phenotypically indistinguishable from the congenic C57BL/6-*Mitf*^{+/+}[Tg]*Dct-LacZ* controls. Melanocytes from

C57BL/6-*Mitf*^{Mi-wh/+}[Tg]*Dct-LacZ* mice contained minimal melanin both visually (Figure 2A right panel) and chemically (Figure 2B).

***Mitf* gene mutations, melanin, and UV-induced cell survival**

Primary melanoblasts/melanocytes from the three genotypes C57BL/6-*Mitf*^{+/+}, C57BL/6-*Mitf*^{Mi-vga9/+}, and C57BL/6-*Mitf*^{Mi-wh/+} were treated with a range of UV exposures to determine their sensitivity to UV-induced apoptosis. Figure 3C demonstrates that cell survival declined after 24 h with increasing doses of UV radiation. Melanocytes from C57BL/6-*Mitf*^{Mi-wh/+} heterozygotes were most sensitive to UV, with decreasing UV sensitivity observed for C57BL/6-*Mitf*^{Mi-vga9/+} cells and wild-type cells, respectively (Figure 3C). All three genotypes showed marked toxicity in response to UV exposure. Activation of caspase 3, as evidenced by the appearance of cleaved caspase 3 (Figure 3A), was induced in a dose-dependent fashion in melanocytic cultures over a range of 0–250 J/m² UV radiation.

The reduced survival of melanocytes heterozygous for mutations in *Mitf* upon UV irradiation (Figure 3C) implied that the effective intracellular dosage of *Mitf* might be a primary factor determining melanocyte survival resistance to UV. However, it remained possible that lower amounts of melanin in the irradiated *Mitf* melanocytes (Figure 2B), rather than *Mitf* dosage itself, were the primary determinant of survival. To distinguish between these possibilities, we isolated both pigmented and non-pigmented, albino primary murine melanocytes. Primary cultures were developed from

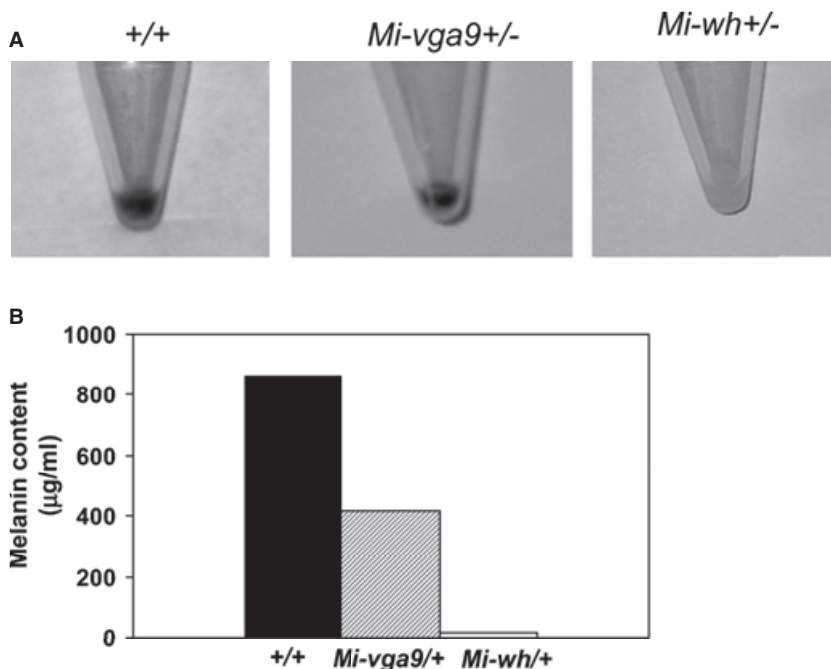


Figure 2. Melanin content of primary melanocyte cultures. Equal numbers (1×10^6) of cells from wild-type C57BL/6J, C57BL/6J-*Mitf*^{Mi-vga9/+}, and C57BL/6J-*Mitf*^{Mi-wh/+} mice were pelleted (A) and melanin extracted. The melanin content of the cells is shown in (B).

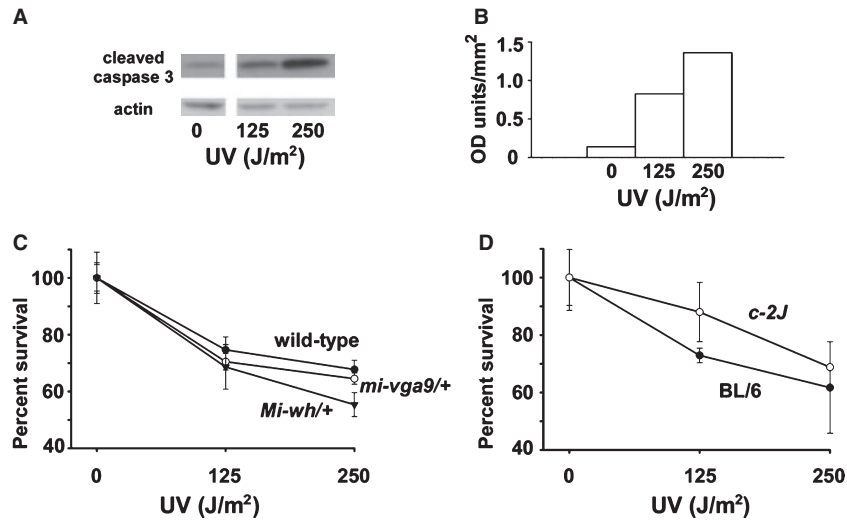


Figure 3. Dose response of melanocytes to UV radiation. Primary cultures of melanocytes and melanoblasts from neonatal mice were exposed in serum-free PBS to 0, 125 or 250 J/m² UV radiation. After washing, fresh media was added and the culture continued for 24 h at 37°C after which the cells were lysed for determination of protein expression with Western blotting (A, B) or utilized in the MTS assay for determination of relative viable cell number. (A) Expression of cleaved caspase 3 in primary C57BL/6 wild-type melanocytes following exposure to increasing doses of UV. β -actin was used as a loading control. (B) Densitometry analysis of (A), with cleaved caspase 3 normalized to actin. (C) Viability of melanocyte cultures from C57BL/6 and congenic C57BL/6J-*Mitf*^{*Mi-wh/+*} and C57BL/6J-*Mitf*^{*mi-vga9/+*} neonatal mice following irradiation with increasing doses of UV, measured with the MTS assay. Viability decreases as a function of increased *Mitf* mutational severity (wild-type > *Mi-wh/+* > *mi-vga9/+*). (D) Viability of melanocyte cultures from C57BL/6 and congenic albino C57BL/6J-*Tyr*^{*c-2J*} neonatal mice following irradiation with increasing doses of UV, measured with the MTS assay. No significant difference was observed between the viability of albino and pigmented melanocytes following UV irradiation. Error bars represent standard error of the mean.

C57BL/6 and congenic, albino C57BL/6-*Tyr*^{*c-2J*} mice to test directly the effect of melanin upon melanocyte survival following UV irradiation. Because these mice lacked the *Dct-lacZ* transgene, we utilized immunofluorescence with an antibody targeting the melanocyte marker *Tyrp1* to assess enrichment of the cultures for melanocytes.

In contrast to the results obtained with *Mitf* mutant melanocytes, UV irradiation of wild-type C57BL/6 primary melanocytes and congenic albino C57BL/6-*Tyr*^{*c-2J*} melanocytes revealed comparable amounts of cell death with increasing UV doses, with a trend to enhanced survival of albino melanocytes under these conditions (Figure 3D, Figure S3). Use of an anti-*Tyrp1* antibody for immunofluorescence identification of melanocytes in these cultures revealed that 70–90% of cells in this experiment exhibited *Tyrp-1* immunoreactivity (Figure S2), comparable to the enrichment observed in the previously described experiments. In a separate experiment, using an independently isolated set of melanocytes enriched at a lower level (40–60%), similar results were obtained (Figure S3). These results establish *Mitf* as a critical determinant of UV-induced melanocyte resistance to death, and also demonstrate that the effect of *Mitf* is a primary effect of *Mitf* on intracellular survival mechanisms rather than a secondary effect due to its regulation of the amount of intracellular melanin.

Mitf gene mutations, melanin, and UV-induced apoptosis

The previous experiments established a primary role for *Mitf* protecting melanocytes from UV-induced death. *Mitf* directly regulates the anti-apoptotic gene *Bcl2* (Mcgill et al., 2002), which is reduced in primary human melanocytes following UV irradiation (Bohm et al., 2005; Kadekaro et al., 2005). Based upon these observations, we proposed that diminished *Mitf*-dependent regulation of *Bcl2* might underlie enhanced cell death in UV-irradiated, *Mitf*-deficient melanocytes, and examined the effect of UV on key apoptotic pathway components in these cells.

Caspase 3, the effector caspase (Youle and Strasser, 2008), was expressed at comparable levels in pigmented C57BL/6 and albino C57BL/6-*Tyr*^{*c-2J*} melanocytes, although following 250 J/m² UV irradiation, slightly more could be detected in each of these cell types (Figure 4A). Consistent with a role for apoptosis in UV-induced cell death, UV enhanced production of cleaved caspase 3 in both pigmented and albino cells (Figure 4B). UV not only consistently diminished amounts of anti-apoptotic *Bcl-2*, as in human melanocytes (Bohm et al., 2005; Kadekaro et al., 2005), but also increased its pro-apoptotic, BH3-only interactor *Bad* in these cells. The similarity between these changes observed in pigmented and albino melanocytes, which also exhibited comparable levels of *Mitf* that may have

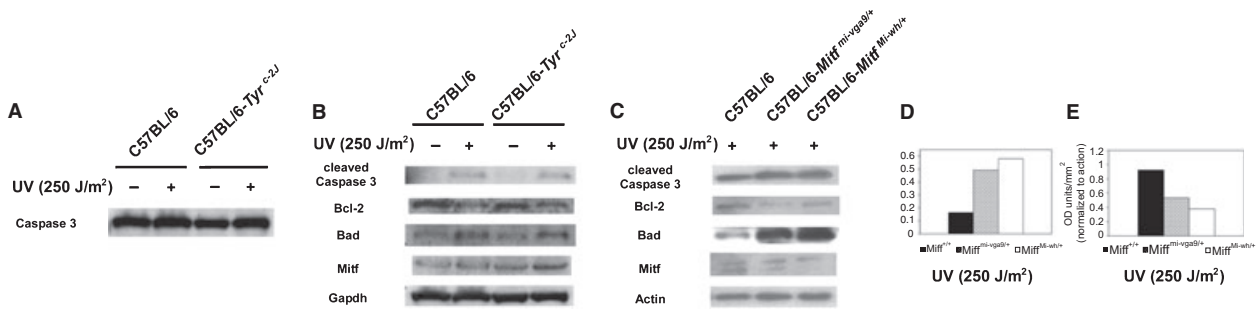


Figure 4. Effect of UV on apoptosis in primary wild-type, albino, and *Mitf*-deficient melanocytes. Primary cultures of C57BL/6, C57BL/6-*Tyr^{c-2J}*, C57BL/6-*Mitf^{mi-vga9/+}*, and C57BL/6-*Mitf^{mi-wh/+}* melanocytes were exposed to 0 or 250 J/m² UV radiation. Expression of apoptotic and anti-apoptotic proteins was studied using Western blotting from cell lysates collected 24 h later. (A) Expression of caspase 3 by C57BL/6 and C57BL/6-*Tyr^{c-2J}* melanocytes. (B) Expression of apoptosis-related proteins caspase 3 (cleaved), Bcl-2, and Bad in irradiated and unirradiated pigmented C57BL/6 and albino C57BL/6-*Tyr^{c-2J}* primary melanocytes. (C) Expression of apoptosis-related proteins in irradiated C57BL/6, C57BL/6-*Mitf^{mi-vga9/+}*, and C57BL/6-*Mitf^{mi-wh/+}* primary melanocytes. (D) Quantification of cleaved caspase 3 expression in irradiated C57BL/6, C57BL/6-*Mitf^{mi-vga9/+}*, and C57BL/6-*Mitf^{mi-wh/+}* primary melanocytes in (C). (E) Ratios of quantified Bcl-2 and Bad expression (Bcl-2:Bad ratio) from irradiated C57BL/6, C57BL/6-*Mitf^{mi-vga9/+}*, and C57BL/6-*Mitf^{mi-wh/+}* primary melanocytes in (C).

been slightly increased in each following UV irradiation (Figure 2B), are consistent with their similar rates of UV-induced cell death (Figure 3D, Figure S3).

In contrast to congenic pigmented and albino melanocytes, UV irradiation induced changes in cleaved caspase 3, Bcl-2, and Bad between pigmented C57BL/6 melanocytes and their congenic, *Mitf*-deficient counterparts C57BL/6-*Mitf^{mi-wh/+}* and C57BL/6-*Mitf^{mi-vga9/+}*. Increased cleaved caspase 3 was generated in C57BL/6-*Mitf^{mi-wh/+}* and C57BL/6-*Mitf^{mi-vga9/+}* melanocytes following UV (Figure 4C, D). Compared to the slightly lowered level of Bcl-2 observed in normal allelic, or wild-type, *Mitf* melanocytes following UV (Figure 4B), Bcl-2 in *Mitf*-deficient melanocytes was decreased even further (Figure 4C). Bad, which was slightly increased in wild-type *Mitf* melanocytes (Figure 4B), was induced disproportionately in *Mitf*-deficient cells (Figure 4C). C57BL/6-*Mitf^{mi-vga9/+}* and C57BL/6-*Mitf^{mi-wh/+}* exhibited a profound (43 and 61% respectively) decrease in the ratio of Bcl-2 to Bad when compared to melanocytes with wild-type *Mitf* following UV exposure (Figure 4E). The magnitude of these changes correlates directly with the propensity of these cells to exhibit enhanced cell death following UV (Figure 3C). These results demonstrate that the intracellular level of *Mitf* regulates the Bcl-2:Bad ratio in melanocytes following UV irradiation.

UV has been reported to activate directly the extrinsic, or death-receptor (Fas/CD95) apoptotic pathway independent of ligand binding (Aragane et al., 1998), prompting us to examine key elements of this pathway as well in *Mitf*-deficient melanocytes. UV exposure slightly induced Fas/CD95, and resulted in the generation of significantly more cleaved caspase 8, in *Mitf*-deficient melanocytes (Figure S4A, B). The expression of pro-apoptotic Bax was unchanged, and the expression of pro-apoptotic Bak varied slightly without a clear trend

(Figure S4). These results suggest, first of all, that extrinsic pathway activation may contribute to enhanced apoptotic cell death in UV-irradiated, *Mitf*-deficient melanocytes. They also suggest that increased induction of pro-apoptotic Bcl-2 homologs is not responsible for enhanced apoptosis in the *Mitf* mutant cells studied. Instead, this data supports a model whereby inactivation of Bcl-2 by increased expression of Bad, and perhaps other Bcl-2-interacting BH3-only family members (Youle and Strasser, 2008), alone is sufficient to trigger increased apoptosis via the intrinsic pathway in *Mitf*-deficient cells.

UV irradiation and MITF-dependent gene expression

The preceding results demonstrate that the resistance of melanocytes to UV-induced death, substantiated by changes in molecular markers of apoptosis, increases according to the order *Mitf^{mi-wh/+}* < *Mitf^{mi-vga9/+}* < +/+, with the least resistance observed in melanocytes heterozygous for the *Mitf^{mi-wh}* mutation which has the most severe heterozygous phenotype and dominant-negative activity in vitro (Hemesath et al., 1994). The limited numbers of cells we obtained in primary cultures restricted the ability to use reporter gene assays to estimate the activity of *Mitf* within these cells in order to correlate precisely the resistance to UV-induced apoptosis with *Mitf* transcriptional activity. However, the melanogenic genes *Tyr*, *Tyrp1*, and *Dct* are known transcriptional targets of *Mitf* (Bentley et al., 1994; Jiao et al., 2004; Yavuzer and Goding, 1994), as are the cell cycle regulatory genes *Cdk2* (Du et al., 2004) and *p21* (Carreira et al., 2005). We examined the expression of these *Mitf* target genes in +/+, *Mitf^{mi-vga9/+}*, and *Mitf^{mi-wh/+}* cells using quantitative RT-PCR to assess their relative levels of *Mitf* activity.

Wild-type, C57BL/6-*Mitf^{mi-vga9/+}*, and C57BL/6-*Mitf^{mi-wh/+}* melanocyte cultures were irradiated and

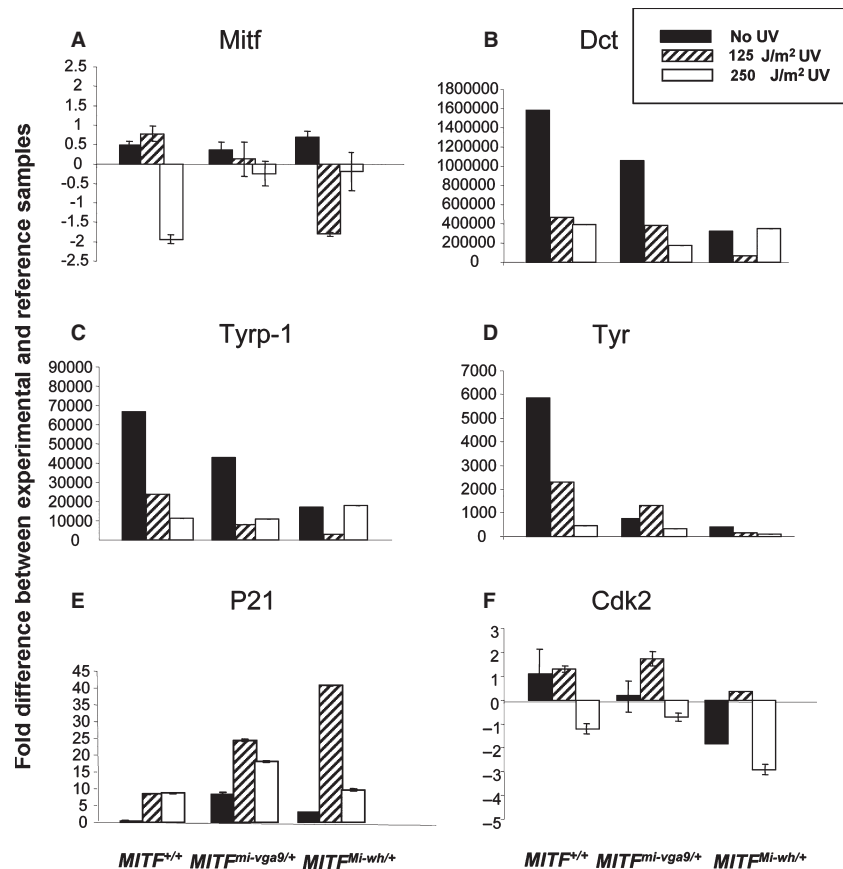


Figure 5. Real-time PCR analysis of RNA from melanocyte cultures. Cultures of melanocytes from the genotypes shown in Figure 1 were subjected to 0, 125 J/m² or 500 J/m² UV radiation and their RNA harvested 24 h later as described in the *Methods*. Data are expressed as the fold difference in gene expression between the experimental sample and a reference sample standard, as described in the *Methods*, using β -actin as an internal control.

RNA extracted 24 h later to measure UV-dependent changes in gene expression. Although the relative level of *Mitf* gene transcription was similar in unirradiated cells regardless of mutational status, the expression of *Mitf* diminished with UV irradiation in the mutant cells and at the higher UV dose in wild-type cells (Figure 5A). Despite the comparable levels of *Mitf* in unirradiated cells, the expression of the *Mitf* target genes *Tyr*, *Dct*/*Tyrp2*, and *Tyrp1* was highest in the cells with wild type *Mitf*, intermediate in *Mitf*^{mi-vga9/+}, and lowest in *Mitf*^{mi-wh/+} mutants (Figure 5B–D). These findings demonstrate a direct correlation between the estimated level of *Mitf* activity in each of these cell types and the expression of melanogenic genes known to be regulated by *Mitf*, and also are consistent with the amounts of melanin produced by the cells of each genotype (Figure 2). The expression of *Cdk2* varied similarly in unirradiated cells (Figure 5F), whereas the expression of *p21* was relatively low in unirradiated cultures and did not follow a similar pattern (Figure 5E).

As with *Mitf*, UV radiation decreased transcription of its melanogenic target genes *Tyr*, *Tyrp1*, and *Dct* in all three genotypes (Figure 5B–D), consistent with the reduction in *Mitf* expression that was observed. In contrast, *p21* expression was strongly induced by UV (Figure 5E), similar to the induction observed previ-

ously following irradiation of fibroblasts (Reinke and Lozano, 1997) and normal human skin (Murphy et al., 2002).

Discussion

Previous studies showed that *Mitf* regulates Bcl-2 expression (McGill et al., 2002) and that Bcl-2 activity is crucial for the establishment of the melanocyte stem cell (Nishimura et al., 2002) in the follicular niche (Nishimura et al., 2005). Primary cultures of murine *Bcl2*^{-/-} melanocytes showed increased cell death following UV irradiation (Gillardon et al., 1999). These observations on the role of *Mitf* and Bcl-2 in melanocyte survival in one context, the maintenance of the melanocyte stem cell, together with the effect of Bcl-2 on melanocyte survival following UV stimulation, suggested that *Mitf* might play a role in melanocyte survival following UV stimulation as well.

The present study examined the apoptotic response to UV radiation by primary murine melanocytes and whether mutations in *Mitf* influence activation of apoptotic pathways. The results show that in melanocytes, UV-induced apoptosis is more complex than previously appreciated and is controlled at multiple points involving both the intrinsic (mitochondrial) and extrinsic

(death receptor) pathways. We found that, in the absence of *Mitf* mutations (i.e., with wild-type *Mitf*), UV induces cell death in primary murine melanocytes, confirming the results demonstrated previously with human (Bohm et al., 2005; Kadokaro et al., 2005; Zhai et al., 1996) and murine (Gillardon et al., 1999; Hill et al., 1997) melanocytes. UV irradiation decreases the expression of Bcl-2 in primary murine melanocytes, extending results obtained previously with primary human melanocytes (Bohm et al., 2005; Kadokaro et al., 2005). In addition to reducing Bcl-2, UV increases expression of the pro-apoptotic BH3-domain protein Bad, a new finding. An increase in the Bad:Bcl-2 ratio is consistent with an apoptotic mechanism of death for these cells. These changes are present and similar in both congenic pigmented and albino melanocytes, correlating with the equivalent amounts of UV cell death we found in these cell types. A previous study (Hill et al., 1997) found a lower rate of UV-induced cell death from combined UVA and UVB (FS20 lamps) in albino melan-c melanocytes, an immortalized murine cell line derived from albino mice, compared to pigmented melan-a melanocytes, immortalized from C57BL/6-derived melanocytes. Our data (Figure 3D, Figure S3) revealed a trend to increased survival of primary albino C57BL/6-*Tyr^{c-2J}* melanocytes, a finding consistent with the aforementioned results from immortalized cells, although this difference did not reach levels of statistical significance. It is possible that the increased range of UV irradiation used in the previous experiments, inherent differences between primary and immortalized cells, or differences in the time course and assay type utilized between these two studies account for the differences.

The enhanced levels of cell death observed in *Mitf*-deficient primary melanocytes (Figure 3C) demonstrate that the amount of functional *Mitf* present intracellularly is a primary determinant of resistance to UV-induced apoptosis. Decreasing amounts of functional *Mitf*, due to haploinsufficiency (*Mitf^{mi-vga9/+}*) or heterozygosity for a dominant-negative mutation (*Mitf^{mi-wh/+}*), translated into reduced melanocyte survival following UV irradiation. We found that in addition to regulating anti-apoptotic Bcl-2, *Mitf* also regulates levels of pro-apoptotic protein Bad. However, we did not detect a significant increase in levels of the pro-apoptotic Bcl-2 homologs Bak or Bax upon UV irradiation in *Mitf*-deficient melanocytes. We propose that an increase in the Bad:Bcl-2 ratio, enabling greater engagement of anti-apoptotic Bcl-2 by the pro-apoptotic BH3 ligand Bad, is sufficient to enhance apoptosis in the *Mitf*-deficient cells independent of increases in Bak or Bax. Such a mechanism is consistent with recent evidence supporting an indirect activation model for BH3-like ligand action (Willis et al., 2007). Consistent with our results, experiments with human melanocytes also found no change at the protein level in the expression of Bax upon UV-irradiation,

although Bad was not examined in this report (Bivik et al., 2005).

The preferential activation of caspase 8 in *Mitf*-deficient melanocytes (Figure S4) also implicates *Mitf* in the regulation of extrinsic apoptotic pathway activity in UV-induced apoptosis in melanocytes. This was unexpected, because, unlike the interaction that has been described between *Mitf* and Bcl-2 in the intrinsic pathway, interactions between *Mitf* and components of the extrinsic apoptotic pathway have not been described. In a previous study, UV irradiation was found not to change the expression of FAS/CD95 in normal human melanocytes (Bohm et al., 2005). Hence, the level of FAS/CD95 expression itself, which may have varied somewhat in our results (Figure S4), is not likely to be relevant to the degree of apoptosis observed. Direct activation of FAS/CD95 in keratinocytes by UV light was observed in a prior study (Aragane et al., 1998), leaving open the possibility that *Mitf* regulates an intermediate step between UV-induced Fas activation and caspase 8 cleavage in melanocytes as well. *Mitf* is a substrate for effector caspases (Larribere et al., 2005), but it is unclear how, for example, preferential depletion of *Mitf* in *Mitf*-deficient cells could, via a feedback loop, induce preferential activation of caspase 8 upstream (Youle and Strasser, 2008). Interestingly, blockade of Kit signaling with the monoclonal antibody ACK2 results in a decline in Bcl-2 and an increase in caspase 8 cleavage (Kimura et al., 2005), findings similar to ours. Because of the dependence of *Mitf* activation upon Kit signaling (Hemesath et al., 1998), this may be another clue that there exists a currently undefined interaction between *Mitf* and the mechanism of caspase 8 activation requiring further study.

Our use of congenic mice heterozygous for distinct mutations in *Mitf* that affect melanocyte development and pigment production allowed us to evaluate its effects using cells progressively less functional for intact *Mitf*. Quantitative measurement of melanin showed that primary melanocyte cultures from C57BL/6-*Mitf^{mi-vga9/+}* heterozygotes produced only half as much melanin as cells from mice with wild-type *Mitf* even though their coat colors were phenotypically indistinguishable. These results suggest that coat color is only a rough indicator of melanin content. Melanin has protective effects on melanocytes (Kadokaro et al., 2003; Yamazaki et al., 2004). The level of *Mitf* activity, inferred both by the relative amount of melanin production in our melanocytes as well as by the relative expression levels of melanogenic enzyme genes driven by *Mitf*, inversely correlated with susceptibility to UV-induced apoptosis. Thus, melanocytes with wild-type *Mitf* were least sensitive to killing by UV and had the lowest amounts of cleaved caspase 8 and caspase 3, cells from C57BL/6-*Mitf^{mi-wh/+}* heterozygotes were the most sensitive to UV and had the highest amounts of these fragments, and cells from C57BL/

6-*Mitf*^{mi-vga9/+} heterozygotes were intermediate in these findings.

Our results demonstrate that *Mitf* expression in melanocytes inhibits UV-induced apoptosis via a mechanism that is independent of melanin production. They extend the role of *Mitf* in the prevention of apoptosis beyond its ability to transcriptionally regulate Bcl-2 to a role in shifting the balance between pro-apoptotic and anti-apoptotic molecules, which has been shown to determine a cell's apoptotic fate (Korsmeyer, 1999; Willis et al., 2007). In wild-type mice with the highest relative level of *Mitf* expression, this balance leans toward survival while decreasing the levels of *Mitf* expression shifts the balance toward apoptosis.

Materials and methods

Mice

C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). C57BL/6J-*Mitf*^{mi-wh}/*Mitf*^{mi-wh} and C57BL/6J-*Mitf*^{mi-vga9}/*Mitf*^{mi-vga9} were a kind gift from Dr. Lynn Lamoreux, College of Veterinary Medicine, Texas A & M University. CD1 outbred mice containing the *Dct-LacZ* transgene (Hornyak et al., 2001) were backcrossed onto the C57BL/6J genetic background for at least six generations to produce the C57BL/6-[Tg]*Dct-LacZ* animals used in the experiments. Breeding colonies were maintained in our animal facility in accordance with National Institutes of Health and American Association for Assessment and Accreditation of Laboratory Animal Care International Guidelines. The animals were housed in filter-protected cages and provided with National Institutes of Health open formula mouse chow and water ad libitum. All procedures were approved by the Henry Ford Health System Institutional Animal Care and Use Committee. For experiments comparing the effects of UV radiation on albino and wild-type melanocytes, B6(Cg)-*Tyr*^{o-2J}/J mice, obtained from The Jackson Laboratories, and C57BL/6 mice, obtained from the NCI-Frederick Animal Production Program (APA), were housed under similar conditions under an approved animal protocol of the Center for Cancer Research, National Cancer Institute.

Cell Cultures

Melanocytes were isolated from the skin using an adaptation of a previously described method (Abdel-Malek et al., 2001). Details are provided in Appendix S1.

UV radiation treatment

Ultraviolet irradiation was administered using either a single FS40 lamp or dual FS20 lamps (National Biological, Twinsburg, OH, USA). FS40 lamps produce 0.5% UVC (260–280 nm), 62% UVB (280–320 nm), and 38% UVA (320–400 nm) with a peak emission at 313 nm. The average irradiance of the source was approximately 1.58×10^{-4} W/cm² at 17 cm distance, or 1.99×10^{-4} W/cm² at 25 cm distance, as measured by an IL1700 research radiometer (International Light, Newburyport, MA, USA) with a UVB-1 filter (#25628) and SED 240 detector, or a SolaSure Solutell radiometer, respectively. Immediately prior to UV radiation treatment, the media was removed from each culture, the cells washed once with PBS and fresh PBS added. Cultures were exposed to UV radiation (1–2 min depending upon dose and device) under sterile conditions, in the absence of the plastic dish cover. Following UV exposure the PBS was removed and replaced with serum-containing culture medium. Control cultures were identically treated but were not UV

irradiated. The effect of UV radiation on cell proliferation and survival was measured by plating equal numbers of transplanted melanocyte cultures from each genotype into 24 flat-bottom well tissue culture plates (Costar, Corning, NY, USA). Two to three days later the culture medium was removed and replaced with PBS and the cells exposed to UV radiation as described above. Control cultures were treated identically but not irradiated. The PBS was replaced with 0.5 ml media per well and the incubation continued. After 24 h, 0.1 ml MTS reagent (Promega Corp., Madison, WI, USA) was added to each well of quadruplicate cultures and the absorbance of the culture supernatants was measured in a Beckman Coulter AD340 ELISA Reader (Beckman Coulter, Fullerton, CA, USA) at 490 nm 1–20 h later, depending upon the experiment and number of plated cells, according to the manufacturer's instructions.

Melanin measurement

Melanin was measured using the method kindly provided by Dr. Zalfa Abdel-Malek, Department of Dermatology, The University of Cincinnati, Cincinnati, OH. Details are provided in Appendix S1.

Western blotting

Twenty four hours after UV exposure cells were scraped from the dishes, replicate cultures pooled and the cells were pelleted by centrifugation at 500 *g* for 10 min. The supernatants were discarded and cell pellets lysed using ice-cold RIPA buffer with enzyme inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 2 mM sodium orthovanadate (Na₂VO₄), and 1 mM sodium fluoride (NaF). Cell debris was pelleted by centrifugation at 13 400 *g* for 3 min. The supernatants were collected and protein measured using a Bradford assay (BioRad, Hercules, CA) according to the manufacturer's instructions. 10–20 μ g of protein per sample was loaded in a denaturing 12.5% or 15% SDS-PAGE gel. Following electrophoretic separation, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) and blocked with 5% non-fat milk (Carnation, Glendale, CA, USA) in 20 mM Tris-buffered saline containing 0.05% Tween-20 (TBST) buffer overnight at 4°C. The membrane was washed $\times 3$ with TBST and incubated with primary antibody overnight at 4°C. Details of antibodies used and detection are presented in Appendix S1.

Quantitative real-time PCR analysis of gene expression

Melanocyte-containing cultures from newborn wild-type, C57BL/6-*Mitf*^{mi-wh/+}[Tg]*Dct-LacZ* and C57BL/6-*Mitf*^{mi-vga9/+}[Tg]*Dct-LacZ* heterozygous mice were exposed to 0, 125 J/m², or 250 J/m² UV radiation as described above. 24 h later the cells were removed from their culture dishes using a cell scraper and the cells counted and sedimented by centrifugation at 500 *g* for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1 ml ice-cold RLT lysis buffer (Qiagen Sciences, Valencia, CA, USA) and stored at –80°C. RNA was isolated and quantitative real-time PCR performed essentially as previously described (Urzua et al., 2006), but with *β -actin* as a reference transcript assayed under identical conditions respective to the gene of interest. Additional details of the analysis of the quantitative real-time PCR analysis are provided in Appendix S1.

Beta-galactosidase (β -gal) staining

Cells were fixed in 0.4% paraformaldehyde/PBS for 15 min at room temperature and washed thrice with PBS for 5 min. each. The slides were incubated in a solution of 100 mM Tris pH 7.5

containing 0.1 M phosphate buffer pH 7.3, 2 mM magnesium chloride, 0.1% sodium deoxycholate, 0.02% NP40, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide and 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal, Gold BioTechnologies, St. Louis, MO, USA). X-gal was prepared as a 20 mg/ml solution in dimethylformamide and stored at -20°C until use. The tissue was incubated in the staining solution in the dark at room temperature overnight or until a blue signal appeared. Cells were post-fixed for 15 min in a 4% paraformaldehyde/PBS solution and photographed.

Acknowledgements

We thank Alice Croxen and Payal Shah for excellent technical assistance. We also thank Kenneth N. Barton for assistance in image analyses. We are grateful to Ronald P. Pelley for critical reading of this manuscript and Dr. Colin Goding for advice and assistance with the manuscript. We also thank Dr. Edward DeFabo for use and calibration of an ultraviolet light box. This work was supported by grants R01-AR47951 (FMS) from the National Institutes, DAMD17-01-1-1709 (FMS) from the Department of Defense, NIH K08 AR01992 (to TJH) and (in part) by the Intramural research program of the NIH, National Cancer Institute, Center for Cancer Research.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Melanin content, phenotype and β -galactosidase expression in primary melanocyte cultures shown in Figure 1. C57BL/6J-*Mitf*^{Mi-wh/+} [Tg]*Dct-LacZ* melanocytes shown in Figure 1D; C57BL/6J *Mitf*^{Mi-vga9/+} [Tg]*Dct-LacZ* heterozygous melanocytes shown in Figure 1F, and C57BL/6J[Tg]*Dct-LacZ* melanocytes with wild type *Mitf* shown in Figure 1H. Arrows indicate melanin-containing cells that are losing their *Dct-LacZ* expression. Arrowhead indicates a melanin-containing melanocyte with lower [Tg]*Dct-LacZ* expression. All melanocyte cultures were stained for β -galactosidase (blue). Bar = 100 μm .

Figure S2. Anti-Tyrp1 (Mel-5) immunostaining of pigmented C57BL/6 and albino C57BL/6-*Tyr*^{c-2J} primary melanocytes. (A) Bright field image of pigmented C57BL/6 primary melanocytes. (B) Anti-Tyrp1 immunofluorescent image of pigmented C57BL/6 primary melanocytes. (C) DAPI staining of pigmented C57BL/6 primary melanocytes. Arrowheads in A, B, and C denote the same cell in respective panels. (D) Bright field image of albino C57BL/6-*Tyr*^{c-2J} primary melanocytes. (E) Anti-Tyrp1 immunofluorescent image of albino C57BL/6-*Tyr*^{c-2J} primary melanocytes. (F) DAPI staining of albino C57BL/6-*Tyr*^{c-2J} primary melanocytes. Arrowheads in E and F denote the same cell in respective panels. (Cell is not visible in D due to lack of pigment).

Figure S3. Viability of melanocyte cultures from C57BL/6 and congenic albino C57BL/6J-*Tyr*^{c-2J} neonatal mice following irradiation with increasing doses of UV, measured with the MTS assay. In this primary culture, 40–60% of the cells were Tyrp1⁺ as determined

by anti-Tyrp1 immunofluorescence as in Figure S2. No significant difference was observed between the viability of albino and pigmented melanocytes following UV irradiation.

Figure S4. Effects of UV irradiation on extrinsic apoptotic pathway components and pro-apoptotic Bcl-2 family members in wild-type and *Mitf*-deficient melanocytes. Expression of apoptosis-related proteins was studied using Western blotting from cell lysates collected 24 h later. (A) Expression of extrinsic pathway proteins Fas, caspase 8, cleaved caspase 8, and pro-apoptotic Bcl-2 family members Bak and Bax in C57BL/6, C57BL/6-*Mitf*^{Mi-vga9/+}, and C57BL/6-*Mitf*^{Mi-wh/+} primary melanocytes 24 h after irradiation with 250 J/m² UV. (B) Quantification of Western blotting data from A.

Appendix S1. Supplementary Methods.

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References

- Abdel-Malek, Z.A., Scott, M.C., Furumura, M., Lamoreux, M.L., Ollmann, M., Barsh, G.S., and Hearing, V.J. (2001). The melanocortin 1 receptor is the principal mediator of the effects of agouti signaling protein on mammalian melanocytes. *J. Cell Sci.* **114**, 1019–1024.
- Aragane, Y., Kulms, D., Metzke, D., Wilkes, G., Poppelmann, B., Luger, T.A., and Schwarz, T. (1998). Ultraviolet light induces apoptosis via direct activation of CD95 (Fas/APO-1) independently of its ligand CD95L. *J. Cell Biol.* **140**, 171–182.
- Bentley, N.J., Eisen, T., and Goding, C.R. (1994). Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. *Mol. Cell Biol.* **14**, 7996–8006.
- Bismuth, K., Skuntz, S., Hallsson, J.H., Pak, E., Dutra, A.S., Steingrimmsson, E., and Arnheiter, H. (2008). An unstable targeted allele of the mouse *Mitf* gene with a high somatic and germline reversion rate. *Genetics* **178**, 259–272.
- Bivik, C.A., Andersson, E.B., and Rosdahl, I.K. (2005). Wavelength-specific effects on UVB-induced apoptosis in melanocytes. A study of Bcl-2/Bax expression and keratinocyte rescue effects. *Melanoma Res.* **15**, 7–13.
- Blume-Jensen, P., Jiang, G., Hyman, R., Lee, K.F., O’gorman, S., and Hunter, T. (2000). Kit/stem cell factor receptor-induced activation of phosphatidylinositol 3'-kinase is essential for male fertility. *Nat. Genet.* **24**, 157–162.
- Bohm, M., Wolff, I., Scholzen, T.E., Robinson, S.J., Healy, E., Luger, T.A., Schwarz, T., and Schwarz, A. (2005). alpha-Melanocyte-stimulating hormone protects from ultraviolet radiation-induced apoptosis and DNA damage. *J. Biol. Chem.* **280**, 5795–5802.
- Busca, R., Berra, E., Gaggioli, C., Khaled, M., Bille, K., Marchetti, B., Thyss, R., Fitsialos, G., Larrubere, L., Virolle, T., Barbry, P., Pouyssegur, J., Ponzio, G., and Ballotti, R. (2005). Hypoxia-inducible factor 1a is a new target of microphthalmia-associated transcription factor (MITF) in melanoma cells. *J. Cell Biol.* **170**, 49–59.
- Carreira, S., Goodall, J., Aksan, I., La Rocca, S.A., Galibert, M.D., Denat, L., Larue, L., and Goding, C.R. (2005). *Mitf* cooperates

- with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. *Nature* 433, 764–769.
- Corre, S., Primot, A., Sviderskaya, E., Bennett, D.C., Vaulont, S., Goding, C.R., and Galibert, M.D. (2004). UV-induced expression of key component of the tanning process, the POMC and MC1R genes, is dependent on the p-38-activated upstream stimulating factor-1 (USF-1). *J. Biol. Chem.* 279, 51226–51233.
- De Leeuw, S.M., Smit, N.P., Van Veldhoven, M., Pennings, E.M., Pavel, S., Simons, J.W., and Schothorst, A.A. (2001). Melanin content of cultured human melanocytes and UV-induced cytotoxicity. *J. Photochem. Photobiol. B* 61, 106–113.
- Du, J., Miller, A.J., Widlund, H.R., Horstmann, M.A., Ramaswamy, S., and Fisher, D.E. (2003). MLANA/MART1 and SILV/P-MEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. *Am. J. Pathol.* 163, 333–343.
- Du, J., Widlund, H.R., Horstmann, M.A., Ramaswamy, S., Ross, K., Huber, W.E., Nishimura, E.K., Golub, T.R., and Fisher, D.E. (2004). Critical role of CDK2 for melanoma growth linked to its melanocyte-specific transcriptional regulation by MITF. *Cancer Cell* 6, 565–576.
- Galibert, M.D., Carreira, S., and Goding, C.R. (2001). The Usf-1 transcription factor is a novel target for the stress-responsive p38 kinase and mediates UV-induced Tyrosinase expression. *EMBO J.* 20, 5022–5031.
- Garraway, L.A., Widlund, H.R., Rubin, M.A. et al. (2005). Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. *Nature* 436, 117–122.
- Gillardot, F., Moll, I., Meyer, M., and Michaelidis, T.M. (1999). Alterations in cell death and cell cycle progression in the UV-irradiated epidermis of bcl-2-deficient mice. *Cell Death Differ.* 6, 55–60.
- Goding, C.R. (2000). Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage. *Genes Dev.* 14, 1712–1728.
- Halaban, R., and Alfano, F.D. (1984). Selective elimination of fibroblasts from cultures of normal human melanocytes. *In Vitro* 20, 447–450.
- Hemesath, T.J., Steingrímsson, E., McGill, G., Hansen, M.J., Vaught, J., Hodgkinson, C.A., Arnheiter, H., Copeland, N.G., Jenkins, N.A., and Fisher, D.E. (1994). *Microphthalmia*, a critical factor in melanocyte development, defines a discrete transcription factor family. *Genes Dev.* 8, 2770–2780.
- Hemesath, T.J., Price, E.R., Takemoto, C., Badalian, T., and Fisher, D.E. (1998). MAP kinase links the transcription factor *Microphthalmia* to c-Kit signalling in melanocytes. *Nature* 391, 298–301.
- Hill, H.Z., Hill, G.J., Cieszka, K., Plonka, P.M., Mitchell, D.L., Meyenhofer, M.F., Xin, P., and Boissy, R.E. (1997). Comparative action spectrum for ultraviolet light killing of mouse melanocytes from different genetic coat color backgrounds. *Photochem. Photobiol.* 65, 983–989.
- Hodgkinson, C.A., Moore, K.J., Nakayama, A., Steingrímsson, E., Copeland, N.G., Jenkins, N.A., and Arnheiter, H. (1993). Mutations at the mouse *microphthalmia* locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. *Cell* 74, 395–404.
- Hoogduijn, M.J., Cemeli, E., Ross, K., Anderson, D., Thody, A.J., and Wood, J.M. (2004). Melanin protects melanocytes and keratinocytes against H₂O₂-induced DNA strand breaks through its ability to bind Ca²⁺. *Exp. Cell Res.* 294, 60–67.
- Hornyak, T.J., Hayes, D.H., Chiu, L.-Y., and Ziff, E.B. (2001). Transcription factors in melanocyte development: distinct roles for Pax-3 and Mitf. *Mech. Dev.* 101, 47–59.
- Jiao, Z., Mollaaghbabab, R., Pavan, W.J., Antonellis, A., Green, E.D., and Hornyak, T.J. (2004). Direct interaction of Sox10 with the promoter of murine Dopachrome Tautomerase (Dct) and synergistic activation of Dct expression with Mitf. *Pigment Cell Res.* 17, 352–362.
- Kadekaro, A.L., Kavanagh, R.J., Wakamatsu, K., Ito, S., Pipitone, M.A., and Abdel-Malek, Z.A. (2003). Cutaneous photobiology. The melanocyte vs. the sun: who will win the final round? *Pigment Cell Res.* 16, 434–447.
- Kadekaro, A.L., Kavanagh, R., Kanto, H. et al. (2005). alpha-Melanocortin and endothelin-1 activate antiapoptotic pathways and reduce DNA damage in human melanocytes. *Cancer Res.* 65, 4292–4299.
- Karlsson, R., Engstrom, M., Jonsson, M., Karlberg, P., Pronk, C.J., Richter, J., and Jonsson, J.I. (2003). Phosphatidylinositol 3-kinase is essential for kit ligand-mediated survival, whereas interleukin-3 and flt3 ligand induce expression of antiapoptotic Bcl-2 family genes. *J. Leukoc. Biol.* 74, 923–931.
- Kim, T.J., Cho, M.K., Lee, J.S., Whang, K.U., Jin, S.Y., and Hoshino, T. (2003). The expression of melanogenic proteins in Korean skin after ultraviolet irradiation. *J. Dermatol.* 30, 665–672.
- Kimura, S., Kawakami, T., Kawa, Y., Soma, Y., Kushimoto, T., Nakamura, M., Watabe, H., Ooka, S., and Mizoguchi, M. (2005). Bcl-2 reduced and fas activated by the inhibition of stem cell factor/KIT signaling in murine melanocyte precursors. *J. Invest. Dermatol.* 124, 229–234.
- Korsmeyer, S.J. (1999). BCL-2 gene family and the regulation of programmed cell death. *Cancer Res.* 59, 1693s–1700s.
- Larribere, L., Hilmi, C., Khaled, M., Gaggioli, C., Bille, K., Auberger, P., Ortonne, J.P., Ballotti, R., and Bertolotto, C. (2005). The cleavage of microphthalmia-associated transcription factor, MITF, by caspases plays an essential role in melanocyte and melanoma cell apoptosis. *Genes Dev.* 19, 1980–1985.
- Lin, C.B., Babiarz, L., Liebel, F., Roydon Price, E., Kizoulis, M., Gendimenico, G.J., Fisher, D.E., and Seiberg, M. (2002). Modulation of microphthalmia-associated transcription factor gene expression alters skin pigmentation. *J. Invest. Dermatol.* 119, 1330–1340.
- Lindholm, C., Andersson, R., Dufmats, M., Hansson, J., Ingvar, C., Moller, T., Sjodin, H., Stierner, U., and Wagenius, G. (2004). Invasive cutaneous malignant melanoma in Sweden, 1990–1999. A prospective, population-based study of survival and prognostic factors. *Cancer* 101, 2067–2078.
- Ludwig, A., Rehberg, S., and Wegner, M. (2004). Melanocyte-specific expression of dopachrome tautomerase is dependent on synergistic gene activation by the Sox10 and Mitf transcription factors. *FEBS Lett.* 556, 236–244.
- McGill, G.G., Horstmann, M., Widlund, H.R. et al. (2002). Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. *Cell* 109, 707–718.
- Murphy, M., Mabruk, M.J., Lenane, P., Liew, A., Mccann, P., Buckley, A., Billet, P., Leader, M., Kay, E., and Murphy, G.M. (2002). The expression of p53, p21, Bax and induction of apoptosis in normal volunteers in response to different doses of ultraviolet radiation. *Br. J. Dermatol.* 147, 110–117.
- Nishimura, E.K., Jordan, S.A., Oshima, H., Yoshida, H., Osawa, M., Moriyama, M., Jackson, I.J., Barrandon, Y., Miyachi, Y., and Nishikawa, S. (2002). Dominant role of the niche in melanocyte stem-cell fate determination. *Nature* 416, 854–860.
- Nishimura, E.K., Granter, S.R., and Fisher, D.E. (2005). Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. *Science* 307, 720–724.
- Ono, K., and Han, J. (2000). The p38 signal transduction pathway: activation and function. *Cell. Signal.* 12, 1–13.
- Plettenberg, A., Ballaun, C., Pammer, J., Mildner, M., Strunk, D., Weninger, W., and Tschachler, E. (1995). Human melanocytes and melanoma cells constitutively express the Bcl-2 proto-oncogene in situ and in cell culture. *Am. J. Pathol.* 146, 651–659.

- Price, E.R., and Fisher, D.E. (2001). Sensorineural deafness and pigmentation genes: melanocytes and the *Mitf* transcriptional network. *Neuron* 30, 15–18.
- Reinke, V., and Lozano, G. (1997). Differential activation of p53 targets in cells treated with ultraviolet radiation that undergo both apoptosis and growth arrest. *Radiat. Res.* 148, 115–122.
- Rosette, C., and Karin, M. (1996). Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science* 274, 1194–1197.
- Sakata, S., Sakamaki, K., Watanabe, K., Nakamura, N., Toyokuni, S., Nishimune, Y., Mori, C., and Yonehara, S. (2003). Involvement of death receptor Fas in germ cell degeneration in gonads of Kit-deficient *Wv/Wv* mutant mice. *Cell Death Differ.* 10, 676–686.
- Schwahn, D.J., Timchenko, N.A., Shibahara, S., and Medrano, E.E. (2005). Dynamic regulation of the human dopachrome tautomerase promoter by MITF, ER- α and chromatin remodelers during proliferation and senescence of human melanocytes. *Pigment Cell Res.* 18, 203–213.
- Smit, N.P., Vink, A.A., Kolb, R.M., Steenwinkel, M.J., Van Den Berg, P.T., Van Nieuwpoort, F., Roza, L., and Pavel, S. (2001). Melanin offers protection against induction of cyclobutane pyrimidine dimers and 6-4 photoproducts by UVB in cultured human melanocytes. *Photochem. Photobiol.* 74, 424–430.
- Steingrimsson, E., Copeland, N.G., and Jenkins, N.A. (2004). Melanocytes and the microphthalmia transcription factor network. *Annu. Rev. Genet.* 38, 365–411.
- Tachibana, M., Hara, Y., Vyas, D., Hodgkinson, C., Fex, J., Grundfast, K., and Arnheiter, H. (1992). Cochlear disorder associated with melanocyte anomaly in mice with a transgenic insertional mutation. *Mol. Cell. Neurosci.* 3, 433–445.
- Tadokoro, T., Kobayashi, N., Zmudzka, B.Z., Ito, S., Wakamatsu, K., Yamaguchi, Y., Korossy, K.S., Miller, S.A., Beer, J.Z., and Hearing, V.J. (2003). UV-induced DNA damage and melanin content in human skin differing in racial/ethnic origin. *FASEB J.* 17, 1177–1179.
- Tadokoro, T., Yamaguchi, Y., Batzer, J., Coelho, S.G., Zmudzka, B.Z., Miller, S.A., Wolber, R., Beer, J.Z., and Hearing, V.J. (2005a). Mechanisms of skin tanning in different racial/ethnic groups in response to ultraviolet radiation. *J. Invest. Dermatol.* 124, 1326–1332.
- Tadokoro, T., Yamaguchi, Y., Batzer, J., Coelho, S.G., Zmudzka, B.Z., Miller, S.A., Wolber, R., Beer, J.Z., and Hearing, V.J. (2005b). Mechanisms of skin tanning in different racial/ethnic groups in response to ultraviolet radiation. *J. Invest. Dermatol.* 124, 1326–1332.
- Tassabehji, M., Newton, V.E., and Read, A.P. (1994). Waardenburg syndrome type 2 caused by mutations in the human microphthalmia (MITF) gene. *Nat. Genet.* 8, 251–255.
- Tomita, Y., Takeda, A., Okinaga, S., Tagami, H., and Shibahara, S. (1989). Human oculocutaneous albinism caused by single base insertion in the tyrosinase gene. *Biochem. Biophys. Res. Commun.* 164, 990–996.
- Urzua, U., Roby, K.F., Gangi, L.M., Cherry, J.M., Powell, J.I., and Munroe, D.J. (2006). Transcriptomic analysis of an in vitro murine model of ovarian carcinoma: functional similarity to the human disease and identification of prospective tumoral markers and targets. *J. Cell. Physiol.* 206, 594–602.
- Widlund, H.R., and Fisher, D.E. (2003). Microphthalmia-associated transcription factor: a critical regulator of pigment cell development and survival. *Oncogene* 22, 3035–3041.
- Willis, S.N., Fletcher, J.I., Kaufmann, T. et al. (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* 315, 856–859.
- Yamazaki, F., Okamoto, H., Miyauchi-Hashimoto, H., Matsumura, Y., Itoh, T., Tanaka, K., Kunisada, T., and Horio, T. (2004). XPA gene-deficient, SCF-transgenic mice with epidermal melanin are resistant to UV-induced carcinogenesis. *J. Invest. Dermatol.* 123, 220–228.
- Yasumoto, K.-I., Yokoyama, K., Takahashi, K., Tomita, Y., and Shibahara, S. (1997). Functional analysis of microphthalmia-associated transcription factor in pigment cell-specific transcription of the human tyrosinase family genes. *J. Biol. Chem.* 272, 503–509.
- Yavuzer, U., and Goding, C.R. (1994). Melanocyte-specific gene expression: role of repression and identification of a melanocyte-specific factor, MSF. *Mol. Cell. Biol.* 14, 3494–3503.
- Youle, R.J., and Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nat. Rev. Mol. Cell Biol.* 9, 47–59.
- Young, A.R. (2004). Tanning devices—fast track to skin cancer? *Pigment Cell Res.* 17, 2–9.
- Zhai, S., Yaar, M., Doyle, S.M., and Gilchrist, B.A. (1996). Nerve growth factor rescues pigment cells from ultraviolet-induced apoptosis by upregulating BCL-2 levels. *Exp. Cell Res.* 224, 335–343.