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Bcl2 Regulation by the Melanocyte Master Regulator Mitf Modulates Lineage Survival and Melanoma Cell Viability

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Summary

Kit/SCF signaling and Mitf-dependent transcription are both essential for melanocyte development and pigmentation. To identify Mitf-dependent Kit transcriptional targets in primary melanocytes, microarray studies were undertaken. Among identified targets was *BCL2*, whose germline deletion produces melanocyte loss and which exhibited phenotypic synergy with *Mitf* in mice. *BCL2*'s regulation by Mitf was verified in melanocytes and melanoma cells and by chromatin immunoprecipitation of the *BCL2* promoter. Mitf also regulates *BCL2* in osteoclasts, and both $Mitf^{mi/mi}$ and $Bcl2^{-/-}$ mice exhibit severe osteopetrosis. Disruption of Mitf in melanocytes or melanoma triggered profound apoptosis susceptible to rescue by *BCL2* overexpression. Clinically, primary human melanoma expression microarrays revealed tight nearest neighbor linkage for *MITF* and *BCL2*. This linkage helps explain the vital roles of both Mitf and *Bcl2* in the melanocyte lineage and the well-known treatment resistance of melanoma.

Introduction

The *microphthalmia* gene encodes Mitf a basic helix-loop-helix leucine zipper (b-HLH-Zip) transcription factor essential for the melanocyte lineage in the developing and adult mouse. Mitf expression is also clinically employed in the diagnosis of human melanocytic neoplasms, especially melanoma (Chang and Folpe, 2001; Dorvault et al., 2001; King et al., 1999, 2001; Koch et al., 2001; Miettinen et al., 2001). Mutations in *Mitf* produce loss of viable melanocytes, a phenotype similar to mice or humans harboring mutations in the Kit receptor and its ligand stem cell factor (SCF). *MITF* mutations in humans produce Waardenburg Syndrome type IIa, a condition characterized by melanocyte deficiencies in the skin and inner ear (Hughes et al., 1994; Price and Fisher, 2001; Tassabehji et al., 1994). In mice, structure-function studies have defined a series of recessively inherited *Mitf* alleles that are functionally null, as well as dominant alleles containing basic region mutations that function as dominant negatives through sequestration of wild-type partners in non-DNA binding dimers (Hemesath et al., 1994; Steingrimsson et al., 1994). In addition to complete absence of melanocytes, *Mitf* dominant-negative mutants exhibit osteopetrosis, manifested by hyperdense bones, due to defective osteoclasts (Moore, 1995; Weilbaecher et al., 2001).

The complete absence of melanocytes in neonatal *Mitf* mutant mice could arise either from transdifferentiation during migration of neural crest-derived precursors or from loss of survival (or both). Alleles like $Mitf^{vit/vit}$, which exhibit essentially normal pigmentation at birth followed by graying due to melanoycte loss (Lerner et al., 1986), suggest that Mitf plays an active role in modulating postdevelopmental melanocyte survival. However, transcriptional target genes of Mitf that regulate melanocyte survival have not yet been elucidated. This is of particular importance because Mitf could also modulate survival in melanomas, virtually all of which retain Mitf expression and exhibit notorious resistance to antineoplastic therapies.

Kit and *Scf* mutant mice, like *Mitf* mutants, exhibit striking coat color phenotypes due to loss of viable melanocytes (Russell, 1979; Witte, 1990). Although Kit activates the PI3-Kinase-Akt survival pathway, genetically engineered mouse mutants revealed that this pathway was dispensable for melanocyte development, though it was important for germ cell survival (Blume-Jensen et al., 2000), suggesting that Kit's melanocyte survival effect is mediated by a different pathway. Kit may reside upstream of Mitf via at least two potential mechanisms in melanocytes. Kit stimulation triggers MAPK-mediated phosphorylation of Mitf, which in turn recruits the p300 transcriptional coactivator (Hemesath et al., 1998; Price et al., 1998; Weilbaecher et al., 2001) as well as ubiquitination (Wu et al., 2000; Xu et al., 2000). Moreover, MAPK-dependent Rsk activation may produce CREB phosphorylation and upregulation of the *Mitf* promoter. The phenotypic overlap of mice harboring mutations in *Scf*, *c-Kit*, *Mitf*, and *Bcl2* (whose homozygous knockout also produces melanocyte loss [Kamada et al., 1995; Nakayama et al., 1994; Veis et al., 1993; Yamamura et al., 1996]) suggests that these genes might coordinately modulate the development and survival of the melanocyte lineage. It is plausible that survival path-

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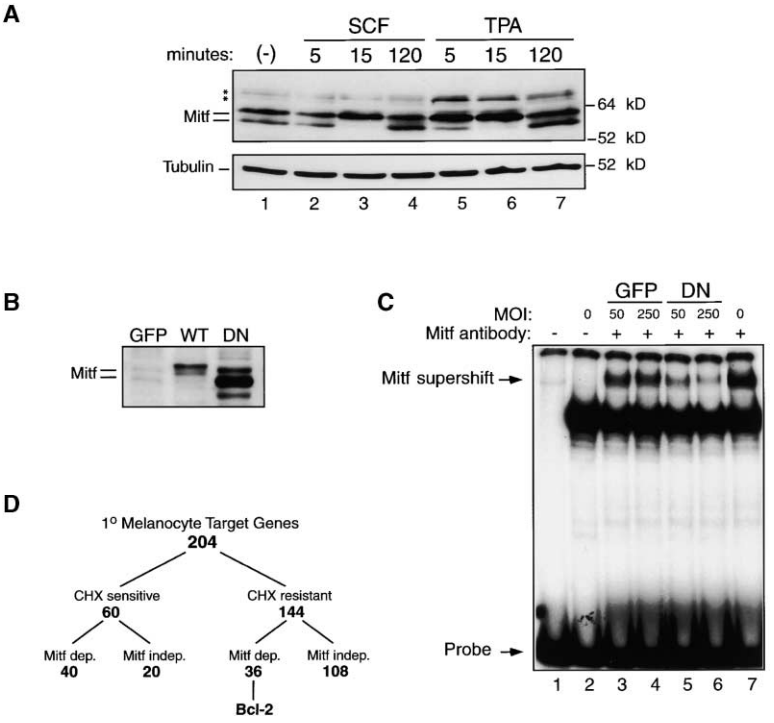

Figure 1. Microarray Screen for Mitf-Dependent Targets of the SCF Pathway

(A) Treatment with either SCF or TPA leads to a phosphorylation-induced Mitf mobility shift. Primary human melanocytes were stimulated with either stem cell factor (SCF) or TPA for indicated times and harvested for Western blot analysis using monoclonal αMitf. Faint upper bands (labeled with asterisks) represent alternate forms of Mitf transcribed from separate promoters.

(B) Expression of adenovirus-encoded proteins in primary human melanocytes infected for 48 hr with control (GFP), wild-type (wt), or dominant-negative (DN) Mitf viruses.

(C) Disruption of endogenous Mitf:DNA complexes by dnMitf adenovirus. Nuclear extracts from control (GFP) or dnMitf (DN)-infected primary human melanocytes at different multiplicities of infection (MOI) were incubated with a ^{32}P-labeled Mitf DNA probe and supershifted with a monoclonal antibody against Mitf.

(D) Summary of screen results from expression profiling of melanocytes stimulated by SCF or TPA ± cycloheximide (CHX) and ± dnMitf (see Experimental Procedures).

ways may operate in melanocytes, which are unique for the requirement to survive UV exposure to ensure melanin synthesis. The notorious treatment resistance of melanoma suggests that melanocyte-specific survival pathway(s) might thus operate in a clinically relevant fashion.

Here, a microarray-based screen was undertaken to identify Mitf-dependent SCF/Kit target genes in primary human melanocytes. Among the collection of identified targets was *BCL2*, the antiapoptotic factor (Gross et al., 1999; Reed, 1998; Vander Heiden and Thompson, 1999) whose germline deletion results in early loss of the melanocyte lineage. *Mitf* and *Bcl2* were found to genetically interact in vivo as demonstrated by profound melanocyte loss in compound heterozygous mice. In addition, we show that Mitf occupies the *BCL2* promoter, regulates endogenous levels of *BCL2* in melanocytes and melanomas, and is coregulated with *BCL2* in primary human melanoma samples analyzed in microarray nearest neighbor analyses. Finally, modulation of *BCL2* expression is shown to be critical for melanocyte and melanoma survival.

Results

Microarray Screen for SCF/Kit/Mitf Target Genes

To identify SCF/Kit transcriptional targets, primary human melanocytes were stimulated with recombinant SCF, and a time course of mRNAs was isolated up to 6 hr later. Kit stimulation produces a well-characterized MAP kinase-mediated phosphorylation of Mitf, which is manifested as a protein mobility shift by Western blot (Figure 1A; Hemesath et al., 1998). TPA is a commonly employed growth factor for primary melanocytes (Eisinger and Marko, 1982; Halaban et al., 1986) and may substitute for SCF in these cultures (data not shown). TPA stimulation produces the identical MAPK-mediated Mitf phosphorylation as for SCF in both primary melanocytes and melanoma cells (Figure 1A; Hemesath et al., 1998; Wu et al., 2000). We chose to identify target genes of SCF/Kit as well as TPA, whose upregulation is dependent upon the presence of endogenous Mitf. Dependence on Mitf was revealed by analyzing expression data from stimulated melanocytes that were previously infected by adenoviruses expressing a specific dominant-negative Mitf mutant (or control GFP virus). The dominant-negative Mitf mutation contains a basic domain amino acid deletion and thereby sequesters wild-type Mitf partners, but not other HLH proteins, into dimers incapable of binding DNA (Hemesath et al., 1994). Because adenoviruses disrupted SCF signaling events, we employed TPA stimulation, which was not measurably affected by viral infection (data not shown). The adenovirus vectors infected >98% of cultured primary melanocytes or melanoma cells (data not shown) and measurably expressed the encoded proteins (Figure 1B). Dominant-negative Mitf adenovirus produced dose-dependent inhibition of DNA binding by endogenous Mitf (revealed as monoclonal antibody-induced supershift) in nuclear extracts of infected melanocytes (Figure 1C). Using this system, expression profiling was undertaken.

Target genes in primary, early passage human melanocytes were identified for SCF, TPA, the overlap of SCF and TPA, and the subgroups of SCF/TPA targets whose activation is dependent or independent of Mitf, as well as those whose induction by SCF or TPA is sensitive versus resistant to cycloheximide (raw data are available as Supplemental Data at http://www.cell.com/cgi/content/full/109/6/707/DC1). As summarized in Figure 1D, a total of 204 genes were upregulated at multiple

time points by SCF/TPA (≥2-fold), with 128 displaying independence of Mitf (resistance to overexpression of dnMitf) and 76 displaying Mitf dependence (induction that was lost in the presence of dnMitf). Of the Mitf-independent targets, 20 were sensitive to cycloheximide treatment (likely indirect targets of SCF/TPA), while 108 were resistant to cycloheximide. Of the Mitf-dependent targets, 40 were sensitive to cycloheximide, and 36 were resistant.

A group of SCF/TPA targets that internally validates the experiment contains the immediate-early genes *JUN*, *FOS*, *EGR1*, and *MYC*. These genes were all significantly induced, and their induction was cycloheximide resistant, as anticipated for immediate-early genes. Furthermore, whereas induction of *MYC* and *EGR1* was unaffected by dnMitf, both *JUN* and *FOS* induction were blocked by dnMitf, suggesting a connection (either direct or indirect) between Mitf activity and the signaling pathway through which these factors are induced downstream of Kit and TPA in melanocytes. Importantly, the resistance of *MYC* (as well as the vast majority of genes on the microarray) to dnMitf helps to validate a specific role of Mitf for the genes whose expression is modulated by dnMitf, though the directness of the relationships cannot be assessed from these data alone. Ultimately, independent verification of target gene relationships is important (as shown for *BCL2* below); however, to qualify as a target, a gene must be upregulated independently by both SCF and TPA (in separate experiments) and then at multiple time points.

Table 1 shows selected sets of Mitf-dependent and -independent SCF/TPA target genes identified by this screen. Among the potential targets (further analysis will be presented elsewhere, M.H. and D.E.F., unpublished data) the antiapoptotic factor *Bcl2* was noteworthy because *Bcl2* homozygous knockout mice (Veis et al., 1993) exhibit dramatic depigmentation due to loss of melanocytes shortly after birth (Kamada et al., 1995; Yamamura et al., 1996). Therefore, a possible relationship between Mitf and *Bcl2* was further investigated.

Mitf and *Bcl2* Interact Genetically In Vivo

To examine genetic interactions between *Mitf* and *Bcl2* in vivo, we generated compound heterozygote crosses for a *Bcl2* null allele (which is recessive) as well as the vitiligo mutation of *Mitf*, $Mitf^{vit}$ (which is also recessive). The $Mitf^{vit}$ mutation weakly diminishes the DNA binding affinity of Mitf (Steingrimsson et al., 1994; Hemesath et al., 1994). $Mitf^{vit/vit}$ mice are born black with occasional white spots and display postnatal melanocyte loss, whereas $Mitf^{vit/+}$ (heterozygous) mice are black and show no melanocyte loss over time. Similarly, mice homozygous for the targeted mutation $Bcl2^{tm1Sjk}$ (hereafter $Bcl2^{-/-}$) display profound melanocyte loss shortly after birth, whereas $Bcl2^{+/-}$ (heterozygous) mice are fully pigmented and display no age-dependent changes (Kamada et al., 1995; Yamamura et al., 1996).

$Mitf^{vit/+}$, $Bcl2^{+/-}$ compound heterozygotes displayed a striking pattern of melanocyte loss over time (Figure 2). Melanocyte loss began shortly after birth ventrally and was accompanied by slower generalized graying throughout (Figures 2A and 2B). Compound heterozygotes also displayed white snout whiskers by 8 weeks (Figure 2C). Early-onset graying was also observed in $Mitf^{vit/vit}$;$Bcl2^{-/+}$ mice (Figure 2D).

To assess whether coat color whitening resulted from melanocyte loss (versus depigmentation of viable melanocytes), a *Dct-LacZ* transgene that tags the melanocyte lineage (Mackenzie et al., 1997) was employed. In unpigmented hair follicles of mice triply heterozygous for $Mitf^{vit}$, *Bcl2*, and the *Dct-LacZ* transgene, a striking lack of LacZ-positive cells (melanocytes) was seen, relative to age-matched littermate controls (Figure 2E). Hair follicles from regions that had not yet depigmented did contain LacZ-positive melanocytes (data not shown), demonstrating that depigmentation in these mice is associated with loss of viable melanocytes. These observations demonstrate genetic cooperativity between Mitf and *Bcl2* for melanocytes.

Mitf Binding and Regulation of the *BCL2* Promoter

We next examined the *BCL2* promoter for potential Mitf binding sites that fit the E box consensus sequence CA[C/T]GTG and identified a CATGTG element 220 base pairs upstream of the transcriptional start (Figure 3A). Chromatin immunoprecipitation revealed that this E box-containing region of the *BCL2* promoter was amplified from crosslinked chromatin isolated from 501mel human melanoma cells and immunoprecipitated with anti-Mitf antibody but not from multiple controls, including an unrelated antibody, the same anti-Mitf antibody tested for binding to the 3′ untranslated region of the *BCL2* gene, or no template control (Figure 3B). In addition, the specificity of this interaction was observed under more stringent immunoprecipitation wash conditions containing lithium chloride (lanes 4 and 6). EMSA was also performed, using the E box-containing promoter region as probe and 501mel human melanoma nuclear extract as a source of MITF protein, together with αMITF monoclonal antibody for supershift. We observed an MITF-specific supershifted complex (Figure 3C) and confirmed sequence specificity of this complex by cold competition assays in which wild-type probe competed the supershift, whereas a double-point mutant probe did not. These results indicate that MITF directly occupies the *BCL2* promoter in vivo and suggest that *BCL2* may be a direct transcriptional target of MITF.

To confirm and extend the microarray observation that MITF regulates endogenous *BCL2* gene expression, primary human melanocytes and human melanoma cells were infected with adenoviruses expressing either nuclear-targeted GFP (control), wild-type MITF, or dominant-negative MITF. In melanocytes, wild-type MITF significantly upregulated *BCL2* mRNA relative to dnMITF by quantitative RT-PCR (Figure 4A). Correspondingly, in 501mel human melanoma, wild-type MITF induced and dnMITF significantly reduced endogenous *BCL2* relative to controls (Figures 4B and 4C). Finally, transient transfections revealed that the wild-type *BCL2* promoter was upregulated by MITF in a manner that was lost upon mutating the E box (Figure 4D).

Mitf Regulates *Bcl2* Levels in Osteoclasts

In addition to loss of viable melanocytes, a number of strong alleles of Mitf display defects in osteoclasts that manifest as osteopetrosis, a condition of shortened stat-

Table 1. SCF/TPA Target Genes

Select Cycloheximide-Resistant Targets

Mitf Dependent			Mitf Independent		
Acc. Number	Gene	(Fold)	Acc. Number	Gene	(Fold)
D90209	ATF4 (CREB2)	10	M65214	TCF3	2
M25269	ELK1	5.7	X96381	Erm	4.8
U85658	Transcription factor ERF-1	3.5	L19067	P65 (NF kappa B subunit)	2.2
U69126	FUSE binding protein 2 (FBP2)	4.1	M29551	PP2B	3.1
U28049	TBX2	3.5	L40027	GSK-3	2.4
U48807	Dual specific protein phosphatase	8.3	X59932	CSK	2.5
L04733	KINESIN LIGHT CHAIN	4	V00574	c-Ha-ras1	2.1
X91247	TXNRD1 Thioredoxin reductase	3.1	X98001	Geranylgeranyl transferase II	2.3
X51345	JUNB	27	J03805	Phosphatase 2A	2.9
V01512	c-fos	34	U19796	Melanoma antigen p15	2.6
X55504	Proliferating Cell Nucleolar Antigen (HG1116-HT1116)	4.1	U91930	AP-3 complex delta subunit	2.9
			X06985	HMOX1 Heme oxygenase 1	3.2
AB000584	Prostate differentiation factor mRNA	11	X99720	TPRC gene	2
X76771	Rad2 (HG4074-HT4344)	4.1	J04444	CYC1 Cytochrome c-1	2.4
X93499	RAB7	2.2	X66945	FGFR1	6
X57522	TAP1 Transporter 1, ABC	3.1	L08246	MCL1	4.5
			J03161	SRF	2.4
M14745	BCL-2	2.5	L76191	IL-1 receptor-associated kinase	2.7
			X56681	JunD	2.8
			S81914	IEX-1	55
			X52541	EGR1	29
			X59798	CCND1 Cyclin D1	10
			M13930	C-Myc, Alt. Splice 3 (HG3523-HT4899)	2.5
			M58286	TNFR1	12.1
			L07395	PP1, Alpha Catalytic Subunit (HG1614-HT1614)	2
			X06614	Receptor of retinoic acid	10.8
			D78577	YWHAH	3.4
			M13194	ERCC1	3.4

Select Cycloheximide-Sensitive Targets

Mitf Dependent			Mitf Independent		
Acc. Number	Gene	(Fold)	Acc. Number	Gene	(Fold)
S87759	PP2C alpha	2.1	L20298	CBFB	2.1
L38810	TRIP1	2.4	M95929	PHOX1	2.2
M12128	Tropomyosin Tm30nm (HG3514-HT3708)	2.2	U29680	Bfl-1	2.6
L11066	Mitochondrial Stress-70 Protein	2.2	U84388	CRADD	2.7
Z49099	Spermine synthase	3.2	D17547	TRP 2/DCT	2.6
J03407	Ret finger protein (HG2825-HT2949)	2.2	U18934	TYRO3	3.2
Z24725	mig-2	4.1	U21090	DNA polymerase delta small subunit	3.4
D14878	Protein D123	2.8			
U14603	PTPCAAX2	2.9			
U50523	BRCA2 region, sequence CG037	2.2			

Selected genes from each category shown in Figure 1D (raw data available as Supplemental Data at http://www.cell.com/cgi/content/full/109/6/707/DC1.

ure due to hyperdense bones with partial ablation of the marrow cavity (Moore, 1995; Weilbaecher et al., 1998). Osteopetrosis in *Mitf* mutants is associated with diminished numbers of fully developed, multinucleated osteoclasts. Whereas melanocytes are entirely absent in *Mitf*$^{mi/mi}$ mutant mice, osteoclasts can be isolated from these mice (likely due to partial compensation by the Mitf family member TFE3), and these osteoclasts permit analysis of whether *Bcl2* expression is diminished in the setting of Mitf deficiency.

Bcl2 protein in *Mitf*$^{mi/mi}$ osteoclasts is greatly reduced in comparison to wild-type littermate controls (Figure 5A). This decrease is not due to an overall defect or reduction in cell number, because expression of the osteoclast marker genes RANK and Fms were comparable in both osteoclast types (data not shown). Moreover, levels of Bad, a proapoptotic member of the Bcl2 family, and α-tubulin were comparable. In addition, using the same adenoviral infection system, we observed that Mitf regulates endogenous *BCL2* mRNA levels in primary human osteoclasts (Figure 5B). This response was specific to *BCL2* since levels of *GAPDH* and the osteoclast receptor *FMS* (data not shown) were unchanged.

Since *Mitf*$^{mi/mi}$ mice display severe osteopetrosis, we examined the possibility that loss of *Bcl2*, as a target of Mitf, might similarly produce osteopetrosis, a phenotype potentially consistent with the short stature and "hunchback" appearance originally described for these mice (Kamada et al., 1995; Nakayama et al., 1994; Veis et al., 1993). Bone densities of *Bcl2*$^{-/-}$ mice and wild-type

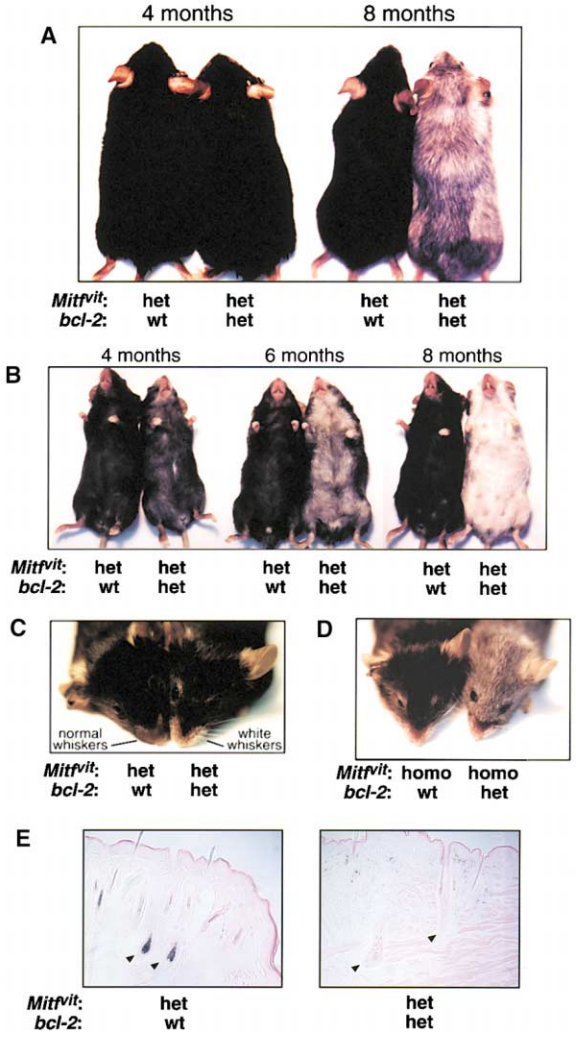

Figure 2. *Mitf* and *Bcl2* Genetically Interact In Vivo

Mitf-vitiligo and *Bcl2* double heterozygotes (n = 49) exhibit dorsal (A) and ventral (B) depigmentation compared to *Mitf-vitiligo* heterozygous control littermates (n = 63).

(C) Whisker depigmentation was seen within 8 weeks in compound heterozygotes.

(D) $Mitf^{vit/vit}$ /$Bcl2^{+/-}$ animals (n = 29) also exhibited depigmentation relative to *Mitf-vitiligo* littermate controls (n = 11).

(E) X-Gal staining of skin sections prepared from the whisker region of 8-week-old *Dct-lacZ* transgenic mice either heterozygous for *Mitf-vitiligo* (left) or compound heterozygous for *Mitf-vitiligo* and *Bcl2* (right). Hair follicles (indicated by arrow heads) contain $LacZ^{+}$ cells (melanocytes) in *Mitf-vitiligo* heterozygotes (controls), but not in compound *Mitf-vitiligo*/+;$Bcl2^{-/+}$ heterozygotes.

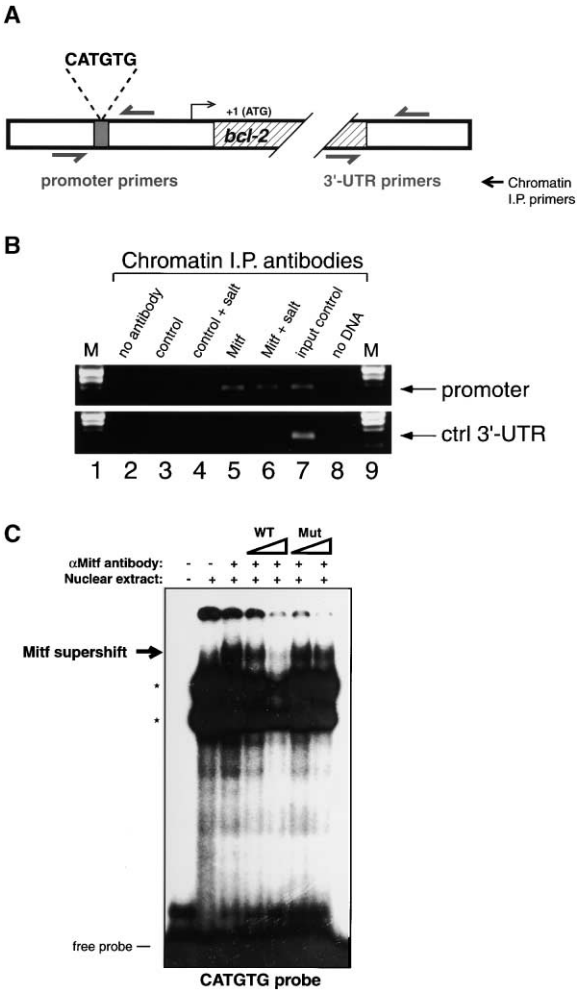

Figure 3. Mitf Binds the *BCL2* Promoter

(A) Diagram of the human *BCL2* gene promoter region showing putative Mitf binding site (filled gray box).

(B) Chromatin immunoprecipitations were performed from log-phase 501mel human melanoma cells. No DNA (lane 8); protein:chromatin-crosslinked complexes immunoprecipitated with either control antibody (lanes 3 and 4), Mitf antibody (lanes 5 and 6), or no antibody (lane 2); and input genomic DNA (lane 7) were run with DNA markers (M, lanes 1 and 9) and stained with EtBr. PCR primers spanning either promoter or 3′ UTR (control) regions were employed.

(C) EMSA was performed on the putative Mitf binding site shown in (A) using 501mel nuclear extract with ^{32}P-labeled DNA probe. Mitf bound complexes were identified by supershift, and cold competition was performed in 5- or 25-fold excess unlabeled wild-type or mutant DNA probes. Asterisks show non-Mitf-containing complexes.

littermate controls were inspected radiographically as well as histologically. Significantly increased bone density was observed in the femurs of 2-week-old $Bcl2^{-/-}$ mice (Figure 5C). Histologically, osteopetrosis was prominent in the metaphysis of long bones (Figure 5C), where considerable chondro-osseous trabeculae (primary spongiosa) accumulated with extensive thickening compared to age-matched wild-type littermates. Marrow spaces were diminished in size, and the cortical plate of the long bones was thicker than normal. These observations suggest that, in addition to other organ systems affected, $Bcl2^{-/-}$ mice also display significant bone resorption defects similar to those seen in $Mitf^{mi/mi}$ mice.

Mitf Is Critical for Melanocyte and Melanoma Cell Survival

If modulation of *BCL2* expression by Mitf were functionally relevant for melanocyte or melanoma cell viability, then interference with Mitf activity may affect survival in

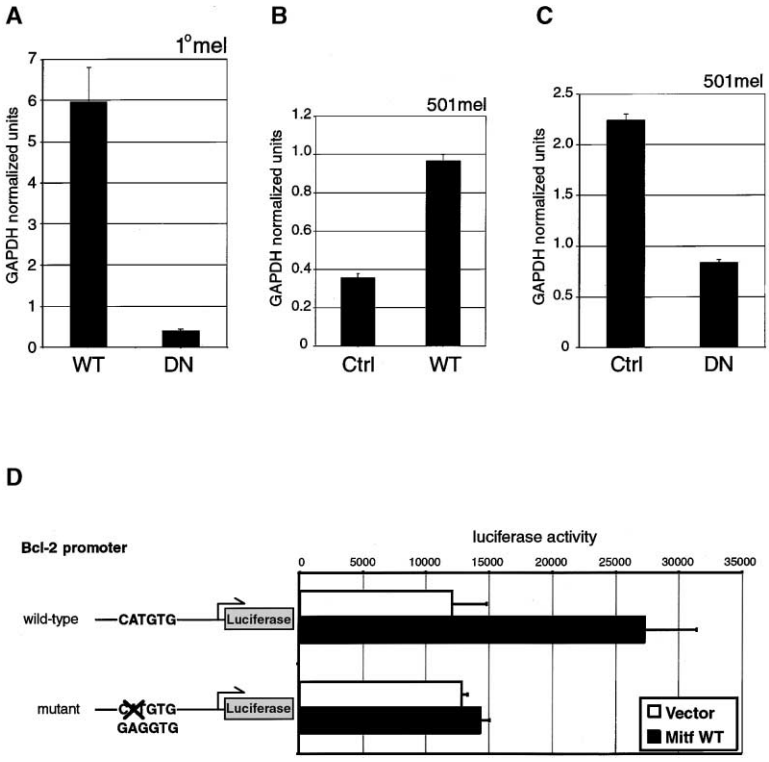

Figure 4. Mitf Regulates Endogenous *BCL2* in Melanocytes and Melanoma

Quantitative RT-PCR analysis of *BCL2* RNA from (A) primary human melanocytes infected with either Mitf wild-type (wt) or dominant-negative (DN) adenovirus (MOI: 200) after 72 hr, (B) 501mel human melanoma infected with either control (Ctrl) or Mitf wild-type (wt) adenovirus (MOI: 200) after 48 hr, or (C) 501mel cells infected with control (Ctrl) or Mitf dominant-negative (DN) adenovirus (MOI: 500) after 72 hr. *BCL2* levels were normalized to *GAPDH* and performed in triplicate.

(D) Luciferase reporter assays demonstrate E box-dependent responsiveness of the human *BCL2* reporter to Mitf.

these cells. To address this question, we asked whether dominant-negative Mitf impacts on viability of primary melanocytes and melanoma cells. Using the same adenovirus system described above, we observed that dominant-negative Mitf produced a significant increase in primary melanocyte death in culture over wild-type infected cells (Figure 6A). A similar pattern of cell death occurred in human melanoma cells. The effects of dnMitf were compared to a different mutant of Mitf defective in dimerization and functionally null (Hemesath et al., 1994). dnMitf, but not the recessive allele, produced substantial apoptosis in human melanoma cells (Figure 6B). To determine whether *BCL2* overexpression from a constitutive promoter could rescue dnMitf-induced apoptosis in melanoma cells, we quantitated the subdiploid (apoptotic) population in melanoma cells that stably overexpress *BCL2* off the LTR of pBABE-*BCL2*-puro. Moreover, cell death induced by dnMitf was significantly rescued by constitutive *BCL2* overexpression (Figure 6C). Of note, although *BCL2* measurably rescued apoptosis in the presence of dnMitf, it could not rescue colony formation in separate experiments (data not shown), consistent with the possibility that Mitf regulates additional target genes that are essential to proliferation or survival. The identification of *BCL2* as an Mitf target, but not the only important target, is also consistent with the observation that *Bcl2* null mice lose pigmentation after birth, whereas *Mitf* null mice are devoid of melanocytes already at birth.

If the Mitf:*BCL2* relationship were maintained in melanomas, then their expression patterns in patient samples should correlate. Alternatively, *BCL2* expression might be disconnected from its regulators, as in t14;18 of follicular lymphoma. Expression profiles of patient-derived primary human melanomas were examined for *MITF* and *BCL2* levels. As shown in Figure 6D, a very tight correlation was seen in their expression patterns. Among these tumors, several display relatively low *MITF* expression, yet *MITF* expression even in these samples is significantly higher than in most nonmelanomas examined in identical microarray analyses (data not shown). Thus, expression of *MITF* and *BCL2* is tightly correlated in human melanomas.

Discussion

Microarray Screen for Stem Cell Factor/Kit Target Genes

The present study provides a systematic array-based approach to the identification of SCF/Kit-induced transcriptional targets in the melanocytic lineage. SCF signaling has a substantial impact on the transcriptional repertoire of primary melanocytes, affecting diverse target gene functional categories such as survival, proliferation, signaling, redox, DNA repair, motility, membrane trafficking, and other aspects of melanocytic differentiation. Genes that had been previously implicated as SCF/Kit-responsive in other cell types (such as *FOS*, *JUNB*, *EGR1*, and *MYC* genes in hematopoietic cells) were confirmed as Kit targets in primary melanocytes. This list also includes *BCL2*, now shown to specifically involve Mitf. Human natural killer cells have been shown to express *BCL2* in response to SCF as a survival mechanism (Carson et al., 1994). This pathway may similarly involve Mitf because NK cell defects have previously been described in Mitf-deficient mice (Ito et al., 2001). In addition, sur-

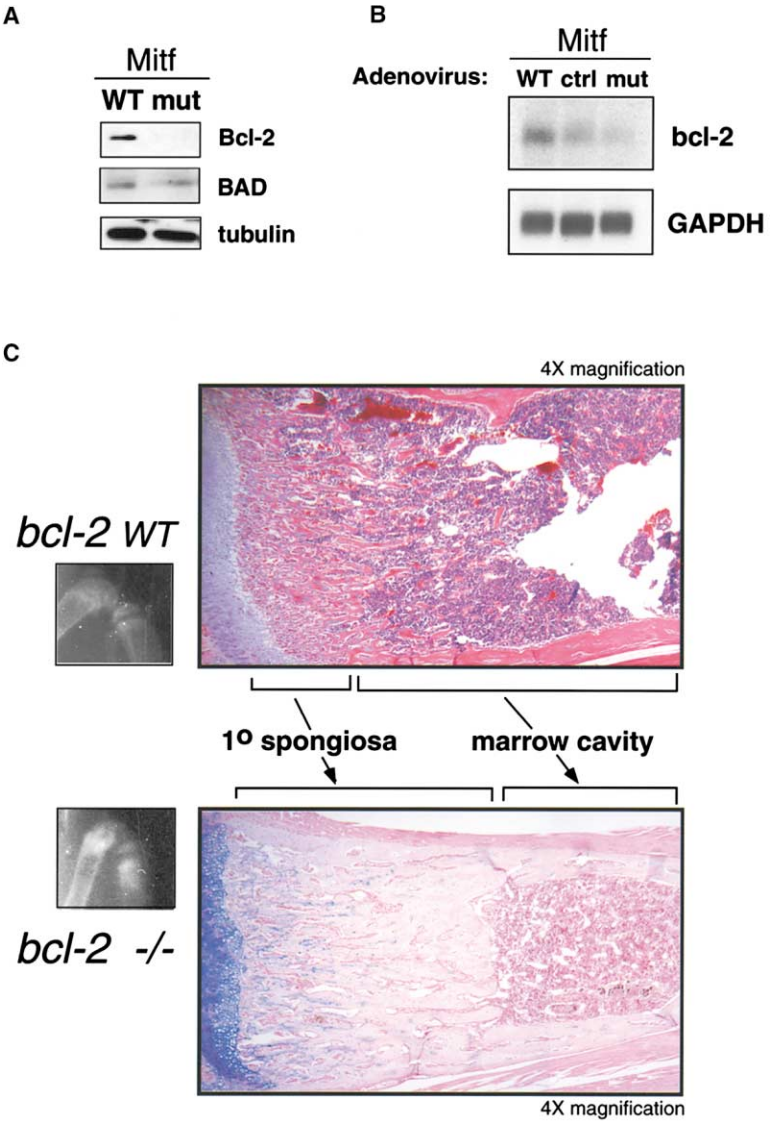

Figure 5. Mitf Regulates *Bcl2* Levels in Osteoclasts

(A) Lysates were prepared from primary murine osteoclasts isolated from the spleen of either Mitf wild-type (wt) or mutant (mut) mice and blotted for Bcl2, Bad, or tubulin.

(B) Northern blot analysis of primary human osteoclasts infected with Mitf wild-type (wt), control (Ctrl), or dnMitf (mut) adenovirus.

(C) Bone changes in 2-week-old *Bcl2*$^{-/-}$ or wild-type (wt) mice was assessed by X-ray and histochemistry (H&E, 4× magnification) and revealed expanded 1° spongiosa in *Bcl2*$^{-/-}$ mice, consistent with osteopetrosis.

vival of mast cells, another of the few Mitf-expressing lineages, appears to be modulated by Kit-induced *Bcl2* upregulation (Bullock and Johnson, 1996; Mekori et al., 2001).

A number of genes that are thought to be posttranslationally modified by SCF/Kit signaling were found to be transcriptionally regulated as well, potentially consistent with a feedback pathway that might replenish signaling intermediates whose activation also targets them for degradation. Among these are *Cdkn1a(p21), Hras1*, *Raf1*, *Src*, *Elk1*, and other members of the *Ets* family. Based on our data, *Dct/Trp2* appears to be regulated by Kit signaling, independently of Mitf, a finding consistent with maintenance of *Dct/Trp2* expression in the eyes (RPE) of *Mitf*$^{mi/mi}$ mutant mice (Nakayama et al., 1998). It is interesting that more pigment genes did not appear as Kit targets, since it has been shown that chronic subcutaneous administration of recombinant SCF to humans results in hyperpigmented melanocytes (Costa et al., 1996). It is important to consider that the current microarray screen did not extend to time points beyond 6 hr and consisted of only a single cytokine stimulation event. Therefore, it may be premature to conclude that pigment enzyme genes are not targeted by SCF/Kit (or by Mitf). The unexpected Mitf dependence of *FOS* and *JUNB* induction occurs through an unclear mechanism that may be indirect. The T box binding protein Tbx2 was also identified in this screen and was previously described as an Mitf target gene in melanocytes (Carreira et al., 2000). While a more detailed discussion of these data is beyond the scope of this report, the complete data set is available online and will be further analyzed separately (M.H. and D.E.F., unpublished data).

Kit's survival function in melanocytes appears to be independent of the PI3K/Akt pathway (Blume-Jensen et al., 2000), a finding consistent with the unique requirement for *Mitf* and *Bcl2* in this lineage. Kit potently activates MAPK, which may upregulate Mitf by two mechanisms: (1) direct Mitf phosphorylation and recruitment of p300/CBP (Hemesath et al., 1998; Price et al., 1998),

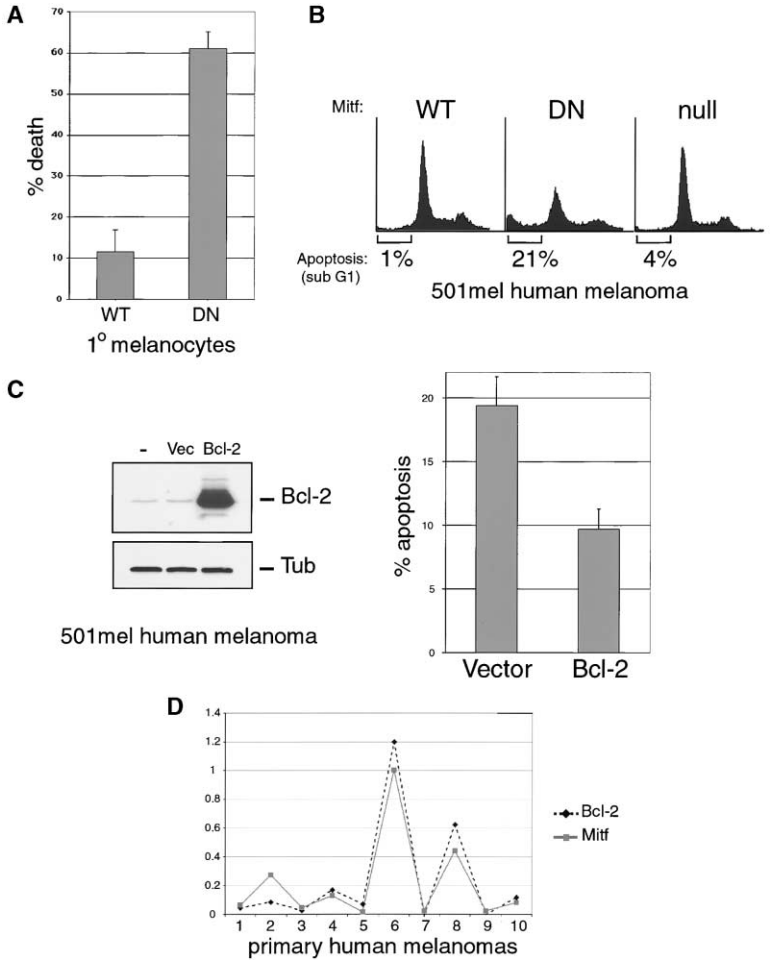

Figure 6. Mitf and *Bcl2* Are Critical for Melanocyte and Melanoma Survival

(A) Primary human melanocytes were infected (M.O.I of 1000) with Mitf wild-type (wt) or dnMitf (DN) adenovirus and harvested 48 hr later. Cell death was measured by trypan blue in triplicate.

(B) FACS analysis of 501mel human melanoma cells transfected with E-GFP and either Mitf wild-type (wt), Mitf dominant-negative (DN), or Mitf-*cloudy eyes* null mutant (null). Fixed and propidium iodine-stained cells were gated for GFP expression and sub-G1 DNA content quantitated to assess percent apoptosis.

(C) Enforced *BCL2* overexpression rescues 501mel human melanoma apoptosis induced by dnMitf. Puromycin-selected, pooled populations of 501mel cells stably overexpressing either vector control (Vec) or *Bcl2* were transfected with E-GFP and Mitf dominant-negative. Percent apoptosis in GFP-gated cells was measured as in (B).

(D) Nearest-neighbor analysis of *MITF* (squares) and *BCL2* (lozenges) mRNA levels performed on a series of primary human melanomas analyzed by Affymetrix GeneChip microarray.

and (2) CREB-mediated increased *Mitf* expression. The observation that interference with Mitf ablates Kit-induced *Bcl2* expression (Figure 1) is thus consistent with the possibility that Mitf is a central effector of this pathway.

Genetic Interaction between *Mitf* and *Bcl2*

It is noteworthy that homozygous *Bcl2* deficiency produces more gradual melanocyte loss (Kamada et al., 1995; Veis et al., 1993; Yamamura et al., 1996) than complete absence of functional *Mitf*, *Kit*, or *Scf*, which produce mice devoid of melanocytes from birth. *Bcl2* may not play a critical role in melanocyte survival early in development, or this role may be redundant with other family members or survival pathways. It is also possible that Mitf regulates other essential targets, such as cell cycle components, and that Kit signaling activates additional survival pathways to account for the delayed phenotype of *Bcl2* deficiency. In support of this, we have been unable to rescue pigment cells from *Kit* deficiency by transgenic expression of *Bcl2* in melanoblasts and melanocytes (S.A.J. and I.J.J., unpublished). In the context of $Mitf^{vit/+}$ heterozygotes, additional loss of a single *Bcl2* allele produced depigmentation, which always initiated on the belly. Ventral spotting is usually a manifestation of a neural crest migration defect, suggesting a measurable role for *Bcl2* in melanoblasts during neural crest migration. Notwithstanding their black coat color at birth, *Bcl2* homozygous null mice might harbor diminished numbers of cutaneous melanocytes, whose further loss postnatally becomes phenotypically evident only following subsequent hair follicle cycles.

BCL2 Is a Direct Target of Mitf

Chromatin IP revealed that Mitf protein occupies the endogenous *BCL2* promoter in melanoma cells. This interaction was specific both for the antibody used and for the *BCL2* promoter position and Mitf protein in melanoma nuclear extract specifically bound to the E box element in the *BCL2* promoter in EMSA. Combined with data showing that up- or downregulation of *Mitf* modulates endogenous *BCL2* expression in melanocytes, melanoma cells, and osteoclasts, these studies suggest that *BCL2* is a direct transcriptional target of Mitf. Other pathways are known to result in *BCL2* upregulation in a variety of contexts. These include CREB-mediated stimulation of the *BCL2* promoter, which regulates certain examples of neuronal signal-dependent survival (Pugazhenthi et al., 1999, 2000). This pathway might be utilized as well in melanocytes or melanoma cells and could operate either through direct effects of CREB on *BCL2* expression or the known role of CREB in upregu-

lating *Mitf* expression (Bertolotto et al., 1998; Price et al., 1998). These data are also consistent with the observation that CREB acts as a survival factor in human melanoma cells (Jean et al., 1998).

Osteopetrosis in *Bcl2*-Deficient Mice

Modulation of *Bcl2* levels by Mitf in primary osteoclasts as well as Bcl2 deficiency within $Mitf^{mi/mi}$ osteoclasts suggest that Mitf regulates *Bcl2* in these cells, a finding also consistent with the osteopetrosis discovered in $Bcl2^{-/-}$ mice, though additional cell:cell signaling could also contribute to the phenotype. Additionally, $Mitf^{mi/+}$, $Bcl2^{+/-}$ compound hets exhibited gross craniofacial and skeletal abnormalities (data not shown). Recent studies have demonstrated that M-CSF, the cytokine deficient in $Csf1^{op/op}$ osteopetrotic mice, resides upstream of Mitf within osteoclasts in a manner similar to SCF/Kit in melanocytes (Weilbaecher et al., 2001). Prior studies by Weissman and colleagues demonstrated partial rescue of the $Csf1^{op/op}$ osteoclast lineage by targeted *Bcl2* overexpression (Lagasse and Weissman, 1997). In those studies, targeted *Bcl2* expression may have coincidentally replaced a specific *Bcl2* deficiency, rather than providing purely generic survival signals.

Mitf as a Survival Factor in Melanocytes and Melanoma

A number of additional observations appear to link *MITF* and *BCL2* within human melanoma. For example, *MITF* and *BCL2* levels are maintained in most melanomas despite the common loss of many melanocytic markers, such as melanin and Kit. A number of studies have reported that *BCL2* is widely expressed in human melanoma (Cerroni et al., 1995; Ramsay et al., 1995; Selzer et al., 1998) without known *BCL2* translocations or amplification, implying a role for this antiapoptotic protein in these notoriously treatment-resistant tumors (Grover and Wilson, 1996; Selzer et al., 1998). These observations have prompted clinical trials using *BCL2* antisense oligonucleotides (Banerjee, 2001; Jansen et al., 2000), and particular efficacy in melanoma has prompted advancement to phase III trials.

In this study we confirm and extend these observations by demonstrating that *MITF* and *BCL2* are tightly correlated in a nearest neighbor analysis performed on primary human melanoma expression microarrays. It is possible that one of Mitf's critical roles in melanoma could be to regulate *BCL2* expression, thereby enhancing survival. If this were the case, disruption of the Mitf-*BCL2* pathway provides a potential tissue-restricted means of targeting melanoma antineoplastic therapy.

Homozygous *Bcl2* deficiency produces a variety of tissue-restricted phenotypes. One potentially interesting similarity between lymphocytes and melanocytes that correlates with their shared dependence on *Bcl2* is their unique exposure to DNA damage/translocation intermediates. The importance of *Bcl2* in lymphocyte survival may relate to the activation of apoptosis by recombination intermediates, a response that may similarly occur in melanocytes following UV-induced DNA damage. Just as lymphocytes must survive genomic recombinations to produce immune responses, melanocytes must survive UV to produce melanin. Given the evidence that Mitf is a central transcriptional regulator of pigmentation (Goding, 2000), it is plausible that Mitf links *Bcl2* expression to the pigmentation response. *Bcl2*'s protective effect may also be directed against the chemical stress associated with pigmentation itself, a possibility which may help explain $Bcl2^{-/-}$ melanoblast loss during neural crest migration. Unfortunately, one of the deleterious consequences of this Mitf-*Bcl2* linkage is likely to be the extreme treatment resistance of melanoma in humans.

Experimental Procedures

Cell Culture

Primary human melanocytes between passages 2 and 5 from neotal foreskins (provided by Dr. Ruth Halaban, Yale University) were grown in TICVA medium containing Ham's F10 (GIBCO-BRL), 7% FBS, penicillin/streptomycin/glutamine (GIBCO-BRL), 1×10^{-4} M IBMX (Sigma), 50 ng ml^{-1} TPA (Sigma), 1 μM Na_3VO_4, and 1×10^{-3} M dbcAMP (Sigma). Purity of cultures was 95%–99% based upon immunofluorescent staining for the D5 melanocytic marker. For microarrays, primary melanocytes were stimulated with 20 ng ml^{-1} human SCF (R&D Systems) or 10 ng ml^{-1} TPA with or without 20 μg ml^{-1} cycloheximide for indicated times after an overnight starvation in F10 medium supplemented only with penicillin/streptomycin/glutamine. 501mel human melanoma cells (gift of Dr. Halaban, Yale Univ.) and osteoclasts were cultured as described (Motyckova et al., 2001; Wu et al., 2000).

Adenoviral Infections

Adenoviruses were previously described (Motyckova et al., 2001) and overexpress either wild-type human Mitf, R215del (dnMitf) or a GFP/wee1-truncation hybrid (which targets GFP to the nucleus as vector control) under control of the EF-1α promoter. Subconfluent primary human melanocytes and 501mel human melanoma cells were incubated with concentrated adenoviruses in serum-free F10 supplemented with 10 mM $MgCl_2$ for 30 min at a multiplicity of infection of 200 and 500, respectively. After infection the medium was replaced by fresh, fully supplemented growth medium and cultured for indicated times until stimulation or harvest.

High-Density Oligonucleotide Array-Based Expression Profiling

RNA was extracted at 0, 0.5, 1, 2, 4, and 6 hr after SCF or TPA treatment of primary human melanocytes. SCF stimulation was carried out in the absence of adenovirus, while TPA stimulation occurred 64 hr after infection with control or dnMITF adenoviruses. RNA was prepared and hybridized as described (Coller et al., 2000; Lockhart et al., 1996). Each RNA sample was hybridized to a single high-density oligonucleotide array (HU_6800, Affymetrix) that enabled interrogation of the expression levels of 6800 human genes and ESTs. With slight modifications, expression data were analyzed as previously described (Tamayo et al., 1999). Overall intensity for each chip was equivalent by scaling of intensity values. Fluorescence intensities were detected with a laser confocal scanner (Hewlett-Packard) and processed with Genechip software (Affymetrix). Small and negative values were thresholded to 100 units. Raw expression values were normalized within an experiment to a mean of zero and a standard deviation of 1.

Significant induction of transcription was defined as: (1) an increase of $\geq$2-fold relative to the 0 hr baseline; (2) for genes exhibiting a trough pattern of gene expression after SCF, induction criteria were $\geq$2-fold relative to the time point with lowest expression prior to the induction during the time course (with at least 1.5-fold induction relative to the 0 hr baseline); (3) induction was present in two or more time points following stimulation; (4) a target gene was considered Mitf-dependent if the ratio of its maximum induction in the presence of control virus to the maximum induction in the presence of dnMitf was $\geq$2; (5) a target gene was considered cycloheximide-sensitive if the ratio of its maximal signal in the absence of cycloheximide relative to the presence of cycloheximide was $\geq$2.

Mouse Crosses and Histology

Heterozygous *Bcl2*$^{-/-}$ null mice (B6,129S-*Bcl2*^{tm1Sjk}) were bred to *Mitf*$^{vit/vit}$ mice (C57,BL/6J-*Mitf*^{vit}) to generate compound heterozygotes. F1 animals were crossed to obtain *Mitf*$^{vit/vit}$ mice heterozygous for *Bcl2* deficiency. Genotyping was performed as described (Veis et al., 1993). X-rays of 2-week-old *Bcl2*$^{-/-}$ mice and controls were taken at Boston's Children's Hospital Radiology Department. For histological studies, bones were removed, fixed in 4% paraformaldehyde/PBS for 12 hr, decalcified in Morse's solution (10% sodium citrate and 22.5% formic acid) for 12 hr, dehydrated through a graded alcohol series, cleared in xylene, and embedded in paraffin. Sections of 6–8 μm were stained with alcian blue and with hematoxylin and eosin using standard protocols. LacZ histology was performed as described (Nishimura et al., 2002).

EMSA and Luciferase Assays

Nuclear extracts were prepared from 501mel melanoma cells as described (Wu et al., 2000). A *BCL2*-specific double-stranded probe spanning the E box was prepared using the following sequences for sense oligos: wildtype probe, 5′-CCCCGCGCGCCATGTGCCC CCGGCGG; mutant probe, 5′-CCCCGCGCGCCATATACCCCCG GCGG.

For Luciferase reporter assays, a ~1.8 Kb fragment of the human *BCL2* promoter and 5′ UTR was fused in-frame to the luciferase initiation ATG in pGL3-basic (Promega) and mutated using Quickchange (Stratagene) with 1 M betaine (Sigma). NIH-3T3 cells were transfected as described (Wu et al., 2000) using Dual-Luciferase (Promega) with normalization to Renilla luciferase, and mean ± standard error was determined from at least three data points.

Chromatin Immunoprecipition (ChIP)

Chromatin immuoprecipitation of human *BCL2* sequences from 501mel human melanoma cells was performed as in Strahl-Bolsinger et al. (1997). PCR (iCycler, BioRad) was carried out using primers specific to the promoter region of human *BCL2* (FWD, 5′-TCCGCAG GCCTGAGCAGAA-3′; and REV, 5′-AAGAAAGAGCCCTCCTCTGAG CCA-3′) or spanning the human *BCL2* coding region/3′-UTR boundary (FWD, 5′-GATGCCTTTGTGGAACTGTACGGCCC-3′; and REV, 5′-TTTTCTTAAACAGCCTGCAGCTTT-3′).

RNA and Protein Analyses

RNA was isolated using the Ambion RNAqueous kit (Ambion) and quantitated by spectrophotometry (Beckman). TaqMan One-Step RT-PCR Master Mix Reagent as well as *GAPDH* Control Reagents (Applied Biosystems, CA) were used for quantitative RT-PCR reactions each containing 100 ng of total sample RNA. Reactions were run for 40 cycles under the following conditions: stage 1, 48°C, 30 min; stage 2, 95°C, 10 min; stage 3, 94°C, 20 s; stage 4, 62°C, 1 min. Human *BCL2* message was detected using the primers forward 5′-CGCCCTGTGGATGACTGAGT-3′ and reverse 5′-CCCAGCCTC CGTTATCCTG-3′ (IDT) and TaqMan probe 6FAM-ACCGCCACCTG CACACCTGGAT-TAMRA (Applied Biosystems, CA). All reactions were run in triplicate on an ABI-PRISM 7700 instrument (Applied Biosystems, CA), and *BCL2* message levels were normalized to *GAPDH* expression. Northern blots were carried out as previously described (Motyckova, 2001). Western blots were performed on 10 day osteoclast cultures as described (Motyckova, 2001) using anti-Bcl2 at 1:2000 dilution (C-2, Santa Cruz, CA).

Apoptosis Analysis

501mel melanoma cells were infected with retrovirus encoding pBABE-*Bcl2*-puro or pBABE-puro control. Following selection with 2 μg/ml puromycin (GIBCO) for 2 days, mixed populations of clones were analyzed by FACS. Vector or *Bcl2*-expressing lines of 501mel were plated at 10^6 cells per 10 cm dish and transfected with lipofectamine using 4 μg dnMitf or vector control and 1 μg EGFP-ER. Attached and floating cells were harvested 48 hr after transfection, fixed in 70% ethanol, and resuspended in 1% PI, and 10,000 GFP-expressing cells were analyzed by FACSCaliber (Becton Dickinson) to quantify the sub-G1 population.

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