Microphthalmia-Associated Transcription Factor Is a Critical Transcriptional Regulator of Melanoma Inhibitor of Apoptosis in Melanomas

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Abstract
Melanoma inhibitor of apoptosis (ML-IAP) is a potent inhibitor of apoptosis, which is highly expressed in melanomas and likely contributes to their resistance to chemotherapeutic treatments. Herein, we show that the lineage survival oncogene microphthalmia-associated transcription factor (MITF) is a critical regulator of ML-IAP transcription in melanoma cells. The ML-IAP promoter contains two MITF consensus sites, and analysis of MITF and ML-IAP mRNA levels revealed a high correlation in melanoma tumor samples and cell lines. In reporter assays, MITF promoted a strong stimulation of transcriptional activity from the ML-IAP promoter, and MITF bound the endogenous ML-IAP promoter in melanoma cells by chromatin immunoprecipitation and electrophoretic mobility shift assay. Strikingly, small interfering RNA (siRNA)–mediated knockdown of MITF in melanoma cells led to a dramatic decrease in ML-IAP mRNA and protein levels, establishing that ML-IAP expression in melanoma cells is MITF dependent. Additionally, cyclic AMP–mediated induction of MITF expression in melanocytes resulted in increased ML-IAP expression, suggesting that melanocytes can express ML-IAP when MITF levels are heightened. Disruption of MITF by siRNA led to a decrease in melanoma cell viability, which could be rescued by ectopic expression of ML-IAP. Collectively, these findings implicate MITF as a major transcriptional regulator of ML-IAP expression in melanomas, and suggest that ML-IAP contributes to the prosurvival activity of MITF in melanoma progression. [Cancer Res 2008;68(9):3124–32]

Introduction
Inhibitor of apoptosis (IAP) proteins are a family of antiapoptotic regulators that block cell death in response to diverse stimuli through interactions with inducers and effectors of apoptosis (1). Melanoma IAP (ML-IAP; also known as Livin, KiAP, and BIRC7) is a potent antiapoptotic protein that is highly expressed in melanomas and other cancers, but not in most adult tissues (2–5). IAP proteins inhibit apoptotic stimuli that signal either intrinsically, such as intracellular damage, or extrinsically, in the case of signaling via death receptor complexes, pathways (1). These stimuli lead to activation of caspases, cysteine-dependent aspartyl-specific proteases that are critical for the execution of programmed cell death (6, 7). X chromosome–linked IAP (XIAP) protein is an IAP family member that can bind to and potently inhibit caspase-3, caspase-7, and caspase-9 (8). After cells receive death stimuli, second mitochondria-derived activator of caspases (SMAC) is released from mitochondria into the cytosol where it binds to XIAP and abrogates its caspase-inhibitory activity (9, 10). ML-IAP, on the other hand, seems to primarily exert its prosurvival properties by binding to SMAC via its conserved baculovirus IAP repeat (BIR) domain, overcoming SMAC antagonism of XIAP-mediated caspase inhibition, and thus effectively halting apoptosis (2, 4, 11).

At present, little is known about how ML-IAP tissue- and tumorspecific expression is achieved. In this study, we focused specifically on elucidation of a molecular mechanism for transcriptional regulation of ML-IAP in melanoma. Melanoma is a neoplasm that originates from melanocytes and is highly resistant to chemotherapeutic and radiological treatments (12). ML-IAP has been shown to be highly expressed in the majority of melanoma patient samples surveyed, as well as in a number of melanoma cell lines (2, 13). However, in the same studies, ML-IAP expression was not detected in benign melanocytic proliferation or normal melanocytes (2, 13). Mechanisms describing how ML-IAP expression may be up-regulated during melanocytic transformation have not yet been reported, although it is noteworthy that ML-IAP maps to 20q13.3, a region frequently amplified in melanomas (14). Understanding the regulation of ML-IAP expression in melanomas may contribute to the ongoing efforts to overcome resistance to apoptosis in melanoma through apoptosis-based therapeutics (15).

Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix-leucine zipper protein (16). Nine different isoforms of MITF with various tissue specific expression patterns have been reported, including the melanocyte-specific isoform MITF-M (17). The MITF-M isoform is expressed specifically in melanocytes and melanomas where it transcriptionally regulates genes for melanogenesis, cell survival, and differentiation (16). A nonfunctional mutation of the Mitf gene in mice leads to a complete absence of melanocytes and consequent lack of coat color (16). In humans, MITF mutation causes Waardenburg syndrome type IIA, resulting in melanocyte deficiencies in the skin and inner ear, hypopigmentation, and deafness (18). The MITF gene is amplified in 15% to 20% of metastatic melanomas (19), and it is considered a lineage survival oncogene due to its requirement for melanocyte proliferation and differentiation and high expression level in the majority of melanomas (16, 20). As a member of the MYC family of transcription factors, MITF binds to the consensus E box sequence CA(C/T)GTG (21–23) within target genes such as cyclin-dependent kinase 2 (CDK2) and the antiapoptotic factor BCL-2 (24, 25). To date, both CDK2 and Bcl-2 have been implicated as important mediators of the effects of
MITF on melanoma proliferation and survival (24, 25). However, there are likely additional MITF targets that also contribute to the role of MITF in melanomas.

Here we report that the ML-IAP promoter contains two functional MITF binding sites and that ML-IAP and MITF-M mRNA levels are well correlated in melanoma patient samples and cell lines. We show that MITF binds to the ML-IAP promoter and transcriptionally regulates ML-IAP expression using luciferase reporter assays, chromatin immunoprecipitation, electrophoretic mobility shift assay, and small interfering RNA (siRNA) knockdown of MITF in melanoma cells. Additionally, up-regulation of MITF expression in melanocytes and melanoma cells was sufficient to elevate ML-IAP mRNA levels. Lastly, MITF modulation of ML-IAP expression was shown to play a vital role in melanoma cell survival.

Materials and Methods

Cell culture and media. A375, A2058, SK-MEL-28, and UACC62 human melanoma and Hek293T human embryonic kidney cells were obtained from American Type Culture Collection and maintained in 50% RPMI 1640/DMEM with 10% fetal bovine serum (FBS). 888 and 624 melanoma lines were maintained in DMEM with 10% FBS in the absence of antibiotics (as described in ref. 2). Normal human epidermal melanocytes were purchased from Cambrex and maintained in MMB-4 medium supplemented with the MGM-4 bullet kit (all Cambrex).

In situ hybridization. PCR primers were designed to amplify a 447-bp fragment of human ML-IAP spanning nucleotides (nt) 21 to 447 of NM_139317.1 (upper, 5′-CAAGTGCTGCACGGGCACC-3′; lower, 5′-GGGAAACCACCTGGCATGCTC-3′) or a 451-bp fragment of human MITF spanning nt 1 to 450 of NM_000248 (upper, 5′-ATGCTGGAAAGATCTGGAATAT-3′; lower, 5′-GACGGGCAAGTATTGGTCC-3′). Primers included extensions encoding 27-nt T7 or T3 RNA polymerase initiation sites, which were removed by incubation in 20 μg/ml RNase A for 30 min at 37°C, followed by a strong stringency wash at 53°C in 0.1× SSC for 2 h and dehydration through a graded ethanol series. The slides were dipped in NTB nuclear track emulsion (Eastman Kodak), exposed in sealed plastic slides, and dehydrated through a graded ethanol series. The slides were dipped in NTB nuclear track emulsion (Eastman Kodak), exposed in sealed plastic slides, and dehydrated through a graded ethanol series. The slides were dipped in NTB nuclear track emulsion (Eastman Kodak), exposed in sealed plastic slides, and dehydrated through a graded ethanol series.

Bioinformatic analysis. The indicated cell lines were treated with 10 μmol/L forskolin (Sigma) for 24 h, 30 μg/ml Wnt3a for 24 h, 10 μmol/L Letoposide (Sigma) for 5 h, or 50 ng/μl tumor necrosis factor α (TNFα; Genentech, Inc.) for 5 h. RNA was extracted with QiAshedder columns and the RNeasy MiniKit (all Qiagen). For real-time PCR analysis, 100 ng of total RNA were used per standard 50-μl reaction volume. Fold change in gene expression was calculated using the comparative CT method of relative quantitation. Standard curves were generated for all primer probe sets to confirm linearity of signals and relative efficiency over the experimentally measured ranges. The following sequences were used: for detection of ML-IAP, 5′-TTCGCCAGACCGCC (F) and 5′-AAAGGTGCTAGGTTGACTCC (R); and 5′-FAM-AGCAGGCTCGTCTGTGGCGCT-TAMRA (probe); for MITF-M, 5′-TCTCTTTGGCAGTTACCTCCA (R), 5′-FAM-ACGGCTTCTCAGTGGGATTGG-TGCGA-TAMRA (probe) for Aoxin2, 5′-CTCCGGTGTCCTGCAT (F), 5′-CATGCAAAATGCTTATTTGACAG (R), and 5′-FAM-TTGAGGCT-TCAAGCTTTCTCCTGGGATGTTA-TAMRA (probe); for MCP-1, 5′-GGGAAATGCTTCTCCTGTCG (F), 5′-CTTGGATATATTTGAGCAGAAC (R), and 5′-FAM-TGGCCTGGACTTTCCTTCCTCGG-TAMRA (probe); and for hRPL19, 5′-ACGGGATCTCCTGAGGAAAC (F), 5′-CTGGTCAGCCGAGAGG (R), and 5′-FAM-TCCAAACAGGGCAGAG-GAAG-TAMRA (probe).

Construction and mutagenesis of ML-IAP reporter constructs. A 2.8-kb fragment containing the 5′-untranslated region and upstream sequence of ML-IAP was amplified from human BAC clone 3243M2 (Invitrogen) using primers 5′-CACCCCAAGCTTCACTCTGGCAACGTG (F) and 5′-CTTGCCACTGCTTCTGTGGCTG (R). The smaller 1.6-kb fragment ML-IAP Pro 4 was subsequently amplified from the same BAC clone or genomic DNA (Roche) using the same reverse primer as used for ML-IAP Pro 4 and the following forward primers: ML-IAP Pro 4, 5′-GACTTCTAGGAGCCCTCCTGCTCC (F) and 5′-GCTAAGGTTTGAGCGCAGCATGGCTTG (R). The other ML-IAP promoter constructs were amplified from either BAC clone or genomic DNA (Roche) using the same reverse primer as used for ML-IAP Pro 4 and the following forward primers: ML-IAP Pro 3, 5′-GACTTCTAGGAGCCCTCCTGCTCC (F) and 5′-GCTAAGGTTTGAGCGCAGCATGGCTTG (R). ML-IAP Pro 2, 5′-GCTACTGAGGTTTGAGCGCAGCATGGCTTG (F) and 5′-GCTACTGAGGTTTGAGCGCAGCATGGCTTG (R). ML-IAP Pro 1, 5′-GACTTCTAGGAGCCCTCCTGCTCC (F) and 5′-GCTACTGAGGTTTGAGCGCAGCATGGCTTG (R).

 Luciferase reporter assay. Hek293T cells, seeded at 8 × 10⁴ per well in six-well dishes, were transfected the following day using Lipofectamine 2000 with ML-IAP reporter constructs, pEGFP-N1, or pGL4.1 together with either pGFP-MITF or pEGFP alone, or in combination with lymphoid enhancer factor-1 (LEF-1) or β-catenin S45Y constructs and pRL-Renilla (Promega). A total of 4 μg of DNA were used for each transfection. Cell lysates were prepared 48 h posttransfection; the activities of firefly and Renilla luciferase were assayed using a Dual Luciferase kit (Promega) according to the manufacturer's recommendations. Luciferase assays were performed as described (27).

Electrophoretic mobility shift assays. Nuclear extracts from 624 cells were prepared using the NE-PER kit (Pierce) according to the manufacturer's instructions. Synthesis and biotin end-labeling of probes were carried out at Genentech, and single-stranded complementary oligos were annealed to produce the double-stranded probe. The sequences of the probe containing the MITF E1 site in the ML-IAP promoter used to detect sequence-specific MITF binding in vitro were 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG and 5′-CCGTCGACAGAGCATGTCGACAGCGGCAGAGCGCAG.
5’-GGCCTCTGGGGTCACATGCTCTGTGCAGCG. Binding reactions were done using the LightShift kit (Pierce). Briefly, 4 µg of 624 nuclear extract and binding buffer were incubated on ice for 20 min in a volume of 20 µL, then the labeled probe (20 fmol) was added, and the reaction was allowed to incubate for an additional 20 min at room temperature. For competition and supershift analyses, unlabeled DNA probe (in 25- to 200-fold excess), anti-MITF antibody (Calbiochem; 1 µg per reaction), or mouse immunoglobulin IgG1 (BD Biosciences; 1 µg per reaction) was added to the reaction mixture during the initial 20-min incubation. The reaction products were separated on a 4% native polyacrylamide gel run in 0.5 Tris-borate EDTA. Following electrophoresis, the DNA-protein complexes were transferred onto nylon membranes and detected by chemiluminescence (LightShift kit, Pierce).

Expression vectors, siRNAs, and transfection. pEgFP-MITF was constructed by transferring the ultimate ORF clone IOH45654 insert into pcDNA-DEST53 using LR clonase (all Invitrogen) according to the manufacturer’s protocol. pCANmycLEF1, pCANmycβ-catenin S45Y, and pTopglow were kindly provided by Bonnee Rubinfeld. Constructs expressing Myc-ML-IAP, FLAG-ML-IAP, and FLAG-ML-IAP K138A have previously been described (2, 11). For siRNA and DNA transfections, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s instructions. siRNA oligonucleotides were synthesized at Genentech. The following siRNA pairs were used for MITF knockdown experiments: 1, 5’-GAACGAAGAAGAAGATTTAdTdT and 5’-TAAATCTTCTTCTTCGTTCdTdT; 2, 5’-GCAGATGGATGATGTAATCdTdT and 5’-GCAGATGGATGATGTAATCdTdT; 3, 5’-GACCTAACCTGTACAA-CAAdTdT and 5’-TTGTGTCAACGTTAGGTCdTdT; 4, 5’-AGACGGAGCACA-CTTGTTGATdTdT and 5’-TACAAAGTGTTGCTCCGTCdTdT; and 5, 5’GGTGAATCGGATCATCAAGdTdT and 5’-CTTGATGATCCGATCCACCdTdT. Scramble II (Dharmacon) siRNA was used for a negative control in siRNA transfections.

Western blot analysis, immunoprecipitation, and antibodies. Western blot analyses and immunoprecipitation were done as previously described (2). Monoclonal antibodies against α-actin (ICN Biomedicals), CDK2 (Lab Vision), MITF (Calbiochem), M S (Upstate), and FLAG (Sigma) were purchased. Monoclonal antibodies against human ML-IAP were generated at Genentech.

Viability and apoptosis assays. 886 melanoma cells were seeded at a density of 5 × 10^4 per well in 24-well plates or 1 × 10^5 per well in 96-well plates and cotransfected with expression vectors and siRNA as described above. Twenty-four hours posttransfection, apoptosis was measured by analyzing caspase-3/caspase-7 activity using the Homogeneous ApoOne Caspase-3/7 Assay kit (Promega) according to the manufacturer’s instructions. Cell viability was measured 48 h posttransfection by Neutral Red uptake as described (28).

Results

MITF is a candidate transcriptional regulator of ML-IAP. To identify transcription factors that could influence the melanoma-specific expression of ML-IAP, the putative promoter region encompassing 1 kb upstream of the translation start site.

Figure 1. MITF is a candidate transcriptional regulator of the human ML-IAP locus. A, schematic representation of the promoter region upstream of the human ML-IAP locus containing two MITF consensus E box elements, named E1 and E2. B, ML-IAP and MITF mRNA levels are well correlated in human melanomas, but not in normal skin. mRNA expression levels of MITF and ML-IAP from microarray data values were analyzed as described in Materials and Methods. Data plots of MITF versus ML-IAP expression in melanoma (open circles) and normal skin (crosses) samples are shown. The straight line represents the least squares regression line of MITF expression on ML-IAP expression in melanoma samples. Pearson’s correlation coefficient (r) = 0.68, P = 0.00006; melanoma sample, n = 29; normal skin sample, n = 51. C, Correlation of ML-IAP and MITF-F mRNA levels in human melanoma cell lines. Total RNA was extracted, ML-IAP and MITF-F transcript levels were measured by real-time PCR analysis, expression levels were normalized to that of hRPL19 (human ribosomal protein L19), and relative abundance levels were calculated with 886 melanoma cells as the reference.
of the ML-IAP gene was searched against the TRANSFAC gene regulation database using the TRANSFAC patch (pattern-based) program. Two sites that matched the E box consensus-binding site CA(C/T)GTG of MITF were identified within the ML-IAP promoter region. One site resides 46 bp and another 282 bp upstream of the ML-IAP translation start site (Fig. 1A), herein referred to as site E1 and E2, respectively.

Because ML-IAP and MITF are both highly expressed in the majority of melanomas studied thus far (2, 13, 29), we sought to explore a potential similarity between ML-IAP and MITF transcript expression in normal skin and melanoma samples. Analysis of microarray expression data using Pearson's correlation coefficient revealed that there was a statistically significant linear relationship between ML-IAP and MITF expression in melanoma samples (r = 0.68, P = 0.0000003, n = 29), whereas no correlation was detected in normal skin samples (Fig. 1B; Supplementary Table S1). In addition, specifically in melanomas but not in normal skin samples, transcript levels of ML-IAP correlated strongly with those of CDK2, a known transcriptional target of MITF in melanomas (ref. 24; Supplementary Table S1). To further investigate the correlation between MITF and ML-IAP expression patterns, mRNA levels of ML-IAP and MITF-M, the melanocyte-specific isoform of MITF, were surveyed in a panel of six melanoma cell lines by real-time PCR analysis. This analysis revealed a strong correlation between ML-IAP and MITF-M expression levels (Fig. 1C).

We extended our correlation analysis by evaluating the status of ML-IAP and MITF expression in archived melanoma patient samples by in situ hybridization. The concordance of MITF and ML-IAP expression was defined as follows: when (a) both genes are negative; (b) both genes are positive, irrespective of levels of positive signals; and (c) both genes are equivocal for expression. Samples positive for one gene but negative for the other are deemed nonconcordant. In 81% (58 of 72 total) of the melanoma cases surveyed, ML-IAP and MITF expression patterns were found to be significantly concordant (P = 0.00000003, Fisher's exact test; Fig. 2; Table 1). For the 19 cases that were scored as 2+ for MITF, 17 (89%) of those were also positive for ML-IAP (Supplementary Table S2). These data, together with the experimental findings presented below, strongly suggest that MITF is a critical regulator of ML-IAP expression. However, whereas 15% (11 of 72) of cases positive for MITF were negative for ML-IAP, 4% (3 of 72) of cases negative for MITF were positive for ML-IAP, indicating the possibility of additional mechanisms in the regulation of ML-IAP expression (Table 1 and Supplementary Table S2). Our discovery that MITF and ML-IAP expression is significantly correlated in human melanoma cells and tumor samples, together with the classification of MITF as a lineage survival oncogene, particularly in melanomas, suggested that MITF is a transcription factor well poised to regulate ML-IAP expression in melanoma.

MITF binds and regulates ML-IAP promoter. To assess whether MITF-M can directly regulate ML-IAP expression, luciferase reporter constructs containing the upstream ML-IAP region were transiently transfected into Hek293T cells with or without MITF-M. Cotransfection of MITF-M with ML-IAP promoter constructs containing either one (ML-IAP Pro 1) or two (ML-IAP Pro 2) MITF consensus sites resulted in strong reporter activation (Fig. 3A). This MITF-dependent transcriptional regulation required the presence of intact E box MITF binding sites because disrupting both E boxes simultaneously (ML-IAP Pro 2 M1 M2) or mutating the single E1 site in the shorter construct (ML-IAP Pro 1 M1) resulted in a complete loss of MITF-M–dependent regulation (Fig. 3A). It seems that both MITF sites contribute equally to the transcriptional regulation of ML-IAP, as evidenced by the similar MITF-M responsiveness of the two
MITF through the melanocortin receptor that up-regulates the cAMP pathway. Forskolin mimics the action of natural signaling molecules and was used to treat melanocytes. Treatment with the cyclic AMP (cAMP) agonist forskolin led to increased cell proliferation and differentiation (30). In normal melanocytes, forskolin stimulation caused a >2-fold increase in ML-IAP mRNA levels. In contrast, ML-IAP mRNA levels were unchanged in melanoma cells treated with the DNA-damaging agent etoposide or TNFα, which are known to induce apoptosis (37–39). These results suggest that ML-IAP is a target of MITF-M in melanocytes in response to signaling stimuli.

**Expression of ML-IAP is not regulated by Wnt, DNA damage, or TNF-mediated signaling in melanoma cells.** The Wnt/β-catenin signaling pathway has been implicated in the formation of malignant melanoma (31), in part, through its transcriptional regulation of the MITF-M promoter (32–34). MITF has also been shown to bind to and cooperate with β-catenin and LEF-1 in transcriptional regulation of target genes (27, 35). Thus, we investigated whether Wnt signaling could contribute to ML-IAP expression in melanomas. Two potential LEF-1 consensus-binding sites CTTTG[A/T][A/T] were identified in the ML-IAP promoter region upstream from the MITF E1 and E2 sites at 483 bp (CTCTGAT; note mismatch at the third nucleotide position) and 1,303 bp (CTTTGAT) away from the MITL translation start site. ML-IAP reporter constructs containing either one (ML-IAP Pro 3) or both LEF-1 sites (ML-IAP Pro 4 and ML-IAP Pro 1), did not show any responsiveness when either a stabilized β-catenin mutant or LEF-1 was ectopically expressed alone, together, or with MITF-M (Fig. 4A). Moreover, treatment of melanoma cell lines with Wnt3a led to no appreciable change in ML-IAP mRNA levels as assessed by real-time PCR analysis (Supplementary Table S3). At the same time, mRNA levels of a bona fide target of Wnt signaling, Axin2 (36), were significantly elevated (Supplementary Table S3).

Table 1. Prevalence of MITF and ML-IAP expression in melanomas

<table>
<thead>
<tr>
<th>MITF ISH cases (n = 72)</th>
<th>ML-IAP ISH cases (n = 72)</th>
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<tbody>
<tr>
<td>No. positive cases</td>
<td>No. negative cases</td>
</tr>
<tr>
<td>No. positive cases</td>
<td>39*</td>
</tr>
<tr>
<td>No. negative cases</td>
<td>3</td>
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<td>No. positive/negative cases</td>
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*Indicates concordant expression of both genes in identical tumor samples.
gene CDK2, were observed with four of five MITF siRNAs tested or with a pool of all five MITF siRNAs (Fig. 5A). The siRNA-mediated knockdown of MITF was confirmed for the four successful individual and pool siRNAs by real-time PCR to measure MITF-M mRNA levels (Fig. 5A). ML-IAP mRNA levels were also lowered significantly as assessed by real-time PCR analysis, as were those of CDK2 (Fig. 5A). MITF siRNA transfection of another melanoma cell line, 624, led to a sizeable decrease in ML-IAP protein and RNA levels as well (Fig. 5B).

Additionally, transient overexpression of MITF-M in A2058 and UACC62 melanoma cells, both of which have very low expression levels of ML-IAP and MITF, was sufficient to induce increased ML-IAP mRNA levels (Supplementary Table S4). Thus, ML-IAP expression is MITF dependent and regulated by MITF at the transcriptional level in melanoma cells.

MITF and ML-IAP are critical for melanoma survival. Loss of MITF function, either by siRNA knockdown or expression of a dominant negative allele, leads to reduced melanoma growth and decreased melanocyte cell viability (25, 40). If regulation of ML-IAP expression by MITF is required for melanoma cell viability, then overexpression of ML-IAP from a constitutive promoter should be able to rescue MITF siRNA–induced cell death. Indeed, transient cotransfection of ML-IAP measurably rescued the cell death induced by MITF siRNA in 888 melanoma cells (Fig. 5C). MITF knockdown–stimulated cell death was accompanied by elevated caspase activity and nuclear condensation, confirming its apoptotic nature (Fig. 5C and Supplementary Fig. S3A). Again, ectopic expression of ML-IAP inhibited these apoptotic features of MITF siRNA–induced cell death (Fig. 5C and Supplementary Fig. S3A). For ML-IAP to exert its
antiapoptotic function, it must retain a functional interaction with SMAC, which can be abolished by the mutation of aspartate to alanine at position 138 within the ML-IAP BIR domain (2, 41). Notably, cotransfection of a flag-tagged ML-IAP harboring the D138A mutation was unable to rescue MITF siRNA–induced cell death in 888 melanoma cells, whereas in the same experiment, cotransfection of wild-type FLAG-ML-IAP led to a significant rescue of melanoma cell viability (Supplementary Fig. S3). These data establish ML-IAP as a functionally important target of MITF, and suggest that ML-IAP contributes to the prosurvival effects of MITF-M in melanoma cells.

Discussion

In the present work, we identify ML-IAP as a new transcriptional target of MITF, a melanocyte master regulator and lineage survival oncogene in melanoma cells.

We report that MITF binds to and activates transcription from the ML-IAP promoter, and that ML-IAP expression is dependent on MITF-M in melanoma cells. Other candidate transcriptional regulators of ML-IAP including p53, nuclear factor κB, β-catenin, and LEF-1 were also explored. This is particularly relevant for β-catenin and LEF-1 because a recent study investigating the regulation of ML-IAP expression in non–small-cell lung cancer cells reported that ML-IAP is a target of β-catenin/T-cell factor signaling (42). However, the Wnt pathway did not enhance the transcriptional activation of ML-IAP or alter ML-IAP mRNA levels in reporter assays or by stimulation of the Wnt pathway in melanoma cells, likely reflecting differential regulation of ML-IAP in melanomas versus lung cancer cells.

Up-regulation of MITF expression, through stimulation of the cAMP pathway, led to a significant increase in ML-IAP mRNA levels in normal melanocytes. Treatment of melanocytes with cAMP-elevating agents has previously been shown to result in increased MITF expression (43), as well as up-regulation of target genes with somewhat paradoxically opposing functions. On one hand, cAMP pathway stimulation of MITF activity in melanocytes up-regulates the expression of hypoxia-inducible factor 1α (HIF1α) and the hepatocyte growth factor receptor MET, which promote cell survival (44, 45). On the flip side, MITF also up-regulates the inhibitors of cell cycle progression, p21CIP1 and p16INK4A (46, 47). Likewise, in melanomas, MITF targets a number of genes with antagonistic behaviors, including genes such as CDK2 and BCL-2, which promote cell cycle progression and survival, as well as p21CIP1 and p16INK4A, which halt the cell cycle (24, 25, 46, 47). The decision of whether MITF will exert a

![Figure 4](https://example.com/figure4.png)

**Figure 4.** ML-IAP expression is not influenced by Wnt pathway transcription factors, DNA damage, or TNF-mediated signaling. A, neither β-catenin nor LEF-1 activates MITF-M–dependent or MITF-M–independent transcription of ML-IAP. ML-IAP promoter constructs were cotransfected with the indicated plasmids for 48 h in HEK293T cells. Firefly luciferase activities were normalized to Renilla luciferase activities, and the ratios between firefly and Renilla luciferase normalized to vector-transfected samples are shown. The β-catenin/LEF-1 responsive Topglow reporter is included as a positive control for transcriptional activation by β-catenin and LEF-1. B, DNA damage does not stimulate ML-IAP expression in melanoma cells. Indicated cell lines were treated with 10 μmol/L etoposide for 5 h. Total RNA was extracted, ML-IAP and p21 transcript levels were measured by real-time PCR analysis, and expression levels were normalized to that of hRPL19. n.d., undetectable mRNA levels in both untreated and treated samples. C, TNF signaling does not regulate ML-IAP levels in melanoma cells. Indicated cell lines were treated with 20 ng/mL TNFα for 5 h. Real-time PCR analysis for ML-IAP and MCP-1 mRNA levels was done as in B. n.d., mRNA levels in both untreated and treated samples was not detected.
prosurvival or growth inhibitory effect in melanocytes and melanoma growth is not fully understood at present, but it is likely that the cellular context and microenvironment are important factors. Our studies suggest that MITF regulation of ML-IAP favors a prosurvival outcome in melanoma, and that ML-IAP may play a role in melanocytic transformation.

The high degree of correlation of ML-IAP and MITF mRNA levels we report in this study provokes the question about whether ML-IAP may be a useful biomarker in melanoma. Currently, a MITF-specific antibody is clinically used as a highly sensitive immunohistochemical marker in melanoma diagnosis (48). However, the expression of MITF only serves as a melanocytic lineage marker and has no prognostic value in discriminating benign from malignant melanoma lesions (16). Perhaps, given the specific expression of ML-IAP in the majority of primary and metastatic lesions and the very infrequent expression of ML-IAP in benign melanocytic proliferation, using a combination of both anti–ML-IAP and anti-MITF antibodies could prove useful in the clinical diagnosis of melanoma. Interestingly, in primary cultures derived from melanoma patients, a correlation between ML-IAP expression and chemotherapeutic drug resistance has been reported (49). Whether the expression of ML-IAP in melanomas has any prognostic or therapeutic value needs to be addressed by future studies examining the influence of ML-IAP expression in melanoma on survival outcome in patients.

Melanoma is a highly aggressive cancer that is resistant to current anticancer treatments including chemotherapy and other strategies that require induction of apoptosis (12). We propose that the transcriptional regulation of ML-IAP by MITF plays an important role in promoting survival during melanocytic

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**Figure 5.** MITF-M transcriptionally regulates endogenous ML-IAP in melanoma cells and MITF transcriptional regulation of ML-IAP is critical for melanoma survival. A, down-regulation of ML-IAP protein and mRNA levels by siRNA-mediated depletion of MITF in 888 melanoma cells. Melanoma cells were transfected with individual siRNA duplexes 1 to 5, or a pool of siRNAs 1 to 5 (denoted P), targeting MITF, or control siRNA. Left, 48 h posttransfection, cells were harvested and lysates were subject to Western blot analysis with anti-ML-IAP, anti-CDK2, and anti-actin antibodies. Right, 24 h posttransfection, total RNA was extracted. ML-IAP, CDK2, and MITF-M transcript levels were measured by real-time PCR analysis, and expression levels were normalized to hRPL19. B, ML-IAP protein and mRNA levels are lowered by siRNA-mediated depletion of MITF in 624 melanoma cells. Melanoma cells were transfected with a pool of MITF siRNAs (1–3 and 5 from A and B), targeting MITF, or control siRNA. Left, 48 h posttransfection, cells were harvested and lysates were subject to Western blot analysis as in A. Right, 24 h posttransfection, total RNA was extracted and real-time PCR analysis was done as in A. C, overexpression of a ML-IAP rescues loss of melanoma cell viability and increased caspase activity induced by MITF siRNA. Left, 888 melanoma cells were cotransfected with MITF or control siRNA and myc-tagged ML-IAP or vector DNA. Forty-eight hours posttransfection, lysates were prepared for Western blot analysis with anti–ML-IAP, anti-MYC, and anti-actin antibodies. Middle, cell viability was assessed by Neutral Red staining as described in Materials and Methods. Columns, mean from at least five independent experiments normalized to control siRNA- and vector DNA–transfected cells; bars, SE. Right, caspase-3/caspase-7 activity was assayed 24 h after transfection. Columns, mean from at least five independent experiments normalized to control siRNA- and vector DNA–transfected cells expressed as relative fluorescent units (RFLU); bars, SE.
transformation and melanoma progression. ML-IAP has been proposed as a promising tumor-specific target in cancer therapy due to its powerful antiapoptotic and preferential expression in a number of tumor types (15, 50). Understanding the mechanism(s) of ML-IAP up-regulation during melanocytic transformation as well as the modulation of ML-IAP expression in melanoma cells may aid in designing therapies targeting ML-IAP in malignant melanomas.

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References


