MicroRNA-340-mediated Degradation of Microphthalmia-associated Transcription Factor mRNA Is Inhibited by the Coding Region Determinant-binding Protein

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Alternative cleavage and polyadenylation generate multiple transcript variants of mRNA isoforms with different length of 3'-untranslated region (UTR). Alternative cleavage and polyadenylation enable differential post-transcriptional regulation of transcripts via the availability of different cis-acting elements in 3'-UTRs. Microphthalmia-associated transcription factor mRNA (MITF) is a master regulator of melanocyte development and melanogenesis. It has also been implicated in melanoma development. Here we show that melanoma cells favor the expression of MITF mRNA with shorter 3'-UTR. This isoform of mRNA is regulated by microRNA, miR-340. miR-340 interacts with two of its target sites on the 3'-UTR of MITF mRNA, causing degradation and decreased expression and activity. On the other hand, the RNA-binding protein coding region determinant-binding protein, shown to be highly expressed in melanoma, directly binds to the 3'-UTR of MITF mRNA and prevents the binding of miR-340. This stabilization of the MITF mRNA isoform is a key post-transcriptional regulatory step affecting the availability of the MITF protein and may uncover novel targets for the treatment and/or prevention of melanomas.

Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix leucine zipper transcription factor belonging to the MYC superfamily of proteins (1). MITF forms dimers and binds to specific sequence motifs present in the promoter of its target genes to activate transcription. Various studies have documented the role of MITF in the induction of genes required for melanin formation as well as normal melanocyte development. It regulates the transcription of three major pigmentation enzymes, tyrosinase (TYR), tyrosinase related protein-1 (TYRP-1), and dopachrome-tautomerase (DCT) (2–4). MITF is also an amplified oncogene in a subset of melanomas and has been shown to regulate a distinct set of target genes that in turn are responsible for neoplasia-related phenotype in these types of cancers. Primarily, this action of MITF in melanomas is via increasing cell proliferation and triggering apoptosis (5). The c-MET proto-oncogene is a target of MITF and suppresses senescence via down-regulation of p21 (5, 6). On the other hand, phosphorylation of the Ser298 residue in MITF by glycogen synthase kinase 3β (GSK3β) results in its ubiquitination and subsequent degradation (16, 17). On the other hand, phosphorylation of the Ser298 residue in MITF by glycogen synthase kinase 3β is believed to increase the DNA binding activity of MITF (18). However, apart from transcriptional and post-translational regulation, another important mode of regulating gene expression is by manipulating the stability of the mature mRNA. Recent reports indicated that MITF is a target of several microRNAs (miRNAs), including the miR-96/183/182 cluster (19) and miR-137 (20), suggesting the importance of a post-transcriptional regulatory step.

miRNAs are small ~22-nucleotide noncoding RNAs known to act as an important class of gene regulators. They are present abundantly in plant and animal genome, and after being transcribed and processed, the final forms of the mature miRNAs bind to their target sites on the mRNAs present mostly in the
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![Diagram of MITF mRNA isoforms with varying lengths of 3′-UTR](image)

**A** Coding sequence 3′-UTR
- miR-182 binding sites
- miR-137 binding site
- miR-340 binding sites

**B** Relative amount of RNA
- Long
- Medium
- Short

![Graph showing the relative amount of MITF mRNA isoforms with varying lengths](image)

**FIGURE 1.** MITF mRNA with short 3′-UTR is more abundant in melanoma cell lines. (A) Diagram of MITF mRNA isoforms with varying lengths of 3′-UTR (19, 20). In this study, we investigated the expression pattern of three such mRNA isoforms in melanoma cell lines and normal human melanocytes, finding that MITF mRNAs with short 3′-UTRs are more abundant in melanoma cell lines. We identified that the mRNA isoform with the short 3′-UTR also undergoes miRNA-mediated regulation, and the miR-340 destabilizes the mRNA of MITF in melanoma cell lines. Furthermore, we set out to decipher the role of a RNA-binding protein, coding region determinant-binding protein (CRD-BP, IMP-1, IGF2BP1) in the regulation of MITF. This protein has been shown to attenuate miRNA-dependent degradation of different mRNA (21) and has also been found to be overexpressed in melanoma (24). Our results showed that CRD-BP restricts the action of miR-340 by preventing its access to the mRNA of MITF, thereby proposing a novel mode of regulation of MITF.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Culture, and Transfection Conditions**—Normal human melanocytes (NHM) were maintained in Ham’s F-10 medium (Mediatech Inc., Manassas, VA) supplemented with human melanocyte growth supplement (Cascade Biologics Inc., Portland, OR), 5% fetal bovine serum, and PSM solution containing penicillin, streptomycin, and amphotericin B. 451Lu cell lines were kindly provided by Dr. Paul Robbins (Center of Cancer Research, NCI, Frederick, MD). The cells were maintained Eagle’s medium, supplemented with 10% bovine calf serum and 1% PSM solution. NHM cells were electroporated using AmaxaTM Bc1606 cell line electroporator (Lonza, Switzerland) according to the manufacturer’s instructions, and transfections of all other cells were performed using Lipofectamine 2000 reagent, according to the manufacturer’s recommendations (Invitrogen).

**Expression Vectors**—Full-length MITF cDNA was purchased from Open Biosystems Inc. (clone identification number 6066096, accession number BC065243, cloned in pCMVSPORT6) and amplified using Fragment 1 forward and 3′-UTR reverse primers (supplemental Table S1). The PCR product was then cloned in pBIG vector (Clontech) and digested with NotI and SalI and end-filled with Klenow enzyme, and a clone with the right orientation was selected. Three fragments of the MITF mRNA coding region (nucleotides 1–421, 422–841, and 842–1260) as well as full-length cDNA (nucleotides 1–1260) having 3′-UTRs of different lengths depending on the position of the alternative cleavage and polyadenylation signals. The 3′-UTR but also were several protein factors having 3′-UTRs of different lengths were identified in the alternative cleavage and polyadenylation complex, ultimately leading to the translation or to the degradation of mRNA.

Alternative cleavage and polyadenylation generate multiple transcript variants of a particular gene with similar mRNA coding region (nucleotides 1–421, 422–841, and 842–1260) as well as full-length cDNA (nucleotides 1–1260). 293T cells were obtained from the ATCC. All of the cell lines were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% PSM solution. NHM cells were electroporated using AmaxaTM Bc1606 cell line electroporator (Lonza, Switzerland) according to the manufacturer’s instructions, and transfections of all other cells were performed using Lipofectamine 2000 reagent, according to the manufacturer’s recommendations (Invitrogen).

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obtain the deletion mutants for miR-340 sites based on pBIG or pBI-GL construct, we performed blunt end ligation of PCR products, treated with DpnI as recommended for the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Plasmids for CRD-BP expression or production CRD-BP shRNA were characterized in (26). The pcdNA5-CMV-d2eGFP vector and control sponge-CXCR4 construct were kindly provided by Dr. P. Sharp (MIT, Cambridge, MA). The constructs of sponge-340 and sponge-584c-3p were designed as described (27). Oligonucleotides having seven bulged binding sites for miR-340 or p81-GL construct, were annealed, gel-purified, cloned into pcdNA5-CMV-d2eGFP vector, and linearized with XhoI and ApaI.

mRNA Degradation in Vivo—To investigate the stability of mRNA transcripts, we used the Tet-Off gene expression system (Clontech) as described elsewhere (26). Melanoma or colorectal cell lines or 293T cells were transfected with 4–6 μg of Tet-Off and 2–3 μg of response plasmid p81-d2eGFP expressing mRNA transcripts of our interest. Transcription was stopped by adding doxycycline (1 mg/ml) into the media 48 h after transfection with other expression vectors. The cells were harvested at different time points after treatment, and the total RNA was extracted as described above. The levels of mRNA were analyzed by real time RT-PCR.

Antibodies and Immunoprecipitation Techniques—Mouse anti-FLAG M2 antibody (Sigma-Aldrich), antibody against β-actin, and MITF (Santa Cruz Biotechnology) as well as secondary antibodies conjugated with horseradish peroxidase...
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FIGURE 3. MITF mRNA is a target of miR-340. A, MITF WT plasmid with short 3'-UTR transcripts was analyzed by real-time RT-PCR following transfection with the previously described shRNA constructs, pSV40 β-galactosidase plasmid (26) or miR-Sponge constructs, pBGI M-MITF wt or pBGI M-MITF 340 plasmids. 48 h after transfection, luciferase activity was measured using luciferase reporter assay reagents, according to the manufacturer’s recommendations (Promega).

senescence-associated β-galactosidase staining—adult zebrafish were maintained according to established methods (32). wild type (AB strain) embryos were obtained from natural mating and staged according to Kimmel et al. (33). the CRD-BP antisense morpholino (MO) designed to block translation of CRD-BP (5'-GAAGTTCAGAAGCAGTCTGTTCATC-3') was purchased from geneTools (philomath, OR). MOs were diluted in 1× danieau buffer (34) to 10 ng/µl. Standard control MO (GeneTools) was diluted 2–8 ng/µl. 0.5–1 nl of MO/embryo was injected at the one- to two-cell stage. Embryos at 24 h post-fertilization were lysed for RNA extraction with TRIzol (Invitrogen).

senescence-associated β-galactosidase staining—The cells were fixed with fixative solution (20% formaldehyde, 2% glutaraldehyde) for 20 min, washed with phosphate-buffered saline, and stained with 5-bromo-4-chloro-3-indolyl-β-d-galactopyrano-
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RESULTS

MITF mRNA with Short 3′-UTR Is Preferentially Expressed in Melanoma Cell Lines—As discussed earlier, several full-length cDNA isoforms of MITF-M were found having the same coding sequence but with 3′-UTRs of varying lengths. Two recent studies that showed regulation of MITF mRNA by two different miRNAs used two different isoforms of MITF mRNA. The study by Bemis et al. (20) showed a functional miR-137-binding site in the 3′-UTR using a full-length cDNA with 1,143 bp of the 3′-UTR sequence (termed hereafter MITF-long 3′-UTR). Segura et al. (21) on the other hand, used the same full-length cDNA with a 3′-UTR of 1,700 bp (termed the long 3′-UTR) and found a functional miR-15a binding site (21). We obtained the full-length transcripts of three fragments of the MITF mRNA coding region, full-length mRNA, and the short 3′-UTR (termed hereafter MITF-short 3′-UTR). Because we found the mRNA isoform with the shorter 3′-UTR was predominately expressed in both melanoma cell lines and also from NHMs (Fig. 1A). The phenomenon of the presence of the same mRNA isoform being regulated by different miRNAs led us to investigate the abundance of MITF mRNAs with different 3′-UTRs in different melanoma cell lines. We designed primers unique to the short, medium, and long 3′-UTRs and used them to detect the relative abundance of the individual isoforms by quantitative real time PCR using RNA isolated from four different melanoma cell lines and also from NHMs (Fig. 1B). The results showed that in NHMs the levels of all of the three isoforms were comparable, but in all of the four melanoma cell lines tested, the relative proportion of MITF mRNA with short 3′-UTR was predominating. This suggested that fast proliferating melanoma cell lines preferentially express MITF mRNA with shorter 3′-UTR.

MITF mRNA with Short 3′-UTR Is Also Regulated by miRNA—As discussed before, miRNAs have been shown to act on the MITF mRNA with medium and long 3′-UTR. Because we found the mRNA isoform with the shorter 3′-UTR is predominantly expressed in both melanoma cell lines and also from NHMs, we investigated whether the short 3′-UTR is also regulated by miRNA(s). In order to test this, we measured the relative degradation rates of the full-length MITF mRNA and the short 3′-UTR transcript. The levels of MITF-M and MITF-M′+CRD-BP plasmids were assessed by real-time RT-PCR after stopping transcription by doxycycline treatment for the indicated time points. Normalization was done with respect to GAPDH expression. All of the results are representative of three separate experiments and are expressed as the mean values ± S.D. (error bars). The average half-lives of mRNAs are presented in supplemental Table S3. The phenomenon of the presence of the same mRNA isoform having two different 3′-UTRs and also being regulated by different miRNAs is unique to the short, medium, and long 3′-UTRs. The study by Bemis et al. showed regulation of MITF mRNA is mediated by two different miRNAs used in the same melanoma cell lines and also from NHMs (Fig. 1B).

Anoside (X-gal) solution overnight at 37 °C. The cells stained blue were counted under a microscope (20×), and the percentages of stained cells were estimated.

FIGURE 4. CRD-BP is a positive regulator of MITF expression. A, 1241 Mel and Mel IM cells were co-transfected with the indicated shRNA-expressing plasmids, β-galactosidase-expressing plasmid, and pHTRPL4, where the luciferase gene is expressed under the MITF-dependent promoter. The values correspond to luciferase activity normalized to β-galactosidase activity, and presented as percentages of control (pBABE). B, 1241 Mel and Mel IM cells were co-transfected with the indicated shRNA-expressing plasmids, β-galactosidase-expressing plasmid, and pHTRPL4, where the luciferase gene is expressed under the MITF-dependent promoter. The values correspond to luciferase activity normalized to β-galactosidase activity, and presented as percentages of control (pBABE). D, NHM cells were electroporated using AMAXA™ with indicated plasmids. 48 h after electroporation, the cells were collected, assayed for levels of MITF mRNA by quantitative RT-PCR normalized to GAPDH expression, and presented as percentages of control (pBABE). D, NHM cells were electroporated using AMAXA™ with indicated plasmids. 48 h after electroporation, the cells were collected, assayed for levels of MITF mRNA by quantitative RT-PCR normalized to GAPDH expression, and presented as percentages of control (pBABE). D, NHM cells were electroporated using AMAXA™ with indicated plasmids. 48 h after electroporation, the cells were collected, assayed for levels of MITF mRNA by quantitative RT-PCR normalized to GAPDH expression, and presented as percentages of control (pBABE). 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short 3'-UTR most prevalent in melanomas, we investigated whether this isoform is also being regulated by miRNAs. We measured the half-life of that mRNA in cells defective in Dicer1 function and thus deficient in miRNA maturation (25). It was observed that the half-life of MITF mRNA with short 3'-UTR was 2–3-fold higher in all three Dicer1Ex5/Ex5 hypomorphic cell lines examined as compared with their normal counterpart where functional miRNAs were present (Fig. 2A–C). This indicated that MITF with short 3'-UTR is also regulated by miRNAs. We then used a bioinformatic approach (microRNA.org web site) to find out the possible miRNA-binding site in that region. Two miRNAs, miR-340 and miR-548c-3p, were selected for further investigation after short listing of the miRNAs based on their alignment score (Fig. 2D). To inhibit the function of these miRNAs in melanoma cells, we used miR-Sponge constructs as described previously (27) for both of them. Our results showed that the amount of the MITF mRNA isoform with short 3'-UTR increased significantly only when miR-340 function was inhibited (Fig. 2E). Moreover, inhibition of miR-340 function in 451Lu cells also increased the MITF protein level (Fig. 2F). These data demonstrated that MITF mRNA with short 3'-UTR is regulated by miR-340.

**miR-340 Destabilizes MITF mRNA**—After the initial observations regarding the involvement of miR-340 in the regulation of MITF mRNA, we set out to investigate whether miR-340 regulates the turnover of MITF mRNA. The result showed a 2-fold increase in half-life upon miR-340 inhibition, indicating that miR-340 is responsible for regulating MITF mRNA. We further tested the action using a reporter construct. The MITF mRNA was cloned just after the pBI-GL (Clontech). In melanoma cell lines significantly increase 3'-UTR of MITF as well as the reporter mRNA with its generation. Furthermore, the deleted version of the full-length MITF mRNA was sufficient for the observed effect. Next, both of the sites of miR-340 were deleted from the full-length MITF mRNA as well as the reporter construct. The luciferase activity of the deleted version of the full-length mRNA as well as the reporter construct was increased significantly (Fig. 3D). As expected, the mutated chimeric mRNA was also found to be increased 2.5-fold, in a manner comparable with the luciferase when miR-340 function was inhibited. On the other hand, miR-340 inhibition had no additional effect on the luciferase activity of the mutant mRNA (Fig. 3C). Together these data suggested that miR-340 acts through its target sites on 3'-UTR of MITF mRNA, leading to its destabilization.

**CRD-BP Stabilizes MITF mRNA**—CRD-BP is a multifunctional mRNA-binding protein, modulating the stability, localization, and translation of several RNAs (c-myc, IGF-II, H19, CD4, MDR-1, βTrCP1, etc.) (35–41). As discussed earlier, CRD-BP protects another mRNA from miRNA-mediated destabilization (21). Moreover, CRD-BP has also been shown to be overexpressed in melanoma cell lines and human melanoma samples contributing to oncogenesis (24). Knockdown of CRD-BP in 451Lu cells decreased the level of endogenous MITF mRNA by more than 5-fold (Fig. 4A). Moreover, MITF-protein expression was also decreased (Fig. 4B). These results strongly suggest that CRD-BP prevents miR-340-mediated degradation of MITF mRNA.

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earlier for other mRNA (21). Munro et al. (42) have characterized the sequence motif required for the binding of IMP in Drosophila as UUUAY, and we found the presence of that motif either within or juxtaposed to both of the miR-340-binding sites (supplemental Fig. S1B). Moreover, overexpression of CRD-BP increased the half-life of MITF mRNA similar to that of the inhibition of miR-340 function. CRD-BP overexpression, on the other hand, could not increase the half-life further when inhibition of miR-340 function already stabilized the mRNA (Fig. 6A).

Similarly, when miR-340 function was inhibited, there was an increase in the endogenous MITF mRNA level that was not altered because of CRD-BP overexpression (Fig. 6B). Furthermore, CRD-BP decreased the rate of MITF mRNA transcription by 50%, but it had no effect when miR-340 function was already inhibited (Fig. 6C). These data suggested that CRD-BP exerts its effect on MITF mRNA by preventing miR-340 action. When miR-340 action is blocked, the mRNA is stable by itself and becomes unresponsive to the availability of CRD-BP. To further confirm the mechanism of interference of CRD-BP with the miR-340 function, we measured the half-life of the MITF mRNAs with deleted miR-340 sites in the similar experiments. We found that the half-life of the mutated MITF mRNA was increased significantly compared with the wild type MITF mRNA, and overexpression of CRD-BP had no effect on the stability of MITF mRNA that lacks miR-340-binding sites (Fig. 6D).

The manifestation that CRD-BP could stabilize MITF mRNA only when miR-340 is functional demonstrates that CRD-BP acts by preventing miR-340 action. To analyze the effect of inhibition of miR-340 on cell proliferation, we have performed colony formation assays and found that inhibition of miR-340 function using specific sponge construct resulted in a significant increase in the number of colonies formed by both 928 mel and 451Lu melanoma cells (Fig. 6E). A similar increase was detected when CRD-BP was overexpressed, but...
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no additive effect on colony formation was observed when CRD-BP and sponge-340 were co-expressed. As expected, knockdown of CRD-BP resulted in drastic inhibition of a number of colonies; however, inhibition of mir-340 rescued the effect of CRD-BP knockdown on the growth of 928 mel and 451Lu melanoma cells (Fig. 6E).

DISCUSSION

MITF is widely regarded as the master regulator of melanocyte biology because of its involvement in the regulation of important melanogenic proteins as well as because of its contribution to melanoblast survival, melanocyte lineage commitment, and also melanoma (reviewed in Ref. 43). Therefore, it is the importance of MITF that potentiates the necessity to study its regulatory mechanisms. Post-transcriptional regulation of gene expression involving RNA-binding proteins has been pivotal in the regulation of expression of several genes, and the discovery of miRNAs has added a new dimension to that. MITF is also post-transcriptionally regulated, and two recent studies showed evidence of miR-137 and miR-182 targeting MITF mRNA (19, 20). In the context of this study, we set out to investigate the post-transcriptional regulation of MITF mRNA expression in further detail.

One of the key mechanisms underlying the process of malignant transformation is the activation of oncogenes. The presence of miRNA-binding sites from the 3′-UTRs of the oncogenic mRNAs is considered to be an important modulator in post-transcriptional regulation and activation (44, 45). This was supported by observations that cancer cells and other proliferating cells express mRNA isoforms with shorter 3′-UTRs because of miRNA-mediated regulation. In this report, we identified several isoforms with different miRNA-binding sites, and the missing part was that the isoform with short 3′-UTR had the binding sites for the previously reported miRNAs (Fig. 1B). We show here that melanoma cells preferentially express the MITF mRNA isoform with short 3′-UTR (Fig. 1C), as do other fast proliferating cells in terms of regulation of oncogenic mRNAs. This finding thus designates the MITF mRNA isoform with short 3′-UTR (Fig. 1C) as the most prevalent isoform among melanoma cell lines. Furthermore, our results show that MITF mRNA isoform with short 3′-UTR (Fig. 1C) escapes regulation by previously reported miRNAs (Fig. 1B). Being regulated by a different miRNA, miR-340-3p, repression of miR-340 with two target sites present on the short 3′-UTR of MITF mRNA results in destabilization of MITF mRNA and inhibition of MITF expression and transcriptional activity (Figs. 2 and 3). Interestingly, miR-340 has been shown to be expressed in primary melanoma cell lines, suggesting its importance in the regulation of melanoma-specific target mRNAs (46). Because the fragment of the MITF mRNA 3′-UTR that contains miR-340-binding sites is a part of all three known 3′-UTRs of MITF, the regulation of MITF by miR-340 appears to be independent of alternative cleavage and polyadenylation and therefore more universal (shared by both melanocytes and melanoma cells).

The results of this study establish CRD-BP as an important positive regulator of MITF expression and function. CRD-BP stabilizes MITF mRNA and increases MITF expression as well as its transcriptional activity (Fig. 4). This effect of CRD-BP is mediated by counteracting the miR-340-mediated degradation of MITF mRNA (Fig. 6). This is not surprising, because we have shown before that CRD-BP also interferes with miR-183 function, resulting in stabilization of BTrCP1 mRNA (21). Our findings here are in line with the recent reports of interplay between RNA-binding proteins and miRNAs, where RNA-binding proteins were shown to interfere with the mRNA function, thereby highlighting a novel mode of post-transcriptional regulation of gene expression (47, 48).

CRD-BP has been shown to be important for the growth and survival of many types of cancer cells (26, 49, 50). It has also been reported to be involved in mammalian development and linked to an effect on cellular adhesion and invasion that takes place during development and malignancy (39, 51). These effects of CRD-BP have been attributed to its regulation of different target mRNAs (i.e. c-myc, BTrCP1, Gli1, etc.). We have also shown previously that CRD-BP is overexpressed in the majority of malignant melanomas (24). However, knockdown of CRD-BP in melanoma caused a much more robust effect than in other tumors, where involvement of an additional melanoma-specific factor was hypothesized. The data provided here about the post-transcriptional regulation of MITF by CRD-BP further support our previous findings and also point to CRD-BP as a potential therapeutic target for intervention of this deadly disease.

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