Oncogenic MITF dysregulation in clear cell sarcoma: Defining the MiT family of human cancers

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Summary

Clear cell sarcoma (CCS) harbors a pathognomonic chromosomal translocation fusing the Ewing's sarcoma gene (EWS) to the CREB family transcription factor ATF1 and exhibits melanocytic features. We show that EWS-ATF1 occupies the *MITF* promoter, mimicking melanocyte-stimulating hormone (MSH) signaling to induce expression of MITF, the melanocytic master transcription factor and an amplified oncogene in melanoma. Knockdown/rescue studies revealed that MITF mediates the requirement of EWS-ATF1 for CCS survival in vitro and in vivo as well as for melanocytic differentiation. Moreover, MITF and TFE3 reciprocally rescue one another in lines derived from CCS or pediatric renal carcinoma. Seemingly unrelated tumors thus employ distinct strategies to oncogenically dysregulate the MiT family, collectively broadening the definition of MiT-associated human cancers.

Introduction

MITF, TFE3, TFEB, and TFEC comprise a family of transcription factors that share a highly homologous basic-helix-loop-helix-leucine zipper (bHLHzip) DNA binding and dimerization domain (reviewed in Steingrimsson et al., 2004). These proteins homo- or heterodimerize in all combinations and bind identical DNA elements (Beckmann and Kadesch, 1991; Fisher et al., 1991; Hemesath et al., 1994), suggesting that they may activate common downstream targets. Indeed, elegant knockout studies have demonstrated the functional redundancy of MITF and TFE3 in modulating murine osteoclast development (Steingrimsson et al., 2002).

The MiT family is critical for the normal development of several cell lineages (reviewed in Steingrimsson et al., 2004). In melanocytes, MITF is a target of the melanocyte-stimulating hormone (MSH) pathway. MSH stimulation increases intracellular cAMP, resulting in CREB/ATF1-mediated activation of the MITF promoter (Bertolotto et al., 1998; Price et al., 1998). Consistent with evidence that MITF is a master regulator of differentiation in the melanocyte lineage, MITF may mediate the well-documented role of MSH in the modulation of pigmentation by regulating many of the genes involved in melanin biosynthesis, including tyrosinase, tyrosinase-related protein 1, dopachrome tautomerase, and AIM-1 (Bentley et al., 1994; Du and Fisher, 2002; Hemesath et al., 1994; Yasumoto et al., 1994).

However, homozygous MITF deficiency in the mouse results in loss of the melanocyte lineage, rather than albinism (pigment deficiency in viable melanocytes), suggesting that the functions of MITF extend beyond regulation of pigmentation. In humans, heterozygous mutation of MITF produces Waardenburg syndrome IIa, which is characterized by white spotting due to melanocyte deficiencies (Hughes et al., 1994; Moore, 1995; Price and Fisher, 2001; Tachibana, 1997; Tassabehji et al., 1994).

SIGNIFICANCE

MITF, TFE3, TFEB, and TFEC comprise a conserved and discrete bHLHzip transcription factor family (MiT). *MITF* amplification in melanoma and *TFEB* or *TFE3* translocation in certain pediatric solid tumors suggest a role for the MiT family in oncogenesis. This study extends the definition of MiT malignancies by demonstrating a mechanism for oncogenic *MITF* dysregulation without amplification or translocation: direct targeting by the EWS-ATF1 fusion protein. Families composed of seemingly unrelated tumors employing distinct strategies of oncogene activation may occur more broadly than previously recognized. For the MiT family, this mechanism suggests that common transcriptional targets are important for oncogenic growth/survival.

Consistent with its vital role, MITF has been suggested to regulate genes important for cell proliferation and survival including *BCL2*, *CDK2*, *p16/Ink4a*, c-*MET*, and $p21^{CIP}$ (Carreira et al., 2005; Du et al., 2004; Loercher et al., 2005; McGill et al., 2002, 2006). *TFEB* knockout produces early embryonic lethality with placental defects (Steingrimsson et al., 1998).

Recently, MiT family gain of function has been directly implicated in tumorigenesis. Approximately 20% of melanomas harbor amplification of the *MITF* gene, in some cases exceeding 100 copies, and disruption of MITF activity is lethal to melanoma cells (Garraway et al., 2005). Furthermore, MITF overexpression, in combination with other oncogenic lesions in human melanocytes, enhances growth factor-independent transformation and confers anchorage-independent growth. *TFEB* and *TFE3* translocations have been identified in pediatric renal carcinoma and alveolar soft part sarcoma (Argani et al., 2001, 2003; Clark et al., 1997; Davis et al., 2003; Heimann et al., 2001; Kuiper et al., 2003b; Sidhar et al., 1996; Weterman et al., 1996). The *TFEB* translocation is particularly informative, since its 5′ fusion partner provides a strong promoter but contributes no protein-encoding sequence, suggesting an oncogenic role arising solely from dysregulated expression. Thus, two mechanisms—amplification or translocation—dysregulate MiT oncogenes in human cancers.

Clear cell sarcoma (CCS) is a soft tissue malignancy of tendons and aponeuroses in young adults (Enzinger, 1965) that is typically unresponsive to chemotherapy or radiotherapy (Deenik et al., 1999; Ferrari et al., 2002; Finley et al., 2001). Although a sarcoma, CCS contains premelanosomes, expresses immunohistochemical markers of melanocytic differentiation (e.g., HMB45), and in some cases produces melanin (Antonescu et al., 2002; Chung and Enzinger, 1983; Granter et al., 2001; Swanson and Wick, 1989). CCS is defined molecularly by a unique cytogenetic translocation joining the Ewing's sarcoma-associated gene (*EWS*) to activating transcription factor 1 (*ATF1*) (Balaban et al., 1984; Langezaal et al., 2001; Zucman et al., 1993). ATF1 is a member of the CREB transcription factor family, whose activity is regulated by protein kinase A or RSK-mediated serine phosphorylation of the kinase-inducible domain (KID) (reviewed in Mayr and Montminy, 2001; Shaywitz and Greenberg, 1999). In CCS, an EWS-derived domain with strong activation activity replaces the KID of ATF1, resulting in a chimeric transcription factor that retains normal cAMP-responsive element (CRE) DNA binding but transactivates in a cAMP-independent fashion (Brown et al., 1995; Fujimura et al., 1996; Lessnick et al., 1995).

In melanocytes, MSH-induced MITF expression requires a CRE located within the melanocyte-specific promoter (M-MITF) that directs expression of a melanocyte-specific first exon (Figure 1A) (Bertolotto et al., 1998; Price et al., 1998). Additional MITF isoforms derive from alternative upstream promoter/first exon units, some (i.e., A-MITF) located as far as ~200 kb upstream of shared exon 2 (Fuse et al., 1996; Hershey and Fisher, 2005; Shibahara et al., 1999). CREB/ATF1-mediated M-MITF activation displays lineage restriction characterized by an inability to respond to cAMP signaling in nonmelanocytic cells (Bertolotto et al., 1998; Price et al., 1998). Tissue-specific M-MITF expression involves cooperativity between CREB/ATF1 and the neural crest-restricted transcription factor SOX10 (Huber et al., 2003). As with *MITF*, *SOX10* mutation results in Waardenburg syndrome (Price and Fisher, 2001).

Several previous studies have identified the expression of MITF protein or mRNA in CCS (Granter et al., 2001; Koch et al., 2001; Antonescu et al., 2002; Li et al., 2003; Schaefer et al., 2004; Segal et al., 2003). These have included immunohistochemical staining as well as RNA and Western blotting analyses, the latter two demonstrating presence of the melanocytic MITF isoform (Antonescu et al., 2002; Li et al., 2003). However, the mechanism of M-MITF regulation in CCS as well as its function are unknown.

Results

MITF expression in clear cell sarcoma

Immunohistochemical analysis revealed nuclear MITF in the CCS primary tumor from which the cell line CCS292 was derived (Figure 1B), in agreement with previous reports for melanoma and CCS (Antonescu et al., 2002; Granter et al., 2001; King et al., 1999; Koch et al., 2001). RNA and protein were examined from three human CCS lines, and all were found to express the melanocyte-specific MITF isoform protein and mRNA (Figures 1C and 1D), in agreement with RNA and protein expression results from primary tumor samples and other cell lines (Antonescu et al., 2002; Li et al., 2003; Schaefer et al., 2004; Segal et al., 2003). CCS cells also express variable quantities of a larger isoform that comigrates with A-MITF, a more ubiquitously expressed isoform seen in a variety of cell types including melanoma and the RAW264.7 leukemia cell line (Figure 1C) (Weilbaecher et al., 2001). In contrast, neither MITF isoform is detected in the Ewing's sarcoma cell line, which expresses the EWS-FLI1 oncoprotein. The identification of the melanocyte-specific M-MITF isoform in several CCS cell lines prompted examination of the mechanism underlying its expression in this tumor.

EWS-ATF1 regulates the MITF promoter

The melanocyte-specific *MITF* promoter contains a CRE site and recognition elements for SOX10, PAX3, and TCF/LEF (Figure 1A) (reviewed in Steingrimsson et al., 2004). To examine the possibility that EWS-ATF1 may bind the endogenous M-isoform promoter, chromatin from three CCS cell lines (expressing EWS-ATF1; Figure S1A in the Supplemental Data available with this article online and data not shown) or a Ewing's sarcoma cell line (control) was immunoprecipitated using EWS-selective antibody. As shown in Figure 2A, EWS-ATF1 occupied the *M-MITF* promoter in all three CCS lines, whereas chromatin from Ewing's sarcoma failed to show similar occupancy for EWS-FLI1. Furthermore, *MITF* promoter occupancy was demonstrated by ChIP using N-terminus-specific EWS antibodies but not C-terminal specific antibodies, consistent with the EWS domain preserved in the EWS-ATF1 fusion (Figure 2B).

If EWS-ATF1 regulates *MITF* through the melanocyte-specific *MITF* promoter, the *MITF* promoter should be constitutively active in a manner dependent upon its CRE sequence but independent of cAMP levels. To test this, the *M-MITF* promoter directing luciferase expression (Price et al., 1998) was used in reporter assays. This reporter was active in all three CCS lines and dependent upon its CRE (Figure 2C). In contrast, the same *M-MITF* promoter was relatively inactive in Ewing's sarcoma cells, and its basal activity was not affected by CRE mutation.

We then examined *M-MITF* promoter responsiveness to cAMP induction in both CCS and melanoma cells (as positive control). In melanoma cells, *MITF* promoter activity was significantly stimulated by the cAMP agonist forskolin (Figure 2D), mimicking MSH signaling, as previously reported (Price et al., 1998).

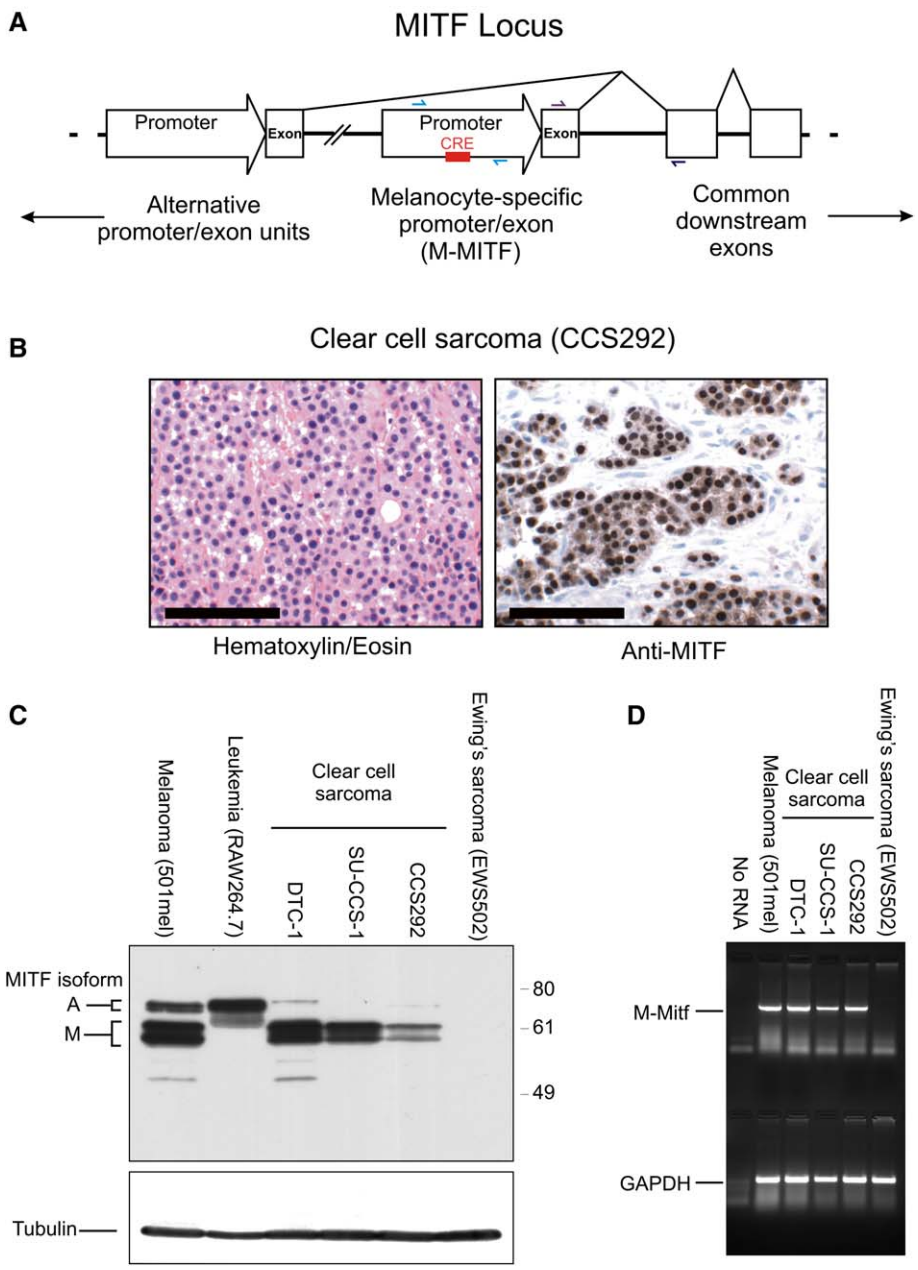

Figure 1. M-MITF expression in clear cell sarcoma

A: The *MITF* locus showing the melanocyte-specific promoter/exon-1 (M-MITF) with its consensus CRE. Location of primers used for MITF chromatin immunoprecipitation (blue) and RT-PCR (purple) are shown.

B: Hematoxylin and eosin and MITF immunostain of primary tumor from which the CCS292 line was derived shows a sheet of tumor cells with strong nuclear reactivity for MITF (bar = 0.1 mm).

C: Extracts of CCS (DTC-1, SU-CCS-1, and CCS292), melanoma (501mel), myelomonocytic leukemia (RAW264.7), or Ewing's sarcoma (EWS502) lines were subjected to Western blot analysis with monoclonal anti-MITF antibody (Weilbaecher et al., 1998).

D: RNA from indicated cells was subjected to RT-PCR with M-form MITF-specific and GAPDH (control) primers.

In CCS cells the *MITF* promoter was constitutively active (Figure 2D) and was not further stimulated by forskolin treatment, despite the requirement of the CRE element (Figure 2D and Figure S1A). Nonetheless, forskolin induced CREB phosphorylation in CCS cells (Figures S1A and S1C). Moreover, inhibition of PKA by treatment with H89 had little effect on *MITF* promoter activity despite inhibiting CREB activation (Figures S1C and S1D). cAMP induction did not significantly affect EWS or EWS-ATF1 levels (Figure S1A). These results demonstrate that the *M-MITF* promoter is active in CCS cells in a CRE-dependent but cAMP-independent manner, a combination of behaviors perfectly matching the predicted activity of EWS-ATF1 on the *MITF* promoter.

Regulation of endogenous MITF by EWS-ATF1

Given the CRE dependence of the *M-MITF* promoter for constitutive activation in CCS cells (Figures 2C and 2D), we asked whether endogenous MITF expression was similarly regulated by EWS-ATF1. Inhibition of EWS-ATF1 was achieved through use of dominant-negative CREB (DNCREB). Mutation of CREB at serine 133, the phosphorylation site that mediates coactivator recruitment and transactivation (Gonzalez and Montminy, 1989), results in strong dominant-negative activity because it retains the dimerization interface but is transcriptionally inactive (Lamph et al., 1990). Expression of DNCREB in CCS cells resulted in potent and selective inhibition of M-MITF expression (Figure 2E). Notably, the ubiquitous A form not only continued to be expressed but was somewhat increased in the presence of DNCREB-encoding adenovirus. These results demonstrate the selective dependence of the *M-MITF* promoter on CREB-like activity and, together with reporter-based assays, that EWS-ATF1 is a necessary transactivator of the *M-MITF* promoter in CCS.

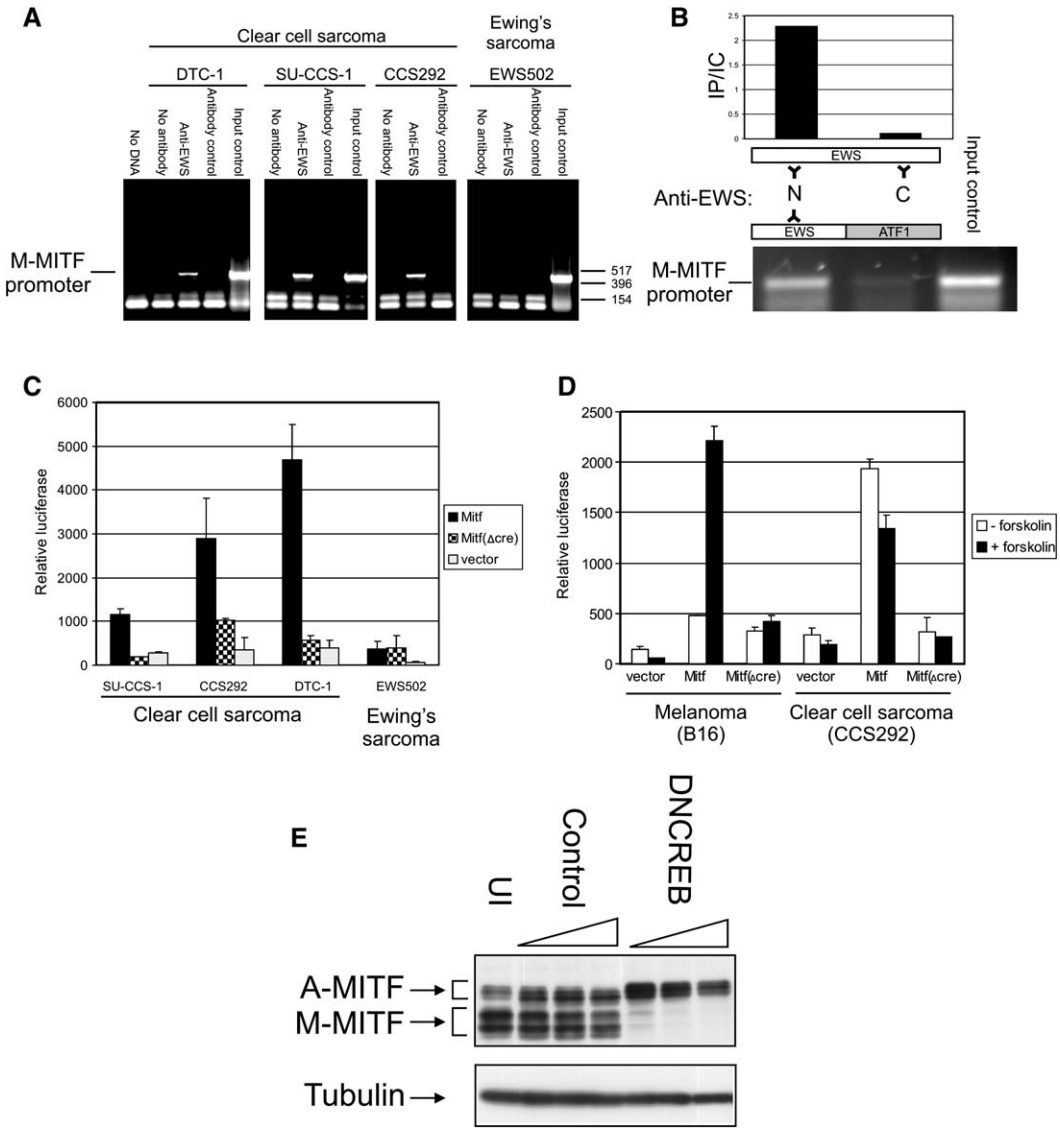

Figure 2. Transcriptional regulation of the *MITF* promoter

A: Chromatin isolated from CCS (DTC-1, SU-CCS-1, or CCS292) or Ewing's sarcoma (EWS502) cells was immunoprecipitated with anti-EWS antibody, anti-CDK2 antibody (control), or no antibody and then amplified with M-MITF promoter-specific primers. Input control indicates amplified total DNA.

B: CCS (DTC-1) chromatin was immunoprecipitated with antibody directed to either the amino (N) or the carboxyl (C) domain of EWS and then quantitatively amplified with M-MITF-specific primers. The ratio of immunoprecipitated versus input control and nonquantitative electrophoresis results are shown.

C: The activity of the M-MITF promoter (Mitf), the identical sequence with mutated CRE [Mitf(Δcre)] or vector control (pGL2) were assayed after transfection by luciferase quantification and normalized to an internal control (pRL).

D: Melanoma (B16) or CCS (CCS292) cell lines were transfected with the M-MITF promoter (Mitf), mutant CRE [Mitf(Δcre)], or vector control. Cells were treated with forskolin for 24 hr prior to luciferase assay.

E: CCS cells were infected with an MOI of 50, 100, or 200 of either control adenovirus or adenovirus encoding dominant-negative CREB (DNCREB). Tubulin serves as a control for cell viability. UI, uninfected cells.

Error bars represent ± standard deviation (SD).

MITF activation requires SOX10 expression

In melanocytes, tissue-specific CRE response of the *MITF* promoter requires cooperativity with the transcription factor SOX10 (Huber et al., 2003). We therefore asked whether SOX10 also participates in *MITF* expression in CCS cells. Three CCS cell lines and a melanoma cell line—but not Ewing's sarcoma or neuroblastoma cell lines—were found to express SOX10 (Figure 3A). In a dose-dependent fashion, a naturally occurring dominant-negative mutant, $SOX10^{dom}$ (Pingault et al., 1998; Southard-Smith et al., 1998), specifically abrogated *MITF* promoter activity (Figure 3B). In 293 cells (lacking endogenous SOX10), ectopic EWS-ATF1 failed to activate the *M-MITF* promoter (Figure 3C), whereas ectopic SOX10 enabled EWS-ATF1-mediated activation. SOX10 alone also measurably activated the *MITF* promoter, likely via cooperativity with endogenous activated CREB, as previously described (Huber et al., 2003). These data demonstrate dependence on SOX10 for CRE-mediated transactivation of the *M-MITF* promoter in CCS and also suggest that the normal cellular counterpart of CCS may be a SOX10-expressing lineage (i.e., neural crest derivative).

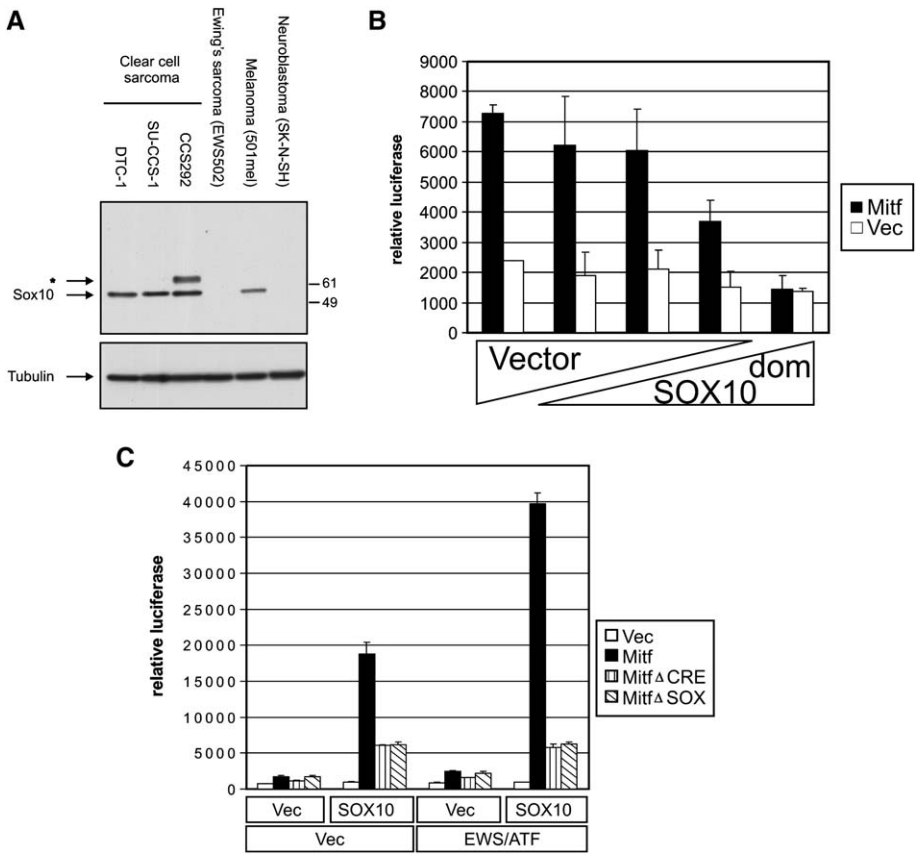

Figure 3. EWS-ATF1 activity on the M-MITF promoter is SOX10 dependent

A: Extracts of CCS, Ewing's sarcoma, melanoma, or neuroblastoma cells were immunoblotted with anti-SOX10 antibody. An uncharacterized cross-reactive band is shown in CCS292 (*).

B: CCS cells (DTC-1) were transfected with increasing amounts of vector (pCDNA3) directing expression of an inactive form of SOX10 ($SOX10^{dom}$) together with either vector (pGL2) or the M-MITF promoter (Mitf). Total DNA content was identical for all conditions. Luciferase activity is shown relative to an internal control (pRL).

C: Vector (pcDNA3) or wild-type SOX10 and either vector (pcDNA3) or EWS-ATF1 type I (EWS/ATF) were cotransfected into HEK293 cells together with vector (pGL2), M-MITF promoter (Mitf), CRE mutant (MitfΔCRE), or SOX10 binding site mutant (MitfΔSOX).

Error bars represent ± SD.

MITF confers melanocytic differentiation in clear cell sarcoma

To examine the role of MITF in the melanocytic differentiation of CCS, we suppressed endogenous EWS-ATF1 activity with DNCREB. In addition, MITF activity was modulated with either full-length MITF or a dominant-negative mutant lacking the 5′ transactivation domain and incorporating a basic region mutation that prevents DNA binding (Widlund et al., 2002). This MITF mutant retains the ability to dimerize through an intact HLHzip domain and sequesters wild-type partners in non-DNA binding complexes but has no effect on the MYC family of E box binding transcription factors. Modulation of MITF activity did not affect EWS-ATF1 levels (Figure S1E). To test whether EWS-ATF1 or MITF inhibition affects endogenous MITF target gene expression, quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assays were performed on RNA derived from adenovirus-infected CCS cells. Given the significance of HMB45 immunostaining for the pathological identification of CCS and the identification of PMEL17 (the gene encoding the HMB45 antigen) as an MITF target (Du et al., 2003), we examined PMEL17 expression. As shown in Figure 4A, PMEL17 expression was modestly increased by MITF overexpression, whereas DNMITF significantly reduced PMEL17 mRNA levels. Inhibition of EWS-ATF1 similarly decreased PMEL17 expression, whereas coexpression of MITF overcame this effect in a dose-dependent fashion (Figure 4B). DNMITF also inhibited expression of the MITF target gene and melanocytic marker MLANA (Figure S1E). These experiments suggest that pigment gene expression in CCS is downstream of EWS-ATF1 via direct regulation by MITF.

We then examined the roles of MITF and EWS-ATF1 on the pigmented phenotype. To quantitatively assay pigmentation, we spectrophotometrically monitored the conversion of uncolored 3,4-dihydroxy-L-phenylalanine (L-DOPA) substrate to darkly colored dopaquinone, a direct measure of tyrosinase activity, the rate-limiting enzyme in melanin biosynthesis, in CCS cell extracts (Cooksey et al., 1997; Oetting et al., 1998). As shown in Figure 4C, disruption of endogenous MITF decreased tyrosinase activity, whereas overexpression of wild-type MITF induced tyrosinase activity. Similarly, disruption of EWS-ATF1 resulted in significantly diminished pigmentation (Figure 4D). Importantly, coexpression of MITF in EWS-ATF1-inhibited cells significantly rescued pigmentation (Figure 4E). Although dominant-negative CREB lacks specificity for EWS-ATF1, that MITF can rescue pigmentation in the context of CREB family inhibition demonstrates the importance of MITF for pigmentation and suggests that EWS-ATF1 acts through MITF to mediate the melanocytic differentiation characteristic of CCS.

MITF activity is necessary for tumor cell proliferation

We hypothesized that the targeting of MITF by EWS-ATF1 may be critical for proliferation or survival of CCS. To test this, we inhibited EWS-ATF1 or MITF activity in CCS cells using retrovirally transduced dominant-negative forms of these proteins as well as shRNA. Infected cells were cultured in soft agar under conditions selective for viral transduction. Whereas CCS colonies developed from cells infected with the parental virus, colony formation was virtually abrogated in CCS cells expressing either DNCREB or DNMITF (Figure 5A). The integrity of the viruses was confirmed by performing identical growth assays on

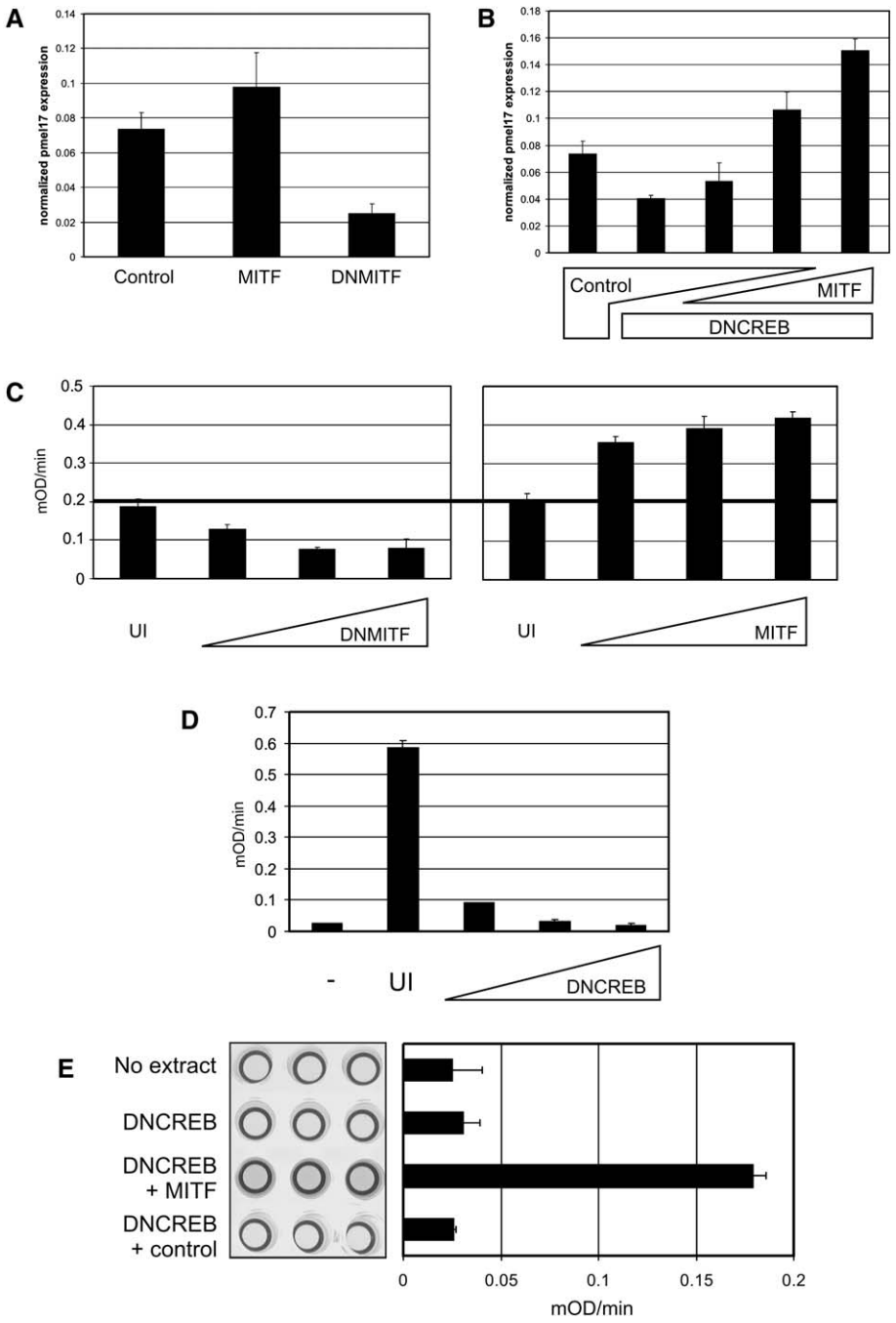

Figure 4. MITF regulates CCS pigmentation

A: PMEL17 expression from CCS (CCS292) cells infected with adenovirus expressing a control polypeptide (Control), MITF, or DNMITF was quantitated by RT-PCR and normalized to GAPDH RNA levels.

B: CCS cells were infected with adenovirus encoding DNCREB and increasing MOI of adenovirus encoding MITF.

C: Extracts of uninfected (UI) or CCS cells infected with an MOI of 50 to 200 of adenovirus expressing either MITF or DNMITF were assayed for enzymatic conversion of DOPA to dopaquinone at 48 and 72 hr, respectively. Slope of the OD versus time curve is plotted.

D: No extract (−), extracts of uninfected (UI) cells, or extracts of CCS292 cells infected with an MOI of 50 to 200 of DNCREB-expressing adenovirus were assayed for DOPA conversion at 72 hr.

E: Extracts of CCS292 cells infected with the highest MOI of DNCREB from **D** were coinfected with MITF or control vector and assayed for DOPA conversion at 72 hr. Representative triplicate microtiter well assays are shown to the left. Quantitation of DOPA conversion is shown to the right. Error bars represent ± SD.

similarly infected Ewing's sarcoma cells as controls. This experiment demonstrates that both EWS-ATF1 and MITF activity are necessary for CCS survival and/or proliferation.

To specifically examine the role of MITF in tumor cell survival, CCS cells were transfected with plasmid expressing the MITF-directed shRNA or a control shRNA (Figure 5B). MITF knockdown significantly decreased cell viability (Figure 5C). To examine MiT family functional redundancy in this setting, we assessed the ability of alternate MiT family members to rescue the lethal effects of MITF knockdown by cotransfecting plasmids encoding TFEB or TFE3 together with MITF shRNA. TFEB expression completely rescued viability in a dose-dependent fashion (Figure 5C), as did TFE3 cotransfection (data not shown). Taken together with the effect of dominant-negative MITF on growth in soft agar, these data demonstrate that MITF is necessary for CCS viability in a manner that can be functionally replaced by other MiT members.

As a complementary test of the functional overlap of MiT oncogenes, we utilized TFE3-directed shRNA (Figure 5D) to determine the requirement for TFE3 for the growth/survival of a *TFE3*-translocated renal carcinoma cell line. Transfection of TFE3-directed shRNA into a renal cell carcinoma cell line that harbors the NonO-TFE3 translocation but fails to express wild-type TFE3 (UOK109; Clark et al., 1997) prevented virtually all colony growth (Figure 5E). However, cotransfection of ectopic MITF efficiently rescued colony formation, demonstrating that, in the context of translocation-associated renal cell carcinoma, MITF can replace translocated TFE3.

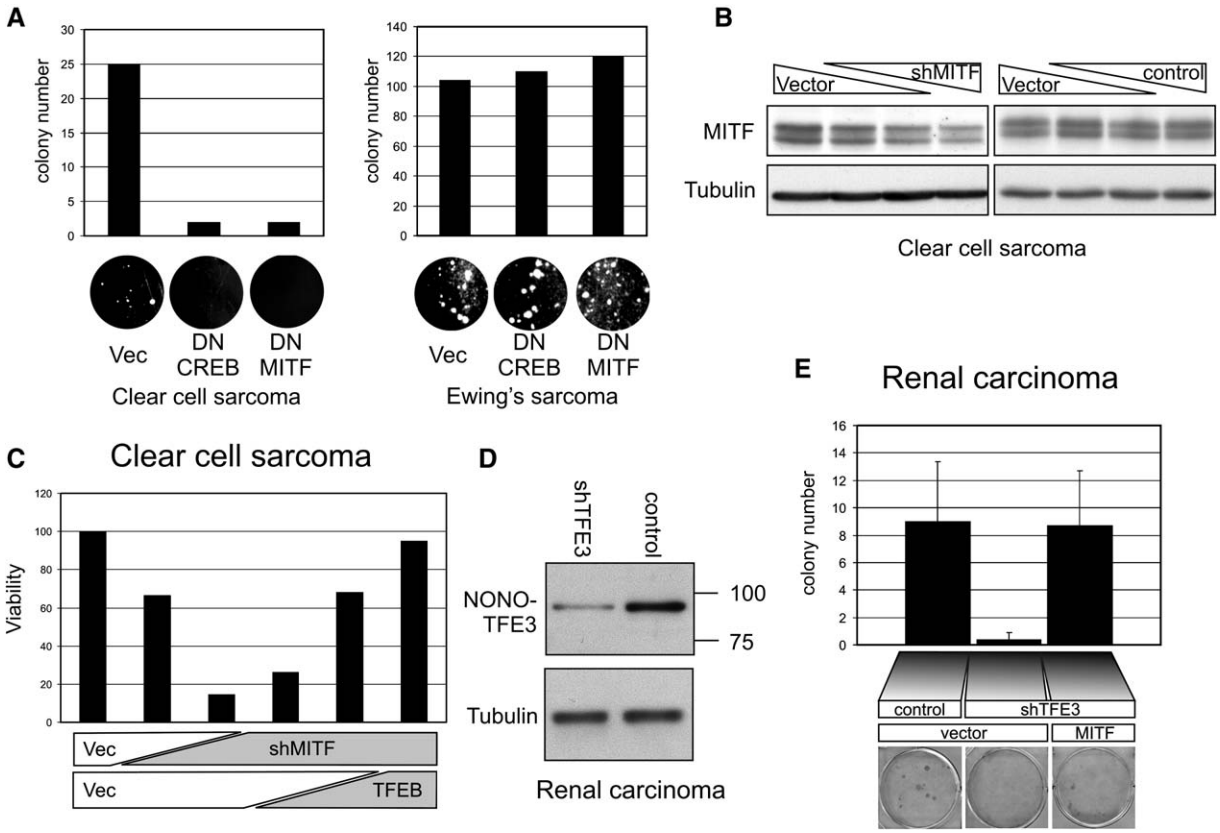

Figure 5. MiT family members demonstrate functionally redundant oncogenic activity in CCS and renal carcinoma

A: CCS292 or Ewing's sarcoma (EWS502) cells were infected with either empty retrovirus (Vec) or retrovirus expressing either DNCREB or DNMITF. Cells were briefly cultured, then plated in soft agar containing G418. Visible colonies were counted.

B: CCS (SU-CCS-1) cells were transfected with 1–3 μg plasmid (pSuper; Vec) expressing either MITF-directed (shMITF) or luciferase-directed (control) shRNA. Extracts of unselected cells were analyzed for MITF or tubulin (control) expression.

C: CCS cells (SU-CCS-1) were transfected with 1–3 μg pSuper (Vec) or pSuper expressing shMITF and either pcDNA3 (Vec) or pCDNA3 encoding TFEB, as well as 0.5 μg pcDNA3 encoding GFP. Two days after transfection, trypan-negative cells were counted, and transfection efficiency was assayed by FACS. The percent GFP-positive and trypan-negative is shown.

D: Lysates of GFP-cotransfected, sorted UOK109 cells transfected with plasmid expressing TFE3 shRNA or control shRNA were analyzed with anti-TFE3 or anti-tubulin (control) antibody.

E: TFE3-translocated renal carcinoma cells (UOK109) were transfected with TFE3-directed or luciferase-directed (control) shRNA together with either vector (pcDNA) or plasmid encoding MITF as well as pBABEpuro (4 μg total plasmid/well). Puromycin-resistant colonies were stained with crystal violet and counted. Photographs of representative wells are shown.

Error bars represent ± SD.

MITF mediates EWS-ATF1-induced tumor growth

Although it is likely that the EWS-ATF1 translocation product aberrantly regulates numerous CREB/ATF-regulated genes, we hypothesized that MITF may be both necessary and sufficient to explain the effect of EWS-ATF1 on CCS cell growth and survival. To test this hypothesis, we designed shRNA directed toward the EWS domain of EWS-ATF1 (Figure 6A). Retroviral transduction of CCS with EWS-directed shRNA resulted in a significant decrease in the number of colonies in soft agar (Figure 6B) with incomplete suppression of colony formation likely reflecting partial EWS-ATF1 knockdown. Importantly, exogenous MITF expression in the presence of the EWS-targeted shRNA significantly rescued colony formation. Control cells (HeLa) lacking an EWS translocation, when transduced with EWS-directed or control shRNA, failed to demonstrate a similar decrease in cell viability.

To extend these analyses to CCS tumor growth in vivo, we generated a xenograft model. Luciferase-expressing CCS tumor cells implanted into mice reproducibly demonstrated measurable and sustained logarithmic tumor growth (Figure 6C). Equal numbers of CCS cells were transduced with retrovirus expressing EWS-directed shRNA and retrovirus encoding MITF (or controls). After 48 hr of ex vivo puromycin selection, the cell population was subcutaneously implanted, and tumor growth was monitored quantitatively. EWS shRNA potently inhibited in vivo tumor growth, whereas coexpression of MITF rescued the growth-suppressive effect of EWS-ATF1 knockdown (Figure 6D). Taken together with the direct targeting of MITF by EWS-ATF1, these data strongly suggest that MITF is both a necessary and sufficient target gene mediating the oncogenic activity of EWS-ATF1 on CCS growth.

Discussion

Our studies demonstrate that the melanocyte-specific *MITF* promoter is occupied and constitutively activated by EWS-ATF1 in CCS. M-MITF promoter activity is CRE dependent in

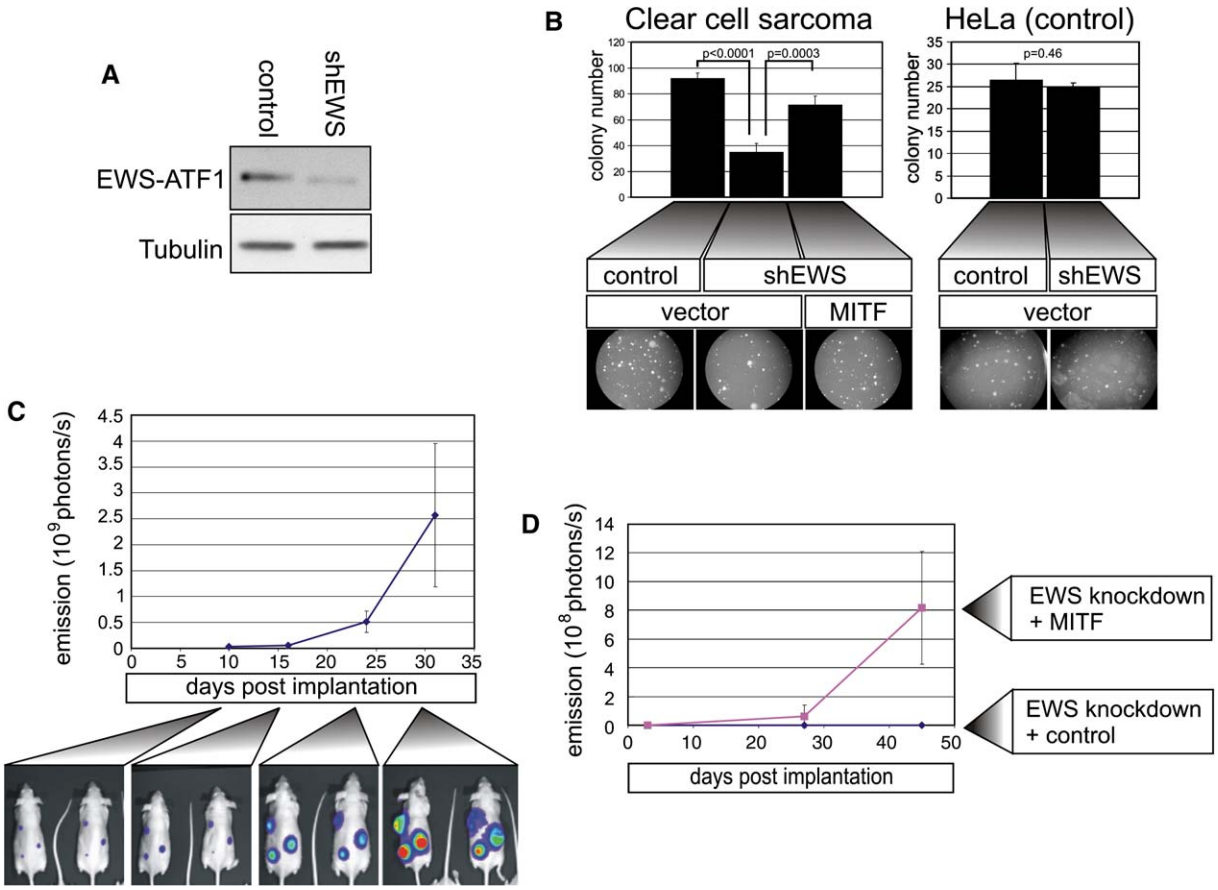

Figure 6. MITF rescues EWS knockdown-mediated loss of cell viability in soft agar and in vivo

A: CCS292 cells transfected with luciferase-directed (control) or EWS-directed shRNA together with GFP were sorted and analyzed for knockdown efficiency with anti-EWS or anti-tubulin (control) antibody.

B: CCS292 or HeLa (control) cells were infected with retrovirus expressing luciferase-directed (control) or EWS-directed (shEWS) shRNA in the presence of either control or MITF-expressing retrovirus (vector). Cells were cultured under selective conditions in soft agar, and colonies were counted.

C: Luciferase-expressing CCS cells (CCS292) were injected subcutaneously into nude mice. Mice were imaged at the indicated days, and photonic emission was quantitated. False-color heat map representations of photonic emission overlaying photographs of mice are shown.

D: Luciferase-expressing CCS292 cells were transduced with retrovirus expressing EWS-directed shRNA and puromycin N-acetyl transferase together with either control retrovirus or retrovirus encoding MITF. After a 48 hr period of antibiotic selection, cells were injected subcutaneously into nude mice and then imaged on the indicated days.

Error bars represent ± SD.

the absence of a cAMP requirement, an unusual scenario explained by the cAMP independence but CRE dependence of the EWS-ATF1 fusion oncoprotein (Brown et al., 1995). These findings contrast those of Li et al., who failed to identify measurable *M-MITF* promoter activity using reporter plasmid-based assays in transfected CCS cells, despite demonstrating that the same cells express M-MITF protein (Li et al., 2003). This discrepancy likely results from significant differences in reporter construction, transfection techniques, and assay conditions. Our data are complemented by the demonstration that endogenous MITF expression levels are coordinately regulated in response to modulation of EWS-ATF1 activity. Furthermore, we show that MITF is critical for both melanocytic differentiation and growth/survival of CCS.

The relationship between CCS and melanoma has been uncertain, since the initial characterization of CCS (Chung and Enzinger, 1983; Enzinger, 1965). Given that many of the genes associated with melanocytic differentiation are targets of MITF, EWS-ATF1-mediated activation of MITF likely explains the melanocyte differentiation profile exhibited by these tumors. Prior reports are consistent with this observation. Microarray-based gene expression studies in CCS confirmed MITF expression in primary tumor material and cell lines (Schaefer et al., 2004; Segal et al., 2003). Moreover, these studies demonstrated the characteristic MITF target gene expression signature in CCS when compared to other sarcomas, supporting a functional role for MITF. Precedence for MITF to impose a pattern of melanocytic differentiation derives from studies of murine fibroblasts (Tachibana et al., 1996). However, it is likely that certain context-dependent features contribute importantly to the expression and differentiation program specified by MITF, such as the presence of SOX10, which is typically restricted to cells derived from the neural crest and central and peripheral nervous system (Kuhlbrodt et al., 1998; Pusch et al., 1998; Southard-Smith et al., 1998). As reported here, SOX10 was required for MITF expression in CCS. Cotransfection experiments suggest that CRE-regulated genes are not uniformly activated by chimeric EWS-ATF1, implying that additional mechanisms specify

target gene activation (Li and Lee, 1998). MITF may be one of a restricted group of target genes that exhibit promoter/enhancer context specificity, in this case constrained by SOX10. The apparent absence of M-MITF expression in a single case of angiomatoid fibrous histiocytoma that expresses EWS-ATF1 may reflect the absence of requisite SOX10 coexpression (Hallor et al., 2005) and suggests the existence of alternative targets of the fusion protein in a different cell lineage.

We demonstrate here that in CCS the EWS-ATF1 fusion protein acts by targeting MITF to promote tumor cell survival/proliferation. The amplification of MITF in melanoma and its ability to participate in melanocyte transformation further support the role of MITF in oncogenesis. It may be interesting in future studies to examine the effects of EWS-ATF1 expression in melanoma cells, although it is unclear how its actions may be modulated by homeostatic mechanisms.

Dysregulation of *MITF* by EWS-ATF1 in CCS and of *TFE3* or *TFEB* by translocation in pediatric renal carcinomas suggests the existence of a discrete family of related human cancers. The functional relatedness of the MiT oncogenes is supported by the ability of TFE3 or TFEB to rescue MITF deficiency in CCS and the reciprocal ability of MITF to rescue TFE3 deficiency in pediatric renal cell carcinoma. Melanoma, pediatric renal cell carcinoma, clear cell sarcoma, and alveolar soft part sarcoma represent clinically and morphologically distinct malignancies that would not otherwise be coclassified. However, it is notable that many of these tumors share not only dysregulated expression of an MiT transcription factor, but also previously documented (mis)expression of melanocytic histopathologic markers, such as HMB45 and melanA/MART1. Since these markers are now known to be direct transcriptional targets of MITF in melanocytes and melanoma (Du et al., 2003), it is likely that they are similarly regulated by TFE3 or TFEB in the other MiT-associated cancers. The MiT tumors share the characteristic of particular refractoriness to traditional chemotherapeutics and radiation therapy (Deenik et al., 1999; Kuiper et al., 2003a; Pappo et al., 1996; Thompson et al., 2005). However, mechanistic insight into these tumors may suggest therapeutic approaches. For example, a fraction of melanoma patients respond strikingly to various immunotherapeutic modalities, and many of the antigens implicated as the successful targets of these immune responses are melanocytic differentiation factors (Bakker et al., 1994; Marincola et al., 1996; Schmollinger et al., 2003). Several of these are known transcriptional targets of MITF. The expression of some of these targets in nonmelanoma MiT-associated tumors provides a rationale for an immunotherapeutic approach to treat these diseases. Another theoretical approach would directly target MiT oncoproteins, recognizing clear challenges in producing inhibitors of non-ligand-associated transcription factors. Finally, the discovery of MiT family target genes important for viability may identify proteins suitable for pharmacological suppression. Although likely lacking intrinsic mutations, these proteins may contribute directly to tumor growth given their hardwired connection to MITF, TFEB, or TFE3.

We have thus demonstrated that MITF is the critical oncogenic target of EWS-ATF1 in CCS. This observation, taken together with MiT family amplification and translocations, supports the hypothesis that multiple mechanisms arise to dysregulate MiT family members, thereby conferring oncogenic properties to a transcription factor family otherwise associated with lineage differentiation. Directed expression of EWS-ATF1 to generate a mouse model of CCS could provide additional insight into the roles of EWS-ATF1 and the MiT family in solid tumor development. The recognition of shared biologic features in these seemingly diverse malignancies will hopefully enhance the discovery of improved therapeutic approaches.

Experimental procedures

Cell culture

DTC-1 (Brown et al., 1995), SU-CCS-1 (Epstein et al., 1984; Zucman et al., 1993), CCS292, and EWS502 cells were grown in RPMI-1640 supplemented with 15% fetal bovine serum (FBS) and penicillin and streptomycin. 501mel cells were grown in Ham's F-10 with 10% FBS. B16, HEK293, HeLa, UOK109, and RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS.

RNA identification and quantitation

Total RNA was purified with Trizol (Gibco-BRL). RT-PCR was performed using Tth DNA polymerase (Roche Molecular Biochemicals) as suggested by the manufacturer. The 5′ primer was specific for the M-MITF isoform RNA (sense, 5′-CCTTCTCTTTGCCAGTCCATCTTC-3′; antisense, 5′-GATCAATCAAGTTTCCCGAGACAG-3′). For quantitative assessment of RNA, 48 hr after cells were infected RNA was isolated (RNeasy, Qiagen). RNA was subjected to quantitative PCR using PMEL17 and GAPDH primers as described (Du et al., 2003).

Chromatin immunoprecipitation

Nuclear extracts were prepared from tissue culture cells as described (Du et al., 2003). Chromatin immunoprecipitated with polyclonal anti-EWS/FLI1, anti-CDK2 polyclonal antibody (Santa Cruz), or anti-EWS (BL1083 and BL1085, Bethyl) were amplified as described (Du et al., 2003, 2004) using primers directed to the melanocyte-specific MITF promoter (sense, 5′-CTTGAACATTCAGCACAGAGTCTCTT-3′; antisense, 5′-ACTTTAGCACAGAACCCTGCTTATA-3′). SYBR Green (Applied Biosystems) was used to quantitate recovered chromatin.

Transfection, reporter assays, and statistical analyses

Transfections using GeneJammer (Stratagene) or Fugene 6 (Roche) at 3:1 ratio relative to DNA were performed using wild-type or mutant MITF promoter (−387 to +97) in pGL2basic (Promega) as described (Huber et al., 2003; Price et al., 1998). Where indicated, cells were treated with forskolin (20 μM; Sigma), 3-isobutyl-1-methylxanthine (10 μM; Sigma), or H89 (30 μM; Calbiochem). SU-CCS-1 cells were transfected by nucleofection (Amaxa) using 3 μg plasmid DNA in solution R using program T20. Two days after transfection, cells and debris were fixed with 0.5% paraformaldehyde in PBS. GFP expression was analyzed by fluorescence activated cell sorting (FACS, Facscalibur, Becton Dickinson). Trypan-excluding cells were counted. SOX10 and SOX10^{dom} expression constructs were previously described (Huber et al., 2003). Transfected CCS292 cells were sorted based on GFP cotransfection, using a DakoCytomation High Speed MoFlo Sorter.

Adenovirus generation and infection

To generate dominant-negative CREB adenovirus, the Hind III-XbaI fragment from FlagCREB M1 was cloned into a CMV-driven shuttle vector containing IRES-hrGFP and recombined with pAdEasy-1 (He et al., 1998) using BJ5183 cells. The same shuttle vector was used to generate adenoviruses used in Figure 4 that express murine wild-type MITF or a dominant-negative MITF lacking amino acids 1–195 (includes transactivation domain) and deleting the critical basic domain Arg217 (Hemesath et al., 1994; Widlund et al., 2002). Cells were infected at multiplicities of infection (MOI) between 50 and 200 with CsCl gradient-purified adenovirus as previously described (Wu et al., 2000).

Tyrosinase pigmentation assay, immunohistochemistry, and Western blot analysis

Forty-eight or seventy-two hours after adenoviral infection, CCS292 cells were washed and lysed in PBS/1% Triton X-100 with protease inhibitors (Complete, Boehringer Manheim). Centrifuged supernatants normalized for protein content (Bio-Rad) were added to 1 mM 3,4-dihydroxy-L-phenylalanine

(L-DOPA; Sigma) in PBS, incubated at room temperature, and OD490 was measured over an 8–12 hr interval using a microplate reader (Model 3550, Bio-Rad). The slope of the OD versus time plot indicates rate of dopaquinone pigment formation. For Western blot analyses, anti-SOX10 (Cemines), anti-MITF (C5), anti-tubulin (Sigma), antiphospho-CREB (9191, Cell Signaling Technology), CREB (9192, Cell Signaling Technology), or anti-EWS (BL1083, Bethyl) antibodies were used. Immunohistochemical staining for MITF was carried out as described (Granter et al., 2001).

Retroviral infection, soft agar, and in vivo growth assays

CCS, Ewing's sarcoma cells, or HeLa cells were incubated with the appropriate retrovirus prepared as described (Du et al., 2004) in the presence of polybrene (8 μg/ml) for 2–16 hr. Two to three days after infection, cells were collected and resuspended in media containing 0.4% noble agar, 1× RPMI, 20% FBS, and puromycin (2 μg/ml) over cell-free bottom agar containing 0.5% noble agar in RPMI (or DMEM for HeLa) with 20% FBS. Cells were cultured several weeks, during which time standard growth media overlaid on the agar was replaced every 3–5 days. Colonies greater than 1 mm were counted. UOK109 cells were transfected with relevant plasmids including a constant amount of pBabe-Puro with Fugene (Roche) at a ratio of 3:2. Forty-eight hours after transfection, cells were selected with puromycin (3 mg/ml). For Western blot analyses, extracts of UOK109 cells were made after 24 hr of selection. CCS cells were transduced with retrovirus encoding MMTV-directed expression of luciferase-aminoglycoside phosphotransferase and selected with Geneticin (0.5 mg/ml; Calbiochem). Luc/Neo CCS cells were transduced with retrovirus expressing EWS-directed shRNA and puromycin N-acetyl transferase as well as LNCX2-MITF retrovirus or LNCX2 retrovirus daily for 2 days, followed by selection in puromycin for 48 hr, after which all the cells were collected and injected subcutaneously into NCR nude mice (Taconic). Animals were anesthetized with ketamine and xylazine and treated with luciferin (Promega) peritoneally followed by imaging (IVIS, Xenogen). All mouse experimentation was performed under ACUC-approved DFCI protocol 02-030.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures and one supplemental figure and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/6/473/DC1/.

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