

β -Catenin–induced melanoma growth requires the downstream target *Microphthalmia*-associated transcription factor

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The transcription factor *Microphthalmia*-associated transcription factor (MITF) is a lineage-determination factor, which modulates melanocyte differentiation and pigmentation. MITF was recently shown to reside downstream of the canonical Wnt pathway during melanocyte differentiation from pluripotent neural crest cells in zebrafish as well as in mammalian melanocyte lineage cells. Although expression of many melanocytic/pigmentation markers is lost in human melanoma, MITF expression remains intact, even in unpigmented tumors, suggesting a role for MITF beyond its role in differentiation. A significant fraction of primary human melanomas exhibit deregulation (via aberrant

nuclear accumulation) of β -catenin, leading us to examine its role in melanoma growth and survival. Here, we show that β -catenin is a potent mediator of growth for melanoma cells in a manner dependent on its downstream target MITF. Moreover, suppression of melanoma clonogenic growth by disruption of β -catenin–T-cell transcription factor/LEF is rescued by constitutive MITF. This rescue occurs largely through a prosurvival mechanism. Thus, β -catenin regulation of MITF expression represents a tissue-restricted pathway that significantly influences the growth and survival behavior of this notoriously treatment-resistant neoplasm.

Introduction

Among cancers, melanoma is well known both for its rapidly increasing incidence and its resistance to virtually all but surgical therapies. Melanoma arises from melanocytes, neural crest derived pigment cells in the skin and eye. During melanoma carcinogenesis, many of the normal markers of the melanocyte lineage are lost, but most if not all melanomas retain expression of the basic/helix-loop-helix/leucine-zipper (bHLHzip)* transcription factor *Microphthalmia*-associated transcription factor (MITF) (King et al., 1999). MITF was

first identified in mouse as a locus whose mutation results in absence of pigment cells causing white coat color and deafness due to melanocyte deficiency in the inner ear (Hodgkinson et al., 1993). In humans, mutation of MITF results in Waardenburg Syndrome IIa, a condition characterized by white forelock and deafness (Hughes et al., 1994). Recent studies have elucidated a network of genes thought to regulate expression or activity of MITF (for review see Goding, 2000). Mutation of many of these genes, including Pax3, Sox10, endothelin-3, c-Kit, or endothelin receptor B, produces very similar pigmentation deafness syndromes in humans due to loss of viable melanocytes (for review see Price and Fisher, 2001).

A role for MITF in pigment gene regulation has been strongly suggested (Bentley et al., 1994; Hemesath et al., 1994; Yasumoto et al., 1994, 1997) based on the existence of highly conserved MITF consensus DNA binding elements in the promoters of the three major pigment enzyme genes: tyrosinase, Tyrp1, and Dct. Mounting evidence also suggests a role for MITF in the commitment, proliferation, and survival of melanocytes before and/or during neural crest cell migration (Opdecamp et al., 1997). Moreover, examination of heterozygous mutant MITF mice shows that abso-

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*Abbreviations used in this paper: APC, adenomatous polyposis coli; bHLHzip, basic/helix-loop-helix/leucine-zipper; dn, dominant-negative; GSK, glycogen synthase kinase; hr, humanized Renilla; LEF, lymphoid enhancer binding factor; MITF, *Microphthalmia*-associated transcription factor; TCF, T-cell transcription factor.

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lute numbers of melanoblasts are reduced (Hornyak et al., 2001), suggesting a dosage dependence on MITF. Collectively, these studies suggest that MITF, in addition to its role in differentiation pathways such as pigmentation, may serve an important role in the proliferation and/or survival of developing melanocytes. The retention of MITF expression in the vast majority of human primary melanomas, including nonpigmented tumors, is consistent with this possibility and has also led to the widespread use of MITF as a diagnostic tool in this malignancy (King et al., 1999; Salti et al., 2000; Chang and Folpe, 2001; Miettinen et al., 2001).

The Wnt signaling pathway plays an integral role throughout development (for review see Wodarz and Nusse, 1998), particularly in the neural crest (for review see Dorsky et al., 2000a), and has also been strongly implicated in human tumorigenesis (for reviews see Bienz and Clevers, 2000; Peifer and Polakis, 2000). Wnt signaling plays a pivotal role in neural crest population expansion (Ikeya et al., 1997) and melanocyte lineage-specific expansion and differentiation (Dorsky et al., 1998; Dunn et al., 2000; Jin et al., 2001). A key downstream effector of this pathway is β -catenin, a multifunctional protein which can either bind to E-cadherins as an integral part of the actin cytoskeleton which anchors cell-cell adhesion, or can act as a transcriptional coactivator by interacting with the T-cell transcription factor (TCF)/lymphoid enhancer binding factor (LEF) family of DNA binding proteins in the nucleus. In the absence of Wnt-signals, β -catenin is targeted for degradation via phosphorylation by a complex consisting of glycogen synthase kinase (GSK3 β), axin, and adenomatous polyposis coli protein (APC). Wnt signals lead to inactivation of GSK3 β , and thus stabilize β -catenin levels in the cell which increase transcription of downstream target genes. Mutations in multiple components of the Wnt pathway have been identified in many human cancers, and share the property of inducing nuclear accumulation of β -catenin (Polakis, 2000). Dysregulation of β -catenin levels has been shown to produce aberrant transactivation of downstream proto-oncogenes such as cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999) and c-Myc (He et al., 1998). In human melanoma, stabilizing mutations of β -catenin have been found in a significant fraction of established cell lines (Rubinfeld et al., 1997). Importantly, in primary human melanoma specimens, almost one third display aberrant nuclear accumulation of β -catenin, although generally without evidence of direct mutations within the β -catenin gene itself (Rimm et al., 1999). These observations are consistent with the hypothesis that this pathway contributes to behavior of melanoma cells and might be inappropriately deregulated in the genesis of this disease.

During zebrafish development, Wnt signaling has been demonstrated to be both necessary and sufficient to promote pigment cell fate of neural crest cells through its effector β -catenin at the expense of neural and glial lineages (Dorsky et al., 1998). The zebrafish MITF homologue, *nacre*, was shown to mediate this fate decision as a downstream target of Wnt signaling (Dorsky et al., 2000b). In mammalian cells, Wnt-3a, was also shown to induce MITF expression (Takeda et al., 2000). These reports indicate that the MITF promoter is transactivated by β -catenin in a manner dependent

on TCF/LEF DNA consensus elements. Based on their pivotal requirements during melanocyte development, it is of interest to speculate that MITF and Wnt/ β -catenin may also play central roles in the behavior of melanoma cells.

Here we show that β -catenin is a significant regulator of melanoma cell growth, with MITF as a critical downstream target. Importantly, disruption of the canonical Wnt pathway abrogates growth of melanoma cells, and constitutive overexpression of MITF rescues the growth suppression. These observations establish a critical role for both β -catenin and MITF in cell growth and survival of human melanoma.

Results

Proliferation of melanoma cells can be altered by overexpression of β -catenin/dnTCF

To assess the functional relevance of Wnt/ β -catenin signals governing the proliferation and survival of melanoma cells, we began by ectopically expressing β -catenin in mouse B16 melanoma cells. Additionally, we expressed a dominant-negative (dn) allele of TCF-1 (dnTCF) that is incapable of interacting with β -catenin, but retains TCF/LEF DNA binding specificity (van de Weterling et al., 1996). B16 melanoma cells were cotransfected with a vector that directs the expression of humanized Renilla (hr) GFP in order to monitor the transfected cells during flow cytometry by gating on GFP-positive cells. Overexpression of β -catenin resulted in an increase of the S-phase fraction as compared with vector control (26–34.5%; Fig. 1 A), whereas ectopic expression of dnTCF resulted in a significant decrease in S-phase content (26–17%). In parallel experiments, we performed colony-forming assays, substituting hrGFP with a puromycin resistance vector, and selected the cells for clonogenic growth (Fig. 1 B). Ectopic expression of β -catenin significantly stimulated colony formation relative to vector control. Conversely, addition of dnTCF was deleterious to colony formation. Thus, β -catenin stimulates proliferation and clonogenic growth in melanoma cells, and blockade of this pathway by dnTCF appears to be growth suppressive.

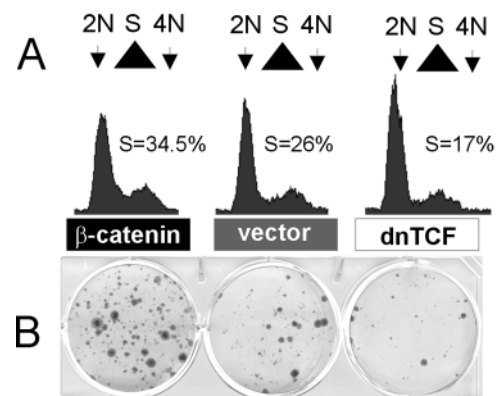


Figure 1. Overexpression of β -catenin is proliferative and pro-survival in melanoma cells and dnTCF is inhibitory. (A) Cell cycle profiles of mouse B16 melanoma cells 48 h after transient transfection by indicated vectors and GFP as analyzed by propidium iodide staining and flow cytometry. (B) Representative colony-forming assays in mouse B16 melanoma cells.

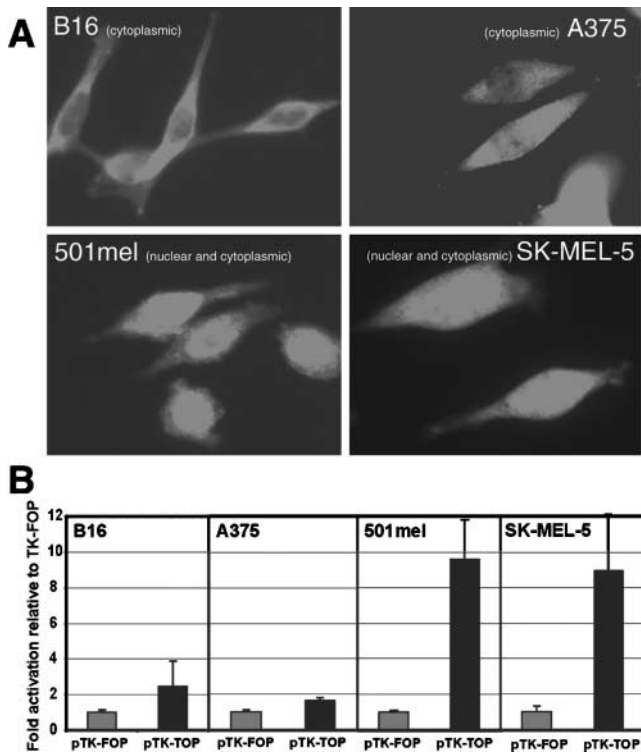


Figure 2. Localization of endogenous β -catenin in melanoma cells and corresponding TCF/LEF transcriptional activity. (A) Melanoma cells (mouse B16 melanoma, human A375, human 501mel, and human SK-MEL-5) were assayed by immunofluorescence for the localization of β -catenin. As shown, B16 and A375 do not display aberrant nuclear accumulation of β -catenin in comparison with 501mel and SK-MEL-5, which display both cytoplasmic and nuclear β -catenin. (B) Bar graphs of the relative activity of TK-TOP, with optimal TCF/LEF binding sites and TK-FOP which carries mutated sites for TCF/LEF as an enhancer for the TK immediate early promoter driving luciferase.

We analyzed endogenous β -catenin localization in the melanomas used in this and subsequent experiments. Using immunofluorescence we found that mouse B16 melanoma and human A375 melanoma cells exhibit mostly cytoplasmic β -catenin staining, whereas human 501mel and SK-MEL-5 melanomas exhibit aberrant nuclear accumulation in addition to cytosolic staining (Fig. 2 A). This finding is comparable to the previous observations that demonstrated β -catenin within the nucleus of $\sim 1/3$ of primary melanoma specimens (Rimm et al., 1999). Reporter assays were performed and showed that cells without aberrant nuclear accumulation of β -catenin retain a modest (approximately twofold) but measurable degree of endogenous TCF/LEF

transcriptional activity (Fig. 2 B). The aberrant nuclear accumulation of β -catenin in 501mel and SK-MEL-5 is associated with significantly higher activity for the reporter containing optimal TCF/LEF binding sites (TK-TOP) relative to the one with mutated TCF/LEF sites (TK-FOP). These experiments suggest that the β -catenin–TCF/LEF transcriptional pathway is activated to varying degrees among melanoma cell lines, a finding which is consistent with data from primary human melanomas (Rimm et al., 1999). Moreover, the β -catenin–TCF/LEF pathway appears to be active, although to a lesser extent, even in those melanomas without excessive quantities of β -catenin in the nucleus. Ectopic expression of β -catenin in these human melanoma lines revealed a correlation between absence of aberrant nuclear β -catenin and a mitogenic effect from exogenous β -catenin (Table I). The lack of a mitogenic effect for exogenous β -catenin in cells with aberrant nuclear accumulated β -catenin is consistent with the possibility that these cells are already maximally stimulated through this pathway, and therefore less responsive to additional (exogenous) β -catenin. However, expression of dnTCF does profoundly disrupt proliferation in all tested melanoma lines (see below), suggesting that the pathway remains important in cells exhibiting either nuclear plus cytoplasmic or mostly cytoplasmic β -catenin.

The MITF promoter is bound and regulated in a lineage specific manner by β -catenin

The MITF promoter has recently been shown to be a downstream target of Wnt signaling in melanocytes (Dorsky et al., 2000b; Takeda et al., 2000). To extend these observations, we analyzed β -catenin responsiveness of the MITF promoter in B16 melanoma cells (Fig. 3 A). The MITF promoter is potently activated by β -catenin in a manner dependent upon the TCF/LEF binding site based on deletion mutagenesis, consistent with point mutagenesis studies of Takeda et al. (2000). A comparison between the β -catenin responsiveness of B16 melanoma cells and epithelial BHK cells using luciferase reporters revealed that the MITF promoter is regulated in a melanocyte restricted fashion (Fig. 3 B), extending previous observations in zebrafish to melanoma cells (Dorsky et al., 2000b). Also in agreement with previous reports (Dorsky et al., 2000b; Takeda et al., 2000), the TCF/LEF site was capable of binding TCF/LEF– β -catenin proteins in vitro as shown by DNA binding studies using nuclear extracts from B16 (mouse) or 501mel (human) melanoma cells, as well as Jurkat (T cells) as a control (Fig. 3 B). We utilized a consensus MITF promoter fragment probe for electrophoretic mobility shift assays. As shown in Fig. 3 C, formation of complexes could be visualized on both the

Table I. Correlation between nuclear β -catenin and induced proliferation in melanoma

Cell line	Species	Endogenous nuclear β -catenin staining	Proliferation induced by constitutive β -catenin
B16	Mouse	No	Yes, S = +23% ($\pm 7\%$)
A375	Human	No	Yes, S = +25% ($\pm 8\%$)
501mel ^a	Human	Yes	No
SK-MEL-5	Human	Yes	No

^aHarbors stabilizing β -catenin mutation (Rubinfeld et al., 1997).

Average proliferation is measured as induced S-phase accumulation relative to vector control transfected cells.

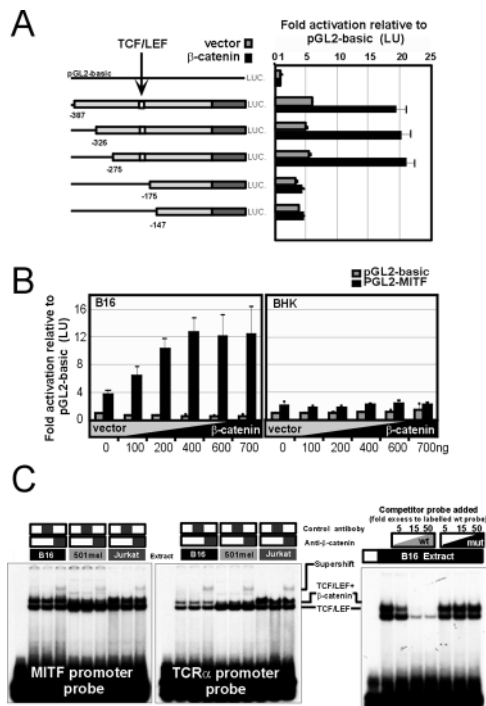


Figure 3. β -catenin transactivates and binds the MITF promoter in vitro. (A) The TCF/LEF binding site is critical for β -catenin induced transactivation of the MITF promoter in B16 cells. (B) β -Catenin transactivation of the MITF promoter is tissue restricted, as in epithelial BHK cells the promoter is unresponsive to β -catenin. (C) Electrophoretic mobility shift assay showing that a complex formed from mouse B16, human 501mel, or Jurkat cell extracts on the MITF TCF/LEF probe can be specifically supershifted using an antibody against β -catenin. As a control, the same extracts were used to supershift a probe of the previously characterized TCR α TCF/LEF binding site. The complex visualized by EMSA is competed away by adding a wild-type MITF probe but not a mutant probe.

MITF promoter and TCR α control probes previously identified as TCF/LEF and TCF/LEF with β -catenin (Korinek et al., 1997). In addition, inclusion of a polyclonal antibody directed at β -catenin produces a supershifted complex, highlighting the identity of this complex. The shifted complex was competed with a wild-type MITF probe but not with a probe in which the TCF/LEF site is mutated. Importantly, nuclear extracts from melanomas with or without aberrant nuclear β -catenin both produce supershiftable β -catenin complexes, further supporting the conclusion that the Wnt/ β -catenin/LEF pathway is functional even in melanomas exhibiting predominantly cytoplasmic β -catenin. To assess whether β -catenin is endogenously bound to the MITF promoter in melanoma cells, we conducted chromatin immunoprecipitation assays in B16 melanoma cells. As shown in Fig. 4 A, these studies revealed occupancy of the endogenous melanocyte-specific MITF promoter by β -catenin with absence of signal from control antibody, no-antibody control, or anti- β -catenin tested on a remote transcriptionally active genomic location (the tyrosinase promoter). This observation on the endogenous MITF gene is in agreement with the previously reported ability of the architectural TCF/LEF proteins to bind the MITF promoter in gel shift assays (Takeda et al., 2000) and extends this further to show that

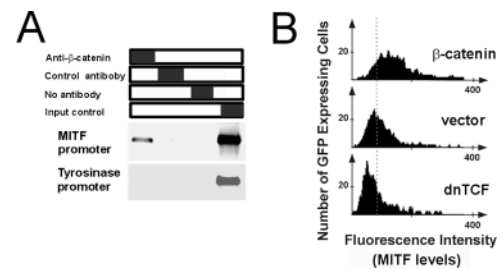


Figure 4. β -catenin is associated with the endogenous MITF promoter and modulates MITF expression levels. (A) In vivo association of β -catenin with the mouse MITF promoter by chromatin immunoprecipitation. The mouse tyrosinase promoter is a transcriptionally active negative control. (B) Ectopic expression of β -catenin leads to endogenous upregulation of MITF protein levels, and conversely, dnTCF downregulates MITF protein as quantitated by flow cytometry and analysis of transfected (gated GFP-positive) cells.

endogenous β -catenin is truly found at the MITF-promoter in melanoma cells.

Endogenous MITF levels are altered by constitutive expression of β -catenin and dnTCF

To determine whether endogenous levels of MITF expression were positively and negatively affected via β -catenin or dnTCF in melanoma cells, we cotransfected β -catenin or dnTCF vectors together with GFP into B16 melanoma cells, and analyzed endogenous MITF protein expression levels by FACS using a monoclonal antibody against MITF. Ectopic expression of β -catenin led to a significant and reproducible increase in MITF protein levels compared with vector control (Fig. 4 B). In contrast, expression of dnTCF led to a measurable reduction of MITF protein levels. Taken together with recent work (Dorsky et al., 2000b; Takeda et al., 2000), these data demonstrate that MITF expression is targeted by the Wnt pathway in a manner that measurably alters endogenous MITF protein levels, and therefore might contribute to the phenotypic effects of β -catenin–TCF/LEF on melanoma cell growth or survival.

A novel MITF(dn) abrogates β -catenin-induced proliferation

During melanocyte development, MITF appears to be a functionally critical target of Wnt signaling, as demonstrated using rescue assays in zebrafish (Dorsky et al., 2000b). Although β -catenin signaling is heavily implicated in human malignancies, few identified downstream targets of this pathway have been found that are lineage specific and functionally important in the neoplastic state. In order to conduct loss-of-function experiments, we made use of a novel dn mutation of MITF (MITF[dn]) based on a mutant mouse allele, which deletes a critical basic domain arginine while retaining an intact HLH-Zip dimerization motif. This mutation is dn through sequestration of a wild-type partner into a heterodimer that is incapable of binding DNA due to the arginine deletion (Hemesath et al., 1994). Without affecting its specificity, a further NH₂-terminal truncation was made which removes the transactivation domain. Immunofluorescence revealed that the dominant negative protein lo-

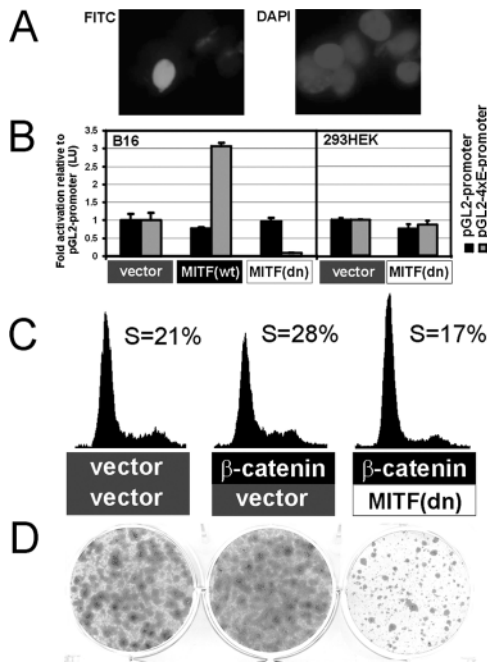


Figure 5. MITF(dn) blocks β -catenin-induced cell proliferation and growth in B16 melanoma cells. (A) Ectopic expression of an HA-tagged MITF(dn) in B16 melanoma cells is nuclear as visualized by FITC-anti-HA staining (compared with nuclear staining with DAPI in the same high power field). (B) MITF(dn) represses the activity of a $4 \times$ E-box reporter that is transactivated by MITF(wt) in melanoma cells and this repression does not occur in the non-MITF expressing HEK293 cell line. (C and D) Expression of MITF(dn) abrogates β -catenin induced proliferation and growth in cell cycle analysis and clonogenic assay.

calizes to the nucleus in transfected melanoma cells (Fig. 5 A). In vitro transcriptional assays using an E-box-dependent reporter demonstrated that the dn construct potently represses basal activity, whereas wild-type MITF stimulates

the reporter in B16 melanoma cells (Fig. 5 B). Importantly, the MITF(dn) construct is unable to repress the same E-box reporter in non-MITF-expressing HEK293 cells, consistent with its repressive effect being due to sequestration of endogenous MITF away from DNA.

Because the Wnt pathway is likely important for both proliferation and survival, we wanted to explore the consequences of interfering with its downstream target MITF by inclusion of the dn mutant, MITF(dn). To examine whether MITF plays an important role in β -catenin's mitogenic effects in melanoma cells, we transfected B16 melanoma cells with β -catenin in the presence of either MITF(dn) or vector control. As before, cell cycle analysis revealed that β -catenin overexpression leads to an increase in S-phase relative to vector control (21–28%), but that coexpression of MITF(dn) abrogates this effect (down to 17%; Fig. 5 C). Moreover, colony formation was also examined to measure broader growth/survival effects and also demonstrated both stimulation by β -catenin and potent repression by MITF(dn) (Fig. 5 D), suggesting a critical dependence of MITF for this pathway in melanoma cells.

MITF specifically rescues apoptosis induced by dnTCF

If MITF were a functionally important mediator of β -catenin signaling in melanoma, then transfection of MITF-encoding plasmid may be able to rescue the phenotype generated by blockade of the Wnt pathway. We recapitulated β -catenin blockade using dnTCF in multiple melanoma lines and examined the effects of simultaneous MITF expression from a constitutive promoter. Constitutive MITF expression significantly rescued colony formation in human 501mel and SK-MEL-5 melanoma cells (Fig. 6 A), both of which exhibit nuclear accumulated β -catenin (Table I). The same experiment was also carried out in B16 melanoma cells, displaying mostly cytoplasmic β -catenin. In this setting as well, growth suppression imposed by dnTCF was similarly res-

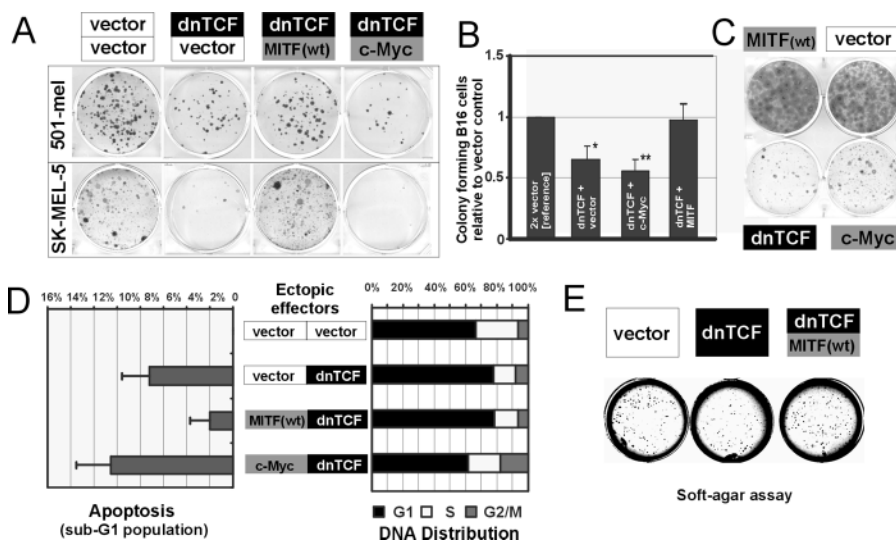


Figure 6. MITF can rescue growth suppression of dnTCF in melanoma cells. (A) Colony-forming assays in two human melanoma cell lines (501mel and SK-MEL-5) show rescue of clonogenic survival in the presence of dnTCF by wild-type MITF, whereas c-Myc is further growth inhibitory (B) Clonogenic survival in B16 melanoma demonstrates suppression by dnTCF and rescue by wild-type MITF, but not c-Myc (quantitative results are normalized to vector control). Experiment done in triplicate and quantitated results are graphed. Indicated with * is significant with $P < 0.03$ and ** significant with $P < 0.01$ as statistically calculated using Student's t test for unpaired samples. (C) Control colony formation assays in B16 cells showing growth suppressive effects of dnTCF and c-Myc in relation to MITF and vector

control. (D) MITF rescues apoptosis induced by dnTCF but cannot relieve cell cycle inhibition in B16 melanoma cells. In contrast c-Myc bypasses dnTCF antiproliferative effects (S-phase repression), but significantly increases the apoptotic population. Apoptosis is measured as sub-G1 peak from propidium iodide staining. The quantitation was performed using ModFit v2.0 and represents values normalized to vector control as baseline. Averages from four independent experiments are presented (including error bars for standard deviation of mean). (E) Soft agar assay showing a representative example of macroscopic colonies of SK-MEL-5 cells 20 d after infection with the indicated retroviruses.

cued by constitutive MITF expression, as displayed in a quantitative summary of clonogenic growth assays (Fig. 6 B). In contrast, *c-Myc*, a different downstream target of the Wnt pathway (He et al., 1998), did not rescue growth suppression by dnTCF in melanoma cells, and in some cases even further suppressed clonogenic growth (Fig. 6, A and B). Control experiments in B16 melanoma revealed measurable growth suppression by both dnTCF and *c-Myc* (Fig. 6 C).

Cell cycle analysis was performed on the transfected populations to further delineate the consequences of manipulating β -catenin–TCF/LEF with MITF or *c-Myc*. As shown in Fig. 6 D, expression of dnTCF produces not only a relative G1 arrest, but also a dramatic increase in apoptotic death. Co-expression of MITF, however, produces a significant rescue of this apoptosis while slightly increasing the S phase cell-cycle profile. These data suggest a role for MITF in survival and are consistent with the possibility that other targets of the Wnt-pathway contribute to the proliferative response in melanoma cells. Coexpression of Cyclin D1, another downstream target of β -catenin (Shtutman et al., 1999; Tetsu and McCormick, 1999), did not rescue dnTCF's cell cycle effects (unpublished data). In contrast, coexpression of *c-Myc* with dnTCF more significantly rescues the S phase decrease observed with dnTCF, but leads to an even greater apoptotic response than dnTCF alone (Fig. 6 D). Despite *c-Myc*'s apparent mitogenic effect, its growth suppression (Fig. 6, A and B) is likely achieved through an augmented apoptotic response. Thus, the rescue of growth observed by expression of MITF with dnTCF appears to be predominantly accounted for by reducing apoptosis, although MITF may contribute to β -catenin's proliferative effects as well, because MITF(dn) abrogates β -catenin–induced cell cycle stimulation (Fig. 5, C and D). Soft agar growth assays were also performed to test whether MITF's ability to rescue dnTCF occurred for multiple functional readouts. As shown in Fig. 6 E, MITF also rescued dnTCF for soft agar growth. Collectively, measurements of colony growth (on plastic), apoptosis, and soft agar growth, all suggest that regulation of melanoma growth by the β -catenin–TCF/LEF pathway is functionally dependent on the MITF transcription factor.

Discussion

This report presents data indicating that the effector of the Wnt signaling pathway, β -catenin, is able to stimulate growth of melanoma cells. Coupled to the finding that nearly one third of human primary melanoma specimens as well as melanoma cell lines exhibit nuclear accumulation of β -catenin (Rubinfeld et al., 1997; Rimm et al., 1999), these data imply a significant functional role for this pathway in human melanoma. Utilizing ectopic expression of dnTCF we revealed an upstream-downstream relationship between β -catenin–TCF/LEF and MITF. Abrogation of TCF/LEF-dependent transcription suppresses growth and triggers apoptosis accompanied by inhibition of MITF expression. MITF is likely a critical survival factor in the Wnt pathway, as the apoptosis triggered by abrogating the Wnt pathway can be rescued by reintroduction of MITF. Additionally, introduction of a dominant-negative allele of MITF blocks

proliferation induced by β -catenin. These data, coupled to the developmental rescue studies in zebrafish (Dorsky et al., 2000b), place MITF as an essential lineage specific target of Wnt signaling both in melanocyte development, and post-developmentally, in melanoma.

MITF as a survival factor in the melanocyte lineage

It is noteworthy that MITF expression is retained in the vast majority of human melanomas, including the many tumors in which other differentiation markers, such as pigmentation, are lost (King et al., 1999). Furthermore, MITF deficiency in mouse or man results in melanocyte loss (rather than depigmentation), and one mutant mouse allele (*Mitf^{fl}*) displays most melanocyte loss postnatally (Lerner et al., 1986). These observations suggest that MITF may be functionally important for melanoma growth or survival, a premise that is tested here.

In addition to its Wnt responsiveness, the MITF promoter responds to melanocyte stimulating hormone via a CRE element (Bertolotto et al., 1998; Price et al., 1998). The observation that melanoma cells are sensitized towards apoptosis by introduction of a dn CREB (Jean et al., 1998) is consistent with the possibility that the response may be at least partially mediated by disruption of MITF expression. Although the Wnt/ β -catenin and CREB signaling pathways are both ubiquitous among cell types, it is striking that their effects on MITF expression are restricted to the melanocyte lineage, providing an example of ubiquitous pathways which are harnessed to regulate a tissue-restricted promoter both during normal development and in malignancy. Recent data have also shown direct transcriptional regulation of the pro-survival gene *BCL2* by MITF (McGill et al., 2002). Furthermore, the Wnt pathway has been shown to protect against induction of apoptosis in an *in vitro* fibroblast system dependent on β -catenin/TCF-mediated transcriptional activity (Chen et al., 2001). *In vivo* inactivation of β -catenin has also been shown to increase apoptosis in migrating neural crest cells (Brault et al., 2001), suggesting that in non-melanocytic neural crest derivatives, Wnt/ β -catenin likely employs different (non-MITF) targets to mediate anti-apoptotic signaling.

MITF as a downstream effector of developmental and oncogenic pathways

As β -catenin has been shown in other systems to augment growth, it is not surprising to find that this also pertains to melanoma cells, where β -catenin overexpression or disruption are shown to significantly modulate both cell cycle and survival (Figs. 1, 5, and 6). In melanomas with aberrant nuclear accumulation of β -catenin, further ectopic β -catenin expression does not lead to augmented proliferation (Table I). This could be due to the maximized basal activity of β -catenin in these cells, as proliferation and growth is still abrogated by introduction of dnTCF irrespective of β -catenin status. Of note, there is also a correlation between the endogenous MITF level and presence of aberrant nuclear β -catenin in the human melanomas employed here, where A375 (with mostly cytoplasmic β -catenin) has significantly less MITF than the human melanoma cell lines which lack strong nuclear β -catenin staining (unpublished data). Still,

MITFs transcriptional ability may be modulated by post-translational events so that quantitative measures of MITF may not necessarily correlate perfectly with its transcriptional activity. We have also shown that β -catenin leads to increased growth that is potently abrogated by MITF(dn), both measured as decreased proliferation via cell cycle profiles and clonogenic growth. The blockade of β -catenin-induced growth of melanoma cells by MITF(dn) suggests an implicit need for MITF to sustain the growth of these cells. MITF(dn) has been found to suppress melanoma growth regardless of whether β -catenin's basal activity is low or high. Interestingly, reintroduction of MITF(wt) minimally reverses the alterations in cell cycle caused by suppressing the Wnt pathway by ectopic dnTCF. This observation argues that whereas MITF may be necessary for cell cycle progression induced by β -catenin, MITF is not sufficient to stimulate cell cycle progression. Therefore, it will be of interest to determine whether there are MITF target genes, which influence cell cycle progression to examine the possibility that MITF contributes to maintenance of the cell cycle machinery while perhaps not directly participating in the mitogenic response. Cell cycle targets of Wnt signaling such as *c-Myc*, *Cyclin D1* (He et al., 1998; Tetsu and McCormick, 1999; Shtutman et al., 1999), and others may more directly mediate β -catenin's mitogenic effects. Such a model would predict that MITF, as a highly tissue-restricted factor, or its downstream target genes, may represent attractive drug targets in melanoma, a disease for which effective therapies are severely lacking. Towards this end, it will also be important to examine these activities using in vivo tumor models.

Concluding remarks

The importance of MITF in the establishment of the melanocyte lineage is clear, but its role postdevelopmentally is incompletely understood at this time. These data suggest an important role for MITF in survival downstream of the canonical Wnt pathway. Here we show that MITF is able to rescue abrogation of the canonical Wnt pathway by dnTCF in cells irrespective of their β -catenin status. However, as MITF is retained in virtually all melanomas, including those with deregulated β -catenin, its role might be broader than as purely an effector for survival in the Wnt pathway. Thus, the many signaling pathways that converge on MITF (Goding, 2000; Price and Fisher, 2001) might reveal additional cues involved in the genesis of melanomas similar to that of the Wnt pathway.

Materials and methods

Cloning, reporter, and expression vectors

The vector pcDNA3.1- β -catenin(wt) was made by excising β -catenin-FLAG fragment from vector pCS-2- β -catenin(wt)-FLAG (Liu et al., 1999) using *SpeI/NcoI*, filling in with Klenow and then ligating into the EcoRV site of pcDNA3.1(+) (Invitrogen). The pcDNA3-dnTCF1 was obtained from Dr. H. Clevers (University Medical Center Utrecht, Utrecht, Netherlands) (van de Weterling et al., 1996) and pRC/CMV-cMyc from Dr. S. Hann (Vanderbilt University School of Medicine, Nashville, TN) (Hann et al., 1994). pRC/CMV-MITF(wt) have been described earlier (Hemesath et al., 1994) and the pcDNA3.1-HA/MITF(dn) was made by PCR amplification of the basic region (beginning ARALAKER) and to the last codon of mouse MITF(del) and ligated into pcDNA3.1 with an HA-tag in frame with the MITF coding region between the *BamHI* and *EcoRI* sites. pcDNA3.1-hrGFP was made by excising the hrGFP coding sequence by *MscI/KpnI* di-

gestion from pIRES-hrGFP 2a (Stratagene) and ligated into EcoRV/*KpnI* of pcDNA3.1(-) (Invitrogen). The human MITF promoter reporter (pGL2-hMiP) consists of 484 bp of the melanocyte specific MITF promoter (-387 to +97) as described previously (Price et al., 1998). Deletion constructs were made by PCR and verified by sequencing. The reporter vectors TK-TOP and TK-FOP driving luciferase were obtained commercially (Upstate Biotechnology).

Cell cycle, MITF endogenous expression, colony-forming, and reporter assays

For cell cycle experiments, B16 cells and A375 (maintained in DME supplemented with 10% FBS) were seeded in serum-free RPMI-1640 media 24 h before transfection in 100-mm plates. Cells were transfected using Lipofectamine2000 (GIBCO BRL) according to the manufacturer's recommendations with the indicated vectors (4 μ g) and 0.3 μ g pcDNA3.1-hrGFP. Cells were refed with RPMI-1640 supplemented with 10% FBS 2 h after transfection, and on the following day they were washed and refed. 48 h after transfection, cells were harvested, fixed, and permeabilized for flow cytometry analysis using propidium iodide to stain for DNA content. Similarly, 501mel and SK-MEL-5 cells were also assayed using their respective media preferences (see below). Endogenous MITF expression was performed similarly as for cell cycle but the cells were harvested earlier, at 28 h posttransfection. MITF protein was stained with mouse monoclonal C5 anti-MITF antibody, washed repeatedly, and then stained secondarily with a R-phycoerythrin conjugated goat-anti-mouse antibody (Molecular Probes). All samples for flow cytometry were analysed on a Becton-Dickinson FACS instrument using CellQuest software and each sample comprised of at least 10^4 gated GFP-positive events. Cell cycle and apoptosis data were calculated using ModFit v2.0 (Becton-Dickinson). Colony-forming assays were conducted in 6-well plates (2 μ g of total DNA/well) and transfected with indicated vectors including 0.25 μ g (12.5%) of pBABE-puro. 501mel were grown in Ham's F10 media, SK-MEL-5 and HEK293 cells in DME, all supplemented with 10% FBS. In experiments with two effector vectors, the ratio of dnTCF to the other vector was 4:1 as indicated. 24 h after transfection cells were refed with DME/10% FBS containing 1 μ g/mL puromycin for antibiotic selection. Cells were then refed with the same media every 2 d for 10–14 d and subsequently colonies were stained with crystal violet, photographed and measured by absorbance for relative cell numbers. Reporter assays were made in 24-well plates (0.8 μ g DNA/well) using Lipofectamine2000 (GIBCO BRL) or Fugene6 (Roche) according to the manufacturer's instructions. 24 h after transfection, the cells were lysed and assayed using Dual Luciferase reagents (Promega). Luciferase values were normalized to constitutive Renilla luciferase generated by cotransfected pRL-CMV plasmid (Promega) and for the TK-FOP/TK-TOP experiments normalized to pRL-TK (Promega).

Soft agar assays

The respective constructs were subcloned into pLHCX-retroviral backbones and replication incompetent virus produced in 293-EBNA cells (Invitrogen) by cotransfection of GagPol and VSV-G expression vectors. High-titer viral supernatants were used to infect SK-MEL-5 in the presence of polybrene (8 μ g/ml) and 48 h after infection cells were seeded at 10^5 cells per 60-mm dishes and refed every 2–3 d until macroscopic colonies were visible. Infection was controlled by coinfection of hrGFP-expressing retrovirus which was equal in all samples 48 h after infection.

Electrophoretic mobility shift assays and ChIPs

Electrophoretic mobility shift assays (EMSA) were performed by incubating (total volume of 20 μ L) 3 μ g of nuclear protein, 0.2 ng of radiolabeled probe, and 100 ng of sonicated herring sperm DNA in 10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 12.5% glycerol. For competition experiments, 5-, 15-, or 50-fold excess unlabeled MITF oligo (wild-type GAGTTTGACTTTGATAGCTCGTCAC or mutant GAGTTTGACTTTGGCAGCTCGTCAC in double-stranded configurations) was incubated with the extract for 5 min before the addition of labeled oligo and the incubation proceeded for an additional 20 min at room temperature. In supershift experiments, 1 μ L of anti- β -catenin antibody (Upstate Biotechnology) or control rabbit polyclonal antisera were added subsequently and incubated for an additional 20 min at room temperature. Samples were analysed on a native 30:1 (acrylamide:bis) polyacrylamide gel run in $0.5 \times$ TBE at 4°C. ChIPs was performed in mouse B16 cells grown in logarithmic phase in DME/10% FBS. Cells were harvested by scraping, homogenized in a low-salt buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 60 mM KCl, 5% sucrose, 0.1% NP-40, 1 mM EDTA, proteinase inhibitors) on ice using a Dounce homogenizer. The nuclei were isolated by centrifugation onto a 10% sucrose pad, crosslinked for 15 min at room temperature

with 1% formaldehyde in PBS, and subsequently, the reaction quenched by the addition of glycine to a final 125-mM concentration. Nuclei were then spun down and resuspended in ChIPs buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 60 mM KCl, 0.1% NP-40, proteinase inhibitors) and sonicated by three 30-s pulses using a Fisher dismembrator fitted with a microtip on ice. The samples were then precleared by an extensive high-speed centrifugation (14,000 g, 10 min) and then precleared additionally with Ultralink protein A/G beads (Pierce Chemical Co.). Anti- β -catenin antibody (06-734; Upstate Biotechnology), and control anti-NFATc1 (7A6; Santa Cruz Biotechnology, Inc.) was then added to a tenfold ChIPs buffer diluted sample and incubated on a rotator for 3 h at room temperature. Subsequently, Ultralink protein A/G beads were added to the samples and an additional control sample without antibody and subsequently incubated for an additional hour. Immunoprecipitates were washed twice with ChIPs buffer, twice with 500 mM NaCl containing ChIPs buffer and once with TE, pH 8. The immunoprecipitates were released from the beads by incubating at 65°C for 20 min in 1%SDS/TE, pH 6.8, and protein digested by proteinase K treatment side by side with an additional unprecipitated sample as input control. Crosslinks were released by heating at 70°C for 10 h and DNA subsequently recovered by extraction with phenol and chloroform at high salt (0.6 M NaAcetate, pH 8). Semiquantitative PCR was then performed on samples using primers FWD-5'-TGGCCATTAGGGAAGTCTCATGTG and REV-5'-AATTCCGAA-GAGCGACCCGCATAAAG covering the TCF/LEF binding site in the mouse MITF promoter (EMBL/GenBank/DBJ) under accession no. AC021060). Mouse tyrosinase promoter specific primers FWD-5'-GAG-GCAACTATTTAGACTGATTACTTT and REV-5'-AGGTTAATGAGTGT-CACAGACTTC were used as a control.

Immunofluorescence

Melanoma cells were seeded onto coverslips in 24-well plates. In transfection experiments cells were transfected 18 h postplating with pcDNA3.1-HA/MITF(dn) using Lipofectamine2000. The day after transfection (or plating alone), cells were fixed with 3% formaldehyde and stained with polyclonal anti- β -catenin antibody (Upstate Biotech) and secondarily with FITC-conjugated goat-anti-rabbit antibody (Jackson ImmunoResearch Laboratories) or for transfection experiments with FITC-conjugated rat high-affinity anti-HA monoclonal antibody (Roche), all in 10% normal goat serum. Subsequently, the cells were washed, counterstained with DAPI, and photographed through a Nikon fluorescence microscope.

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