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## In melanoma, beta-catenin acts as suppressor of invasion through a cell-type specific mechanism

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### Abstract

Cell-type specific signalling determines cell fate under physiological conditions, but it is increasingly apparent that also in cancer development the impact of any given oncogenic pathway on the individual cancer pathology is dependent on cell-lineage specific molecular traits. For instance in colon and liver cancer canonical Wnt signalling produces increased cytoplasmic and nuclear localised beta-catenin, which correlates with invasion and poor prognosis. In contrast, in melanoma increased cytoplasmic and nuclear beta-catenin is currently emerging as a marker for good prognosis and thus appears to have a different function compared to other cancer types; however this function is unknown. We discovered that in contrast to its function in other cancers, in melanoma, beta-catenin blocks invasion. We demonstrate that this opposing role of nuclear beta-catenin in melanoma is mediated through MITF, a melanoma-specific protein that defines the lineage background of this cancer type. Downstream of beta-catenin MITF not only suppresses the Rho-GTPase regulated cell-morphology of invading melanoma cells, but also interferes with beta-catenin induced expression of the essential collagenase MT1-MMP, thus affecting all aspects of an invasive phenotype. Importantly, overexpression of MITF in invasive colon cancer cells modifies beta-catenin directed signalling and induces a 'melanoma-phenotype'. In summary, the cell type specific presence of MITF in melanoma affects beta-catenin's pro-invasive properties otherwise active in colon or liver cancer. Thus our study reveals the general importance of considering cell-type specific signalling for the accurate interpretation of tumour markers and ultimately for the design of rational therapies.

### Keywords

melanoma; invasion; Rho; ROCK; MMP; MITF

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## Introduction

Deregulations in the Wnt pathway stabilise the overall protein-level of beta-catenin, resulting in an increase in cytoplasmic and nuclear localised beta-catenin compared to the normally membrane bound protein (Giles *et al.*, 2003). Increased cytoplasmic and nuclear beta-catenin is frequently found in different cancer types, but its impact on the individual tumour pathology can differ strikingly. In over 90% of colon cancers the Wnt pathway is deregulated (Giles *et al.*, 2003) and increased nuclear beta-catenin can be found at the invasive front of adenocarcinomas (Jung *et al.*, 2001). Various studies have identified beta-catenin target genes that contribute to colon cancer progression. Amongst these are EphB4, whose expression is increased in colorectal lymph node or liver metastases, or MMP-7 (Matrilysin) and S100A4, markers for invasiveness and poor survival (Adachi *et al.*, 2001; Boye *et al.*, 2010; Kumar *et al.*, 2009; Stein *et al.*, 2006; Wagenaar-Miller *et al.*, 2004). Also, in liver cancer nuclear beta-catenin is correlated within invasion, enhanced metastasis, poor prognosis and reduced disease free survival (Liu *et al.*, 2010; Zulehner *et al.*, 2010). In contrast, in melanoma it has been suggested that increased cytoplasmic/nuclear beta-catenin expression is linked to a good prognosis and increased overall survival (Bachmann *et al.*, 2005; Chien *et al.*, 2009; Gould Rothberg *et al.*, 2009). Furthermore, cytoplasmic/nuclear beta-catenin expression has been found to be reduced during progressive disease (Bachmann *et al.*, 2005; Kageshita *et al.*, 2001; Maelandsmo *et al.*, 2003; Omholt *et al.*, 2001; Pecina-Slaus *et al.*, 2007; Rimm *et al.*, 1999). Thus, in melanoma beta-catenin appears to have a different function compared to other cancer types, but this function is unknown.

Melanoma cells are extremely motile and invasive cells, and like other tumour cells they either invade predominantly by modulating the extracellular matrix (ECM) through protease activities (such as matrix-metalloproteinases (MMPs)) or they invade by undergoing cell-shape changes in order to adjust to the architecture of the 3D-environment (Sabeh *et al.*, 2009; Sahai & Marshall, 2003; Wolf *et al.*, 2003). The latter mode is less dependent on protease activity and is represented by a round cell-shape, Rho-dependent actin cortex contractions and formation of membrane blebs (Fackler & Grosse, 2008; Sahai & Marshall, 2003).

The round mode of invasion has also been observed *in vivo*, and for melanoma cells this morphology appears to be characteristic for cells that are more motile (Pinner *et al.*, 2009; Wolf *et al.*, 2003). In addition, these more motile melanoma cells are less differentiated (Pinner *et al.*, 2009). Differentiation of melanocytes and melanoma cells is controlled by the melanocyte specific transcription factor MITF (Levy *et al.*, 2006), and transformed melanocytes generally show reduced expression of MITF (Wellbrock *et al.*, 2004; Wellbrock *et al.*, 2002). Importantly, *MITF* is a beta-catenin target gene in pigment cells (Takeda *et al.*, 2000), but although beta-catenin expression has been widely analysed in melanoma tissues, the correlation between beta-catenin and MITF expression during human melanoma development is not clear.

For the design of rational therapies it is crucial to understand mechanisms that underlie the metastatic behaviour of melanoma cells and to characterise high-risk melanoma. Recent studies identify beta-catenin as a promising candidate for a prognostic marker that could help to distinguish between low-risk and high-risk melanoma. However, the function of beta-catenin particularly during the invasive progression of melanoma is unclear, which hampers the precise interpretation of this marker. Here we show that in contrast to colon or liver cancer, in melanoma high expression of nuclear beta-catenin produces a non-invasive phenotype, which explains beta-catenin's correlation with good prognosis. We identify a melanoma-specific mechanism that clarifies the opposite function of beta-catenin and

demonstrates the relevance of a cell-type specific background for the cancer-type specific role of an oncogenic protein.

## Results

### Increased cytoplasmic/nuclear beta-catenin suppresses melanoma cell invasion

We analysed a panel of melanoma biopsies to assess the correlation of beta-catenin expression with tumour progression, and confirming previous reports (Kageshita *et al.*, 2001; Maelandsmo *et al.*, 2003) we find that there is a decrease in tumours expressing nuclear beta-catenin during tumour progression (Suppl. Fig.1). However, the most striking finding is that beta-catenin expression is significantly reduced within primary tumours in areas of dermal invasion before cells cluster to nests of cells (Fig1a). To further investigate this observation we used melanoma cell lines expressing high or low levels of cytoplasmic and nuclear localised beta-catenin compared to the predominantly membrane bound beta-catenin in melanocytes (Fig1b).

When in an approach to mimic the *in vivo* situation, these cells are plated on a thick gel of dermal collagen, high beta-catenin expressing cells (shown are 501mel cells) display high proliferative activity, whereas low beta-catenin expressing WM266-4 cells divide less frequently (Fig.1c). Most strikingly though, 501mel cells show an increase in upward growth with cells piling on top of each other on the collagen gel, whereas WM266-4 cells invade the gel (Fig.1c, bottom panel). Likewise, WM266-4 cells show high invasiveness in a quantitative collagen-invasion assay, but all three high nuclear beta-catenin expressing cell lines display very poor invasive activity (Fig.1d). This invasive behaviour is not restricted to collagen, and we observe a similar trend when invasion into matrigel (basement membrane) or a collagen/matrigel mixture is analysed (Suppl Fig.2a).

Importantly, when beta-catenin expression is reduced in 501mel cells using two different siRNAs, invasion into 3D collagen is increased (Fig.2a). Furthermore, up-regulation of beta-catenin in invasive WM266-4 cells by GSK-3 inhibition suppresses invasion, and this can be significantly rescued when beta-catenin up-regulation is prohibited by RNAi (Fig.2b,c, Suppl. Fig.2). Together this reveals an anti-invasive function of beta-catenin in melanoma cells.

### MITF suppresses invasion downstream of beta-catenin

The particular behaviour of beta-catenin in melanoma suggests that it might function through a target gene specific to melanocytic cells. Such a lineage specific beta-catenin target is MITF (Takeda *et al.*, 2000), and indeed, confirming previous findings (Hoek *et al.*, 2006) we find a correlation of MITF expression with beta-catenin expression in melanoma biopsies (Fig.2d). A correlation of high MITF expression with the presence of high beta-catenin levels is also preserved in the nuclear beta-catenin expressing cell lines (Fig.2d), and further analysis confirmed that beta-catenin regulates MITF expression in these cells (Suppl. Fig.2e).

These data suggest a link between up-regulated MITF expression in nuclear beta-catenin expressing cells and suppression of invasion. Indeed, when we reduce MITF expression in 501mel cells using a validated MITF specific siRNA (Wellbrock *et al.*, 2008), we observe a strong increase in their invasive activity in 3D collagen (Fig.2e). In contrast, pre-treatment of WM266-4 with forskolin (FO), a differentiation-inducing agent (Busca & Ballotti, 2000) that up-regulates MITF (Fig.2f), results in significantly decreased invasive activity in a dose dependent manner (Fig.2f). This observation is in line with a previous report describing that differentiated melanoma cells are less motile (Pinner *et al.*, 2009). Although Forskolin elicits numerous effects, when we prevent the forskolin-induced up-regulation of MITF by RNAi,

this results in a partial rescue of the suppressed invasion (Fig.2g). This clearly demonstrates that up-regulation of MITF contributes to the suppression of invasion in differentiated cells. Importantly, when we ectopically over-express MITF, invasion of WM266-4 cells is suppressed as well, confirming MITF's inhibitory function on invasion (suppl. Fig.2b).

### MITF suppresses actin-cortex contraction and membrane-bleb formation in 3D

Considering their anti-invasive function, we next assessed the effect of beta-catenin and MITF on the regulation of the cytoskeleton dynamics of invading cells. In vivo, motile melanoma cells show a more rounded morphology, whereas non-motile cells are extended and occasionally form 'dendrite' like extensions (Pinner *et al.*, 2009). Consistent with this we find that the majority of the highly invasive WM266-4 cells invade with a round morphology in our 3D-collagen system (Fig.3a, b, suppl.Fig.3a). However, when WM266-4 cells are treated with beta-catenin up-regulating GSK3 inhibitors, we observe a significant change in morphology with an over 50% reduction in the round phenotype (Fig.3a). Importantly, treatment of WM266-4 cells with forskolin stimulates a similar morphology change (Fig.3b, suppl.Fig.3b,c). This morphology change is accompanied by the complete loss of membrane blebs and F-actin cortex-contraction (Fig.3b), which otherwise produces the round shape. Instead we observe an extended F-actin cortex with filopodia-type protrusions that interact with the collagen through actin-rich focal adhesions (Fig.3b). These findings suggest that there is a correlation of nuclear beta-catenin, high MITF expression and the suppression of the round bleb-associated phenotype. Consistent with this, the majority of high beta-catenin, high MITF expressing 501mel cells show an elongated filopodia phenotype interacting through focal adhesion with the collagen gel (Fig.3c, suppl. Fig.3c). However, when MITF expression is reduced this significantly increases the number of round blebbing cells (Fig.3c and suppl.Fig.3c), clearly demonstrating that MITF expression levels control the contraction of the F-actin cortex and the formation of membrane blebs.

### MITF regulates the round bleb-phenotype in a ROCK-dependent manner

At the molecular level the round bleb-associated cell morphology is regulated by Rho/ROCK mediated myosin light chain (MLC) phosphorylation, which induces the contraction of the cortical actin-meshwork and regulates bleb retraction of cells in 3D (Fackler & Grosse, 2008). We detect phosphorylated MLC in the F-actin cortex underlying the plasma membrane and within fully formed membrane blebs in WM266-4 cells cultured in 3D collagen (Fig.4a). However, in the presence of forskolin and increased MITF expression phospho-MLC in WM266-4 cells is reduced (Fig.4a). Furthermore, phospho-MLC is low in all nuclear beta-catenin, high MITF expressing melanoma cell lines (Fig.4b), but when MITF expression is reduced in 501mel cells, we see a significant increase in phospho-MLC (Fig.4c). This shows that high MITF levels suppress upstream events leading to phosphorylation of MLC.

An important upstream regulator of MLC phosphorylation is the Rho-associated kinase ROCK. Inhibiting ROCK with the small molecule inhibitor Y27632 completely abolishes the positive effect of the MITF knock-down on phospho-MLC (Fig.4c), which demonstrates that this effect was produced through ROCK, and that high MITF levels suppress ROCK activity towards MLC.

It has been previously proposed that MITF might regulate ROCK through the actin nucleator DIA1 (Carreira *et al.*, 2006). However, we do not find that depleting DIA1 using an ON-TARGET<sup>plus</sup> siRNA can phenocopy MITF induced effects on the cell morphology in 3D (Fig.4d) or ROCK mediated MLC phosphorylation (Fig.4e). Thus we find that DIA1 does not regulate ROCK downstream of MITF. The discrepancy between our findings and the

previously suggested link might be due to the fact that in the former study actin-cytoskeleton related effects produced by DIA1 over-expression were analysed in cells on coverslips/2D (Carreira *et al.*, 2006), which does not necessarily reflect a situation related to 3D (Abbott, 2003). In line with this, DIA1 has been ruled out to be involved in the regulation of membrane blebs suggesting that other actin nucleators are playing a role (Charras & Paluch, 2008). Thus, the detailed mechanism, as to how MITF suppresses ROCK activity in melanoma cells in a 3D-environment is still unclear, and is subject of current investigations.

### **MMP expression is reduced in high MITF expressing melanoma cells**

In line with the observation that high MITF levels suppress ROCK activity towards MLC, in 501mel cells ROCK activity towards MLC is low and Y27632 does not have any significant effect on MLC phosphorylation or the phenotype of 501mel cells (Fig.5a,b). However, inhibition of ROCK in WM266-4 cells has a significant effect on phospho-MLC and converts the morphology from a round bleb-phenotype to the more elongated phenotype (Fig.5a,b), indicating that ROCK activity is required for the phospho-MLC mediated cortex contraction and the round bleb-associated phenotype. The difference in ROCK activity in 501mel and WM266-4 cells is also seen in the ability of Y27632 to block the invasion of WM266-4 cells, whereas the invasion of 501mel cells is not significantly affected (Fig.5c). Cells that do not invade via Rho/ROCK activities use proteases such as matrix metalloproteinases (MMPs) in order to modulate the ECM for invasion, and indeed the invasion of 501mel cells is strongly reduced when proteolytic activities are inhibited (Fig. 5d). This indicates that 501mel cells use proteases for invasion, but this does not explain why WM266-4 cells are still more invasive than 501mel cells, even when their ROCK mediated cytoskeleton dynamics are blocked by Y27632 (Fig.5c). This led us to analyse MMP activity in the two cell lines. Zymography of WM266-4 and 501mel cells shows that MMP-2 is present in the medium of WM266-4 cells at much higher levels when compared to 501mel cells (Fig.5e). In addition we detect predominantly cleaved active MMP-2 in the medium of WM266-4 cells, whereas 501mel cells secrete mainly the non-processed inactive form proMMP-2 (Fig.5e).

Cleaved, activated proMMP-2 is produced by the membrane-anchored MT1-MMP, but importantly, MT1-MMP is also a collagenase and as such essential for cancer cell invasion (Itoh, 2006; Sabeh *et al.*, 2004). We find that *MT1-MMP* expression is significantly lower in 501mel than in WM266-4 cells (Fig.5f), which is in line with the amount of active MMP-2 levels detected in the medium of the cells (Fig.5e). Moreover, lower *MT1-MMP* expression is detectable in all nuclear beta-catenin cell lines (Fig.5f).

### **Increased MITF expression suppresses beta-catenin induced MT1-MMP expression in melanoma cells**

The low amounts of MT1-MMP mRNA in all nuclear beta-catenin cell lines is rather surprising, since in colon cancer cells *MT1-MMP* has been identified as a beta-catenin dependent TCF/LEF target gene (Hlubek *et al.*, 2004). More strikingly, we find that also in WM266-4 cells *MT1-MMP* expression is dependent on beta-catenin (Fig.6a), suggesting that its regulation is conserved in melanoma cells. Accordingly, in 501mel cells, beta-catenin-depletion also reduces the levels of *MT1-MMP* expression (Fig.6a). However, despite the fact that these cells express higher amounts of nuclear beta-catenin, its contribution to *MT1-MMP* expression is much lower than in WM266-4 cells.

This seemingly contradicting difference between melanoma and colon cancer cells could be due to the presence of MITF in melanoma cells, because MITF can recruit beta-catenin to MITF target genes by directly binding to it (Schepsky *et al.*, 2006). As a result of this recruitment and interaction, MITF could interfere with the regulation of beta-catenin at TCF/



LEF target genes. Because MITF expression is highly increased in 501mel cells, we asked whether such a mechanism could explain the regulation of *MT1-MMP* in these cells. We can confirm that beta-catenin is present in MITF immuno-precipitates from 501mel cells (Fig. 6b). Furthermore, MITF depletion from 501mel cells results in a significant increase in the transcriptional activity from a generic TCF/LEF regulated promoter (Fig.6c). Most importantly however, a similar situation is found for endogenous *MT1-MMP* expression (Fig.6d). In line with this inhibitory effect of MITF in 501mel cells, we find that when WM266-4 cells are treated with forskolin -which increases MITF expression- *MT1-MMP* expression is significantly reduced (Fig.6e). Together these data show that high MITF expression levels can interfere with beta-catenin directed transcription, and this results in suppression of *MT1-MMP* expression by MITF.

### MITF induces a 'melanoma-phenotype' in nuclear beta-catenin expressing colon cancer cells

Finally, we wanted to investigate whether MITF's 'modifier' function can explain the difference of beta-catenin's role in melanoma compared to other cancer types such as colon cancer. We therefore analysed the effect of ectopic MITF expression on SW480 colon cancer cells, which are homozygous for an APC mutation (Kawasaki *et al.*, 2003). As a consequence SW480 cells express increased amounts of nuclear beta-catenin (Fig.7a) and importantly, ectopically expressed MITF co-localises with nuclear beta-catenin in SW480 cells (Fig.7a). Like WM266-4 cells, SW480 cells display a round phenotype with the formation of membrane-bleb protrusions when cultured on a 3D collagen gel (Fig.7b). However, when MITF is expressed in SW480 cells this results in a complete change in morphology to an elongated cell body and the formation of focal adhesion like protrusions in a significant proportion of cells (Fig.7b), suggesting that also in colon cancer cells MITF suppresses membrane-bleb protrusions and affects the actin-cytoskeleton. Furthermore, we find that MITF interferes with beta-catenin directed transcription in SW480 cells (Fig.7c) and therefore also suppresses the beta-catenin target gene *MT1-MMP* expression (Fig.7d). Thus, MITF regulates cellular events in nuclear beta-catenin expressing colon cancer cells comparable to melanoma cells.

## Discussion

We have identified a mechanism that demonstrates that in order to understand the cellular outcome of a genetic lesion in cancer cells, it is essential to consider the cell-type specific background. In particular, our data provide an explanation for the opposing function of beta-catenin in malignant melanoma when compared to colon or liver cancer.

The role of beta-catenin in melanoma has been a matter of debate for a long time, because initial findings proposed a situation similar to colon cancer, where mutated activated beta-catenin drives tumour progression (Rubinfeld *et al.*, 1997). However, it is now convincingly established that increased cytoplasmic and nuclear beta-catenin expression is linked to a good prognosis (Bachmann *et al.*, 2005; Chien *et al.*, 2009; Gould Rothberg *et al.*, 2009; Omholt *et al.*, 2001). Nevertheless, we and others find nuclear beta-catenin in a considerable amount of primary melanomas (Bachmann *et al.*, 2005; Kageshita *et al.*, 2001; Maelandsmo *et al.*, 2003; Omholt *et al.*, 2001), which suggests that it might contribute to early events in melanoma development. However, the role of beta-catenin in melanoma initiation is not clear, because suggested functions in bypassing senescence (Delmas *et al.*, 2007) and regulating proliferation (Widlund *et al.*, 2002) appear to be not prevalent in the human disease situation, where increased nuclear beta-catenin is found in the majority of benign 'senescent' nevi and in tumours with low proliferative index (Bachmann *et al.*, 2005; Chien *et al.*, 2009; Demirkan *et al.*, 2007; Kageshita *et al.*, 2001). It thus appears that the presence of increased cytoplasmic and nuclear beta-catenin in early stages of melanoma development

might not be the cause but rather the consequence of this cancer type originating from melanocytic cells. Here, it is important to mention that canonical Wnt signalling is an essential regulator of melanocyte proliferation and differentiation in the epidermis (Yamaguchi *et al.*, 2004). The fact that the majority of benign nevi express increased cytoplasmic and nuclear beta-catenin could thus simply reflect that differentiation signalling is strongly switched on in these benign lesions, which usually are highly pigmented. This would suggest that canonical Wnt signalling predominantly regulates differentiation in melanocytic cells, and is in line with the fact that Wnt3a induces the expression of differentiation related genes even in melanoma cells (Chien *et al.*, 2009). In this context it is important to mention that non-canonical Wnt signalling (specifically WNT5A) is activated during melanoma progression and most crucially, non-canonical Wnt signalling can suppress canonical Wnt signalling (O'Connell & Weeraratna, 2009), which as we have shown results in increased invasiveness.

As mentioned above cytoplasmic and nuclear beta-catenin is frequently expressed in primary melanomas, but in line with others we find that this expression decreases during progressive disease (Bachmann *et al.*, 2005; Kageshita *et al.*, 2001; Maelandsmo *et al.*, 2003). Importantly, this mirrors the pattern described for MITF expression (Nazarian *et al.*, 2010; Salti *et al.*, 2000). Moreover, we see that MITF expression levels correlate with beta-catenin in melanoma samples, which is consistent with the fact that beta-catenin regulates MITF expression in melanoma cells (Widlund *et al.*, 2002). Furthermore, in line with the reported detection of reduced beta-catenin and MITF expression during tumour progression, we show that lowering beta-catenin expression in melanoma cells increases their invasiveness, and we identify MITF downstream of beta-catenin as suppressor of invasion. This finding is entirely in agreement with the fact that very low MITF expression levels are associated with a more invasive phenotype and an invasive gene signature in human melanoma (Hoeck *et al.*, 2008).

Our data demonstrate that MITF can interfere with the cellular program of a cancer cell. In particular we reveal a mechanism in which MITF controls the signalling output downstream of beta-catenin (Fig.8). In cells expressing low amounts of cytoplasmic/nuclear beta-catenin, MITF expression is low and does not interfere with beta-catenin directed transcription of *MT1-MMP*, this results in high protease activity (Fig.8b). In addition, ROCK is not inhibited, which results in MLC phosphorylation, actin-cortex contraction and a round, membrane-bleb phenotype (Fig.8b). Cells with this morphology are more motile and their invasion is less restricted by proteolytic activities (Sahai & Marshall, 2003). However, the expression of increased nuclear beta-catenin results in high MITF levels (Fig.8a), which blocks beta-catenin induced *MT1-MMP* expression and inhibits ROCK activity, resulting in an elongated morphology (Fig.8a). Cells with this morphology require proteolytic activity for invasion (Sahai & Marshall, 2003) and the low expression of *MT1-MMP* results in cells with low invasive potential. Importantly, while these processes are seen in melanoma cells, the same mechanisms apply to colorectal cancer cells after forced over-expression of MITF, thus demonstrating the crucial role of MITF in regulating the cellular program of a cancer cell.

## Materials and Methods

### Cell culture, reagents and transfection procedures

Primary human melanocytes were grown as described (Wellbrock *et al.*, 2008). 501mel, 888mel, 1290mel cells (Robbins *et al.*, 1995), A375, WM266-4 and SW480 cells were grown in DMEM/10% FCS (Invitrogen). All melanoma cell lines were established from metastatic tumours. Forskolin and Hoechst-53342 were from Sigma, Cy-5-Phalloidin from Invitrogen and Y27632 from Tocris. RNAi transfections used 10-40nM siRNA

oligonucleotides. The verified MITF siRNA has been described (Wellbrock *et al.*, 2008). Further siRNA oligonucleotides were from Qiagen (bcat1: AACCACUAAUGUCCAGCGUUU and bcat2: AAGUGGGUGGUAUAGAGGCUC). The ON-TARGET-*plus* DIA1 SMART-pool was from Dharmacon. SW480 cells were transfected with pEF-MITF (Wellbrock *et al.*, 2008). To culture cells on thick collagen layers, cells were seeded on a 12 well containing 0.6 ml (2.3 mg/ml) fibrillar bovine dermal collagen (Nutacon, 2.3mg/ml).

### Immunoblots and antibodies

Cell lysates were prepared as described (Wellbrock *et al.*, 2008) and analysed by standard Western-blotting protocols. For immunoprecipitation, MITF was precipitated from 1mg of total protein using 2 $\mu$ g of MITF (D5) antibody. The primary antibodies used were: MITF: D5, C5 (Neomarkers, USA); beta-catenin: E-5, ERK2: C-14 and beta-tubulin: H-235 (Santa Cruz), phospho-MLC2 (Ser19) (Cell Signalling).

### Immunofluorescence and microscopy

Cells on cover slips were stained as described (Wellbrock & Marais, 2005). Cells on collagen were formalin fixed and incubated with antibodies or phalloidin for 3h respectively. Nuclei were stained with Hoechst 53342, and collagen plugs were mounted on slides using DABCO. For conventional fluorescence microscopy a Zeiss Axiovert 200 equipped with epifluorescence was used; images were taken by a Photometrics Cool Snap HQ CCD camera driven by Metamorph software (Universal Imaging). GFP expressing cells were monitored using a Leica DM IL HC inverted microscope and a FC340 Cooled Monochrome camera. For 3D reconstructions cells were analysed using a Delta Vision RT (Applied Precision) restoration microscope. Images were collected by a Coolsnap HQ (Photometrics) camera with a Z optical spacing of 1 $\mu$ m. Raw images were then deconvolved using the Softworx software, and ImageJ was used for the 3D reconstruction.

### In vitro Invasion assays

Invasion assays analysing melanoma cell invasion into collagen (Nutacon, 2.3 mg/ml) or Cultrex basement membrane (R&D, 15.83 mg/ml) were performed as described (Arozarena *et al.*, 2011). The cocktail of protease inhibitors was prepared as described (Sahai & Marshall, 2003): 20 $\mu$ M GM6001 (Calbiochem), 10 $\mu$ M calpeptin (Calbiochem) and 10 $\mu$ g/ml aprotinin and leupeptin (Sigma). Data are from at least three independent measurements and are shown as mean  $\pm$  SEM; if not otherwise indicated, differences between treatments and controls were analysed by an unpaired t-test.

### Gelatin zymography

An equal number of 501mel or WM266.4 cells were seeded on top of collagen gels and after 72h aliquots of conditioned media were mixed with non-reducing sample buffer and analysed on gelatin containing (1.2mg/ml) acrylamide gels. Gels were stained with 0.025% Coomassie and then destained until bands were visualized.

### Quantitative Real-Time PCR

RNA was isolated with TRIZOL® and selected genes were amplified by quantitative real time PCR using SYBR green (Qiagen). Primers sequences were *MITF*: CCGTCTCTCACTGGATTGGT, TACTTGGTGGGGTTTTTCGAG; *MT1-MMP*: CCGAGGGCTTCCATGGCGAC, ATTCTGACAGTCCAAGGCTCGGC; *GAPDH*: CAATGACCCCTTCATTGACC, GACAAGCTTCCCGTTCTCAG. Data are from three independent experiments and are shown as mean  $\pm$  SEM; differences between treatments and controls were analysed by an unpaired t-test.



## Luciferase Reporter Assays

Cells were transfected with 0.6  $\mu$ g of TOPFLASH-reporter construct (van de Wetering *et al.*, 1997), 0.3  $\mu$ g of pEF-MITF and 0.3  $\mu$ g of pcmv-renilla. For RNAi 80nM siRNA was co-transfected with the plasmids. Cells were analysed for luciferase activity after 48h using a Firefly and Renilla Luciferase kit (Cambridge). The data are corrected for Renilla luciferase and represent the activity for assays performed in triplicate, with error bars to represent standard errors from the mean. Data are from three independent experiments and are shown as mean  $\pm$  SEM; differences between treatments and controls were analysed by an unpaired t-test.

## Immunohistochemistry

From sample archives of USZ 21 blocks of paraffin-embedded formalin-fixed melanoma tissues were selected including primary tumours (9), skin metastases (6), one lymphnode metastasis and one lung metastasis. Tissue sections of 1 $\mu$ m were obtained, and immunostaining for beta-Catenin (Transduction Laboratories 1:200) and MITF (Zytomed Systems 1:60) was assessed using the APAAP method on DAKO Autostainer. Antibodies were visualised using DAKO Real Detection System kit (Code K5005). Slides were counterstained with haematoxylin. Nuclear beta-catenin was quantified for intensity (absent, weak, moderate) and area (<10%, 10-50%, >50%).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

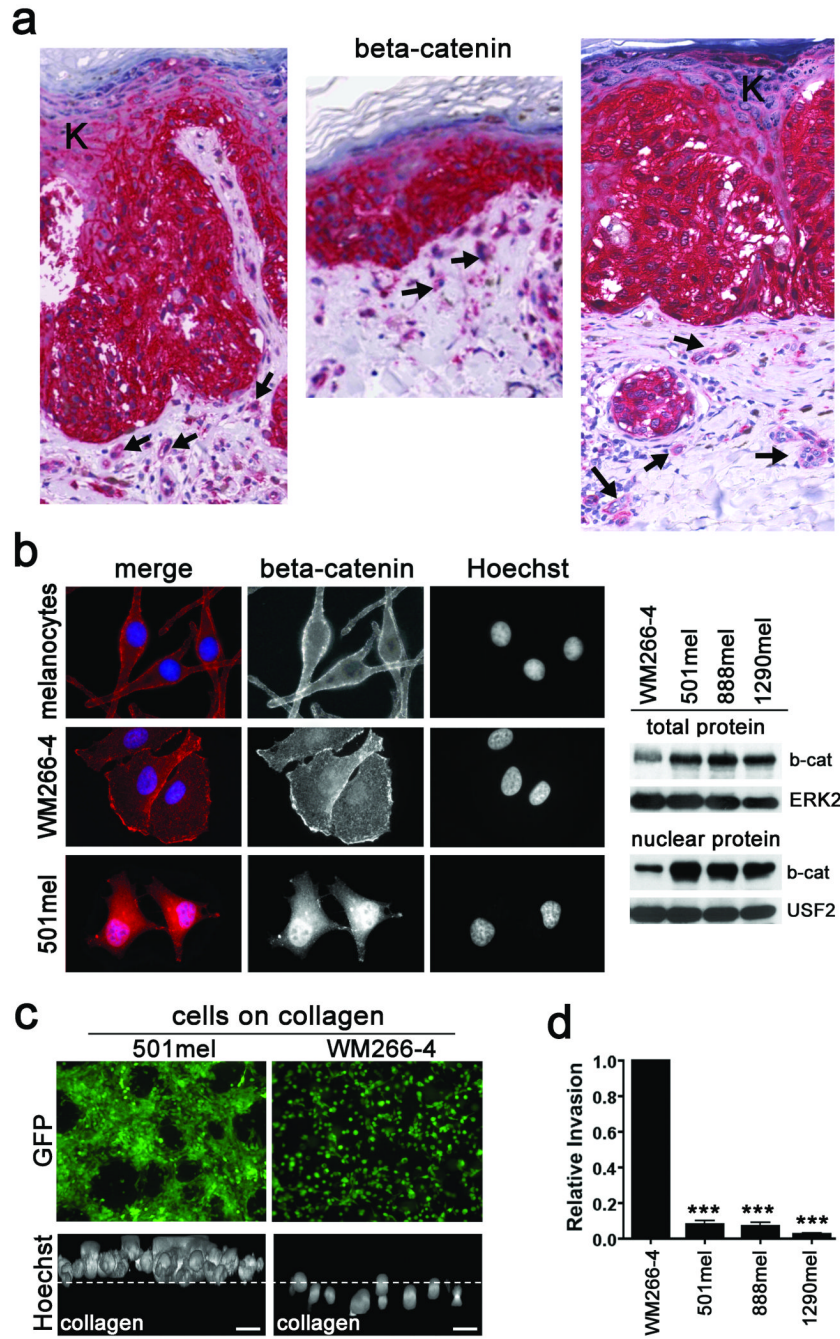
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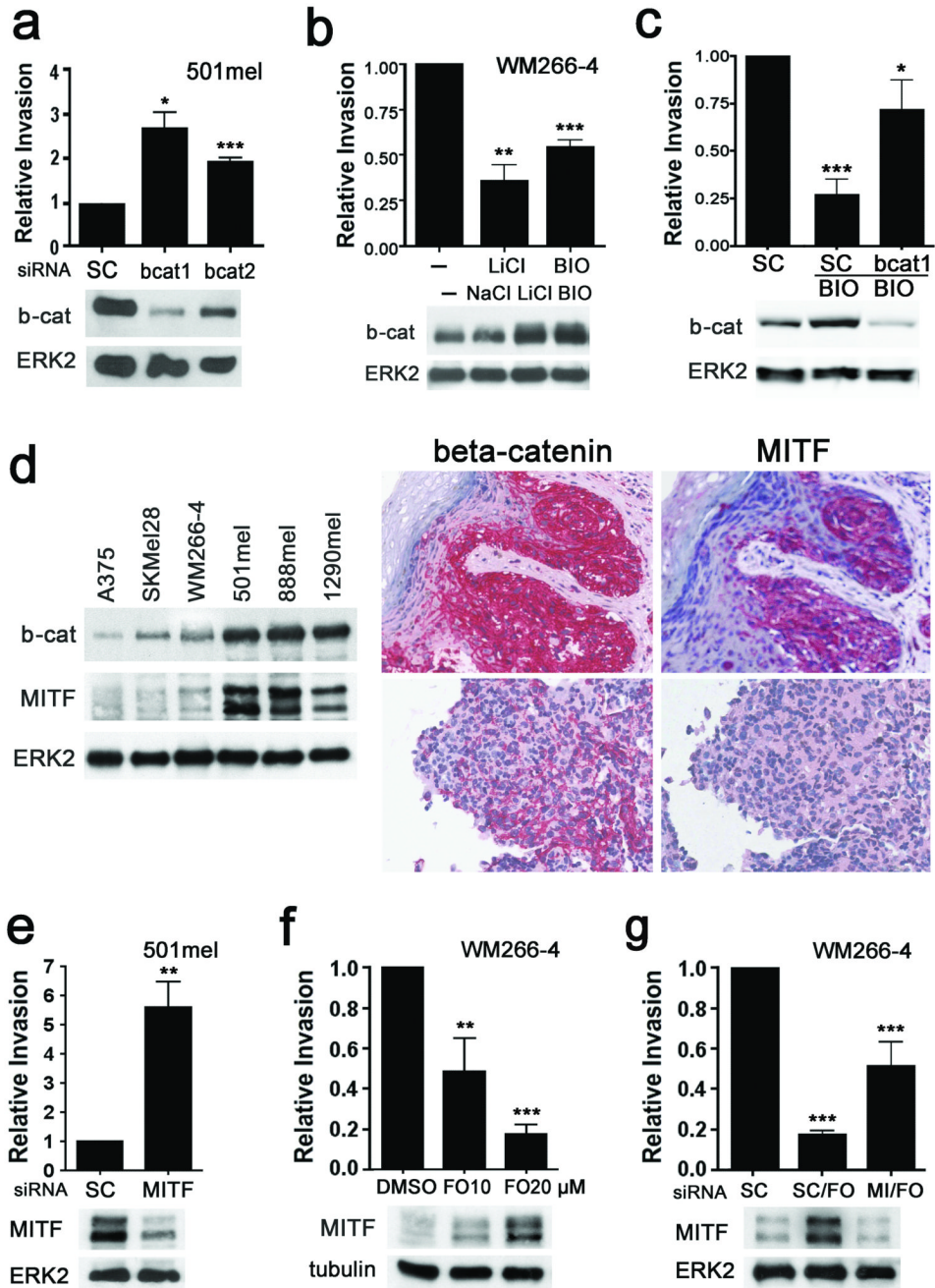
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**Figure 1.** Beta-catenin suppresses melanoma cell invasion. (a) Paraffin embedded samples of primary melanoma were analysed by histochemical staining for beta-catenin. Arrows indicate invading melanoma cells. Note membrane beta-catenin staining in the dermal keratinocytes (K). (b) Beta-catenin immunofluorescence in primary human melanocytes, and WM266-4 and 501mel melanoma cell lines using Cy3-labelled secondary antibodies. Nuclei were stained with Hoechst-53342. Western blot of total and nuclear beta-catenin for the indicated cell lines; ERK2 and USF2 were used as loading control. (c) Top panel: 10,000 GFP expressing 501mel or WM266-4 cells were cultured in a 12 well on top of a collagen gel (thickness ~1500µm) for 7 days and photographed. Bottom panel: 3D reconstruction of

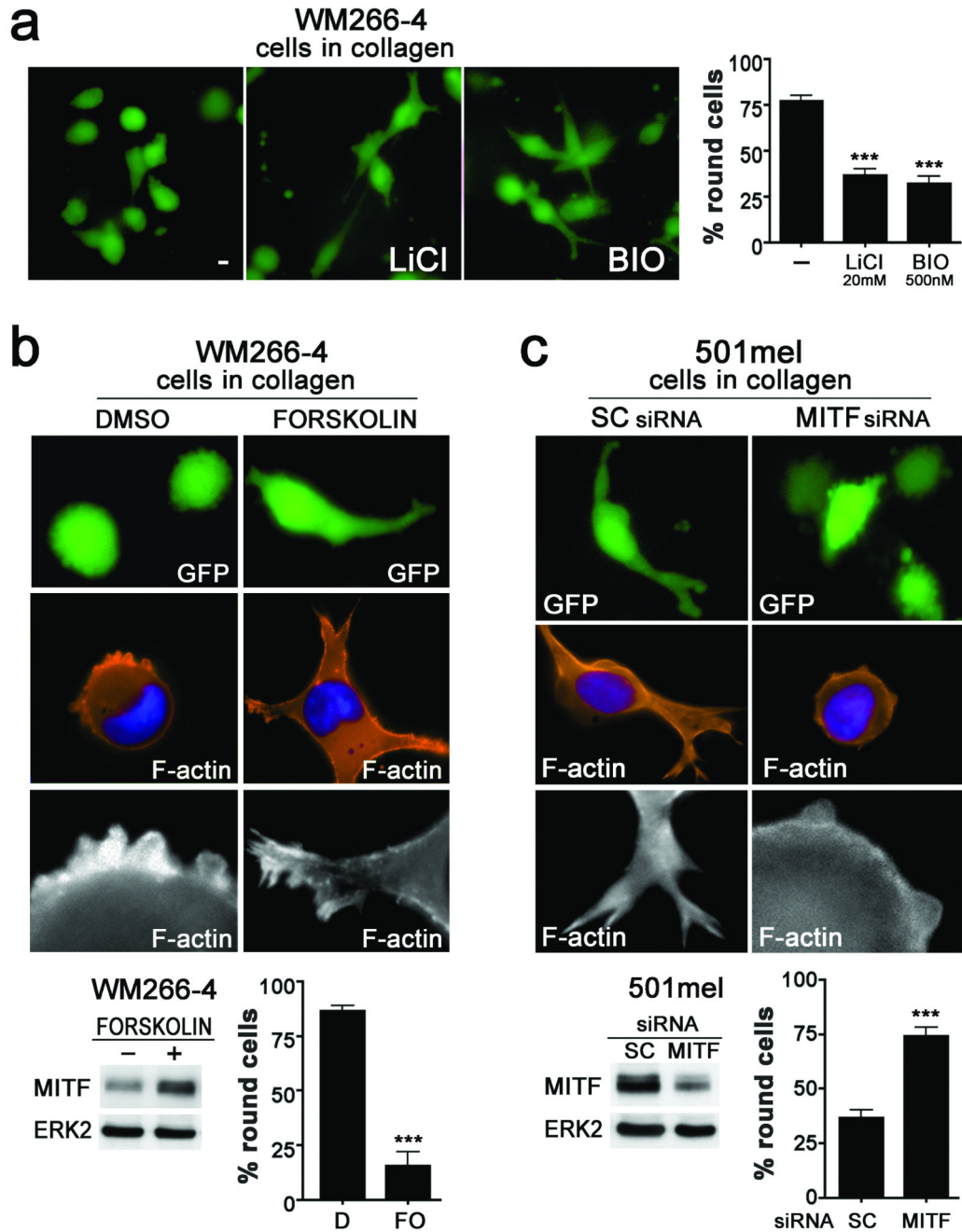
Hoechst 53342 stained nuclei of GFP-501mel and GFP-WM266-4 cells cultured on top of collagen for 7 days. Scale bar, 20 $\mu$ m. (d) Analysis of invasion of the indicated melanoma cell lines into type I collagen. The mean and SEM is shown,  $p < 0.0001$ .



**Figure 2.**

MITF levels correlate with beta-catenin expression and regulate melanoma cell invasion. (a) Collagen-invasion assay of 501mel cells treated with either a scrambled control (SC) or beta-catenin specific (bcat1, bcat2) siRNAs. The efficiency of knock down at the start of the assay is shown by Western blot. ERK2 was used a loading control. bcat1:  $p=0.012$ ; bcat2:  $p=0.0002$ . (b) After pre-treatment with the GSK3 inhibitors LiCl (20mM) or BIO (500nM), WM266-4 cells were analysed in a collagen invasion assay. LiCl:  $p=0.0017$ ; BIO:  $p=0.0003$ . Beta-catenin expression levels in either untreated or GSK3 inhibitor pre-treated WM266-4 cells were analysed by Western blotting before the assay. (c) Collagen-invasion assay of WM266-4 cells transfected with either a control (SC) or beta-catenin specific

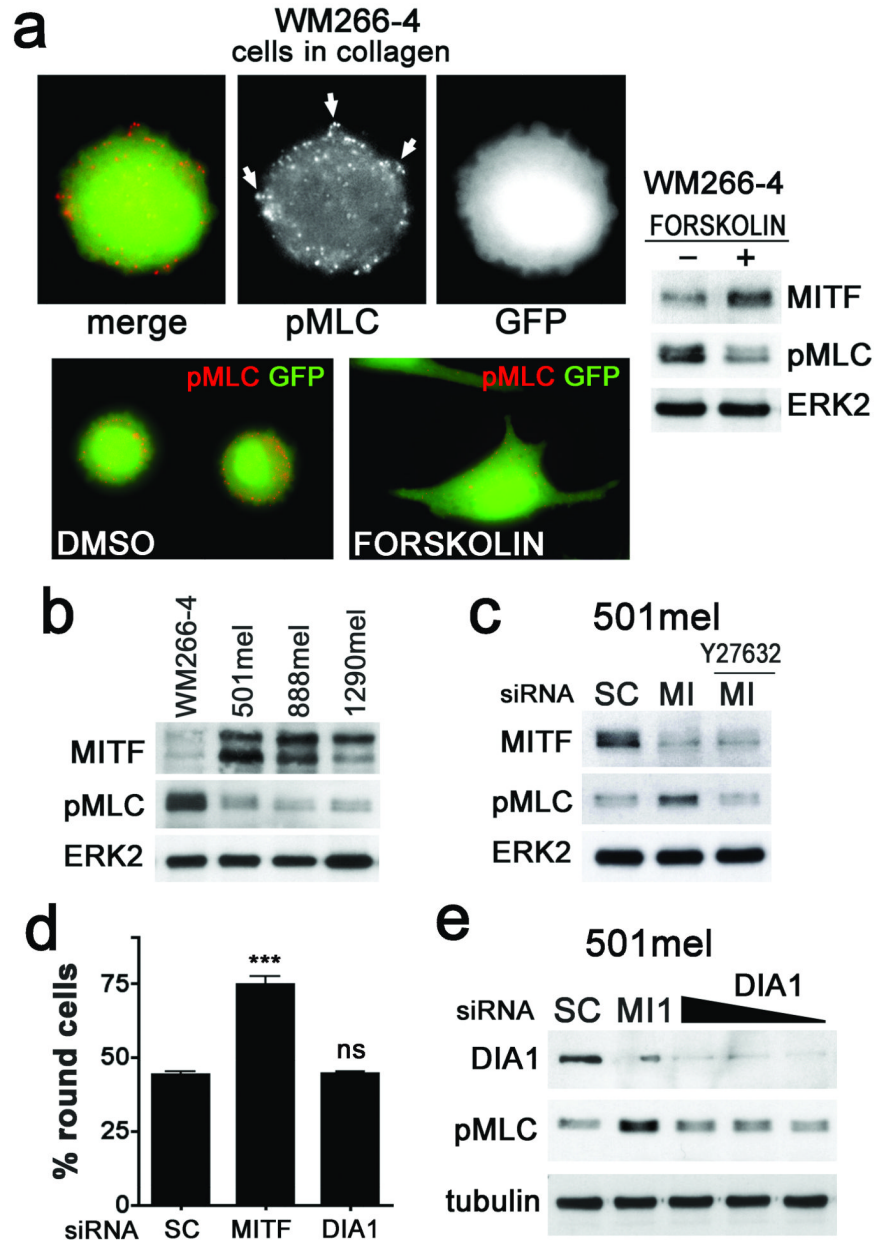
siRNA and treated with BIO (500nm) as in (b). BIO:p<0.0001; bcat/BIO:p=0.0197. Beta-catenin expression in cells used for the assay is shown by Western blot. ERK2 was used as a loading control. (d) Western blot of the indicated either low or high nuclear beta-catenin expressing melanoma cell lines for beta-catenin and MITF. ERK2 was used as loading control. Paraffin embedded tissue samples were analysed by histochemical staining for beta-catenin and MITF. (e) Collagen-invasion assay of 501mel cells treated with control (SC) or MITF specific siRNA. p= 0.061. The efficiency of the knock is shown by Western blot. ERK2 was used as loading control. (f) Collagen invasion of WM266-4 cells in the presence of increasing concentrations of forskolin. 10 $\mu$ M, p= 0.059; 20 $\mu$ M, p<0.0001. The cells were pre-treated for 4h before they were analysed; a Western blot of MITF expression at 4h is shown; tubulin was used as loading control. (g) Collagen invasion of WM266-4 cells treated with either scrambled control (SC) or MITF specific siRNA in the absence or presence of 20 $\mu$ M forskolin. SC/FO, p<0.0001; MI/FO p<0.0001. A Western blot of MITF expression in cells at the start of the assay is shown. ERK2 was used as loading control.



**Figure 3.**

High MITF levels suppress F-actin cortex contraction. (a) GFP-WM266-4 cells in 3D collagen in the presence of DMSO, 20mM LiCl or 500nM BIO were photographed and the number of round cells was quantified. LiCl:  $p=0.0003$ ; BIO:  $p=0.0003$ . (b) GFP-WM266-4 cells in 3D collagen in the presence of 20 $\mu$ M forskolin or DMSO were fixed, stained with Cy5-labelled phalloidin and photographed; the number of round cells was quantified.  $p=0.0005$ . In parallel cells were analysed for MITF on a Western blot. ERK2 serves as loading control. (c) GFP-501mel cells transfected with either control (SC) or MITF specific siRNA were cultured in 3D collagen. After 24h cells were fixed, stained with Cy5-labelled

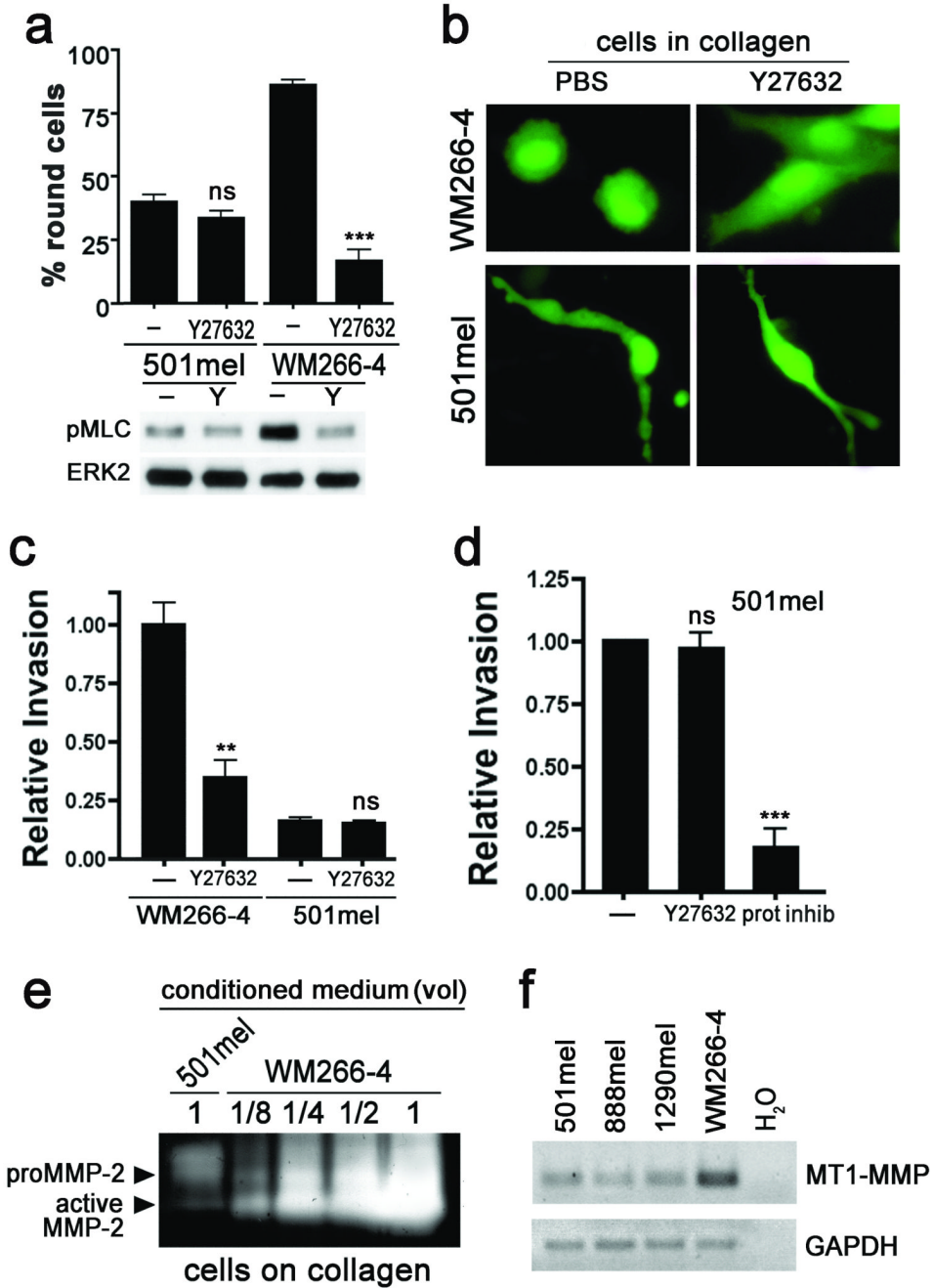
phalloidin and photographed. The number of round cells was quantified,  $p=0.0004$ . In parallel cells were analysed for MITF on a Western blot. ERK2 serves as loading control.



**Figure 4.** MITF regulates cortex contraction and membrane blebbing in a phospho-MLC dependent manner. (a) GFP-WM266-4 cells cultured in 3D collagen were untreated or treated with 20 $\mu$ M forskolin or DMSO for 24hours, before they were stained for phospho-MLC (pMLC). pMLC staining and GFP are shown. Arrows indicate pMLC staining in membrane blebs. In parallel cells were analysed for MITF and pMLC on a Western blot. ERK2 was used as loading control. (b) WM266-4, 501mel, 888mel, 1290mel cells were analysed for pMLC on a Western blot. ERK2 was used as loading control. (c) 501mel cells were transfected with either control (SC) or MITF specific siRNA in the absence or presence of 10 $\mu$ M Y27632. Cells were analysed for MITF and pMLC on a Western blot. ERK2 was used as loading control. (d) 501mel cells transfected with either control (SC) or MITF specific siRNA, or decreasing amounts (40nM, 20nM, 10nM) of DIA1 ON-TARGET $plus$  siRNA were cultured

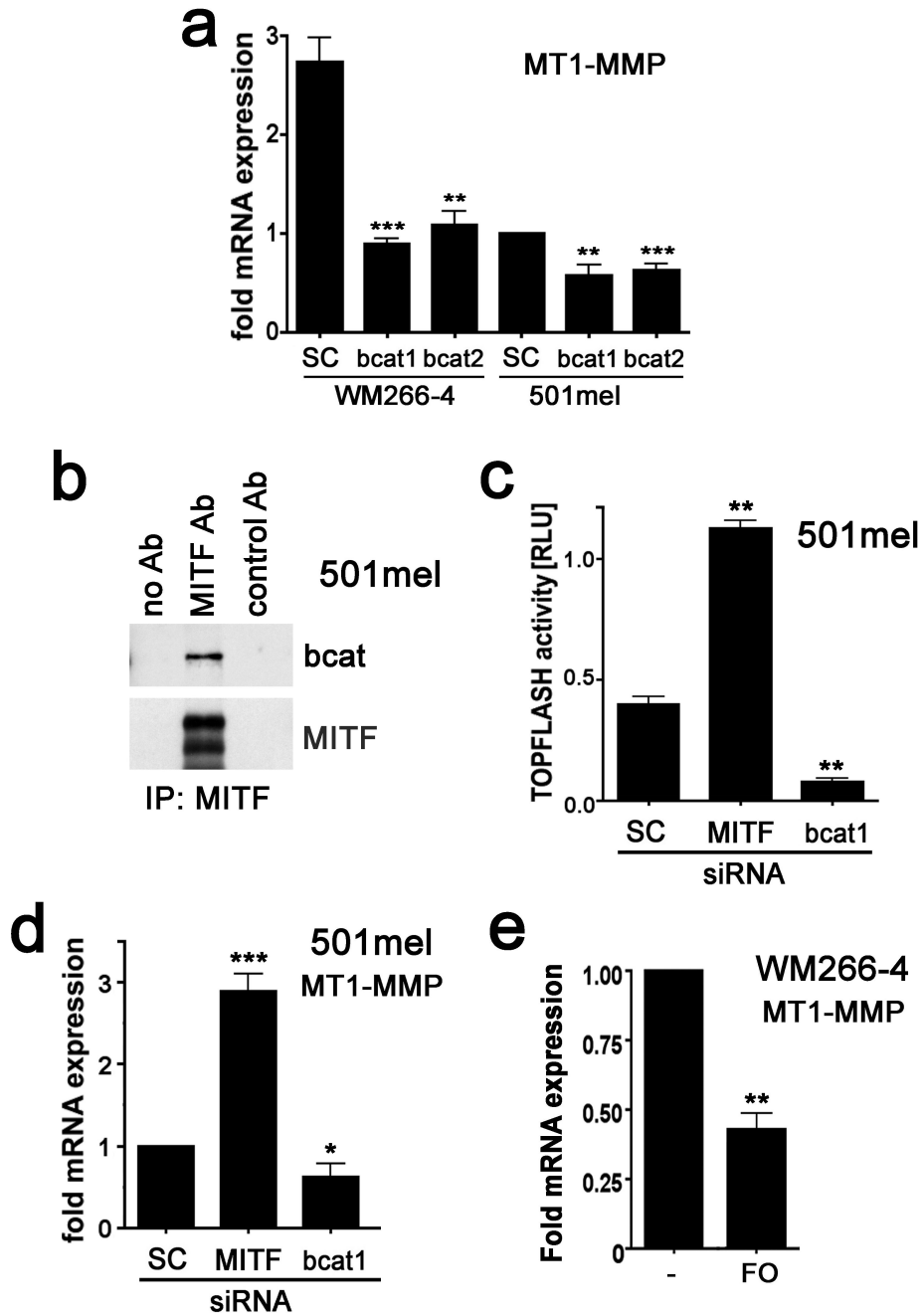


on 3D collagen. After 24h cells were fixed, and the number of round cells (SC, MITF, 10nM DIA1 siRNA) was quantified.  $p=0.0001$ . (e) In parallel cells were analysed for DIA1 and pMLC on a Western blot. Beta-tubulin serves as loading control.



**Figure 5.** High MITF expression correlates with protease driven invasion. (a) GFP-WM266-4 and GFP-501mel cells were cultured on 3D collagen with either 10  $\mu$ M Y27632 or the vehicle PBS. After 24h cells were photographed and the number of round cells was quantified. WM266-4, Y27632:  $p = 0.0001$ . In parallel inhibitor treated or untreated (-) cells were analysed for pMLC on a Western blot. ERK2 was used as loading control. (b) WM266-4-GFP and 501mel-GFP cells in 3D collagen in the presence of either 10  $\mu$ M Y27632 or the vehicle PBS were photographed after 24hours. (c) WM266-4-GFP and 501mel-GFP cells in 3D collagen in the presence of either 10  $\mu$ M Y27632 or the vehicle PBS were analysed in a collagen-invasion assay. WM266-4, Y27632:  $p = 0.0015$  (d) Collagen invasion assay of

501mel cells either untreated, treated with 10 $\mu$ M Y27632 or a protease inhibitor cocktail. p=0.0001 (e) Zymogram of conditioned media from 501mel and WM266-4 cells. The conditioned medium was analysed at various dilutions, and undiluted medium was set 1. proMMP-2 and cleaved/activated MMP2 are shown. (f) RT-PCR for MT1-MMP expression in WM266-4, 501mel, 888mel and 1290mel cells. Amplification of GAPDH was used as control.

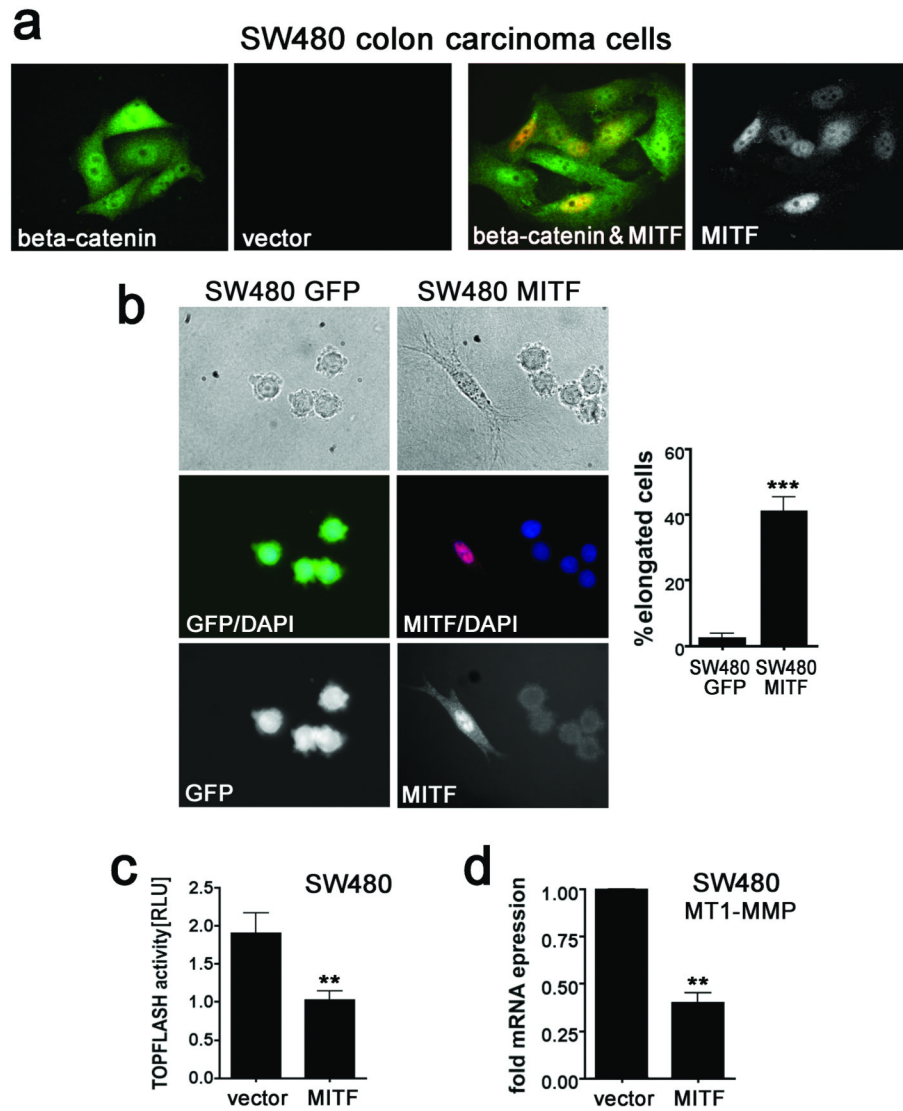


**Figure 6.**

MITF and beta-catenin regulate MT1-MMP expression. (a) Real-time PCR of MT1-MMP expression in 501mel and WM266-4 cells transfected with either control (SC) or beta-catenin specific (bcat1, bcat2) siRNAs. bcat1:  $p=0.003$  and  $0.0021$ , bcat2:  $p=0.0012$  and  $0.0001$  (b) Immunoprecipitation of MITF from 501mel cells. The precipitates were analysed for beta-catenin and MITF on a Western blot. (c) Luciferase activity (RLU) from a TCF/LEF reporter construct (TOPFLASH) in 501mel cells transfected with either control siRNA (SC), or MITF specific ( $p=0.0039$ ) or beta-catenin specific (bcat,  $p=0.0066$ ) siRNA. (d) Real-time PCR of MT1-MMP expression in 501mel cells transfected with either control (SC), MITF specific ( $p=0.0003$ ) or beta-catenin specific (bcat1,  $p=0.0132$ ) siRNAs. The

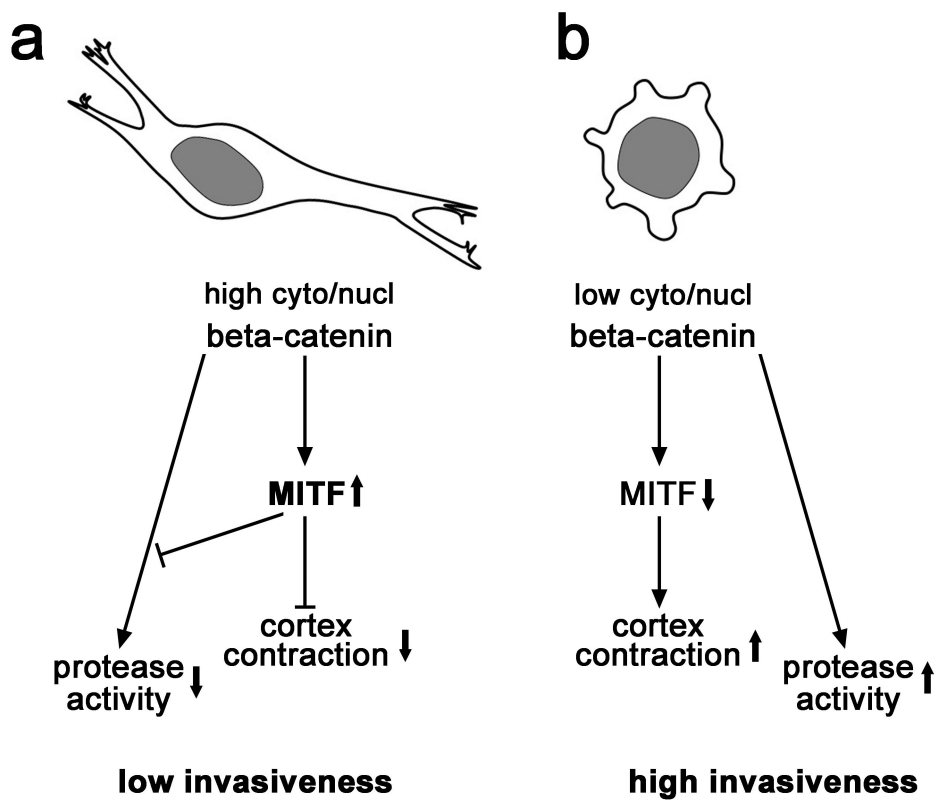
expression in 501mel control treated cells (SC) was set 1. (e) Real-time PCR of MT1-MMP expression in WM266-4 cells treated with forskolin (20  $\mu$ M) or DMSO (-);  $p= 0.0097$ .





**Figure 7.**

MITF alters the actin cytoskeleton and MT1-MMP expression in SW480 cells. (a) Immunofluorescence of SW480 cells for beta-catenin and MITF using Cy2- and Cy3-labelled secondary antibodies. (b) Immunofluorescence for MITF (Cy3) in SW480 cells cultured on collagen. Cells were either transfected with pMAX-GFP or pEFMITF and analysed for GFP expression or MITF expression. Nuclei were stained with Hoechst 53342. The number of elongated cells was quantified;  $p = 0.0001$  (c) Luciferase activity (RLU) from a TCF/LEF reporter (TOPFLASH) in SW480 cells transfected with either pEFMITF or an empty vector;  $p = 0.0091$  (d) Real-time PCR of MT1-MMP expression in SW480 cells transfected with either pEFMITF or empty vector;  $p = 0.0028$ .



**Figure 8.**  
Model showing how high or low levels of nuclear beta-catenin modulate the invasive behaviour of melanoma cells through MITF.