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Oncogenic B-RAF^{V600E} signaling induces the T-box3 transcriptional repressor to repress E-cadherin and enhance melanoma cell invasion

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Abstract

Approximately 50% of melanomas require oncogenic B-RAF^{V600E} signaling for proliferation, survival and metastasis, and the use of highly selective B-RAF inhibitors has yielded remarkable, albeit short term, clinical responses. Re-activation of signaling downstream of B-RAF is frequently associated with acquired resistance to B-RAF inhibitors, and the identification of B-RAF targets may therefore provide new strategies for managing melanoma. In this report, we applied whole genome expression analyses to reveal that oncogenic B-RAF^{V600E} regulates genes associated with epithelial-mesenchymal transition in normal cutaneous human melanocytes. Most prominent was the B-RAF-mediated transcriptional repression of E-cadherin, a keratinocyte-melanoma adhesion molecule whose loss is intimately associated with melanoma invasion and metastasis. Here we identify a link between oncogenic B-RAF, the transcriptional repressor Tbx3 and E-cadherin. We show that B-RAF^{V600E} induces the expression of Tbx3, which potently represses E-cadherin expression in melanocytes and melanoma cells. Tbx3 expression is normally restricted to developmental embryonic tissues, promoting cell motility but is also aberrantly increased in various cancers and has been linked to tumor cell invasion and metastasis. We propose that this B-RAF/Tbx3/E-cadherin pathway plays a critical role in promoting the metastasis of B-RAF mutant melanomas.

Introduction

Cutaneous melanoma is a highly aggressive cancer and until recently, the majority of patients with visceral metastases had survival rates of less than one year (Balch *et al.*, 2001). The use of targeted inhibitors in particular inhibitors of oncogenic B-RAF, have produced remarkable, albeit short-lived clinical responses (Flaherty *et al.*, 2010; Kefford *et al.*, 2010). Developing an effective treatment for metastatic melanoma therefore remains a major challenge, and requires a thorough understanding of the events occurring downstream of B-RAF.

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Conflict of Interest
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Constitutively activating mutations affecting the serine/threonine kinase, B-RAF, predominately the oncogenic B-RAF^{V600E}, are found in approximately 50% of metastatic melanomas (reviewed in Platz *et al.*, 2008). Oncogenic B-RAF signals via the mitogen activated protein kinase (MAPK) cascade to promote the proliferation and survival of transformed melanocytes. There is also mounting evidence that oncogenic B-RAF contributes to tumor aggressiveness by regulating tumor cell morphology, adhesion, migration and invasion. Indeed, B-RAF^{V600E} in human melanoma is associated with loss of cell cohesion and the upward migration of melanoma cells into the epidermis (Broekaert *et al.*, 2010; Viros *et al.*, 2008). Moreover, B-RAF^{V600E} is strongly associated with lymph node metastasis in papillary thyroid carcinomas (Basolo *et al.*, 2010) and there is some evidence that this may reflect altered expression of extracellular matrix genes, such as integrins, laminin and fibronectin and epithelial to mesenchyme transition (EMT) associated adhesion factors (Knauf *et al.* 2011; Nucera *et al.*, 2010). EMT involves disassembly of cellular junctional structures, which characteristically involves the loss of E-cadherin expression and accumulation of mesenchymal proteins. Loss of E-cadherin expression is common in melanoma and plays a critical role in altering melanoma cell interactions and promoting tumor cell invasion and metastasis (Ikoma *et al.*, 2005; Kreizenbeck *et al.*, 2008; Tucci *et al.*, 2007). However, how B-RAF regulates an EMT-like transition in melanoma cells is not known, although it presumably involves the activation of transcription factors able to down-regulate adhesion molecules to promote invasiveness.

Here we applied a genome-wide expression profiling approach to identify downstream B-RAF^{V600E} signaling changes that modulate melanocyte morphology and migration. As predicted oncogenic B-RAF signaling altered the expression of genes involved in cell morphology, cell adhesion, migration, ECM remodelling and epithelial to mesenchymal transition. Importantly, we show that B-RAF represses E-cadherin transcription by promoting the expression of Tbx3. Thus, Tbx3 acts as critical regulator of oncogenic B-RAF signaling, promoting cell proliferation, migration and metastasis by repressing the transcription of tumor suppressor genes including E-cadherin.

Results

Oncogenic B-RAF deregulates genes involved in melanocyte adhesion and cytoskeleton remodelling

To evaluate the influence of B-RAF^{V600E} signaling on the transcriptome of primary human melanocytes, B-RAF^{V600E} was stably transduced into primary melanocytes using lentiviral vectors co-expressing Copepod GFP (copGFP). Viral titres were selected to provide an efficiency of infection above 90% and activation of the ERK pathway that was comparable to human melanoma cells expressing endogenous B-RAF^{V600E} (Figure 1). We then performed genome wide expression analysis using the high throughput Illumina HumanRef-6 platform to compare the transcriptome signatures of human epidermal melanocytes with or without oncogenic B-RAF^{V600E}. Among the set of 1650 genes that were differentially expressed (P value <0.01 from duplicate experiments) genes involved in integrin-mediated cell adhesion and migration (p-value 6.3e-9), ECM remodelling (p-value 1.7e-7) cytoskeleton remodelling (p-value 2e-7) and EMT (p-value 1.480e-3) were highly enriched. As shown in Table 1, this gene set included many genes involved in cell adhesion (VEGFA-C, ITGA3, ITGA5), cytoskeleton remodelling (EZR, PLAT, MSN) and extracellular matrix remodelling (TIMP1-3, IL8, KLK2) including an extensive range of molecules associated with EMT (AP1, FOSL1, CTGF, CDH1). Of particular interest for melanoma progression and metastasis was the reduction of the E-cadherin transcript (CDH1) upon B-RAF^{V600E} signaling (Table 1). Consistent with the observed transcription profile, melanocytes expressing B-RAF^{V600E} show rounded cell morphology and diminished substrate and cell adhesion as reported previously (Becker *et al.*, 2010).

B-RAF^{V600E} diminishes E-cadherin expression in melanocytes

To confirm the impact of mutant B-RAF^{V600E} on E-cadherin transcript expression, we used quantitative real time PCR and confirmed that exogenous B-RAF^{V600E} signaling reduced melanocyte E-cadherin transcript levels by more than 90%, three days post transduction (Figure 2a) and B-RAF^{V600E} repressed the E-cadherin promoter similarly in promoter reporter assays (data not shown). Moreover, E-cadherin protein levels were significantly diminished in B-RAF^{V600E} transduced normal melanocytes derived from three individuals (Figure 2b) and E-cadherin protein levels increased when B-RAF^{V600E} was specifically silenced by shRNA or inhibited using the targeted B-RAF inhibitor Vemurafenib (PLX4032) in two B-RAF^{V600E}-positive melanoma cell lines (NM176 and ME1042) (Figure 2c).

B-RAF^{V600E} upregulates the Tbx3 transcriptional repressor

To define the mechanism by which oncogenic B-RAF suppressed E-cadherin transcription, we re-analyzed our transcriptome arrays for changes in the expression of established regulators of E-cadherin transcription. Although EMT has traditionally been associated with up-regulation of the E-cadherin regulators SLUG and SNAIL (Bolos *et al.*, 2003; Cano *et al.*, 2000; Conacci-Sorrell *et al.*, 2003; Hajra *et al.*, 2002; Poser *et al.*, 2001), in melanoma an additional tissue restricted repressor of E-cadherin, Tbx3, has been identified (Rodriguez *et al.*, 2008). Tbx3, a member of the T-box family of developmental regulators (Bamshad *et al.*, 1997; Lee *et al.*, 2007; Rowley *et al.*, 2004; Suzuki *et al.*, 2008) has been strongly implicated in cancer (Hoek *et al.*, 2004; Peres *et al.*, 2010; Rodriguez *et al.*, 2008). Indeed, as shown in Figure 3a, Tbx3 was significantly upregulated (~10-fold) in melanocytes with B-RAF^{V600E} signaling, while no other known E-cadherin regulator in our Illumina dataset was significantly altered. We confirmed that B-RAF^{V600E} upregulated Tbx3 transcription using qRT-PCR analyses (Figure 3b). It is noteworthy that the Tbx3 primers used in these experiments amplified both splice variants of Tbx3 (Lee *et al.*, 2007) and expression of both variants was elevated by B-RAF^{V600E} (Figure 3c). Moreover the activity of the Tbx3 promoter was strongly upregulated by exogenous B-RAF^{V600E} when measured using promoter reporter assays (Figure 4a). Consistent with these data the expression of Tbx3 correlated with B-RAF mutation status in our microarray analyses of 60 stage III excised human melanoma lymph node metastases. In particular, the expression of Tbx3 was significantly increased in B-RAF mutant tumors (Mann-Whitney p=0.01) (Figure 4b, Table 2). Significantly, we confirmed that oncogenic B-RAF also promoted the accumulation of Tbx3 protein as well as the reduction of E-cadherin in three independent melanocytes strains and as expected E-cadherin repression correlated with N-cadherin accumulation (Figure 2b). We also confirmed that the B-RAF-mediated effects on E-cadherin and Tbx3 expression were not due to B-RAF^{V600E}-induced melanocyte senescence (Scurr *et al.*, 2010) as the introduction of B-RAF^{V600E} into the NM179 melanoma cells (wild type B-RAF), also regulated the expression of Tbx3 and E-cadherin (Figure 2b) in the absence of proliferative arrest (data not shown). Finally, the specific silencing or inhibition of BRAF^{V600E} expression in the NM176 and ME1042 melanoma cells decreased Tbx3 protein expression and this correlated with the concomitant increased E-cadherin (Figure 2c).

B-RAF^{V600E} mediated Tbx3 upregulation causes E-cadherin repression

To confirm that Tbx3 was the critical effector of B-RAF^{V600E}-mediated E-cadherin transcriptional repression in melanocytes, we applied two highly specific Tbx3 silencing molecules. Each silencer was introduced into melanocytes for five days, followed by transduction with oncogenic B-RAF. The Tbx3 silencers partially suppressed the B-RAF-mediated induction of Tbx3 and this led to the partial restoration of E-cadherin expression in the presence of B-RAF^{V600E} (Figure 5). As expected, E-cadherin transcript levels, when compared to expression levels in melanocytes (data not shown), were universally low in our

patient cohort of stage III lymph node metastatic melanomas. Consequently, although there was a positive relationship between oncogenic B-RAF and Tbx3 (see Figure 4b, Table 2), we could not detect an association between oncogenic B-RAF or Tbx3 with E-cadherin expression in these samples (Table 2).

B-RAF^{V600E} signaling and Tbx3 promote melanoma cell invasion

To examine the role of the B-RAF/Tbx3/E-cadherin cascade on melanoma cell invasion, we silenced B-RAF^{V600E} or Tbx3 in the B-RAF-dependent NM176 and ME1042 melanoma cells. As expected, suppression of B-RAF^{V600E} decreased the invasion of both melanoma cell lines through matrigel. Similarly, silencing Tbx3 expression also dramatically diminished the invasive potential of the NM176 and ME1042 melanoma cells (Figure 6).

Discussion

B-RAF^{V600E} deregulates many genes involved in cell adhesion, extracellular matrix formation and cytoskeletal integrity (Nucera *et al.*, 2010; Knauf *et al.*, 2011). The combined downstream effects suggest that oncogenic B-RAF promotes EMT and thereby enhances the migratory and metastatic capacity of transformed cells. EMT involves the disassembly of junctional structures typically via the loss of E-cadherin expression, loss of cellular polarity and increased migration. Two studies have shown that B-RAF signaling is associated with reduced levels of E-cadherin in breast epithelial and colon cancer cells (Brummer *et al.*, 2006; Minoo *et al.*, 2007), and we now report that B-RAF^{V600E} inhibits the transcription of E-cadherin in human melanocytes and melanoma cells. Although melanocytes are not epithelial cells, E-cadherin is required for their critical interaction with keratinocytes. The loss of E-cadherin produces an EMT-like melanocytic phenotype, is required for invasion and is associated with melanoma progression and metastasis by preventing keratinocyte-regulation of melanocytes (reviewed in Haass *et al.*, 2005; Hsu *et al.*, 2000).

Here, we provide evidence that Tbx3 is strongly upregulated by oncogenic B-RAF in human melanocytes and melanoma cells, which is inhibited by PLX4032 treatment. Furthermore, we show that Tbx3 upregulation correlated with B-RAF mutation status in 60 metastatic melanoma samples. This data from fresh-frozen, uncultured melanoma biopsies support a recent report showing increased levels of Tbx3 in B-RAF mutant melanoma cell lines (Johansson *et al.*, 2007).

Importantly we have confirmed that partial depletion of Tbx3 opposed the suppression of E-cadherin expression by oncogenic B-RAF in melanocytes and this has important implications in melanoma and other cancers including, ovarian carcinoma, pancreatic cancer, breast cancer and cervical cancer that overexpress Tbx3 (reviewed in Lu *et al.* 2010). We have shown previously that melanoma progression is associated with increased expression of Tbx3 protein and significantly that silencing of Tbx3 in melanoma cells decreases melanoma invasiveness (Rodriguez *et al.*, 2008). Our data link oncogenic B-RAF to the Tbx3/E-cadherin cascade and confirm that Tbx3 and B-RAF^{V600E} silencing suppress melanoma invasion through matrigel. The importance of the B-RAF/Tbx3/E-cadherin network is further highlighted by several studies, which show independently that E-cadherin loss and increased Tbx3 are associated with the progression of melanoma, invasiveness and poor prognosis (Hoek *et al.*, 2004; Kreizenbeck *et al.*, 2008; Mowla *et al.*, 2010; Rodriguez *et al.*, 2008; Tucci *et al.*, 2007). It is not surprising that E-cadherin levels were generally low in our tumor samples and thus did not correlate with B-RAF mutation status. E-cadherin loss is critical for metastasis and while we have provided evidence that B-RAF^{V600E}-induced Tbx3 represses E-cadherin expression, E-cadherin promoter silencing (Tsutsumida *et al.*, 2004) and expression of alternate transcriptional repressors (Kuphal and Bosserhoff, 2011;

Poser *et al.*, 2001) have also been reported in melanoma and may account for the low E-cadherin levels in tumors without mutant B-RAF.

Although we found no evidence that other transcriptional repressors of E-cadherin, including SLUG and SNAIL participated in B-RAF^{V600E}-mediated repression of E-cadherin in melanocytes, this does not preclude their involvement. For instance, our microarray analyses did not yield high signal data for the SNAIL transcript, which is a predicted B-RAF^{V600E} target (Lin *et al.*, 2010) and may contribute to the suppression of E-cadherin in B-RAF^{V600E}-positive melanoma cell lines (Poser *et al.*, 2001). Thus, although we do no exclude the involvement of other E-cadherin regulators, our data indicate that Tbx3 is a critical mediator of the transcriptional repression of E-cadherin by oncogenic B-RAF.

It is also worth noting that Tbx3 may prevent the onset of senescence via its ability to repress the expression of the p16^{INK4a}, p14ARF and p21^{Waf1} tumor suppressors (Brummelkamp *et al.*, 2002; Hoogaars *et al.*, 2008; Mowla *et al.*, 2010). Nevertheless, expression of oncogenic B-RAF^{V600E} in human melanocytes promotes senescence that is associated with increased levels of p16^{INK4a} (Scurr *et al.*, 2010) despite Tbx3 accumulation. This most likely reflects the complex network of regulatory and negative feedback pathways activated in response to oncogenic stimulation and this network favors p16^{INK4a} accumulation and enforces proliferative arrest in primary cells, but also primes damaged cells for transformation. Thus, the combined loss of p16^{INK4a} and the induced accumulation of Tbx3 by B-RAF^{V600E} may favor proliferation and transformation of melanocytes.

In conclusion, we report that B-RAF^{V600E} represses E-cadherin in melanocytes and this is consistent with data showing that B-RAF^{V600E} contributes to the epithelial to mesenchymal transition of transformed cells {Riesco-Eizaguirre, 2009 #193; Minoo, 2007 #61; Lin, 2010 #186). We show that B-RAF induces the transcriptional repressor Tbx3, which results in diminished E-cadherin expression in human melanocytes and increased invasiveness of B-RAF^{V600E}-mutant melanoma cells. These results highlight a key role of Tbx3, which is commonly upregulated in melanomas, in enhancing B-RAF-dependent melanoma progression, and suggest that inhibiting Tbx3 expression or activity may represent a therapeutic target downstream of B-RAF.

Materials and Methods

Cell culture and lentiviral transduction

Primary human dermal melanocytes (Cell Applications, San Diego, CA) were cultured as reported previously (Haferkamp *et al.*, 2009; Scurr *et al.*, 2010). Melanoma cell lines NM39, NM176, NM179, Sk-Mel28, ME1042, WMM1175, 501mel were cultured in DMEM media with 10% FCS. Cells were treated with 10μm Vemurafinib (PLX4032) (Selleckchem, Houston, Texas) for two days. Lentiviral particles were generated and used to transduce cells as reported previously (Haferkamp *et al.*, 2009; Scurr *et al.*, 2010). The following shRNA sequences were cloned into pSIH1-H1-copGFP to produce lentiviral particles: TBX3#2: GATCCCTGCCTATAGAGATATATTCACTTCCTGTCAGATGAATAT ATCTCTATAGGCATT, TBX3#3 GATCCGCTACAGAGAAATCTCGATCTTCCTGTCAGAATCGAGATTTCTCTG TAGCTTTTTG, B-RAF^{V600E} GATCCGCTACAGAGAAATCTCGATCTTCCTGTCAGAATCGAGATTTCTCTG TAGCTTTTTG or control shRNA (no homology to any known human transcript): GATCCTTAGAGGCGAGCAAGACTACTTCCTGTCAGATAGTCTTGCTCGCCT CTAATTTTTG.

Immunoblotting

For detection of E-cadherin, cells were harvested with EDTA (2 mM in PBS). Equal amounts of total cellular proteins (30-50μg), extracted at 4°C with RIPA lysis buffer containing protease inhibitors (Roche, Mannheim, Germany) and phosphatase inhibitors (Roche, Mannheim, Germany), were resolved on 12% SDS-polyacrylamide gels, transferred to Immobilon-P membranes (Millipore, Bedford, MA) and probed with antibodies against E-cadherin (SHE78-7, Zymed), total ERK (137F5, Cell Signaling), phosphorylated ERK (E-4, Santa Cruz), Tbx3 (ZMD.569, Zymed), endogenous B-RAF (L12G7, Cell Signaling) and ß-actin (AC-74, Sigma). MYC-tagged B-RAF was detected with anti-MYC (A-14, Santa Cruz).

qRT-PCR

Total RNA was extracted from melanocytes using TRI Reagent (Sigma) with an additional purification step with RNAeasy kits (QIAGEN, Valencia, CA, USA). Samples were collected from two separate experiments, and each sample amplified in duplicate in at least two independent experiments. 1 μg RNA was used for cDNA synthesis using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) with the supplied Oligo(dT)$_{20}$ primer. qRT-PCR products were amplified in 25 μl from 2 μl cDNA with SYBR-Green incorporation (Power SYBR-Green PCR Master Mix, Applied Biosystems) using a Corbett Rotorgene3000 and a final concentration of 0.3 μM of qRT-PCR primers: E-cadherin fwd: TGAAGGTGACAGAGCCTCTGGAT and E-cadherin rev: TGGGTGAATTCGGGCTTGTT (Tsai *et al.*, 2002), Tbx3 fwd: CGAAATGCCAAAGAGGATGT and Tbx3 rev: GAATTCAGTTTCGGGGAACA, note that this primer combination is known to amplify two splice variants of TBX3 (Lee *et al.*, 2007), GAPDH fwd: CTCTCTGCTCCTCCTGTTCGAC, GAPDH rev: TGAGCGATGTGGCTCGGCT and TBP (TATA box protein) fwd: TGCACAGGAGCCAAGAGTGAA TBP rev: CACATCACAGCTCCCCACCA. Melting curve analysis and agarose gel separation ensured product specificity. Relative gene expression was calculated from a standard curve included with each run. Expression data were normalized separately against the housekeeping genes GAPDH and TBP, which were not affected by B-RAF^{V600E} expression. Gene expression levels of B-RAF^{V600E} expressing melanocytes are presented as fold change in comparison to expression levels derived from melanocytes only expressing copGFP control, which was set at 1.

RNA Extraction and Microarray Gene Expression Analysis

Total RNA was extracted from melanocytes or from 10–20 mg of fresh-frozen, homogenized tumor sample with Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA). Chloroform was added and after spinning the upper phase was mixed with 70% ethanol and RNA was further purified with an RNeasy kits with DNase I digestion. 250 ng total RNA (RNA integrity number: 9–10) was amplified and labeled with biotin (Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX). Gene expression analysis was performed using the Sentrix HumanRef-6 v.3.0 Expression BeadChip (Illumina, San Diego, CA, USA) and BeadStation system from Illumina according to manufacturer's instructions.

For analysis average signal intensities were background subtracted and normalized using the cubic spline function in GenomeStudio and the Illumina Custom function was used to assign a differential expression score and *P* value to each gene. Transcripts with detection and differential expression $P < 0.01$ were considered significantly different. The Metacore analysis software package, version 6.8, was used to identify gene ontology groups associated with oncogenic B-RAF-signaling in melanocytes. For tumor samples (stage III melanoma lymphnode metastasis) expression of Tbx3 and E-cadherin (CDH1) were assessed in correlation to their B-RAF mutation status in a univariate analysis by logistic regression.

Scatter plots were used to illustrate the distribution of gene expression by B-RAF mutation status (tumors with other known MAPK pathway mutations as determined using the Sequenom OncoCarta panel of 19 oncogenes and 238 mutations were excluded from this analysis). Medians and interquartile ranges were applied to summarize the distributions, and the Mann-Whitney test was used to determine the differences between the B-RAF wild-type and B-RAF^{V600E} mutant populations.

Promoter reporter assays

200ng of the human Tbx3 promoter (−249 to +168), cloned into the luciferase promoter reporter vector pGL3-basic or vector alone was transfected into 501mel cells with 25 or 100ng of wild type or V600E Myc epitope-tagged B-RAF expression vector. Extracts were processed and assayed for luciferase. Western probing for the Myc-tag of B-RAF and total ERK as a loading control confirmed similar expression of the B-RAF constructs.

Transwell matrigel invasion assays

Matrigel invasion chambers (BD Biosciences, Bedford MA) were rehydrated for 2 hours with low serum (0.1% FCS) DMEM. 5×10^4 melanoma cells transduced with Tbx3 shRNA #3, B-RAF^{V600E} shRNA or control shRNA for five days and suspended in low serum media were added to each 24 well insert and media containing 10% FCS was added to the bottom chamber. Approximately, 24 h post seeding the invading melanoma cells were quantified by Diff Quick stain (Lab aids, Narabeen, Australia) and microscopy for cells adhering to the bottom of the membrane. Since some melanoma cell lines grow in suspension we also tested cells that had invaded into media in the bottom chamber. The latter were harvested and spiked with 2×10^5 HEK293T cells and the number of copGFP expressing transduced melanoma cells per 5×10^4 unstained HEK293T cells was determined by FACS analysis and normalized on the input. ME1042 produced a negligible proportion of bottom chamber suspension cells (<4% of membrane bound control cells) and these were not included in calculating relative invasion, while NM176 suspension cells (>48% of membrane bound control cells) were included in calculating the relative invasion. Significance of the decrease in invasion was determined by Student's T-test.

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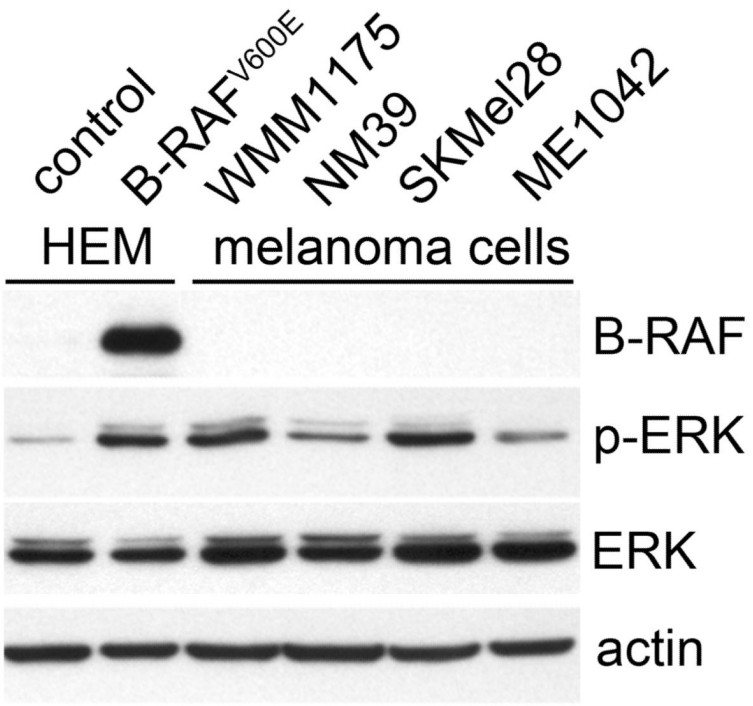

Figure 1. Activity of exogenous B-RAF^{V600E} in human melanocytes

Primary human melanocytes were infected with lentiviruses encoding B-RAF^{V600E} with copGFP or copGFP alone (control) for three days. Expression levels of phosphorylated ERK (p-ERK) were compared to those observed in a series of melanoma cell lines with known B-RAF/N-RAS genotypes (WMM1175: N-RAS^{G13R}; NM39, SKMel28 and ME1042: B-RAF^{V600E}). Ectopic B-RAF expression was detected by probing for the Myc-tag of B-RAF^{V600E}.

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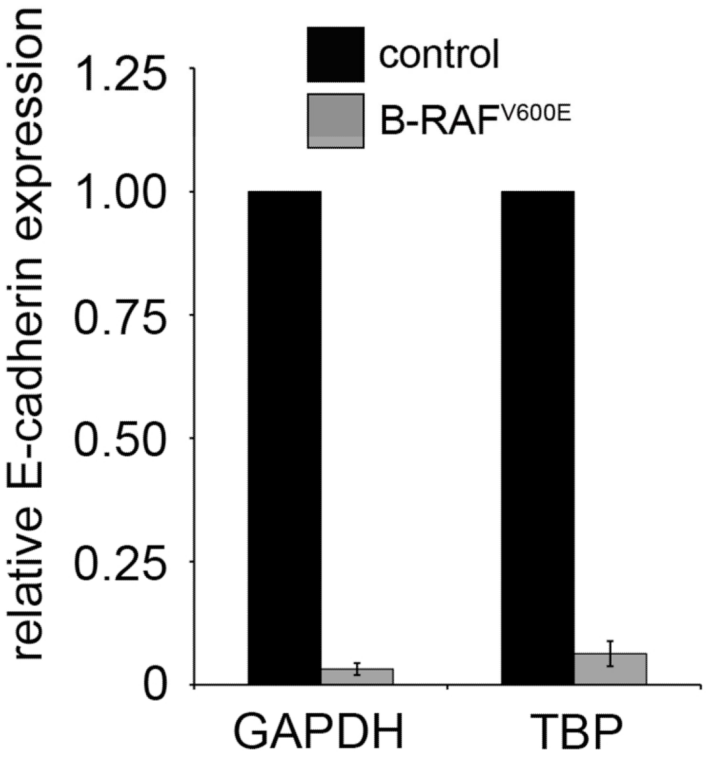

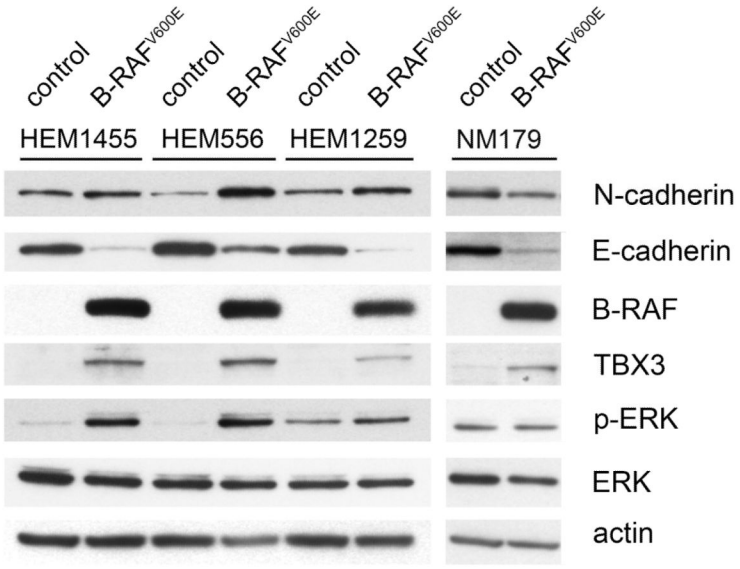

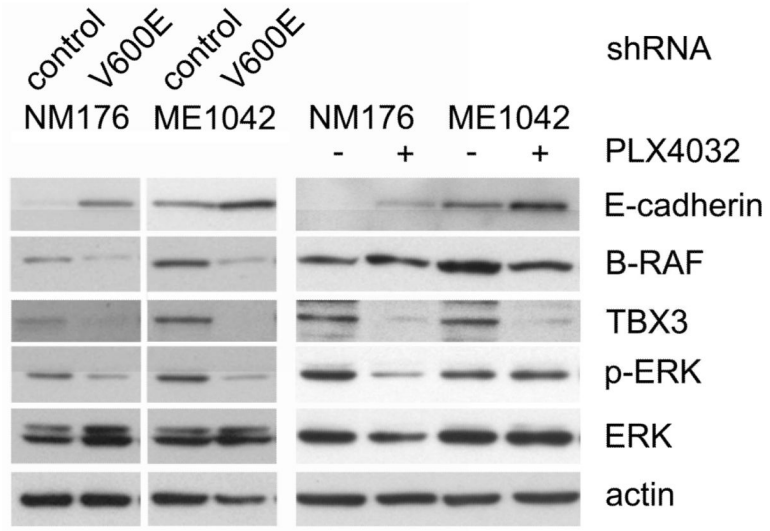

Figure 2. B-RAF^{V600E} signaling represses E-cadherin

(a) Melanocytes were infected with lentiviruses encoding B-RAF^{V600E} with copGFP or copGFP alone (control) for three days. Total RNA was analysed by qRT-PCR. E-cadherin transcript levels in B-RAF^{V600E}-transduced melanocytes were normalised to levels of GAPDH or TBP housekeeping genes and expressed relative to normalized E-cadherin transcript levels in control-transduced cells.

(b) Three independent melanocyte strains and NM179 melanoma cells were infected with lentiviruses encoding B-RAF^{V600E} with copGFP or copGFP alone (control) for three days. Total protein was immunoblotted for the indicated proteins.

(c) B-RAF signaling was inhibited in the B-RAF mutant melanoma cell lines, NM176 and ME1042, using the B-RAF^{V600E}-specific silencing molecule for five days or 10μM PLX4032 for two days. Total protein was immunoblotted for the indicated proteins.

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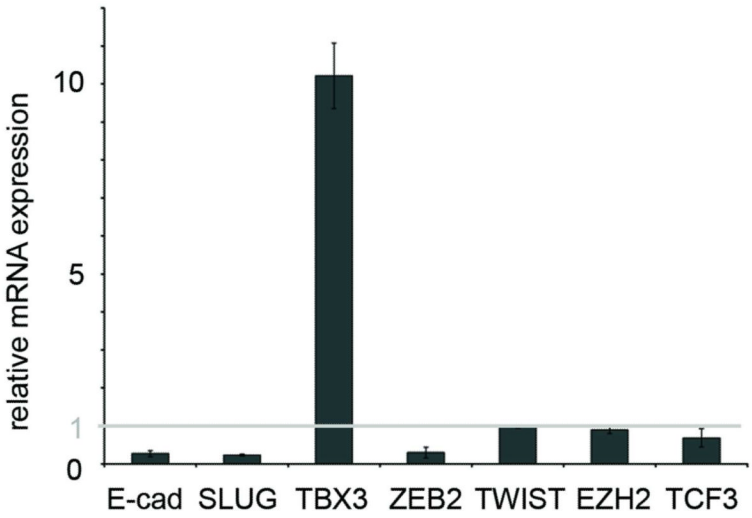

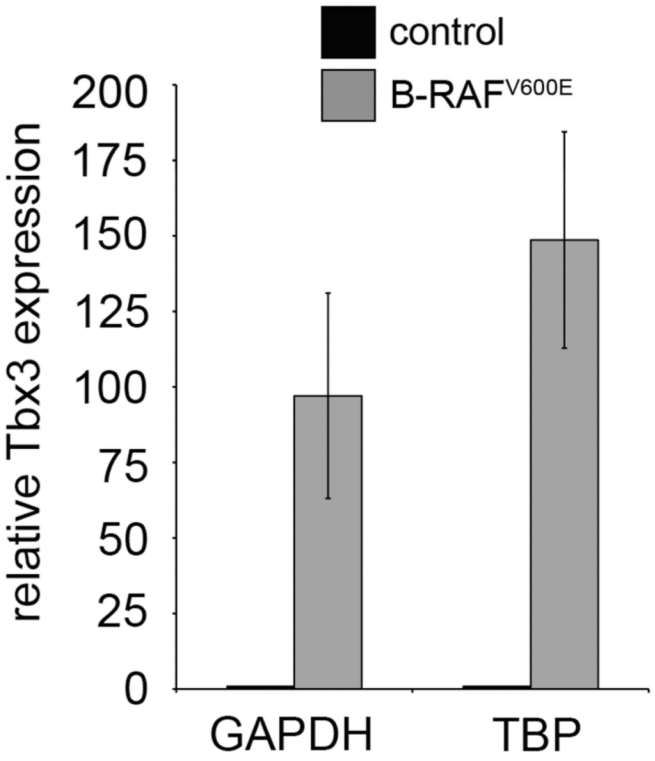

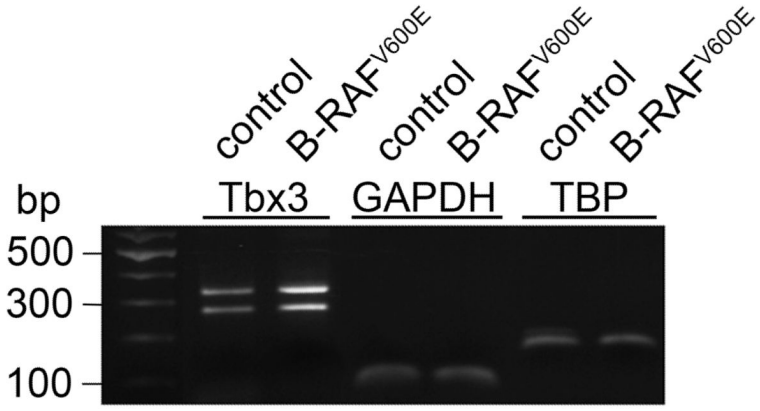

Figure 3. B-RAF^{V600E} regulates Tbx3 expression in melanocytes

Primary human melanocytes were infected with lentiviruses encoding B-RAF^{V600E} with copGFP or copGFP alone (control) for three days.

(a) Transcript expression levels of established E-cadherin transcriptional regulators in B-RAF^{V600E}-transduced melanocytes measured in gene expression arrays. Transcript levels are expressed relative to control transduced melanocytes (indicated by grey line).

(b) Total RNA derived from a minimum of three independent transduction experiments were analysed by qRT-PCR. Tbx3 transcript levels in B-RAF^{V600E}-transduced melanocytes were normalised to levels of GAPDH or TBP housekeeping genes and expressed relative to normalised Tbx3 transcript levels in control-transduced cells.

(c) Tbx3 cDNA derived from B-RAF^{V600E} or control-transduced melanocytes was amplified for 25 PCR cycles and products analysed using agarose gel electrophoresis.

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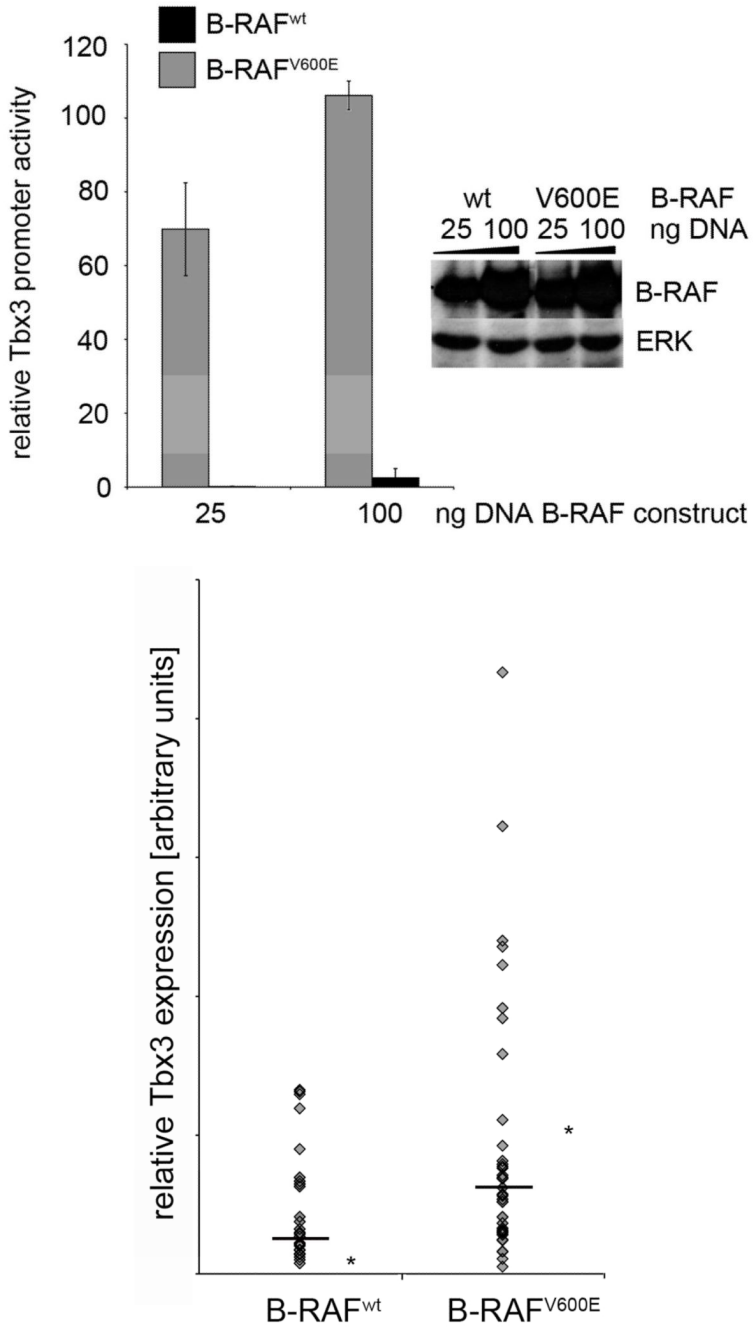

Figure 4. B-RAF^{V600E} regulates Tbx3 expression in melanoma

(a) 501mel melanoma cells were transfected with the human Tbx3 promoter (−249 to +168 in pGL3basic) or the pGL3 basic vector alone together with 25 or 100 ng of B-RAF wild type or V600E expression plasmid. Promoter activity was derived form the measured luciferase activity normalized to the promoter activity of pGL3 basic transfected cells. Immunoblotting confirmed similar expression of the Myc epitope-tagged B-RAF constructs using anti-Myc antibody.

(b) Total RNA from stage III melanoma lymph node metastasis was analysed by gene expression arrays. The relative median Tbx3 transcript expression levels are shown (n=27 B-RAF wild type (wt) tumors and n=33 tumors expressing B-RAF^{V600E}). For comparison

expression levels of Tbx3 in cultured normal melanocytes transduced to express control or B-RAF^{V600E} are presented (black asterisk).

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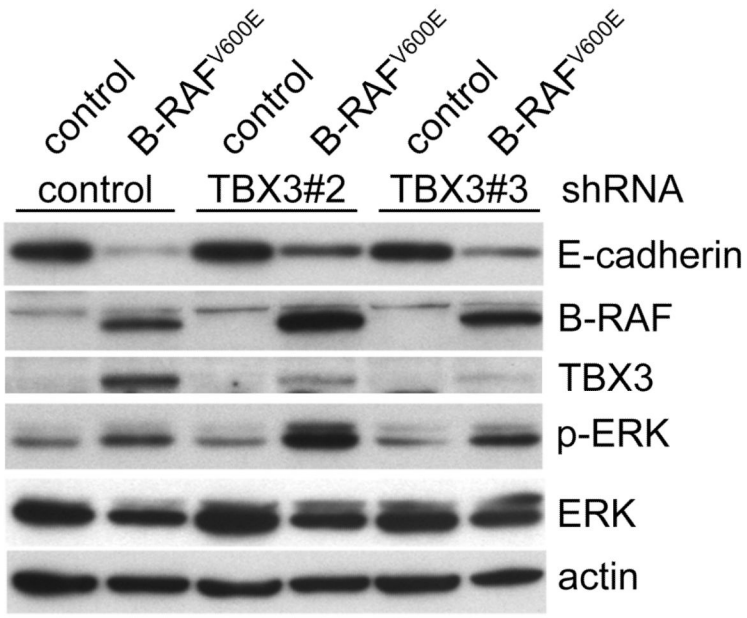

Figure 5. B-RAF mediates regulation of E-cadherin via Tbx3

Melanocytes were transduced with lentiviruses containing the indicated shRNA constructs. Five days post infection the cells were re-transduced with copGFP control lentivirus or lentiviruses expressing B-RAF^{V600E} or copGFP, as shown. Total protein was immunoblotted for the indicated proteins. This figure is compiled from duplicate immunoblots.

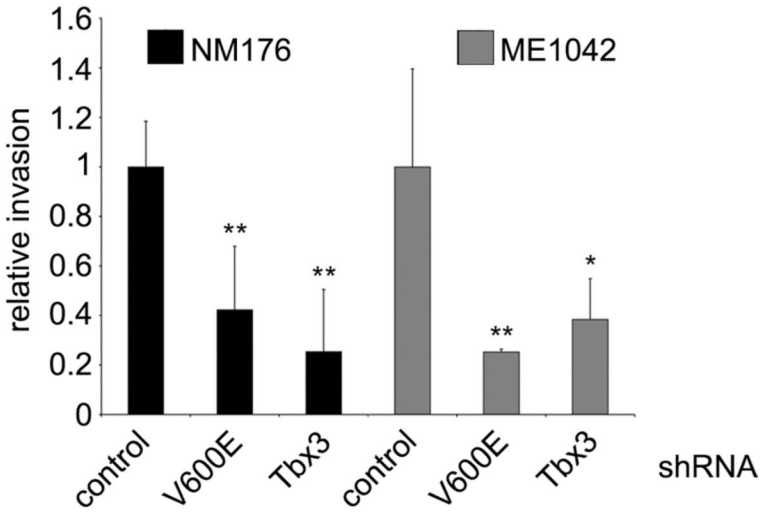

Figure 6. B-RAF^{V600E} promotes melanoma cell invasion

The V600E B-RAF mutant allele or Tbx3 were specifically silenced in the NM176 and ME1042 melanoma cell lines for five days. The invasion of melanoma cells was determined by transwell matrigel invasion assays and is presented as relative invasion compared to control silenced melanoma cells. Knockdown of BRAF^{V600E} and Tbx3 induced a significant (**$p<0.05$) or near significant (*$p=0.05$) decrease in cell invasion (p-values: NM176 V600E: 0.003, Tbx3: 0.0007; ME1042 V600E: 0.04, Tbx3: 0.05).

Table 1

Fold gene expression changes in melanocytes (V600E/control) presented in common gene ontology groups

Gene Symbol	Protein Name	Fold change (V600E+/control)
Focal Adhesion		
Jun	Transcription factor AP-1 subunit Jun	10.7±1
JunB	Transcription factor AP-1 subunit jun-B	9.6±0.8
VEGFA	Vascular endothelial growth factor A	7.1±0.7
VEGFC	Vascular endothelial growth factor C	5.0±0.1
MAPK8IP3	C-Jun-amino-terminal kinase-interacting protein 3	4.9±1.3
ITGA5	Integrin alpha-5	4.2±0.9
GSK3B	Glycogen synthase kinase-3 beta	3.5±0.7
ZYX	Zyxin	3.1±0.4
ACTN4	Alpha-actinin-4	3.0±0.6
ITGB5	Integrin beta-5	2.9±0.3
BCAR1	Breast cancer anti-estrogen resistance protein 1	2.7±0.1
MAP2K1	Dual specificity mitogen-activated protein kinase kinase 1	2.5±0.3
MAPK1	Mitogen-activated protein kinase 1	2.4±0.2
SRC	Proto-oncogene tyrosine-protein kinase Src	2.3±0.5
ITGA3	Integrin alpha-3	2.3±0.5
CRKL	Crk-like protein	2.0±0.0
TLN1	Talin-1	2.0±0.2
ACTN1	Alpha-actinin-1	1.7±0.3
ITGB1	Integrin beta-1	1.5±0.3
PTEN	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN	0.4±0.1
PIK3R2	Phosphatidylinositol 3-kinase regulatory subunit beta	0.4±0.1
PRKCA	Protein kinase C alpha type	0.4±0.1
VEGFB	Vascular endothelial growth factor B	0.4±0.1
PRKCB1	Protein kinase C beta type	0.3±0.0
COL5A2	Collagen alpha-2(V) chain	0.3±0.1
PPP1CB	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	0.3±0.1
Regulation of actin cytoskeleton/ cytoskeleton remodeling		
PLAUR	Urokinase plasminogen activator surface receptor	14.6±0.2
EZR	Ezrin	8.1±0.0
PLAT	Tissue-type plasminogen activator	7.7±0.4
INSIG1	Insulin-induced gene 1 protein	5.4±0.3
RRAS2	Ras-related protein R-Ras2	3.7±1.4
PAK3	Serine/threonine-protein kinase PAK 3	3.4±0.1
MSN	Moesin	2.1±0.3

Gene Symbol	Protein Name	Fold change (V600E+/control)
ACTB	beta cytoskeletal actin	0.7±0.2
ACTA2	alpha-actin-2	0.5±0.1
CYFIP2	Cytoplasmic FMR1-interacting protein 2	0.5±0.0
WASF3	Wiskott-Aldrich syndrome protein family member 3	0.4±0.1
MYH10	Myosin-10	0.3±0.0
Adherens Junctions		
PVRL2	Poliovirus receptor-related protein 2	2.6±0.3
FERMT3	Fermitin family homolog 3	2.1±0.1
MET	Hepatocyte growth factor receptor	0.5±0.0
SNAI2	Zinc finger protein SNAI2 (SLUG)	0.3±0.0
CDH1	Cadherin-1 (E-cadherin)	0.3±0.1
METTL9	Methyltransferase-like protein 9	0.2±0.0
Cell adhesion molecules		
CD82	CD82 molecule	4.9±0.4
CD8A	T-cell surface glycoprotein CD8 alpha chain	4.1±0.2
F11R	Junctional adhesion molecule A	3.8±0.1
MPZL1	Myelin protein zero-like protein 1	3.3±0.6
HLA-F	HLA class I histocompatibility antigen, alpha chain F	2.8±0.1
HLA-B	HLA class I histocompatibility antigen, B-7 alpha chain	2.6±0.1
PVR	Poliovirus receptor	2.6±0.3
HLA-A	HLA class I histocompatibility antigen, A-1 alpha chain	2.5±0.4
HLA-G	HLA class I histocompatibility antigen, alpha chain G	2.4±0.1
CD151	Membrane glycoprotein SFA-1	2.2±0.2
MAGED4	Melanoma-associated antigen	0.4±0.1
CD47	Leukocyte surface antigen CD47	0.3±0.0
ECM Remodelling		
IL8	Interleukin-8	39.9±13.2
HBEGF	Heparin-binding EGF-like growth factor	17.0±0.7
Il6	Interleukin-6	9.2±0.4
TIMP3	Metalloproteinase inhibitor 3	4.6±1.6
IGFBP4	Insulin-like growth factor-binding protein 4	3.2±1.2
KLK2	Kallikrein-2	3.0±0.5
TIMP1	Metalloproteinase inhibitor 1	2.2±0.1
MMP12	Macrophage metalloelastase	0.4±0.0
TIMP2	Metalloproteinase inhibitor	2 0.3±0.1
Epithelial Mesenchymal Transition		
PTGS2	prostaglandin-endoperoxide synthase 2	56.2±4.3

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Gene Symbol	Protein Name	Fold change (V600E+/control)
FOSB	oncogene FOS-B	45.8±1.9
JUN	Transcription factor AP-1 subunit Jun	10.7±1.0
RPS6KA1	ribosomal protein S6 kinase	10±1.1
JunB	Transcription factor AP-1 subunit jun-B	9.6±0.8
EGR1	early growth response protein 1	7.7±2.2
CTGF	insulin-like growth factor-binding protein 8	6.3±0.3
FOSL1	FOS-like antigen 1	6.0±1.2
FOS	cellular oncogene c-fos	5.0±0.5
CD8A	T-cell surface glycoprotein CD8 alpha chain	4.1±0.2
TNFRSF1A	tumor necrosis factor receptor type 1	3.9±0.1
MAP2K3	dual specificity mitogen-activated protein kinase kinase 3	3.8±1.4
AXIN1	axis inhibitor 1	3.5±0.0
GSK3B	Glycogen synthase kinase-3 beta	3.5±0.7
RPS6KA2	ribosomal protein S6 kinase	3.5±0.7
RPS6KA3	ribosomal protein S6 kinase	3.0±0.2
MAP2K1	mitogen-activated protein kinase kinase 1	2.5±0.3
MAPK1	mitogen-activated protein kinase 1	2.4±0.2
SRC	Proto-oncogene tyrosine-protein kinase Src	2.3±0.5
NES	nestin	2.2±0.4
ACVR1	activin A receptor, type I	1.8±0.2
ITGB1	Integrin beta-1	1.5±0.3
ACTB	beta cytoskeletal actin	0.7±0.2
CREB1	cAMP responsive element binding protein 1	0.6±0.1
ACTA2	alpha-actin-2	0.5±0.1
STAT1	signal transducer and activator of transcription 1	0.5±0.1
MET	Hepatocyte growth factor receptor	0.5±0.0
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1	0.4±0.1
PIK3R2	phosphoinositide-3-kinase, regulatory subunit 2	0.4±0.1
CALD1	caldesmon 1	0.4±0.2
JAG1	jagged 1	0.3±0.1
CDH1	Cadherin-1 (E-cadherin)	0.3±0.1
SNAI2	Zinc finger protein SNAI2 (SLUG)	0.3±0.0
ZEB2	zinc finger E-box binding homeobox 2	0.2±0.0

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Table 2

Expression changes in excised human melanoma stage III (lymph node) metastases according to B-RAF-status

	Wild type		B-RAF^{V600E}		p-value
Transcript	**Median**	**LQ/UQ**	**Median**	**LQ/UQ**	
CDH-1	930.9	29/9240.7	704.2	72.2/7004.6	1.0
Tbx3	144.5	38.1/662.7	310.4	25.8/2166.4	0.01

LQ: lower quartile, UQ upper quartile

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