

**KIT<sup>D816V</sup> induces SRC-mediated tyrosine phosphorylation of MITF and  
altered transcription program in melanoma**

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## **Abstract**

The oncogenic D816V mutation of the KIT receptor is well characterized in systemic mastocytosis and acute myeloid leukemia. Although KIT<sup>D816V</sup> has been found in melanoma, its function and involvement in this malignancy is not understood. Here we show that KIT<sup>D816V</sup> induces tyrosine phosphorylation of MITF through a triple protein complex formation between KIT, MITF and SRC family kinases. In turn, phosphorylated MITF activates target genes that are involved in melanoma proliferation, cell cycle progression, suppression of senescence, survival and invasion. By blocking the triple protein complex formation, thus preventing MITF phosphorylation, the cells became hypersensitive to SRC inhibitors. We have therefore delineated a mechanism behind the oncogenic effects of KIT<sup>D816V</sup> in melanoma and provided a rationale for the heightened SRC inhibitor sensitivity in KITD816V transformed cells.

Implications: This study demonstrates that an oncogenic tyrosine kinase mutant, KIT-D816V, can alter the transcriptional program of the transcription factor MITF in melanoma

## Introduction

The receptor tyrosine kinase KIT is important for melanocyte and mast cell development. Upon binding of the KIT ligand (also known as Stem Cell Factor, SCF), KIT auto-phosphorylates its intracellular domain leading to recruitment of signaling proteins to the receptor and activation of downstream signaling pathways. Mutations in certain residues of KIT lead to a constitutively active and ligand-independent receptor and are found in several types of cancer including gastrointestinal stromal tumors, testicular seminomas, systemic mastocytosis, acute myeloid leukemia and melanoma (1). Previously, it was believed that the loss of KIT expression facilitated melanomagenesis and increased metastasis (2)(3). However, more recently The Cancer Genome Atlas Network identified *KIT* mutations and focal amplifications as major element of the melanoma subtype, triple wild-type (4). Moreover, using imatinib to target melanoma harboring *KIT* alterations in clinical trials shows promising results (5-7).

The D816V KIT mutation is well characterized in systemic mastocytosis and acute myeloid leukemia. In contrast, although  $KIT^{D816V}$  was identified in melanoma many years ago it remains uncharacterized (8). The  $KIT^{D816V}$  receptor shows a pattern of intracellular localization compared to wild-type KIT (9, 10). Interestingly, it has been shown that  $KIT^{D816V}$  is able to generate oncogenic signaling without the need to reach the cell membrane (10). Antibody staining of KIT in mucosal melanoma tissue samples carrying the D816V mutation showed intracellular localization [4]. Moreover,  $KIT^{D816V}$  aberrantly activates oncogenic signaling pathways partially by virtue of its altered substrate specificity (11). The nuclear transcription factors STAT-1, STAT-3 and STAT-5 are recruited by  $KIT^{D816V}$  to the

cytoplasm to amplify oncogenic signaling in leukemic cells. In this case the activation of the survival factor AKT is crucial for cellular transformation (12, 13).

Wild-type KIT has been shown to signal to the melanocyte master regulator microphthalmia-associated transcription factor (MITF) that is known to control critical melanoma functions including metabolism and resistance to MAP kinase pathway inhibition (14, 15). Wild-type KIT engages the MAP kinase pathway leading to MITF phosphorylation at S73 and S409, thus affecting the transcription activation potential of MITF (16). However, *in vivo* studies have shown that these phosphorylation events are not important for coat color and eye development in mice (17), suggesting a more complex role of MITF phosphorylation *in vivo*. Like KIT, MITF is an essential mediator of melanocyte and mast cell development(18) and its involvement in melanomagenesis is well established (reviewed in (19)). Interestingly, in transformed mast cells, KIT<sup>D816V</sup> has been shown to upregulate MITF protein levels, resulting in abnormal mast cell proliferation and malignant mast cell disease (20). Collectively, these data suggest that MITF might be an important target of KIT<sup>D816V</sup> in melanoma.

Here we show that a triple protein complex formation between KIT<sup>D816V</sup>, MITF and SRC family kinases results in tyrosine phosphorylation of MITF and subsequent effects on MITF-dependent gene expression. Phosphorylation of MITF was shown to be a crucial event mediating the oncogenic effects of KIT<sup>D816V</sup>. Thus, we have defined a major oncogenic pathway involving MITF tyrosine phosphorylation in KIT<sup>D816V</sup>-positive melanoma cells. More importantly, our findings suggest KIT<sup>D816V</sup>-transformed melanoma cells to be sensitive to Src family kinase inhibitors.

## **Materials and methods**

### **Cell culture**

The HEK293T cells (Thermo Scientific Open Biosystems), B16F0 melanoma cells (LGC Standards AB) and A375 melanoma cells (ATCC) and 501 mel cells were cultured according to manufacturers' recommendations. All cell lines were obtained between 2009 and 2013 and routinely tested negative for Mycoplasma using the MycoAlert mycoplasma detection kit (Lonza). Cells were cultured for less than 2 months in our laboratory. Cell line authentication was not routinely performed.

### **Proliferation assay**

B16F0 and A375 melanoma cells stably transfected (through G418 selection, 900 µg and 600 µg, respectively, after transfection) with empty CMV vector or KIT<sup>D816V</sup> were plated (5000 cells/ well) in 96-well plates and assayed with PrestoBlue cell viability reagent (Life Technologies).

### **Survival assay**

The number of live B16F0 or A375 cells were determined using PrestoBlue (see previous section) 48 h after plating (5000 cells/ well in 96-well plates). Cells were treated with SU6656 (Sigma Aldrich) or dasatinib (Shanghai Yingxuan) at the following concentrations: 10 nM, 50 nM, 70 nM, 150 nM, and 200 nM. As a control, cells were also treated with DMSO in a volume equivalent to the drug used (never exceeding 0.1 % of the total volume). CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used to determine vemurafenib (Selleckchem) dose escalation (72 h) assay.

## Mutagenesis and constructs

The QuikChange mutagenesis kit (Stratagene) was used, according to the manufacturer's recommendations, to generate tyrosine-to-phenylalanine substitution mutants of MITF. The following primers, designed at [bioinformatics.org/primerx](http://bioinformatics.org/primerx), were used for this reaction:

(MITF Y22F) Forward- 5' CCCCACCAAGTTCCACATACAGCAAGC 3'

(MITF Y22F) Reverse- 5' GCTTGCTGTATGTGGAAGTTGGTGGGG 3'

(MITF Y35F) Forward- 5' GCACCAGGTAAAGCAGTTCCTTTCTACCAC 3'

(MITF Y35F) Reverse- 5' GTGGTAGAAAGGAACTGCTTTACCTGGTGC 3'

(MITF Y90F) Forward- 5' CTGTGAAAAAGAGGCATTTTTTAAGTTTGAGGAGCAGAGC 3'

<b>(MITF</b>	<b>Y90F)</b>	<b>Reverse-</b>	<b>5'</b>
<b>GCTCTGCTCCTCAAAGTTAAAAAATGCCTCTTTTTTCACAG 3'</b>			

Isolated gel (SDS-PAGE) bands were minced, destained, and subjected to in-gel digestion by trypsin (sequencing grade; Promega) as previously described (21). The resulting peptides were analyzed by online reversed-phase C18 nanoscale liquid chromatography (LC) tandem mass spectrometry (MS). The experiments were performed on an EASY-nLC™ system (Proxeon Biosystems) connected to a LTQ-Orbitrap Velos (Thermo Electron, Bremen, Germany) through a nano-electrospray ion source, as described previously (22). The peptides were separated by a linear gradient of increasing acetonitrile in 0.5 % acetic acid. The mass spectrometer was operated in data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. Survey full-scan MS spectra were acquired in the orbitrap with resolution of 70,000 after accumulation to a target value of 1e6 in the linear ion trap. The ten most intense peptide ions with charge states 2 were sequentially isolated to a target value of 5e4 and fragmented with higher-energy collisional dissociation in the octopole collision cell and analyzed in the orbitrap with a resolution of 7,500. All LC-MS/MS files were processed with the MaxQuant software suite, as described (23). Tandem mass spectra were initially

matched with a mass tolerance of 7 ppm on precursor masses and 0.02 Da for fragment ions, strict trypsin specificity and allowing for up to three missed tryptic cleavage sites. Cysteine carbamidomethylation (Cys +57.021464 Da) was searched as a fixed modification, whereas N-acetylation of protein (N-term +42.010565 Da), N-pyro-glutamine (Gln -17.026549), oxidized methionine (+15.994915 Da) and phosphorylation of serine, threonine and tyrosine (Ser/Thr/Tyr +79.966331 Da) were searched as variable modifications. Identified peptides were filtered to an estimated false discovery rate < 0.01.

### **Antibody generation, immunoblotting, immunoprecipitation and protein expression quantitation**

The phospho-specific antibodies against MITF were generated by immunizing rabbits with synthetic peptides (JPT Peptide Technology, Berlin Germany) with the following sequences; CLENPTK-pY-HIQQAQR, CRHQVKQ-pY-LSTTLA and CEKEAF-pY-KFEEQS corresponding to mouse MITF-M phosphorylated tyrosine 22, 35 and 90, respectively. The resulting antibodies were purified with affinity chromatography. Immunoblotting and immunoprecipitation was performed as described previously (24).

Transfection was performed using JetPEI (PolyPlus) transfection reagent according to manufacturer's recommendation with mouse MITF-M in P3XFLAG-CMV-14 and either KIT wild-type or KIT<sup>D816V</sup> mutant. Anti-FLAG antibodies (Sigma-Aldrich) were used for immunoprecipitation and protein G beads (Dynabeads, Life Technology) were added to pull down bound proteins. In addition, protein G Sepharose beads from GE Healthcare were employed as an extra control in co-immunoprecipitation experiments. Relative protein expression was quantified with ImageJ ([Schneider et al 2012](#)) using the optical density signals obtained from western blotting.

Co-immunoprecipitation of protein in the SFKs was performed in the lysate of transfected HEK293T cells using either anti-FLAG antibodies (Sigma-Aldrich), anti-c-Myc (clone 9E10, Roche), anti-V5 antibody (Sigma-Aldrich)) to immunoprecipitate the respective SFK. The anti-FLAG antibodies were then used for immunoblotting to detect MITF-FLAG. Co-immunoprecipitation of endogenous SRC was carried out by adding anti-v-SRC antibodies (Ab-1, Calbiochem) to A375 cell lysate and processed as described above.

## Results

### **KIT<sup>D816V</sup> mediates tyrosine phosphorylation of MITF**

We investigated the phosphorylation state of MITF in cells expressing KIT<sup>D816V</sup>. To do this, we co-expressed KIT<sup>D816V</sup> and FLAG-tagged mouse MITF in HEK293T cells and used high-resolution orbitrap tandem mass spectrometry (MS) to identify phosphorylation sites on the MITF protein after immunoprecipitation using anti-FLAG antibody. This resulted in identification of three tryptic phosphopeptides derived from the N-terminal part of MITF that were tyrosine phosphorylated in the presence of KIT<sup>D816V</sup> on Y22, Y35 and Y90, respectively (Supplementary Figure 1). Multiple alignment of MITF proteins from different species, including vertebrates and invertebrates, revealed that all three tyrosine sites are highly conserved and likely to have functional impact on MITF; the domain containing Y90 is missing from the zebrafish and Drosophila MITF genes (Supplementary Figure 2).

To investigate if phosphorylation is induced by KIT<sup>D816V</sup> kinase activity and not by wild-type KIT, we overexpressed a FLAG-tagged MITF construct together with either wild-type KIT or KIT<sup>D816V</sup> in B16F0 mouse melanoma cells. The MITF protein was isolated by immunoprecipitation of FLAG-tagged MITF and western blots performed with the 4G10 anti-phosphotyrosine antibody. The results showed that KIT<sup>D816V</sup> induced tyrosine phosphorylation of MITF (Figure 1a, top panel, lane 1) whereas no phosphorylation was



observed upon activation of wild-type KIT with KIT ligand (KITLG) (Figure 1a). KIT phosphorylation was also assessed by immunoprecipitating KIT and staining with the 4G10 antibody. This showed that KIT<sup>D816V</sup> was tyrosine phosphorylated similar to KITLG stimulated wild-type KIT (Figure 1a).

To further characterize these tyrosine phosphorylation sites, we generated and affinity purified phosphospecific antibodies against the phosphorylated Y22, Y35 and Y90 tyrosine residues of MITF. The antibodies only recognized the MITF protein when the respective sites were phosphorylated and not when they were mutated to a non-phosphorylatable phenylalanine, thus demonstrating their specificity (Figure 1b). More importantly, these tyrosine residues were only phosphorylated in the presence of KIT<sup>D816V</sup> and not in the presence of wild-type KIT. For example, using the pY22 antibodies, phosphorylation of Y22 of MITF was only detected in the presence of KIT<sup>D816V</sup> and only when this residue was tyrosine, never when it was mutated to phenylalanine (Y22F; Figure 1b). Similarly, the phosphospecific pY35 and pY90 MITF antibodies only recognized MITF in the presence of KIT<sup>D816V</sup> when the respective residues were tyrosine and not when they were mutated to phenylalanine (Figure 1b). These results were observed in B16F0 melanoma cells (Figure 2b) as well as in HEK293T cells and A375 human malignant melanoma cells (Supplementary Figure 3).

### **SRC family kinases mediate MITF tyrosine phosphorylation**

The SRC family kinases (SFKs) are non-receptor tyrosine kinases and prominent downstream signaling components of KIT (1). The KIT phosphorylation sites Y568 and Y570 are crucial for SFK binding and activation. To determine if SFKs are involved in KIT<sup>D816V</sup>-induced tyrosine phosphorylation of MITF, B16F0 melanoma cells were transfected with KIT<sup>D816V</sup> or KIT<sup>D816V, Y568/570F</sup> that lacks the SFK binding sites, together with FLAG-tagged MITF and tyrosine phosphorylation determined using the 4G10 antibodies. Our results show that

tyrosine phosphorylation of MITF was triggered by KIT<sup>D816V</sup> but not by KIT<sup>D816V, Y568/570F</sup> (Figure 1c). Furthermore, treating the cells with the SFK inhibitor SU6656, with or without KITLG, blocked the ability of KIT<sup>D816V</sup> to mediate MITF tyrosine phosphorylation (Figure 1c). These experiments suggest that SFKs are involved in mediating the signals from KIT<sup>D816V</sup> to MITF.

### **KIT<sup>D816V</sup> and MITF interact through direct protein association that is dependent on SFKs**

Interestingly, when A375 melanoma cells were transfected simultaneously with FLAG-tagged MITF and KIT<sup>D816V</sup> expression constructs, the KIT<sup>D816V</sup> protein was co-immunoprecipitated with MITF, suggesting direct interactions between these proteins (Figure 2a). However, the SFK non-binding KIT<sup>D816V, Y568/570F</sup> mutant did not co-immunoprecipitate with MITF (Figure 2a), suggesting that KIT interaction with SFKs is required for the binding of MITF to the KIT<sup>D816V</sup> protein. To further verify this, we used the same approach and immunoprecipitated the endogenous SRC protein and showed that MITF co-immunoprecipitated with SRC but only in the presence of KIT<sup>D816V</sup> and not KIT<sup>D816V, Y568/570F</sup> (Figure 2b). Moreover, the level of MITF co-immunoprecipitation was severely decreased in cells treated with the SFK inhibitor SU6656 (Figure 2b). This suggests direct binding between KIT<sup>D816V</sup>, MITF and SFKs.

In order to determine which SFK was involved in this interaction, we transiently transfected HEK293T cells with the different SFKs including BLK, BRK, SRC, FGR, FYN, HCK, LCK, LYN and RAK, and showed that all these kinases were able to bind to MITF in the KIT<sup>D816V</sup> transfected samples (Supplementary Figure 4). The binding was severely reduced in the presence of SU6656 or KIT<sup>D816V, Y568/570F</sup> (Supplementary Figure 4). This suggests that oncogenic KIT<sup>D816V</sup> interacts directly with MITF and that any of the above SFKs are needed for this association. The results also suggest that the binding between the SFKs and KIT depends on the kinase activity of SFKs.

### **Tyrosine phosphorylation affects nuclear localization of MITF**

Previous reports have shown that wild-type KIT does not influence the nuclear localization of MITF (25). However, as the SFK and KIT<sup>D816V</sup> proteins are located in the cytosol and since they mediate MITF phosphorylation, we investigated the subcellular localization of MITF. B16F0 melanoma cells were transfected with either wild-type KIT or KIT<sup>D816V</sup> together with FLAG-tagged wild-type MITF or MITF containing the different combinations of the Y22/35/90F mutations. The cells were then stained with PE-conjugated anti-KIT and Alexa Fluor 647-conjugated anti-FLAG antibodies against MITF-FLAG. Consistent with previous reports in melanoma cells (25), our results show that the wild-type melanocyte specific MITF-M isoform was exclusively localized to the nucleus (Figure 3a). The presence of wild-type KIT did not affect the localization of MITF (Figure 3a and Supplementary Figure 5). However, when co-expressed with KIT<sup>D816V</sup>, a large proportion of the wild-type MITF protein was localized to the cytosol as well as in the nucleus (Figure 3a). Similarly, when the Y22, Y35 and Y90 phosphorylation sites of MITF were mutated singly or in double mutant combinations, the MITF protein was localized to both the nucleus and the cytosol when co-expressed with KIT<sup>D816V</sup> but was nuclear in presence of wild-type KIT (Fig. 3a and Supplementary Figure 5). However, the MITF<sup>Y22/35/90F</sup> triple mutant showed exclusive nuclear localization, indicating that all three tyrosine phosphorylation sites are important for the KIT<sup>D816V</sup>-mediated cytoplasmic retention of MITF (Figure 3a and Supplementary Figure 5).

To further confirm that a proportion of MITF is retained in the cytosol in the presence of KIT<sup>D816V</sup>, we fractionated transfected B16F0 melanoma cells into a cytosolic and a nuclear portion. Co-expression with KIT<sup>D816V</sup> resulted in elevated levels of MITF protein in both the cytosolic and nuclear fractions, whereas wild-type KIT did not affect the level of MITF and the protein was only found in the nucleus (Figure 3b). These results confirm our confocal

microscopy findings that only KIT<sup>D816V</sup> and not wild-type KIT can mediate cytosolic localization of MITF.

In order to show that endogenous MITF also partially localizes to the cytosol in the presence of KIT<sup>D816V</sup>, we stained for the endogenous MITF protein in 501Mel melanoma cells transfected with either wild-type KIT or KIT<sup>D816V</sup>. This showed that MITF was located exclusively in the nucleus (Figure 3c) whereas in the KIT<sup>D816V</sup> transfected cells, MITF was localized both in the nucleus and the cytosol (Figure 3c). Our results indicate that the endogenous MITF protein is partially retained in the cytosol in the presence of KIT<sup>D816V</sup>, presumably because of its interaction with KIT and SFKs.

### **KIT<sup>D816V</sup> differentially affects MITF targets genes**

To determine whether KIT<sup>D816V</sup> affects MITF-mediated target gene expression, we performed qPCR analysis of MITF target genes (see Supplementary Table 1). SiRNA against MITF (siMITF) in B16F0 cells resulted in knockdown of endogenous MITF expression to around 40 % compared with cells treated with negative control siRNA (Figure 4a sample 4). The knockdown also reduced expression of a number of genes confirming that these genes are indeed MITF targets (Figure 4b, sample 4, Supplementary Figure 6a and Supplementary Table 1). B16F0 cells stably transfected with KIT<sup>D816V</sup> negatively affected the expression of genes involved in melanoma/melanocyte differentiation, cell cycle inhibition and tumor suppression (gene names indicated in green), whereas expression of genes involved in melanoma proliferation, survival, angiogenesis and cell cycle initiation (gene names in red) was increased (Figure 4b, sample 2 and Supplementary Figure 6b). The addition of siMITF reduced the overall effects of KIT<sup>D816V</sup> on the melanoma genes, indicating that the effects observed were mediated by MITF (Figure 4b, sample 4 and Supplementary Figure 6c).

The addition of human KITLG to cells stably expressing KIT<sup>D816V</sup> did not alter the gene expression pattern observed with KIT<sup>D816V</sup> without treatment (Supplementary Table 1). In addition, mouse KITLG treatment that activates endogenous KIT resulted in increased expression of melanocyte differentiation genes. In summary, KIT<sup>D816V</sup>, via MITF, negatively affects the expression of genes controlling melanocyte differentiation, cell cycle inhibition and tumor suppression, and positively regulates the expression of genes involved in proliferation, survival, angiogenesis and cell cycle initiation.

**The effects of KIT<sup>D816V</sup> on MITF gene regulation depend on tyrosine phosphorylation**

To determine if MITF tyrosine phosphorylation is involved in the KIT<sup>D816V</sup>-mediated effects on gene regulation, we repeated the gene expression analysis after transfecting the B16F0 cells with wild-type and mutant MITF constructs. Transfection of the different MITF constructs led to an increase in MITF mRNA levels (Figure 4c) (for all data values see Supplementary Table 2). Transient transfection of wild-type MITF also increased expression of all the target genes as compared to control cells transfected with empty vector (Figure 4d, sample 4 and Supplementary Figure 6d).

Transfection of the triple MITF<sup>Y22F/Y35F/Y90F</sup> mutant construct in the absence of KIT<sup>D816V</sup> had similar effects on gene expression as wild-type MITF, suggesting that the transcription activation function of the triple mutant is similar to wild-type MITF (Figure 4d, sample 5 and Supplementary Figure 6e). Transfection of wild-type MITF in cells stably expressing KIT<sup>D816V</sup> increased expression of genes involved in survival, proliferation, angiogenesis and cell cycle activation (indicated in red), whereas genes controlling differentiation, tumor suppression and cell cycle inhibition were repressed (indicated in green in Figure 4d, sample 2 and Supplementary Figure 6). However, KIT<sup>D816V</sup> expressing cells transfected with the triple MITF<sup>Y22/35/90F</sup> mutant resulted in overall enhanced expression of all genes and did not

selectively affect a specific class of genes (Figure 4d, sample 3). Importantly, it did not lead to an increase in expression of genes responsible for melanoma survival or proliferation to the same level as wild-type MITF in the presence of KIT<sup>D816V</sup> (Figure 4d, sample 3 and Supplementary Figure 6f). Indeed, the gene expression profile of the MITF<sup>Y22/35/90F</sup> triple mutant in the presence of KIT<sup>D816V</sup> was comparable to cells transfected with only wild-type MITF (Figure 4d, samples 3 and 4 and Supplementary Figure 6g). Thus, the results indicate that KIT<sup>D816V</sup> signaling requires intact tyrosine phosphorylation sites of MITF in order to mediate the differential effects on gene expression.

The addition of human KITLG to cells stably expressing KIT<sup>D816V</sup> showed the same gene expression pattern as cells without treatment (Supplementary table 2). In addition, mouse KITLG treatment that activated endogenous KIT resulted in increased expression of melanocyte differentiation genes.

Hierarchical clustering analysis revealed that the two main clusters of genes tested were differentially affected by the presence of KIT<sup>D816V</sup>. The first cluster of genes is involved in tumor suppression, differentiation and cell cycle inhibition (gene names indicated in green in Figure 4b and d) whereas the second cluster drives melanoma proliferation, survival, angiogenesis and cell cycle initiation (gene names indicated in red). Moreover, KIT<sup>D816V</sup> transfected samples clustered into separate groups (Figure 4b), illustrating that oncogenic KIT has different effects on MITF-dependent genes than wild-type KIT. Our results suggest that the effects of KIT<sup>D816V</sup> on gene regulation depend on tyrosine phosphorylation of MITF.

#### **Effects of KIT<sup>D816V</sup> on melanoma cell proliferation depend on MITF phosphorylation**

The above results suggest that KIT<sup>D816V</sup> signaling leads to MITF tyrosine phosphorylation which then has differential effects on the expression of MITF-target genes. In order to determine if this affects cell function, we measured the proliferation of B16F0 and A375

melanoma cells stably transfected with KIT<sup>D816V</sup> over three days. The proliferation assay showed that KIT<sup>D816V</sup> expressing cells proliferated more than two fold faster than non-transfected and CMV empty vector transfected cells (Figure 5a-b). Treatment with siMITF over 48-72 h suppressed proliferation of non-transfected, CMV transfected and KIT<sup>D816V</sup> transfected B16F0 and A375 melanoma cells (Figure 5a-b). This shows that MITF is necessary for proliferation of melanoma cells, including cells expressing KIT<sup>D816V</sup>.

Cells stably transfected with either KIT<sup>D816V</sup>, wild-type MITF or MITF<sup>Y22/35/90F</sup> alone exhibited a significantly enhanced proliferation rate compared with normal B16F0 and A375 cells (Figure 5c-d). Cells transfected with both KIT<sup>D816V</sup> and wild-type MITF resulted in the greatest proliferative effects. By contrast, cells co-transfected with MITF<sup>Y22/35/90F</sup> and KIT<sup>D816V</sup> showed similar proliferative ability as cells transfected with either construct alone (Figure 5c-d). This suggests that the Y22, Y35 and Y90 phosphorylation sites of MITF are essential for the KIT<sup>D816V</sup>-mediated effects on melanoma proliferation.

#### **KIT<sup>D816V</sup> transformed melanoma cells are sensitive to SRC family kinase inhibitors**

Our results demonstrate that MITF phosphorylation is dependent on the activity of both KIT<sup>D816V</sup> and SFKs. To determine if SFK inhibitors have an impact on the signaling between KIT<sup>D816V</sup> and MITF, and consequently on the biological function of melanoma cells, we treated B16F0 and A375 cells with the SFK inhibitors SU6656 and dasatinib. Cells stably transfected with KIT<sup>D816V</sup> and treated with DMSO, as a control condition, displayed increased viability compared to normal untransfected cells or cells transfected with an empty vector (Figure 6a-b). Treating normal and CMV transfected cells with increasing doses (10-200 nM) of SU6656 or dasatinib did not significantly decrease the number of living cells. However, the viability of KIT<sup>D816V</sup> transformed cells was significantly reduced by treatment with either SU6656 or dasatinib (Figure 6a-b). To study the effects of the BRAF<sup>V600E</sup> mutation that is present in the 501 mel and A375 cell lines, we treated these cells with vemurafenib.

Interestingly, the stably KIT<sup>D816V</sup> expressing cells were resistant to vemurafenib treatment (Figure 6c-d). However, cell viability was significantly reduced in the presence of dasatinib. As expected the wild-type KIT expressing cells were sensitive to vemurafenib and co-treatment with dasatinib did not yield any synergistic effects.

## Discussion

We have shown that KIT<sup>D816V</sup> forms a protein complex with MITF and any of the multiple members of the SRC family of kinases. As a result, MITF is tyrosine phosphorylated on at least three sites leading to differential effects on transcription such that proliferation and survival of melanoma cells is increased.

The presence of KIT<sup>D816V</sup> leads to increased expression of MITF target genes including *Tbx2*, *Bcl2*, *Sox10*, *Cdk2*, *Hif1a*, *p35*, and *Diaph1* all of which have been implicated in melanoma proliferation, cell cycle progression, suppression of senescence, survival and invasion. Interestingly, the presence of KIT<sup>D816V</sup> not only increases the expression of these genes, it also reduced expression of melanocytic marker genes such as *Tyr*, *Tyrp1*, *Dct* and *Mlana*. It has been reported that overexpression of MLANA is reduced during melanoma progression (26). MLANA is also observed to be downregulated by HIF1 $\alpha$  in melanoma cells that have switched to an invasive phenotype (27). Both of these reports fit with our study in that phosphorylated MITF increases HIF1 $\alpha$  expression whereas MLANA expression is reduced. Thus, it is tempting to speculate that the MITF-mediated suppression of *Mlana* is caused by the increase in *Hif1a* expression. Our proliferation assays showed that KIT<sup>D816V</sup>, via MITF phosphorylation, enhances melanoma proliferation and survival, lend further support to the conclusion that KIT<sup>D816V</sup> affects proliferation via MITF phosphorylation. It is not clear how the tyrosine phosphorylation of MITF differentially affects target gene expression. Perhaps



the phosphorylated MITF affects interactions with important gene-specific co-factors such that some targets are avoided or preferred.

The melanocyte-specific MITF-M isoform is localized to the nucleus in melanoma cells (28). However, alternative subcellular location of MITF has been reported to be regulated in some cell types. For example, in osteoclast precursors, MITF is cytosolic but translocates to the nucleus in response to treatment with colony stimulating factor-1 (CSF-1) and receptor-activator of nuclear factor- $\kappa$ B ligand (RANKL) (29, 30). These factors lead to signaling to MITF, thus disrupting the association of MITF with the signaling regulatory 14-3-3 complex, which keeps it in the cytosol (30). The 1B1b exon of MITF, which is present in all MITF isoforms except for the MITF-M isoform, has been postulated to regulate cytoplasmic shuttling (31). Recently, the TFEB and TFE3 transcription factors, which are closely related to MITF, have been shown to be important for regulating autophagy and lysosomal function. These proteins are phosphorylated by the mTOR pathway and maintained in the cytoplasm by interactions with the 14-3-3 complex in nutrient rich conditions. However, upon starvation, mTOR becomes inactive, the TFEB/TFE3 proteins are not phosphorylated and are therefore sent to the nucleus where they activate expression of lysosomal and autophagy genes (32). In addition, the subcellular localization of MITF has also been suggested to be regulated in a similar fashion in pancreatic cancer cells (33). Our results suggest that, in the presence of KIT<sup>D816V</sup>, MITF is at least partly cytoplasmic when it is tyrosine phosphorylated. Presumably the increased cytoplasmic presence of MITF is due to interactions with SFKs and KIT<sup>D816V</sup>. Unfortunately, our phospho-tyrosine antibodies against MITF cannot be used to determine whether the cytoplasmic fraction or nuclear fraction of MITF is phosphorylated. However, as the subcellular location of the Y22/35/90F MITF triple mutant was not affected by KIT<sup>D816V</sup> whereas that of wild-type MITF was, it seems likely that subcellular localization is affected

by tyrosine phosphorylation. Although it is possible that nuclear MITF is not influenced by KIT<sup>D816V</sup>, transcriptional activation of selected MITF targets was significantly reduced by mutating the tyrosine phosphorylation sites of MITF. This suggests that a major effect of the tyrosine phosphorylation is to modulate gene transcription such that only a certain set of genes are transcribed.

SFKs are important signaling components in many types of human tumors, where they regulate proliferation, invasion, angiogenesis and motility of cancer cells (34). Several investigators have reported increased SRC kinase activity in melanoma cells (35-37). Among the many pathways activated by SFKs are STAT3 and STAT5 that are important for melanoma cell proliferation and cell survival (35, 38, 39). SFKs have been suggested as promising pharmacological targets in melanoma (35, 36, 40). More specifically, BRAF inhibitor resistant melanoma tumors, in which increased SFK signaling is driven by the EGF receptor, are especially sensitive to dasatinib treatment. Treatment with dasatinib decreases both tumor growth and proliferation (41, 42). Although dasatinib was originally described to be a dual-specificity SRC/ABL inhibitor (43), it was later shown also to inhibit KIT, including KIT<sup>D816V</sup>. We have previously demonstrated that the D816V mutation in KIT leads similar kinase specificity of KIT as that of SRC (11). Indeed, we found that KIT<sup>D816V</sup> transformed melanoma cells were hypersensitive to SFK inhibitor treatment. Our results thus suggest that melanoma cells in which the KIT<sup>D816V</sup> mutation is present are sensitive to SRC inhibitors or dasatinib.

### **Conflict of interest**

The authors declare not conflicts of interest

## **Acknowledgements**

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## Figures

**Figure 1. KIT<sup>D816V</sup> induces tyrosine phosphorylation of MITF in a SFK-dependent manner.** (a) Western blots showing immunostaining with general tyrosine phosphorylation (pY (4G10)) antibodies after immunoprecipitation of the MITF-FLAG protein (using anti-FLAG antibodies) from B16F0 mouse melanoma cells transfected with MITF-FLAG together with either KIT or KIT<sup>D816V</sup>, with or without KITLG stimulation (50 ng/ ml). After the MITF immunoprecipitation the same samples were subjected to immunoprecipitation with KIT antibodies. Subsequently, anti-pY antibodies were used against immunoprecipitated KIT to determine receptor phosphorylation. The KIT and MITF proteins migrate as double bands on western blots whereas KIT<sup>D816V</sup> runs as a single band. Whole cell lysates (WCL) taken prior to immunoprecipitation and stained with anti-FLAG and KIT antibodies to determine protein levels. (b) Western blots showing immunostaining with antibodies developed against phosphorylated tyrosine residues 22, 35 and 90 of MITF. B16F0 cells were transfected with wild-type or mutant MITF-FLAG constructs, in which the indicated tyrosine residues were mutated to phenylalanine, with or without KIT<sup>D816V</sup>, lysed and the MITF-FLAG protein immunoprecipitated with anti-FLAG antibodies before western blot analysis. The blots were also stained with anti-FLAG antibodies in order to determine total protein levels. (c) Similar to a, but including SU6656 (SFK inhibitor) treatment and transfection with the KIT<sup>D816V</sup>, Y568/570F SFK non-binding mutant.

**Figure 2. MITF directly interacts with KIT<sup>D816V</sup> in a SRC-dependent manner.** (a) Western blot of a co-immunoprecipitation experiment. A375 human malignant melanoma cells were transfected with the indicated constructs and the lysates immunoprecipitated with the anti-FLAG antibodies against FLAG-MITF and stained with the indicated antibodies.

Whole cell lysate (WCL) provides protein loading controls. **(b)** Western blot of a co-immunoprecipitation experiment. A375 cells were transfected with the indicated constructs and lysates immunoprecipitated with a c-SRC antibody and the western blot stained with the indicated antibodies. Prior to lysis, cells were treated with either KITLG (50 ng/ ml, 15 minutes, lane 2) and or with SU6656 (2 or 5  $\mu$ M, 1 h, lane 3, 4 and 6). **(c)** Graph showing the densitometric quantification of the MITF band on the western blot in b. Error bars show standard deviation from three independent measurements. IP= immunoprecipitation, KITLG= KIT ligand

**Figure 3. KIT<sup>D816V</sup> mediates cytosolic retention of MITF through tyrosine phosphorylation.** **(a)** Confocal images of B16F0 cells transfected with the indicated MITF and KIT expression constructs and stained for nucleus (DAPI, blue), MITF-FLAG with Alexa Fluor 647-conjugated FLAG antibodies (green) and KIT with PE-conjugated anti-KIT antibodies (red). Scale bar indicates 5  $\mu$ m. **(b)** B16F0 cells transfected with increasing amounts of either wild-type KIT or KIT<sup>D816V</sup> and with MITF-FLAG were fractionated into cytosolic and nuclear fractions. Immunoblotting against the cytosolic protein tubulin and the nuclear TATA binding protein was performed as controls to verify that there was no cross contamination from the different fractions. **(c)** Antibody staining of the endogenous MITF (red) and KIT (green) proteins and the TO-PRO stain for the nucleus (blue) performed on KIT or KIT<sup>D816V</sup> transfected 501 mel cells. Scale bar indicates 5  $\mu$ m.

**Figure 4. Effects of KIT<sup>D816V</sup> on MITF-dependent gene regulation.** **(a)** Graph showing the relative expression of MITF in the samples assayed in b. **(b)** Hierarchical clustering of expression levels of 15 MITF target genes in B16F0 mouse melanoma cells after the treatment indicated on right. Gene names in red are involved in melanocyte/melanoma

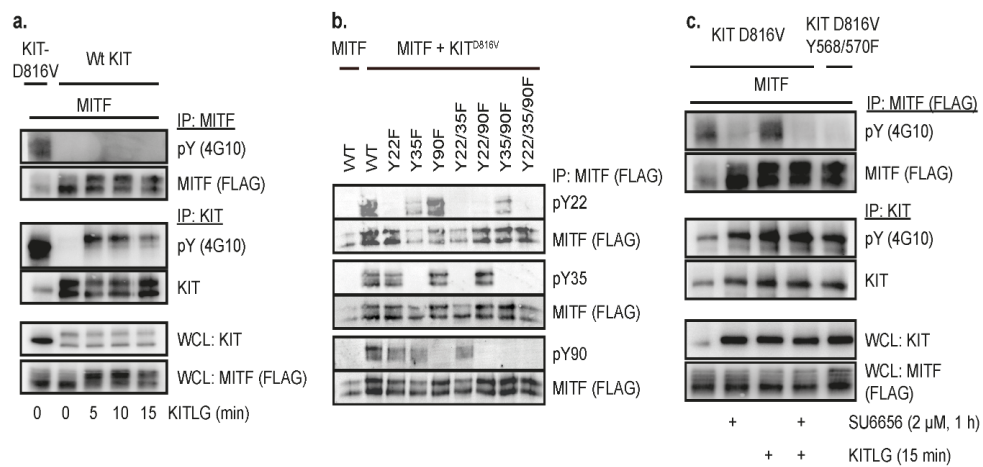


proliferation, survival, angiogenesis and cell cycle initiation and genes in green are involved in differentiation, tumor suppression and cell cycle inhibition. Heat map and graphs represent average values from three independent experiments and error bars indicate standard deviation. All data shown here are presented in Supplementary Table 1. **(c)** Graph showing relative expression of MITF in the samples assayed in d. **(d)** Hierarchical clustering of expression levels of 15 MITF target genes in B16F0 melanoma cells after the treatment indicated on the right. The KIT<sup>D816V</sup> construct was stably transfected into the cells whereas MITF was transiently overexpressed. Heat maps and graphs represent average values from 3 independent experiments and error bars indicate standard deviation. All data shown here are included in Supplementary Table 2.

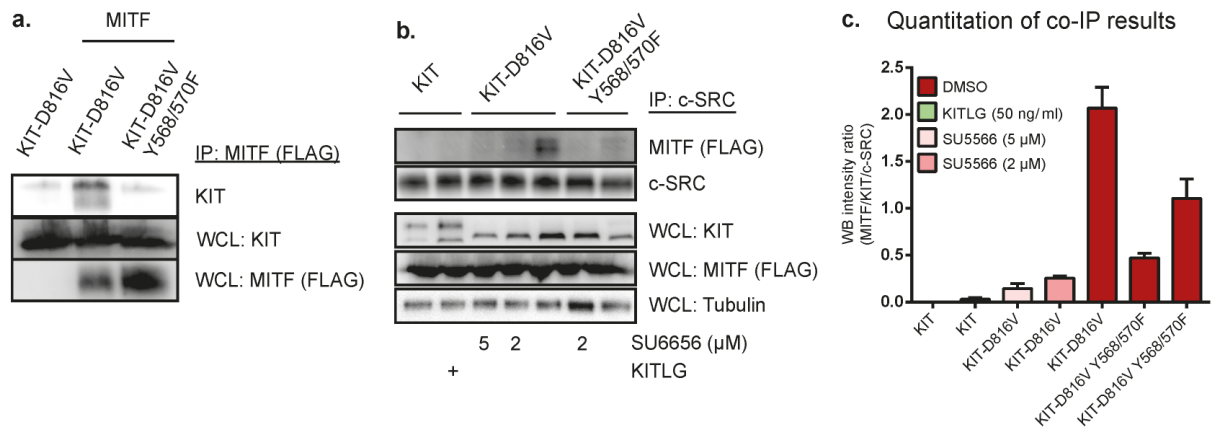
**Figure 5. KIT<sup>D816V</sup> affects proliferation by phosphorylating MITF.** Proliferation (fold change) of **(a)** B16F0 and **(b)** A375 melanoma cells, either not transfected (normal), stably transfected with empty vector (CMV) or stably transfected with KIT<sup>D816V</sup> measured with the PrestoBlue cell viability assay over three days. In addition, the cells were either treated with negative control siRNA or siRNA against MITF (siMITF) on day 0. Proliferation (fold change) of **(c)** B16F0 and **(d)** A375 cells stably transfected with the indicated KIT and MITF or control constructs for 48 h. Two-way ANOVA was applied on a. and b. and one-way ANOVA on c. and d. Significant results ( $P < 0.05$ ) in a. and b. were further processed with Bonferroni post-tests whereas c. and d. were calculated with Dunnett's Multiple Comparison Test. Post-hoc analysis of all graphs was compared with respective normal cells. Error bars represent standard error of the mean from at least three independent experiments. Statistical significance is illustrated by \* ( $P \leq 0.05$ ), \*\* ( $P \leq 0.01$ ) and \*\*\* ( $P \leq 0.001$ ). Data illustrated in a. and b. are further presented in Supplementary Table 3 and data shown in c. and d. are described in more detail in Supplementary Table 4.

**Figure 6. KIT<sup>D816V</sup> transformed melanoma cells are hypersensitive to SFK inhibitors.**

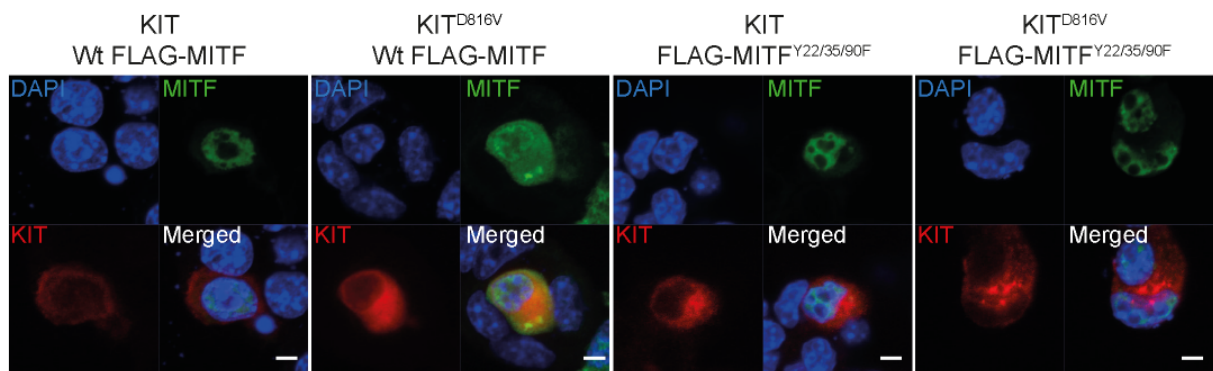
Cell viability of (a) B16F0 and (b) A375 melanoma cells either non-transfected (normal) or stably transfected with empty CMV vector (controls) or transfected with KIT<sup>D816V</sup>, and then treated with increasing concentrations of DMSO (control), SU6656 (10-200 nM) or dasatinib (10-200 nM). Cell viability was measured after 48 h. Statistical analysis included Two-way ANOVA and post-hoc Bonferroni tests. Statistical significance from normal cells is illustrated by \* ( $P \leq 0.05$ ), \*\* ( $P \leq 0.01$ ) and \*\*\* ( $P \leq 0.001$ ). Error bars represent standard error of the mean from at least three independent experiments. Data of a-b are included in Supplementary Table 5. (c) 501 mel and (d) A375 cells stably expressing either wild-type KIT or KIT<sup>D816V</sup> were treated with increasing doses of the BRAF<sup>V600E</sup> inhibitor (BRAFi) vemurafenib for 72 h. Co-treatment was performed with 100 nM of dasatinib in combination with escalating concentrations of BRAFi.



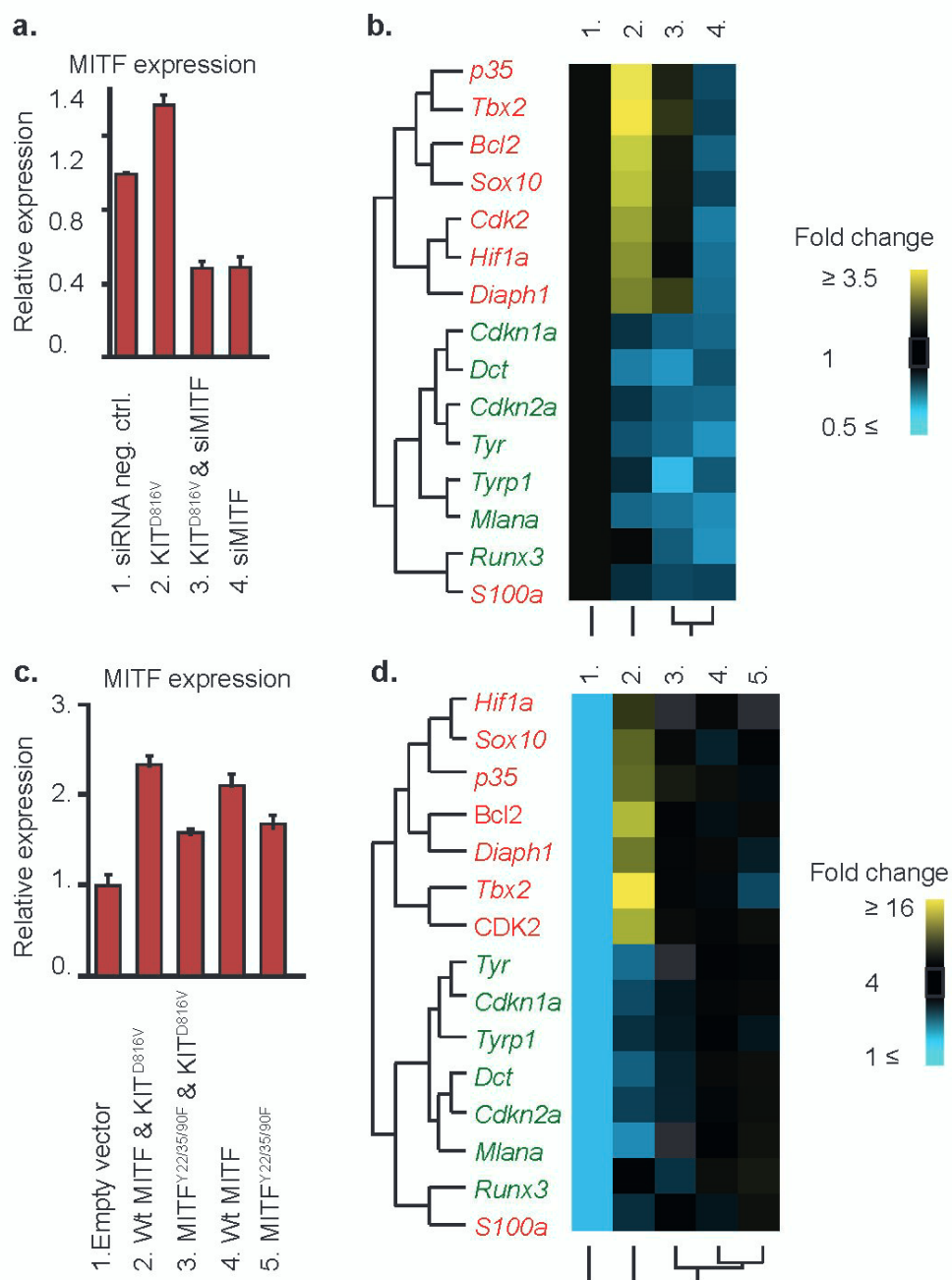
Phung et al., Figure 1



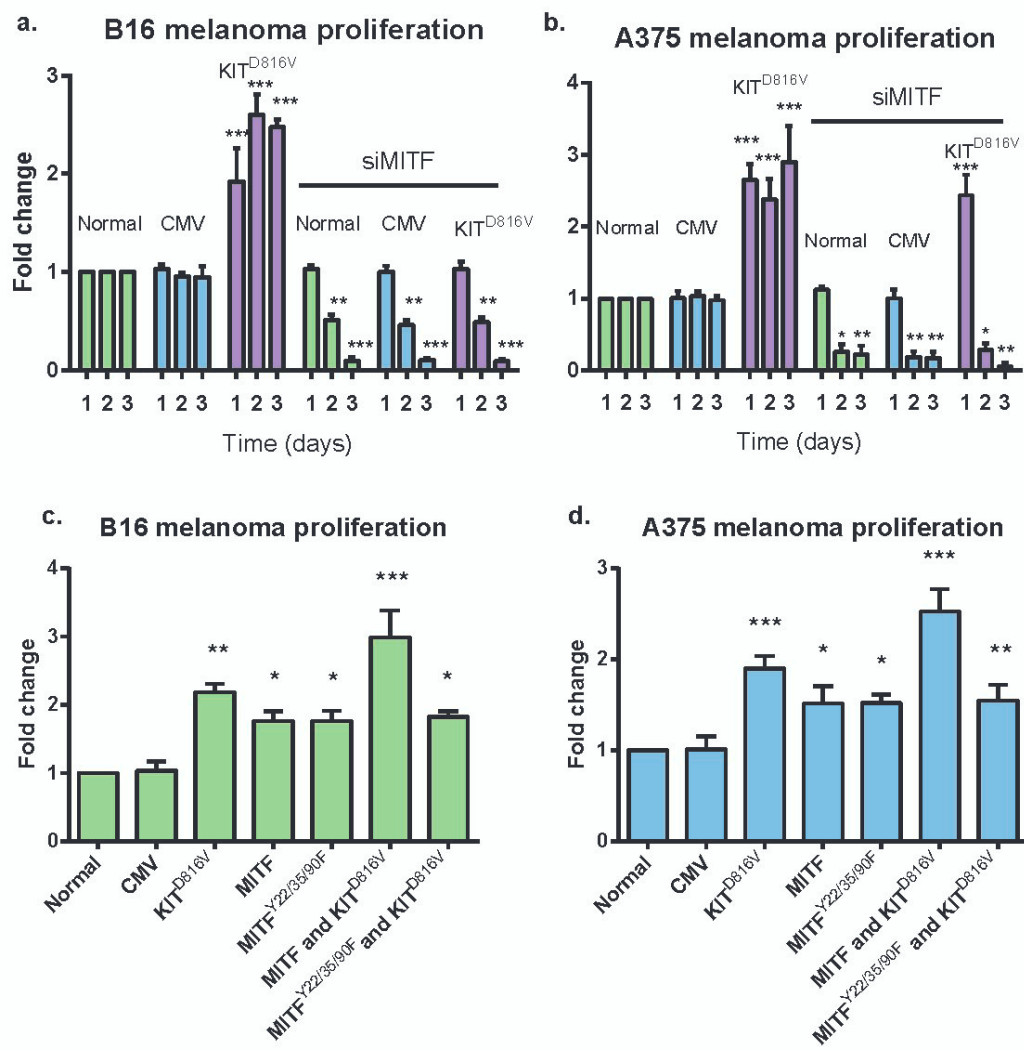
Phung et al., Figure 2



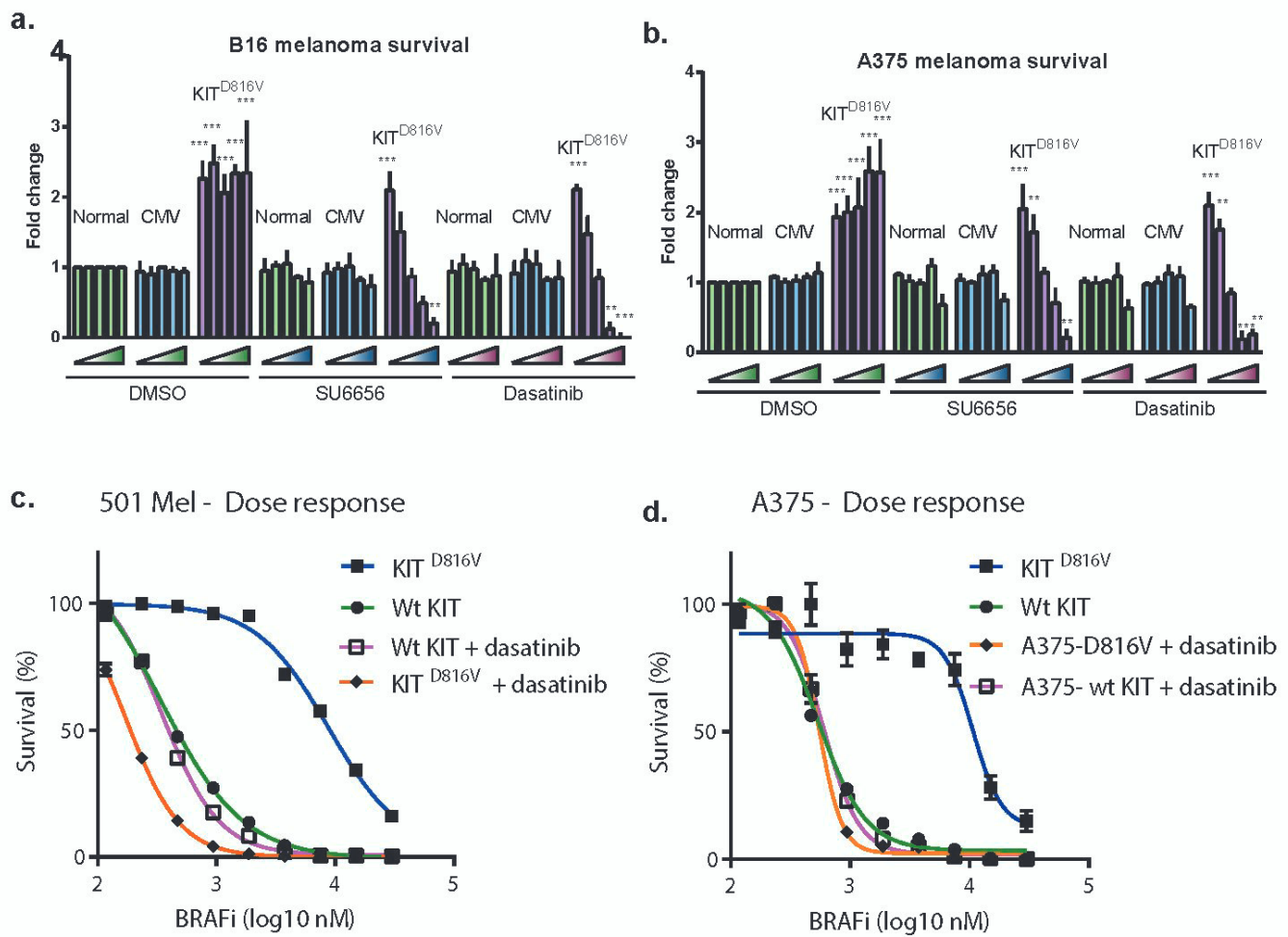
Phung et al., Figure 3



Phung et al., Figure 4



Phung et al., Figure 5



Phung et al., Figure 6