

***ABCB5* is activated by MITF and  $\beta$ -catenin and is associated with melanoma differentiation**

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**Abstract**

Defining markers of different phenotypic states in melanoma is important for understanding disease progression, determining the response to therapy, and defining the molecular mechanisms underpinning phenotype-switching driven by the changing intratumor microenvironment. The ABCB5 transporter is implicated in drug-resistance, and has been identified as a marker of melanoma-initiating cells. Indeed ongoing studies are using ABCB5 to define stem cell populations. However, we show here that the *ABCB5* is a direct target for the Microphthalmia-associated transcription factor MITF and its expression can be induced by  $\beta$ -catenin, a key activator and co-factor for MITF. Consequently *ABCB5* mRNA expression is primarily associated with melanoma cells exhibiting differentiation markers. The results suggest first that ABCB5 is unlikely to represent a marker of dedifferentiated melanoma stem cells, and second that ABCB5 may contribute to the non-genetic drug-resistance associated with highly differentiated melanoma cells. To reconcile the apparently conflicting observations in the field we propose a model in which ABCB5 may mark a slow-cycling differentiated population of melanoma cells.

**Significance**

The ABCB5 transporter has been linked to slow cycling melanoma-initiating cells, but its role as a marker for this population has been controversial. We show that *ABCB5* is an MITF and  $\beta$ -catenin target gene and that its expression is associated with melanoma differentiation rather than de-differentiated melanoma-initiating cells described by others. The results raise the possibility that both differentiated and undifferentiated slow-cycling cells are tumorigenic and drug resilient.

The microphthalmia-associated transcription factor (MITF) (Goding and Arnheiter, 2019) plays a crucial role in melanocyte development where its expression defines the lineage and promotes melanoblast survival (Hodgkinson et al., 1993) and differentiation (Carreira et al., 2005; Gaggioli et al., 2003; Loercher et al., 2005) by activating many of the genes implicated in production of melanin and formation and intracellular transport of melanosomes (Cheli et al., 2010), melanocyte-specific organelles. MITF also plays a key role in melanoma biology where it suppresses invasion (Carreira et al., 2006) and senescence (Giuliano et al., 2010) while at the same time promoting cell division (Garraway et al., 2005; Carreira et al., 2006; Du et al., 2004; Widlund et al., 2002), efficient DNA damage repair (Strub et al., 2011), and lysosome biogenesis and autophagy (Ploper et al., 2015; Zhang et al., 2014). At least part of MITF's pro-proliferative role may be attributed to its regulation of metabolism since it stimulates mitochondrial biogenesis (Haq et al., 2013; Vazquez et al., 2013) and the TCA cycle (Louphrasitthiphol et al., 2019). Given its central role in melanoma biology, MITF has been the focus of considerable attention as a key melanoma biomarker for specific phenotypic subpopulations of cells generated as a consequence of the impact of the intratumor microenvironment (Rambow et al., 2019; Hoek and Goding, 2010). Notably a combination of gene expression profiling of cell lines (Hoek et al., 2006; Tsoi et al., 2018) as well as single cell RNA expression analysis has identified 6 melanoma phenotypic states including MITF-negative or poorly expressing undifferentiated and Neural Crest Stem-like Cells (NCSCs) that are drug tolerant (Ennen et al., 2017; Ennen et al., 2015; Rambow et al., 2019; Rambow et al., 2018; Tirosh et al., 2016). Drug tolerance has also been linked to cells with high MITF expression (Johannessen et al., 2013; Muller et al., 2014; Smith et al., 2016). Yet, how MITF might control drug tolerance is poorly understood.

The ABCB5 (ATP-binding cassette, subfamily B member 5) gene encodes a membrane glycoprotein and member of the ATP-binding cassette transporter superfamily implicated in drug tolerance in melanoma (Chen et al., 2009). Although it can promote drug-resistance, for example by promoting efflux of doxorubicin (Frank et al., 2005), ABCB5 came to prominence when it was shown that cells expressing ABCB5 exhibited enhanced tumor-initiation capacity (Schatten et al., 2008). By contrast, Quintana et al (2010) showed no correlation between ABCB5 expression, or other proposed stem cell markers, and tumor-initiating capacity (Quintana et al., 2010) and suggested instead that melanoma cells did not exhibit a hierarchical organisation. Moreover, Cheli et al (2014) failed to detect a significant overlap in expression of ABCB5 and CD271(NGFR) which had also been identified as a marker for melanoma-initiating cells (Boiko et al., 2010). Nevertheless, ABCB5 was identified independently as a marker of slow cycling melanoma cells (Luo et al., 2012), a property associated with tumor-initiation capacity in melanoma (Cheli et al., 2011; Falletta et al., 2017; Roesch et al., 2010). The potential of ABCB5 as a marker of a specific subpopulation of melanoma cells endowed with tumor-initiation capacity has therefore remained controversial.

Initial attempts to use anti-ABCB5 antibodies were unsuccessful; using 4 different siRNAs we were able to deplete ABCB5 mRNA (Supporting information Fig. 1A), but were unable to detect substantial changes in levels of the major bands detected by western blotting using 3 different commercially available anti-ABCB5 antibodies (see Supporting information Materials and methods for information) over time up to 96 h (Supporting information Fig. 1B). It was possible that ABCB5 has an extremely long half-life or that the antibodies cross-react with other proteins. To test this we used the goat anti-ABCB5 antibody in 501mel cells and showed that the protein decayed over the course of 24 h with a half-life of around 8 h, using anti-MITF as a control (Supporting information Fig. 1C). Given that 96 h after siRNA-transfection ABCB5 mRNA was severely reduced (Supporting information Fig. 1A), we might have expected that by this time the levels of the major proteins detected using the goat anti-ABCB5 antibody would have been reduced. The fact that they remained high (Supporting information Fig. 1B) raises the possibility that the proteins detected might not be ABCB5, though further work will be needed to verify the specificity of this antibody. We then performed a similar analysis using the mouse anti-ABCB5 antibody. In this case, the results (Supporting information Fig. 1D) revealed a single major band by western blotting that was unaffected by cycloheximide treatment and which was also readily detected in IGR39 cells that express very little ABCB5 mRNA (Supporting information Fig. 1E), using anti-SCD as a control. These data suggest that the protein detected using the mouse antibody is unlikely to represent ABCB5. Note that the antibody used in the original study (Schatton et al., 2008) was not examined here. Nevertheless, the potential issues surrounding the use of antibodies to detect ABCB5 led us instead to focus on determining ABCB5 mRNA expression.

Given the role of MITF in promoting differentiation and melanoma proliferation, and that MITF<sup>Low</sup> cells exhibit high tumor-initiating capacity, as a melanoma-initiating cell marker we would expect *ABCB5* to be expressed in MITF<sup>Low</sup> cells. To determine the relationship between *ABCB5* expression and melanoma phenotype, we examined the 471 human melanomas in the TCGA database for ABCB5 mRNA expression, and compared it to 4 melanoma phenotypic markers: *MITF*, a marker of proliferative/differentiated melanomas (Goding and Arnheiter, 2019); *AXL* encoding a receptor tyrosine kinase expressed in MITF<sup>Low</sup>, invasive and drug resilient cells (Dugo et al., 2015; Konieczkowski et al., 2014; Muller et al., 2014); and the invasive and proliferative melanoma gene expression signatures described by Verfaillie et al. (2015). The results (Fig. 1A-D) revealed that *ABCB5* mRNA expression correlated very well with that of *MITF* (Fig.1A), and the Verfaillie proliferative signature (Fig.1B), but strongly anti-correlated with both *AXL* expression (Fig.1C) and the Verfaillie invasive gene expression signature (Fig. 1D). These results were surprising, given that MITF expression is associated with proliferation and differentiation, rather than stemness or tumor-initiating capacity.

Since tumors in the TCGA dataset comprise not only melanoma cells, but stromal cell types including fibroblasts and infiltrating immune cells we wished to verify the correlation with *MITF* expression using independent datasets. Examining the Cancer Cell Line Encyclopedia (CCLE) melanoma cell line database

ranked by expression of *MITF*, *ABCB5* was preferentially expressed in cell lines expressing high levels of *MITF* that also exhibited a high Verfaillie proliferative gene expression signature score (Fig. 1E). By contrast *MITF*<sup>Low</sup> cell lines exhibited low *ABCB5* mRNA expression, but expressed high levels of *AXL* mRNA and the Verfaillie invasive gene expression signature.

Recent RNA-seq analysis of 53 melanoma cell lines led to their being assigned into 4 sub-classes related to the trajectory of cells as they undergo a transition from embryonic stem cells to differentiated melanocytes via intermediate neural crest like cell and transitory/melanoblast states (Tsoi et al., 2018). In this panel of lines (Fig. 1F), high *ABCB5* mRNA expression was largely restricted to cell lines of the *MITF*-positive melanocytic subtype, with low expression also being detected in some lines exhibiting the transitory phenotype. By contrast *ABCB5* was very poorly expressed in the *MITF*<sup>Low</sup> cell lines corresponding to the Neural Crest-like or undifferentiated states that express *AXL*, *NGFR* (CD271) and the Verfaillie invasive gene expression signature.

Collectively the data obtained from the cell lines indicate that *ABCB5* is preferentially expressed in *MITF*-positive, differentiated melanocytic melanoma cells (both the TCGA melanoma cohort as well as in cell lines) but not in any invasive or undifferentiated populations. Indeed examining the correlation between the expression of *MITF*, *ABCB5* and a number of other reported stem cell markers in the TCGA melanoma cohort (Fig. 1G) indicated that while *ABCB5* expression was correlated with that of *CD133* (PROM1) as reported (Schatton and Frank, 2008; Schatton et al., 2008), *CD133/PROM1* was, like *ABCB5*, expressed preferentially in *MITF*<sup>High</sup> melanomas and was associated with the Verfaillie proliferative gene expression signature. By contrast, expression of the proposed *CD34* (Held et al., 2010) and *NGFR* (CD271) (Boiko et al., 2010; Cheli et al., 2014) stem cell markers were anti-correlated with *ABCB5* or *MITF* expression and also correlated with *AXL* and the Verfaillie invasive gene expression signature. An additional marker of tumor initiating cells *KDM5B* (JARID1B) (Held and Bosenberg, 2010; Roesch et al., 2010; Roesch et al., 2013), did not show any strong correlation with any specific phenotype marker.

Given the correlation between *ABCB5* mRNA expression and that of *MITF* we next asked whether *ABCB5* was an *MITF* target gene. Preliminary evidence to suggest that *MITF* might regulate *ABCB5* has been reported previously (Bertolotto et al., 2011). In this study, adenovirus mediated ectopic overexpression of *MITF* up-regulated *ABCB5* mRNA expression around 2-fold in RCC4 renal carcinoma cells, and in a melanoma cell line chromatin-immunoprecipitation followed by RNA-sequencing (ChIP-seq) indicated that a sumoylation-defective E318K *MITF* mutant was able to bind the *ABCB5* gene. However binding by WT *MITF* was very weak and regulation of *ABCB5* by overexpressed *MITF* in melanoma cells was not observed, nor was depletion of *MITF* used to interrogate the possibility that *ABCB5* was an *MITF* target. Using a previously published ChIP-seq dataset from 501mel cells (Louphrasitthiphol et al, 2019), a cell line that expresses substantial levels of *MITF* and which therefore should also express the *ABCB5* gene, we found that *MITF* was bound at two sites upstream from *ABCB5* containing consensus *MITF* recognition elements (TCACATGA and CACGTGA) (Fig. 2A) with a peak score well above that of several well-

characterised MITF target genes (Fig. 2B), raising the possibility that *ABCB5* was indeed a direct MITF target gene in melanoma. To confirm this we tested three siRNAs, including two specific for the human *MITF* gene, for their ability to deplete MITF in the same cell line (Fig. 2C). We then used a 3'RNA-seq approach to determine the gene expression profile of cells treated with control siRNA or depleted for MITF using two different siRNAs. The results (Fig. 2D; Supporting Information Table 1) revealed that for both siRNAs used, depletion of MITF led to a reduction in *ABCB5* mRNA expression. This result was confirmed using two different human-specific siRNAs to deplete MITF followed by RT-qPCR to detect *MITF* and *ABCB5* mRNA levels in 3 MITF-positive cell lines. In all cases, transfection of siMITF led to reduced *MITF* mRNA levels and also a reduction in *ABCB5* mRNA expression (Supporting information Fig. 2). Combined with the observation (Fig. 2A) that MITF binds upstream from *ABCB5*, and the correlations between *MITF* expression and that of *ABCB5* in cell lines and in melanomas *in vivo* (Fig.1), our results strongly suggest that *ABCB5* is expressed in MITF-positive differentiated melanoma cells, and not in undifferentiated invasive melanoma cells.

Previous work (Bertolotto et al., 2011) showed that ectopic MITF expression in A375 melanoma cells failed to up-regulate known endogenous target genes, a result reflecting previous work showing that ectopic MITF is unable to stimulate endogenous gene expression (Gaggioli et al., 2003; Vachtenheim et al., 2001). Although there may be several explanations for these observations, one possibility is that ectopically expressed MITF lacks an essential and limiting cofactor that enables it to turn on its endogenous targets. Alternatively activation of endogenous MITF should facilitate activation of its endogenous genes. We therefore engineered cell lines to express an inducible epitope-tagged nuclear form of  $\beta$ -catenin (Fig. 2E), a well-characterised activator of the endogenous *MITF* promoter (Dorsky et al., 2000; Takeda et al., 2000) that also acts as a co-factor for MITF in activation of differentiation-associated target genes (Schepsky et al., 2006). In the melanocyte lineage  $\beta$ -catenin activates melanocyte stem cells to trigger proliferation, and via its interaction with MITF also promotes differentiation (Guo et al., 2016; Yamada et al., 2013; Schepsky et al., 2006). Western blotting revealed that expression of  $\beta$ -catenin using this system could be induced (Fig. 2F) and immunofluorescence confirmed that ectopic, doxycycline-induced  $\beta$ -catenin was localised to the nucleus (Fig. 2G). Using 3' RNA-seq we were able to demonstrate that induction of  $\beta$ -catenin in IGR37 melanoma cells, a cell line expressing ample endogenous MITF, led to robust up-regulation of MITF and *BRN2* (*POU3F2*), a second melanoma-specific  $\beta$ -catenin target gene (Goodall et al., 2004), as well as *ABCB5* (Fig. 2H). Consistent with  $\beta$ -catenin increasing MITF activity, we observed a substantial increase in a repertoire of differentiation-associated MITF targets, including *ABCB5*, on induction of  $\beta$ -catenin (Fig. 2I; Supporting Information Table 2).

The results reported here indicate that at the mRNA level, *ABCB5* expression is up-regulated by MITF and  $\beta$ -catenin and is associated with melanomas cells exhibiting a more differentiated phenotype. The fact that genetic variants in *ABCB5* correlating with diminished *ABCB5* transporter activity exhibit increased pigmentation and reduced melanoma risk (Lin et al., 2013), suggests that one role for *ABCB5* may be to

prevent premature terminal differentiation of MITF-positive cells. This interpretation would be consistent with the increased binding to *ABCB5* by the sumoylation defective MITF E318K mutant that is associated with increased melanoma risk (Bertolotto et al., 2011; Yokoyama et al., 2011).

*ABCB5* has been associated with label retaining cells (Schatten et al., 2008), with label retention being a hallmark of slow-cycling or cell cycle arrested cells. However, although label retention has been associated with MITF<sup>Low</sup> de-differentiated cells (Cheli et al. 2014), it is also possible that some MITF<sup>High</sup> cells could be label retaining; MITF can promote a differentiation-associated cell cycle arrest (Carreira et al., 2005; Loercher et al., 2005), and reducing MITF activity in zebrafish can enable differentiated melanocytes to reenter the cell cycle (Lister et al., 2013). Since it is clear that slow cycling cells in melanoma are associated with tumor-initiation capacity (Cheli et al., 2011; Falletta et al., 2017; Perego et al., 2018; Roesch et al., 2010), one possibility that is consistent with the observations from many groups is that slow-cycling cells, irrespective of their differentiation status, have an increased capacity to initiate tumors. In this scenario, *ABCB5* and *CD133*, whose expression correlates in the TCGA melanoma cohort, would be associated with slow cycling MITF<sup>High</sup> tumor-initiating cells exhibiting a more differentiated (but not terminally differentiated) phenotype. By contrast, other markers such as NGFR/CD271 or CD34 would be expressed on slow cycling MITF<sup>Low</sup> tumor initiating cells. Although this explanation is speculative, such a model would be compatible with the observation that up to 28% of single melanoma cells have tumor-initiating capacity (Quintana et al., 2008) and the reports that there is no single marker of melanoma initiating cells (Quintana et al., 2010). What is clear is that the *ABCB5* gene, by being an MITF and  $\beta$ -catenin target, is more associated with melanocyte/melanoma differentiation than with undifferentiated MITF<sup>Low</sup> cells. Importantly, accumulating evidence suggests that MITF<sup>High</sup> cells may be drug-resilient. For example, in a mouse melanoma model following BRAF and MEK inhibitor combination therapy, both MITF<sup>High</sup> and MITF<sup>Low</sup> cells were present at the minimal residual disease state, with drug resilience being conferred by non-genetic mechanisms (Rambow et al., 2018). One interesting possibility is that when confronted with MAPK pathway inhibitors survival would be enhanced by transporters able to promote drug efflux, such as *ABCB5* in the MITF<sup>High</sup> population. As such the identification of mechanisms promoting drug efflux in phenotypically drug-resilient populations may provide opportunities to develop small molecules able to target phenotypic drug-resistant populations.

### Data availability

RNA seq data for the IGR37 cells expressing inducible  $\beta$ -catenin have been deposited at the Gene Expression Omnibus with accession number GSE136803.

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### Author Contribution

PL performed the experimentation and PL and JC undertook all bioinformatics analysis. CRG provided funding and supervision. PL and CRG wrote the manuscript.

**Conflicts of interest**

The authors declare no conflict of interest

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## FIGURE LEGENDS

**Figure 1.** ABCB5 is associated with a differentiated melanoma phenotype. The 471 melanomas in the TCGA human melanoma cohort were ranked for expression of MITF (A), ABCB5 (B,D), or AXL (C) (black lines). Each melanoma sample was then interrogated for expression of ABCB5 (A, C), or the Verfaillie proliferative (B) or invasive (D) signatures (grey lines) and the moving average of each presented as a colored line). (E) Relative expression of the indicated genes in the CCLE set of melanoma cell lines. (F) Relative expression of the indicated genes in the set of melanoma cell lines described by Tsoi et al (2018) grouped according to their phenotype. (G) Heatmap showing Spearman correlation of expression of indicated genes and gene expression signatures in the TCGA melanoma cohort (NGFR, CD271; PROM1, CD133; KDM5B, JARID1B).

**Figure 2.** ABCB5 expression is regulated by MITF. (A) UCSC genome-browser screenshot showing a duplicate MITF ChIP-seq profile and control at the ABCB5 locus. (B) Peak scores from a duplicate MITF ChIP-seq in 501mel cells for the indicated genes. (C) Western blot from 501mel cells showing depletion of MITF using three different MITF-specific siRNAs including two that are human specific (sihsMITF). (D) Results from 3'RNA-seq determination of the relative levels of *MITF* and *ABCB5* mRNAs 48 h after transfection into 501mel cells of two MITF siRNAs. (E) Schematic of the Doxycyclin-inducible MYC-, HA- and FLAG-epitope-tagged  $\beta$ -catenin used. NLS = SV40 nuclear localization signal. (F) Western blot showing induction of HA-tagged  $\beta$ -catenin over time using 20 ng doxycycline for 16 h. Extracts were probed using either anti-HA or anti- $\beta$ -catenin antibody as indicated. GAPDH was used as a loading control. (G) Immunofluorescence using anti-HA antibody of cells stably expressing epitope-tagged NLS- $\beta$ -catenin with and without doxycycline. (H, I) Heatmaps derived from triplicate 3' RNA-seq of IGR37 cells stably expressing epitope-tagged NLS- $\beta$ -catenin with and without doxycycline (20 ng 24 h) as indicated.

## Supporting information Materials and Methods

### Supporting Information Figure 1

Quality control of anti-ABCB5 antibodies. (A) RT-qPCR of mRNA from 501mel cells transfected with 4 different anti-ABCB5 siRNAs over time. Results are presented relative to expression using a control siRNA. (B) Western blot using three different anti-ABCB5 antibodies (goat, rabbit and mouse as indicated) of 501mel cells transfected with indicated ABCB5 siRNAs or control siRNA over time. (C) Western blot using goat anti-ABCB5 antibody of 501mel cells treated with 50 µg/ml cycloheximide over time. Anti-MITF was used as a control. (D) Western blots using mouse anti-ABCB5 antibody in 3 different melanoma cell lines as indicated treated with 50 µg/ml cycloheximide over time. Anti-SCD was used as a control. For IGR39 cells SCD is poorly expressed and the blot was therefore exposed longer. (E) Triplicate 3' RNA-seq data showing relative normalized expression of MITF and ABCB5 in 3 melanoma cell lines.

### Supporting Information Figure 2

Depletion of MITF suppresses *ABCB5* mRNA expression. RT-qPCR of mRNA from cells transfected with indicated human-specific MITF siRNA using RTPCR primers specific for *MITF* or *ABCB5* mRNA. Expression normalised to *ACTB* expression; n=4, error bars indicate S.D..

### Supporting information Table 1

Biological triplicate 3' RNA-seq data showing relative *ABCB5* mRNA expression following depletion of MITF using two different siRNAs.

### Supporting information Table 2

Relative expression of listed genes determined using 3'RNA-seq of IGR37 cells in which the epitope-tagged, nuclear localized β-catenin was induced for 24 h using 20 ng doxycycline and compared to expression without doxycycline treatment.

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## **Materials and Methods**

### **Cell culture**

Cell lines (501mel, 501mel HA-MITF, IGR37, IGR37-iHA-CTNNB1) were mycoplasma free, authenticated using STR profiling, and grown under 10% CO<sub>2</sub> in RPMI 1640 (Gibco) supplemented with 10% FBS (Biosera) and 100 U/ml Pen Strep (Gibco). Inducible  $\beta$ -catenin cell lines were generated using a modified PiggyBac transposon vector system where the SV40 Large T-antigen nuclear localisation signal was introduced at the N-terminus of the insertion site. Plasmids were transfected using FuGENE 6 (Promega) as per supplier recommendation. Stable cell lines were generated by selecting cells under 1 mg/ml Geneticin (Gibco). MITF knock downs via siRNA were carried out using Lipofectamine RNAiMAX (Invitrogen) according to the supplier's recommendation. The siRNAs used were sihsMITF#1 (UGGCUAUGCUUACGCUUAA), sihsMITF#3 (AGACGGAGCACACUUGUUA) (Dharmacon), siMITF1 (AGCAAGUACCUUUCUACCAC) (custom order, QIAGEN) at 20 nM concentration for 48 h. siRNAs from ABCB5 were ON-TARGETplus Human ABCB5 (Dharmacon): siABCB5#1 (J-007303-09-0005), siABCB5#2 (J-007303-10-0005), siABCB5#3 (J-007303-11-0005), siABCB5#4 (J-007303-12-0005)

### **Gene expression analysis of melanoma tumor sample and cell line data**

Clinical melanoma RNA-seq expression data from human melanomas from The Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>) was accessed through the cBioportal for Cancer Genomics (<http://www.cbioportal.org>) using the R-based package CGDS-R (Cerami et al. 2012; Gao et al. 2013) following the TCGA guidelines for the use of TCGA data (<http://cancergenome.nih.gov/publications/publicationguidelines>). Expression data from melanoma cell lines was downloaded from the Cancer Cell Line Encyclopedia (CCLE; <http://www.broadinstitute.org/ccle/home>) and RNAseq data from the cell lines described in Tsoi et al (2018) was downloaded from the Gene Expression Omnibus (GEO) database accession number GSE80829. For the correlation analysis and heatmaps and we calculated the average expression of the proliferative and invasive signatures from Verfaillie et al. (2013). All heatmaps were generated using the R package pheatmap. The correlations between expression of *NGFR*, *CD34*, *ABCB5*, *PROM1*, *KDM5B*, *AXL*, *MITF*, and the proliferative and invasive gene expression signatures were calculated using Spearman's correlation analysis. For the moving average plots, the moving average expression of individual genes of interest or averaged signatures was calculated using a sample window size of n=20 and trendlines were added to the bar plots. An R-script for calculating and generating moving average plots of TCGA cancer cohorts implementing TCGA access via cBioportal was provided in our previous study (Riesenberg et al.



2015). The significance of the Spearman rank correlation was determined by an asymptotic Spearman correlation test using the original log<sub>2</sub> expression values and not the moving average values.

### **RNA-seq**

RNAs for transcriptomic analysis were prepared using RNeasy Mini kit (Qiagen) as per supplier recommendation for extraction from cell culture. Only samples with RIN values  $\geq 9.5$  (as assessed using a Bioanalyzer (Agilent)) were carried forward for library prep using QuantSeq Forward kit (Lexogen) with 500 ng input material. Sequencing was carried out on a HiSeq4000 (Illumina) by the Oxford Genomics Centre, Wellcome Trust Centre for Human Genetics. Fastq files were mapped using STAR (Dobin et al., 2013) against hg38 (GRCh38, 2015) with feature counts on. Differential gene expression analyses were carried out using function glmQLFTest of the package EdgeR (Robinson et al., 2010). Heatmaps of edgeR-normalised gene expression were generated using the package Pheatmap (<https://cran.r-project.org/web/packages/pheatmap/index.html>).

### **Western blotting**

Lysates for Western blotting were prepared by directly lysing the samples in LDS sample buffer (Invitrogen) and boiled at 95°C for 10 min before SDS-PAGE on Bis-Tris gel in MES running buffer (Invitrogen). Transfer to PVDF membrane (GE) was carried out using Tris-Glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3 with 20% methanol (vol/vol)). Blocking was done at room-temperature for 2 h in 10% non-fat dried-milk-TBS supplemented with 0.25% Tween-20 (TBST). Incubation of primary antibodies (mouse anti-MITF (C5, Millipore), mouse anti-GAPDH (6C5, Santa Cruz Biotechnologies), mouse-anti  $\beta$ -catenin (610454, BD), rabbit anti-HA tag (C29F4, Cell-signalling technology) and anti-SCD (HPA063921, Sigma) were carried out in 5% BSA dissolved in TBST overnight, room temperature. Incubation with HRP-conjugated secondary antibodies (Bio-Rad) were carried out for 1 h at room temperature and washed in excess TBST for 30 minutes prior to visualisation on X-ray films (Fuji) using an ECL detection kit (GE). The anti-ABCB5 antibodies used were mouse anti-ABCB5 monoclonal antibody (5H3C6, Life Technology), rabbit anti-ABCB5 (N-term) antibody (SAB1300315, Sigma), and goat anti-ABCB5 polyclonal antibody (ab77549, Abcam).

### **RT-qPCR**

Total RNA from cultured cells was purified using RNeasy Mini Kit (Qiagen #74104). gDNA-contaminants were removed using gDNA Wipeout Buffer (Qiagen #205313) for RT-qPCR prior to reverse-transcription step using QuantiTect Reverse Transcription Kit (Qiagen #205313) according to the supplier's protocol. 50 ng starting RNA per quantitative real-time PCR (qPCR) reaction was used to avoid sampling problems. qPCR analysis was carried out in biological triplicates on the Rotor Gene

Q (Qiagen) using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent #600883) and 66.7 nM primers with cycling parameters of 95°C, 2 min enzyme activation and 60 cycles of 95°C, 3 s; 60°C, 15 s; 72°C, 5 s. The melting curve was determined by ramping temperature from 60°C to 95°C in steps of 1°C.

#### qPCR primers

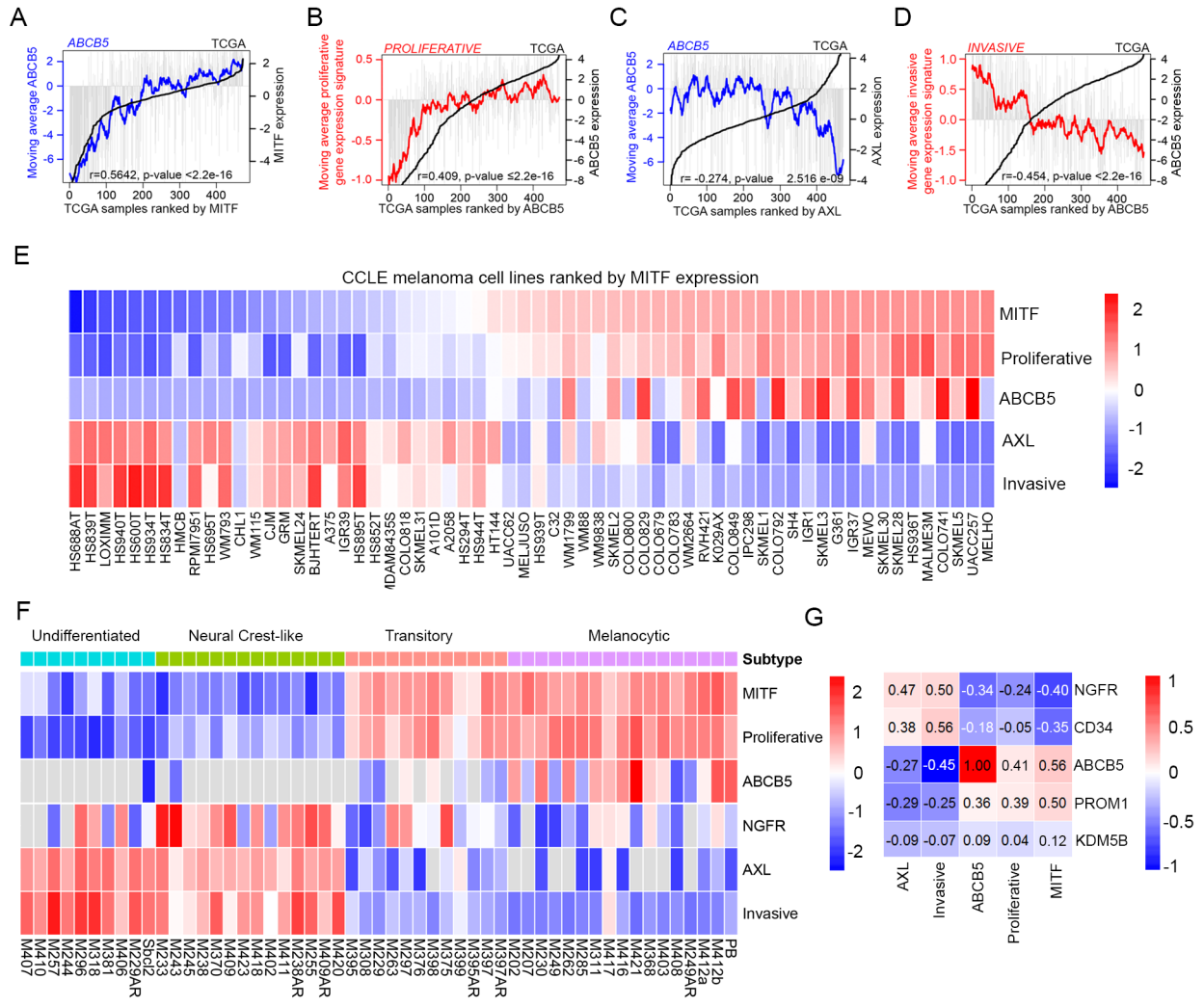
Primer names	Sequence
ACTB_GEX F#3	GCACTCTTCCAGCCTTCC
ACTB_GEX R#3	TGTCCACGTCACACTTCATG
MITF_GEX F#4	CATCCGTGGACTATATCCGAAAG
MITF_GEX R#4	CGAGCCTGCATTTCAAGTTC
ABCB5_GEX F#2	CCACTTCAGCCCTCGATAATG
ABCB5_GEX R#2	CCACTATCAAATCTGCGTTCTG

#### Immunofluorescence

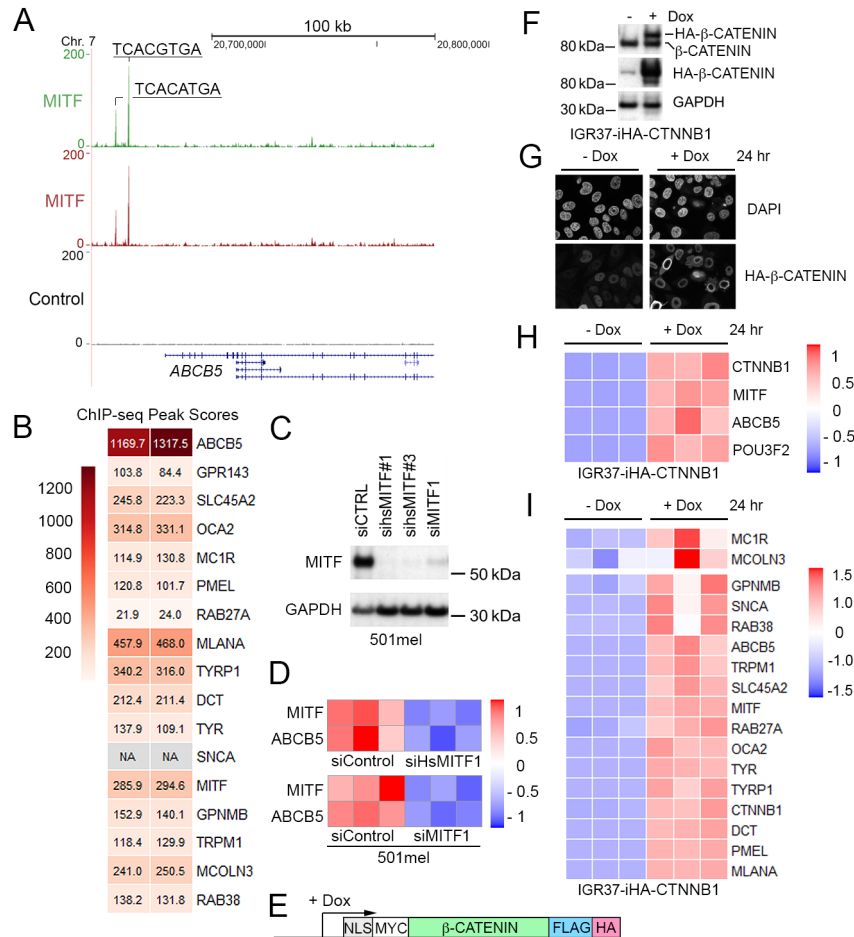
Cells grown and treated on coverslips were fixed in 3.7% formaldehyde dissolved in PBS supplemented with 0.1% Triton-X 100 (PBST) for 10 minutes at room temperature prior to sequential incubation with primary and secondary antibodies (AlexaFluor, Invitrogen) suspended in 5% BSA and dissolved in PBST for 1 h, room temperature. Washing was done in an excess volume of PBST for 30 minutes at room temperature before mounting on poly-lysine slides using Vectashield (Vectorlabs).

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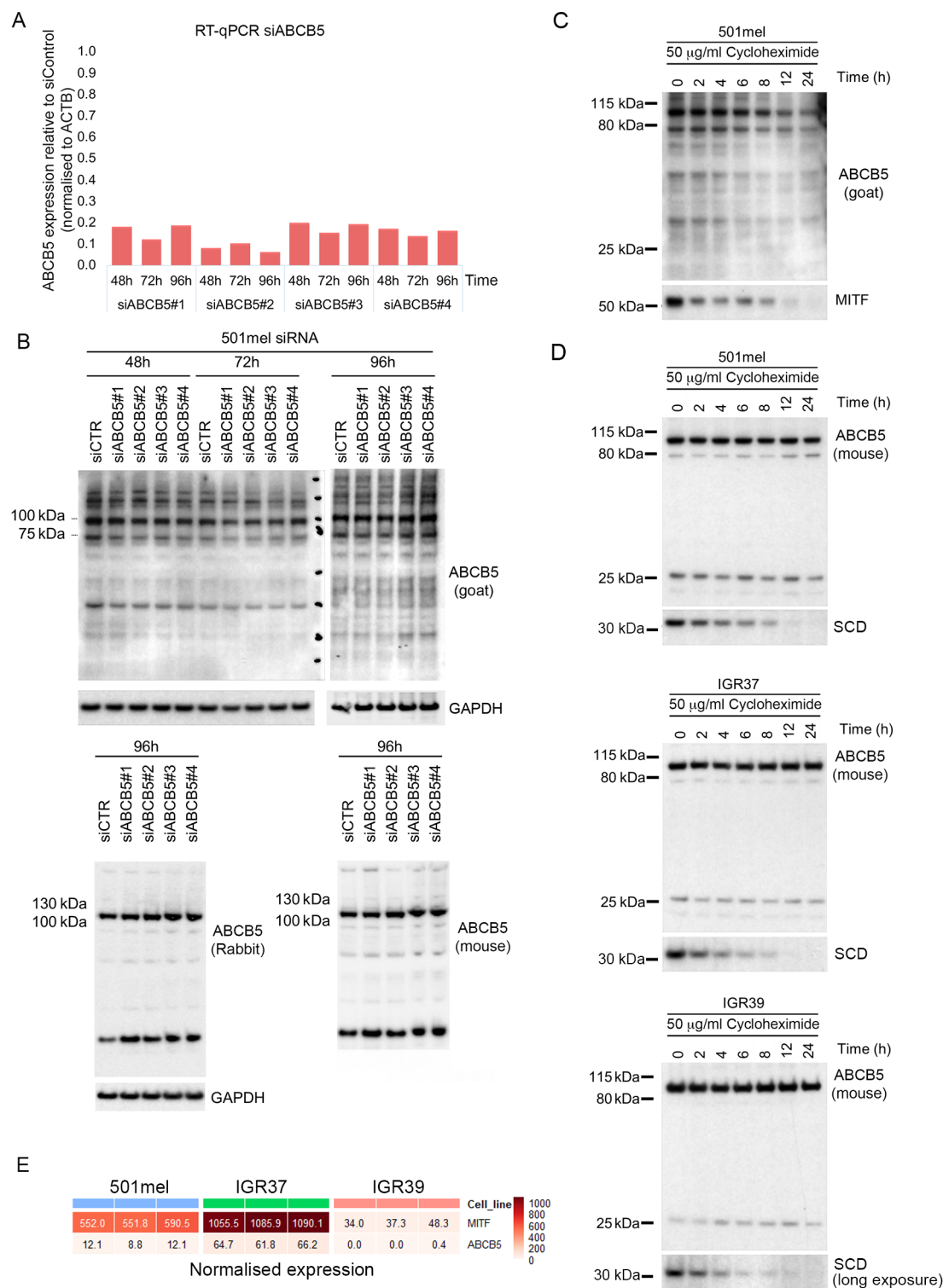




**Figure 1.** ABCB5 is associated with a differentiated melanoma phenotype. The 471 melanomas in the TCGA human melanoma cohort were ranked for expression of *MITF* (A), *ABCB5* (B,D), or *AXL* (C) (black lines). Each melanoma sample was then interrogated for expression of *ABCB5* (A, C), or the Verfaillie proliferative (B) or invasive (D) signatures (grey lines) and the moving average of each presented as a colored line. (E) Relative expression of the indicated genes in the CCLE set of melanoma cell lines. (F) Relative expression of the indicated genes in the set of melanoma cell lines described by Tsoi et al (2018) grouped according to their phenotype. (G) Heatmap showing Spearman correlation of expression of indicated genes and gene expression signatures in the TCGA melanoma cohort (*NGFR/CD271*; *PROM1/CD133*; *KDM5B/JARID1B*).

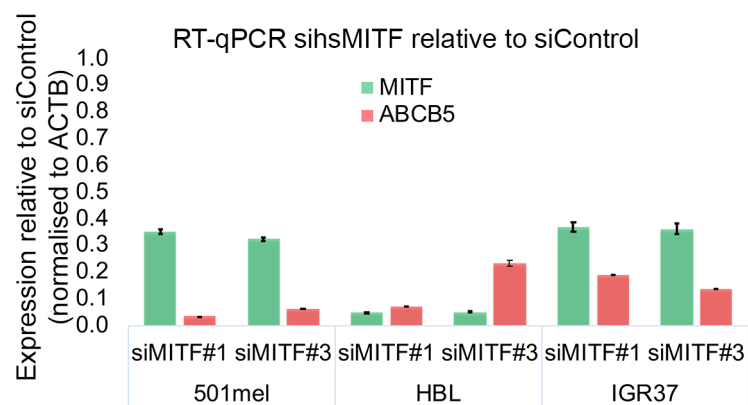


**Figure 2.** ABCB5 expression is regulated by MITF. (A) UCSC genome-browser screenshot showing a duplicate MITF ChIP-seq profile and control at the ABCB5 locus. (B) Peak scores from a duplicate MITF ChIP-seq in 501mel cells for the indicated genes. (C) Western blot from 501mel cells showing depletion of MITF using three different MITF-specific siRNAs including two that are human specific (sihsMITF). (D) Results from 3'RNA-seq determination of the relative levels of *MITF* and *ABCB5* mRNAs 48 h after transfection into 501mel cells of two MITF siRNAs. (E) Schematic of the Doxycyclin-inducible MYC-, HA- and FLAG-epitope-tagged β-catenin used. NLS = SV40 nuclear localization signal. (F) Western blot showing induction of HA-tagged β-catenin over time using 20 ng doxycycline for 16 h. Extracts were probed using either anti-HA or anti-β-catenin antibody as indicated. GAPDH was used as a loading control. (G) Immunofluorescence using anti-HA antibody of cells stably expressing epitope-tagged NLS-β-catenin with and without doxycycline. (H, I) Heatmaps derived from triplicate 3' RNA-seq of IGR37 cells stably expressing epitope-tagged NLS-β-catenin with and without doxycycline (20 ng, 24 h) as indicated.



### Supporting Information Figure 1

Quality control of anti-ABCB5 antibodies. (A) RT-qPCR of mRNA from 501mel cells transfected with 4 different anti-ABCB5 siRNAs over time. Results are presented relative to expression using a control siRNA. (B) Western blot using three different anti-ABCB5 antibodies (goat, rabbit and mouse as indicated) of 501mel cells transfected with indicated ABCB5 siRNAs or control siRNA over time. (C) Western blot using goat anti-ABCB5 antibody of 501mel cells treated with 50 mg/ml cycloheximide over time. Anti-MITF was used as a control. (D) Western blots using mouse anti-ABCB5 antibody in 3 different melanoma cell lines as indicated treated with 50 µg/ml cycloheximide over time. Anti-SCD was used as a control. For IGR39 cells SCD is poorly expressed and the blot was therefore exposed longer. (E) Triplicate 3' RNA-seq data showing relative normalized expression of *MITF* and *ABCB5* in 3 melanoma cell lines.



**Supporting Information Figure 2**

Depletion of *ABCB5* mRNA expression on depletion of MITF. RT-qPCR of mRNA from cells transfected with indicated human-specific MITF siRNA using RT-PCR primers specific for *MITF* or *ABCB5* mRNA. Expression normalised to *ACTB* expression; n=4; error bars indicate S.D.

	<b>ABCB5</b>					
	<b>logFC</b>	<b>FC</b>	<b>logCPM</b>	<b>F</b>	<b>PValue</b>	<b>FDR</b>
siMITF1	-1.48539	0.357153	5.879096	65.84352	5.90E-12	1.53E-10
sihsMITF	-1.48179	0.358045	3.532258	46.56024	5.45E-07	2.00E-05

### Supporting information Table 1

Biological triplicate 3' RNA-seq data showing mRNA expression of ABCB5 following depletion of MITF using two different siRNAs compared to expression in cells transfected with a control siRNA.

<b>Symbol</b>	<b>logFC</b>	<b>FC</b>	<b>logCPM</b>	<b>F</b>	<b>PValue</b>	<b>FDR</b>
CTNNB1	1.961599	3.894936	9.565001	241.9045	3.06E-10	1.24E-08
MITF	4.220099	18.63702	8.293994	867.0431	5.14E-14	5.41E-11
ABCB5	7.220107	149.097	5.175824	400.4551	1.03E-11	8.89E-10
POU3F2	6.79098	110.7359	2.144761	133.6282	1.48E-08	2.90E-07
MC1R	2.439903	5.426051	4.163394	78.216	4.11E-07	4.59E-06
MCOLN3	1.1404	2.204422	2.414407	7.843992	0.014137	0.03035
GPNMB	0.995255	1.993433	7.925996	42.81241	1.29E-05	8.13E-05
SNCA	3.838294	14.30347	6.553129	256.092	2.09E-10	9.09E-09
RAB38	4.912897	30.12517	6.776138	221.4145	5.50E-10	2.02E-08
TRPM1	6.18578	72.79565	5.010822	570.0775	9.23E-13	2.22E-10
SLC45A2	6.311091	79.40132	8.77358	663.6784	3.25E-13	1.58E-10
RAB27A	1.531249	2.89036	6.650688	98.83346	9.86E-08	1.38E-06
OCA2	6.203176	73.67869	5.892275	525.4125	1.61E-12	2.95E-10
TYR	6.466282	88.41886	7.247978	809.239	8.28E-14	8.04E-11
TYRP1	6.136467	70.34945	9.196695	466.0835	3.66E-12	4.27E-10
DCT	6.669343	101.7823	10.30775	897.5049	4.05E-14	5.41E-11
PMEL	6.30452	79.04051	10.12749	592.0892	7.12E-13	2.10E-10
MLANA	6.068968	67.13382	7.972438	872.397	4.93E-14	5.41E-11

### Supporting information Table 2

Relative expression of listed genes determined using 3'RNA-seq of IGR37 cells in which the epitope-tagged, nuclear localized  $\beta$ -catenin was induced for 24 h using 20 ng doxycycline and compared to expression without doxycycline treatment.



