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1 **LOSS OF CITED1, AN MITF REGULATOR, DRIVES A PHENOTYPE SWITCH IN VITRO**  
2 **AND CAN PREDICT CLINICAL OUTCOME IN PRIMARY MELANOMA TUMOURS**

3

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28 ABSTRACT

29 CITED1 is a non-DNA binding transcriptional co-regulator whose expression can  
30 distinguish the 'proliferative' from 'invasive' signature in the *phenotype-switching*  
31 model of melanoma. We have found that CITED1 expression is repressed by TGF $\beta$  in  
32 addition to other 'proliferative' signature genes while the 'invasive' signature genes  
33 are upregulated. In agreement, CITED1 positively correlates with MITF expression  
34 and can discriminate the MITF-high/pigmentation tumor molecular subtype in a  
35 large cohort (120) of melanoma cell lines. Interestingly, CITED1 overexpression  
36 significantly suppressed MITF promoter activation, mRNA and protein expression  
37 levels while MITF was transiently upregulated following siRNA mediated CITED1  
38 silencing. Conversely, MITF siRNA silencing resulted in CITED1 downregulation  
39 indicating a reciprocal relationship. Whole genome expression analysis identified a  
40 phenotype shift induced by CITED1 silencing and driven mainly by expression of MITF  
41 and a cohort of MITF target genes that were significantly altered. Concomitantly, we  
42 found changes in the cell-cycle profile that manifest as transient G1 accumulation,  
43 increased expression of CDKN1A and a reduction in cell viability. Additionally, we  
44 could predict survival outcome by classifying primary melanoma tumors using our *in*  
45 *vitro* derived 'CITED1-silenced' gene expression signature. We hypothesize that  
46 CITED1 acts a regulator of MITF, functioning to maintain MITF levels in a range  
47 compatible with tumourigenesis.

48

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50 KEYWORDS – CITED1, MITF, MELANOMA, PHENOTYPE-SWITCHING MODEL

## 51 INTRODUCTION

52 CITED1 is the founding member of the CITED (CBP/p300-interacting transactivator  
53 with glutamic acid [E]/aspartic acid [D]-rich C-terminal domain) family of  
54 transcriptional co-regulators and was originally cloned from a differential display  
55 screen between pigmented mouse B16 melanoma cells and their dedifferentiated  
56 non-pigmented derivative, B16F10s. This led to speculation that CITED1 or *msg1*  
57 (melanocyte specific gene 1) as it was known at that time, was involved in the  
58 process of pigmentation<sup>1</sup>. Subsequently, Nair et al. reported that stable  
59 overexpression of CITED1 increased the levels of tyrosinase, dopachrome  
60 tautomerase (Dct) and melanin in B16 cells, reinforcing the idea that it had a role in  
61 melanogenesis<sup>2</sup>. By 2005, as gene expression profiling became relatively  
62 commonplace, CITED1 was identified in several new screens: in two studies CITED1  
63 was identified as a gene whose expression distinguished nevi from primary  
64 melanoma, while another study found CITED1 to be upregulated in advanced stage  
65 melanomas in comparison to benign nevi or melanoma *in situ*<sup>3-5</sup>.

66

67 Based on extensive gene expression profiling of melanoma cell lines *in vitro*, Hoek et  
68 al. proposed the 'phenotype-switching' model of melanoma that was independent of  
69 the degree of transformation or disease progression and sought to explain the  
70 observation that melanoma cells altered between two states: those with high  
71 proliferative potential that are less invasive and those with high metastatic potential  
72 that are less proliferative. These separate but alternating states are controlled by  
73 different transcriptional programs and can be defined by specific gene signatures<sup>6</sup>.

74 MITF expression and many of its known targets (TYR, MLANA) define the

‘proliferative’ group, while the ‘invasive’ signature group is characterized by expression of negative regulators of the Wnt signaling pathway (WNT5A, DKK1, CTGF). CITED1 expression was associated with the proliferative pathway signature and subsequently confirmed in an updated and expanded data set to be significantly correlated with the proliferative phenotype ( $P < 1.00E-05$ , <http://www.jurmo.ch/hopp>, accessed 19 March 2013) <sup>7,8</sup>.

Studies on CITED1 suggest that it is a non-DNA binding nuclear transcriptional co-regulator capable of influencing TGF $\beta$  induced transcription mediated by ligand-induced SMAD hetero-oligomerization; estrogen-dependent transcription mediated by ER $\alpha$ , and Wnt/ $\beta$ -Catenin-dependent transcription. These effects are dependent on CITED1-CBP/P300 binding via the conserved CITED family CR2 domain and while in the case of ER $\alpha$ , CITED1 is thought to act by stabilizing the CBP/P300-ER $\alpha$  interaction, in the case of  $\beta$ -Catenin it acts to repress transcription by competing for binding with CBP/P300 transcriptional co-activators <sup>9-12</sup>.

Microphthalmia-associated transcription factor, MITF, acts as a master-regulator of melanocyte differentiation and as a result has been intensely studied in the field of melanoma research <sup>13,14</sup>. It is a basic helix-loop-helix leucine zipper transcription factor that recognizes E-box and M-box sequences in the promoter regions of its target genes. Highlighting its importance in the disease, amplification of MITF locus has been found in >15% of metastatic melanomas and germline mutations in MITF that predispose carriers to melanoma development have also been found <sup>15-17</sup>. In

98 melanoma cells the target genes of MITF include most notably TYR, MCIP, DCT,  
 99 MLANA involved in the process of pigmentation; cell cycle regulators such as CDK2  
 100 and CDKN1A and the more recently identified BRCA1 gene that has, with other  
 101 target DNA repair genes, defined a role for MITF in the DNA damage response (DDR)  
 102 <sup>18-20</sup>.

103

104 The regulation of MITF is complex and tightly controlled, exhibiting both  
 105 transcriptional and post-translational regulation. There are several transcript  
 106 isoforms, of which MITF-M is the dominant form expressed in melanocytes. Multiple  
 107 signaling pathways converge on the MITF-M specific promoter that harbors binding  
 108 sites for PAX3, SOX10, CREB, FOXD3, LEF-1 and BRN2 among other transcription  
 109 factors <sup>14,21</sup>. Additionally, the MITF target gene CDKN1A/P21 has been shown to act  
 110 as reciprocal transcriptional cofactor independently of its CDK inhibitor function,  
 111 suggesting the existence of at least one positive feedback loop <sup>22</sup>.

112 MITF post-translational activity can be affected by phosphorylation, sumoylation,  
 113 ubiquitination and by binding with proteins that block access to the DNA binding  
 114 domain such as PIAS3 <sup>21,23</sup>. Oncogenic BRAF (but not wildtype BRAF), which is  
 115 mutated in up to 50% of melanomas, also regulates MITF via simultaneously  
 116 stimulating MITF activation through ERK phosphorylation, which leads to its  
 117 subsequent degradation, and by inducing transcription of MITF via BRN2  
 118 upregulation <sup>24,25</sup>.

119 The consensus regarding why the cell invests such effort in maintaining control of  
 120 MITF levels and why there are so many regulatory mechanisms, is that melanocytes

and melanoma are exquisitely sensitive to even small variations in MITF expression. Ultimately its activity must be sustained within the narrow window permissive for continued survival and proliferation. In this study, we characterise the role of CITED1 as a novel regulator of MITF in melanoma.

## RESULTS

### **TGF $\beta$ induces expression of the invasive signature genes while suppressing a cohort of proliferative signature genes including CITED1**

Hoek et al. noted that many of the genes that defined the invasive phenotype were commonly TGF $\beta$  driven while at the same time only the proliferative signature phenotype cells were sensitive to TGF $\beta$  growth inhibition *in vitro*<sup>7</sup>. In agreement, we have previously shown that the melanoma cell line A2058 upregulates WNT5A in response to TGF $\beta$  stimulation and that exogenous Wnt-5a increased their invasive potential<sup>26</sup>. In an effort to examine what other phenotype specifying genes were directly regulated by TGF $\beta$  in these cells we performed gene expression analysis and found that while TGF $\beta$  treatment resulted in upregulation of many of the invasive signature genes it also suppressed expression of many of the genes characterizing the proliferative phenotype (Fig. 1a). The effect is more pronounced if only those signature genes that were deemed significantly altered by TGF $\beta$  treatment are examined. The original signature set defined by Hoek et al., was redefined as more public datasets became available and has a slightly different but overlapping gene profile based on the top ranked differentially expressed genes (Fig. 1b). Both MITF and CITED1 are in the proliferative cohort and their response to TGF $\beta$  treatment was

confirmed at protein level in A2058 cells (Fig. 1C).

### **CITED1 expression positively correlates with the expression of MITF**

Examination of publically available gene expression data on 120 melanoma cell lines demonstrated a consistent positive correlation between CITED1 and MITF expression ( $r=0.6543$ ). Each cell line was assigned as either 'proliferative' or 'invasive' based on a score derived from the averaged expression values of the approximately 50 genes in each defining signature set that had matching gene symbols in our data (Fig. 2a). We also confirmed the correlation in cell lines derived from our own lab (Fig. S1). This was important as inconsistency in interlaboratory phenotype signatures has previously been reported<sup>8</sup>. We could additionally confirm expression at the protein level (Fig. 2b)

### **Gene expression analysis reveals CITED1 silencing can induce a phenotype-switch**

To investigate the function of CITED1 in melanoma, we transiently downregulated its expression using CITED1 targeting siRNA. We choose the HT144 cell line as it had a relatively high level of detectable CITED1 mRNA and protein expression. A scatterplot of the 120 cell lines assigned as either 'proliferative' or 'invasive' based on the maximum matching gene signature score demonstrates the shift in phenotype that occurs following CITED1 downregulation (Fig. 3a,b). A heatmap of the expression profiles clearly illustrates that the shift is due to a general induction of the 'proliferative' and suppression of the 'invasive' cohort (Fig. 3c). It was apparent that the #3 siCITED1 siRNA was not as effective at switching the cells as the #1 siCITED1, this was observed consistently throughout our experiments and may be



167 due to the fact that #3 siCITED1 was not as successful at silencing CITED1 (Fig. 3b,  
168 *inset*).

169

#### 170 **CITED1 is a reciprocal regulator of MITF and impacts MITF target gene expression**

171 A heatmap of only the significantly differentially induced transcripts between siNEG  
172 and both #1 & #3 siCITED1 highlights the identity of the genes responsible for the  
173 expression switch (Fig. 4a). Of most relevance, we found MITF, a known driver of the  
174 proliferative phenotype switch and many of its previously known downstream  
175 targets, these also included genes categorized by Gene Ontology annotation (GO) as  
176 related to pigmentation and UV/DNA damage response (Fig. 4a)<sup>18,27-29</sup>. We could  
177 confirm that indeed MITF protein levels were affected by siCITED1 in HT144 cells and  
178 that conversely, overexpression of CITED1 in A2058 cells, resulted in downregulation  
179 of MITF (Fig. 4b). Strub et al. identified a large number of genomic targets of MITF by  
180 ChIP-seq analysis<sup>18</sup>. A comparison of the genes differentially expressed by siCITED1  
181 compared to siNEG, revealed that there was significant enrichment of these  
182 potential targets (Fig. S2a). Notably, genes both up and down regulated by siCITED1  
183 are represented among genes defined as having MITF-occupied promoters (Fig. S2b).  
184 We also found that downregulation of MITF using siRNA in HT144 cells (Fig. 4c) and  
185 in WM293A, and SKMEL5 cells (Fig. S3a,b) resulted in decreased protein expression  
186 of CITED1 suggesting reciprocity between these factors.

187

#### 188 **Induction of MITF by CITED1 silencing transiently restrains cell cycle progression** 189 **and impacts cell viability**

To investigate the effect of CITED1 silencing on melanoma cells behaviour we analysed the cell cycle distribution following siRNA treatment, by flow cytometry. In siCITED1 treated HT144 cells we saw G1 accumulation as indicated by an increase in the diploid G1 fraction and a concomitant reduction in the total S-phase fraction peaking at 33 hours but also observed at 48 and 72 hours post-transfection in comparison to siRNA control HT144 cells. Again, the effect was apparent but not as pronounced using the #3 siCITED1 (Fig S4a). Similar effects were seen in #1 and #3 siCITED1 treated A2058 and A375 cells (Fig. S4b,c).

Owing to the previously reported dependency of MITF-induced cell cycle arrest on CDKN1A/P21 we investigated the levels of several cyclin-dependant kinase inhibitors following CITED1 silencing<sup>30</sup>. We found that CDKN1A/P21 was transiently increased in siCITED1 treated HT144 cells relative to the siNEG treated HT144 cells. In contrast, in A2058 cells, which do not have detectable levels of CDKN1A/P21 (Fig. S5), the levels of CDKN1C/P57 were suppressed in response to CITED1 overexpression (Fig 5b). We hypothesised therefore that melanoma cells can utilise either CDKN1A/P21 or CDKN1C/P57 to mediate cell cycle arrest induced by MITF and this is reflected in the expression levels of the alternate CDK inhibitors in different melanoma cell lines (Fig. S5).

In agreement with the cell cycle data, an Alamar Blue assay revealed a significant reduction in cell metabolic activity over 5 days in HT144 cells treated with siCITED1 (Fig 5c). The effect was apparent but not as pronounced in the #3 siCITED1 sample.

## **The effect of CITED1 silencing on MITF is transient and mediated via promoter activation**

We observed that the peak upregulation of MITF and CDKN1A/P21 protein following siCITED1 treatment varied from transfection to transfection, being seen between 24-48 hours post-transfection but appearing as unchanged or even downregulated after this time (Fig. 6a, *upper panel*). In agreement, later timepoints of the cell cycle analysis ( $\geq 72$  hours) exhibited little or no change in G1/S-phase distribution or even a reverse pattern (Fig. 5a HT144, and data not shown: A2058, A375). We therefore sought to examine the transcriptional dynamics more closely, map the changes in MITF following CITED1 silencing and see if they corresponded to cell behaviour and changes at the protein level. We used a quantitative droplet digital PCR based assay (Biorad) to measure mRNA in HT144 cells transfected with siCITED1#1 and siNEG as well as A2058 cells transiently overexpressing CITED1 compared to an empty vector control. MITF, CITED1 and IPO8 specific primers and probes were used to measure exact copies/ $\mu$ l of each mRNA from aliquots of the same cDNA solution. Plots of  $\text{siCITED1}(\text{copies}/\mu\text{l})/\text{siNEG}(\text{copies}/\mu\text{l})$  and  $\text{EV}(\text{copies}/\mu\text{l})/\text{pCITED1}(\text{copies}/\mu\text{l})$  show the directional change in MITF and CITED1 relative to the housekeeper IPO8. CITED1 expression is rapidly suppressed following siCITED1 treatment of HT144 cells, concomitant with an upregulation of MITF that diminishes over time and in fact is suppressed by 100 hours in accordance with observations at the protein level (Fig 6a, *lower panel*). In contrast, overexpression of CITED1 in A2058 cells results in transient suppression of MITF at both protein and transcript level (Fig. 6b, *upper and lower panels*).

The rapid MITF transcriptional response to CITED1 manipulation suggested to us that the effect could be directly mediated at the promoter level. To test this hypothesis, we over expressed an MITF-M promoter-reporter construct and CITED1 in A375 cells. We chose A375 cells, as while they had less endogenous CITED1 and MITF than HT144 or A2058 so as not to cause interference with the assay, we also knew that they could respond adequately as they had an identical G1 accumulation/S-phase decrease to both HT144 and A2058 cells following CITED1 silencing (Fig S4c). TGF $\beta$  treatment was used as a positive control for repression of the MITF-M promoter. CITED1 transfection led to significant suppression of the MITF-M promoter luciferase activity relative to the empty vector control, as did TGF $\beta$  treatment alone or combination with CITED1 overexpression (Fig. 6c). There did not appear to be an additive or synergistic effect using both TGF $\beta$  treatment and CITED1 overexpression suggesting TGF $\beta$  may be dependent on CITED1 for MITF suppression.

### **The CITED1-silenced gene signature predicts outcome in primary melanoma**

The ‘proliferative’ and ‘invasive’ signature phenotypes have served to define the gene expression classification of melanoma cell lines. However, primary tumours and metastatic lesions have also been molecularly classified into several distinct groups by gene expression profiling<sup>31,32</sup>. The four-class structure found in tumours consists of the ‘pigmentation’, ‘proliferative’, ‘high-immune’ and ‘normal-like’ subgroups with a subset falling into an unclassifiable cohort<sup>32</sup>. We used the same tumour classification to subtype the 120 cell lines that had publically available expression data and could show that the tumour ‘pigmentation’ subgroup that highly expresses MITF, corresponds to the cell line ‘proliferative’ phenotype described by Hoek et al.

Accordingly, the tumour ‘proliferative’ and ‘high-immune’ subgroups comprise the cell line ‘invasive’ phenotype (Fig. 7a). It is worth noting that the names of the tumour subgroups were derived from a description of the differentially expressed genes that comprised each molecular classification while the ‘invasive-proliferative’ switching phenotypes were named to reflect the *behaviour* exhibited by lines classified by one or other signature. This distinction helps to explain the confusing occurrence that both classifications have a group referred to as ‘proliferative’ although they are not equivalent.

The overlap between the primary tumour classifying and cell line classifying systems allows us to infer that CITED1 expression is most likely restricted to a subset of MITF high ‘pigmentation’ subtype tumours. As the tumour subtype classification was shown to be prognostically significant in primary melanomas we were interested to know if CITED1 expression itself was independently predictive of outcome. Previously we reported on the analysis of 223 primary lesions using the Illumina WG-DASL protocol<sup>31</sup>. As the CITED1 probe in this assay did not produce reliable data we instead derived a CITED1-silenced gene signature score based on the differentially expressed genes from the HT144 siCITED1 experiment (Fig. 3) We subsequently interrogated the gene expression data on the primary melanoma lesions using a nearest centroid approach derived from the CITED1-silenced gene signature. This revealed that primary melanomas with a gene expression signature most similar to the CITED1-silenced signature ([CITED1low class](#)) had a significantly better outcome than those with a signature most disparate from the CITED1-silenced signature ([CITED1high class](#)) (Fig. 7b). Importantly, the CITED1 signature [classing](#) had

286 independent prognostic information (HR 1.85, CI 0.30-0.98, p=0.044) from the AJCC  
287 staging system (HR 5.05, CI 2.42-10.55, p=1.64x10<sup>-5</sup>). These data indirectly imply  
288 that CITED1 expression itself is a potential prognostic indicator in primary  
289 melanomas and the transcriptional program influenced by CITED1 expression  
290 determines tumour behaviour *in vivo*.

291

## 292 DISCUSSION

293 One seemingly paradoxical observation from our study and previous investigations is  
294 that although CITED1 behaves as a negative regulator of MITF, both their expression  
295 levels appear positively correlated across cells lines and tumours. We maintain that  
296 this observation simply reflects the fact that where there are high levels of MITF,  
297 high levels of its negative regulator are also required. The evidence of the tight  
298 control exerted over MITF levels in melanocytes and melanoma simply speaks to the  
299 necessity of the cell to maintain a level compatible with survival and proliferation, in  
300 a type of biological 'sweet-spot' facilitating tumour progression. The cellular effects  
301 of both extremes i.e: very low or high levels of MITF, have been elegantly described  
302 by a rheostat model in order to reconcile the conflicting observations of the effects  
303 of manipulating MITF *in vitro*, and the fact that counter-intuitively, a lineage-  
304 specifying differentiation factor can behave as a potent oncogene<sup>33-35</sup>. The rheostat  
305 model (Fig. S6) explains why MITF silencing can block cells in G1 and induce  
306 senescence, while it is also possible to induce a G1 arrest by MITF overexpression via  
307 CDKN2A/P16 or CDKN1A/P21 and, as we now propose, potentially also via  
308 CDKN1C/P57<sup>20,30,34,36</sup>. At the extreme high end of MITF expression lies differentiated  
309 melanocytic cells, while the lowest levels can lead to senescence and irreversible cell

death. Between these two extremes however it is thought that melanoma cells can oscillate from a low-MITF 'invasive' to a high-MITF 'proliferative' state via phenotype-switching.

We hypothesise that the role of CITED1 in melanoma is to maintain levels of MITF compatible with tumour progression and effectively tip the balance in favour of cell cycle progression rather than MITF-induced G1-arrest. This is supported by our findings that downregulation of CITED1 using siRNA results in a phenotype switch to a more pigmented state driven by increased MITF expression and concomitant upregulation of CDKN1A/P21. Conversely, we could observe that downregulation of MITF resulted in suppression of CITED1 in several cell lines suggesting the existence of a classical feedback loop where low MITF levels result in inhibition of its negative regulator. MITF induced cell cycle arrest was previously shown to be dependent on CDKN1A/P21 and it was demonstrated that MITF does not cause a cell cycle arrest in CDKN1A-deficient mouse embryo fibroblasts (MEF) cells<sup>30</sup>. However, our data indicate that in melanoma cells deficient in CDKN1A/P21, the alternative CDK inhibitor CDKN1C/P57 is expressed and responsive to MITF.

Interestingly, while we observed upregulation of most MITF targets following CITED1 silencing, we found that BRCA1 and other DNA damage response (DDR) genes were suppressed, suggesting that CITED1 downregulation does not necessarily facilitate transcription of all MITF targets. It is thus tempting to speculate that rather than simply acting to induce MITF and thereby indirectly enhance transcription of its target genes, that CITED1 may also act as co-factor for MITF at various genomic

locations differentially modulating the MITF target gene response at individual promoters. One way that this might be achieved is via MITF-CITED1 competition for CBP/P300 binding as CBP/P300 is a known transcriptional coregulator for MITF, although it is not required for transcription of all MITF targets<sup>37-39</sup>.

As suggested by Sáez-Ayala et al., anti-cancer therapy should be ideally independent of dominant or 'driver' genetic alterations so that subclonal populations do not gain a subsequent advantage and the same holds true in the case of targeting a specific phenotype. Successful therapy will necessarily need to switch or push the subdominant phenotype into the susceptible state or eradicate the phenotype resistant to treatment. This approach was initially championed by Cheli et al., who proposed the eradication of low-MITF cells as a therapeutic strategy<sup>40</sup>. Indeed the idea of lineage-specific therapy has been subsequently proved in principle using methotrexate (MTX) to first activate MITF expression, in turn activating the tyrosinase enzyme, and thereby sensitising tumour cells to a tyrosinase-processed anti-folate prodrug (TMECG)<sup>41</sup>. However, even without drug targeting, induction of MITF, above what is tolerated by even the highly pigmented tumor cell types, i.e: levels reaching that of melanocytes, would seem to be incompatible with melanoma progression as it inhibits cell cycle progression. Our assertion is that CITED1 acts to repress MITF in order to maintain its level in a range compatible with tumorigenesis. This assertion as a consequence naturally suggests CITED1 as therapeutic target for genetic manipulation. Successful implementation of such a strategy would result in cell specific enhancement of MITF expression and increased susceptibility to the type



of chemotherapeutic eradication demonstrated by Sáez-Ayala et al. or potentially induction of CDKN1A/p21 or CDKN1C/p57-dependent cell growth arrest even without further intervention (Fig. S6) <sup>41</sup>.

## MATERIALS AND METHODS

### Cell lines

Cell lines were obtained from ATCC. HT144 and SKMEL3 cells were cultured in McCoy's5A supplemented with 10% and 15% fetal bovine serum (FBS), respectively. A2058, WM852 and WM239 were cultured in RPMI 1640 supplemented with 10% FBS; A375 and HMBC cells were cultured in DMEM supplemented with 10% FBS and SKMEL5 cells were cultured in MEM media supplemented with 10% FBS. Cells were grown in the presence of penicillin and streptomycin (50 I.U./mL).

### Gene expression analysis

RNA was isolated (4 replicates for each treatment) using a Qiagen RNeasy Plus mini-kit and the quality determined using a Bioanalyser (Agilent). Gene expression experiments were performed using the Illumina HT12 array covering more than 47,000 transcripts and known splice variants across the human transcriptome. The raw data was quantile normalized and Illumina control probes were removed from subsequent analysis using BASE <sup>42</sup>. The data were exported to MeV, log2 transformed and gene and sample centered<sup>43</sup>. SAM (significance of microarray analysis) was performed using a two-group comparison; for the siRNA experiment

the groups corresponded to siNEG vs #1 & #3 siCITED1 and for the TGFβ1 experiment the groups corresponded to cells with or without TGFβ1 treatment. In both cases there was a median false discovery risk of 10 false-positive transcripts. Hierarchical clustering was performed to visualize the data. 312 probes were found to be significantly altered in the siRNA experiment (208 upregulated and 104 downregulated) while 1009 probes were significantly altered by TGFβ1 treatment. DAVID was used to assist in functional annotation of the final gene lists<sup>44</sup>

For the publically available data cited, 120 melanoma cell lines from three cohorts (PMID: 17516929, 16827748, 20406975) analyzed by Affymetrix gene expression microarrays were collected, individually MAS5 normalized, and merged into a single cohort. Probe sets were collapsed into single genes and mean-centered across the entire cohort. Data from Harbst et al. were classified using nearest centroid and pearson correlation. Survival analysis and multivariate cox regression methods were performed in R.

# **Transient transfections, promoter-reporter assay and TGFβ1-treatment**

Transient transfections were performed using Lipofectamine2000 and Opti-MEM reduced serum media (Life Technologies) according to the manufactures recommendations. siRNA was purchased from Applied Biosystems and the notations in the text: siNEG, #1 siCITED1 and #3 siCITED1 correspond to the catalogue ID numbers #4390843, #s8965 and #s224062 respectively. For the MITF targeting siRNA; N, siM1 and siM3 correspond to the catalogue ID numbers #4390843, #s8790 and #ss8792, respectively. For the luciferase reporter assay, a Dual-Luciferase

Reporter assay system #E1910 (Promega) was used to measure relative reporter activity on a FLUOstar Omega microplate reader (BMG Labtech). A375 cells were transfected with a luciferase reporter construct harboring 2.3kb of the MITF-M specific promoter in a PGL2 vector<sup>25</sup>. A pRL-Renilla Luciferase reporter vector was used as a control for each transfection. CITED1 was overexpressed using a pRc/CMV containing a N-terminal HA-tagged human CITED1 (transcript isoform 1) referred to as pCITED1 in the text. An empty CMV-promoter expression plasmid, pcDNA3.1 (+) was used as a negative control. Recombinant human transforming growth factor- $\beta$ 1 (TGF $\beta$ 1), #PHG9203 was purchased from Invitrogen. For the A2058 gene expression experiment, cells were exposed to either 5 or 10ng/ml TGF $\beta$ 1 in serum-free media for 24 hours. In the case of the Luciferase reporter assay, cells were serum starved the day after transfection for 3 hours and exposed to 5ng/ml TGF $\beta$ 1 in serum free media for 24 hours prior to harvesting.

417

#### 418 **Antibodies and Immunoblotting**

The following antibodies were used: anti-CITED1, #AB15096 from Abcam; anti-MITF (C5 clone), # MA5-14146 from ThermoScientific; anti-MITF (D5 clone) from Dako, #M3621, (used in Fig. 4c); anti-CDKN1A/P21, #2947 and anti-CDKN1C/P57, #2557 were purchased from Cell Signaling Technology and anti-  $\beta$ -Actin (AC-15), #A5441 from Sigma-Aldrich. Cell lysates were resolved by SDS-PAGE and transferred to 0.45  $\mu$ m PVDF membranes by electroblotting. The membranes were blocked in 5% non-fat milk in TBST prior to incubation with primary antibodies diluted 2.5% non-fat milk. The blots were probed with the appropriate secondary antibodies (Pierce

427 Biotechnology) in 5% non-fat milk. The membranes were developed using ECL (GE  
428 Healthcare).

429

#### 430 **Cell cycle analysis**

431 Flow cytometry was performed on a FACSCalibur (BD Biosciences) and subsequently  
432 analysed using ModFit (Verity House Software). Briefly, following transfection,  
433 confluent cells were detached, washed in 1XPBS and fixed in 70% ethanol. Prior to  
434 analysis they were stained with a propidium iodide solution and a 20G syringe was  
435 used to obtain a homogenous single cell solution. All events were saved (up to  
436 20,000 events per replicate) ungated, using BD Cell Quest and the data exported to  
437 ModFit where following selection of the appropriate ploidy status, a standard auto-  
438 analysis fit using autolinerarity was performed. We found that a 2-cycle aneuploid-  
439 dip/tetraploid was appropriate for HT144 and A2058 while 1-cycle diploid was  
440 suitable for A375.

441

#### 442 **Alamar Blue Assay**

443 The Alamar blue assay reagent was purchased from Invitrogen and used according to  
444 the manufactures' instructions. Briefly, following transfection cells were seeded into  
445 96-well plates at 5000cells/well. In each experiment, for each of the treatments i.e.:  
446 siNEG, #1 siCITED1 and #3 siCITED1, 8 wells spread over 3 rows were used. At the  
447 indicated time points (4, 72, 96 and 120 hours post-transfection), Alamar blue was  
448 added and the cells incubated at 37°C for 2 hours. Fluorescence was measured  
449 (544nm) on a FLUOstar Omega microplate reader (BMG Labtech). The values  
450 obtained at the 4-hour time point were used to normalize the fluorescence readings

to account for any initial cell counting error. Cells were also seeded in parallel for Western blot analysis (72, 96, 120 hours) to ensure successful CITED1 down regulation.

# **Droplet digital PCR**

RNA was isolated from cells using a Qiagen RNeasy Plus mini-kit and quantified using a Nanodrop spectrophotometer (ThermoScientific). cDNA was generated from 50-100ng total RNAs using 'iScript Advanced cDNA synthesis for RT-qPCR' (Bio-Rad). Bio-RAD's 'ddPCR Supermix for Probes' was then used with predesigned TaqMan gene expression assays (Applied Biosystems) consisting of specific primers and FAM labelled probes for MITF (#Hs01117294\_m1), MITF-M isoform specific transcript (Hs00165165\_m1)\*, CITED1 (#Hs00918445\_g1) and IPO8 (#Hs00183533\_m1). (\*There appeared to be no advantage in using the MITF-M isoform specific transcript over the MITF probe that could measure multiple isoforms). A manual cut-off for positive/negative droplets was selected using the Biorad QuantaSoft™ data analysis suite to calculate the relative copies/μl of each transcript.

# **Conflict of interest**

The authors have no conflicts of interest to declare.

# **Acknowledgements**

We would like to thank Dr. Claudia Wellbrock (University of Manchester, UK) for the kind gift of the PG2-MITF promoter-reporter construct and Prof. Toshi Shioda (MGH, Harvard) for the human CITED1 pRc/CMV expression vector. We also would like to

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## Figure Legends

**Figure 1:** TGF $\beta$  induced gene expression in A2058 melanoma cells (a) Distribution of the proliferative and invasive signature score genes relative to the heatmap of gene expression changes induced by TGF $\beta$  treatment. (b) Gene expression heatmap of the proliferative and invasive signature within those genes significantly altered by TGF $\beta$  treatment (1009 transcripts following SAM, median FDR q-value=1%. ‘2006’ refers to the signature list (motif1 and motif2) while ‘2012’ refers to the updated signature derived from further datasets <sup>8</sup>. (c) Western blot of MITF and CITED showing both proteins are suppressed by TGF $\beta$  treatment.  $\beta$ -Actin is used as a loading control.

**Figure 2:** CITED1 expression correlates with MITF expression. (a) The relative MITF and CITED1 expression levels from the publically available gene expression data of 120 melanoma cell lines (Pearson correlation  $r=0.6543$ ,  $p < 0.001$ ). Arrows indicate



the cell lines used in this study. The cell lines are further subdivided into one of either 'invasive' or 'proliferative' phenotype based on expression signature score. (b) A Western blot is shown of the relative protein expression levels of both MITF and CITED1 in our cell lines in good agreement with the transcript levels.

**Figure 3:** CITED1 silencing induces a phenotype switch. (a) 120 melanoma cell lines are shown distributed on the basis of the phenotype score. The HT144 cells chosen to study the effects of CITED1 downregulation are indicated. (b) Following CITED1 downregulation a phenotype shift is observed indicated by their scatter position change according to the average expression score of genes that distinguish invasive from proliferative phenotype. For the 120 melanoma cell lines (Affymetrix platform) the expression score was derived from expression levels of 50 and 54 proliferative and invasive genes with matching genes symbols, respectively, while for the HT144 experiment (Illumina platform), 51 and 54 proliferative and invasive genes with matching genes symbols were retrieved. A Western blot of the degree of protein downregulation of CITED1 at the time of the expression analysis is also shown.  $\beta$ -Actin is used as a loading control (inset). (c) A heatmap comprising the 'invasive' and 'proliferative' signature genes illustrating how they are altered by CITED1 silencing; #1 and #3 denote two separate siRNAs targeting CITED1.

**Figure 4:** CITED1 regulates MITF and its targets genes. (a) A heatmap showing the 312 transcripts identified as significantly changed using a SAM 2-way comparison between siNEG and siCITED1 (#1 & #3 were combined), median FDR q-value =3%). MITF, as well as a cohort of significantly enriched MITF targets, genes associated

with pigmentation, and genes involved in the UV/DNA damage response are highlighted on the right. (b) Western blot confirmation of the effect of silencing CITED1, using siRNA (#1, #3) relative to a negative control siRNA (N), on MITF protein expression in HT144 cells at 24 and 48 hours post-transfection, and the effect of overexpressing CITED1 (pCITED1) relative to an empty vector control (EV) in A2058 cells at 24 hours post-transfection.  $\beta$ -Actin is used as a loading control in each case. (c) Western blot showing the effect of silencing MITF using two siRNAs (siM1, siM3) on both MITF and CITED1 levels in HT144 cells at 48 hours post-transfection relative to a negative control siRNA (N).  $\beta$ -Actin is used as a loading control.

**Figure 5:** CITED1 silencing restrains cell cycle progression and reduces cell viability.

(a) A bar chart showing the % change in cell cycle distribution in #1 siCITED1 treated HT144 cells relative to siNEG treated HT144 cells. The reduction in the total S-phase is shown at 33 hours, 48 hours and 72 hours post-transfection in addition to the corresponding increase in the diploid G1 fraction. (b) Western blots showing upregulation of CDKN1A/P21 following CITED1 silencing in HT144 cells and suppression of CDKN1C/P57 following CITED1 overexpression in A2058 cells. (c) An Alamar Blue based metabolic assay shows a reduction in cell viability over 5 days in HT144 cells treated with siCITED1 relative to those treated with siNEG. Stars indicate significance for siNEG vs. #1 siCITED1 where \*\*\* $p \leq 0.0005$ , \*\* $p \leq 0.005$  and \* $p \leq 0.05$ . In the case of siNEG vs. #1 siCITED1, the difference is significant (\*) at 96 and 120 hours.

**Figure 6:** CITED1 silencing transiently upregulates MITF via promoter activation. (a) A Western blot of HT144 cell lysate samples taken at the indicated time points post-transfection and showing the corresponding levels of MITF protein in #1 siCITED1 and siNEG treated cells. Shown underneath are the changes in mRNA levels of MITF-M, CITED1 and a housekeeper gene IPO8, as measured by specific ddPCR assays over a time course of 4-100 hours following transfection of HT144 cells with either siCITED1 or siNEG. (b) A Western blot of A2058 cell lysate samples taken at the indicated time points post-transfection and showing the corresponding levels of MITF protein between CITED1 overexpression (pCITED1) and empty vector (EV) control. Shown underneath are the changes in mRNA levels of MITF and a housekeeper gene IPO8, as measured by specific ddPCR assays over a time course of 33-72 hours following transfection of A2058 with either pCITED1 or an empty vector control. (c) The relative luciferase activity of the MITF-M promoter reporter measured in lysates of A375 cells transfected with the pCITED1 expression plasmid or empty vector (EV) control and treated with or without TGF $\beta$  for 24 hours (\*\* $p \leq 0.0005$ , \*\* $p \leq 0.005$  and NS= not significant).

**Figure 7:** The CITED1-silenced gene signature predicts patient outcome. (a) In the leftmost panel a scatter plot of the 120 melanoma cell lines are shown distributed on the basis of their 'invasive' or 'proliferative' phenotype signature score and coloured according to the tumour molecular subtypes as defined by Jönsson et al. to illustrate the overlap between the two classification systems<sup>32</sup> In the rightmost panel the data is presented as a heatmap where each gene of the proliferative or invasive signature genes is represented by a horizontal line and the 120 individual cells lines are

676 grouped by molecular tumour subtype (coloured blocks) and shown vertically. (b)  
677 Recurrence free survival (RFS) of primary melanoma patients grouped by gene  
678 expression similarity to the CITED1 (siCITED1) silenced gene signature.

679

#### 680 **Supplementary Figure Legends S1-5**

681 **S1:** A scatter plot of CITED1 expression versus MITF expression in our in-house cell  
682 lines subjected to gene expression analysis in agreement with the observation in the  
683 public data set (see Fig 2a).

684 **S2:** (a) Of the 5578 unique occupied genes (those associated with genomic regions  
685 bound by MITF) identified by Strub et al., 5169 could be found in the Illumina HT12  
686 gene set, mapping to 8272 probes by searching on gene symbol<sup>18</sup>. Of the 312 probes  
687 significantly changed by siCITED1, 41% or 128 are found in this list. (b) A Venn  
688 diagram indicating the number of genes significantly up or down regulated by  
689 siCITED1 represented in the MITF occupied gene list.

690 **S3:** (a) Western blot showing the effect of silencing MITF using two siRNAs (siM1,  
691 siM3) on both MITF and CITED1 levels in WM239A cells relative to a negative control  
692 siRNA (N) at 48 hours post-transfection.  $\beta$ -Actin is used as a loading control. (b)  
693 Western blot showing the effect of silencing MITF using the siRNA siM3 on both  
694 MITF and CITED1 levels in SKMEL5 cells relative to a negative control siRNA (N) at 48  
695 hours post-transfection.  $\beta$ -Actin is used as a loading control.

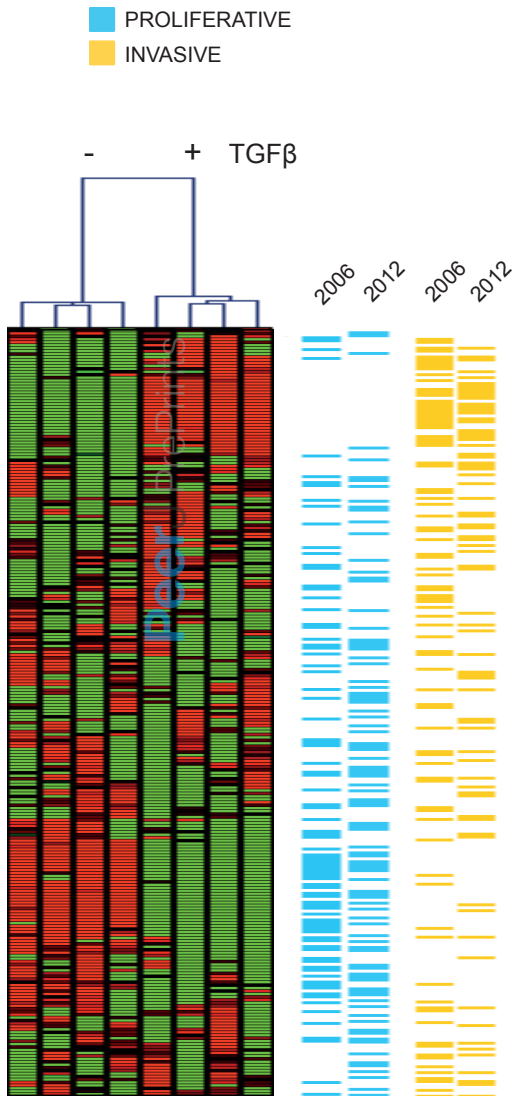
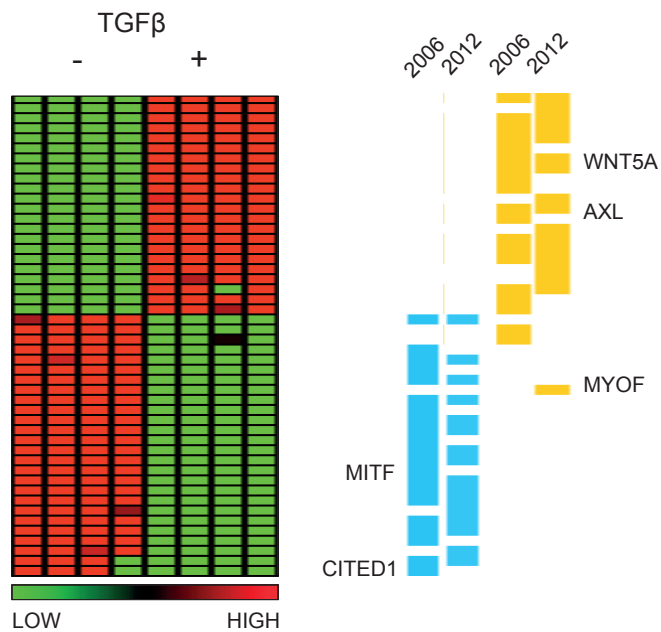
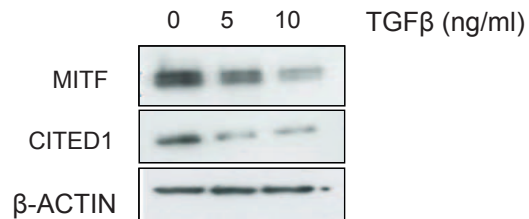
696 **S4:** (a) A bar chart showing the % change in cell cycle distribution in both #1 and #3  
697 siCITED1 treated HT144 cells relative to siNEG treated HT144 cells. The reduction in  
698 total S-phase is shown at 33 hours, 48 hours and 72 hours post-transfection in

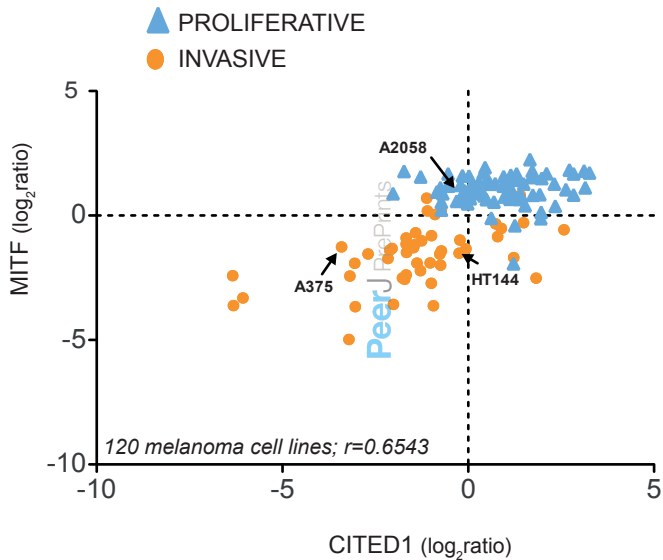
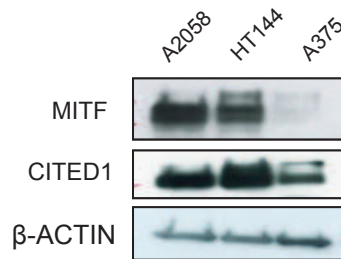
699 addition to the corresponding increase in the diploid G1 fraction. (b) A bar chart  
700 showing the % change in cell cycle distribution in both #1 and #3 siCITED1 treated  
701 relative to siNEG treated A2058 cells. The reduction in total S-phase and  
702 corresponding increase in G1 is shown at 48 hours post-transfection. (c) A bar chart  
703 showing the % change in cell cycle distribution in both #1 and #3 siCITED1 treated  
704 relative to siNEG treated A375 cells. The reduction in total S-phase and  
705 corresponding increase in G1 is shown at 48 hours post-transfection.

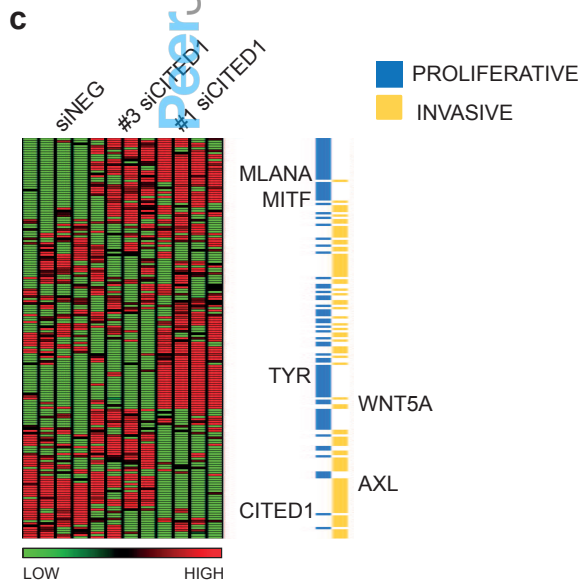
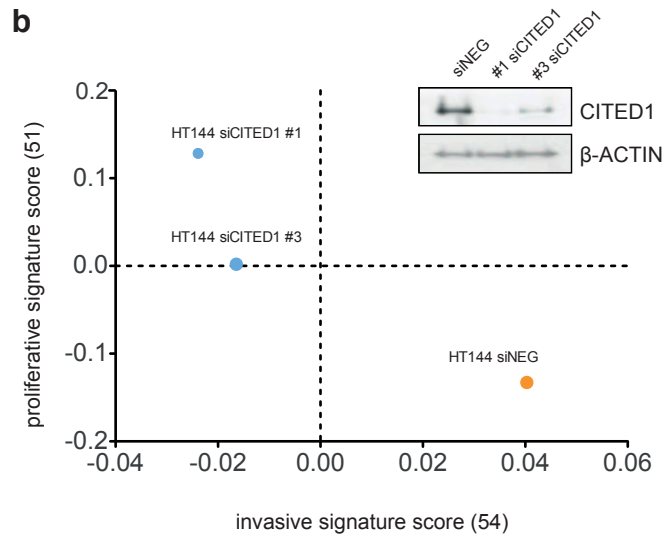
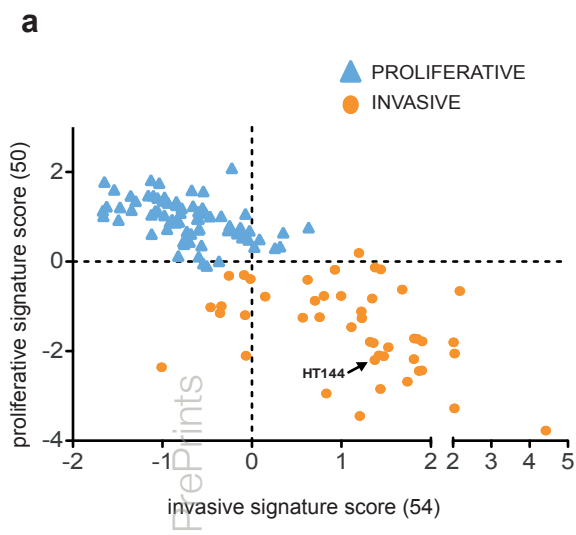
706 **S5:** Western blot of melanoma cells lines indicating the differential expression of the  
707 cell cycle regulator CDKN1A/P21 and CDKN1C/P57 in addition to MITF and CITED1.  $\beta$ -  
708 Actin is used as a loading control.

709 **S6:** The rheostat model of MITF action in melanoma indicating the hypothesised role  
710 of CITED1 in the regulation of MITF expression and melanoma cell behaviour. The  
711 model is an original adaptation of those previously proposed by others, now  
712 incorporating our data<sup>33-35</sup>.

713

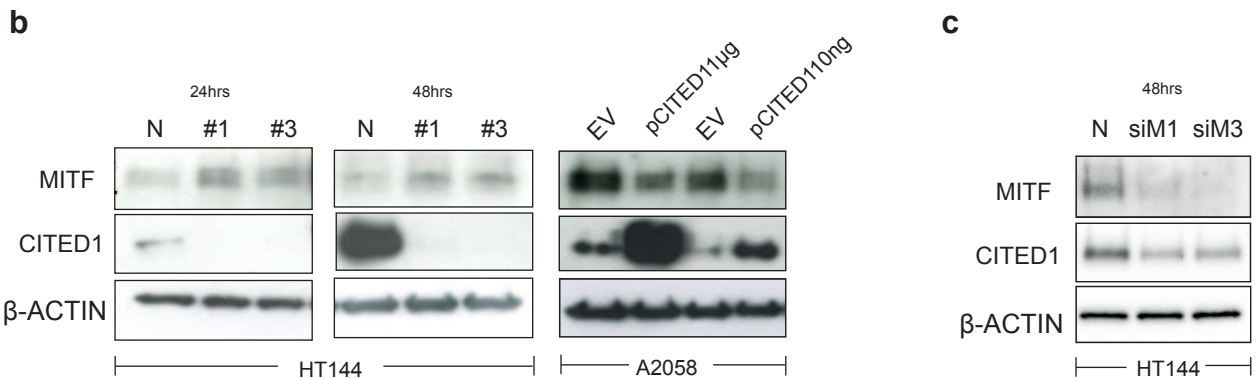
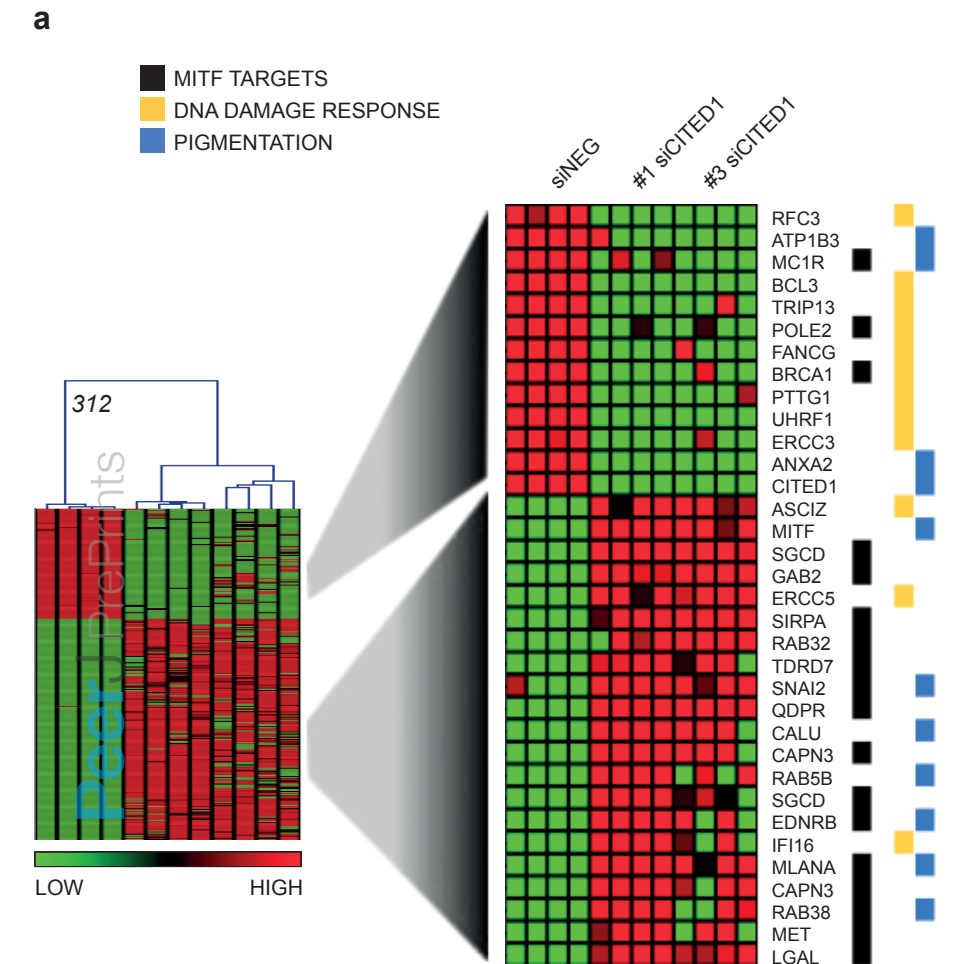
**a****b****c****figure 1**

**a****b****figure 2**

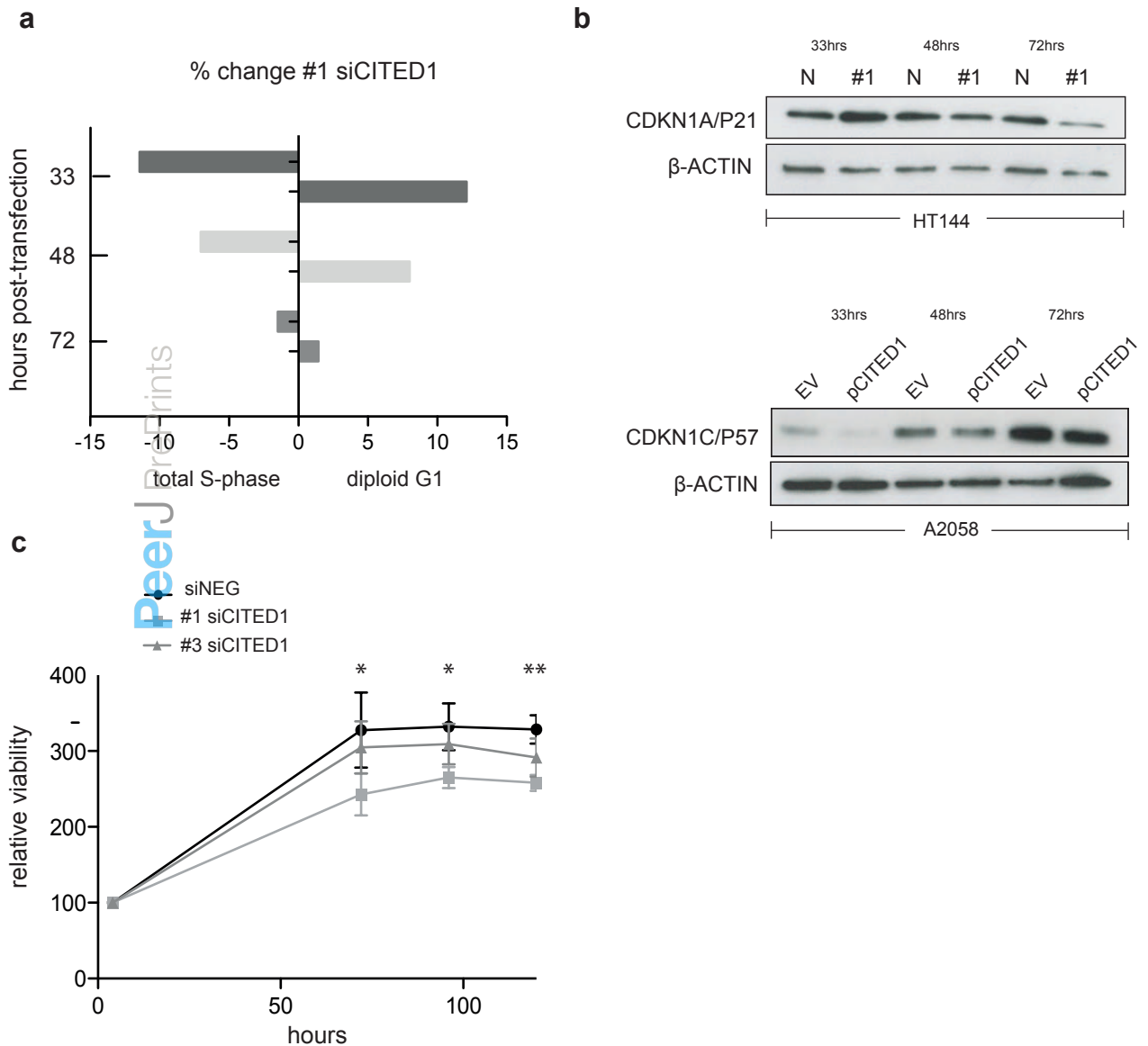


**figure 3**



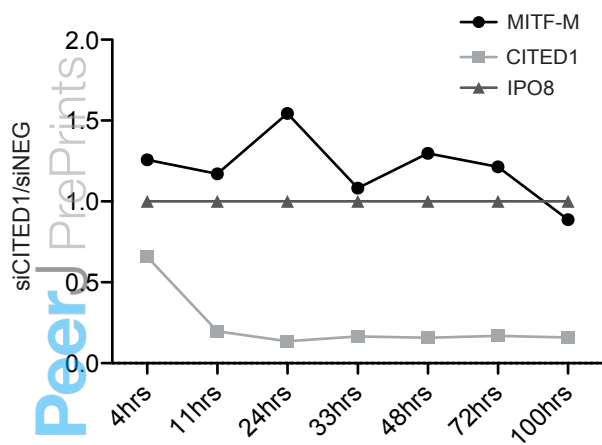
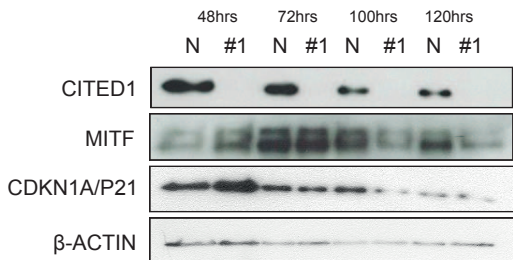


**figure 4**

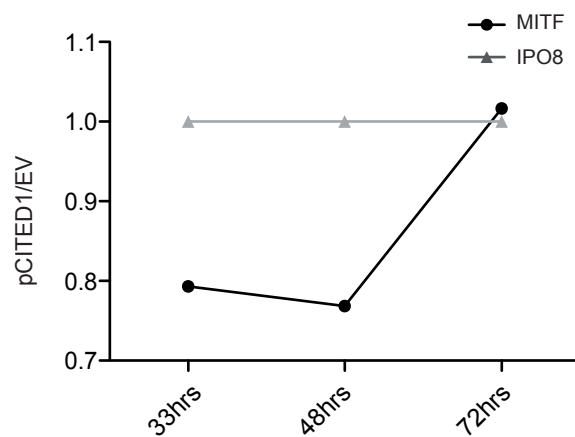
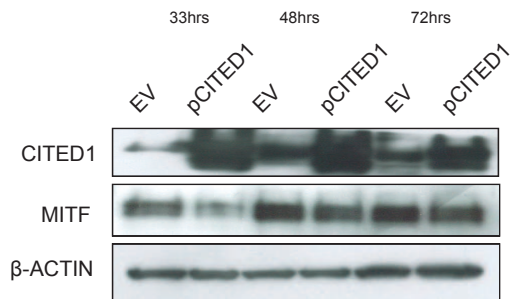


**figure 5**

a



b



c

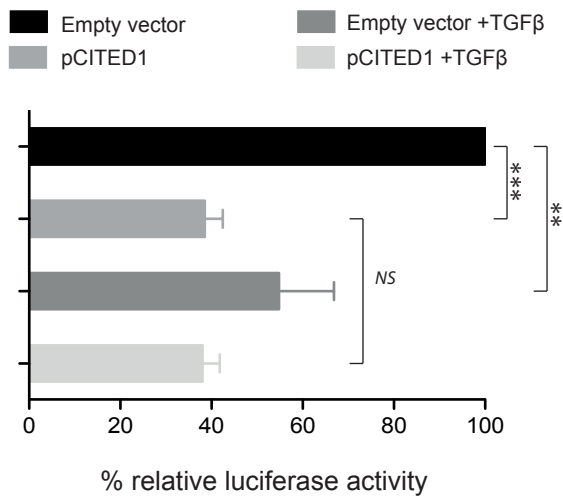
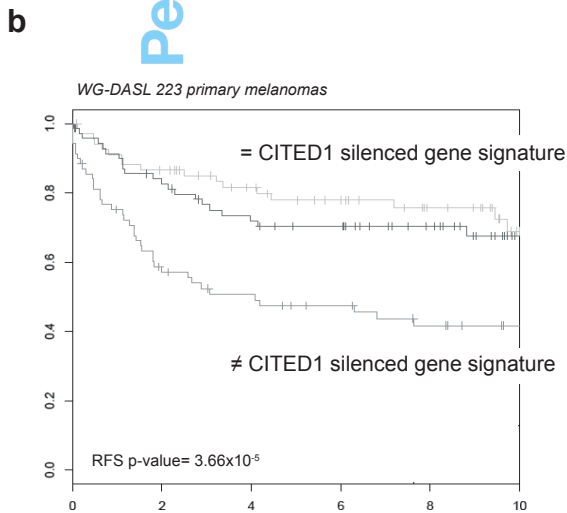
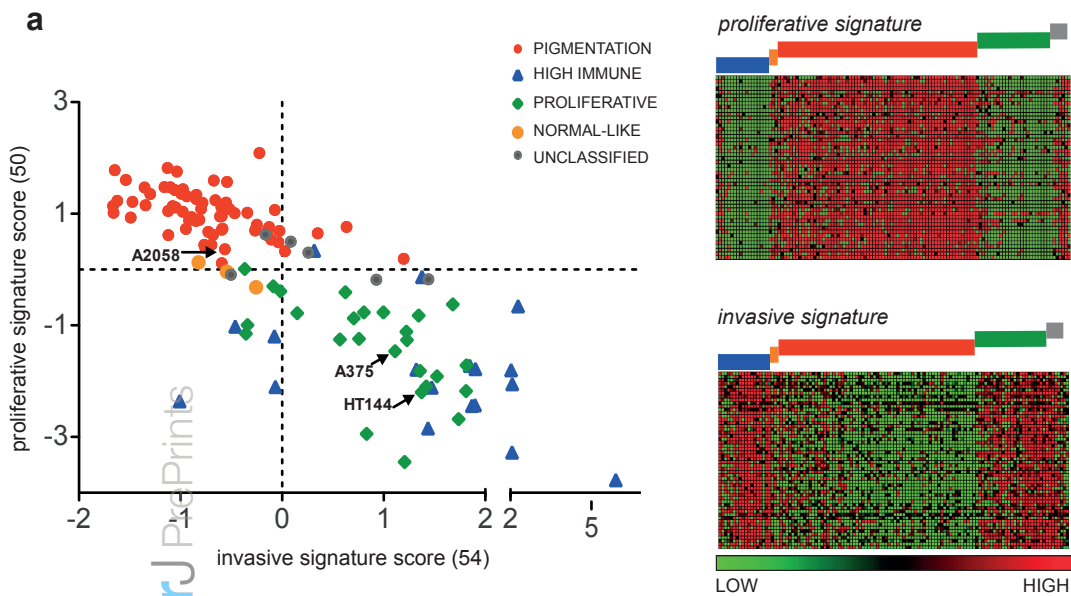
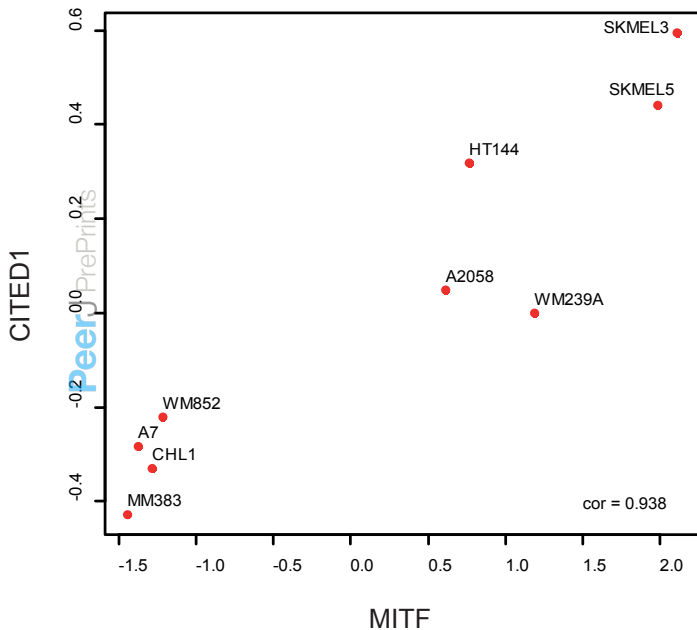


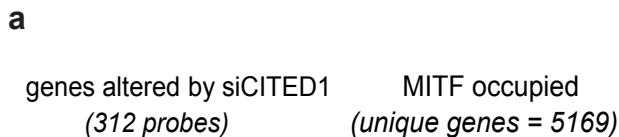
figure 6



**figure 7**

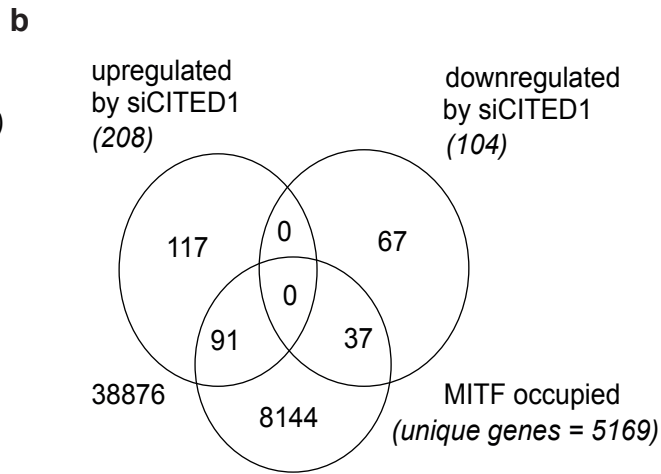


**supplementary figure S1**

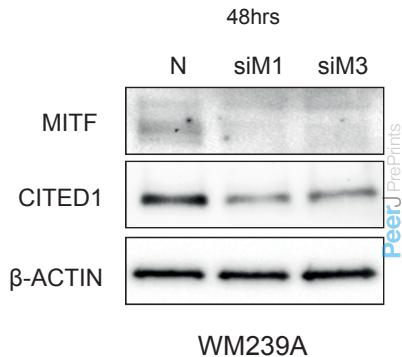
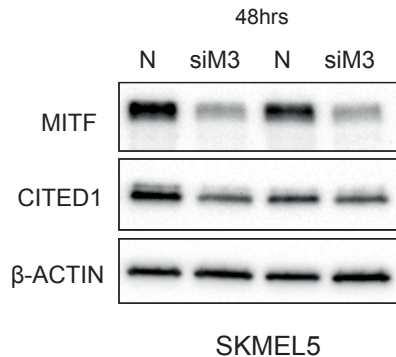


### Illumina probes

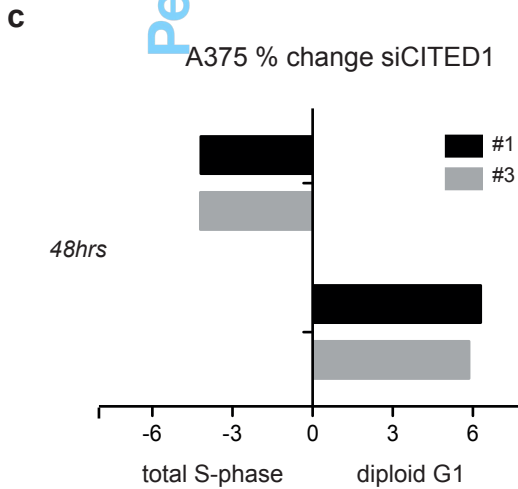
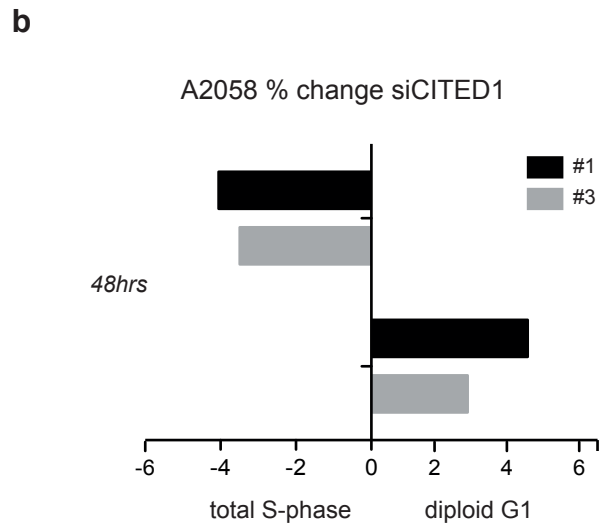
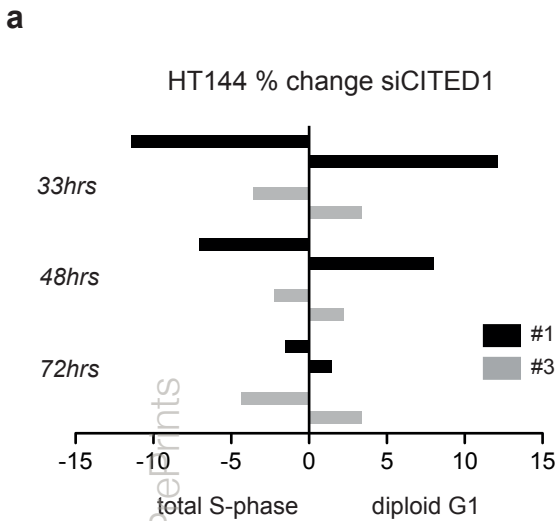
	MITF occupied	Not occupied	Total
sig changed with siCITED1	128	184	312
not signif	8144	38876	47020
Total	8272	39060	47332



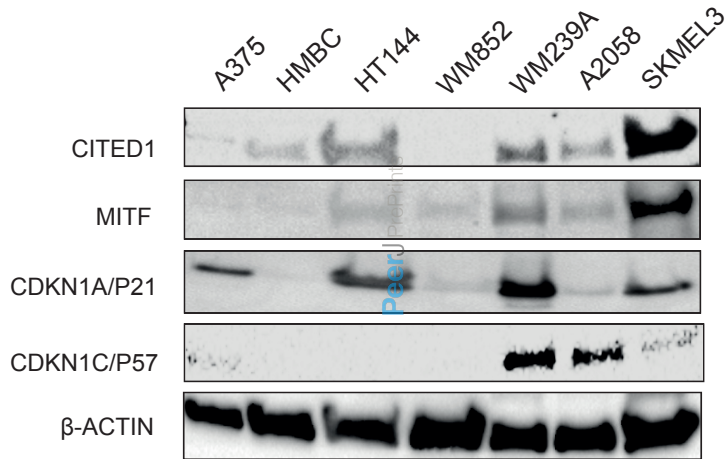
## supplementary figure S2

**a****b**

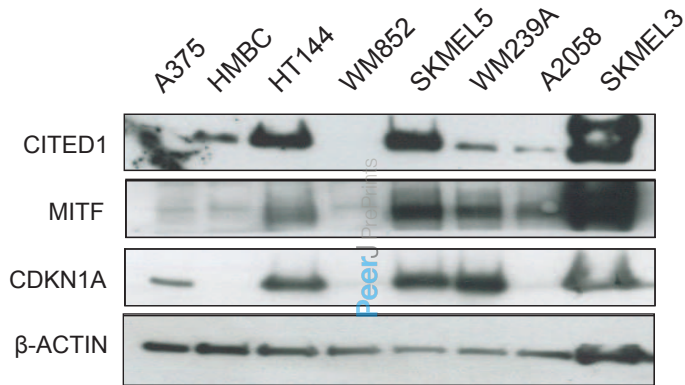
**supplementary figure S3**



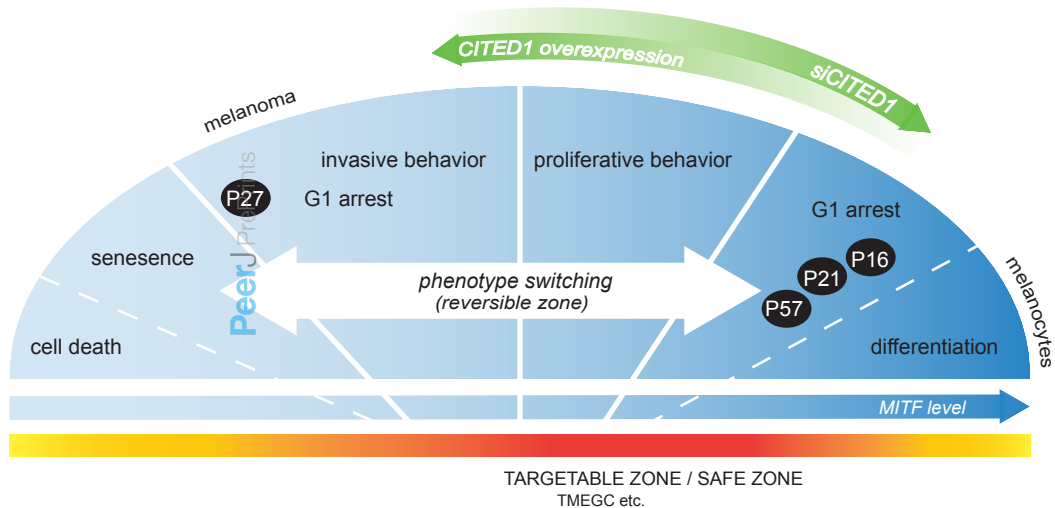




**supplementary figure S5**



**supplementary figure S5**



supplementary figure S6