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Loss of CITED1, an MITF regulator, drives a phenotype switch *in vitro* and can predict clinical outcome in primary melanoma tumours

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

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14 INTRODUCTION

15 CITED1 is the founding member of the CITED (CBP/p300-Interacting
16 transactivator with glutamic acid [E]/aspartic acid [D]-rich C-terminal
17 domain) family of transcriptional co-regulators and was originally cloned from
18 a differential display screen between pigmented mouse B16 melanoma cells
19 and their dedifferentiated weakly-pigmented derivative, B16F10s. This led to
20 speculation that CITED1 or *msg1* (melanocyte specific gene 1) as it was
21 known at that time, was involved in the process of pigmentation (Shioda,
22 Fenner and Isselbacher, 1996). Subsequently, Nair et al. reported that stable
23 overexpression of CITED1 increased the levels of tyrosinase, dopachrome
24 tautomerase (Dct) and melanin in B16 cells, reinforcing the idea that it had a
25 role in melanogenesis (Nair et al., 2001). By 2005, as gene expression
26 profiling became relatively commonplace, CITED1 was identified in several
27 new screens of tumours and cell lines: two studies identified CITED1 as a
28 gene whose expression distinguished nevi from primary melanoma, another
29 found CITED1 to be upregulated in advanced stage melanomas in comparison
30 to benign nevi or melanoma *in situ*, while expression profiling of an *in vitro*
31 progression model identified CITED1 among a signature of genes lost in
32 aggressive melanoma lines relative to primary melanocytes in culture (Ryu et
33 al., 2007; Haqq et al., 2005; Talantov, 2005; Smith, Hoek and Becker, 2005).

34 Based on extensive gene expression profiling of melanoma cell lines *in vitro*,
35 Hoek et al. proposed the 'phenotype-switching' model of melanoma that was
36 independent of the degree of transformation or disease progression, and

sought to explain the observation that melanoma cells altered between two states: those with high proliferative potential that are less invasive and those with high metastatic potential that are less proliferative. These separate but alternating states are controlled by different transcriptional programs and can be defined by specific gene signatures (Hoek, Eichhoff, et al., 2008). 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 signalling 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) (Hoek et al., 2006); (Widmer et al., 2012).

Studies on CITED1 suggest that it is a non-DNA binding nuclear transcriptional co-regulator capable of influencing TGF β induced transcription mediated by ligand-induced SMAD hetero-oligomerization; estrogen-dependent transcription mediated by ER α , and Wnt/ β -Catenin-dependent transcription. These effects are dependent on CITED1-CBP/P300 binding via the conserved CITED family CR2 domain and while CITED1 is thought to act by stabilizing the CBP/P300-ER α interaction, in the case of β -Catenin it acts to repress transcription by competing for binding with CBP/P300 transcriptional co-activators (Shioda et al., 1998; Yahata et al., 2001; 2000; Plisov, 2005).

59 Microphthalmia-associated transcription factor, MITF, acts as a master-
 60 regulator of melanocyte differentiation and as a result has been intensely
 61 studied in the field of melanoma research (Widlund and Fisher, 2003; Levy,
 62 Khaled and Fisher, 2006). It is a basic helix-loop-helix leucine zipper
 63 transcription factor that recognizes E-box and M-box sequences in the
 64 promoter regions of its target genes. Highlighting its importance in the
 65 disease, amplification of MITF locus has been found in >15% of metastatic
 66 melanomas and germline mutations in MITF that predispose carriers to
 67 melanoma development have also been found (Garraway et al., 2005;
 68 Bertolotto et al., 2011; Yokoyama et al., 2011). In melanoma cells the target
 69 genes of MITF include most notably TYR, MC1R, DCT, MLANA involved in the
 70 process of pigmentation; cell cycle regulators such as CDK2 and CDKN1A and
 71 the more recently identified BRCA1 gene that has, with other target DNA
 72 repair genes, defined a role for MITF in the DNA damage response (DDR)
 73 (Strub et al., 2011; Beuret et al., 2011; Giuliano et al., 2010).

74 The regulation of MITF is complex and tightly controlled, exhibiting both
 75 transcriptional and post-translational regulation. There are several transcript
 76 isoforms, of which MITF-M is the dominant form expressed in melanocytes.
 77 Multiple signalling pathways converge on the MITF-M specific promoter that
 78 harbours binding sites for PAX3, SOX10, CREB, FOXD3, LEF-1 and BRN2
 79 among other transcription factors (Yokoyama and Fisher, 2011; Levy, Khaled
 80 and Fisher, 2006). Additionally, the MITF target gene CDKN1A/P21 has been
 81 shown to act as reciprocal transcriptional cofactor independently of its CDK

82 inhibitor function, suggesting the existence of at least one positive feedback
83 loop (Sestáková, Ondrusová and Vachtenheim, 2010).

84 MITF post-translational activity can be affected by phosphorylation,
85 sumoylation, ubiquitination and by binding with proteins that block access to
86 the DNA binding domain such as PIAS3 (Yokoyama and Fisher, 2011; Levy,
87 2001). Oncogenic BRAF (but not wildtype BRAF), which is mutated in up to
88 50% of melanomas, also regulates MITF via simultaneously stimulating MITF
89 activation through ERK phosphorylation, which leads to its subsequent
90 degradation, and by inducing transcription of MITF via BRN2 upregulation
91 (Davies et al., 2002; Wellbrock et al., 2008).

92 The consensus regarding why the cell invests such effort in maintaining
93 control of MITF levels and why there are so many regulatory mechanisms, is
94 that melanocytes and melanoma are exquisitely sensitive to even small
95 variations in MITF expression. Ultimately its activity must be sustained within
96 the narrow window permissive for continued survival and proliferation. In this
97 study, we characterise the role of CITED1 as a novel regulator of MITF in
98 melanoma.

99 MATERIALS AND METHODS

100 **Cell lines**

101 Cell lines were obtained from ATCC. HT144 and SKMEL3 cells were cultured in

102 McCoy's5A supplemented with 10% and 15% foetal bovine serum (FBS),
 103 respectively. A2058, WM852 and WM239 were cultured in RPMI 1640
 104 supplemented with 10% FBS; A375 and HMBC cells were cultured in DMEM
 105 supplemented with 10% FBS and SKMEL5 cells were cultured in MEM media
 106 supplemented with 10% FBS. Cells were grown in the presence of penicillin
 107 and streptomycin (50 I.U./mL) purchased from Invitrogen. Cell media and FBS
 108 were purchased from ThermoScientific, HyClone range. As of March 2014,
 109 these are part of the Life Technologies (Thermo Fisher Scientific) product
 110 portfolio.

111 **Gene expression analysis**

112 RNA was isolated (4 replicates for each treatment) using a Qiagen RNeasy
 113 Plus mini-kit and the quality determined using a Bioanalyser (Agilent).
 114 Replicates were cell samples from separate wells, but plated on the same day
 115 and derived from the same passage number. Gene expression experiments
 116 were performed using the Illumina HT12 array covering more than 47,000
 117 transcripts and known splice variants across the human transcriptome. The
 118 raw data was quantile normalized and Illumina control probes were removed
 119 from subsequent analysis using BASE (Vallon-Christersson et al., 2009). The
 120 data were exported to MeV, log2 transformed and gene and sample centred
 121 (Saeed et al., 2006). SAM (significance of microarray analysis) was performed
 122 using a two-group comparison; for the siRNA experiment the groups
 123 corresponded to siNEG vs. #1 & #3 siCITED1 and for the TGFβ1 experiment
 124 the groups corresponded to cells with or without TGFβ1 treatment. In both

125 cases there was a median false discovery risk of 10 false-positive transcripts.
 126 Hierarchical clustering was performed to visualize the data. 1009 probes
 127 were significantly altered by TGF β 1 treatment while 312 probes were found to
 128 be significantly altered in the siRNA experiment (208 upregulated and 104
 129 downregulated). These data can be found in Supplemental files S7 and S8,
 130 respectively. DAVID was used to assist in functional annotation of the final
 131 gene lists (Huang et al., 2007)

132 For the publically available data cited, 120 melanoma cell lines from three
 133 cohorts ((Johansson, Pavey and Hayward, 2007); (Hoek et al., 2006);
 134 (Greshock et al., 2010)) analysed by Affymetrix gene expression microarrays
 135 were collected, individually MAS5 normalized, and merged into a single
 136 cohort. Probe sets were collapsed into single genes and mean-centred across
 137 the entire cohort. These 120 cell lines and their associated normalized
 138 expression data can be found in supplemental data S10. Data from Harbst et
 139 al. were classified using nearest centroid and Pearson correlation. Survival
 140 analysis and multivariate cox regression methods were performed in R.

141 **Transient transfections, promoter-reporter assay and TGF β 1-** 142 **treatment**

Transient transfections were performed using
 143 Lipofectamine2000 and Opti-MEM reduced serum media (Life Technologies)
 144 according to the manufactures recommendations. siRNA was purchased from
 145 Applied Biosystems and the notations in the text: siNEG, #1 siCITED1 and #3
 146 siCITED1 correspond to the catalogue ID numbers #4390843, #s8965 and

147 #s224062 respectively. For the MITF targeting siRNA; N, siM1 and siM3
 148 correspond to the catalogue ID numbers #4390843, #s8790 and #ss8792,
 149 respectively. For the luciferase reporter assay, a Dual-Luciferase Reporter
 150 assay system #E1910 (Promega) was used to measure relative reporter
 151 activity on a FLUOstar Omega microplate reader (BMG Labtech). A375 cells
 152 were transfected with a luciferase reporter construct harbouring 2.3kb of the
 153 MITF-M specific promoter in a PGL2 vector (Wellbrock et al., 2008). A pRL-
 154 Renilla Luciferase reporter vector was used as a control for each transfection.
 155 CITED1 was overexpressed using a pRc/CMV containing a N-terminal HA-
 156 tagged human CITED1 (transcript isoform 1) referred to as pCITED1 in the
 157 text (Shioda, Fenner and Isselbacher, 1996). An empty CMV-promoter
 158 expression plasmid, pcDNA3.1 (+) was used as a negative control. Recombinant
 159 human transforming growth factor- β 1 (TGF β 1), #PHG9203 was purchased
 160 from Invitrogen. For the A2058 gene expression experiment, cells were
 161 exposed to either 5 or 10ng/ml TGF β 1 in serum-free media for 24 hours. In
 162 the case of the Luciferase reporter assay, cells were serum starved the day
 163 after transfection for 3 hours and exposed to 5ng/ml TGF β 1 in serum free
 164 media for 24 hours prior to harvesting.

165 **Antibodies and Immunoblotting**

166 The following antibodies were used: anti-CITED1, #AB15096 from Abcam;
 167 anti-MITF (C5 clone), # MA5-14146 from ThermoScientific; anti-MITF (D5
 168 clone) from Dako, #M3621, (used in Fig. 4c); anti-CDKN1A/P21, #2947 and

169 anti-CDKN1C/P57, #2557 were purchased from CellSignaling Technology and
 170 anti- β -Actin (AC-15), #A5441 from Sigma-Aldrich. Cell lysates were resolved
 171 by SDS-PAGE (pre-cast gels purchased from Life Technologies) and
 172 transferred to 0.45 μ m PVDF membranes purchased from Millipore by
 173 electroblotting. The membranes were blocked in 5% non-fat milk in TBST
 174 prior to incubation with primary antibodies diluted 2.5% non-fat milk. The
 175 blots were probed with the appropriate secondary antibodies (Pierce
 176 Biotechnology) in 5% non-fat milk. The membranes were developed using
 177 ECL (GE Healthcare).

178 **Cell cycle analysis**

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

190 **Alamar Blue Assay**

191 The Alamar blue assay reagent was purchased from Invitrogen (Life
192 Technologies) and used according to the manufactures' instructions. Briefly,
193 following transfection cells were seeded into 96-well plates at 5000cells/well.
194 In each experiment, for each of the treatments i.e.: siNEG, #1 siCITED1 and
195 #3 siCITED1, 8 wells spread over 3 rows were used. At the indicated time
196 points (4, 72, 96 and 120 hours post-transfection), Alamar blue was added
197 and the cells incubated at 37°C for 2 hours. Fluorescence was measured
198 (544nm) on a FLUOstar Omega microplate reader (BMG Labtech). The
199 values obtained at the 4-hour time point were used to normalize the
200 fluorescence readings to account for any initial cell counting error. Cells were
201 also seeded in parallel for Western blot analysis (72, 96, 120 hours) to ensure
202 successful CITED1 down regulation.

203 **Droplet digital PCR**

204 RNA was isolated from cells using a Qiagen RNeasy Plus mini-kit and
205 quantified using a Nanodrop spectrophotometer (ThermoScientific). cDNA
206 was generated from 50-100ng total RNAs using 'iScript Advanced cDNA
207 synthesis for RT-qPCR' (Bio-Rad). Bio-RAD's 'ddPCR Supermix for Probes' was
208 then used with predesigned TaqMan gene expression assays (Applied
209 Biosystems) consisting of specific primers and FAM labelled probes for MITF
210 (#Hs01117294_m1), MITF-M isoform specific transcript (Hs00165165_m1)*,
211 CITED1 (#Hs00918445_g1) and IPO8 (#Hs00183533_m1). (*There appeared
212 to be no advantage in using the MITF-M isoform specific transcript over the
213 MITF probe that could measure multiple isoforms). A manual cut-off for

214 positive/negative droplets was selected using the Bio-Rad QuantaSoft™ data
215 analysis suite to calculate the relative copies/μl of each transcript.

216 RESULTS

217 **TGFβ induces expression of the invasive signature genes while** 218 **suppressing a cohort of proliferative signature genes including** 219 **CITED1**

220 Hoek et al. noted that many of the genes that defined the invasive
221 phenotype were commonly TGFβ-driven while at the same time only the
222 proliferative signature phenotype cells were sensitive to TGFβ growth
223 inhibition *in vitro* (Hoek et al., 2006). That MITF levels decrease and
224 invasiveness is enhanced in response to TGFβ stimulation was also confirmed
225 subsequently (Pierrat et al., 2012; Pinner et al., 2009). In agreement, we
226 showed that the melanoma cell line A2058 upregulates WNT5A in response to
227 TGFβ exposure and that exogenous Wnt-5a in turn, increased their invasive
228 potential (Jenei et al., 2009). For the present study, in an effort to examine
229 what other phenotype specifying genes were directly regulated by TGFβ, we
230 performed gene expression analysis and found TGFβ treatment resulted in
231 both upregulation of invasive signature genes and suppression of genes
232 characterizing the proliferative phenotype (Fig. 1a). The effect is most
233 pronounced if only those signature genes that were deemed significantly
234 altered by TGFβ treatment are examined. The original signature set defined
235 by Hoek et al., was redefined as more public datasets became available and

236 has a slightly different but overlapping gene profile based on the top ranked
237 differentially expressed genes (Fig. 1b). Both MITF and CITED1 are in the
238 proliferative cohort and their response to TGF β treatment was confirmed at
239 protein level in A2058 cells (Fig. 1C).

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

241 Examination of publically available gene expression data on 120 melanoma
242 cell lines demonstrated a consistent positive correlation between CITED1 and
243 MITF expression ($r=0.6543$). Each cell line was assigned as either
244 'proliferative' or 'invasive' based on a score derived from the averaged
245 expression values of the approximately 50 genes in each defining signature
246 set that had matching gene symbols in our data (Fig. 2a). We also confirmed
247 the correlation in cell lines derived from our own lab (Fig. S1). This was
248 important as inconsistency in interlaboratory phenotype signatures has
249 previously been reported (Widmer et al., 2012). We could additionally confirm
250 expression at the protein level (Fig. 2b)

251 **Gene expression analysis reveals CITED1 silencing can induce a** 252 **phenotype-switch**

253 To investigate the function of CITED1 in melanoma, we transiently
254 downregulated its expression using CITED1 targeting siRNA. We choose the
255 HT144 cell line as it had a relatively high level of detectable CITED1 mRNA
256 and protein expression. A scatterplot of the 120 cell lines assigned as either
257 'proliferative' or 'invasive' based on the maximum matching gene signature
258 score, demonstrates the shift in phenotype that occurs following CITED1

259 downregulation (Fig. 3a,b). A heatmap of the expression profiles clearly
 260 illustrates that the shift is due to a general induction of the ‘proliferative’ and
 261 suppression of the ‘invasive’ cohort (Fig. 3c). It was apparent that the #3
 262 siCITED1 siRNA was not as effective at switching the cells as the #1
 263 siCITED1, this was observed consistently throughout our experiments and
 264 may be due to the fact that #3 siCITED1 was not as successful at silencing
 265 CITED1 (Fig. 3b, *inset*).

266 **CITED1 is a reciprocal regulator of MITF and impacts MITF target** 267 **gene expression**

268 A heatmap highlights the identity of the only the significantly differentially
 269 induced transcripts between siNEG and both #1 & #3 siCITED1 (Fig. 4a). Of
 270 most relevance, we found MITF, a known driver of the proliferative phenotype
 271 switch and many of its previously known downstream targets, these also
 272 included genes categorized by Gene Ontology annotation (GO) as related to
 273 pigmentation and UV/DNA damage response (Fig. 4a) (Hoek, Schlegel, et al.,
 274 2008; McGill et al., 2006; Sánchez-Martín et al., 2002; Strub et al., 2011). We
 275 could confirm that indeed MITF protein levels were affected by siCITED1 in
 276 HT144 cells and that conversely, overexpression of CITED1 in A2058 cells,
 277 resulted in downregulation of MITF (Fig. 4b). Strub et al. identified a large
 278 number of genomic targets of MITF by ChIP-seq analysis (Strub et al., 2011).
 279 A comparison of the genes differentially expressed by siCITED1 compared to
 280 siNEG, revealed that there was significant enrichment of these potential
 281 targets (Fig. S2a). Notably, genes both up and down regulated by siCITED1

282 are represented among genes defined as having MITF-occupied promoters
283 (Fig. S2b). We also found that downregulation of MITF using siRNA in HT144
284 cells (Fig. 4c) and in WM293A, and SKMEL5 cells (Fig. S3a, b) resulted in
285 decreased protein expression of CITED1 suggesting reciprocity between these
286 factors.

287 **Induction of MITF by CITED1 silencing transiently restrains cell cycle** 288 **progression and impacts cell viability**

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

298 Owing to the previously reported dependency of MITF-induced cell cycle
299 arrest on CDKN1A/P21 we investigated the levels of several cyclin-dependant
300 kinase inhibitors following CITED1 silencing (Carreira et al., 2005). We found
301 that CDKN1A/P21 was transiently increased in siCITED1 treated HT144 cells
302 relative to the siNEG treated HT144 cells (Fig 5b). In contrast, in A2058 cells,
303 which do not have detectable levels of CDKN1A/P21 (Fig. S5), the levels of

304 CDKN1C/P57 were suppressed in response to CITED1 overexpression (Fig 5b).
 305 We hypothesised therefore that melanoma cells can utilise either
 306 CDKN1A/P21 or CDKN1C/P57 to mediate cell cycle arrest induced by MITF and
 307 this is reflected in the expression levels of the alternate CDK inhibitors in
 308 different melanoma cell lines (Fig. S5).

309

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

314 **The effect of CITED1 silencing on MITF is transient and mediated via** 315 **promoter activation**

316 We observed that the peak upregulation of MITF and CDKN1A/P21 protein
 317 following siCITED1 treatment varied from transfection to transfection, being
 318 seen between 24-48 hours post-transfection but appearing as unchanged or
 319 even downregulated after this time (Fig. 6a, *upper panel*). In agreement, later
 320 timepoints of the cell cycle analysis (≥ 72 hours) exhibited little or no
 321 change in G1/S-phase distribution or even a reverse pattern (Fig. 5a HT144,
 322 and data not shown: A2058, A375). We therefore sought to examine the
 323 transcriptional dynamics more closely, map the changes in MITF following
 324 CITED1 silencing and see if they corresponded to cell behaviour and changes
 325 at the protein level. We used a quantitative droplet digital PCR based assay
 326 (Bio-Rad) to measure mRNA in HT144 cells transfected with siCITED1#1 and

327 siNEG as well as A2058 cells transiently overexpressing CITED1 compared to
 328 an empty vector control. MITF, CITED1 and IPO8 specific primers and probes
 329 were used to measure exact copies/ μ l of each mRNA from aliquots of the
 330 same cDNA solution. Plots of siCITED1(copies/ μ l)/siNEG(copies/ μ l) and
 331 EV(copies/ μ l)/pCITED1(copies/ μ l) show the directional change in MITF and
 332 CITED1 relative to the housekeeper IPO8. CITED1 expression is rapidly
 333 suppressed following siCITED1 treatment of HT144 cells, concomitant with an
 334 upregulation of MITF that diminishes over time and in fact is suppressed by
 335 100 hours in accordance with observations at the protein level (Fig 6a, *lower*
 336 *panel*). In contrast, overexpression of CITED1 in A2058 cells results in
 337 transient suppression of MITF at both protein and transcript level (Fig. 6b,
 338 *upper and lower panels*).

339 The rapid MITF transcriptional response to CITED1 manipulation suggested to
 340 us that the effect could be directly mediated at the promoter level. To test
 341 this hypothesis, we over expressed an MITF-M promoter-reporter construct
 342 and CITED1 in A375 cells. We chose A375 cells, as while they had less
 343 endogenous CITED1 and MITF than HT144 or A2058 so as not to cause
 344 interference with the assay, we also knew that they could respond
 345 adequately as they had an identical G1 accumulation/S-phase decrease to
 346 both HT144 and A2058 cells following CITED1 silencing (Fig S4c). TGF β
 347 treatment was used as a positive control for repression of the MITF-M
 348 promoter. CITED1 transfection led to significant suppression of the MITF-M
 349 promoter luciferase activity relative to the empty vector control, as did TGF β

350 treatment alone or combination with CITED1 overexpression (Fig. 6c). There
351 did not appear to be an additive or synergistic effect using both TGF β
352 treatment and CITED1 overexpression suggesting TGF β may be dependent on
353 CITED1 for MITF suppression.

354 **The CITED1-silenced gene signature predicts outcome in primary** 355 **melanoma**

356 The 'proliferative' and 'invasive' signature phenotypes have served to define
357 the gene expression classification of melanoma cell lines. However, primary
358 tumours and metastatic lesions have also been molecularly classified into
359 several distinct groups by gene expression profiling (Harbst et al., 2012;
360 Jonsson et al., 2010). The four-class structure found in tumours consists of
361 the 'pigmentation', 'proliferative', 'high-immune' and 'normal-like' subgroups
362 with a subset falling into an unclassifiable cohort (Jonsson et al., 2010). We
363 used the same tumour classification to subtype the 120 cell lines that had
364 publically available expression data and could show that the tumour
365 'pigmentation' subgroup that highly expresses MITF, corresponds to the cell
366 line 'proliferative' phenotype described by Hoek et al. Accordingly, the
367 tumour 'proliferative' and 'high-immune' subgroups comprise the cell line
368 'invasive' phenotype (Fig. 7a). It is worth noting that the names of the tumour
369 subgroups were derived from a description of the differentially expressed
370 genes that comprised each molecular classification while the 'invasive-
371 proliferative' switching phenotypes were named to reflect the *behaviour*
372 exhibited by lines classified by one or other signature. This distinction helps

373 to explain the confusing occurrence that both classifications have a group
374 referred to as 'proliferative' although they are not equivalent.

375 The overlap between the primary tumour classifying and cell line classifying
376 systems allows us to infer that CITED1 expression is most likely restricted to
377 a subset of MITF high 'pigmentation' subtype tumours. As the tumour
378 subtype classification was shown to be prognostically significant in primary
379 melanomas we were interested to know if CITED1 expression itself was
380 independently predictive of outcome. Previously we reported on the analysis
381 of 223 primary lesions using the Illumina WG-DASL protocol (Harbst et al.,
382 2012). As the CITED1 probe in this assay did not produce reliable data we
383 instead derived a CITED1-silenced gene signature score based on the
384 differentially expressed genes from the HT144 siCITED1 experiment (Fig. 3).
385 We therefore effectively created a multi-gene surrogate expression signature
386 rather than using CITED1 gene expression itself. We subsequently
387 interrogated the gene expression data on the primary melanoma lesions
388 using a nearest centroid approach derived from the CITED1-silenced gene
389 signature. This revealed that primary melanomas with a gene expression
390 signature most similar to the CITED1-silenced signature (CITED1low-class)
391 had a significantly better outcome than those with a signature most disparate
392 from the CITED1-silenced signature (CITED1high-class) (Fig. 7b). Importantly,
393 the CITED1 signature classing had independent prognostic information (HR
394 1.85, CI 0.30-0.98, $p=0.044$) from the AJCC staging system (HR 5.05, CI 2.42-
395 10.55, $p=1.64 \times 10^{-5}$). Accepting the caveat that we depend here on a proxy

396 gene-signature, these data indirectly imply that CITED1 expression is a
397 potential prognostic indicator in primary melanomas and the transcriptional
398 program influenced by CITED1 expression determines tumour behaviour *in*
399 *vivo*.

400 DISCUSSION

401 One seemingly paradoxical observation from our study and previous
402 investigations is that although CITED1 behaves as a negative regulator of
403 MITF, both their expression levels appear positively correlated across cells
404 lines and tumours. We maintain that this observation simply reflects the fact
405 that where there are high levels of MITF, high levels of its negative regulator
406 are also required. The evidence of the tight control exerted over MITF levels
407 in melanocytes and melanoma simply speaks to the necessity of the cell to
408 maintain a level compatible with survival and proliferation, in a type of
409 biological 'sweet-spot' facilitating tumour progression. The cellular effects of
410 both extremes i.e.: very low or high levels of MITF, have been elegantly
411 described by a rheostat model in order to reconcile the conflicting
412 observations of the effects of manipulating MITF *in vitro*, and the fact that
413 counter-intuitively, a lineage-specifying differentiation factor can behave as a
414 potent oncogene (Hoek and Goding, 2010; Carreira et al., 2006; Cheli,
415 Giuliano, et al., 2011). The rheostat model (Fig. S6) attempts to explain why
416 MITF silencing can block cells in G1 and induce senescence, while it is also
417 possible to induce a G1 arrest by MITF overexpression via CDKN2A/P16 or
418 CDKN1A/P21 and, as we now propose, potentially also via CDKN1C/P57

419 (Carreira et al., 2006; Loercher et al., 2005; Carreira et al., 2005). At the
 420 extreme high end of MITF expression lies differentiated melanocytic cells,
 421 while the lowest levels can lead to senescence and irreversible cell death.
 422 Between these two extremes however it is thought that melanoma cells can
 423 oscillate from a low-MITF 'invasive' to a high-MITF 'proliferative' state via
 424 phenotype-switching.

425 We hypothesise that the role of CITED1 in melanoma is to maintain levels of
 426 MITF compatible with tumour progression and effectively tip the balance in
 427 favour of cell cycle progression rather than MITF-induced G1-arrest. This is
 428 supported by our findings that downregulation of CITED1 using siRNA results
 429 in a phenotype switch to a more pigmented state driven by increased MITF
 430 expression and concomitant upregulation of CDKN1A/P21. Conversely, we
 431 could observe that downregulation of MITF resulted in suppression of CITED1
 432 in several cell lines suggesting the existence of a classical feedback loop
 433 where low MITF levels result in inhibition of its negative regulator. MITF
 434 induced cell cycle arrest was previously shown to be dependent on
 435 CDKN1A/P21 and it was demonstrated that MITF does not cause a cell cycle
 436 arrest in CDKN1A-deficient mouse embryo fibroblasts (MEF) cells (Carreira et
 437 al., 2005). However, our data indicate that in melanoma cells deficient in
 438 CDKN1A/P21, the alternative CDK inhibitor CDKN1C/P57 is expressed and
 439 responsive to MITF.

440 Interestingly, while we observed upregulation of most MITF targets following

441 CITED1 silencing, we found that BRCA1 and other DNA damage response
 442 (DDR) genes were suppressed, suggesting that CITED1 downregulation does
 443 not necessarily facilitate transcription of all MITF targets. It is thus tempting
 444 to speculate that rather than simply acting to induce MITF and thereby
 445 indirectly enhance transcription of its target genes, that CITED1 may also act
 446 as co-factor for MITF at various genomic locations differentially modulating
 447 the MITF target gene response at individual promoters. One way that this
 448 might be achieved is via MITF-CITED1 competition for CBP/P300 binding as
 449 CBP/P300 is a known transcriptional coregulator for MITF, although it is not
 450 required for transcription of all MITF targets (Vachtenheim, Šestáková and
 451 Tuháčková, 2007; Yan et al., 2013).

452 As suggested by Sáez-Ayala et al., anti-cancer therapy should be ideally
 453 independent of dominant or 'driver' genetic alterations so that subclonal
 454 populations do not gain a subsequent advantage and the same holds true in
 455 the case of targeting a specific phenotype. Successful therapy will
 456 necessarily need to switch or push the subdominant phenotype into the
 457 susceptible state or eradicate the phenotype resistant to treatment. This
 458 approach was initially championed by Cheli et al., who proposed the
 459 eradication of low-MITF cells as a therapeutic strategy (Cheli, Guiliano, et al.,
 460 2011). Indeed the idea of lineage-specific therapy has been subsequently
 461 proved in principle using methotrexate (MTX) to first activate MITF
 462 expression, in turn activating the tyrosinase enzyme, and thereby sensitising
 463 tumour cells to a tyrosinase-processed anti-folate prodrug (TMECG) (Sáez-

464 Ayala et al., 2013). However, even without drug targeting, induction of MITF,
 465 to levels seen in melanocytes or above what is tolerated by even the highly
 466 pigmented tumour cell types, would seem to be incompatible with melanoma
 467 progression as it can inhibit cell cycle progression (Goding, 2013). Our
 468 assertion is that CITED1 acts to repress MITF in order to maintain its level in a
 469 range compatible with tumorigenesis. This assertion as a consequence
 470 naturally suggests CITED1 as therapeutic target for genetic manipulation.
 471 Successful implementation of such a strategy would result in cell specific
 472 enhancement of MITF expression and increased susceptibility to the type of
 473 chemotherapeutic eradication demonstrated by Sáez-Ayala et al. or
 474 potentially induction of CDKN1A/p21 or CDKN1C/p57-dependent cell growth
 475 arrest even without further intervention (Fig. S6) (Sáez-Ayala et al., 2013).

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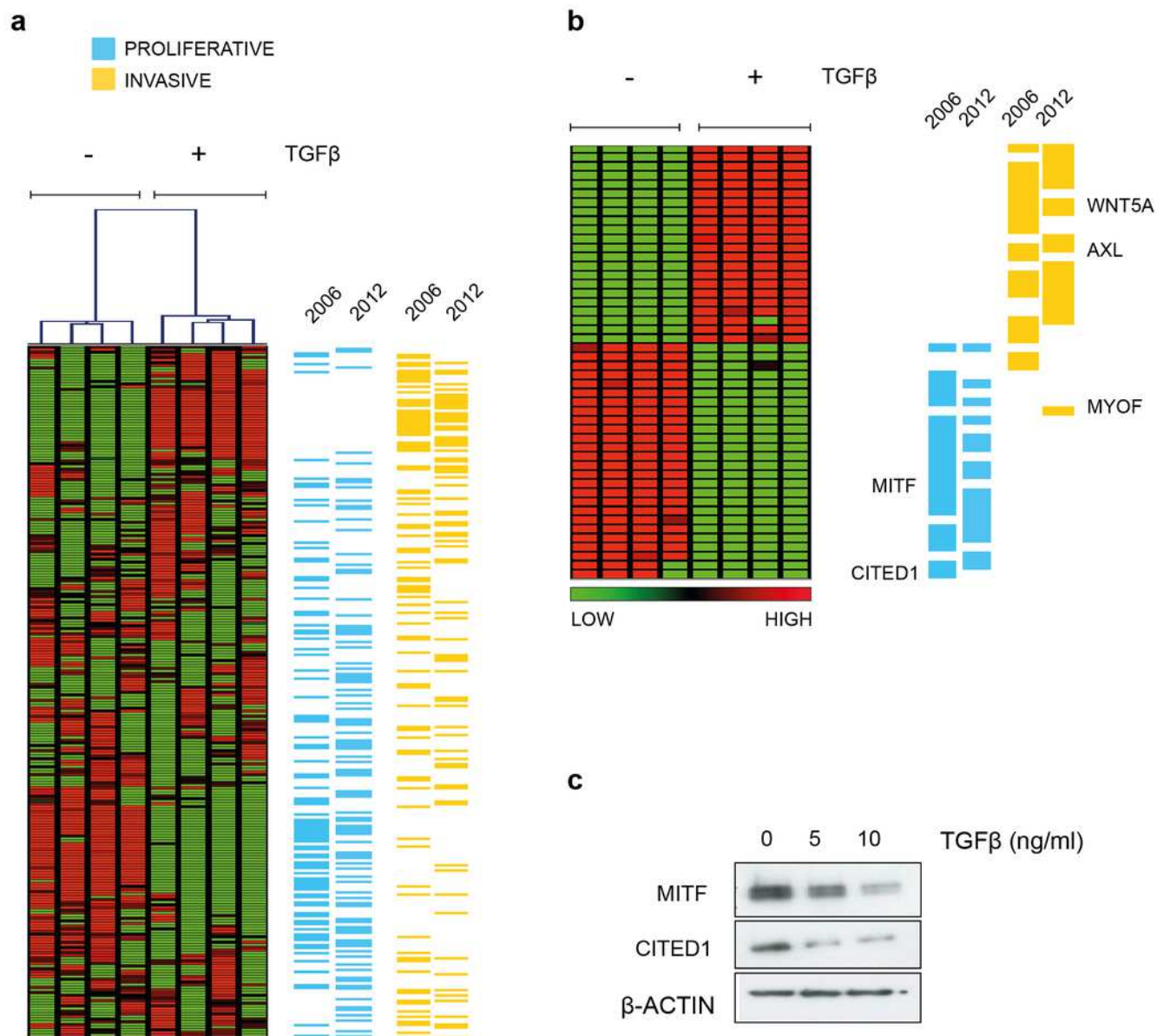
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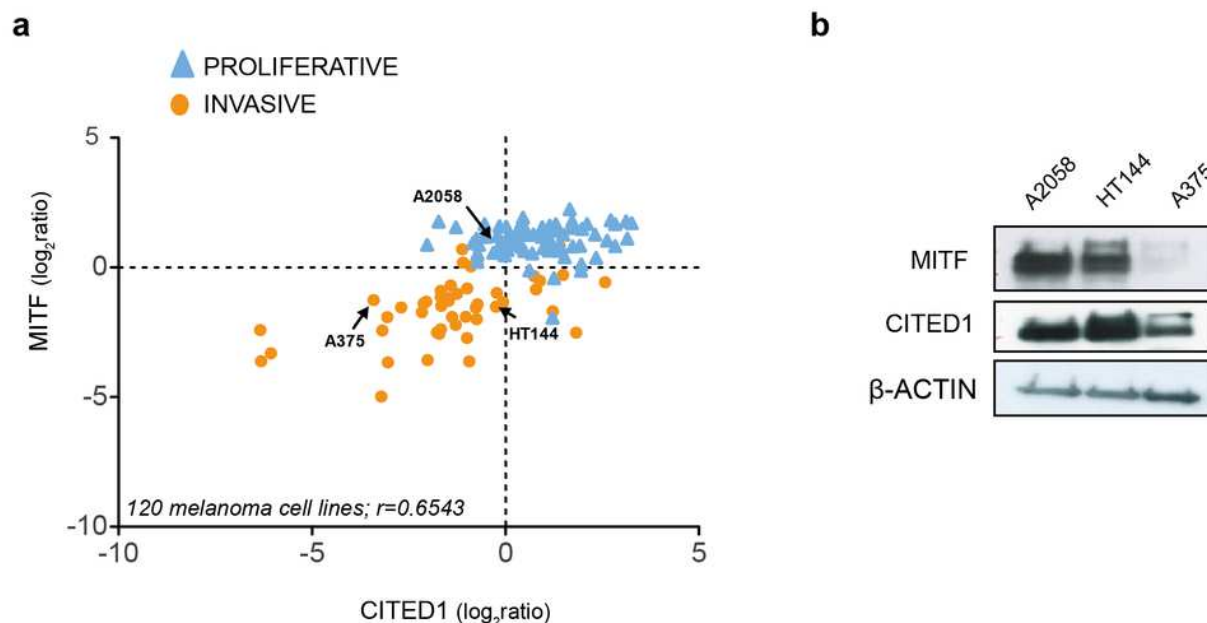
TGF β induced gene expression in A2058 melanoma cells

(a) Distribution of the proliferative and invasive signature score genes relative to the heatmap of gene expression changes induced by TGF β treatment. Vertical lanes in the hierarchical cluster represent TGF β treated or untreated replicates in the SAM 2-group comparison b) Gene expression heatmap of the proliferative and invasive signature within those genes significantly altered by TGF β treatment (1009 transcripts following SAM, median FDR q-value=1%, the full list can be found in S7). '2006' refers to the original signature list (motif1 and motif2, see Hoek et al. 2006) while '2012' refers to the updated signature derived from further datasets (Widmer et al. 2012). These lists can also be found in supplementary data S9. (c) Western blot of MITF and CITED showing both proteins are suppressed by TGF β treatment. β -Actin is used as a loading control.



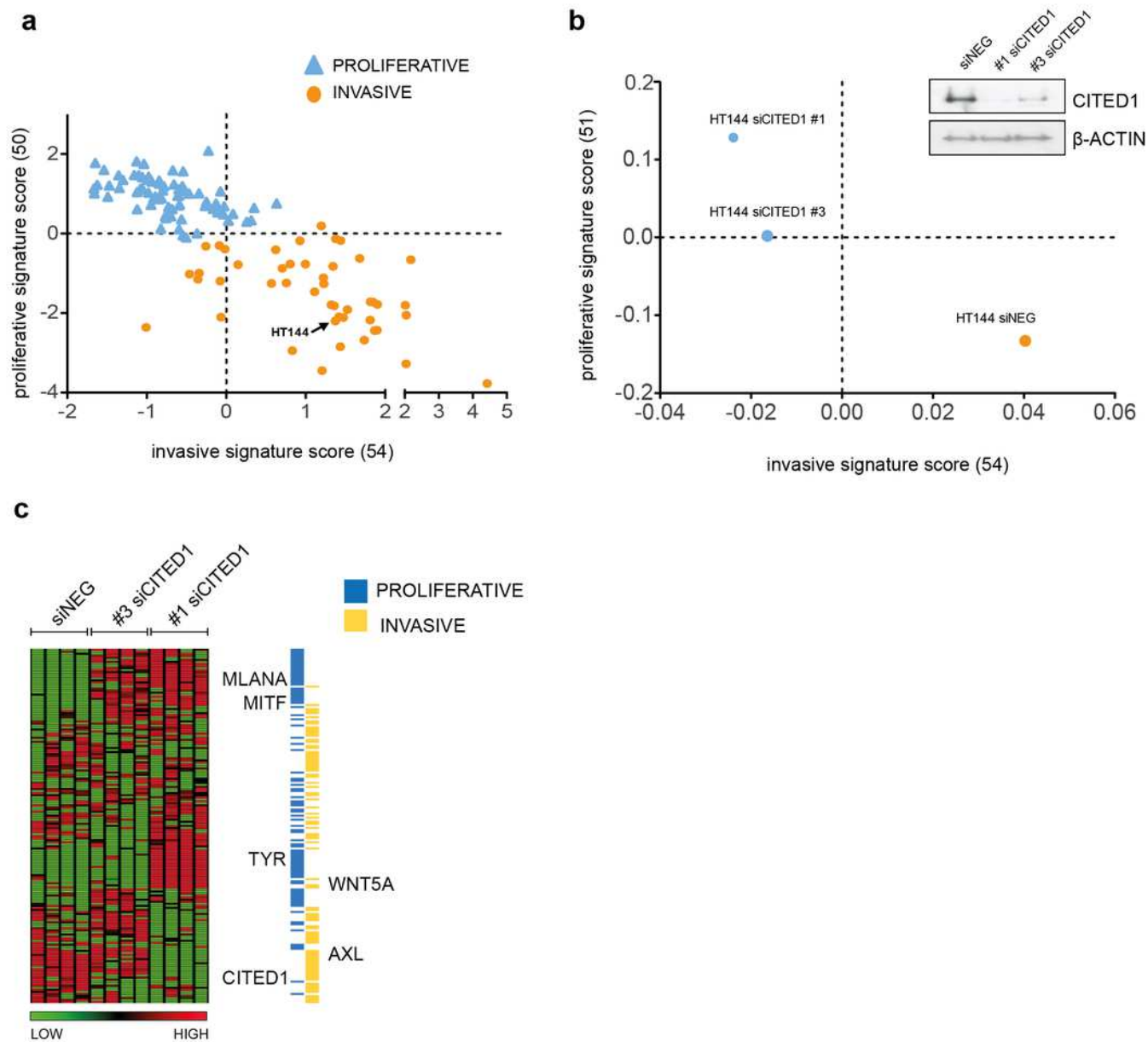
CITED1 expression correlates with MITF expression

(a) The relative MITF and CITED1 expression levels from the publicly available gene expression data of 120 melanoma cell lines (Pearson correlation $r=0.6543$, $p < 0.001$). The full list of cells lines and expression data can be found in S10. Arrows indicate the cell lines used in this study. The cell lines are further subdivided into one of either 'invasive' of '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.



CITED1 silencing induces a phenotype switch

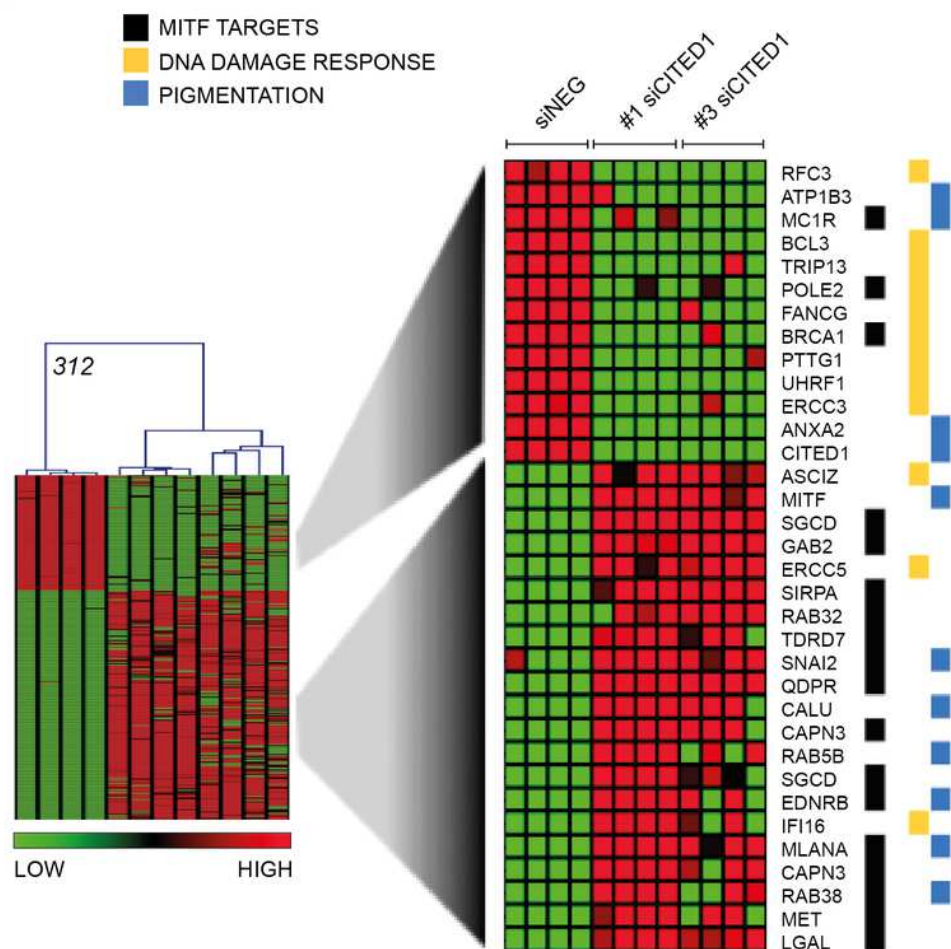
(a) 120 melanoma cell lines are shown distributed on the basis of the phenotype score. The HT144 cells chosen to study the effects of CITED1 downregulation are indicated. (b) Following CITED1 downregulation a phenotype shift is observed indicated by their scatter position change according to the average 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. β -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 and the vertical lanes represent the 4 replicates per treatment.



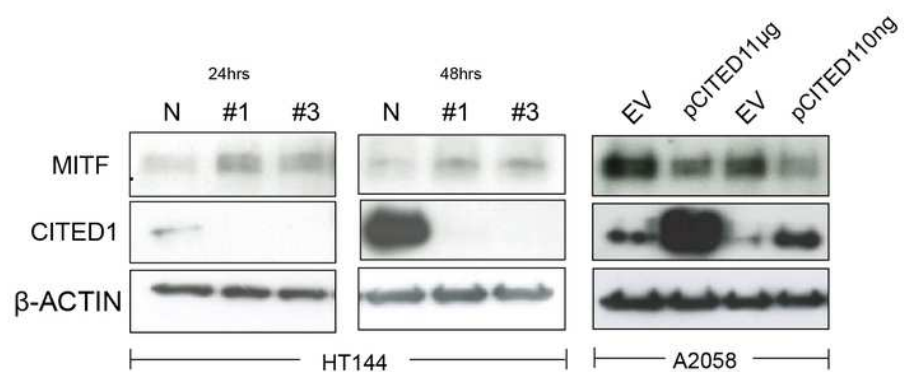
CITED1 regulates MITF and its targets genes

(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%. The four replicates from each condition, siNEG and, siCITED1#1 and siCITED1#3, respectively, group together in the hierarchical cluster shown. The full list of genes can be found in S8. 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. β -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). β -Actin is used as a loading control.

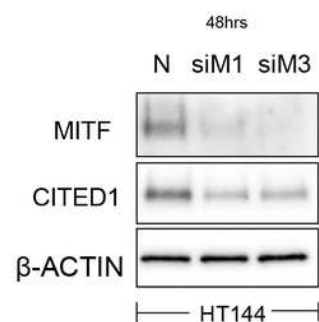
a



b

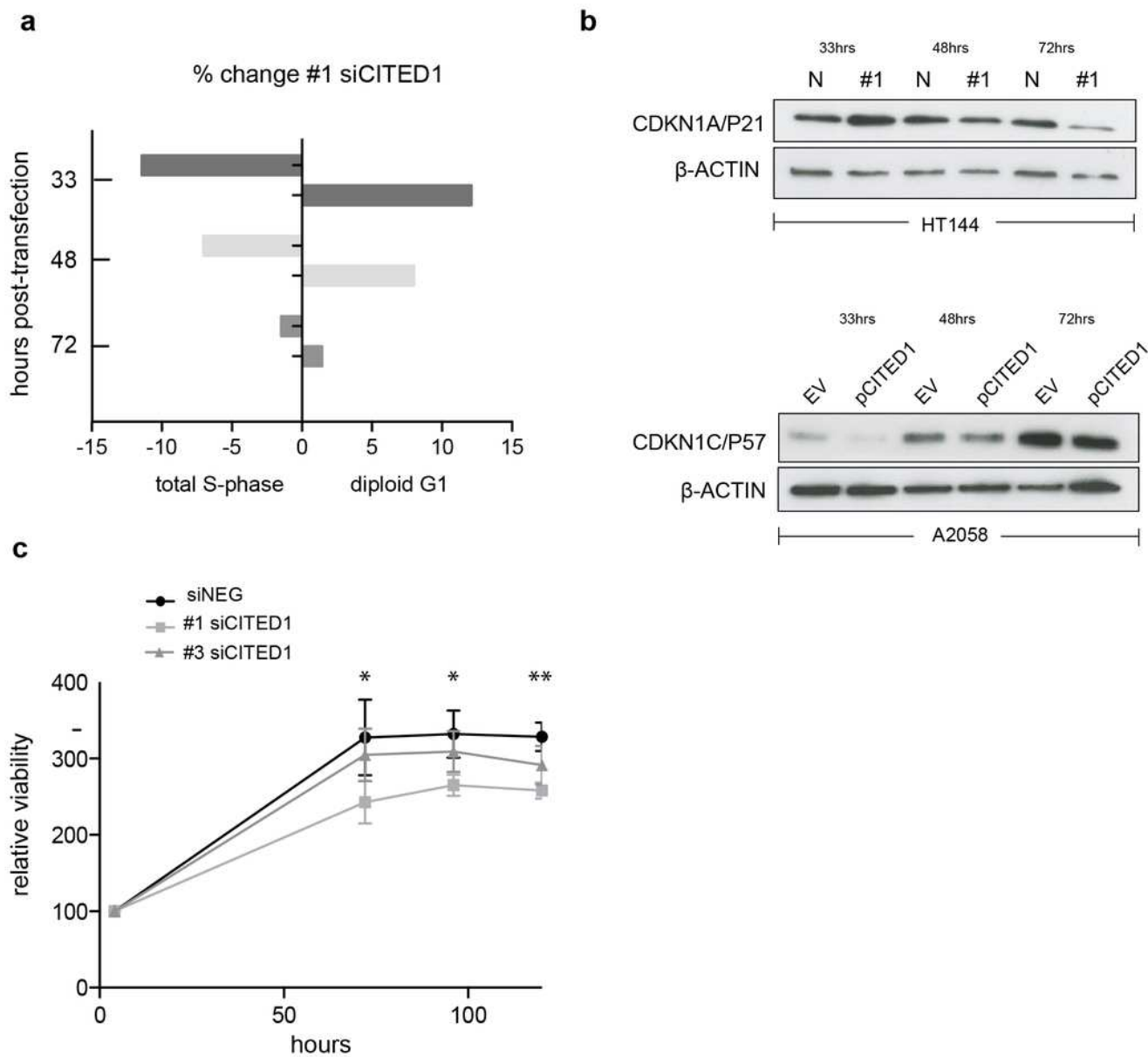


c



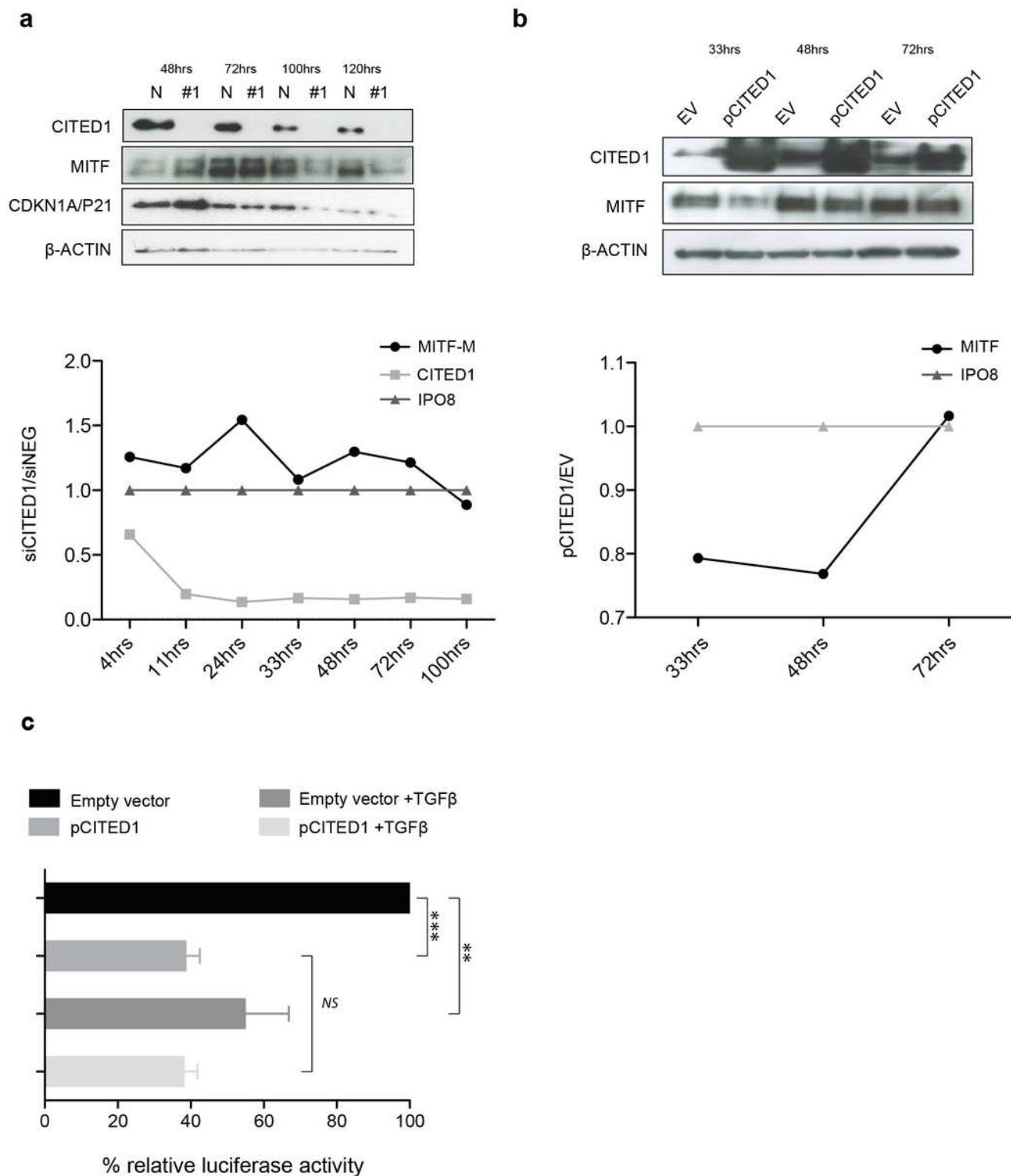
CITED1 silencing restrains cell cycle progression and reduces cell viability

(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.



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 β for 24 hours (***p $\leq$ 0.0005, **p $\leq$ 0.005 and NS= not significant).



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 (Jonsson et al. 2010) 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 grouped by molecular tumour subtype (coloured blocks) and shown vertically. (b) Recurrence free survival (RFS) of primary melanoma patients grouped by gene expression similarity to the CITED1 (siCITED1) silenced gene signature.

