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

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 CITED1 expression is repressed by TGFβ in addition to other 'proliferative' signature genes 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 (**C**BP/p300-**i**nteracting

16   **t**ransactivator 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 non-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 et al.

22   1996). Subsequently, Nair et al. reported that stable overexpression of

23   CITED1 increased the levels of tyrosinase, dopachrome tautomerase (Dct)

24   and melanin in B16 cells, reinforcing the idea that it had a role in

25   melanogenesis(Nair et al. 2001). By 2005, as gene expression profiling

26   became relatively commonplace, CITED1 was identified in several new

27   screens of tumors and cell lines: two studies identified CITED1 as a gene

28   whose expression distinguished nevi from primary melanoma, another found

29   CITED1 to be upregulated in advanced stage melanomas in comparison to

30   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

33   et al. 2007; Haqq et al. 2005; Talantov 2005; Smith et al. 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

37 sought to explain the observation that melanoma cells altered between two

38 states: those with high proliferative potential that are less invasive and those

39 with high metastatic potential that are less proliferative. These separate but

40 alternating states are controlled by different transcriptional programs and

41 can be defined by specific gene signatures (Hoek, Eichhoff, et al. 2008a).

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

43 'proliferative' group, while the 'invasive' signature group is characterized by

44 expression of negative regulators of the Wnt signaling pathway (WNT5A,

45 DKK1, CTGF). CITED1 expression was associated with the proliferative

46 pathway signature and subsequently confirmed in an updated and expanded

47 data set to be significantly correlated with the proliferative phenotype

48 (P<1.00E-05, http://www.jurmo.ch/hopp, accessed 19 March 2013) (Hoek et

49 al. 2006),(Widmer et al. 2012).

50 Studies on CITED1 suggest that it is a non-DNA binding nuclear

51 transcriptional co-regulator capable of influencing TGFβ induced transcription

52 mediated by ligand-induced SMAD hetero-oligomerization; estrogen-

53 dependent transcription mediated by ERα, and Wnt/β-Catenin-dependent

54 transcription. These effects are dependent on CITED1-CBP/P300 binding via

55 the conserved CITED family CR2 domain and while in the case of ERα, CITED1

56 is thought to act by stabilizing the CBP/P300-ERα interaction, in the case of β-

57 Catenin it acts to repress transcription by competing for binding with

58 CBP/P300 transcriptional co-activators (Shioda et al. 1998; Yahata 2001;

59 Yahata 2000; Plisov 2005).

60 Microphthalmia-associated transcription factor, MITF, acts as a master-
61 regulator of melanocyte differentiation and as a result has been intensely
62 studied in the field of melanoma research(Widlund & Fisher 2003; Levy et al.
63 2006). It is a basic helix-loop-helix leucine zipper transcription factor that
64 recognizes E-box and M-box sequences in the promoter regions of its target
65 genes. Highlighting its importance in the disease, amplification of MITF locus
66 has been found in >15% of metastatic melanomas and germline mutations in
67 MITF that predispose carriers to melanoma development have also been
68 found (Garraway et al. 2005; Bertolotto et al. 2011; Yokoyama et al. 2011). In
69 melanoma cells the target genes of MITF include most notably TYR, MCIP,
70 DCT, MLANA involved in the process of pigmentation; cell cycle regulators
71 such as CDK2 and CDKN1A and the more recently identified BRCA1 gene that
72 has, with other target DNA repair genes, defined a role for MITF in the DNA
73 damage response (DDR) (Strub et al. 2011; Beuret et al. 2011; Giuliano et al.
74 2010).

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

82 reciprocal transcriptional cofactor independently of its CDK inhibitor function,

83 suggesting the existence of at least one positive feedback loop (Sestáková et

84 al. 2010).

85 MITF post-translational activity can be affected by phosphylation,

86 sumoylation, ubiquitination and by binding with proteins that block access to

87 the DNA binding domain such as PIAS3 (Yokoyama & Fisher n.d.; Levy 2001).

88 Oncogenic BRAF (but not wildtype BRAF), which is mutated in up to 50% of

89 melanomas, also regulates MITF via simultaneously stimulating MITF

90 activation through ERK phosphorylation, which leads to its subsequent

91 degradation, and by inducing transcription of MITF via BRN2 upregulation

92 (Davies et al. 2002; Wellbrock et al. 2008).

93 The consensus regarding why the cell invests such effort in maintaining

94 control of MITF levels and why there are so many regulatory mechanisms, is

95 that melanocytes and melanoma are exquisitely sensitive to even small

96 variations in MITF expression. Ultimately its activity must be sustained within

97 the narrow window permissive for continued survival and proliferation. In this

98 study, we characterise the role of CITED1 as a novel regulator of MITF in

99 melanoma.

100 MATERIALS AND METHODS

101 **Cell lines**

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

103 McCoy's5A supplemented with 10% and 15% fetal bovine serum (FBS),

104 respectively. A2058, WM852 and WM239 were cultured in RPMI 1640

105 supplemented with 10% FBS; A375 and HMBC cells were cultured in DMEM

106 supplemented with 10% FBS and SKMEL5 cells were cultured in MEM media

107 supplemented with 10% FBS. Cells were grown in the presence of penicillin

108 and streptomycin (50 I.U./mL).

109 **Gene expression analysis**

110 RNA was isolated (4 replicates for each treatment) using a Qiagen RNeasy

111 Plus mini-kit and the quality determined using a Bioanalyser (Agilent). Gene

112 expression experiments were performed using the Illumina HT12 array

113 covering more than 47,000 transcripts and known splice variants across the

114 human transcriptome. The raw data was quantile normalized and Illumina

115 control probes were removed from subsequent analysis using BASE (Vallon-

116 Christersson et al. 2009). The data were exported to MeV, log2 transformed

117 and gene and sample centered(AI et al. 2003). SAM (significance of

118 microarray analysis) was performed using a two-group comparison; for the

119 siRNA experiment the groups corresponded to siNEG vs #1 & #3 siCITED1

120 and for the TGFβ1 experiment the groups corresponded to cells with or

121 without TGFβ1 treatment. In both cases there was a median false discovery

122 risk of 10 false-positive transcripts. Hierarchical clustering was performed to

123 visualize the data. 312 probes were found to be significantly altered in the

124 siRNA experiment (208 upregulated and 104 downregulated) while 1009

125 probes were significantly altered by TGFβ1 treatment. DAVID was used to

126 assist in functional annotation of the final gene lists (Huang et al. 2007)

127 For the publically available data cited, 120 melanoma cell lines from three

128 cohorts (PMID: 17516929, 16827748, 20406975) analyzed by Affymetrix

129 gene expression microarrays were collected, individually MAS5 normalized,

130 and merged into a single cohort. Probe sets were collapsed into single genes

131 and mean-centered across the entire cohort. Data from Harbst et al. were

132 classified using nearest centroid and pearson correlation. Survival analysis

133 and multivariate cox regression methods were performed in R.

134 **Transient transfections, promoter-reporter assay and TGFβ1-**

135 **treatment**   Transient transfections were performed using

136 Lipofectamine2000 and Opti-MEM reduced serum media (Life Technologies)

137 according to the manufactures recommendations. siRNA was purchased from

138 Applied Biosystems and the notations in the text: siNEG, #1 siCITED1 and #3

139 siCITED1 correspond to the catalogue ID numbers #4390843, #s8965 and

140 #s224062 respectively. For the MITF targeting siRNA; N, siM1 and siM3

141 correspond to the catalogue ID numbers #4390843, #s8790 and #ss8792,

142 respectively. For the luciferase reporter assay, a Dual-Luciferase Reporter

143 assay system #E1910 (Promega) was used to measure relative reporter

144 activity on a FLUOstar Omeaga microplate reader (BMG Labtech). A375 cells

145 were transfected with a luciferase reporter construct harboring 2.3kb of the

146   MITF-M specific promoter in a PGL2 vector (Wellbrock et al. 2008). A pRL-

147   Renilla Luciferase reporter vector was used as a control for each transfection.

148   CITED1 was overexpressed using a pRc/CMV containing a N-terminal HA-

149   tagged human CITED1 (transcript isoform 1) referred to as pCITED1 in the

150   text. An empty CMV-promoter expression plasmid, pcDNA3.1 (+) was used a

151   negative control. Recombinant human transforming growth factor-β1 (TGFβ1),

152   #PHG9203 was purchased from Invitrogen. For the A2058 gene expression

153   experiment, cells were exposed to either 5 or 10ng/ml TGFβ1 in serum-free

154   media for 24 hours. In the case of the Luciferase reporter assay, cells were

155   serum starved the day after transfection for 3 hours and exposed to 5ng/ml

156   TGFβ1 in serum free media for 24 hours prior to harvesting.

157   **Antibodies and Immunoblotting**

158   The following antibodies were used: anti-CITED1, #AB15096 from Abcam;

159   anti-MITF (C5 clone), # MA5-14146 from ThemoScientific; anti-MITF (D5

160   clone) from Dako, #M3621, (used in Fig. 4c); anti-CDKN1A/P21, #2947 and

161   anti-CDKN1C/P57, #2557 were purchased from Cell Signaling Technology and

162   anti- β-Actin (AC-15), #A5441 from Sigma-Aldrich. Cell lysates were resolved

163   by   SDS-PAGE   and   transferred   to   0.45 μm   PVDF   membranes   by

164   electroblotting. The membranes were blocked in 5% non-fat milk in TBST

165   prior to incubation with primary antibodies diluted 2.5% non-fat milk. The

166   blots   were   probed   with   the   appropriate   secondary   antibodies   (Pierce

167 Biotechnology) in 5% non-fat milk. The membranes were developed using

168 ECL (GE Healthcare).

Cell cycle analysis

170 Flow cytometry was performed on a FACSCalibur (BD Biosciences) and

171 subsequently analysed using ModFit (Verity House Software). Briefly,

172 following transfection, confluent cells were detached, washed in 1XPBS and

173 fixed in 70% ethanol. Prior to analysis they were stained with a propidium

174 iodide solution and a 20G syringe was used to obtain a homogenous single

175 cell solution. All events were saved (up to 20,000 events per replicate)

176 ungated, using BD Cell Quest and the data exported to ModFit where

177 following selection of the appropriate ploidy status, a standard auto-anaylsis

178 fit using autolinerarity was performed. We found that a 2-cycle aneuploid-

179 dip/tetraploid was appropriate for HT144 and A2058 while 1-cycle diploid was

180 suitable for A375.

Alamar Blue Assay

182 The Alamar blue assay reagent was purchased from Invitrogen and used

183 according to the manufactures' instructions. Briefly, following transfection

184 cells were seeded into 96-well plates at 5000cells/well. In each experiment,

185 for each of the treatments i.e.: siNEG, #1 siCITED1 and #3 siCITED1, 8 wells

186 spread over 3 rows were used. At the indicated time points (4, 72, 96 and

187 120 hours post-transfection), Alamar blue was added and the cells incubated

188 at 37°C for 2 hours. Fluorescence was measured (544nm) on a FLUOstar

189 Omeaga microplate reader (BMG Labtech). The values obtained at the 4-hour

190 time point were used to normalize the fluorescence readings to account for

191 any initial cell counting error. Cells were also seeded in parallel for Western

192 blot analysis (72, 96, 120 hours) to ensure successful CITED1 down

193 regulation.

194 **Droplet digital PCR**

195 RNA was isolated from cells using a Qiagen RNeasy Plus mini-kit and

196 quantified using a Nanodrop spectrophotometer (ThermoScientific). cDNA

197 was generated from 50-100ng total RNAs using 'iScript Advanced cDNA

198 synthesis for RT-qPCR' (Bio-Rad). Bio-RAD's 'ddPCR Supermix for Probes' was

199 then used with predesigned TaqMan gene expression assays (Applied

200 Biosystems) consisting of specific primers and FAM labelled probes for MITF

201 (#Hs01117294_m1), MITF-M isoform specific transcript (Hs00165165_m1)*,

202 CITED1 (#Hs00918445_g1) and IPO8 (#Hs00183533_m1). (*There appeared

203 to be no advantage in using the MITF-M isoform specific transcript over the

204 MITF probe that could measure multiple isoforms). A manual cut-off for

205 positive/negative droplets was selected using the Biorad QuantaSoft™ data

206 analysis suite to calculate the relative copies/µl of each transcript.

207 RESULTS

TGFβ 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 TGFβ-driven while at the same time only the proliferative signature phenotype cells were sensitive to TGFβ growth inhibition *in vitro* (Hoek et al. 2006). That MITF levels increase and invasiveness is enhanced in response to TGFβ stimulation was also confirmed subsequently (Pierrat et al. 2012; Pinner et al. 2009). In agreement, we showed that the melanoma cell line A2058 upregulates WNT5A in response to TGFβ exposure and that exogenous Wnt-5a in turn increased their invasive potential(Jenei et al. 2009). For the present study, in an effort to examine what other phenotype specifying genes were directly regulated by TGFβ, we performed gene expression analysis and found TGFβ treatment resulted in both upregulation of invasive signature genes and suppression of genes characterizing the proliferative phenotype (Fig. 1a). The effect is most pronounced if only those signature genes that were deemed significantly altered by TGFβ 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 TGFβ treatment was confirmed at protein level in A2058 cells (Fig. 1C).

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

232 Examination of publically available gene expression data on 120 melanoma

233 cell lines demonstrated a consistent positive correlation between CITED1 and

234 MITF expression (r=0.6543). Each cell line was assigned as either

235 'proliferative' or 'invasive' based on a score derived from the averaged

236 expression values of the approximately 50 genes in each defining signature

237 set that had matching gene symbols in our data (Fig. 2a). We also confirmed

238 the correlation in cell lines derived from our own lab (Fig. S1). This was

239 important as inconsistency in interlaboratory phenotype signatures has

240 previously been reported (Widmer et al. 2012). We could additionally confirm

241 expression at the protein level (Fig. 2b)

242 **Gene expression analysis reveals CITED1 silencing can induce a**

243 **phenotype-switch**

244 To investigate the function of CITED1 in melanoma, we transiently

245 downregulated its expression using CITED1 targeting siRNA. We choose the

246 HT144 cell line as it had a relatively high level of detectable CITED1 mRNA

247 and protein expression. A scatterplot of the 120 cell lines assigned as either

248 'proliferative' or 'invasive' based on the maximum matching gene signature

249 score demonstrates the shift in phenotype that occurs following CITED1

250 downregulation (Fig. 3a,b). A heatmap of the expression profiles clearly

251 illustrates that the shift is due to a general induction of the 'proliferative´ and

252 suppression of the 'invasive' cohort (Fig. 3c). It was apparent that the #3

253 siCITED1 siRNA was not as effective at switching the cells as the #1

254 siCITED1, this was observed consistently throughout our experiments and

255 may be due to the fact that #3 siCITED1 was not as successful at silencing

256 CITED1 (Fig. 3b, *inset*).

257 CITED1 is a reciprocal regulator of MITF and impacts MITF target

258 gene expression

259 A heatmap of only the significantly differentially induced transcripts between

260 siNEG and both #1 & #3 siCITED1 highlights the identity of the genes

261 responsible for the expression switch (Fig. 4a). Of most relevance, we found

262 MITF, a known driver of the proliferative phenotype switch and many of its

263 previously known downstream targets, these also included genes categorized

264 by Gene Ontology annotation (GO) as related to pigmentation and UV/DNA

265 damage response (Fig. 4a) (Hoek, Schlegel, et al. 2008b; McGill et al. 2006;

266 Sánchez-Martín et al. 2002; Strub et al. 2011). We could confirm that indeed

267 MITF protein levels were affected by siCITED1 in HT144 cells and that

268 conversely, overexpression of CITED1 in A2058 cells, resulted in

269 downregulation of MITF (Fig. 4b). Strub et al. identified a large number of

270 genomic targets of MITF by ChIP-seq analysis (Strub et al. 2011). A

271 comparison of the genes differentially expressed by siCITED1 compared to

272 siNEG, revealed that there was significant enrichment of these potential

273 targets (Fig. S2a). Notably, genes both up and down regulated by siCITED1

274 are represented among genes defined as having MITF-occupied promoters

275 (Fig. S2b). We also found that downregulation of MITF using siRNA in HT144

276 cells (Fig. 4c) and in WM293A, and SKMEL5 cells (Fig. S3a,b) resulted in

277 decreased protein expression of CITED1 suggesting reciprocity between these

278 factors.

279 Induction of MITF by CITED1 silencing transiently restrains cell cycle

280 progression and impacts cell viability

281 To investigate the effect of CITED1 silencing on melanoma cells behaviour we

282 analysed the cell cycle distribution following siRNA treatment, by flow

283 cytometry. In siCITED1 treated HT144 cells we saw G1 accumulation as

284 indicated by an increase in the diploid G1 fraction and a concomitant

285 reduction in the total S-phase fraction peaking at 33 hours but also observed

286 at 48 and 72 hours post-transfection in comparison to siRNA control HT144

287 cells. Again, the effect was apparent but not as pronounced using the #3

288 siCITED1 (Fig S4a). Similar effects were seen in #1 and #3 siCITED1 treated

289 A2058 and A375 cells (Fig. S4b,c).

290 Owing to the previously reported dependency of MITF-induced cell cycle

291 arrest on CDKN1A/P21 we investigated the levels of several cyclin-dependant

292 kinase inhibitors following CITED1 silencing(Carreira et al. 2005). We found

293 that CDKN1A/P21 was transiently increased in siCITED1 treated HT144 cells

294 relative to the siNEG treated HT144 cells. In contrast, in A2058 cells, which

295 do not have detectable levels of CDKN1A/P21 (Fig. S5), the levels of

296 CDKN1C/P57 were supressed in response to CITED1 overexpression (Fig 5b).

297 We hypothesised therefore that melanoma cells can utilise either

298 CDKN1A/P21 or CDKN1C/P57 to mediate cell cycle arrest induced by MITF and

299 this is reflected in the expression levels of the alternate CDK inhibitors in

300 different melanoma cell lines (Fig. S5).

301

302 In agreement with the cell cycle data, an Alamar Blue assay revealed a

303 significant reduction in cell metabolic activity over 5 days in HT144 cells

304 treated with siCITED1 (Fig 5c). The effect was apparent but not as

305 pronounced in the #3 siCITED1 sample.

306 **The effect of CITED1 silencing on MITF is transient and mediated via**

307 **promoter activation**

308 We observed that the peak upregulation of MITF and CDKN1A/P21 protein

309 following siCITED1 treatment varied from transfection to transfection, being

310 seen between 24-48 hours post-transfection but appearing as unchanged or

311 even downregulated after this time (Fig. 6a, *upper panel*). In agreement, later

312 timepoints of the cell cycle analysis (=/>72 hours) exhibited little or no

313 change in G1/S-phase distribution or even a reverse pattern (Fig. 5a HT144,

314 and data not shown: A2058, A375). We therefore sought to examine the

315 transcriptional dynamics more closely, map the changes in MITF following

316 CITED1 silencing and see if they corresponded to cell behaviour and changes

317 at the protein level. We used a quantitative droplet digital PCR based assay

318 (Biorad) to measure mRNA in HT144 cells transfected with siCITED1#1 and

319 siNEG as well as A2058 cells transiently overexpressing CITED1 compared to

320 an empty vector control. MITF, CITED1 and IPO8 specific primers and probes

321 were used to measure exact copies/µl of each mRNA from aliquots of the

322 same cDNA solution. Plots of siCITED1(copies/µl)/siNEG(copies/µl) and

323 EV(copies/µl)/pCITED1(copies/µl) show the directional change in MITF and

324 CITED1 relative to the housekeeper IPO8. CITED1 expression is rapidly

325 supressed following siCITED1 treatment of HT144 cells, concomitant with an

326 upregulation of MITF that diminishes over time and in fact is supressed by

327 100 hours in accordance with observations at the protein level (Fig 6a, *lower*

328 *panel*). In contrast, overexpression of CITED1 in A2058 cells results in

329 transient suppression of MITF at both protein and transcript level (Fig. 6b,

330 *upper and lower panels*).

331 The rapid MITF transcriptional response to CITED1 manipulation suggested to

332 us that the effect could be directly mediated at the promoter level. To test

333 this hypothesis, we over expressed an MITF-M promoter-reporter construct

334 and CITED1 in A375 cells. We chose A375 cells, as while they had less

335 endogenous CITED1 and MITF than HT144 or A2058 so as not to cause

336 interference with the assay, we also knew that they could respond

337 adequately as they had an identical G1 accumulation/S-phase decrease to

338 both HT144 and A2058 cells following CITED1 silencing (Fig S4c). TGFβ

339 treatment was used as a positive control for repression of the MITF-M

340 promoter. CITED1 transfection led to significant suppression of the MITF-M

341 promoter luciferase activity relative to the empty vector control, as did TGFβ

342 treatment alone or combination with CITED1 overexpression (Fig. 6c). There

343 did not appear to be an additive or synergistic effect using both TGFβ

344 treatment and CITED1 overexpression suggesting TGFβ may be dependent on

345 CITED1 for MITF suppression.

346 The CITED1-silenced gene signature predicts outcome in primary

347 melanoma

348 The 'proliferative' and 'invasive' signature phenotypes have served to define

349 the gene expression classification of melanoma cell lines. However, primary

350 tumours and metastatic lesions have also been molecularly classified into

351 several distinct groups by gene expression profiling (Harbst et al. 2012;

352 Jonsson et al. 2010). The four-class structure found in tumours consists of the

353 'pigmentation', 'proliferative', 'high-immune' and 'normal-like' subgroups

354 with a subset falling into an unclassifiable cohort(Jonsson et al. 2010). We

355 used the same tumour classification to subtype the 120 cell lines that had

356 publically available expression data and could show that the tumour

357 'pigmentation' subgroup that highly expresses MITF, corresponds to the cell

358 line 'proliferative' phenotype described by Hoek et al. Accordingly, the

359 tumour 'proliferative' and 'high-immune' subgroups comprise the cell line

360 'invasive' phenotype (Fig. 7a). It is worth noting that the names of the tumour

361 subgroups were derived from a description of the differentially expressed

362 genes that comprised each molecular classification while the 'invasive-

363 proliferative' switching phenotypes were named to reflect the *behaviour*

364 exhibited by lines classified by one or other signature. This distinction helps

365 to explain the confusing occurrence that both classifications have a group

366 referred to as 'proliferative' although they are not equivalent.

367 The overlap between the primary tumour classifying and cell line classifying

368 systems allows us to infer that CITED1 expression is most likely restricted to

369 a subset of MITF high 'pigmentation' subtype tumours. As the tumour

370 subtype classification was shown to be prognostically significant in primary

371 melanomas we were interested to know if CITED1 expression itself was

372 independently predictive of outcome. Previously we reported on the analysis

373 of 223 primary lesions using the Illumina WG-DASL protocol (Harbst et al.

374 2012). As the CITED1 probe in this assay did not produce reliable data we

375 instead derived a CITED1-silenced gene signature score based on the

376 differentially expressed genes from the HT144 siCITED1 experiment (Fig. 3)

377 We subsequently interrogated the gene expression data on the primary

378 melanoma lesions using a nearest centroid approach derived from the

379 CITED1-silenced gene signature. This revealed that primary melanomas with

380 a gene expression signature most similar to the CITED1-silenced signature

381 (CITED1low-class) had a significantly better outcome than those with a

382 signature most disparate from the CITED1-silenced signature (CITED1high-

383 class) (Fig. 7b). Importantly, the CITED1 signature classing had independent

384 prognostic information (HR 1.85, CI 0.30-0.98, p=0.044) from the AJCC

385 staging system (HR 5.05, CI 2.42-10.55, p=1.64x10-5). These data indirectly

386 imply that CITED1 expression itself is a potential prognostic indicator in

387 primary melanomas and the transcriptional program influenced by CITED1

388 expression determines tumour behaviour *in vivo*.

389 DISCUSSION

390 One seemingly paradoxical observation from our study and previous

391 investigations is that although CITED1 behaves as a negative regulator of

392 MITF, both their expression levels appear positively correlated across cells

393 lines and tumours. We maintain that this observation simply reflects the fact

394 that where there are high levels of MITF, high levels of its negative regulator

395 are also required. The evidence of the tight control exerted over MITF levels

396 in melanocytes and melanoma simply speaks to the necessity of the cell to

397 maintain a level compatible with survival and proliferation, in a type of

398 biological 'sweet-spot' facilitating tumour progression. The cellular effects of

399 both extremes i.e: very low or high levels of MITF, have been elegantly

400 described by a rheostat model in order to reconcile the conflicting

401 observations of the effects of manipulating MITF *in vitro*, and the fact that

402 counter-intuitively, a lineage-specifying differentiation factor can behave as a

403 potent oncogene(Hoek & Goding 2010; Carreira et al. 2006; Cheli, Giuliano,

404 et al. 2011a). The rheostat model (Fig. S6) explains why MITF silencing can

405 block cells in G1 and induce senescence, while it is also possible to induce a

406 G1 arrest by MITF overexpression via CDKN2A/P16 or CDKN1A/P21 and, as we

407 now propose, potentially also via CDKN1C/P57 (Carreira et al. 2006; Loercher

408 et al. 2005; Carreira et al. 2005). At the extreme high end of MITF expression

409 lies differentiated melanocytic cells, while the lowest levels can lead to

410 senescence and irreversible cell death. Between these two extremes however

411 it is thought that melanoma cells can oscillate from a low-MITF 'invasive' to a

412 high-MITF 'proliferative' state via phenotype-switching.

413 We hypothesise that the role of CITED1 in melanoma is to maintain levels of

414 MITF compatible with tumour progression and effectively tip the balance in

415 favour of cell cycle progression rather than MITF-induced G1-arrest. This is

416 supported by our findings that downregulation of CITED1 using siRNA results

417 in a phenotype switch to a more pigmented state driven by increased MITF

418 expression and concomitant upregulation of CDKN1A/P21. Conversely, we

419 could observe that downregulation of MITF resulted in suppression of CITED1

420 in several cell lines suggesting the existence of a classical feedback loop

421 where low MITF levels result in inhibition of its negative regulator. MITF

422 induced cell cycle arrest was previously shown to be dependent on

423 CDKN1A/P21 and it was demonstrated that MITF does not cause a cell cycle

424 arrest in CDKN1A-deficient mouse embryo fibroblasts (MEF) cells(Carreira et

425 al. 2005). However, our data indicate that in melanoma cells deficient in

426 CDKN1A/P21, the alternative CDK inhibitor CDKN1C/P57 is expressed and

427 responsive to MITF.

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

429 CITED1 silencing, we found that BRCA1 and other DNA damage response

430 (DDR) genes were supressed, suggesting that CITED1 downregulation does

431 not necessarily facilitate transcription of all MITF targets. It is thus tempting

432 to speculate that rather than simply acting to induce MITF and thereby

433 indirectly enhance transcription of its target genes, that CITED1 may also act

434 as co-factor for MITF at various genomic locations differentially modulating

435 the MITF target gene response at individual promoters. One way that this

436 might be achieved is via MITF-CITED1 competition for CBP/P300 binding as

437 CBP/P300 is a known transcriptional coregulator for MITF, although it is not

438 required for transcription of all MITF targets (Vachtenheim et al. 2007)⁻(Yan et

439 al. 2013).

440 As suggested by Sáez-Ayala et al., anti–cancer therapy should be ideally

441 independent of dominant or 'driver' genetic alterations so that subclonal

442 populations do not gain a subsequent advantage and the same holds true in

443 the case of targeting a specific phenotype. Successful therapy will

444 necessarily need to switch or push the subdominant phenotype into the

445 susceptible state or eradicate the phenotype resistant to treatment. This

446 approach was initially championed by Cheli et al., who proposed the

447 eradication of low-MITF cells as a therapeutic strategy (Cheli, Guiliano, et al.

448 2011b). Indeed the idea of lineage-specific therapy has been subsequently

449 proved in principle using methotrexate (MTX) to first activate MITF

450 expression, in turn activating the tyrosinase enzyme, and thereby sensitising

451 tumour cells to a tyrosinase-processed anti-folate prodrug (TMECG) (Sáez-

452 Ayala et al. 2013). However, even without drug targeting, induction of MITF,

453 above what is tolerated by even the highly pigmented tumor cell types, i.e:

454 levels reaching that of melanocytes, would seem to be incompatible with

455 melanoma progression as it inhibits cell cycle progression. Our assertion is

456 that CITED1 acts to repress MITF in order to maintain its level in a range

457 compatible with tumorigenesis. This assertion as a consequence naturally

458 suggests CITED1 as therapeutic target for genetic manipulation. Successful

459 implementation of such a strategy would result in cell specific enhancement

460 of MITF expression and increased susceptibility to the type of

461 chemotherapeutic eradication demonstrated by Sáez-Ayala et al. or

462 potentially induction of CDKN1A/p21 or CDKN1C/p57-dependent cell growth

463 arrest even without further intervention (Fig. S6) (Sáez-Ayala et al. 2013).

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Figure 1

TGFβ induced gene expression in A2058 melanoma cells

(a) Distribution of the proliferative and invasive signature score genes relative to the heat map of gene expression changes induced by TGFβ treatment. (b) Gene expression heatmap of the proliferative and invasive signature within those genes significantly altered by TGFβ 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 (Widmer et al. 2012). (c) Western blot of MITF and CITED showing both proteins are suppressed by TGFβ treatment. β-Actin is used as a loading control.

a

PROLIFERATIVE
INVASIVE

b

c
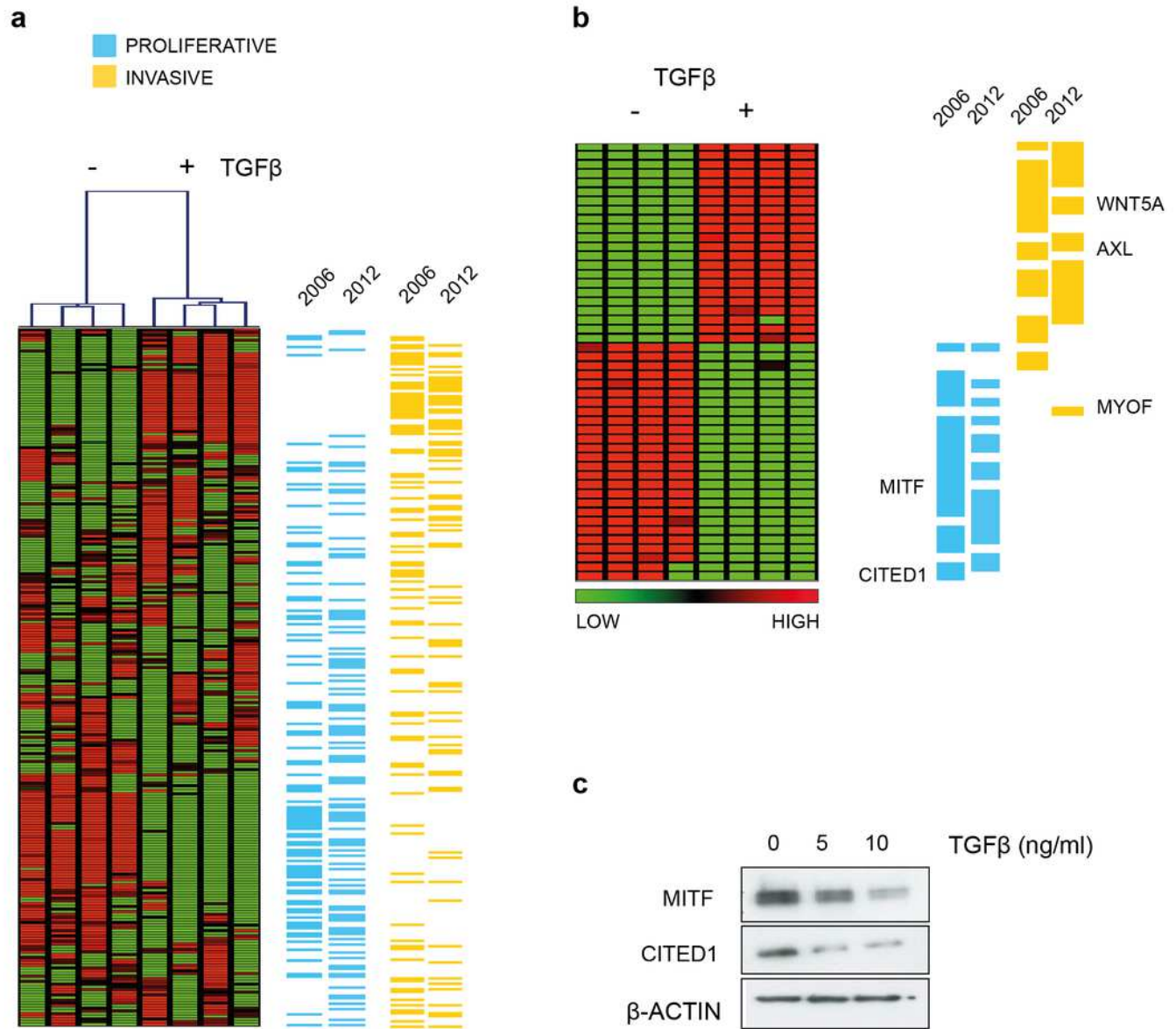
figure 1

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' 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.
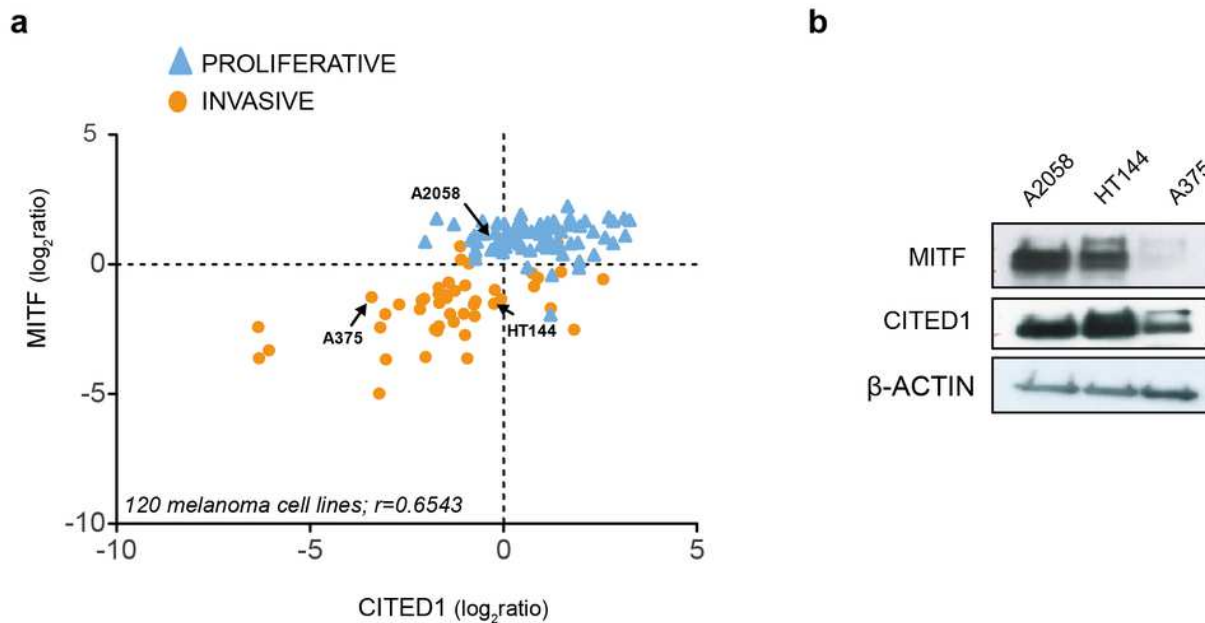
figure 2

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. 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.
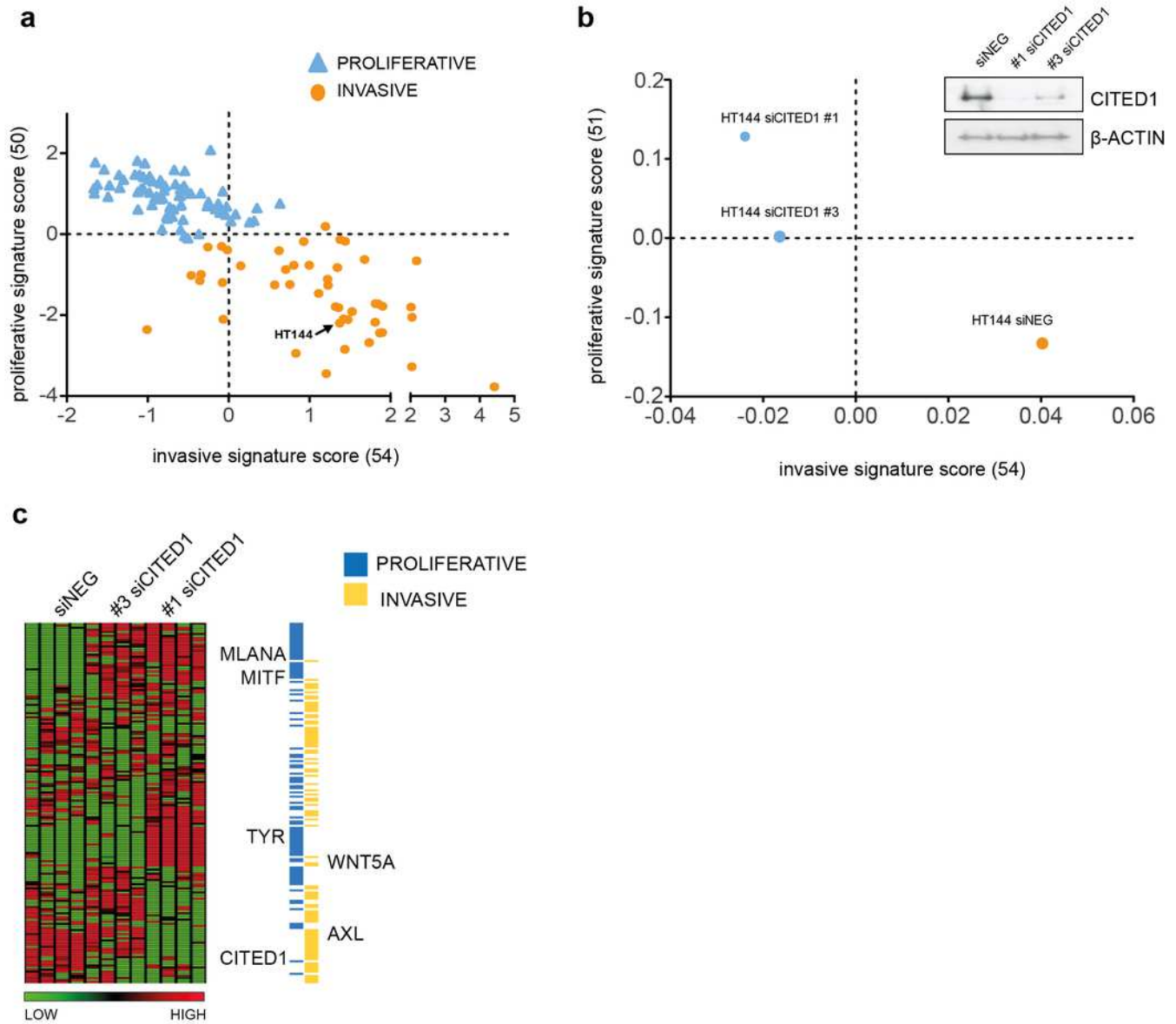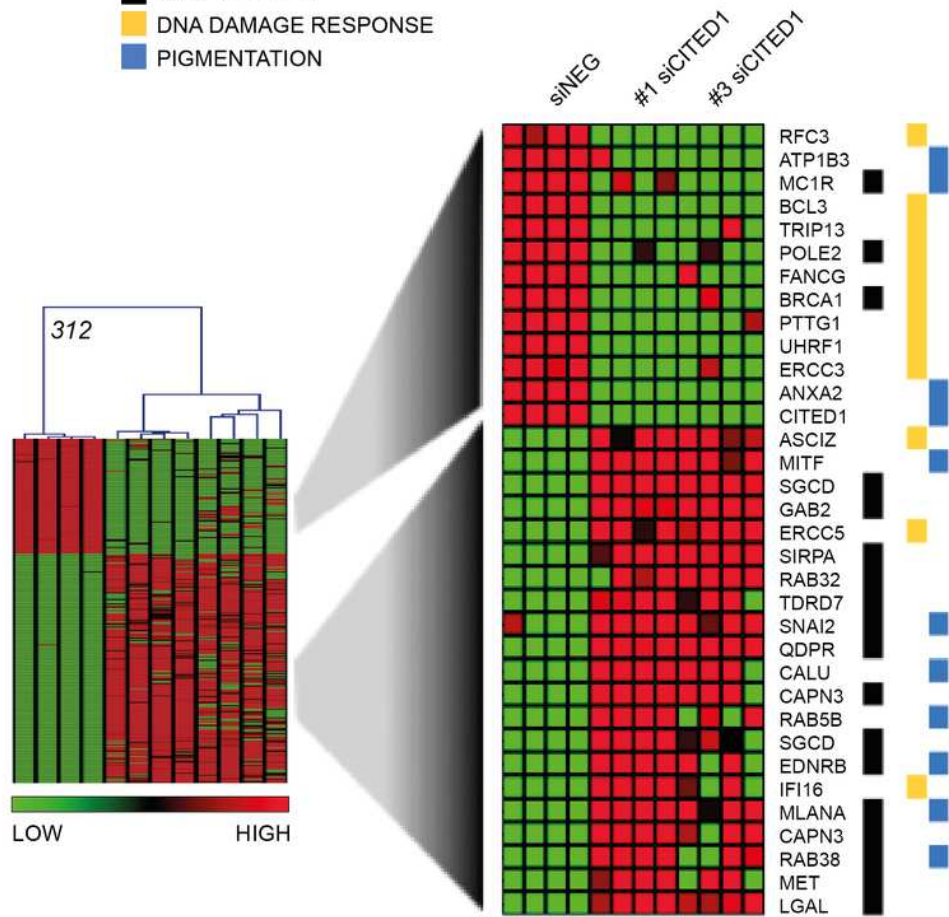
figure 3

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. 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.
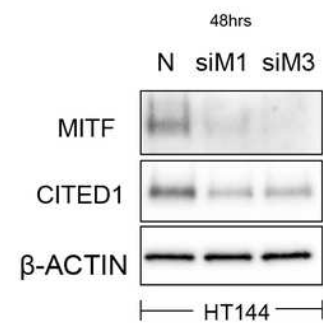
a

b
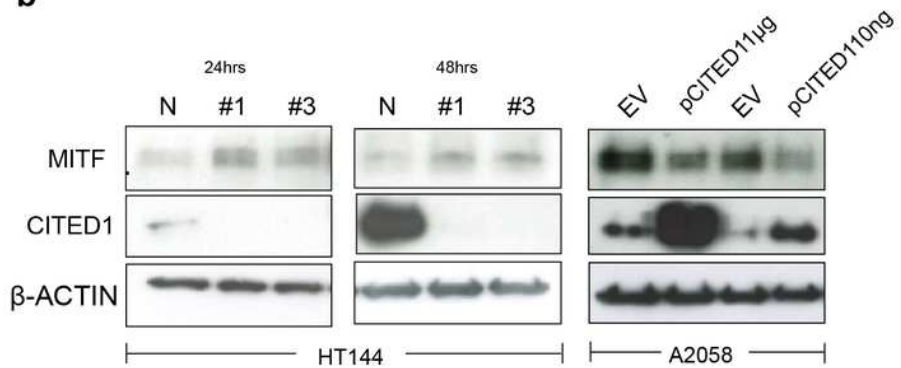
c

figure 4

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 ***$p <= 0.0005$, **$p <= 0.005$ and *$p <= 0.05$. In the case of siNEG vs. #1 siCITED1, the difference is significant (*) at 96 and 120 hours.

a

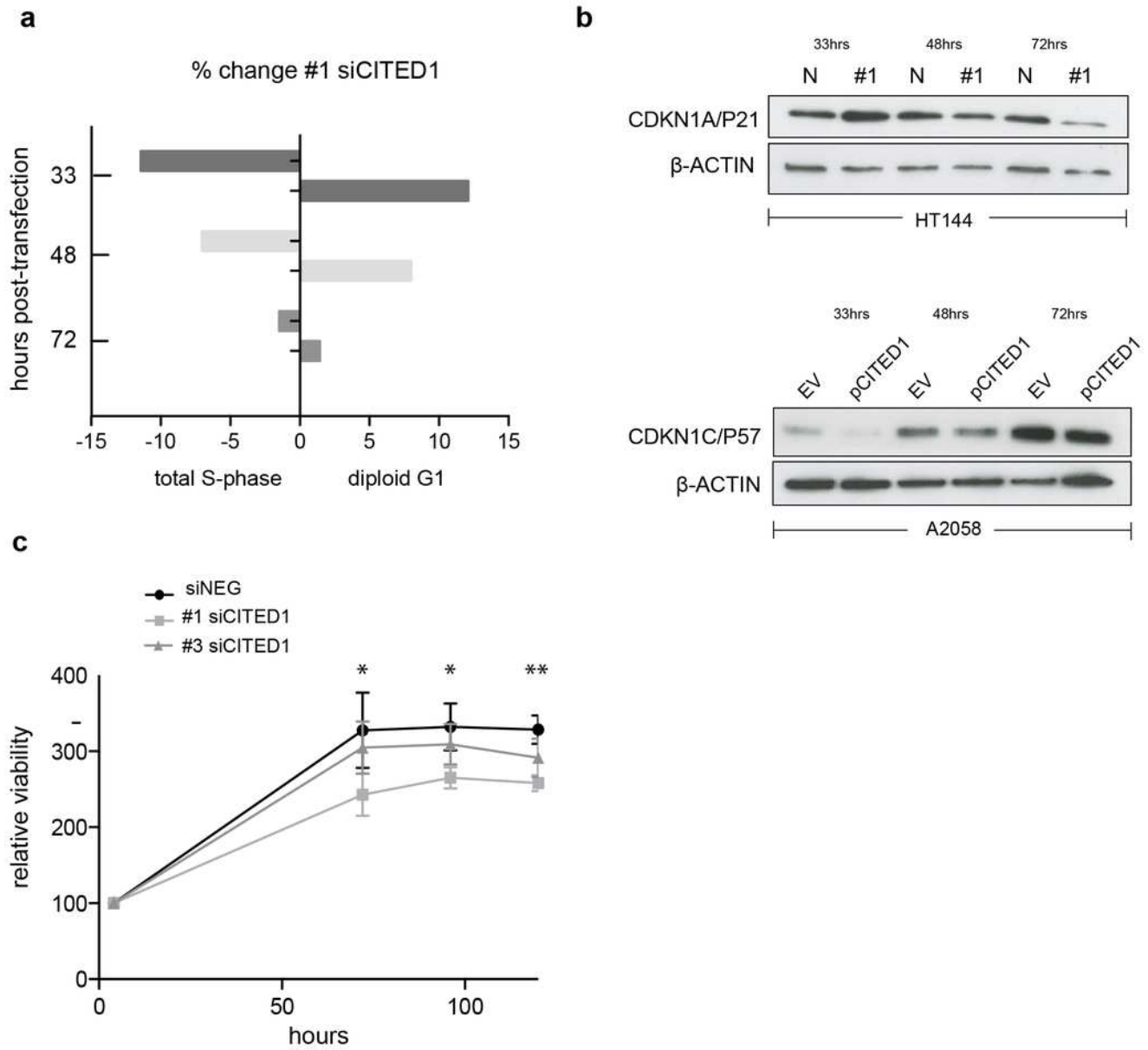% change #1 siCITED1

b

c

figure 5

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 TGFβ for 24 hours (***p<= 0.0005, **p<= 0.005 and *NS*= not significant).
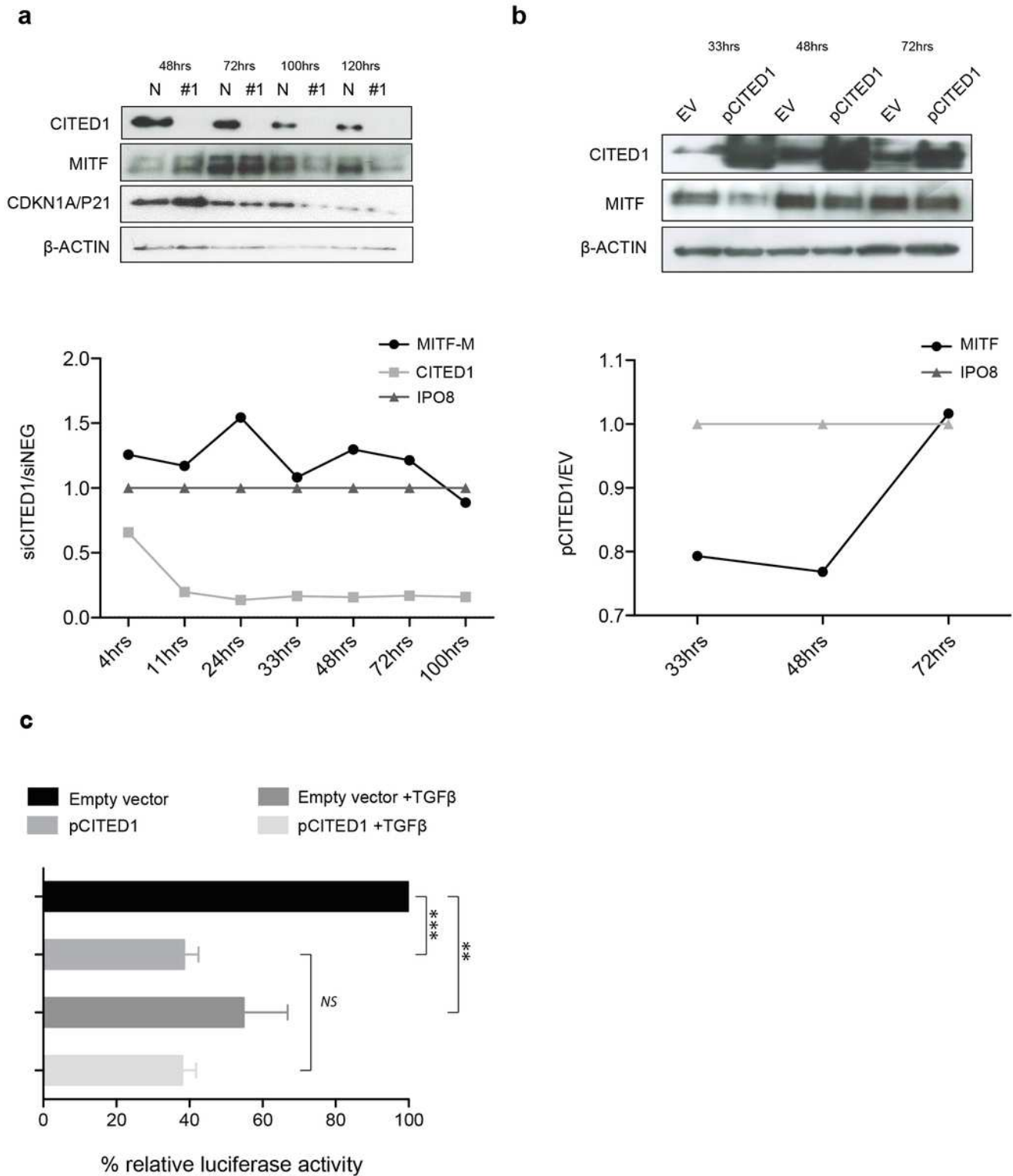
figure 6

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 tumor 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 heat map 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.
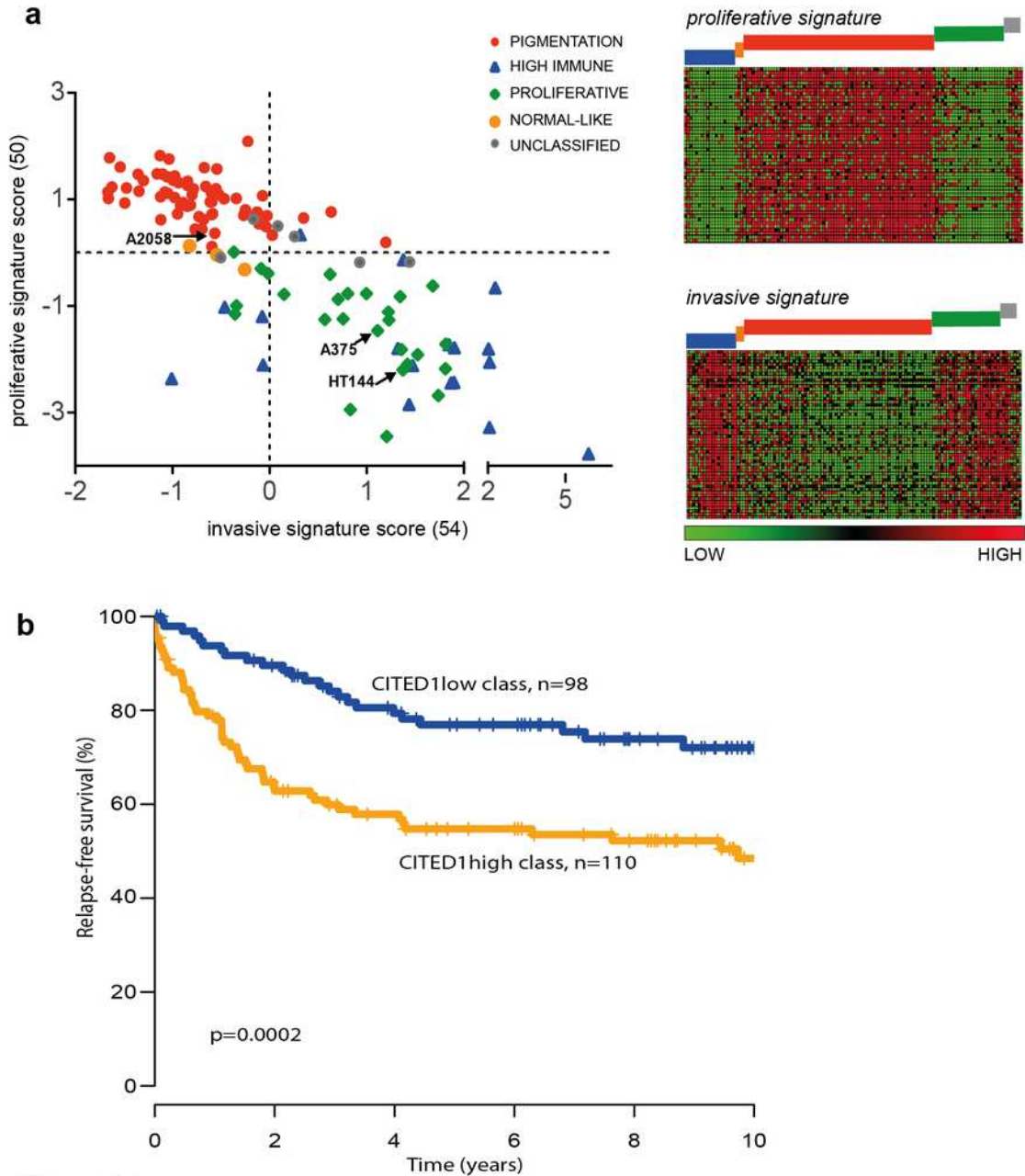
figure 7