

# CITED2 functions as a molecular switch of cytokine-induced proliferation and quiescence

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Transforming growth factor- $\alpha$  (TGF- $\alpha$ )-induced proliferation and transforming growth factor- $\beta$  (TGF- $\beta$ )-mediated quiescence are intricately balanced in normal lung-tissue homeostasis but are deregulated during neoplastic progression of lung cancer. Here, we show that Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (CITED2), a novel MYC-interacting transcriptional modulator, responds to TGF- $\alpha$  induction and TGF- $\beta$  suppression to orchestrate cellular proliferation and quiescence, respectively. Upon TGF- $\alpha$  induction, CITED2 was induced by MYC and further modulated MYC-mediated transcription in a feed-forward manner. CITED2 recruited p300 to promote MYC-p300-mediated transactivation of *E2F3*, leading to increased G1/S cell cycle progression. Moreover, CITED2 inhibited cellular quiescence by enhancing MYC-mediated suppression of *p21<sup>CIP1</sup>*. CITED2 interacted with histone deacetylase 1 (HDAC1) and potentiated MYC-HDAC1 complex formation. TGF- $\beta$  stimulation provoked downregulation of CITED2, which abrogated MYC-HDAC1-mediated *p21<sup>CIP1</sup>* suppression, causing cellular quiescence. Ectopic CITED2 expression enhanced tumor growth in nude mice; furthermore, CITED2 knockdown caused tumor shrinkage and increased overall host mouse survival rates. Expression of CITED2/MYC/E2F3/*p21<sup>CIP1</sup>* signaling molecules was associated with poor prognosis of lung cancer patients. Thus, CITED2 functions as a molecular switch of TGF- $\alpha$  and TGF- $\beta$ -induced growth control, and MYC-CITED2 signaling axis provides a new index for predicting clinical outcome.

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Transforming growth factor (TGF)- $\alpha$  and transforming growth factor (TGF)- $\beta$  are two pivotal cytokines that regulate proliferation and quiescence, respectively, of lung epithelial cells during both normal and neoplastic lung development.<sup>1</sup> Epidermal growth factor receptor (EGFR) is often amplified or mutated in non-small-cell lung cancer (NSCLC),<sup>2</sup> and TGF- $\alpha$ , one of the EGFR cognate ligands, is frequently overexpressed and causes receptor hyperactivity in autocrine loops in cancer cells isolated from patients with NSCLC.<sup>3</sup> In contrast, TGF- $\beta$  induces quiescence of lung epithelial cells.<sup>4</sup> TGF- $\beta$  signaling is mediated through the TGF- $\beta$  type II (TGFBR2) and type I (TGFBR1) receptors; methylation silencing of TGFBR2 is observed in some NSCLC cancer cells,<sup>5</sup> rendering tumor cells refractory to TGF- $\beta$ -induced growth arrest.<sup>6</sup>

Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (CITED2), which was originally identified from the studies of differential gene expression by cytokine stimulation, encodes a transcriptional modulator

without DNA-binding domains.<sup>7</sup> Mice without CITED2 display abnormal fetal lung development, including reduced terminal sacs and aberrant differentiation of alveolar epithelial cells.<sup>8</sup> CITED2 is induced by cytokines such as interleukin-1 $\alpha$ , -2, -4, -6, -9 and -11; granulocyte/macrophage colony-stimulating factor; interferon- $\gamma$ ; platelet-derived growth factor and insulin.<sup>7</sup> In contrast, CITED2 is inhibited by TGF- $\beta$  through a post-transcriptional control mechanism.<sup>9</sup> CITED2 interacts with p300/CBP, Lhx2, TFAP2, Smad2/Smad3 and PPAR $\gamma$ , thereby modulating transcription mediated by these transcription factors.<sup>10–14</sup>

MYC, a potent oncogene, is highly expressed in bronchial epithelium basal cells and hyperplastic alveolar type II pneumocytes.<sup>15</sup> Through the regulation of E2F transcription factor expression, MYC has a critical role in cell cycle control.<sup>16,17</sup> Among the E2Fs, E2F3 is overexpressed in most lung cancers<sup>18</sup> and is induced by EGFR to transactivate genes involved in the G1/S transition.<sup>19,20</sup> In addition, MYC binds to the *p21<sup>CIP1</sup>* promoter, causing

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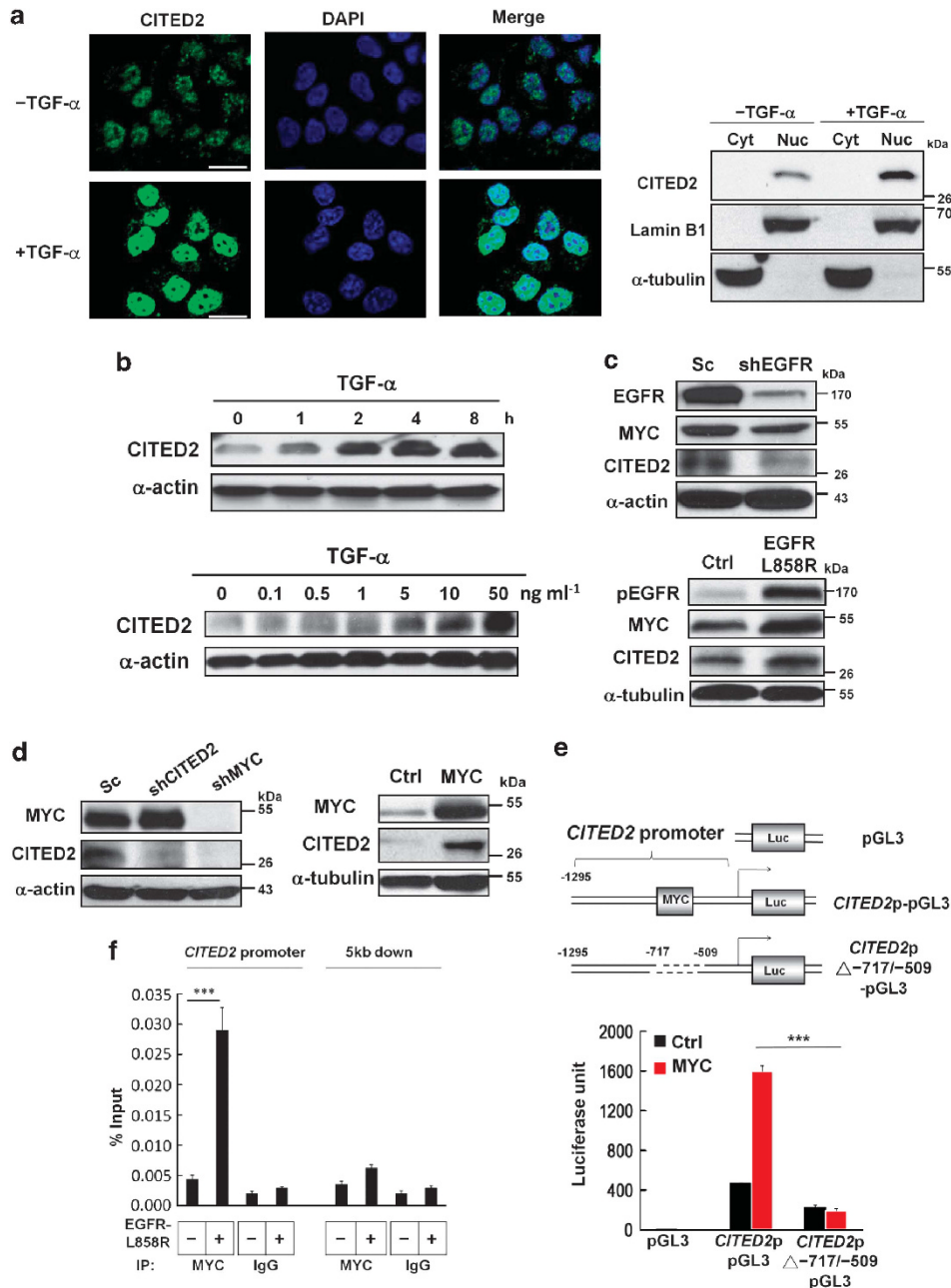
**Keywords:** CITED2; MYC; cytokine; transcriptional modulator; lung cancer

**Abbreviations:** CITED2, Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2; TGF- $\alpha$ , transforming growth factor alpha; TGF- $\beta$ , transforming growth factor beta; NSCLC, non-small-cell lung cancer; EGFR, epidermal growth factor receptor; TGFBR1, transforming growth factor beta receptor I; TGFBR2, transforming growth factor beta receptor II; p300, E1A binding protein p300; HDAC1, histone deacetylase 1; Dox, doxycycline; eGFP, enhanced green fluorescent protein; shRNA, short hairpin RNA; GST, glutathione S-transferase

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transcriptional suppression of  $p21^{CIP1}$ .<sup>21,22</sup> TGF- $\beta$  induces quiescence of epithelial cells through suppression of MYC and activation of the  $p21^{CIP1}$ -mediated senescence pathway.<sup>23</sup>

Although it is known that MYC mediates abnormal cytokine signaling to induce tumorigenesis, how MYC can respond to signals from diverse cytokines and function as both a transcriptional activator and repressor to regulate cell cycle

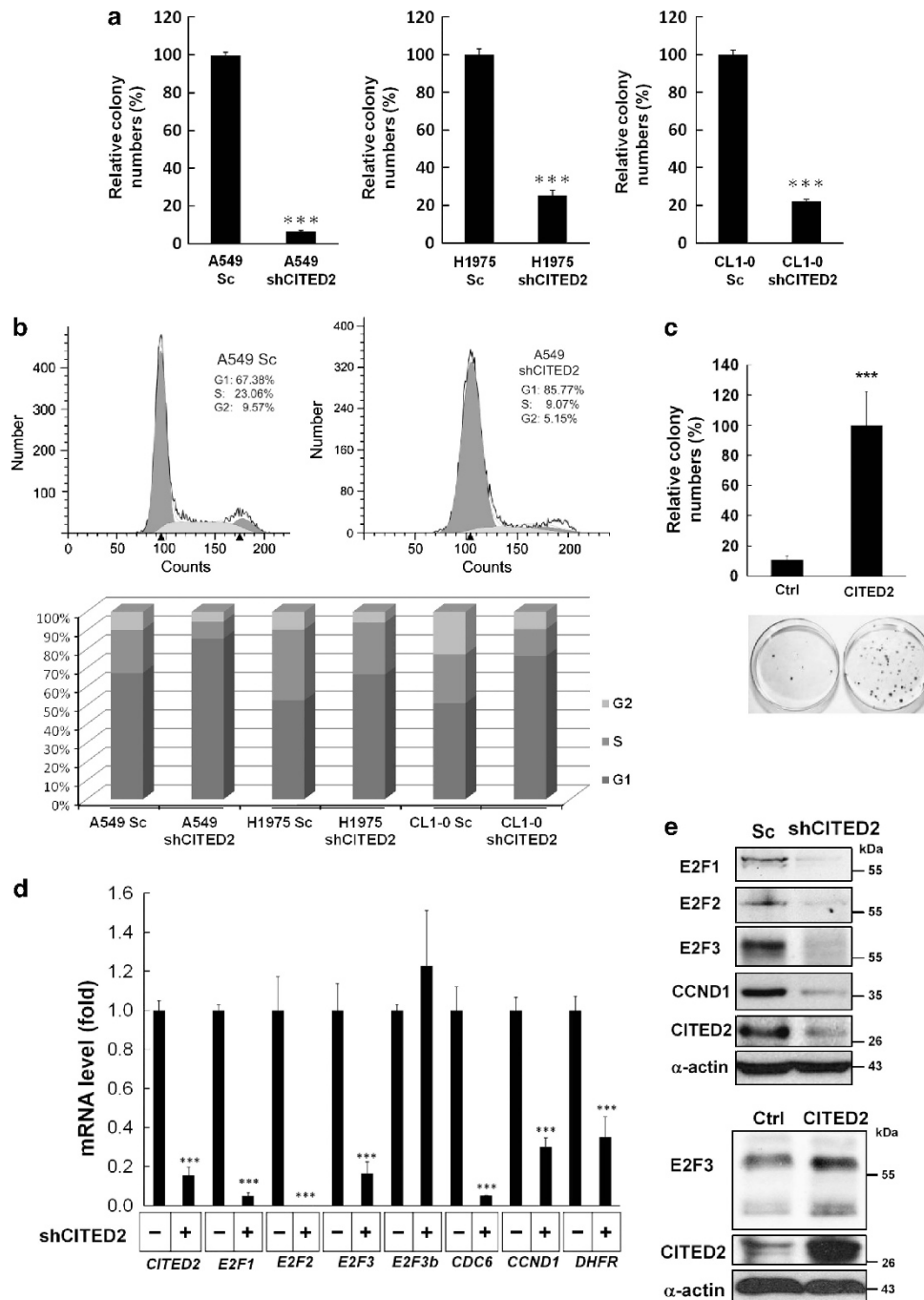


**Figure 1** CITED2 is induced by TGF- $\alpha$ /EGFR signaling. (a) Representative image of confocal immunofluorescence staining of CITED2 expression in A549 cells treated with or without TGF- $\alpha$  (50 ng/ml) for 6 h (left). Scale bars, 10  $\mu$ m. Immunoblot analysis of nuclear and cytoplasmic fractions of A549 cells treated with or without TGF- $\alpha$  (50 ng/ml) for 6 h (right). (b) Immunoblot analysis of CITED2 expression in A549 cells treated with TGF- $\alpha$  (50 ng/ml) for the indicated times (top) or with the indicated concentrations of TGF- $\alpha$  for 6 h (bottom). (c) Immunoblot analysis of total cell lysates from A549 cells infected with scramble control (Sc) or shEGFR lentiviral particles (top). Immunoblot analysis of total cell lysates from A549 cells infected with control or EGFR-L858R-expressing lentiviral particles (bottom). pEGFR, phosphorylated EGFR. (d) Immunoblot analysis of total cell lysates from A549 cells infected with Sc, shCITED2 or shMYC lentiviral particles (left). Immunoblot analysis of total cell lysates from A549 cells infected with control or MYC-expressing lentiviral particles (right). (e) Schematic of the pGL3 vector and the CITED2 promoter reporters CITED2p-pGL3 and CITED2p( $\Delta$  -717/ -509)-pGL3, in which MYC-binding site was deleted (top). Luciferase reporter assays with these three vectors were performed in HEK293T cells transfected with pcDNA-MYC or pcDNA control vector (bottom). Results are representative of at least three independent experiments and are expressed as the mean  $\pm$  S.D. \*\*\* $P$  < 0.001. (f) Binding of MYC to the CITED2 promoter (-544 to -480 bp) was determined by chromatin immunoprecipitation (ChIP) in A549 cells infected with control or EGFR-L858R-expressing lentiviral particles. ChIP with primers for the site located 5 kb downstream from CITED2 was performed as a negative control. Results are expressed as the mean  $\pm$  S.D. \*\*\* $P$  < 0.001. IP, immunoprecipitation

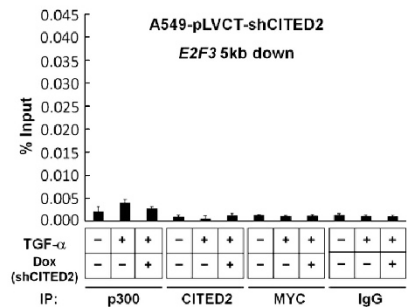
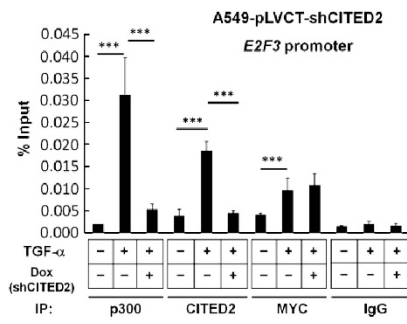
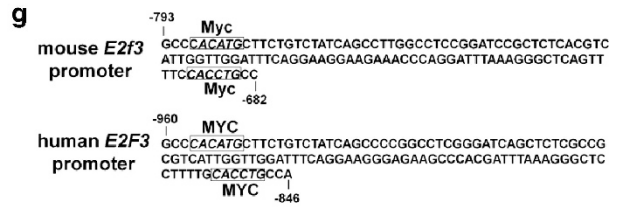
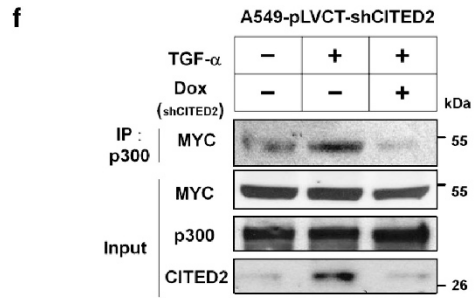
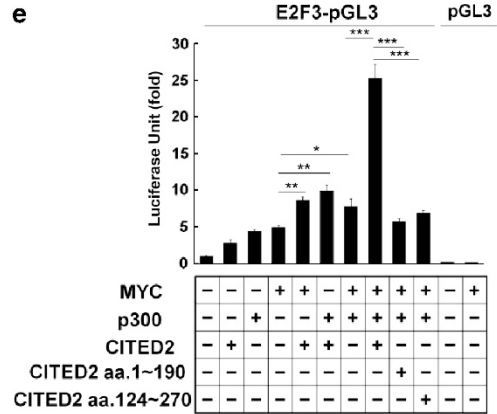
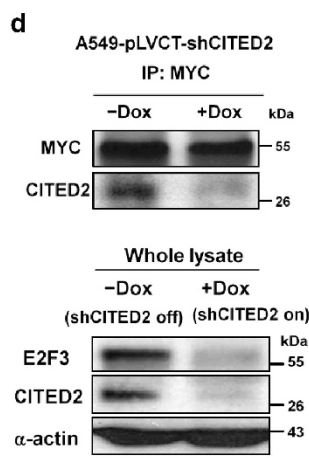
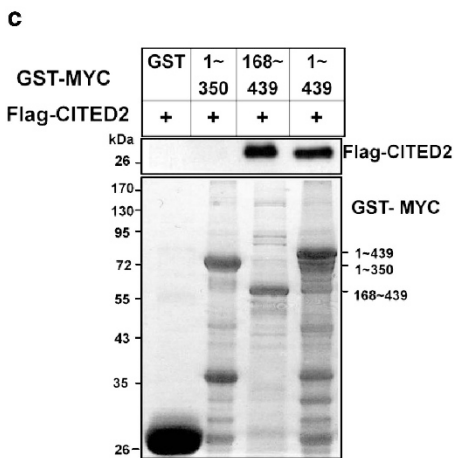
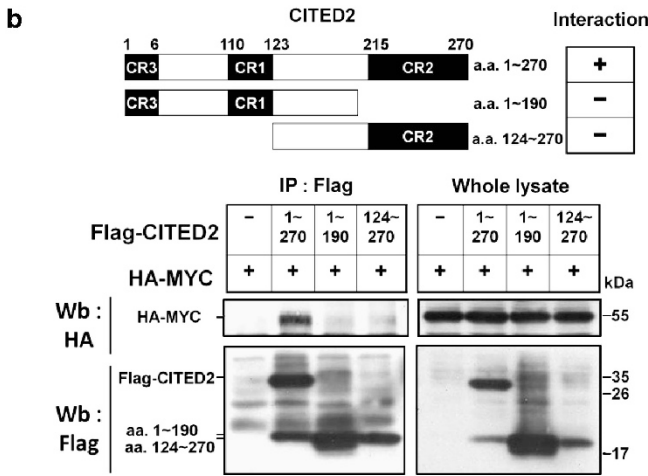
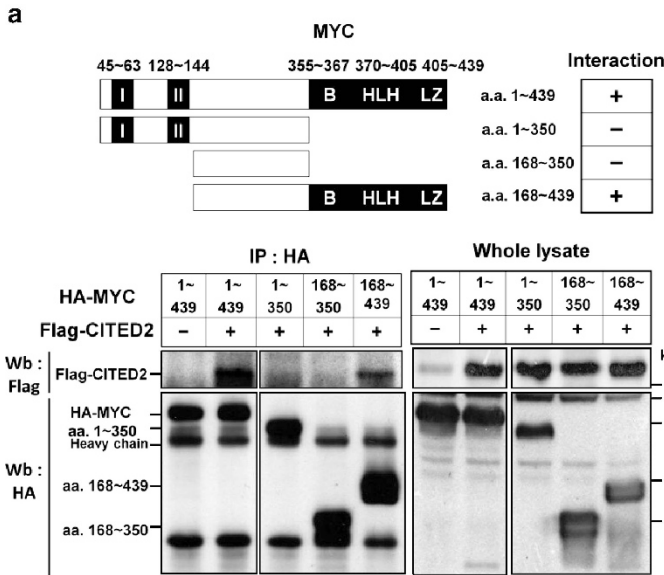
progression is still elusive. In this study, we show that CITED2 functions as a molecular switch for TGF- $\alpha$  proliferation and TGF- $\beta$  quiescence stimuli by orchestrating MYC-mediated transcription.

## Results

**CITED2 expression is induced by TGF- $\alpha$ /EGFR signaling through a MYC-dependent pathway.** As TGF- $\alpha$ /EGFR



**Figure 2** CITED2 regulates E2F3 expression. (a) A549, H1975 and CL1-0 cells were infected with scramble control (Sc) or shCITED2 lentiviral particles for 5 days and subjected to clonogenic assays. Results represent triplicate counts per treatment and are representative of three independent experiments. The relative colony number of Sc cells was set to 100% and error bars represent mean  $\pm$  S.D. \*\*\* $P$  < 0.001. (b) Flow cytometry cell cycle analysis with propidium iodide staining of A549, H1975 and CL1-0 cells infected with Sc or shCITED2 lentiviral particles. A549 data are shown at the top, and the results from all three cell lines are summarized at the bottom. (c) A clonogenic assay of CL1-0 cells infected with control or CITED2-expressing lentiviral particles and maintained under serum-free conditions. The colony number resulting from infection with CITED2 was set to 100% (top) based on crystal violet staining (bottom). Results are representative of three independent experiments and are expressed as the mean  $\pm$  S.D. \*\*\* $P$  < 0.001. (d) Q-PCR analysis of total RNA from A549 cells infected with Sc (-) or shCITED2 (+) lentiviral particles. Results are representative of three independent experiments and are expressed as the mean  $\pm$  S.D. \*\*\* $P$  < 0.001. (e) Immunoblot analysis of total cell lysates from cells as in d (top). Immunoblot analysis of total cell lysates from CL1-0 cells infected with control or CITED2-expressing lentiviral particles (bottom)



signaling contributes to lung cancer progression<sup>3</sup> and because CITED2 can be induced by various cytokines,<sup>7</sup> we examined the effect of TGF- $\alpha$ /EGFR signaling on CITED2 expression in lung cancer cells. Immunofluorescence (IF) staining of A549 lung-cancer cells showed strong nuclear localization of CITED2 after TGF- $\alpha$  induction (Figure 1a). Analysis of CITED2 expression in isolated nuclear and cytoplasmic fractions of A549 cells showed that CITED2 was predominantly localized in the nucleus, and expression of nuclear CITED2 could be further enhanced by TGF- $\alpha$  stimulation (Figure 1a). Immunoblotting demonstrated that TGF- $\alpha$  induced CITED2 expression in A549 cells in a time-dependent and dose-dependent manner (Figure 1b). Furthermore, overexpression of the active L858R mutant EGFR increased the expression of CITED2, as well as MYC (Figure 1c). Knockdown of EGFR by short hairpin RNA (shRNA) against EGFR (shEGFR) attenuated the expression of CITED2 and MYC (Figure 1c). These data demonstrate that TGF- $\alpha$ /EGFR signaling induces CITED2 expression in lung cancer cells.

As MYC is a critical effector of EGFR downstream signaling,<sup>24,25</sup> we examined the effect of MYC on CITED2 expression. Overexpression of MYC increased CITED2 levels, and knockdown of MYC with MYC-specific shRNA (shMYC) significantly blocked CITED2 expression comparable to that of CITED2-silenced cells, indicating that MYC induces CITED2 expression (Figure 1d). To study the effect of MYC on *CITED2* transcription, the 1.3-kb promoter of *CITED2* was cloned and used in a promoter reporter assay. The promoter reporter analysis indicated that MYC activated *CITED2* transcription (Figure 1e). Analysis of the *CITED2* promoter sequence identified a possible MYC-binding site in the *CITED2* promoter region (Figure 1e). When the MYC-binding sequence in the *CITED2* promoter reporter was deleted, overexpression of MYC did not activate the mutated *CITED2* promoter (Figure 1e), suggesting that MYC activates *CITED2* transcription through the MYC-binding site. Chromatin immunoprecipitation indicated that the constitutively active EGFR-L858R mutant promoted the binding of MYC to the *CITED2* promoter but not to the region 5 kb downstream from the *CITED2* promoter (Figure 1f). Similarly, increased MYC binding to the *CITED2* promoter was observed upon TGF- $\alpha$  stimulation (Supplementary Figure S1). Taken together, our data support

the notion that TGF- $\alpha$ /EGFR signaling stimulates CITED2 expression via MYC.

### CITED2 regulates the E2F-mediated G1/S transition during the cell cycle.

To investigate the role of CITED2 in lung cancer cells, CITED2 was knocked down in A549, H1975 and CL1-0 lung cancer cells. CITED2 silencing significantly attenuated the clonogenic activity of all tested lung cancer cells (Figure 2a). We further carried out cell cycle analysis on CITED2-silenced cells and discovered that knockdown of CITED2 caused G1/S cell cycle arrest (Figure 2b and Supplementary Figure S2). As CITED2 can be induced by serum,<sup>7</sup> to investigate the effect of CITED2 in cellular proliferation, CITED2 was ectopically expressed in CL1-0 cells. Ectopic expression of CITED2 results in sustained growth in serum-free media (Figure 2c). As E2F1, E2F2 and E2F3 are pivotal regulators of G1/S-phase progression, we performed quantitative RT-PCR (Q-PCR) analysis of *E2F1*, *E2F2* and *E2F3* expression in CITED2 knockdown cells. We found that *E2F1*, *E2F2* and *E2F3* and its downstream targets such as *CDC6*, *CCND1* and *DHFR* were downregulated in CITED2-silenced A549 cells; in contrast, the short isoform of E2F3 (referred to as E2F3b) was not affected by CITED2 silencing (Figure 2d). Western blotting further demonstrated that CITED2 silencing inhibited E2F1, E2F2 and E2F3 expression in A549 cells (Figure 2e). Overexpression of CITED2 not only promoted clonogenic growth but also enhanced the amount of E2F3 in CL1-0 cells (Figure 2c and e). These results indicate that CITED2 controls cell cycle progression.

### CITED2 interacts with MYC and potentiates MYC transactivation activity by recruiting p300.

MYC directly regulates the expression of E2F1-3.<sup>16,26</sup> As CITED2 regulates E2F1-3 levels, it is possible that CITED2 regulates E2F1-3 by modulating MYC-mediated transcription. To test this hypothesis, we first investigated whether CITED2 interacts with MYC using co-immunoprecipitation. We constructed MYC truncation mutants to map the CITED2-interacting region in MYC. CITED2 interacted with full-length MYC (residues 1-439) and residues 168-439 of MYC, but not with residues 1-350 or 168-350, indicating that CITED2 interacts with the C terminus but not the N terminus of MYC (Figure 3a). Conversely, CITED2 truncation mutants were

**Figure 3** CITED2 promotes MYC-mediated transactivation of *E2F3*. (a) Schematic illustration of the functional domains in wild-type MYC and MYC deletion mutants (top). B, HLH and LZ represent basic, helix-loop-helix and leucine zipper domains, respectively. Western blotting analysis (Wb) of hemagglutinin (HA)-immunoprecipitated products or whole-cell lysates from HEK293T cells transfected with pFlag-CMV2-CITED2 (Flag-CITED2) and pcDNA-HA-MYC (HA-MYC) or HA-tagged MYC deletion mutants (bottom). (b) Schematic illustration of the conserved regions (CR1, CR2 and CR3) in wild-type CITED2 and CITED2 deletion mutants (top). Western blotting analysis of Flag-immunoprecipitated products or whole-cell lysates from HEK293T cells transfected with HA-MYC and Flag-tagged CITED2 or Flag-tagged CITED2 deletion mutants (bottom). (c) *In vitro*-transcribed and -translated Flag-CITED2 was mixed with GST-tagged MYC deletion mutants and subjected to a GST pull-down assay. Immunoblotting and Coomassie blue staining show the resulting expression. (d) A549-pLVCT-shCITED2 cells were treated with or without Dox (1  $\mu$ g/ml) for 24 h and subjected to western blotting to monitor CITED2 and E2F3 expression (bottom). Whole-cell lysates were immunoprecipitated with antibodies against endogenous MYC followed by immunoblotting for MYC and CITED2 (top). (e) A luciferase reporter assay for *E2F3* promoter activity was carried out in HEK293T cells transfected with pcDNA-MYC, pcDNA-p300 and/or Flag-CITED2 deletion mutants together with the *E2F3* promoter reporter E2F3-pGL3 or pGL3. Results are representative of at least three independent experiments and are expressed as the mean  $\pm$  S.D. \* $P$  < 0.05; \*\* $P$  < 0.01; \*\*\* $P$  < 0.001. (f) A549-pLVCT-shCITED2 cells were treated with or without TGF- $\alpha$  (50 ng/ml) for 8 h in the presence or absence of Dox (1  $\mu$ g/ml) and monitored for expression of p300, MYC and CITED2 in cells (input). Binding of endogenous p300 to MYC was determined by co-immunoprecipitation with anti-p300, followed by immunoblotting for MYC. (g) *E2F3* promoter sequences from mouse and human showing conserved MYC-binding sequences (top). A549-pLVCT-shCITED2 cells were treated with or without TGF- $\alpha$  (50 ng/ml) for 8 h in the presence or absence of Dox (1  $\mu$ g/ml) and were subjected to ChIP analysis with the *E2F3* promoter (middle) or with 5 kb downstream from the promoter (bottom) with antibodies against MYC, CITED2 and p300. Results are expressed as the mean  $\pm$  S.D. \*\*\* $P$  < 0.001

also constructed to map the MYC-interacting region of CITED2. MYC interacted with full-length CITED2 but not with the N- or C-terminal deletion mutants (Figure 3b). These results indicate a specific interaction between MYC and CITED2. To further confirm the physical interaction between CITED2 and MYC, a glutathione *S*-transferase (GST) pull-down assay was performed. GST-tagged MYC deletion constructs were expressed *in vitro*, and full-length CITED2 was transcribed and translated *in vitro*. CITED2 interacted with GST-MYC residues 168–439 but not with GST-MYC residues 1–350 (Figure 3c). These results indicate that the C-terminal region of MYC is necessary for the interaction with CITED2.

To obtain cells in which endogenous CITED2 can be manipulated, we generated A549 cells that stably express doxycycline (Dox)-inducible shRNA against CITED2 (A549-pLVCT-shCITED2). Among E2Fs, E2F3 is highly expressed in lung cancer;<sup>18</sup> therefore, we focused on E2F3 expression. After endogenous CITED2 was knocked down, the expression of E2F3 decreased (Figure 3d). Moreover, endogenous CITED2 was co-immunoprecipitated by anti-MYC, supporting a specific interaction between MYC and CITED2 (Figure 3d).

As CITED2 interacts with MYC (which regulates *E2F3* transcription), we performed a promoter reporter assay to investigate the role of CITED2 in MYC-mediated *E2F3* expression. We found that MYC stimulated *E2F3* promoter activity, and CITED2 promoted MYC-mediated transactivation of the *E2F3* promoter (Figure 3e). As CITED2 interacts with p300,<sup>14</sup> we further investigated whether CITED2 promoted MYC-mediated transactivation by recruiting p300. Through co-immunoprecipitation analysis with antibodies against p300, we found that treating A549-pLVCT-shCITED2 cells with TGF- $\alpha$  induced CITED2 expression and enhanced the interaction between p300 and MYC; this interaction was attenuated by knockdown of CITED2 with Dox treatment (Figure 3f). Moreover, a reporter assay indicated that the expression of CITED2, but not of N- or C-terminal deletion mutants of CITED2, promoted MYC-mediated *E2F3* promoter activity in the presence of ectopic p300 (Figure 3e). These data suggest that CITED2 interacts with MYC and recruits p300 to activate *E2F3* expression. Myc binding sequences have been identified in the mouse *E2f3* promoter.<sup>16</sup> By comparing the sequences between the mouse and human *E2F3* promoters, we identified putative MYC-binding sequences in the human *E2F3* promoter (Figure 3g). Following chromatin immunoprecipitation analysis with antibodies against MYC, CITED2 and p300, we discovered that increased CITED2, MYC and p300 bound to the *E2F3* promoter upon TGF- $\alpha$  stimulation, and knockdown of CITED2 not only inhibited CITED2 but also attenuated p300 binding to the *E2F3* promoter. (Figure 3g). These data support that CITED2 binds to MYC and recruits p300 to the *E2F3* promoter to enhance *E2F3* expression.

**TGF- $\beta$  downregulates CITED2, leading to cellular quiescence.** In contrast to TGF- $\alpha$ , TGF- $\beta$  promotes quiescence in epithelial cells, and TGF- $\beta$ -mediated downregulation of MYC is thought to be responsible for TGF- $\beta$ -induced cellular quiescence.<sup>23</sup> We examined the effects of TGF- $\beta$  on the

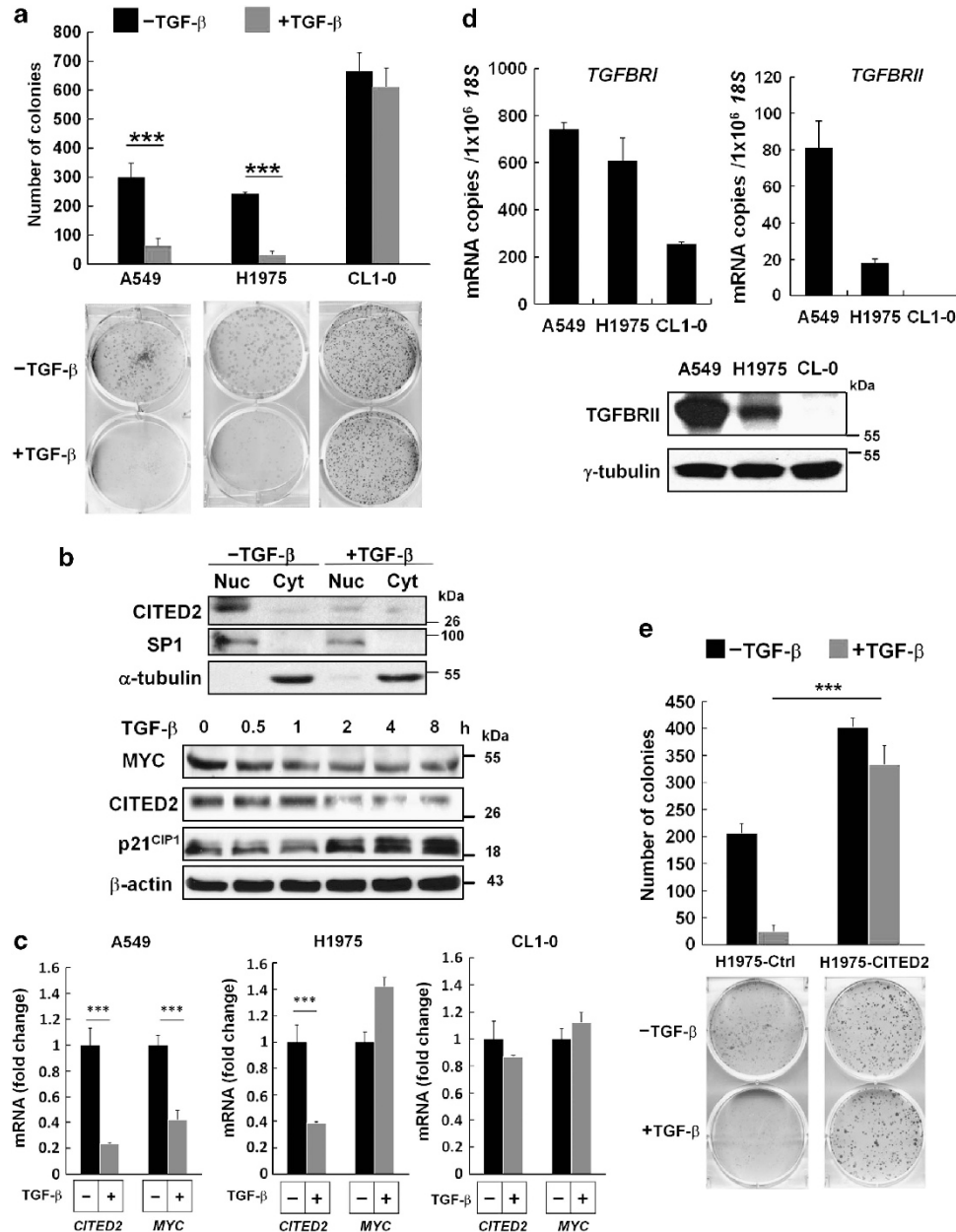
clonogenic growth of lung adenocarcinoma cells. TGF- $\beta$ -mediated cellular quiescence was observed in A549 and H1975 cells but not in CL1–0 cells (Figure 4a). As CITED2-silencing induced cellular quiescence of these cells (Figures 2a and b), we also examined whether CITED2 expression is affected by TGF- $\beta$  stimulation. Upon TGF- $\beta$  treatment, nuclear CITED2 was decreased in A549 cells (Figure 4b). Furthermore, TGF- $\beta$  downregulated both MYC and CITED2 but induced p21<sup>CIP1</sup> in A549 cells in a time-dependent manner (Figure 4b). The Q-PCR data supported that TGF- $\beta$  induced MYC downregulation in A549 cells; however, TGF- $\beta$ -mediated downregulation of MYC was not observed in H1975 cells (Figure 4c). Interestingly, CITED2 expression was inhibited by TGF- $\beta$  in both A549 and H1975 cell lines, which responded to TGF- $\beta$ -induced cellular quiescence (Figures 4a and c). As defective expression of TGF- $\beta$  receptors (TGFBR1 and TGFBR2) is found in cells from patients with lung cancer,<sup>5,6</sup> expression of *TGFBR1* and *TGFBR2* messenger RNA (mRNA) was investigated in these cells. Absolute Q-PCR analysis and western blot indicated loss of *TGFBR2* in CL1–0 cells (Figure 4d). Furthermore, methylation status of the *TGFBR2* promoter in these lung cancer cells was analyzed by bisulfite sequencing. The *TGFBR2* promoter in CL1–0 cells, but not in A549 and H1975 cells, was highly methylated (Supplementary Figure S3). To investigate whether downregulation of CITED2 is responsible for TGF- $\beta$ -mediated cellular quiescence, CITED2 was ectopically expressed in H1975 cells, followed by TGF- $\beta$  stimulation. We observed that CITED2-overexpressing H1975 cells were resistant to TGF- $\beta$ -mediated cell growth arrest (Figure 4e). These data suggest that downregulation of CITED2 contributes to TGF- $\beta$ -mediated cellular quiescence.

**CITED2 regulates the p21<sup>CIP1</sup> senescence pathway by enhancing the MYC–histone deacetylase 1 (HDAC1) interaction.** As p21<sup>CIP1</sup> is induced by TGF- $\beta$  to drive cellular quiescence,<sup>27</sup> which is mediated by downregulation of CITED2, we examined the expression of p21<sup>CIP1</sup> and its correlation with CITED2 levels in TGF- $\beta$ -treated cells. We found that both protein and mRNA levels of p21<sup>CIP1</sup> were negatively correlated with CITED2 expression after TGF- $\beta$  stimulation in A549 cells (Figure 4b and Figure 5a). In addition, p21<sup>CIP1</sup> mRNA was induced by TGF- $\beta$  in H1975 cells; moreover, overexpression of CITED2 in H1975 cells not only rendered cells resistant to TGF- $\beta$ -mediated growth arrest but also attenuated p21<sup>CIP1</sup> expression (Figure 4d and Figure 5b). These results suggest a possible role for CITED2 in the regulation of the p21<sup>CIP1</sup> senescence pathway.

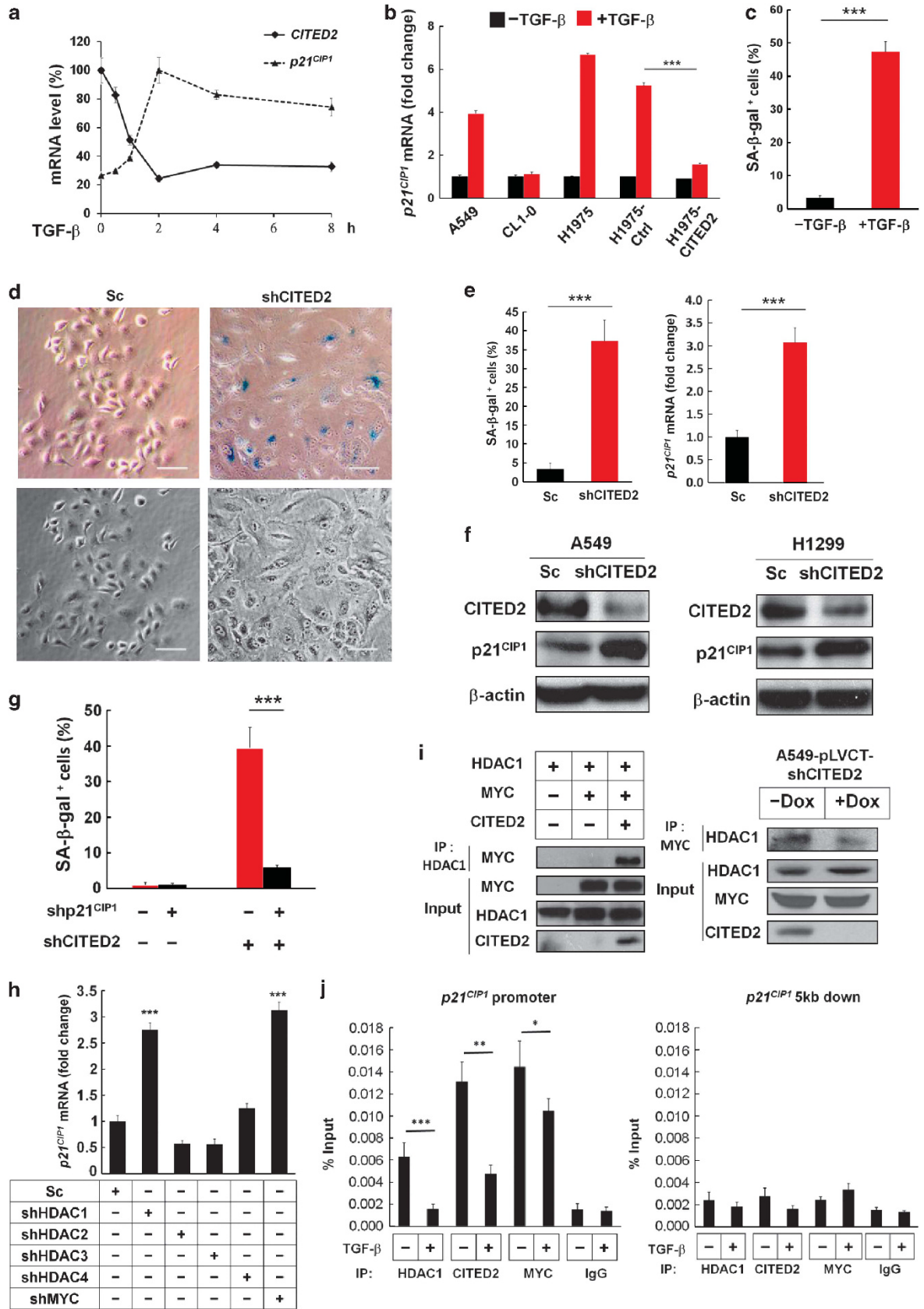
As TGF- $\beta$  induces p21<sup>CIP1</sup> to establish cellular senescence,<sup>28</sup> we investigated the role of CITED2 in the senescence of lung cancer cells. Whereas TGF- $\beta$  downregulated CITED2 and induced senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) in A549 cells (Figures 5a and c), we found that knockdown of CITED2 increased the activity of SA- $\beta$ -gal in A549 with a senescence phenotype, including a flattened and enlarged morphology (Figures 5d and e and Supplementary Figure S4). Additionally, CITED2 silencing in A549 cells induced expression of p21<sup>CIP1</sup> mRNA (Figure 5e and Supplementary Figure S4). These data support that

CITED2 regulates  $p21^{CIP1}$  expression. As  $p21^{CIP1}$  can be induced by a p53-dependent or p53-independent pathway,<sup>29</sup> CITED2 was knocked down in both A549 (wild-type p53) and H1299 (p53 null) cells, followed by immunoblotting to detect  $p21^{CIP1}$  expression. We observed that  $p21^{CIP1}$  was induced in both cell types regardless of p53 expression (Figure 5f), suggesting that  $p21^{CIP1}$  induction in CITED2-silenced cells is

through a p53-independent pathway. To investigate whether increased  $p21^{CIP1}$  expression is responsible for senescence in CITED2-silenced cells, both  $p21^{CIP1}$  and CITED2 were knocked down in A549 cells and the cells were then subjected to the SA- $\beta$ -gal assay (Figure 5g). Although CITED2-silencing induced cellular senescence, knockdown of  $p21^{CIP1}$  rescued the CITED2-silenced cells from senescence, indicating that



**Figure 4** CITED2 downregulation contributes to TGF- $\beta$ -induced quiescence. (a) Clonogenic assays of A549, H1975 and CL1-0 cells treated with or without TGF- $\beta$  (2 ng/ml) for 14 days (top) and stained with crystal violet (bottom). Results are representative of three independent experiments. Error bars represent mean  $\pm$  S.D. \*\*\* $P$  < 0.001. (b) Immunoblot analysis of nuclear and cytoplasmic fractions of A549 cells treated with or without TGF- $\beta$  (2 ng/ml) for 6 h (top). Immunoblot analysis of total cell lysates from A549 cells treated with TGF- $\beta$  (2 ng/ml) for the indicated times (bottom). (c) Q-PCR analysis for the expression of MYC and CITED2 mRNA in A549, H1975 and CL1-0 cells treated with (+) or without (-) TGF- $\beta$  (2 ng/ml) for 6 h (bottom). Results are representative of at least three independent experiments and are expressed as the mean  $\pm$  S.D. \*\*\* $P$  < 0.001. (d) Absolute Q-PCR analysis of TGFBR1 (upper left) and TGFBR2 (upper right) mRNA in A549, H1975 and CL1-0 cells. Immunoblot analysis of total cell lysates from A549, H1975 and CL1-0 cells (lower). (e) H1975 cells were infected with control or CITED2-expressing lentiviral particles for 5 days followed by treatment with or without TGF- $\beta$  (2 ng/ml) for 14 days. Clonogenic assays were carried out (top) by staining with crystal violet (bottom). Results are representative of three independent experiments and are expressed as the mean  $\pm$  S.D. \*\*\* $P$  < 0.001





p21<sup>CIP1</sup> mediates the senescence (Figure 5g). MYC and HDACs inhibit p21<sup>CIP1</sup> expression;<sup>23,30</sup> consistent with other reports, we found that knockdown of MYC or HDAC1 induced p21<sup>CIP1</sup> expression (Figure 5h). Furthermore, we observed that CITED2 interacted with HDAC1 (Supplementary Figure S5). As CITED2 interacts with MYC and HDAC1, it is possible that CITED2 mediates p21<sup>CIP1</sup> suppression by recruiting HDAC1 to the MYC-interacting complex. To test this hypothesis, we first investigated whether CITED2 promotes MYC interaction with HDAC1. Through co-immunoprecipitation, we found that ectopically expressed MYC in HEK293T cells did not interact with HDAC1, but co-expression of CITED2 enriched the MYC–HDAC1-interacting complex (Figure 5i). Similarly, the endogenous MYC–HDAC1-interacting complex was observed through a co-immunoprecipitation assay; knockdown of CITED2 decreased the formation of the MYC–HDAC1-interacting complex (Figure 5i). These data support that CITED2 promotes MYC interactions with HDAC1. Moreover, chromatin immunoprecipitation showed that although both CITED2 and HDAC1 were recruited to the MYC-binding region of the p21<sup>CIP1</sup> promoter, TGF- $\beta$  stimulation attenuated the binding of CITED2 and HDAC1 to the p21<sup>CIP1</sup> promoter (Figure 5j). These data demonstrate that CITED2 modulates MYC-mediated p21<sup>CIP1</sup> suppression by recruiting HDAC1.

#### CITED2 promotes the growth of lung tumor xenografts.

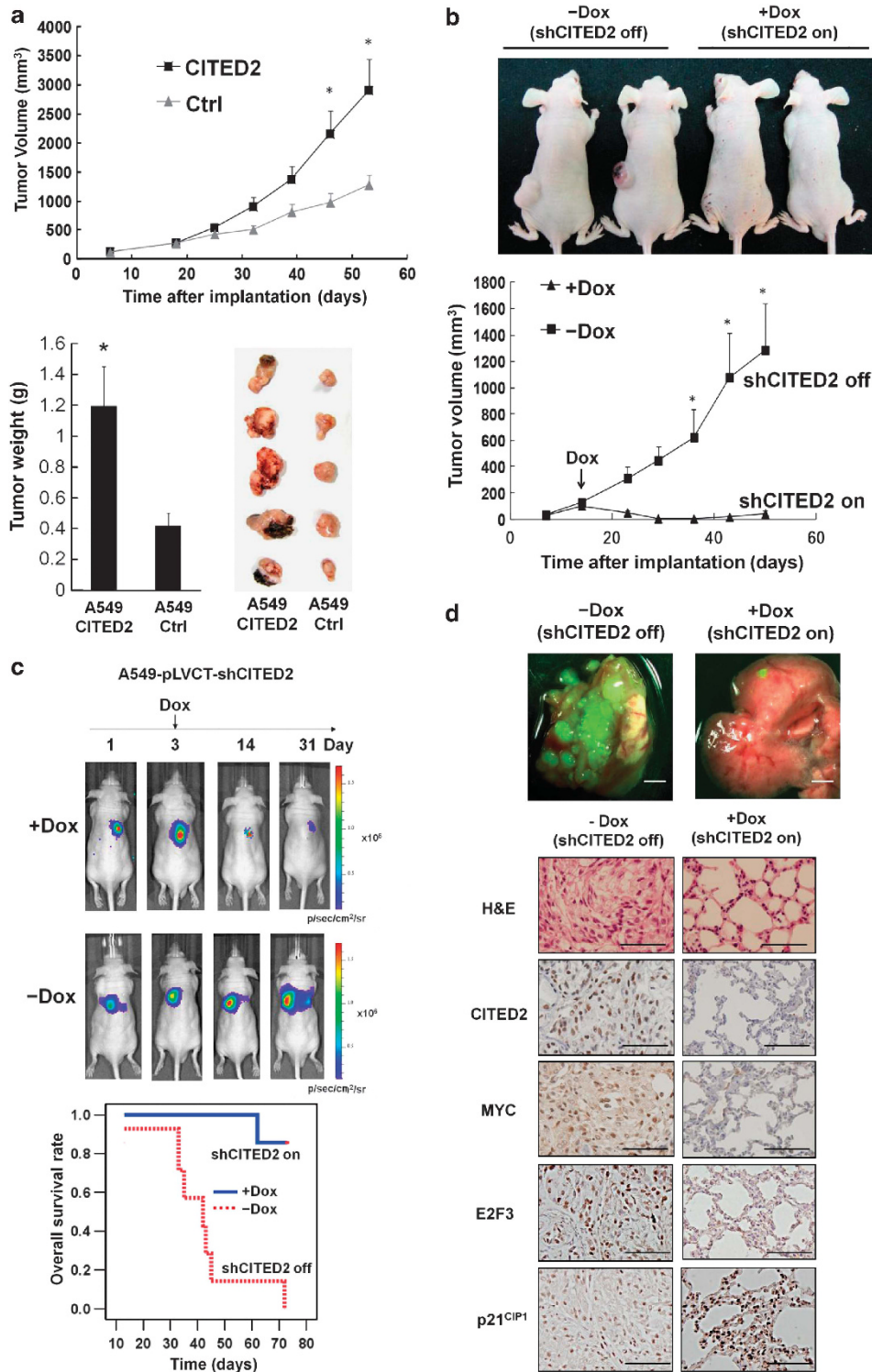
To investigate the role of CITED2 *in vivo*, we injected CITED2-overexpressing or control A549 cells subcutaneously into athymic nude mice. We examined tumor volume over time and observed that CITED2 overexpression significantly enhanced tumor growth rates and increased tumor weight (Figure 6a). To evaluate the significance of endogenous CITED2 in tumor formation, we used A549-pLVCT-shCITED2 cells in a xenograft animal model. When palpable tumor bulges were observed in the host mice, shCITED2 was induced in the xenograft tumors through Dox treatment (Figure 6b). We found that knockdown of endogenous CITED2 with Dox treatment caused significant tumor shrinkage (Figure 6b). To examine whether CITED2 could function as a therapeutic target for lung cancer treatment, luciferase/enhanced green fluorescent protein

(eGFP)-labeled A549 cells with Dox-inducible shCITED2 were orthotopically implanted into the lungs of nude mice and monitored by live imaging. We observed that knockdown of CITED2 significantly decreased the growth of orthotopically implanted lung tumors and increased the overall survival rate of recipient mice ( $P=0.0004$ ; Figure 6c). To examine the expression of CITED2, MYC, E2F3 and p21<sup>CIP1</sup> during tumorigenesis, orthotopically implanted lung tumors were excised and examined for tumor growth using fluorescence microscopy (upper images) and analyzed by immunohistochemistry (IHC) (lower images) for CITED2, MYC, E2F3 and p21<sup>CIP1</sup> expression (Figure 6d). We found that CITED2, MYC and E2F3 were highly expressed in neoplastic cells of lung xenografts (Figure 6d). In contrast, normal alveolar ducts and alveoli were observed in the CITED2 knockdown xenografts, and high p21<sup>CIP1</sup> expression was detected in normal alveolar cells (Figure 6d). These data support that CITED2 regulates lung tumor development.

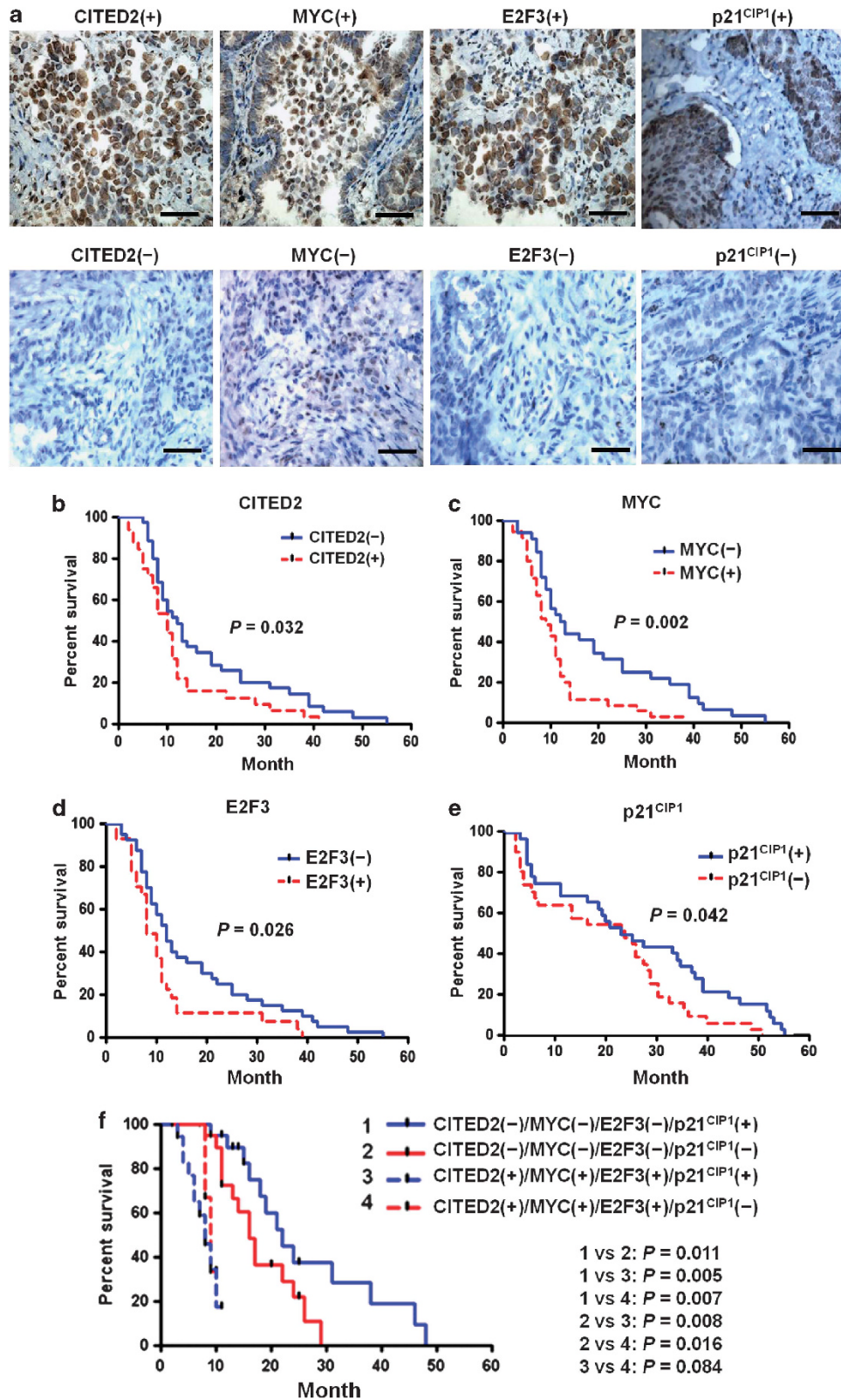
#### Poor overall survival rate of patients with NSCLC was associated with expression of the CITED2/MYC/E2F3/p21<sup>CIP1</sup> pathway.

The above results suggest that the CITED2/MYC/E2F3/p21<sup>CIP1</sup> pathway is activated by TGF- $\alpha$  but inhibited by TGF- $\beta$  cytokine signaling. As abnormal cytokine signaling such as EGFR activation and TGFBR1 silencing occurs in most patients with NSCLC,<sup>2,5</sup> we studied the levels of MYC, CITED2, E2F3 and p21<sup>CIP1</sup> proteins by IHC of a panel of specimens from 72 individuals with NSCLC (Figure 7a). Patient characteristics are summarized in Supplementary Table S1. Kaplan–Meier survival analysis was then conducted to determine the prognostic significance of CITED2, MYC, E2F3 and p21<sup>CIP1</sup> expression in patients with NSCLC. First, the results showed that the CITED2-positive cases were associated with a considerably worse overall survival rate as compared with CITED2-negative ones (Figure 7b). Second, patients with lower MYC expression had a better survival prognosis as compared with patients who expressed high levels of MYC (Figure 7c). Third, patients with high E2F3 expression had a poor survival prognosis (Figure 7d). Fourth, the overall survival rate of p21-positive patients was higher than that of p21-negative patients (Figure 7e). Finally, CITED2 + /MYC + /E2F3 +

**Figure 5** CITED2 modulates MYC-mediated suppression of the p21<sup>CIP1</sup> senescence pathway. (a) Q-PCR analysis of *CITED2* and *p21<sup>CIP1</sup>* mRNA in A549 cells treated with TGF- $\beta$  (2 ng/ml) for the indicated times. (b) Q-PCR analysis of *p21<sup>CIP1</sup>* mRNA in A549, CL1–0, H1975 and H1975-CITED2 cells treated with or without TGF- $\beta$  (2 ng/ml) for 6 h. Results are representative of at least three independent experiments and are expressed as the mean  $\pm$  S.D. \*\*\* $P < 0.001$ . (c) A549 cells treated with or without TGF- $\beta$  (2 ng/ml) for 5 days were subjected to a senescence assay by measuring SA- $\beta$ -gal positive cells. Results are representative of three independent experiments and are expressed as the mean  $\pm$  S.D. \*\*\* $P < 0.001$ . (d) The photographs show SA- $\beta$ -gal activity at pH 6.0, stained in blue color (upper images), and changes in morphology (lower images) of A549 cells 5 days after infection with pLKO.1-Scramble (Sc) or pLKO.1-shCITED2. Scale bars, 20  $\mu$ m. (e) SA- $\beta$ -gal positive cells in d were quantified (left). *p21<sup>CIP1</sup>* mRNA in A549 cells infected with pLKO.1-Sc or pLKO.1-shCITED2 was determined by Q-PCR (right). Results are representative of three independent experiments and are expressed as the mean  $\pm$  S.D. \*\*\* $P < 0.001$ . (f) CITED2 and p21<sup>CIP1</sup> were analyzed by immunoblotting in A549 (left) and H1299 (right) cells infected with Sc or shCITED2 lentiviral particles. (g) A549 cells were first infected with pLKO.1-Sc (shp21<sup>CIP1</sup> –) or pLKO.1-shp21<sup>CIP1</sup> (shp21<sup>CIP1</sup> +) and were subsequently infected with (shCITED2 +) or without (shCITED2 –) pLKO.1-shCITED2 and were assayed as in c. Results are representative of three independent experiments and are expressed as the mean  $\pm$  S.D. \*\*\* $P < 0.001$ . (h) Q-PCR analysis of total RNA derived from A549 cells infected with Sc, shHDAC1, shHDAC2, shHDAC3, shHDAC4 or shMYC lentiviral particles. Results are representative of at least three independent experiments and are expressed as the mean  $\pm$  S.D. \*\*\* $P < 0.001$ . (i) Immunoblotting of HDAC1-immunoprecipitated products or whole-cell lysates from HEK293T cells transfected with pcDNA-HDAC1, pcDNA-HA-MYC and/or Flag-CITED2 (left). A549-pLVCT-shCITED2 cells were treated with or without Dox (1  $\mu$ g/ml) for 24 h, and the expression of HDAC1, MYC and CITED2 in cells was monitored. Binding of endogenous HDAC1 to MYC was determined by co-immunoprecipitation with anti-HDAC1, followed by immunoblotting for MYC (right). (j) Binding of MYC, CITED2 and HDAC1 to the p21<sup>CIP1</sup> promoter region (–301 to –242 bp) and the 5 kb downstream region was analyzed by chromatin immunoprecipitation with antibodies against MYC, CITED2 and HDAC1 in A549 cells treated with or without TGF- $\beta$  (2 ng/ml) for 6 h. Results are expressed as the mean  $\pm$  S.D. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$



**Figure 6** CITED2 enhances lung tumor xenograft growth. (a) Tumor growth of CITED2-overexpressing and vector-only control A549 cells in a subcutaneous xenograft model in nude mice. Tumor volume (top) and tumor weight (bottom left) are shown. The photographs illustrate representative features of tumor growth 8 weeks after injection (bottom right). Error bars indicate the S.E.M. ( $n = 10$  mice/group;  $*P < 0.05$ ). (b) A549-pLVCT-shCITED2 cells were injected subcutaneously into nude mice. Two weeks after cancer cell injection, mice were treated with or without Dox (200  $\mu\text{g}/\text{ml}$  in drinking water). The photographs illustrate tumor growth 42 days after injection (top). Tumor volume was monitored over time as indicated (bottom). Error bars indicate the S.E.M. ( $n = 10$  mice/group;  $*P < 0.05$ ). (c) Luciferase/eGFP-labeled A549-pLVCT-shCITED2 cells were orthotopically implanted into the lungs of nude mice. Three days after implantation, mice were treated with or without Dox (200  $\mu\text{g}/\text{ml}$ ) in drinking water ( $n = 7$  mice/group). Tumor growth was monitored based on luciferase activity over time as indicated by IVIS imaging (top), followed by Kaplan–Meier analysis of the overall survival (bottom) of the recipient mice. (d) Excised lungs from the mice in c were further photographed under bright-field/fluorescence modes (merged image; white scale bars, 0.5 mm; top) and subjected to haematoxylin and eosin (H and E) staining combined with immunohistochemistry analysis (bottom). Black scale bars, 25  $\mu\text{m}$



**Figure 7** Correlation of CITED2/MYC/E2F3/p21<sup>CIP1</sup> signaling with the survival rate among patients with NSCLC. (a) Representative results of immunohistochemical staining for CITED2, MYC, E2F3 and p21<sup>CIP1</sup> in 72 patients with NSCLC. (b–e) Kaplan–Meier analyses of overall survival of patients analyzed in a based on (b) CITED2 expression, (c) MYC expression, (d) E2F3 expression and (e) p21<sup>CIP1</sup> expression. (f) The mean survival times analyzed with different combinations of the four markers CITED2, MYC, E2F3 and p21<sup>CIP1</sup> were measured by Kaplan–Meier analysis

triple positive patients, regardless of  $p21^{CIP1}$  status, exhibited a significantly worse prognosis with an exponential decrease in survival as compared with other patients (Figure 7f).

## Discussion

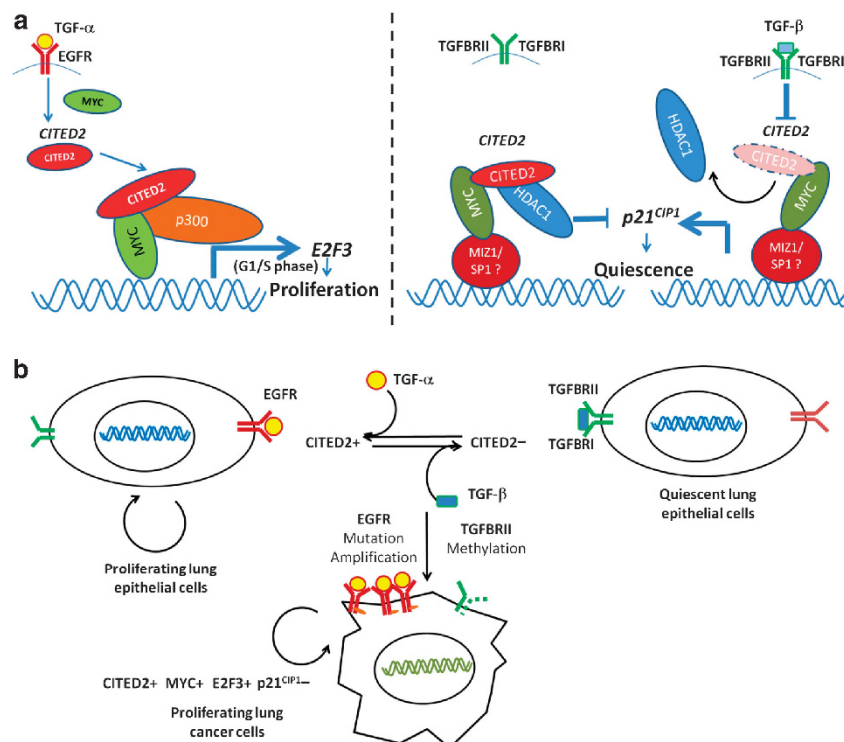
Abnormal cytokine signaling such as that caused by the activating mutation of *EGFR* or silencing of *TGFBR2* is often observed in cells from patients with NSCLC.<sup>2,5</sup> We found that CITED2 responds to TGF- $\alpha$ /EGFR induction and TGF- $\beta$ /TGFBR suppression to orchestrate MYC-mediated proliferation and quiescence, respectively. In addition, CITED2 is induced by many other cytokines.<sup>7</sup> This cytokine-inducible characteristic of CITED2 and its critical role in cell cycle control lead it to function as a molecular switch for cytokine-induced proliferation and quiescence, and abnormal cytokine signaling could turn on the switch, leading to deregulated growth of neoplastic cells (Figure 8).

It is reported that MYC interacts with p300, and MYC-p300 interaction enhances MYC-mediated transactivation of *hTERT* promoter.<sup>31</sup> Consistently, we observed that p300 enhanced MYC-mediated transactivation of *E2F3* promoter, and CITED2 expression boosted MYC-p300-mediated transactivation of *E2F3* promoter (Figure 3e). We found that MYC bound CITED2 through its C-terminus, and

knocking down CITED2 attenuated MYC-p300 interaction. These findings indicate that CITED2 promotes MYC-p300 complex formation and enhances MYC-p300-mediated transactivation of *E2F3*.

Although it is known that MYC activates E2Fs but represses  $p21^{CIP1}$  expression,<sup>22,26</sup> how MYC can function as both a transcriptional activator and repressor has proven elusive. Here, we showed that CITED2 recruited p300 and HDAC1 to promote MYC-mediated transactivation of *E2F3* and transrepression of  $p21^{CIP1}$ . As the binding of MIZ1 and SP1 to the  $p21^{CIP1}$  promoter are essential for MYC-mediated suppression,<sup>21,22</sup> whether the MIZ1/SP1/MYC complex predetermines the CITED2-HDAC1 molecules, but not the CITED2-p300 molecules, that are recruited to the  $p21^{CIP1}$  promoter requires further investigation. The presence of a CITED2-MYC feed-forward network provides an elegant regulatory mechanism to turn on/off or fine-tune a magnitude of MYC-transcriptional activity by modulating CITED2 levels upon cytokine stimulation.

Although we observed that both A549 and H1975 cells responded to TGF- $\beta$ -induced cellular quiescence, MYC was downregulated by TGF- $\beta$  in A549 cells but not in H1975 cells. In contrast, *CITED2* was downregulated in both A549 and H1975 cells, suggesting that TGF- $\beta$ -induced cellular quiescence is mediated by a CITED2-dependent pathway.



**Figure 8** Cytokine-induced CITED2/MYC/E2F3/ $p21^{CIP1}$  signaling in lung cancer. (a) Model of CITED2/MYC/E2F3/ $p21^{CIP1}$  signaling. CITED2 expression is induced by TGF- $\alpha$ /EGFR through MYC. Elevated CITED2 levels further recruit p300 to the *E2F3* promoter to enhance MYC-mediated transactivation, leading to increased G1/S cell cycle transition (left). TGF- $\beta$  downregulates CITED2, and loss of CITED2 prevents MYC from interacting with HDAC1, causing activation of the  $p21^{CIP1}$  promoter and cellular quiescence (right). (b) Model of cytokine-induced homeostasis and neoplastic development of lung epithelial cells. Homeostasis of normal lung epithelial cells is intricately balanced by TGF- $\alpha$ /EGFR proliferation and TGF- $\beta$ /TGFBRI quiescence signaling, both of which are fine-tuned by CITED2 expression. Activation of MYC/CITED2/E2F3/ $p21^{CIP1}$  pathway by abnormal cytokine signaling such as EGFR mutation/amplification-activation and TGFBRI silencing contributes to neoplastic development of lung epithelial cells

Consistent with the previous observation that defective repression of MYC is associated with the loss of TGF- $\beta$ -mediated growth arrest,<sup>32</sup> overexpression of MYC in A549 cells induced CITED2 expression and rendered the cells resistant to TGF- $\beta$ -mediated cell growth arrest (Supplementary Figure S6), indicating that the development of TGF- $\beta$  resistance is in part through the induction of CITED2 expression in tumor cells that overexpress MYC. Supporting this notion, knockdown of endogenous CITED2 by Dox-induced shRNA caused cell growth arrest in MYC-overexpressing A549 cells (Supplementary Figure S6). It is indicated that TGF- $\beta$ -mediated CITED2 downregulation in MDA-MB-231 cells occurs post-transcriptionally.<sup>9</sup> Thus, TGF- $\beta$  mediated downregulation of CITED2 is regulated through a MYC-dependent transcriptional control and a MYC independent post-transcriptional mechanism (Supplementary Figure S7).

Knockout of CITED2 induces cellular senescence in mouse embryonic fibroblasts through a *Bmi-1/CDKN2A*-dependent pathway.<sup>33</sup> Nonetheless, *CDKN2A* levels were not detected in human lung-cancer cell lines in the presence or absence of shRNA against CITED2; an effect on the expression of *Bmi-1* was also not observed after CITED2 knockdown (data not shown). In contrast, p21<sup>CIP1</sup> did increase in A549 cells after CITED2 knockdown, and p21<sup>CIP1</sup> silencing partially rescued the senescence that was induced by CITED2 knockdown, suggesting that p21<sup>CIP1</sup> pathway is dominant in lung cancer cells. p21<sup>CIP1</sup> blocks the function of E2Fs through direct interaction with E2Fs<sup>34</sup> or by inhibiting Cyclin/Cdk-mediated phosphorylation of retinoblastoma protein.<sup>35</sup> The observation that CITED2 modulates both E2F3 and p21<sup>CIP1</sup> indicates a role for CITED2 in synchronizing E2F3 proliferation and p21<sup>CIP1</sup> quiescence pathways.

Finally, we observed that molecules in the CITED2/MYC/E2F3/p21<sup>CIP1</sup> pathway are elevated in patients with NSCLC who have a poor prognosis. As MYC, E2F3 and p21<sup>CIP1</sup> have been used as prognostic or diagnostic markers in lung cancer,<sup>18,36–38</sup> our observation that hyperactive CITED2/MYC/E2F3/p21<sup>CIP1</sup> signaling is associated with overall survival of patients with NSCLC provides a novel index for predicting disease progression and clinical outcome.

## Materials and Methods

**Reagents.** Recombinant human TGF- $\alpha$  and TGF- $\beta$ 1 were purchased from PeproTech EC (London, UK). Dox (doxycycline hyclate) was ordered from Sigma-Aldrich (St. Louis, MO, USA).

**Cell culture.** A549 cells were obtained from the American Type Culture Collection. H1975 cells were kindly provided by Dr. Wayne Chang in the National Health Research Institutes, Taiwan. CL1–0 and CL1–5 cells were established in our laboratory as described.<sup>39</sup> All lung cancer cell lines tested positive for human origin and were grown in RPMI-1640 medium with 10% fetal bovine serum.

**Plasmid construction.** Detailed procedures for construction of CITED2, MYC or EGFR-L858R-expressing lentiviral vectors, CITED2 or E2F3 promoter reporters and shRNA lentiviral vectors can be found in Supplementary Information.

**Q-PCR.** The Q-PCR assay was performed as described.<sup>25</sup> Relative gene expression was analyzed using the  $2^{-\Delta\Delta CT}$  method using the LightCycler 480 Real-Time PCR System (Roche Applied Science, Indianapolis, IN, USA) combined with the Universal Probe Library (Roche Applied Science). Absolute Q-PCR was carried out as described.<sup>37</sup> 18S rRNA was used as a reference transcript. Primer sequences designed to detect specific genes are listed in Supplementary Table S2.

**Luciferase reporter assay.** The luciferase assays were performed according to the standard protocols (Dual luciferase reporter assay, Promega, Madison, WI, USA). Renilla luciferase plasmid (pRL-SV40) was co-transfected as an internal control.

**Chromatin immunoprecipitation (ChIP).** ChIP assay were performed according to the protocol for the LowCell# ChIP kit (Diagenode, Liege, Belgium) and quantified by Q-PCR. To increase the specificity of Q-PCR in ChIP assay, UPL probe system (Roche Applied Science) was applied in the assay. Antibodies are described in the Supplementary Information. Primer sequences and the corresponding probes designed to detect specific promoters are listed in Supplementary Table S2.

**Clonogenic assay.** For clonogenic assay with lung cancer cells, 1000 cells/well in a 6-well plate or 3000 cells/well in a 100 mm Petri dish were seeded. Colonies were fixed and stained with 3.7% formaldehyde/80% methanol/0.25% crystal violet (Fisher Scientific, Fairlawn, NJ, USA) and counted 2 weeks after plating.

**Co-immunoprecipitation assay and western blot analysis.** Co-immunoprecipitation assays and western blots were carried out as described.<sup>40</sup> Antibodies are described in the Supplementary Information.

**IF and IHC.** IF was performed as previously described.<sup>40</sup> Antibodies are described in the Supplementary Information.

**GST pull-down assay.** Flag-tagged CITED2 was transcribed *in vitro* and translated using TNT Coupled Reticulocyte Lysate Systems (Promega). GST-fused MYC proteins were immortalized on glutathione-Sepharose beads. The GST pull-down assay was performed as described.<sup>13</sup>

**Senescence assay.** The senescence assay was performed according to the protocol for the Cellular Senescence Detection Kit (Cell Biolabs Inc., San Diego, CA, USA). Cells were washed and transferred to PBS, viewed with an inverted transmission microscope (OLYMPUS IX71, Tokyo, Japan) and documented with a digital imaging system (OLYMPUS C50502, Tokyo, Japan). SA- $\beta$ -gal positive cells were quantified by counting positive and negative cells in five random visual fields and averaging the results.

**Patients.** Patient samples were obtained at the time of surgery in Tri-Service General Hospital and granted by the Institutional Review Board protocol number 099–05–183. All patients gave their informed consent, and the ethics and scientific committees of the participating institutions approved the study. Tumor types were determined according to WHO classification.

**Xenograft tumorigenicity assay.** Xenograft tumorigenicity was evaluated as described.<sup>25</sup> Detailed procedures for xenograft tumorigenicity assay and orthotopic implantation can be found in Supplementary Information.

**Statistical analysis.** Statistical analysis was performed using Student's *t*-test or a one-way or two-way analysis of variance followed by Turkey's test, as appropriate.  $P < 0.05$  was considered to be statistically significant. Overall survival rate was assessed using the Kaplan–Meier method and compared using the log-rank test.

## Conflict of Interest

The authors declare no conflict of interest.

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