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ORIGINAL ARTICLE

The nuclear receptor TR3 regulates mTORC1 signaling in lung cancer cells expressing wild-type p53

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The orphan nuclear receptor TR3 (NR41A and Nur77) is overexpressed in most lung cancer patients and is a negative prognostic factor for patient survival. The function of TR3 was investigated in non-small-cell lung cancer A549 and H460 cells, and knockdown of TR3 by RNA interference (siTR3) inhibited cancer cell growth and induced apoptosis. The prosurvival activity of TR3 was due, in part, to formation of a p300/TR3/ specificity protein 1 complex bound to GC-rich promoter regions of survivin and other Sp-regulated genes (mechanism 1). However, in p53 wild-type A549 and H460 cells, siTR3 inhibited the mTORC1 pathway, and this was due to activation of p53 and induction of the p53-responsive gene *sestrin 2*, which subsequently activated the mTORC1 inhibitor AMP-activated protein kinase α (AMPKα) (mechanism 2). This demonstrates that the pro-oncogenic activity of TR3 in lung cancer cells was due to inhibition of p53 and activation of mTORC1. 1,1-Bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH) is a recently discovered inhibitor of TR3, which mimics the effects of siTR3. DIM-C-pPhOH inhibited growth and induced apoptosis in lung cancer cells and lung tumors in murine orthotopic and metastatic models, and this was accompanied by decreased expression of survivin and inhibition of mTORC1 signaling, demonstrating that inactivators of TR3 represent a novel class of mTORC1 inhibitors.
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Introduction

NR4A1 (TR3 and Nur77), NR4A2 (Nurr1) and NR4A3 (Nor-1) are orphan nuclear receptors (Gronemeyer *et al*., 2004; McKenna *et al*., 2009) and were initially identified as immediate-early genes induced by nerve growth factor in PC-12 cells (Milbrandt, 1988). *NR4A* genes are induced by multiple stressors in various tissues, and there is increasing evidence that NR4A receptors are essential for maintaining cellular homeostasis and they have a role in vascular function, metabolic pathways, inflammation, steroidogenesis and the central nervous system (reviewed in Maxwell and Muscat, 2006; Pearen and Muscat, 2010).

TR3 is highly expressed in multiple cancer cell lines and tumors, and higher levels of nuclear TR3 have been observed in colon, bladder and pancreatic tumors compared with non-tumor tissues (Maruyama *et al*., 1995; Ke *et al*., 2004; Chintharlapalli *et al*., 2005; Li *et al*., 2006; Cho *et al*., 2007, 2010; Zhang, 2007; Lee *et al*., 2010b). With few exceptions, knockdown of TR3 by RNA interference or a related technique in cancer cells resulted in growth inhibition, induction of apoptosis or decreased angiogenesis, indicating that TR3 is a pro-oncogenic factor (Bras *et al*., 2000; Kolluri *et al*., 2003; Ke *et al*., 2004; Zeng *et al*., 2006; Azoitei *et al*., 2010; Wu *et al*., 2010; Lee *et al*., 2010b). Many different classes of apoptotic agents induce apoptosis in cancer through TR3-dependent pathways (reviewed in Zhang, 2007). One of the major mechanisms associated with these effects involves nuclear export of TR3 and formation of a proapoptotic TR3–bcl-2 complex, which can decrease mitochondrial membrane potential, resulting in the release of cytochrome *c* and activation of the extrinsic apoptosis pathway (Li *et al*., 2000; Lin *et al*., 2004; Kolluri *et al*., 2008). Cytosporone B and related analogs inhibit cancer cell and tumor growth through both nuclear export of TR3 and activation of nuclear TR3 and, unlike most other apoptosis-inducing agents, cytosporone B binds directly to TR3 (ligand-binding domain) (Zhan *et al*., 2008; Liu *et al*., 2010).

1,1-Bis(3'-indolyl)-1-(*p*-substituted phenyl)methanes (C-DIMs) activate multiple nuclear receptors, and the *p*-methoxyl derivative (DIM-C-pPhOCH_3) activates nuclear TR3 and induces proapoptotic genes, including *p21*, *fas* and *TRAIL*, and apoptosis (Chintharlapalli *et al*., 2005; Cho *et al*., 2007, 2010; Lee *et al*., 2009). In contrast, the *p*-hydroxy C-DIM analog (1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane, DIM-C-pPhOH) deactivates nuclear TR3 and also induces apoptosis and inhibits pancreatic cancer cell and tumor growth (Lee *et al*., 2010b). Both TR3 knockdown (siTR3) and DIM-C-pPhOH decreased expression of survivin and induced

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apoptosis in pancreatic cancer cells. Constitutive expression of survivin and other specificity protein 1 (Sp1)-regulated genes (for example, bcl-2) involved a p300/TR3/Sp1 complex bound to the proximal GC-rich region of the survivin promoter (Lee *et al*., 2010b). Deactivation of TR3 and downregulation of survivin were due to loss of p300 from this complex, and similar results were observed in this study in lung cancer cells (mechanism 1). In this study, another TR3-dependent pro-oncogenic pathway was identified in non-small-cell lung cancer (NSCLC) A549 and H460 cells that are wild-type for p53. siTR3 and DIM-C-pPhOH inhibited mTORC1 signaling through activation of AMP-activated protein kinase α (AMPKα), and this was due to upstream activation of p53 and sestrin 2. Thus, endogenous TR3 maintains p53, sestrin 2 and AMPKα in a repressed state, thereby facilitating enhanced mTORC1 activity in cancer cells. These results are consistent with the pro-oncogenic function of TR3; however, this study also demonstrates the potential clinical importance of drugs, such as DIM-C-pPhOH that block mTORC1 signaling through deactivation of TR3.

Results

TR3 expression and prognostic significance in lung cancer patients

The orphan nuclear receptor TR3 is overexpressed in several different cancer cell lines and tumors and, in this study we investigated expression of this receptor in NSCLC tumors by immunostaining and also determined the prognostic significance of TR3 expression. Figures 1a–c show the typical immunostaining of TR3 in normal lung tissue and adenocarcinoma (A), squamous-cell carcinoma and large cell carcinoma (B). Relatively low TR3 staining was observed in normal lung tissue compared with lung tumors. Moreover, among the 59 control and 59 lung cancer patients (Table 1), many of the lung cancer patients (62.7%) exhibited high TR3 staining, whereas high TR3 staining was not observed in normal lung tissue (Figure 1c). Lung tumor tissue samples also exhibited moderate (33.9%) and low (3.4%) staining, whereas in normal lung tissue non-detectable (61.8%) TR3 staining was observed (Supplementary Table S1). A Kaplan–Meier plot of survival probability over time for 58 lung cancer patients with high TR3 expression versus ⩽moderate TR3 showed that high expression of TR3 was associated with increased mortality for NSCLC patients (Figure 1d). Moreover, univariate and multivariate analyses of TR3 expression versus survival probability for patients with different stages of lung cancer invariably showed that high TR3 expression was inversely correlated with patient survival (Table 1; Supplementary Figure S1 and Supplementary Table 2).

Table 1 Cox proportional hazards regression model analysis of prognostic factors in patients with NSCLC

Variables	*Hazards ratio (95% CI)*	*Comparison*	P-*value*
Univariate analysis			
TR3	2.57 (1.21–5.47)	High/⩽moderate	0.0151[a]
Gender	1.42 (0.67–3.02)	Female/male	NS
Age	0.82 (0.40–1.65)	⩾65/<65	NS
PT factor	3.24 (1.45–7.27)	T_{3-4}/T_{1-2}	0.0045[a]
PN factor	2.45 (1.27–4.75)	Positive/negative	0.0082[a]
Cancer type	1.67 (0.73–3.81)	Non-ADC/ADC	NS
Cancer stage	2.64 (1.34–5.20)	II–IV/I	0.0052[a]
Multivariate analysis			
TR3	2.59 (1.05–6.38)	High/⩽moderate	0.0397[a]
Gender	1.78 (0.75–4.20)	Female/male	NS
Age	1.12 (0.49–2.55)	⩾65/<65	NS
PT factor	5.96 (1.53–23.27)	T_{3-4}/T_{1-2}	0.0106[a]
PN factor	3.16 (0.78–12.87)	Positive/negative	NS
Cancer type	2.09 (0.13–1.75)	Non-ADC/ADC	NS
Cancer stage	0.77 (0.16–3.78)	II–IV/I	NS

Abbreviations: ADC, adenocarcinoma; non-ADC, carcinosarcoma, large-cell carcinoma and squamous-cell carcinoma; CI, confidence interval; NS, no significance; NSCLC, non-small-cell lung cancer.
[a]Statistically significant. PT and PN refers to tumor size and degree of metastasis, respectively.

TR3 knockdown inhibits mTORC1 signaling

TR3 is highly expressed in pancreatic cancer cells, and knockdown of the receptor by RNA interference (siTR3) decreased cell growth and induced apoptosis (Lee *et al*., 2010b). siTR3 also decreased proliferation of A549 and H460 NSCLC cell lines (Figure 2a). In pancreatic cancer cells, siTR3 also decreased survivin, bcl-2 and other growth-promoting and prosurvival genes, and it was shown that expression of survivin was dependent on formation of a p300/TR3/Sp1 complex at the proximal GC-rich survivin promoter. Knockdown of TR3, Sp1 or p300 by RNA interference decreased survivin and induced apoptosis (Lee *et al*., 2010b). Transfection of A549 or H460 cells with siTR3 also decreased expression of survivin and other *Sp1*-regulated genes (EGFR, bcl-2 and c-myc) (Figure 2b) as previously reported in pancreatic cancer cells, and the decreased survivin expression was not due to decreased Sp1 protein (data not shown). siTR3 decreased transactivation in H460 cells transfected with two GC-rich survivin promoter constructs, a GAL4–Sp1 chimera, a GAL4–luc reporter plasmid and a consensus GC-rich construct (Supplementary Figures S2A and B). Gel mobility shift assays using a consensus GC-rich oligonucleotide showed that Sp1 binding using nuclear extracts from H460 cells transfected with siTR3 was unchanged, whereas there was a significant decrease in p300 binding (Supplementary Figure S2C), and this was consistent with results previously observed in pancreatic cancer cells (Lee *et al*., 2010b). Knockdown of Sp1 (siSp1) or p300 (sip300) decreased transactivation in cells transfected with GC-rich constructs (Supplementary Figure S2D), decreased expression of survivin and induced poly (ADP-ribose) polymerase cleavage in H460 cells (Supplementary Figure S2E) and p53-null H1299 lung cancer cells (Supplementary Figure S2F). These data support the role of p300/TR3/Sp1 complex in expression of survivin and maintenance of lung cancer survival as previously observed in pancreatic cancer cells (Lee *et al*., 2010b).

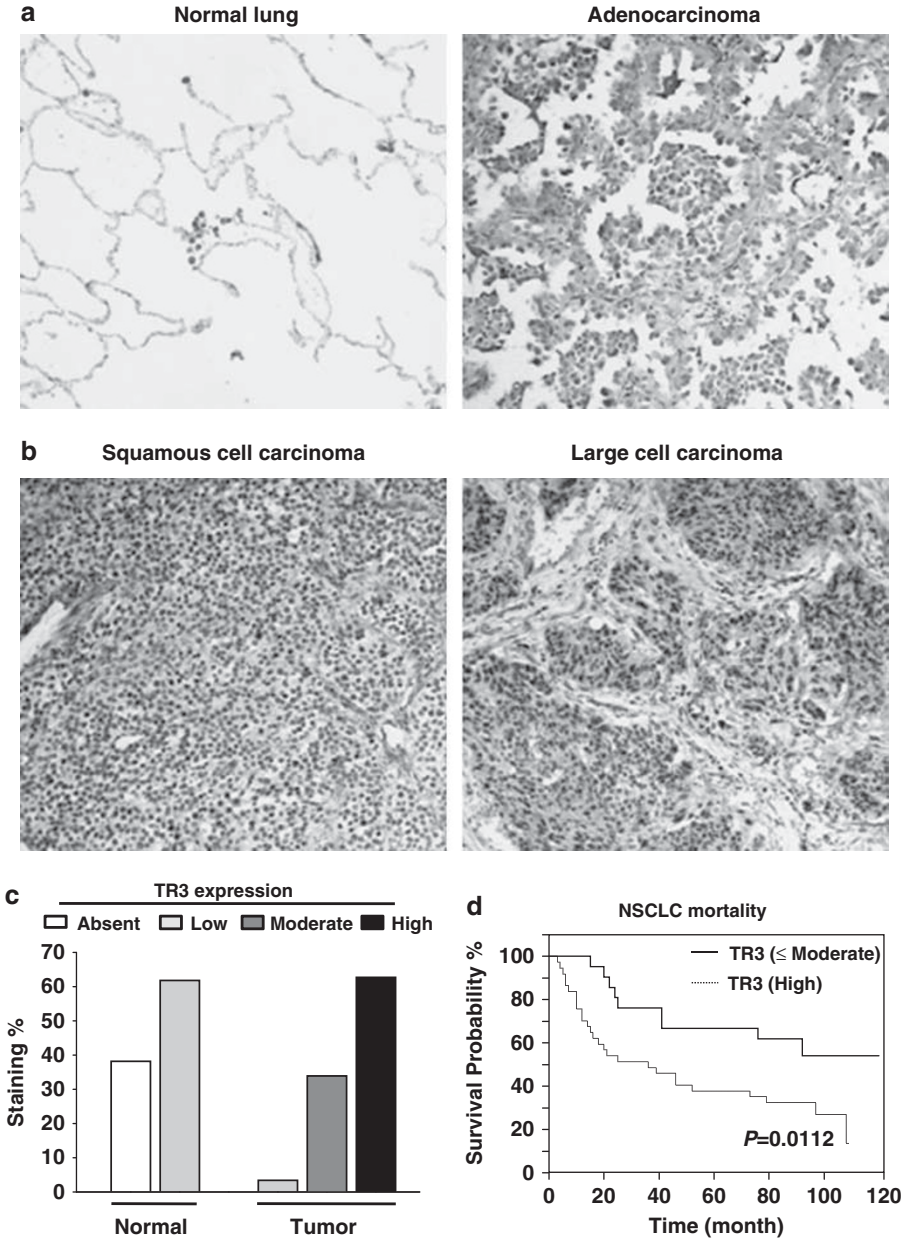

Figure 1 Immunohistochemical analysis of TR3 in 59 human non-small-cell lung carcinomas and 59 self-matching normal adjacent lung tissues. (**a**, **b**) Representative TR3 staining is shown for normal lung, adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma ($\times 100$). (**c**) Histogram of immunohistochemical score distribution obtained from the analysis showing enhanced expression of TR3 in lung tumors, but low or non-detectable TR3 staining was observed in normal lung tissues. (**d**) Association of TR3 overexpression with poor clinical outcome for NSCLC patients. Kaplan–Meier survival analysis in patients with NSCLC ($n = 58$) according to TR3 expression.

The induction of apoptosis in A549 and H460 cells transfected with siTR3 was significant (Figure 2b) but appreciably lower than observed in pancreatic cancer cells (Lee *et al*., 2010b); we hypothesized that TR3 may also regulate other growth-promoting and anti-apoptotic pathways and, based on results of preliminary screening, we focused on the mTORC1 pathway. Figure 2c illustrates that in A549 and H460 cells transfected with siTR3, there was significantly decreased phosphorylation of three key phospho-proteins regulated by mTORC1, namely, phosphorylated p70S6K, pS6RP and 4E-BP1, whereas levels of the corresponding unphosphorylated proteins were unchanged. Moreover, the effects of siTR3 on the mTORC1 pathway were different from the p300/TR3/Sp1-dependent regulation of survivin and apoptosis (Lee *et al*., 2010b; Supplementary Figure S2), as knockdown of Sp1 in A549 cells did not affect expression of p70S6K, S6RP or 4E-BP1 (phosphorylated/unphosphorylated; Figure 2d).

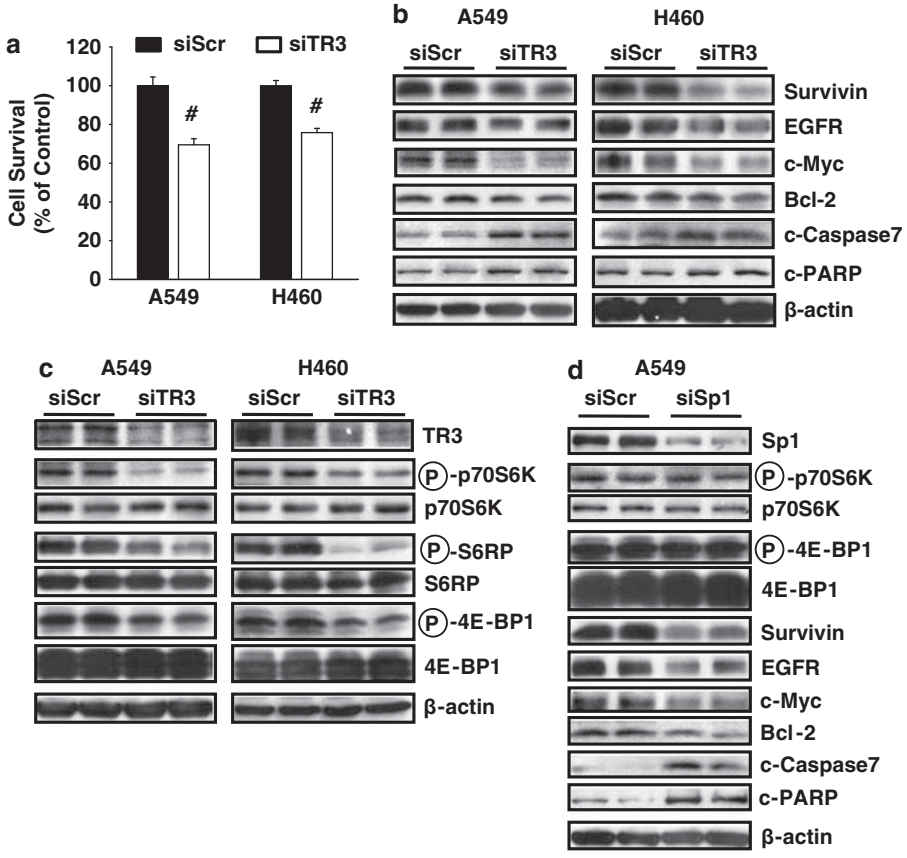

Figure 2 Knockdown of TR3 inhibits cell growth and induces apoptosis in NSCLC cells. (**a**) Cell survival. After transfection with either siScr or siTR3 for 4 days, the number of cells in each well was counted. The experiment was repeated three times with four replicates each and the data are presented as means with s.d. $^{\#}P<0.001$ versus siScr. (**b**–**d**) Cells were transfected with an indicated small interfering RNA for 72 h, and whole-cell lysates were analyzed by western blot analysis. β-Actin was used as a loading control and the experiment was repeated three times with similar results. TR3 knockdown (**c**) varied between 60 and 80% in this study, and similar results were observed in subsequent experiments using siTR3.

TR3 inhibits p53→sestrin (induction)→AMPKα (activation) by inactivating 53

Phosphorylated AKT and AMPKα are potential upstream activators and deactivators, respectively, of mTORC1, and transfection of siTR3 into A549 and A460 cells did not affect expression of phospho-AKT, whereas there was an increase in phospho-AMPKα in both cell lines (Figure 3a). Moreover, in A549 cells transfected with siTR3, siTR3 increased phosphorylation of AMPKα and decreased phosphorylation of 4E-BP1 and p70S6K, whereas knockdown of AMPKα alone had minimal effects on phospho-p70S6K or 4E-BP1 (Figures 2c and 3a). However, siAMPKα inhibited the effects of siTR3 on phosphorylation of 70S6K and 4E-BP1, confirming that inactivation of TR3 resulted in activation of AMPKα and inhibition of the mTORC1 pathway.

Both A549 and H460 cells are LKB1 null, which cannot be an upstream regulator of mTORC1; therefore, the effects of siTR3 on expression of sestrin 2, another potential upstream regulator of AMPKα, were examined (Budanov and Karin, 2008). Transfection of A549 and H460 cells with siTR3 resulted in increased levels of sestrin protein, mRNA and luciferase activity in cells transfected with pSESN2–luc, a construct containing the −730 to +190 regions from the sestrin 2 promoter (Figure 3c). The role of sestrin 2 in mediating TR3-dependent activation of AMPKα and inhibition of mTORC1 was determined by RNA interference in A549 cells transfected with siTR3 and siSENS2 alone or in combination (Figure 3d). The effects of siTR3 alone were consistent with activation and inactivation of AMPKα and mTORC1 signaling, respectively. Sestrin 2 knockdown alone had minimal effects, whereas in cells co-transfected with siTR3 plus siSENS2 the loss of sestrin 2 expression abrogated the effects of siTR3- on AMPKα-independent inhibition of the mTORC1 pathway. In addition, we also showed that overexpression of TR3 in H460 cells decreased SESN2 and phosphorylation of AMPKα, and activated mTORC1 signaling (Supplementary Figure S3A); however, the effects of TR3 overexpression were not observed in cells co-transfected with siSESN2 (Supplementary Figure S3B).

p53 activates sestrin 2 (Budanov and Karin, 2008), and TR3 directly interacts with p53 and inhibits the transcriptional activity by blocking p53 acetylation (Budanov and Karin, 2008). Functional interactions between TR3 and p53 were examined in A549 cells (p53

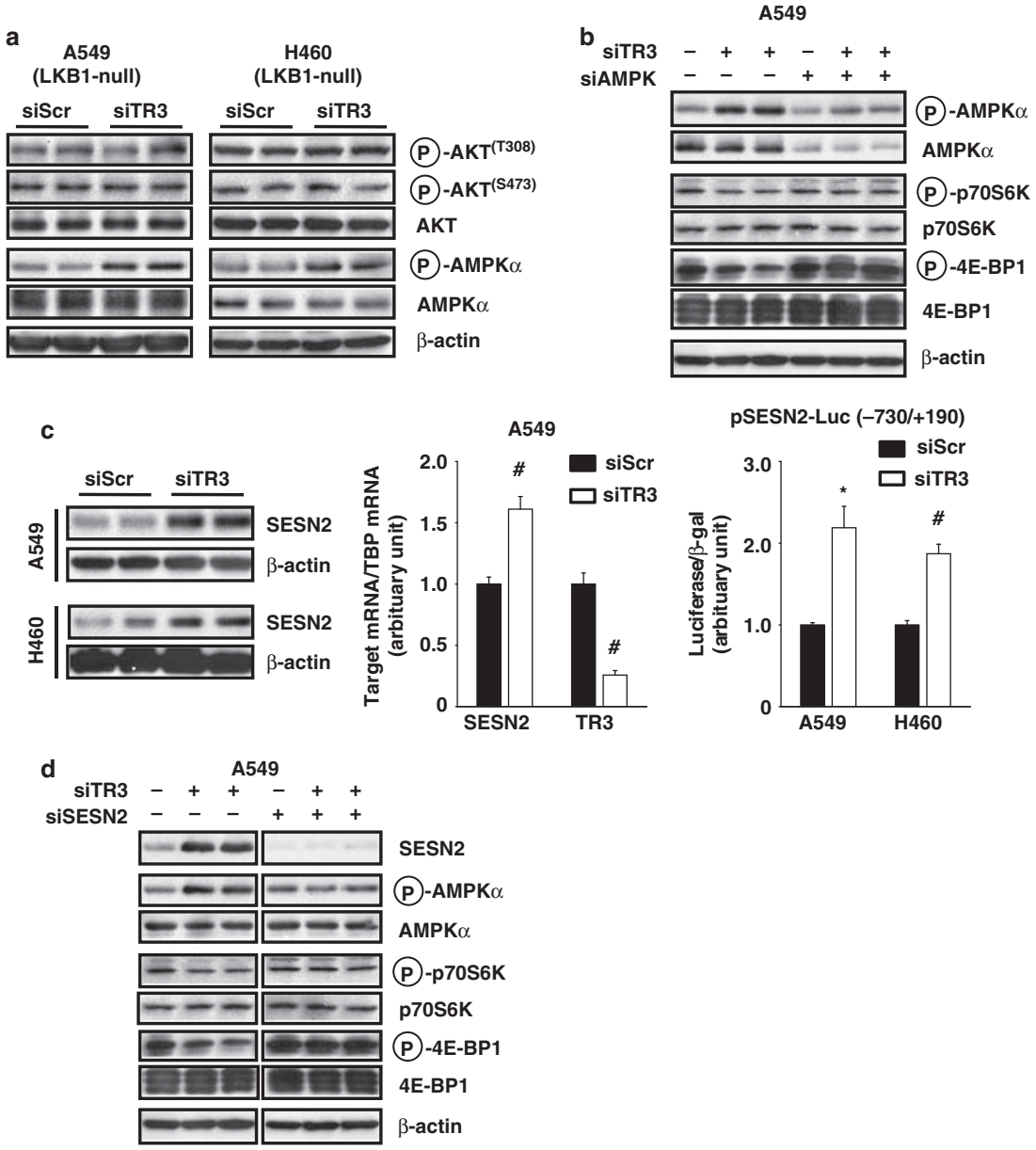

Figure 3 Knockdown of TR3 inhibits mTORC1 signaling through sestrin-2-dependent but LKB1-independent activation of AMPKα in NSCLC cells. (**a**, **b**, **d**) Cells were transfected with indicated small interfering RNAs (siRNAs) for 72 h, and whole-cell lysates were analyzed by western blot analysis. (**c**) Cells were transfected with either siScr or siTR3 for 72 h (left panel) or for 60 h (middle panel), and sestrin 2 protein and mRNA levels were determined by western blot analysis and real-time PCR, respectively, as described in the Materials and methods. β-Actin was used as a loading control, and TATA-binding protein (TBP) was used as an internal control. Sestrin 2 and TR3 mRNA levels are presented as means with s.d. of three experiments. $^{\#}P<0.001$ versus siScr. (**c**; right panel) Cells were co-transfected with each siRNA and pSESN2–luc (−730/+190), and luciferase activity (relative to β-galactosidase activity) was determined. The corresponding empty vector was used as a control, and the results are presented as means with s.d. of three experiments. $^{*}P<0.005$ and $^{\#}P<0.001$ versus siScr.

wild type) transfected with siTR3 and sip53 alone or in combination (Figure 4a). Transfection with siTR3 induced sestrin 2 and only slightly increased p53 protein levels and affected phosphorylation of AMPKα, p70S6K and S6RP, as indicated in Figures 2c, 3b and d. sip53 alone decreased p70S6K and S6RP phosphorylation. In combination with siTR3, sip53 also decreased the effects of siTR3 on sestrin 2 protein expression and phosphorylation of AMPKα, 70S6K and S6RP. In contrast, transfection of siTR3 into p53-null H1299 lung cancer cells did not affect expression of sestrin 2 or the phospho-proteins associated with AMPKα-dependent inhibition of mTORC1 (Figure 4b), whereas p53-independent inactivation of the TR3/p300/Sp1 complex by siTR3 resulted in downregulation of survivin and induction of apoptosis (Supplementary Figure S4A). However, we observed that overexpression of p53 in H1299 cells co-transfected with siTR3 increased SESN2/

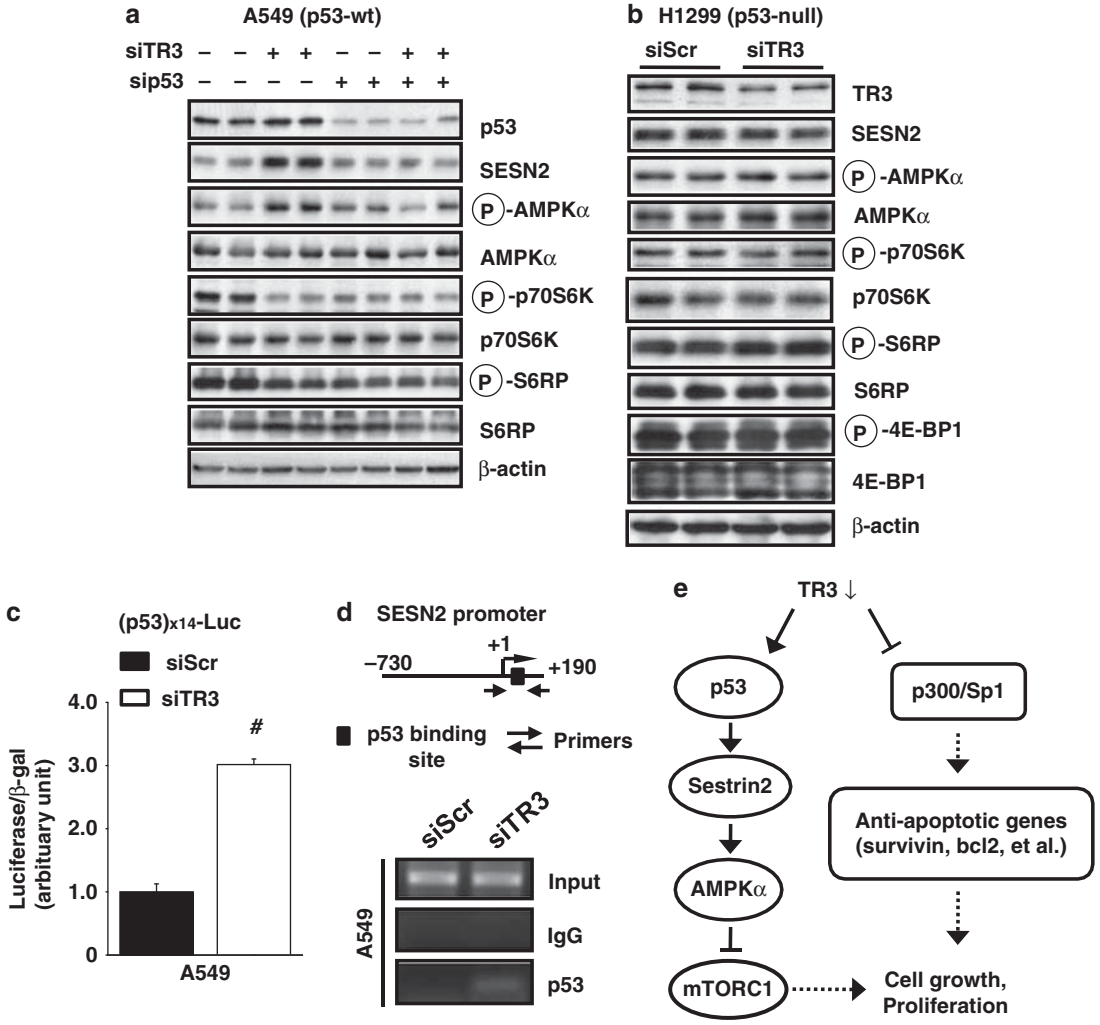

Figure 4 Knockdown of TR3 regulates sestrin 2–AMPKα–mTORC1 through induction of p53 transcriptional activity. (**a**, **b**) Cells were transfected with the indicated small interfering RNAs (siRNAs) for 72 h, and whole-cell lysates were analyzed by western blot analysis. β-Actin was used as a loading control, and the experiment was repeated three times with similar results. (**c**) Cells were co-transfected with each siRNA and $p53_{x14}$–luc, and luciferase activity (relative to β-galactosidase activity) was determined. The corresponding empty vector was used as a control, and the results are presented as means with s.d. of three experiments. $^{\#}P<0.001$ versus siScr. (**d**) ChIP assay. Cells were transfected with either siScr or siTR3 for 60 h, and binding of p53 with the sestrin 2 promoter region containing p53-binding site as indicated was determined as described in the Materials and methods. The experiment was repeated three times with similar results. (**e**) Schematic diagram summarizing knockdown of TR3 inhibits cell growth through dual targeting of mTORC1 signaling and Sp1 in human lung cancer cells expressing wild-type p53.

phospho-AMPKα and inhibited mTORC1 signaling, whereas this was not observed in cells co-transfected with siTR3-overexpressing mutant p53 (Supplementary Figure S4B). Inactivation of TR3 by RNA interference also significantly induced luciferase activity in cells transfected with a p53-responsive construct (Figure 4c), and in A549 cells transfected with siTR3, results of a chromatin immunoprecipitation (ChIP) assay showed enhanced recruitment of p53 to the p53 response element in the sestrin 2 promoter (Figure 4d). Supplementary Figure S5A confirms that both p53 and TR3 are co-immunoprecipitated in A549 and H460 cells as previously reported (Zhao *et al*., 2006). We also demonstrate that TR3 overexpression (a) decreased luciferase activity in A549 and H460 cells transfected with the p53–luc construct, (b) decreased p53 binding to the SESN2 promoter in a ChIP assay in H460 cells and (c) decreased luciferase activity in H460 cells transfected with the SESN2–luc construct (Supplementary Figures S5B–D). Thus, TR3 is not only required for basal expression of survivin and survival genes via a p300/TR3/Sp1 complex, but also activates the mTORC1 pathway through inactivation of p53 (Figure 4e), suggesting that agents or drugs that inactivate or block TR3 signaling will be highly effective as inhibitors of cancer cell and tumor growth and survival.

DIM-C-pPhOH inactivates TR3 and TR3-dependent responses

DIM-C-pPhOH inactivates TR3 in pancreatic cancer cells and the effects of this compound and TR3 knockdown (siTR3) were comparable (Lee *et al*.,

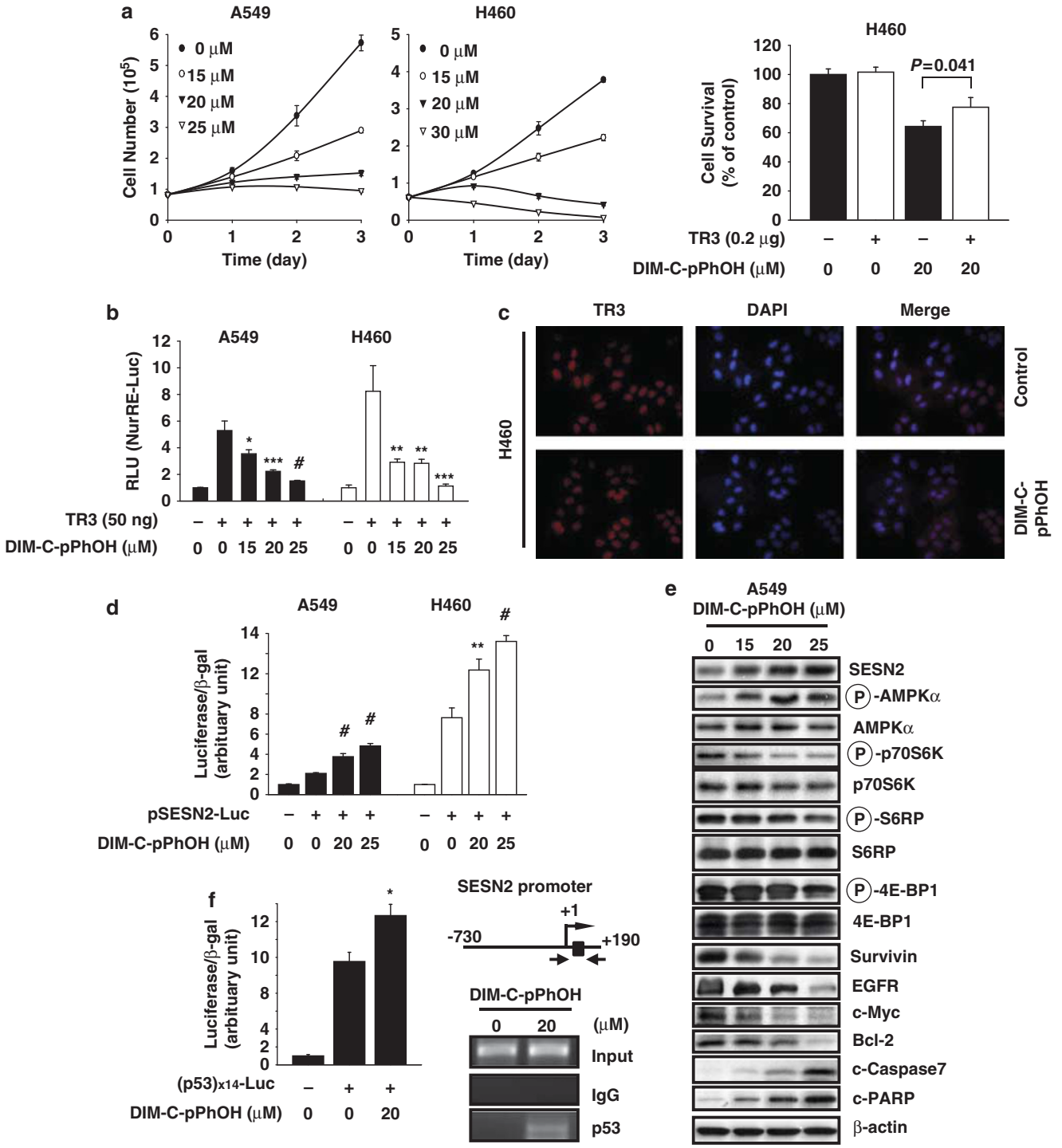

Figure 5 DIM-C-pPhOH, the TR3 deactivator, inhibits mTORC1 signaling through activation of the p53/sestrin 2/AMPKα axis in NSCLC cells. (**a**; left panel) Cells were treated with either dimethyl sulfoxide (DMSO) or DIM-C-pPhOH for 3 days, and the number of cells in each well was counted on days 1, 2 and 3. (**a**; right panel) Cells were transfected with either Flag-empty or Flag-TR3 for 24 h, and treated with DIM-C-pPhOH (20 μM) for another 24 h. The number of cells in each well was counted, and the data are presented as means with s.d. of three experiments. (**b**) Cells were co-transfected with NurRE–luc (0.1 μg) and 50 ng of Flag-TR3 for 5 h, and treated with DIM-C-pPhOH for 18 h. Luciferase activity (relative to β-galactosidase activity) was determined, and the corresponding empty vector was used as a control. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ and $^{\#}P<0.001$ versus DMSO + TR3. (**c**) Subcellular localization of TR3. Cells were treated with either DMSO or 20 μM of DIM-C-pPhOH for 12 h, and endogenous TR3 was stained and visualized as described in the Materials and methods. (**d**, **f**; left panel) Cells were transfected with pSESN2–luc (−730/+190) or $p53_{x14}$–luc for 5 h, and treated with DIM-C-pPhOH for another 18 h. $^{*}P<0.05$, $^{**}P<0.005$ and $^{\#}P<0.001$ versus DMSO. (**e**) Cells were treated with DIM-C-pPhOH for 24 h, and whole-cell lysates were analyzed by western blot analysis. (**f**; right panel) Cells were treated with DIM-C-pPhOH for 12 h, and the ChIP assay was performed.

2010b). Results in Figure 5a show that DIM-C-pPhOH inhibited A549 and H460 cell proliferation, and overexpression of TR3 partially reversed the growth inhibitory effects of DIM-C-pPhOH. DIM-C-pPhOH also decreased transactivation in A549 and H460 cells transfected with NuRE–luc and TR3 (Figure 5b), and immunostaining demonstrated that DIM-C-pPhOH did not affect TR3 expression or induce nuclear translocation (Figure 5c). Moreover, western blot analysis of lysates from cells treated with dimethyl sulfoxide or 20 μM DIM-C-pPhOH showed that TR3, p300 and Sp1 expression were observed only in the nucleus, and levels were similar in both treatment groups (data not shown). We also observed similar effects of DIM-C-pPhOH and siTR3 on disruption of the TR3/p300/Sp1-mediated expression of survivin and survival in lung cancer cells. DIM-C-pPhOH decreased transactivation in cells transfected with Gal4–Sp1/Gal4–luc or GC-rich constructs (GC_3–TATA and GC_3–TK–luc; Supplementary Figure S6A). DIM-C-pPhOH also decreased survivin mRNA levels and luciferase activity in cells transfected with survivin constructs (Supplementary Figure S6B). In a gel shift assay, DIM-C-pPhOH decreased p300 but not Sp1 binding to a GC-rich probe (Supplementary Figure S6C), and the effects of DIM-C-pPhOH on inhibition of cell growth decreased survivin expression. Induction of apoptosis was also observed in p53-null H1299 cells (Supplementary Figure S6D). Thus, similar to siTR3, DIM-C-pPhOH inactivates the TR3/p300/Sp1 survival complex (Figure 4e). The complementarity between siTR3 and DIM-C-pPhOH was also observed for the activation of sestrin 2 and AMPKα and the subsequent inhibition of the mTORC1 pathway. DIM-C-pPhOH induced luciferase activity in A549 and H460 cells transfected with pSENS2–luc (Figure 5d), as well as induced sestrin 2 and phospho-AMPKα and decreased phosphorylated p70S6K, S6RP and 4E-BP1 expression in A549 cells (Figure 5e). DIM-C-pPhOH also induced luciferase activity in cells transfected with p53–luc constructs, and in a ChIP assay DIM-C-pPhOH enhanced recruitment of p53 to the p53 response element region of the sestrin 2 promoter (Figure 5f) as observed for siTR3 (Figure 4d). Results in Supplementary Figures S7A and B also show in H460 cells transfected with siTR3 that DIM-C-pPhOH in combination with siTR3 enhances inhibition of growth and mTORC1 signaling. Thus, both siTR3 and DIM-C-pPhOH decrease or deactivate TR3, respectively, to inhibit growth and survival of lung cancer cells by inhibition of the mTORC1 pathway via p53/sestrin-dependent activation of AMPKα.

DIM-C-pPhOH inhibits lung tumor growth

The effects of DIM-C-pPhOH on the growth of orthotopic lung tumors derived from A549 cells were carried out to complement the *in vitro* studies with siTR3 and DIM-C-pPhOH in lung cancer cells (Figures 2–5). DIM-C-pPhOH (30 mg/kg/day) decreased lung tumor weights and volumes, and this was accompanied by increased apoptosis (terminal deoxynucleotidyl transferase dUTP nick-end labeling staining) in the tumors from animals treated with DIM-C-pPhOH compared with tumors from the control (corn oil) mice (Figure 6a and Supplementary Table S3). Treatment with DIM-C-pPhOH also decreased survivin and increased cleavage of caspases 3 and 7 and poly (ADP-ribose) polymerase (Figure 6b), which is associated with inactivation of the p300/TR3/Sp1 complex (Figure 2, Supplementary Figures S2 and S3). DIM-C-pPhOH also inhibited mTORC1 signaling through activation of sestrin 2 and AMPKα, and this was accompanied by decreased phosphorylation of 4E-BP1 and p7056K (Figure 6c). The effects of DIM-C-pPhOH (30 mg/kg/day) were also investigated in a metastatic mouse model for lung cancer where cells were introduced by tail vein injection (Figures 6c and d). In this study, DIM-C-pPhOH also decreased tumor weights, volumes and burden (Figure 6d and Supplementary Table S4). These data clearly demonstrate that *in vivo* deactivation of TR3 by DIM-C-pPhOH results in tumor growth inhibition by inhibiting at least two TR3-mediated pro-oncogenic pathways (Figure 4e).

Discussion

TR3 and other NR4A receptors are involved in multiple pathways involved in cellular homeostasis and are particularly important in response to different forms of stress (Maxwell and Muscat, 2006; Pearen and Muscat, 2010). There is also increasing evidence that TR3 is overexpressed in multiple tumor types and exhibits pro-oncogenic activities (Maruyama *et al*., 1995; Bras *et al*., 2000; Kolluri *et al*., 2003; Ke *et al*., 2004; Chintharlapalli *et al*., 2005; Li *et al*., 2006; Zeng *et al*., 2006; Cho *et al*., 2007, 2010; Zhang, 2007; Azoitei *et al*., 2010; Wu *et al*., 2010; Lee *et al*., 2010b), and results summarized in Figure 1 and Supplementary Figure 1 suggest that overexpression of TR3 is a negative prognostic factor for lung cancer patient survival. Thus, drugs that target TR3 may be important for treating cancer, and the effective use of TR3 agonists or antagonists will be dependent on understanding the role of these receptors in critical disease-specific pathways.

Among the earliest studies on the role of TR3 in cancer was the observation that various apoptosis-inducing agents induced nuclear export of TR3 and this resulted in formation of a proapoptotic mitochondrial TR3–bcl-2 complex (Li *et al*., 2000; Kolluri *et al*., 2003, 2008; Lin *et al*., 2004; Zhang, 2007; Wu *et al*., 2010). This pathway is being extensively exploited for drug development. The importance of TR3 nuclear export has been confirmed in recent studies in liver cancer that show that endogenous suppression of TR3 nuclear export by the chromodomain helicase/adenosine triphosphatase DNA-binding protein-1-like oncogene is an important determinant for liver cancer (Chen *et al*., 2009, 2010). A recent study showed that TR3 knockdown or treatment with DIM-C-pPhOH inhibited growth and induced apoptosis in pancreatic cancer cells and downregulated expression of survivin (Lee *et al*.,

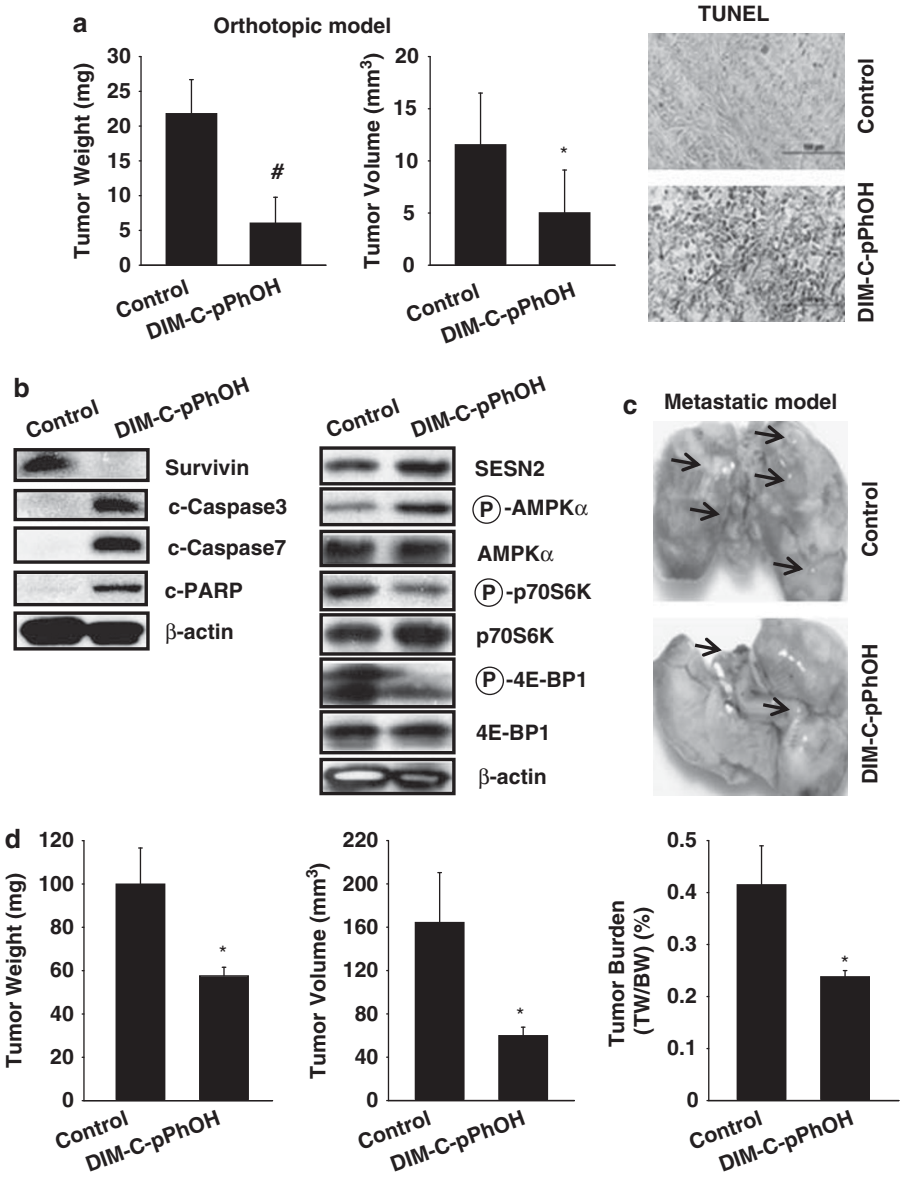

Figure 6 DIM-C-pPhOH inhibits tumor growth and lung metastasis *in vivo*. (**a**, **b**) The orthotopic mouse model of lung cancer. A549 cells were orthotopically implanted into athymic nude mice, and each mouse was dosed three times a week by oral gavage with either corn oil (control) or DIM-C-pPhOH (30 mg/kg/day) for 4 weeks starting 7 days after implantation. Median tumor weights and volumes (**a**; left panel) were calculated as described in the Materials and methods. The data are presented as means with s.d. ($n = 10$ per group). $^{*}P < 0.05$ and $^{\#}P < 0.001$ versus control group. (**a**; right panel) Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining. Tumor sections were stained using the DeadEnd colorimetric kit (Promega, Madison, WI, USA) as described in the Materials and methods. The apoptotic tumor cells are stained. Images were collected at high ($\times 100$) magnification. (**b**) Protein expression in tumor lysates. Tumor lysates from tumor samples were further analyzed by western blot analysis, and β-actin was used as a loading control. (**c**, **d**) The metastatic mouse model of lung cancer. A549 (2×10^6) cells were inoculated into athymic nude mice via the tail vein for 4 weeks before treatment, and each mouse was dosed three times a week by oral gavage with either corn oil (control) or DIM-C-pPhOH (30 mg/kg/day) for 4 weeks. (**c**) Lung micrographs show development of multiple tumor foci (arrows). (**d**) Metastatic tumor weight, volume and burden were calculated.

2010b). Mechanistic studies showed that endogenous expression of survivin was dependent on formation of p300/TR3/Sp1 complex bound to the proximal GC-rich region of the survivin gene promoter, and inactivation of this complex by siTR3 or DIM-C-pPhOH resulted in loss of p300 and decreased survivin expression. Similar results were observed in p53-positive A549 and H460 and p53-negative H1299 lung cancer cells where both siTR3 and DIM-C-pPhOH inhibited cell growth, induced apoptosis and decreased expression of survivin and other *Sp1*-regulated genes (Figures 2b, d and 5c, Supplementary Figures S2 and S6). DIM-C-pPhOH was also highly effective as an inhibitor of lung tumor growth in orthotopic and metastatic *in vivo* models (Figure 6). Thus, identification of a novel endogenous p300/TR3/Sp1-dependent prosurvival pathway in

pancreatic (Lee *et al*., 2010b) and lung cancer cells is consistent with the anticancer activities observed for DIM-C-pPhOH as a TR3 deactivator.

TR3 binds and inactivates p53 (Zhao *et al*., 2006), and the role of this interaction in A549 and H460 cells that express wild-type p53 was investigated. Results of preliminary pathway screening studies with siTR3 identified the mTORC1 signaling pathway as another TR3-regulated circuit in p53 wild-type lung cancer cells, and siTR3 decreased phosphorylation of the diagnostic mTORC1 downstream targets, namely, p70S6K, S6RP and 4E-BP1 (Figure 2c). mTORC1 has become an increasingly important target for anticancer drugs such as rapamycin because of its central role in regulating multiple downstream pro-oncogenic factors (Bjornsti and Houghton, 2004; Hay and Sonenberg, 2004; Shaw and Cantley, 2006; Guertin and Sabatini, 2007; Yang and Guan, 2007). The phosphorylated forms of 4E-BP1 and p70S6K are key downstream targets of mTORC1 that regulate initiation and translation of multiple genes, such as *cyclin D1* and *cyclin A*, that are critical for normal cell growth, and in a cancer context many of the the *4E-BP1*- and *p70S6K*-regulated genes are pro-oncogenic (Hay and Sonenberg, 2004; She *et al*., 2010). Dephosphorylation of 4E-BP1 and p70S6K in cells transfected with siTR3 is consistent with a role for TR3 in regulating mTORC1 in lung cancer cells, and our results for DIM-C-pPhOH (Figure 5), a TR3 deactivator, demonstrate that this compound represents a novel inhibitor of mTORC1 that acts by deactivation of TR3.

mTORC1 can be inhibited by LKB1 or by inactivation of PI3K; however, both A549 and H460 cells are LKB1 negative, and siTR3 did not affect phosphorylation of AKT (Figure 3a). In the lung cancer cell lines, siTR3 activated AMPKα and inhibited mTORC1 through induction of sestrin 2, and these responses were blocked in cells co-transfected with siAMPKα (Figure 3b), thus demonstrating a connection between TR3- and AMPKα-dependent inhibition of mTORC1. In contrast, siTR3 did not affect AMPKα phosphorylation in p53-null H1299 lung cancer cells (Figure 4b); however, overexpression of p53 in H1299 cells co-transfected with siTR3 inhibited mTORC1 signaling (Supplementary Figure S4B). These results indicate that the effects of siTR3 and DIM-C-pPhOH on activation of AMPKα and inhibition of mTORC1 are p53 dependent.

TR3 binds to and inactivates p53 (Zhao *et al*., 2006); however, loss of TR3 (by siTR3) or inactivation of TR3 in lung cancer cells treated with DIM-C-pPhOH resulted in increased p53-dependent transactivation (Figures 4a, c and 5f) and this was not due to increased acetylation (data not shown). Loss of or inactivation of TR3 was accompanied by activation of the p53-responsive gene *sestrin 2* and recruitment of p53 to the p53-responsive region of the sestrin 2 promoter (Figures 3–5). A recent report showed that regulation of mTORC1 signaling by p53 is through p53-dependent activation of sestrins, which, in turn, activate AMPKα (Budanov and Karin, 2008; Lee *et al*., 2010a). Results of studies in lung cancer cells transfected with siTR3 alone or siAMPKα, siSESN2 and sip53 alone or in combination with siTR3 (Figures 3b, d and 4a) demonstrate that TR3 regulates the mTORC1 pathway and this is due to direct interactions of TR3 with p53 (Supplementary Figure S5A), which inactivate the tumor suppressor gene (Zhao *et al*., 2006). Moreover, overexpression of TR3 blocks interaction of p53 with the sestrin 2 promoter, inhibits activation of constructs containing p53 and sestrin 2 promoter inserts (Supplementary Figures S5B and D), and activates mTORC1 signaling (Supplementary Figure S3A). Thus, inactivation of p53 by TR3 blocks the activation of sestrin 2 and AMPKα, resulting in activation of mTORC1 (Figure 4e), and this defines another pro-oncogenic function of TR3 in p53 wild-type cancer cells.

As endogenous TR3 activates mTORC1 through inhibition of p53/sestrin-2-dependent activation of AMPKα, an mTORC1 inhibitor, this suggests that drugs, such as DIM-C-pPhOH, that inactivate TR3 and thereby enhance p53-dependent inhibition of mTORC1 *in vitro* (Figure 5e) and *in vivo* (Figure 6b) will be highly effective anticancer agents. Thus, identification of the role of TR3 as a prognostic factor (Figure 1) and as an important regulator of mTORC1 signaling and survival pathways in lung cancer (Figure 4e) suggests that subsets of lung cancer patients that overexpress TR3 and are wild type for p53 would benefit from clinical treatment with TR3 inactivators, such as DIM-C-pPhOH alone or in combination therapy. Drugs such, as DIM-C-pPhOH, that inactivate TR3 represent a new class of mTORC1 inhibitors, and our ongoing studies are focused on developing other novel potent inhibitors of this orphan receptor and its downstream pro-oncogenic pathways.

Materials and methods

Immunohistochemical analysis

The tissue microarray slides containing 59 cases of human NSCLC tissues (IMH-305) and 59 cases of self-matching normal adjacent lung tissues (IMH-340) were obtained from Imgenex (San Diego, CA, USA). Immunohistochemical staining for TR3 was performed on paraffin-embedded specimens by using standard avidin–biotin complex method described previously (Lee *et al*., 2010b). Immunostaining intensity was scored as absent, low, moderate or high by three independent investigators without prior knowledge of the clinical follow-up data. There was no specific staining when secondary antibody was used alone as a negative control.

Cell lines and plasmids

A549, H460, and H1299 human NSCLC cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 ml/l 100 × antibiotic antimycotic solution (Sigma-Aldrich, St Louis, MO, USA). Cells were maintained at 37 °C in the presence of 5% CO_2. All the plasmids used in this study, except the sestrin 2 promoter (−730/+190) reporter construct (pSESN2–luc) and $p53_{X14}$-luciferase reporter con-

struct ($p53_{X14}$–luc), pCMV–p53 wild type and pCMV–p53–mt135 mutant (p53–mt135), were previously described (Lee *et al*., 2010b). The pSESN2–luc and $p53_{X14}$–luc were purchased from SwitchGear Genomics Inc. (Menlo Park, CA, USA) and Stratagene (Cedar Creek, TX, USA), respectively, and the wild-type and mutant p53 expression plasmids were purchased from Clontech (Mountain View, CA, USA).

Transfection of small interfering RNA oligonucleotides
Cells (1.5×10^5 cells/well) were plated in six-well plates in Dulbecco's modified Eagle's medium media supplemented with 10% fetal bovine serum. After 16 h, the cells were transfected with 200 nM of each small interfering RNA duplex for 6 h using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. After transfection, cells were collected for cell proliferation assay, western blot analysis and quantitative real-time PCR assay. The sequences of small interfering RNA oligonucleotides used were as follows: TR3-1, 5′-CAGUCCAGCCAUGC UCCUdTdT-3′; TR3-2, 5′-CGCUCAUGCCAGCAUUAU-3′; TR3-3, 5′-GGCUUGAGCUGCAGAAUGA-3′; Sp1, 5′-AU CACUCCAUGGAUGAAAUGAdTdT-3′; and p300, 5′-AAC CCCUCCUCUUCAGCACCA dTdT-3′. The small interfering RNA for AMPKα (sc-45312), sestrin 2 (sc-106544), and p53 (sc-29435) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). As a negative control, a nonspecific scrambled small inhibitory RNA (siScr) oligonucleotide was used (Qiagen, Valencia, CA, USA).

Subcellular localization assay
Cells were seeded onto coverslips in 12-well plates, and then treated with either the vehicle (dimethyl sulfoxide) or the compound. After 12 h, cells were fixed in 1% formalin in phosphate-buffered saline (pH 7.4) after washing with phosphate-buffered saline and permeabilized by immersing the cells in 0.2% Triton X-100 solution in phosphate-buffered saline for 10 min. Cells were then incubated with anti-TR3 rabbit immunoglobulin G, followed by anti-rabbit immunoglobulin G conjugated with FITC (Santa Cruz Biotechnology). For nuclear counterstaining, cells were mounted in mounting medium including DAPI (Vector Lab, Burlingame, CA, USA). Fluorescent images were collected and analyzed using a Zeiss Axioplan2 fluorescence microscope (Carl Zeiss, Jena, Germany).

ChIP assay
The ChIP assay was performed using the ChiP-IT Express Magnetic Chromatin Immunoprecipitation kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer′s protocol. After treatment with the compound or small interfering RNAs, cells were fixed with 1% formaldehyde for 10 min, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were then hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to desired chromatin length (~500 bp). The chromatin was immunoprecipitated with each target antibody and protein G magnetic beads at 4 °C overnight with gentle agitation. The beads were collected and washed, and the chromatin–protein complex was eluted. Cross-linking was then reversed and purified DNA was subjected to PCR amplification. The sestrin 2 primers that contain a p53-binding site were 5′-CAGACCTCTGATTGGCTGGACCG-3′ (sense) and 5′-CAGGGGTTTTCACGGCCTCGGAA-3′ (antisense), and encompass a region containing a p53-binding site. PCR products were analyzed by electrophoresis on a 2% agarose gel in the presence of ethidium bromide.

In vivo *experiments*
A549 human NSCLC cells were used in both the orthotopic and metastatic models, and a detailed description of the procedures is provided in the Supplementary Materials and methods section.

Statistical analysis
The significance of correlations was determined using the χ^2-test and Cox proportional hazard regression model. To evaluate the degree of association between variables, hazard ratio, odds ratio and corresponding 95% confidence intervals were analyzed. Association of TR3 expression with clinical outcome for NSCLC patients was examined using Kaplan–Meier survival analysis, and $P<0.05$ was considered significant. Statistical significance of differences in protein levels, luciferase activity and cell and tumor growth between groups was analyzed using either Student's *t*-test or analysis of variance with Scheffe's test. The results are expressed as means with s.d. for three experiments for each group, unless otherwise indicated, and a $P<0.05$ was considered statistically significant. All statistical tests were two sided.

All other Materials and methods are described in the Supplementary Materials and Methods.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)