Cancer Research

Tid1-L Inhibits EGFR Signaling in Lung Adenocarcinoma by Enhancing EGFR Ubiquitinylation and Degradation

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Abstract

Tid1 (DNAJA3), a DnaJ cochaperone, may promote degradation of oncogenic kinases. Tid1 has 2 isoforms, Tid1-L and Tid1-S, that may function differently. In this study, we investigated the role of the Tid1 isoforms in regulating EGF receptor (EGFR) signaling and lung cancer progression. We found that both Tid1-L and Tid1-S expressions were reduced in patients with non–small cell lung cancer compared with normal counterparts. Tid1-L expression correlated inversely with EGFR expression. Low Tid1-L/high EGFR expression predicted poor overall survival in patients with lung adenocarcinoma. Tid1-L overexpression in lung cancer cells attenuated EGFR signaling and inhibited cell proliferation, colony formation, and tumor growth in subcutaneous and orthotropic xenograft models. Conversely, depletion of Tid1 restored EGFR signaling and increased cell proliferation and colony formation. Tid1-L, but not Tid1-S, interacted with EGFR/HSP70/HSP90 through the DnaJ domain, counteracting the EGFR regulatory function of HSP90 by causing EGFR ubiquitinylation and proteasomal degradation. Tid1-L inhibited EGFR signaling even more than the HSP90 inhibitor 17-allylamino-demethoxy geldanamycin. We concluded that Tid1-L acted as a tumor suppressor by inhibiting EGFR signaling through interaction with EGFR/HSP70/HSP90 and enhancing EGFR ubiquitinylation and degradation. *Cancer Res; 73(13); 4009–19. ©2013 AACR.*

Introduction

Lung cancer is the leading cause of cancer deaths worldwide, and non–small cell lung cancer (NSCLC) is the dominant cell type (1). Recent epidemiologic studies have shown a global trend of a decrease in squamous cell carcinoma and a sharp rise in adenocarcinoma (1). EGF receptor (EGFR), a receptor tyrosine kinase (RTK), is the major driver pathway of lung adenocarcinoma, regulating important tumorigenic processes including proliferation, apoptosis, angiogenesis, and invasion (2). EGFR is frequently overexpressed and mutated in lung adenocarcinoma (3); however, the detailed mechanism of how EGFR signaling regulates oncogenic pathways is unclear.

The major signaling pathways activated by EGFR are the mitogen-activated protein kinase, phosphatidylinositol 3-kinase–AKT, and the STAT pathways (4). Although ligand-induced kinase activation normally targets EGFR for ubiquitinylation and subsequent degradation in lysosomes (5), several studies have reported that certain EGFR mutants and amplification may escape this regulation (3, 6).

EGFR is a client protein for HSP90 (7), which commonly functions in concert with various cochaperones including HSP70 and HSP40 (8). These chaperone proteins form a complex with the client protein and assist protein folding, activation, and degradation. Therefore, these chaperones or cochaperones are anticipated to participate in the regulation of EGFR signaling. Interruption of the HSP70/HSP90-folding cycle through the addition of a HSP90-specific inhibitor such as 17-allylamino-demethoxy geldanamycin (17-AAG) leads to ubiquitinylation and degradation of EGFR (9).

Tid56, the Drosophila lethal (2) tumorous imaginal disc gene, was the first gene encoding a member of the DnaJ cochaperone family to be classified as a tumor suppressor (10). *Tid1*, the human homologue of the *Tid56* gene, was recently shown to be a tumor suppressor in head and neck cancer (11). *Tid1* encodes 2 alternative splicing isoforms (Tid1 long form, Tid1-L; and Tid1 short form, Tid1-S) that are involved in the destabilization of hypoxia-inducible factor-1α and ErbB2 (12, 13) as well as the attenuation of EGFR–AKT signaling (11). However, the molecular basis for the

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involvement of Tid1 in EGFR regulation and in lung adenocarcinoma tumorigenesis remains largely unknown.

Because Tid1 is a DnaJ family protein that may interact with HSP70 through its DnaJ-domain, and because EGFR is a client protein of HSP90 (14, 15), we investigated whether the 2 isoforms of Tid1 function as tumor suppressors in lung adenocarcinoma by affecting EGFR signaling through modulation of the EGFR/HSP90/HSP70 complex- and EGFR-dependent pathways.

Materials and Methods

Patients and tumor specimens

Two independent cohorts were recruited for this study. The first cohort consisted of 20 patients with histologic confirmed NSCLC who underwent surgical resection at the National Taiwan University Hospital (Taipei, Taiwan, ROC) between 2000 and 2003. The tumors and adjacent normal tissues from this cohort were used for the analysis of Tid1 mRNA expression. The second cohort was derived from 55 patients with confirmed lung adenocarcinoma who underwent surgical resection at China Medical University and Hospital (Taichung, Taiwan, ROC) between November 19, 2004 and August 15, 2011. The tumors and adjacent normal tissue specimens from this cohort were used for immunohistochemical staining. This study was reviewed and approved by the Institutional Review Board and Ethics Committee of National Taiwan University Hospital and China Medical University and Hospital. Written informed consent was obtained from all patients.

Immunohistochemistry

Immunohistochemistry (IHC) was conducted as described previously (16). The primary antibodies used for staining were targeted against Tid1-L (clone C-15) and wild-type EGFR (clone EGFR.25).

Cell lines

A panel of human lung adenocarcinoma cell lines, CL1-0, CL1-1, and CL1-5, in ascending order of invasiveness, was established in our laboratory using a Transwell invasion chamber (17). Primary lung adenocarcinoma cell lines, CL83, CL152, and CL141, established in our laboratory, were derived from the clinical patients. A549 cells (American Type Culture Collection) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS. Primary NSCLC cells were cultured in RPMI-1640 medium containing 10% FBS. The virus-packaging cell line 293T was cultured in DMEM containing 10% FBS. Culture media, chemical compounds, and FBS were purchased from Life Technologies.

Assays for cell proliferation and anchorage-independent growth

Assays for cell proliferation by MTT and anchorage-independent growth were conducted as described previously (11).

Reverse transcriptase PCR

RNA extraction and regular reverse transcriptase PCR (RT-PCR) were conducted as previously described (18). The sequences of the oligonucleotide primers are listed in Supplementary Table S2. Detection of Tid1-L and Tid1-S by quantitative RT-PCR was conducted using product numbers Hs00911265_m1, CHA1270000, and HSP40_TRANS_2, AIT956T, respectively, from Applied BioSystems. Commercially available primers and probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Hs99999905, Applied Biosystems) were used for the normalization. Fluorescence emitted by the reporter dye was detected online in real time using an ABI prism 7900 sequence detection system. The quantities of Tid1-L and Tid1-S mRNA were analyzed in triplicate, normalized against the GAPDH control, and expressed in relation to a calibrator sample.

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were conducted as described previously (11).

Subcutaneous and orthotropic xenograft tumor growth *in vivo*

The effect of Tid1-L overexpression on tumorigenic activity *in vivo* was studied using 6-week-old nude BALB/c nu/nu mice ($n = 8$ per group). CL1-5 cells were infected with lentiviral Tid1-L-GFP or vector for 48 hours before injection into the flanks of nude mice. Detailed analyses were conducted as previously described (11). For orthotropic tumor implantation assays, CL1-5 cells infected with lentiviral Tid1-L-GFP or vector were injected into the pleural cavity of 6-week-old nude BALB/c nu/nu mice ($n = 6$ per group). Detailed analyses were conducted as previously described (19). All animal experiments were carried out in accordance with the guidelines of the Department of Animal Care, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China.

Statistical analysis

For nonparametric analysis, the Student *t* test, Fisher exact test, and the Kaplan–Meier method were conducted using Prism 5.0 (GraphPad Software) or the Statistical Package for the Social Sciences version 12.0 (SPSS, Inc.). Differences between the variables were considered significant for $P < 0.05$.

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

Results

Expression level of Tid1-L and Tid1-S in lung adenocarcinoma and cell lines

To determine, which isoform of Tid1 is the main tumor suppressor in lung adenocarcinoma, the mRNA levels of splicing variants Tid1-L and Tid1-S were examined by quantitative RT-PCR of 20 surgically resected paired samples of tumor and adjacent normal tissues from patients with NSCLC. The clinical characteristics of the patients are summarized in Supplemental Table S1. Lung adenocarcinoma was the most common type in these patients with lung cancer. The regular and quantitative RT-PCR results are shown in Fig. 1A and Supplementary Fig. S1A, respectively. In both cases, the expression level of both Tid1-L and Tid1-S in the tumors was lower than that in the adjacent normal tissue in majority of the patients with NSCLC (95% in Tid1-S and 100% in Tid1-L).

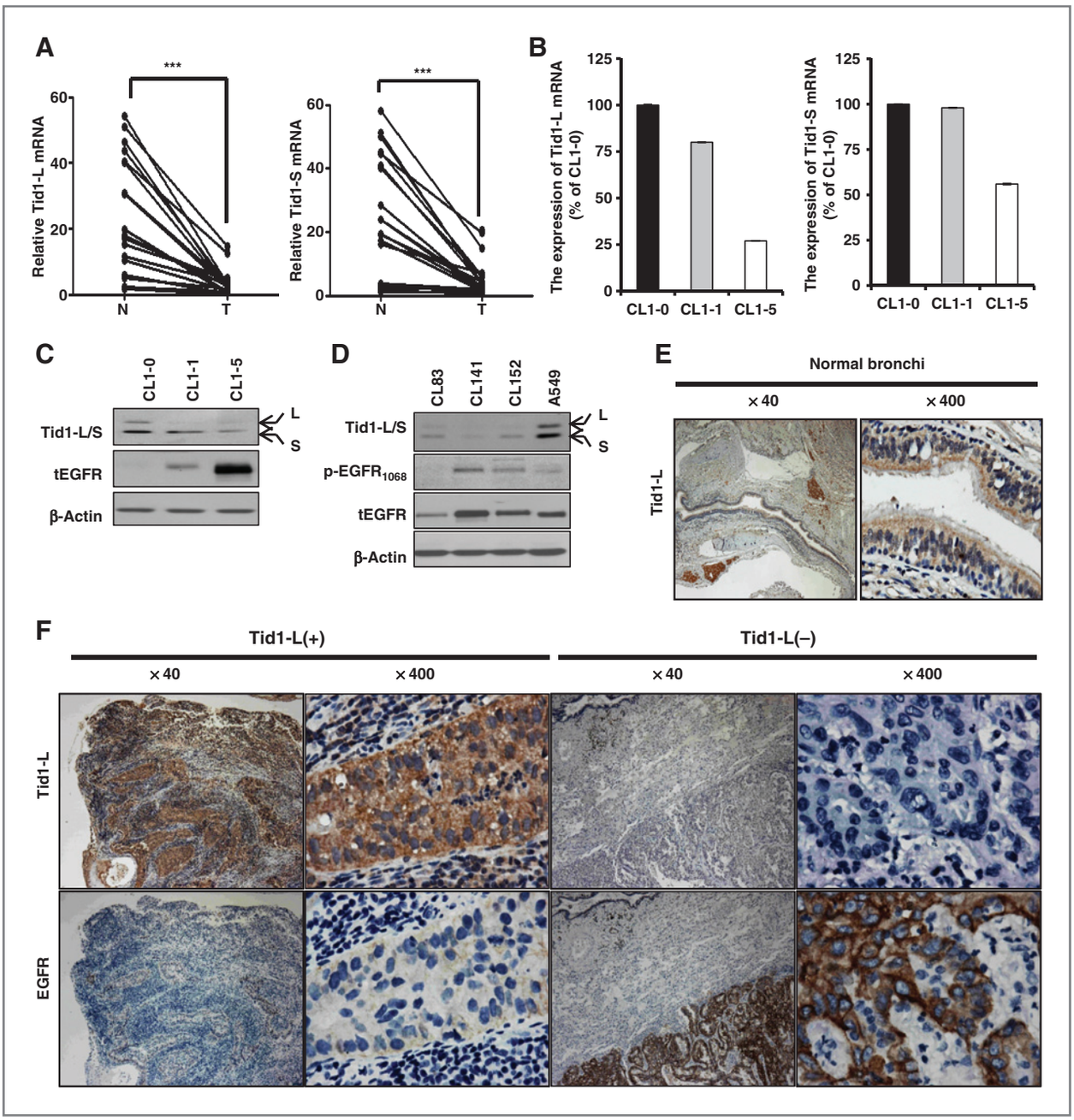


Figure 1. Expression levels of Tid1-L and EGFR in lung adenocarcinoma cell lines and tissues. A, quantitative RT-PCR of Tid1-L/S expression in lung adenocarcinoma tissues. The Tid1-L and Tid1-S levels were determined by quantitative RT-PCR and the mRNA level of Tid1-L and Tid1-S was normalized to GAPDH expression in each sample. Tumor (T), NSCLC; normal (N), matched noncancerous tissues. ***, $P < 0.001$ based on the Student t test. B, analysis of Tid1-L/S expression in a panel of lung adenocarcinoma cells with increasing invasiveness by quantitative RT-PCR. The relative expressions of Tid1-L and Tid1-S in CL1-5 and CL1-1 cells are expressed as percent of the CL1-0 cells. Bars are means ± SD from 3 experiments. C, Western blot analyses of the expression levels of Tid1-L/S and tEGFR in CL1-0, CL1-1, and CL1-5 cells. The endogenous long form (L) and short form (S) of Tid1 are indicated by arrows marked L and S, respectively. β-actin served as a loading control. D, Western blot analyses of the expression levels of Tid1-L/S, phospho-EGFR (pEGFR), and tEGFR in CL83, CL141, CL152, and A549 cells. E, IHC staining of Tid1-L of a representative normal bronchi from 1 patient. F, IHC staining of Tid1-L (top) and EGFR (bottom) expressions in lung adenocarcinoma tissues that stained positive with Tid1-L [Tid1-L (+)] and that stained negative with Tid1-L [Tid1-L (–)].

We further evaluated the Tid1-L and Tid1-S mRNA and protein levels in a panel of lung adenocarcinoma cell lines of increasing invasiveness (13). Similar to the results obtained with the resected tissues, the expression level of Tid1-S was higher than that of Tid1-L in all cell lines tested (Fig. 1B and Supplementary Fig. S1B). The Tid1 mRNA and protein

expressions, especially Tid1-L expression, were reduced in the highly invasive cells (Fig. 1B and C).

Expression of Tid1-L was inversely correlated with the expression of EGFR in lung adenocarcinoma and cell lines

Because EGFR signaling is the key oncogenic driver pathway in lung adenocarcinoma (2, 6), we hypothesized that the tumor suppressor function of Tid1 in lung adenocarcinoma may be related to its ability to attenuate the EGFR network. To test this hypothesis, we first examined the correlation between Tid1-L expression level and EGFR activation. As shown in Fig. 1C, an inverse correlation was observed between the expression level of Tid1-L and EGFR in a panel of lung adenocarcinoma cells and increasing invasiveness. Furthermore, Tid1-L expression level was also inversely correlated with EGFR phosphorylation in several other lung adenocarcinoma cell lines (Fig. 1D).

The expression levels of Tid1-L and EGFR were further examined by IHC of paired tumor and adjacent normal tissues from 55 patients with lung adenocarcinoma. The clinical features of these patients with lung adenocarcinoma are summarized in Table 1. A computerized image analysis system was used for IHC scoring as shown in Supplementary Fig. S2. The normal bronchi of all patients were stained positive for Tid1-L (Fig. 1E). In cancerous lesions, a high expression level of Tid1-L was usually associated with a low expression level of EGFR (Fig. 1F, top left). In contrast, a low expression level of Tid1-L was associated with a high expression level of EGFR (Fig. 1F, top right). Statistical analysis showed a significant inverse correlation between the expression level of Tid1-L and the expression level of EGFR ($P < 0.05$; Table 1).

Table 1. Characteristics of 55 patients with lung adenocarcinoma with high or low Tid1-L expressions[a]

Characteristics	Tid1-L High[b] (N = 18)	Tid1-L Low[c] (*N* = 37)	*P*
Age, y			
<60	11	17	
>60	7	20	0.39
Gender			
Male	5	18	
Female	13	19	0.16
Stage			
I/II	12	12	
III/IV	6	25	0.02[f]
Infiltrating lymphoid cells			
High	14	16	
Low	4	21	0.02[f]
Lymphovascular invasion			
Absent	13	17	
Present	5	20	0.08
EGFR expression			
High[d]	7	26	
Low[e]	11	11	0.04[f]

[a]By Fisher test.
[b]High, representative lung adenocarcinoma with intense cytosolic Tid1-L immunoreactivity (+, score 2).
[c]Low, representative lung adenocarcinoma showing negative expression and almost absent Tid1-L immunoreactivity (−, scores 0, 1).
[d]High, representative lung adenocarcinoma with intense cytosolic EGFR immunoreactivity (+, scores 2, 3).
[e]Low, representative lung adenocarcinoma showing negative expression and almost absent EGFR immunoreactivity (−, scores 0, 1).
[f]$P < 0.05$.

Low Tid1-L/high EGFR expressions predicted poor overall survival of patients with lung adenocarcinoma

We examined the Tid1-L and EGFR expressions and correlated them with the clinical outcomes. Kaplan–Meier survival analysis showed that patients with a higher expression of Tid1-L had a better overall survival than those with a low or undetectable expression of Tid1-L (Fig. 2A). Likewise, patients with a high expression of EGFR had a poorer overall survival compared with those having a low expression of EGFR (Fig. 2B). To further analyze the expressions of Tid1-L and EGFR and the influence of the expressions on patient survival, the scoring systems of Tid1-L and EGFR were divided into 2 categories: positive and negative expression. Patients with Tid1-L (+)/EGFR (−) had better overall survival rates than those with Tid1-L (−)/EGFR (+; Fig. 2C).

Tid1-L expression inhibited cell proliferation and promoted apoptosis in lung adenocarcinoma cells

The N-terminus of Tid1 bears a mitochondrial targeting sequence, and a major fraction of endogenous or ectopically expressed Tid1 proteins is known to reside in the mitochondrial matrix (20). We confirmed that both endogenous and exogenously expressed Tid1 was located mostly in the mitochondria of cytosol (Supplementary Fig. S3). To evaluate whether Tid1-L is involved in lung adenocarcinoma tumorigenesis, we overexpressed Tid1-L in human lung cancer cell line CL1-5. We found that transient transfection of Tid1-L inhibited cell proliferation (Fig. 3A, left) and anchorage-independent growth (Fig. 3A, right). Conversely, depletion of endogenous Tid1 expression in CL1-0 increased cell proliferation (Fig. 3B, left) and anchorage-independent growth (Fig. 3B, right). The Tid1-L–overexpressed CL1-5 cells displayed a loss of adherence, condensed cytoplasm, and the formation of apoptotic bodies, which are all indicative of apoptosis (Fig. 3C). Cleavage of the caspase substrate PARP also increased in the lysate of Tid1-L–overexpressed CL1-5 cells, whereas the expression of antiapoptotic Bcl-2 decreased (Fig. 3D). We further confirmed these results in lung adenocarcinoma cell line CL141 (Fig. 3D). Together, these results suggest that Tid1-L plays an important role in regulating cell proliferation and apoptosis in lung adenocarcinoma cells.

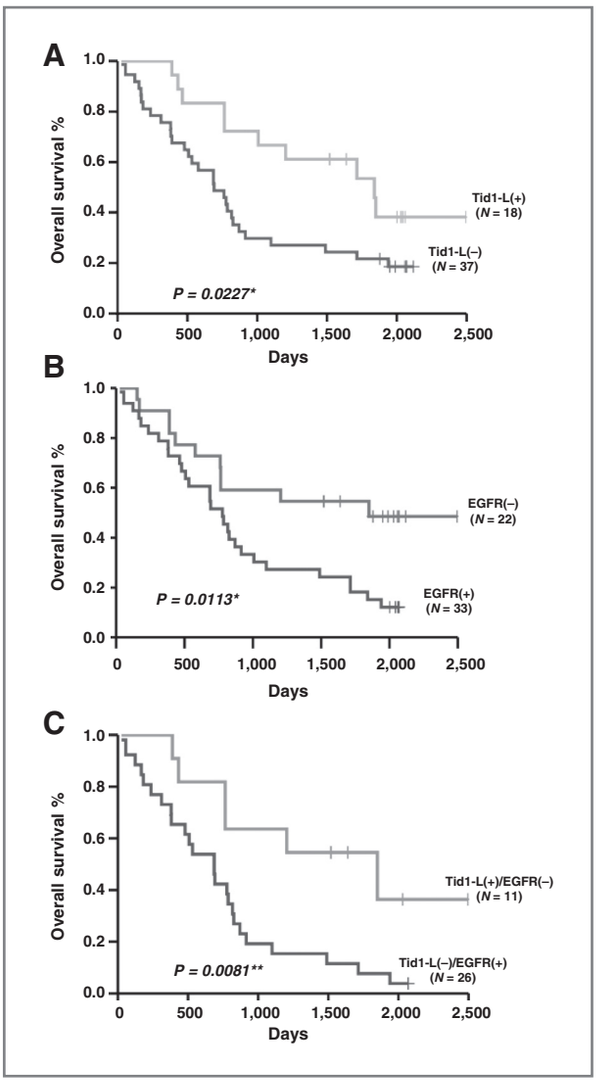


Figure 2. Kaplan–Meier analysis of overall survival. A–C, overall survival of the patients stratified according to the Tid1-L score (A), EGFR score (B), and representative multivariate data (C), grouped into Tid1-L (+)/EGFR(–) or Tid1(–)/EGFR(+). *, $P < 0.05$; **, $P < 0.01$ based on the Student *t* test.

Overexpression of Tid1-L reduced tumor growth of subcutaneous and orthotropic xenografts *in vivo*

To investigate the antitumorigenicity of Tid1-L on lung adenocarcinoma cells *in vivo*, 5×10^6 CL1-5 cells, infected with recombinant lentiviruses expressing GFP or Tid1-L, were subcutaneously injected into the flanks of nude mice. As shown in Fig. 3E, the growth of the tumors injected with Tid1-L–overexpressed CL1-5 cells was more retarded than the growth of tumors with GFP-overexpressed CL1-5 cells. We also carried out the same experiment using orthotropic injections into the thoracic cavity. As shown in Fig. 3F, orthotropic tumor growth of Tid1-L–overexpressed CL1-5 cells was significantly reduced compared with the controls. IHC showed that Tid1-L was expressed in the orthotropic lung tumors that had been injected with Tid1-L–overexpressed cells (Fig. 3G, right). These results confirmed that Tid1-L might attenuate the tumorigenicity of lung adenocarcinoma cells *in vivo*.

Tid1-L/EGFR complex could form constitutively and EGF stimulation increased the complex formation

The DnaJ-domain of HSP40 contains an His/Pro/Asp (HPD) motif that is thought to stabilize the interaction between client proteins and the HSP70/90 complex (21, 22). Because EGFR is a client protein for HSP90, we investigated the possibility that Tid1-L modulates EGFR signaling through interaction with the HSP70/HSP90 complex, CL1-5 cells were transfected with hemagglutinin (HA)-tagged Tid1-L or Tid1-S, and the cell extracts were immunoprecipitated with anti-HA antibodies. As shown in Fig. 4A, HSP90, HSP70, and EGFR were readily detected in the immunoprecipitate from cells transfected with Tid1-L-HA; however, only HSP70 was detected in cells transfected with Tid1-S-HA, indicating that Tid1-L interacts with HSP90 and EGFR.

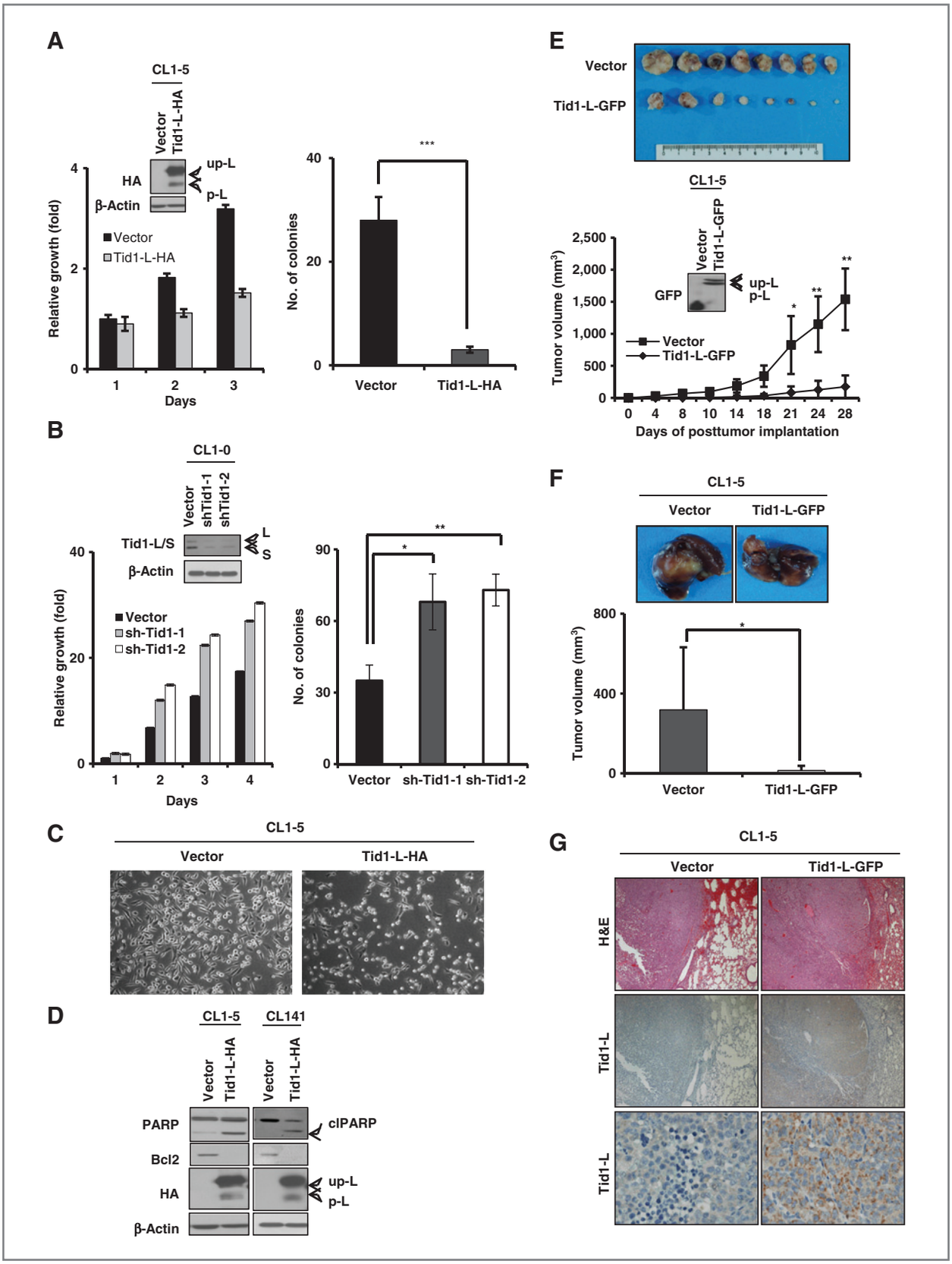
To investigate whether endogenous Tid1-L interacts with the EGFR/HSP90/HSP70 complex in lung adenocarcinoma cells, we examined the effect of EGF stimulation on EGFR-dependent signaling and the interaction of Tid1-L with the EGFR/HSP90/HSP70 complex in A549 cells. As shown in the left panel of Fig. 4B, EGF greatly increased the EGFR-related signaling. Under these conditions, EGFR, HSP70, and HSP90 were detected in both the nonstimulated and EGF-stimulated cell extracts immunoprecipitated with anti-Tid1-L antibodies (Fig. 4B, right), indicating that endogenous Tid1-L may interact with HSP70, HSP90, and EGFR constitutively.

Tid1-L attenuated EGFR signaling in lung adenocarcinoma cells

We next investigated whether overexpression of Tid1-L affects EGFR-dependent signaling in lung adenocarcinoma. CL1-5 cells were transfected with Tid1-L-HA, and the transfected cells were starved for 24 hours in the absence of serum before stimulation with 10 ng/mL EGF for 5 minutes. The Tid1-L–overexpressed cells had less total EGFR (tEGFR) and exhibited decreased activation of EGFR, AKT, extracellular signal-regulated kinase (ERK), and STAT3 compared with cells transfected with the empty vector (Fig. 4C). Moreover, we confirmed that stable Tid1-L overexpression decreased the EGFR level in both lung adenocarcinoma cell lines CL1-5 and CL141 (Supplementary Fig. S4). In contrast, knockdown of Tid1 in CL1-0 cells resulted in increased expressions of phosphor-STAT3 and -ERK (Fig. 4D), even though these cells expressed low levels of EGFR. Taken together, these results indicate that Tid1-L may attenuate the EGFR-dependent signaling pathway in lung adenocarcinoma cells.

Tid1-L counteracted the function of HSP90 in regulating EGFR expression

Given that RTKs comprise the largest category of HSP90 client proteins, one approach to suppress angiogenic signaling and ameliorate therapeutic resistance is to use HSP90-targeted inhibition of RTKs. To test the hypothesis that Tid1-L may regulate EGFR expression through HSP90 inhibition, we



examined the effects of HSP90 overexpression and HSP90 inhibitor 17-AAG (9) on the Tid1-L–mediated inhibition of EGFR. The CL1-5 cells were transfected with Tid1-L-HA, pcDNA3-HSP90, or empty vector, and then treated with 17-AAG. As shown in Fig. 5A, tEGFR expression level was greatly reduced in the cells overexpressing Tid1-L. Overexpression of HSP90, however, resulted in restoration of the high expression level of tEGFR in the Tid1-L–overexpressed cells. Next, we examined the effect of 17-AAG on the expression level of EGFR in the Tid1-L–expressing CL1-5 cells. As shown in Fig. 5B, the level of EGFR was reduced in the vector-transfected CL1-5 cells only after treatment with 17-AAG at higher concentrations. In the Tid1-L–expressing cells, the already reduced expression level of EGFR in the nontreated cells was further reduced following exposure to low concentrations of 17-AAG. Overexpression of HSP90 not only restored the levels of tEGFR but also increased the resistance of EGFR to 17-AAG inhibition in the Tid1-L–expressing cells (Fig. 5C). The relative EGFR levels under these experimental conditions were quantified by densitometry and are summarized in Fig. 5D. From this analysis, it is clear that Tid1-L overexpression and 17-AAG treatment exerted a synergistic effect on reducing the EGFR level, and overexpression of HSP90 seemed to counteract the synergistic effect of Tid1-L overexpression on the inhibition expression level of EGFR by 17-AAG. Therefore, we suggest that Tid1-L can counteract HSP90 in regulating EGFR expression. The level of EGFR messenger RNA was not significantly changed in Tid1-L–overexpressed CL1-5 cells or in the Tid1–depleted CL1-0 cells (Supplementary Fig. S5).

To further evaluate the effect of HSP90 inhibition on EGFR-related signaling, we compared the effects of Tid1-L overexpression and 2 μmol/L 17-AAG treatment on basal EGFR-related signaling in CL1-5 cells without the stimulation of EGF. As shown in Fig. 5E, although treatment of cells with 2 μmol/L of 17-AAG seemed to downregulate the level of EGFR more than the overexpression of Tid1-L, the overexpression of Tid1-L reduced the activation of AKT, ERK, and STAT3 more than treatment with 17-AAG. Apoptosis induction as measured by PARP cleavage was also more evident in cells overexpressing Tid1-L than with 17-AAG treatment. Notably, the EGFR level and the activation of AKT were more dramatically reduced in the Tid1-L–overexpressed cells upon treatment with 17-AAG (Fig. 5E).

The DnaJ-domain of Tid1-L was required for polyubiquitination and proteasome degradation of EGFR

The conserved DnaJ-domain of Tid1 forms a complex with HSP70 to exert a cochaperone function (11, 13). The HPD in the amino acids 121–123 of Tid1 is known to play a critical function for the DnaJ-domain (13, 23). Previous studies have indicated that the Tid1 mutant with a single replacement H121Q still retains the activity to interact with HSP70 (13). To ensure that the function of the DnaJ-domain was inactivated, we generated a Tid1-L–triple mutant (Tid1-L-Mut) with Q121N122A123 replacing H121P122D123 in the DnaJ-domain. Plasmids encoding HA-tagged wild-type Tid1-L (Tid1-L-Wt) or the HA-tagged DnaJ domain mutant of Tid1-L (Tid1-L-Mut; Fig. 6A) were transfected into CL1-5 cells, and the presence of tEGFR, HSP90, and HSP70 in the immunoprecipitates was examined by Western blotting using anti-HA antibodies. As shown in Fig. 6B, tEGFR, HSP70, and HSP90 were only detected in immunoprecipitates from cells transfected with Tid1-L-Wt, indicating that a functional DnaJ-domain is required for the interaction between Tid1-L and tEGFR, HSP70, and HSP90.

To determine whether the cochaperone function of Tid1-L is critical for the regulation of EGFR-dependent signaling, CL1-5 cells were transfected with Tid1-L-Wt or Tid1-L-Mut, and the transfected cells were then analyzed for the effects of Tid1-L expreesion on basal EGFR-related signaling without the stimulation of EGF. As shown in Fig. 6C, the tEGFR level and STAT3, AKT, and ERK activation were reduced in CL1-5 cells overexpressing Tid1-L-Wt. In contrast, a reduced level of EGFR and a reduced activation of ERK, AKT, and STAT were not detected in the cells overexpressing Tid1-L-Mut. These results suggest that the DnaJ-domain of Tid1-L is crucial for the regulation of EGFR-dependent signaling.

To determine whether the cochaperone function of Tid1-L regulates EGFR-dependent signaling by affecting the stability of EGFR, CL1-5 cells were transfected with Tid1-L-Wt or Tid1-L-Mut, and the transfected cells were then incubated with cycloheximide. As shown in Fig. 6D, the stability of EGFR was reduced in CL1-5 cells overexpressing Tid1-L-Wt. Finally, to

Figure 3. Effect of Tid1-L on the proliferation and tumorigenesis in lung adenocarcinoma cell lines and nude mice. A, left, effect of Tid1-L expression on cell proliferation. CL1-5 cells were transfected with Tid1-L-HA or the empty vector and cultured for the indicated times. Cell proliferation was determined by MTT assay. Right, effect of Tid1-L expression on anchorage-independent growth of CL1-5 cells. B, left, effect of Tid1 depletion on cell proliferation. CL1-0 cells were infected with sh-Tid1 or the empty vector (control) and cultured for the indicated times. Assay for cell proliferation was the same as described in A. Right, effect of Tid1 depletion on anchorage-independent growth of CL1-0 cells. C and D, left, CL1-5 cells were transfected with the empty vector or Tid1-L-HA and cultured for 48 hours. Phase contrast images of the transfected cells are shown in C. Apoptosis induction as analyzed by detection of the PARP cleavage product (clPARP) and Bcl-2 in transfected cells are shown in D. D, right, CL141 cells were transfected and analyzed for apoptosis induction as C above. The arrows indicated by up-L and p-L show the positions of the unprocessed form (up) and the processed form (p) of Tid1-L-HA (L), respectively. β-actin was used as a loading control. E, effect of Tid1-L on tumorigenesis by xenotransplantation *in vivo*. CL1-5 cells infected with Tid1-L-GFP or the empty vector were injected subcutaneously into the flanks of nude mice ($n = 8$ per group). Tumor volumes were determined twice weekly. Top, sizes of tumors excised from the sacrificed mice at day 28. Bottom, average volumes of tumors of the 8 mice in each group. The figure inset shows the expression of Tid1-L-GFP in CL1-5 cells infected with Tid1-L-GFP or the empty vector. F, effect of Tid1-L on tumorigenesis by orthotransplantation *in vivo*. Infected cells were orthotropically inoculated directly into the pleural cavities of nude mice ($n = 6$ per group), and the tumor volumes were measured from the excised lungs after 4 weeks of inoculation. Top, representative lungs were excised from mice injected with CL1-5 cells infected with Tid1-L-GFP or the empty vector. Bottom, quantitative evaluation of lung tumor volumes (the mean ± SD, $n = 6$) in each group. G, tumors in the lung tissues were examined by hematoxylin and eosin staining (magnification, ×40, top) or IHC for determination of the expression of Tid1-L (magnification, ×40, middle and ×400, bottom). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ based on the Student *t* test.

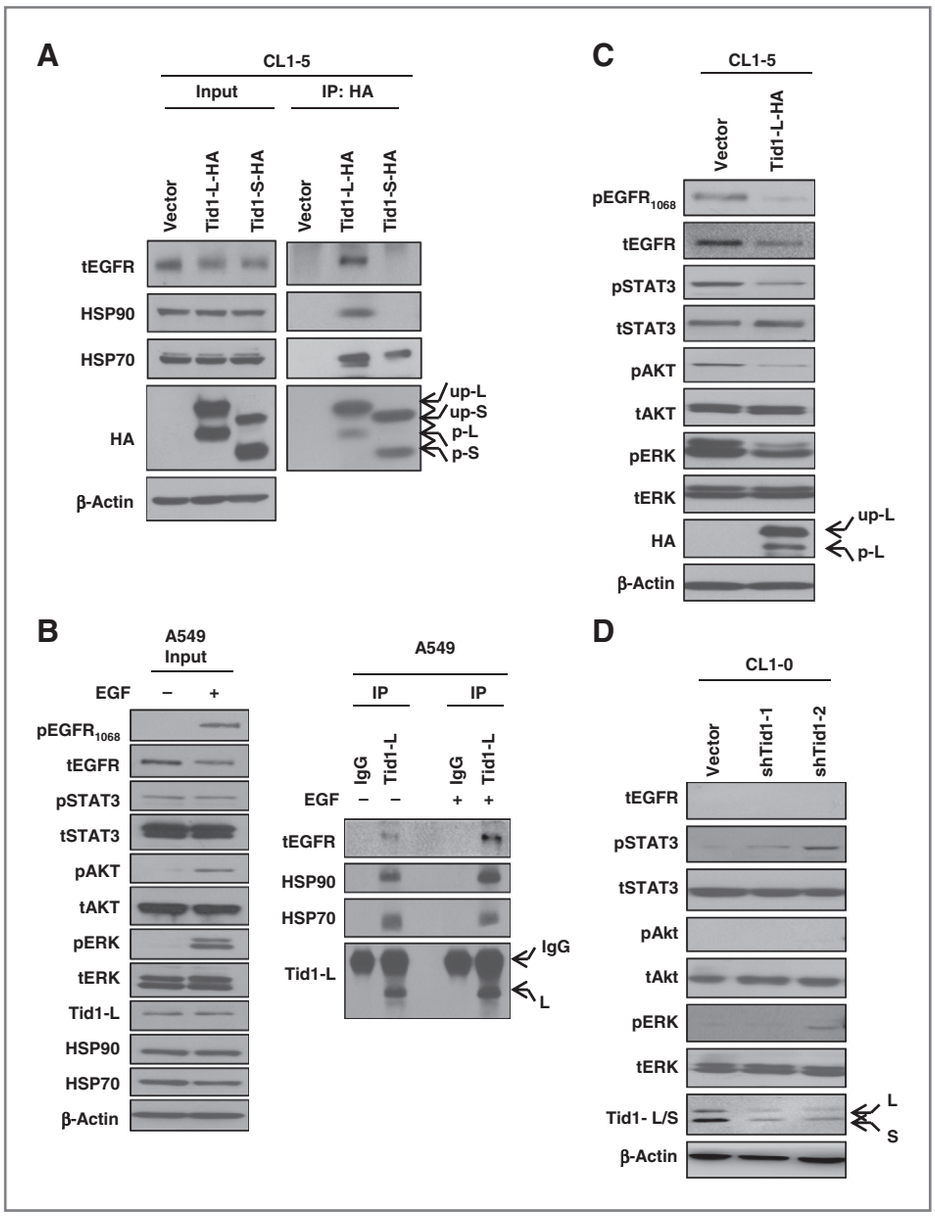


Figure 4. Tid1-L interacted with the EGFR/HSP90/HSP70 complex and attenuated the EGFR-dependent pathway in lung adenocarcinoma cells. A, CL1-5 cells were transfected with Tid1-L-HA and Tid1-S-HA, and the cell extracts were immunoprecipitated with anti-HA–conjugated agarose beads. The proteins in the immunoprecipitates were analyzed by Western blotting. B, interaction of endogenous Tid1-L, EGFR, HSP70, and HSP90 in A549 cells in response to EGF stimulation. A549 cells were serum-starved for 24 hours before treatment with 10 ng/mL EGF at 37°C for 5 minutes. Cell lysates of the A549 cells or A549 cells treated with EGF were immunoprecipitated (IP) with antibodies directed against Tid1-L, and analyzed as A above. C, CL1-5 cells were transfected with Tid1-L-HA and cultured for 24 hours. The EGF stimulation was as described in the legend of Fig. 4B. Cell lysates were analyzed for the levels of total (t) and phosphorylated proteins (p). D, CL1-0 cells were infected with empty vector (control) or 2 different shTid1 lentiviruses (sh-Tid1-1 and sh-Tid1-2). The expression of Tid1 in the infected cells was analyzed by Western blotting. The arrows in A, C, and D indicate the processed form (p) or the unprocessed form (up) of Tid1-L (L) and Tid1-S (S). β-actin served as a loading control in A to D. IgG, immunoglobulin G.

address whether the reduced stability of EGFR in Tid1-L–overexpressed cells enhanced EGFR ubiquitination, the proteasome inhibitor MG-132 was added to the transfected cells. As shown in Fig. 6E, ectopic expression of Tid1-L-Wt significantly promoted ubiquitination of EGFR as revealed by anti-ubiquitin immunoblot analysis. Collectively, these results showed that Tid1-L regulated EGFR stability in CL1-5 cells via the DnaJ-domain, leading to ubiquitination and proteasome degradation of EGFR.

Discussion

Two alternatively spliced forms of Tid1 are expressed in humans, Tid1-L and Tid1-S, which differ only at their C-termini. Tid1 proteins are synthesized as cytosolic precursor proteins, which are proteolytically cleaved by metalloproteases at the consensus mitochondrial targeting and cleavage sequences to allow for the translocation of Tid1 to the mitochondria. Tid1-L has a greater cytosolic stability and reduced rate of mitochondrial import compared with Tid1-S, resulting in higher levels of Tid1-L in the cytoplasm (20). The different subcellular localization of Tid1-L and Tid1-S may influence the ability of these cochaperone isoforms to associate with HSP70 and other cytosolic proteins, and hence influence their biologic interactions and functions.

The *Tid1* gene has been mapped to human chromosome band 16p13.3 (24). On the basis of array-comparative genomic hybridization data collected by Gallegos Ruiz and colleagues, loss of this chromosomal band is found in at least 20% of the patients with lung adenocarcinoma (25). In this study, we showed that Tid1-L likely functions as a tumor suppressor in

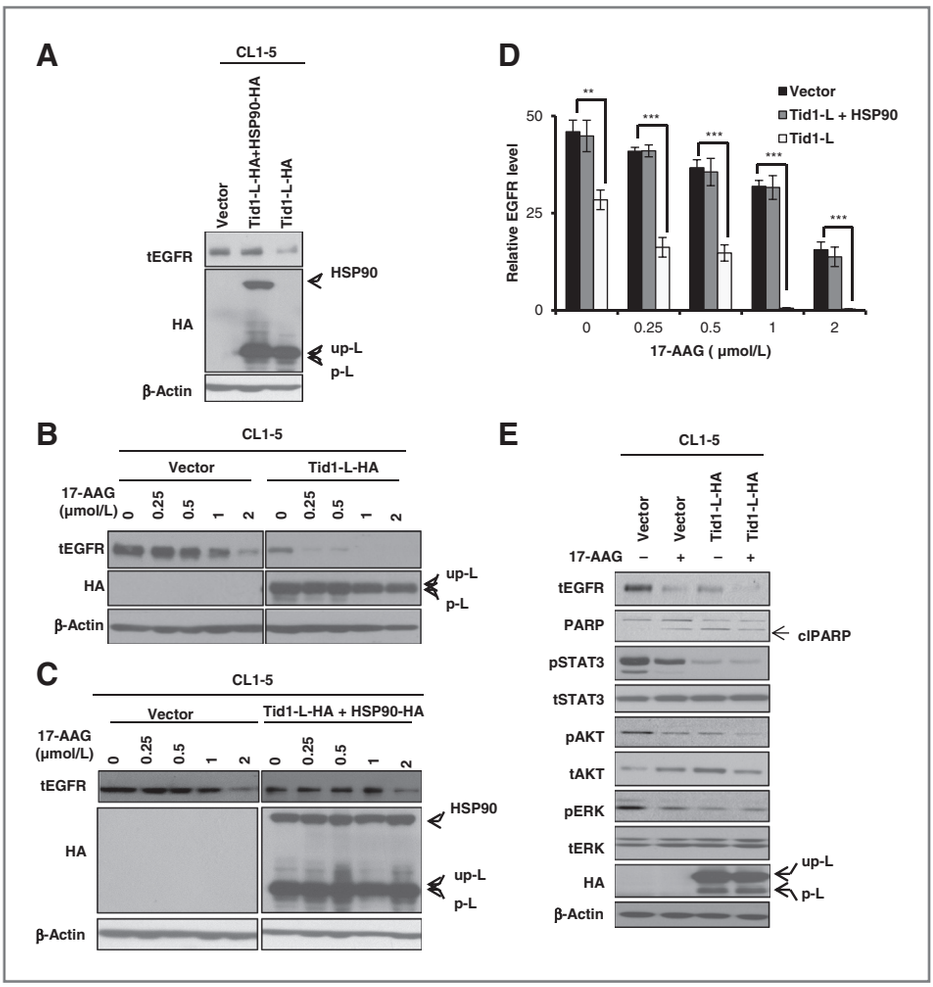


Figure 5. Effects of Tid1-L, HSP90, and 17-AAG on expressions levels of EGFR and EGFR-related signaling. A, CL1-5 cells were cotransfected with Tid1-L-HA and HSP90-HA and cultured for 48 hours. The levels of tEGFR and HA were assessed by Western blotting. B and C, effect of the proteasome inhibitor 17-AAG on the stability of EGFR. CL1-5 cells were transfected with Tid1-L-HA (B) or with both Tid1-L-HA and HSP90-HA (C) as described above. Forty-eight hours after transfection, the cells were treated with different concentrations of 17-AAG for 16 hours. The levels of tEGFR and HA were assessed by Western blotting. D, quantitative analysis of the relative EGFR levels. The results shown in B and C were quantified by densitometry analysis and expressed as relative EGFR levels. **, $P < 0.01$; ***, $P < 0.001$ based on the Student *t* test. E, effect of the Tid1-L overexpression and 17-AAG treatment on the activation of EGFR-related signaling. CL1-5 cells were transfected with Tid1-L-HA as described above. Forty-eight hours after transfection, the cells were treated with 2 μmol/L 17-AAG for 16 hours. The levels of total (t), phosphorylated proteins (p), HA, and clPARP were assessed by Western blotting. The positions of HSP90, cleaved PARP (clPARP), the unprocessed form of Tid1-L-HA (up-L), and the processed form of Tid1-L-HA (p-L) are indicated by the arrows.

lung adenocarcinoma. Tid1-L is expressed in normal bronchial epithelial cells, and the expression of Tid1-L in lung adenocarcinoma is inversely correlated with the expression of EGFR. Interestingly, survival analysis indicated that the overall survival of patients was negatively correlated with the expression of EGFR but positively correlated with the expression of Tid1-L. A low expression level of Tid1-L and high expression of EGFR were associated with poor overall survival of patients with lung adenocarcinoma, and vice versa. This clinical correlation may support the role of Tid1-L in regulating EGFR expression *in vivo*, and the Tid1-L/EGFR expression signature may represent a useful biomarker to predict clinical outcomes.

We also showed that Tid1-L overexpression induced lung adenocarcinoma cell apoptosis and inhibited lung tumor growth *in vitro* and *in vivo*. Tid1-L may interact with EGFR/HSP70/HSP90 through the DnaJ-domain and downregulate EGFR signaling by induction of EGFR ubiquitinylation and proteasome degradation. In this regard, it should be pointed out that the majority of Tid1 is localized in the mitochondria (Supplementary Fig. S3). At present, it is not known whether the inhibition of EGFR signaling is resulted from the action of cytosolic Tid1-L or mitochondrial Tid1-L or both. In any case, the inhibition of EGFR signaling by Tid1-L is comparable with or even stronger than that of the proven HSP90 inhibitor 17-AAG. Our results suggest that Tid1-L is a potential therapeutic target to treat lung adenocarcinoma.

Tid1 functions both as a chaperone and a cochaperone for a variety of proteins, and Tid1 is similar to another lung

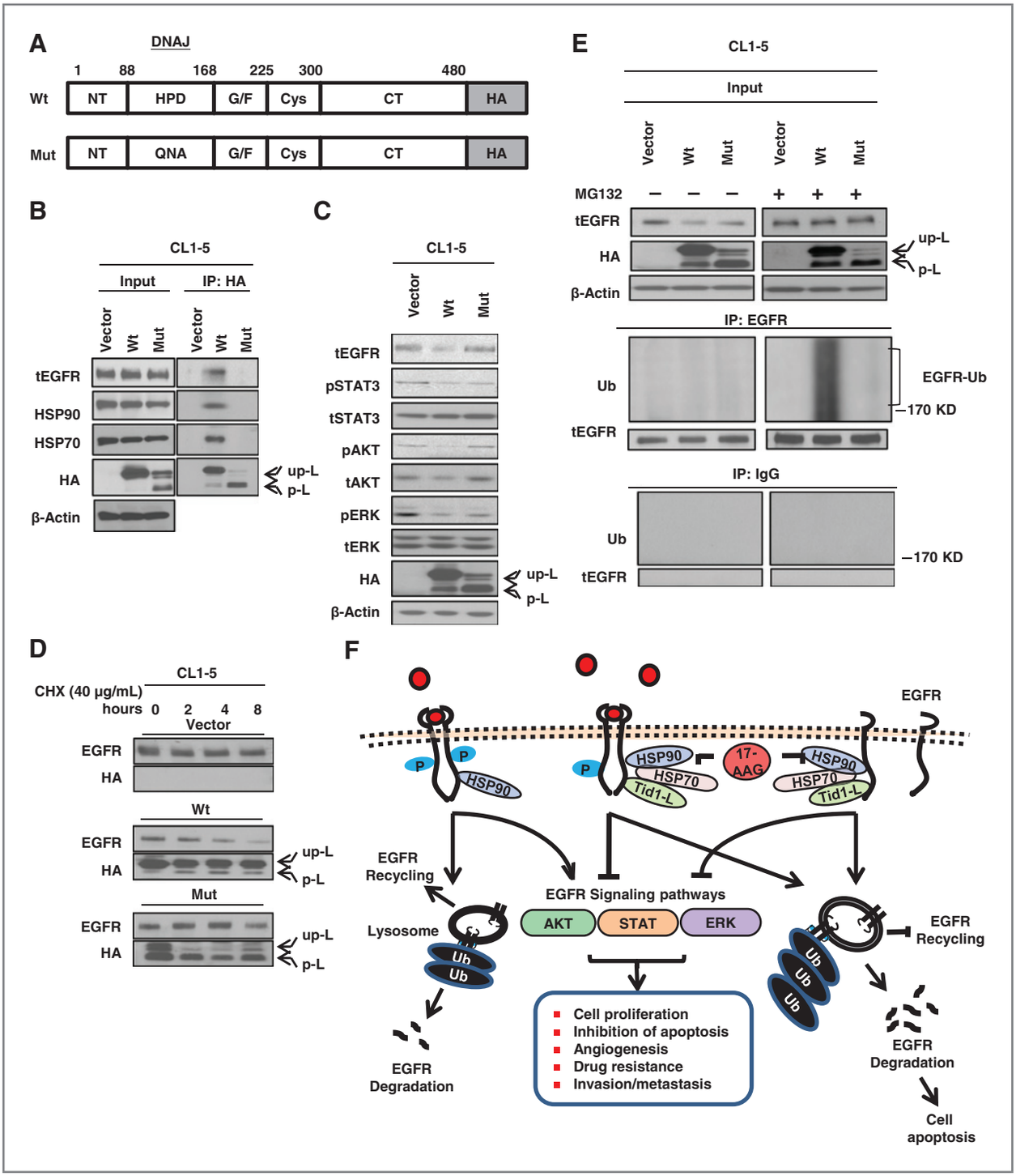


Figure 6. The DnaJ-domain of Tid1-L was required for polyubiquitination and proteasome degradation of EGFR. A, diagram of wild-type Tid1-L-HA (Wt) and the DnaJ-domain mutant Tid1-L (Mut) indicating the replacement of H121P122D123 of the wild-type with Q121N122A123 of the mutant. B, influence of the DnaJ-domain mutations on the interaction between Tid1-L and EGFR/HSP90/HSP70. CL1-5 cells were transfected with Tid1-L-HA (Wt), Tid1-L-HA (Mut), and cultured for 24 hours. Analyses of the proteins in the immunoprecipitates (IP) were as described in the legend of Fig. 4A. C, CL1-5 cells were transfected with the plasmids as described in B. The levels of total (t) and phosphorylated (p) proteins in the cell lysates were determined by Western blotting. D, CL1-5 cells were transfected and cultured as B. Transfected cells were then incubated with cycloheximide for 2 hours before sampling at the indicated times. The level of EGFR in the cell lysates was analyzed by Western blotting. E, effect of Tid1-L-HA (Wt) and Tid1-L-HA (Mut)overexpression on the polyubiquitination of EGFR. CL1-5 cells were transfected with the plasmids as in B. After 48 hours of transfection, the cells were treated with MG132 or dimethyl sulfoxide for 4 hours. The polyubiquitination of EGFR was analyzed by Western blotting of the proteins in the immunoprecipitates. F, model for the regulation of EGFR signaling and tumor suppression mediated by Tid1-L. IgG, immunoglobulin G.

cancer tumor suppressor, HLJ1 (26). Both proteins are of the same family of HSPs that function as molecular chaperones. HLJ1 overexpression retards cell-cycle progression by affecting the STAT1/P21 pathway, and this is accompanied by a decrease in cyclin D1 expression. We showed that overexpression of Tid1-L attenuated EGFR-dependent signaling. Therefore, these 2 molecular chaperones seem to exert their tumor suppressor functions by affecting different targets. Additional understanding of the role of the HSP40 family of proteins in oncogenesis is essential and may lead to the identification of novel targets for cancer therapy.

In summary, we showed that Tid1-L likely functions as a tumor suppressor in lung adenocarcinoma by downregulating EGFR signaling through interaction with the HSP70/HSP90 chaperone (Fig. 6F). Design of new strategies to unregulated Tid1-L expression and inhibit EGFR signaling may be useful for the treatment of lung adenocarcinoma because adenocarcinoma is driven by the EGFR pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C.-Y. Chen, J.-F. Lo, P.-C. Yang
Development of methodology: C.-Y. Chen, S.-C. Yang, S.-H. Pan, P.-C. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-Y. Chen, C.-I. Jan, P.-C. Yang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.-Y. Chen, C.-I. Jan, P.-C. Yang
Writing, review, and/or revision of the manuscript: C.-Y. Chen, C.-I. Jan, P.-C. Yang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.-Y. Chen, S.-C. Yang, Y.-L. Chang, W.-L. Wang, T.-M. Hong, P.-C. Yang
Study supervision: C.-Y. Chen, P.-C. Yang

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