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TRIM59 promotes gefitinib resistance in EGFR mutant lung adenocarcinoma cells

Zhilei Cui[a,1], Zhen Liu[b,1], Junxiang Zeng[c], Shulin Zhang[d], Lei Chen[e], Guorui Zhang[a], Weiguo Xu[a], Lin Song[a,*], Xuejun Guo[a,*]

[a] Department of Respiratory Medicine, XinHua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, China
[b] State Key Laboratory of Microbial Metabolism, School of Life Science and Biotechnology, Shanghai Jiao Tong University, China
[c] Department of Laboratory Medicine, XinHua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, China
[d] Department of Immunology and Microbiology, Shanghai Jiao Tong University School of Medicine, China
[e] Department of Pathology, XinHua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, China

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ABSTRACT

Aims: The relationship between TRIM59 and drug resistance is elusive despite of its multiple uncovered roles in human cancers. Here we aimed to characterize the expression status of TRIM59 in gefitinib-resistant EGFR mutant lung adenocarcinoma cells and elucidate its mechanism underlying the drug resistance.

Main methods: Gefitinib-resistant cell lines were established by progressive dosage. Relative expression of TRIM59 was determined by both real-time PCR and Western blot. Target gene knockdown was achieved by specific shRNAs. Cell viability was measured by MTT assay. Cell apoptosis was analyzed by flow cytometry with Annexin V/7-AAD double staining. Cell proliferation was determined by clonogenic formation assay. Migration and invasion capacities were detected using transwell chamber assay. Direct interaction between TRIM59 and STAT3 was analyzed by co-immunoprecipitation assay.

Key findings: We first observed overexpression of TRIM59 in gefitinib-resistant EGFR mutant lung adenocarcinoma cells. ShRNA-mediated knockdown of TRIM59 significantly inhibited cell viability and stimulated apoptosis. Meanwhile, TRIM59-deficiency suppressed cell migration and invasion. We further identified the interaction between TRIM59 and STAT3. TRIM59-deficiency remarkably impaired the activation of STAT3 signaling. STAT3-specific shRNAs significantly re-sensitized TRIM59-proficient EGFR mutant lung adenocarcinoma cells to gefitinib.

Significance: Our data characterized aberrant TRIM59 overexpression in gefitinib-resistance EGFR mutant lung adenocarcinoma cells, and indicated the potential involvement of TRIM59-STAT3 signaling in the occurrence of gefitinib-resistance.

1. Introduction

Lung cancer is one of the most common human malignancies and ranks the first place in causing cancer-related death in males, and second in females only behind breast cancer [1]. Globally, lung cancer has been diagnosed in 1.8 million people and approximately 1.6 million deaths were claimed in 2012 [2]. The majority of lung cancer can be histologically categorized into two main types including small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) [3]. NSCLC is further divided into adenocarcinoma and squamous cell carcinoma, which are treated as different diseases in the clinic. Long-term tobacco smoking accounts for the majority of lung cancer cases, whereas only 10–15% of diseases occur in population free-of-cigarette. Incidences of the latter often associate with genetic disorders and occupational exposure to radon gas, asbestos and air pollution particles [4]. Lung cancer can practically be diagnosed by chest radiograph and computed tomography scan, and confirmed by biopsy through bronchoscopy [5]. Clinical treatments and long-term outcomes of this disease greatly associate with the subtypes of cancer, progressive status and individual health condition. The mainstay therapeutics include surgical resection, chemotherapy and radiotherapy [6]. However, most lung cancers are not curable with unfavorable prognosis. In the United States, the five-

Abbreviations: TRIM59, Tripartite Motif Containing 59; EGFR, epidermal growth factor receptor; SCLC, small-cell lung cancer; NSCLC, non-small-cell lung cancer

* Corresponding authors.
E-mail addresses: everlasting1981@126.com (L. Song), guoxuejun@xinhuamed.com.cn (X. Guo).

[1] These authors contributed equally to this work.

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year survival rate is only approximately 15%.

Currently, several targeted drugs are clinically employed to treat lung cancer, especially in advanced cases [7], among which, erlotinib, gefitinib and afatinib constitute the membrane receptor tyrosine kinase inhibitor family which specifically inhibits the epidermal growth factor receptor (EGFR) [8]. Gefitinib (trade name Iressa) is the first selective inhibitor of EGFR tyrosine domain and was approved by FDA in 2003 as monotherapy for patients with locally advanced or metastatic EGFR mutant lung adenocarcinoma after platinum-based and docetaxel chemotherapy. More recently, gefitinib was approved by the FDA as the first-line treatment for EGFR mutant lung adenocarcinoma in 2015. The gefitinib target protein EGFR is frequently mutated in lung and breast cancers, which leads to inappropriate activation of the anti-apoptotic Ras signaling cascade and uncontrolled cell proliferation [9]. Research on gefitinib sensitivity in lung adenocarcinoma identified the mutational spectrum in the EGFR tyrosine kinase domain (L858R, del747), which is responsible for the activation of ATP binding and downstream signaling cascades [10]. Despite the marvelous therapeutic effects achieved so far, emerging evidences have reported acquired resistance to gefitinib in a considerable portion of recipient patients. A number of underlying mechanisms related to this acquired drug resistance have been investigated and proposed. For instance, Engelman et al. reported that amplification in MET led to gefitinib resistance in lung cancer through activation of ERBB3 signaling [11]. Pao et al. identified that acquired resistance of lung adenocarcinoma to gefitinib or erlotinib was associated with a second mutation in the EGFR kinase domain [12]. Sequist et al. uncovered the transformation from lung adenocarcinoma to SCLC contributed to a portion of acquired EGFR inhibitor resistance [13]. Ochi et al. demonstrated that Src mediated ERK re-activation in gefitinib-resistant EGFR mutant lung adenocarcinoma [14]. Kitamura et al. proposed that miR-134/487b/655 cluster regulated TGF-β-induced epithelial-mesenchymal transition and resistance to gefitinib by targeting MAGI2 in lung adenocarcinoma cells [15]. β-catenin overexpression was shown to associate with gefitinib resistance in lung adenocarcinoma as well [16]. Therefore, multiple mechanisms of action might individually or convergently contribute to drug resistance in different contexts.

TRIM59 (Tripartite Motif Containing 59) is a protein coding gene involved in ubiquitin-protein transfer process and may serve as a multifunctional regulator in the innate immune signaling pathways. Several investigations indicated potential functions of this protein in human cancers. For example, Zhan et al. reported that TRIM59 promoted the proliferation and migration of EGFR mutant lung adenocarcinoma cells by upregulating cell cycle-related proteins [17]. Zhou et al. showed that TRIM59 was up-regulated in gastric tumors and promoted ubiquitination and degradation of p53 [18]. Khatamianfar et al. proposed TRIM59 as a novel multiple cancer biomarker for immunohistochemical detection of tumorigenesis [19]. In our study, we, for the first time, characterized the aberrant overexpression of TRIM59 in gefitinib-resistant and EGFR mutant adenocarcinoma cells and elucidated the underlying mechanism.

2. Materials and methods

2.1. Cell culture

Human EGFR mutant lung adenocarcinoma cell lines HCC827 (an adenocarcinoma cell line with EGFR deletion from E746 to A750) and PC9 (an adenocarcinoma cell line with EGFR exon 19 deletion) were ordered from the American Type Culture Collection (Manassas, VA) and subjected to mycoplasma detection and DNA fingerprinting. All cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin/streptavidin (Gibco, Grand Island, NY). The gefitinib-resistant cells (HCC827GR and PC9GR) were established by progressive dosage of drug: parental cells were cultured with stepwise escalation of concentration of gefitinib from 5 nM to 5 μM over six months. All cells were cultured at 37 °C in the humidified incubator supplied with 5% CO_2. Gefitinib was purchase from Selleck (Shanghai, China).

2.2. Transfection

Cell transfection was conducted with Lipofectamine 3000 following the manufacturer's manual. Briefly, the exponential cells were cultured in 6-well plate the day prior to transfection. 2 μg of indicated plasmid was packaged by Lipofectamine 3000 diluted in 125 μL of Opti-MEM medium at room temperature for 5 min. The mixture was then added into each well dropwise. The transfection efficiency was evaluated by Western blot.

2.3. MTT assay

Cell proliferation was measured with the MTT assay kit (ab211091, Abcam, Cambridge, MA, USA) following the provider's manual. The parental and gefitinib-resistant HCC827 and PC9 cells were seeded into 96-well plate in triplicate and treated with different concentration of gefitinib (0, 2.5, 5 and 10 μM) for 96 h. The culture medium was cautiously aspirated and replaced with 50 μL of serum-free medium plus 50 μL of MTT working solution. The culture plate was incubated at 37 °C for 3 h and then supplemented with 150 μL of MTT solvent. After incubation on shaker at ambient temperature for 15 mins, the absorption at 590 nm was recorded by the SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, CA, USA).

2.4. Western blot

Cell lysate was prepared in ice-cold RIPA lysis buffer. Protein content was quantitated with the BCA Protein Assay Kit (ThermoFisher, Waltham, MA USA) following the manufacturer's guide. Protein was resolved by the SDS-PAGE gel and then transferred onto PVDF membrane on ice (300 mA, 2 h). Blocking was performed with 5% nonfat milk dissolved in the TBST buffer for 1 h at room temperature on the shaker. The indicated primary antibodies were hybridized overnight at 4 °C. Excessive antibodies were completely washed off with TBST (6 × 5 min) and the membrane was subjected to the horseradish peroxidase-conjugated secondary antibody incubation for another hour at room temperature. The protein blot was then visualized using the enhanced chemiluminescence kit (ECL, Millipore, Billerica, MA, USA). TRIM59 antibodies was purchased from Sangon Biotech (Shanghai, China). STAT3, p-STAT3, EGFR and β-actin primary antibodies, as well as secondary antibodies were purchased from Proteintech Group (Danvers, MA, USA).

2.5. Real-time PCR

Total RNA was extracted from cells using the TriZol reagent (Invitrogen, Waltham, MA USA) according to the provider's recommendations. RNA integrity and concentration were determined by the agarose electrophoresis and Nanodrop 2000 (ThermoFisher, Waltham, MA USA). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, Waltham, MA USA). Quantitative PCR was performed with PowerUp SYBR Green Master Mix (ThemoFisher, Waltham, MA USA) on CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The expression of target genes was analyzed by the $2^{-\Delta\Delta Ct}$ method. The primer sequence information used in Real-time PCR analysis were: TRIM59-F (ACGAATTCCACTCAAGTGCC), TRIM59-R (GTA ATGTTCAGGGCAGGTGAC), GAPDH-F (ACCACAGTCCATGCCATCAC),

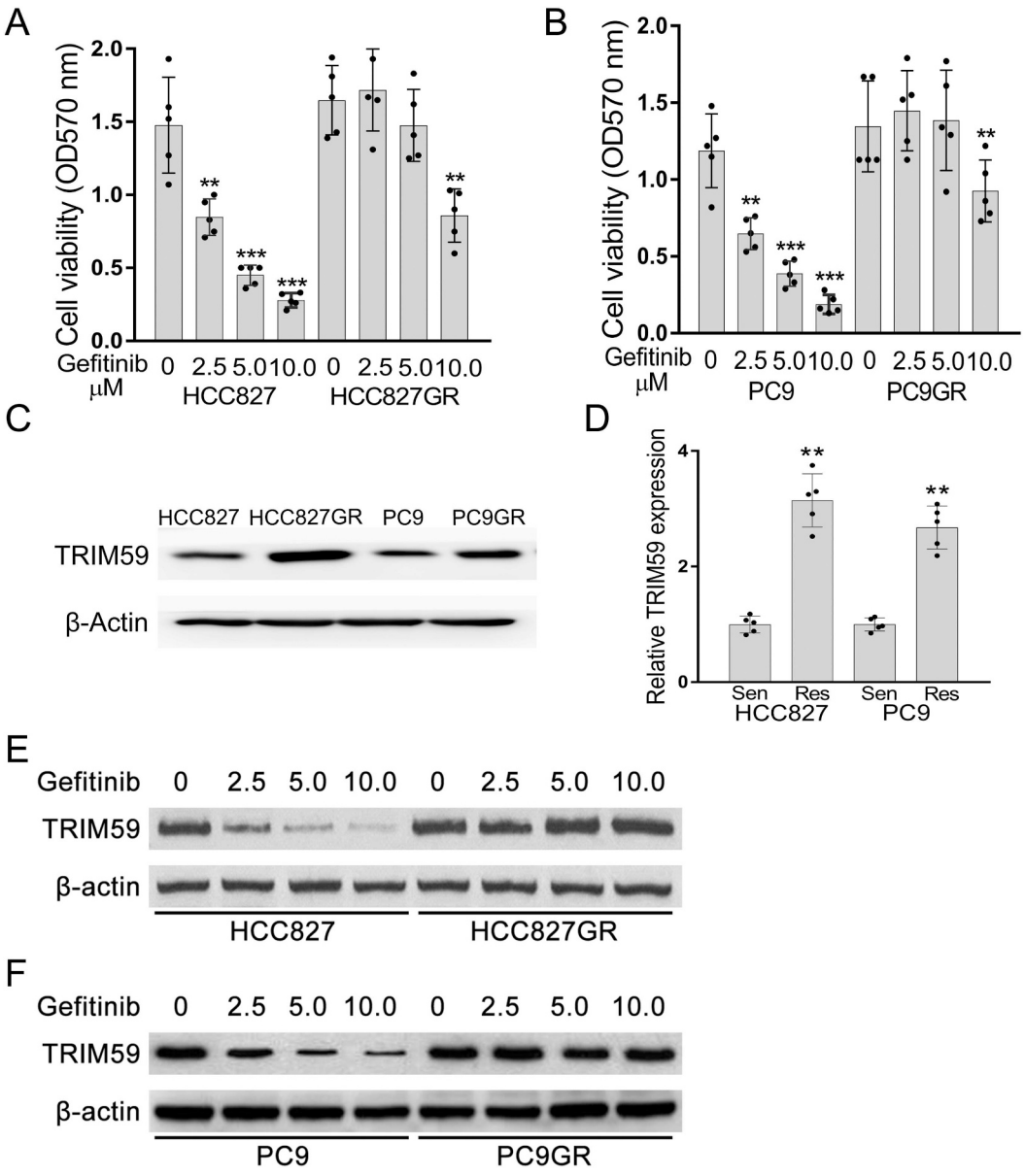

Fig. 1. TRIM59 is upregulated in gefitinib-resistant EGFR mutant lung adenocarcinoma cell lines. Cell viability assay of (A) HCC827 and HCC827/GR, (B) PC9 and PC9/GR cells measured at 96 h by MTT. (C) WB detection of TRIM59 protein in HCC827 and HCC827/GR cells (left panel) and PC9 and PC9/GR cells (right panel). β-actin served as loading controls. (D) Quantitative RT-PCR analysis of the level of TRIM59 RNA in HCC827 and HCC827/GR cells (left panel) and PC9 and PC9/GR cells (right panel). GAPDH served as a housekeeping gene. (E) WB detection of TRIM59 protein in HCC827 and PC9 cells after Gefitinib treated for 48 h. β-actin served as loading controls. (F) WB detection of TRIM59 protein in HCC827/GR and PC9/GR cells after Gefitinib treated for 48 h. β-actin served as loading controls. Data were presented as mean ± SD of three independent experiments. For all panels: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

GAPDH-R (TCAGGTCCACCACTGACACG).

2.6. Apoptosis

HCC827 and PC9 cells were seeded into 6-well plate and dosed with 5 μM gefitinib for 48 h. Apoptosis was performed with the PE Annexin V Apoptosis Detection Kit 1 (BD Pharmingen, CA, USA) in accordance with the manufacturer's instructions. Single-cell suspension was prepared in 1 × binding buffer at a concentration of 10^6 cells/mL. 100 μL of solution was transferred into 5 mL tube and subjected to PE Annexin V (5 μL) and 7-AAD (5 μL) staining for 15 min at room temperature in the dark. After adding 400 L of 1 × binding buffer, the cells were analyzed on the BD FACSCelesta Flow Cytometer (BD BioSciences, Franklin Lakes, NJ, USA).

2.7. Clonogenic assay

1000 cells were seeded into 6-well plate in triplicate and treated with 5 μM gefitinib for 2 weeks. After complete removal of culture medium, the formed colonies were fixed with ice-cold methanol for 10 min and stained by 0.25% crystal violet at room temperature for 10 min. The images were captured under the optical microscope and colonies were counted in five random areas.

2.8. Migration and invasion assay

Migration and invasion were measured using the transwell chamber assay (Corning, NY, USA). For invasive assay, the transwell chamber was pre-coated with 0.1% basal membrane extracts (R&D Systems, Minneapolis, MN, USA). Single-cell suspension was prepared from the

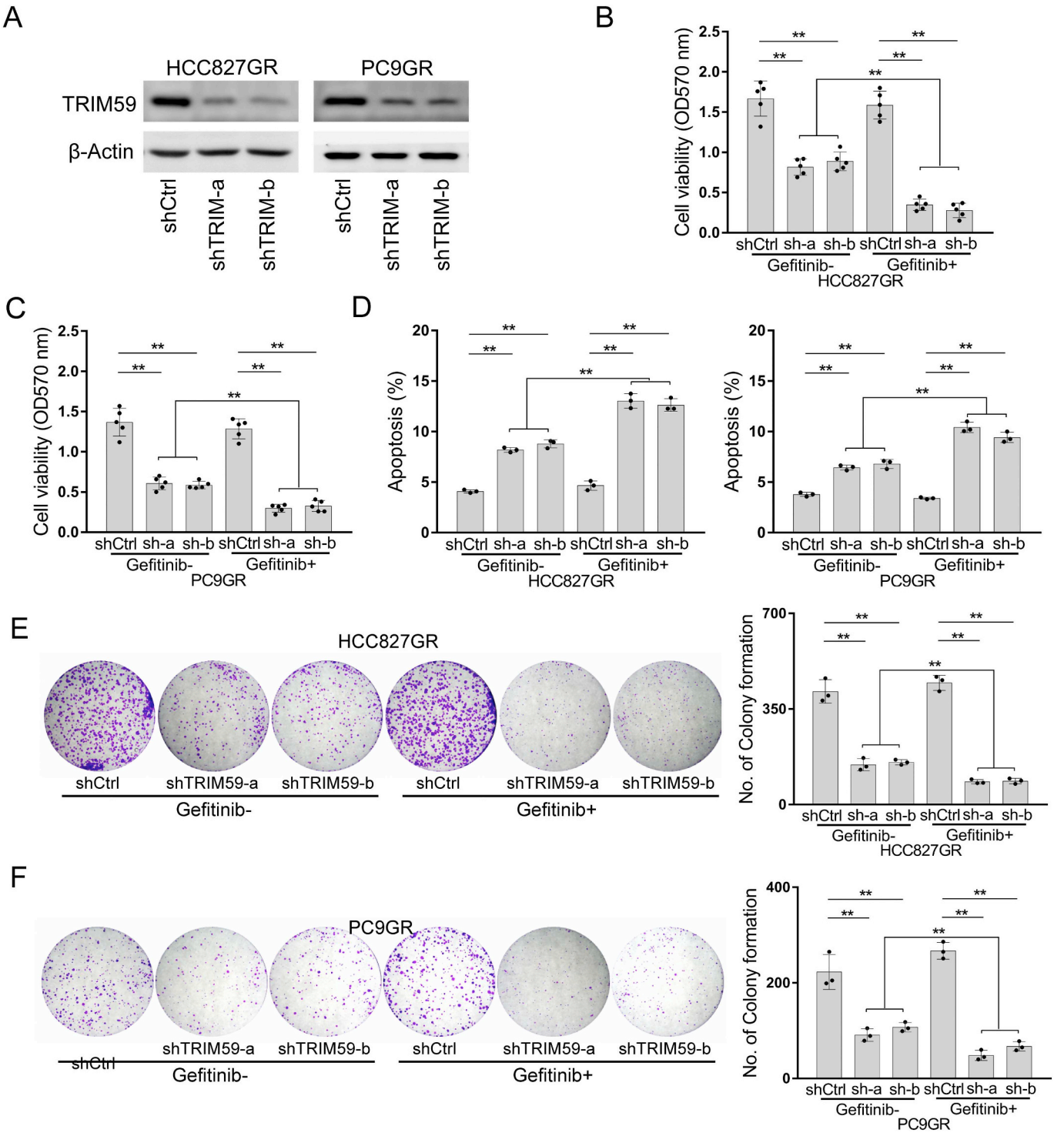

Fig. 2. TRIM59 inhibits cell viability in gefitinib-resistant EGFR mutant lung adenocarcinoma cell lines. (A) Western blotting assay for the levels of TRIM59 in HCC827 and PC9 cells stably expressing shCtrl or shTRIM59. β-actin served as loading controls. Cell viability assay of (B) HCC827/GR and (C) PC9/GR cells were measured at 96 h by MTT. Cell apoptosis assay of (D) HCC827/GR and PC9/GR cells were measured by flow cytometry analysis after cells were treated with gefitinib for 48 h. (E) HCC827/GR and (F) PC9/GR cells were treated with gefitinib for two weeks and subjected to cell colony formation assay. Gefitinib + and Gefitinib- represent the culture media with and without 5 μM gefitinib, respectively. Data were presented as mean ± SD of three independent experiments. For all panels: $^{*}P < 0.05$; $^{**}P < 0.01$.

indicated cells and adjusted the concentration to 5×10^5/mL. 5×10^4 cells were added to the insert dropwise and 650 L of complete culture medium was supplemented into the lower compartments. After 24 h, the uninvited cells were cautiously and completely wiped off with cotton swab. After fixation with 4% paraformaldehyde at room temperature for 10 min and staining with 0.25% crystal violet for 20 min, the representative images were captured and invaded cells were counted.

2.9. Immunoprecipitation

HCC827GR and PC9GR cells were lysed with low-salt buffer (0.5%

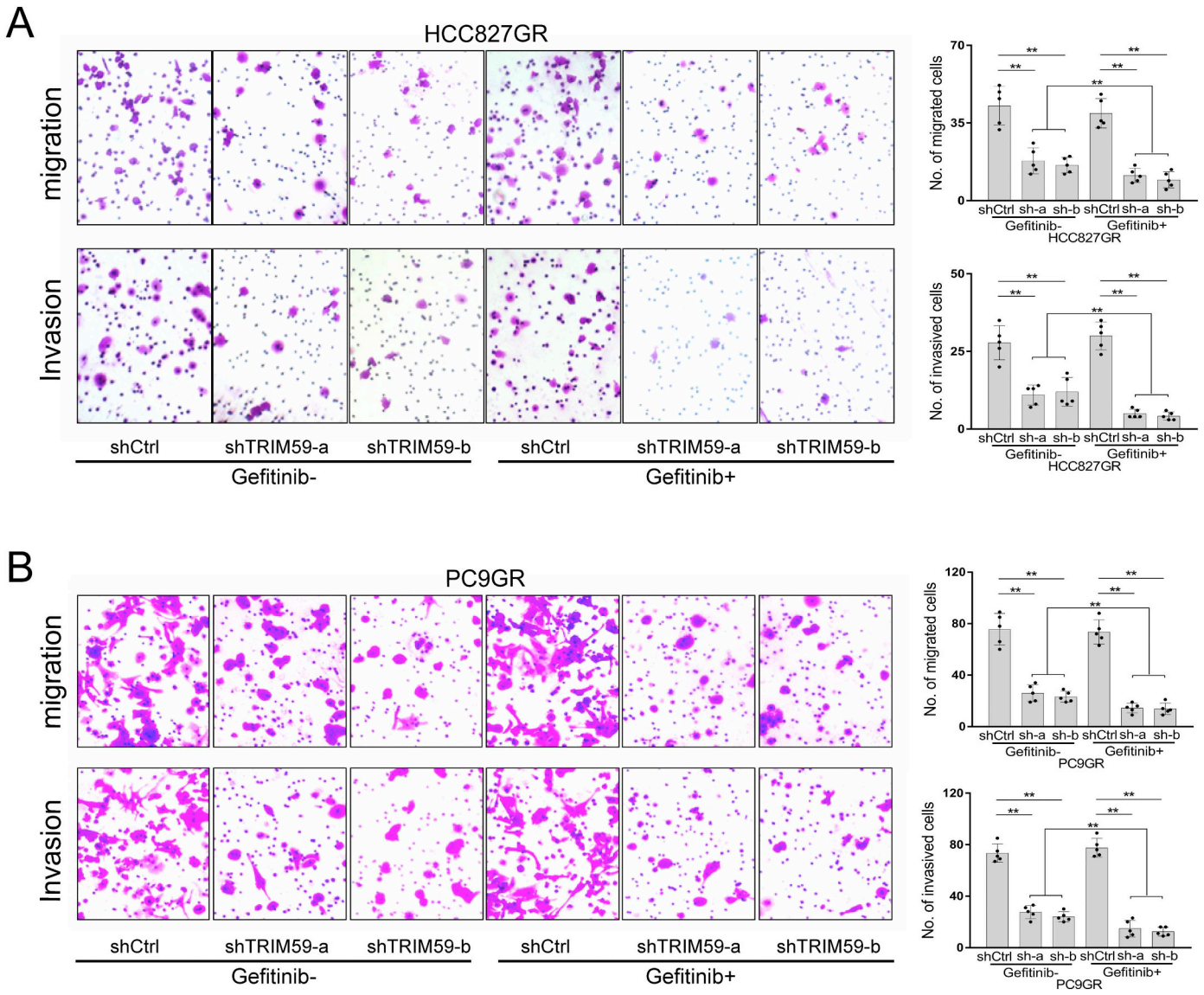

Fig. 3. TRIM59 inhibits cell migration and invasion in gefitinib-resistant EGFR mutant lung adenocarcinoma cell lines with or without gefitinib treatment (5 μM). (A) HCC827/GR and (B) PC9/GR cells were subjected to transwell migration and invasion assay. The number of transwelled cells was counted from at least five independent microscopic fields. Data were presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

NP-40, 20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM EDTA, 1.5 mM $MgCl_2$ plus protease inhibitor cocktail and protein phosphatase inhibitor cocktail) for 30 min on ice. The cell debris was discarded after centrifugation at 13,000 rpm for 15 min at 4 °C. The supernatants were incubated with indicated antibodies overnight at 4 °C followed by incubation with Protein A/G magnetic beads (ThermoFisher, Waltham, MA USA) for 2 h at room temperature. After rigorous wash with IP buffer, the beads were boiled in SDS-PAGE loading buffer. The co-immunoprecipitated complex was analyzed by Western blot.

2.10. Statistical analysis

All experiments presented in study were repeated at least three times unless otherwise indicated. Data was processed and analyzed with the PRISM 6.0 software. One-way ANOVA followed by the Turkey's test was employed for the statistical comparison. The statistical significance was calculated as P value, and $P < 0.05$ was considered as significantly different.

3. Results

3.1. TRIM59 is upregulated in gefitinib-resistant EGFR mutant lung adenocarcinoma cell lines

We first set out to investigate the expression status of TRIM59 in gefitinib-resistant EGFR mutant lung adenocarcinoma cells. To this purpose, we established gefitinib-resistant HCC827 and PC9 cells *via* progressive dosage. The successful establishment of resistant cell lines was experimentally confirmed by the MTT assay. As shown in Fig. 1A and B, gefitinib inhibited cell viability in a dose-dependent manner in both wild-type HCC827 and PC9 cells. However, no significant inhibitory effect was observed with 5 μM of gefitinib in the resistant progenitor cells, despite of marginal effects elicited by 10 μM of gefitinib. We then compared the relative expression of TRIM59 in the resistant cells to that in parental cells at both protein and transcript levels. TRIM59 protein was significantly increased in gefitinib-resistant HCC827 and PC9 cells (Fig. 1C). Consistently, the mRNA contents of TRIM59 were 2.5–3-fold higher in the resistant cells than naïve counterparts (Fig. 1D), which suggested a potentially inducing mechanism at

the transcription level. In the naïve cells, gefitinib induced significant decrease in TRIM59 protein in a dose-dependent manner in both HCC827 and PC9 cells, while no noticeable changes were observed in the resistant counterparts (Fig. 1E and F). Our data demonstrated up-regulation of TRIM59 in gefitinib-resistant EGFR mutant lung adenocarcinoma cells and indicated potential association between aberrant overexpression of TRIM59 and gefitinib resistance in this disease.

3.2. TRIM59-deficiency inhibits cell viability in gefitinib-resistant EGFR mutant lung adenocarcinoma cell lines

Next, we evaluated the potential impact of TRIM59-deficiency on gefitinib-resistant EGFR mutant lung adenocarcinoma cell lines. To this purpose, we first established stable TRIM59-knockdown cell lines in both HCC827/GR and PC9/GR cells. The successful establishment of subject cell lines was confirmed by Western blot (Fig. 2A). Notably, to exclude any artifact associated with off-target effect of shRNA, here we employed two shRNAs for TRIM59 in each cell line. The viability of TRIM59-deficient HCC827/GR and PC9/GR cells in response to either mock or gefitinib treatment was evaluated by the MTT assay. As shown in Fig. 2B and C, TRIM59 knockdown significantly compromised the viability of both HCC827/GR and PC9/GR cells, which re-sensitized the resistant cells to gefitinib treatment as well. Meanwhile, TRIM59-deficiency stimulated remarkable spontaneous cell apoptosis, which was further aggravated by gefitinib (Fig. 2D). Consistently, colony formation capacity was tremendously impaired by TRIM59 knockdown in both HCC827/GR and PC9/GR cells, wherein treatment with gefitinib manifested more inhibitory effects on colony formation (Fig. 2E, F). Therefore, our data highlighted the importance of TRIM59 in gefitinib-resistant EGFR mutant lung adenocarcinoma cells, deficiency of which significantly impaired cell viability and induced evident cell apoptosis. Noteworthily, TRIM59 knockdown re-sensitized the resistant cells to gefitinib treatment.

3.3. TRIM59-deficiency inhibits cell migration and invasion in gefitinib-resistant EGFR mutant lung adenocarcinoma cells

Next, we further evaluated potential impact of TRIM59-deficiency on the malignant behaviors of gefitinib-resistant EGFR mutant lung adenocarcinoma cells. Both migration and invasion of HCC827/GR and PC9/GR cells were interrogated by the transwell chamber assay. As shown in Fig. 3A, application of both independent shRNAs suppressed migration and invasion capacity in the host cells, which was further enhanced by co-treatment of gefitinib. Similar results were observed in PC9/GR cells (Fig. 3B), which indicated that aberrant overexpression of TRIM59 contributed to the malignant metastatic process, in addition to cell growth, in gefitinib-resistant EGFR mutant lung adenocarcinoma cells.

3.4. TRIM59 knockdown re-sensitizes resistant cell to gefitinib in EGFR mutant lung adenocarcinoma cell lines

Meanwhile, we evaluated the gefitinib sensitivity in either naïve or resistant EGFR mutant lung adenocarcinoma cells in response to TRIM59 deficiency. To this end, we specifically silenced TRIM59 in both HCC827/GR and PC9/GR cells with two independent shRNAs, which were subsequently subjected to treatments with different doses of gefitinib. As a result, cell viability was significantly decreased in TRIM59-deficient gefitinib-resistant cells to the extent comparable with naïve parental cells (Fig. 4A, B). In contrast, cell apoptosis was dramatically induced by gefitinib in the resistant cells in response to TRIM59 knockdown in comparison with parental cells (Fig. 4C, D). Colony formation capacity was greatly compromised in response to TRIM59 knockdown in resistant cells exposed to low concentration of gefitinib (Fig. 4E, F). In addition, the migrative and invasive behaviors were evidently inhibited by TRIM59 knockdown in gefitinib-resistant cell lines (Fig. 4G–J). Therefore, our data indicated that TRIM59 knockdown re-sensitized resistant cells to gefitinib treatment in the EGFR mutant lung adenocarcinoma cells.

3.5. TRIM59 interacts with STAT3

Next, we sought to understand the signaling pathway implicating TRIM59 in gefitinib-resistant EGFR mutant lung adenocarcinoma cells. To this end, we set out to identify potential TRIM59-interacting proteins in gefitinib-resistant HCC827/GR and PC9/GR cells. Intriguingly, we have identified STAT3 in the immunoprecipitated complex of HA-tagged TRIM59 in HCC827/GR cells (Fig. 5A), which indicated possible involvement of TRIM59 in the STAT3 signaling pathway. We consolidated this observation in PC9/GR cells as well (Fig. 5B), which implied the common interaction between TRIM59 and STAT3 in the gefitinib-resistant EGFR mutant lung adenocarcinoma cells. Furthermore, activation of STAT3 signaling, as indicated by phosphorylation, was significantly abrogated by TRIM59-deficiency in both HCC827/GR and PC9/GR cells (Fig. 5C, D). Consistent with previous report, we further confirmed that the interaction between TRIM59 and STAT3 was strengthened by EGF stimulation (Fig. 5E), which indicated the contributing effect of EGFR in this scenario [20]. Therefore, our data demonstrated the important role of TRIM59 in the activation of STAT3 pathway in the gefitinib-resistant EGFR mutant lung adenocarcinoma cells.

3.6. STAT3 is critical for TRIM59-mediated cancer progression

Next, we sought to evaluate whether STAT3 predominantly mediated the TRIM59-provoked gefitinib-resistance in EGFR mutant lung adenocarcinoma. The STAT3 pathway was evidently inhibited in HCC827/GR cells by STAT3-specific shRNA (Fig. 6A). Exogenous introduction of TRIM59 into HCC827/GR cells stimulated overtly increased cell viability, which was completely abolished by simultaneous STAT3-knockdown (Fig. 6B). On the contrary, ectopic expression of TRIM59 remarkably suppressed cell apoptosis, which was readily reversed by STAT3-deficiency (Fig. 6C). Likewise, TRIM59-proficiency promoted colony formation and STAT3-deficiency compromised this phenotype in HCC827/GR cells (Fig. 6D). Both migrative and invasive capacities were exacerbated by overexpression of TRIM59, which were blocked by simultaneous inhibition of STAT3 (Fig. 6E, F). Notably, STAT3-deficiency re-sensitized the TRIM59-proficient HCC827/GR cells to gefitinib with respect to cell viability, apoptosis, clonogenic capacity, migration and invasion. Therefore, we provided evidences showing that STAT3 predominantly mediated TRIM59-associated gefitinib-resistance in EGFR mutant lung adenocarcinoma.

4. Discussion

Despite of its promising clinical effectiveness against EGFR mutant lung adenocarcinoma, frequently provoked resistance disastrously compromised the therapeutic outcome of gefitinib in EGFR mutant lung adenocarcinoma patients. Therefore, deep insight into the underlying mechanism leading to drug resistance is the emerging focus for the scientific community. In this study, we for the first time characterized the aberrant overexpression of TRIM59 in gefitinib-resistant EGFR mutant lung adenocarcinoma cells at both transcript and protein levels. We further demonstrated that shRNA-mediated deficiency of TRIM59 in gefitinib-resistant EGFR mutant lung adenocarcinoma cells significantly decreased cell viability. Meanwhile, spontaneous cell apoptosis was tremendously stimulated in the drug resistant cells in response to TRIM59 knockdown. Clonogenic capacity was compromised as well in the TRIM59-deficient HCC827/GR and PC9/GR cells. In addition to the fundamental influences on cell growth, we further uncovered that defects in TRIM59 remarkably inhibited both migration and invasion of gefitinib-resistant EGFR mutant lung adenocarcinoma cells. Notably,

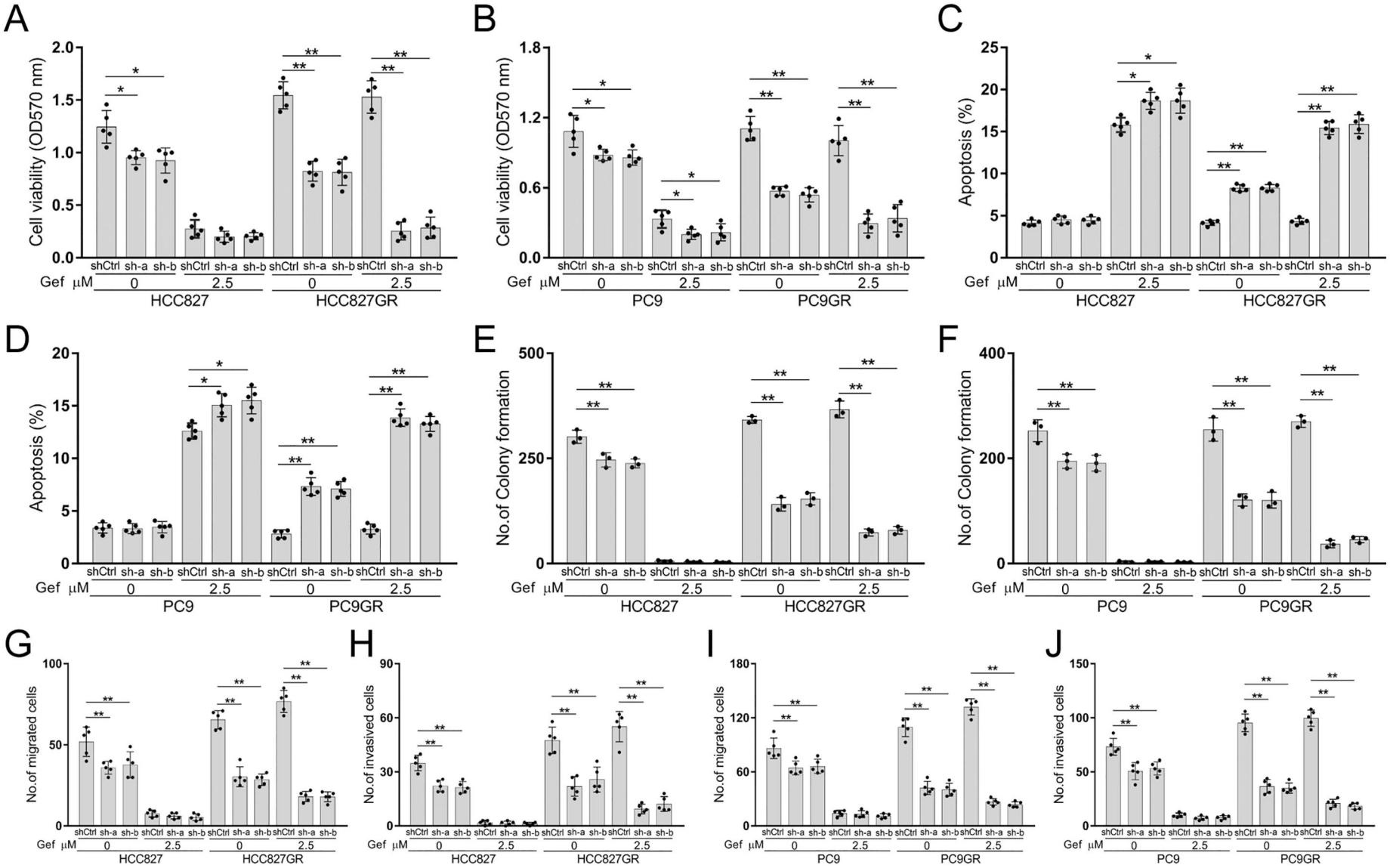

Fig. 4. TRIM59 knockdown increase gefitinib sensitivity in both gefitinib sensitive and resistant EGFR mutant lung adenocarcinoma cell lines. (A) HCC827, HCC827GR and (B) PC9, PC9GR cells were measured after cells were treated with gefitinib for 72 h by MTT. Cell apoptosis assay of (C) HCC827, HCC827GR and (D) PC9, PC9GR cells were measured by flow cytometry analysis after cells were treated with or without gefitinib for 48 h. (E) HCC827, HCC827GR and (F) PC9, PC9GR cells were treated with or without gefitinib and subjected to cell colony formation assay. (G) (H) HCC827, HCC827GR and (I) (J) PC9, PC9GR cells were subjected to transwell migration and invasion assay after cells were treated with or without gefitinib. Data were presented as mean ± SD of three independent experiments. For all panels: $*P < 0.05$; $**P < 0.01$.

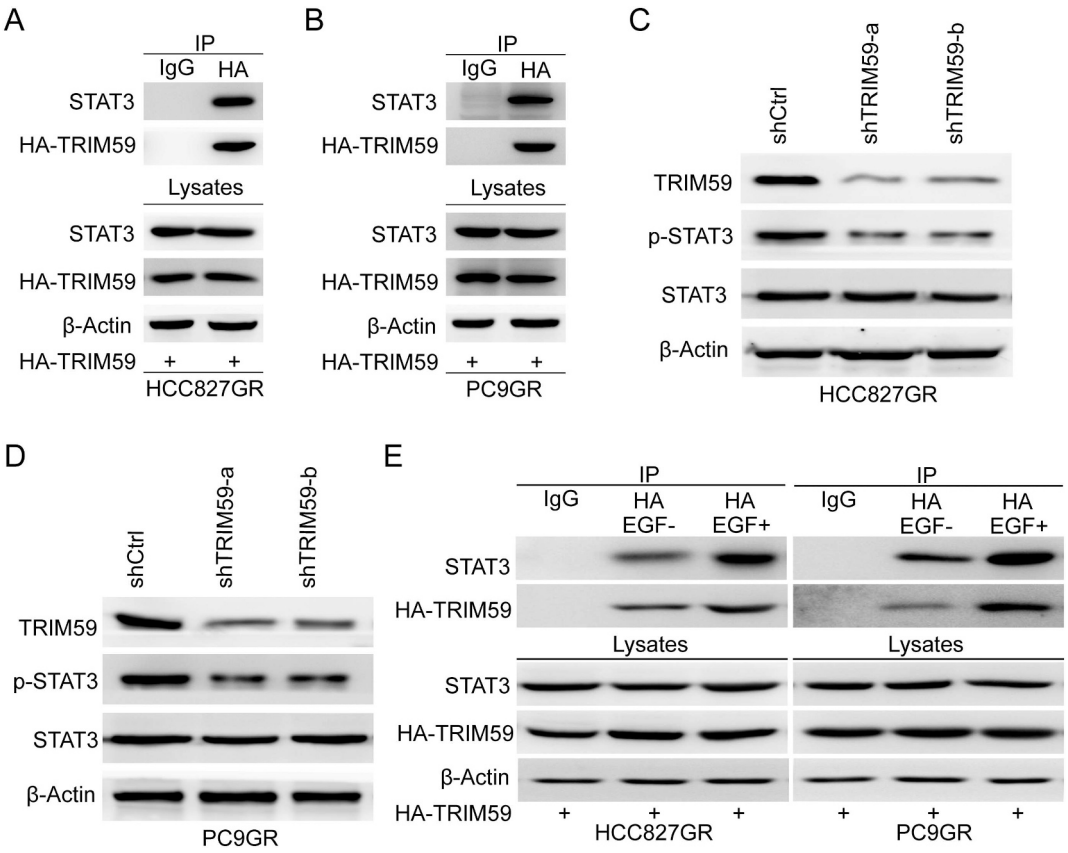

Figure 5. TRIM59 interacts with STAT3. IP analyses of the association of STAT3 with ectopic HA-TRIM59 in (A) HCC827/GR and (B) PC9/GR cells. IgG was used as a control. (C) (D)WB detection of TRIM59, p-STAT3 and STAT3 protein in HCC827/GR cells and PC9/GR cells. IP analyses of the association of STAT3 with ectopic HA-TRIM59 in (E) HCC827/GR and PC9/GR cells after EGF treated for 24 h. IgG was used as a control. β-actin served as loading controls.

TRIM59-deficiency significantly re-sensitized the host cells to gefitinib, which evidently suppressed the migrative and invasive capacities of the gefitinib-resistant cells, suggesting acquired gefitinib resistance in EGFR mutant lung adenocarcinoma cells. Mechanistically, we identified STAT3 as the direct interacting partner of TRIM59 in both gefitinib-resistant cell lines, and TRIM59-deficiency markedly suppressed STAT3 activation. Our data indicated that TRIM59 intimately associated with STAT3 activation, which might consequently contribute to the acquired drug resistance in EGFR mutant lung adenocarcinoma cells. To further estimate the importance of STAT3 signaling pathway in mediating TRIM59-associated gefitinib-resistance, we ectopically overexpressed TRIM59 in both HCC827/GR and PC9/GR cells. Consistent with previous observations, forced overexpression of TRIM59 significantly stimulated cell viability and suppressed cell apoptosis. Likewise, malignant capacities such as migration and invasion were deteriorated by exogenous introduction of TRIM59. Most importantly, all the above-mentioned phenotypes were completely abrogated by co-knockdown of STAT3, which highlighted the critical and predominant role of STAT3 pathway in mediating the TRIM59-related drug resistance in EGFR mutant lung adenocarcinoma cells. Therefore, we for the first time uncovered that aberrant overexpression of TRIM59 associated with gefitinib-resistance in EGFR mutant lung adenocarcinoma cells, which greatly depended on activation of STAT3 signaling pathway. Our study demonstrated the potential prognostic value of TRIM59-STAT3 signaling axis with respect to gefitinib response in EGFR mutant lung adenocarcinoma cells, which also held invaluable promise for therapeutic exploitation to conquer the acquired gefitinib resistance.

In agreement with previous investigations into the intimate linkage of aberrant TRIM59 expression with diverse human malignancies, here we characterized overexpression of TRIM59 in gefitinib-resistant EGFR mutant lung adenocarcinoma cells and provided proof-of-concept that dysregulated TRIM59 associated with gefitinib resistance. Despite of the experimentally validated upregulation of TRIM59 in gefitinib-resistant EGFR mutant lung adenocarcinoma cells, the precise mechanism underlying the observed high TRIM59 level was still elusive currently. Epigenetic mechanism was proposed for modulation of TRIM59 expression in familial Alzheimer's disease, where hypermethylation of TRIM59 and KLF14 influenced cell death signaling [21]. Jin et al. reported that Bacillus Calmette-Guerin increased membrane expression of TRIM59 through the TLR2/TLR4/IRF5 pathway in RAW264.7 macrophages [22]. However, whether the identical mode of action operated in TRIM59 regulation in gefitinib-resistance was still be to addressed.

Our study further pinpointed the critical role of dysregulated STAT3 pathway at the downstream of TRIM59 in the occurrence of gefitinib resistance. Accumulative evidences have unraveled the importance of STAT3 in drug resistance in cancer therapies. For instance, Spitzner et al. demonstrated that STAT3 inhibition sensitized colorectal cancer to chemotherapy both *in vivo* and *in vitro* [23]. Van Schaeybroeck et al. reported that ADAM17-dependent c-MET-STAT3 signaling mediated resistance to MEK inhibitors in KRAS mutant colorectal cancer [24]. Li et al. showed that nuclear PKM2 contributed to gefitinib resistance *via* regulating STAT3 activation in colorectal cancer [25]. Liu et al. demonstrated that inhibition of constitutively active STAT3 reversed enzalutamide resistance in LNCaP derivative prostate cancer cells [26]. Also in prostate cancer, Zhou et al. proposed loss of DAB2IP conferred resistance to androgen deprivation therapy through activating STAT3 and inhibiting apoptosis [27]. Yeom et al. showed that RRAD promoted EGFR-mediated STAT3 activation and induced temozolomide resistance in malignant glioblastoma [28]. In addition, STAT3-targeting drugs manifested promising therapeutic effects in a number of human cancers. Pandey et al. employed Berberine and Curcumin, which targeted Survivin and STAT3, respectively, and demonstrated their synergistic

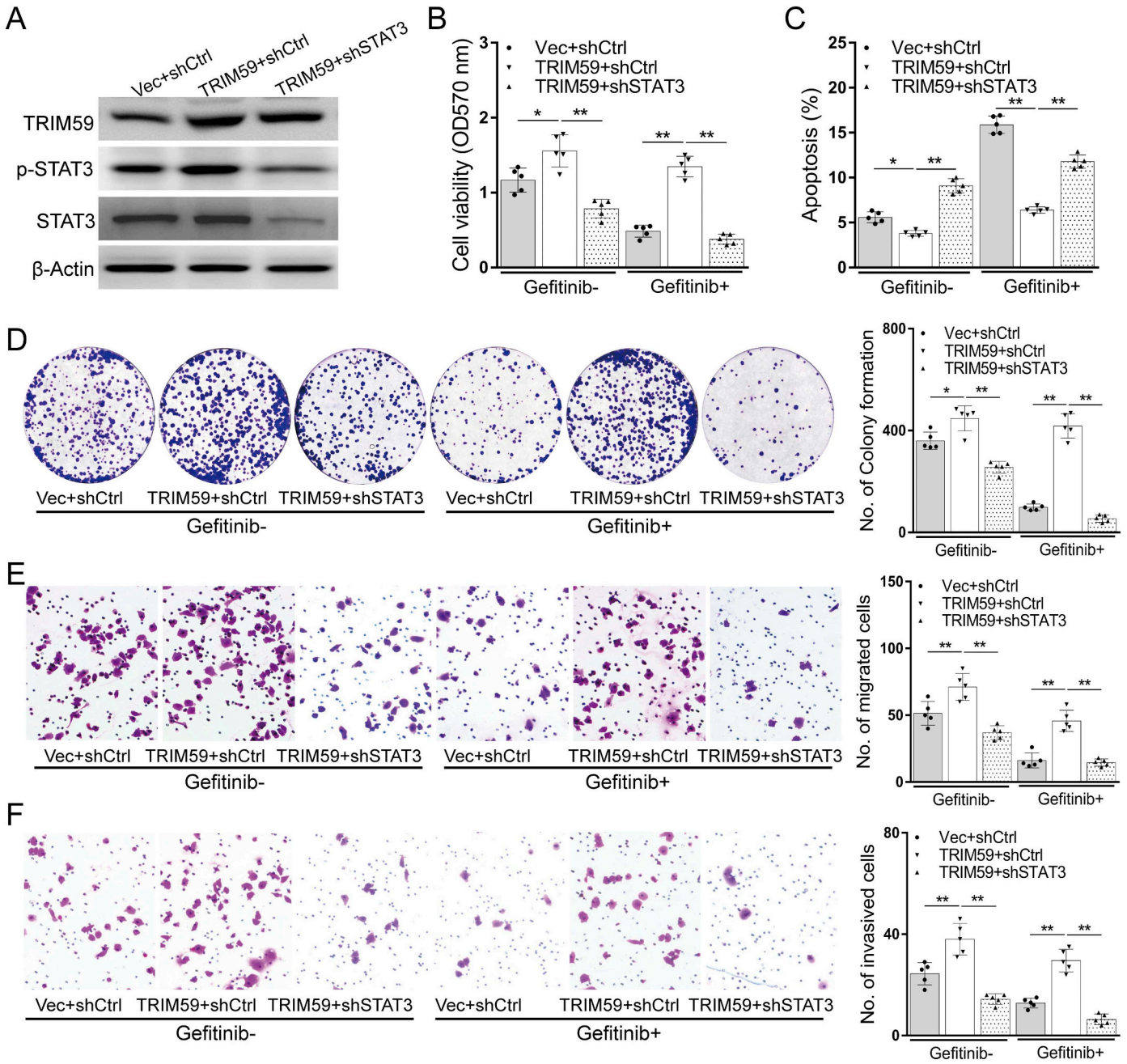

Fig. 6. STAT3 is critical for TRIM59-mediated cancer progression. TRIM59 was over-expressed and STAT3 was silenced either in combination or alone in HCC827/GR cells (A). Cell viability assay of (B) HCC827/GR cells were measured at 96 h by MTT. Cell apoptosis assay of (C) HCC827/GR cells were measured by flow cytometry analysis after cells were treated with gefitinib for 48 h. (D) HCC827/GR cells were treated with gefitinib for two weeks and subjected to cell colony formation assay. HCC827/GR cells were subjected to transwell (E) migration and (F) invasion assay. Gefitinib + and Gefitinib- represent the culture media with and without 5 μM gefitinib, respectively. Data were presented as mean ± SD of three independent experiments. For all panels: $^{*}P < 0.05$; $^{**}P < 0.01$.

action with standard chemotherapeutic 5-Fluorouracil in gastric cancer cells [29]. Disruption of STAT3 by niclosamide reversed radiation resistance of human lung cancer [30]. In support of our findings, Wu et al. showed that STAT3-mediated Akt activation resulted in gefitinib resistance in lung cancer cells [31]. Dysregulated STAT3 signaling mechanistically contributed to gefitinib resistance and might serve as a potential target for therapeutic exploitations.

5. Conclusion

In summary, in this study we have characterized aberrant over-expression of TRIM59 in gefitinib-resistant EGFR mutant lung adenocarcinoma cells, which directly activates STAT3 signaling, contributing to drug resistance. Our data indicate the potential involvement of TRIM59-STAT3 axis in the occurrence of gefitinib-resistance, which could serve as a prognostic marker and/or therapeutic target.

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Conflict of interests

The authors declare that they have no conflict of interest.

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