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Published in:
Cancer Letters

DOI:
10.1016/j.canlet.2017.02.029

Publication date:
2017

Document Version
Peer reviewed version

DDX17 nucleocytoplasmic shuttling promotes acquired gefitinib resistance in non-small cell lung cancer cells via activation of β-catenin

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Running title: DDX17 nucleocytoplasmic shuttling contributes to gefitinib resistance

Keywords: Non-small cell lung cancer, DDX17, Chemoresistance, Nucleocytoplasmic shuttle, β-catenin

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Abstract

Although epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are effective for non-small cell lung cancer (NSCLC) patients with EGFR mutations, almost all these patients will eventually develop acquired resistance to EGFR-TKI. However, the molecular mechanisms responsible for gefitinib resistance remain still not fully understood. Here, we report that elevated DDX17 levels are observed in gefitinib-resistant NSCLC cells than gefitinib-sensitive cells. Upregulation of DDX17 enhances the gefitinib resistance, whereas DDX17-silenced cells partially restore gefitinib sensitivity. Mechanistically, we demonstrate that DDX17 disassociates the E-cadherin/β-catenin complex, resulting in β-catenin nuclear translocation and subsequently augmenting the transcription of β-catenin target genes. Moreover, we identify two nuclear localization signal (NLS) and four nuclear export signal (NES) sequences mediated DDX17 nucleocytoplasmic shuttling via an exportin/importin-dependent pathways. Interruption of dynamic nucleocytoplasmic shuttling of DDX17 impairs DDX17-mediating the activation of β-catenin and acquired resistance in NSCLC cells. In conclusion, our findings reveal a novel and important mechanism by which DDX17 contributes to acquired gefitinib resistance through exportin/importin-dependent cytoplasmic shuttling and followed by activation of β-catenin, and DDX17 inhibition may be a promising strategy to overcome acquired resistance of gefitinib in NSCLC patients.

1. Introduction

Lung cancer is one of the mostly common malignancy and the leading cause of cancer-related deaths worldwide, with a five-year overall survival rate of only 15% [1]. Patients with non-small cell lung cancer (NSCLC), which accounts for approximately 80% of all lung cancer cases, are often diagnosed at advanced stages of the disease, leading to poor prognosis in lung cancer patients [2, 3]. Recent studies have indicated that the epidermal growth factor receptor (EGFR) signalling is frequently overexpressed or aberrantly activated in NSCLC and has been as an attractive target for cancer therapy [4, 5]. Somatic mutations including in-frame deletion mutation in exon 19 and the L858R mutation in exon 21 of the EGFR gene, are associated with favorable response to the EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib [6-8]. Despite good initial responses to EGFR-TKIs, most lung adenocarcinoma patients eventually develop resistance to anti-EGFR agents within 12 months through development of a secondary mutation in EGFR that reduces its binding affinity for TKIs or constitutive activation of downstream molecules to raise the compensatory survival signals [9, 10]. Overexpression or constitutive phosphorylation of HER3 can lead to significant resistance to EGFR-TKIs by activation of downstream PI3K/AKT pathways, which is independent of EGFR kinase activity [11, 12]. Moreover, abnormal activation of c-Met was significantly associated with poor response to EGFR-TKIs treatment, regardless of the EGFR status in NSCLC patients [13, 14]. A randomized phase II trial has demonstrated that dual inhibition of EGFR and c-Met can overcome resistance of EGFR-TKIs and improve outcomes in the MET-positive NSCLC patients [15, 16]. However, the mechanisms responsible for intrinsic resistance and other acquired resistance to EGFR-TKI are not fully understood.

DEAD box helicase 17 (DDX17) belongs to the DEAD box family of RNA helicases and is a transcriptional co-regulator required for the action of diverse transcription factors that are critical for normal biologic processes as well as cancer development [17]. DDX17 coactivates oestrogen receptor alpha (ERα) and is required for oestrogen-dependent expression of ERα-responsive genes and breast cancer cell growth [18]. Furthermore, DDX17 dysregulation is associated with the tumorigenesis of meibomian cell carcinoma [19]. In mouse mammary tumor cells, DDX17 regulates the alternative splicing of the chromatin-binding factor macroH2A1 histone gene, leading to transcriptional alterations to a set of genes involved in redox metabolism [20]. Additionally, DDX17 subunit in the mouse Drosha complex is indispensable for survival in mice and is required for primary miRNA and rRNA processing [21]. However, the role of DDX17 in the susceptibility to EGFR-TKIs in NSCLC cells remains unknown.

In the present study, we provided the first evidence that increased expression of DDX17 in gefitinib-resistant NSCLC cells than gefitinib-sensitive NSCLC cells. DDX17 downregulation promotes the apoptosis of gefitinib-resistant NSCLC cells exposed to gefitinib, whereas DDX17 overexpression protects gefitinib-sensitive NSCLC cells against this effect. Mechanistically, we demonstrate that DDX17 interacts with cytoplasmic β-catenin, facilitates the dissociation of β-catenin from the E-cadherin/β-catenin complex, enhances β-catenin nuclear accumulation, subsequently augments the transcription of β-catenin target genes, and ultimately leads to acquired resistance to gefitinib. Moreover, we found that DDX17 was a nucleocytoplasmic shuttling protein that was mediated by two NLS and four NES sequence elements. Interrupting DDX17 nucleocytoplasmic shuttling impairs DDX17-mediated activation of β-catenin and resistance to gefitinib in NSCLC cells. Taken together, our study highlights the significance of

DDX17 in gefitinib-resistant NSCLC and implicates DDX17 as a potential therapeutic target to enhance the efficacy of gefitinib in NSCLC patients.

2. Materials and Methods

2.1 *Cell culture and establishment of gefitinib resistant cell lines*

A431 and A431-GR cell lines were gifts from Prof. Zeng Cai (Sichuan Provincial People's Hospital, China). A549 and A549-GR cell lines were gifts from Prof. Feng Bi (Sichuan University, China). HCC827 and PC9 NSCLC cell lines were maintained at 37°C and 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco) and 100 units/ml penicillin, and 100 mg/ml streptomycin. To establish gefitinib resistant cell lines, parental cells were exposed to gradually elevated concentrations of gefitinib for two months as reported previously [13, 22].

2.2 *Reagents*

Gefitinib and XAV-939 were purchased from Selleck chemicals, and recombinant human EGF was purchased from PeproTech. The primary antibodies included AKT (Cell signalling Technology, #4691), p-AKT (Ser473) (Cell signalling Technology, #4060), cleaved caspase-3 (Cell Signaling Technology, #9664), cleaved PARP (Cell signalling Technology, #5625), DDX17 (Santa Cruz Biotechnology, sc-271112), E-cadherin (Abcam, ab1416), **EGFR (Cell signalling Technology, #4267), p-EGFR (Tyr1068) (Cell signalling Technology, #3777),** ERK (Cell signalling Technology, #9102), p-ERK (Thr202/Tyr204) (Cell signalling Technology, #4370), Flag (Cell signalling Technology, #8146), Ki67 (Abcam, ab15580), KPNA1 (Sangon Biotech, D154120), KPNB1 (Sangon Biotech, D161792), PCNA (Santa Cruz Biotechnology, sc-56), XPO1 (Sangon

Biotech, D221884), α-Tubulin (Santa Cruz Biotechnology, sc-5286), β-actin (Santa Cruz Biotechnology, sc-47778), β-catenin (Abcam, ab32572), **p-β-Catenin (Ser33/37/Thr41) (Cell signalling Technology, #9561)**.

2.3 *Stable cell line generation for DDX17 knockdown or overexpression*

The DDX17 lentiviral expression vector was constructed by inserting expanded DDX17 cDNA (NM_006386.4) fragments into a lentiviral shuttle vector. DDX17 knockdown was accomplished using a specific shRNA targeting DDX17. The shRNA sequences were as follows: shRNA-DDX17, 5'-CAA GGG UAC CGC CUA UAC C-3'; shRNA-NC, 5'-TTC TCC GAA CGT GTC AGG T-3'. The packing and purification of the recombinant lentiviral vector were performed by the GenePharma Company (Shanghai, China). The indicated NSCLC cells infected with the recombinant lentiviral vectors were selected with puromycin for 2 weeks.

2.4 *RNA extraction and real-time PCR*

Total RNA was extracted using RNAiso Plus (TAKARA) according to the manufacturer's instructions. Real-time PCR was performed with SYBR® Premix Ex Taq™ II (TAKARA) using an iCycler iQ™ Multicolor Real-Time Detection System (BIO-RAD) as previously described [23, 24]. The following primers were used: DDX17 forward 5'-GAACATCCGGAAGTAGCAAGG-3', reverse 5'-GATCCATCAACACATCCATTACATAT-3'; GAPDH forward 5'-ACCACAGTCCATGCCATCAC-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'. The relative expression levels were determined using Gene Expression Macro Version 1.1 software (BIO-RAD).

2.5 *TOP/FOP flash assay*

For cotransfection, the indicated NSCLC cells were transfected with TOPflash plasmid plus pRL-TK plasmid or FOPflash plasmid plus pRL-TK plasmid in 48-well plates. Twenty-four hours post-transfection, the cells were rinsed twice with PBS and then lysed in Passive Lysis Buffer, and the dual-luciferase reporter assay was performed according to the manufacturer's instructions (Promega) using a Multi-Mode Microplate Reader (Synergy 2, BioTek).

2.6 *In vivo assays for tumor growth*

Female BALB/c nude mice (6-week-old) were raised in specific pathogen-free conditions. Animal care and experimental protocols were in accordance with guidelines established by the Institutional Animal Care and Use Committee of Sichuan University. The indicated NSCLC cells were suspended in 150 μL serum-free DMEM and implanted subcutaneously into the right flanks of nude mice. When subcutaneous tumor reached approximately 100 mm^3, the tumor-bearing mice were treated with gefitinib (10 mg/kg/day, by a gavage) for 15 days. The tumor volumes were measured by calipers every 3 days, and calculated using the following formula: tumor volume (mm^3) = $\pi/6 \times \text{length} \times \text{width}^2$.

2.7 *Statistical analysis*

All the experiments were performed at least three times independently, and all data are expressed as "mean ± SD". A one-way ANOVA test was used to analyse quantitative data between groups. The data were analyzed using SPSS statistical software version 22.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1 *Upregulation of DDX17 correlates with gefitinib resistance in NSCLC cells*

To explore the role of DDX17 in the acquired resistance to gefitinib, we first evaluated the expression of DDX17 in different NSCLC cell lines. As shown in Figure 1A, the level of DDX17 was markedly higher in gefitinib-insensitive NSCLC cell lines (PC9-GR and HCC827-GR) than that in gefitinib-sensitive NSCLC cell lines (PC9 and HCC827), respectively. **A similar pattern of increased DDX17 expression level was observed in A549-GR and A431-GR cells compared with their parental A549 and A431 cells (Supplementary Figure S1).** These data suggest that DDX17 may be positively correlated with gefitinib resistance in NSCLC cells. Next, we engineered stable upregulation of DDX17 expression in gefitinib-sensitive PC9 and HCC827 cells, and stable shRNA-mediated knockdown of DDX17 in gefitinib-insensitive PC9-GR and HCC827-GR cells (Figure 1B and **Supplementary Figure S2A**). Enforced DDX17 expression significantly increased the cell viability of PC9 and HCC827 cells in response to gefitinib (Figure 1C), whereas DDX17-silenced cells partially restored gefitinib sensitivity of PC9-GR and HCC827-GR cells **(Supplementary Figure S2B)**. Moreover, upregulated DDX17 enhanced the resistance to gefitinib in PC9 and HCC827 cells compared to negative controlled cells (Figure 1D). Consistent with the MTT assay results, the colony formation in DDX17 overexpression NSCLC cells was significantly more compared to Mock cells (Figure 1E). The converse results were observed in DDX17-deficient NSCLC cells **(Supplementary Figure S2C)**. **Gefitinib treatment reduced p-EGFR and downstream signaling proteins p-Akt and p-ERK expressions, and meanwhile increased the levels of two apoptosis markers, cleaved caspase 3 and cleaved PARP**

(Figure 1F). However, the upregulation of DDX17 partially overcame the gefitinib-inhibited EGFR, AKT and ERK activation, and suppressed cell apoptosis (Figure 1F). Conversely, knockdown of DDX17 caused decreased phosphorylation of EGFR, AKT and ERK, while increased expression of cleaved caspase 3 and cleaved PARP in gefitinib-resistant NSCLC cells treated with gefitinib (Supplementary Figure S2D). Taken together, our data indicate that DDX17 contributes to the development of acquired drug resistance to gefitinib in NSCLC cells.

3.2 *DDX17 disassociates the E-cadherin/β-catenin complex and promotes β-catenin nuclear translocation*

Recent evidence indicates that constitutive activation of Wnt/β-catenin signalling is associated with the acquired drug resistance to EGFR-TKIs in NSCLC [25-27]. To understand the molecular mechanism by which DDX17 promotes gefitinib resistance, we explored the effect of DDX17 on the nuclear translocation and activation of β-catenin. **As shown in Figure 2A, β-catenin was located primarily in the plasma membrane in Mock PC9 cells; however, upregulated DDX17 led to the nuclear accumulation of β-catenin. Next, we evaluated the phosphorylation status of β-catenin. Consistently, phosphorylation of the residues that target β-catenin for proteasomal degradation (S33/S37/T41) was reduced in response to DDX17 overexpression (Figure 2B).** Conversely, DDX17 knockdown significantly inhibited the levels of nuclear β-catenin in PC9-GR cells **(Figure 2C).** EGF, a potent activator of Wnt/β-catenin signalling as described previously [28-30], promotes β-catenin nuclear translocation in PC9-GR cells, however, DDX17 deletion markedly repressed the increased nuclear accumulation of β-catenin induced by EGF **(Figure 2C).** TOP/FOP-Flash assay showed that down-regulation of

DDX17 impaired the transcriptional activity of β-catenin/T-cell factor (TCF) complex regardless of EGF treatment **(Figure 2D). Moreover, we found increased nuclear localization of β-catenin as well as decreased p-β-catenin (S33/S37/T41) in PC9-GR cells compared with PC9 cells (Supplementary Figure S3).** Beta-catenin bound to the E-cadherin/catenin adhesion complex is mainly localizes to cell-cell adherent junctions at membranes lacking Wnt signalling, and Wnt signalling promotes the disassociation of E-cadherin/β-catenin complex and subsequently β-catenin nuclear translocation. Therefore, we next assessed whether DDX17 influenced E-cadherin/β-catenin complex stability. As shown in **Figure 2E**, overexpression of DDX17 downregulated E-cadherin/β-catenin complex formation, whereas knockdown of DDX17 augmented the association of β-catenin and E-cadherin. Considering the role of DDX17 in the nuclear accumulation and activation of the β-catenin, we next explored whether DDX17 can interact with β-catenin. Reciprocal immunoprecipitation studies revealed that endogenous DDX17 bound to endogenous β-catenin directly **(Figure 2F).** Moreover, increased interaction between DDX17 and β-catenin was observed in response to EGF stimulus, whereas EGF repressed β-catenin binding to E-cadherin **(Figure 2G).** Notably, we found that DDX17 predominantly interacted with β-catenin in the cytoplasm and that EGF enhanced both the cytoplasmic and nuclear interaction of β-catenin with DDX17 in a time-dependent manner **(Figure 2H). To further investigate whether DDX17-regulated gefitinib resistance involves β-catenin activation, a specific Wnt/β-catenin signalling pathway inhibitor (XAV-939) was used. XAV-939 treatment effectively reversed DDX17-induced gefitinib resistance in PC9 and HCC827 cells (Figure 2I), leading to the increased levels of cleaved caspase 3 and cleaved PARP (Figure 2J).** These data indicate that elevated DDX17 level leads to release and nuclear translocation of

β-catenin from the E-cadherin/β-catenin complex and thereby resulting in the activation of Wnt/β-catenin signalling and acquired resistance to gefitinib.

3.3 *Two NLSs mediated DDX17 nuclear transport by an importin-dependent pathway*

Because DDX17 interacted with β-catenin in the cytoplasm and nucleus, we presumed that DDX17 might be a nucleocytoplasmic shuttling protein. To test this hypothesis, we first examined the sublocalization of DDX17 in PC9 and PC9-GR cells using an immunofluorescence assay. Interestingly, DDX17 was predominantly localized to the nucleus in PC9 cells, whereas DDX17 was present in the cytoplasm and nucleus in PC9-GR cells (Supplementary Figure S2A). Immunoblotting analysis also showed that more DDX17 was accumulated in both the cytoplasmic and nuclear fractions of PC9-GR cells than of PC9 cells (Supplementary Figure S2B). These results indicate that DDX17 may be a nucleocytoplasm shuttling protein.

Most nucleocytoplasm shuttling proteins carry sequence elements of both nuclear localization signal (NLS) and (nuclear exporting signal) NES. We analysed the DDX17 amino acid sequence and identified two putative NLSs based on cNLS Mapper analysis (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (Figure 3A). Because the classical NLSs rich in basic amino acids are known as NLSs recognized by importin, we generated mutations in NLS1 (K50A, K53A, R74A, R75A, K76A and K77A) or NLS2 (K349A, R350A and R351A) of DDX17 fused with a GFP fluorescent protein. As shown in Figure 3B, the DDX17-WT protein showed both cytoplasmic and nuclear fluorescence. However, the NLS 1 mutant showed strong cytoplasmic fluorescence, and the NLS 2 mutant exhibited a complex distribution in both the nucleus and cytoplasm (Figure 3B). Moreover, no significant nuclear

localization of DDX17 was observed in NLS mutant (NLS M, mutated both NLS 1 and NLS 2) (Figure 3B). The similar result was also confirmed by immunoblot analyses (Figure 3C). As the nuclear transport of most nucleocytoplasmic proteins is mediated by importin complex which formed a hetero-dimer, we next explored whether DDX17 interacts with importin. Reciprocal immunoprecipitation studies revealed that endogenous DDX17 co-precipitated with endogenous KPNA1 (also known as Importin subunit alpha-5) and KPNB1 (also known as Importin subunit beta-1) (Figure 3D). However, DDX17 NLS mutant did not co-precipitate with KPNA1 and KPNB1 (Figure 3E). To further determine whether importin signalling mediated DDX17 nuclear localization, we treated cells with Ivermectin, which is a potent inhibitor of importin α/β-dependent transport [31, 32]. As shown in Figure 3F, ivermectin treatment caused the main cytoplasmic fluorescence of GFP-DDX17 fusion protein. Similarly, immunoblot analysis also showed the decreased nuclear accumulation of DDX17 in the presence of ivermectin (Figure 3G). Notably, ivermectin blocked the association of DDX17 with importin complex (Figure 3H). These data indicate that two NLSs of DDX17 recognized by importin complex mediated DDX17 nuclear transport.

3.4 *Four NESs mediated DDX17 nuclear export by an exportin-dependent pathway*

For the classical nuclear export pathway, XPO1 binds directly hydrophobic residue-rich NES sequence in the cargo protein and directs the export of the complex from the nucleus [33]. According to this theory, we found four putative NESs in DDX17 and constructed a series of site directed mutagenesis fused to the C-terminus of GFP (Figure 4A). As shown in Figure 4B, both cytoplasmic and nuclear localizations of DDX17-WT protein were observed in PC9 cells. The NES

1 mutant (L144A, L147A, L148A, I151A, V152A and I154A), NES 2 mutant (L226A, I227A, F229A, L230A, L237A, L243A, V244A, L245A) and NES 3 mutant (V284A, L284A, L292A, Y295A, I298A, L303A, L305A) showed major nuclear localization, whereas mutations on NES 4 (L448A, L454A, I455A, V457A, L458A) localized in dispersed subnuclear speckles (Figure 4B). In addition, a complete NES mutant (mutated all four NESs) resulted in exclusive nuclear localization of DDX17, suggesting that these four NESs function as DDX17 nuclear export signals (Figure 4B). Immunoblot analysis also confirmed the subcellular localization of DDX17-WT and DDX17 NES mutant (Figure 4C). To confirm the effects of XPO1 on export of DDX17, we probed the interaction between DDX17 and XPO1 via co-immunoprecipitation. It was clear that endogenous DDX17 co-immunoprecipitated with endogenous XPO1 (Figure 4D). In addition, we found that the DDX17 NES mutant impaired the binding of DDX17 and XPO1 (Figure 4E). To further verify that the DDX17 cytoplasmic shuttling is mediated by XPO1, PC9 cells were treated with leptomycin B (LMB), a potent and specific nuclear export inhibitor. LMB effectively suppressed the level of cytoplasmic DDX17 in PC9 cells (Figures 4F and 4G). Moreover, LMB treatment significantly disrupted the interaction between DDX17 and XPO1 (Figure 4H). These observations suggest that four NESs are required for DDX17 cytoplasmic shuttling mediated by the classical exportin-dependent pathway.

3.5 *The integrity of DDX17 nucleocytoplasmic shuttling is indispensable for mediating the acquired resistance and activation of β-catenin*

We next evaluated the impact of DDX17 nucleocytoplasmic shuttling on cellular resistance to gefitinib. As shown in Figure 5A, ectopic expression of DDX17-WT significantly increased the

289 cell viability of PC9 and HCC827 cells upon gefitinib treatment. The decreased resistances to
290 gefitinib were observed in DDX17-NLS mutant and DDX17-NES mutant PC9 cells compared to
291 DDX17-WT PC9 cells, respectively (Figure 5A). Interestingly, DDX17-NLS mutant PC9 cells
292 showed a similar sensitivity as mock PC9 cells, whereas DDX17-NES mutant PC9 cells were
293 more resistant to gefitinib than mock PC9 cells (Figure 5A). Consistently, less colony formations
294 were found in DDX17-NLS mutant and DDX17-NES mutant PC9 cells than DDX17-WT PC9 cells
295 (Figure 5B). The immunoblotting test showed that cleaved caspase 3 and cleaved PARP were
296 significantly enhanced in both DDX17-NLS mutant and DDX17-NES mutant PC9 cells compared
297 to DDX17-WT PC9 cells (Figure 5C). To further determine the effect of DDX17 nucleocytoplasmic
298 shuttling on the gifitinib resistance *in vivo*, indicated PC9 cells were injected subcutaneously into
299 the flanks of BALB/c nude mice. Overexpression of DDX17-WT exerted obvious gefitinib
300 insensitivity in tumor xenografts model compared to mock xenografts in the present of gefitinib (10
301 mg/kg per day, gavaged orally) (Figure 5D). However, DDX17-NLS mutant and DDX17-NES
302 mutant xenografts was partially rescued gefitinib sensitivity compared with DDX17-WT xenografts
303 (Figure 5D). The primary tumors from the DDX17-WT xenografts exhibited increased expression
304 of Ki67 while decreased expression of cleaved caspase 3 compared with tumors originating from
305 DDX17-NLS mutant and DDX17-NES mutant xenografts (Figure 5E).

306 Based on the aforementioned observations, we became interested in exploring the effect of
307 DDX17 nucleocytoplasmic shuttling on the activation of β-catenin. As shown in Figure 6A, DDX17
308 NLS mutant showed a comparable association of β-catenin, whereas DDX17 NES mutant
309 showed a slightly decreased association of β-catenin compared with DDX17 WT. DDX17 WT and
310 DDX17 NLS mutant caused significant disassociation of E-cadherin/β-catenin complex, however,

DDX17 NES mutant displayed modest inhibition of the interaction between β-catenin and E-cadherin compared to control (Figure 6B). Moreover, less nuclear β-catenin was observed in the DDX17 NLS mutant and NES mutant group compared to that of the wild type (Figure 6C). In addition, DDX17 NLS mutant and NES mutant groups showed significantly decreased luciferase activity compared to wild type (Figure 6D). Overall, the above experiments suggest that interruption of DDX17 nucleocytoplasmic shuttling impairs DDX17-mediating the acquired resistance and activation of β-catenin in NSCLC cells.

4. Discussion

EGFR-mutant NSCLC patients who benefited from EGFR-TKI eventually develop acquire resistance to these therapies and the median duration of response is about 10 to 14 months [34, 35]. Although accumulating studies revealed that a variety of mechanisms can stimulate acquired resistance to EGFR-TKI including secondary mutations within EGFR at position T790, activation of parallel receptor tyrosine kinases (such as ALK, MET and RET), and mutation or upregulation of EGFR effector proteins [36-38], the mechanisms responsible for acquired resistance to EGFR-TKIs are still large unknown. **In this study, we showed that DDX17 levels were increased in gefitinib resistant cells compared with gefitinib sensitive cells.** Overexpression of DDX17 significantly increased tolerance of PC9 and HCC827 cells in the present of gefitinib, whereas DDX17 suppression resulted in reduced cell viability of gefitinib-resistant PC9-GR and HCC827-GR cells. These data revealed that DDX17 expression was associated with tumor sensitivity to gefitinib in NSCLC cells.

The Wnt/β-catenin signaling is one of the most critical signaling transduction pathways

during embryonic development and the stemness maintenance, and has become a hot topic in tumor research [39, 40]. In recent years, accumulating evidence reported that Wnt/β-catenin signalling has been implicated in the chemoresistance of varied cancers [41, 42]. Here, we demonstrated that DDX17 directly bound and dissociated the E-cadherin/β-catenin complex to release β-catenin, subsequently leading to β-catenin nuclear accumulation. Moreover, we found that EGF augmented the interaction between β-catenin and DDX17 both in the cytoplasm and nucleus, whereas DDX17 repression abolished the EGF-induced nuclear translocation and activation of β-catenin. Therefore, we postulated that DDX17-dependent nuclear accumulation of β-catenin released from the E-cadherin/β-catenin adhesion complex was an important mechanism driving acquired resistance to gefitinib in NSCLC cells.

To enhance cytoplasmic β-catenin nuclear translocation, DDX17, which is predominantly localized in the nucleus [43], must shuttle to the cytoplasm. Interestingly, increased DDX17 levels were observed in both the cytoplasmic and nuclear fractions of PC9-GR cells than of PC9 cells. Moreover, DDX17 interacted with β-catenin both in the cytoplasm and nucleus. These data supported DDX17 as a nucleocytoplasmic protein. Most nucleocytoplasmic protein movement through the nuclear pore complex is mediated by a nuclear receptor system [44, 45]. In this study, we identified two NLSs and four NESs required for DDX17 nucleocytoplasmic shuttling. Mutation of the NLSs significantly inhibited the DDX17 nuclear localization and association of DDX17 and import complex. Analogously, NESs mutant caused nuclear accumulation of DDX17 and disrupted the interaction between DDX17 and XPO1. These results indicated that the nucleocytoplasmic shuttling of DDX17 followed a classical exportin/importin-dependent pathway. Interestingly, several DEAD box RNA helicases shuttle between the nucleus and cytoplasm via

XPO1-dependent nuclear export pathway, including DDX3, DDX25, and DDX48 [46-48]. Furthermore, block of DDX17 nucleocytoplasmic shuttling significantly reduced DDX17-mediated activation of β-catenin and gefitinib sensitivity in NSCLC cells, suggesting that dynamic nucleocytoplasmic shuttling of DDX17 is essential for its function. Interestingly, a decreased tolerance was observed in DDX17-NES mutant cells compared with DDX17-WT cells, however, DDX17-NES mutant cells were more resistant to gefitinib than mock PC9 cells. Because DDX17 can act as co-transcriptional regulator, one possible explanation is that nuclear DDX17 regulated the transcription of target genes that lead to the activation of Wnt/β-catenin signalling independent cytoplasmic function of DDX17. Although DDX17-NLS mutant has no significant effect on the association of DDX17 and β-catenin, the nuclear accumulation of β-catenin was depressed in DDX17-NLS mutant cells compared with DDX17-WT cells. Considering the main interaction of DDX17 and β-catenin in the cytoplasm, we presume that DDX17 might function as a ‘chaperone’ to aid β-catenin nuclear import; however, the detailed mechanism requires further exploration.

In summary, our study provides the first evidence that upregulated DDX17 expression is associated with gefitinib resistance in NSCLC cells and DDX17 is a nucleocytoplasmic protein mediated by two NLSs and Four NESs. We demonstrate a new molecular mechanism by which the exportin/importin-dependent nucleocytoplasmic translocation of DDX17 disassociates the E-cadherin/β-catenin complex, resulting in β-catenin nuclear translocation and subsequently augmenting the transcription of β-catenin target genes, ultimately driving gefitinib resistance in NSCLC cells. Interruption of dynamic nucleocytoplasmic shuttling of DDX17 impairs DDX17-mediating the activation of β-catenin and acquired resistance in NSCLC cells. In conclusion, we propose that DDX17 is an attractive and potential target for overcoming gefitinib

resistance in NSCLC therapy

5. Acknowledgements

This project was supported by the National Natural Science Foundation of China (81401979, 81402944 and 81572604); the Science and Technology Department of Sichuan Province Foundation (2014SZ0020); and the China Postdoctoral Science Foundation (2014M552367).

6. Conflict of Interest:

The authors disclose no potential conflicts of interest.

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8. FIGURE LEGENDS

8.1 Figure 1. Upregulated DDX17 is associated with gefitinib resistance in human NSCLC cells. (A) Quantitative real-time PCR and immunoblot analysis of DDX17 expression in gefitinib sensitive and insensitive NSCLC cells. (B) Quantitative real-time PCR and immunoblot analysis showed the successful lentiviral infections of DDX17 in PC9 and HCC827 cells. (C) Stably expressing DDX17 or mock NSCLC cells were treated with 1 μM gefitinib for indicated time and analyzed for cell viability by MTT assay. (D) Effect of DDX17 overexpression on gefitinib efficacy in HCC827 and PC9 cells was detected by MTT assay. (E) Representative photographs of the colony formation of indicated NSCLC cells treated with gefitinib for 14 days after culture of cells. (F) The indicated NSCLC cells were treated with or without gefitinib and then subjected to immunoblot analysis using the indicated antibodies. Data represent the mean ± standard deviation (SD). Each experiment was performed at least in triplicate, producing consistent results. *$P < 0.05$.

8.2 Figure 2. DDX17 disassociates the E-cadherin/β-catenin complex and promotes β-catenin nuclear translocation. (A) Immunofluorescence analysis of β-catenin (red) in indicated PC9 cells. Merged images represent overlays of β-catenin (red) and nuclear staining by DAPI (blue). (B) Immunoblotting for phosphorylated β-catenin (S33/S37/T41) in indicated PC9 cells. (C) Immunoblotting for β-catenin in the nuclear extracts of indicated PC9-GR cells in the present of EGF. PCNA was used as the control. **(D)** TOP/FOP flash assay in the indicated PC9-GR cells treated with or without EGF. **(E)** The effect of DDX17 on the association of β-catenin with E-cadherin was detected by western blot analysis. **(F)** The

endogenous interaction of DDX17 and β-catenin was detected by immunoprecipitation with indicated antibodies. **(G)** The impact of EGF on the interaction of DDX17 and β-catenin/E-cadherin complex. **(H)** The cytoplasmic and nuclear extracts of PC9-GR cells stimulated with EGF were isolated. Immunoprecipitation were performed with anti-DDX17 antibody. α-Tubulin and PCNA were control. **(I) Effect of XAV-939 on gefitinib efficacy in indicated NSCLC cells was detected by MTT assay. (J) The indicated NSCLC cells were treated with gefitinib in the presence or absence of XAV-939, and then subjected to immunoblot analysis using the indicated antibodies.** Data represent the mean ± SD of three independent experiments. *$P < 0.05$.

8.3 Figure 3. Two NLSs mediated DDX17 nuclear transport by an importin-dependent pathway. (A) Putative sequence segments of DDX17 NLSs. (B) Representative of fluorescent microscopy images show the localizations of the exogenously expressed DDX17 wild type (WT) and putative NLSs mutants fused with GFP in PC9 cells. (C) The levels of exogenously expressed DDX17 WT or NLS mutant in the extracts made from the cytoplasm or the nucleus of PC9 cells were examined by immunoblotting analysis. PCNA and α-Tubulin were used as the control. (D) The endogenous association of DDX17 and importin complex was detected by immunoprecipitation with indicated antibodies. (E) The impact of NLS mutant on the interaction between DDX17 and importin complex. (F) Representative of fluorescent microscopy images show the sub-localizations of DDX17 in PC9 cells treated with or without ivermectin. (G) The effect of Ivermectin on the distribution of DDX17 was performed by western blot analysis in PC9 cells. (H) The effect of Ivermectin on the binding of DDX17 and importin complex. Data represent

the mean ± SD of three independent experiments.

8.4 Figure 4. Four NESs mediated DDX17 nuclear export by an exportin-dependent pathway. (A) Putative sequence segments of DDX17 NESs. (B) Representative of fluorescent microscopy images show the localizations of the exogenously expressed DDX17 WT and putative NESs mutants fused with GFP in PC9 cells. (C) Immunoblotting for DDX17 in PC9 cytoplasmic and nuclear extracts of cells transfected with the indicated plasmids. PCNA and α-Tubulin were used as the control. (D) The endogenous association of DDX17 and XPO1 was detected by immunoprecipitation with indicated antibodies. (E) The impact of NES mutant on the interaction between DDX17 and XPO1. (F) Representative of fluorescent microscopy images show the sub-localizations of DDX17 in PC9 cells treated with or without LMB. (G) The effect of LMB on the distribution of DDX17 was performed by western blot analysis in PC9 cells. (H) The effect of LMB on the binding of DDX17 and XPO1. Data represent the mean ± SD of three independent experiments.

8.5 Figure 5. Interruption of DDX17 nucleocytoplasmic shuttling impairs DDX17-mediated gefitinib resistance *in vitro* and *in vivo*. (A) Indicated NSCLC cells were treated with varied concentration of gefitinib, and the cell viability were analysed by MTT assay. (B) Representative photographs of the colony formation of indicated NSCLC cells treated with gefitinib for 14 days after culture of cells. (C) The indicated NSCLC cells were treated with gefitinib and then subjected to immunoblot analysis using the indicated antibodies. (D) The indicated NSCLC cells were transplanted into nude mice. When subcutaneous tumor reached approximately 100 mm^3, the

tumor-bearing mice were treated with gefitinib (10 mg/kg/day, by a gavage) for 15 days. The tumor volumes were measured by calipers every 3 days. (E) Tumor xenograft tissues were fixed with 4% paraformaldehyde, processed, embedded in paraffin wax and then assessed for immunohistochemical analyses with indicated antibodies. Data represent the mean ± SD of three independent experiments. *$P < 0.05$.

8.6 Figure 6. The integrity of DDX17 nucleocytoplasmic shuttling is essential for DDX17-mediating the activation of β-catenin in NSCLC cells. (A) The impact of NLS mutant and NES mutant on the interaction of DDX17 and β-catenin. (B) The effect of NLS mutant and NES mutant on the association of E-cadherin/β-catenin complex. (C) Immunoblotting for β-catenin in the nuclear extracts of indicated PC9 cells. PCNA was used as the control. (D) TOP/FOP flash assay were performed in the indicated PC9 cells. Data represent the mean ± SD of three independent experiments. *$P < 0.05$.

Figure 1

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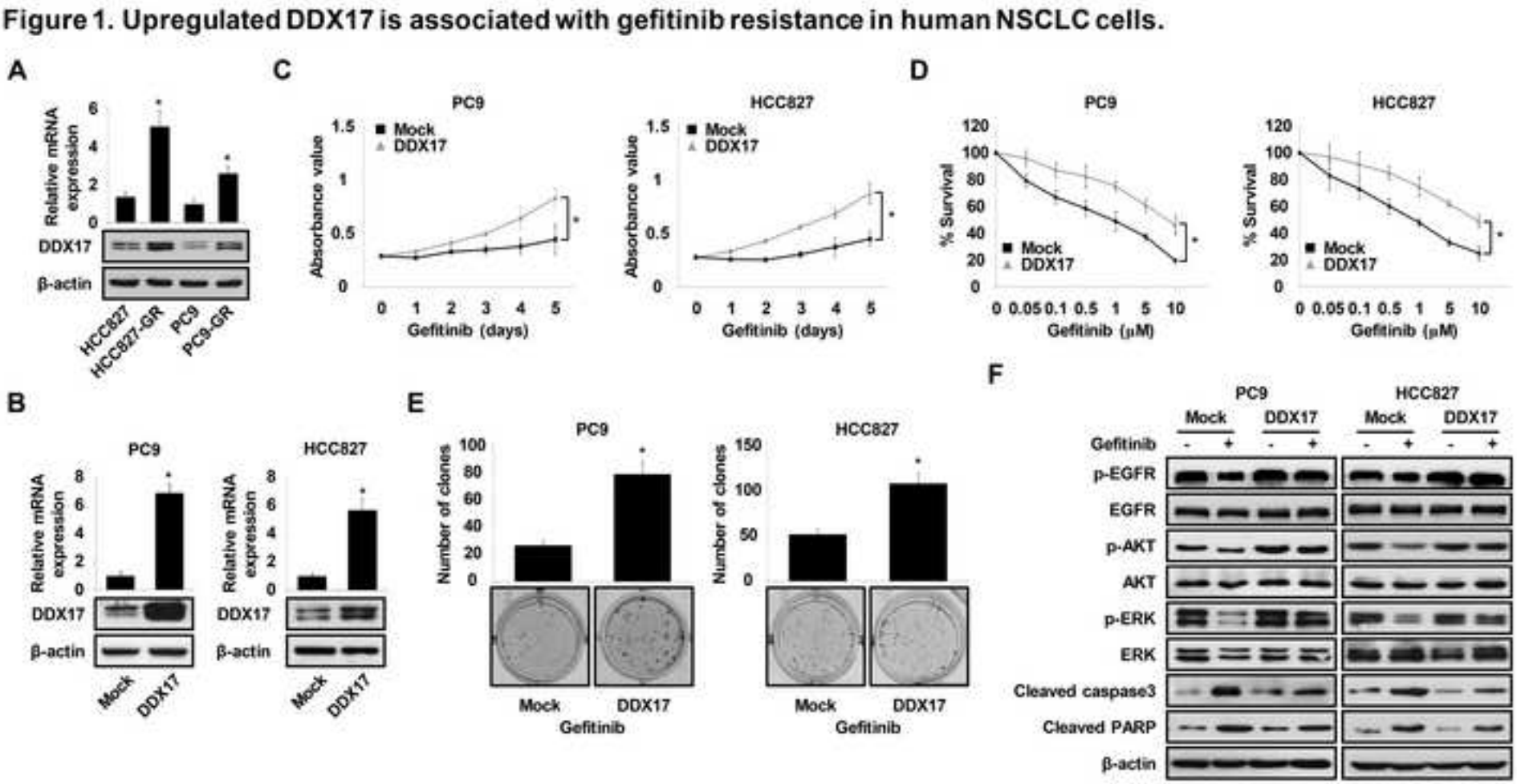

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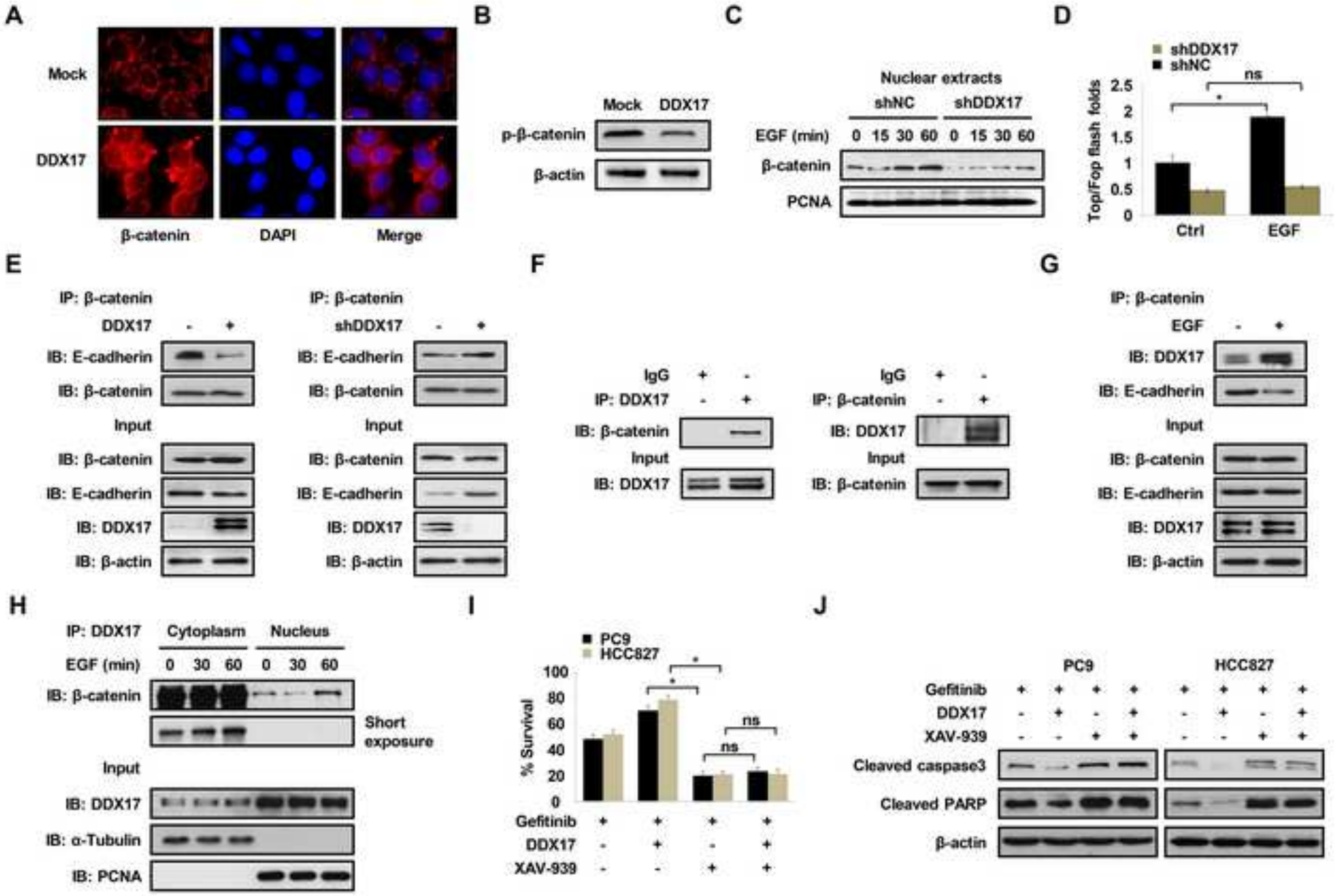

Figure 3

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Figure 3. Two NLSs mediated DDX17 nuclear transport by an importin-dependent pathway

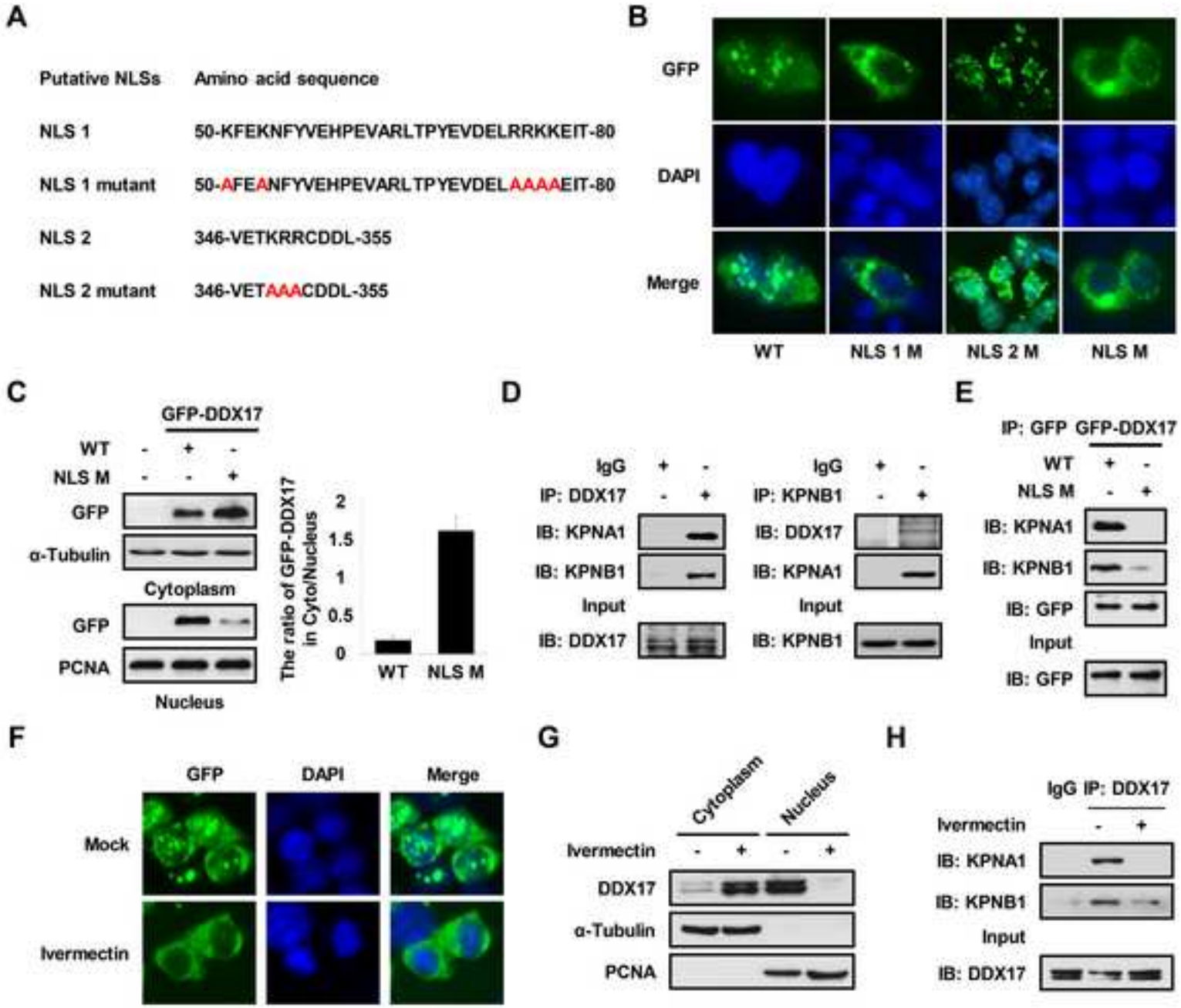

Figure 4

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Figure 4. Four NESs mediated DDX17 nuclear export by an exportin-dependent pathway.

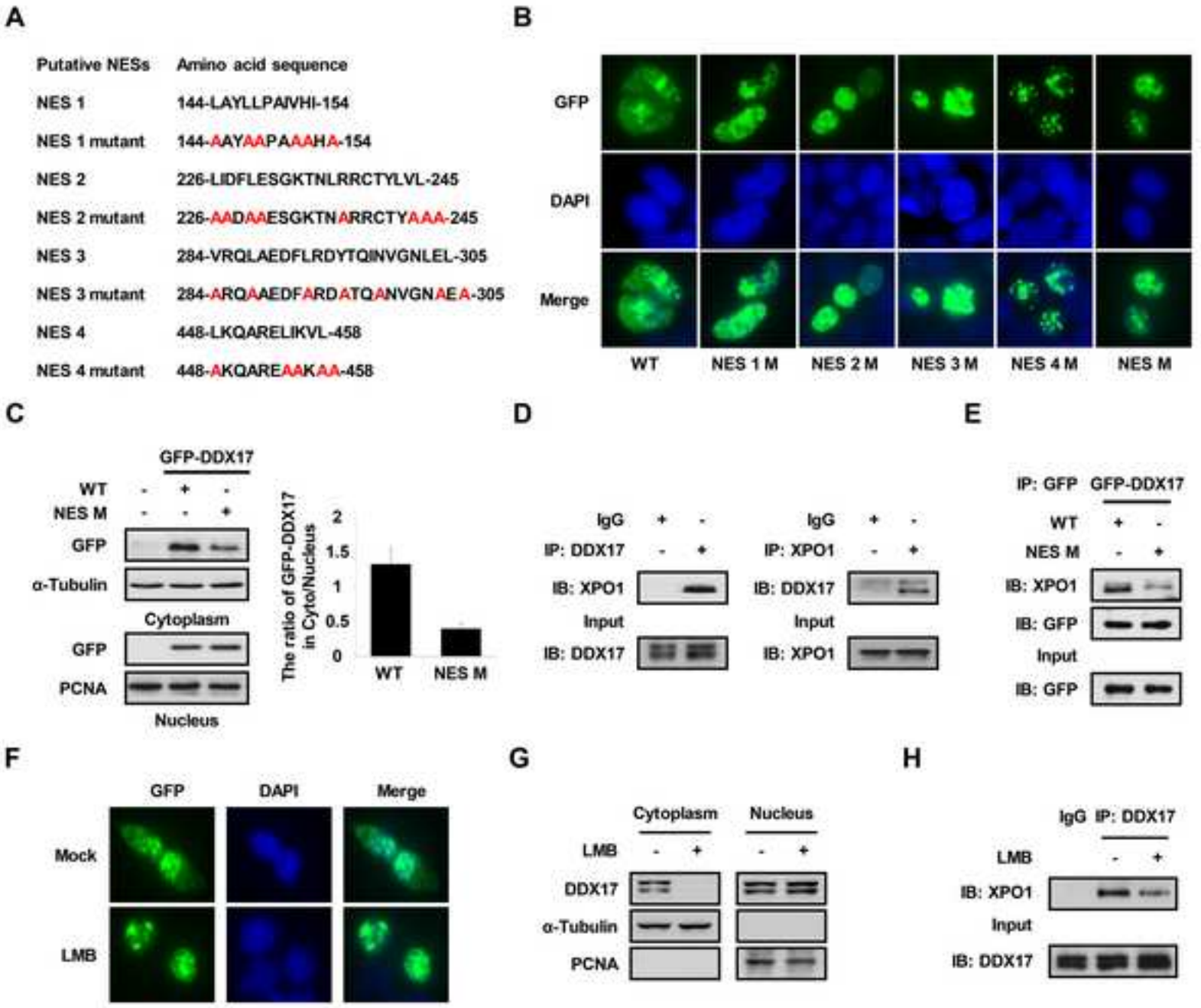

Figure 5
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Figure 5. Interruption of DDX17 nucleocytoplasmic shuttling impairs DDX17-mediated gefitinib resistance *in vitro* and *in vivo*.

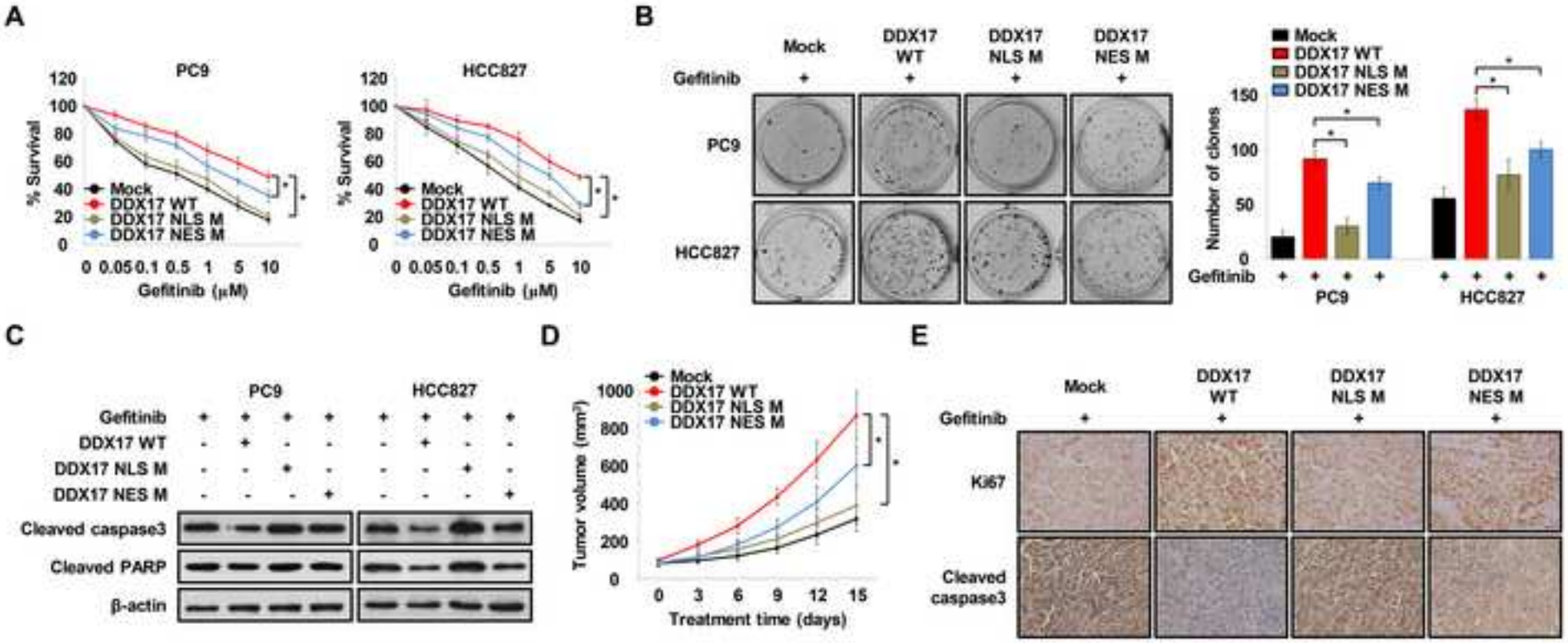

Figure 6
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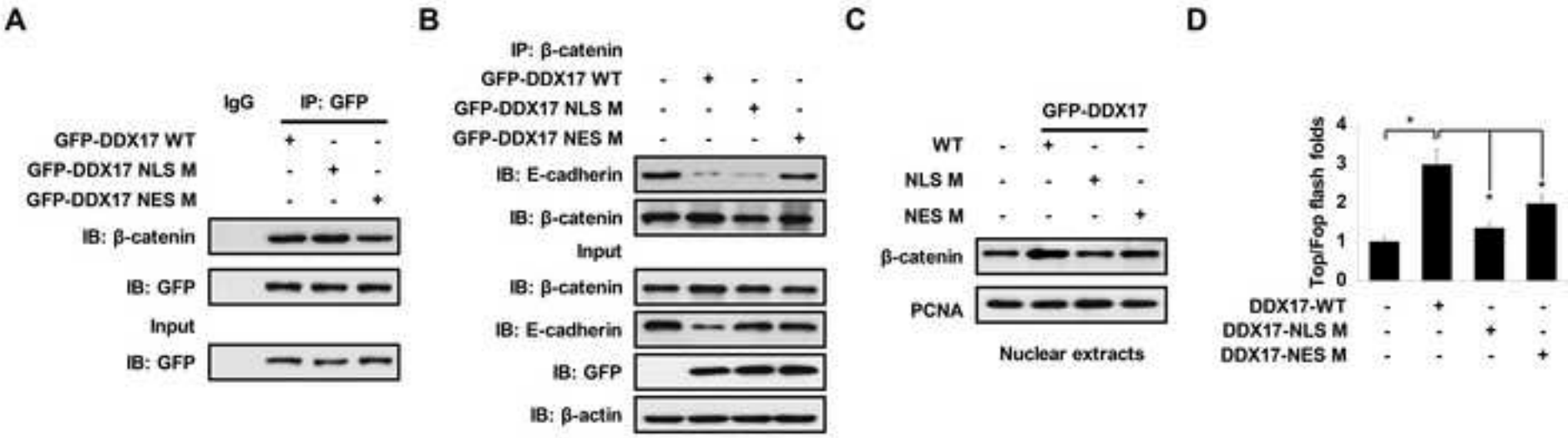
Figure 6. The integrity of DDX17 nucleocytoplasmic shuttling is essential for DDX17-mediating the activation of β-catenin in NSCLC cells.



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DDX17 nucleocytoplasmic shuttling promotes acquired gefitinib resistance in non-small cell lung cancer cells via activation of β-catenin

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Running title: DDX17 nucleocytoplasmic shuttling contributes to gefitinib resistance

Keywords: Non-small cell lung cancer, DDX17, Chemoresistance, Nucleocytoplasmic shuttle, β-catenin

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Abstract

Although epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are effective for non-small cell lung cancer (NSCLC) patients with EGFR mutations, almost all these patients will eventually develop acquired resistance to EGFR-TKI. However, the molecular mechanisms responsible for gefitinib resistance remain still not fully understood. Here, we report that elevated DDX17 levels are observed in gefitinib-resistant NSCLC cells than gefitinib-sensitive cells. Upregulation of DDX17 enhances the gefitinib resistance, whereas DDX17-silenced cells partially restore gefitinib sensitivity. Mechanistically, we demonstrate that DDX17 disassociates the E-cadherin/β-catenin complex, resulting in β-catenin nuclear translocation and subsequently augmenting the transcription of β-catenin target genes. Moreover, we identify two nuclear localization signal (NLS) and four nuclear export signal (NES) sequences mediated DDX17 nucleocytoplasmic shuttling via an exportin/importin-dependent pathways. Interruption of dynamic nucleocytoplasmic shuttling of DDX17 impairs DDX17-mediating the activation of β-catenin and acquired resistance in NSCLC cells. In conclusion, our findings reveal a novel and important mechanism by which DDX17 contributes to acquired gefitinib resistance through exportin/importin-dependent cytoplasmic shuttling and followed by activation of β-catenin, and DDX17 inhibition may be a promising strategy to overcome acquired resistance of gefitinib in NSCLC patients.

1. Introduction

Lung cancer is one of the mostly common malignancy and the leading cause of cancer-related deaths worldwide, with a five-year overall survival rate of only 15% [1]. Patients with non-small cell lung cancer (NSCLC), which accounts for approximately 80% of all lung cancer cases, are often diagnosed at advanced stages of the disease, leading to poor prognosis in lung cancer patients [2, 3]. Recent studies have indicated that the epidermal growth factor receptor (EGFR) signalling is frequently overexpressed or aberrantly activated in NSCLC and has been as an attractive target for cancer therapy [4, 5]. Somatic mutations including in-frame deletion mutation in exon 19 and the L858R mutation in exon 21 of the EGFR gene, are associated with favorable response to the EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib [6-8]. Despite good initial responses to EGFR-TKIs, most lung adenocarcinoma patients eventually develop resistance to anti-EGFR agents within 12 months through development of a secondary mutation in EGFR that reduces its binding affinity for TKIs or constitutive activation of downstream molecules to raise the compensatory survival signals [9, 10]. Overexpression or constitutive phosphorylation of HER3 can lead to significant resistance to EGFR-TKIs by activation of downstream PI3K/AKT pathways, which is independent of EGFR kinase activity [11, 12]. Moreover, abnormal activation of c-Met was significantly associated with poor response to EGFR-TKIs treatment, regardless of the EGFR status in NSCLC patients [13, 14]. A randomized phase II trial has demonstrated that dual inhibition of EGFR and c-Met can overcome resistance of EGFR-TKIs and improve outcomes in the MET-positive NSCLC patients [15, 16]. However, the mechanisms responsible for intrinsic resistance and other acquired resistance to EGFR-TKI are not fully understood.

DEAD box helicase 17 (DDX17) belongs to the DEAD box family of RNA helicases and is a transcriptional co-regulator required for the action of diverse transcription factors that are critical for normal biologic processes as well as cancer development [17]. DDX17 coactivates oestrogen receptor alpha (ERα) and is required for oestrogen-dependent expression of ERα-responsive genes and breast cancer cell growth [18]. Furthermore, DDX17 dysregulation is associated with the tumorigenesis of meibomian cell carcinoma [19]. In mouse mammary tumor cells, DDX17 regulates the alternative splicing of the chromatin-binding factor macroH2A1 histone gene, leading to transcriptional alterations to a set of genes involved in redox metabolism [20]. Additionally, DDX17 subunit in the mouse Drosha complex is indispensable for survival in mice and is required for primary miRNA and rRNA processing [21]. However, the role of DDX17 in the susceptibility to EGFR-TKIs in NSCLC cells remains unknown.

In the present study, we provided the first evidence that increased expression of DDX17 in gefitinib-resistant NSCLC cells than gefitinib-sensitive NSCLC cells. DDX17 downregulation promotes the apoptosis of gefitinib-resistant NSCLC cells exposed to gefitinib, whereas DDX17 overexpression protects gefitinib-sensitive NSCLC cells against this effect. Mechanistically, we demonstrate that DDX17 interacts with cytoplasmic β-catenin, facilitates the dissociation of β-catenin from the E-cadherin/β-catenin complex, enhances β-catenin nuclear accumulation, subsequently augments the transcription of β-catenin target genes, and ultimately leads to acquired resistance to gefitinib. Moreover, we found that DDX17 was a nucleocytoplasmic shuttling protein that was mediated by two NLS and four NES sequence elements. Interrupting DDX17 nucleocytoplasmic shuttling impairs DDX17-mediated activation of β-catenin and resistance to gefitinib in NSCLC cells. Taken together, our study highlights the significance of

DDX17 in gefitinib-resistant NSCLC and implicates DDX17 as a potential therapeutic target to enhance the efficacy of gefitinib in NSCLC patients.

2. Materials and Methods

2.1 *Cell culture and establishment of gefitinib resistant cell lines*

A431 and A431-GR cell lines were gifts from Prof. Zeng Cai (Sichuan Provincial People's Hospital, China). A549 and A549-GR cell lines were gifts from Prof. Feng Bi (Sichuan University, China). HCC827 and PC9 NSCLC cell lines were maintained at 37°C and 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco) and 100 units/ml penicillin, and 100 mg/ml streptomycin. To establish gefitinib resistant cell lines, parental cells were exposed to gradually elevated concentrations of gefitinib for two months as reported previously [13, 22].

2.2 *Reagents*

Gefitinib and XAV-939 were purchased from Selleck chemicals, and recombinant human EGF was purchased from PeproTech. The primary antibodies included AKT (Cell signalling Technology, #4691), p-AKT (Ser473) (Cell signalling Technology, #4060), cleaved caspase-3 (Cell Signaling Technology, #9664), cleaved PARP (Cell signalling Technology, #5625), DDX17 (Santa Cruz Biotechnology, sc-271112), E-cadherin (Abcam, ab1416), EGFR (Cell signalling Technology, #4267), p-EGFR (Tyr1068) (Cell signalling Technology, #3777), ERK (Cell signalling Technology, #9102), p-ERK (Thr202/Tyr204) (Cell signalling Technology, #4370), Flag (Cell signalling Technology, #8146), Ki67 (Abcam, ab15580), KPNA1 (Sangon Biotech, D154120), KPNB1 (Sangon Biotech, D161792), PCNA (Santa Cruz Biotechnology, sc-56), XPO1 (Sangon Biotech,

D221884), α-Tubulin (Santa Cruz Biotechnology, sc-5286), β-actin (Santa Cruz Biotechnology, sc-47778), β-catenin (Abcam, ab32572), p-β-Catenin (Ser33/37/Thr41) (Cell signalling Technology, #9561).

2.3 *Stable cell line generation for DDX17 knockdown or overexpression*

The DDX17 lentiviral expression vector was constructed by inserting expanded DDX17 cDNA (NM_006386.4) fragments into a lentiviral shuttle vector. DDX17 knockdown was accomplished using a specific shRNA targeting DDX17. The shRNA sequences were as follows: shRNA-DDX17, 5'-CAA GGG UAC CGC CUA UAC C-3'; shRNA-NC, 5'-TTC TCC GAA CGT GTC AGG T-3'. The packing and purification of the recombinant lentiviral vector were performed by the GenePharma Company (Shanghai, China). The indicated NSCLC cells infected with the recombinant lentiviral vectors were selected with puromycin for 2 weeks.

2.4 *RNA extraction and real-time PCR*

Total RNA was extracted using RNAiso Plus (TAKARA) according to the manufacturer's instructions. Real-time PCR was performed with SYBR® Premix Ex Taq™ II (TAKARA) using an iCycler iQ™ Multicolor Real-Time Detection System (BIO-RAD) as previously described [23, 24]. The following primers were used: DDX17 forward 5'-GAACATCCGGAAGTAGCAAGG-3', reverse 5'-GATCCATCAACACATCCATTACATAT-3'; GAPDH forward 5'-ACCACAGTCCATGCCATCAC-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'. The relative expression levels were determined using Gene Expression Macro Version 1.1 software (BIO-RAD).

2.5 *TOP/FOP flash assay*

For cotransfection, the indicated NSCLC cells were transfected with TOPflash plasmid plus pRL-TK plasmid or FOPflash plasmid plus pRL-TK plasmid in 48-well plates. Twenty-four hours post-transfection, the cells were rinsed twice with PBS and then lysed in Passive Lysis Buffer, and the dual-luciferase reporter assay was performed according to the manufacturer's instructions (Promega) using a Multi-Mode Microplate Reader (Synergy 2, BioTek).

2.6 *In vivo assays for tumor growth*

Female BALB/c nude mice (6-week-old) were raised in specific pathogen-free conditions. Animal care and experimental protocols were in accordance with guidelines established by the Institutional Animal Care and Use Committee of Sichuan University. The indicated NSCLC cells were suspended in 150 μL serum-free DMEM and implanted subcutaneously into the right flanks of nude mice. When subcutaneous tumor reached approximately 100 mm^3, the tumor-bearing mice were treated with gefitinib (10 mg/kg/day, by a gavage) for 15 days. The tumor volumes were measured by calipers every 3 days, and calculated using the following formula: tumor volume (mm^3) = $\pi/6 \times \text{length} \times \text{width}^2$.

2.7 *Statistical analysis*

All the experiments were performed at least three times independently, and all data are expressed as "mean ± SD". A one-way ANOVA test was used to analyse quantitative data between groups. The data were analyzed using SPSS statistical software version 22.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1 *Upregulation of DDX17 correlates with gefitinib resistance in NSCLC cells*

To explore the role of DDX17 in the acquired resistance to gefitinib, we first evaluated the expression of DDX17 in different NSCLC cell lines. As shown in Figure 1A, the level of DDX17 was markedly higher in gefitinib-insensitive NSCLC cell lines (PC9-GR and HCC827-GR) than that in gefitinib-sensitive NSCLC cell lines (PC9 and HCC827), respectively. A similar pattern of increased DDX17 expression level was observed in A549-GR and A431-GR cells compared with their parental A549 and A431 cells (Supplementary Figure S1). These data suggest that DDX17 may be positively correlated with gefitinib resistance in NSCLC cells. Next, we engineered stable upregulation of DDX17 expression in gefitinib-sensitive PC9 and HCC827 cells, and stable shRNA-mediated knockdown of DDX17 in gefitinib-insensitive PC9-GR and HCC827-GR cells (Figure 1B and Supplementary Figure S2A). Enforced DDX17 expression significantly increased the cell viability of PC9 and HCC827 cells in response to gefitinib (Figure 1C), whereas DDX17-silenced cells partially restored gefitinib sensitivity of PC9-GR and HCC827-GR cells (Supplementary Figure S2B). Moreover, upregulated DDX17 enhanced the resistance to gefitinib in PC9 and HCC827 cells compared to negative controlled cells (Figure 1D). Consistent with the MTT assay results, the colony formation in DDX17 overexpression NSCLC cells was significantly more compared to Mock cells (Figure 1E). The converse results were observed in DDX17-deficient NSCLC cells (Supplementary Figure S2C). Gefitinib treatment reduced p-EGFR and downstream signaling proteins p-Akt and p-ERK expressions, and meanwhile increased the levels of two apoptosis markers, cleaved caspase 3 and cleaved PARP (Figure 1F). However, the

upregulation of DDX17 partially overcame the gefitinib-inhibited EGFR, AKT and ERK activation, and suppressed cell apoptosis (Figure 1F). Conversely, knockdown of DDX17 caused decreased phosphorylation of EGFR, AKT and ERK, while increased expression of cleaved caspase 3 and cleaved PARP in gefitinib-resistant NSCLC cells treated with gefitinib (Supplementary Figure S2D). Taken together, our data indicate that DDX17 contributes to the development of acquired drug resistance to gefitinib in NSCLC cells.

3.2 *DDX17 disassociates the E-cadherin/β-catenin complex and promotes β-catenin nuclear translocation*

Recent evidence indicates that constitutive activation of Wnt/β-catenin signalling is associated with the acquired drug resistance to EGFR-TKIs in NSCLC [25-27]. To understand the molecular mechanism by which DDX17 promotes gefitinib resistance, we explored the effect of DDX17 on the nuclear translocation and activation of β-catenin. As shown in Figure 2A, β-catenin was located primarily in the plasma membrane in Mock PC9 cells; however, upregulated DDX17 led to the nuclear accumulation of β-catenin. Next, we evaluated the phosphorylation status of β-catenin. Consistently, phosphorylation of the residues that target β-catenin for proteasomal degradation (S33/S37/T41) was reduced in response to DDX17 overexpression (Figure 2B). Conversely, DDX17 knockdown significantly inhibited the levels of nuclear β-catenin in PC9-GR cells (Figure 2C). EGF, a potent activator of Wnt/β-catenin signalling as described previously [28-30], promotes β-catenin nuclear translocation in PC9-GR cells, however, DDX17 deletion markedly repressed the increased nuclear accumulation of β-catenin induced by EGF (Figure 2C). TOP/FOP-Flash assay showed that down-regulation of DDX17 impaired the transcriptional

activity of β-catenin/T-cell factor (TCF) complex regardless of EGF treatment (Figure 2D). Moreover, we found increased nuclear localization of β-catenin as well as decreased p-β-catenin (S33/S37/T41) in PC9-GR cells compared with PC9 cells (Supplementary Figure S3). Beta-catenin bound to the E-cadherin/catenin adhesion complex is mainly localizes to cell-cell adherent junctions at membranes lacking Wnt signalling, and Wnt signalling promotes the disassociation of E-cadherin/β-catenin complex and subsequently β-catenin nuclear translocation. Therefore, we next assessed whether DDX17 influenced E-cadherin/β-catenin complex stability. As shown in Figure 2E, overexpression of DDX17 downregulated E-cadherin/β-catenin complex formation, whereas knockdown of DDX17 augmented the association of β-catenin and E-cadherin. Considering the role of DDX17 in the nuclear accumulation and activation of the β-catenin, we next explored whether DDX17 can interact with β-catenin. Reciprocal immunoprecipitation studies revealed that endogenous DDX17 bound to endogenous β-catenin directly (Figure 2F). Moreover, increased interaction between DDX17 and β-catenin was observed in response to EGF stimulus, whereas EGF repressed β-catenin binding to E-cadherin (Figure 2G). Notably, we found that DDX17 predominantly interacted with β-catenin in the cytoplasm and that EGF enhanced both the cytoplasmic and nuclear interaction of β-catenin with DDX17 in a time-dependent manner (Figure 2H). To further investigate whether DDX17-regulated gefitinib resistance involves β-catenin activation, a specific Wnt/β-catenin signalling pathway inhibitor (XAV-939) was used. XAV-939 treatment effectively reversed DDX17-induced gefitinib resistance in PC9 and HCC827 cells (Figure 2I), leading to the increased levels of cleaved caspase 3 and cleaved PARP (Figure 2J). These data indicate that elevated DDX17 level leads to release and nuclear translocation of β-catenin from the E-cadherin/β-catenin complex and thereby resulting in the activation of

Wnt/β-catenin signalling and acquired resistance to gefitinib.

3.3 *Two NLSs mediated DDX17 nuclear transport by an importin-dependent pathway*

Because DDX17 interacted with β-catenin in the cytoplasm and nucleus, we presumed that DDX17 might be a nucleocytoplasmic shuttling protein. To test this hypothesis, we first examined the sublocalization of DDX17 in PC9 and PC9-GR cells using an immunofluorescence assay. Interestingly, DDX17 was predominantly localized to the nucleus in PC9 cells, whereas DDX17 was present in the cytoplasm and nucleus in PC9-GR cells (Supplementary Figure S2A). Immunoblotting analysis also showed that more DDX17 was accumulated in both the cytoplasmic and nuclear fractions of PC9-GR cells than of PC9 cells (Supplementary Figure S2B). These results indicate that DDX17 may be a nucleocytoplasm shuttling protein.

Most nucleocytoplasm shuttling proteins carry sequence elements of both nuclear localization signal (NLS) and (nuclear exporting signal) NES. We analysed the DDX17 amino acid sequence and identified two putative NLSs based on cNLS Mapper analysis (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (Figure 3A). Because the classical NLSs rich in basic amino acids are known as NLSs recognized by importin, we generated mutations in NLS1 (K50A, K53A, R74A, R75A, K76A and K77A) or NLS2 (K349A, R350A and R351A) of DDX17 fused with a GFP fluorescent protein. As shown in Figure 3B, the DDX17-WT protein showed both cytoplasmic and nuclear fluorescence. However, the NLS 1 mutant showed strong cytoplasmic fluorescence, and the NLS 2 mutant exhibited a complex distribution in both the nucleus and cytoplasm (Figure 3B). Moreover, no significant nuclear localization of DDX17 was observed in NLS mutant (NLS M, mutated both NLS 1 and NLS 2)

(Figure 3B). The similar result was also confirmed by immunoblot analyses (Figure 3C). As the nuclear transport of most nucleocytoplasmic proteins is mediated by importin complex which formed a hetero-dimer, we next explored whether DDX17 interacts with importin. Reciprocal immunoprecipitation studies revealed that endogenous DDX17 co-precipitated with endogenous KPNA1 (also known as Importin subunit alpha-5) and KPNB1 (also known as Importin subunit beta-1) (Figure 3D). However, DDX17 NLS mutant did not co-precipitate with KPNA1 and KPNB1 (Figure 3E). To further determine whether importin signalling mediated DDX17 nuclear localization, we treated cells with Ivermectin, which is a potent inhibitor of importin α/β-dependent transport [31, 32]. As shown in Figure 3F, ivermectin treatment caused the main cytoplasmic fluorescence of GFP-DDX17 fusion protein. Similarly, immunoblot analysis also showed the decreased nuclear accumulation of DDX17 in the presence of ivermectin (Figure 3G). Notably, ivermectin blocked the association of DDX17 with importin complex (Figure 3H). These data indicate that two NLSs of DDX17 recognized by importin complex mediated DDX17 nuclear transport.

3.4 *Four NESs mediated DDX17 nuclear export by an exportin-dependent pathway*

For the classical nuclear export pathway, XPO1 binds directly hydrophobic residue-rich NES sequence in the cargo protein and directs the export of the complex from the nucleus [33]. According to this theory, we found four putative NESs in DDX17 and constructed a series of site directed mutagenesis fused to the C-terminus of GFP (Figure 4A). As shown in Figure 4B, both cytoplasmic and nuclear localizations of DDX17-WT protein were observed in PC9 cells. The NES 1 mutant (L144A, L147A, L148A, I151A, V152A and I154A), NES 2 mutant (L226A, I227A, F229A,

L230A, L237A, L243A, V244A, L245A) and NES 3 mutant (V284A, L284A, L292A, Y295A, I298A, L303A, L305A) showed major nuclear localization, whereas mutations on NES 4 (L448A, L454A, I455A, V457A, L458A) localized in dispersed subnuclear speckles (Figure 4B). In addition, a complete NES mutant (mutated all four NESs) resulted in exclusive nuclear localization of DDX17, suggesting that these four NESs function as DDX17 nuclear export signals (Figure 4B). Immunoblot analysis also confirmed the subcellular localization of DDX17-WT and DDX17 NES mutant (Figure 4C). To confirm the effects of XPO1 on export of DDX17, we probed the interaction between DDX17 and XPO1 via co-immunoprecipitation. It was clear that endogenous DDX17 co-immunoprecipitated with endogenous XPO1 (Figure 4D). In addition, we found that the DDX17 NES mutant impaired the binding of DDX17 and XPO1 (Figure 4E). To further verify that the DDX17 cytoplasmic shuttling is mediated by XPO1, PC9 cells were treated with leptomycin B (LMB), a potent and specific nuclear export inhibitor. LMB effectively suppressed the level of cytoplasmic DDX17 in PC9 cells (Figures 4F and 4G). Moreover, LMB treatment significantly disrupted the interaction between DDX17 and XPO1 (Figure 4H). These observations suggest that four NESs are required for DDX17 cytoplasmic shuttling mediated by the classical exportin-dependent pathway.

3.5 *The integrity of DDX17 nucleocytoplasmic shuttling is indispensable for mediating the acquired resistance and activation of β-catenin*

We next evaluated the impact of DDX17 nucleocytoplasmic shuttling on cellular resistance to gefitinib. As shown in Figure 5A, ectopic expression of DDX17-WT significantly increased the cell viability of PC9 and HCC827 cells upon gefitinib treatment. The decreased resistances to

gefitinib were observed in DDX17-NLS mutant and DDX17-NES mutant PC9 cells compared to DDX17-WT PC9 cells, respectively (Figure 5A). Interestingly, DDX17-NLS mutant PC9 cells showed a similar sensitivity as mock PC9 cells, whereas DDX17-NES mutant PC9 cells were more resistant to gefitinib than mock PC9 cells (Figure 5A). Consistently, less colony formations were found in DDX17-NLS mutant and DDX17-NES mutant PC9 cells than DDX17-WT PC9 cells (Figure 5B). The immunoblotting test showed that cleaved caspase 3 and cleaved PARP were significantly enhanced in both DDX17-NLS mutant and DDX17-NES mutant PC9 cells compared to DDX17-WT PC9 cells (Figure 5C). To further determine the effect of DDX17 nucleocytoplasmic shuttling on the gifitinib resistance *in vivo*, indicated PC9 cells were injected subcutaneously into the flanks of BALB/c nude mice. Overexpression of DDX17-WT exerted obvious gefitinib insensitivity in tumor xenografts model compared to mock xenografts in the present of gefitinib (10 mg/kg per day, gavaged orally) (Figure 5D). However, DDX17-NLS mutant and DDX17-NES mutant xenografts was partially rescued gefitinib sensitivity compared with DDX17-WT xenografts (Figure 5D). The primary tumors from the DDX17-WT xenografts exhibited increased expression of Ki67 while decreased expression of cleaved caspase 3 compared with tumors originating from DDX17-NLS mutant and DDX17-NES mutant xenografts (Figure 5E).

Based on the aforementioned observations, we became interested in exploring the effect of DDX17 nucleocytoplasmic shuttling on the activation of β-catenin. As shown in Figure 6A, DDX17 NLS mutant showed a comparable association of β-catenin, whereas DDX17 NES mutant showed a slightly decreased association of β-catenin compared with DDX17 WT. DDX17 WT and DDX17 NLS mutant caused significant disassociation of E-cadherin/β-catenin complex, however, DDX17 NES mutant displayed modest inhibition of the interaction between β-catenin and

E-cadherin compared to control (Figure 6B). Moreover, less nuclear β-catenin was observed in the DDX17 NLS mutant and NES mutant group compared to that of the wild type (Figure 6C). In addition, DDX17 NLS mutant and NES mutant groups showed significantly decreased luciferase activity compared to wild type (Figure 6D). Overall, the above experiments suggest that interruption of DDX17 nucleocytoplasmic shuttling impairs DDX17-mediating the acquired resistance and activation of β-catenin in NSCLC cells.

4. Discussion

EGFR-mutant NSCLC patients who benefited from EGFR-TKI eventually develop acquire resistance to these therapies and the median duration of response is about 10 to 14 months [34, 35]. Although accumulating studies revealed that a variety of mechanisms can stimulate acquired resistance to EGFR-TKI including secondary mutations within EGFR at position T790, activation of parallel receptor tyrosine kinases (such as ALK, MET and RET), and mutation or upregulation of EGFR effector proteins [36-38], the mechanisms responsible for acquired resistance to EGFR-TKIs are still large unknown. In this study, we showed that DDX17 levels were increased in gefitinib resistant cells compared with gefitinib sensitive cells. Overexpression of DDX17 significantly increased tolerance of PC9 and HCC827 cells in the present of gefitinib, whereas DDX17 suppression resulted in reduced cell viability of gefitinib-resistant PC9-GR and HCC827-GR cells. These data revealed that DDX17 expression was associated with tumor sensitivity to gefitinib in NSCLC cells.

The Wnt/β-catenin signaling is one of the most critical signaling transduction pathways during embryonic development and the stemness maintenance, and has become a hot topic in

tumor research [39, 40]. In recent years, accumulating evidence reported that Wnt/β-catenin signalling has been implicated in the chemoresistance of varied cancers [41, 42]. Here, we demonstrated that DDX17 directly bound and dissociated the E-cadherin/β-catenin complex to release β-catenin, subsequently leading to β-catenin nuclear accumulation. Moreover, we found that EGF augmented the interaction between β-catenin and DDX17 both in the cytoplasm and nucleus, whereas DDX17 repression abolished the EGF-induced nuclear translocation and activation of β-catenin. Therefore, we postulated that DDX17-dependent nuclear accumulation of β-catenin released from the E-cadherin/β-catenin adhesion complex was an important mechanism driving acquired resistance to gefitinib in NSCLC cells.

To enhance cytoplasmic β-catenin nuclear translocation, DDX17, which is predominantly localized in the nucleus [43], must shuttle to the cytoplasm. Interestingly, increased DDX17 levels were observed in both the cytoplasmic and nuclear fractions of PC9-GR cells than of PC9 cells. Moreover, DDX17 interacted with β-catenin both in the cytoplasm and nucleus. These data supported DDX17 as a nucleocytoplasmic protein. Most nucleocytoplasmic protein movement through the nuclear pore complex is mediated by a nuclear receptor system [44, 45]. In this study, we identified two NLSs and four NESs required for DDX17 nucleocytoplasmic shuttling. Mutation of the NLSs significantly inhibited the DDX17 nuclear localization and association of DDX17 and import complex. Analogously, NESs mutant caused nuclear accumulation of DDX17 and disrupted the interaction between DDX17 and XPO1. These results indicated that the nucleocytoplasmic shuttling of DDX17 followed a classical exportin/importin-dependent pathway. Interestingly, several DEAD box RNA helicases shuttle between the nucleus and cytoplasm via XPO1-dependent nuclear export pathway, including DDX3, DDX25, and DDX48 [46-48].

Furthermore, block of DDX17 nucleocytoplasmic shuttling significantly reduced DDX17-mediated activation of β-catenin and gefitinib sensitivity in NSCLC cells, suggesting that dynamic nucleocytoplasmic shuttling of DDX17 is essential for its function. Interestingly, a decreased tolerance was observed in DDX17-NES mutant cells compared with DDX17-WT cells, however, DDX17-NES mutant cells were more resistant to gefitinib than mock PC9 cells. Because DDX17 can act as co-transcriptional regulator, one possible explanation is that nuclear DDX17 regulated the transcription of target genes that lead to the activation of Wnt/β-catenin signalling independent cytoplasmic function of DDX17. Although DDX17-NLS mutant has no significant effect on the association of DDX17 and β-catenin, the nuclear accumulation of β-catenin was depressed in DDX17-NLS mutant cells compared with DDX17-WT cells. Considering the main interaction of DDX17 and β-catenin in the cytoplasm, we presume that DDX17 might function as a 'chaperone' to aid β-catenin nuclear import; however, the detailed mechanism requires further exploration.

In summary, our study provides the first evidence that upregulated DDX17 expression is associated with gefitinib resistance in NSCLC cells and DDX17 is a nucleocytoplasmic protein mediated by two NLSs and Four NESs. We demonstrate a new molecular mechanism by which the exportin/importin-dependent nucleocytoplasmic translocation of DDX17 disassociates the E-cadherin/β-catenin complex, resulting in β-catenin nuclear translocation and subsequently augmenting the transcription of β-catenin target genes, ultimately driving gefitinib resistance in NSCLC cells. Interruption of dynamic nucleocytoplasmic shuttling of DDX17 impairs DDX17-mediating the activation of β-catenin and acquired resistance in NSCLC cells. In conclusion, we propose that DDX17 is an attractive and potential target for overcoming gefitinib resistance in NSCLC therapy

5. Acknowledgements

This project was supported by the National Natural Science Foundation of China (81401979, 81402944 and 81572604); the Science and Technology Department of Sichuan Province Foundation (2014SZ0020); and the China Postdoctoral Science Foundation (2014M552367).

6. Conflict of Interest:

The authors disclose no potential conflicts of interest.

7. References

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8. FIGURE LEGENDS

8.1 Figure 1. Upregulated DDX17 is associated with gefitinib resistance in human NSCLC cells. (A) Quantitative real-time PCR and immunoblot analysis of DDX17 expression in gefitinib sensitive and insensitive NSCLC cells. (B) Quantitative real-time PCR and immunoblot analysis showed the successful lentiviral infections of DDX17 in PC9 and HCC827 cells. (C) Stably expressing DDX17 or mock NSCLC cells were treated with 1 μM gefitinib for indicated time and analyzed for cell viability by MTT assay. (D) Effect of DDX17 overexpression on gefitinib efficacy in HCC827 and PC9 cells was detected by MTT assay. (E) Representative photographs of the colony formation of indicated NSCLC cells treated with gefitinib for 14 days after culture of cells. (F) The indicated NSCLC cells were treated with or without gefitinib and then subjected to immunoblot analysis using the indicated antibodies. Data represent the mean ± standard deviation (SD). Each experiment was performed at least in triplicate, producing consistent results. *$P < 0.05$.

8.2 Figure 2. DDX17 disassociates the E-cadherin/β-catenin complex and promotes β-catenin nuclear translocation. (A) Immunofluorescence analysis of β-catenin (red) in indicated PC9 cells. Merged images represent overlays of β-catenin (red) and nuclear staining by DAPI (blue). (B) Immunoblotting for phosphorylated β-catenin (S33/S37/T41) in indicated PC9 cells. (C) Immunoblotting for β-catenin in the nuclear extracts of indicated PC9-GR cells in the present of EGF. PCNA was used as the control. (D) TOP/FOP flash assay in the indicated PC9-GR cells treated with or without EGF. (E) The effect of DDX17 on the association of β-catenin with E-cadherin was detected by western blot analysis. (F) The endogenous interaction of DDX17

and β-catenin was detected by immunoprecipitation with indicated antibodies. (G) The impact of EGF on the interaction of DDX17 and β-catenin/E-cadherin complex. (H) The cytoplasmic and nuclear extracts of PC9-GR cells stimulated with EGF were isolated. Immunoprecipitation were performed with anti-DDX17 antibody. α-Tubulin and PCNA were control. (I) Effect of XAV-939 on gefitinib efficacy in indicated NSCLC cells was detected by MTT assay. (J) The indicated NSCLC cells were treated with gefitinib in the presence or absence of XAV-939, and then subjected to immunoblot analysis using the indicated antibodies. Data represent the mean ± SD of three independent experiments. *$P < 0.05$.

8.3 Figure 3. Two NLSs mediated DDX17 nuclear transport by an importin-dependent pathway. (A) Putative sequence segments of DDX17 NLSs. (B) Representative of fluorescent microscopy images show the localizations of the exogenously expressed DDX17 wild type (WT) and putative NLSs mutants fused with GFP in PC9 cells. (C) The levels of exogenously expressed DDX17 WT or NLS mutant in the extracts made from the cytoplasm or the nucleus of PC9 cells were examined by immunoblotting analysis. PCNA and α-Tubulin were used as the control. (D) The endogenous association of DDX17 and importin complex was detected by immunoprecipitation with indicated antibodies. (E) The impact of NLS mutant on the interaction between DDX17 and importin complex. (F) Representative of fluorescent microscopy images show the sub-localizations of DDX17 in PC9 cells treated with or without ivermectin. (G) The effect of Ivermectin on the distribution of DDX17 was performed by western blot analysis in PC9 cells. (H) The effect of Ivermectin on the binding of DDX17 and importin complex. Data represent the mean ± SD of three independent experiments.

8.4 Figure 4. Four NESs mediated DDX17 nuclear export by an exportin-dependent pathway. (A) Putative sequence segments of DDX17 NESs. (B) Representative of fluorescent microscopy images show the localizations of the exogenously expressed DDX17 WT and putative NESs mutants fused with GFP in PC9 cells. (C) Immunoblotting for DDX17 in PC9 cytoplasmic and nuclear extracts of cells transfected with the indicated plasmids. PCNA and α-Tubulin were used as the control. (D) The endogenous association of DDX17 and XPO1 was detected by immunoprecipitation with indicated antibodies. (E) The impact of NES mutant on the interaction between DDX17 and XPO1. (F) Representative of fluorescent microscopy images show the sub-localizations of DDX17 in PC9 cells treated with or without LMB. (G) The effect of LMB on the distribution of DDX17 was performed by western blot analysis in PC9 cells. (H) The effect of LMB on the binding of DDX17 and XPO1. Data represent the mean ± SD of three independent experiments.

8.5 Figure 5. Interruption of DDX17 nucleocytoplasmic shuttling impairs DDX17-mediated gefitinib resistance *in vitro* and *in vivo*. (A) Indicated NSCLC cells were treated with varied concentration of gefitinib, and the cell viability were analysed by MTT assay. (B) Representative photographs of the colony formation of indicated NSCLC cells treated with gefitinib for 14 days after culture of cells. (C) The indicated NSCLC cells were treated with gefitinib and then subjected to immunoblot analysis using the indicated antibodies. (D) The indicated NSCLC cells were transplanted into nude mice. When subcutaneous tumor reached approximately 100 mm^3, the tumor-bearing mice were treated with gefitinib (10 mg/kg/day, by a gavage) for 15 days. The

tumor volumes were measured by calipers every 3 days. (E) Tumor xenograft tissues were fixed with 4% paraformaldehyde, processed, embedded in paraffin wax and then assessed for immunohistochemical analyses with indicated antibodies. Data represent the mean ± SD of three independent experiments. *$P < 0.05$.

8.6 Figure 6. The integrity of DDX17 nucleocytoplasmic shuttling is essential for DDX17-mediating the activation of β-catenin in NSCLC cells. (A) The impact of NLS mutant and NES mutant on the interaction of DDX17 and β-catenin. (B) The effect of NLS mutant and NES mutant on the association of E-cadherin/β-catenin complex. (C) Immunoblotting for β-catenin in the nuclear extracts of indicated PC9 cells. PCNA was used as the control. (D) TOP/FOP flash assay were performed in the indicated PC9 cells. Data represent the mean ± SD of three independent experiments. *$P < 0.05$.