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

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22

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29 **Abstract**

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

47 **1. Introduction**

48 Lung cancer is one of the mostly common malignancy and the leading cause of
49 cancer-related deaths worldwide, with a five-year overall survival rate of only 15% [1]. Patients
50 with non-small cell lung cancer (NSCLC), which accounts for approximately 80% of all lung
51 cancer cases, are often diagnosed at advanced stages of the disease, leading to poor prognosis
52 in lung cancer patients [2, 3]. Recent studies have indicated that the epidermal growth factor
53 receptor (EGFR) signalling is frequently overexpressed or aberrantly activated in NSCLC and has
54 been as an attractive target for cancer therapy [4, 5]. Somatic mutations including in-frame
55 deletion mutation in exon 19 and the L858R mutation in exon 21 of the EGFR gene, are
56 associated with favorable response to the EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as
57 gefitinib and erlotinib [6-8]. Despite good initial responses to EGFR-TKIs, most lung
58 adenocarcinoma patients eventually develop resistance to anti-EGFR agents within 12 months
59 through development of a secondary mutation in EGFR that reduces its binding affinity for TKIs or
60 constitutive activation of downstream molecules to raise the compensatory survival signals [9, 10].
61 Overexpression or constitutive phosphorylation of HER3 can lead to significant resistance to
62 EGFR-TKIs by activation of downstream PI3K/AKT pathways, which is independent of EGFR
63 kinase activity [11, 12]. Moreover, abnormal activation of c-Met was significantly associated with
64 poor response to EGFR-TKIs treatment, regardless of the EGFR status in NSCLC patients [13,
65 14]. A randomized phase II trial has demonstrated that dual inhibition of EGFR and c-Met can
66 overcome resistance of EGFR-TKIs and improve outcomes in the MET-positive NSCLC patients
67 [15, 16]. However, the mechanisms responsible for intrinsic resistance and other acquired
68 resistance to EGFR-TKI are not fully understood.

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

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

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

93

94 **2. Materials and Methods**

95 *2.1 Cell culture and establishment of gefitinib resistant cell lines*

96 **A431 and A431-GR cell lines were gifts from Prof. Zeng Cai (Sichuan Provincial**
97 **People's Hospital, China). A549 and A549-GR cell lines were gifts from Prof. Feng Bi**
98 **(Sichuan University, China).** HCC827 and PC9 NSCLC cell lines were maintained at 37°C and 5%
99 CO₂ in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco) and 100 units/ml penicillin,
100 and 100 mg/ml streptomycin. To establish gefitinib resistant cell lines, parental cells were exposed
101 to gradually elevated concentrations of gefitinib for two months as reported previously [13, 22].

102

103 *2.2 Reagents*

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

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

116

117 2.3 Stable cell line generation for DDX17 knockdown or overexpression

118 The DDX17 lentiviral expression vector was constructed by inserting expanded DDX17 cDNA
119 (NM_006386.4) fragments into a lentiviral shuttle vector. DDX17 knockdown was accomplished
120 using a specific shRNA targeting DDX17. The shRNA sequences were as follows: shRNA-DDX17,
121 5'-CAA GGG UAC CGC CUA UAC C-3'; shRNA-NC, 5'-TTC TCC GAA CGT GTC AGG T-3'. The
122 packing and purification of the recombinant lentiviral vector were performed by the GenePharma
123 Company (Shanghai, China). The indicated NSCLC cells infected with the recombinant lentiviral
124 vectors were selected with puromycin for 2 weeks.

125

126 2.4 RNA extraction and real-time PCR

127 Total RNA was extracted using RNAiso Plus (TAKARA) according to the manufacturer's
128 instructions. Real-time PCR was performed with SYBR[®] Premix Ex Taq[™] II (TAKARA) using an
129 iCycler iQ[™] Multicolor Real-Time Detection System (BIO-RAD) as previously described [23, 24].
130 The following primers were used: DDX17 forward 5'-GAACATCCGGAAGTAGCAAGG-3', reverse
131 5'-GATCCATCAACACATCCATTACATAT-3'; GAPDH forward 5'-ACCACAGTCCATGCCATCAC-3',
132 reverse 5'-TCCACCACCCTGTTGCTGTA-3'. The relative expression levels were determined
133 using Gene Expression Macro Version 1.1 software (BIO-RAD).

134

135 *2.5 TOP/FOP flash assay*

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

141

142 *2.6 In vivo assays for tumor growth*

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

151

152 *2.7 Statistical analysis*

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

157

158 **3. Results**

159 *3.1 Upregulation of DDX17 correlates with gefitinib resistance in NSCLC cells*

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

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

185

186 *3.2 DDX17 disassociates the E-cadherin/ β -catenin complex and promotes β -catenin nuclear* 187 *translocation*

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

201 DDX17 impaired the transcriptional activity of β -catenin/T-cell factor (TCF) complex regardless of
202 EGF treatment **(Figure 2D). Moreover, we found increased nuclear localization of β -catenin**
203 **as well as decreased p- β -catenin (S33/S37/T41) in PC9-GR cells compared with PC9 cells**
204 **(Supplementary Figure S3).** Beta-catenin bound to the E-cadherin/catenin adhesion complex is
205 mainly localizes to cell-cell adherent junctions at membranes lacking Wnt signalling, and Wnt
206 signalling promotes the disassociation of E-cadherin/ β -catenin complex and subsequently
207 β -catenin nuclear translocation. Therefore, we next assessed whether DDX17 influenced
208 E-cadherin/ β -catenin complex stability. As shown in **Figure 2E**, overexpression of DDX17
209 downregulated E-cadherin/ β -catenin complex formation, whereas knockdown of DDX17
210 augmented the association of β -catenin and E-cadherin. Considering the role of DDX17 in the
211 nuclear accumulation and activation of the β -catenin, we next explored whether DDX17 can
212 interact with β -catenin. Reciprocal immunoprecipitation studies revealed that endogenous DDX17
213 bound to endogenous β -catenin directly **(Figure 2F)**. Moreover, increased interaction between
214 DDX17 and β -catenin was observed in response to EGF stimulus, whereas EGF repressed
215 β -catenin binding to E-cadherin **(Figure 2G)**. Notably, we found that DDX17 predominantly
216 interacted with β -catenin in the cytoplasm and that EGF enhanced both the cytoplasmic and
217 nuclear interaction of β -catenin with DDX17 in a time-dependent manner **(Figure 2H)**. **To further**
218 **investigate whether DDX17-regulated gefitinib resistance involves β -catenin activation, a**
219 **specific Wnt/ β -catenin signalling pathway inhibitor (XAV-939) was used. XAV-939 treatment**
220 **effectively reversed DDX17-induced gefitinib resistance in PC9 and HCC827 cells (Figure**
221 **2I), leading to the increased levels of cleaved caspase 3 and cleaved PARP (Figure 2J).**
222 These data indicate that elevated DDX17 level leads to release and nuclear translocation of

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

225

226 3.3 *Two NLSs mediated DDX17 nuclear transport by an importin-dependent pathway*

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

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

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

260

261 *3.4 Four NESs mediated DDX17 nuclear export by an exportin-dependent pathway*

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

267 1 mutant (L144A, L147A, L148A, I151A, V152A and I154A), NES 2 mutant (L226A, I227A, F229A,
268 L230A, L237A, L243A, V244A, L245A) and NES 3 mutant (V284A, L284A, L292A, Y295A, I298A,
269 L303A, L305A) showed major nuclear localization, whereas mutations on NES 4 (L448A, L454A,
270 I455A, V457A, L458A) localized in dispersed subnuclear speckles (Figure 4B). In addition, a
271 complete NES mutant (mutated all four NESs) resulted in exclusive nuclear localization of DDX17,
272 suggesting that these four NESs function as DDX17 nuclear export signals (Figure 4B).
273 Immunoblot analysis also confirmed the subcellular localization of DDX17-WT and DDX17 NES
274 mutant (Figure 4C). To confirm the effects of XPO1 on export of DDX17, we probed the interaction
275 between DDX17 and XPO1 via co-immunoprecipitation. It was clear that endogenous DDX17
276 co-immunoprecipitated with endogenous XPO1 (Figure 4D). In addition, we found that the DDX17
277 NES mutant impaired the binding of DDX17 and XPO1 (Figure 4E). To further verify that the
278 DDX17 cytoplasmic shuttling is mediated by XPO1, PC9 cells were treated with leptomycin B
279 (LMB), a potent and specific nuclear export inhibitor. LMB effectively suppressed the level of
280 cytoplasmic DDX17 in PC9 cells (Figures 4F and 4G). Moreover, LMB treatment significantly
281 disrupted the interaction between DDX17 and XPO1 (Figure 4H). These observations suggest
282 that four NESs are required for DDX17 cytoplasmic shuttling mediated by the classical
283 exportin-dependent pathway.

284

285 *3.5 The integrity of DDX17 nucleocytoplasmic shuttling is indispensable for mediating the* 286 *acquired resistance and activation of β -catenin*

287 We next evaluated the impact of DDX17 nucleocytoplasmic shuttling on cellular resistance
288 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 gefitinib 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,

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

318

319 **4. Discussion**

320 EGFR-mutant NSCLC patients who benefited from EGFR-TKI eventually develop acquire
321 resistance to these therapies and the median duration of response is about 10 to 14 months [34,
322 35]. Although accumulating studies revealed that a variety of mechanisms can stimulate acquired
323 resistance to EGFR-TKI including secondary mutations within EGFR at position T790, activation
324 of parallel receptor tyrosine kinases (such as ALK, MET and RET), and mutation or upregulation
325 of EGFR effector proteins [36-38], the mechanisms responsible for acquired resistance to
326 EGFR-TKIs are still large unknown. **In this study, we showed that DDX17 levels were**
327 **increased in gefitinib resistant cells compared with gefitinib sensitive cells.** Overexpression
328 of DDX17 significantly increased tolerance of PC9 and HCC827 cells in the present of gefitinib,
329 whereas DDX17 suppression resulted in reduced cell viability of gefitinib-resistant PC9-GR and
330 HCC827-GR cells. These data revealed that DDX17 expression was associated with tumor
331 sensitivity to gefitinib in NSCLC cells.

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

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

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

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

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

377 resistance in NSCLC therapy

378

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383

384 **6. Conflict of Interest:**

385 The authors disclose no potential conflicts of interest.

386

387 **7. References**

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551

552 **8. FIGURE LEGENDS**

553 **8.1 Figure 1. Upregulated DDX17 is associated with gefitinib resistance in human NSCLC**
554 **cells.** (A) Quantitative real-time PCR and immunoblot analysis of DDX17 expression in gefitinib
555 sensitive and insensitive NSCLC cells. (B) Quantitative real-time PCR and immunoblot analysis
556 showed the successful lentiviral infections of DDX17 in PC9 and HCC827 cells. (C) Stably
557 expressing DDX17 or mock NSCLC cells were treated with 1 μ M gefitinib for indicated time and
558 analyzed for cell viability by MTT assay. (D) Effect of DDX17 overexpression on gefitinib efficacy
559 in HCC827 and PC9 cells was detected by MTT assay. (E) Representative photographs of the
560 colony formation of indicated NSCLC cells treated with gefitinib for 14 days after culture of cells.
561 (F) The indicated NSCLC cells were treated with or without gefitinib and then subjected to
562 immunoblot analysis using the indicated antibodies. Data represent the mean \pm standard
563 deviation (SD). Each experiment was performed at least in triplicate, producing consistent results.
564 * $P < 0.05$.

565
566 **8.2 Figure 2. DDX17 disassociates the E-cadherin/ β -catenin complex and promotes**
567 **β -catenin nuclear translocation. (A) Immunofluorescence analysis of β -catenin (red) in**
568 **indicated PC9 cells. Merged images represent overlays of β -catenin (red) and nuclear**
569 **staining by DAPI (blue). (B) Immunoblotting for phosphorylated β -catenin (S33/S37/T41) in**
570 **indicated PC9 cells. (C) Immunoblotting for β -catenin in the nuclear extracts of indicated**
571 **PC9-GR cells in the present of EGF. PCNA was used as the control. (D) TOP/FOP flash assay in**
572 **the indicated PC9-GR cells treated with or without EGF. (E) The effect of DDX17 on the**
573 **association of β -catenin with E-cadherin was detected by western blot analysis. (F) The**

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

583

584 **8.3 Figure 3. Two NLSs mediated DDX17 nuclear transport by an importin-dependent**
585 **pathway.** (A) Putative sequence segments of DDX17 NLSs. (B) Representative of fluorescent
586 microscopy images show the localizations of the exogenously expressed DDX17 wild type (WT)
587 and putative NLSs mutants fused with GFP in PC9 cells. (C) The levels of exogenously expressed
588 DDX17 WT or NLS mutant in the extracts made from the cytoplasm or the nucleus of PC9 cells
589 were examined by immunoblotting analysis. PCNA and α -Tubulin were used as the control. (D)
590 The endogenous association of DDX17 and importin complex was detected by
591 immunoprecipitation with indicated antibodies. (E) The impact of NLS mutant on the interaction
592 between DDX17 and importin complex. (F) Representative of fluorescent microscopy images
593 show the sub-localizations of DDX17 in PC9 cells treated with or without ivermectin. (G) The
594 effect of Ivermectin on the distribution of DDX17 was performed by western blot analysis in PC9
595 cells. (H) The effect of Ivermectin on the binding of DDX17 and importin complex. Data represent

596 the mean \pm SD of three independent experiments.

597

598 **8.4 Figure 4. Four NESs mediated DDX17 nuclear export by an exportin-dependent pathway.**

599 (A) Putative sequence segments of DDX17 NESs. (B) Representative of fluorescent microscopy
600 images show the localizations of the exogenously expressed DDX17 WT and putative NESs
601 mutants fused with GFP in PC9 cells. (C) Immunoblotting for DDX17 in PC9 cytoplasmic and
602 nuclear extracts of cells transfected with the indicated plasmids. PCNA and α -Tubulin were used
603 as the control. (D) The endogenous association of DDX17 and XPO1 was detected by
604 immunoprecipitation with indicated antibodies. (E) The impact of NES mutant on the interaction
605 between DDX17 and XPO1. (F) Representative of fluorescent microscopy images show the
606 sub-localizations of DDX17 in PC9 cells treated with or without LMB. (G) The effect of LMB on the
607 distribution of DDX17 was performed by western blot analysis in PC9 cells. (H) The effect of LMB
608 on the binding of DDX17 and XPO1. Data represent the mean \pm SD of three independent
609 experiments.

610

611 **8.5 Figure 5. Interruption of DDX17 nucleocytoplasmic shuttling impairs DDX17-mediated**

612 **gefitinib resistance *in vitro* and *in vivo*.** (A) Indicated NSCLC cells were treated with varied
613 concentration of gefitinib, and the cell viability were analysed by MTT assay. (B) Representative
614 photographs of the colony formation of indicated NSCLC cells treated with gefitinib for 14 days
615 after culture of cells. (C) The indicated NSCLC cells were treated with gefitinib and then subjected
616 to immunoblot analysis using the indicated antibodies. (D) The indicated NSCLC cells were
617 transplanted into nude mice. When subcutaneous tumor reached approximately 100 mm³, the

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

623

624 **8.6 Figure 6. The integrity of DDX17 nucleocytoplasmic shuttling is essential for**

625 **DDX17-mediated the activation of β -catenin in NSCLC cells.** (A) The impact of NLS mutant
626 and NES mutant on the interaction of DDX17 and β -catenin. (B) The effect of NLS mutant and
627 NES mutant on the association of E-cadherin/ β -catenin complex. (C) Immunoblotting for β -catenin
628 in the nuclear extracts of indicated PC9 cells. PCNA was used as the control. (D) TOP/FOP flash
629 assay were performed in the indicated PC9 cells. Data represent the mean \pm SD of three
630 independent experiments. * P < 0.05.

Figure 1
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Figure 1. Upregulated DDX17 is associated with gefitinib resistance in human NSCLC cells.

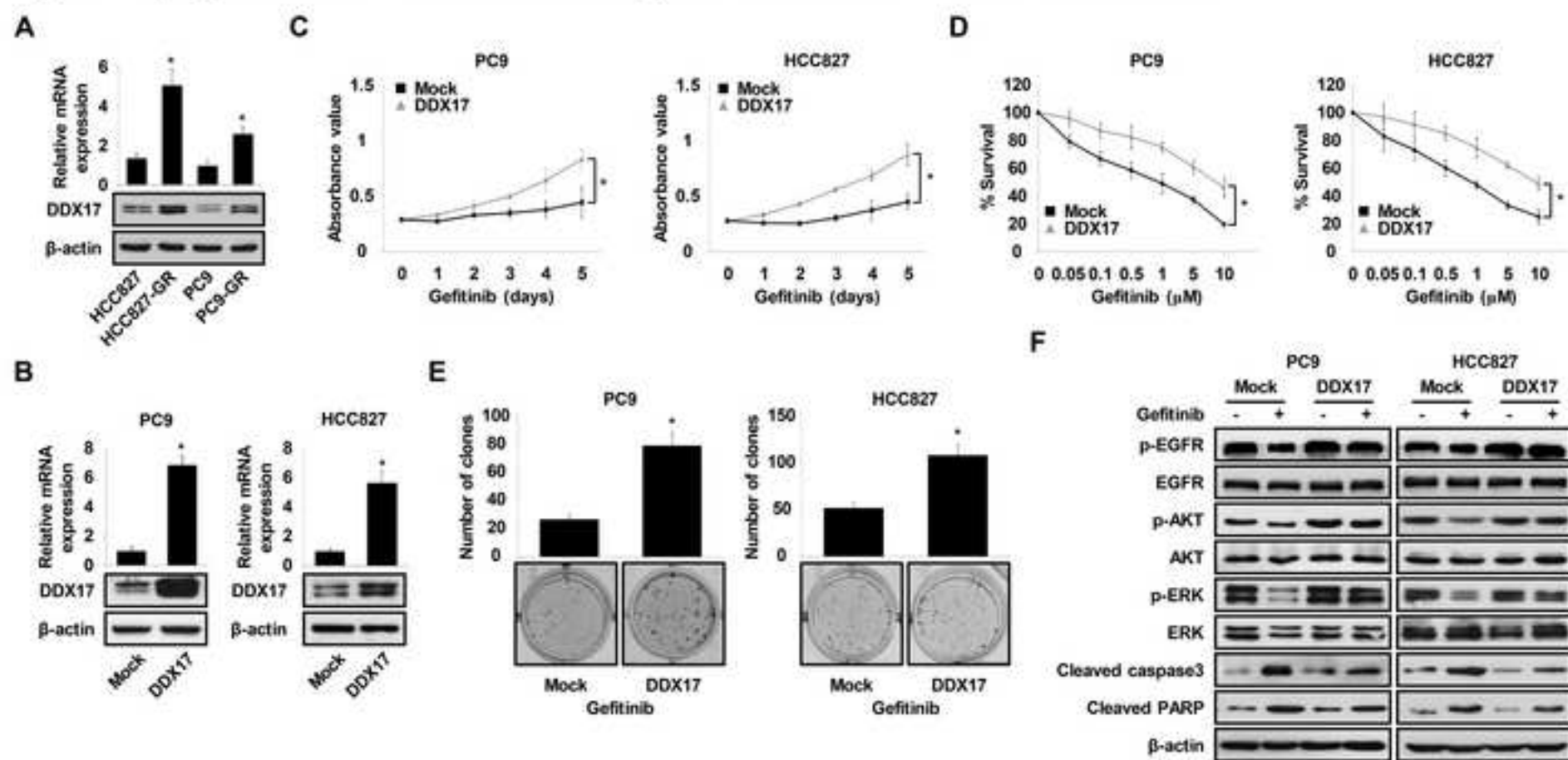


Figure 2. DDX17 disassociates the E-cadherin/ β -catenin complex and promotes β -catenin nuclear translocation.

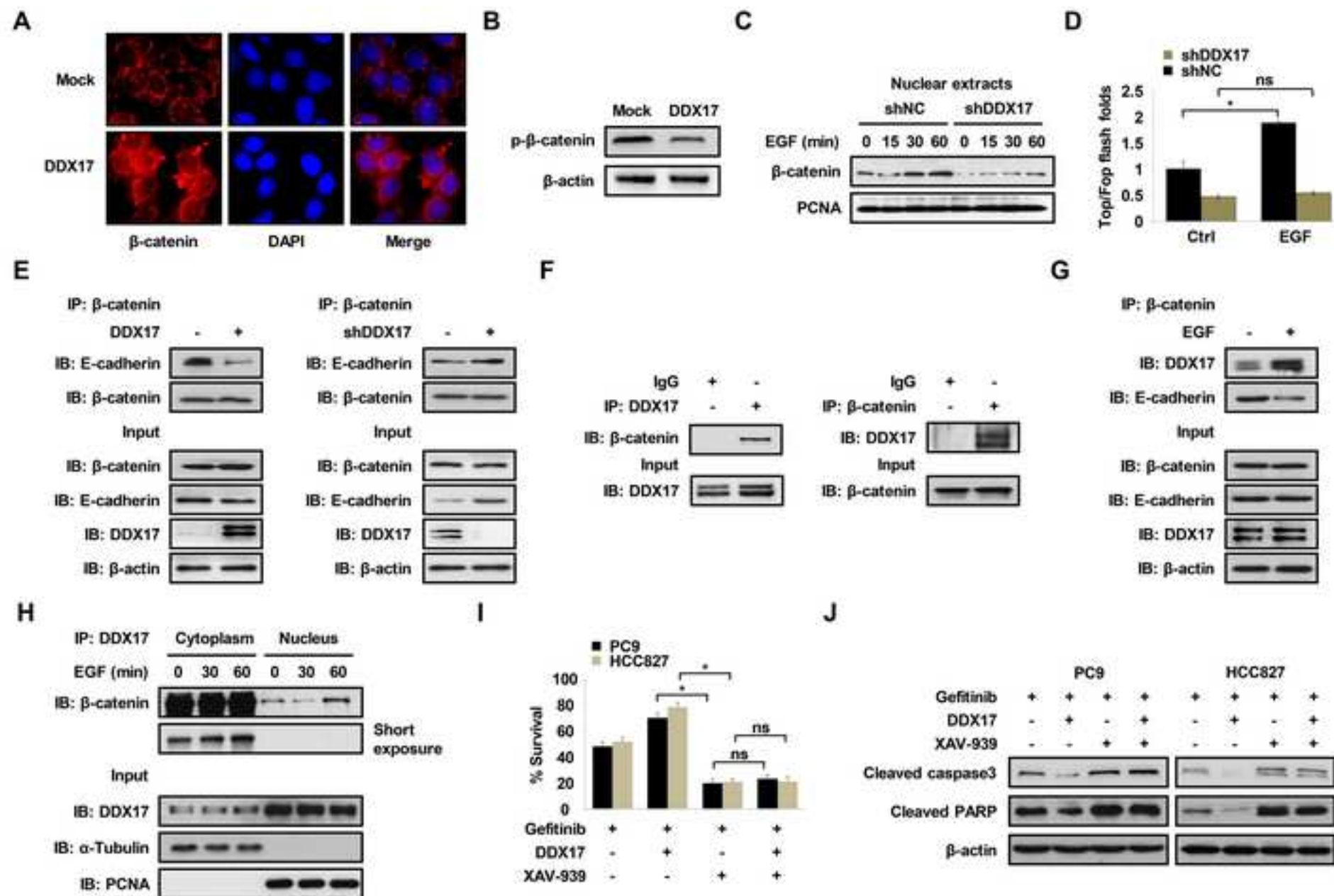


Figure 3. Two NLSs mediated DDX17 nuclear transport by an importin-dependent pathway

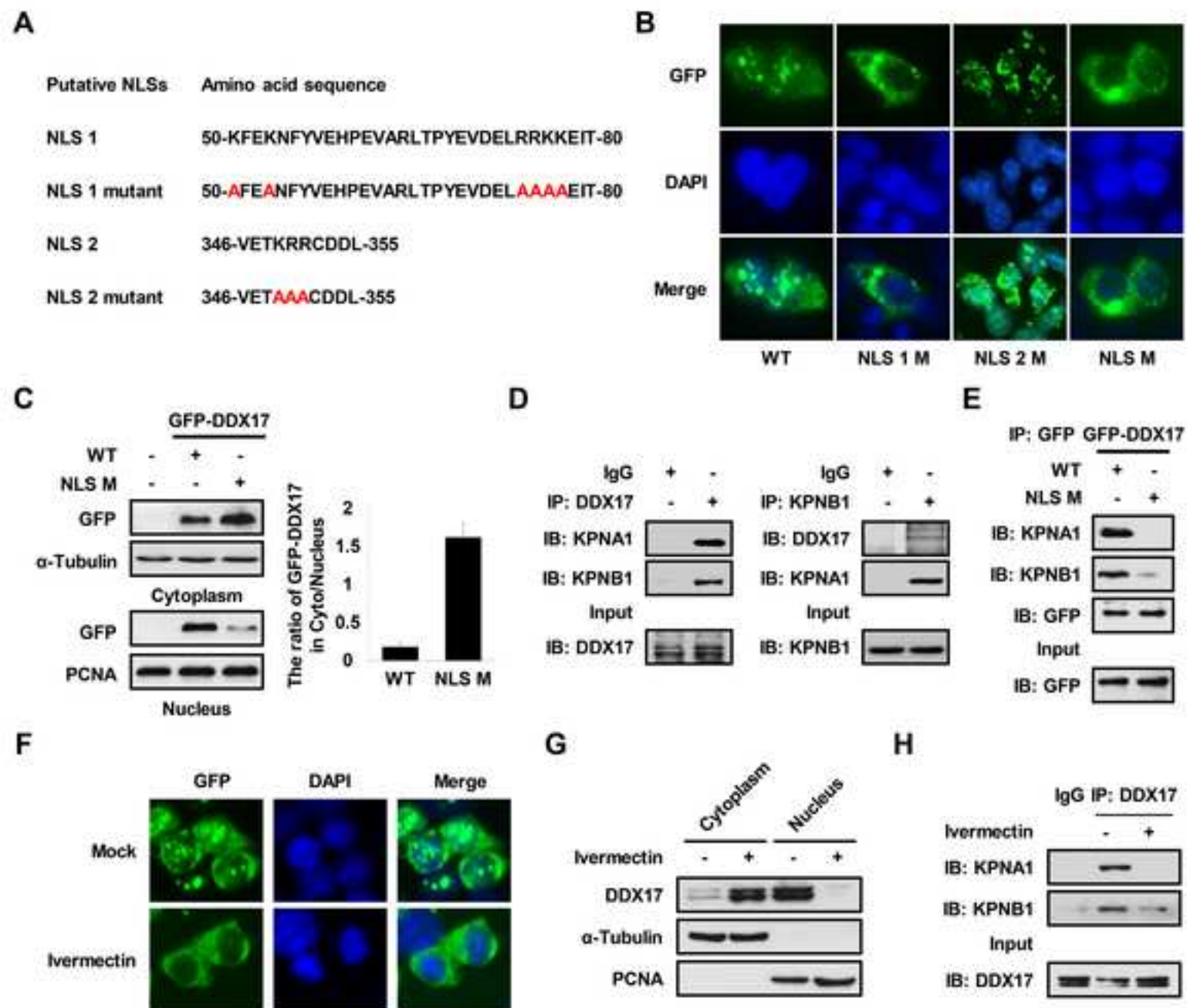


Figure 4

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

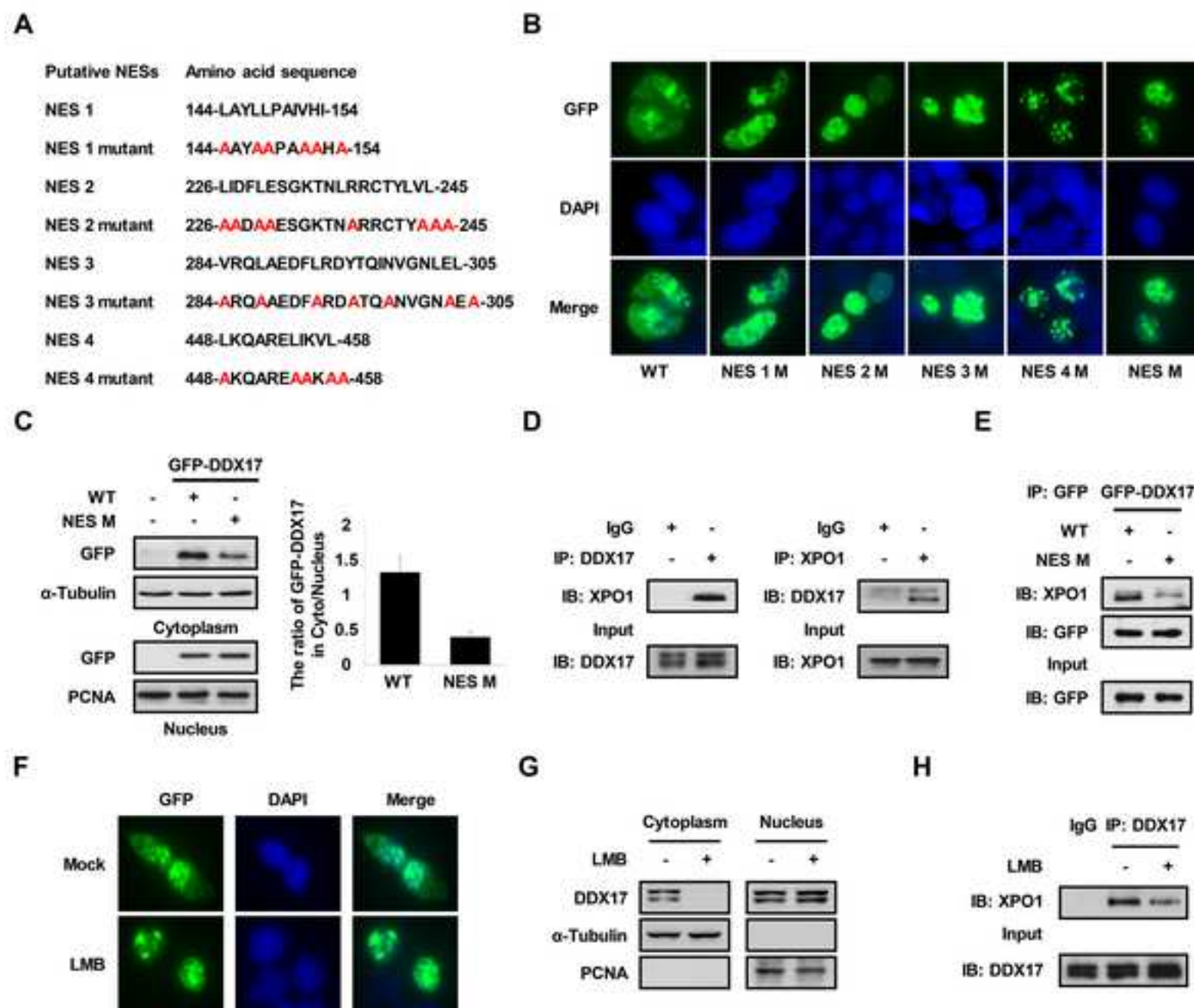


Figure 5
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Figure 5. Interruption of DDX17 nucleocytoplasmic shuttling impairs DDX17-mediated gefitinib resistance *in vitro* and *in vivo*.

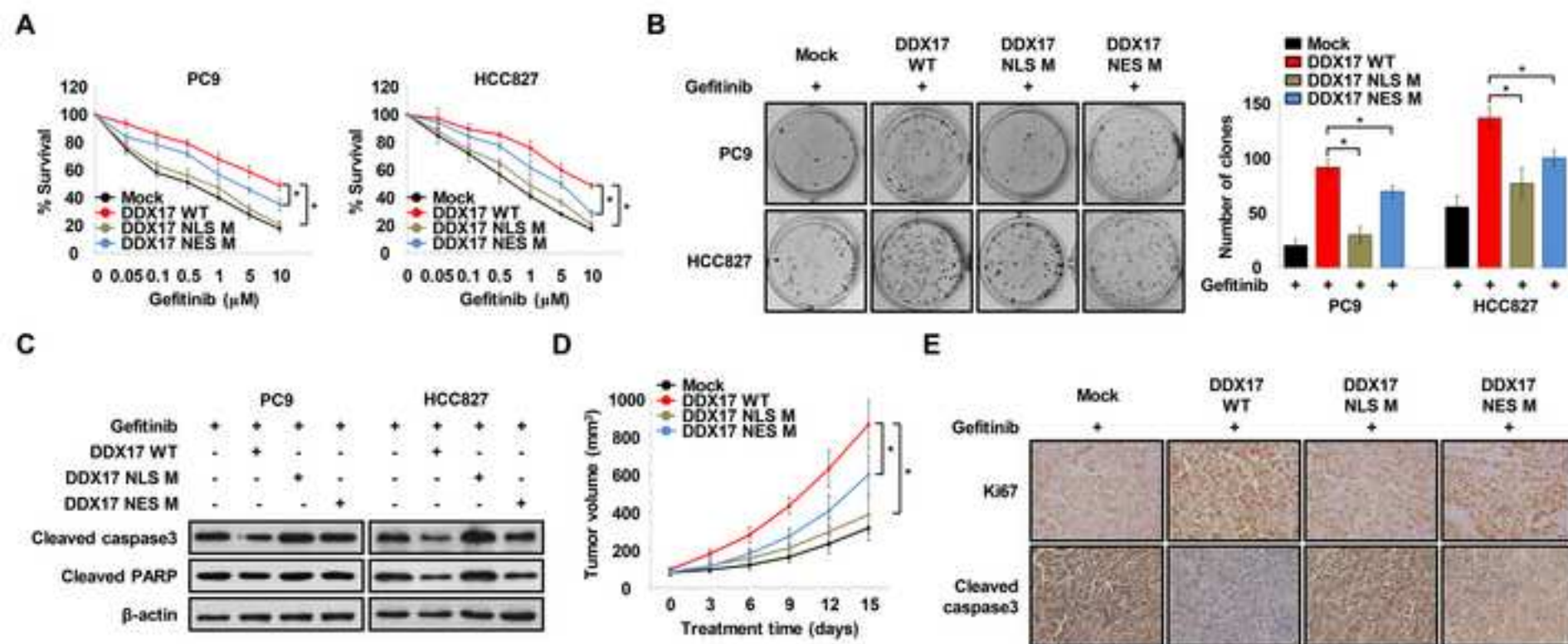
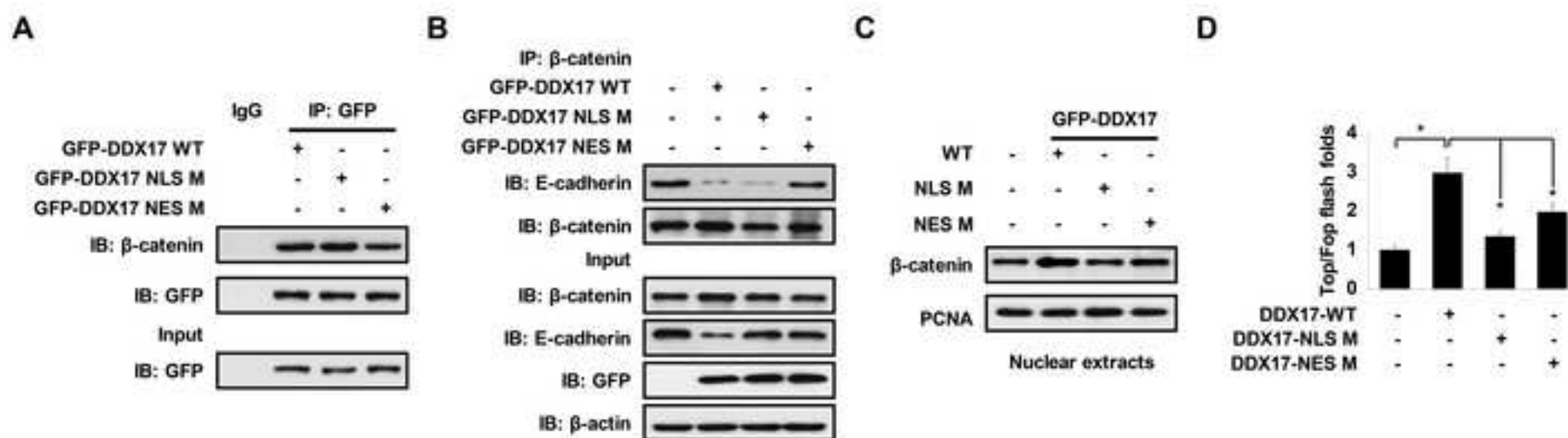


Figure 6. The integrity of DDX17 nucleocytoplasmic shuttling is essential for DDX17-mediated activation of β -catenin in NSCLC cells.



Supplementary Figure 1

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Conflicts of Interest

We confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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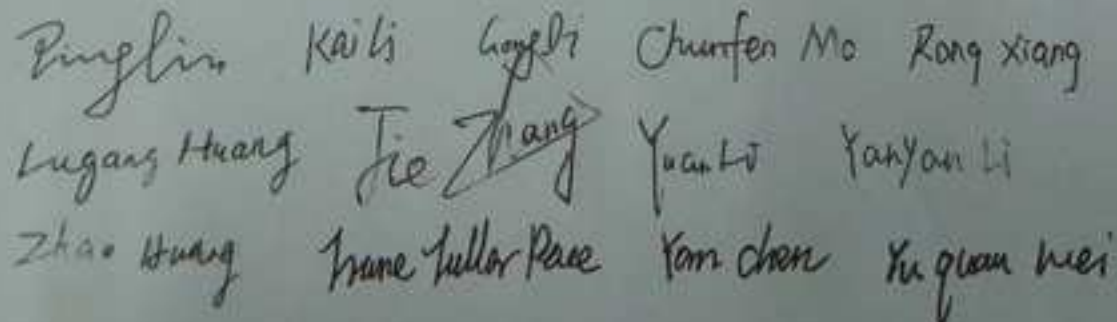
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1 **DDX17 nucleocytoplasmic shuttling promotes acquired gefitinib resistance in non-small**
2 **cell lung cancer cells via activation of β -catenin**

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17
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19
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22

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29 **Abstract**

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

47 **1. Introduction**

48 Lung cancer is one of the mostly common malignancy and the leading cause of
49 cancer-related deaths worldwide, with a five-year overall survival rate of only 15% [1]. Patients
50 with non-small cell lung cancer (NSCLC), which accounts for approximately 80% of all lung
51 cancer cases, are often diagnosed at advanced stages of the disease, leading to poor prognosis
52 in lung cancer patients [2, 3]. Recent studies have indicated that the epidermal growth factor
53 receptor (EGFR) signalling is frequently overexpressed or aberrantly activated in NSCLC and has
54 been as an attractive target for cancer therapy [4, 5]. Somatic mutations including in-frame
55 deletion mutation in exon 19 and the L858R mutation in exon 21 of the EGFR gene, are
56 associated with favorable response to the EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as
57 gefitinib and erlotinib [6-8]. Despite good initial responses to EGFR-TKIs, most lung
58 adenocarcinoma patients eventually develop resistance to anti-EGFR agents within 12 months
59 through development of a secondary mutation in EGFR that reduces its binding affinity for TKIs or
60 constitutive activation of downstream molecules to raise the compensatory survival signals [9, 10].
61 Overexpression or constitutive phosphorylation of HER3 can lead to significant resistance to
62 EGFR-TKIs by activation of downstream PI3K/AKT pathways, which is independent of EGFR
63 kinase activity [11, 12]. Moreover, abnormal activation of c-Met was significantly associated with
64 poor response to EGFR-TKIs treatment, regardless of the EGFR status in NSCLC patients [13,
65 14]. A randomized phase II trial has demonstrated that dual inhibition of EGFR and c-Met can
66 overcome resistance of EGFR-TKIs and improve outcomes in the MET-positive NSCLC patients
67 [15, 16]. However, the mechanisms responsible for intrinsic resistance and other acquired
68 resistance to EGFR-TKI are not fully understood.

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

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

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

93

94 **2. Materials and Methods**

95 *2.1 Cell culture and establishment of gefitinib resistant cell lines*

96 A431 and A431-GR cell lines were gifts from Prof. Zeng Cai (Sichuan Provincial People's
97 Hospital, China). A549 and A549-GR cell lines were gifts from Prof. Feng Bi (Sichuan University,
98 China). HCC827 and PC9 NSCLC cell lines were maintained at 37°C and 5% CO₂ in RPMI 1640
99 supplemented with 10% fetal bovine serum (Gibco) and 100 units/ml penicillin, and 100 mg/ml
100 streptomycin. To establish gefitinib resistant cell lines, parental cells were exposed to gradually
101 elevated concentrations of gefitinib for two months as reported previously [13, 22].

102

103 *2.2 Reagents*

104 Gefitinib and XAV-939 were purchased from Selleck chemicals, and recombinant human
105 EGF was purchased from PeproTech. The primary antibodies included AKT (Cell signalling
106 Technology, #4691), p-AKT (Ser473) (Cell signalling Technology, #4060), cleaved caspase-3 (Cell
107 Signaling Technology, #9664), cleaved PARP (Cell signalling Technology, #5625), DDX17 (Santa
108 Cruz Biotechnology, sc-271112), E-cadherin (Abcam, ab1416), EGFR (Cell signalling Technology,
109 #4267), p-EGFR (Tyr1068) (Cell signalling Technology, #3777), ERK (Cell signalling Technology,
110 #9102), p-ERK (Thr202/Tyr204) (Cell signalling Technology, #4370), Flag (Cell signalling
111 Technology, #8146), Ki67 (Abcam, ab15580), KPNA1 (Sangon Biotech, D154120), KPNB1
112 (Sangon Biotech, D161792), PCNA (Santa Cruz Biotechnology, sc-56), XPO1 (Sangon Biotech,

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

116

117 *2.3 Stable cell line generation for DDX17 knockdown or overexpression*

118 The DDX17 lentiviral expression vector was constructed by inserting expanded DDX17 cDNA
119 (NM_006386.4) fragments into a lentiviral shuttle vector. DDX17 knockdown was accomplished
120 using a specific shRNA targeting DDX17. The shRNA sequences were as follows: shRNA-DDX17,
121 5'-CAA GGG UAC CGC CUA UAC C-3'; shRNA-NC, 5'-TTC TCC GAA CGT GTC AGG T-3'. The
122 packing and purification of the recombinant lentiviral vector were performed by the GenePharma
123 Company (Shanghai, China). The indicated NSCLC cells infected with the recombinant lentiviral
124 vectors were selected with puromycin for 2 weeks.

125

126 *2.4 RNA extraction and real-time PCR*

127 Total RNA was extracted using RNAiso Plus (TAKARA) according to the manufacturer's
128 instructions. Real-time PCR was performed with SYBR[®] Premix Ex Taq[™] II (TAKARA) using an
129 iCycler iQ[™] Multicolor Real-Time Detection System (BIO-RAD) as previously described [23, 24].
130 The following primers were used: DDX17 forward 5'-GAACATCCGGAAGTAGCAAGG-3', reverse
131 5'-GATCCATCAACACATCCATTACATAT-3'; GAPDH forward 5'-ACCACAGTCCATGCCATCAC-3',
132 reverse 5'-TCCACCACCCTGTTGCTGTA-3'. The relative expression levels were determined
133 using Gene Expression Macro Version 1.1 software (BIO-RAD).

134

135 2.5 *TOP/FOP flash assay*

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

141

142 2.6 *In vivo assays for tumor growth*

143 Female BALB/c nude mice (6-week-old) were raised in specific pathogen-free conditions.
144 Animal care and experimental protocols were in accordance with guidelines established by the
145 Institutional Animal Care and Use Committee of Sichuan University. The indicated NSCLC cells
146 were suspended in 150 μ L serum-free DMEM and implanted subcutaneously into the right flanks
147 of nude mice. When subcutaneous tumor reached approximately 100 mm³, the tumor-bearing
148 mice were treated with gefitinib (10 mg/kg/day, by a gavage) for 15 days. The tumor volumes were
149 measured by calipers every 3 days, and calculated using the following formula: tumor volume
150 (mm³) = $\pi/6 \times \text{length} \times \text{width}^2$.

151

152 2.7 *Statistical analysis*

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

157

158 **3. Results**

159 *3.1 Upregulation of DDX17 correlates with gefitinib resistance in NSCLC cells*

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

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

185

186 *3.2 DDX17 disassociates the E-cadherin/ β -catenin complex and promotes β -catenin nuclear* 187 *translocation*

188 Recent evidence indicates that constitutive activation of Wnt/ β -catenin signalling is
189 associated with the acquired drug resistance to EGFR-TKIs in NSCLC [25-27]. To understand the
190 molecular mechanism by which DDX17 promotes gefitinib resistance, we explored the effect of
191 DDX17 on the nuclear translocation and activation of β -catenin. As shown in Figure 2A, β -catenin
192 was located primarily in the plasma membrane in Mock PC9 cells; however, upregulated DDX17
193 led to the nuclear accumulation of β -catenin. Next, we evaluated the phosphorylation status of
194 β -catenin. Consistently, phosphorylation of the residues that target β -catenin for proteasomal
195 degradation (S33/S37/T41) was reduced in response to DDX17 overexpression (Figure 2B).
196 Conversely, DDX17 knockdown significantly inhibited the levels of nuclear β -catenin in PC9-GR
197 cells (Figure 2C). EGF, a potent activator of Wnt/ β -catenin signalling as described previously
198 [28-30], promotes β -catenin nuclear translocation in PC9-GR cells, however, DDX17 deletion
199 markedly repressed the increased nuclear accumulation of β -catenin induced by EGF (Figure 2C).
200 TOP/FOP-Flash assay showed that down-regulation of DDX17 impaired the transcriptional

201 activity of β -catenin/T-cell factor (TCF) complex regardless of EGF treatment (Figure 2D).
202 Moreover, we found increased nuclear localization of β -catenin as well as decreased p- β -catenin
203 (S33/S37/T41) in PC9-GR cells compared with PC9 cells (Supplementary Figure S3).
204 Beta-catenin bound to the E-cadherin/catenin adhesion complex is mainly localizes to cell-cell
205 adherent junctions at membranes lacking Wnt signalling, and Wnt signalling promotes the
206 disassociation of E-cadherin/ β -catenin complex and subsequently β -catenin nuclear translocation.
207 Therefore, we next assessed whether DDX17 influenced E-cadherin/ β -catenin complex stability.
208 As shown in Figure 2E, overexpression of DDX17 downregulated E-cadherin/ β -catenin complex
209 formation, whereas knockdown of DDX17 augmented the association of β -catenin and E-cadherin.
210 Considering the role of DDX17 in the nuclear accumulation and activation of the β -catenin, we
211 next explored whether DDX17 can interact with β -catenin. Reciprocal immunoprecipitation studies
212 revealed that endogenous DDX17 bound to endogenous β -catenin directly (Figure 2F). Moreover,
213 increased interaction between DDX17 and β -catenin was observed in response to EGF stimulus,
214 whereas EGF repressed β -catenin binding to E-cadherin (Figure 2G). Notably, we found that
215 DDX17 predominantly interacted with β -catenin in the cytoplasm and that EGF enhanced both the
216 cytoplasmic and nuclear interaction of β -catenin with DDX17 in a time-dependent manner (Figure
217 2H). To further investigate whether DDX17-regulated gefitinib resistance involves β -catenin
218 activation, a specific Wnt/ β -catenin signalling pathway inhibitor (XAV-939) was used. XAV-939
219 treatment effectively reversed DDX17-induced gefitinib resistance in PC9 and HCC827 cells
220 (Figure 2I), leading to the increased levels of cleaved caspase 3 and cleaved PARP (Figure 2J).
221 These data indicate that elevated DDX17 level leads to release and nuclear translocation of
222 β -catenin from the E-cadherin/ β -catenin complex and thereby resulting in the activation of

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

224

225 3.3 *Two NLSs mediated DDX17 nuclear transport by an importin-dependent pathway*

226 Because DDX17 interacted with β -catenin in the cytoplasm and nucleus, we presumed that

227 DDX17 might be a nucleocytoplasmic shuttling protein. To test this hypothesis, we first examined

228 the sublocalization of DDX17 in PC9 and PC9-GR cells using an immunofluorescence assay.

229 Interestingly, DDX17 was predominantly localized to the nucleus in PC9 cells, whereas DDX17

230 was present in the cytoplasm and nucleus in PC9-GR cells (Supplementary Figure S2A).

231 Immunoblotting analysis also showed that more DDX17 was accumulated in both the cytoplasmic

232 and nuclear fractions of PC9-GR cells than of PC9 cells (Supplementary Figure S2B). These

233 results indicate that DDX17 may be a nucleocytoplasm shuttling protein.

234 Most nucleocytoplasm shuttling proteins carry sequence elements of both nuclear

235 localization signal (NLS) and (nuclear exporting signal) NES. We analysed the DDX17 amino acid

236 sequence and identified two putative NLSs based on cNLS Mapper analysis

237 (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (Figure 3A). Because the

238 classical NLSs rich in basic amino acids are known as NLSs recognized by importin, we

239 generated mutations in NLS1 (K50A, K53A, R74A, R75A, K76A and K77A) or NLS2 (K349A,

240 R350A and R351A) of DDX17 fused with a GFP fluorescent protein. As shown in Figure 3B, the

241 DDX17-WT protein showed both cytoplasmic and nuclear fluorescence. However, the NLS 1

242 mutant showed strong cytoplasmic fluorescence, and the NLS 2 mutant exhibited a complex

243 distribution in both the nucleus and cytoplasm (Figure 3B). Moreover, no significant nuclear

244 localization of DDX17 was observed in NLS mutant (NLS M, mutated both NLS 1 and NLS 2)

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

259

260 *3.4 Four NESs mediated DDX17 nuclear export by an exportin-dependent pathway*

261 For the classical nuclear export pathway, XPO1 binds directly hydrophobic residue-rich
262 NES sequence in the cargo protein and directs the export of the complex from the nucleus [33].
263 According to this theory, we found four putative NESs in DDX17 and constructed a series of site
264 directed mutagenesis fused to the C-terminus of GFP (Figure 4A). As shown in Figure 4B, both
265 cytoplasmic and nuclear localizations of DDX17-WT protein were observed in PC9 cells. The NES
266 1 mutant (L144A, L147A, L148A, I151A, V152A and I154A), NES 2 mutant (L226A, I227A, F229A,

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

283

284 *3.5 The integrity of DDX17 nucleocytoplasmic shuttling is indispensable for mediating the* 285 *acquired resistance and activation of β -catenin*

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

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

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

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

317

318 **4. Discussion**

319 EGFR-mutant NSCLC patients who benefited from EGFR-TKI eventually develop acquire
320 resistance to these therapies and the median duration of response is about 10 to 14 months [34,
321 35]. Although accumulating studies revealed that a variety of mechanisms can stimulate acquired
322 resistance to EGFR-TKI including secondary mutations within EGFR at position T790, activation
323 of parallel receptor tyrosine kinases (such as ALK, MET and RET), and mutation or upregulation
324 of EGFR effector proteins [36-38], the mechanisms responsible for acquired resistance to
325 EGFR-TKIs are still large unknown. In this study, we showed that DDX17 levels were increased in
326 gefitinib resistant cells compared with gefitinib sensitive cells. Overexpression of DDX17
327 significantly increased tolerance of PC9 and HCC827 cells in the present of gefitinib, whereas
328 DDX17 suppression resulted in reduced cell viability of gefitinib-resistant PC9-GR and
329 HCC827-GR cells. These data revealed that DDX17 expression was associated with tumor
330 sensitivity to gefitinib in NSCLC cells.

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

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

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

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

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

377

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382

383 **6. Conflict of Interest:**

384 The authors disclose no potential conflicts of interest.

385

386 **7. References**

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550

551 **8. FIGURE LEGENDS**

552 **8.1 Figure 1. Upregulated DDX17 is associated with gefitinib resistance in human NSCLC**

553 **cells.** (A) Quantitative real-time PCR and immunoblot analysis of DDX17 expression in gefitinib
554 sensitive and insensitive NSCLC cells. (B) Quantitative real-time PCR and immunoblot analysis
555 showed the successful lentiviral infections of DDX17 in PC9 and HCC827 cells. (C) Stably
556 expressing DDX17 or mock NSCLC cells were treated with 1 μ M gefitinib for indicated time and
557 analyzed for cell viability by MTT assay. (D) Effect of DDX17 overexpression on gefitinib efficacy
558 in HCC827 and PC9 cells was detected by MTT assay. (E) Representative photographs of the
559 colony formation of indicated NSCLC cells treated with gefitinib for 14 days after culture of cells.
560 (F) The indicated NSCLC cells were treated with or without gefitinib and then subjected to
561 immunoblot analysis using the indicated antibodies. Data represent the mean \pm standard
562 deviation (SD). Each experiment was performed at least in triplicate, producing consistent results.
563 * $P < 0.05$.

564

565 **8.2 Figure 2. DDX17 disassociates the E-cadherin/ β -catenin complex and promotes**

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

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

581

582 **8.3 Figure 3. Two NLSs mediated DDX17 nuclear transport by an importin-dependent**

583 **pathway.** (A) Putative sequence segments of DDX17 NLSs. (B) Representative of fluorescent
584 microscopy images show the localizations of the exogenously expressed DDX17 wild type (WT)
585 and putative NLSs mutants fused with GFP in PC9 cells. (C) The levels of exogenously expressed
586 DDX17 WT or NLS mutant in the extracts made from the cytoplasm or the nucleus of PC9 cells
587 were examined by immunoblotting analysis. PCNA and α -Tubulin were used as the control. (D)
588 The endogenous association of DDX17 and importin complex was detected by
589 immunoprecipitation with indicated antibodies. (E) The impact of NLS mutant on the interaction
590 between DDX17 and importin complex. (F) Representative of fluorescent microscopy images
591 show the sub-localizations of DDX17 in PC9 cells treated with or without ivermectin. (G) The
592 effect of Ivermectin on the distribution of DDX17 was performed by western blot analysis in PC9
593 cells. (H) The effect of Ivermectin on the binding of DDX17 and importin complex. Data represent
594 the mean \pm SD of three independent experiments.

595

596 **8.4 Figure 4. Four NESs mediated DDX17 nuclear export by an exportin-dependent pathway.**

597 (A) Putative sequence segments of DDX17 NESs. (B) Representative of fluorescent microscopy
598 images show the localizations of the exogenously expressed DDX17 WT and putative NESs
599 mutants fused with GFP in PC9 cells. (C) Immunoblotting for DDX17 in PC9 cytoplasmic and
600 nuclear extracts of cells transfected with the indicated plasmids. PCNA and α -Tubulin were used
601 as the control. (D) The endogenous association of DDX17 and XPO1 was detected by
602 immunoprecipitation with indicated antibodies. (E) The impact of NES mutant on the interaction
603 between DDX17 and XPO1. (F) Representative of fluorescent microscopy images show the
604 sub-localizations of DDX17 in PC9 cells treated with or without LMB. (G) The effect of LMB on the
605 distribution of DDX17 was performed by western blot analysis in PC9 cells. (H) The effect of LMB
606 on the binding of DDX17 and XPO1. Data represent the mean \pm SD of three independent
607 experiments.

608

609 **8.5 Figure 5. Interruption of DDX17 nucleocytoplasmic shuttling impairs DDX17-mediated**

610 **gefitinib resistance *in vitro* and *in vivo*.** (A) Indicated NSCLC cells were treated with varied
611 concentration of gefitinib, and the cell viability were analysed by MTT assay. (B) Representative
612 photographs of the colony formation of indicated NSCLC cells treated with gefitinib for 14 days
613 after culture of cells. (C) The indicated NSCLC cells were treated with gefitinib and then subjected
614 to immunoblot analysis using the indicated antibodies. (D) The indicated NSCLC cells were
615 transplanted into nude mice. When subcutaneous tumor reached approximately 100 mm³, the
616 tumor-bearing mice were treated with gefitinib (10 mg/kg/day, by a gavage) for 15 days. The

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

621

622 **8.6 Figure 6. The integrity of DDX17 nucleocytoplasmic shuttling is essential for**

623 **DDX17-mediated the activation of β -catenin in NSCLC cells.** (A) The impact of NLS mutant
624 and NES mutant on the interaction of DDX17 and β -catenin. (B) The effect of NLS mutant and
625 NES mutant on the association of E-cadherin/ β -catenin complex. (C) Immunoblotting for β -catenin
626 in the nuclear extracts of indicated PC9 cells. PCNA was used as the control. (D) TOP/FOP flash
627 assay were performed in the indicated PC9 cells. Data represent the mean \pm SD of three
628 independent experiments. * $P < 0.05$.