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YAP mediates resistance to EGF-induced apoptosis in *EGFR*-mutated non-small cell lung cancer cells

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ABSTRACT

Mechanisms underlying the growth and survival of non-small cell lung cancer (NSCLC) cells positive for activating mutations of the epidermal growth factor receptor gene (*EGFR*) have remained unclear. We here examined the functional relation between such mutant forms of EGFR and Yes-associated protein (YAP), a transcriptional coactivator of the Hippo signaling pathway that regulates cell proliferation and survival. Under the condition of serum deprivation, epidermal growth factor (EGF) induced activation of YAP in NSCLC cell lines positive for mutated *EGFR* but not in those wild type (WT) for *EGFR*. Similar EGF-induced activation of YAP was apparent in A549 lung cancer cells forcibly expressing mutant EGFR but not in those overexpressing the WT receptor. Furthermore, EGF induced apoptotic cell death in serum-deprived A549 cells overexpressing the WT form of EGFR but not in those expressing mutant EGFR, and knockdown of YAP rendered the latter cells sensitive to this effect of EGF. Our results thus suggest that activation of YAP mediates resistance of *EGFR*-mutated NSCLC cells to EGF-induced apoptosis and thereby contributes specifically to the survival of such cells.

1. Introduction

Non-small cell lung cancer (NSCLC) is responsible for most cases of lung cancer worldwide and is a leading cause of cancer-related mortality [1]. A substantial proportion of NSCLC tumors harbor activating mutations of the epidermal growth factor receptor (EGFR) gene, most prominently in exons 18 to 21 encoding the tyrosine kinase domain, including exon-19 deletions [2]. *EGFR*-mutated (MT) NSCLC shows different treatment responses and outcomes compared with NSCLC harboring wild-type (WT) *EGFR* alleles, including a poor response to treatment with immune checkpoint inhibitors and a higher risk of postoperative recurrence [3,4]. These differences suggest that *EGFR* mutations may activate specific proliferation and survival mechanisms, but such mechanisms have remained uncharacterized [5].

The Hippo pathway is an essential and evolutionarily conserved signaling pathway that regulates cell proliferation and apoptosis as well as the self-renewal of stem cells [6]. This pathway in mammals is composed of four core components: mammalian Ste20-like kinase (MST) 1/2, large tumor suppressor kinase (LATS) 1/2, Salvador 1 (SAV1), and Mps one binder kinase activator 1 (MOB1). Activation of these upstream molecules results in the phosphorylation and consequent sequestration in the cytoplasm of Yes-associated protein (YAP). Such cytoplasmic sequestration limits the transcriptional coactivator function of YAP, which is mediated through its binding to members of the TEA domain (TEAD) family of transcription factors. YAP has been implicated in tumorigenesis, and has been found to promote both cancer stemness and metastasis [7].

We previously revealed an association of MOB1 expression with early recurrence of early-stage NSCLC, including *EGFR*-MT cases [8]. We have now examined the possible role of YAP in *EGFR*-MT lung cancer. We found that *EGFR*-MT cells manifest YAP activation in response to epidermal growth factor (EGF) stimulation under the condition of serum deprivation, and that such activation contributes to resistance to EGF-induced apoptosis. Our study thus reveals a key role for YAP activation in promoting the survival of *EGFR*-MT lung cancer cells.

2. Materials and methods

2.1. Cell culture and reagents

HEK293T, BEAS-2B, A549, H1299, H1650, H2009, H4006, H1975, and HCC827 cell lines were obtained from American Type Culture

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Collection, PC9 and H322 from European Collection of Authenticated Cell Cultures, and II-18 from RIKEN BioResource Research Center. All cells were maintained under a humidified atmosphere of 5% CO_2 at 37 °C in RPMI 1640 medium or Dulbecco's modified Eagle's medium, each supplemented with 10% fetal bovine serum. Recombinant human EGF (Peprotech) was dissolved in phosphate-buffered saline containing 0.1% bovine serum albumin.

2.2. RNA extraction and RT-qPCR analysis

Total RNA was extracted from cells with the use of an RNeasy Mini Kit (Qiagen) and was subjected to reverse transcription (RT) with the use of PrimeScript RT Master Mix (Takara). Quantitative polymerase chain reaction (qPCR) analysis was performed in triplicate with the use of TB Green Premix Ex Taq II (Takara) and a Thermal Cycler Dice Real Time System (Takara) or CFX Connect R Real Time PCR System (Bio-Rad). The qPCR primers (forward and reverse, respectively) were as follows: YAP, 5′-TGACCCTCGTTTTGCCATGA-3′ and 5′-GTTGCTGCTGGTTGGAGTTG-3′; CTGF, 5′-GTTTGGCCCAGACCCAACTA-3′ and 5′-GGCTCTGCTTCTCTAGCCTG-3′; and 18S rRNA, 5′-ACTCAACACGGGAAACCTCA-3′ and 5′-AACCAGACAAATCGCTCCAC-3′. The abundance of each mRNA was normalized by that of 18S rRNA.

2.3. Immunoblot analysis

Cultured cells were lysed in RIPA buffer (Thermo Fisher Scientific), the lysates were fractionated by SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was incubated overnight at 4 °C with primary antibodies to phospho-YAP (S127), to YAP, to phospho-EGFR (Y1068), to EGFR, and to β-actin (Cell Signaling Technology). Immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies to rabbit immunoglobulin G (Cytiva), Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific), and a ChemiDoc Touch Imaging System (Bio-Rad).

2.4. Plasmid constructs for EGFR expression

The plasmid pBabe-EGFR WT was kindly provided by M. Meyerson (Addgene plasmid #11011). The coding sequence for WT human EGFR was amplified from this plasmid by PCR with PrimeSTAR GXL DNA Polymerase (Takara), and the PCR product was verified by sequencing and then ligated between the NotI and PacI sites of the pQCXIP retroviral vector (Clontech) with the use of an In-Fusion HD Cloning Kit (Clontech). An expression vector for EGFR with an exon-19 deletion (del19, E746–A750) was established as previously described [9].

2.5. Establishment of stable cell lines

The pQCXIP vectors encoding WT or del19 mutant forms of EGFR were introduced into HEK293T cells by transfection for 48 h with the use of the Lipofectamine 3000 reagent (Thermo Fisher Scientific). The culture supernatants were then passed through a 0.45-μm filter, incubated overnight at 4 °C with a Retro-X concentrator (Clontech), and centrifuged at 1500×*g* for 45 min at 4 °C for isolation of retrovirus pellets. A549 cells were infected for 6 h with the retroviruses in the presence of polybrene (Nacalai Tesque) at 4 μg/ml and were then cultured in complete growth medium overnight. The infected cells were then subjected to selection by culture in the presence of puromycin (Invivogen) at 1 mg/ml.

2.6. RNA interference

Cells were transferred to six-well plates for transient transfection with small interfering RNAs (siRNAs) mixed with the RNAiMAX reagent (Thermo Fisher Scientific). After transfection for 24 h, the cells were deprived of serum for 2 h and then stimulated with EGF. The sequences of the siRNA duplexes (Japan Bio Services) were 5′-GACAUCUUCUGGUCAGAGATT-3′ and 5′-UCUCUGACCAGAAGAUGUCTT-3′ for YAP, and 5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′ for a control.

2.7. Cell viability assay

Cells were transferred to 96-well plates (1×10^4 cells per well) and cultured for 24 h. They were then deprived of serum for 2 h before stimulation with EGF at various concentrations for 72 h. Cell viability was then determined with a Cell Counting Kit-8 (Dojindo).

2.8. Flow cytometric analysis

For detection of apoptosis, cells were stained with fluorescein isothiocyanate (FITC)–labeled Annexin V (Biolegend) at 0.45 μg/ml and with propidium iodide (Biolegend) at 5 μg/ml. The stained cells were analyzed with a FACSverse instrument (BD Biosciences) and FlowJo software version 10.9.

2.9. Statistical analysis

Quantitative data are presented as means ± SEM. Statistical analysis was performed with the use of GraphPad Prism 9 software. Data were compared between two groups with the unpaired Student's *t*-test or among more than two groups by one-way or two-way analysis of variance (ANOVA). A p value of <0.05 was considered statistically significant.

3. Results

3.1. YAP activation by EGF in EGFR-MT NSCLC cells

We first evaluated the expression of YAP in *EGFR*-WT and *EGFR*-MT human NSCLC cell lines as well as the nontumor cell line BEAS-2B. Immunoblot analysis revealed that the abundance of YAP tended to be higher in *EGFR*-MT cell lines than in *EGFR*-WT cell lines (Fig. 1). This observation suggested that EGF-EGFR signaling might affect YAP function preferentially in *EGFR*-MT cells. We therefore investigated the possible effect of EGF on YAP activity in serum-deprived *EGFR*-WT (H1299 and A549) and *EGFR*-MT (PC9 and HCC827) cell lines. Immunoblot analysis revealed that EGF reduced the level of inhibitory phosphorylation of YAP at Ser^{127} in PC9 and HCC827 cells, whereas it had no effect on such phosphorylation in H1299 and A549 cells (Fig. 2A). Consistent with these observations, RT-qPCR analysis revealed a robust EGF-induced increase in expression of the YAP target gene for connective tissue growth factor (CTGF) specifically in both *EGFR*-MT cell lines (Fig. 2B). These findings thus indicated that EGF activates YAP signaling in *EGFR*-MT lung cancer cells but not in *EGFR*-WT cells.

3.2. Forced expression of mutant EGFR in A549 cells confers sensitivity to EGF-induced YAP activation

To determine whether the observed difference in YAP activation in response to EGF stimulation between *EGFR*-MT and *EGFR*-WT cells was dependent on mutant EGFR, we established A549 cells (*EGFR*-WT) that express recombinant WT or del19 mutant forms of EGFR by retroviral infection. In the presence of serum, the abundance of YAP protein was greater in A549 cells expressing the exogenous WT or del19 mutant forms of EGFR than in control cells infected with the empty virus (Fig. 3A). In contrast, the amount of YAP mRNA did not differ among the three A549 cell lines (Fig. 3B), suggesting that increased stability of the protein rather than increased transcription was responsible for the increased abundance of YAP in the cells forcibly expressing WT or mutant EGFR. Furthermore, after serum deprivation, EGF stimulation

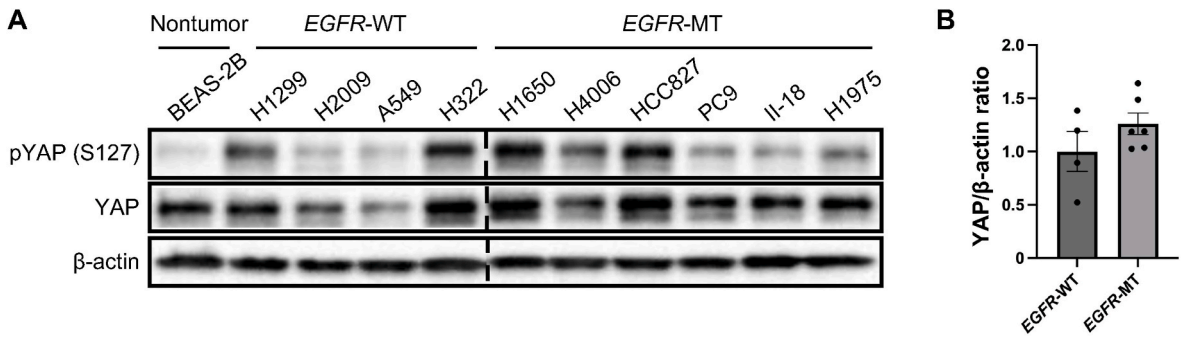

Fig. 1. YAP expression in NSCLC cell lines. **A**) Immunoblot analysis of Ser^{127} (S127)–phosphorylated (p) and total forms of YAP in *EGFR*-WT and *EGFR*-MT NSCLC cell lines and the nontumor cell line BEAS-2B cultured in the presence of serum. β-actin was examined as a loading control. **B**) The YAP/β-actin band intensity ratio determined for *EGFR*-WT and *EGFR*-MT cells as in **A**. Data are means ± SEM for the four and six cell lines, respectively.

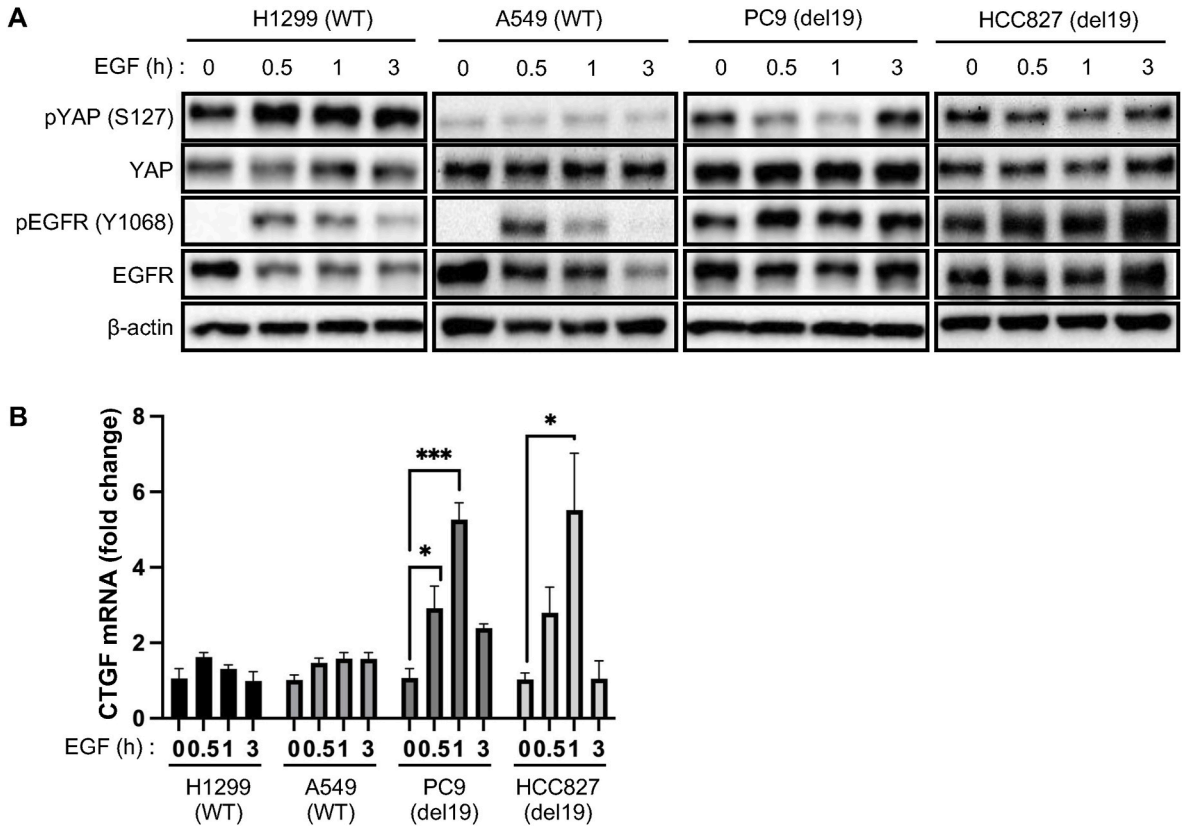

Fig. 2. EGF-induced activation of YAP in *EGFR*-MT cell lines. **A**) H1299, A549, PC9, and HCC827 cells were deprived of serum overnight and then stimulated with EGF (100 ng/ml) for 0–3 h. Cell lysates were then subjected to immunoblot analysis with antibodies to S127-phosphorylated or total forms of YAP, to Y1068-phosphorylated or total forms of EGFR, and to β-actin. **B**) RT-qPCR analysis of CTGF mRNA in cells treated as in **A**. Normalized data are expressed relative to the corresponding value for nonstimulated cells (time 0) and are means + SEM from three independent experiments. *$p < 0.05$, ***$p < 0.001$ (one-way ANOVA).

induced YAP activation in A549 cells expressing the del19 mutant of EGFR, as evidenced by decreased YAP phosphorylation, but not in those overexpressing the WT protein. (Fig. 3C). These data thus indicated that activating mutations of *EGFR* such as del19 render NSCLC cells sensitive to EGF-induced activation of YAP.

3.3. YAP protects cells expressing mutant EGFR from EGF-induced apoptosis

We further investigated the functional relevance of EGF-induced YAP activation in *EGFR*-MT lung cancer. Given that EGF stimulation has been found to induce apoptosis under certain conditions [10], we examined the effect of EGF on the viability of the established A549 cell lines forcibly expressing WT or del19 mutant forms of EGFR. Stimulation of serum-deprived cells with EGF reduced the viability of the cells expressing WT EGFR but not that of those expressing the mutant receptor (Fig. 4A). We then examined whether this effect was due to the induction of apoptosis. Flow cytometric analysis revealed that EGF increased the number of Annexin V-positive (apoptotic) cells for those expressing WT EGFR but not for those expressing the del19 mutant (Fig. 4B). Finally, we investigated the relation of YAP expression to cell survival. Knockdown of YAP with a specific siRNA resulted in an increase in the frequency of apoptosis for cells expressing either the WT or mutant form of EGFR, whereas EGF stimulation induced a significant increase in the number of apoptotic cells not only for the YAP-depleted cells expressing WT EGFR but also for those expressing the mutant

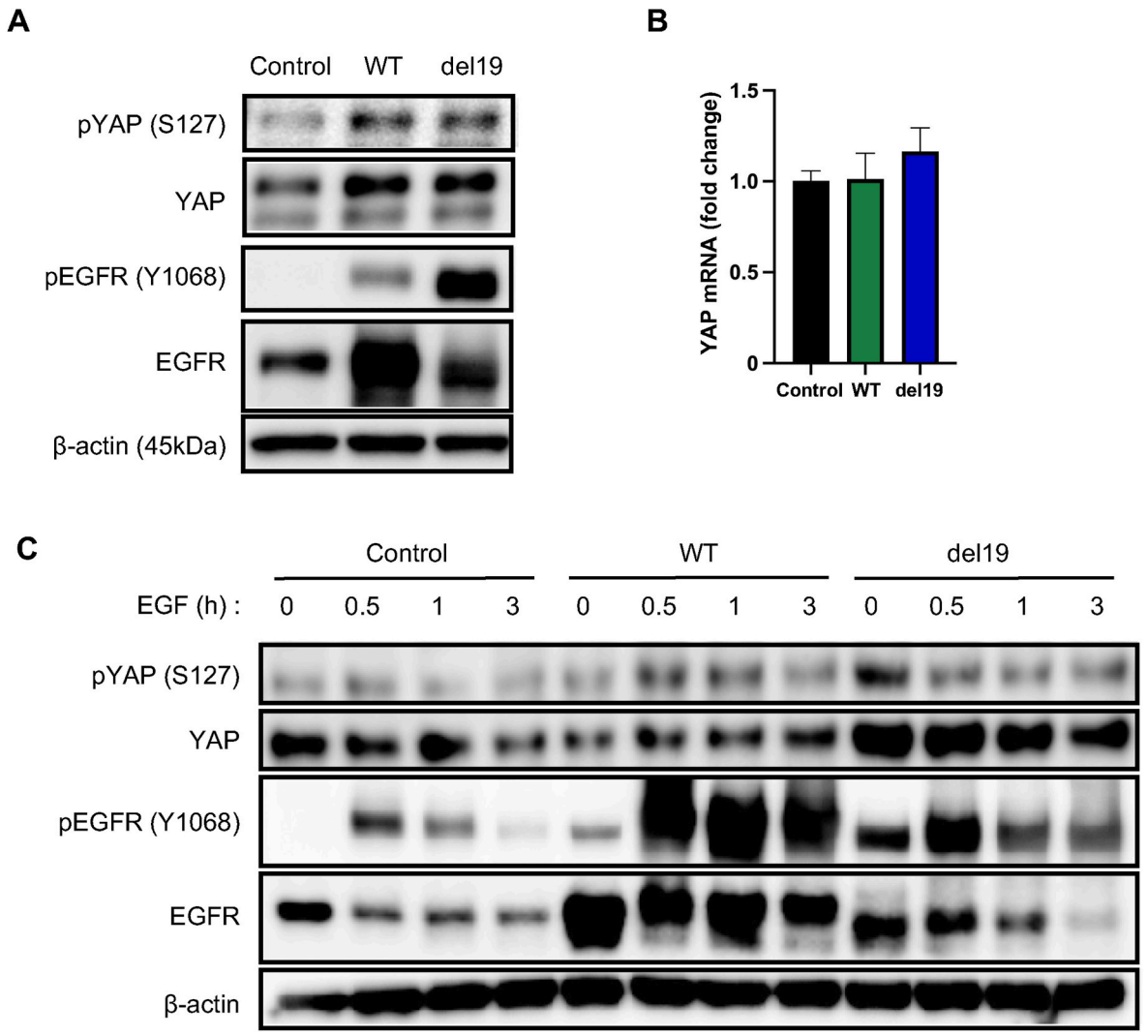

Fig. 3. Forced expression of mutant EGFR in A549 cells confers sensitivity to EGF-induced YAP activation. **A**) Immunoblot analysis of S127-phosphorylated and total YAP as well as of Y1068-phosphorylated and total EGFR in A549 cells infected with retroviruses encoding WT or del19 mutant forms of EGFR or with the empty virus, Control and maintained in the presence of serum. **B**) RT-qPCR analysis of YAP mRNA for cells as in **A**. Normalized data are expressed relative to the value for control cells and are means + SEM from three independent experiments. **C**) Immunoblot analysis as in **A** for cells that had been deprived of serum overnight and then stimulated with EGF (100 ng/ml) for the indicated times.

receptor (Fig. 4C). Two-way ANOVA revealed a significant interaction between EGF stimulation and YAP knockdown only for the cells expressing the mutant receptor, suggesting that the effects of this combination were simply additive for cells expressing WT EGFR but synergistic for those expressing the del19 mutant (Fig. 4D and Supplementary Table S1) [11]. These results thus implicated YAP in the resistance of *EGFR*-MT lung cancer to EGF-induced apoptosis.

4. Discussion

We have here shown that YAP expression tended to be higher in *EGFR*-MT than in *EGFR*-WT NSCLC cell lines cultured in the presence of serum, and that EGF stimulation induced YAP activation specifically in the former cell lines after serum deprivation. To examine whether this activation of YAP was dependent on activating mutation of *EGFR*, we established A549 cells (*EGFR*-WT) that stably express WT or del19 mutant forms of EGFR by retroviral infection. EGF induced activation of YAP only in the cells expressing the mutant form of the receptor. Finally, we found that this mutant form of EGFR conferred resistance to EGF-induced apoptosis, and that this resistance was dependent on the expression of YAP.

Activation of the EGFR signaling pathway was shown to lead to activation of YAP in *Drosophila* [12]. Such an effect is also conserved in humans, with similar observations having been made with the nontumor cell line MCF10A [13] as well as in various cancer types including cervical cancer [14] and hepatocellular carcinoma [15]. In the case of cervical cancer, stimulation of the EGFR pathway by growth factors such as transforming growth factor (TGF)-α or amphiregulin was shown to reduce the level of phosphorylation and activity of LATS1/2 and MOB1 and thereby to activate YAP, which in turn increased the expression of TGF-α, amphiregulin, and EGFR and thereby completed a positive feedback loop [14]. These previous analyses were performed with *EGFR*-WT cells, however, and the effects of *EGFR* mutations on the dynamics and function of YAP have remained unknown.

Activating mutations of *EGFR* in NSCLC result in persistent activation of downstream signaling by the RAS-mitogen-activated protein kinase (MAPK) pathway [16]. Activated RAS induces YAP activation through both Hippo pathway-dependent and -independent mechanisms. Activation of RAS results in the phosphorylation of Wilms tumor protein 1-interacting protein (WTIP), a member of the Ajuba protein family [12]. Phosphorylated WTIP binds to and thereby inhibits the activity of LATS1, an upstream suppressor of YAP, which results in YAP activation.

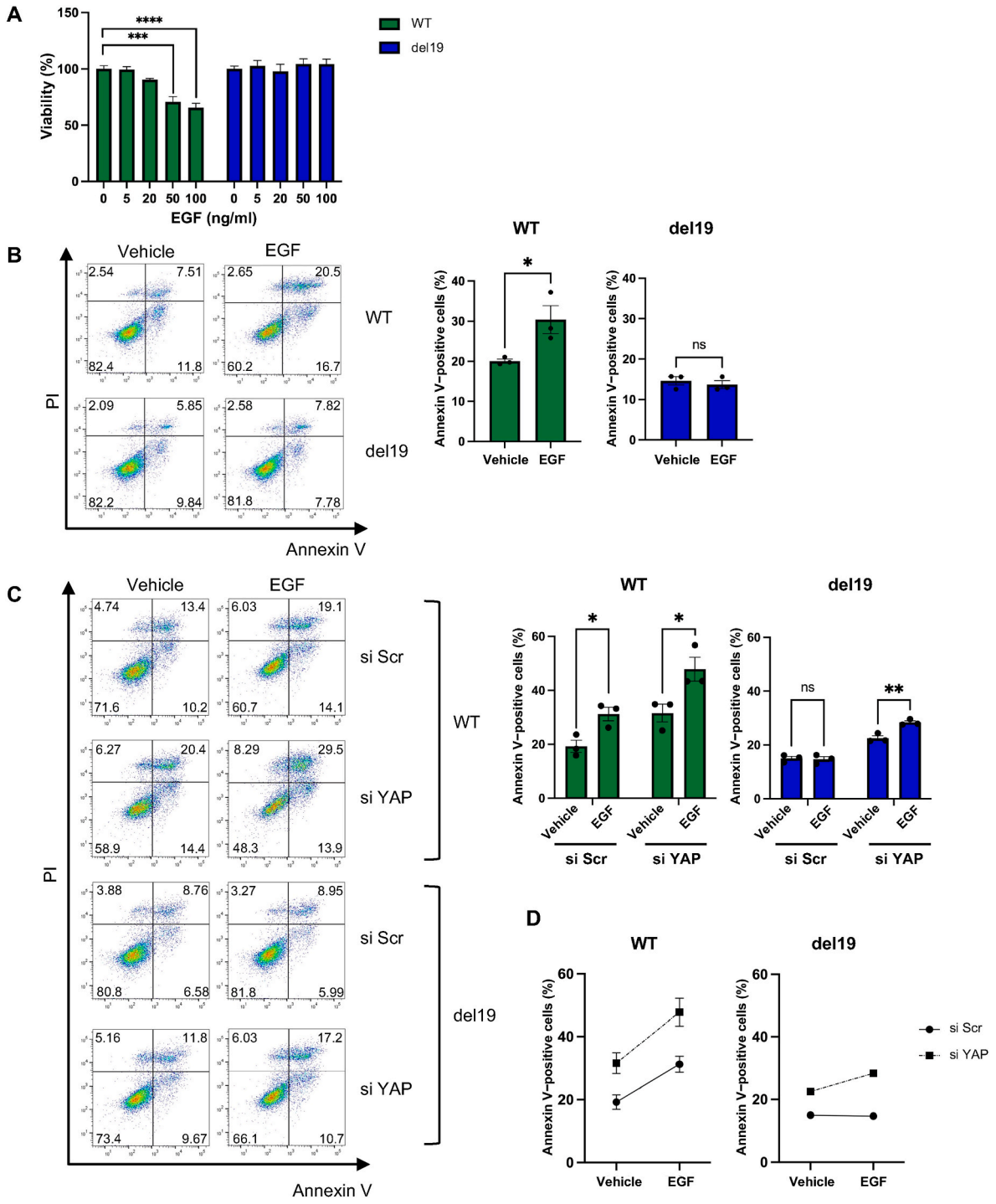

Fig. 4. YAP mediates resistance to EGF-induced apoptosis in NSCLC cells expressing mutant EGFR. **A**) Cell viability assay for A549 cells stably expressing WT or del19 mutant forms of EGFR that had been deprived of serum for 2 h and then stimulated with the indicated concentrations of EGF for 72 h. Data are means + SEM for three independent experiments. **B**) Cells as in **A** were deprived of serum for 2 h, treated with EGF (100 ng/ml) or vehicle for 48 h, and then stained with propidium iodide (PI) and Annexin V for flow cytometric analysis of apoptosis. Representative flow cytometry traces and quantitative data (means ± SEM) from three independent experiments are shown. **C**) Cells as in **A** were transfected with YAP or scrambled (Scr) siRNAs, deprived of serum for 2 h, treated with EGF (100 ng/ml) or vehicle for 48 h, and then subjected to flow cytometric analysis of apoptosis. Representative flow cytometry traces and quantitative data (means ± SEM) from three independent experiments are shown. **D**) The results in **C** are presented by the method of Slinker to reveal an additive effect by parallel lines (WT) and synergism by nonparallel lines (del19). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ by Student's *t*-test (**B** and **C**) or one-way ANOVA (**A**). ns, not significant.

On the other hand, independently of the Hippo pathway, the active form of RAS downregulates the expression of suppressor of cytokine signaling (SOCS)-box proteins including SOCS5/6 and thereby inhibits the ubiquitination and degradation of YAP, leading to YAP activation [17]. Our results now suggest that activating mutations of *EGFR* are essential for RAS-mediated YAP activation in NSCLC cells.

The differences in YAP dynamics between cells expressing WT or mutant forms of EGFR observed in our study were evident under the condition of serum deprivation. Serum deprivation induces stress in the tumor microenvironment and has been found to promote both tumor

invasion and tumor cell apoptosis [18]. The nutrient and energy depletion associated with serum deprivation limit tumor cell survival and proliferation, and proteins that promote cell proliferation such as YAP may need to be retrained under such conditions. Indeed, the activity of YAP has previously been shown to be inhibited by serum deprivation in *EGFR*-WT cancer cells [19]. We have now found that EGF-induced YAP activation was apparent in *EGFR*-MT but not in *EGFR*-WT lung cancer cells after serum deprivation. The detailed mechanism underlying YAP activation by EGF in serum-deprived cells harboring an activating mutation of *EGFR* is unclear, but our findings suggest that YAP is a key survival factor for *EGFR*-MT NSCLC.

Exposure to high (nanomolar) concentrations of EGF induces apoptosis in cells such as A431 epidermoid carcinoma cells [10], and we also observed such an effect in A549 lung cancer cells overexpressing WT EGFR. This response was previously shown to be dependent on tyrosine phosphorylation of signal transducer and activator of transcription 1 (STAT1) [10]. This paradoxical effect was also enhanced by exposure to a MAPK kinase (MEK) inhibitor such as trametinib, highlighting the importance of the balance between the MEK-MAPK and STAT1 signaling pathways in determination of cell fate between survival and apoptosis [20]. The resistance to EGF-induced apoptosis observed in lung cancer cells expressing mutant EGFR may therefore be due to the constitutive activation of RAS-MAPK signaling in these cells.

We found that the resistance to EGF-induced apoptosis in *EGFR*-MT NSCLC was dependent on YAP. Activated YAP directly promotes transcription of the genes for anti-apoptotic proteins such as Bcl-2 [21] and survivin [22], whereas it inhibits that of the gene for the pro-apoptotic protein Bcl-2–modifying factor (BMF) [23], thereby potentially contributing to the development of apoptosis resistance. Given that the proportion of apoptotic cells after YAP knockdown and EGF stimulation was still smaller for A549 cells expressing mutant EGFR than for those expressing the WT receptor, other factors also appear to contribute to apoptosis resistance in *EGFR*-MT cells—possibly including the pro-apoptotic protein Bcl-2–interacting mediator of cell death (BIM) [24] and the anti-apoptotic protein myeloid cell leukemia 1 (MCL1) [25], both of which are regulated by the RAS-MAPK pathway [26]. Our results suggest the possibility that, in the presence of EGF stimulation, RAS and YAP independently regulate apoptosis in *EGFR*-WT cells, whereas YAP may function downstream of activated RAS to inhibit apoptosis in *EGFR*-MT cells.

With regard to limitations of our study, we did not fully investigate the role of cross talk between survival and proliferative signals in the activation of YAP by EGF stimulation in *EGFR*-MT NSCLC under the condition of serum deprivation. The relation between the status of upstream Hippo pathway components as well as that of components of other pathways to YAP activation warrants further investigation. Second, a difference in the expression of WT and mutant forms of EGFR in the engineered A549 cells may have influenced the different responses of these cells to EGF stimulation. Finally, whereas we demonstrated resistance to EGF-induced apoptosis in *EGFR*-MT NSCLC, other types of cell stimulation or cell death (such as pyroptosis or necroptosis) were not examined.

In conclusion, we have shown that EGF activates YAP specifically in *EGFR*-MT lung cancer cells, and that YAP contributes to the resistance of such cells to EGF-induced apoptosis. Our findings provide a basis for further elucidation of the growth and survival mechanisms specific to *EGFR*-MT lung cancer and may contribute to the development of new therapeutic strategies.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kentaro Tanaka reports financial support was provided by Nippon Kayaku Co Ltd. Kentaro Tanaka reports financial support was provided by Nippon Boehringer Ingelheim Co Ltd. Kentaro Tanaka reports financial support was provided by Chugai Pharmaceutical Co Ltd. Kentaro Tanaka reports financial support was provided by Japan Society for the Promotion of Science. Isamu Okamoto reports a relationship with Chugai Pharmaceutical Co Ltd that includes: funding grants and speaking and lecture fees. Isamu Okamoto reports a relationship with AstraZeneca Pharmaceuticals LP that includes: funding grants and speaking and lecture fees. Isamu Okamoto reports a relationship with Daiichi Sankyo Co Ltd that includes: funding grants. Isamu Okamoto reports a relationship with Merck & Co Inc that includes: funding grants. Isamu Okamoto reports a relationship with Takeda Pharmaceutical Company Limited that includes: funding grants and speaking and lecture fees. Isamu Okamoto reports a relationship with Eli Lilly Japan KK that includes: funding grants and speaking and lecture fees. Isamu Okamoto reports a relationship with Ono Pharmaceutical Co Ltd that includes: funding grants and speaking and lecture fees. Isamu Okamoto reports a relationship with Bristol-Myers Squibb Co that includes: funding grants and speaking and lecture fees. Isamu Okamoto reports a relationship with Taiho Pharmaceutical Co Ltd that includes: funding grants and speaking and lecture fees. Isamu Okamoto reports a relationship with Nippon Boehringer Ingelheim Co Ltd that includes: funding grants and speaking and lecture fees. Isamu Okamoto reports a relationship with Novartis Pharmaceuticals that includes: speaking and lecture fees.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.09.067.

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