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Irreversible tyrosine kinase inhibitors induce the endocytosis and downregulation of ErbB2

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ABSTRACT

Erb-b2 receptor tyrosine kinase 2 (ErbB2) is an oncogene that frequently overexpressed in a subset of cancers. *Anti*-ErbB2 therapies have been developed to treat these types of cancers. However, less is known about how *anti*-ErbB2 drugs affect the trafficking and degradation of ErbB2. We demonstrate that the reversible and irreversible tyrosine kinase inhibitors (TKIs) differentially modulate the subcellular trafficking and downregulation of ErbB2. Only the irreversible TKIs can induce the loss of ErbB2 expression, which is not dependent on proteasome or lysosome. The irreversible TKIs promote ErbB2 endocytosis from plasma membrane and enhance the ErbB2 accumulation at endosomes. The endocytosis of ErbB2 is mediated by a dynamin-dependent but clathrin-independent mechanism. Blocking of ErbB2 endocytosis can impair the TKI-induced ErbB2 downregulation.

1. Introduction

The ErbB receptors are a family of receptor tyrosine kinases (RTKs) comprised by four members: the EGFR (also known as ErbB1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) [1]. These ErbB receptors are widely expressed in a variety of tissues and play fundamental roles in regulating cell growth, differentiation, survival, and migration [2]. The dysregulation of ErbB receptor-mediated signaling can lead to diseases such as developmental disorder and cancer [3–5]. The overexpression or overactivation of ErbB receptors, especially EGFR and ErbB2, has been found in many types of cancers. For example, EGFR amplification or mutations have been identified in head and neck, breast, lung, colorectal, prostate, kidney, pancreas, ovary, and brain cancers [6]. The amplification of ErbB2 gene has been found in more than 30% breast cancers [7–9]. Furthermore, ErbB2 amplification or mutations are also frequently happened in many different types of cancers such as pancreatic carcinomas [10], gastric [11,12], ovarian [13], colorectal [14], and lung cancers [15,16]. Elevated ErbB2 overexpression is associated with worse prognosis, and increased recurrence in head and neck squamous cell carcinoma (HNSCC) [17,18]. Therefore, EGFR and ErbB2 have been used as important drug targets to develop anti-cancer therapies [19,20].

The activation of ErbB family receptors must be tightly controlled in normal cells in order to prevent the overwhelming signaling that may cause cancer [21]. It is well established that the downregulation of EGFR is mediated by a series of highly organized membrane trafficking processes [22]. After activated by ligand binding, EGFR is quickly internalized from plasma membrane and accumulated at endosomes, and then sorted to lysosomes for final degradation [21]. However, ErbB2 is resistant to downregulation and the membrane trafficking pathway of ErbB2 are largely unknown [23]. Treatment of *anti*-EGFR drugs, including small molecular tyrosine kinase inhibitors (TKIs) and monoclonal antibodies, can affect EGFR membrane trafficking and regulate EGFR expression levels. The EGFR TKI gefinitib significantly decreases ligand-induced EGFR endocytosis [24]. EGFR monoclonal antibodies such as Cetuximab and necitumumab promote the endocytosis of EGFR, which causes the downregulation of EGFR levels [5,25–27]. Compared with EGFR, the effect of *anti*-ErbB2 drugs on ErbB2 trafficking and expression remains largely unknown.

Here, we reported that the irreversible TKIs could induce the endocytosis and accumulation of ErbB2 at endosomes, which is required for the downregulation of ErbB2 expression. The TKI-induced ErbB2

Abbreviations: ErbB2, Erb-b2 receptor tyrosine kinase 2; TKI, Tyrosine kinase inhibitor; RTK, Receptor tyrosine kinase; HNSCC, Head and neck squamous cell carcinoma; ERK1/2, Extracellular signal-regulated kinase 1/2; LAMP1, Lysosomal Associated Membrane Protein 1.

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endocytosis is mediated via a dynamin-dependent but clathrin-independent manner.

2. Material and methods

2.1. Cell culture

Head and neck squamous cell carcinoma (HNSCC) cell lines UMSCC1 (University of Michigan-Squamous Cell Carcinoma-1) and UMSCC22B (University of Michigan-Squamous Cell Carcinoma-22B) were derived in the lab of Dr. Thomas Carey at the University of Michigan. UMSCC1 and UMSCC22B cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1× Non-Essential Amino Acids Solution (Gibco). The breast cancer cell line MDA-MB-453 (MD Anderson-Metastatic Breast-453, from American Type Culture Collection) cells were cultured in DMEM supplemented with 10% FBS.

2.2. Reagents

ErbB receptors TKIs Gefitinib, Erlotinib, Dacomitinib, Afatinib, TAK165, and Lapatinib were purchased from Selleckchem (Houston, TX). Dimethyl Sulfoxide (DMSO), Chloroquine, PitStop2, and ALLN were from MilliporeSigma (Rockville, MD). Dyngo-4a was from Selleckchem (Houston, TX). Antibodies to EGFR, phosphor-EGFR (Tyr1068), phosphor-ErbB2 (Tyr1196), total AKT, phosphor-AKT, total ERK1/2, phosphor-ERK1/2, LAMP1, and Rab7 were from Cell Signaling Technology (Danvers, MA). Antibodies to ErbB2 and Tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

2.3. Western blot

Lysates were prepared from the indicated cell lines and different treatments by using the RIPA Lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium desoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_3, 1 mM EGTA). 40 μg of protein lysates were separated using 8% SDS-Polyacrylamide Gel Electrophoresis. Gels were subjected to electro blotting on PVDF membrane. Membranes were blocked with 5% Non-fat milk in PBST (1× Phosphate-Buffered Saline, 0.1% Tween® 20 Detergent) for 1hr at room temperature (RT) and then probed with the primary antibody (1:2000) at 4 °C overnight. The membrane was washed with PBST and incubated with secondary antibody (1:4000) for 1 h at RT. The blots were visualized by enhanced chemiluminescence using Syngene G-box (Imgen Technologies, USA). The blots were quantified via ImageJ with at least three independent experiments.

2.4. Real time PCR

Total RNA from UMSCC1 cells (~2 × 10^6) was extracted by using IsolateII RNA mini Kit (Bioline, USA) following the manufacturer's protocol. This was followed by cDNA synthesis using the cDNA Reverse transcription kit (Thermofisher Scientific, USA). The expression of ErbB2 was analyzed by quantitative real-time PCR using SYBR Green (Power-UP SYBR-Green PCR Master Mix; Applied Biosystems, USA) in Quantstudio 3 Real-time PCR system (Applied Biosystems, USA). All the reactions were performed in triplicate and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as endogenous control in this study and mRNA fold changes were calculated using the $2^{-\Delta\Delta CT}$ value [28]. The specific primer sequences used for GAPDH were as follows: 5′-GTCTCCTCTGACTTCAACAGCG-3' (Forward), and 5′-ACCACCC TGTTGCTGTAGCCAA-3' (Reverse). Primer sequences used for ErbB2 were as follows: 5′-GGAAGTACACGATGCGGAGACT-3' (Forward), and 5′-ACCTTCCTCAGCTCCGTCTCTT-3' (Reverse).

2.5. Immunofluorescence

Cells were seeded on coverslips. After treated with different drugs, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. Cells were then blocked with 3% BSA in PBS for 1hr at room temperature and incubated with primary antibodies overnight at 4 °C, washed two times with 0.1% Triton X-100 in PBS, incubated with fluorescence-labeled secondary antibodies for 1hr at room temperature. Cells were then incubated with DAPI for 1 min to stain the cell nucleus and washed three times with 0.1% Triton X-100 in PBS. Cells were examined under a Zeiss LSM 710 laser scanning fluorescence confocal microscope with a 63× NA Plan-Apochromat lens. Images were acquired and processed using ZEN 2012 software (Carl Zeiss Microimaging) and ImageJ (NIH).

2.6. Statistics

All data analysis was performed using Prism (GraphPad). Bar graphs represent means ± SEM., as indicated. Statistical significance was assessed using the Student *t*-test.

3. Results

3.1. Reversible and irreversible TKIs differentially induce the loss of ErbB2 expression

ErbB family receptors have been used as therapeutic targets to develop drugs to treat cancers such as HNSCC, breast cancer, lung cancer, and colorectal cancer [29,30]. Some clinical trials are ongoing to test the effects of different ErbB TKIs in the treatment of HNSCC [31,32]. However, it is not clear how these TKIs affect the ErbB receptors subcellular trafficking and degradation. To compare the effects of different TKIs on ErbB2 or EGFR expression, a HNSCC cell line UMSCC1 was used. Among these TKIs used, Gefitinib and Erlotinib are reversible TKIs that can specifically inhibit EGFR activation. Afatinib and Dacomitinib are irreversible TKIs that can efficiently inhibit both EGFR and ErbB2 activation. As shown in Fig. 1A, the reversible TKIs Gefitinib and Erlotinib dramatically decreased the activation of EGFR (indicated by levels of EGFR Y1068 phosphorylation). These two TKIs had less effect on inhibiting ErbB2 activation (indicated by levels of ErbB2 Y1196 phosphorylation). The irreversible TKIs Afatinib and Dacomitinib strongly inhibited both EGFR and ErbB2 activation. All the TKIs similarly downregulated the ErbB downstream Extracellular signal-regulated kinase 1/2 (ERK1/2) activation (indicated by levels of ERK1/2 Thr202/Tyr204 phosphorylation). The irreversible TKIs showed stronger effect on inhibiting downstream AKT activation (indicated by levels of AKT Ser 473 phosphorylation) compared with reversible TKIs. These results suggest that TKIs differentially modulate ErbB downstream signaling. None of these TKIs affected the EGFR protein levels. The irreversible TKIs significantly decreased ErbB2 protein levels (Fig. 1A–B). A real time PCR approach was used to test whether the treatment of irreversible TKIs can affect the ErbB2 mRNA levels. The Dacomitinib- or Afatinib-treatment did not significantly change the mRNA levels of ErbB2 (Fig. 1C). It suggests that Dacomitinib or Afatinib can induce ErbB2 protein degradation, which is independent on the change of ErbB2 mRNA levels.

To test whether the effect of Afatinib or Dacomitinib on ErbB2 expression is cell line specific, another HNSCC cell line UMSCC22B was used. As ErbB2 is overexpressed in a subtype of breast cancers [9], a breast cancer cell line with high levels of ErbB2 expression, MDA-MB-453, was also used to test the effects of TKIs on ErbB2 expression. Similarly as in UMSCC1 cells, Afatinib and Dacomitinib significantly decreased the ErbB2 protein levels in both UMSCC22B (Fig. 1D–E) and MDA-MB-453 cells (Fig. 1F–G). Gefitinib and Erlotinib had no effect to downregulate ErbB2 expression (Fig. 1E–G). As Gefitinib

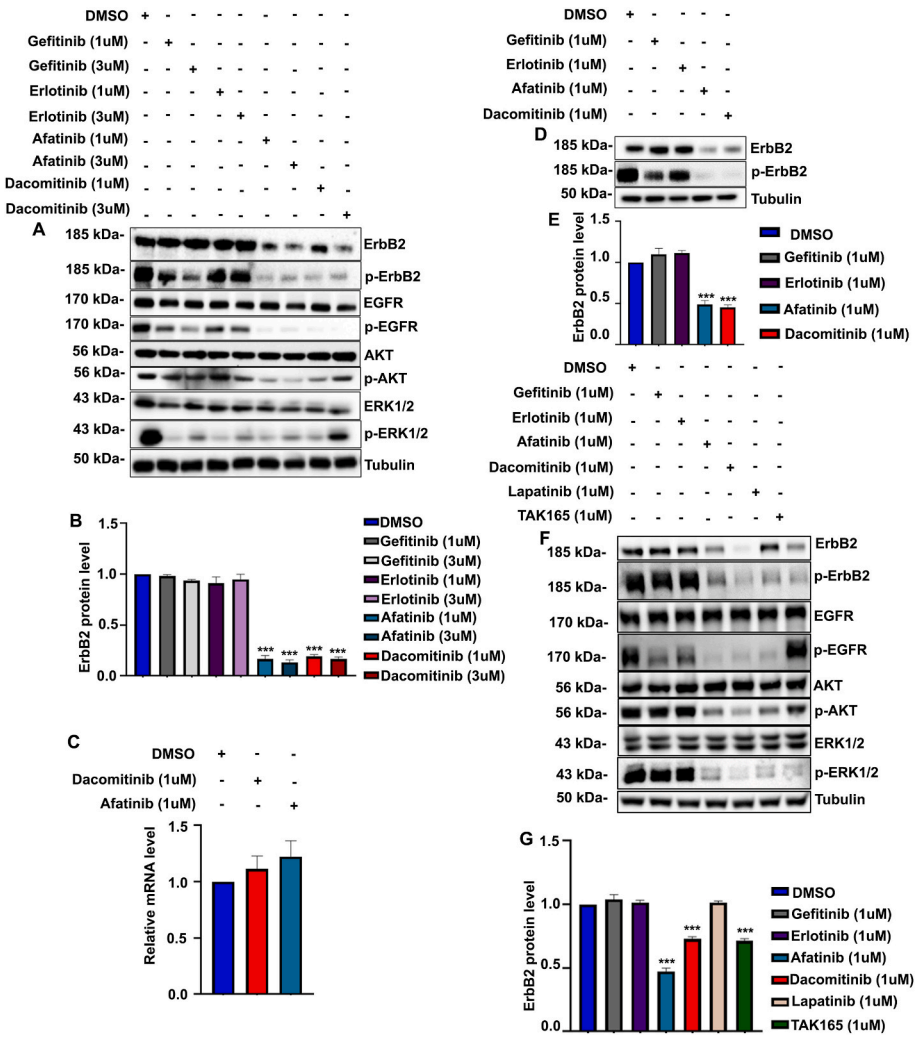

Fig. 1. Irreversible ErbB2 TKIs induce the down-regulation of ErbB2. UMSCC1 cells were treated with vehicle control (DMSO, 1:1000) or four different TKIs at the concentrations as indicated for 6 h, and then the indicated protein levels were detected by Western blot (A) and the ErbB2 levels were quantified (B). UMSCC1 cells were treated with vehicle control (DMSO 1:1000), Dacomitinib (1 μM), or Afatinib (1 μM) for 6 h and the ErbB2 mRNA levels were quantified by Real-time PCR (C). UMSCC22B cells were treated with vehicle control (DMSO 1:1000) or different TKIs as indicated, and then the indicated protein levels were detected by Western blot (D) and the ErbB2 levels were quantified (E). MDA-MB-453 cells were treated with vehicle control (DMSO 1:1000), or different TKIs as indicated, and then the indicated protein levels were detected by Western blot (F) and the ErbB2 levels were quantified (G). Error bars indicate mean ± SEM from three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

and Erlotinib are reversible TKIs mainly targets EGFR, we used Lapatinib, a reversible TKI that can target both EGFR and ErbB2, as a control. Although Lapatinib can dramatically block both EGFR/ErbB2 activation and inhibit downstream ERK/AKT activation, it cannot induce significant change of ErbB2 protein levels (Fig. 1F–G). These results suggest that only irreversible TKIs can induce the reduction of ErbB2 protein levels. TAK-165 is an irreversible TKI that specifically targets ErbB2. Treatment of TAK-165 only blocked ErbB2 activation but not EGFR activation (Fig. 1F). TAK-165 can decrease the ErbB2 expression similarly as Afatinib and Dacomitinib (Fig. 1F–G). This suggests that the co-inhibition of both EGFR and ErbB2 is not required for loss of ErbB2 expression. In MDA-MB-453 cells, Gefitinib and Erlotinib cannot block ERK1/2 and AKT activation (Fig. 1F). This may because MDA-MB-453 cells have high ErbB2 expression and the ERK1/2 and AKT activation are more dependent on ErbB2 but not EGFR.

3.2. The loss of ErbB2 expression is not dependent on lysosome or proteasome

To determine whether the loss of ErbB2 protein is mediated by lysosome or proteasome, UMSCC1 cells were pretreated with lysosome inhibitor chloroquine or proteasome inhibitors Velcade and Pr-171. As shown in Fig. 2A–B, none of these inhibitors can reverse the loss of ErbB2 in Dacomitinib-treated cells. These results suggest that the loss of ErbB2 is not dependent on lysosome or proteasome. The benzoquinoid ansamycin antibiotics geldanamycin can induce ErbB2 degradation by proteolytic fragmentation, which is blocked by proteases inhibitor ALLN [33,34]. However, the treatment of ALLN cannot rescue ErbB2 levels in Dacomitinib-treated cells (Fig. 2C–D). This suggests that Dacomitinib and geldanamycin induce the loss of ErbB2 via distinct mechanisms.

3.3. Irreversible TKI induces the accumulation of ErbB2 at endosomes

The trafficking of ErbB receptors is critical for regulation of their activation and degradation [21]. To test the effects of TKIs treatment on ErbB2 trafficking, the subcellular location of ErbB2 was monitored by immunofluorescence (IF). Before TKIs treatment, most ErbB2 were found on the plasma membrane (Fig. 3A). The treatment of irreversible TKI Dacomitinib significantly decreased the levels of ErbB2 on plasma membrane (Fig. 3A–B). At the same time, the colocalization of ErbB2 with endosome marker Lysosomal Associated Membrane Protein 1 (LAMP1) (Fig. 3A–C) or Rab7 (Fig. 3D–E) was dramatically increased. This suggests that Dacomitinib treatment can promote ErbB2 endocytosis from plasma membrane and increase its translocation to endosomes. The treatment of reversible TKI Lapatinib had no effect on the membrane levels of ErbB2 (Fig. 3A–B). Furthermore, Lapatinib treatment cannot enhance the colocalization of ErbB2 with endosome marker LAMP1 (Fig. 3A–C) or Rab7 (Fig. 3D–E). These results are consistent with that only Dacomitinib but not Lapatinib can induce the down-regulation of ErbB2 expression (Fig. 1F). These results indicate the different effects of Dacomitinib and Lapatinib on ErbB2 subcellular trafficking.

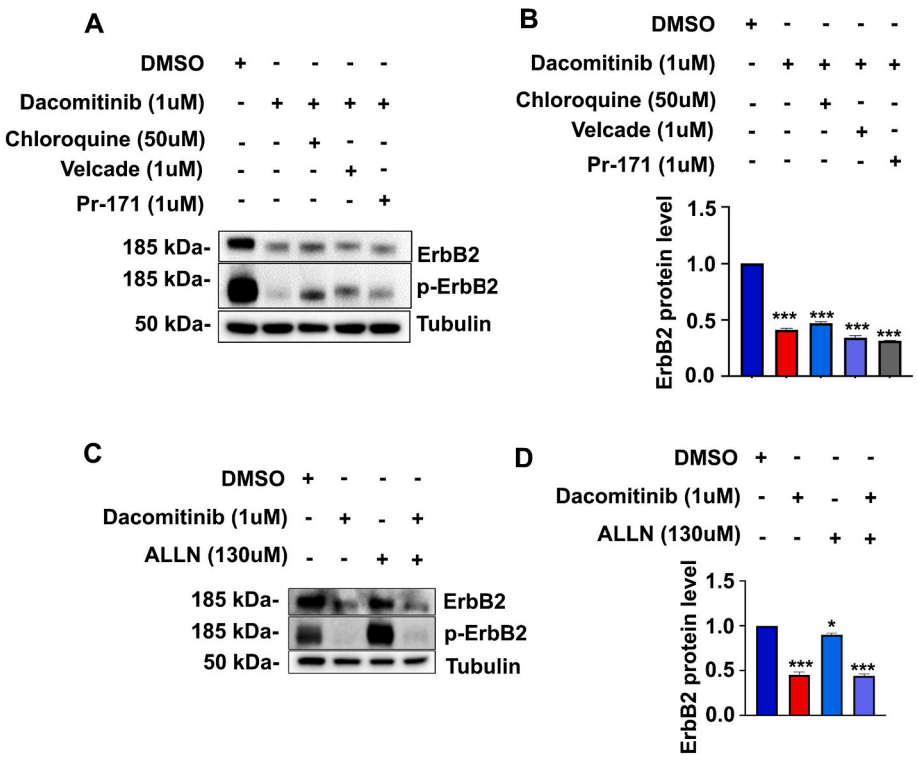

Fig. 2. Irreversible TKI-induced ErbB2 downregulation is not dependent on lysosome or proteasome. UMSCC1 cells were treated with vehicle control (DMSO 1:1000), Dacomitinib (1 μM), combination of Dacomitinib with Chloroquine (50 μM), Velcade (1 μM), or Pr-171 (1 μM) as indicated for 6 h, and then the ErbB2 and *p*-ErbB2 protein levels were detected by Western blot (A) and the ErbB2 levels were quantified (B). UMSCC1 cells were treated with vehicle control (DMSO 1:1000), Dacomitinib (1 μM), ALLN (130 μM) and their combination as indicated for 6 h and then the protein levels of ErbB2 and *p*-ErbB2 were measured by Western blot (C) and the ErbB2 levels were quantified (D). Error bars indicate mean ± SEM from three independent experiments. $^{***}P < 0.0001$.

3.4. Block of ErbB2 endocytosis impairs the TKI-induced ErbB2 downregulation

As the irreversible TKI Dacomitinib can induce ErbB2 endocytosis and the downregulation of ErbB2 expression, endocytosis inhibitors were used to determine whether the Dacomitinib-induced loss of ErbB2 is dependent on endocytosis. Dyngo-4a is a dynamin inhibitor that can block dynamin-dependent endocytosis. The effects of Dyngo-4a treatment on subcellular location of ErbB2 was examined by IF. Dyngo-4a prevented the Dacomitinib-induced loss of ErbB2 from plasma membrane (Fig. 4A and B). Dyngo-4a also blocked the Dacomitinib-induced ErbB2 colocalization with endosome marker LAMP1 (Fig. 4A and C) or Rab7 (Fig. 4D and E). These results indicate that the dynamin function is required for TKI-induced ErbB2 translocation to endosomes. Pitstop2 is a clathrin inhibitor that inhibits clathrin-mediated endocytosis (CME). Pitstop2 cannot block the Dacomitinib-induced loss of ErbB2 from plasma membrane (Fig. 4A and B). Pitstop2 also did not block the Dacomitinib-induced ErbB2 colocalization with endosome marker LAMP1 (Fig. 4A and C) or Rab7 (Fig. 4D and E). These results suggest that TKI-induced ErbB2 endocytosis and endosomal accumulation are not dependent on clathrin. Furthermore, treatment of Dyngo-4a but not Pitstop2 can impair the Dacomitinib-induced ErbB2 downregulation (Fig. 4F and G). This suggests that the TKI-induced loss of ErbB2 is dependent on dynamin-mediated but clathrin-independent endocytosis.

4. Discussion

Unlike the other ErbB family RTKs, ErbB2 has no naturally occurring ligands [35,36]. Therefore, the activation of ErbB2 depends on its formation of heterodimeric complexes with other ErbB family RTK members [37–39]. Compared with other ligand-binding ErbB members, ErbB2 can bind a broader subset of downstream effector proteins and functions like an amplifier in the ErbB signaling network [40,41]. Therefore, ErbB2 is a special member of the ErbB RTKs family. The mechanisms controlling ErbB2 subcellular trafficking and degradation are still in a mystery. Our current research provides a novel mechanism: irreversible TKIs can induce the endocytosis and accumulation of ErbB2 at endosomes, which is required for the downregulation of ErbB2.

The downregulation of EGFR and ErbB2 are mediated by different membrane trafficking mechanisms. Agonist-induced phosphorylation of EGFR significantly promotes its internalization, which is dependent on clathrin-mediated endocytosis [21]. Then EGFR will be sorted from endosome to lysosome for degradation. The TKIs treatment blocks EGFR activation and prevents the endocytosis of EGFR [24]. Unlike EGFR, the activation or phosphorylation of ErbB2 does not promote its endocytosis or degradation [23]. The treatment of irreversible TKIs inhibits ErbB2 phosphorylation (Fig. 1) and promotes the endocytosis and accumulation of ErbB2 at endosomes (Fig. 3).

Different molecular mechanisms have been reported to induce the endocytosis and degradation of ErbB2. The chaperone protein HSP90 binds to ErbB2, which is required to stabilize ErbB2 expression at plasma membrane [23,42]. The HSP90 inhibitor geldanamycin can induce the down-regulation of ErbB2 from the plasma membrane by endocytosis in a clathrin dependent manner [43]. Another research reported that PMA-induced PKC activation could induce ErbB2 endocytosis and sorting to endocytic recycling compartment in a clathrin independent manner [44]. Geldanamycin can induce the degradation of ErbB2 [43]. However, PMA-induced PKC activation cannot lead to the ErbB2 degradation [44]. Our current research suggests that the mechanisms by which TKIs induce ErbB2 downregulation is distinct compared with geldanamycin or PMA. The TKI-induced ErbB2 endocytosis is not dependent on clathrin for the clathrin inhibitor Pitstop2 cannot block ErbB2 endocytosis (Fig. 4). The dynamin inhibitor Dyngo-4a prevents TKI-induced endocytosis of ErbB2 (Fig. 4), which suggests the TKI-induced endocytosis of ErbB2 is mediated via a dynamin-dependent but clathrin-independent mechanism. TKIs and geldanamycin use different mechanisms to induce ErbB2 degradation. Geldanamycin-induced degradation of ErbB2 can be blocked by the proteases inhibitor ALLN [33,45,46]. However, ALLN treatment cannot block the TKIs-induced ErbB2 degradation (Fig. 2). Furthermore, the lysosome and proteasome inhibitors cannot block TKIs-induced ErbB2 degradation (Fig. 2). These results suggest a distinct mechanism for

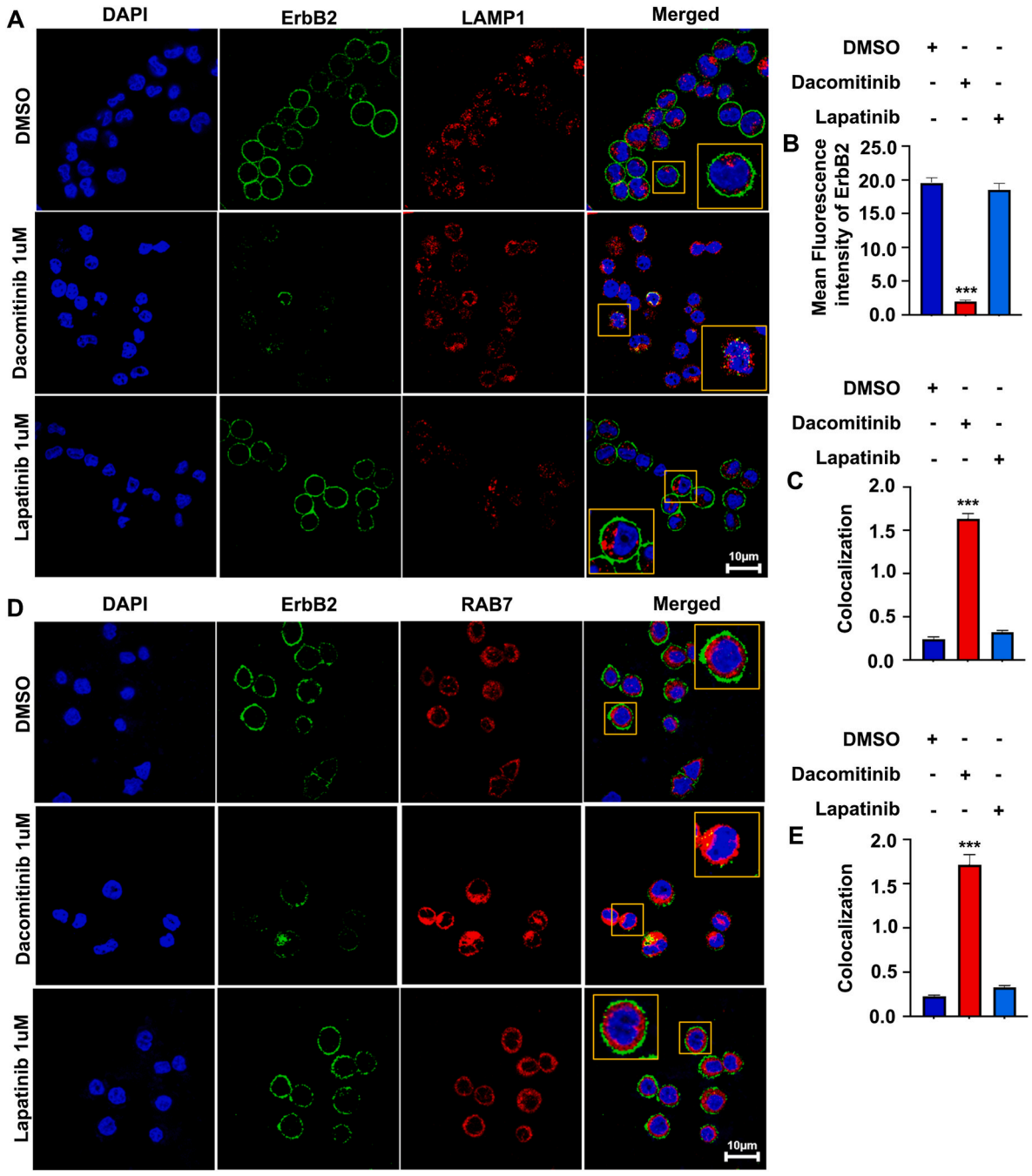

Fig. 3. Irreversible TKI stimulates the endocytosis and accumulation of ErbB2 at endosomes. MDA-MB-453 cells were treated with vehicle control (DMSO 1:1000), Dacomitinib (1 μM), or Lapatinib (1 μM) for 6 h, and then the subcellular location of ErbB2 and LAMP1 were detected by confocal microscopy (A). ErbB2 fluorescence intensity on plasma membrane was quantified (B). Colocalization of ErbB2 with LAMP1 was quantified (C). The subcellular location of ErbB2 and Rab7 were detected by confocal microscopy (D). Colocalization of ErbB2 with Rab7 was quantified (E). Insets show high magnifications of the corresponding framed areas. Scale bars 10 μm. Error bars indicate mean ± SEM from three independent experiments. $^{***}P < 0.0001$.

TKI-induced ErbB2 degradation. By covalently binding to ErbB2, irreversible TKIs achieve a stronger bonding ability to ATP binding pocket than reversible TKIs, which could affect the ErbB2 interaction with effector proteins to regulate ErbB2 degradation. Future studies are warranted to explore the possible effector proteins and specific proteases responsible for TKIs-induced ErbB2 degradation. The current research of TKIs was focused on two HNSCC cell lines and the epithelial-like breast cancer cell line MDA-MB-453. Different mechanisms to induce ErbB2 degradation may exist in different types of cells.

ErbB2 modulates tumor progression not only by stimulating downstream signaling such as ERK and AKT, but also by playing a non-canonical function as a transcriptional regulator in the nucleus [47,48]. For example, nuclear ErbB2 represses DEPTOR transcription to inhibit autophagy [49]. By downregulating the ErbB2 expression, the irreversible TKIs may also block the non-canonical function of ErbB2. Our previous work reported that the irreversible TKIs could induce much stronger autophagy in head and neck cancer cells compared with reversible TKIs [50]. It suggests that the irreversible TKIs can release the ErbB2-mediated autophagy inhibition by leading to the loss of ErbB2. As irreversible TKIs can both block ErbB2 activation and decrease ErbB2 expression levels, the irreversible TKIs should be more efficient to suppress the progression of ErbB2-dependent cancer compared with

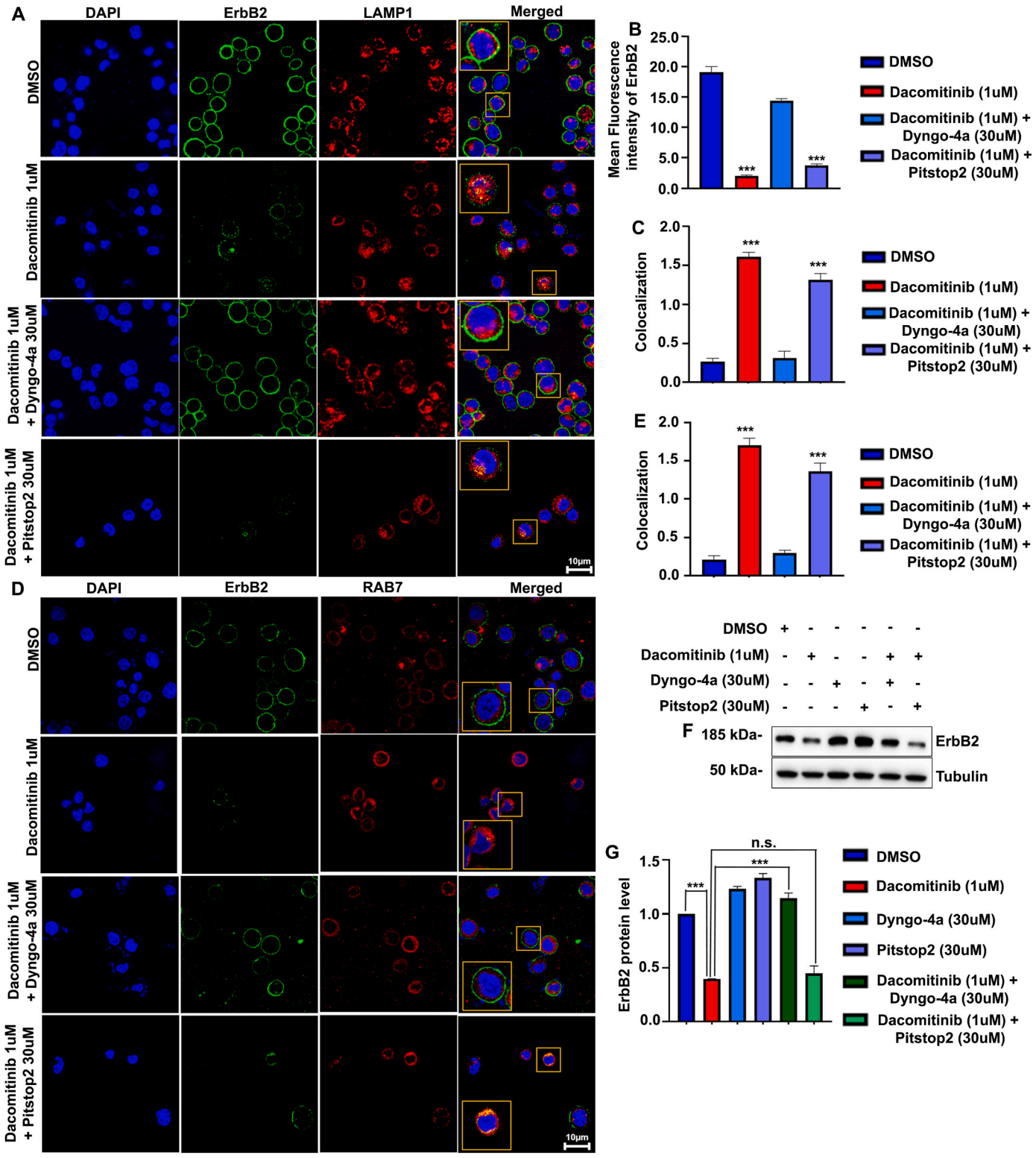

Fig. 4. Irreversible TKI-induced ErbB2 downregulation requires the dynamin-dependent but clathrin-independent endocytosis. MDA-MB-453 cells were treated with vehicle control (DMSO 1:1000), Dacomitinib (1 μM), and the combination of Dacomitinib (1 μM) with Dyngo-4a (30 μM) or with PitStop2 (30 μM) as indicated for 6 h, and then the subcellular location of ErbB2 and LAMP1 were detected by confocal microscopy (A). ErbB2 fluorescence intensity on plasma membrane was quantified (B). Colocalization of ErbB2 with LAMP1 was quantified (C). The subcellular location of ErbB2 and Rab7 were detected by confocal microscopy (D). Colocalization of ErbB2 with Rab7 was quantified (E). MDA-MB-453 cells were treated with vehicle control (DMSO 1:1000), Dacomitinib (1 μM), Dyngo-4a (30 μM), Pitstop2 (30 μM) and their combination as indicated for 6 h, and then ErbB2 protein expression levels were measured by Western blot (F) and quantified (G). Insets show high magnifications of the corresponding framed areas. Scale bars 10 μm. Error bars indicate mean ± SEM from three independent experiments. ***$P < 0.0001$, n.s.-non-significant.

reversible TKIs. It is worthy to further identify the molecular mechanisms mediating ErbB2 degradation, which has the potential to provide new targets to improve current *anti*-ErbB2 therapies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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