

## Review

# Stress-Induced EGFR Trafficking: Mechanisms, Functions, and Therapeutic Implications

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**Epidermal growth factor receptor (EGFR) has fundamental roles in normal physiology and cancer, making it a rational target for cancer therapy. Surprisingly, however, inhibitors that target canonical, ligand-stimulated EGFR signaling have proven to be largely ineffective in treating many EGFR-dependent cancers. Recent evidence indicates that both intrinsic and therapy-induced cellular stress triggers robust, noncanonical pathways of ligand-independent EGFR trafficking and signaling, which provides cancer cells with a survival advantage and resistance to therapeutics. Here, we review the mechanistic regulation of noncanonical EGFR trafficking and signaling, and the pathological and therapeutic stresses that activate it. We also discuss the implications of this pathway in clinical treatment of EGFR-overexpressing cancers.**

## EGFR Trafficking in Cancer Signaling and Therapies

EGFR is a receptor tyrosine kinase with fundamental roles in development and normal physiology of epithelial cells [1], including stimulating cell proliferation, differentiation, and motility. Overexpression and/or hyperactivation of EGFR are a predictor of poor prognosis in many cancers, where it drives tumor initiation and progression [2]. Full activation of EGFR, as well as termination of its signaling, depends on ligand-stimulated endocytosis and intracellular trafficking (Figure 1). It has been proposed that dysregulation of this sorting process contributes to oncogenesis in many carcinomas.

Here, we briefly summarize ligand-stimulated EGFR trafficking pathways; for details, readers are referred to other reviews [3–6]. Upon ligand binding, EGFR is autophosphorylated and initiates signaling at the plasma membrane. The phosphotyrosine residues generated by this activation provide docking sites for downstream effectors, such as the E3 ubiquitin ligase CBL, the phosphoinositide 3-kinase (PI3K) complex, Src kinase, and growth factor receptor-bound protein 2 (GRB2) [7]. Activated EGFR is rapidly internalized to endosomes by clathrin-dependent and -independent routes, where it continues to signal until recycled to the plasma membrane, in the case of TGF $\alpha$  binding, or is extinguished in lysosomes, if occupied by EGF [8]. Lysosomal destruction of activated EGFR requires receptor ubiquitination, which starts at the plasma membrane and continues in endosomes [6], causing it to be recognized by the **endosomal sorting complex required for transport** (ESCRT, see [Glossary](#)) machineries that drive its sorting into intraluminal vesicles (ILVs) at **multivesicular endosomes** (MVEs) and degradation following MVE fusion with lysosomes [9,10]. In addition to these recycling and lysosomal sorting pathways, recent studies have also reported ligand-stimulated EGFR trafficking to mitochondria

### Trends

EGFR is overexpressed and/or hyperactivated in most epithelial cancers, but EGFR-targeting therapies have had limited clinical benefit.

Many intrinsic and iatrogenic cellular stresses stimulate ligand-independent EGFR internalization and signaling that provide a survival advantage to tumor cells.

Cellular stresses stimulate EGFR internalization and intracellular accumulation with a consequent induction of autophagic responses.

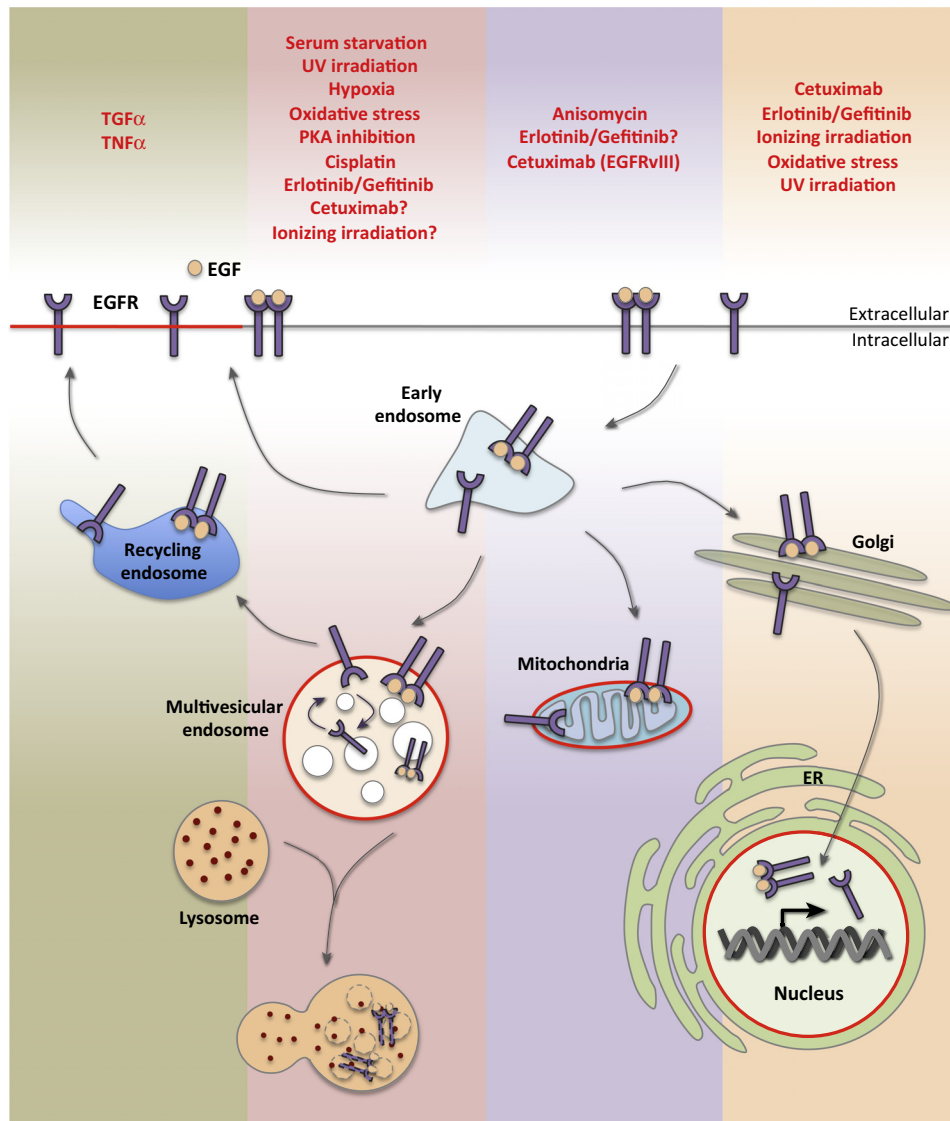
Overcoming therapeutic resistance resulting from ligand-independent EGFR functions promises new treatment strategies for EGFR-overexpressing cancers.

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#### Trends in Cell Biology

**Figure 1. Epidermal Growth Factor (EGF)- and Stress-Induced EGFR Trafficking.** Ligand and various cellular stresses or stress inducers stimulate EGFR internalization and intracellular trafficking. Following EGF treatment or stress stimulation, EGFR is internalized and can then be recycled back to the plasma membrane or sorted to multivesicular endosomes (MVEs), mitochondria, endoplasmic reticulum (ER), and nuclei. Different background colors highlight EGFR trafficking to specific subcellular destinations (red compartments) upon different cellular stresses at the top. At each location, stress-stimulated EGFR has both kinase-dependent and -independent functions. Ligand- or stress-activated EGFR signals from various compartments unless it is sequestered on intraluminal vesicles (ILVs) in the lumen of MVEs or delivered to the lysosome for degradation. While ligand-stimulated EGFR trafficking to MVEs targets the receptor for lysosomal degradation, most stresses cause perinuclear arrest of EGFR at nondegradative MVEs, where EGFR has been shown to undergo intraluminal sorting and back-fusion recycling in response to UV irradiation. Abbreviations: PKA, protein kinase A; TNF, tumor necrosis factor.

[11–13] and nuclei [14–16] (Figure 1). In some situations, nuclear or mitochondrial localization is also induced by cellular stressors, as discussed further below.

Due to its frequent overexpression and hyperactivation, EGFR has been a therapeutic target for many epithelial cancers. Three small-molecule tyrosine kinase inhibitors (TKIs), erlotinib, gefitinib

and lapatinib, and two EGFR-specific antibodies, cetuximab and panitumumab, have been shown to efficiently suppress ligand-stimulated EGFR kinase activity in clinical studies [2]. However, these drugs have little or no effect in most solid tumors, with the exception of nonsmall cell lung cancers (NSCLC) carrying activating mutations in EGFR, which initially respond to the TKIs but eventually develop resistance [2,17–21]. Despite the possible existence of additional oncogenic factors in wild-type EGFR-expressing cancers, the innate resistance to canonical EGFR signaling inhibitors suggests that previously unappreciated noncanonical EGFR signaling pathways, including ligand-independent and potentially tyrosine kinase-independent mechanisms, have a role (Box 1).

Recent advances have shown that many intrinsic and iatrogenic cellular stresses induce ligand-independent EGFR transactivation, internalization, and intracellular trafficking (Figure 1), which are associated with resistance to EGFR-targeting therapies. In addition, kinase-independent EGFR functions have been reported, including, among others, a role for the endosomal-accumulated inactive EGFR in the induction of autophagy [22]. While the other kinase-independent functions for EGFR (Box 1) are less well understood, its role in autophagy initiation is clearer and more targetable for cancer treatments. Although the core autophagy machinery is evolutionarily conserved in all eukaryotes, mammalian cells have evolved more complex regulatory mechanisms for the control of autophagy to maintain cellular homeostasis in response to constantly changing environments and physiological conditions. Misregulation of autophagy is observed in various diseases, including cancers, neurodegenerative diseases, and aging. High autophagy levels, assessed by punctate LC3B staining, are frequently associated with solid tumors and correlate with tumor malignancy, metastasis, and poor outcome [23]. Tumors with hypoxic stress intrinsically upregulate transcriptional EGFR expression through hypoxia-inducible factor (HIF)-2 $\alpha$ , providing an explanation for non-mutational EGFR overexpression in cancers [24]. Given the overexpression of EGFR in many epithelial cancers and the capability of EGFR to respond to many cellular stressors, it is not surprising that cancer cells make use of readily available EGFR molecules to survive stressed

### Box 1. Kinase-Independent EGFR Functions

It has been known for years that there are kinase-independent roles for EGFR in normal physiology. Mice with kinase-dead EGFR survive better than EGFR-knockout mice. The EGFR-knockout mice die before or after birth with severe developmental defects, depending on the genetic background [102–104]. However, mice with kinase-dead EGFR can survive well with obvious defects only in the eyes and skin [105]. It is equally possible that kinase-independent EGFR functions can be essential for cancer cells. In fact, while kinase-activating mutations of EGFR are commonly found in nonsmall cell lung cancers (NSCLCs), kinase-dead mutations are also found [106], although the role for kinase-dead EGFR in these cases are not clear. However, more recent work may help us understand why these kinase-dead EGFR mutants could still have important roles in cancers.

Over the past 20 years, several studies have reported kinase-independent functions of EGFR in cell survival [22,107–109]. Expressing of either wild-type or the K721R kinase-dead mutant of EGFR promotes cell survival in the absence of interleukin 3 (IL3) in 32D murine hematopoietic cells that normally depend on IL3 for growth and survival [109]. Interestingly, another kinase-dead mutant, D813A, does not provide similar survival advantage in the same cells. Similarly, the K721 M mutant can still stimulate the expression of c-fos, a proto-oncogene [110]. However, D813A but not K721 mutants of EGFR are able to stimulate DNA synthesis [108]. These results suggest that specific conformations, but not the kinase activity, of EGFR are important for certain cell survival functions.

At the plasma membrane, EGFR associates with, and stabilizes, the sodium/glucose co-transporter 1 (SGLT1), which is independent of EGFR kinase activity [107]. As such, EGFR facilitates cancer cell survival by maintaining cellular glucose levels even without its tyrosine kinase activity. In fact, SGLT1 expression levels in oral squamous cell carcinoma cell lines and patient tumors are significantly correlated with EGFR levels, and their expression is inversely related to tumor differentiation [111].

With its recently identified role in autophagy, EGFR is now known to have at least four distinct kinase-independent functions, including selective protein expression, DNA synthesis, and intracellular glucose level maintenance, as well as autophagy. There are surely more kinase-independent EGFR functions to be discovered in future studies, but the current data have already transformed our understanding of EGFR in cancer biology, which should now be seriously taken into account when considering EGFR targeting in clinical therapies.

### Glossary

**Back-fusion:** fusion of intraluminal vesicles with the limiting membrane of an MVE.

**EGFRvIII:** the type III EGFR mutation that lacks part of the extracellular ligand-binding domain and is constitutively active. It is the most common deletion mutation of EGFR.

**Endosomal sorting complex required for transport (ESCRT):** a series of multisubunit complexes that mediate the recognition and intraluminal sorting of transmembrane receptor cargos.

**Multivesicular endosome (MVE):** a subset of endosomes with multiple intraluminal vesicles (ILVs).

**Oxidative stress:** cellular exposure to reactive oxygen species (ROS) that are highly reactive oxidizing molecules produced either endogenously or exogenously and can attack and damage biomolecules.

**p38 mitogen-activated protein kinases (p38MAPK):** a class of protein kinases participating in a signaling cascade controlling cellular responses to cytokines and stress.

**Run domain Beclin 1 interacting and cysteine-rich containing protein (Rubicon):** an inhibitor of autophagy primarily found at late endosomes and/or lysosomes that associates with, and suppresses, the activity of the Beclin 1–VPS34 complex.

conditions by activating pathways such as autophagy. Thus, targeting autophagy in combination with canonical EGFR-targeting therapies is likely a promising approach for the treatment of solid tumors with wild-type EGFR overexpression.

Ligand-independent EGFR functions are emerging as resistant mechanisms for canonical therapies of EGFR-driven cancers. Here, we review current understanding of ligand-independent EGFR trafficking and functions stimulated by various cellular stresses or stress inducers, and discuss their implications for new approaches to cancer therapy.

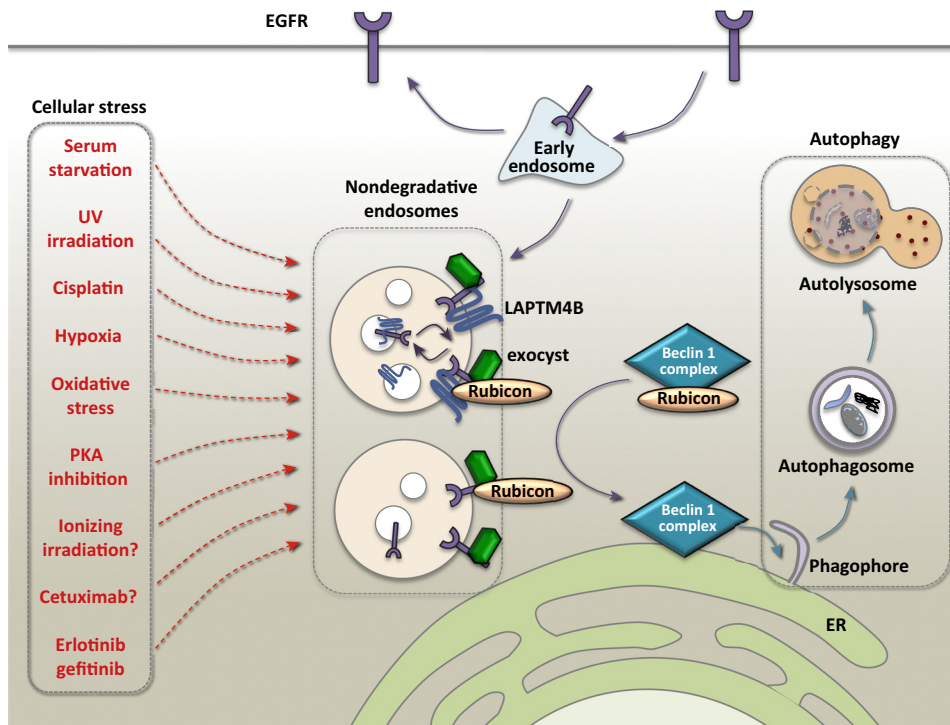
### Endosomal-Accumulated Inactive EGFR in Autophagy Initiation

Growth factor signaling directs the utilization of nutrients to maintain cell survival and growth [25]. Autophagy is a highly conserved self-eating process that maintains cellular homeostasis and functions as a survival mechanism under stressed conditions (for recent reviews, see [26,27]). In nutrient-rich conditions with sufficient growth factors, EGFR activation stimulates cell survival, proliferation, and migration [28]. It also suppresses autophagy by activating the Akt-mechanistic target of rapamycin complex 1 (mTORC1) pathway or by directly phosphorylating and inhibiting Beclin 1 (Atg6 in yeast), a core subunit of the VPS34 autophagy-initiating complex [29]. By contrast, whereas activated EGFR suppresses autophagy, recent studies revealed a role for inactive EGFR in autophagy initiation (Figure 2), which can be stimulated by serum starvation or EGFR TKIs [22].

Endosomal EGFR accumulation during serum starvation is due to a strong interaction between inactive EGFR and lysosomal-associated protein transmembrane 4 beta (LAPTM4B), a four-transmembrane protein that is localized to a fraction of early and late endosomes [30] and is overexpressed in many cancers [31,32]. Surprisingly, inactive EGFR and LAPTM4B stabilize each other at these nondegradative endosomes. This intracellular arrest phenotype appears similar to that observed after UV irradiation or cisplatin treatment [33]. However, serum starvation specifically increases the endosomal pool of EGFR without affecting cell surface EGFR levels [22], whereas UV irradiation or cisplatin treatment triggers acute EGFR internalization [33]. Of note, the K721A kinase-dead EGFR mutant maintains a strong LAPTM4B interaction and still accumulates in LAPTM4B-positive endosomes. It is likely that, during basal ligand-independent EGFR trafficking and turnover, a fraction of inactive EGFR is recognized by LAPTM4B and is then sequestered at endosomes.

At the endosome, inactive EGFR regulates the Beclin1 autophagy-initiating complex (Figure 2). The Beclin1-VPS34 (class III PI3K) complex has an essential role in autophagy initiation by generating phosphatidylinositol 3-phosphate (PI3P) at the endoplasmic reticulum (ER), where PI3P effectors are recruited for phagophore assembly [34–37]. The **Run domain Beclin-1 interacting and cysteine-rich containing protein** (Rubicon) is an autophagy inhibitor that, when associated with the Beclin 1 complex, inhibits PI3P generation [38]. With the help of LAPTM4B and the Sec5 exocyst subcomplex at endosomes, the inactive EGFR complex interacts with Rubicon and promotes its disassociation from Beclin 1, resulting in Beclin 1 activation and autophagy initiation [22]. In support of this mechanism, the exocyst complex was recently reported to regulate autophagy initiation in both mammals and plants [39,40]. Interestingly, this receptor-mediated autophagy pathway appears specific to EGFR, because the loss of other receptors, such as c-Met, PDGFR $\beta$ , or FGFR2, does not cause autophagy defects.

Most solid tumors have innate resistance to EGFR TKIs and receive no clinical benefits from these inhibitors [2]. Recently, many groups have found that EGFR TKIs induce cytoprotective autophagy in cancer cells as an innate TKI-resistance mechanism [17,41–49]. Since EGFR activation suppresses autophagy by multiple mechanisms, the TKI-stimulated autophagy was thought to result from a loss-of-function of EGFR kinase signaling. However, a gain-of-function for EGFR in autophagy initiation upon TKI (erlotinib or gefitinib) treatment has been discovered



Trends in Cell Biology

**Figure 2. Stress-Induced Epidermal Growth Factor Receptor (EGFR) Endosomal Arrest and Autophagy Induction.** Most cellular stress stimuli cause EGFR arrest at nondegradative endosomes, where the receptors have both kinase signaling and kinase-independent functions, although many stressors also trigger EGFR trafficking to other subcellular locations. Serum starvation and EGFR tyrosine kinase inhibitors (erlotinib and gefitinib) have been recently shown to trigger a kinase-independent role for EGFR at endosomes in autophagy initiation. Such a role for EGFR might also be triggered by most other stress stimuli shown in the figure, because they also induce both EGFR endosomal arrest and autophagy upregulation. The inactive EGFR is arrested at endosomes upon serum starvation via a protein–protein interaction with an endosomal protein lysosomal-associated protein transmembrane 4 beta (LAPT4B) or by erlotinib and gefitinib through unknown mechanisms. An exocyst is then recruited and facilitates EGFR-mediated Run domain Beclin-1 interacting and cysteine-rich containing protein (Rubicon) disassociation from the Beclin 1 complex, which releases Beclin 1 from Rubicon inhibition and activates autophagy initiation. Abbreviations: ER, endoplasmic reticulum; PKA, protein kinase A.

(Figure 2). TKIs also trigger EGFR accumulation at endosomes and enhance its association with the exocyst complex and Rubicon, inducing Rubicon disassociation from Beclin 1 and activating autophagy initiation [22]. Therefore, EGFR TKIs mimic the function of LAPT4B in stabilizing EGFR at endosomes and facilitating EGFR recruitment of downstream effectors to modulate autophagy.

It is likely that the role of EGFR in autophagy initiation is not only induced by serum starvation and TKI treatments, but may also act downstream of other stresses (Figure 2). Not only LAPT4B and TKIs arrest EGFR at nondegradative endosomes, but other stress inducers, such as UV irradiation, cisplatin treatment, hypoxia, oxidative stress, and protein kinase A (PKA) inhibition, also stimulate EGFR internalization and endosomal arrest, although the underlying mechanisms are stress dependent. It appears that most stressors stimulate either **p38 mitogen-activated protein kinase** (p38MAPK)- and clathrin-mediated or Src- and caveolin-mediated EGFR internalization (Table 1). Interestingly, EGFR TKIs are known to induce both oxidative stress (which activates Src) and p38 MAPK activation [46,50], suggesting that both pathways are involved in EGFR internalization and intracellular arrest upon TKI treatment [22]. Of note, although two distinct mechanisms are used for the internalization of EGFR, almost all stimuli,



Table 1. Overview of Stress-Induced EGFR Trafficking Pathways

Stress Type	Ubiquitination of EGFR	EGFR Degradation	Endocytosis	Intracellular Trafficking	Autophagy Induction	Refs
TGF $\alpha$	Yes	No	Mainly clathrin mediated	Rapid recycling	No	[8,112]
EGF	Yes	Yes	Mainly clathrin mediated	Partially recycled; mostly degraded in lysosome; mitochondrial and nuclear translocation	No	[6,8,12,81,112,113]
UV	No	No	p38MAPK and clathrin mediated	Endosomal arrest; nuclear translocation	Yes	[33,52–55,58,64–66,89]
Cisplatin	No	No	p38MAPK and clathrin mediated	Endosomal arrest	Yes	[33,52,59–63]
Anisomycin	No	Yes	p38MAPK and clathrin mediated	Rapid degradation; mitochondrial translocation	Yes	[55–57,96]
TNF $\alpha$	No	No	p38MAPK and clathrin mediated	Rapid recycling	Yes	[52]
Serum starvation	?	No	Clathrin-mediated basal endocytosis?	Endosomal arrest	Yes	[22,29]
Erlotinib and gefitinib	?	No	Caveolin mediated?	Endosomal arrest; mitochondria; promotes cetuximab-induced nuclear translocation	Yes	[22,42,43,45–49,96]
Hypoxia	?	No	Src and caveolin mediated?	Endosomal arrest	Yes	[67–69]
Oxidative stress	No	No	Src and caveolin mediated	Endosomal arrest; nuclear translocation	Yes	[46,50,51,70,71,75,89]
PKA inhibition	No	No	Clathrin dependent and independent	Endosomal arrest	Yes	[76–80,100]
Cetuximab	?	Yes	Src and caveolin mediated	Endosomal arrest? Mitochondria translocation of EGFRvIII; ER and nuclear translocation of EGFR	Yes	[2,14,82–85,97]
Ionizing irradiation	No	No	Src and caveolin mediated	Endosomal arrest? Nuclear translocation	Yes	[51,81,86–88,90,91,114,115]

including serum starvation, can induce oxidative stress [51] and endosomal arrest of EGFR (Figure 2), putting them at the forefront of many stress response pathways. Therefore, it is important to know, in each stressed condition, whether the endosomal EGFR gains a function in autophagy initiation.

### p38MAPK-Mediated EGFR Internalization and Endosomal Arrest

Several cellular stress inducers, including UV irradiation, the antibiotic anisomycin, cisplatin, and tumor necrosis factor alpha (TNF $\alpha$ ), have been found to induce ligand-independent EGFR internalization (Table 1). A commonality is that they all trigger activation of p38MAPK, which is

required for EGFR internalization in these conditions. Among them, the best-characterized stimulus is UV irradiation.

UV irradiation induces rapid, ligand-independent, and clathrin-mediated internalization of EGFR, with kinetics comparable to those induced by high concentrations of EGF [33,52]. Interestingly, other receptors, including c-Met, insulin receptor, and transferrin receptor, are not affected by UV irradiation [33,52]. While EGF-stimulated EGFR internalization is tyrosine kinase dependent and requires autophosphorylation of multiple tyrosines, UV-stimulated internalization is tyrosine kinase independent [53]. Instead, it requires phosphorylation of serine and threonine residues in the EGFR cytoplasmic C-terminal tail (C-tail) [54] as well as continual activation of p38MAPK by UV irradiation [52,55]. The antibiotic, anisomycin, similar to UV, also induces p38MAPK-mediated EGFR internalization that is independent of EGFR tyrosine kinase activity, tyrosine phosphorylation, and ubiquitination [55]. EGFR is phosphorylated at Ser1039 and Thr1041, as well as at other Ser/Thr residues downstream of p38MAPK upon anisomycin treatment, and these residues are also phosphorylated at low levels in response to EGF [56,57]. How the differential phosphorylation patterns correlate with the distinct intracellular trafficking routes upon stress or EGF stimulation is an important future direction for study in EGFR trafficking.

Unlike EGF, which stimulates EGFR degradation, UV induces stable EGFR accumulation or arrest in endosomal compartments [53]. Although EGFR degradation is ultimately observed after long-term UV exposure, this may reflect nonspecific cleavage by caspases triggered by the onset of apoptosis rather than sorting to lysosomes [58]. Surprisingly, although the overall response of EGFR to anisomycin and UV appears similar, anisomycin triggers fast EGFR degradation, apparently through ubiquitination- and lysosome-independent mechanisms [55]. Inflammatory cytokines, such as  $\text{TNF}\alpha$ , induce transient p38MAPK activation and EGFR phosphorylation and internalization, followed by rapid recycling once p38MAPK is inactivated [52].

The UV-induced endosomal accumulation of EGFR is due not to persistent internalization and recycling, but rather to p38MAPK-mediated endosomal arrest of EGFR [33]. Arrested EGFR is observed in a population of lysobisphosphatidic acid (LBPA)-positive MVEs that are distinct from the EGF-induced pool of MVEs and do not fuse with lysosomes [33]. UV induces ubiquitination-independent, ALG-2-interacting protein X (ALIX)- and ESCRT-dependent sorting of EGFR onto ILVs at these nondegradative MVEs [33,53]. Interestingly, the intraluminally sorted EGFR can be recycled back to the plasma membrane upon inhibition of p38MAPK, suggesting **back-fusion** of the ILVs with the limiting membrane of MVEs [33]. In support of this, the UV-induced EGFR signaling requires ALIX [33], suggesting that the dynamic subendosomal EGFR trafficking has a key role in regulating receptor signaling.

The chemotherapeutic reagent cisplatin also triggers p38MAPK-mediated EGFR phosphorylation, internalization, and endosomal arrest without EGFR ubiquitination or degradation [33,52]. Interestingly, both UV and cisplatin stimulate cytoprotective autophagy, which is a resistance mechanism for UV- or cisplatin-induced cell death [59–66]. This raises the important question: does endosomally accumulated EGFR mediate UV- or cisplatin-induced autophagy? This question is addressed here and in [22].

### Src- and Caveolin-Mediated EGFR Internalization and Endosomal Accumulation

Hypoxia, as well as nutrient deprivation, is a common condition in solid tumors, and it contributes to the metabolic rewiring of cancer cells, angiogenesis, and metastasis. EGFR also responds to hypoxia to provide a cell survival advantage (Table 1). Hypoxia not only transcriptionally upregulates EGFR expression [24], but also transactivates EGFR and triggers its internalization

and late endosomal accumulation [67]. Hypoxia activates Src [68] and potentially stimulates caveolin-mediated internalization of EGFR, as discussed below. Hypoxia dramatically stimulates EGFR interaction at late endosomes with argonaute 2 (AGO2), a membrane-associated protein involved in miRNA maturation [67]. Hypoxia-transactivated EGFR phosphorylates AGO2, resulting in inhibited maturation of select tumor suppressor miRNAs, which in turn promotes EGFR-mediated cancer cell survival and invasiveness [67]. Interestingly, oxidative stress also stimulates the EGFR-AGO2 interaction, albeit not as well as hypoxia, suggesting that the EGFR-mediated processing of tumor suppressor miRNA is a general stress response. Hypoxia is also a potent stimulator of autophagy through multiple mechanisms [69], but a role for the late endosomally accumulated EGFR in hypoxia-induced autophagy is not defined.

**Oxidative stress** is a normal physiological condition, although it also has roles in numerous diseases, including cancer. Oxidative stress induced by H<sub>2</sub>O<sub>2</sub> (or glucose oxidase that generates H<sub>2</sub>O<sub>2</sub>) stimulates EGFR activation without ubiquitination or degradation of EGFR (Table 1), possibly due to little phosphorylation at Tyr1045, the docking site for the E3 ubiquitin ligase CBL, which ubiquitinates EGFR [70]. Importantly, H<sub>2</sub>O<sub>2</sub> induces conformational changes in EGFR that differ from those induced by EGF. H<sub>2</sub>O<sub>2</sub> causes EGFR phosphorylation but not dimerization, and the phosphorylation is resistant to treatment by the EGFR TKI tyrosophostin (AG1478) [71], which might be partially due to H<sub>2</sub>O<sub>2</sub>-mediated oxidation and inactivation of tyrosine phosphatases that negatively regulate EGFR phosphorylation [72,73] or to EGFR phosphorylation by kinases other than EGFR itself [74]. Similar to the other stressors discussed above, H<sub>2</sub>O<sub>2</sub> also causes EGFR internalization and perinuclear arrest [75]. However, H<sub>2</sub>O<sub>2</sub>-stimulated EGFR internalization is caveolin dependent and likely promoted by Src kinase activity, as discussed below [75]. Oxidative stress is emerging as the converging point for many cellular stresses that stimulate autophagy [51], as is EGFR, as discussed throughout this review (Figure 2).

An additional way to trigger perinuclear arrest of EGFR without causing its lysosomal degradation is by inhibiting PKA signaling (Table 1). Initial studies found that inhibition of phosphatidic acid (PA) phosphohydrolases (PAPs) causes ligand- and tyrosine kinase-independent internalization of EGFR with no evidence of tyrosine phosphorylation, ubiquitination, or degradation of EGFR [76,77]. PA accumulation inhibits PKA signaling, resulting in EGFR internalization through both clathrin-dependent and -independent routes [76]. Although the functional relevance of PKA inhibition-induced EGFR endosomal accumulation is not clear, it might participate in autophagy initiation (Figure 2). PKA is an established negative regulator of autophagy that functions upstream of the ULK1 (atg1 in yeast) complex independently of mTORC1 [78], and more extensive feedback regulation between PKA signaling and autophagy has been recently revealed [79,80].

### Caveolin-Mediated EGFR Internalization and Nuclear Translocation

Cetuximab binds the EGFR extracellular domain, blocks ligand binding, and inhibits EGFR activation. It also induces strong internalization of EGFR, potentially through caveolin-mediated mechanisms (Table 1) [81], but the downstream trafficking events have been uncharacterized to date. However, it has been established that cetuximab can stimulate EGFR trafficking to the ER and the nucleus [82]. Interestingly, erlotinib and gefitinib, but not lapatinib, promote cetuximab-induced nuclear translocation of EGFR, suggesting that EGFR kinase activity is not required, but a unique receptor conformation is crucial because erlotinib and gefitinib keep EGFR in a different conformation, as does lapatinib. Although nuclear EGFR trafficking is associated with cancer resistance to cetuximab therapy, there are also many other resistance mechanisms [83]. Recently, cetuximab has been reported to stimulate cytoprotective autophagy in several cancer cell lines [84]. The underlying mechanisms for autophagy induction have been attributed to cetuximab-stimulated downregulation of HIF1 as well as Bcl-2, which in turn releases Beclin 1 from Bcl-2 suppression [85]. However, whether cetuximab triggers endosomal arrest of EGFR



or whether the cetuximab-bound internalized EGFR itself has a gain-of-function in autophagy have not been explored.

A major therapeutic approach for treating localized tumors is ionizing radiation, a treatment for which EGFR provides cellular resistance (Table 1). EGFR expression levels are upregulated by ionizing irradiation [86]. Radiation also stabilizes and activates Src kinase that, in turn, phosphorylates caveolin-1 and EGFR, resulting in caveolin-mediated EGFR internalization [81]. In addition, radiation triggers PKC $\epsilon$ -mediated EGFR phosphorylation at Thr654 [87], which blocks CBL-mediated EGFR ubiquitination and lysosomal degradation and promotes EGFR nuclear transport [88]. The nuclear EGFR might contribute to enhanced DNA repair [81], and kinase inhibition of Src causes a block of EGFR nuclear transport upon radiation [14]. H<sub>2</sub>O<sub>2</sub>-induced oxidative stress as well as UV irradiation also stimulates rapidly nuclear translocation of EGFR in human keratinocytes [89], which may control resistance to DNA damage. Overexpression, transactivation, and nuclear localization of EGFR are all associated with radioresistance and poor therapeutic outcome; thus, EGFR targeting has been a strategy to resensitize tumors to radiotherapy [90]. However, resistance to EGFR inhibitors is observed when used in combination with radiation, for which autophagy is emerging as a resistance mechanism. Although it is not resolved whether inactive EGFR has a role in radiation-induced autophagy, it is known that autophagy is strongly upregulated by radiation in radio-resistant, but not radio-sensitive cells, and that autophagy inhibition can resensitize resistant cells to radiation [91,92].

### Stress-Induced Mitochondrial Translocation of EGFR

The mitochondrion is positioned at the center of cellular pathways controlling metabolism, survival, and death, where it modulates not only apoptosis, but also autophagy. EGFR has been shown to translocate to mitochondria in multiple conditions, and it potentially regulates mitochondrial pathways in cell survival (Figure 1). Even without mitochondrial localization, both EGFR and **EGFRvIII** (the most common deletion mutant of EGFR in cancers) have been shown to interact with the p53-upregulated modulator of apoptosis (PUMA) to inhibit PUMA translocation to mitochondria and PUMA-mediated apoptosis [93]. It is yet not defined which part of EGFR binds PUMA, but this function does not require EGFR kinase activity.

Both EGF and some stress stimuli induce EGFR translocation to mitochondria (Figure 1). Upon EGF stimulation, mitochondrial EGFR phosphorylates Cytochrome c oxidase subunit II (COXII), resulting in decreased Cox activity and cellular ATP levels, which prevent apoptosis [12]. EGF also stimulates a role for mitochondrial EGFR in the fission and redistribution of mitochondria, which is associated with enhanced cancer cell motility [94]. Alternatively, activation of cell surface EGFR has been proposed to stimulate *de novo* synthesis of palmitate, which in turn activates mitochondrial EGFR to promote mitochondrial fusion and cancer cell survival [95]. Mitochondrial localization of EGFR can also be independent of EGFR endocytosis, suggesting direct EGFR delivery to mitochondria upon synthesis [13].

EGFR also translocates to mitochondria and provides drug resistance in stressed conditions triggered by the apoptotic inducers staurosporine, anisomycin, or gefitinib [96]. Cetuximab treatment does not induce mitochondrial translocation of wild-type EGFR, but does induce translocation of EGFRvIII and increased mitochondrial activity without affecting the kinase activity of EGFRvIII [97], suggesting potential cetuximab resistance mediated by mitochondrial EGFRvIII. How this leads to drug resistance is not yet defined, but it could involve changes in mitochondrial activity or autophagy (Figure 2), the latter of which antagonizes apoptosis and is induced by gefitinib. Thus, EGFR potentially contributes to autophagy at ER-mitochondria and/or ER-endosome contact sites (Box 2). Consistently, the autophagy inducer rapamycin triggers EGFR translocation to mitochondria, which is inhibited by the autophagy inhibitor 3-MA or by knock-down of Beclin 1 [11]. Interestingly, cells with more mitochondrial EGFR are more vulnerable to

### Box 2. A Potential Role for Inactive EGFR at the ER–Mitochondria or ER–Endosome Contact Sites for Autophagy Initiation

Autophagy initiation involves complex membrane trafficking events that deliver membrane vesicles to the phagophore nucleation sites. It is generally agreed that starvation-induced autophagy initiates at ER, but the membrane from not only ER, but also ER–Golgi intermediate compartments (ERGIC), Golgi, early and recycling endosomes, plasma membrane, and mitochondria all contributes to phagophore expansion [26,27]. A role for ER–mitochondria contact sites in phagophore formation was recently established [116]. It is plausible that stress-induced mitochondrial translocation of EGFR [11] has a role in autophagy initiation in these conditions.

Remarkably, endosomal-localized inactive EGFR also has an essential role in autophagy initiation by releasing Rubicon-free Beclin 1 [22], the latter of which is expected to mediate autophagy initiation at ER in close proximity. The MVEs have well-established roles in fusion with autophagosomes as predegradative compartments [117]. However, given the constant contacts between ER and MVEs [118–121], one tends to postulate that the ER–MVE contact sites might provide a platform for the endosomal inactive EGFR to effectively regulate the Beclin 1 complex for autophagy initiation. It will be important to explore whether the endosomes, where EGFR accumulates upon serum starvation, TKI treatment, UV irradiation, or cisplatin treatment, have contacts with ER and, if yes, whether these contacts are involved in autophagy initiation. In fact, there are endosome–ER contact sites specified by an interaction between the ER-localized PTP1B and endosomal EGFR, where PTP1B dephosphorylates and inactivates EGFR [118]. It is yet to be determined whether the PTP1B–EGFR-mediated contact sites have a role in EGFR-mediated autophagy initiation.

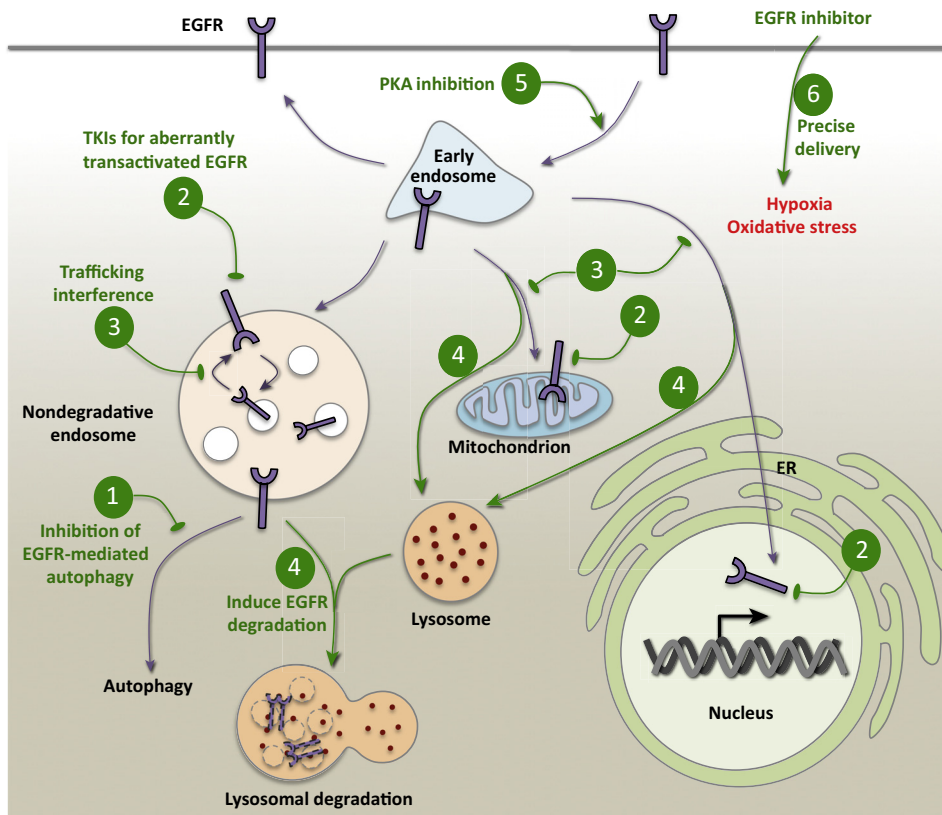
EGF treatment or EGFR knockdown [11], suggesting a dependence on mitochondrial EGFR-mediated survival functions.

### Therapeutic Implications for Stress-Induced EGFR Functions

Since EGFR and autophagy are both at the converging point responding to intrinsic and iatrogenic stress, it would be beneficial to combine autophagy inhibition with other therapeutic approaches for EGFR-overexpressing cancers (Figure 3). In line with this, both EGFR-overexpressing cell lines and xenografts have been found to depend on autophagy for growth and survival, and autophagy inhibition sensitizes them to irradiation [98,99]. However, given that autophagy is also fundamental in normal physiology, general autophagy inhibition might cause serious problems, such as neurodegeneration and immunosuppression. Therefore, developing a list of autophagy inhibitors that specifically target certain types of cancer (e.g., EGFR-overexpressing cancers) is critical for patients. To resolve this problem, detailed mechanisms behind inactive EGFR-mediated autophagy should be further dissected and more specific targets identified. As in cells with higher EGFR, more autophagic activity might depend on the EGFR-mediated autophagy pathway; specific inhibition of this pathway in combination with canonical EGFR targeting approaches as well as general chemo- and radiotherapies would induce more powerful and selective killing of EGFR-overexpressing cancer cells.

In addition, new EGFR TKIs that target not only ligand-activated EGFR, but also transactivated EGFR are needed (Figure 3). Although autophagy is mediated by kinase-independent EGFR functions, many clinically used treatments transactivate a fraction of EGFR that signals at the plasma membrane, endosomes, mitochondria, and/or nuclei, and the transactivated EGFR can be resistant to currently available TKIs. For example, oxidative stress, which is induced by many therapeutic approaches, may not only stimulate a kinase-independent role for EGFR in autophagy, but also transactivate a fraction of EGFR by inducing unique conformational changes, which cannot be targeted by canonical EGFR TKI tyrphostin [71]. Further investigation of the unique EGFR conformations and discovery of new TKIs that effectively inhibit transactivated EGFR might be key to overcoming current clinical resistance.

Moreover, stressed-induced EGFR trafficking could be additionally targeted to antagonize therapeutic resistance. Since stress-induced EGFR functions are tightly bound to EGFR internalization and intracellular trafficking, and certain functions are associated with specific subcellular localizations, such as nondegradative endosomes, mitochondria, and nuclei, therapeutic



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**Figure 3. Therapeutic Strategies to be Combined with Canonical Treatments for Epidermal Growth Factor Receptor (EGFR)-Overexpressing Cancers.** Given the substantial roles for stress-induced EGFR trafficking and signaling in cancer cells, more therapeutic strategies need to be considered in addition to canonical treatments, such as radiotherapy and EGFR-targeting chemotherapy. Major strategies include inhibiting the kinase-independent role of EGFR in autophagy, blocking transactivated EGFR signaling from various compartments, interfering with stress-induced EGFR trafficking or alternatively directing the receptor for lysosomal degradation. Protein kinase A (PKA) inhibition, although it mimics stress-induced EGFR trafficking, can be explored as a supplementary therapeutic strategy because it has been found to have antitumor activities. Additionally, the features of EGFR-overexpressing tumor cells (hypoxia and oxidative stress, etc.) might allow the engineering of EGFR inhibitors for precise delivery to these cells. Abbreviation: TKI, tyrosine kinase inhibitor.

interventions that block these trafficking events or alternatively direct internalized EGFR for lysosomal degradation can be considered as a combined approach with canonical therapies (Figure 3).

Furthermore, the PKA inhibition approach can be explored for the treatment of EGFR-overexpressing cancers (Figure 3). The clinically available PAP inhibitors, propranolol and desipramine, are known to cause PA accumulation and PKA inhibition, resulting in EGFR internalization. Although this mimics EGFR trafficking induced by other cellular stresses, PKA inhibition shows tumor suppression activities in EGFR-driven cancer cells [100]. Thus, PKA inhibition reagents could be further explored in combination with other therapeutic approaches, such as EGFR inhibition, radiation, and autophagy suppression.

Finally, strategies to enhance precise delivery of EGFR-targeting therapies can be used (Figure 3). For example, because hypoxia is a common feature of solid tumors that upregulate and

transactivate EGFR, the recently designed hypoxia-activated EGFR inhibitor [101] could be a promising approach to effectively and specifically inhibit EGFR signaling in hypoxic solid tumors.

### Concluding Remarks

Ligand-stimulated EGFR signaling has pivotal roles in many cancers, and is a crucial target for cancer treatments. However, due to the limited clinical response and frequent patient resistance to current therapies targeting ligand-stimulated EGFR signaling, new strategies to overcome resistance have become a major focus for this field. It is now clear that both intrinsic and iatrogenic cellular stressors can trigger ligand-independent EGFR internalization through p38 MAPK- and clathrin-mediated or Src- and caveolin-mediated mechanisms. The internalized EGFR primarily traffics to endosomes, mitochondria, ER, and nuclei, where ligand-independent EGFR functions are mediated by tyrosine kinase-dependent (transactivation) and -independent pathways, both of which can evade canonical EGFR TKI treatments and could be manipulated to overcome resistance. Here, we have discussed the ligand-independent EGFR trafficking and functions in response to many cellular stress stimuli, with an emphasis on a kinase-independent role for EGFR in autophagy that is potentially stimulated in most stressed conditions. We have also suggested potential therapeutic strategies to be combined with canonical treatments for EGFR-overexpressing cancers, including co-targeting EGFR and autophagy, because both are at the converging point responding to intrinsic and iatrogenic stresses. Clearly, many questions remain (see Outstanding Questions). We hope that this article will stimulate more extensive research on the mechanistic regulation of noncanonical EGFR trafficking and functions and the development of more effective EGFR-targeting therapies.

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### Outstanding Questions

Why does EGFR respond to so many stressors? What makes EGFR unique compared with other receptors that do not respond? Are there other receptors mediating stress responses similarly to EGFR?

How is EGFR post-translationally modified (phosphorylation and ubiquitination) upon different cellular stresses and how does different modification specify EGFR internalization and trafficking routes?

How do different stressors induce endosomal EGFR accumulation and/or arrest? Does stress-induced endosomal EGFR arrest always involve ubiquitination-independent EGFR intraluminal sorting and back-fusion recycling? How is this subendosomal trafficking of EGFR regulated, and how could it be inhibited to block transactivated EGFR signaling and autophagy?

Do all of those stress stimuli trigger the role for EGFR in autophagy? Does EGFR transactivated by all stress stimuli adopt a similar aberrant conformation as induced by oxidative stress and how could it be pharmacologically inhibited? Can the aberrantly activated EGFR stimulate autophagy?

Is there a way to pharmacologically induce a transition of those EGFR-positive nondegradative MVEs into degradative ones or to simply stimulate their fusion with lysosomes? Is there a way to promote lysosomal targeting of the fraction of EGFR that would otherwise translocate to mitochondria or nuclei?

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