Ligand-activated epidermal growth factor receptor (EGFR) signaling governs endocytic trafficking of unliganded receptor monomers by non-canonical phosphorylation

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Running title: Dual-mode regulation of the endocytic trafficking dynamics of EGFR

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Key Words: clathrin, endocytosis, EGFR, p38 MAPK, TNF-α

The canonical description of transmembrane receptor function is initial binding of ligand, followed by initiation of intracellular signaling and then internalization en route to degradation or recycling to the cell surface. It is known that low concentrations of extracellular ligand leads to a higher proportion of receptor that is recycled, and that non-canonical mechanisms of receptor activation, including phosphorylation by the kinase p38, can induce internalization and recycling. However, no connections have been made between these pathways; i.e., it has yet to be established what happens to unbound receptors following stimulation with ligand. We herein demonstrate that a minimal level of activation of EGFR tyrosine kinase by low levels of ligand is sufficient to fully activate downstream MAPK pathways, with most of the remaining unbound EGFR molecules being efficiently phosphorylated at intracellular serine/threonine residues by activated MAPKs. This non-canonical, p38-mediated phosphorylation of the C-tail of EGFR, near Ser1015, induces the clathrin-mediated endocytosis of the unliganded EGFR monomers, which occurs slightly later than the canonical endocytosis of ligand-bound EGFR dimers via tyrosine autophosphorylation. EGFR endocytosed via the non-canonical pathway is largely recycled back to the plasma membrane as functional receptors, whereas p38-independent populations are mainly sorted for lysosomal degradation. Moreover, ligand concentrations balance these endocytic trafficking pathways. These results demonstrate that ligand-activated EGFR signaling controls unliganded receptors through feedback phosphorylation, identifying a dual-mode regulation of the endocytic trafficking dynamics of EGFR.

Epidermal growth factor receptor (EGFR), one of the most characterized receptor tyrosine kinases (RTKs), regulates many cellular functions, including survival, proliferation, and differentiation. The aberrant activation of EGFR by overexpression or activating mutations is a major mechanism underlying the pathogenesis of human cancers, including colorectal and lung cancers, and participates in acquired resistance to anti-cancer agents (1-4).

Ligand-bound EGFR proteins form an asymmetric homodimer on the plasma membrane, which is followed by the activation of its tyrosine kinase. Activated EGFR is then rapidly internalized via clathrin-mediated endocytosis and clathrin-independent endocytosis. Sequential sorting to several vesicular transport systems, including early endosomes, late endosomes, multivesicular bodies (MVBs), and recycling endosomes,
directs the fate of internalized EGFR to lysosomal degradation or recycling to the cell surface (5-7). However, the mechanisms by which structurally identical EGFR proteins are sorted to the different endocytic machineries of clathrin-dependent or -independent endocytosis and recycling or degradation have not yet been elucidated in detail.

Ligand concentrations in the extracellular environment are a key factor affecting EGFR intracellular transportation. Low concentrations mainly induce clathrin-mediated endocytosis, and a large portion of internalized EGFR is recycled back to the plasma membrane. Higher concentrations increase the ratio of lysosomal degradation, instead of recycling endocytosed EGFR (7, 8). Even in the presence of 50,000 molecules of EGFR on a single HeLa cell surface, the binding of 300 molecules of EGF was found to be sufficient to trigger an EGFR response in 50% of cells (9). Thus, the minimal activation of ligand-bound EGFR is sufficient to evoke intracellular signaling, indicating that most cell surface EGFR remain in a ligand-unoccupied state. Nevertheless, previous studies did not examine residual unliganded receptors during a ligand stimulation in adequate detail.

Evidence is increasing for the non-canonical activation of RTKs by the serine/threonine phosphorylation of their intracellular domains in ligand- and tyrosine kinase-independent manners. The phosphorylation of EphA2 Ser-897, for example, plays crucial roles in cell motility and proliferation, and has been correlated with poor prognoses for lung cancer and glioblastoma multiforme (10, 11). The non-canonical regulation of EGFR has also been investigated in the last decade. We and others demonstrated that pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), and other cellular stresses induced the serine/threonine phosphorylation and internalization of EGFR (12-17). This type of EGFR endocytosis depends entirely on p38 activation and clathrin recruitment. Moreover, endocytosed EGFR is completely recycled to the plasma membrane.

In the present study, we attempted to confirm the integrated hypothesis that ligand-activated canonical EGFR signaling provokes the p38-mediated non-canonical regulation of residual unliganded EGFR monomers. The results obtained may resolve some of the controversial issues related to the regulation of EGFR endocytic trafficking including sorting mechanisms for degradation or recycling following a stimulation with different ligand concentrations.

Results

p38-dependent and -independent endocytosis of EGFR

In order to investigate the role of p38 in ligand-induced endocytosis, we examined the effects of a p38 inhibitor (SB203580) and siRNA against p38α, a major subtype of the p38 family, in HeLa cells (Fig. 1, A-D). As shown previously (12), immunofluorescence analysis confirmed that TNF-α-induced EGFR internalization was completely dependent on p38 (Fig. 1, A, B, C and and supplementary Fig. S1A). EGFR endocytosis triggered by low-EGF (3 ng/ml), but not by high-EGF (100 ng/ml) was largely inhibited by SB203580 (Fig. 1, A and B) and the knockdown of p38α (Fig. 1, C, D and supplementary Fig. S1A). Similar results were obtained in HeLa cells treated with the other EGFR ligands, TGF-α and HB-EGF (Fig. 1E and supplementary Fig. S1B), and in EGF-treated A549 lung cancer cells (Fig. 1F and supplementary Fig. S1C). Although it was difficult to detect membrane-expressing EGFR at permeabilized condition, EGFR co-localized with the early endosomal marker EEA1 in HeLa cells, indicating that fluorescence dot signals were derived from endocytosed EGFR (supplementary Fig. S2A).

As reported previously (18), endocytosis by low-EGF was largely dependent on clathrin, while approximately half of EGFR endocytosis by high-EGF occurred independent of clathrin (supplementary Fig. S2, B and C). We next subjected non-permeabilized cells to flow cytometry (Fig. 1G) and immunofluorescence (Fig. 1H and supplementary Fig. S1D) to analyze cell surface EGFR. These analyses also revealed the distinct contributions of p38 to EGFR endocytosis by low-EGF and high-EGF. Collectively, these results demonstrate that EGF induces p38-dependent (non-canonical) as well as -independent (canonical) mechanisms for EGFR endocytosis in a concentration-dependent manner.

Non-canonical phosphorylation of EGFR at
low EGF concentration

It has been shown that p38-dependent phosphorylation of EGFR at C-terminal serine/threonine residues is involved in its cytokine-induced endocytosis. In order to investigate total EGFR phosphorylation, we employed immunoblotting using a Phos-tag, which detects phosphorylated proteins as shifted bands. TNF-α caused band shifts in all EGFR proteins expressed, and these were abolished by SB203580, but not by PD153035 (an EGFR tyrosine kinase inhibitor) or U0126 (a MEK inhibitor), indicating p38-dependent non-canonical EGFR phosphorylation (Fig. 2A). In analyses with various EGF concentrations, most EGFR molecules shifted unexpectedly at 3 ng/ml (Fig. 2B), at which canonical tyrosine autophosphorylation on Tyr-974, Tyr-1045 and Tyr-1068 was only slightly detected (Fig. 2C). Quantitative analysis demonstrated that low-EGF induced the band shift with only slight pY1068 (Fig. 2D). In addition, the shifts induced by low-EGF were also completely dependent on p38, suggesting that low-EGF stimulation mainly caused the non-canonical phosphorylation (Fig. 2E). Taken together, these results show that the minimal tyrosine kinase activation of EGFR by low-EGF leads to the prominent non-canonical phosphorylation of most cell surface EGFR molecules probably in a ligand-unbound form.

p38-dependent endocytosis of ligand-unbound EGFR

Previous studies mainly focused on the internalization of ligand-bound EGFR, described as the canonical endocytosis pathway in this study. We herein investigated whether p38-mediated endocytosis is an event in ligand-unbound EGFR. Ligand-receptor co-localization was monitored by immunofluorescence using rhodamine-conjugated EGF. A large amount of internalized EGFR co-localized with the ligand (yellow dots) in the presence of high-EGF. In contrast, low-EGF induced a small amount of yellow dots, but strongly enhanced green dots, which mainly composed of ligand-unbound EGFR (Fig. 3A). A quantitative analysis showed that although receptor endocytosis by low-EGF was similar to that by high-EGF, endocytosis of the ligand markedly increased (Fig. 3B). Moreover, all endocytic events were canceled by the EGFR inhibitor (Fig. 3C); however, the endocytosis of unliganded EGFR was selectively inhibited by the p38 inhibitor (Fig. 3, D and E). These results clearly demonstrate that ligand-dependent activation of the p38-mediated non-canonical pathway selectively induces the endocytosis of unliganded EGFR.

p38-dependent endocytosis of inactive EGFR monomers

EGFR markedly changes its conformation to initiate the activation of tyrosine kinase by dimerization (19, 20). In order to investigate whether p38-dependent EGFR endocytosis requires dimer formation, we generated a dimer-deficient EGFR mutant (dd-EGFR) with a C-terminal GFP tag, which lacks the CR1 loop in the extracellular dimerization domain and intracellular docking sites (Ile-682 and Val-924) (19, 21) (see also Fig. 4A). We confirmed the non-canonical phosphorylation of dd-EGFR as a similar Phos-tag shift pattern in endogenous EGFR (compare Fig. 4B to Fig. 2B). The shift induced by both low-EGF and high-EGF was dependent on p38 (Fig. 4C). Importantly, pY1068 of GFP-tagged dd-EGFR was not detected even in high-EGF stimulation, whereas GFP-tagged wild type EGFR (wt-EGFR) and endogenous EGFR were effectively activated, indicating that dd-EGFR did not form dimers with endogenous EGFR (Fig. 4D). In addition, phosphorylation of Ser-1046/1047, typical p38 target sites, of dd-EGFR and endogenous EGFR was detected to a similar extent, indicating that dd-EGFR only receives non-canonical regulation from endogenous EGFR (Fig. 4D).

We then examined the endocytosis of dd-EGFR in CHO-K1 cells expressing negligible levels of endogenous EGFR in order to assess its potential as a tool for non-canonical regulation. In contrast to the wt-EGFR, dd-EGFR did not internalize, even in the presence of high-EGF, whereas anisomycin, a p38 activator, efficiently triggered the endocytosis of dd-EGFR and wt-EGFR (Fig. 4E). The TNF-α-induced endocytosis of dd-EGFR was also observed in a p38-dependent manner, indicating that it maintains the potential for non-canonical, but not canonical endocytosis (Fig. 4F and supplementary Fig. S3A). We investigated whether the ligand-induced activation of endogenous EGFR signaling induces the internalization of dd-EGFR in HeLa cells. As expected, low-EGF induced the endocytosis of
wt-EGFR and dd-EGFR to a similar extent (Fig. 4G). Moreover, the endocytosis of dd-EGFR was completely abolished by the inhibition of p38, whereas wt-EGFR remained partially internalized (Fig. 4G and supplementary Fig. S3B). In addition, the endocytosis of dd-EGFR was clathrin-dependent (Fig. 4, H, I and supplementary Fig. S4A), and endocytosed dd-EGFR co-localized with EEA1 (supplementary Fig. S4B). These results demonstrate that ligand-induced and p38-mediated non-canonical EGFR endocytosis occurs in a dimerization-independent manner.

Identification of Ser/Thr sites controlling EGFR endocytosis

Previous studies reported that the p38-mediated phosphorylation of Ser-1015/Thr-1017/Ser-1018 (region 1; R1) or Ser-1046/Ser-1047 (region 2; R2) is important for stress signal-induced EGFR endocytosis (16, 22, 23). As described above, dd-EGFR was employed to identify amino acid residues involved in ligand-induced non-canonical endocytosis. The substitution of Lys-721 to alanine (K721A) did not affect ligand-induced non-canonical endocytosis, confirming that tyrosine kinase activity is not required for the endocytosis of dd-EGFR (Fig. 5B and supplementary Fig. S5A). Crucial phosphorylation sites for endocytosis were identified by the alanine substitution of serine/threonine residues in the R1 and R2 regions. Ligand-induced shifts in bands disappeared in the R1 mutant (R1m), but not R2m (Fig. 5C). In addition, R1m did not internalize following a low-EGF stimulation, although single S1015A mutation did not impair, indicating that the multiple phosphorylation of the R1 region is involved in p38-mediated non-canonical EGFR endocytosis (Fig. 5D and supplementary Fig. S5, B and C). A di-leucine motif (Leu-1010 and Leu-1011) near the R1 region, which was identified as an important site for EGFR endocytosis via an unknown mechanism (24, 25), was also involved in non-canonical phosphorylation and endocytosis, suggesting a functional interaction between the R1 region and neighboring di-leucine motif (supplementary Fig. S5, D and E).

We investigated whether the R1 site also regulates canonical endocytosis using wt-EGFR in CHO-K1 cells. GFP-tagged wt-EGFR was internalized by high-EGF and anisomycin (Fig. 5E). The high-EGF-induced endocytosis of EGFR-R1m was still intact, whereas anisomycin-induced endocytosis was impaired (Fig. 5E and supplementary Fig. S5F). These results demonstrate that the R1 site only regulates non-canonical endocytosis, which further supports the idea that canonical signaling regulates the non-canonical endocytosis of EGFR via the p38-mediated phosphorylation of serine/threonine residues.

Phosphorylation of EGFR at Ser-1015

We generated recombinant monoclonal phospho-specific EGFR (Ser-1015) antibodies to investigate phosphorylation of the R1 region of endogenous EGFR. We obtained five clones available for Western blot (supplemental Fig. S6A). Among them, we selected clone 10 in the following analyses, because it is also available for immunofluorescence. Surface plasmon resonance (SPR) analysis demonstrated specific binding of clone 10 to the phosphorylated antigen peptide with high affinity (KD = 1.25 x 10^-8 M) (supplementary Fig. S6B). Moreover, it could not recognize Ser-1015-mutated EGFR in immunoblotting (supplementary Fig. S6C).

In a time course analysis, both pS1015 and pS1047 in the R1 region and pS1047 in the R2 region were rapidly induced within 5 min in a p38-dependent manner in TNF-α-treated HeLa cells (Fig. 6, A and B). Similarly, low-EGF induced pS1015 in a p38-dependent manner, in which tyrosine autophosphorylation was observed prior to serine phosphorylation (Fig. 6, C, D and supplementary Fig. S6D). In addition, antibodies against pS1015 and pS1047 recognized the shifted bands in Phos-tag gel (supplementary Fig. S6E). Moreover, the mobility of pY1068 band was shifted down when pS1015 was inhibited by SB203580, suggesting that pY and pS were occurred on a single EGFR molecule (supplementary Fig. S6F).

Immunofluorescence analysis demonstrated that pS1015 was transiently induced at 15 min after TNF-α stimulation and overlapped with internalized EGFR (Fig. 6E) and pS1047 (Fig. 6F). TNF-α- and low-EGF-induced pS1015 was inhibited by SB203580 (supplementary Fig. S6G). Moreover, internalized dd-EGFR was also phosphorylated at Ser-1015, indicating that non-canonical phosphorylation was occurred on monomeric
EGFR (Fig. 6G). In a time course analysis, as comparing with wild type EGFR, endocytosis of dd-EGFR was delayed until 15 min (Fig. 6H). In addition, rapid dephosphorylation of Ser-1015 within 30 min in immunoblotting (Fig. 6, A and C) was linked to disappearance of endocytosed pS-EGFR at 30 min in low-EGF stimulation (Fig. 6F). Together, these results demonstrated that pS1015 closely correlated with endocytosis as well as the fate of EGFR after endocytosis.

**Endocytic mechanisms determine post-endocytic pathways of EGFR**

The mechanisms by which internalized EGFR are sorted to different endosomes for degradation or recycling are not fully understood. Since TNF-α induced selective endocytic recycling without degradation (14), we hypothesized that ligand-induced endocytosis via the non-canonical pathway is linked to the preferential recycling of EGFR. On the other hand, the canonically activated ligand-EGFR complex was mainly delivered to degradative lysosomes. Immunofluorescence demonstrated that endocytosed EGFR with a 15-min low-EGF stimulation was strongly recycled at 60 min, whereas high-EGF did not induce recycling (Fig. 7A). These results correlated with ligand-binding capacity to the cell surface, on which binding of rhodamine-conjugated EGF disappeared once after 15 min, but largely recovered at 60 min (Fig. 7B). A flow cytometric analysis clearly demonstrated that the recycling/degradation balance was controlled by the concentrations of EGF and TGF-α (Fig. 7C). Furthermore, the degradation of EGFR and tyrosine phosphorylation of Cbl ubiquitin ligase showed similar concentration dependencies (supplementary Fig. S7, B and C). These results strongly suggest that preferential recycling under low-ligand conditions reflects the phenomenon for p38-regulated EGFR via the non-canonical pathway; therefore, we next attempted to clarify the effects of SB203580 on the TGF-α-induced intracellular trafficking of EGFR. We confirmed that EGF and TGF-α induced similar concentration- and time-dependent activation of EGFR and its downstream pathways (supplementary Fig. S7D). The kinetics of EGFR internalization was examined in more detail by measuring cell surface EGFR using flow cytometry. As expected, EGFR was internalized in the first 15 min, and then approximately 50% of EGFR was recycled back to the cell surface within 30-60 min (Fig. 7D and supplementary Fig. S7E). Delayed internalization from 4-15 min was selectively inhibited by SB203580, whereas early phase internalization within 4 min was not (Fig. 7D). In addition, endocytosed EGFR in the presence of SB203580 was not recycled (Fig. 7D and supplementary Fig. S5B). The p38 inhibitor exerted similar effects on EGF- and TGF-α-treated cells (Fig. 7E).

In order to confirm whether two independent endocytic mechanisms influence the trafficking routes of EGFR, dd-EGFR was expressed at a similar level to endogenous EGFR in HeLa cells and their behaviors were monitored. A stimulation with high-EGF resulted in the sufficient degradation of exogenous GFP-tagged wt-EGFR and endogenous EGFR. In contrast, GFP-tagged dd-EGFR was not degraded until 180 min; it was recycled back to the plasma membrane, even with the high-EGF stimulation (Fig. 7, F-H), indicating that the EGFR status influences endocytic trafficking. Collectively, these results reveal that canonical and non-canonical endocytic mechanisms determine the intracellular trafficking of EGFR, namely, degradation or recycling, respectively. The most important result here is that a major recycling component is ligand-unbound EGFR, which is internalized via a p38-dependent mechanism.

**Transient suppression of ligand-induced EGFR activation**

We previously demonstrated the TNF-α-induced transient suppression of ligand-induced EGFR activation by prior non-canonical phosphorylation (14). In the present study, we showed that low-ligand conditions established a similar intracellular environment; therefore, we attempted to investigate the effects of a 10-min low-ligand pretreatment on high-ligand-induced EGFR activation (Fig. 8A). The pretreatment resulted in a reduction in secondary high-ligand-induced pY-EGFR from the no pretreatment control (Fig. 8B). In contrast, recycled EGFR at 60 min was sufficiently functional to be activated again by the extracellular ligand (Fig. 8C). These results correlated with ligand-binding capacity to the cell surface, on which binding disappeared once after 15 min, but largely recovered at 60 min (Fig. 7B). Collectively, the
ligand-induced non-canonical control of EGFR causes transient feedback inhibition in the early phase, but subsequently may contribute to the sequential activation of growth factor signaling.

Discussion

A major unsolved issue in the ligand-induced endocytic trafficking mechanisms of EGFR is how it is recruited to the different endocytic machineries and then sorted to different routes for recycling or degradation (5-7, 26, 27). In the present study, we demonstrated the dual-mode regulation of the ligand-induced endocytic trafficking dynamics of EGFR (Fig. 9). Since the minimal activation of EGFR was sufficient to fully activate MAPKs, many unliganded cell surface EGFR are targets for feedback phosphorylation by activated MAPKs. Therefore, non-canonical endocytosis is the main event following a stimulation under low-ligand conditions. In contrast, the majority of EGFR may initially be occupied by ligands under high-ligand conditions, which induces the dimerization-dependent tyrosine kinase activation and internalization of a ligand-EGFR complex before the activation of p38. We propose that two different forms of EGFR are internalized following a ligand stimulation: the preceding ligand-bound pY-EGFR dimers and delayed unliganded pST-EGFR monomers, which result in straightforward transport to lysosomal degradation and recycling to the cell surface, respectively. Thus, ligand-induced EGFR trafficking involves the complex parallel events of canonical and non-canonical endocytosis, which are balanced by the number of cell surface EGFR proteins, initial ligand occupation rate, and MAPK activation levels.

Another factor to consider in the post-endocytic fate of EGFR is ligand specificity. TGF-α, for example, appears to dissociate from EGFR at endosomal acidic pH, thereby allowing the receptor to escape from lysosomal degradation and recycling to the cell surface. In contrast, EGFR preferentially causes lysosomal degradation over TGF-α because EGF-EGFR binding remains stable at acidic pH in endosomes (5, 28). An integrated multilayered proteomics approach using a high concentration of ligands (100 ng/ml) recently identified Rab7 tyrosine phosphorylation and the recruitment of the Rab-coupling protein RCP to EGFR as molecular switches dictating TGF-α- and EGF-dependent EGFR trafficking (29). We demonstrated that EGF and TGF-α both provoked the canonical and non-canonical endocytic trafficking of EGFR, indicating that not only the stability of the ligand-EGFR complex in endosomes, but also the balance between canonical and non-canonical trafficking are important mechanisms responsible for ligand specificity.

Ligand-induced endocytosis/recycling of ligand-unbound EGFR, the new setting in this study, is similar to TNF-α-induced clathrin-mediated endocytosis and recycling. The present study clearly demonstrated using dd-EGFR that low-EGF as well as TNF-α induced the tyrosine kinase-independent and clathrin-dependent endocytosis of EGFR monomers through the p38-dependent phosphorylation of C-terminal region 1 around Ser-1015. Low-EGF and TNF-α both induce transient p38 activation; therefore, EGFR undergoes plasma membrane recycling within 30-60 min of the dephosphorylation of region 1. Furthermore, ubiquitination is not involved in p38-dependent endocytic trafficking (14, 17). Therefore, low-EGF and TNF-α appear to drive a common endocytosis/recycling system under the control of p38 activation. A recent study reported that cellular stresses, including ultraviolet C (UVC) and the genotoxic agent cisplatin, trigger sustained p38 activation and the non-canonical endocytosis of EGFR, in which EGFR accumulates in a subset of lysobisphosphatidic acid (LBPA)-rich perinuclear MVBs through a mechanism involving the actin polymerization-promoting protein WASH and endosomal sorting complex containing ALIX and ESCRT (30). The inhibition of p38 results in the recycling of intraluminally sorted EGFR to the cell surface. We also previously demonstrated that high osmotic conditions induced the sustained internalization of EGFR during persistent p38 activation (14). We speculate that non-canonically endocytosed EGFR under low-EGF conditions also accumulate in LBPA-rich MVBs, and are then rapidly sorted to recycling endosomes after the dephosphorylation of region 1 due to the rapid turnover of p38 activation. Thus, p38 activation kinetics may be a critical determinant of the retention time of pST-EGFR in MVBs. In any case, endocytosed
pST-EGFR is not sorted to a distinct subset of degradative MVBs, the main sorting compartments of the activated ligand-EGFR complex with tyrosine phosphorylation and ubiquitination. EEA1 (31), an early endosomal marker, and Eps15 (32), a clathrin-adapter protein, are also phosphorylated by p38, suggesting that the clathrin-dependent endocytosis and endosomal sorting of EGFR are completely controlled by p38 signaling pathways.

Convincing evidence has been reported for the ubiquitin-dependent targeting of EGFR to lysosomal degradation (6, 33, 34). A threshold-controlled ubiquitination model was recently proposed, in which the E3 ligase Cbl is recruited in complex with Grb2 to EGFR with pY1045 (35). We confirmed that high-EGF efficiently induced EGFR degradation, even when p38 was activated. However, p38 is not involved in the early canonical internalization process of the ligand-EGFR complex because canonical endocytosis occurred prior to p38-mediated non-canonical phosphorylation (Fig. 6C). In contrast, the results of the Phos-tag analysis suggest that ligand-activated pY-EGFR is additionally phosphorylated by p38 after internalization (Fig. 2E). An in vitro study previously demonstrated that the phosphorylation of Ser-1046/1047 reduced the binding affinity of Cbl to pY1045 (36). More importantly, the threshold EGF concentration for EGFR ubiquitination (approximately 3 ng/ml) was similar to the minimally effective concentration for the p38-mediated non-canonical regulation of EGFR in HeLa cells, suggesting the role of p38 in the threshold-controlled lysosomal targeting of ubiquitinated EGFR. There is a possibility that Ser-1015/Thr-1017/Ser-1018 in the R1 and Ser-1046/Ser-1047 in the R2 play distinct roles in triggering non-canonical endocytosis and preventing ubiquitination of EGFR. Further characterization is essential for understanding the potential role of p38-mediated serine/threonine phosphorylation in the ubiquitination-dependent degradation of EGFR.

The intensity and duration of receptor activation are known to affect cellular responses to a ligand (37, 38). Low-EGF has been shown to stimulate cell proliferation in a similar manner to high-EGF and sustained EGFR signaling is controlled by clathrin-mediated endocytosis in HeLa cells (8). In the present study, we clarified the importance of the MAPK-mediated feedback phosphorylation of EGFR following a low-EGF stimulation, which may cause transient impairments in association with the extracellular ligand with endocytosed EGFR across the membrane. ERK-mediated Thr-669 phosphorylation in the juxtamembrane domain, a negative feedback site, may also be involved in the inhibition of EGFR tyrosine kinase activity (39). In any case, it is important for recycled EGFR to be sufficiently functional in order to respond to the ligand, and the continuous existence of active ligands in the extracellular environment may be necessary for sustained EGFR signaling (Fig. 7, A and B). These results suggest that EGF induces the multi-phase activation of EGFR on the plasma membrane, which may be beneficial for sustaining receptor signaling.

Mice with kinase-inactive EGFR have some eye and skin defects, but better survival than EGFR-deficient mice, indicating kinase-independent roles for EGFR (15). We previously demonstrated that EGFR plays an anti-apoptotic role in the TNF-α signaling pathway (12). The most notable finding from recent studies is the role of EGFR in autophagy. Serum starvation induces the endosomal arrest of inactive EGFR via an interaction with the endosomal protein lysosomal-associated protein transmembrane 4 beta (LAPTM4B) (40). EGFR then induces the dissociation of the Run domain Beclin-1 interacting and cysteine-rich containing protein (Rubicon) from the Beclin-1 complex, which activates the initiation of autophagy by Beclin-1. EGFR tyrosine kinase inhibitors, including gefitinib and erlotinib, may also trigger autophagy in cancer cells (15). In contrast, EGF, tested at 100 ng/ml, inhibits the initiation of autophagy by decreasing the interaction between EGFR and LAPTM4B (40). In addition, the EGFR-mediated tyrosine phosphorylation of Beclin-1 is also involved in the suppression of autophagy (41). EGFR may be sorted to different endosomes/MVBs in a ligand concentration-dependent manner; therefore, it will be interesting to evaluate the effects of low-EGF, which may induce the endocytosis of many inactive EGFR, on the initiation of autophagy. Further studies are needed in order to fully understand the physiological roles of inactive EGFR in the
ligand-induced dual-mode activation model.

In summary, we herein describe a new concept for the ligand-induced regulation of multiple receptor functions in EGFR systems. The results obtained may be applied to other ligand-receptor systems in cellular signaling. Moreover, a comprehensive understanding of the feedback regulation of receptors is the next important challenge in signal transduction research, and will contribute to the oncology field by providing information for identifying new therapeutic targets and overcoming resistance to anti-cancer agents.

Experimental procedures

Antibodies and reagents

Phospho-specific antibodies against p38 (Thr-180/Tyr-182), ERK (Thr-202/Tyr 204), and EGFR (Tyr-845, Tyr-974, Tyr-1045, Tyr-1068 and Ser-1046/1047) and anti-CHC and EEA1 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against EGFR (1005), c-Cbl (C-15), α-Tubulin (B-7), GFP (B-2), and Actin (C-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An anti-EGFR (Ser-1047, clone 1H9) antibody was purchased from Abcam (Cambridge, MA, USA), which is available for immunofluorescence and immunoblotting. Both of the phospho-specific antibodies against Ser-1046/1047 (Cell Signaling Technology) and Ser-1047 (Abcam) can similarly detect pS-EGFR in immunoblotting. For immunofluorescence, an anti-EGFR antibody (clone LA1; Upstate) was used. Recombinant human EGF, HB-EGF, and TNF-α were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant human TGF-α was obtained from PeproTech (Rocky Hill, NJ, USA). Rhodamine-conjugated EGF was obtained from Life Technologies (Carlsbad, CA, USA). The Phos-tag ligand and anisomycin were from Wako Pure Chemical Industries (Osaka, Japan). SB203580, U0126, and PD153035 were from Merck Biosciences (Darmstadt, Germany). All chemical inhibitors were dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide was less than 0.1%.

Cell culture

HeLa and HEK293 cells were obtained from the American Type Culture Collection (ATCC, Rockville, TX, USA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2. A549 cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2. CHO-K1 cells, a kind gift from Prof. T. Imanaka (University of Toyama, Toyama, Japan), were cultured in Ham F12 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2.

Transfection of plasmid DNAs

HeLa, HEK293, and CHO-K1 cells were transfected using Lipofectamine Reagent, Lipofectamine 2000, or Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA), respectively, in accordance with the manufacturer’s instructions. The GFP-tagged EGFR expression plasmid was described previously (12). The mutations K721A (KD), S1046A/S1047A (R2m), S1015A/T1017A/S1018A (R1m), ΔCR1/I682Q/V924R (dd), and L1010A/L1011A (LLAA) were generated by PCR with KOD FX Neo polymerase or KOD-Plus-Neo polymerase (TOYOBO, Tokyo, Japan).

RNA Interference

Small interfering RNAs (siRNAs) were synthesized by Hokkaido System Science Co., Ltd. (Sapporo, Japan). The target sequences were as follows: 5’-TAATCCAATTGAGACACCAAT-3’ (CHC), 5’-GCAUUAACACCCAGACGUUGAUUU-3’ (p38α), and 5’-CGUACGCGAAUACUUGCAG-3’ (firefly luciferase GL2 as a negative control). HeLa cells were transfected with siRNAs at a final concentration of 20 to 100 nM using Lipofectamine reagent or Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). Cells were used for experiments 48 or 72 h post-transfection.

Immunoblotting

After a stimulation or transfection, whole cell lysates were prepared with lysis buffer (25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 20
mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 µg/ml aprotinin, and 10 µg/ml leupeptin). Cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P nylon membrane (Millipore, Billerica, MA, USA). The membrane was treated with Block Ace (Dainippon Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan) and stained with primary antibodies. Antibodies were detected using horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat immunoglobulin G (Dako, Glostrup, Denmark) and visualized with an enhanced chemiluminescence system (GE Healthcare Bioscience, Piscataway, NJ, USA). Some antibody reactions were performed in Can Get Signal solution (TOYOBO, Tokyo, Japan).

**Immunoprecipitation**

Cell lysates were diluted with an equal volume of dilution buffer (20 mM HEPES pH 7.7, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). After centrifugation, lysates were incubated with antibodies at 4 °C overnight, and then rotated with Dynabeads protein G (Thermo Fisher Scientific) at 4 °C for 1.5 h. The beads were washed three times with wash buffer (1:1 mixture of whole cell lysate buffer and dilution buffer).

**Zn²⁺-Phos-Tag SDS-PAGE**

The procedures for Zn²⁺-Phos-tag SDS-PAGE were described previously (32, 42). Cell lysates were prepared with RIPA buffer (50 mM Tris-HCl (pH7.4), 0.15 M NaCl, 0.25% sodium deoxycholate, 1.0% NP-40, 1.0 mM EDTA, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). Each sample was mixed with a half volume of SDS-PAGE sample buffer (195 mM Tris-HCl (pH 6.8), 3.0% SDS, 15% 2-mercaptoethanol, 30% glycerol, and 0.1% bromophenol blue) and heated at 95 °C for 5 min. The acrylamide pendant Phos-tag ligand and two equivalents of ZnCl₂ were added to the separating gel before polymerization. The running buffer consisted of 100 mM Tris and 100 mM MOPS containing 0.1% SDS and 5 mM sodium bisulfite. After electrophoresis, the gel was washed twice by a solution containing 25 mM Tris, 192 mM glycine, 10% methanol, and 1.0 mM EDTA for 20 min and then washed once with a solution containing 25 mM Tris, 192 mM glycine, and 10% methanol for 20 min. Gel transfer, blocking, antibody reactions, and detection were performed according to the normal immunoblotting protocol described above.

**Flow Cytometry**

HeLa cells were harvested in phosphate-buffered saline (PBS). Cells were fixed with 2% paraformaldehyde at room temperature for 15 min. Cells were resuspended in 100 µl FACS buffer (PBS containing 0.5% bovine serum albumin (BSA)) containing 0.5 µg of an anti-EGFR monoclonal antibody (clone LA1; Upstate) and incubated on ice for 1 h. After being washed with FACS buffer, cells were incubated with a fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G antibody (Dako, Glostrup, Denmark) on ice for 1 h in the dark, and then analyzed using the FACS Calibur system (BD Biosciences).

**Immunofluorescence**

Cells were seeded on cover glasses. Two days after seeding, cells were incubated with inhibitors and ligands or transfected with plasmid DNAs. Cells were rinsed in cold PBS and fixed in 4% paraformaldehyde (PFA) for 15 min or methanol for 10 min. After fixation with PFA, cells were permeabilized in PBS containing 0.5% Triton X-100 and washed by PBS. Cells were incubated for 1 h with a primary antibody and then washed and incubated with isotype-specific secondary antibodies conjugated to Alexa Fluor (Invitrogen) for 30 min. These antibodies were diluted in PBS containing 0.5% BSA. Microscopy was performed using LSM 700 confocal microscope (Zeiss, Oberkochen, Germany).

For quantification, signal intensities of internalized EGFR dots were calculated as a grey value. At least 50 cell profiles were counted and data represent the mean ± S.D. We confirmed the reproducibility of the data in more than two independent experiments and a representative result is shown.

**Generation of rabbit monoclonal antibodies**
against pS1015-EGFR

Phospho-specific monoclonal antibodies were generated using the rabbit-immunospot array assay on a chip (ISAAC) system, as described previously (43, 44). The synthetic peptides, EGFR-peptide (TPLLSSLSATSNNST), EGFR-peptide phosphorylated at Ser-1015 (pS-EGFR; TPLLSSL(pS)ATSNNST), biotinylated pS-EGFR peptide, and KLH conjugated pS-EGFR peptide, were obtained from Eurofins (Tokyo, Japan). A rabbit was immunized with the KLH-conjugated pS-EGFR peptide. Immunoglobulin (IgG) was purified, titrated by an enzyme-linked immunosorbent assay, and applied for Western blotting and immunofluorescence. Experiments using rabbits were approved by the Committee on Animal Experiments at University of Toyama.

Data processing and statistical analysis

We confirmed the reproducibility of the data in more than three independent experiments and a representative result is shown. Quantitative analysis of immunoblots and immunofluorescence was performed using densitometry with ImageJ software. Values are shown as the mean ± S.D.. The significance of differences was assessed by the Student’s t-test. P values of <0.01 were considered to be significant.

Acknowledgements: We are grateful to Drs. Tsuneo Imanaka and Chihiro Tohda for providing materials and technical assistance, respectively. This work was supported in part by JSPS KAKENHI Grant Number JP16H04694 and Platform Project for Supporting Drug Discovery and Life Science Research from Japan Agency for Medical Research and Development (AMED).

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: T.T. contributed to the experimental design, conduction of experiments, data analysis and interpretation, and manuscript writing. Y.Z. contributed to the experimental design, data analysis and interpretation. T.O and A.M. contributed to the antibody production. R.O., A.B., T.Y., and E.O. performed the cell culture experiments. H.S. contributed to the experimental design, data analysis and interpretation, and manuscript writing.

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**Footnotes**

The abbreviations used are: RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; TNF-α, tumor necrosis factor-α; TGF-α, transforming growth factor-α; CME, clathrin-mediated endocytosis; CIE, clathrin-independent endocytosis

**FIGURE LEGENDS**

**Figure 1.** p38-mediated endocytosis of EGFR with a ligand stimulation.

*A,* HeLa cells were pretreated with 10 µM SB203580 or 5 µM U0126 for 30 min and then stimulated with 100 ng/ml TNF-α or 3 or 100 ng/ml EGF for another 15 min. The subcellular localization of EGFR was analyzed by confocal fluorescent microscopy. *B,* Signal intensities of internalized EGFR dots in (*A*) were calculated as a grey value. At least 50 cell profiles were counted and data represent the mean ± S.D. *C, D,* HeLa cells were transfected with siRNAs against p38α or the negative control and incubated for 48 h. Cells were stimulated with 20 ng/ml TNF-α or 3 or 100 ng/ml EGF for 15 min, and the subcellular localization of EGFR (green) was then analyzed by confocal fluorescent microscopy (*C*). Scale bar, 10 µm. The knockdown efficiency of p38 was assessed by immunoblotting (*D*).

**Figure 2.** Ligand-Induced Feedback Phosphorylation of EGFR by MAPKs.

*A,* HeLa cells were pretreated with 1 µM PD153035 (PD), 10 µM SB203580 (SB), and 5 µM U0126 (U) for 30 min, and then stimulated with 20 ng/ml TNF-α for another 10 min. Whole cell lysates were separated by Zn²⁺-Phos-tag SDS-PAGE followed by immunoblotting with an anti-EGFR antibody. *B, C,* Whole cell lysates were prepared from HeLa cells stimulated with the indicated concentration of EGF for 10 min. After Zn²⁺-Phos-tag SDS-PAGE (*B*) and normal SDS-PAGE (*C*), the expression of each protein was detected. *D,* Band densities in (*B*) and (*C*) were quantified by ImageJ software, and the band shift rate of EGFR in the Phos-tag gel and pY1068-EGFR were calculated. Values represent the mean ± S.D. of three independent experiments as fold increases. *E,* HeLa cells were stimulated with the indicated concentration of 3 ng/ml EGF for 10 min. Chemical inhibitors were pretreated for 30 min before the addition of EGF. Whole cell lysates were separated by Zn²⁺-Phos-tag SDS-PAGE followed by immunoblotting with an anti-EGFR antibody.

**Figure 3.** p38-mediated endocytosis of unliganded EGFR.

*A,* HeLa cells were treated with the indicated concentration of rhodamine-EGF (red) for 15 min, fixed, and then stained with an anti-EGFR antibody (green). The subcellular localization of rhodamine and EGFR was examined by confocal fluorescent microscopy. Scale bar, 10 µm. *B,* The signal intensities of EGFR and rhodamine-EGF in (*A*) were calculated independently as grey values. At least 110 cell profiles were counted and values represent the mean ± S.D. *C, D,* HeLa cells were pretreated with 1 µM PD153035 (PD) or 10 µM SB203580 (SB) for 30 min and then treated with 3 or 100 ng/ml rhodamine-EGF (red) for another 15 min. The subcellular localization of EGFR (green) was assessed. Merged photos are shown. *E,* The signal intensities in (*D*) were calculated. At least 110 cell profiles were counted and values represent the mean ± S.D. *: p<0.01.
Figure 4. Endocytosis of EGFR monomers via EGF-induced p38 activation.

A, Schematic diagram of ligand-induced EGFR endocytosis. Ligand (red circle) binding to wild-type EGFR (wt-EGFR) caused the tyrosine phosphorylation (pY) of the EGFR asymmetric dimer. Activated wt-EGFR induced the p38-dependent serine/threonine phosphorylation (pST) of dimer-deficient EGFR (dd-EGFR) harboring a deletion (ΔCR1) and point mutations (I682Q and V924R). B, C, HeLa cells were transiently transfected with GFP-tagged dd-EGFR. Cells were stimulated with the indicated concentrations of EGF for 10 min (B). Transfected cells were pretreated with 10 μM SB203580 for 30 min and then treated with 3 or 100 ng/ml EGF for another 10 min (C). The Phos-tag shift was assessed by immunoblotting with an anti-GFP antibody. D, HeLa cells were transiently transfected with GFP-tagged wt- or dd-EGFR and stimulated with 100 ng/ml EGF for 10 min. The phosphorylation and total expression of endogenous (endo) and exogenous (GFP) EGFR were assessed by immunoblotting. E, CHO-K1 cells were transiently transfected with GFP-tagged wt- or dd-EGFR and then stimulated with 100 ng/ml EGF for 15 min or 50 μM anisomycin for 30 min. The subcellular localization of EGFR-GFP was analyzed. F, G, HeLa cells were transfected with GFP-tagged dd-EGFR with the K721A mutation (dd-K721A), pretreated with 10 µM SB203580 for 30 min, and then stimulated with 3 ng ml⁻¹ EGF or 20 ng/ml TNF-α for 15 min. The subcellular localization of EGFR-GFP was analyzed. H, I, HeLa cells were transfected with siRNAs against CHC or the negative control. After a 48-h incubation, cells were further transfected with GFP-tagged wt- or dd-EGFR and stimulated with 5 ng/ml EGF. The knockdown efficiency of CHC was confirmed by immunoblotting (H). The subcellular localization of EGFR-GFP was analyzed (I).

Figure 5. Phosphorylation of Ser/Thr is essential for non-canonical EGFR endocytosis.

A, The structure and key amino acid residues of EGFR. Tyrosine phosphorylation sites and two p38 target regions are shown in red and green, respectively. TM, transmembrane domain; JM, juxtamembrane domain; TK, tyrosine kinase domain; CT, C-terminal domain. B, HeLa cells were transiently transfected with GFP-tagged dd-EGFR with the K721A mutation (dd-K721A), pretreated with 10 μM SB203580 for 30 min, and then stimulated with 3 ng ml⁻¹ EGF or 20 ng/ml TNF-α for 15 min. The subcellular localization of EGFR-GFP was analyzed. C, CHO-K1 cells were transiently transfected with GFP-tagged wt-EGFR or EGFR-R1m and stimulated with 100 ng/ml EGF for 15 min or anisomycin for 30 min. The subcellular localization of EGFR-GFP was analyzed. Scale bar, 10 μm.

Figure 6. Phosphorylation of EGFR at Ser-1015 in endocytic trafficking.

A, C, HeLa cells were stimulated with 20 ng/ml TNF-α (A) or 3 ng/ml EGF (C) for the indicated time. B, D, HeLa cells were pretreated with 10 μM SB203580 (SB) and 5 μM U0126 (U) for 30 min, and then stimulated with 20 ng/ml TNF-α or 3 ng/ml EGF for another 10 min. A-D, Whole cell lysates were analyzed by immunoblotting with the indicated antibodies. E, F, HeLa cells were stimulated with 20 ng/ml TNF-α for the indicated time. The subcellular localization of total-EGFR, pS1015-EGFR and pS1047-EGFR was analyzed by confocal fluorescent microscopy. G, HeLa cells were transiently transfected with GFP-tagged dd-EGFR and then stimulated with 3ng/ml EGF or 20 ng/ml TNF-α for 15 min. The subcellular localization of EGFR-GFP was analyzed. Scale bar, 10 μm.

Figure 7. Post-endocytic fate of EGFR with low- and high-ligand stimuli.

A, HeLa cells were treated with EGF (3 or 100 ng/ml) or TNF-α (20 ng/ml) for 15 or 60 min. The cell surface expression of EGFR was analyzed by immunofluorescence under non-permeable conditions. B, HeLa cells were pretreated with 3 ng/ml EGF at 37 °C for the indicated time, washed three times with...
cold PBS, and then treated with rhodamine-EGF at 4 °C for 30 min. The cell surface binding of rhodamine-EGF was analyzed by confocal fluorescence microscopy. C, HeLa cells were treated with EGF or TGF-α (3, 10, 30, and 100 ng/ml) for 15 or 60 min. The cell surface expression of EGFR was analyzed by flow cytometry and the recycling ratio was calculated using the median values of fluorescence. Data are shown as the mean ± S.D. of three independent experiments. D, E, HeLa cells were stimulated with 10 ng/ml EGF or TGF-α for the indicated time in the absence or presence of 10 μM SB203580. The percentage of maximal internalization was calculated using the median values of fluorescence in flow cytometric assays. Data represent the mean ± S.D. of four (D) and three (E) independent experiments. *: p<0.01. n.s.: not significant. F, G, HeLa cells were transiently transfected with GFP-tagged wt-EGFR or dd-EGFR and then stimulated with 100 ng/ml EGF for the indicated time. GFP-tagged and endogenous EGFR were detected by immunoblotting with an anti-EGFR antibody (F). The band densities of GFP-tagged and endogenous EGFR in control and stimulated (180 min) cells were measured (G). Data represent the mean ± S.D. of three independent experiments. *: p<0.01. H, HeLa cells were transiently transfected with GFP-tagged wt- or dd-EGFR and then stimulated with 100 ng/ml EGF for 10 and 180 min. The subcellular localization of EGFR-GFP was analyzed. Scale bar, 10 μm.

Figure 8. Transient suppression of ligand-induced EGFR activation. A, The protocol for the experiment in B is shown. B, HeLa cells were pretreated with 3 ng/ml EGF (pre-EGF) or 100 ng/ml TNF-α (pre-TNF-α) for 10 min, and then stimulated with 100 ng/ml EGF for 2 or 5 min (post-EGF). C, HeLa cells were pretreated with 3 ng/ml EGF or 10 ng/ml TGF-α for the indicated time, and then stimulated with 100 ng/ml EGF (post-EGF). Whole cell lysates were immunoblotted with phospho-EGFR (Tyr-845, 1045, and 1068), EGFR, phospho-ERK, and α-Tubulin antibodies.

Figure 9. A model for ligand concentration-dependent dual endocytic trafficking of EGFR. Ligand binding induces dimerization of cell surface EGFR and tyrosine phosphorylation (pY)-dependent endocytosis (canonical endocytosis; shown in red). In addition, canonically activated EGFR induces p38 activation, which leads to serine/threonine phosphorylation (pST) and clathrin-dependent endocytosis of monomeric EGFR (non-canonical pathway; shown in green). Thus, ligand-induced EGFR trafficking involves the complex parallel events of canonical and non-canonical endocytosis, which are balanced by the ligand concentration reflecting initial ligand occupation rate. Under low-ligand condition, a large amount of ligand-unbound EGFR is internalized via non-canonical pathway, and then recycled back to the cell surface. Conversely, ligand-occupied EGFR is internalized mainly via canonical pathway under high-ligand condition, which is preferentially sorted to the lysosome for degradation.
**Fig 1. Tanaka et al.**

A. DMSO, SB203580, U0126

- TNF-α
- EGF (3)
- EGF (100)

EGFR/DAPI

B. Relative intensity (%)

| Inhibitors: - SB U | TNF-α | EGF (3) | EGF (100) |
|-------------------|-------|---------|-----------|
|                   |       | 0       | 50        |
|                   |       | *       | *         |
|                   |       | n.s.    |           |

C. siCont, sip38α

- TNF-α
- EGF (3)
- EGF (100)

EGFR/DAPI

D. p38, α-Tubulin

E. DMSO, SB203580

- TNF-α
- TGF-α (3)
- TGF-α (100)

HB-EGF (3)

EGFR/DAPI

F. A549 cells

- DMSO
- SB203580

EGFR/DAPI

G. A549 cells

- DMSO
- SB203580

EGFR/DAPI

H. Cont, EGFR

- TNF-α
- EGF (3)
- EGF (100)

Counts

Cell surface EGFR

- Cont
- EGF (3)
- EGF (100) + SB

- Cont
- EGF (100)
- EGF (100) + SB
Fig 2. Tanaka et al.

A

TNF-α: -- ++ + +
Inhibitor: -- PD SB U

Phos-tag
IB: EGFR

B

EGF (ng/ml): 0.1 0.3 3 10 30 100

Phos-tag
IB: EGFR

C

EGF (ng/ml): 0.1 0.3 3 10 30 100

pY974
pY1045
pY1068
EGFR
pp38
α-Tubulin

D

Intensity (fold)

EGFR (shifted)

0 3 100

EGF (ng/ml)

E

EGF: -- 3 ng/ml
Inhibitor: -- PD SB U

Phos-tag
IB: EGFR
Fig 3. Tanaka et al.

A

Rh-EGF (3)  Rh-EGF (100)

Rh-EGF

EGFR

Merged (+DAPI)

(Enlarged)

B

EGFR

Rh-EGF

C

Rh-EGF (3)  Rh-EGF (100)

DMSO  PD

DMSO  PD

Merged

Rh-EGF/EGFR/DAPI

D

Rh-EGF (3)  Rh-EGF (100)

DMSO  SB

DMSO  SB

Merged

Rh-EGF/EGFR/DAPI

E

EGFR

Rh-EGF

EGFR

Rh-EGF

Rh-EGF (3)  Rh-EGF (100)

DMSO SB  DMSO SB

DMSO SB  DMSO SB

Relative intensity

Rh-EGF(ng/ml)

Relative intensity

n.s.

*
**Fig 4. Tanaka et al.**

**A**

Wild type (wt) ➔ Dimer deficient (dd)

- I682Q
- V924R
- ΔCR1

Endocytosis ➔ Endocytosis

**B**

EGF (ng/ml): 0 3 100

Phos-tag Phos-tag

IB: GFP (dd)

**C**

EGF (ng/ml): 0 3 100

SB203580: + + +

**D**

EGFR-GFP:

- wt
- dd

EGF (100):

- -
- +
- -
- +

pY1068

GFP

endo

pS1046/7

GFP

endo

EGFR

GFP

endo

α-Tubulin

**E**

CHO-K1 cells

- Cont
- EGF (100)
- Anisomycin

**F**

Cont

TNF-α

- DMSO
- DMSO
- SB

wt

dd

EGFR (GFP)/DAPI

**G**

Cont

EGF (5)

- DMSO
- DMSO
- SB

wt

dd

EGFR (GFP)/DAPI

**H**

wt
dd

siRNA:

- Cont
- CHC

EGFR (GFP)

α-Tubulin

**I**

siCont

siCHC

- EGF (5)

EGFR (GFP)/DAPI
Fig 6. Tanaka et al.
Fig 7. Tanaka et al.

A

|        | Cont | EGF (3) | EGF (100) | TNF-α |
|--------|------|---------|-----------|-------|
| 0 min  |      |         |           |       |
| 15 min |      |         |           |       |
| 60 min |      |         |           |       |

EGFR

DAPI

B

|        | EGF (3) |
|--------|---------|
| 0 min  |         |
| 15 min |         |
| 60 min |         |

Rh-EGF/DAPI

C

|        | EGF | TGF-α |
|--------|-----|-------|
|        | 3   | 10  |
|        | 30  | 100 |
| Recycl. | 40 | 60 |

D

|        |        |
|--------|--------|
|        |        |

Loss of cell surface EGFR expression (%)

E

|        |        |
|--------|--------|
|        |        |

EGF (10)

TGF-α (10)

SB: - +

F

|        |        |
|--------|--------|
|        |        |

EGFR (GFP):

wt
dd

GFP-tagged

Endogenous

IB: EGFR

G

|        |        |
|--------|--------|
|        |        |

Expression (fold)

H

|        |        |
|--------|--------|
|        |        |

0 min 15 min 180 min

wt

dd

EGFR (GFP)/DAPI
Fig 8. Tanaka et al.

A

B

C
Ligand-activated epidermal growth factor receptor (EGFR) signaling governs endocytic trafficking of unliganded receptor monomers by non-canonical phosphorylation

Tomohiro Tanaka, Yue Zhou, Tatsuhiko Ozawa, Ryuya Okizono, Ayako Banba, Tomohiro Yamamura, Eiji Oga, Atsushi Muraguchi and Hiroaki Sakurai

*J. Biol. Chem.* published online December 18, 2017

Access the most updated version of this article at doi: 10.1074/jbc.M117.811299

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