induced the recruitment of cytosolic p53 to the plasma membrane and p53 was co-localized with a complex including CHC, EGF and EGFR, suggesting that the binding of EGF to EGFR leads to post-translational modifications and certain conformational change of the cytoplasmic region of EGFR to allow the recruitment of p53 as well as endocytic apparatus (Fig. 3F). We next examined whether loss-of-function mutants of p53 are recruited to plasma membrane by EGF treatment. For this purpose, we used two cell lines, DLD1 and T98G, bearing p53 mutations, S241F and M237I, respectively. Even though these cell lines were treated with EGF, the recruitment of p53 to plasma membrane was not observed under confocal microscopy (Supplementary Fig. S2A), although the tumor-derived p53 mutants are able to associate with CHC (Supplementary Fig. S2B).

To assess the effect of down-regulation of CHC gene expression by RNAi on EGF-induced translocation of p53 to plasmalemmal sites, two small-interfering RNAs (siRNAs) for different sequences in *chc* mRNA were synthesized and used. Because complete depletion of CHC expression caused cell death and cell growth arrest, CHC expression was partially reduced by restricted amounts of siRNA. CHC down-regulation by RNAi caused the decreased EGF-induced p53 translocation (Supplementary Fig. S3), suggesting that CHC is required for the EGF-induced recruitment of p53 to plasma membrane.

### Depletion of p53 by RNAi delays EGFR internalization

The co-localization of p53 with CHC at the site, where endocytosis occurs promoted us to investigate the role of p53 in clathrin-mediated endocytosis. To test the effect of p53 on clathrin-mediated endocytosis, we knocked down p53 expression using RNAi. For endocytic activity, the uptake of $^{125}$I-labeled EGF into TIG-7 cells was quantified. When cells were infected with lentiviruses encoding short-hairpin RNA (shRNA) against p53, the expression level of p53 was reduced more than 90% compared with control cells (Fig. 4A). These cells were starved in serum-free medium for 3 h, treated with $^{125}$I-labeled EGF on ice to saturate $^{125}$I-labeled EGF binding to EGFR on the cell surface, and uptake of $^{125}$I-labeled EGF was measured by a temperature shift to 37 °C at various time points. In p53-depleted cells, the internalization of $^{125}$I-labeled EGF was delayed compared with that of mock-infected control cells (Fig. 4B, upper panel). We also tested internalization assay using additional two different siRNAs for p53. The rate of EGF uptake was dramatically reduced by down-regulation of p53 expression using other siRNAs, as similar as that in stable p53-depleted cells (Supplementary Fig. S4). To further confirm this, we performed an internalization assay for EGFR using human lung carcinoma A549 cells expressing wild-type p53 under the same conditions as described above. The p53 expression was suppressed using a synthetic siRNA (Fig. 4C). In p53-depleted cells, uptake of $^{125}$I-labeled EGF was obviously decreased by half (Fig. 4D, upper panel).

Transferrin delivers iron to cells via clathrin-mediated endocytosis of TfnR, but several reports have suggested that the sorting mechanism of transferrin is different from that of EGF (Lakadamyali *et al.* 2006). Therefore, to assess whether p53 specifically functions in the signaling pathway for EGF internalization, we next examined the effect of the ablation of p53 expression on the endocytic pathway for transferrin. The uptake of $^{125}$I-labeled transferrin was not affected even though p53 expression was down-regulated (Fig. 4B,D, lower panels), indicating that p53 specifically influenced clathrin-mediated endocytosis of EGFR.

### Ablation of p53 alters EGFR signaling

It has recently been suggested that EGF-induced endocytosis of EGFR regulates the spatial and temporal coordination of accurate signaling hierarchy (Shilo 2005; Kholodenko 2006); therefore, we next asked if the downstream signaling pathways of EGFR are altered by the ablation of p53 expression. In response to EGF stimulation, EGFR is dimerized and autophosphorylated, and activated EGFR transduces signals to multiple pathways including the Ras–MAPK pathway, the PI3K–Akt pathway and the JNK–SAPK pathway (Logan *et al.* 1997; Weston *et al.* 2004). Therefore, we examined the effect of p53 on the signaling pathways for EGFR using phosphorylation-specific antibodies to determine the kinetics of the activation of signaling pathways. Immunoblot analysis revealed that the expression levels of EGFR and CHC proteins were not changed and EGFR was tyrosine-phosphorylated in response to EGF treatment irrespective of p53 expression (Fig. 5A); however, EGF-dependent activation of Akt and JNK was greatly up-regulated in p53-depleted cells (Fig. 5B). In contrast, no significant differences were observed in MAPK activity (Fig. 5B).

It is known that Akt activity is regulated by PTEN, a negative regulator of PI3K/Akt-dependent cellular survival, and that p53 regulates PTEN transcription (Stambolic *et al.* 2001). To exclude the possibility that the significant activation of Akt in p53-depleted cells was due to the down-regulation of PTEN, we analyzed the

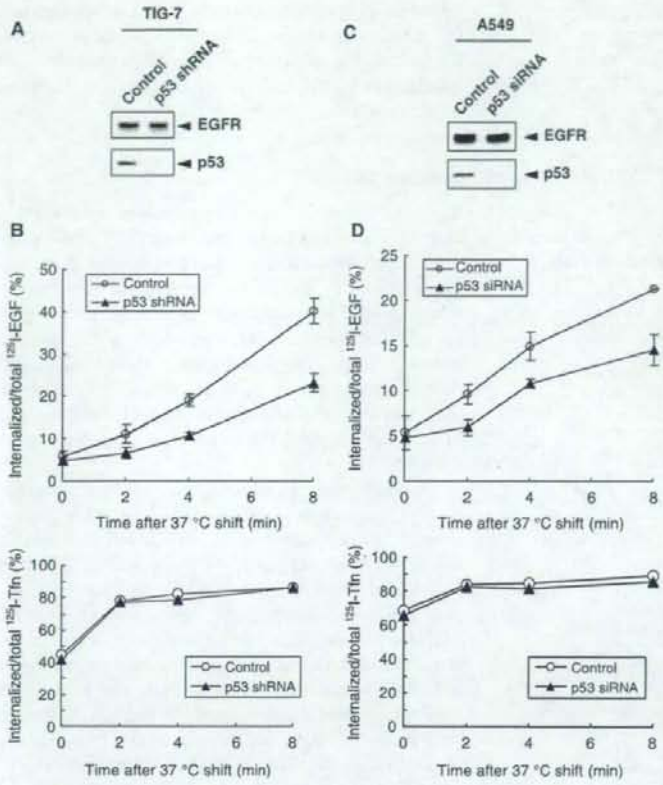


**Figure 4** Uptake of EGF, but not transferrin, delays in p53-depleted cells. (A) p53 was knocked down by stably expressing shRNA targeted to p53 in TIG-7 cells. Equal amounts of cells were lysed, the lysates were resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (B) The internalization of $^{125}$I-EGF (1 ng/mL, upper panel) and $^{125}$I-transferrin (500 ng/mL, lower panel) was measured as described in Experimental procedures. The internalization is shown as the ratio (%) of internalized to total (internalized and surface-bound) $^{125}$I-EGF or $^{125}$I-transferrin at each time point. (C) p53 expression in A549 cells was down-regulated using small-interfering RNA (siRNA). At 72 h post-transfection, equal amounts of cells were lysed and the lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (D) The internalization activity of $^{125}$I-EGF (upper panel) and $^{125}$I-transferrin (lower panel) was measured as in (B).

PTEN expression level. Immunoblotting analysis showed that the expression level of PTEN was comparable among samples even when p53 expression was depleted (Fig. 5B). These data indicate that EGF-dependent activation of Akt in p53-depleted cells was PTEN-independent, at least under our conditions. Taken together, these results suggest that p53 is required for transmitting the correct signaling from EGFR through the modulation of CHC-mediated endocytosis.

### p53 interacts with EGFR complex in response to EGF treatment

To understand the mechanism by which p53 regulates EGFR internalization, we next analyzed the physical association of p53 with EGFR complex upon EGF treatment. Biotin-labeled EGF was used to stimulate EGFR on cell surfaces and to purify a complex containing biotin-labeled EGF/EGFR from cell lysates by streptavidin-conjugated magnetic beads. Cells were starved in serum-free medium for 3 h, treated without or with biotin-labeled EGF on ice for 1 h, and then incubated for indicated times. These cells treated with EGF were immediately placed on ice and homogenized in hypotonic buffer to prepare a fraction containing cytosol and plasma membrane for immunopurification of an EGF-signaling complex. Interestingly, when eluates were immunoblotted with the various antibodies indicated, not only endocytic regulators but also p53 were co-purified with EGFR complex in response to EGF stimulation (Fig. 6A), and p53 transiently associated with EGFR complex unlike endocytic regulators tested (Fig. 6A), suggesting that p53 may act as a catalyst regulating clathrin-mediated endocytosis. In parallel experiments, we checked if p53 still co-localized with CHC in immunofluorescence under same conditions. Even though cells were incubated for various time periods, p53 still co-localized with CHC at plasma membrane

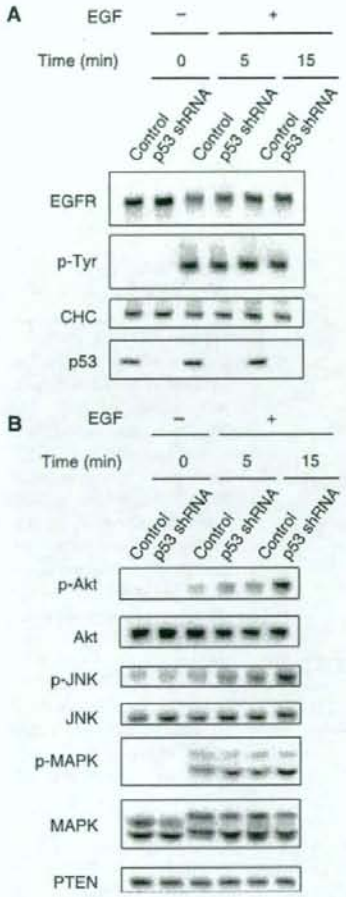


**Figure 5** Ablation of p53 causes aberrant signaling from EGFR. (A) TIG-7 cells were starved overnight and incubated with 100 ng/mL EGF for 1 h on ice and then warmed to 37 °C for various time periods. Whole cell lysates were electrophoresed (15 μg of proteins/lane) and immunoblotted with indicated antibodies. The activation of EGFR (approximately 180 kDa) by EGF treatment was assessed by anti-phospho-tyrosine (p-Tyr) antibody. The p53 expression level was confirmed by anti-p53 antibody (DO-1). (B) EGF-induced phosphorylation of downstream kinases was determined by immunoblotting using active form-specific antibodies. Total Akt, JNK and MAPK levels were also detected by the respective antibodies.

(Fig. 6B), being consistent with the data that p53 is included in EGFR complex in response to EGF stimulation. We next examine the effect of partial ablation of CHC gene expression by RNAi on EGF-induced p53–EGFR complex formation. CHC down-regulation by RNAi caused the reduced p53–EGFR complex formation (Fig. 6C), supporting our findings that CHC contributes to EGF-induced recruitment of p53 at plasma membrane.

## Discussion

This study revealed that p53 associates with CHC in cytosol (Fig. 1) and co-localizes with CHC at the plasma membrane where endocytosis occurs (Fig. 2). We also found that p53 localizes at the plasma membrane in response to EGF treatment (Fig. 3). In p53-depleted cells, EGFR internalization is delayed (Fig. 4B,D) and the intracellular signaling from EGFR is altered compared to that in control cells (Fig. 5). Based on these results, we speculate that p53 probably controls clathrin-mediated internalization of EGFR to transduce correct signaling from EGFR.

Confocal immunofluorescent microscopic analysis shows that co-localization of p53 with EGF–EGFR is observed in cells treated with fluorescent-labeled EGF on ice; however, p53 is rarely detected in the EGF–EGFR complex under the same conditions (Fig. 6). This discrepancy may be caused because the interaction of p53 with EGFR complex is easily dissociated by biochemical fractionation. On the other hand, the association of p53 with EGFR complex was transiently detected at very early time points (Fig. 6A), at which p53 still co-localized with EGF–EGFR–CHC complex (Fig. 6B and data not shown), suggesting that the binding affinity between p53 and EGFR complex increases in an energy-dependent and unknown mechanisms. Presumably, incubation of EGF-treated cells at 18 °C leads to the stabilization of p53–EGFR complex association through energy-dependent modifications or conformational changes of EGFR, p53 or its associated proteins at the plasma membrane. Thus, our findings suggest that p53 regulates the internalization of EGFR by binding to it or associated proteins.

Activated RTKs including EGFR, insulin receptor and TrkA are rapidly internalized and eventually delivered to the lysosome to down-regulate signaling from cell-surface receptors (Di Fiore & Gill 1999; Wiley & Burke 2001). It has been shown that p53 associates with TrkA (high-affinity receptor for nerve growth factor) *in vitro* and *in vivo* (Montano 1997), implying that p53 is involved in its signal transduction pathways, possibly through association with TrkA. Our findings, that signal transduction from EGFR was aberrant when p53 expression was suppressed (Fig. 5B) and that p53 was co-purified with EGF–EGFR complex (Fig. 6), suggest that p53 functions as a spatial and temporal coordinator of correct

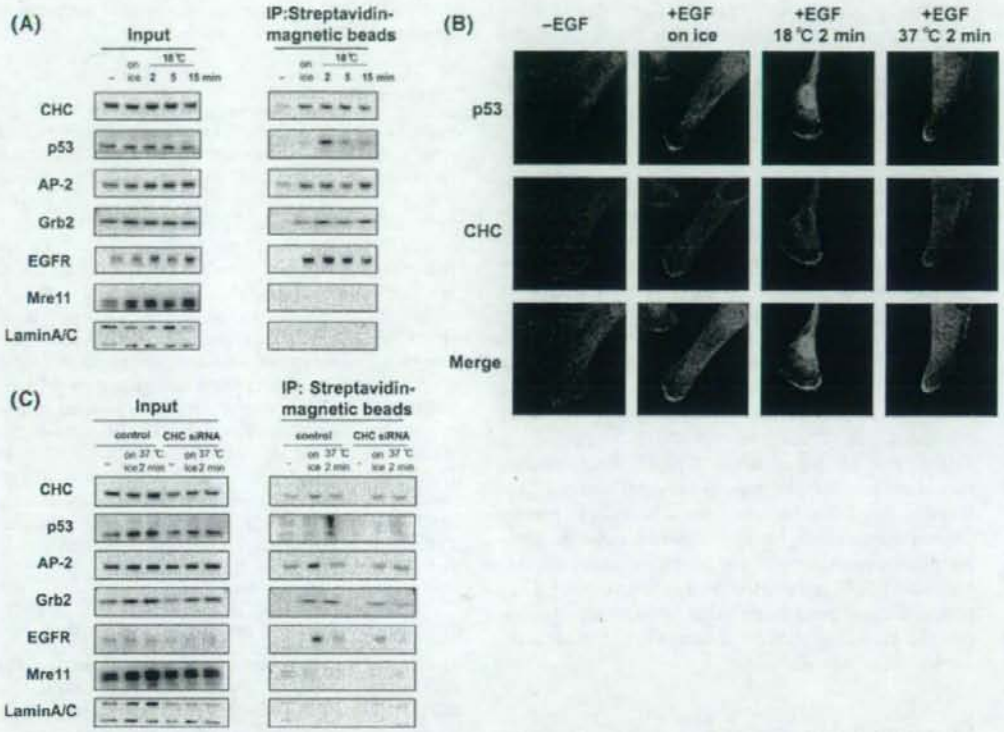


**Figure 6** p53 is co-purified with EGFR complex in response to EGF treatment. (A) Serum-starved TIG-7 cells were incubated with 100 ng/mL biotin-labeled EGF (bio-EGF) for 1 h on ice and then warmed for indicated periods at 18 °C. Cells were suspended in hypotonic buffer (20 mM Hepes-KOH pH 7.9, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) containing 0.1% Triton X-100. Lysates were immunopurified with streptavidin-conjugated magnetic beads and the complex including bio-EGF was eluted with SDS-sample buffer. Eluates were electrophoresed and immunoblotted with indicated antibodies. (B) The localization of CHC (green: AF488 staining) and p53 (red: AF594 staining) in TIG-7 cells upon EGF stimulation was observed by confocal microscopy. The co-localization of these proteins is visible as yellow staining. (C) CHC is partially down-regulated by siRNA targeted to CHC in TIG-7 cells. These cells were treated with bio-EGF and the lysates were immunopurified with streptavidin-magnetic beads as described in (A). Eluates were electrophoresed and immunoblotted with indicated antibodies.

signaling from EGFR at the plasma membrane. The magnitude and kinetics of signal activation in RTK pathways are important for homeostasis. Once this system is dysregulated, it presumably causes various diseases including cancer (Le Roy & Wrana 2005). In the case of EGFR, prevention of EGFR internalization by the suppression of factors responsible for EGFR internalization facilitates cell transformation (Levkowitz *et al.* 1998). Our observations provide the possibility that p53 also coordinates signals from growth factor receptor through the regulation of clathrin-mediated endocytosis to prevent cell transformation. Thus, our findings provide a new insight on p53 as a tumor suppression mechanism and for the possibility that cytosolic p53 participates in the regulation of clathrin-mediated endocytosis in a transcription-independent manner although we cannot exclude the possibility that p53 indirectly regulates these events via the induction of p53-target genes. To address the effect of p53 on the recruitment of endocytic proteins to EGFR complex, we examined immunopurification experiments using p53-depleted cells; however, as far as we assayed whether various endocytic proteins including CHC, AP-2 and Grb2 are contained in the EGFR complex in p53-depleted cells, no alteration of complex formation

of these proteins was observed (data not shown). Presumably, p53 depletion by RNAi causes the altered association or expression of other endocytic proteins. Further analysis will be required to understand the direct contribution of cytosolic p53 to clathrin-mediated endocytosis.

Why are p53-deficient mice viable without any developmental defect (Donehower *et al.* 1992) although p53 regulates clathrin-mediated EGFR internalization? One possible explanation is that the regulation of EGFR internalization by p53 may be restricted to specific areas in specific tissues. Even though hierarchical signaling from EGFR is aberrant in restricted cells, p53-deficient mice would not exhibit lethality. Indeed, various mice deficient in endocytic regulators such as Hip1, c-Cbl and Parkin responsible for EGFR internalization are viable without obvious abnormalities (Murphy *et al.* 1998; Naramura *et al.* 1998; Rao *et al.* 2001; Goldberg *et al.* 2003). Thus, the dysregulation of EGFR internalization may not cause developmental lethality although the function is required for homeostasis in adults to prevent diseases. Alternatively, we also cannot rule out the possibility that the mechanism by which p53 regulates clathrin-mediated EGFR internalization is specific to the human system. Further investigation will be necessary to elucidate the physiological roles of cytosolic p53 in clathrin-mediated endocytosis.

## Experimental procedures

### Cell lines and antibodies

Human lung carcinoma H1299 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). H1299 cells transfected with FLAG-tagged p53 construct were prepared as described previously (Enari *et al.* 2006). Normal human fibroblast TIG-7 cells and human lung carcinoma A549 cells were grown in DMEM containing10% FBS. Anti-p53 (DO-1) and anti-CHC (X.22) antibodies were purchased from Oncogene Science, Cambridge, MA. Anti-CHC (clone 23), anti-EGFR (clone 13) and anti-AP-2 (clone 8) antibodies were purchased from BD Biosciences, Rockville, MD. Anti-Lamin A/C (N-18), anti-β-Tubulin (H-235), anti-Grb2 (C-23), anti-JNK (FL), anti-p53 (FL-393) and horseradish peroxidase (HRP)-conjugated anti-p53 (DO-1) antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-phospho-MAPK (Thr202/Tyr204), anti-MAPK, anti-phospho-Akt (Thr308), anti-Akt and anti-phospho-JNK (Thr183/Tyr185) antibodies were purchased from Cell Signaling Technology, Danvers, MA. Anti-PTEN (6H2.1) antibody was from Cascade BioScience, Winchester, MA. Anti-phospho-tyrosine (4G10) antibody was purchased from Upstate Biotechnology, Lake Placid, NY. Anti-FLAG (M2) antibody was purchased from Sigma, St Louis, MO. HRP-conjugated NeutrAvidin was obtained from Pierce, Rockford, IL and HRP-conjugated secondary antibodies were from Amersham Biosciences, Uppsala, Sweden.

### Fractionation

Cytosolic and nuclear fractions were extracted, as described previously, with some modifications (Dignam *et al.* 1983). Briefly, cells were collected by centrifugation at 600 *g* for 5 min, washed with ice-cold PBS and cell pellets were suspended in hypotonic buffer (20 mM HEPES–KOH, pH 7.9, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM $Na_3VO_4$, 10 mM NaF, 10 μg/mL antipain, 10 μg/mL pepstatin, 10 μg/mL chymostatin, 10 μg/mL leupeptin, 10 μg/mL E-64, 10 μg/mL aPMSF) and incubated for 10 min on ice. The cells were homogenized with 15 strokes of a Dounce homogenizer. The supernatants were collected by centrifugation at 600 *g* for 5 min and used as cytosolic fractions. To prepare nuclear extracts, the pellets were washed twice with hypotonic buffer and resuspended in hypertonic buffer (20 mM HEPES–KOH, pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM DTT, 1 mM $Na_3VO_4$, 10 mM NaF, 10 μg/mL antipain, 10 μg/mL pepstatin, 10 μg/mL chymostatin, 10 μg/mL leupeptin, 10 μg/mL E-64, 10 μg/mL aPMSF) and incubated for 30 min on ice. After centrifugation at 20 000 *g* for 20 min, the supernatants were collected as nuclear fractions.

### Immunoprecipitation and immunoblotting

Cytosolic and nuclear extracts were diluted with lysis buffer (50 mM Tris–HCl, pH 7.2, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 1 mM $Na_3VO_4$, 10 mM NaF, 10 μg/mL antipain, 10 μg/mL pepstatin, 10 μg/mL chymostatin, 10 μg/mL leupeptin, 10 μg/mL E-64, 10 μg/mL aPMSF) and supplemented to final concentrations of 100 mM NaCl and 0.1% Tween-20, respectively. Both fractions were immunoprecipitated with anti-CHC (X.22) or anti-FLAG (M2) antibody-coupled protein A-Sepharose beads (Amersham Biosciences). Immunoprecipitates were then washed 3 times with lysis buffer containing 50 mM NaCl and 0.1% Tween-20, and eluted with SDS sample buffer. Immunoprecipitates were resolved by 5%–20% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-craft, North Wales, PA) and transferred to PVDF membranes (Millipore, Bedford, MA), which were sequentially incubated with primary and secondary antibodies, and the immune-complex was detected using ECL Western blotting detection reagents (Amersham Biosciences).

### EGF or transferrin treatment

Cells were starved in serum-free medium (DMEM containing 0.1% bovine serum albumin (BSA)) for 3 h at 37 °C in a $CO_2$ incubator, washed extensively with pre-chilled serum-free medium, placed on ice for 10 min and then changed to pre-chilled serum-free medium containing EGF or transferrin (various concentrations according to the purpose described below), followed by further incubation on ice for 1 h. For immunofluorescence and

immuno-electron microscopy, 2 μg/mL AlexaFluor488-labeled EGF (AF488-EGF, Molecular Probes, Eugene, OR) and 100 ng/mL human EGF (Roche Applied Science, Basel, Switzerland) were used, respectively. For internalization assay, 1 ng/mL $^{125}$I-labeled human EGF (Amersham Biosciences) or 500 ng/mL $^{125}$I-labeled transferrin (Perkin Elmer, Boston, MA) were used. For immunopurification of EGFR complex, 100 ng/mL biotin-labeled EGF (Molecular Probes) was used. To examine EGFR downstream signaling pathways, cells were starved in serum-free medium overnight, treated with 100 ng/mL human EGF, washed twice with pre-chilled serum-free medium to remove unbound EGF and then kept on ice or warmed to 18 °C or 37 °C for various time periods.

### Immunofluorescence

Cells plated on a 4-well chamber slide (NalgeNunc, Rochester, NY) were starved in serum-free medium and treated with EGF or AF488-EGF as described above. EGF-treated cells were placed on ice to stop the reaction of EGF-induced EGFR-mediated endocytosis. The cells were fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 for 5 min on ice, washed with PBS and blocked with 3% BSA for 30 min, followed by sequential incubation with an antibody against p53 (FL393), CHC (X.22) or Grb2 (C-23) for 1 h at room temperature and with AF488- or AF594-conjugated secondary antibody (Molecular Probes). Confocal imaging was performed using an ECLIPSE E600 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a Radiance 2000 imaging system (Bio-Rad, Hercules, CA).

### Internalization assays

Ligand uptake assays were performed as described previously, with several modifications (Hinrichsen *et al.* 2003; Motley *et al.* 2003). In brief, cells grown on 12-well plates were starved in serum-free medium and treated with $^{125}$I-labeled human EGF or $^{125}$I-labeled transferrin on ice for 1 h, washed twice with pre-chilled serum-free medium and then further incubated with pre-warmed serum-free medium at 37 °C for various periods. The cell surface-bound $^{125}$I-labeled EGF and transferrin were collected by incubation twice with pre-chilled acid buffer (0.2 M acetic acid at pH 4.5, 0.5 M NaCl) on ice for 2–5 min. On the other hand, internalized $^{125}$I-labeled EGF and transferrin were collected by extraction twice with extraction buffer (0.1 M Tris–HCl at pH 8.0, 1% SDS). The radioactivity in each sample was quantified using a gamma counter (Cobra, Packard, Meriden, CT).

### Immunopurification of EGFR complex

Cells were starved in serum-free medium and treated with biotin-labeled EGF as described above. Biotin-labeled EGF-treated cells were lysed with hypotonic buffer containing 0.1% Triton X-100 on ice for 10 min to fractionate cytosolic and nuclear extracts. Biotin-labeled EGF–EGFR complex was immunopurified using Dynabeads M-280 Streptavidin (Dynal, Oslo, Norway) without a centrifugation step (to exclude the loss of fractions containing heavy membrane by centrifugation even at a low speed). Immunopurified complex was washed 3 times with hypotonic buffer and eluted with SDS sample buffer at room temperature.

### RNAi knockdown

Small-interfering RNAs (siRNAs) directed to human p53 (For siRNA sequences, see Supplementary information) and its negative control were purchased from Invitrogen, Carlsbad, CA and transfected in A549 cells with Lipofectamine 2000 according to the manufacturer's protocol. The siRNAs directed to human CHC were purchased from Qiagen, Venlo, the Netherlands for sequences #1 (5′-GUAAUCCAAUUCGAAGACCTT-3′) and Sigma for sequences #2 (5′-AGAGCACCAUGAUUC CAAUTT-3′) and transfected in TIG-7 cells as described above. For the generation of p53-knockdown TIG-7 cells, lentiviruses derived from pLenti6/V5-DEST lentiviral expression vector (Invitrogen), engineered so that shRNA targeted to p53 is expressed in cells, were infected in TIG-7 cells, blasticidinS-resistant cells were selected and used for assays.

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## Supplementary material

The following supplementary materials are available for this article online:

**Figure S1** Observation of EGF-induced p53 localization at plasma membrane by using two different anti-p53 antibodies.

**Figure S2** No or little recruitment of tumor-derived p53 mutants to plasma membrane upon EGF treatment.

**Figure S3** Decreased EGF-induced p53 translocation to plasma membrane by partial reduction of CHC expression.

**Figure S4** Delayed EGF uptake by ablation of p53 expression using another siRNA targeted to p53.

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