Supplementary Data

Figure Legends followed by Supplementary Figures

Supplementary Figure S1

ErbB2 is not down-regulated from the plasma membrane upon incubation with cycloheximide. SKBr3 cells were incubated with or without cycloheximide (CHX, 25 mg/ml) for 6 or 12 h before the cells were trypsinized, fixed and immunostained using antibody e.c.* recognizing the extracellular part of ErbB2 followed by Phycoerythrin-conjugated goat anti-mouse antibody. The amount of ErbB2 at the plasma membrane of a minimum of 10.000 cells was then analyzed by flow cytometry. Unstained cells represent cells stained with only secondary antibody. Control cells were not incubated with CHX.

Supplementary Figure S2

Intact ErbB2 is localized to vesicles upon incubation with GA. SKBr3 cells were incubated without (upper row) or with GA (3 μM) (lower row) for 4 h before the cells were fixed and immunostained using anti-ErbB2 antibodies i.c.* and e.c.* recognizing intracellular - and extracellular parts of ErbB2 respectively. The cells were examined using confocal microscopy. Yellow in the overlay visualizes co-localization between intracellular - and extracelluar part of ErbB2. Bar, 10 μm.

Supplementary Figure S3

ErbB2 is localized to early endosomes upon incubation with GA. SKBr3 cells were incubated with GA (3 μM) for 4 h before the cells were fixed and doubly immunostained

using antibodies recognizing the intracellular part of ErbB2 (antibody i.c.*) and early endosomal antigen 1 (EEA1) respectively. The cells were examined using confocal microscopy. The overlay demonstrates co-localization between ErbB2 and EEA1. Bar, 10 μm.

Supplementary Figure S4

Incubation with GA induces down-regulation of ErbB2 from the plasma membrane in PAE cells stably transfected with EGFR and ErbB2. A. PAE.EGFR.ErbB2 cells (Haslekas *et al.*, 2005) were incubated without (Control) or with GA (3 μM) for 6 h before cells were trypsinized, fixed and immunostained with antibody e.c.* recognizing the extracellular part of ErbB2 followed by phycoerythrin-conjugated goat anti-mouse antibody. The amount of ErbB2 at the plasma membrane of a minimum of 10.000 cells was analyzed by flow cytometry. Unstained cells represent cells stained with secondary antibody only. **B.** PAE.EGFR.ErbB2 cells were incubated with or without GA (3 μM) for 4 h before the cells were fixed and immunostained using antibodiy to the extracellular part of ErbB2 (e.c*) and examined using confocal microscopy. The vesicular labeling pattern in GA-treated cells demonstrates GA-induced endocytosis of ErbB2. **C** and **D.** PAE.EGFR.ErbB2 cells were incubated with GA for increasing times before the cells were lysed and subjected to Western blotting using antibodies recognizing (**C**) the intracellular part of ErbB2 (antibody i.c.) or (**D**) the extracellular part of ErbB2 (antibody e.c.). Western blotting with anti-Erk antibody was used as loading control. C: control cells not incubated with GA.

Supplementary Figure S5

Incubation with GA induces down-regulation of ErbB2 from the plasma membrane in cells not over-expressing ErbB2. HEp2 cells were used to examine the effect of GA in cells not over-expressing ErbB2. **A.** HEp2 cells were incubated without (Control) or with GA (3 μM) for 2 and 6 h before the cells were trypsinized, fixed and immunostained with antibody e.c.* recognizing the extracellular part of ErbB2 followed by phycoerythrin-conjugated goat anti-mouse antibody. Plasma membrane level of ErbB2 was then measured by flow cytometry. Unstained cells are cells stained with secondary antibody only. **B.** HEp2 cells were incubated without (Control) or with GA (3 μM) for 4 h before the cells were fixed and immunocytochemically labeled with mouse anti-ErbB2 antibody (e.c.*) and examined using confocal microscopy. The vesicular labeling pattern in GA-treated cells demonstrates GA-induced endocytosis of ErbB2. **C** and **D.** HEp2 cells were incubated with GA (3 μM) for increasing time periods as indicated in the figure. After incubation the cells were lysed and subjected to Western blotting using antibodies against the intracellular part of ErbB2 (antibody i.c.*) (**C**) and the extracellular part of ErbB2 (antibody e.c.**) (**D**). Western blotting with anti-tubulin antibody was used as loading control. C: control cells not incubated with GA.

Supplementary Figure S6

Incubation with GA induces partial degradation of ErbB2 in cells depleted of CHC. SKBr3 cells transfected without (Control) or with CHC siRNA were incubated with GA (3 μM) for increasing time periods before being lysed and subjected to Western blotting

with antibody e.c. to the extracellular part of ErbB2. Note the GA-induced shift in the molecular weight of ErbB2 in cells depleted of clathrin. Western blotting with mouse anti-tubulin antibody was used as loading control. C: control cells not incubated with GA.

Supplementary Figure S7

GA-induced down-regulation of ErbB2 from the plasma membrane is independent of caspase activity. SKBr3 cells were pre-incubated with the caspase inhibitor z-VAD-fmk for 30 min before further incubation with GA (3 μM) for 6 h. The caspase inhibitor was present during the incubation with GA. The cells were subsequently trypsinized, fixed and immunostained with an antibody e.c. recognizing the extracellular part of ErbB2 followed by phycoerythrin-conjugated goat anti-mouse antibody. The amount of ErbB2 at the plasma membrane of a minimum of 10.000 cells was then analyzed by flow cytometry. Unstained cells represent cells stained with only secondary antibody, control cells are cells incubated with neither GA nor caspase inhibitor.

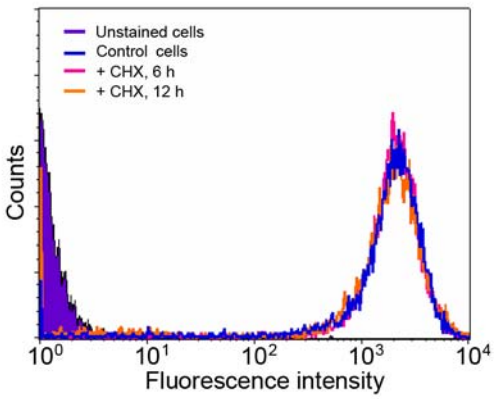
Supplementary Figure S8

ErbB2 is localized to early endosomes upon incubation with lactacystin and GA. SKBr3 cells were pre-incubated with lactacystin for 1 h before incubation with lactacystin and GA for 4 h (upper panel) or 6 h (lower panel). The cells were then fixed and doubly immunostained using antibodies recognizing either the extracellular part of ErbB2 (antibody e.c.*, upper panel) or the intracellular part of ErbB2 (antibody i.c., lower panel), and early endosomal antigen 1 (EEA1), respectively. The cells were

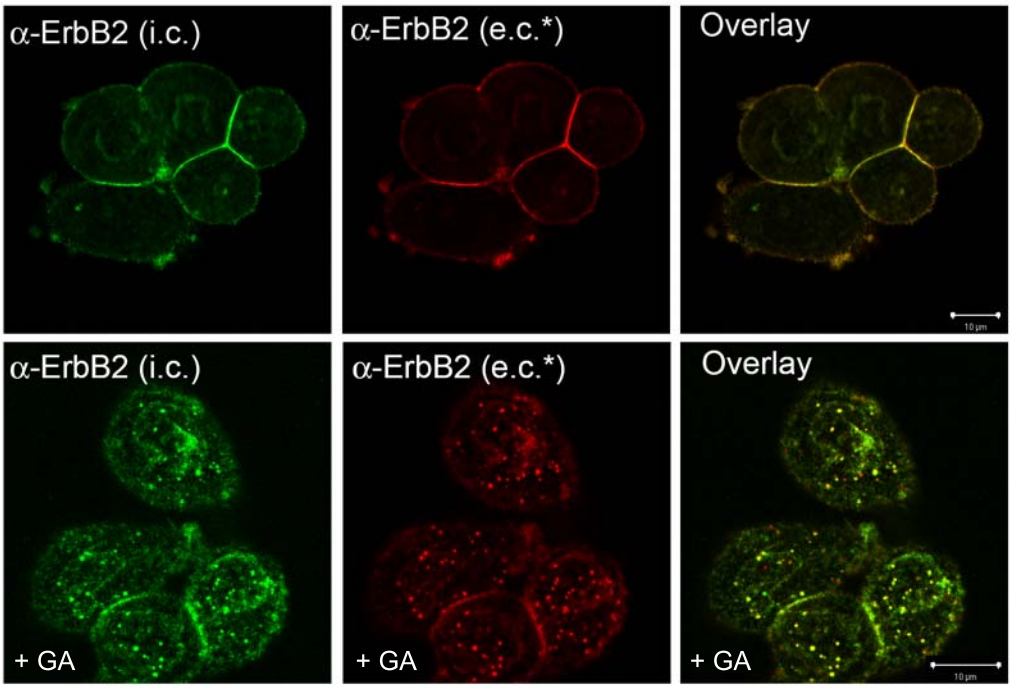
examined using confocal microscopy. The overlay demonstrates co-localization between ErbB2 and EEA1.

Reference

Haslekas, C., Breen, K., Pedersen, K.W., Johannessen, L.E., Stang, E., and Madshus, I.H. (2005). The inhibitory effect of ErbB2 on epidermal growth factor-induced formation of clathrin-coated pits correlates with retention of epidermal growth factor receptor-ErbB2 oligomeric complexes at the plasma membrane. Mol Biol Cell *16*, 5832-5842.



Supplementary Fig. S1



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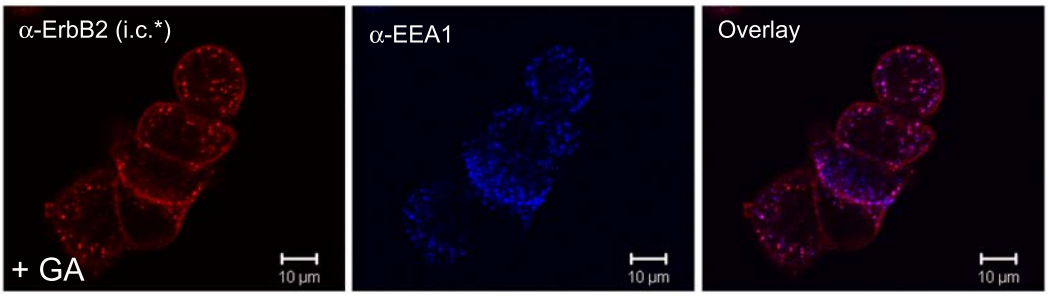

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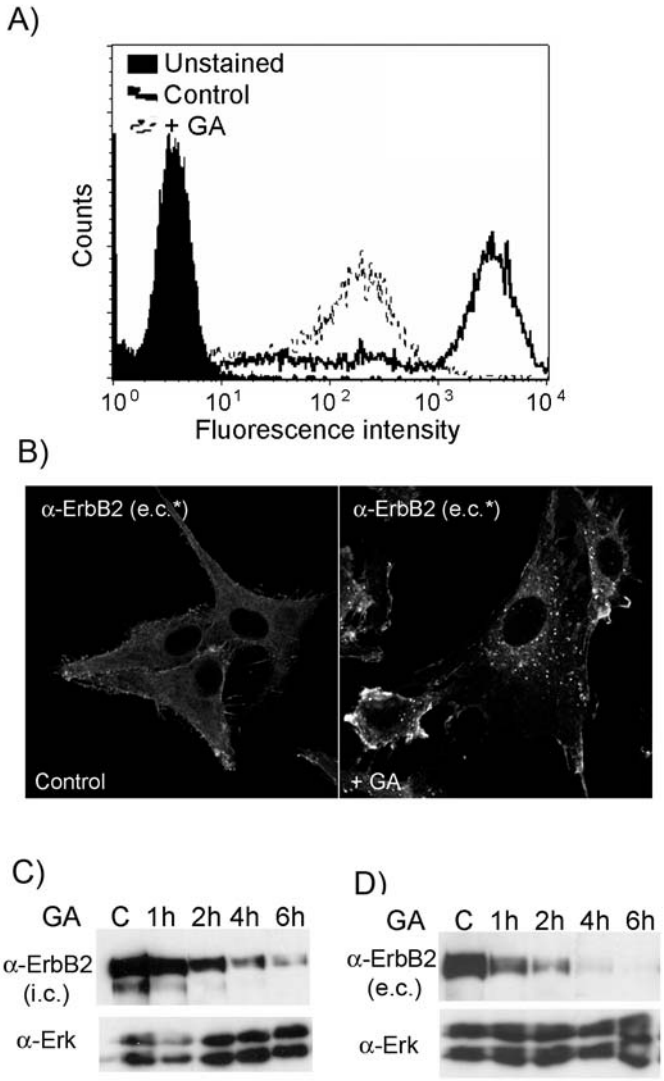

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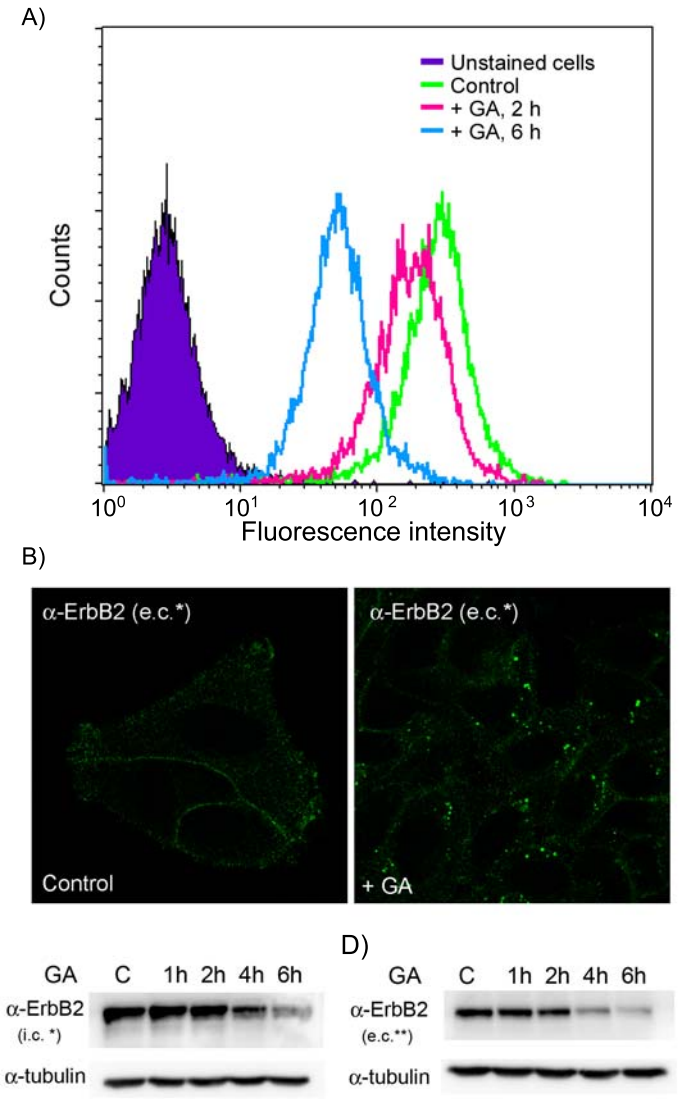

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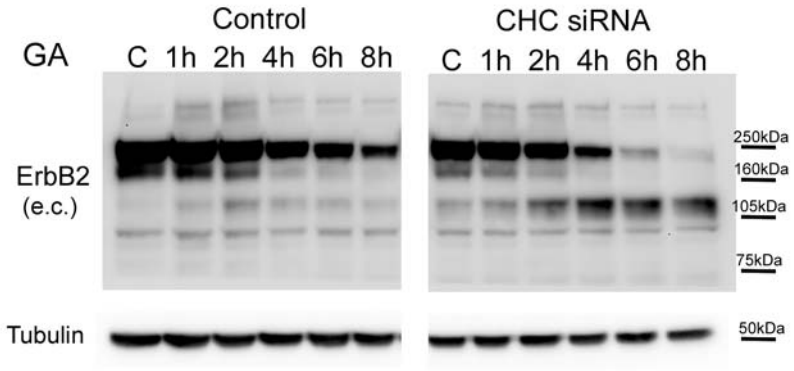

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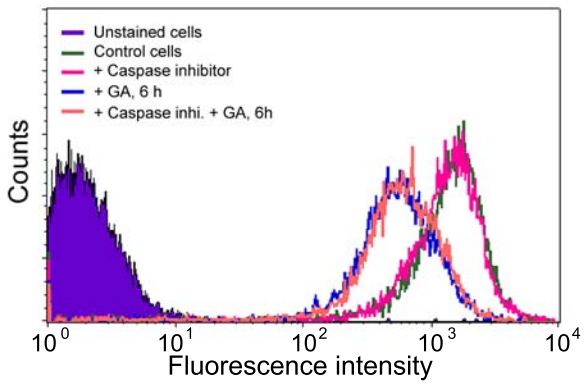
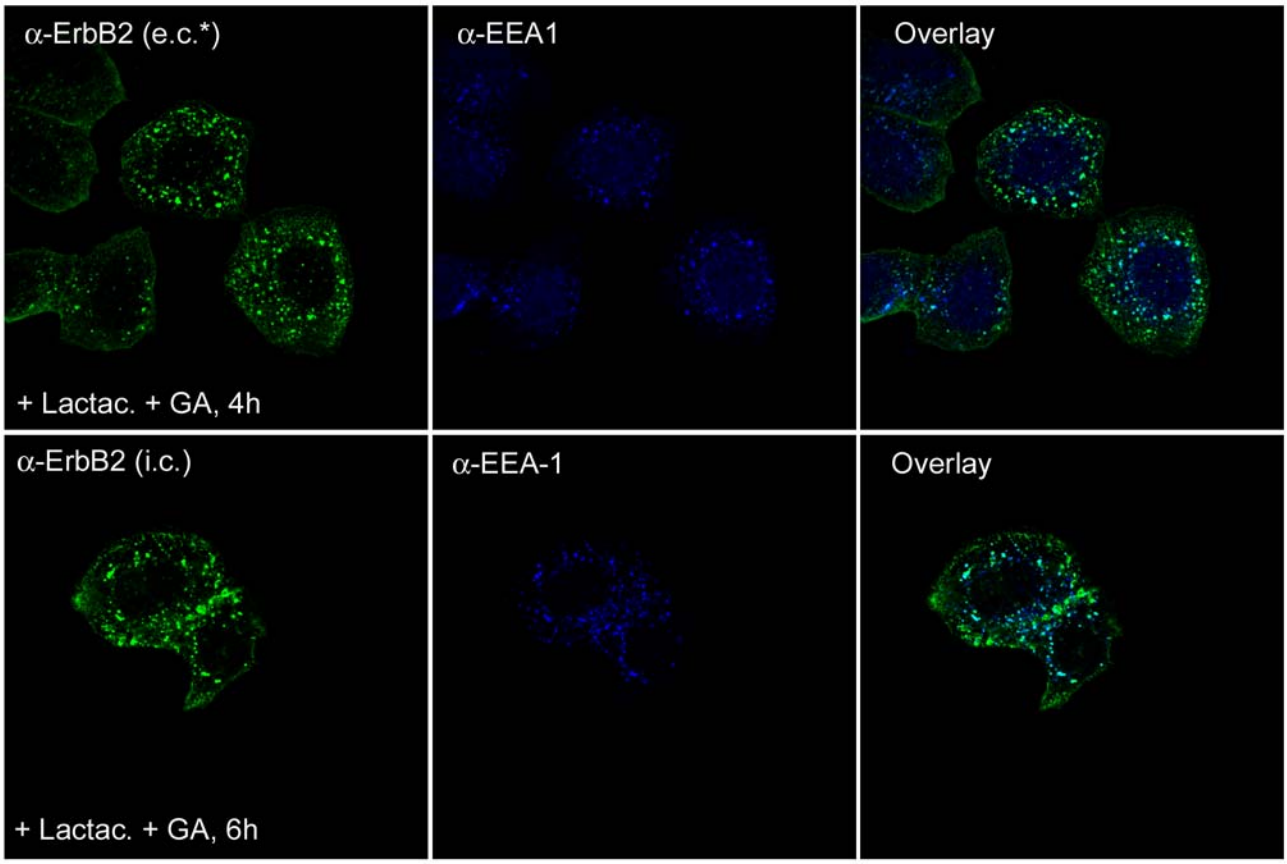


Supplementary Fig. S7



Supplementary Fig. S8