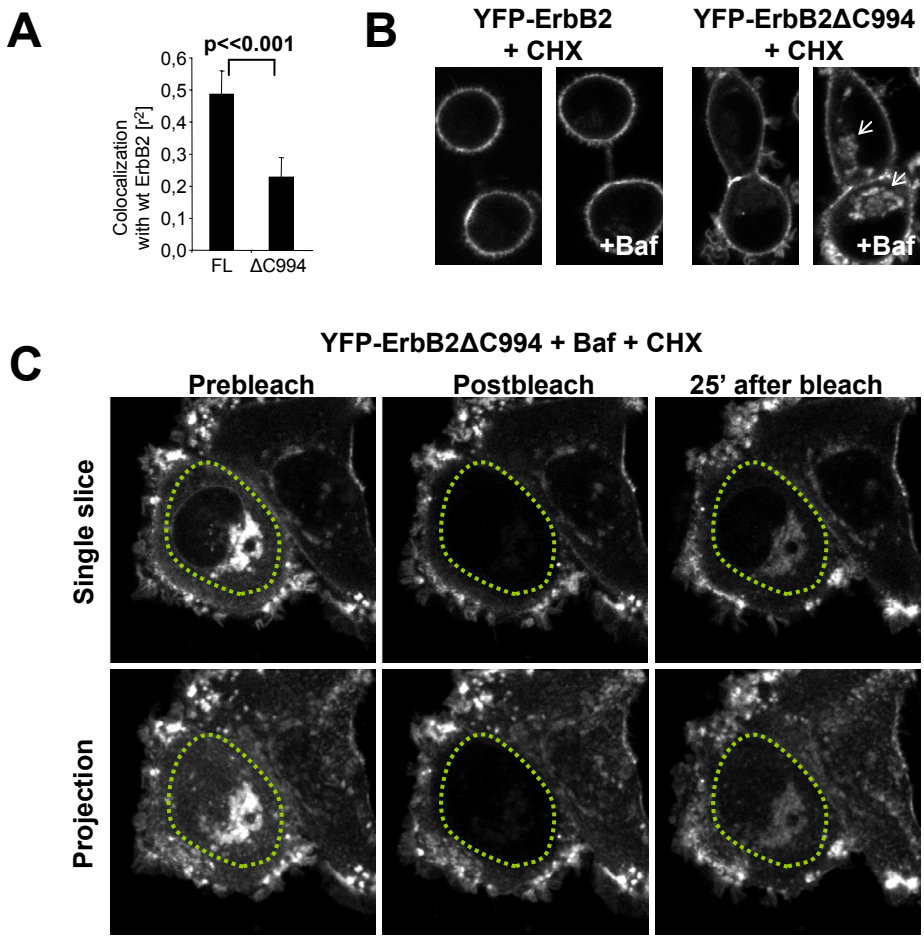
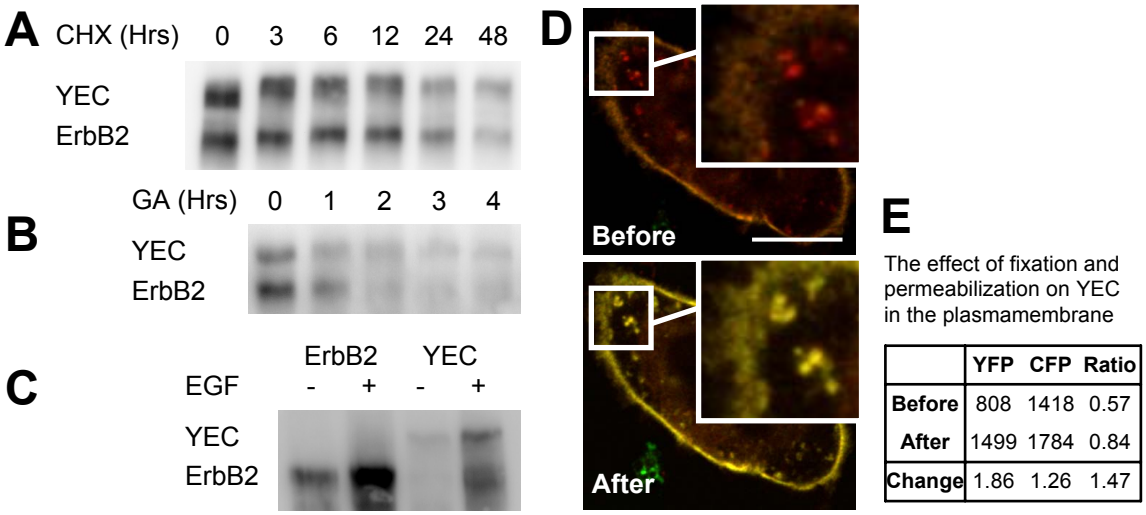


YFP-ErbB2ΔC994 is easily internalized. (A) YFP-ErbB2ΔC994 colocalizes less with endogenous ErbB2 than full-length YFP-ErbB2 (FL). Quantitation of colocalization of images similar to figure 1G+H was performed by calculating the Pearson's correlation coefficients (r^2 values) between the intensities of the two channels (method previously described in Lerdrup et al, 2006). The resulting r^2 values from the YFP-ErbB2ΔC994 transfected cells (ΔC994) were significantly lower than from the YFP-ErbB2 transfected cells ($p<<0.001$, Mann-Whitney two-tailed U-Test). n=24 and 29. (B+C) Intracellular YFP-ErbB2ΔC994 is derived from the plasma membrane. Confocal microscopy images of live SK-BR-3 cells transfected with YFP-ErbB2ΔC994 or full-length YFP-ErbB2 and treated as indicated. (B) YFP-ErbB2ΔC994 or YFP-ErbB2 transfectants were treated with 10µg/ml cycloheximide (CHX, Sigma-Aldricht) 1 hour prior to imaging. After the first image cells were stimulated with 500 nM bafilomycin and imaged after one hour. Please note that YFP-ErbB2ΔC994 still accumulates intracellularly regardless of inhibited protein synthesis, confirming that it is derived from the plasma membrane, whereas little YFP-ErbB2 is accumulated intracellularly after bafilomycin stimulation, demonstrating that its half-life is higher than that of YFP-ErbB2ΔC994. (C) YFP-ErbB2ΔC994 transfectants treated with cycloheximide (CHX) and bafilomycin for one hour before 3D confocal imaging. Upper images are a single confocal plane, while the lower images are 2D reconstructions of confocal scans of the entire cell. A region containing the intracellular YFP fluorescence was bleached using the 514 nm Argon laser line, and the cell was imaged immediately after and following 25 minutes of incubation. Note the marked reappearance of intracellular fluorescence within 25 minutes, suggesting that there is a significant translocation of YFP-ErbB2ΔC994 from the plasma membrane to intracellular compartments.

Supplementary Figure 1

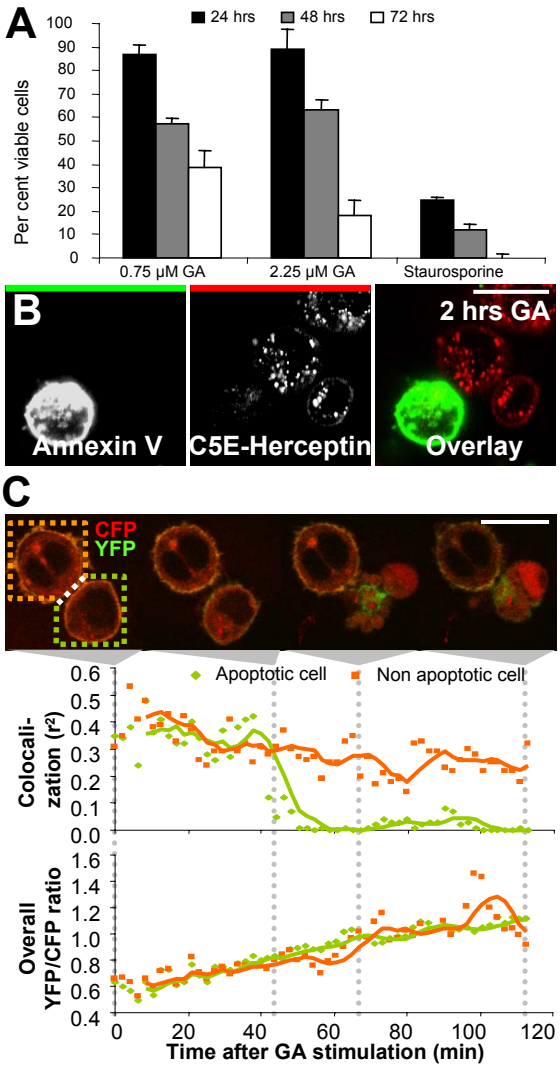


The effect of fixation and permeabilization on YEC in the plasmamembrane

	YFP	CFP	Ratio
Before	808	1418	0.57
After	1499	1784	0.84
Change	1.86	1.26	1.47

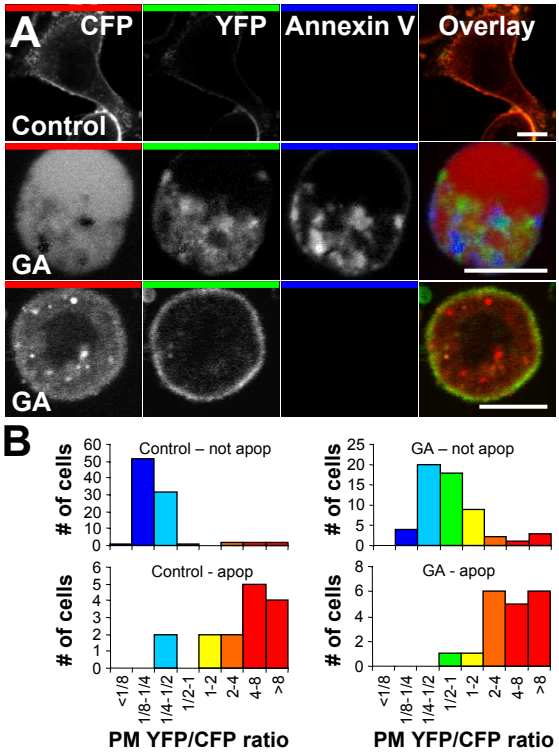
Validation of YFP-ErbB2-CFP (YEC) behaviour. (A) To evaluate YEC stability compared to wildtype ErbB2 both constructs were transfected into Hep2 cells. The cells were subsequently treated for up to 48 hours with the translation inhibitor CycloHeximide (CHX) before lysis in RIPA buffer. Following protein content adjustment of the lysates, the samples were analysed for ErbB2 expression by Western blotting. The blot show that YEC and wildtype ErbB2 have similar half-lifes. (B) After Geldanamycin stimulation most YEC and ErbB2 was degraded within a couple of hours, and importantly the two proteins were degraded equally fast. (C) To evaluate protein phosphorylation both YEC and wildtype ErbB2 the constructs were transfected into Hep2 cells, stimulated with EGF for 10 min and the cells lysed in RIPA buffer. Protein content adjustment was done to assure equal loading and Western-blotting for phosphorylation on Tyrosine-877 revealed that EGF stimulation leads to efficient Y877 phosphorylation on both wild-type and YEC. (D) Fluorescent proteins have a pKa value that allows the low pH in endosomes and lysosomes to attenuate their fluorescence. Accordingly, YFP in YEC was sensitive to pH changes in the vesicular lumen while CFP was insensitive due to its position in the cytoplasm. In situ fixation of a YEC-transfected SK-BR-3 cell where ErbB2 had accumulated intracellularly due to 12 hours incubation at high pH. Upper and lower images show the cell before and after fixation with 2% paraformaldehyde, permeabilization, and pH adjustment to 7.2, respectively (YFP=green and CFP=red, Bar=20µm). (E) Table showing the changes in YFP- and CFP-intensity quantitated from regions on the plasma membrane on the images in D.

Supplementary Figure 2



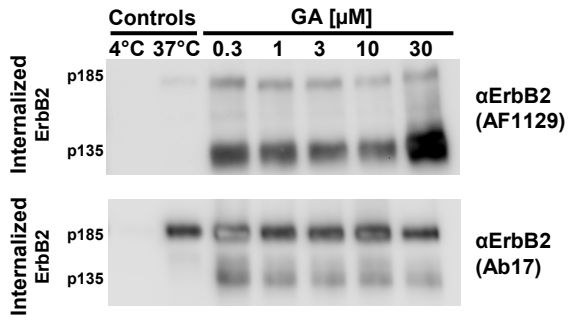
Apoptosis leads to low internalization of ErbB2 and a sudden cleavage of ErbB2 that causing marked reduction in the colocalisation of its termini. (A) Viability assay showing decreased viability of SK-BR-3 cells with increasing concentrations and exposure times of GA. Cells were grown under standard conditions in microtiter wells in 100 µl DMEM. 25 µl MTT (0.5% MTT in PBS +Ca/Mg) was added to each well and cells incubated for 4 hours at 37°C. Samples were frozen at -80°C to measure all samples simultaneously. Before measuring, 100 µl solubilisation buffer (20% SDS, 50% N, N-dimethylformamide) was added to each well and samples incubated overnight at room temperature in darkness. Optical density was measured at 570nm. 1 µM staurosporine is used as positive control for cell death. (B) Confocal images of 3 µM GA treated SK-BR-3 cells. Left image shows the apoptotic marker Alexa-555 labelled Annexin V (Invitrogen), the middle image shows the low-pH activated probe Cypher5E (GE Healthcare) conjugated to Herceptin (C5E-Herceptin), and the right image show an overlay with Annexin V and C5E-Herceptin. Note that the apoptotic cell had no uptake of C5E-Herceptin. (C) Time-lapse microscopy of apoptotic (green) and non-apoptotic (orange) YEC-expressing SK-BR-3 cells. The gray dotted lines below each image indicate the time passed. The pixel colocalization between YFP and CFP (r^2) and the cellular YFP/CFP ratios in the two cells are depicted against time in the two diagrams below. Bars 20 µm.

Supplementary Figure 3



ErbB2 is cleaved both in apoptotic and in non-apoptotic GA-stimulated cells. (A) Confocal images of YEC-transfected SK-BR-3 cells stained with Alexa-555-Annexin V (Invitrogen). Examples of untreated non-apoptotic control cells, 3 μM GA-treated apoptotic cells, and 3 μM GA-treated non-apoptotic cells are shown. Bars, 10 μ. (B) Distribution of the YFP/CFP ratio on the plasma membrane (PM) of treated/non-treated and apoptotic/non-apoptotic (Annexin V or FLICA Red (Invitrogen) positive) cells as in A. Column height represents the number of cells with the particular ratios. It is seen that apoptotic cells in both unstimulated and GA-stimulated cultures had similarly high YFP/CFP ratios, which were significantly higher than that of non-apoptotic cells in the same cultures ($p<<0.001$ in both cases, Mann-Whitney two-tailed U-Test). Importantly, also non-apoptotic GA-stimulated cells had a significantly increased YFP/CFP-ratio of plasma membrane YEC compared to non-apoptotic unstimulated cells ($p<<0.001$, Mann-Whitney two-tailed U-Test).

Supplementary Figure 4



Two western blots made from the same samples from the same biotin internalization assay, but using two different ErbB2 antibodies: AF1129 (upper panel - identical to Fig. 1A upper panel) and Ab17 (lower panel)(Neomarkers). Please note the marked difference between the two antibodies in the relative staining between p185 and p135 ErbB2. AF1129 binds an extracellular epitope, whereas Ab17 is a cocktail of to monoclonal antibodies – one binding an intracellular epitope and another binding an extracellular epitope. From the figure we can reason that the two antibodies in the Ab17 cocktail must bind ErbB2 with different efficiencies, and that the majority of the Ab17 binding comes from the antibody binding the intracellular epitope - as much as 20-30 fold more than the antibody binding the extracellular epitope). Ab17 therefore gives a clear underestimate of ErbB2 intracellular cleavage.

Supplementary Figure 5

```
import ij.*;
import ij.process.*;
import ij.gui.*;
import java.awt.*;
import ij.plugin.filter.*;

public class Ratiometry_ implements PlugInFilter {
    ImagePlus imp;
    int ratiomodifier;
    int intensitetmodifier;
    int farve;
    float ratio;
    int intensitet;
    boolean maxint;
    boolean ratiomodus;
    boolean dynerror;
    public int setup(String arg, ImagePlus imp) {
        this.imp = imp;
        GenericDialog dialog=new GenericDialog("Ratiometry of the Green/Red channels");
        dialog.addNumericField("Ratio modifier",128,0,4,"...128 equals 1:1");
        dialog.addNumericField("Intensity modifier", 100,0,4,"...100 leaves intensity unchanged");
        dialog.addCheckbox("Normalization to maximum intensity (Leave unchecked to normalize to average intensity)",false);
        dialog.addCheckbox("Calculate ratio as Green/(Red+Green) (Leave unchecked for the ratio beeing Green/Red)",false);
        dialog.showDialog();
        ratiomodifier=(int)dialog.getNextNumber();
        intensitetmodifier=(int)dialog.getNextNumber();
        maxint=dialog.getNextBoolean();
        ratiomodus=dialog.getNextBoolean();
        return DOES_ALL+DOES_STACKS+SUPPORTS_MASKING;
    }

    public void run(ImageProcessor ip) {
        IJ.showStatus("Calculating ratios...");
        dynerror=false;
        for(int y=0; y<=ip.getHeight()-1; y++) {
            for(int x=0; x<=ip.getWidth()-1; x++) {
                int farve = ip.getPixel(x,y);
                float roed=(farve & 16711680) >> 16;
                float groen=(farve & 65280) >> 8;
                float blaa=farve & 255;
                if (groen<1) groen=1;
                if (roed<1) roed=1;
                if (ratiomodus) ratio=groen/(roed+groen)*ratiomodifier;
                else ratio=groen/roed*ratiomodifier;
                if (maxint) intensitet=Math.max((int)groen,(int)roed);
                else intensitet=((int)groen+(int)roed)/2;
                if (ratio<=64) {
                    roed=0;
                    groen=ratio*4;
                    blaa=255;
                }
                else if (ratio<=128) {
                    roed=0;
                    groen=255;
                    blaa=256-(ratio-64)*4;
                }
                else if (ratio<=196) {
                    roed=(ratio-128)*4;
                    groen=255;
                    blaa=0;
                }
                else if (ratio<=255) {
                    roed=255;
                    groen=256-(ratio-196)*4;
                    blaa=0;
                }
                else {
                    roed=255;
                    groen=0;
                    blaa=0;
                }
                roed=roed*intensitet/255*intensitetmodifier/100;
                groen=groen*intensitet/255*intensitetmodifier/100;
                blaa=blaa*intensitet/255*intensitetmodifier/100;
                if(roed>255) {
                    dynerror=true;
                    roed=255;
                    }
                if(groen>255) {
                    dynerror=true;
                    groen=255;
                    }
                if(blaa>255) {
                    dynerror=true;
                    blaa=255;
                    }
                ip.putPixel(x,y,(((int)roed & 255) << 16)+(((int)groen & 255) << 8)+((int)blaa & 255));
            }
        }
        imp.updateAndDraw();
        if (dynerror) IJ.showMessage("Some pixels had too high values. This might result in a ratiometric image with a lower dynamic range. To avoid this try again with a lower Intensity modifier.");
    }
}
```

Supplementary Textfile 1