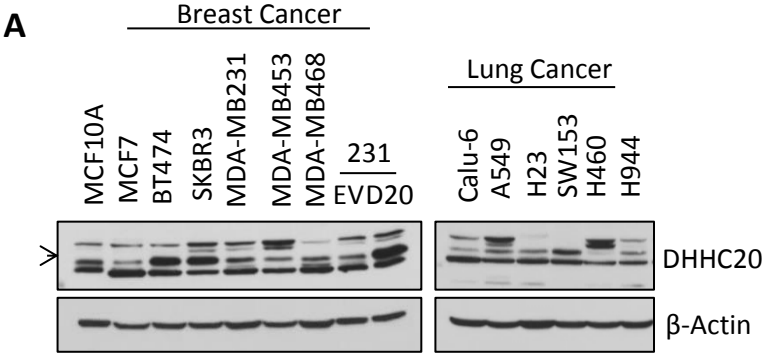
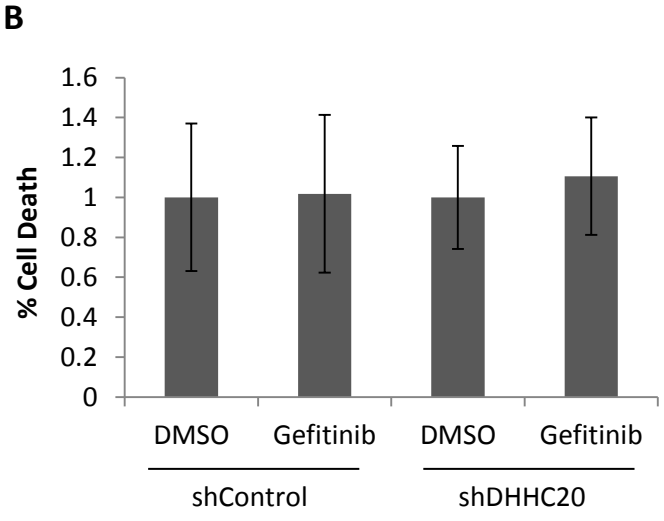
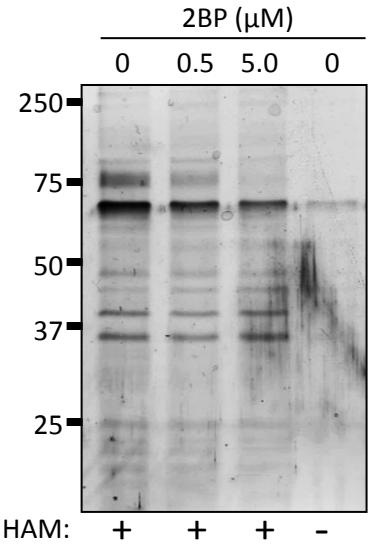
Supplemental Figure 1

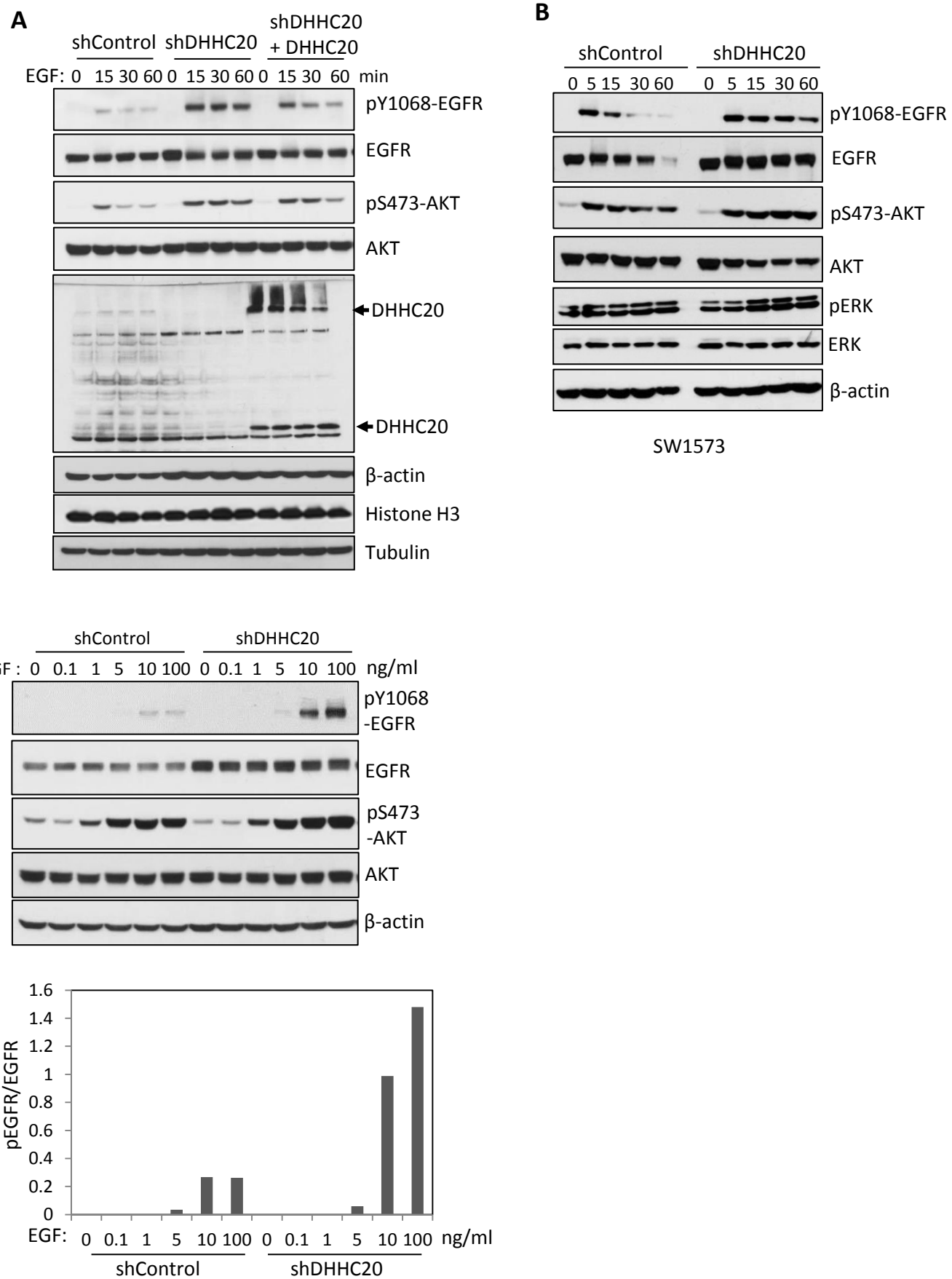




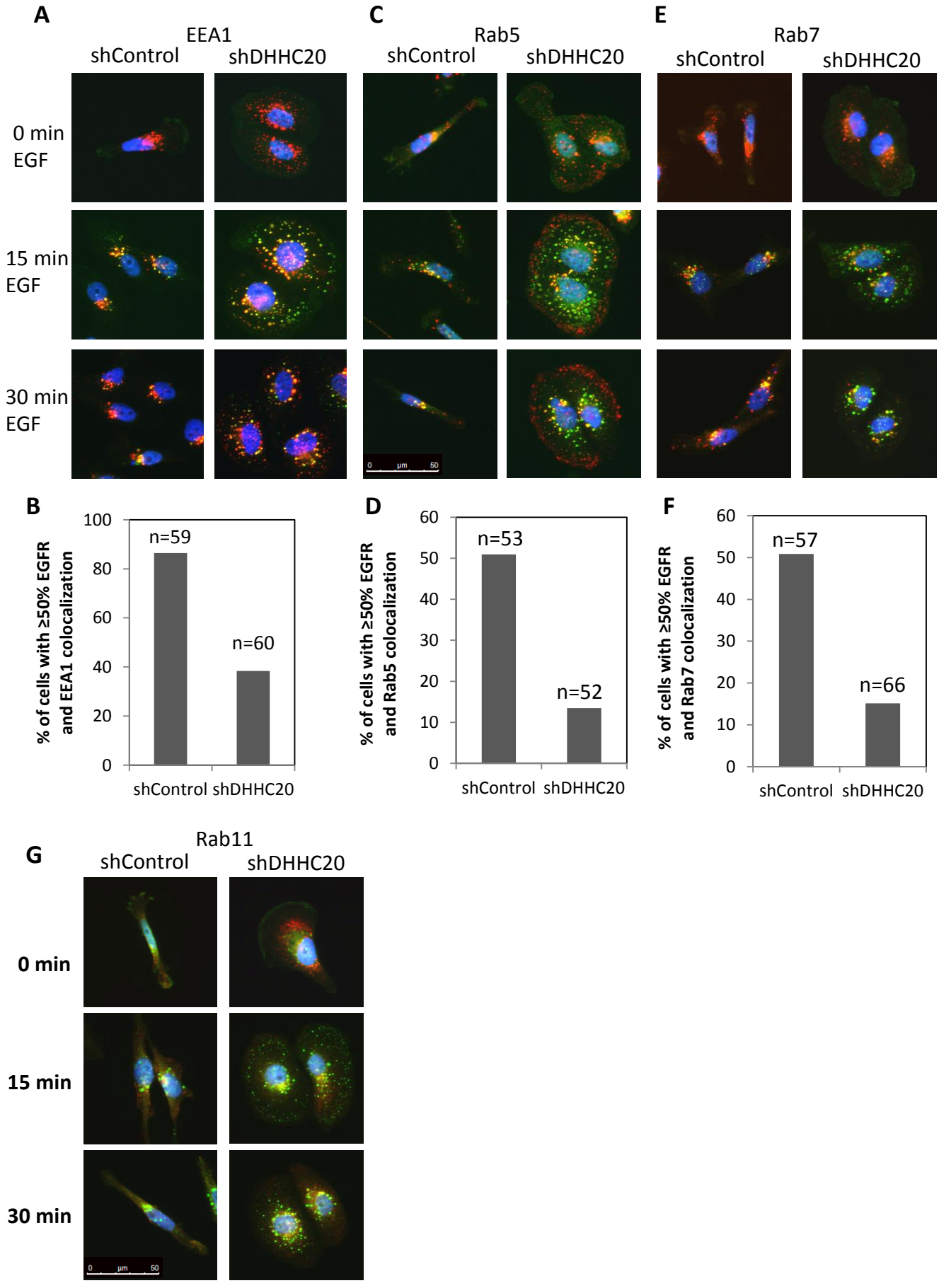
Supplemental Figure 2



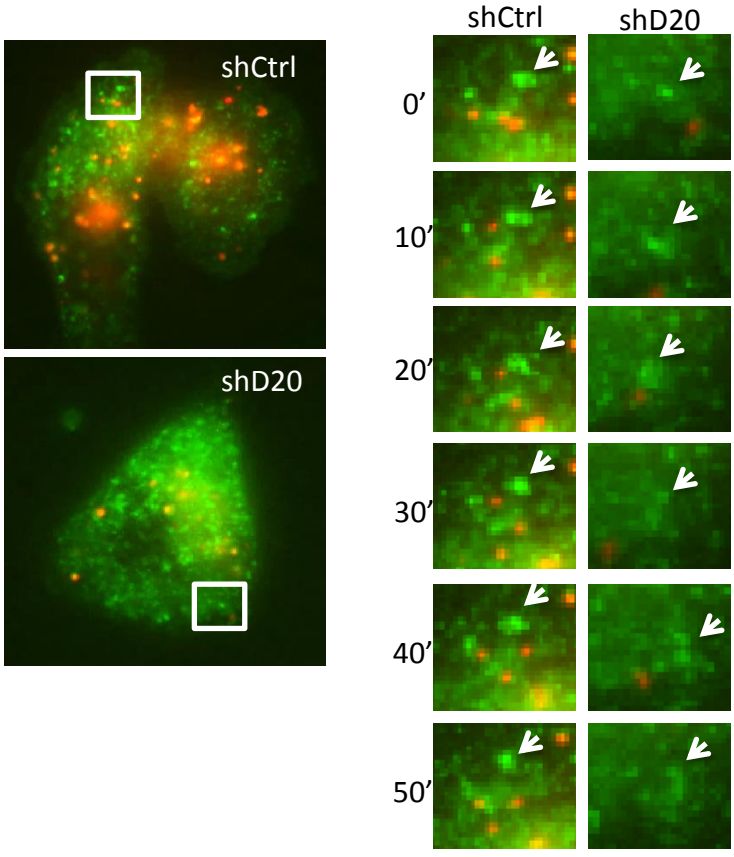
Supplemental Figure 3



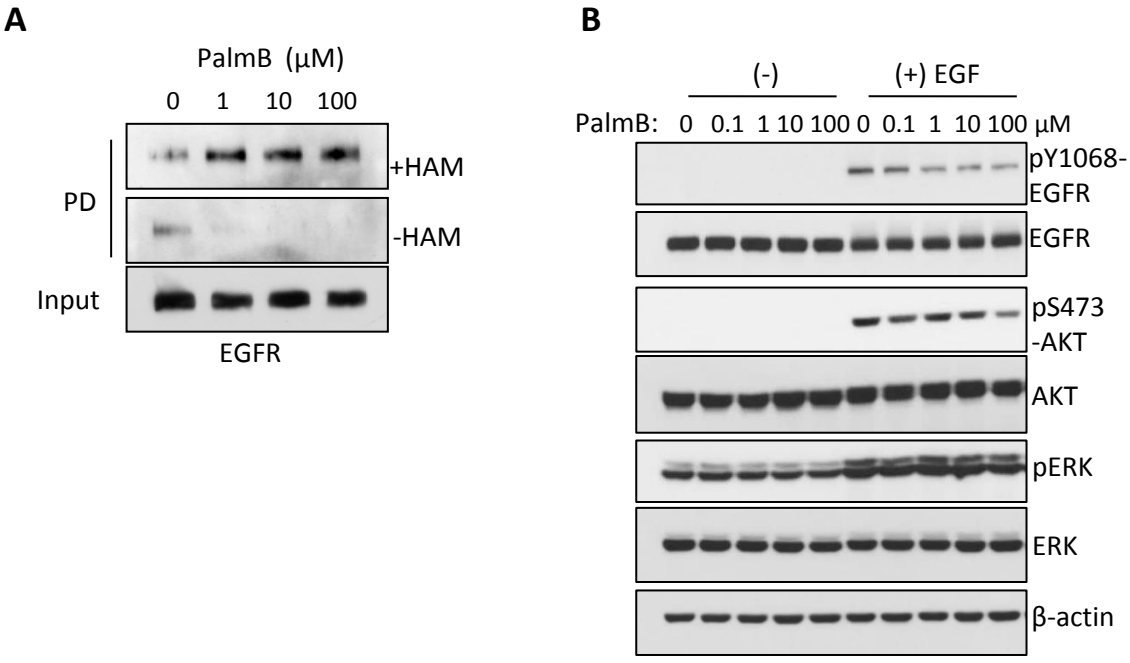
Supplemental Figure 4



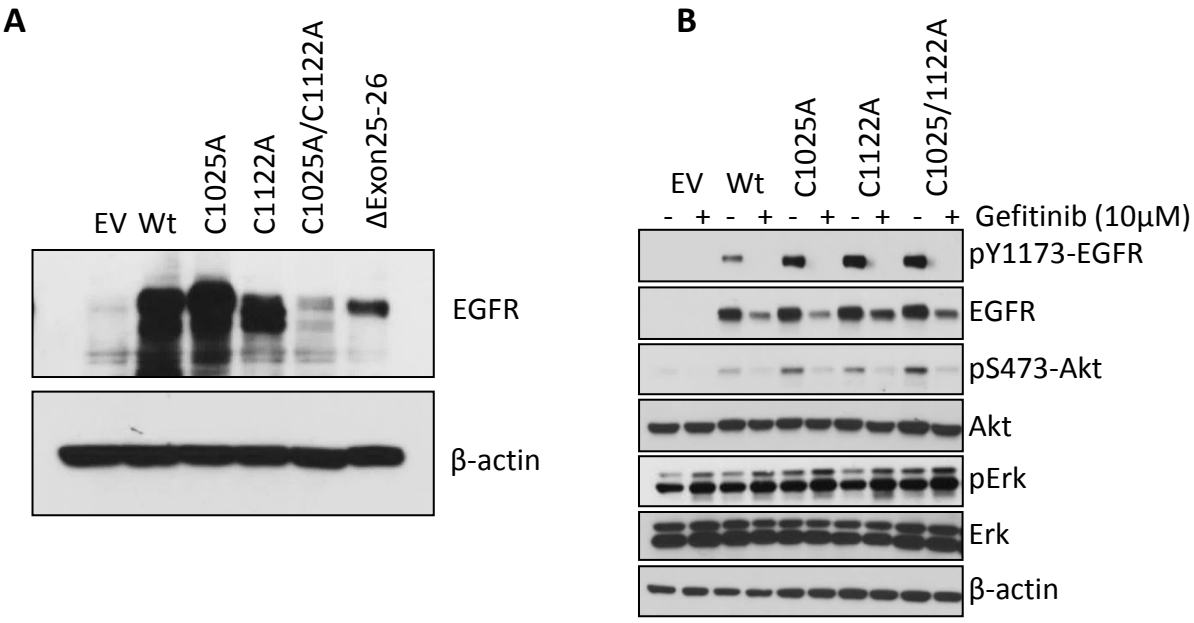
Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7



Supplemental Figure Legends

Figure S1. (A) Related to Figure 1. DHHC20 is expressed in breast cancer and lung cancer cell lines. Cells were lysed and DHHC20 expression was determined by SDS-PAGE.

(B) Related to Figure 1F. Treatment of MDA-MB-231 shControl and shDHHC20 cells with Gefitinib for 18 hours does not induce cell death. MDA-MB-231 cells were treated with 10uM gefitinib for 18 hours and the percent cell death was measured by trypan blue staining.

(C) Related to Figure 1G. DHHC20 expression is reduced in SW1573 lung cancer cells by shRNA. SW1573 cells were infected with shDHHC20 shRNA, selected with puromycin, and protein expression was determined by SDS-PAGE.

Figure S2. Related to Figure 1H. 2BP dose dependently reduces palmitoylation. MDA-MB-231 cells were treated with 0, 0.5 or 5μM 2BP for 24 hours and palmitoylation was determined by ABE and silver stain.

Figure S3. Related to Figure 2. Elevated EGFR signaling is specific to DHHC20 inhibition and is conserved in SW1573 cells. (A) Exogenous expression of DHHC20 partially rescues the elevated EGFR signaling. MDA-MB-231 shControl, shDHHC20 and shDHHC20 cells stably expressing a shRNA resistant DHHC20 construct were serum starved and treated with 100ng/ml EGF for the indicated times. EGFR signaling was determined by SDS-PAGE. (B) EGF-induced activation of EGFR, AKT and ERK is sustained in DHHC20 silenced lung adenocarcinoma cells. SW1573 cells were starved, treated with 100ng/ml EGF for the indicated time points, and activation of EGFR, AKT and ERK was determined by SDS-PAGE.

(C) Related to Figure 2. Silencing DHHC20 increases EGFR activation in response to low and high doses of EGF. MDA-MB-231 shControl and shDHHC20 cells were serum starved, treated with EGF at doses ranging from 0ng/ml - 100ng/ml for 15 minutes, and activation of EGFR and AKT was determined by SDS-PAGE. The phosphorylation of EGFR at Y1068 was normalized to total levels of EGFR by densitrometry using ImageJ.

Figure S4. Related to Figure 3. Silencing DHHC20 decreases EGFR localization to early and late endosome compartments. (A, C, E) MDA-MB-231 shControl and shDHHC20 cells were serum starved in DMEM + 0.2% BSA for 17 hours and treated with 100ng/ml EGF for 0, 15 and 30 minutes. Cells were fixed and stained for EGFR (green), EEA1, Rab5 or Rab7 (red) and DAPI (blue). Images were obtained using the Leica AF6000 microscope and the Hamamatsu ORCA R^2 digital CCD camera at 40x magnification. (B, D, F) Quantification of the percentage of cells with EGFR localized near EEA1, Rab5 or Rab7 positive vesicles was determined using Leica LAS software. (G) Silencing DHHC20 does not enhance EGFR recycling through Rab11 positive endosomes. MDA-MB-231 shControl and shDHHC20 cells were serum starved for 17 hours in DMEM + 0.2% BSA and stimulated with 100ng/ml EGF for the indicated time points. Cells were fixed, stained for EGFR (green), Rab11 (red) and DAPI (blue) and imaged as in A, C, E.

Figure S5. Related to Figure 2C. DHHC20 expression does not affect the intracellular trafficking of the transferrin. MDA-MB-231 shControl and shDHHC20 cells were plated onto glass-bottom imaging dishes and starved in DMEM + 0.2% BSA for 17 hours. Cells were treated with 15ng/ml EGF in combination with 2.5µg/ml Alexa-Fluor488 labeled transferrin and 50nM lysotracker and incubated on ice for 1 hour. Cells were washed with PBS and images were obtained every 10 seconds for 20 minutes using the Leica AF6000 microscope and the Hamamatsu ORCA R^2 digital CCD camera at 40x magnification.

Figure S6. Related to Figure 4. Inhibition of EGFR depalmitoylation decreases EGFR signaling. (A) The depalmitoylation inhibitor palmostatin B increases EGFR palmitoylation. MDA-MB-231 cells were treated with 1, 10, 100 µM palmostatin B for 15 hours and EGFR palmitoylation was determined by ABE. (B) Inhibiting depalmitoylation with palmostatin B attenuates EGFR activation. MDA-MB-231 cells were pretreated with the indicated doses of palmostatin B for 8 hours and serum starved in the presence of palmostatin B for an additional 17 hours. Cells were

treated with 100ng/ml EGF for 15 min, lysed and protein expression and protein phosphorylation was determined by immunoblotting.

Figure S7. (A) Related to Figure 5D and E. NIH 3T3 cells were transfected with EV, EGFR WT or EGFR cysteine mutant constructs and stably selected with puromycin. Cells were lysed and expression of EGFR was determined by SDS-PAGE. (B) Related to Figure 6A. Gefitinib inhibits the activation of EGFR and AKT in cells expressing EGFR cysteine mutants. NIH 3T3 cells were transfected with EGFR WT or cysteine mutant constructs for 30 hours followed by an 18 hour serum starvation in the presence of DMSO or 10µM Gefitinib. Cells were lysed and activation of EGFR, AKT and ERK was determined by SDS-PAGE.

Supplemental Methods

Acyl-biotinyl exchange (ABE) assay

The protocol is adapted from Wan et al., 2007. Cells were harvested in lysis buffer (50mM HEPES pH 7.4, 1% Triton X-100, 150mM NaCl, 5mM EDTA, 50mM N-ethyl-maleimide (NEM), 1µg/ml leupeptin, 1µg/ml aprotinin, 2µg/ml pepstatin A). Lysates were clarified by centrifugation at 15,000 RPM for 10 minutes. 1µg of anti-EGFR (sc-120) was added to 200µl of lysate and incubated overnight on ice. 15µl of protein A sepharose was added to lysates and incubated for 2 hours at 4ºC. Beads were washed in lysis buffer without NEM. The beads were eluted in 4%SDS buffer+50mM NEM (50mM HEPES pH 7.4, 4% SDS, 150mM NaCl, 5mM EDTA). 10µg of acetylated BSA was added as a carrier to the eluate followed by methanol/chloroform (m/c) precipitation. The dried pellet was resuspended in 40µl 4%SDS buffer+50mM NEM and incubated at room temperature for 1 hour. The samples were m/c precipitated twice then resuspended in 80µl 4%SDS buffer. The samples were split in half and 160µl of hydroxylamine buffer (0.7M hydroxylamine pH 7.4, 50mM HEPES pH 7.4, 0.2% Triton X-100, 150mM NaCl, 5M EDTA) was added to one half of the sample and control 0.2% Triton X-100 buffer (50mM HEPES pH 7.4, 0.2% Triton X-100, 150mM NaCl, 5mM EDTA) was added to the remaining

sample and incubated at room temperature for 1hour. The samples were m/c precipitated and resuspended in 40µl 4%SDS buffer containing 10µM Biotin-HPDP. 160µl of 0.2% Triton X-100 buffer +10µM Biotin-HPDP was added and incubated at RT for 1hour. The samples were m/c precipitated and resuspended in 20µl of 4%SDS buffer followed by addition of 800µl of 1% Triton X-100 buffer (50µl removed for analysis as “input”). 30µl of streptavidin agarose beads were added to the samples and incubated overnight at 4ºC rotating. The samples were washed in 1% Triton-X100 buffer and analyzed by SDS-PAGE.

Nano-LC-MS/MS analysis and data analysis

Digestion solution was acidified by 5% formic acid, and peptides were desalted prior to LC-MS/MS analysis using in-house C18 STAGE tips as previously described [PMID: 12585499]. Peptide samples were loaded onto a 75 µm I.D. x 20 cm fused silica capillary column packed with Reprosil-Pur C18-AQ resin (3 µm; Dr. Maisch GmbH, Germany) and resolved by an EASY-nLC 1000 HPLC system (Thermo Scientific) coupled in-line with a Q-Exactive (Thermo Scientific). The HPLC gradient was 2-30% solvent B (A = 0.1% formic acid in water; B = 0.1% formic acid in acetonitrile) for 70 min, followed by 30% to 95% solvent B for 10 min, and then held at 95% solvent B for 10 min, with a constant flow-rate of 300 nL/min. Full MS spectrum scans (m/z 350-1600) were performed at a resolution of 70,000 (at 200 m/z), and the 3 most intense ions were selected for MS/MS performed with high-energy collision dissociation (HCD) with normalized collision energy of 25 at a resolution of 17,500 (at 200 m/z). Five target MS/MS (508.7633, 736.3706, 1103.0043, 1104.0523 and 1470.3366) were set in case they were missed in data-dependent acquisition mode. AGC targets of full MS and MS/MS scans were 1x106 and 5x104, respectively. Unassigned charge states and singly charged species were rejected, dynamic exclusion was set to 30 seconds, and lock mass calibration was implemented using polysiloxane ions 371.10123 and 445.12000. Mascot was used for database searching. Two trypsin miss-cleavage sites were allowed, and precursor ion and fragment ion tolerances were set to 10 ppm and 0.02 Da, respectively. Oxidation (+15.9949) on methionine,

carbamidomethylation (+57.0215) and N-ethylmaleimide (125.0477) on cysteine were set as dynamic modifications. A peptide score of 20 was chosen to filter the peptide identification matches. Peptide quantification was performed on the extracted ion chromatograms (XICs) of peptides with all charge states.