SI Appendix – Huang et al.

Supplementary Materials and Methods

Immunoprecipitation and Western blotting

To examine ubiquitination,and tyrosine phosphorylation of EGFR by Western blotting, PAE cells stably expressing EGFR or EGFR-AMSH chimeric proteins were treated with EGF and processed for EGFR immunoprecipitatation as described in mass-spectrometry experiments.

In experiments examining EGFR degradation, the cells were incubated with EGF (100 ng/ml) for indicated times. The cells were then lysed as described in mass-spectrometry experiments with the exception that OV and NEM were omitted from the lysis buffer. This was necessary to provide equal efficiency of Western blotting detection of inactive and activated EGFR by Ab1005 in cell lysates.

To probe for active EGFR and ERK1/2, cells in 6-well plates were serum-starved overnight, treated with EGF at 37°C for indicated times, and lysed in the presence of OV and NEM.

The precipitates and lysates were resolved on 7.5% SDS-PAGE followed by transfer to the nitrocellulose membrane. Western blotting was performed by incubating with appropriate primary antibodies followed by secondary antibodies conjugated to far-red fluorescent dyes (IRDye-680 and -800) and detection using Odyssey Li-COR system. Quantifications were performed using Li-COR software. PY20 conjugated with horse radish peroxidase was detected using the enhanced chemiluminescence kit was from Pierce (Rockford, IL, USA).

Fluorescence Microscopy

To determine the extent of co-localization of EGF-Rh with EEA.1, PAE/EGFR cells grown on glass coverslips were treated with 100 ng/ml EGF-Rh at 37°C for 10 min, washed with ice-cold phosphate buffer saline and fixed with freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA, USA). The cells were stained with EEA.1 antibody followed by secondary antibody conjugated with Cy5; all incubations in the presence of saponin (0.02%). To compare the localization of EGFR and LysoTrackerRed, the cells were preincubated with leupeptine (21 μM) for 1 hr at 37^{o}C to block lysosomal degradation, and then incubated with 100 ng/ml EGF-FITC in the same medium at 37^{o}C for 2 hrs. LysoTracker (50 nM) was added for the last 30 min of 2-hour incubation with EGF-FITC. The cells were fixed and imaged without permeabilization.

Z-stacks of images were acquired using a spinning disk confocal imaging system based on a Zeiss Axio Observer Z1 inverted fluorescence microscope (with 63x Plan Apo PH NA 1.4), equipped with a computer-controlled Spherical Aberration Correction unit, Yokogawa CSU-X1, Vector photomanipulation module, Photometrics Evolve 16-bit EMCCD camera, HQ2 cooled CCD camera, environmental chamber and piezo stage controller and lasers (405, 445, 488, 515,

561, and 640 nm), all controlled by SlideBook 5 software (Intelligent Imaging Innovation, Denver, CO). All image acquisition settings were identical in each experiment.

The quantification of the relative amount of EGF-Rh or EGF-FITC co-localized with EEA.1 or LysoTrackerRed, respectively, was performed using the statistics module of SlideBook. Briefly, the background-subtracted 3-D images were segmented using a minimal intensity of EEA.1- or LysoTracker-labeled vesicles as a low threshold. The integrated voxel intensity of EGF-Rh or EGF-FC in the resulting mask (mask#1) was considered as EGF-Rh or EGF-FITC localized in EEA.1- or LysoTracker-labeled vesicles, respectively. Mask#2 corresponding to the total cellular amount of EGF-Rh or EGF-FITC was generated by image segmentation using a minimal intensity of EGF-Rh or EGF-FITC as a low threshold. The extent of co-localization was calculated as the ratio of the integrated fluorescence intensity of EGF-Rh or EGF-FITC of mask #1 to that intensity of mask#2 in each image. Statistical significance (P value) was calculated using unpaired two-tailed Student's t tests (Prism 5 and Excel).

LC-MS/MS analysis of tryptic peptides

Tryptic digests were analyzed by reverse-phased LC-MS/MS using a nanoflow LC (Waters nanoACQUITY UPLC system, Waters Corp., Milford, MA) coupled online to LTQ/Orbitrap Velos hybrid mass spectrometer (Thermo-Fisher, San Jose, CA). Separations were performed using a C18 column (nanoACQUITY UPLC column, 180 µm inner diameter x 250 mm length, 1.7µm particle size, BEH300 C18, Waters Corp., Milford, MA). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Samples were injected onto a trap column (nanoACQUITY UPLC trap column, Waters Corp., Milford, MA) and washed with 1% mobile phase B at a flow rate of 5ul/min for 3 min. Peptides were eluted off the column using a 90 minute gradient running at 300nl/min (5% B for 3 min, 5-30% B in 60 min, 30-95% B in 1 min, 95% B for 5 min, 95%-5% B in 1min, 5% B for 20 min). The LTQ/Orbitrap instrument was operated in a data-dependent MS/MS mode in which each high resolution broad-band full MS spectra (R = 60,000 at mass to charge (*m/z*) 400, precursor ion selection range of *m/z* 300 to 2000) was followed by 9 MS/MS scans in the linear ion trap where the 9 most abundant peptide molecular ions dynamically determined from the MS scan were selected for tandem MS using a relative collision-induced dissociation (CID) energy of 35%. Dynamic exclusion was enabled to minimize redundant selection of peptides previously selected for CID.

Peptide identification by database search

MS/MS spectra were searched with the SEQUEST search engine (Thermo-Fisher Proteome Discoverer 1.3) against a UniProt human proteome database (June 2012 release) from the European Bioinformatics Institute (http://www.ebi.ac.uk/integr8) merged with a contaminant database from AB Sciex (Framingham, MA) with the following modifications: static modification of cysteine (carboxyamidomethylation, +57.0214 Da), variable modification of methionine (oxidation, +15.9949 Da) and variable modification of lysine (ubiquitination,

+114.0429 Da). The mass tolerance was set at 10 ppm for the precursor ions and 0.5 Da for the fragment ions. Peptide identifications were filtered using Percolator with a q-value cutoff of 0.05 based on reversed database search (5% global false discovery rate, FDR).

Label-free quantitation for ubiquitinated EGFR peptides

Extracted ion chromatograms from full scan spectral data (MS1) were used for label-free quantitation. LC-MS/MS data acquired with LTQ/Orbitrap Velos operated in data-dependent mode was imported into Skyline with predicted *m/z* values for identified ubiquitinated EGFR peptides. The count for isotope peaks included was set to 4, the precursor mass analyzer was set to Orbitrap and the resolving power was set at 100,000 at *m/z* 400. The extracted ion chromatogram peaks were manually inspected to make sure that the proper peaks were selected. The observed full MS peaks for ubiquitinated EGFR peptides were all within 5 parts per million (ppm; Figure S2A).

Table S1: List of measured peptides and transitions.

Protein	Peptide name	Peptide sequence	Heavy SRM (m/z)	Light SRM (m/Z)	Spiked-in amount (fmol)
Ubiquitin	K63_3	TLSDYNIQK^{GG}ESTLHLV<u>L</u>R	751.1 → 1074.6 945.6 644.4 507.4	748.7 → 1067.6 938.6 637.4 500.4	2500
Ubiquitin	K63_4	TLSDYNIQK^{GG}ESTLHLV<u>L</u>R	563.6 → 1074.6 945.6 644.4 507.4	561.8 →1067.6 938.6 637.4 500.4	2500
Ubiquitin	K48	LIFAGK^{GG}Q<u>L</u>EDGR	489.9 → 1023.5 724.4 596.3 621.3	487.6 →1016.5 717.4 589.3 617.8	600
Ubiquitin	TLC	T<u>L</u>SDYNIQK	544.8 → 867.4 780.4 665.4 502.3	541.3 → 867.4 780.4 665.4 502.3	500
Ubiquitin	EST	ESTLHLV<u>L</u>R	358.9 → 507.4 394.3 295.2 429.8	356.5 → 500.4 387.3 288.2 426.3	500
EGFR	NLQ	NLQEILHGAV<u>R</u>	630.4 → 1032.6 904.5 775.5 549.3	625.4 → 1022.6 894.5 765.5 539.3	500
EGFR	YLV	YLVIQGDE<u>R</u>	551.8 → 826.4 727.4 614.3 486.2	546.8 → 816.4 717.4 604.3 476.2	500

* Heavy amino acids were underlined (13C, 15N).

Supplemental Equations:

Equation 1:

Use the measured AUC values to calculate the amount of each peptide in the sample

$fmol\ peptide = AUC_{light} / AUC_{heavy} \times fmol_{heavy}\ peptide$

Equation 2: Calculation of the amount of total ubiquitin in the sample using the TLS, EST, and K63 peptides

$Ub_{Total} = (fmol\ TLS + fmol\ EST) / 2 + (\ fmol\ K63\_3 + fmol\ K63\_4) / 2$

Equation 3: Calculation of the approximate number of ubiquitins bound to each molecule of EGFR using EGFR peptides and Ub_{Total}

$Ub/EGFR = fmol\ Ub_{Total} / fmol\ EGFR\ [(\ NLQ + YLV)/2]$

Equation 4: Calculation of the total ubiquitin in the form of poly-ubiquitin chains

$Total\ Chains = fmol\ K48 + [(fmol\ K63\_3 + K63\_4) / 2]$

Equation 5: Calculation of the total ubiquitin in the form of mono-ubiquitin and endcaps

$MonoUb\ \&\ Endcap = fmol\ UbTotal\ -\ Total\ Chains$

Equation 6: The number of ubiquitination sites per molecule of EGFR

$\#\ Ub'n\ sites = fmol\ MonoUb\ \&\ Endcap/fmol\ EGFR$

Equation 7: Average number of Ub per chain

$\#Ub/cnain = (Ub/EGFR)/\#Ub'n\ sites$

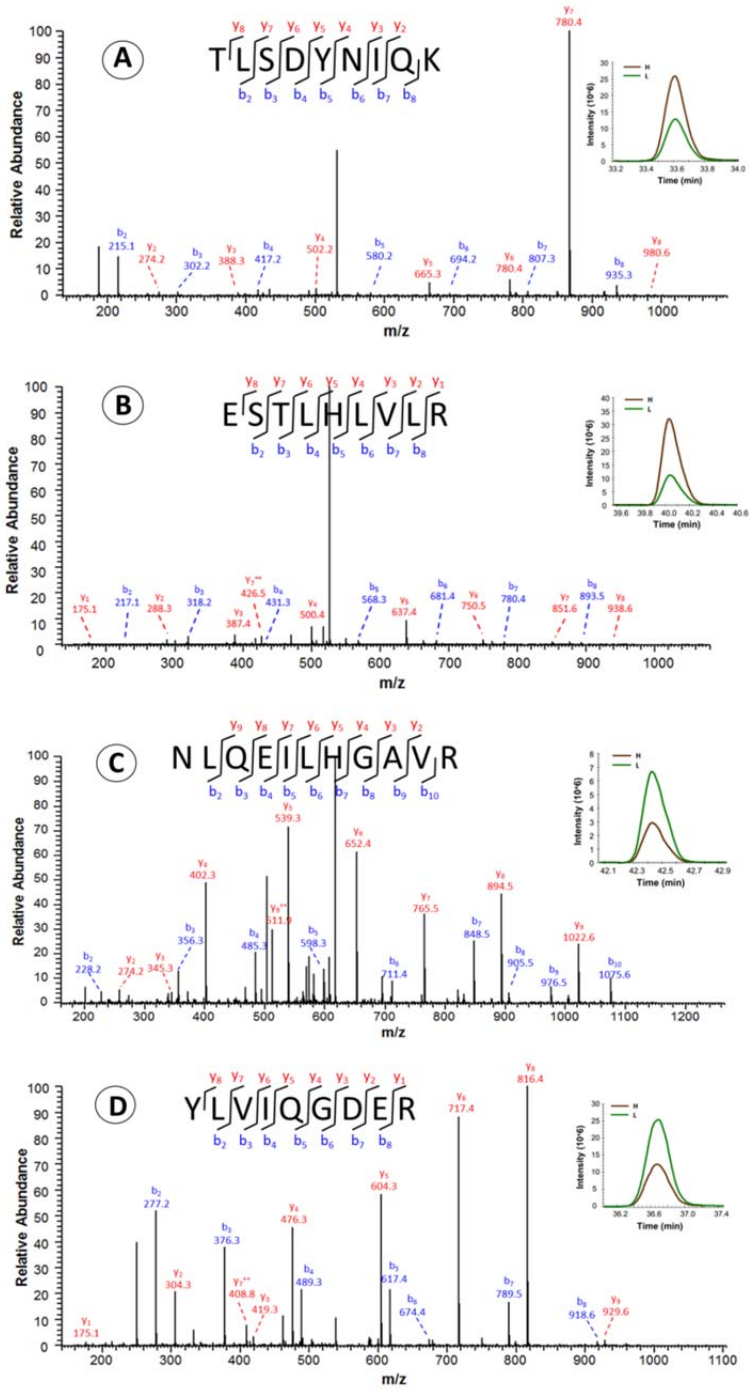

Figure S1. Representative tandem mass spectra for ubiquitin and EGFR peptides. Peaks matching expected singly- and doubly-charged (++) b- and y-ions are labeled. Insets showed representative mass chromatograms from SRM analysis.

A

Ubiquitination site	Peptide sequence	Mass Deviation (ppm)
K692	IK^{GG}VLGSGAFGTVYK	0.6
K713	GLWIPEGEK^{GG}VK	1.3
K843	LLGAEEK^{GG}EYHAEGGK	0.3
K851	EYHAEGGK^{GG}VPIK	0.2
K905	PYDGIPASEISSILEK^{GG}GER	1.8
K946	ELIIEFSK^{GG}MAR	1.2

B

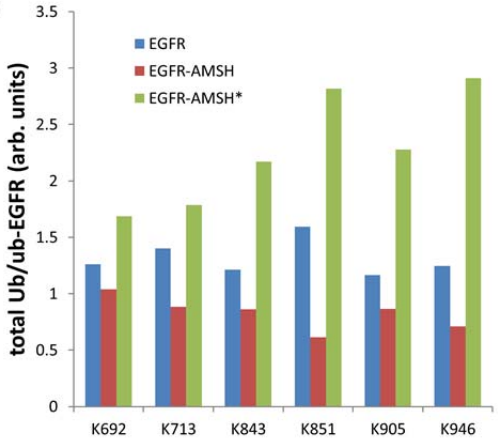

Figure S2. Label-free quantitation of ubiquitinated EGFR peptides. (A) List of identified EGFR ubiquitin sites, characterized by the presence of a –GG modified lysine (K^{GG}). (B) Relative abundance of ubiquitin normalized by the amount of ubiquitinated EGFR. The area of extracted ion chromatograms for each peptide was adjusted based on the mean area across samples. The total ubiquitin for each sample was based on the amount determined from absolute quantitation using SRM assays. The value for peptide *m* in sample *n* the bar chart was calculated as follow: $\text{totalUb}_n\text{(pmol)}/((\text{MS area})_{m,n}/\text{mean(MS area)}_m)$.

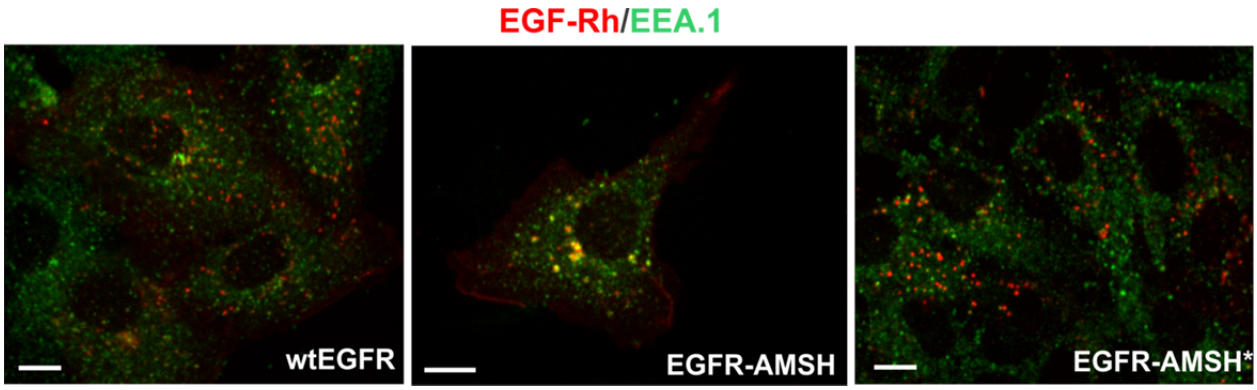

Figure S3. Prolonged residence of EGFR-AMSH in early endosomes.

Cells stably expressing similar levels of wtEGFR, EGFR-AMSH or EGFR-AMSH* were preincubated with leupeptin for 1 hr and incubated with EGF-Rh (100 ng/ml) for 2 hrs at 37^{o}C. After fixation, the cells were stained with antibody to EEA.1. A z-stack of confocal images were acquired though 561nm (EGF-Rh) and 640nm (EEA.1) channels. Confocal sections through the middle of the cell are shown. “Yellow” signifies the overlap of red and green fluorescence. Scale bars, 10 μm.

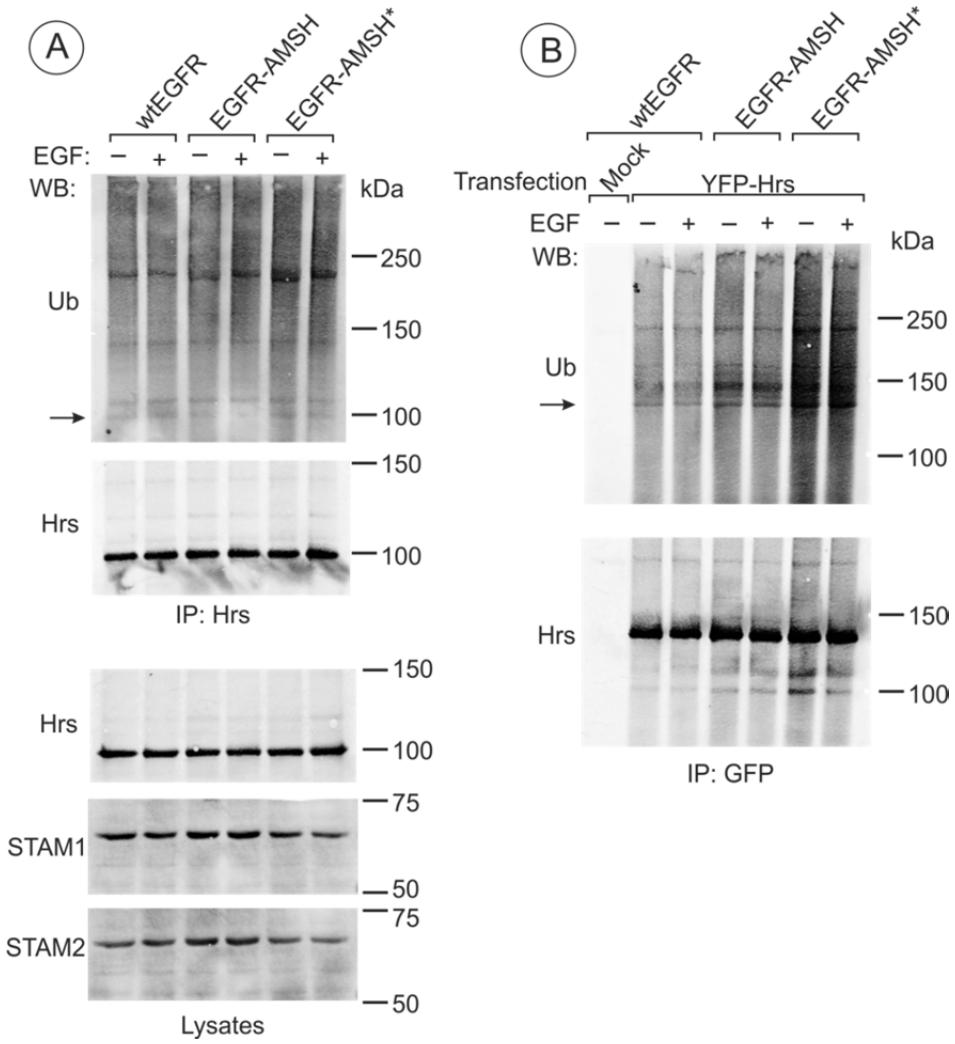

Figure S4. Expression of EGFR-AMSH does not affect Hrs ubiquitination and cellular concentrations of Hrs, STAM1 and STAM2.

(**A**) PAE cells expressing wtEGFR, EGFR-AMSH (clone #17) or EGFR-AMSH* (clone #27) were treated or not with 20 ng/ml EGF for 15 min at 37^{o}C. The cells were lysed and Hrs was immunoprecipitated as described for the EGFR immunoprecipitation section in "Materials and Methods". Immunoprecipitates and aliquots of lysates were resolved by SDS-PAGE and probed with Ub, Hrs, STAM1 and STAM2 antibodies. The results are representative of 3 independent experiments. No statistically significant differences in the amounts of Ub, Hrs and STAM between various cell lines and cells treated versus untreated with EGF were found.

(**B**) PAE cells expressing wtEGFR, EGFR-AMSH (clone #17) or EGFR-AMSH* (clone #27) were transfected with Hrs fused to yellow fluorescent protein (YFP-Hrs) (provided by Dr. H. Stenmark, Radium Institute, Oslo, Norway). After 2 days the cells were treated or not with EGF and lysed as in (***A***). YFP-Hrs was immunoprecipitated using GFP antibody. Immunoprecipitates were resolved by SDS-PAGE and probed with Ub and Hrs. Note, the ubiquitin signal is EGF-independent and proportionally corresponding to the amount of immunoprecipitated YFP-Hrs in different cell lines. The results are representative of 2 independent experiments.

Arrows point at the position of Hrs on ubiquitin blots.