Supporting Information

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SI Materials and Methods

Antibodies. Primary antibodies used were: anti-GAPDH (*Proteus* Biosciences), anti-tubulin (Sigma), anti-dynamin (BD), anti-AP-2 (ABR), anti-Hip1R (Chemicon), polyclonal anti-epsin 1 (1), monoclonal anti-epsin 1 (from our laboratory), anti-epsin 2 (2), anti-ezrin (Upstate), anti-clathrin TD1 (ATCC), anti-clathrin 4045 (Chemicon), and monoclonal anti-HA tag (Covance). The following antibodies were kind gifts: anti-EHD1 and EHD2 (from Bettina Winckler, University of Virginia, Charlottesville, VA, and Steve Caplan, University of Nebraska Medical Center, Omaha, NE, respectively); anti-H/K ATPase (HK9) (from Michael Caplan, Yale University, New Haven, CT); and anti-H/K ATPase (12.18) (from James Goldenring, Vanderbilt University, Nashville, TN). Secondary antibodies and protein A: HRP-conjugated antibodies for Western blotting (Biorad), Alexa Fluor-conjugated antibodies for immunofluorescence (Invitrogen), and 5- or 10-nm protein A gold for electron microscopy (Utrect-UMC).

Immunogold Labeling for Electron Microscopy. Immunogold labeling of ultrathin frozen sections for electron microscopic analysis was carried out by standard procedures, according to: http://medicine.yale.edu/cellbio/ccmi/em/protocols/index.aspx.

Reagents used for single labeling were: rabbit polyclonal anti-clathrin antibodies, rabbit polyclonal anti-epsin 3 antibodies, rabbit polyclonal anti-H/K ATPase antibodies. Secondary reagent was 10-nm protein A gold.

Reagents and steps (in sequence) used for double labeling were as follows: (*i*) mouse monoclonal anti-epsin 1 antibodies; (*ii*) rabbit anti-mouse IgGs (JacksonImmuno); (*iii*) 5-nm protein A gold; (*iv*) rabbit polyclonal anti-epsin 3 antibodies; (*v*) 10-nm protein A gold. Samples were viewed on FEI Tencai Biotwin TEM at 80 Kv. Images were taken using a Morada CCD camera fitted with iTEM (Olympus) software. For electron microscopy morphometry, a total of 293 gold particles from 30 micrographs were counted and classified into one of the four groups listed in Fig. S1.

Plasmids. To generate GST-EHD1 and GST-EHD2 (EH domains) plasmids, amino acid sequences 439 to 535 and 444 to 544 of mouse EHD1 and EHD2, respectively, were amplified from GFP-tagged EHD plasmids using EcoRI/XhoI (for EHD1) and BamHI/XhoI (for EHD2) restriction sites, and then subcloned into the pGEX-6P-1 vector.

Analysis of Human Tissues. Immunohistochemistry tissue microarray analysis (IHC-TMA) was designed and prepared as described (3, 4). Formalin-fixed, paraffin-embedded gastric tissue blocks of primary tumors, paired lymph node, and normal tissue were from the same group of patients described in Vecchi et al. (4). Tissue section arrays, which comprised two representative cores of all samples, were processed by immunoperoxidase staining using anti-epsin 3 monoclonal antibodies followed by detection with the EnVision Plus/HRP detection system (DAKO). Based on visual inspection of the intensity of the immunoreactive signal, tissues were classified in two groups: one with negative or low-moderate reactivity and one with a high level of reactivity similar to that of normal parietal cells. Statistical analyses of IHC-TMA results were performed using JMP IN 5.1 software (SAS).

Epsin 3 Affymetrix Microarray analysis was performed on our previously published data set of gastric tumors (4). Data were normalized using the MAS5.0 and processed in GeneSpring 7.3 (Agilent). The fold-change of epsin 3 expression in each tissue sample is relative to the ratio of the average signal of the two Affymetrix EPN3 probesets divided by their median across the entire dataset. Scatter plots were generated using Prism software (GraphPad Software, Inc.) and *P* values calculated by the two-tailed Welch's *t* test on a $\log_2$ expression ratio.

1. Chen H, et al. (1998) Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature* 394:793–797.
2. Rosenthal JA, et al. (1999) The epsins define a family of proteins that interact with components of the clathrin coat and contain a new protein module. *J Biol Chem* 274: 33959–33965.
3. Kononen J, et al. (1998) Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 4:844–847.
4. Vecchi M, et al. (2007) Gene expression analysis of early and advanced gastric cancers. *Oncogene* 26:4284–4294.

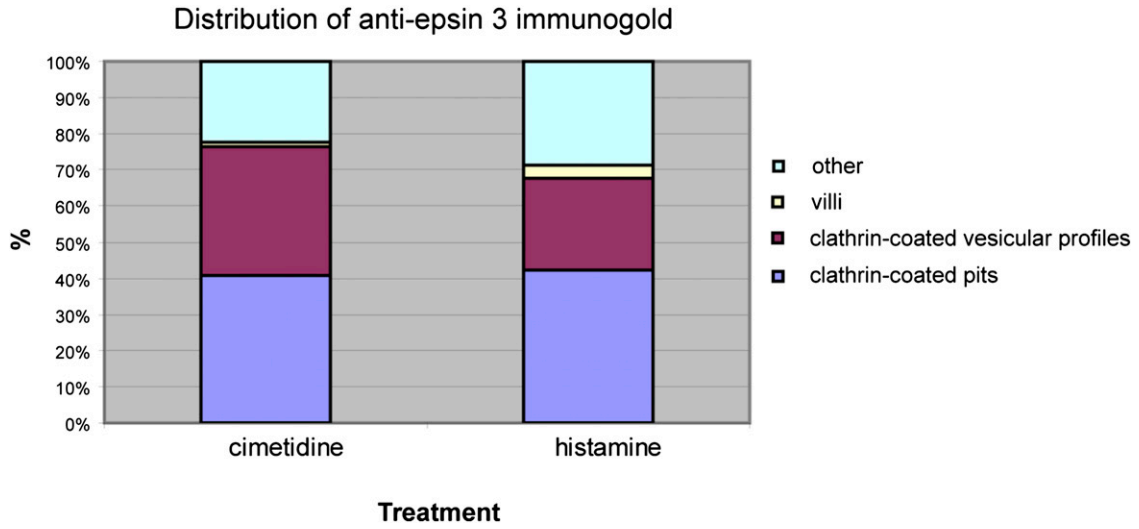


Fig. S1. Morphometric analysis of anti-epsin 3 immunogold on gastric parietal cell compartments. Ultrathin frozen sections of mouse stomachs were labeled with anti-epsin 3 polyclonal antibodies.

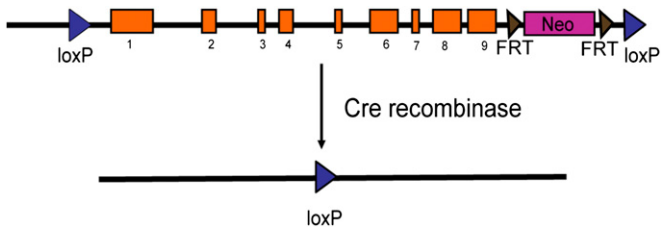
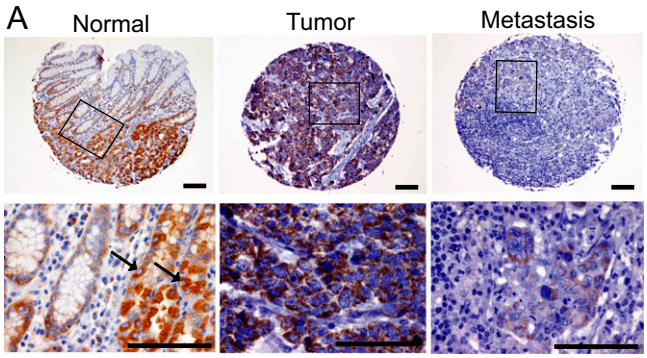


Fig. S2. Strategy used for the deletion of the epsin 3 gene. The entire coding region was removed.

A



B

Specimen	epsin 3 immunoreactivity		P-value
	negative/low	high	
Gastric cancer (n=27)	6 (22%)	21 (78%)	**0.006**
Metastasis (n=13)	9 (69%)	4 (31%)	

C

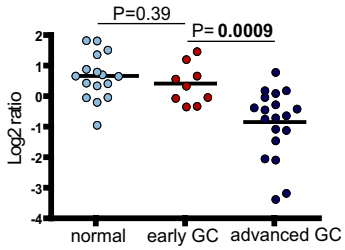


Fig. S3. Epsin 3 expression in human gastric tumors. (*A*) Epsin 3 immunoreactivity (brown color superimposed on hematoxylin staining) in specimens from a representative patient: normal gastric mucosa, primary tumor, and metastatic specimens. Boxed areas in images of the top row are shown at higher magnification in the bottom row. Arrows point to the intensely epsin 3-positive normal parietal cells. (Scale bars, 100 μm.) (*B*) Epsin 3 immunoreactivity in human gastric tumors and metastases as assessed by immunohistochemistry on tissue microarrays. Number in parentheses indicates the number of specimens for gastric carcinomas (GC) and available paired metastases. The *P* value was calculated with the Fisher's exact test. (*C*) Gene expression analysis of epsin 3 in the Affymetrix gastric cancer screening. The vertical scatter plot shows the distribution of the Log_2 expression ratios of epsin 3 in normal, early GC, and advanced GC stomach samples. The expression ratios between normal and early GC, and between advanced GC and early GC, are indicated, as well as the corresponding *P* values (Welch's *t* test).