Supporting Information

Rhodes et al. 10.1073/pnas.1003421107

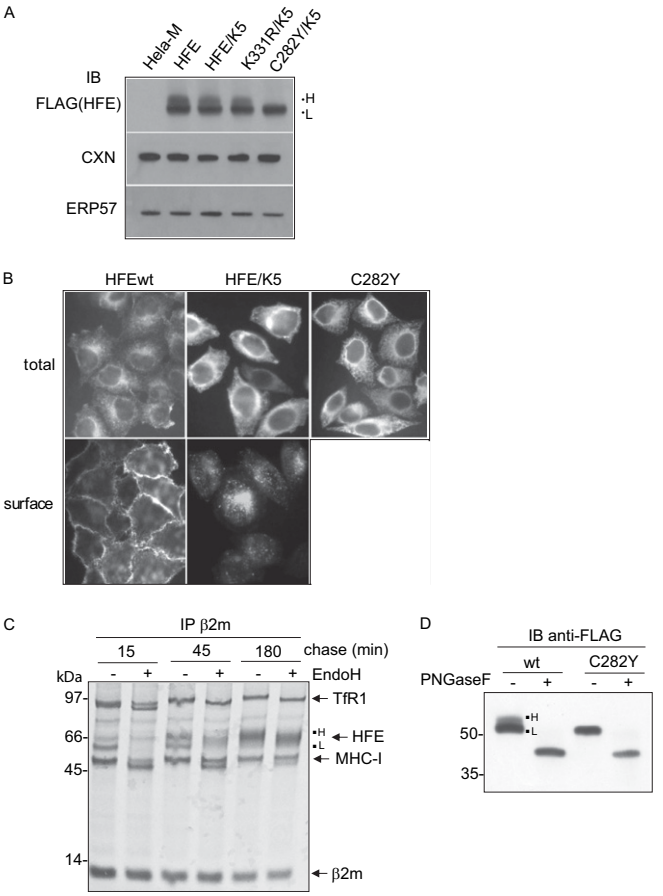

Fig. S1. HFE H "high" is endoglycosidase H resistant but PNGase F sensitive. (*A*) Immunoblot of total cell protein (10 μg) using anti-FLAG M2, anti-calnexin (CXN), and anti-ERP57 primary and anti-mouse HRP-conjugated secondary antibodies. Lanes: 1, Hela-M; 2, Hela/HFE; 3, HFE/K5; 4, K331R/K5; 5, C282Y/K5. (*B*) Confocal microscopy using anti-FLAG M2 primary and anti-mouse Alexa 568 secondary antibody on FLAG-HFE wild-type, HFE wild-type/K5, and C282Y cells. Total staining (*Upper*) shows antibody binding to cells fixed and permeabilized. Surface staining (*Lower*) shows antibody binding to fixed cells. Wild-type HFE showed cell surface staining and total staining consistent with Golgi and secretory vesicles. The C282Y mutant exhibited reticular intracellular staining. HFE/K5 cells exhibited faint, speckled residual surface staining and intracellular staining that did not conform strictly to that of wild-type or C282Y. (*C*) Hela-M FLAG-HFE cells were metabolically labeled and subjected to immunoprecipitation using anti-b2microglobulin antibody at different chase time points. Samples were left untreated or treated with endoglycosidase H and analysed by SDS-PAGE. (*D*) Two forms of HFE were observed, L (low) and H (high), by modified N-linked glycosylation. The panel shows anti-FLAG immunoblot of HFE wild-type and C282Y Hela-M cells with/without PNGase F treatment. The C282Y mutant is trapped in the endoplasmic reticulum so did not exhibit HFE H (high) form.

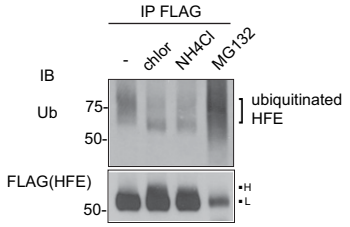

Fig. S2. Detection of ubiquitinated HFE in the presence of lysosomal inhibitors chloroquine (chlor) and ammonium chloride (NH4Cl) and inhibitor of proteosomes MG132. HeLa-M FLAG-HFE cells were treated with the indicated inhibitors for 24 h. Cell lysates were subjected to IP using anti-FLAG M2 antibody, followed by IB for Ub. The blot was reprobed using rabbit anti-FLAG antibody as a loading control. Chloroquine and ammonium chloride were effective at rescuing HFE "high". MG132 treatment was ineffective despite high levels of ubiquitination. This ubiquitination was not dependent on K331.

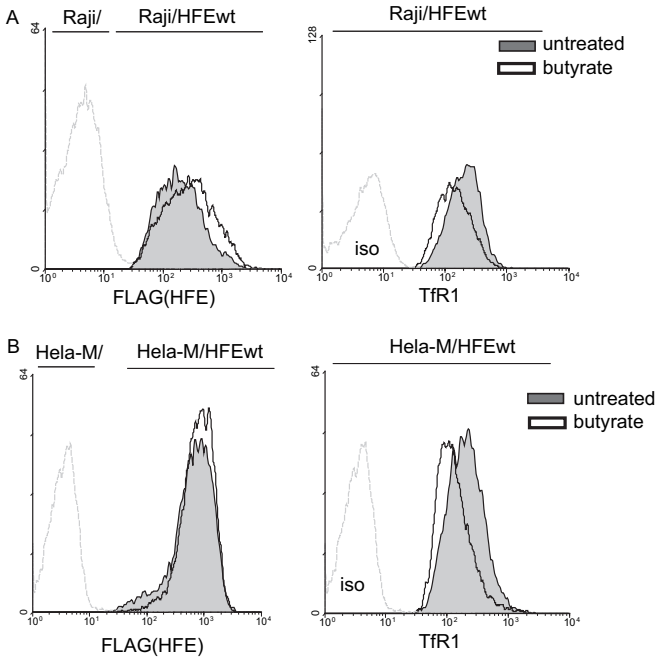

Fig. S3. Effect of butyrate on FLAG(HFE) and TfR1 in KSHV negative cells. (*A*) Lymphoblastoid B cell line Raji transduced with lentivirus containing FLAG-HFE wild type and (*B*) HeLa-M FLAG-HFE cells were left untreated (shaded histogram) or treated with sodium butyrate (1 mM, solid line) for 24 h. Cells were stained with anti-FLAG or anti-TfR1 primary and anti-mouse Alexa 633 secondary antibody and analyzed by flow cytometry.

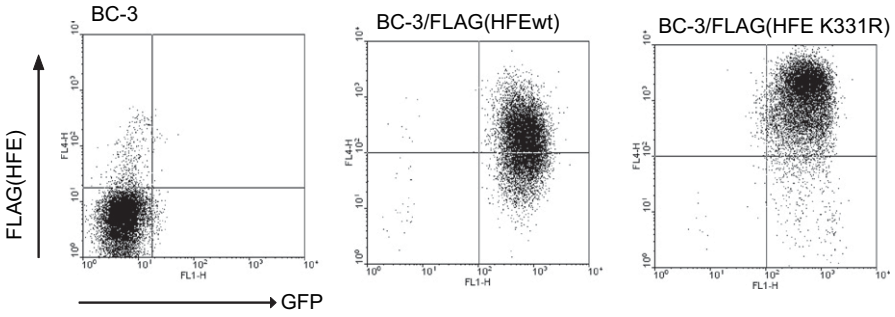

Fig. S4. FLAG-HFE wild type shows intermediate surface staining compared with K331R in BC-3 cells. Plots show anti-FLAG M2 antibody staining in flow cytometry on untransduced BC-3 cells (*Left*), BC-3 transduced with FLAG-HFE wild type (*Center*), and FLAG-HFE K331R (*Right*) lentivirus. The lentiviral vector contains a second promoter driving expression of GFP, which showed no variation in expression levels.

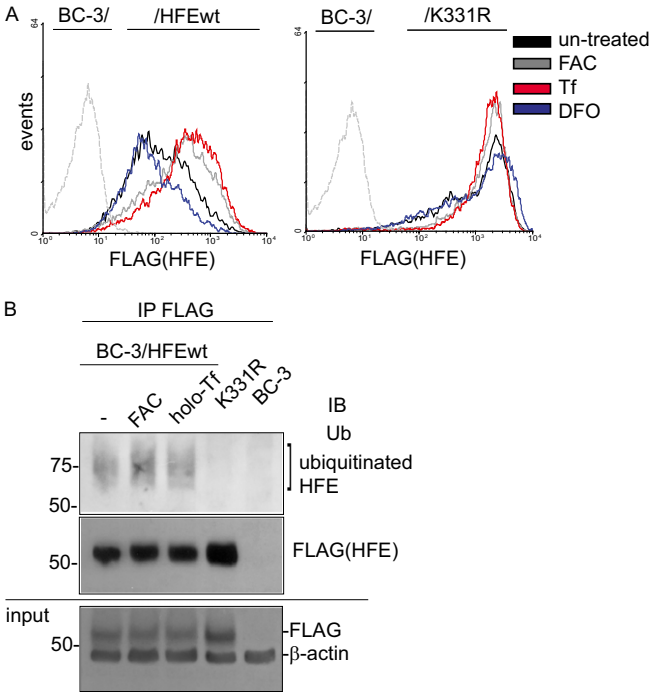

Fig. S5. (*A*) Effect of iron delivery on expression of FLAG-HFE wild-type (*Left*) and K331R (*Right*) BC-3 cells. Cells were incubated for 24 h as in Fig. 6*A*, stained with anti-FLAG antibody, and then analyzed by flow cytometry. Cells were untreated (black histogram), treated with ferric ammonium citrate (FAC, 1 mM, gray), treated with holo-transferrin (10 μM Tf, red), or treated with desferrioxamine (DFO, 10 μM, blue). The gray dashed line shows FLAG antibody binding to parental BC-3 cells. Surprisingly, FLAG antibody staining was increased in BC-3/HFE wild-type cells treated with FAC and Tf, suggesting that HFE was up-regulated by iron (*A Left*). No change in antibody staining was seen in K331R cells, which retained high surface expression, acting as a control for anti-FLAG antibody binding to iron-treated FLAG(HFE) cells (*A Right*). (*B*) We looked for any variation in HFE ubiquitination to account for this increase, by IP from HFE wild-type BC-3 cells treated with FAC or Tf. Lysates from BC-3/HFE wild-type cells left untreated or treated with ferric ammonium citrate (FAC, 1 mM) or transferrin (10 μM Tf), and untreated K331R and parental BC-3 cell lysates, were subjected to IP using FLAG M2 antibody followed by IB using anti-Ub HRP. Blots were reprobed using rabbit anti-FLAG. Input panel shows IB using anti-FLAG and β-actin antibodies as loading controls, which represent 10% of lysate used in IP. No change in total cell protein or polyubiquitination was detected for HFE wild type (*B*). K331R was not ubiquitinated as expected, but increased surface expression in flow cytometry did correlate with increased total protein for this variant, relative to control IB using β-actin. These results were consistent with increased recycling of wild-type HFE in iron-treated BC-3 cells.

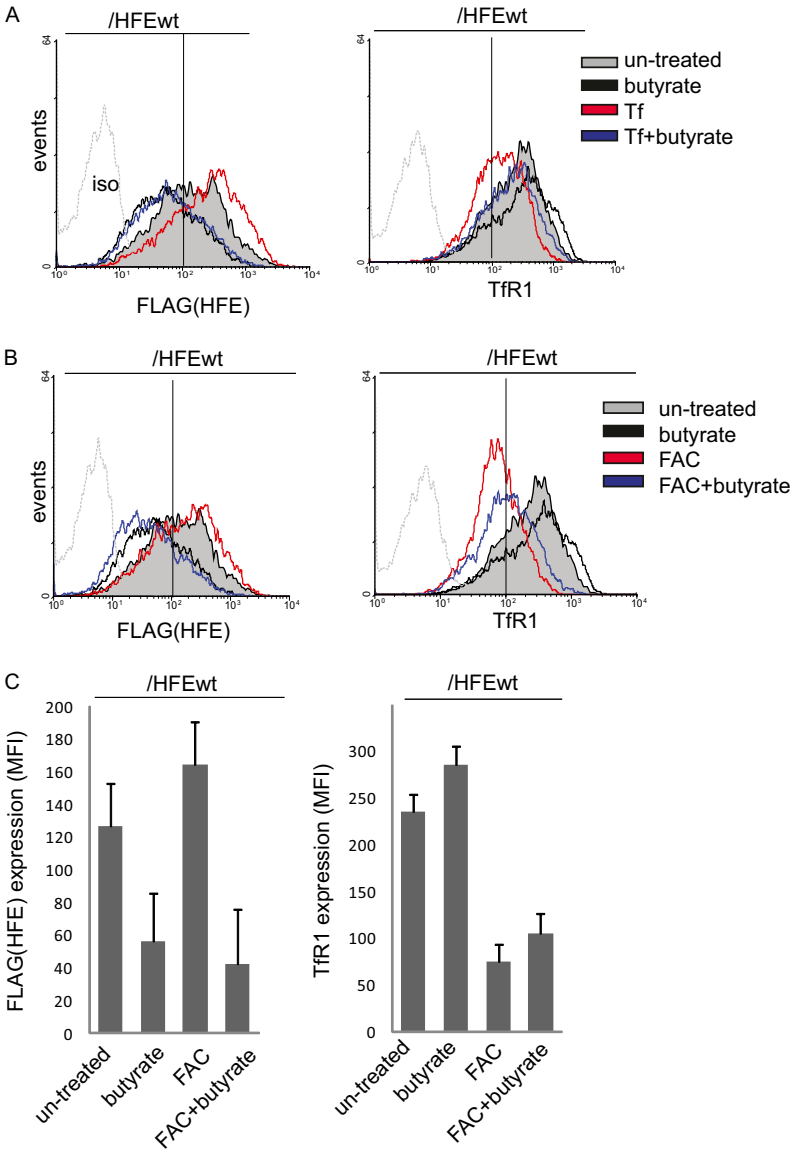

Fig. S6. (*A*) Effect of lytic KSHV in the presence of iron on expression of FLAG(HFE) (*Left*) and TfR1 (*Right*). BC-3/HFE wild-type cells were left untreated (gray solid histogram) or treated with butyrate (1 mM, black line), Tf (10 μM, red) or butyrate and Tf together (blue) for 24 h; stained with anti-FLAG and anti-TfR1 antibodies; and then analyzed by flow cytometry. (*B*) Similar results were obtained using ferric ammonium citrate (FAC, 1 mM, red). (*C*) Combined mean fluorescence from duplicate experiments assessing the effect of lytic KSHV in the presence FAC.