Dores et al., http://www.jcb.org/cgi/content/full/jcb.201110031/DC1

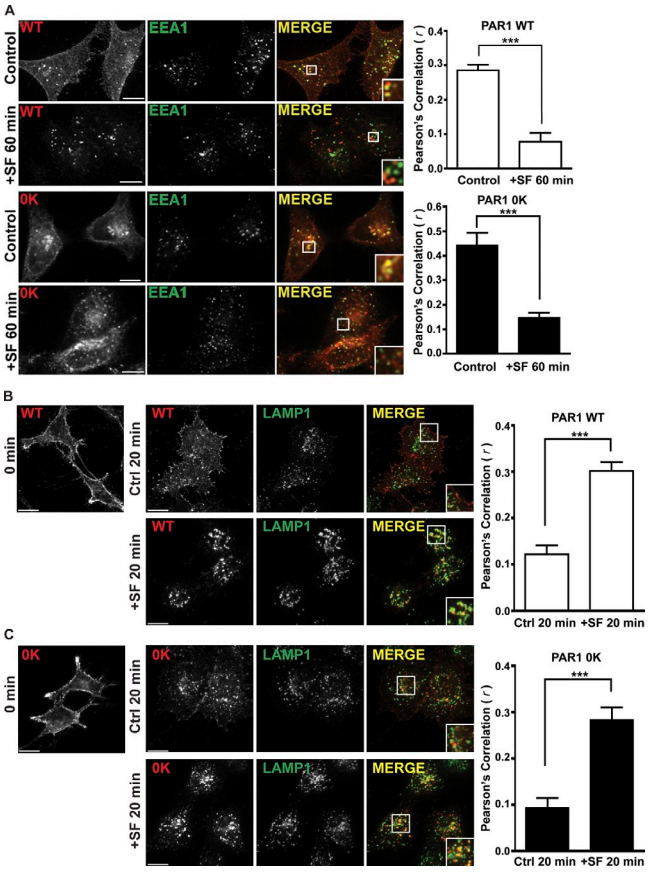

Figure S1. **PAR1 WT and 0K mutant are sorted from endosomes to lysosomes after agonist stimulation.** (A) HeLa cells expressing FLAG-PAR1 WT or 0K mutant were pretreated with 0.2 mM leupeptin. Cells were then incubated without (Control) or with 100 µM SFLLRN (SF) for 60 min at 37°C, fixed, permeabilized, and incubated with anti-FLAG and -EEA1 antibodies. Immunolabeled cells were processed and imaged by confocal microscopy. Colocalization of PAR1 and EEA1 is revealed by the yellow color in the merged image and is representative of many cells examined in three independent experiments. Bars, 10 µm. The data (mean ± SD) represent Pearson's correlation coefficients for quantification of colocalization that were calculated from dual-channel images of whole cells (n = 6) and were significantly different, as calculated by Student's t test (***, $P < 0.001$; n = 3). (B and C) HeLa cells expressing either FLAG-PAR1 WT or 0K mutant were prelabeled with anti-FLAG antibody at 4°C to label the surface cohort of receptor. Cells were warmed to 37°C and incubated for 20 min with (+SF 20 min) or without (control) 100 µM SFLLRN agonist, fixed, permeabilized, and coimmunostained for LAMP1. Images are representative of multiple cells from three independent experiments. Bars, 10 µm. The data (mean ± SD) represent Pearson's correlation coefficients for quantification of colocalization that were calculated from dual-channel images of whole cells (n = 6) and were significantly different, as calculated by Student's t test (***, $P < 0.001$; n = 3).

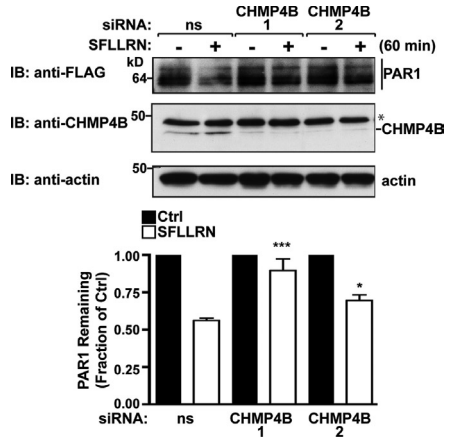

Figure S2. **Depletion of CHMP4B by individual siRNAs inhibited PAR1 degradation.** HeLa cells expressing FLAG-PAR1 were transfected with either nonspecific (ns) or two individual siRNAs targeting CHMP4B, designated 1 and 2. Cells were stimulated with 100 µM SFLLRN for 60 min at 37°C. The amount of PAR1 remaining was determined from an equivalent amount of cell lysates by immunoblotting (IB). Membranes were stripped and probed for endogenous CHMP4B and actin. The asterisk denotes a nonspecific band in the anti-CHMP4B immunoblot. The data (mean ± SD) are expressed as a fraction of untreated control (Ctrl) from three different experiments. The amount of PAR1 remaining in CHMP4B siRNA–treated cells was significantly different compared with nonspecific siRNA-treated cells, as determined by a two-way analysis of variance (*, $P < 0.01$; ***, $P < 0.001$; $n = 3$).

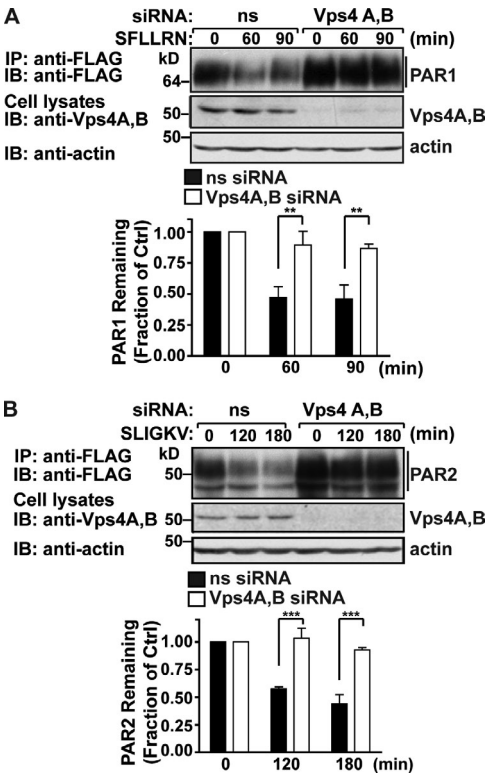

Figure S3. **Vps4 mediates PAR1 and PAR2 lysosomal degradation.** (A and B) HeLa cells expressing FLAG-PAR1 or FLAG-PAR2 were transfected with 100 nM of nonspecific (ns) or Vps4A and B SMARTpool siRNAs and then stimulated with 100 µM SFLLRN or 100 µM SLIGKV at 37°C, as indicated. The amount of PAR1 or PAR2 remaining was detected by immunoblotting (IB). Cell lysates were immunoblotted with anti-Vps4 and -actin antibodies. IP, immunoprecipitation. The data (mean ± SD) are expressed as the fraction of PAR1 or PAR2 remaining compared with untreated control (Ctrl) and were analyzed by a two-way analysis of variance (PAR1: **, $P < 0.01$, $n = 3$; PAR2: ***, $P < 0.001$, $n = 3$).

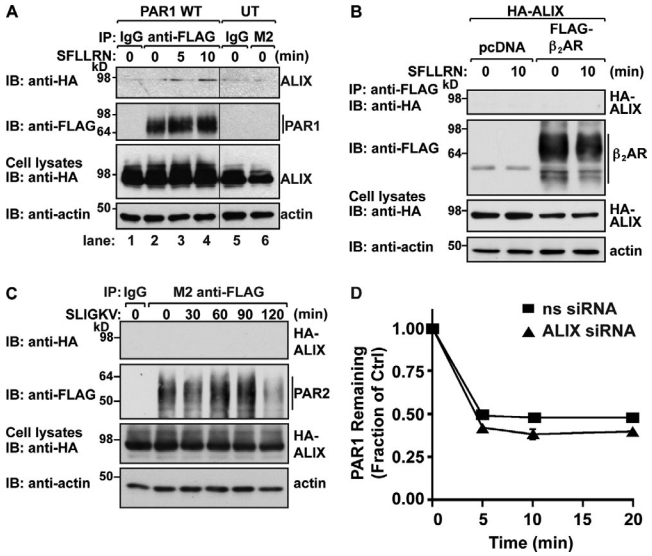

Figure S4. **HA-ALIX coimmunoprecipitates with PAR1, and ALIX depletion does not affect PAR1 internalization.** (A) Untransfected (UT) HeLa cells or HeLa cells coexpressing PAR1 WT and HA-ALIX were stimulated for the indicated times with 100 µM SFLLRN at 37°C. Cell lysates were immunoprecipitated (IP) with either IgG or anti-FLAG antibodies, and immunoprecipitates were analyzed by immunoblotting (IB) using anti-HA antibodies. Cell lysates were immunoblotted with anti-HA and -actin antibodies as controls. (B) PAR1-expressing HeLa cells were cotransfected with FLAG-tagged β_2-adrenergic receptor (β_2AR) and either HA-ALIX or pcDNA empty vector and stimulated for 10 min with 100 µM SFLLRN at 37°C. FLAG-β_2AR was immunoprecipitated with anti-FLAG antibody, and immunoprecipitates were analyzed by immunoblotting using anti-HA antibodies. Cell lysates were immunoblotted with anti-HA and -actin antibodies as controls. (C) HeLa cells expressing FLAG-PAR2 were transfected with HA-ALIX, stimulated with 100 µM SLIGKV, lysed, and immunoprecipitated with anti-FLAG antibody or IgG control, and the presence of PAR2 and ALIX was assessed by immunoblotting. (D) PAR1-expressing HeLa cells were transfected with either 100 nM of nonspecific (ns) siRNA or ALIX-specific siRNA. Cells were then prelabeled at 4°C with anti-FLAG antibody before incubation with 100 µM SFLLRN for the indicated times at 37°C. Cells were fixed, and the amount of antibody remaining on the cell surface was quantitated by ELISA. The data are expressed as the fraction of surface receptor remaining as compared with control (Ctrl) untreated cells (mean ± SD) from three independent experiments.

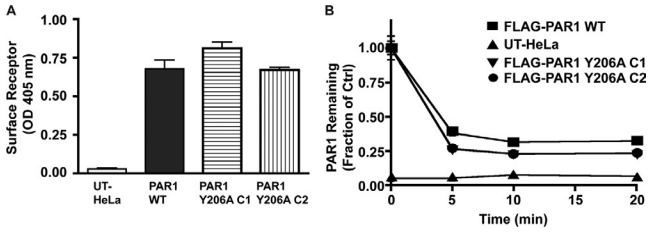

Figure S5. **ALIX depletion and mutation of the PAR1 YPX$_3$L motif do not affect PAR1 internalization.** (A) HeLa cells stably expressing either FLAG-tagged PAR1 WT or Y206A mutant clone 1 (C1) or 2 (C2) were prelabeled with anti-FLAG antibody at 4°C. Cells were fixed, and the amount of antibody remaining at the cell surface was determined by ELISA. The data (mean ± SD) are expressed as the absorbance determined at OD at 405 nm from three separate experiments. UT, untransfected. (B) HeLa cells stably expressing either FLAG-PAR1 WT or Y206A mutant were prelabeled with anti-FLAG antibody for 1 h at 4°C and then incubated with 100 µM SFLLRN for the indicated times at 37°C. Cells were fixed, and the amount of antibody remaining at the cell surface was determined by ELISA. The data (mean ± SD) are expressed as the fraction of cell surface receptor remaining as compared with control (Ctrl) untreated cells from three independent experiments.