SUPPLEMENTARY INFORMATION

Supplementary Methods

Antibodies– AU1 monoclonal antibody, Covance. EPS15 rabbit polyclonal antibody, Santa Cruz. EEA1 monoclonal antibody, BD. S5a monoclonal antibody and anti-ubiquitin conjugate monoclonal antibody (FK2), Cedarlane. GFP monoclonal antibody, Roche. β-tubulin rabbit polyclonal antibody, Sigma. MYC tagged proteins were detected with the anti-MYC clone 9E10. Rabbit PLIC-1 antibodies as described in Bedford et al., 2001. Donkey anti-mouse and anti-rabbit antibodies conjugated to fluorescein, rhodamine or peroxidase, Jackson Labs.

Plasmids– MYC-PLIC-1 representing isoform 2 was created by PCR from rat brain cDNA and cloned into pRK5-MYC. MYC-PLIC-1$^{\Delta UBL}$ (codons 110-561) and MYC-PLIC-1$^{\Delta UBA}$ (codons 2-514) were created by PCR using MYC-PLIC-1 as a template and cloned into pRK5-MYC. GFP-PLIC-1^{UBL} and GST-PLIC-1^{UBL} were created by PCR of the UBL domain (1-109) using pRK5-MYC-PLIC-1 as a template and cloned into pEGFP-C1 (BD Biosciences) and pGEX-4T-3 (Amersham Biosciences) respectively. pEGFP-EPS15 was kindly provided by Dr. P. McPherson. pGEX-EPS15^{UIM1+2} and pDEST15-S5a used for the preparation of GST-EPS15^{UIM1+2} and GST-S5a fusion proteins respectively, as well as HIS-EPS15, HIS-EPS15$^{\Delta UIM}$ were kindly provided by Dr. E.A. Fon. pCMVTag3a-HSJ1a and pCMVTag3-HSJ1a$^{\Delta UIM}$ were kindly provided by Dr. M. Cheetham. pMYC-ataxin-3 and pAUI-Q78 were kindly provided by Dr. G. Rouleau.

Immunoprecipitation of Ubiquitinated Proteins– HEK293 cells were plated on 10 cm dishes and transfected with MYC-tagged PLIC-1 constructs. 24 hours post-transfection, cells were lysed in buffer containing 25 mM Hepes pH 7.4, 125 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA and 0.5% NP-40 and 10mM NEM at 4ºC for 1h. Nuclei and insoluble material were removed by centrifugation at 13,000 rpm. Antigen-antibody complexes were immunoprecipitated with anti-MYC clone 9E10 and Protein-G-Sepharose beads (Amersham Biosciences) at 4ºC for 2h. Beads were then washed in lysis buffer three times before analysis by Western blot with anti-Myc and anti-ubiquitin (FK2) antibodies.

Semi-quantitative PCR– cDNA was synthesized from total RNA (1μg) by reverse transcription using 70 U of Superscript Reverse Transcriptase (Invitrogen) and oligo(dt)12–18 primer (Invitrogen) in a 10μl reaction mixture containing Superscript buffer (Invitrogen), 0.5 mM dNTP mix, 10 mM DTT, and 40 U RNase inhibitor. Total RNA and oligo(dt)12–18 primers

were incubated at 65°C for 5 min prior to the reverse transcription reaction. After incubation for 1 h at 42 °C, the reaction was stopped by incubation with RNAseH (1U) digestion 30 min at 37 °C. For PCR amplification, 0.5 μl of cDNA was added to a 20μl reaction containing 25 μM each primer, 0.2 mM of dNTP mix, 1 U Taq polymerase (Invitrogen) in 1X mix in Thermopol buffer (Invitrogen). PCR was performed in a DNA Thermal Cycler (PTC-200, MJ research). The following primers were used: PLIC-1 sense 5'-CCC AGG GGG ATA TAA TGC TT-3; PLIC-1 antisense 5'-TAC CAG TAG TGC CAC CCA CA-3', CHOP sense 5'-GGC AGC TGA GTC ATT GCC 3'; CHOP antisense, 5'- GCA GAT TCA CCA TTC GGT CA -3', GAPDH sense, 5'-GAA GGT GAA GGT CGG AGT C-3'; and GAPDH antisense 5'-GAA GAT GGT GAT GGG ATT TC-3'. The PCR products (9.5 μl) were resolved by electrophoresis in a 1% agarose gel in TAE buffer. The gels were stained with ethidium bromide and then photographed under ultraviolet light. cDNA for PLIC-1 was amplified for 28 (94 °C 45 sec, 60 °C 45 sec, and 72 °C 45 sec) cycles, for CHOP 25 cycles and for GAPDH 28 cycles.

Live Imaging and Nocodozole Treatment– HEK293 cells were transfected with GFP-EPS15 and MYC-PLIC-1 and imaged. Images of GFP fluorescence were taken once every 90 seconds for 30 minutes. Nocodazole (1μM) was added to the media 40 min prior and during imaging. Images were acquired on a Zeiss Axiovert 135 microscope.

LEGENDS TO FIGURES

Supplementary Figure 1. Cellular localization of transfected PLIC-1 and EPS15. HEK293 cells were transfected with (**A**) MYC-PLIC-1 or (**B**) HIS-EPS15 and stained for immunoflourescence with anti-MYC or anti-EPS15 antibodies. Scale bar is 10μM.

Supplementary Figure 2. Cytoplasmic structures are not expanded endosomes. HEK293 cells were transfected with MYC-PLIC-1 and HIS-EPS15 and stained for immunoflourescence with anti-EPS15 and anti-EEA1 antibodies. Scale bar is 10μm.

Supplementary Figure 3. Cytoplasmic structures merge in a microtubule dependent manner. HEK293 cells were transfected with GFP-EPS15 and MYC-PLIC-1 and images acquired in (**A**) the absence or (**B**) the presence of 1μM nocodazole. Images of GFP fluorescence were acquired every 90 seconds, four images covering 9 minutes are shown. Note in (**A**) perinuclear-localized fusion events at 6 min and 9 min time points. Arrows point to a smaller structure fusing with a larger structure. In (**B**) note a larger number of smaller aggregates as well as fewer fusion events. *Scale bar* is 10 μm. (**C**) The change in number of cytoplasmic

aggregates per cell over a 30 minute time period. For untreated cells, change in the number of was –2.57±0.72, n=7 and for nocodazole treated cells, the change in the number of was -0.25±0.88, n= 8.

Supplementary Figure 4. PLIC-1Δ^{UBL} binds ubiquitinated proteins. HEK293 cells were untransfected (1), or transfected with MYC-PLIC-1 (2), MYC-PLIC-1Δ^{UBA} (3), MYC-PLIC-1Δ^{UBL} (4). PLIC-1 proteins were immunoprecipitated with anti-MYC (9E10) antibodies and probed by Western blot for (**A**) ubiquitinated proteins (Ub^n) or (**B**) the MYC-tagged PLIC-1 proteins, arrow denotes the heavy chain of immunoglobulin (IgG). (**C**) Expression of the MYC-PLIC-1 constructs was also confirmed by Western blotting of lysates from transfected cells with an antibody to the MYC epitope. Polyubiquitinated (Ub^n) protein binding is seen as a high molecular weight smear with full length PLIC-1 and PLIC-1Δ^{UBL} but not MYC-PLIC-1Δ^{UBA}.

Supplementary Movie 1. Formation of EPS15 and PLIC-1 dependent structures. HEK293 cells co-transfected with GFP-EPS15 and MYC-PLIC-1 were imaged by time-lapse microscopy for the GFP signal. Images taken every 90 seconds for 30 minutes are assembled sequentially in time.

Supplementary Movie 2. Intact microtubules are required for the formation of EPS-15 and PLIC-1 structures. HEK293 cells co-transfected with GFP-EPS15 and MYC-PLIC-1, treated with nocodazole (1μM) were imaged by time-lapse microscopy for the GFP signal. Images taken every 90 seconds for 30 minutes are assembled sequentially in time. Small ALIS are seen moving but not merging into larger ALIS.