Developmental Cell, Volume *36*

Supplemental Information

Phosphorylation-Dependent Activation of the ESCRT Function of ALIX in Cytokinetic Abscission and Retroviral Budding

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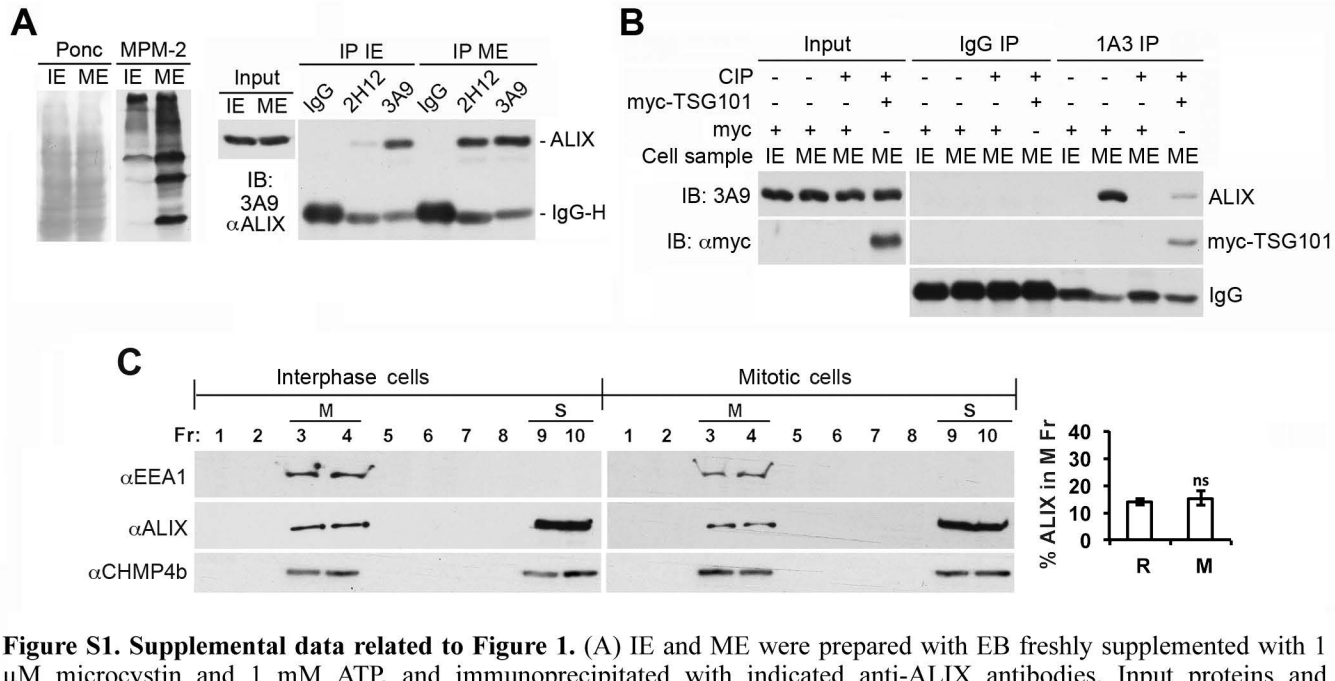

Figure S1. Supplemental data related to Figure 1. (A) IE and ME were prepared with EB freshly supplemented with 1 μM microcystin and 1 mM ATP, and immunoprecipitated with indicated anti-ALIX antibodies. Input proteins and immunocomplexes were immunoblotted with indicated antibodies. (B) ME was first incubated with the TNT product of myc or myc-TSG101 at 4ºC for 2 h, and then treated with CIP. IE, ME and the two samples of differently treated ME were immunoprecipitated with indicated antibodies, followed by immunoblotting with indicated antibodies. (C) PNSs from asynchronously growing HEK293 cells (Interphase cells) or mitotically arrested HEK293 cells (Mitotic cells) were fractionated by membrane flotation centrifugation. Same volumes of aliquots were taken and immunoblotted with indicated antibodies; membrane (M) and soluble (S) protein fractions are indicated. The average percentages of ALIX in the M fraction and SDs were determined from three independent experiments and plotted.

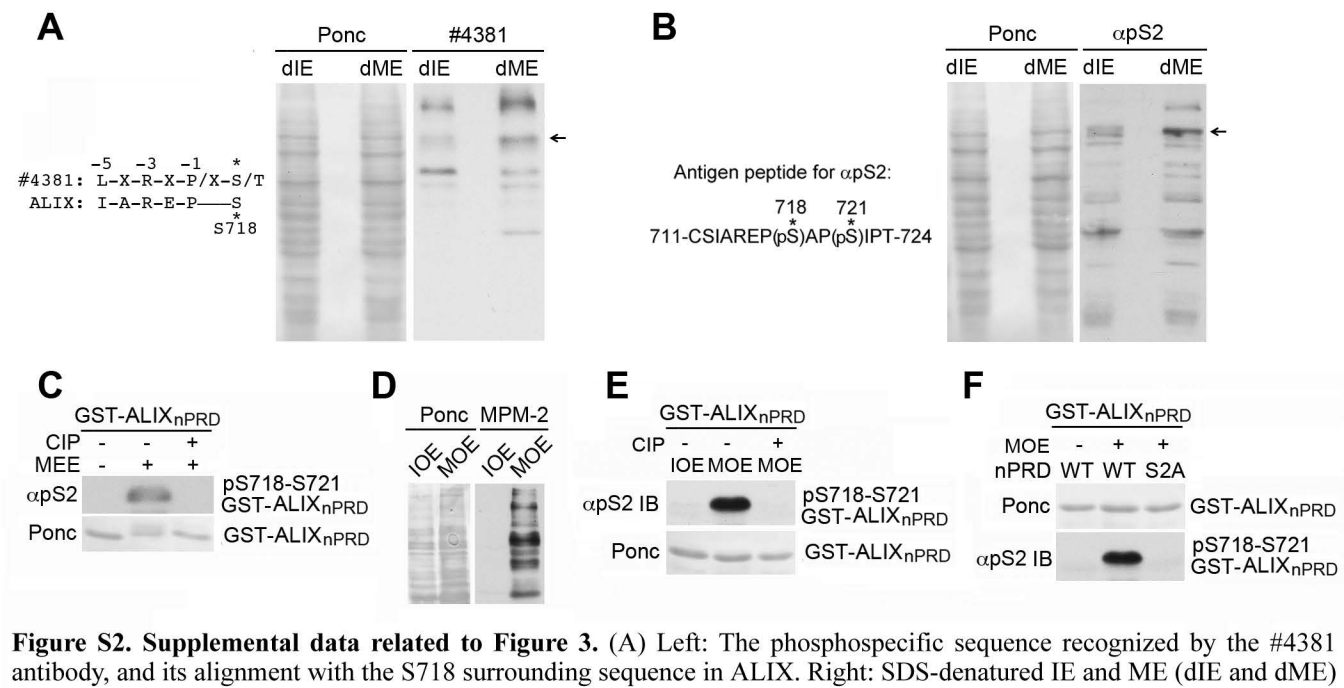

Figure S2. Supplemental data related to Figure 3. (A) Left: The phosphospecific sequence recognized by the #4381 antibody, and its alignment with the S718 surrounding sequence in ALIX. Right: SDS-denatured IE and ME (dIE and dME) were immunoblotted with the #4381 antibody after Ponc staining, and the arrow indicates the position of ALIX. (B) Left: Sequence of the phosphorylated ALIX peptide used for production of the anti-pS2 antibody. Right: The dIE and dME were immunoblotted with the anti-pS2 antibody after Ponc staining, and the arrow indicates the position of ALIX. (C) GST-ALIX$_{nPRD}$ was mock-treated or phosphorylated with MEE or MEE plus CIP, and then immobilized onto GSH beads. Bound proteins were immunoblotted with the anti-pS2 antibody. (D) Immature oocytes and progesterone-matured oocytes were extracted with EB supplemented with ATP, and freshly prepared immature oocyte extracts (IOE) and mature oocyte extracts (MOE) were immunoblotted with MPM2. (E) GST-ALIX$_{nPRD}$ was phosphorylated with freshly prepared IOE, MOE, or MOE plus CIP, and then immobilized onto GSH beads. Bound proteins were immunoblotted with the anti-pS2 antibody. (F) WT and S2A GST-ALIX$_{nPRD}$ were mock treated or phosphorylated with MOE, and then immobilized onto GSH beads. Bound proteins were immunoblotted with the anti-pS2 antibody.

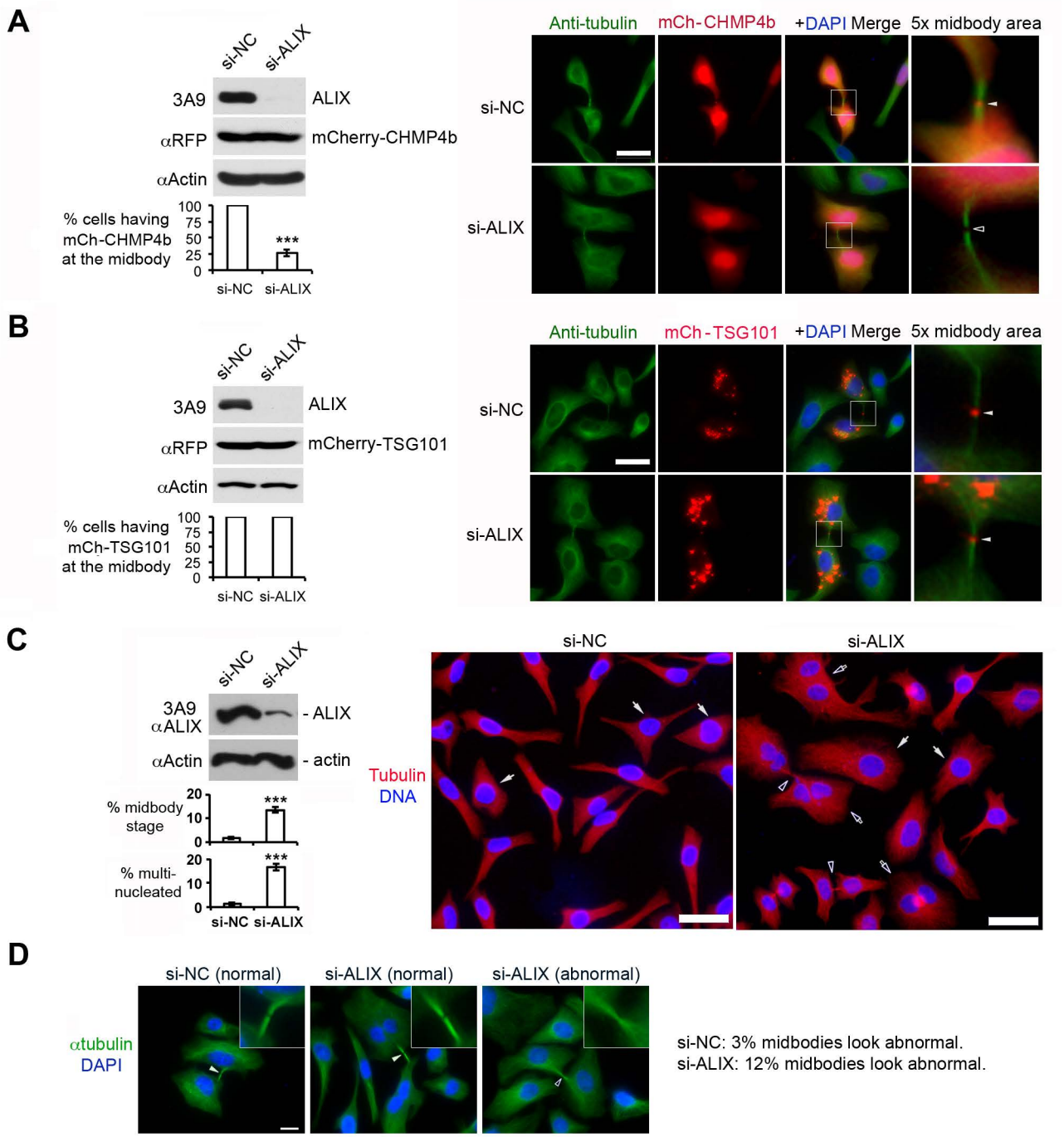

Figure S3. Supplemental data related to Figure 5. (A&B) HeLa cells were transfected with indicated agents and cultured as done for Fig. 5A. Cell lysates were immunoblotted with indicated antibodies to visualize ALIX, mCherry-CHMP4b (A), mCherry-TSG101 (B) and actin. Fixed cells were immunostained with anti-tubulin antibody (green), and counterstained with DAPI (blue). The average percentages of mCherry positive cells with midbody localization of mCherry-CHMP4b and SDs (A) or mCherry-TSG101 (B) were determined from three independent experiments and plotted. Representative images are shown; the squares show the midbody areas to be enlarged. Solid and hollow arrowheads indicate the presence and absence of mCherry-CHMP4b (A) or mCherry-TSG101 (B) at the midbody, respectively. Scale bar: 50 μm. (C) HeLa cells were transfected with indicated siRNAs for 72 h, and cell lysates were immunoblotted with indicated antibodies to visualize ALIX and actin. Fixed cells were immunostained with an anti-tubulin antibody (red), and counterstained with DAPI (blue). The average percentages of midbody-stage cells or multinucleated cells and SDs were determined from three independent experiments and plotted. Representative images are shown; solid and hollow arrows indicate mononucleated and multinucleated cells, respectively, and hollow arrowheads indicate midbodies between daughter cells. Scale bar: 50 μm. (D) HeLa cells were transfected with indicated siRNAs for 72 h, and fixed cells were immunostained with an anti-tubulin antibody (green), and counterstained with DAPI (blue). Representative images are shown in which solid and hollow arrowheads indicate midbodies with normal and abnormal morphology, respectively. The squares on the right corner show the 3x enlarged midbody area. The percentages of abnormal midbodies were determined from at least 50 midbody-stage cells. Scale bar: 15 μm.

Figure S4. Supplemental data related to Figure 6. IE from HEK293 cells ectopically expressing GFP-ALIX was processed as diagrammed (left panel). Input proteins (middle panel) and immunocomplexes (right panel) were immunoblotted with indicated antibodies to visualize GFP-ALIX, GST, GST-p9 and IgG-H.

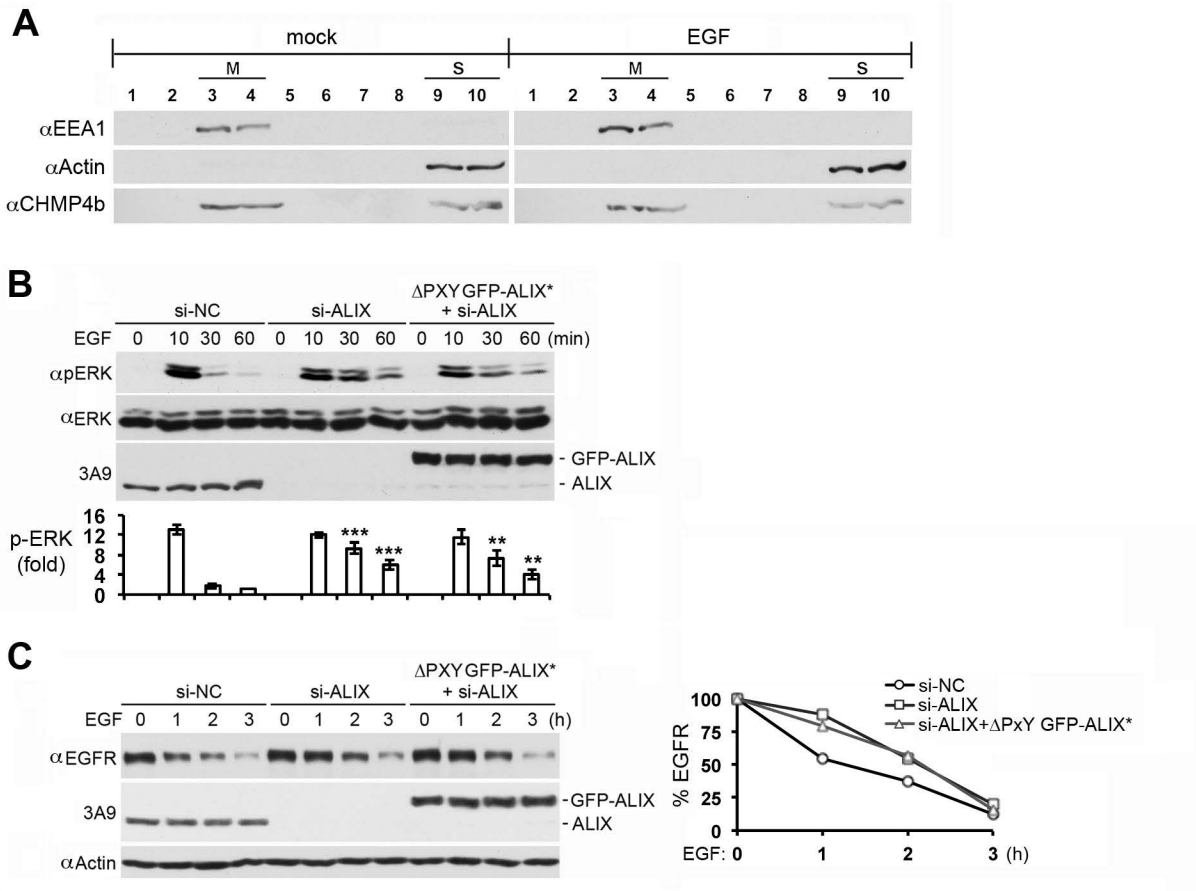

Figure S5. Supplemental data related to Figure 7. (A) HEK293 cells ectopically expressing indicated forms of GFP-ALIX were processed as described for Fig. 7A. Same volumes of aliquots were taken and immunoblotted with indicated antibodies. (B) HEK293 cells transfected with indicated siRNAs and the plasmid for ΔPxY GFP-ALIX* were processed as described for Fig. 7 C&D. (C) HEK293 cells transfected with indicated siRNAs and the plasmid for ΔPxY GFP-ALIX* were processed as described as Fig. 7 E&F.

Table S1. Sequences of siRNAs used in this study - related to Experimental Procedures

Target	Name	Sequence	Source
ALIX	si-ALIX	5'-GAGAAGAAAUUGCAAGGUUdTdT-3'	Sigma-Genosys
Firefly GL3 luciferase	si-NC	5'-CUUACGCUGAGUACUUCGAdTdT-3'	Sigma-Genosys

Table S2. Mammalian and bacterial expression vectors used in this study – related to Experimental Procedures

Vector	Source	Reference
1. pEGFP-C3-based mammalian expression vector for GFP-ALIX	A gift from Dr. Masatoshi Maki (Nagoya, Japan)	(Shibata et al., 2004)
1a. pEGFP-C3-based mammalian expression vector for ALIX-siRNA-insensitive GFP-ALIX (GFP-ALIX*)	Site-directed mutagenesis of vector 1	new
1b. pEGFP-C3-based mammalian expression vector for S718A GFP-ALIX	Site-directed mutagenesis of vector 1	new
1c. pEGFP-C3-based mammalian expression vector for S721A GFP-ALIX	Site-directed mutagenesis of vector 1	new
1d. pEGFP-C3-based mammalian expression vector for S718A-S721A GFP-ALIX	Site-directed mutagenesis of vector 1	new
1e. pEGFP-C3-based mammalian expression vector for S712A-S729A GFP-ALIX	Site-directed mutagenesis of vector 1	new
1f. pEGFP-C3-based mammalian expression vector for S718A-S721A ALIX-siRNA-insensitive GFP-ALIX (S2A GFP-ALIX*)	Site-directed mutagenesis of vector 1a	new
1g. pEGFP-C3-based mammalian expression vector for S712A-S729A ALIX-siRNA-insensitive GFP-ALIX (S2A- GFP-ALIX*)	Site-directed mutagenesis of vector 1e	new
1h. pEGFP-C3-based mammalian expression vector for S718D-S721D GFP-ALIX	Site-directed mutagenesis of vector 1	new
2. pCMV-based mammalian expression vector for FLAG-CHMP4b	A gift from Dr. Masatoshi Maki (Nagoya, Japan)	(Katoh et al., 2003)
3. pIRES2 based mammalian expression vector for FLAG–TSG101	A gift from Dr. Wesley I. Sundquist (Salt Lake City, UT)	(von Schwedler et al., 2003)
4. pmCherry-C1-based expression vector for mCherry-CHMP4b	Cut the CHMP4b fragment in vector 2 by EcoRI and XhoI and insert it into the pmCherry-C1 vector (Clontech) after digestion with EcoRI and SalI.	new
5. pmCherry-C1-based expression vector for mCherry-TSG101	PCR amplification of coding region of TSG101 from pIRES2-FLAG–TSG101, followed by subcloning into pmCherry-C1 vector (clontech).	new
6. pEV53B-based mammalian expression vector for infection defective EIAV	A gift from Dr. John Olsen (Chapel Hill, NC)	(Olsen, 1998)
7. pCS2-MT based TNT expression vector for myc-ALIX$_{nPRD}$	PCR amplification of coding region of amino acid 706-786 of WT GFP-ALIX, followed by subcloning into pCS2-MT vector (clontech)	new
8. pCS2-MT based TNT expression vector for S718A-S721A myc-ALIX$_{nPRD}$	PCR amplification of coding region of amino acid 706-786 of S718A-S721A GFP-ALIX, followed by subcloning into pCS2-MT vector (clontech)	new
9. pCS2-MT based TNT expression vector for S712A-S729A myc-ALIX$_{nPRD}$	PCR amplification of coding region of amino acid 706-786 of S712A-S729A GFP-ALIX, followed by subcloning into pCS2-MT vector (clontech)	new
10. pCS2-MT based TNT expression vector for S718D-S721D myc-ALIX$_{nPRD}$	PCR amplification of coding region of amino acid 706-786 of S718D-S721D GFP-ALIX, followed by subcloning into pCS2-MT vector (clontech)	new
11. pCS2-MT based TNT expression vector for myc-TSG101	PCR amplification of coding region of TSG101 from pIRES2-FLAG–TSG101, followed by subcloning into pCS2-MT vector (clontech)	new

12. pCS2-HA based expression vector for HA-Plx1 (Xenopus)	PCR amplification of coding region of Plx1 from pBluescript-Plx1(A gift from Dr. William G. Dunphy (Kumagai and Dunphy, 1996), followed by subcloning into pCS2-HA vector (clontech)	new
13. pCS2-HA based expression vector for HA-K82R Plx1 (Xenopus)	PCR amplification of coding region of K82R Plx1 from pBluescript-K82R Plx1, followed by subcloning into pCS2-HA vector (clontech)	new
14. pGEX-4T3 based bacterial expression vector GST-$ALIX_{nPRD}$	PCR amplification of coding region of amino acid 706-786 of WT GFP-ALIX, followed by subcloning into pGEX-4T3 vector (Amersham Biosciences)	new
15. pGEX-4T3 based bacterial expression vector for GST-$ALIX_{Bro1}$	Generated in our previous studies	(Pan et al., 2006)
16. pGEX-4T3 based bacterial expression vector for GST-$ALIX_{1-746}$	Generated in our previous studies	(Zhou et al., 2010)
17. pGEX-4T3 based bacterial expression vector for GST-CHMP4b	PCR amplification of coding region of CHMP4b from FLAG-CHMP4b, followed by subcloning into pGEX-4T3 vector (Amersham Biosciences)	new
18. pGEX-4T3 based bacterial expression vector for GST-$p9^{Gag}$	A gift from Dr. Wesley I. Sundquist (Salt Lake City, UT)	(Fisher et al., 2007)

Table S3. PCR primers used for site-directed mutagenesis and making vectors – related to Experimental Procedures

Product	Primers (Forward/Reverse)	Template
1a. ALIX-siRNA-insensitive GFP-ALIX	5'-GAAGAAATTTGGGGAGGAGATCGCGAGATTACAGCATGCAGCAG-3' 5'-CTGCTGCATGCTGTAATCTCGCGATCTCCTCCCCAAATTTCTTC-3'	1. WT GFP-ALIX
1b. S718A GFP-ALIX	5'-CATTGCCAGAGAACCTGCTGCTCCTTCAATTCCTACAC-3' 5'-GTGTAGGAATTGAAGGAGCAGCAGGTTCTCTGGCAATG-3'	1. WT GFP-ALIX
1c. S721A GFP-ALIX	5'-GAACCTAGTGCTCCTGCAATTCCTACACCTGC-3' 5'-GCAGGTGTAGGAATTGCAGGAGCACTAGGTTC-3'	1. WT GFP-ALIX
1d. S718A-S721A GFP-ALIX	5'-CATTGCCAGAGAACCTGCTGCTCCTGCAATTCCTACACCTG-3' 5'-CAGGTGTAGGAATTGCAGGAGCAGCAGGTTCTCTGGCAATG-3'	1. WT GFP-ALIX
1e. S712A-S729A GFP-ALIX	WT to S712A: 5'-CTTAAAGGACTTGCAACAAGCCATTGCCAGAGAACCTAGTG-3' 5'-CACTAGGTTCTCTGGCAATGGCTTGTTGCAAGTCCTTTAAG -3' S712A to S712A/S729A: 5'-CTACACCTGCGTATCAGGCCTCACCAGCAGGAGGAC-3' 5'-GTCCTCCTGCTGGTGAGGCCTGATACGCAGGTGTAG-3'	1. WT GFP-ALIX
1f. S718A-S721A ALIX-siRNA-insensitive GFP-ALIX	5'-CATTGCCAGAGAACCTGCTGCTCCTGCAATTCCTACACCTG-3' 5'-CAGGTGTAGGAATTGCAGGAGCAGCAGGTTCTCTGGCAATG-3'	1a. ALIX-siRNA-insensitive GFP-ALIX
1g. S712A-S729A ALIX-siRNA-insensitive GFP-ALIX	5'-GAAGAAATTTGGGGAGGAGATCGCGAGATTACAGCATGCAGCAG-3' 5'-CTGCTGCATGCTGTAATCTCGCGATCTCCTCCCCAAATTTCTTC-3'	1e. S712A-S729A GFP-ALIX
1h. S718D-S721D GFP-ALIX	5'-GCATTGCCAGAGAACCTGATGCTCCTGATATTCCTACACCTGCG-3' 5'-CGCAGGTGTAGGAATATCAGGAGCATCAGGTTCTCTGGCAATGC-3'	1. WT GFP-ALIX
5. mCherry-TSG101	5'-TAACTCGAGCT ATGGCGGTGTCGGAGAG-3' (Xho I) 5'- TAAGAATTCTCAGTAGAGGTCACTGAGACCG-3' (EcoRI)	3. pIRES2-FLAG-TSG101
7. pCS2-MT-ALIX$_{nPRD}$	5'-TAAGAATTCATTAAAGGACTTGCAACAAAGCATTG-3' (EcoRI) 5'-TAACTCGAGTGGCGCAGCAGTCCC-3' (Xho I)	1. WT GFP-ALIX
8. pCS2-MT-S718A-S721A ALIX$_{nPRD}$	5'-TAAGAATTCATTAAAGGACTTGCAACAAAGCATTG-3' (EcoRI) 5'-TAACTCGAGTGGCGCAGCAGTCCC-3' (Xho I)	1d. S718A-S721A GFP-ALIX
9. pCS2-MT-S712A-S729A ALIX$_{nPRD}$	5'-TAAGAATTCATTAAAGGACTTGCAACAAGCCATTG-3' (EcoRI) 5'-TAACTCGAGTGGCGCAGCAGTCCC-3' (Xho I)	1e. S712A-S729 A GFP-ALIX
10. pCS2-MT-S718D-S721D myc-ALIX$_{nPRD}$	5'-TAAGAATTCATTAAAGGACTTGCAACAAAGCATTG-3' (EcoRI) 5'-TAACTCGAGTGGCGCAGCAGTCCC-3' (Xho I)	1h. S718D-S721D GFP-ALIX
11. pCS2-MT-TSG101	5'- TAAGAATTCAATGGCGGTGTCGGAGAG-3' (EcoRI) 5'- TAACTCGAGTCAGTAGAGGTCACTGAGACCG-3' (Xho I)	3. pIRES2-FLAG-TSG101
12. pCS2-HA-Plx1 (*Xenopus*)	5'-AATGGGCCCTCAAGTGGCCGGTAAGAAAC-3' (Apa I) 5'-GCCTCTAGAGCCGAGGCCTTTACGTGTGC-3' (Xba I)	pBluescript-Plx1
13. pCS2-HA-Plx1 K82R (*Xenopus*)	5'-AATGGGCCCTCAAGTGGCCGGTAAGAAAC-3' (Apa I) 5'-GCCTCTAGAGCCGAGGCCTTTACGTGTGC-3' (Xba I)	pBluescript-Plx1 K82R
14. pGEX-4T3-ALIX$_{nPRD}$	5'-TAAGAATTCCTTAAAGGACTTGCAACAAAGCATTG-3' (EcoRI) 5'-TAACTCGAGTGGCGCAGCAGTCCC-3' (Xho I)	1. WT GFP-ALIX
17. pGEX-4T3-CHMP4b	5'- TAAGAATTCCATGTCGGTGTTCGGGAAG-3' (EcoRI) 5'- TAACTCGAGTTACATGGATCCAGCCCAG-3' (Xho I)	2. FLAG-CHMP4b

Table S4. Antibodies used in this study – related to Experimental Procedures

Antibody	Recognition	Type	Source	Use
1A3 anti-ALIX	ALIX (Y319)/ Xp95(Y318)	Mouse monoclonal	Made in our previous studies	Immunoblotting Immunoprecipitation
1A12 anti-ALIX	$ALIX_{605-709}$	Mouse monoclonal	Made in our previous studies	Immunoprecipitation
2H12 anti-ALIX	$ALIX_{F676\ pocket}$	Mouse monoclonal	Made in our previous studies	Immunoprecipitation
3A9 anti-ALIX	$ALIX_{605-709}$	Mouse monoclonal	Made in our previous studies	Immunoblotting Immunoprecipitation
anti-actin	Actin	Mouse monoclonal	Sigma-Aldrich Cat#: A5441	Immunoblotting
anti-tubulin	Alpha-tubulin	Rabbit monoclonal	Cell Signaling Cat#: 2125S	Immunostaining
anti-CA	EIAV capsid antigen (CA)	Mouse monoclonal	A gift from Dr. Robert Mealey (Pullman, WA)	Immunoblotting
anti-CHMP4b	CHMP4b	Rabbit polyclonal	Santa Cruz Cat#: sc-134946	Immunoblotting
anti-EEA1	EEA1	Rabbit monoclonal	Epitomics Cat#: 3704-1	Immunoblotting
anti-EGFR	EGFR	Rabbit monoclonal	Epitomics Cat#: 1902-1	Immunoblotting
anti-ERK1	ERK1	Rabbit polyclonal	Santa Cruz Cat#:sc-94	Immunoblotting
anti-ERK2	ERK2	Rabbit polyclonal	Santa Cruz Cat#:sc-154	Immunoblotting
anti-FLAG	FLAG epitope	Mouse monoclonal	Pierce Cat#: MA1-918781	Immunoblotting
anti-FLAG	FLAG epitope	Rabbit polyclonal	Santa Cruz Cat#: sc-807	Immunoblotting Immunoprecipitation
anti-GFP	GFP	Mouse monoclonal	Santa Cruz Cat#: sc-9996	Immunoblotting Immunoprecipitation Immunostaining
anti-GST	GST	Rabbit polyclonal	Santa Cruz Cat#: sc-459	Immunoblotting
anti-HA	HA epitope	Rabbit polyclonal	Santa Cruz Cat#: sc-805	Immunoblotting Immunoprecipitation
IgG	IgG	Mouse	Sigma-Aldrich Cat#: I5381-10MG	Immunoprecipitation
IgG	IgG	Rabbit	Sigma-Aldrich Cat#: I5006-10MG	Immunoprecipitation
anti-myc	myc epitope	Rabbit polyclonal	Santa Cruz Cat#: sc-789	Immunoblotting Immunoprecipitation
MPM2	Mitotic phosphoproteins	Mouse monoclonal	Lab reserve	Immunoblotting
#4381 antibody	PKD substrates	Rabbit polyclonal	Cell Signaling Cat#: 4381	Immunoblotting Immunoprecipitation
anti-pS2 antibody	p-S718-S721-ALIX	Rabbit polyclonal	Made in this study	Immunoblotting Immunoprecipitation
anti-p-ERK	p-ERK1/2 at Tyr 204	Mouse monoclonal	Santa Cruz Cat#: sc7383	Immunoblotting
anti-RFP	mCherry epitope	Mouse monoclonal	Pierce Cat#: MA5-15257	Immunoblotting
anti-p-Tyr	phosphotyrosine	Mouse monoclonal	Cell Signaling Cat#: 9416	Immunoblotting
anti-TSG101	TSG101	Rabbit monoclonal	Epitomics Cat#: 5377-1	Immunoblotting

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

In vitro phosphorylation of ALIX fragments with *Xenopus* extracts and GST pull-down

In vitro transcription and linked translation of proteins was performed by using TNT® Quick Coupled Transcription/Translation System (Promega) according to the manufacturers' instructions. GST and GST-tagged proteins were produced and purified using our standard procedures (Che et al., 1997). Phosphorylation reaction included one volume of substrate proteins and three volumes of IOE or MEE. The reaction was performed at 22ºC for 2 h unless otherwise indicated, and terminated by adding SDS-PAGE sample buffer. The Plk1 inhibitor BI-2536 (Axon Medchem), the PKD inhibitor CID755673 (BioVision Inc.), and the pan-kinase inhibitor staurosporine (LC Laboratories) were all dissolved in Dimethyl sulfoxide (DMSO), and added to MEE at 4ºC whenever indicated 15 min prior to the phosphorylation reaction to reach a final concentration of 2 μM, 5 μM and 5 μM, respectively. GST tagged proteins were immobilized onto glutathione (GSH) beads (GenScript), and GST pull-down was performed at 4°C for 2 h. After GSH beads were washed five times with EB, proteins remaining on the beads were eluted with SDS-PAGE sample buffer for immunoblotting.

Immunostaining and fluorescence microscopy

Transfected HeLa cells were subcultured into chamber slides (Nunc Lab-Tek) coated with poly-D-Lysine (Cultrex) and cultured for 48 h before being fixed with 4% (w/v) of Paraformaldehyde at room temperature for 20 min. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS followed by blocking with 1x blocking buffer (1% BSA, 0.25% horse serum, 0.2% Triton X-100 in PBS). Blocked cells were first stained with primary antibodies in 0.1x blocking buffer at 4ºC overnight, and then with Alexa Fluor 568, Alexa Fluor 488 or Alexa Fluor 647 conjugated secondary antibodies in TBST (0.1% Triton X-100 in TBS) at room temperature for 1 h. Nuclei were stained with DAPI (Sigma). Images were acquired using MetaMorph software (7.7.5.0) on ZEISS Axioplan2 image system (Objective: plan-NEOFLUAR 20×/0.50). For obtaining the percentages of the midbody localization of mCherry-CHMP4b or mCherry-TSG101 in midbody-staged cells with knockdown of ALIX, at least 10 clearly mCherry positive cells were counted for each experiment. For obtaining the percentages of the midbody localization of mCherry-CHMP4b in midbody-staged cells ectopically expressing both GFP-ALIX* and mCherry-CHMP4b, at least 10 clearly double positive cells were counted for each experiment. For obtaining the percentages of multinucleated or midbody-staged cells induced by ALIX knockdown, at least 200 cells were counted for each experiment. For obtaining the percentages of multinucleated or midbody-staged cells ectopically expressing GFP or GFP-ALIX*, at least 100 clearly GFP-positive cells were counted for each experiment. There are four types of GFP-ALIX transfected cells under our fluorescence microscope. (i) No over-background green signal. (ii) Low over-background green signal without clear cell contour. (iii) Easily discernible over-background green signal with clear cell contour and specific localization in the cytoplasm. (iv) Very bright green cells with rounded cell shape, suggesting cell toxicity. We specifically examined the third type of cells.

Generation of rabbit polyclonal antibodies recognizing phosphorylated Ser718 and Ser721 at ALIX

To prepare antigen, a synthetic phosphopeptide consisting of the residues 711 to 724 of ALIX and phosphorylated at both Ser718 and Ser721 (CSIAREP(pS)AP(pS)IPT) was conjugated to keyhole limpet hemocyanin (KLH). To generate rabbit polyclonal antibodies, rabbits were immunized with the conjugated phosphopeptide for 42 days, and immunesera were collected. The IgG fraction of the antibodies was purified by protein G affinity chromatography. The phosphospecificity of the purified antibodies were evaluated with the enzyme-linked immune sorbent assay (ELISA).

SUPPLEMENTAL REFERENCES

Che, S., Weil, M.M., Nelman-Gonzalez, M., Ashorn, C.L., and Kuang, J. (1997). MPM-2 epitope sequence is not sufficient for recognition and phosphorylation by ME kinase-H. FEBS Letter *413*, 417-423.

Fisher, R.D., Chung, H.Y., Zhai, Q., Robinson, H., Sundquist, W.I., and Hill, C.P. (2007). Structural and biochemical studies of ALIX/AIP1 and its role in retrovirus budding. Cell *128*, 841-852.

Katoh, K., Shibata, H., Suzuki, H., Nara, A., Ishidoh, K., Kominami, E., Yoshimori, T., and Maki, M. (2003). The ALG-2-interacting protein Alix associates with CHMP4b, a human homologue of yeast Snf7 that is involved in multivesicular body sorting. J Biol Chem *278*, 39104-39113. Epub 32003 Jul 39114.

Kumagai, A., and Dunphy, W.G. (1996). Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts. Science *273*, 1377-1380.

Olsen, J.C. (1998). Gene transfer vectors derived from equine infectious anemia virus. Gene Ther *5*, 1481-1487.

Pan, S., Wang, R., Zhou, X., He, G., Koomen, J., Kobayashi, R., Sun, L., Corvera, J., Gallick, G.E., and Kuang, J. (2006). Involvement of the adaptor protein Alix in actin cytoskeleton assembly. J Biol Chem *285*, 34640-34650.

Shibata, H., Yamada, K., Mizuno, T., Yorikawa, C., Takahashi, H., Satoh, H., Kitaura, Y., and Maki, M. (2004). The penta-EF-hand protein ALG-2 interacts with a region containing PxY repeats in Alix/AIP1, which is required for the subcellular punctate distribution of the amino-terminal truncation form of Alix/AIP1. J Biochem (Tokyo) *135*, 117-128.

von Schwedler, U.K., Stuchell, M., Muller, B., Ward, D.M., Chung, H.Y., Morita, E., Wang, H.E., Davis, T., He, G.P., Cimbora, D.M*., et al.* (2003). The protein network of HIV budding. Cell *114*, 701-713.

Zhou, X., Si, J., Corvera, J., Gallick, G.E., and Kuang, J. (2010). Decoding the intrinsic mechanism that prohibits ALIX interaction with ESCRT and viral proteins. Biochem J *432*, 525-534.