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Unraveling the pivotal role of ALIX in MVB sorting and silencing of activated EGFR

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Abstract

ESCRT-III mediated membrane invagination and scission is a critical step in MVB sorting of ubiquitinated membrane receptors and generally thought to be required for degradation of these receptors in lysosomes. The adaptor protein ALIX is critically involved in multiple ESCRT-III-mediated membrane remodeling processes in mammalian cells. However, ALIX knockdown does not inhibit degradation of activated EGFR in mammalian cell lines, leading to a widely held notion that ALIX is not critically involved in MVB sorting of ubiquitinated membrane receptors in mammalian cells. In this study, we demonstrate that despite its non-essential roles in degradation of activated EGFR, ALIX plays a critical role in MVB sorting and silencing of activated EGFR. EGF stimulation of mammalian cell lines induces ALIX interaction with ubiquitinated EGFR through the ALIX V domain and increases ALIX association with membrane-bound CHMP4 through the ALIX Bro1 domain. Under both continuous and pulse-chase EGF stimulation conditions, inhibition of ALIX interaction with membrane-bound CHMP4, inhibition of ALIX dimerization through the V domain or ALIX knockdown dramatically inhibits MVB sorting of activated EGFR and promotes sustained activation of ERK1/2. Under the continuous EGF stimulation conditions, these cell treatments also retard degradation of activated EGFR. These findings indicate that ALIX is critically involved in MVB sorting of ubiquitinated membrane receptors in mammalian cells.

Keywords

ALIX; EGFR; MVB sorting; ESCRT; CHMP4; ERK activation

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AUTHOR CONTRIBUTION

Sheng Sun designed and performed most of the experiments; Xi Zhou initiated the project, created multiple crucial reagents and performed some of the pilot experiments; Wei Zhang created crucial reagents and participated in manuscript preparation; Gary E. Gallick participated in designing the research and writing the paper; and Jian Kuang directed the project and wrote the paper.

INTRODUCTION

Ligand binding to membrane receptors on the cell surface triggers receptor internalization by endocytosis. Some of these internalized receptors are sorted into the intraluminal vesicles (ILVs) of multivesicular bodies (MVBs) through membrane invagination and abscission. Sorting of these receptors into the ILVs of MVBs, often called MVB sorting, prevents their recycling to the plasma membrane and stops their signaling functions. Moreover, as the ILVs formed within MVBs are destined for lysosomes, MVB sorting leads to lysosome-dependent degradation of internalized membrane receptors [1–3].

MVB sorting of endocytosed membrane receptors requires coordinated cargo recognition and assembly of membrane sculpturing machinery that buds cargo-loaded membrane vesicles into the lumen of endosomes. Yeast genetic and cell biology studies have established a framework that MVB sorting of ubiquitinated membrane receptors is driven by the coordinated action of five distinct ESCRT complexes (ESCRTs-0, -I, -II, -III, and the Vps4 complex) and associated proteins [4–7]. The ubiquitinated cargo is recognized and clustered by ESCRTs-0, -I, and -II. Membrane invagination and scission is driven by the assembly of the highly oligomerized ESCRT-III complex from four core subunits (Vps20, Snf7/Vps32, Vps24, and Vps2 in yeasts) followed by the timely disassembly of this complex by Vps4. The coordination of cargo recognition and membrane remodeling is achieved by the ability of ESCRT-II complex to both recognize the cargo and activate the assembly of ESCRT-III.

BRO1 is a well-recognized ESCRT-III associated protein in yeast, which interacts with the ESCRT-III component Snf7 and promotes MVB sorting of ubiquitinated membrane receptors through ESCRT-III [8–11]. Apoptosis-linked gene-2 product (ALG-2) interacting protein X (ALIX) [12], also termed ALG-2 interacting protein 1 (AIP1) [13] and Hp95 [14], is the mammalian ortholog of yeast BRO1, which interacts with the mammalian ortholog of Snf7, CHMP4 [15–18]. Prompted by the MVB sorting functions of BRO1, CHMP4-bound ALIX has been demonstrated to be critically involved in a variety of ESCRT-III-mediated membrane remodeling processes in mammalian cells, including retroviral budding [15, 16, 19], cytokinetic abscission [20, 21], biogenesis of exosomes [22], ubiquitin-independent MVB sorting of the GPCR PAR1 [23] and plasma membrane repair [24]. Based on these findings, it is logical to predict that ALIX interaction with CHMP4 also plays an important role in MVB sorting of ubiquitinated membrane receptors in mammalian cells. However, multiple previous studies demonstrated that ALIX knockdown produced no or only a minor inhibitory effect on degradation of internalized EGF or EGFR in HeLa cells [25–29]. Since ESCRT-mediated MVB sorting of activated EGFR is generally thought to be required for trafficking of activated EGFR to lysosomes for degradation, these results led to a widely held notion that ALIX does not play a critical role in ESCRT-mediated MVB sorting of ubiquitinated membrane receptor in mammalian cells [30].

Although ESCRT-III mediated MVB sorting of ubiquitinated membrane receptors is required for their degradation in yeasts, inhibition of MVB sorting of activated EGFR by the PI3-kinase inhibitor wortmannin [31] or by depletion of annexin 1 [32] was found to have

no effect on degradation of internalized EGF and cause only a small delay in degradation of activated EGFR in mammalian cell lines, indicating that this rule may not always apply to mammalian cells. With the possible presence of redundant mechanisms that traffic activated EGFR to lysosomes, the lack of obvious effects of ALIX knockdown on degradation of activated EGFR does not necessarily indicate that CHMP4-bound ALIX does not play a critical role in ESCRT-mediated MVB sorting of ubiquitinated membrane receptors in mammalian cells.

His-domain protein tyrosine phosphatase (HD-PTP) of ~180 kDa is an ALIX paralog that contains an ALIX homologous domain in its N-terminus. While ALIX knockdown slightly delayed the degradation of internalized EGF in HeLa cells, HD-PTP knockdown dramatically inhibited the EGF degradation [25]. These findings led to a proposal that HD-PTP is the major functional counterpart of yeast BRO1 in mammalian cells. However, although HD-PTP interacts with CHMP4 through a similar mechanism as ALIX does, full-length HD-PTP does not require its interaction with CHMP4 to promote MVB sorting and degradation of activated EGFR. On the other hand, HD-PTP, but not ALIX, also interacts with the ESCRT-0 component STAM2 [33] and the ESCRT-I component UBAP1 [34] and promotes endolysosomal trafficking of activated EGFR through these interactions. ALIX, but not HD-PTP, also interacts with the unconventional phospholipid lysobisphosphatidic acid (LBPA) enriched in late endosomes [35]. This ALIX-lipid interaction inhibits the formation of multivesicular liposomes in vitro [36] and promotes back fusion of the ILVs with the limiting membrane of late endosomes in intact cells [35]. Based on these non-shared functions of HD-PTP and ALIX, it is possible that the much more dramatic effects of HD-PTP knockdown on EGF degradation than ALIX knockdown were due to the ability of HD-PTP to promote endolysosomal trafficking of activated EGFR through both ALIX/BRO1-related and non-ALIX/BRO1-related mechanisms and/or the opposing effects of ALIX on MVB sorting at different stages of endosomes.

In all defined ESCRT-III mediated membrane remodeling processes that involve ALIX function, ALIX connects the sorting cargo to ESCRT-III through multiple molecular interactions. Thus another potential explanation for the notion that ALIX is not involved in MVB sorting of ubiquitinated membrane receptors is that ALIX does not recognize ubiquitinated membrane receptors. However, yeast BRO1 was recently shown to function as an ubiquitin receptor in MVB sorting of ubiquitinated membrane receptors [37]. Mammalian ALIX was also recently discovered to interact with ubiquitinated proteins through the V domain [37–39]. Thus ALIX is probably able to recognize ubiquitinated membrane receptors.

In this study, we made a comprehensive analysis of the role of ALIX in recognition, MVB sorting, silencing and degradation of activated EGFR in mammalian cell lines. Our results demonstrate that ALIX recognizes activated EGFR and plays an essential role in MVB sorting and rapidly silencing of activated EGFR. However, ALIX does not play an essential role in trafficking of activated EGFR to lysosomes for degradation. These findings identify ALIX as an important regulator of the signaling output of activated EGFR and possibly other receptor tyrosine kinases.

EXPERIMENTAL

Cell culture, transfection and EGF stimulation

HEK293 were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (Mediatech Inc) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Atlanta Biologicals). Subconfluent cultures of cells in 60-mm or 35-mm culture dishes were transfected with siRNAs or expression vectors using PolyJet™ DNA In Vitro Transfection Reagent or GenMute™ siRNA Transfection Reagent (SignaGen Laboratories) according to manufacturer's instructions. Transfected cells were cultured for additional 24 to 48 h before experimental analyses. siRNAs used in this study are summarized in Table S1. Due to high abundance of ALIX, transfection with ALIX-specific siRNAs was done twice (at 0 and 24 h) as performed in multiple previous studies [25, 27]. Mammalian expression vectors used in this study are summarized in Table S2, and PCR primers used for site-directed mutagenesis and making vectors are summarized in Table S3.

Sub-confluent cultures of HEK293 cells in 35-mm dishes were switched to serum free medium and cultured for ~12 h. For continuous EGF stimulation, recombinant EGF (Sigma) was added to the medium at a final concentration of 100 ng/ml, and cells were cultured further for indicated lengths of time. For pulse-chase EGF stimulation, serum-starved cells were first incubated with 100 ng/ml EGF in DMEM at 4°C for 30 min and then cultured in EGF-free DMEM at 37°C for indicated lengths of time. The lysosome inhibitor chloroquine (CQ) was obtained from Sigma, and whenever indicated, used at a final concentration of 25 mM.

Protein extraction, immunoblotting and immunoprecipitation

To prepare crude cell lysates for immunoblotting, cells scraped from culture plates were pelleted and extracted with cell lysis buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 (TX), 0.1% SDS, 0.5 mM EDTA, 100 µM sodium orthovanadate, 100 µM sodium fluoride, 100 µM sodium pyrophosphate, 1 mM dichlorodiphenyltrichloroethane (DTT) and proteinase inhibitor cocktail (Sigma); 100–200 µl of cell lysis buffer was used to extract cells from one 60-mm dish. Cell lysates were cleared by centrifugation at 16,000 g for 10 min at 4°C. To prepare membrane solubilized cell lysates for immunoprecipitation, pelleted cells were extracted by sonication in 50–100 µl of cell lysis buffer that omits 0.1% SDS and includes 10 mM N-Ethylmaleimide (NEM) (Sigma) whenever indicated. Cell lysates were cleared by centrifugation at 16,000 g for 10 min at 4°C and diluted 10 fold before immunoprecipitation. Immunoblotting and immunoprecipitation were performed according to our standard protocols [40]. Relative signals on immunoblots were quantified by analyzing scanned images with NIH ImageJ 1.41o. Antibodies used in immunoblotting and immunoprecipitation are summarized in Table S4.

GST pull-down

GST and GST tagged proteins were produced and purified using our standard procedures [41] and immobilized onto Glutathione beads (GenScript). Isolation of membrane-associated proteins was performed as previously described [42] with minor modifications. Briefly, mock-treated and EGF-stimulated HEK293 cells were lysed by sonication in cold TBS,

which was supplemented with 10 mM NEM, 100 μ M sodium orthovanadate, 100 μ M sodium fluoride, 100 μ M sodium pyrophosphate, 1 mM DTT and proteinase inhibitor cocktail, and cell lysates were centrifuged at 130,000 g for 30 min. After supernatants were discarded and pellets were washed with TBS, membrane-associated proteins in the pellets were extracted with TBS supplemented with 0.1% TX and 1 mM NEM. The extracts were cleared by centrifugation at 130,000 g for 30 min. Immobilized GST and GST tagged proteins were incubated with dissolved membrane proteins at 4°C overnight, and then washed with 0.1% TX in TBS five times. Proteins remaining on the beads were eluted with SDS-PAGE sample buffer and subject to immunoblotting.

Membrane flotation centrifugation

Preparation and membrane flotation centrifugation of the post-nuclear supernatant (PNS) of cell lysates through a step sucrose gradient was performed according to published protocols [43, 44] with minor modifications as described previously [45]. In brief, HEK293 cells collected from one 60-mm dish were pelleted and resuspended in 100 μ l of 10% (w/v) sucrose in TE buffer (TBS plus 1 mM EDTA) supplemented with proteinase inhibitor cocktail. After cells were lysed by sonication, cell lysates were centrifuged at 1800 g for 5 min at 4°C, and the PNS was recovered. From each PNS, 0.1 ml aliquot was taken and mixed with 0.4 ml of 85.5% (w/v) sucrose in TE buffer to give a final concentration of 73% (w/v) sucrose. The mixture was then placed at the bottom of a 4-ml ultracentrifuge tube, above which 2.3 ml of 65% (w/v) sucrose and 1.2 ml of 10% (w/v) sucrose in TE buffer were sequentially overlaid. The formed step sucrose gradients were ultracentrifuged at 100,000 g for 18 h at 4°C in a Beckman SW55-Ti rotor. After centrifugation, ten 0.4-ml fractions were manually collected by pipetting, and equivalent aliquots were taken from collected fractions for immunoblotting. In a typical execution of this protocol, fractions 3 and 4 contained membrane vesicles floating to the boundary of the 10% (w/v) and 65% (w/v) sucrose layers, whereas fractions 9 and 10 contained soluble proteins unable to float up.

Proteinase K protection assay

Proteinase K protection assay was performed as described in previous studies [23, 46, 47]. In brief, mock treated or EGF stimulated cells were collected and pelleted by centrifugation at 1,800 g for 5 min. Pelleted cells were resuspended in 10 volumes of 6.5 μ g/mL digitonin (Sigma) in PBS, followed by incubation first at room temperature for 5 min and then on ice for 30 min. Samples were then centrifuged at 16,000 g for 5 min, and pellets were resuspended in 10 volumes of homogenization buffer containing 100 mM K_2HPO_4 / KH_2PO_4 , 5 mM $MgCl_2$ and 250 mM sucrose. Three equal aliquots were taken from each sample, which were mock-treated or digested with 4 ng proteinase K/ μ g sample protein at room temperature for 10 min in the presence or absence of 0.1% TX. Proteinase K digestion was stopped by adding SDS-PAGE sample buffer, and proteins were subject to SDS-PAGE and immunoblotting. Proteinase K should digest all EGFR in the presence of 0.1% TX. When this condition was met, the percentage of proteinase K-resistant EGFR in the absence of 0.1% TX relative to total EGFR in mock-treated sample was taken as the percentage of EGFR sorted into MVBs.

Statistical analysis

Statistical analyses were performed using Students t-test. 0.01 p -value <0.05 was considered significant (*). 0.001 p -value <0.01 was considered highly significant (**). p -value <0.001 was considered very highly significant (***).

RESULTS

ALIX interacts with activated and ubiquitinated EGFR through the ALIX V domain

To determine the ability of ALIX to recognize activated and ubiquitinated EGFR, we ectopically expressed GFP-ALIX in HEK293 cells and examined GFP-ALIX interaction with EGFR in control and EGF-stimulated cells by co-immunoprecipitation. Under our cell transfection conditions, the ectopically expressed GFP-ALIX was 1 to 2.5 fold of endogenous ALIX, as determined by immunoblotting of GFP-ALIX and endogenous ALIX on the same blot with an anti-ALIX monoclonal antibody (Fig. 2C, Fig. 3E, Fig. 5D, and Fig. S4C–E). Cell lysates were prepared from mock-treated and EGF-stimulated cells with a membrane solubilizing cell lysis buffer in the presence or absence of the deubiquitinase inhibitor N-ethylmaleimide (NEM). The cell lysis buffer not only solubilizes EGFR but also relieves the intramolecular interaction that inhibits ALIX interaction with CHMP4 [45]. Immunoblotting of EGFR with a monoclonal antibody that recognizes a cytoplasmic epitope surrounding Tyr1068 of human EGFR showed that the inclusion of NEM in the cell lysis buffer was required for detection of the EGF-induced upward gel mobility shift of EGFR (Fig. 1A), indicating that the shift was due to activation-associated ubiquitination of EGFR. The cell lysates were then immunoprecipitated with an anti-GFP antibody or a mouse IgG (negative control antibody), and the precipitated proteins were subject to immunoblotting to visualize GFP-ALIX, EGFR, ubiquitinated EGFR and CHMP4b (Fig. 1B). In the presence of NEM, the association of GFP-ALIX with EGFR increased by 4 fold in cells stimulated with EGF for 10 or 20 min relative to control cells, and the newly recruited EGFR was readily detectable by anti-ubiquitin antibodies. In contrast, GFP-ALIX interaction with CHMP4b did not change as anticipated. In the absence of NEM, however, GFP-ALIX interacted with a low level of non-shifted and non-ubiquitinated EGFR irrespective of EGF stimulation. In contrast to GFP-ALIX, ectopically expressed GFP did not interact with EGFR under both NEM conditions (Fig. S1). These results demonstrate that ALIX is capable of two modes of interaction with EGFR: a low level of basal, constitutive interaction, and a high level of EGF-induced interaction that depends on the activation-associated ubiquitination of EGFR. The low level of the constitutive interaction of ALIX with EGFR is consistent with the previous observation that ectopically expressed ALIX interacts with endogenous EGFR in HEK293 cells [28].

To identify the domain in ALIX that is responsible for ALIX interaction with EGFR, we ectopically expressed FLAG-ALIX_{Br01} or HA-ALIX_V in HEK293 cells and used co-immunoprecipitation to examine their interaction with EGFR in membrane-solubilized lysates in the presence of NEM. FLAG-ALIX_{Br01} had a low level of interaction with EGFR irrespective of EGF stimulation (Fig. 1C), indicating that ALIX_{Br01} mediates the constitutive ALIX interaction with EGFR. In contrast, HA-ALIX_V preferentially interacted with EGFR from EGF-stimulated cells (Fig. 1D), indicating that ALIX_V mediates the

induced ALIX interaction with activated and ubiquitinated EGFR. We also isolated membrane proteins from control and EGF-stimulated cells in the presence of NEM and used the GST pull-down assays to examine the ability of purified GST-ALIX_{Bro1} or GST-ALIX_V to interact with EGFR. While GST-ALIX_{Bro1} did not interact with a detectable level of EGFR irrespective of EGF stimulation (Fig. 1E), GST-ALIX_V pulled down readily detectable levels of EGFR from EGF-stimulated cells but not control cells (Fig. 1F). These results further demonstrate that ALIX_V mediates the EGF-induced ALIX interaction with activated and ubiquitinated EGFR.

Multiple recent studies have demonstrated that the ALIX V domain directly binds ubiquitin and ubiquitinated proteins [37–39]. Thus direct recognition of ubiquitinated EGFR by the ALIX V domain is likely to be one of the major causes for EGF-induced ALIX interaction with EGFR. However, ubiquitinated EGFR is known to be recognized and clustered by ESCRT-0, -I and -II. ALIX is known to interact with ESCRT-I. ALIX phosphorylation may increase in response to EGF stimulation, which in turn affects ALIX interaction with EGFR. Thus it is possible that multiple factors contribute to increased ALIX interaction with activated and ubiquitinated EGFR in EGF stimulated cells.

EGFR activation induces increased ALIX interaction with membrane-bound CHMP4

ALIX interaction with membrane-bound CHMP4 is required for ALIX involvement in all ESCRT-mediated membrane remodeling processes. Thus to define the role of ALIX in MVB sorting of activated EGFR, we first characterize ALIX interaction with membrane-bound CHMP4 in control and EGF-stimulated HEK293 cells. For this objective, the post-nuclear supernatant (PNS) of cell lysates was fractionated by membrane flotation centrifugation through a three-step sucrose gradient. This established procedure separates membrane vesicles from soluble proteins in the PNS. Collected fractions were then subjected to immunoblotting to visualize the distribution of ALIX and CHMP4b, as well as the membrane marker protein EEA1, between the membrane (M) and soluble protein (S) fractions. The percentage of ALIX recovered in the M fraction was determined by quantifying immunoblot signals.

As shown in Fig. 2A, ~13% of endogenous ALIX from control cells was localized in the M fraction, which increased to ~35% in cells stimulated with EGF for 1 h. In contrast, CHMP4b was evenly distributed between the M and S fractions irrespective of EGF stimulation. Wild type (WT) and a CHMP4 interaction-defective mutant I212D GFP-ALIX were then ectopically expressed in HEK293 cells and examined as described above. As shown in Fig. 2B, the WT GFP-ALIX behaved similarly as endogenous ALIX; i.e., ~13% and ~33% of the protein was membrane-associated in control and EGF-stimulated cells, respectively. In contrast, only ~3% of the I212D GFP-ALIX was membrane associated irrespective of EGF stimulation. Together, these results demonstrate that EGFR activation induces increased ALIX interaction with membrane-bound CHMP4.

An antiparallel dimerization of ALIX through the middle V domain has been demonstrated to be required for ALIX involvement in ESCRT-III-mediated retroviral budding [48]. To determine whether membrane-associated ALIX in HEK293 cells is able to form a dimer through the V domain, we first verified that ALIX is able to dimerize in HEK293 cells by

coimmunoprecipitation of endogenous ALIX with ectopically expressed GFP-ALIX. Immunoblotting of input cell lysates with the 1A3 anti-ALIX antibody, which recognizes both ALIX and HD-PTP through an identical epitope surrounding Y319 of ALIX [45], showed that HD-PTP expression is at least 10 fold less than ALIX expression. Immunoblotting of immunocomplexes with the 1A3 antibody showed that while ALIX interaction with GFP-ALIX was robust, HD-PTP interaction with GFP-ALIX was undetectable (Fig. 2C). ALIX dimerization was further corroborated by reciprocal co-immunoprecipitation of ectopically expressed FLAG-ALIX and GFP-ALIX (Fig. S2A&B). We then ectopically expressed FLAG-ALIX_V in HEK293 cells and determined its interaction with membrane-associated ALIX by the membrane flotation centrifugation. Immunoblotting of the pooled M and S fractions showed that despite the lack of a CHMP4 binding site in ALIX_V, ~20% of FLAG-ALIX_V associated with the membrane (Fig. 2D). Moreover, immunodepletion of FLAG-ALIX_V from the M fraction removed the endogenous ALIX (Fig. 2E). Thus the CHMP4-bound ALIX at the membrane is able to form a dimer through the V domain-mediated self-interaction.

The CHMP4-bound ALIX dimer plays an essential role in MVB sorting of activated EGFR

The ability of ALIX to recognize activated EGFR and the positive effect of EGFR activation on ALIX interaction with membrane-bound CHMP4 point to the possibility that the CHMP4-bound ALIX at the membrane is positively involved in MVB sorting of activated EGFR. To test this possibility, we first used the proteinase K protection assay to measure MVB sorting of activated EGFR during the first 30 min of EGF stimulation as outlined in Fig. 3A. The assay involves isolation of membrane vesicles after the plasma membrane is dissolved, and proteins are digested with proteinase K in the presence or absence of the non-ionic detergent Triton X-100. Because proteinase K cannot reach the membrane receptors localized within the lumen of intact endosomes, the percentage of a proteinase K-insensitive receptor in the absence of Triton X-100 is indicative of the amount of this receptor sorted into ILVs of MVBs. This assay has been previously used to measure MVB sorting of activated EGFR and PAR1 [23, 46] and has the advantage of being efficient and quantitative. By this assay, ~5% of EGFR in control cells was proteinase K-insensitive, but this pool increased to ~60% in cells stimulated with EGF for 30 min (Fig. 3B). In contrast, the entire pool of the peripheral membrane protein EEA1 was proteinase K-sensitive irrespective of EGF stimulation. Treating cells with the microtubule poison nocodazole to block early to late endosome trafficking [49] did not reduce the percentage of the protected EGFR in EGF-stimulated cells (Fig. S3E). These results demonstrate a fast and robust MVB sorting of activated EGFR at early endosomes.

To characterize the role of ALIX interaction with membrane-bound CHMP4 in MVB sorting of activated EGFR, we ectopically expressed high levels of FLAG-ALIX_{Bro1} to compete with endogenous ALIX to interact with membrane-bound CHMP4 and performed the proteinase K protection assay to determine the effect on MVB sorting of activated EGFR. Initially, the FLAG-ALIX_{Bro1} used for competition was constructed to contain Y319F mutation to eliminate its interaction with Src [50]; FLAG-ALIX_{Bro1} titration of endogenous Src may affect normal cellular physiology. The negative control for Y319F FLAG-ALIX_{Bro1} was a FLAG-ALIX_{Bro1} containing both Y319F and I212D mutations; the

latter mutation eliminated ALIX_{Bro1} interaction with CHMP4 [19]. Immunoblots of cell lysates showed that the two proteins were expressed at comparable levels (Fig. 3C, left panel). The proteinase K protection assay revealed that the expression of Y319F FLAG-ALIX_{Bro1}, capable of interaction with CHMP4, reduced the percentage of protected EGFR from ~60% to the basal level of ~5%, whereas the expression of the CHMP4-noninteractive Y319F-I212D FLAG-ALIX_{Bro1} had little effect (Fig. 3C, middle and right panels). After these results, we also performed the proteinase K protection assay on HEK293 cells ectopically expressing WT FLAG-ALIX_{Bro1} and the I212D FLAG-ALIX_{Bro1} and obtained similar results (Fig. S3A). Together, these results demonstrate that ALIX interaction with membrane-bound CHMP4 plays an essential role in MVB sorting of activated EGFR at early endosomes.

To determine whether the V domain-mediated ALIX dimerization is required for the CHMP4-bound ALIX to promote MVB sorting of activated EGFR, we further determined the effect of inhibition of the V domain-mediated ALIX dimerization on MVB sorting of activated EGFR. For this objective, GFP-ALIX was co-expressed with WT or dimerization site-mutated (DM) HA-ALIX_V in HEK293 cells, and the heterodimerization was determined by co-immunoprecipitation. DM HA-ALIX_V interacted with GFP-ALIX 5 fold less efficiently than WT HA-ALIX_V did (Fig. S3B), making DM HA-ALIX_V a proper but an incomplete negative control agent for WT HA-ALIX_V. Importantly, while WT HA-ALIX_V robustly interacted with endogenous ALIX, it did not interact with endogenous HD-PTP (Fig. S3C). This makes it unlikely that ectopic expression of WT HA-ALIX_V may inhibit MVB sorting of activated EGFR through inhibiting HD-PTP. Since ALIX_V interacts with activated EGFR, we also coexpressed GFP-ALIX with WT or DM HA-ALIX_V and examined GFP-ALIX interaction with EGFR in control and EGF-stimulated cells by co-immunoprecipitation. Neither WT nor DM HA-ALIX_V expression reduced the constitutive or EGF-induced GFP-ALIX interaction with EGFR (Fig. S3D). The proteinase K protection assay was then used to measure MVB sorting of activated EGFR in control, the WT HA-ALIX_V expressing or the DM HA-ALIX_V expressing cells. As shown in Fig. 3D, the WT HA-ALIX_V expression caused a ~14-fold reduction in the percentage of the proteinase K-insensitive EGFR as observed with the Y319F FLAG-ALIX_{Bro1} expression, whereas the DM HA-ALIX_V expression caused a 2-fold decrease in the percentage of the protected EGFR. The weak inhibitory effect of DM HA-ALIX_V could be due to its residual ability to interact with full-length ALIX. These results indicate that the CHMP4-bound ALIX must be dimerized through the V domain in order to promote MVB sorting of activated EGFR at early endosomes.

To further test the hypothesis that the CHMP4-bound ALIX dimer promotes MVB sorting of activated EGFR, we transfected HEK293 cells with an ALIX siRNA and determined its effect on MVB sorting of activated EGFR with or without co-expression of the ALIX siRNA-insensitive GFP-ALIX (GFP-ALIX*). The ALIX siRNA transfection resulted in >95% reduction in ALIX expression, and ectopically expressed GFP-ALIX was 2.5 fold of the original endogenous ALIX (Fig. 3E). Such ALIX knockdown reduced the percentage of the proteinase K-insensitive EGFR from >50% to ~10%. The inhibitory effect of ALIX knockdown was not affected by pretreating cells with nocodazole (Fig. S3E), indicating that ALIX knockdown inhibits MVB sorting at early endosomes. Most importantly, the

inhibitory effect of ALIX knockdown could be rescued by expression of the WT GFP-ALIX* but not by the I212D or DM GFP-ALIX* (Fig. 3E). These results lend a strong support to the hypothesis that the CHMP4-bound ALIX dimer plays a critical role in MVB sorting of activated EGFR at early endosomes.

The CHMP4-bound ALIX dimer plays an important role in rapidly silencing of activated EGFR before EGFR degradation

EGF-induced activation of ERK1/2 has been shown to be highly transient in HEK293 cells [51]. Consistent with these previous results, immunoblotting of phosphorylated ERK1/2 in cells stimulated with EGF for 10, 20, 30 and 60 min showed that EGF stimulation induced a peak activation of ERK1/2 at 10 min, which quickly dropped to ~10% of its peak level at 20 min and remained at low levels afterwards (Fig. 4A&B). Given that the level of EGFR does not change during the first 20 min of EGF stimulation, this sharp peak kinetics of ERK1/2 activation indicates that (i) the activating phosphorylation of ERK1/2 is in rapid turnover; and (ii) the signaling function of activated EGFR is rapidly terminated long before EGFR degradation.

To determine the role of the ALIX-supported MVB sorting in the rapid silencing of activated EGFR, we characterized the effect of the Y319F FLAG-ALIX_{Bro1} expression, the HA-ALIX_V expression or ALIX knockdown on activation-associated phosphorylation of ERK1/2 in EGF-stimulated HEK293 cells. Neither the Y319F FLAG-ALIX_{Bro1} nor the HA-ALIX_V expression affected the amplitude of ERK1/2 activation; however, both treatments prevented the quick and dramatic inactivation of ERK1/2 and sustained the ERK1/2 activation at 80% and 50% of the peak level at 30 and 60 min (Fig. 4C&4E). Further examination of the relative levels of activated ERK1/2 at 1, 2 or 3 h showed that the low level of activated ERK1/2 in control cells further decreased to hardly detectable levels from 1 to 3 h, whereas the elevated level of activated ERK1/2 in the Y319F FLAG-ALIX_{Bro1} or HA-ALIX_V expressing cells only moderately decreased during this period (Fig. 4D&4F). ALIX knockdown cells behaved similarly as the Y319F FLAG-ALIX_{Bro1} expressing or the HA-ALIX_V expressing cells in sustaining the EGF-induced ERK1/2 activation (Fig. 4G&4H). Consistent with each other, these results indicate that the ALIX-supported MVB sorting plays an important role in rapidly silencing of activated EGFR before EGFR degradation.

Elimination of the CHMP4-bound ALIX dimer retards degradation of activated EGFR

To characterize the role of the ALIX-supported MVB sorting in lysosome-dependent degradation of activated EGFR, we first determined the kinetics of EGF-induced EGFR degradation in the presence or absence of the lysosome inhibitor chloroquine (CQ) [52]. HEK293 cells that had been serum-starved for 12 h were stimulated with EGF for 1, 2, 3 or 4 h, and the relative levels of EGFR at different time points were determined by immunoblotting with the anti-EGFR antibody described earlier that recognizes a cytoplasmic epitope surrounding Tyr1068 of human EGFR. As shown in Fig. 5A, a progressive reduction in the protein level of EGFR was observed, with 50% EGFR remaining at approximately 1.5 h. When cells were stimulated with EGF in the presence of

CQ, EGFR degradation was inhibited, confirming that the observed EGFR degradation is mainly due to lysosome activity.

We then determined the effect of the Y319F FLAG-ALIX_{Bro1} expression, the WT HA-ALIX_V expression or ALIX knockdown on the kinetics of EGF-induced EGFR degradation. The Y319F FLAG-ALIX_{Bro1} expression eliminated the slight (~15%) EGFR reduction during the first 30 min and increased the percentage of remaining EGFR by ~2 fold at 1, 2, 3 and 4 h. This postponed the 50% EGFR reduction time from 1 h to ~2.5 h. In contrast, the expression of the double-mutant form of FLAG-ALIX_{Bro1} had little effect (Fig. S4A, Fig. 5B). The WT HA-ALIX_V expression retarded the EGFR degradation similarly as the FLAG-ALIX_{Bro1} expression did, whereas the DM HA-ALIX_V expression had little effect (Fig. S4B, Fig. 5C). Less potent than the FLAG-ALIX_{Bro1} or HA-ALIX_V expression, siRNA-mediated ALIX knockdown eliminated the slight EGFR reduction during the first 30 min and increased the percentage of remaining EGFR by ~2 fold at 1 and 2 h but had no significant effects afterwards. The retardation effect of ALIX-siRNA on EGFR degradation was specific as the effect was rescued by expressing the WT GFP-ALIX* (Fig. S4C, Fig. 5D) but not by expressing the I212D GFP-ALIX* (Fig. S4D) or the DM GFP-ALIX* (Fig. S4E). These results demonstrate that the ALIX-supported MVB sorting accelerates but is not required for trafficking of activated EGFR to lysosomes for degradation.

The CHMP4-bound ALIX dimer may promote MVB sorting and silencing of activated EGFR without accelerating EGFR degradation

Multiple previous studies using pulse-chase EGF conditions showed little or no effects of ALIX knockdown on the rate of degradation of internalized EGF [25, 27, 28]. To determine whether the CHMP4-bound ALIX dimer may promote MVB sorting of activated EGFR without accelerating EGFR degradation, we determined the effect of the Y319F FLAG-ALIX_{Bro1} expression, the HA-ALIX_V expression or ALIX knockdown on MVB sorting, silencing and degradation of activated EGFR under pulse-chase EGF conditions. Serum-starved cells were incubated with EGF at 4°C for 30 min (EGF pulse) and then cultured at 37°C in the absence of EGF (chase) for different lengths of time. Since activation and endocytosis of EGFR is largely inhibited at 4°C, MVB sorting, silencing and degradation of activated EGFR can only occur during the chase.

Different from the results obtained under continuous EGF conditions, ALIX knockdown increased the percentage of remaining EGFR only at 1 h by 1.3 fold but had no effects at 2, 3 and 4 h (Fig. 6A). These results concurred with the negative results from previous studies. Notably, even when pulse EGF stimulation was conducted at 37°C, similar results were obtained (Fig. S5A); so cold temperature was not the reason why ALIX knockdown had little effect on EGFR degradation upon pulse-chase EGF conditions. Although the Y319F FLAG-ALIX_{Bro1} or HA-ALIX_V expression still retarded the EGFR degradation (Fig. 6B), the effects were more transient and less severe than those observed under continuous EGF conditions. Thus elimination of the CHMP4-bound ALIX dimer is less inhibitory on EGFR degradation under pulse-chase EGF conditions than under continuous EGF conditions. Despite this difference, however, the proteinase K protection assay revealed that the expression of HA-ALIX_V or Y319F FLAG-ALIX_{Bro1} reduced the percentage of the

protected EGFR from ~35% to the basal level of ~2%, and that ALIX knockdown reduced the percentage of the protected EGFR from ~35% to ~8% (Fig. 6C, Fig. S5B–D). Immunoblots of phosphorylated ERK1/2 showed that the kinetics of ERK1/2 activation of non-transfected cells is similar to that observed under continuous EGF condition (Fig. 6D). Most importantly, all three treatments greatly prolonged the EGF-induced activation of ERK1/2 (Fig. 6E–G), also consistent with the results obtained under continuous EGF conditions. These results indicate that the CHMP4-bound ALIX dimer promotes MVB sorting of activated EGFR without significantly accelerating EGFR degradation under pulse-chase EGF conditions.

DISCUSSION

Multiple previous studies demonstrated that ALIX knockdown has no or only a minor inhibitory effect on trafficking of internalized EGF or activated EGFR to lysosomes for degradation under the EGF pulse-chase conditions [25–29]. These findings led to a widely held notion that different from the yeast ALIX, BRO1, mammalian ALIX is not critically involved in ESCRT-mediated MVB sorting of ubiquitinated membrane receptors. In this study, we demonstrate that despite the validity of these previous observations, ALIX directly interacts with activated and ubiquitinated EGFR and plays an essential role in MVB sorting of activated EGFR under both continuous and pulse-chase EGF conditions. These findings reconcile the roles of BRO1 and ALIX in MVB sorting of ubiquitinated membrane receptors and unify the role of ALIX in different ESCRT-III mediated membrane remodeling processes in mammalian cells.

The level of HD-PTP expression is very low in HEK293 cells as compared to that of ALIX expression (Fig. 2C, Fig. S3C). This raises the possibility that the essential role of ALIX in MVB sorting and silencing of activated EGFR observed in this study is due to deficient function of HD-PTP in HEK293 cells. Although we cannot exclude this possibility, we should note that among multiple established cell lines we have examined, more than half of them are similar to HEK293 cells in the relative expression of ALIX and HD-PTP (unpublished results). This makes it unlikely that the findings made in this study are a rare exception.

MVB formation may occur at early endosomes and/or late endosomes. Unique to MVB formation by LPBA-enriched late endosomes is the potential to occur spontaneously without requiring the ESCRT function and the presence of a topologically opposing process, i.e., back fusion of ILVs with the limiting membranes. Although the proteinase K protection assay used in this study did not distinguish MVB sorting at early or late endosomes, treating cells with the microtubule poison NC to block early to late endosome trafficking did not significantly change the rate of MVB sorting in control or ALIX knockdown cells (Fig. S3E). Thus it seems likely that the ALIX-supported MVB sorting of activated EGFR unravelled in this study primarily occurs at early endosomes.

While none of previous studies have examined the role of ALIX in MVB sorting of activated EGFR at early endosomes, one of the previous studies has specifically examined the role of ALIX in MVB sorting of activated EGFR at late endosomes by using a

reconstituted cell free system [53]. In contrast to the findings in this study, ALIX knockdown was found to increase the protease-insensitive EGFR at late endosomes from ~50% to ~85%. Consistent with the finding from the same group that ALIX interaction with LBPA inhibits the formation of multivesicular liposomes in vitro [36] and promotes back fusion of the ILVs with the limiting membrane of late endosomes in intact cells [35], this finding indicates an inhibitory role of ALIX in the overall rate of MVB sorting at late endosomes. The presence of inhibitory roles of ALIX in MVB sorting at late endosomes offers a potential explanation as to why ALIX knockdown consistently inhibits MVB sorting less dramatically than the Y319F FLAG-ALIX_{Bro1} or HA-ALIX_V overexpression (Fig. 3, Fig. 6).

To reconcile the essential role of ALIX in MVB sorting but a non-essential role of ALIX in degradation of activated EGFR, a logical explanation is that trafficking of activated EGFR to lysosomes in mammalian cells can be achieved through both MVB sorting-dependent and an alternative mechanism, a notion also suggested by previous studies [31, 32], and that ALIX is only involved in the MVB sorting-dependent mechanism. Following the presence of redundant mechanisms, specific inhibition of the ALIX-supported MVB sorting should moderately retard the EGFR degradation if the MVB sorting mechanism is faster than the alternative mechanism in trafficking activated EGFR to lysosomes. This may be the case when we examined the effect of ALIX knockdown/perturbation on the rate of EGFR degradation under continuous EGF conditions. On the other hand, if the ALIX-supported MVB sorting and the alternative mechanism traffic activated EGFR to lysosomes with similar rates, inhibition of the ALIX-supported MVB sorting should produce little effect on the rate of EGFR degradation. This may be the scenario when we and others examined the effect of ALIX knockdown/perturbation on the rate of EGFR degradation under pulse-chase EGF conditions.

We have yet to understand how activated EGFR may be trafficked to lysosomes without going through the MVB sorting step. However, when ALIX knockdown was found not to inhibit degradation of activated EGFR or internalized EGF, knockdown of the ESCRT-I component Vps37/HCRP1 [26], the ESCRT-I component Vps23/TSG101 [27], or HD-PTP [25] potentially inhibited degradation of activated EGFR or internalized EGF under the same experimental conditions. These previous results raise the possibility that ESCRT-0, ESCRT-I and HD-PTP not only promote MVB sorting of the ubiquitinated EGFR through the sequential functions of ESCRT-II, ESCRT-III and Vps4 as is currently understood, but also triggers a different pathway that is able to traffic the receptor to lysosomes without going through the MVB sorting process.

Although lysosomal trafficking of activated EGFR can occur without MVB sorting in mammalian cells, MVB sorting terminates the signaling function of activated EGFR much earlier than EGFR degradation [54]. However, EGFR can also be inactivated by a protein tyrosine phosphatase, and so it is unclear how important MVB sorting is in rapidly terminating the signaling function of activated EGFR. Our results show that under both continuous and pulse-chase EGF conditions, inhibition of the ALIX-supported MVB sorting of activated EGFR by each of the three different approaches employed converts the sharp peak kinetics of the EGF-induced activation of ERK1/2 into a slow slope kinetics,

lengthening the EGF-induced ERK1/2 activation from <20 min to >2 h. These results indicate that MVB sorting is one of the major players in rapidly silencing the signaling function of activated EGFR and predict that cells may regulate the signaling output of activated EGFR through regulating whether activated EGFR is degraded through MVB sorting or the alternative mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Katzmann DJ, Odorizzi G, Emr SD. Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol.* 2002; 3:893–905. [PubMed: 12461556]
2. Gruenberg J, Stenmark H. The biogenesis of multivesicular endosomes. *Nat Rev Mol Cell Biol.* 2004; 5:317–323. [PubMed: 15071556]
3. Wegner CS, Rodahl LM, Stenmark H. ESCRT proteins and cell signalling. *Traffic.* 2011; 12:1291–1297. [PubMed: 21518165]
4. Henne WM, Buchkovich NJ, Emr SD. The ESCRT pathway. *Dev Cell.* 2011; 21:77–91. [PubMed: 21763610]
5. Henne WM, Stenmark H, Emr SD. Molecular mechanisms of the membrane sculpting ESCRT pathway. *Cold Spring Harb Perspect Biol.* 2013; 5
6. Hurley JH, Hanson PI. Membrane budding and scission by the ESCRT machinery: it's all in the neck. *Nat Rev Mol Cell Biol.* 2010; 11:556–566. [PubMed: 20588296]
7. Lata S, Schoehn G, Solomons J, Pires R, Gottlinger HG, Weissenhorn W. Structure and function of ESCRT-III. *Biochem Soc Trans.* 2009; 37:156–160. [PubMed: 19143622]
8. Kim SY, Song EJ, Lee KJ, Ferrell JE Jr. Multisite M-phase phosphorylation of *Xenopus* Wee1A. *Mol Cell Biol.* 2005; 25:10580–10590. [PubMed: 16287869]
9. Springael JY, Nikko E, Andre B, Marini AM. Yeast Npi3/Bro1 is involved in ubiquitin-dependent control of permease trafficking. *FEBS Lett.* 2002; 517:103–109. [PubMed: 12062418]
10. Nikko E, Marini AM, Andre B. Permease recycling and ubiquitination status reveal a particular role for Bro1 in the multivesicular body pathway. *J Biol Chem.* 2003; 278:50732–50743. [PubMed: 14523026]
11. Odorizzi G, Katzmann DJ, Babst M, Audhya A, Emr SD. Bro1 is an endosome-associated protein that functions in the MVB pathway in *Saccharomyces cerevisiae*. *J Cell Sci.* 2003; 116:1893–1903. [PubMed: 12668726]
12. Missotten M, Nichols A, Rieger K, Sadoul R. Alix, a novel mouse protein undergoing calcium-dependent interaction with the apoptosis-linked-gene 2 (ALG-2) protein. *Cell Death Differ.* 1999; 6:124–129. [PubMed: 10200558]
13. Vito P, Pellegrini L, Guet C, D'Adamio L. Cloning of AIP1, a novel protein that associates with the apoptosis-linked gene ALG-2 in a Ca²⁺-dependent reaction. *J Biol Chem.* 1999; 274:1533–1540. [PubMed: 9880530]

14. Wu Y, Pan S, Che S, He G, Nelman-Gonzalez M, Weil MM, Kuang J. Overexpression of Hp95 induces G1 phase arrest in confluent HeLa cells. *Differentiation*. 2001; 67:139–153. [PubMed: 11683497]
15. von Schwedler UK, Stuchell M, Muller B, Ward DM, Chung HY, Morita E, Wang HE, Davis T, He GP, Cimbara DM, Scott A, Krausslich HG, Kaplan J, Morham SG, Sundquist WI. The protein network of HIV budding. *Cell*. 2003; 114:701–713. [PubMed: 14505570]
16. Strack B, Calistri A, Craig S, Popova E, Gottlinger HG. AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. *Cell*. 2003; 114:689–699. [PubMed: 14505569]
17. Katoh K, Shibata H, Suzuki H, Nara A, Ishidoh K, Kominami E, Yoshimori T, Maki M. 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*. 2003; 278:39104–39113. Epub 2003 Jul 39114. [PubMed: 12860994]
18. McCullough J, Fisher RD, Whitby FG, Sundquist WI, Hill CP. ALIX-CHMP4 interactions in the human ESCRT pathway. *Proc Natl Acad Sci USA*. 2008; 105:7687–7691. [PubMed: 18511562]
19. Fisher RD, Chung HY, Zhai Q, Robinson H, Sundquist WI, Hill CP. Structural and biochemical studies of ALIX/AIP1 and its role in retrovirus budding. *Cell*. 2007; 128:841–852. [PubMed: 17350572]
20. Carlton JG, Martin-Serrano J. Parallels between cytokinesis and retroviral budding: a role for the ESCRT machinery. *Science*. 2007; 316:1908–1912. [PubMed: 17556548]
21. Morita E, Sandrin V, Chung HY, Morham SG, Gygi SP, Rodesch CK, Sundquist WI. Human ESCRT and ALIX proteins interact with proteins of the midbody and function in cytokinesis. *EMBO J*. 2007; 26:4215–4227. [PubMed: 17853893]
22. Baietti MF, Zhang Z, Mortier E, Melchior A, Degeest G, Geeraerts A, Ivarsson Y, Depoortere F, Coomans C, Vermeiren E, Zimmermann P, David G. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat Cell Biol*. 2012; 14:677–685. [PubMed: 22660413]
23. Dore MR, Chen B, Lin H, Soh UJ, Paing MM, Montagne WA, Meerloo T, Trejo J. ALIX binds a YPX3L motif of the GPCR PAR1 and mediates ubiquitin-independent ESCRT-III/MVB sorting. *J Cell Biol*. 2012; 197:407–419. [PubMed: 22547407]
24. Jimenez AJ, Maiuri P, Lafaurie-Janvare J, Divoux S, Piel M, Perez F. ESCRT machinery is required for plasma membrane repair. *Science*. 2014; 343:1247136. [PubMed: 24482116]
25. Doyotte A, Mironov A, McKenzie E, Woodman P. The Bro1-related protein HD-PTP/PTPN23 is required for endosomal cargo sorting and multivesicular body morphogenesis. *Proc Natl Acad Sci USA*. 2008; 105:6308–6313. [PubMed: 18434552]
26. Cabezas A, Bache KG, Brech A, Stenmark H. Alix regulates cortical actin and the spatial distribution of endosomes. *J Cell Sci*. 2005; 118:2625–2635. [PubMed: 15914539]
27. Bowers K, Piper SC, Edeling MA, Gray SR, Owen DJ, Lehner PJ, Luzio JP. Degradation of endocytosed epidermal growth factor and virally ubiquitinated major histocompatibility complex class I is independent of mammalian ESCRTII. *J Biol Chem*. 2006; 281:5094–5105. [PubMed: 16371348]
28. Schmidt MH, Hoeller D, Yu J, Furnari FB, Cavenee WK, Dikic I, Bogler O. Alix/AIP1 antagonizes epidermal growth factor receptor downregulation by the Cbl-SETA/CIN85 complex. *Mol Cell Biol*. 2004; 24:8981–8993. [PubMed: 15456872]
29. Luyet PP, Falguieres T, Pons V, Pattnaik AK, Gruenberg J. The ESCRT-I subunit TSG101 controls endosome-to-cytosol release of viral RNA. *Traffic*. 2008; 9:2279–2290. [PubMed: 18817529]
30. Bissig C, Gruenberg J. ALIX and the multivesicular endosome: ALIX in Wonderland. *Trends Cell Biol*. 2014; 24:19–25. [PubMed: 24287454]
31. Futter CE, Collinson LM, Backer JM, Hopkins CR. Human VPS34 is required for internal vesicle formation within multivesicular endosomes. *J Cell Biol*. 2001; 155:1251–1264. [PubMed: 11756475]
32. White IJ, Bailey LM, Aghakhani MR, Moss SE, Futter CE. EGF stimulates annexin 1-dependent inward vesiculation in a multivesicular endosome subpopulation. *EMBO J*. 2006; 25:1–12. [PubMed: 16052208]

33. Ali N, Zhang L, Taylor S, Mironov A, Urbe S, Woodman P. Recruitment of UBPY and ESCRT exchange drive HD-PTP-dependent sorting of EGFR to the MVB. *Curr Biol.* 2013; 23:453–461. [PubMed: 23477725]
34. Stefani F, Zhang L, Taylor S, Donovan J, Rollinson S, Doyotte A, Brownhill K, Bennion J, Pickering-Brown S, Woodman P. UBAP1 is a component of an endosome-specific ESCRT-I complex that is essential for MVB sorting. *Curr Biol.* 2011; 21:1245–1250. [PubMed: 21757351]
35. Bissig C, Lenoir M, Velluz MC, Kufareva I, Abagyan R, Overduin M, Gruenberg J. Viral infection controlled by a calcium-dependent lipid-binding module in ALIX. *Dev Cell.* 2013; 25:364–373. [PubMed: 23664863]
36. Matsuo H, Chevallier J, Mayran N, Le Blanc I, Ferguson C, Faure J, Blanc NS, Matile S, Dubochet J, Sadoul R, Parton RG, Vilbois F, Gruenberg J. Role of LBPA and Alix in multivesicular liposome formation and endosome organization. *Science.* 2004; 303:531–534. [PubMed: 14739459]
37. Pashkova N, Gakhar L, Winistorfer SC, Sunshine AB, Rich M, Dunham MJ, Yu L, Piper RC. The yeast Alix homolog Bro1 functions as a ubiquitin receptor for protein sorting into multivesicular endosomes. *Dev Cell.* 2013; 25:520–533. [PubMed: 23726974]
38. Dowlatshahi DP, Sandrin V, Vivona S, Shaler TA, Kaiser SE, Melandri F, Sundquist WI, Kopito RR. ALIX is a Lys63-specific polyubiquitin binding protein that functions in retrovirus budding. *Dev Cell.* 2012; 23:1247–1254. [PubMed: 23201121]
39. Keren-Kaplan T, Attali I, Estrin M, Kuo LS, Farkash E, Jerabek-Willemsen M, Blutraich N, Artzi S, Peri A, Freed EO, Wolfson HJ, Prag G. Structure-based in silico identification of ubiquitin-binding domains provides insights into the ALIX-V:ubiquitin complex and retrovirus budding. *EMBO J.* 2013; 32:538–551. [PubMed: 23361315]
40. Pan S, Wang R, Zhou X, He G, Koomen J, Kobayashi R, Sun L, Corvera J, Gallick GE, Kuang J. Involvement of the adaptor protein Alix in actin cytoskeleton assembly. *J Biol Chem.* 2006; 285:34640–34650. [PubMed: 16966331]
41. Che S, Weil MM, Nelman-Gonzalez M, Ashorn CL, Kuang J. MPM-2 epitope sequence is not sufficient for recognition and phosphorylation by ME kinase-H. *FEBS Lett.* 1997; 413:417–423. [PubMed: 9303547]
42. Zhou X, Pan S, Sun L, Corvera J, Lin SH, Kuang J. The HIV-1 p6/EIAV p9 docking site in Alix is autoinhibited as revealed by a conformation-sensitive anti-Alix monoclonal antibody. *Biochem J.* 2008; 414:215–220. [PubMed: 18476810]
43. Spearman P, Horton R, Ratner L, Kuli-Zade I. Membrane binding of human immunodeficiency virus type 1 matrix protein in vivo supports a conformational myristyl switch mechanism. *J Virol.* 1997; 71:6582–6592. [PubMed: 9261380]
44. Ono A, Freed EO. Binding of human immunodeficiency virus type 1 Gag to membrane: role of the matrix amino terminus. *J Virol.* 1999; 73:4136–4144. [PubMed: 10196310]
45. Zhou X, Pan S, Sun L, Corvera J, Lee YC, Lin SH, Kuang J. The CHMP4b and Src docking sites in the Bro1 domain are autoinhibited in the native state of Alix. *Biochem J.* 2009; 418:277–284. [PubMed: 19016654]
46. Malerod L, Stuffers S, Brech A, Stenmark H. Vps22/EAP30 in ESCRT-II mediates endosomal sorting of growth factor and chemokine receptors destined for lysosomal degradation. *Traffic.* 2007; 8:1617–1629. [PubMed: 17714434]
47. Taelman VF, Dobrowolski R, Plouhinec JL, Fuentealba LC, Vorwald PP, Gumper I, Sabatini DD, De Robertis EM. Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. *Cell.* 2010; 143:1136–1148. [PubMed: 21183076]
48. Pires R, Hartlieb B, Signor L, Schoehn G, Lata S, Roessle M, Moriscot C, Popov S, Hinz A, Jamin M, Boyer V, Sadoul R, Forest E, Svergun DI, Gottlinger HG, Weissenhorn W. A crescent-shaped ALIX dimer targets ESCRT-III CHMP4 filaments. *Structure.* 2009; 17:843–856. [PubMed: 19523902]
49. Aniento F, Emans N, Griffiths G, Gruenberg J. Cytoplasmic dynein-dependent vesicular transport from early to late endosomes. *J Cell Biol.* 1993; 123:1373–1387. [PubMed: 8253838]

50. Schmidt MH, Dikic I, Bogler O. Src phosphorylation of Alix/AIP1 modulates its interaction with binding partners and antagonizes its activities. *J Biol Chem.* 2005; 280:3414–3425. [PubMed: 15557335]
51. Xu TR, Lu RF, Romano D, Pitt A, Houslay MD, Milligan G, Kolch W. Eukaryotic translation initiation factor 3, subunit a, regulates the extracellular signal-regulated kinase pathway. *Mol Cell Biol.* 2012; 32:88–95. [PubMed: 22025682]
52. Marchese A, Benovic JL. Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J Biol Chem.* 2001; 276:45509–45512. [PubMed: 11641392]
53. Falguieres T, Luyet PP, Bissig C, Scott CC, Velluz MC, Gruenberg J. In vitro budding of intraluminal vesicles into late endosomes is regulated by Alix and Tsg101. *Mol Biol Cell.* 2008; 19:4942–4955. [PubMed: 18768755]
54. Eden ER, White IJ, Futter CE. Down-regulation of epidermal growth factor receptor signalling within multivesicular bodies. *Biochem Soc Trans.* 2009; 37:173–177. [PubMed: 19143625]

SUMMARY STATEMENT

The adaptor protein ALIX directly interacts with activated EGFR and promotes sorting of activated EGFR into the lumen of endosomes. Sorting of activated EGFR into the lumen of endosomes is a critical determinant of the functional duration of activated EGFR.

Figure 1. ALIX interacts with activated and ubiquitinated EGFR through the ALIX V domain (A&B) Serum starved HEK293 cells ectopically expressing GFP-ALIX were stimulated with EGF for indicated minutes. Cell lysates prepared in the presence or absence of NEM were either directly immunoblotted (A) or immunoprecipitated with anti-GFP antibody or mouse IgG at first and then immunoblotted with indicated antibodies to visualize EGFR, ubiquitinated EGFR, GFP-ALIX and CHMP4b (B). Results represent two independent experiments. (C&D) Serum starved HEK293 cells ectopically expressing FLAG-ALIX_{Bro1} (C) or HA-ALIX_V (D) were stimulated with EGF for indicated minutes. Cell lysates prepared in the presence of NEM were immunoprecipitated with anti-FLAG antibody and mouse IgG (C) or anti-HA antibody and mouse IgG (D). Crude cell lysates and immunocomplexes were immunoblotted with indicated antibodies to visualize FLAG-ALIX_{Bro1} and EGFR in (C) or HA-ALIX_V, EGFR and ubiquitinated EGFR in (D). (E&F) Solubilized membrane proteins were isolated in the presence of NEM from HEK293 cells stimulated with EGF for indicated minutes and incubated in parallel with immobilized GST and GST-ALIX_{Bro1} in (E) or GST and GST-ALIX_V in (F). Input and bound membrane proteins were immunoblotted with indicated antibodies to visualize EGFR, GST and GST-ALIX_{Bro1} in (E) or EGFR, GST and GST-ALIX_V in (F). Results represent two independent experiments.

Figure 2. EGFR activation leads to increased ALIX interaction with membrane-bound CHMP4
 (A) Serum-starved HEK293 cells were mock-treated or stimulated with EGF for 1 h, and the PNS was fractionated by membrane flotation centrifugation. Same volumes of aliquots were taken from collected fractions and immunoblotted with indicated antibodies to visualize EEA1, ALIX and CHMP4b; membrane (M) and soluble (S) protein fractions are indicated (left panel). The average percentage of ALIX in the M fractions and SDs were determined from three independent experiments and plotted (right panel). (B) Serum-starved HEK293 cells ectopically expressing wild type (WT) or I212D GFP-ALIX were processed as described for (A). Proteins in fractions collected from membrane flotation centrifugation were immunoblotted with indicated antibodies to visualize GFP-ALIX (left panel), and the average percentage of WT or I212D GFP-ALIX in the M fraction and SDs were determined from three independent experiments and plotted (right panel). (C) Lysates of HEK293 cells ectopically expressing GFP or GFP-ALIX were immunoprecipitated with IgG or anti-GFP antibody, and immunocomplexes were immunoblotted with indicated antibodies to visualize GFP and GFP-ALIX in the top panel and endogenous ALIX, GFP-ALIX and endogenous HD-PTP. (D) The PNS of HEK293 cells ectopically expressing FLAG-ALIX_v was fractionated by membrane flotation centrifugation. Proteins in the pooled M and S protein fractions were immunoblotted with indicated antibodies to visualize FLAG-ALIX_v, EEA1 and actin. (E) The pooled M fractions from (D) were extracted with 1% TX, and the solubilized proteins were immunoprecipitated with monoclonal anti-FLAG antibody. Proteins in the input, pellets (P) and supernatants (S) were immunoblotted with indicated antibodies to visualize FLAG-ALIX_v and endogenous ALIX.

Figure 3. The CHMP4-bound ALIX dimer plays an essential role in MVB sorting of activated EGFR

(A) A flowchart for measurement of EGF-induced MVB sorting of EGFR by the proteinase K protection assay. (B) The proteinase K protection assay was performed on mock-treated and EGF-stimulated (30 min) HEK293 cells (upper panel), and the average percentage of protected EGFR and SDs were determined from three independent experiments (lower panel). (C) The proteinase K protection assay was performed on mock transfected, the Y319F FLAG-ALIX_{Bro1} expressing or the Y319F-I212D FLAG-ALIX_{Bro1} expressing HEK293 cells after a 30-min EGF stimulation (left and middle panels). The average percentage of protected EGFR and SDs were determined from three independent experiments (right panel). (D) The proteinase K protection assay was performed on mock transfected, the WT HA-ALIX_v expressing or the DM HA-ALIX_v expressing cells after a 30-min EGF stimulation (left and middle panels). The average percentage of protected EGFR and SDs were determined from three independent experiments (right panel). (E) HEK293 cells were first transfected with indicated siRNA and cultured for 48 h. These cells were then transfected with expression plasmids for indicated proteins and cultured for another 24 h. After being treated with EGF for 30 min, these cells were assayed for MVB sorting of activated EGFR by the proteinase K protection assay (left and middle panel). The average percentage of protected EGFR and SDs were determined from three independent experiments (right panel).

Figure 4. Elimination of the CHMP4-bound ALIX dimer promotes sustained activation of ERK1/2

(A&B) Non-transfected HEK293 cells were stimulated with EGF for indicated minutes (A) or hours (B), and cell lysates were immunoblotted with indicated antibodies to visualize phosphorylated ERK1/2 (p-ERK1/2), ERK1/2 and actin. The relative levels of p-ERK at different time points were determined, normalized against the level at 60 min in (A) and 1 h in (B) and plotted. (C&D) Mock transfected and the Y319F FLAG-ALIX_{Bro1} expressing cells were stimulated with EGF for indicated minutes (C) or hours (D) and analyzed for EGF-induced ERK1/2 activation as described for (A&B) except that immunoblotting of Y319F FLAG-ALIX_{Bro1} with an anti-FLAG antibody was included and the relative levels of p-ERK at different time points were determined from two independent experiments with error bars indicating the range of the data. (E&F) Mock transfected and the HA-ALIX_v expressing cells were stimulated with EGF for indicated minutes (E) or hours (F) and

analyzed for EGF-induced ERK1/2 activation as described for (C&D) except that immunoblotting of Y319F FLAG-ALIX_{Bro1} with an anti-FLAG antibody was changed to immunoblotting of HA-ALIX_V with an anti-HA antibody. (G&H) HEK293 cells transfected with indicated siRNAs were stimulated with EGF for indicated minutes (G) or hours (H) and analyzed for EGF-induced ERK activation as described for (C&D) except that immunoblotting of Y319F FLAG-ALIX_{Bro1} with an anti-FLAG antibody was changed to immunoblotting of endogenous ALIX with 3A9 anti-ALIX antibody.

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Figure 5. Elimination of the CHMP4-bound ALIX dimer retards degradation of activated EGFR

(A) Serum starved HEK293 cells were stimulated with EGF for indicated hours in the presence or absence of chloroquine (CQ). Cell lysates were immunoblotted with indicated antibodies to visualize EGFR and actin (left panel). The average % of remaining EGFR at different time points were determined from two independent experiments; error bars indicate the range of the data (right panel). (B) Mock transfected and indicated forms of FLAG-ALIX_{Bro1} expressing HEK293 cells were stimulated with EGF for 1, 2, 3 or 4 h, and cell lysates were immunoblotted with indicated antibodies to visualize EGFR, FLAG-ALIX_{Bro1} and actin (left panel). The average % of remaining EGFR and SDs at different time points for each cell condition were determined from three independent experiments and plotted (right panel). (C) Mock transfected and indicated forms of HA-ALIX_v expressing cells were analyzed as described for (B) except that immunoblotting of FLAG-ALIX_{Bro1} with an anti-FLAG antibody was changed to immunoblotting of HA-ALIX_v with an anti-HA antibody. (D) HEK293 cells were transfected with indicated siRNA and cultured for 48 h. These cells were then transfected with indicated plasmids and cultured further for another 24 h. These HEK293 cells were analyzed for EGF-induced EGFR degradation as described for (B) except that immunoblotting of Y319F FLAG-ALIX_{Bro1} with an anti-FLAG antibody was changed to immunoblotting of endogenous ALIX and GFP-ALIX* with 3A9 anti-ALIX antibody.

Figure 6. The role of the CHMP4-bound ALIX dimer in MVB sorting, silencing and degradation of activated EGFR under EGF pulse-chase conditions

(A) HEK293 cells transfected with indicated siRNAs were analyzed for EGFR degradation after a 30-min EGF pulse followed by chase for 1, 2, 3 or 4 h (left panel). The average % of remaining EGFR and SDs at different time points for each cell condition were determined from three independent experiments and plotted (right panel). (B) Mock transfected, the Y319F FLAG-ALIX_{Bro1} expressing and the HA-ALIX_v expressing HEK293 cells were analyzed for EGFR degradation after a 30-min EGF pulse followed by chase for 1, 2, or 3 h (left panel). The average % of remaining EGFR and SDs at different time points for each cell condition were determined from three independent experiments and plotted (right panel). (C) HEK293 cells were transfected as indicated, and the proteinase K protection assay was performed on these cells after a 30-min EGF pulse followed by a 30-min chase (left panel). The average % of protected EGFR for each cell condition was determined from two independent experiments; error bars indicate the range of the data (right panel). (D) HEK293 cells were analyzed for ERK1/2 activation after EGF pulse for 30 min followed by chase for indicated minutes. (E) Mock transfected and the Y319F FLAG-ALIX_{Bro1}

expressing cells were analyzed for ERK1/2 activation after EGF pulse for 30 min followed by chase for indicated hours. The relative levels of p-ERK at different time points were averaged from two independent experiments; error bars indicate the range of the data (bottom panel). (F) Mock transfected and the HA-ALIX_V expressing cells were analyzed for EGF-induced ERK1/2 activation as described for (E) except that immunoblotting of Y319F FLAG-ALIX_{Bro1} with an anti-FLAG antibody was changed to immunoblotting of HA-ALIX_V with an anti-HA antibody. (G) HEK293 cells transfected with indicated siRNA were analyzed for EGF-induced ERK1/2 activation after EGF pulse for 30 min as described for (E) except that immunoblotting of Y319F FLAG-ALIX_{Bro1} with an anti-FLAG antibody was changed to immunoblotting of endogenous ALIX with 3A9 anti-ALIX antibody.