LTK is an ER-resident receptor tyrosine kinase that regulates secretion

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The endoplasmic reticulum (ER) is a key regulator of cellular proteostasis because it controls folding, sorting, and degradation of secretory proteins. Much has been learned about how environmentally triggered signaling pathways regulate ER function, but only little is known about local signaling at the ER. The identification of ER-resident signaling molecules will help gain a deeper understanding of the regulation of ER function and thus of proteostasis. Here, we show that leukocyte tyrosine kinase (LTK) is an ER-resident receptor tyrosine kinase. Depletion of LTK as well as its pharmacologic inhibition reduces the number of ER exit sites and slows ER-to-Golgi transport. Furthermore, we show that LTK interacts with and phosphorylates Sec12. Expression of a phosphoablating mutant of Sec12 reduces the efficiency of ER export. Thus, LTK-to-Sec12 signaling represents the first example of an ER-resident signaling module with the potential to regulate proteostasis.

Introduction

The secretory pathway handles a third of the proteome (Sharpe et al., 2010), and it is becoming increasingly clear that its functional organization is regulated by a wide range of signaling pathways (Pulvirenti et al., 2008; Farhan et al., 2010; Farhan and Rabouille, 2011; Zacharogianni et al., 2011; Giannotta et al., 2012; Cancino and Luini, 2013; Scharaw et al., 2016). Much has already been learned about how the secretory pathway responds to external stimuli. However, our understanding of its autoregulation, i.e., about its homeostasis-maintaining responses to stimuli from within the endomembrane system, is less developed. This is mainly due to our ignorance of signaling cascades operating locally on the secretory pathway. The probably best-understood example for autoregulation of the secretory pathway is the unfolded protein response (UPR). The UPR is induced by an accumulation of unfolded proteins in the ER, which results in increasing the expression of chaperones as well as the accumulation of unfolded proteins in the ER, which results in increasing the expression of chaperones as well as the machinery for protein degradation, vesicle budding, tethering, and fusion (Gardner et al., 2013). A major characteristic of the UPR is that its signaling mediators localize permanently to the ER. However, this is not the case with other signaling molecules identified so far. Very recently, Gα12 was shown to be active at the ER (Subramanian et al., 2019), but only a minor fraction of Gα12 localizes to this organelle. The small GTPase Rac1 was also shown to be activated at the nuclear envelope, which is part of the ER (Woroniuk et al., 2018). Again, the vast majority of Rac1 is either in endosomes or the plasma membrane. Mutant variants of the kinase FLT3 were shown to be permanently ER localized, but these are confined to cancer driving mutants and thus not useful to decipher physiological ER-based signaling (Choudhary et al., 2009; Schmidt-Arras et al., 2009). Thus, signaling at the ER remains poorly understood, which emphasizes the importance of the quest for ER-localized or -resident signaling molecules.

COPII vesicles form at ER exit sites (ERESs) and are responsible for ferrying secretory cargo out of the ER. The COPII coat is composed of the small GTPase Sar1, the Sec23-Sec24 heterodimer, and the Sec13-Sec31 heterotetramer (Zanetti et al., 2011). Activation of Sar1 is mediated by its exchange factor, Sec12, a type II transmembrane protein, which localizes to the general ER as well as to ERESs (Montegna et al., 2012; Saito et al., 2014). ERESs were discovered as COPII decorated sites that often localize in close vicinity to the ER Golgi intermediate compartment (ERGIC; Orci et al., 1991; Appenzeller-Herzog and Hauri, 2006). Previous siRNA screens uncovered a collection of kinases that regulate ERESs (Farhan et al., 2010; Simpson et al., 2012). Among the hits shared between the two RNAi screens, we focused on leukocyte tyrosine kinase (LTK), because it was previously reported to partially localize to the ER (Bauskin et al., 1991). Our current work identifies LTK as the first ER-resident receptor tyrosine kinase that regulates COPII-dependent function.
 trafficking and thus represents a potential druggable proteostasis regulator.

Results and discussion
LTK localizes to the ER
LTK is a receptor tyrosine kinase that is highly homologous to the anaplastic lymphoma kinase (ALK; Fig. 1 A). While their cytoplasmic kinase domain is 79% identical, the extracellular domain of ALK is much larger than that of mammalian LTK as it contains two MAM domains (acronym derived from meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu). Analysis of LTK and ALK evolution shows that deletions of the largest part of the extracellular domain of LTK occurred only in mammals (Fig. 1 B). Non-mammalian LTK rather resembles ALK than human LTK. According to The Human Protein Atlas, LTK mRNA is found in most tissues except muscle.

LTK was reported to localize to the ER (Bauskin et al., 1991), but this was questioned by recent findings showing LTK activation by extracellular ligands (Zhang et al., 2014; Reshetnyak et al., 2015). Overexpressed flag-tagged LTK, but not ALK, colocalized with the ER marker CLIMP63 (Fig. 1 C and Fig. S1 A). Endogenous LTK also localized to the ER (Fig. 1 D). The specificity of the antibody was tested by showing that the fluorescence signal is weaker in LTK-depleted cells (Fig. S1 B). We also noticed in 10% of cells a weak colocalization of LTK with the ERES marker Sec31 (Fig. S1 C). Immunofluorescence of endogenous LTK was performed in HepG2 cells because they express high levels of LTK but are essentially ALK negative (Fig. S2 A), limiting the possibility of antibody cross-reactivity.

To corroborate the immunofluorescence results, we subjected intact cells expressing flag-tagged ALK or LTK to PNGase F treatment. PNGase F is an enzyme that cleaves glycans and is therefore expected to cause a shift in electrophoretic mobility of proteins exposed to extracellular milieu. Consistent with its absence at the cell surface, we found that LTK was insensitive to treatment of cells with PNGase F (Fig. 1 E). On the contrary, ALK, which is expressed at the cell surface, was sensitive to digestion with PNGase F (Fig. 1 E). We next treated cell lysates expressing flag-tagged LTK or ALK with endoglycosidase H (EndoH), which only digests core-glycosylated proteins that have not entered the ER. Alectinib and crizotinib were added to the culture media. We determined that crizotinib affects ERESs by inhibiting LTK (Fig. 2 C). Notably, crizotinib had no effect on ERESs in LTK-depleted cells, supporting the notion that crizotinib affects ERESs by inhibiting LTK (Fig. 2 C). We also noticed in 10% of cells a weak colocalization of LTK with the ERES marker Sec31 (Fig. S1 C). Immunofluorescence of endogenous LTK was performed in HepG2 cells because they express high levels of LTK but are essentially ALK negative (Fig. S2 A), limiting the possibility of antibody cross-reactivity.

To determine the effect on ER-to-Golgi trafficking, we used the RUSH (rapid ultraviolet-dependent secretory apparatus tracking) system, which monitors the trafficking of a fluorescently labeled reporter protein out of the ER. This reporter is retained in the ER through a streptavidin-based interaction with an ER-resident hook. Treatment with biotin relieves retention and allows the reporter to exit toward post-ER compartments. We engineered GFP-tagged LTK into the RUSH system and expressed it together with a well-described secretory RUSH reporter, Mannosidase-II (Man-II), tagged with mCherry. Initially, LTK and Man-II colocalized in the ER (Fig. 1 H). We fixed and imaged cells 30 min after biotin addition, a time point at which Man-II was entirely in the Golgi. However, LTK was still ER-localized (Fig. 1 H). Even after 2 h, LTK showed no signs of leaving the ER (Fig. 1 H), making it highly unlikely that it ever leaves the ER. Altogether, our results show that LTK is an ER-resident receptor tyrosine kinase, making it a promising candidate to regulate secretion by local ER-based signaling.

LTK regulates ER export
We next asked whether LTK regulates ER-to-Golgi trafficking. We chose to test this in HeLa and HepG2 cells, which are LTK positive but negative for its close relative ALK (Fig. S2 A). Knockdown of LTK (Fig. S2 B; siRNA #3 was used for all further experiments) resulted in a reduction of the number of ERESs by 30–40% in HepG2 (Fig. 2 A, and B) and HeLa cells (Fig. S2 C). To support the results of the knockdown experiments, we treated HepG2 cells with two LTK inhibitors, alectinib and crizotinib. Because HepG2 cells are ALK negative (Fig. S2 A), any effect of these drugs is due to LTK inhibition. Treatment with both drugs for 30 min resulted in a reduction in the number of ERES comparable to LTK knockdown (Fig. 2 B). Notably, crizotinib had no effect on ERESs in LTK-depleted cells, supporting the notion that crizotinib affects ERESs by inhibiting LTK (Fig. 2 C).

To determine the effect on ER-to-Golgi trafficking, we used the RUSH assay with Man-II as a RUSH cargo (RUSH-Man-II; Boncompain et al., 2012). Silencing LTK expression or its pharmacologic inhibition resulted in a clear retardation of trafficking to the Golgi (Fig. 3 A, Video 1, and Video 2). This effect was a retardation of traffic rather than a total inhibition, because when we allowed the RUSH cargo to traffic for two hours, there was no difference between control and LTK-inhibited cells (Fig. 3 B). The effect of LTK knockdown was not limited to Man-II, but was also observed with another RUSH cargo, namely collagen X (Fig. 3 C), indicating that the effect of LTK is not limited to one type of cargo. Our results so far indicate that LTK is an ER-resident receptor tyrosine kinase that regulates ER export.

LTK interacts with and phosphorylates Sec12
We next sought to mechanistically uncover how LTK regulates ER export. To this end, we mapped the interactome of flag-tagged LTK expressed in HEK293 cells, because they do not express endogenous LTK and can be transfected easily. The most notable enrichment within the LTK interactome is proteins of the early secretory pathway (Fig. 4, A and B; and Table S1). This is consistent with the localization of LTK to the ER. The top associated gene ontology (GO) term among the LTK interactome was “Endoplasmic reticulum” (Fig. 4 A). Among the potential

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Figure 1. **Subcellular localization of LTK.** (A) Schematic illustrating the domains of LTK and ALK. LDLα, low-density lipoprotein (LDL) receptor class A repeat. (B) Phylogenetic tree of ALK and LTK kinase domains. Color ranges highlight invertebrate LTK/ALK-like proteins (yellow), vertebrate ALK proteins (orange), and vertebrate LTK proteins (green). Red squares indicate the presence of one or two MAM domains. Black circles mark branches with bootstrap support above 50%. Human ROS1 and placozoan insulin receptor–like kinase domains are used as outgroup. The list of abbreviations used in the figure can be found in the Materials and methods section. (C) Immunostaining of flag-tagged human LTK and endogenous CLIMP63 in HeLa cells. (D) Immunofluorescence staining of endogenous LTK and CLIMP63 in HepG2 cells. (E) HeLa cells expressing flag-tagged LTK or ALK were treated with PNGase F followed by lysis and immunoblotting against flag to detect ALK or LTK. (F) HeLa cells expressing flag-tagged LTK or ALK were lysed and the lysate treated with EndoH followed by immunoblotting against flag to detect ALK or LTK. (G) HeLa cells expressing GFP-tagged LTK were imaged using live microscopy. (H) HeLa cells expressing GFP-tagged LTK and mCherry-tagged Man-II in the RUSH system were treated for 0 or 2 h with biotin followed by fixation. Scale bars are 10 mm.
LTK interaction partners identified, we focused on Sec12 (also known as PREB), due to its well-established role in ER export and the biogenesis of ERESs (Barlowe and Schekman, 1993; Montegna et al., 2012; Saito et al., 2014). Sec12 is a type II transmembrane protein that acts as a guanine nucleotide exchange factor for Sar1. Using coimmunoprecipitation, we confirmed that LTK interacts with Sec12, but not with an unrelated transmembrane protein of the ER that was not recovered in the interactome (Fig. 4 and Fig. S3 A). Co-expression with LTK resulted in an increase in tyrosine phosphorylation of Sec12.

Figure 2. LTK regulates ER export and ERESs. (A) HepG2 cells were either subjected to LTK silencing with siRNA followed by fixation and staining after 72 h, or treated with crizotinib and alectinib (1 µM) for 30 min before fixation and immunostaining against Sec31 to label ERESs. (B) Quantification of ERES number per cell displayed as percentage of control (all values are set as percentage of siControl). Data are from three independent experiments with at least 35 cells per experiment per condition. Asterisk indicates statistically significant differences at P < 0.05. (C) HepG2 cells were transfected with control or LTK siRNA. After 72 h, cells were treated with solvent or with crizotinib (1 µM) for 30 min before staining for Sec31 to determine ERES number. (D) HeLa cells expressing flag-tagged LTK were treated with solvent or with crizotinib or alectinib for 30 min before lysis and immunoblotting as indicated. P-LTK indicates immunoblotting against an antibody that detects phosphorylation on Y672. Flag immunoblotting was performed to determine equal loading. (E) HeLa cells expressing GFP-Sec16A treated with 1 µM crizotinib followed by confocal live imaging. Stills of the indicated time points are depicted. The ERESs in the boxed area are depicted in black and white to enhance visibility. The number of ERESs was counted and is displayed in the lower graph. siLTK, LTK silenced. Scale bars are 10 mm.
which was crizotinib sensitive (Fig. 4 D). Two tyrosine residues in Sec12 (Y177 and Y10) were predicted by databases to be phosphorylated (PhosphoSitePlus and NetPhos3.1). Therefore, we mutated both tyrosine residues to phenylalanine, creating Sec12-Y10F and Sec12-Y177F. Sec12-Y10F was markedly less tyrosine-phosphorylated than wild-type Sec12, indicating the Y10 residue is a strong candidate site for phosphorylation by LTK (Fig. 4 D). Mutation of tyrosine 177 had no effect. We also noted that the Y10 residue in Sec12 is conserved in mammals but not in nonvertebrates (Fig. 4 E). We next purified the cytosolic domain of Sec12 and incubated it with an immunoprecipitate containing flag-LTK. Addition of ATP to the mix resulted in a crizotinib-sensitive increase of Sec12 phosphorylation (Fig. 4 F), supporting the notion that Sec12 phosphorylation is LTK-dependent. Inhibition of Src family kinases had only a marginally effect on Sec12 tyrosine phosphorylation (Fig. S3 B).

Because Sec12 is the exchange factor for Sar1, we next tested the effect of LTK inhibition on the dynamics of YFP-tagged Sar1A using FRAP microscopy. Cells were pretreated with solvent or with crizotinib for 20 min before FRAP microscopy. Inhibition of LTK reduced the mobile fraction of Sar1, indicative of a reduced exchange activity on single ERES (Fig. 5 A). No effect of crizotinib on general ER structure was detected (Fig. S3 C). We tried using the tryptophan fluorescence assay to monitor GTP exchange in Sar1 and its modulation by LTK. However, this assay cannot be used because the inclusion of ATP in the reaction (to promote Sec12 phosphorylation) distorted the assay (data not shown). Thus, we used a different approach to support the results of the FRAP assay, namely by immunostaining for Sar1-GTP-positive ERESs. This approach has been used by others previously (Venditti et al., 2012). Treatment of cells with crizotinib resulted in cells with fewer and fainter Sar1-GTP-positive puncta (Fig. 5 B), indicating that inhibition of LTK negatively affects the levels of active Sar1 on ERESs.

To obtain further support for a role of Sec12 phosphorylation in ERES function, we determined the number of peripheral ERGIC-53 structures in cells expressing the phosphoablating mutant of Sec12 (Sec12-Y10F). Peripheral ERGIC structures are good indicators of ERES function (Ben-Tekaya et al., 2005; Farhan et al., 2010). Expression of Sec12-Y10F resulted in a decrease in the number of ERGIC-53 puncta (Fig. 5 C). No effect of Sec12-Y10F expression was detected in LTK knockdown cells (Fig. 5 C). Altogether, we propose that Sec12 is phosphorylated in a manner dependent on LTK and that this phosphorylation affects ERES function.
Figure 4. LTK interacts with and phosphorylates Sec12. (A) Volcano plot of the interactome of HA-tagged LTK revealed by immunoprecipitation MS from HEK293 cells. The red labeled candidate interacting proteins are related to receptor tyrosine kinase signaling or ER-associated processes. The table indicates the top-scoring biological processes enriched among the LTK interaction partners. (B) Schematic representation of key components of the ER folding, quality control, and export machinery with red-highlighted interaction partners. Red box highlights ER export. (C) Flag-tagged LTK was immunoprecipitated followed by immunoblotting against Sec12, which was identified in the interactome and against RTN4, a transmembrane protein that we did not find in the LTK interactome. (D) In vitro kinase assays were performed on GST-Sec12, GST-Sec12-Y10F, or vector alone. The Y10 site on Sec12 was phosphorylated by LTK in the presence of ATP.

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Over the past decade, mounting evidence has indicated that endomembranes house a wide variety of signaling molecules such as GTPases, kinases, and phosphatases (Farhan and Rabouille, 2011; Cancino and Luini, 2013; Baschieri et al., 2014). An emerging concept of endomembrane signaling is autoregulation, which is defined as a response of a biological system that helps reestablish homeostasis. The UPR is the best understood and characterized autoregulatory response of the secretory pathway (Ron and Walter, 2007; Gardner et al., 2013), making it a useful template to compare other autoregulatory circuits with. The UPR is induced by misfolded or unfolded proteins, and its main purpose is to globally up-regulate the capacity of the endomembrane system to promote folding or degradation of these misfolded proteins. Such a broad response is expected because the purpose of the UPR is to maintain or reestablish global homeostasis of the ER. Another feature of the UPR is that its main sensors and mediators such as IRE1, ATF6, and PERK are resident to the ER. Contrary to the response of the ER to misfolded proteins, we know very little about whether and how local signaling at the ER controls the capacity of the ER to unload of folded proteins, i.e., of ER export. Very recently, a signaling cascade including Gα12 was shown to operate at ERESs...
(Subramanian et al., 2019). However, this signaling circuit controls the export of a small subset of proteins, and interfering with it had no effect on general protein secretion or on global ERES number. Thus, this novel pathway represents a tailored response of the ER, which is unlike the more global response of the UPR. Another difference to the UPR is that the main signaling mediator of this pathway Ga12 is not resident at the ER or ERES.

As far as LTK is concerned, our results indicate that it might be more similar to the UPR. First, LTK is resident in the ER, and second, the effect of LTK is a global regulation of trafficking and ERES number. This in line with the observation that LTK phosphorylates Sec12, a general regulator of ERES biogenesis. Thus, LTK is a strong candidate to be a general autoregulator of ER export. Future work will need to address the question concerning what stimuli activate LTK. Because the LTK interactome contained several cargo receptors such as ERGIC-53, VIF36, ERGL, ERGIC1, and SURF4, we speculate that these cargo receptors might represent stimuli that induce LTK activity to positively regulate ER export. Previous work has suggested that secreted ligands called FAM150A and FAM150B might act as ligands for LTK and ALK (Zhang et al., 2014; Guan et al., 2015; Reshetnyak et al., 2015). However, this is not compatible with our observation that LTK is resident to the ER. A potential reason for this discrepancy is that FAM150A and FAM150B are ligands for ALK and that none of the aforementioned papers tested the effects in an ALK-free background, or they have been used with fish LTK that rather resembles ALK than mammalian LTK (Fadeev et al., 2018).

Another important question for future investigations is how LTK is deactivated. In principle, receptor tyrosine kinases can be deactivated either by dephosphorylation or by degradation. A number of ER-resident phosphatases have been described, and our LTK interactome also contains few phosphatases. Because LTK is an ER export regulator, identifying a phosphatase that regulates LTK will further expand our understanding of the regulation of ER export by signaling molecules.

The role of LTK in secretion might also be relevant for human diseases. Gain of function mutations in LTK have been observed in patients and mice with systemic lupus erythematosus (Li et al., 2004). We speculate that this gain of function mutation confers a selective advantage to autoimmune plasma cells as it allows them to cope with a higher secretory load. LTK might also represent a suitable drug target in cancer therapy, especially since cancer cells are considered to be addicted to secretion due to a high proteostatic challenge (Dejeans et al., 2015; Urra et al., 2016). This notion is supported by our observation that LTK inhibition increases the ER stress response (as measured by increased XBPI levels) in cells treated with thapsigargin (Fig. S3 D). The investigation of the potential of LTK as a drug target will be an interesting area of future investigation.

**Materials and methods**

**Mass spectrometry (MS)**

Immunoprecipitated proteins were eluted from beads using repeated incubations with 8 M Guanidinhydrochloride at pH 8.0 and subjected to reductive alkylation (using 15 mM iodoacetamide and 5 mM DTT) and methanol/chloroform extraction followed by digestion with sequencing-grade trypsin (Promega) overnight at 37°C. Tryptic peptides were desalted and analyzed by liquid chromatography tandem MS using a NanoLC 1200 coupled via a nano-electrospray ionization source to a Q Exactive HF mass spectrometer. Peptide separation was performed according to their hydrophobicity on an in-house packed 18-cm column with 3-mm C18 beads (Dr. Maisch) using a binary buffer system consisting of solutions A (0.1% formic acid) and B (80% acetonitrile, 0.1% formic acid). Linear gradients from 7 to 38% B in 35 min were applied with a following increase to 95% B within 5 min and a reequilibration to 5% B. MS spectra were acquired using 36e as an AGC target, a maximal injection time of 20 ms, and a 60,000 resolution at 200 m/z. The mass spectrometer operated in a data-dependent Top15 mode with subsequent acquisition of higher energy collisional dissociation fragmentation MS/MS spectra of the top 15 most intense peaks. Resolution for MS/MS spectra was set to 30,000 at 200 m/z. AGC target to 1e5, maximum injection time to 64 ms, and the isolation window to 1.6 Th. Raw data files were processed with MaxQuant (1.6.0.1) as described previously (Cox and Mann, 2008; Cox et al., 2011) using human (UP000005640) UniProt databases, tryptic specifications, and default settings for mass tolerances for MS and MS/MS spectra. Carbamidomethylation at cysteine residues was set as a fixed modification, while oxidations at methionine and acetylation at the N terminus were defined as variable modifications. The minimal peptide length was set to seven amino acids, and the false discovery rate for proteins and peptide-spectrum matches to 1%. Perseus (1.5.8.5) was used for further analysis (Pearson’s correlation, two-sample t test) and data visualization. Functional annotation enrichment analysis was performed using the DAVID database (Huang et al., 2007) coupled to significance determination using Fisher’s exact test and correction for multiple hypothesis testing by the Benjamini and Hochberg false discovery rate.

**Immunofluorescence**

Cells were fixed in 3% paraformaldehyde for 20 min at room temperature. Afterward, cells were washed in PBS with 20 mM glycine followed by incubation in permeabilization buffer (PBS with 0.2% Triton X-100) for 5 min at room temperature. Subsequently, cells were incubated for 1 h with the primary antibody and after washing for another 1 h in secondary antibody diluted in 3% BSA in PBS. Cells were mounted in polyvinyl alcohol with DABCO antifade and imaged.

For Sar1-GTP staining, cells were fixed and permeabilized in ice-cold 50% methanol–50% acetone for 10 min at -20°C. Subsequently, cells were incubated for 1 h with the blocking buffer (PBS with 10% goat serum) at room temperature followed by incubation with primary antibody for 2 h and another 1 h with secondary antibody, both diluted in 3% BSA in PBS.

**Cell culture and transfection**

HeLa, HEK293T, and HepG2 cells were cultured in DMEM (GIBCO) supplemented with 10% FCS and 1% penicillin/streptomycin (GIBCO).
For overexpression of plasmids, cells were transfected with either Fugene 6 or with TransIT-LT1 (Mirus). For knockdown experiments, cells were reverse-transfected with 10 nM siRNA (final concentration) using HiPerfect (Qiagen) according to the manufacturer’s instructions.

Cell lysis, immunoblotting, and immunoprecipitation
Cells were washed twice with PBS and collected in lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 0.1% SDS, and 1% NP-40) supplemented with proteinase and phosphatase inhibitor (Pierce Protease and Phosphatase Inhibitor Mini Tablets, EDTA free). Lysates were incubated on ice for 10 min followed by clearing centrifugation at 20,000 xg at 4°C for 10 min. Supernatants were transferred into a fresh tube, and reducing loading buffer was added. Lysates were subjected to SDS-PAGE and transferred on a nitrocellulose membrane using semidy transfer. The membrane was blocked (in ROTI buffer [Roth] or 5% milk in PBS with 0.1% Tween) and probed with the appropriate primary antibodies. Subsequently, membranes were incubated with HRP-conjugated secondary antibody. Immunoblots were developed using a chemiluminescence reagent (ECL Clarity; BioRad) and imaged using ChemiDoc (BioRad). For immunoprecipitation experiments, cells were lysed in immunoprecipitation buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 10% glycerol, 0.5% NP-40, and n-dodecyl-B-D-maltoside).

PGNase F digestion and EndoH
For PGNase F digestion, 3.2 × 10⁵ HeLa cells were seeded into 6-well plates and the next day transfected with 1 µg plasmid DNA. 24 h later, cells were washed with PBS, and the cells were incubated in 1 ml serum-free medium and 250 U/ml PGNase F (P0704S; NEB) for 6 h. Subsequently, cells were lysed as described above.

For EndoH digestion, 2 × 10⁶ cells were plated into a 10-cm dish. After 24 h, cells were transfected with 3 µg plasmid DNA. The next day, cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 10% glycerol, 150 mM NaCl, 2 mM EDTA, and 0.5% Triton X-100) supplemented with proteinase and phosphatase inhibitor (Pierce Protease and Phosphatase Inhibitor Mini Tablets, EDTA free). Immunoprecipitation against flag was performed using EZview Red ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich) overnight at 4°C followed by washing in immunoprecipitation buffer. Beads were incubated with 1,000 U EndoH (P0702S; NEB) according to the manufacturer’s instructions for 90 min at 37°C. Subsequently, the reaction was stopped by adding reducing sample buffer.

Microscopes and image acquisition
Imaging was performed on laser scanning confocal microscopes: LeicaSP5 and Zeiss LSM700. All images were acquired using a 63× oil immersion objective (NA 1.4).

FRAP was performed on a LeicaSP5 confocal microscope using a 63×/1.4 NA oil-immersion objective at threefold digital magnification. All experiments were performed at 37°C, and cells were maintained in complete medium supplemented with 25 mM Hepes, pH 7.4. After acquisition of a prebleach image, the ERES was bleached at 100% laser intensity for 750 ms. After bleaching, images were acquired at one image per second. Images were analyzed using ImageJ. The fluorescence intensity of the ERES before bleaching was set to 100%, and all subsequent values were normalized to it. The mobile fraction was calculated as MF = (F∞−F∞₀)/(F₀−F∞₀), where F∞ is fluorescence in the bleached region after recovery, F₀ is the fluorescence in the bleached region before bleaching, and F∞₀ is the fluorescence in the bleached region directly after bleaching.

EREs were quantified as described previously (Tillmann et al., 2015).

Sequence analysis
The sequences of ALK and LTK kinase domains were aligned using the Muscle algorithm and Jalview environment (Edgar, 2004; Waterhouse et al., 2009). Phylogenetic trees were built using the PhyML program (Guindon et al., 2009) on the phylogeny.fr server (Dereeper et al., 2008) and visualized with the help of the iTol server (Letunic and Bork, 2016). Protein domains were detected using a conserved domain search (Marchler-Bauer et al., 2017). The following abbreviations were used in Fig. 1 B: alligator Am, Alligator mississippiensis; brachiopod La, Lingula anatina; chicken Gg, Gallus gallus; finch Tg, Taeniopygia guttata; frog Xt, Xenopus tropicalis; fruit fly Dm, Drosophila melanogaster; hemichordate Sk, Saccoglossus kowalevskii; lancelet BF, Branchiostoma floridae; lizard Ac, Anolis carolinensis; mouse Mm, Mus musculus; nematode Bm, Brugia malayi; plocozoan Ta, Trichoplax adhaerens; possum Md, Monodelphis domestica; sea urchin Sp, Strongylocentrotus purpuratus; shark Cm, Callorhinichus milii; starfish Ap, Acanthaster planci; and zebrafish Dr, Danio rerio.

Purification of GST-tagged Sec12 cytosolic domain
Escherichia coli BL21 (DE3) was transformed with GST-tagged Sec12 construct. Bacteria were cultured in HSG growth medium, and induction was performed with 0.4 mM IPTG. The bacterial pellet was dissolved in lysis buffer (50 mM Tris, pH 8, 150 NaCl, 10% glycerol, 0.1% Triton X-100, and 100 µg/ml lysozyme supplemented with proteinase and phosphatase inhibitor), sonified and centrifuged at 32,000 xg for 30 min at 8°C. Supernatant was incubated with Glutathione Sepharose 4 Fast Flow (GE Healthcare) for 2 h at 4°C and washed with PBS, and beads were resuspended in buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5% glycerol).

Kinase assay
HeLa cells expressing flag-tagged LTK were lysed, and LTK was immunoprecipitated using anti-flag M2 beads. The immunoprecipitate was resuspended in buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, and 10% glycerol). Typically, 5 × 10⁶ cells were used. The immunoprecipitate was incubated with 1.5 µg of GST or GST-tagged Sec12 cytosolic domain for 30 min at 30°C. To induce kinase activity, 400 µM ATP was included. The reaction was stopped by adding sample buffer.

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Quantitative PCR (qPCR)
The levels of LTK and ALK were determined by qRT-PCR. Total RNA was extracted using a Direct-Zol RNA kit (Zymo Research), and cDNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The expression of LTK and ALK was determined using the LightCycler 480 SYBR Green I Master Mix (Roche Life Science) and normalized to GAPDH using commercially available primers (Qiagen; for LTK, QT00219877; ALK, QT00028847; and GAPDH, QT00079247).

Reagents

Antibodies
A list of all used antibodies is provided in Table S2.

Primers
A list of all used primers is provided in Table S3.

Online supplemental material

Fig. S1 (related to Fig. 1) shows the plasma membrane localization of ALK as well as a reduction of endogenous LTK labeling in LTK-depleted cells to test for antibody specificity. Finally, this figure shows coimmunoprecipitation between LTK and Sec12 siRNAs and their effects on ERES numbers. Fig. S3 (related to Fig. 1 and Fig. 2) shows qPCR data of LTK and ALK expression in HepG2 and HeLa cells. In addition, it shows the results of the MS experiment of the LTK interactome cells as well as XBP1s levels in crizotinib-treated cells. Table S1 shows a FRAP of general ER in crizotinib-treated and crizotinib-treated cells. A list of all primers used is shown in Table S3. Video 1 and Video 2 show live imaging of a RUSH experiment in control and crizotinib-treated cells.

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Author contributions: F.G. Centonze and V. Reiterer performed and analyzed most experiments. K. Nalbach and C. Behrends performed and analyzed the MS experiments. K. Saito provided reagents and help conceive the Sec12 experiments. K. Pawlowski performed the evolutionary LTK analysis and produced the sequence logo. H. Farhan conceived the project, acquired the funding, planned and analyzed experiments, and wrote the manuscript with input from all authors.

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