Elucidation of the receptor tyrosine kinase LTK as a regulator of Proteostasis in response to folded proteins

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Per aspera, ad astra

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List of publications

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Abbreviations

AAT	alpha1-antitrypsin
ALCL	Anaplastic Large-Cell Lymphoma
ALK	Anaplastic Lymphoma Kinase
ALKAL	ALK-Activating Ligand
AML	Acute Myeloid Leukemia
APF1	ATP-dependent Proteolysis Factor-1
AREX	Auto-Regulation of ER export
ASK1	Apoptosis Signal-regulating Kinase 1
ATF6α	Activating Transcription Factor 6α
ATP	Adenosine triphosphate
ATZ	Alpha1-antitrypsin Z
BiP	Immunoglobulin binding protein
CatZr	Cathepsin Z related protein
CFFL	Coherent Feed-Forward Loop
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
ChIP	Chromatin Immunoprecipitation
CHIP	C- terminus of Hsp70 Interacting Protein
ChIP-seq	Chip sequencing
CHO	Chinese Hamster Ovary
CLSD	Craniolenticulo-Sutural Dysplasia
CMA	Chaperone-Mediated Autophagy
CNX	Calnexin
COPII	Coat protein complex type II
CRT	Calreticulin
DR5	Death Receptor 5
ECD	Extracellular Domain

EGF	Epidermal Growth Factor
EGFR	EGF Receptor
eIF2α	Eukaryotic Initiation Factor 2 α
ER	Endoplasmic Reticulum
ERAD	ER-Associated Degradation
ERES	ER Exit Sites
ERGIC	ER-Golgi Intermediate Compartment
ERGL	ERGIC-53-Like protein
ERK	Extracellularly Regulated Kinase
FKBP	FK506-Binding Proteins
Gab1	Grb2-Associated Binder 1
GABA	γ-aminobutyric acid
GABARAP	GABA Receptor-Associated Protein
GAP	GTPase-Activating Protein
GDP78	Glucose-regulated protein of 78 KDa
GEF	Guanine nucleotide Exchange Factor
GnRHR	Gonadotropin-Releasing Hormone Receptor
GT	Glycoprotein glucosyltransferase
Hsp	Heat shock proteins
IGF1	Insulin-like Growth Factor 1
Ig-HC	Immunoglobulin Heavy Chain
IgM	Immunoglobulin M
IRE1α	Inositol-Requiring Enzyme 1α
IRS1	Insulin Receptor Substrate-1
ITD	Internal Tandem Duplication
JNK	c-Jun NH2-terminal Kinase
LAMP-2a	Lysosome-Associated Membrane Protein 2a
LC3	Light Chain 3

LDLa	Low-Density Lipoprotein receptor class A domain
LIR	LC3-Interaction Region
LTK	Leucocyte Tyrosine Kinase
MAD	Mitochondria-Associated Degradation
MAM	Meprin/A5-protein/PTPmu
MAPK	Mitotic-Associated Protein Kinase
MDCK	Madin-Darby Canine Kidney
MM	Multiple Myeloma
mTOR	Mechanistic Target Of Rapamycin
MVB	Multi-Vesicular Body
NBD	Nucleotide Binding Domain
NGF	Nerve Growth Factor
NHK-AAT	Null Hong Kong-mutant of alpha1-antitrypsin
NPC	Nuclear Pore Complex
NPM	Nucleophosmin
NSCLC	Non-Small-Cell Lung Carcinoma
NTKR1	Neutrophic Tyrosine Kinase Receptor type 1
NZB	New Zealand Black
PC-I	Pro-Collagen I
PCTV	Pre-Chylomicron Transport Vesicles
PDI	Protein Disulphide Isomerase
PDIA4	Protein Disulfide Isomerase A4
PE	PhosphatidylEthanolamine
PERK	PRK-like ER Kinase
PI3KC3–C1	class III PI3-Kinase Complex I
PKA	Protein Kinase A
PKA-RIIa	PKA Regulatory subunit IIa
PKC	Protein-Kinase-C

PLC-γ	Phospholipase C-γ
PN	Proteostasis Network
PPI	Peptidyl-Prolyl Isomerases
PTB	Phosphotyrosine-Binding
PTP	Protein Tyrosine Phosphatase
RIDD	Regulated IRE1-Dependent Decay
RTK	Receptor Tyrosine Kinase
SBD	Substrate Binding Domain
SCF	Stem Cell Factor
SERT	Serotonine Transporter
SH2	Src Homology-2
Shc1	Src Homology 2 domain Containing transforming protein 1
SLE	Systemic Lupus Erythematosus
SREBP-2	Sterol Regulatory Element Binding Protein 2
tER	Transitional ER
TFG	Trk-Fused Gene
TKD	Tyrosine Kinase Domain
TKI	Tyrosine Kinase Inhibitor
TORC1	TOR Complex 1
TTR	Transthyretin
TXNIP	Thioredoxin-Interacting Protein
UBC6	Ubiquitin Conjugating enzyme 6
UDP-Glc	Uridine Diphosphate Glucose
ULK	Unc-51-Like autophagy-activating Kinase
UPR	Unfolded Protein Response
UPS	Ubiquitin-Proteasome System
VEGF	Vascular Endothelial Growth Factor
VIP36	Vesicular Integral-membrane Protein of 36 KDa

VIPL	VIP36-Like protein
VSV-G	Vesicular Stomatitis Virus ts045 G protein
XBP1	X-Box Binding Protein 1
XBP1s	XBPI spliced

Introduction

The Proteostasis Network (PN)

Proteins play roles in almost every biological process in a cell. From the moment the genome encodes the amino acid sequence of a polypeptide to the final stage when a properly folded and assembled protein performs its biological function, it is surely a long way. Although the amino acid sequence dictates a protein's native structure, the folding state can dynamically change in response to many environmental alterations, resulting in changes in protein biological activity. Accordingly, it is a big challenge for a cell to ensure proteome integrity. Therefore, all organisms from Bacteria to Eukarya have evolved a molecular network aimed to maintain protein homeostasis (proteostasis), the so-called Proteostasis Network (PN) (Powers & Balch, 2013). In the case of Eukarya, the components of the PN play direct role in protein synthesis, folding, trafficking, aggregation, disaggregation and degradation. According to this definition, the PN comprises the translational machinery, molecular chaperones and co-chaperones, the degradative pathways such as the ubiquitin-proteasome system (UPS) and autophagy and finally the machinery for membrane trafficking (Labbadia & Morimoto, 2015). Because the endoplasmic reticulum (ER) is a major site for proteostasis, it is conceivable that the machinery involved in export from ER plays a major role in orchestrating the PN (Routledge, Gupta, & Balch, 2010). Moreover, signaling pathways as the Unfolded Protein Response (UPR) are important modifiers of the PN and therefore play a critical role in proteostasis (Walter & Ron, 2011) (Fig.1).

Molecular chaperones and co-chaperones

The ER is the place in the cell where protein synthesis takes place. In order to function, newly synthesized proteins need to fold to their native three-dimensional structure (Dobson, Sali, & Karplus, 1998) and (in case of oligomeric proteins) assemble into higher-order complexes (Reddy & Corley, 1998). Once this is achieved, secretory proteins are then ready for export from the ER (Fig.1). Folding is an error-prone process that can lead to formation of cytotoxic aggregates. On top of that, contrary to small-single domain proteins that acquire their native structure immediately upon translation (Radford, 2000), the folding of large proteins is a very slow process. Therefore, cells have collected a set of dynamic molecular chaperones and co-chaperones which assist proteins during their folding process, promoting correct folding and preventing aggregation. This "proof-reading" system was termed as the ER quality control (Ellgaard & Helenius, 2003).

Chaperones were first defined as a family of additional proteins required for the correct assembly of other proteins, but not themselves part of their final structure (Ellis & Hemmingsen, 1989). In general they bind protein folding intermediates but not correctly folded proteins. This binding is an evaluation process to make sure that only native structures pass through to the secretory journey, while the non-

native either get corrected or eventually degraded, if terminally misfolded. The major part of chaperones is represented by the three families of the heat shock proteins (Hsp) Hsp70, Hsp40 and Hsp90. They generally bind their substrates via exposed hydrophobic domains which represent the signal for the chaperone indicating that the substrate is not in its native structure (Horwich, Neupert, & Hartl, 1990). Among the components of the Hsp70 family, BiP (immunoglobulin binding protein), also called glucose-regulated protein of 78 KDa (GDP78) (Ellgaard, Molinari, & Helenius, 1999), is the most studied and most abundant chaperone which plays several roles in the ER. BiP is involved in protein translocation of newly synthesized proteins into the ER by binding to them and preventing them from slipping back through the Sec61 translocation channel (Vogel, Misra, & Rose, 1990). Moreover, BiP plays roles in protein folding (Knittler & Haas, 1992) and is able to bind unfolded proteins and aggregates (Gething, McCammon, & Sambrook, 1986) (Bertolotti, Zhang, Hendershot, Harding, & Ron, 2000). Two domains of BiP determine its function as a chaperone: the N-terminal ATPase domain, also named nucleotide binding domain (NBD) and the C-terminal substrate binding domain (SBD). These domains are conserved among the Hdp70s family members (Saibil, 2008). When the NBD is loaded with ATP, BiP exhibits a low substrate affinity and in the ADP-bound state, BiP acquires a higher affinity for its substrates (Behnke, Feige, & Hendershot, 2015). BiP interacts with many co-factors, or co-chaperones that are able to regulate the binding of BiP to its substrates. For example, the ERdjs which are part of the HsP40s/DNAJ family (Qiu, Shao, Miao, & Wang, 2006) interact with BiP via their J-domain. They are able to induce BiP ATPase activity and to assist the recruitment of substrates to BiP (Kampinga et al., 2019).

Other two families of chaperone co-factors are represented by the thiol-disulfide oxidoreductases and the peptidyl-prolyl-isomerases. The former catalyse oxidation, isomerization and reduction of disulphide bonds whose formation is considered a crucial step during protein folding (Hatahet & Ruddock, 2007). The protein disulphide isomerase (PDI) is a large family of ER proteins with more than twenty members that play essential roles in disulfide bond formation and maintenance (Appenzeller-Herzog & Ellgaard, 2008). A member of the PDI family is ERp57 which associates with the chaperones calnexin and calreticulin (Ellgaard et al., 2001) (Frickel et al., 2002) (Leach, Cohen-Doyle, Thomas, & Williams, 2002), in order to recruit glycosylated substrates (Molinari & Helenius, 1999) (Oliver, Roderick, Llewellyn, & High, 1999). ERp57 acts as an oxidoreductase in close contact with glycoprotein folding intermediates to ensure proper disulfide bonding (Jessop et al., 2007). Peptidyl-prolyl isomerases (PPI) catalyse the cis-trans isomerization of peptidyl-prolyl bonds (Gothel & Marahiel, 1999) (Christis, Lubsen, & Braakman, 2008). There three main families of PPI: the cyclophilins, the FK506-binding proteins (FKBP) and the parvulins. Cyclophilins and

FKBP interact with chaperones in the ER. For example, in mouse, FKBP32 was shown to interact with BiP in a calcium dependent manner and to modulate its ATPase activity (X. Zhang et al., 2004) (Y. Wang et al., 2007). Cyclophilins were shown to be part of the chaperoning network in the ER (Meunier, Usherwood, Chung, & Hendershot, 2002).

Most secretory proteins undergo N-linked glycosylation as they are translated and translocated into the ER (Huh et al., 2003) (Apweiler, Hermjakob, & Sharon, 1999). As a glycoprotein folds and matures, the modifications of the glycans affect the glycoprotein's interaction with carbohydrate- binding proteins resident to the ER, thereby altering folding (Zielinska, Gnad, Wisniewski, & Mann, 2010). The lectin chaperones calreticulin (CRT) and calnexin (CNX) represent the N-glycan- dependent quality control system (Caramelo & Parodi, 2015) and ensure correct folding of glycosylated cargo in the ER. CRT is a luminal protein and CNX is a type I membrane protein. They are both composed of a P-domain and a globular domain which is responsible for sugar binding, CNX and CNX bind ATP but do not possess ATPase activity (Ou, Bergeron, Li, Kang, & Thomas, 1995) (Corbett et al., 2000). The initial step in the N-glycan quality control is the addition of the 14 saccharide core unit (Glc3Man9GlcNAc2) from a dichol P-P derivative to an Asn residue in an N-X- S/T motif in a nascent polypeptide chain (C. Hammond & Helenius, 1994). Once attached to the protein, the glycan is rapidly restructured by ER resident glycosidases (I and II) that further modify this glycan chain in order to obtain the one that serves as substrate for calnexin and calreticulin. The composition of glycans act as tag indicating the status and age of the protein to which they are attached (Helenius & Aebi, 2004) (Hebert, Garman, & Molinari, 2005). The mechanism of how CNX nd CRT interact with their folding substrates is debatable. Indeed, two models have been proposed: the "lectin-only" and the "dual binding" model. According to the "lectin only" model (Hebert, Lamriben, Powers, & Kelly, 2014) (C. Hammond, Braakman, & Helenius, 1994), CNX and CRT initial binding to substrates occurs following the trimming of the precursor form by the action of glucosidases I and II. If the protein is not correctly folded, it undergoes reglucosylation by the UDP-Glc: glycoprotein glucosyltransferase (GT), which acts as folding sensor (Hebert, Foellmer, & Helenius, 1995) (Sousa & Parodi, 1995). According to this model, CNX and CRT interaction with their substrates is primarily mediated by monoglucosylated glycans, independent of whether the proteins are in their native structure or not. CNX or CRT would also not be able to suppress aggregation according to this model. Instead, they recruit other folding factors such as ERp57 which was shown in vitro to promote the formation of disulfide bonds within monoglucosylated RNase B that is bound to the lectin site of CNX or CRT (Trombetta & Helenius, 2000). The "dual binding" model follows pretty much the central principle of the lectin-only model but, in addition, proposes the existence of a second substrate binding site on CNX/CRT that recognizes exposed hydrophobic patches of non-native glycoproteins (Zapun et al., 1998) (Ihara, Cohen-Doyle, Saito, & Williams, 1999). According to this model, substrate and chaperones interaction depends not only on the action of glucosidase II but a change in affinity of the

polypeptide binding site, possibly regulated by a shift from an ATP-bound to an ADP-bound or unbound state (Ware et al., 1995) (Y. Saito, Ihara, Leach, Cohen-Doyle, & Williams, 1999). The main difference between the models is that, in the second model, CNX and CRT acquire the classical function of molecular chaperones by suppressing aggregation in addition to being capable of recruiting folding factors such as ERp57, as stated in the first model as well. If correct folding does not happen, GT reglucosylates the non-native glycoproteins back to its monoglucosylated form, allowing it to re-enter the CNX/CRT cycle and obtain the correct folding. In case the protein is terminally misfolded, it will be targeted to ER associated degradation (ERAD).

ER-associated degradation (ERAD)

In general, short-lived proteins are degraded by proteasomes, whereas lysosomes are responsible for the degradation of long-lived proteins. Proteins that fail to fold correctly in the ER were shown to be largely degraded by targeting to the proteasome, a process referred to as ERAD (Fig.1). The discovery of the lysosome (De Duve, Gianetto, Appelmans, & Wattiaux, 1953) (Gianetto & De Duve, 1955), an organelle that contains a broad array of proteases with different specificities, led to the assumption that cellular proteins must be degraded in this organelle. However, later on, several experimental evidences indicated that degradation of most cellular proteins was actually mediated by a non-lysosomal machinery. This revolutionary hypothesis was further supported by the discovery that rabbit reticulocytes, that do not contain lysosomes, efficiently degrade abnormal hemoglobin (Rabinovitz & Fisher, 1964). When later, a cell-free proteolytic preparation from reticulocytes was isolated, it was found that the degrading extract was functioning at a neutral pH, suggesting that the proteolytic activity was not lysosomal (Etlinger & Goldberg, 1977). Shortly afterwards, it was discovered that ubiquitin, initially designated ATP-dependent proteolysis factor-1 (APF1), is covalently conjugated to protein substrates and stimulates their proteolysis in the presence of ATP (Ciehanover, Hod, & Hershko, 1978) (Ciechanover, Heller, Elias, Haas, & Hershko, 1980) (Hershko, Ciechanover, Heller, Haas, & Rose, 1980). Ubiquitylation is a cascade of reactions starting with an enzyme-1 (E1) which represents the ubiquitin-activating enzyme, E2, which transfers ubiquitin and E3, the ubiquitin-protein ligase and specific substrate-binding component of the cascade (Ciechanover, Elias, Heller, & Hershko, 1982) (Hershko, Heller, Elias, & Ciechanover, 1983). While ubiquitylation can have various biologic consequences, the one relevant to my work is that it targets proteins to the 26S proteasome (Hough, Pratt, & Rechsteiner, 1986) which consist of a 19S regulatory cap and a 20S proteolytic core. The 19S cap recognizes ubiquitylated substrates, removes ubiquitin chains, and unfolds the client to allow entry into the 20S core, where it is degraded (Coux, Tanaka, & Goldberg, 1996). The proteasomes localizes to both cytosol and nucleus, and plays a key role in proteostasis. Around 30% of all newly synthesized proteins were shown to be degraded through ERAD (Schubert et al., 2000). First

evidence supporting this hypothesis came from findings that unassembled subunits of the T cell receptor were degraded in a lysosomal-independent way (Lippincott-Schwartz, Bonifacino, Yuan, & Klausner, 1988). This led to the idea that the ER itself would house unidentified protease targeting misfolded proteins. Subsequent work in yeast showed that a loss of function mutants of an ubiquitin conjugating enzyme (UBC6) suppress the protein translocation defect caused by a mutation in SEC61 (Sommer & Jentsch, 1993). This implied a role of the proteasome in ERAD. Later work showed that the ubiquitin–proteasome system is involved in ER protein quality control in mammalian cells as well. These studies were conducted cystic fibrosis transmembrane conductance regulator (CFTR), a polytopic membrane protein with an inefficient folding process. Inhibition of proteasome function led to accumulation of polyubiquitinated CFTR, suggesting that its degradation is dependent on ubiquitination (Jensen et al., 1995) (C. L. Ward, Omura, & Kopito, 1995). Soon after, it became clear this mechanism also applied to the degradation of the luminal misfolded proteins CPY* (Hiller, Finger, Schweiger, & Wolf, 1996).

ER molecular chaperones are required for ERAD and facilitate the process. Indeed, molecular chaperones as BiP maintain the solubility of the ERAD substrates keeping them in a retrotranslocation-competent state (Nishikawa, Fewell, Kato, Brodsky, & Endo, 2001). BiP substrates subsequently interact with the cytosolic protein Herp. It was shown that depletion of Herp inhibits the degradation of the non-glycosylated BiP substrates, whereas it had no effect on the degradation of the glycosylated calnexin substrates (Okuda-Shimizu & Hendershot, 2007). This suggests that there is some difference in how ERAD deals with these two types of substrates. Chaperones can help ERAD in other situations as well. It's the case of the misfolded membrane proteins with a large cytosolically localized portion as CFTR that associates with cytoplasmic Hsc70. Dissociation of CFTR from Hsc70 leads to an increased export of the protein. Here, the chaperone Hsc70 interacts with its co-factor, the E3-ubiquitin-protein ligase CHIP to mediate the degradation of CFTR (Jiang et al., 1998) (Rubenstein & Zeitlin, 2000). In yeast, these E3 ligase complexes are well characterized. Doa10 (Swanson, Locher, & Hochstrasser, 2001) and Hrd1 (Bordallo, Plemper, Finger, & Wolf, 1998) (Bays, Gardner, Seelig, Joazeiro, & Hampton, 2001) which assemble into the Doa10 and the Hrd1 complexes, respectively, are responsible for the degradation of different classes of ERAD substrates (Carvalho, Goder, & Rapoport, 2006). Proteins with misfolded domains in the cytoplasmic side of the membrane (ERAD-C substrates) are degraded via the Doa10 complex. Proteins with luminal (ERAD-L substrates) or intramembrane (ERAD-M substrates) misfolded domains are targeted to the Hrd1 complex (Taxis et al., 2003) (Vashist & Ng, 2004) (Schulze et al., 2005). In mammalian cells the best-studied E3 ligases are Hrd1 and Gp78 which are both homologous to yeast Hrd1 but assemble into different E3 ligase complexes that target different substrates (Mueller, Klemm, Spooner, Claessen, & Ploegh, 2008) (Bernasconi, Galli, Calanca, Nakajima, & Molinari, 2010) (Christianson et al., 2011) (Dai & Li, 2001).

Once selected, ERAD substrates are retrotranslocated from the ER lumen or membrane back to the cytosol where they are delivered to and degraded by the 26S proteasome. Initially, the Sec61 translocation channel was the best candidate for a retrotranslocation channel (Pilon, Schekman, & Romisch, 1997) (Romisch, 1999). Two more recent publications question the contribution of Sec61 to protein export from the ER and propose instead that a protein with four transmembrane domains, Derlin-1 (Der1p in yeast), forms the export channel (Lilley & Ploegh, 2004) (Y. Ye, Shibata, Yun, Ron, & Rapoport, 2004). Indeed, two transmembrane proteins, Derlin-1 and VCP-interacting membrane protein (VIMP) were found to interact with the hexameric AAA-ATPase chaperone complex formed by p97, also called VCP (Cdc48p in yeast), which is required for ERAD of many substrates (Y. Ye et al., 2004). The AAA+ ATPase (VCP)/p97 complex forms an hexameric ring and each subunit contains a regulatory N-terminal domain and two ATPase domains, D1and D2 which empower p97 to impose conformational changes on substrate proteins (Peters, Walsh, & Franke, 1990). This complex is involved in many cellular processes and its best-studied role is linked to ERAD. Indeed, p97 associates with the ER membrane and pulls the ERAD substrates through its central pore out of the ER. The ring is able to recognize directly the ubiquitin tag on substrates (Rape et al., 2001) (Rabinovich, Kerem, Frohlich, Diamant, & Bar-Nun, 2002) (Jarosch et al., 2002) and a chain of at least four ubiquitins is needed for the substrate to be sent out (Kim, Mi, & Rao, 2004). This polyubiquitin tag is then recognized by other proteins that escort the substrate to the proteosome (Medicherla, Kostova, Schaefer, & Wolf, 2004) (Richly et al., 2005) (Kim et al., 2006) (Raasi & Wolf, 2007) (Marza et al., 2015). VCP/p97 also extracts ubiquitin-modified proteins from mitochondrial outer membrane in a process termed mitochondria-associated degradation (MAD) (Heo et al., 2010) (S. Xu, Peng, Wang, Fang, & Karbowski, 2011) (Karbowski & Youle, 2011). In addition to these pathways, p97 also facilitates degradation of soluble proteins in the cytosol (Y. Xu, Anderson, & Ye, 2016). Besides proteasomal pathways, p97 also has an impact on the lysosomal system and autophagy. Several reports indicate that p97 and its cofactors are involved in sorting ubiquitinated cargo along the endolysosomal pathway (Ritz et al., 2011) (Ramanathan & Ye, 2012) (Ren, Pashkova, Winistorfer, & Piper, 2008). P97 seems to be involved, through many ways, in maintenance of proteostasis mainly through promoting protein degradation.

Autophagy

Although the proteasome can degrade a broad array of proteins and thereby represents the primary player for protein degradation in the cell, it shows many limitations when it comes to degradation of large protein aggregates. Proteins need to be unfolded before they can access the proteasome's catalytic core. Therefore, protein aggregates that resist unfolding by chaperone activities must be degraded elsewhere. For example, disease-associated forms of huntingtin with long polyglutamine

tracts are degraded in the lysosome (Bhutani, Piccirillo, Hourez, Venkatraman, & Goldberg, 2012). Other examples related to mutant forms of gonadotropin-releasing hormone receptor (GnRHR) (Houck et al., 2014) and mutant alpha1-antitrypsin Z (ATZ) (Hidvegi et al., 2010) prefer a lysosome-dependent way of degradation. Lysosomes are membrane-bound organelles containing an array of proteases, such as cathepsins (Turk et al., 2012). Here proteins are hydrolysed and free amino acids can then be re-used in cellular metabolism. Cytoplasmic proteins and organelles are trafficked to lysosomes by autophagy, which was first described as a cellular process where a membrane-bound structure (the autophagosome) engulfs and recycle cytoplasmic components (De Duve & Wattiaux, 1966). Autophagy can be divided into three different pathways: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy (Kroemer, Marino, & Levine, 2010). Extracellular proteins and misfolded plasma membrane proteins are directed to lysosomes through the endocytic pathway. The detection of misfolded plasma membrane proteins is mediated by the CHIP (C- terminus of Hsp70 interacting protein) ubiquitin ligase, which promotes the ubiquitination of misfolded substrates and is thus required for subsequent endocytosis and lysosomal trafficking events (MacGurn, 2014).

Macroautophagy (referred to as autophagy) is the best-studied autophagic pathway and is characterized by the sequestration of organelles or cytosolic macromolecules into a double-membrane vesicle structure known as an autophagosome. It can be divided into two subtypes: non selective (bulk) and selective autophagy. Through nonselective autophagy, cells engulf a portion of their cytoplasmic content in response to deprivation of nutrients in order to recycle building blocks of macromolecules such as amino acids and compensate for the lack of nutrients. Autophagy was first believed to be nonselective toward its substrates, being a bulk response to starvation. However, the molecular mechanisms of selectivity started recently to emerge. Autophagy can selectively target cellular components for degradation and depending on the type of cargo, it can mediate the degradation of protein aggregates (Aggrephagy), damaged organelles such as peroxisomes (Pexophagy), the ER (ER- phagy), or mitochondria (Mitophagy) (Farre & Subramani, 2016) (Svenning & Johansen, 2013).

During the initiation step in autophagy, a conserved and mostly cytosolic machinery orchestrates the biogenesis of the autophagosome. This machinery which consists of the Ser/Thr kinase ULK (unc-51-like autophagy-activating kinase) complex (Atg1 in yeast) and the class III PI3-kinase complex I (PI3KC3–C1), interacts with membranes and shapes them to generate an autophagosome (Carlsson & Simonsen, 2015). Mammalian ULK1 forms a complex together with the noncatalytic subunits ATG13, ATG101, and FIP200. In yeast, the kinase Atg1 forms a complex with Atg13, Atg17, and the yeast-specific subunits Atg29 and Atg31. Inhibition of mTOR (mechanistic target of rapamycin) activates the ULK1/Atg1 complex leading to recruitment of the class III phosphatidylinositol 3 kinase complex which comprises the PI3-kinase Vps34/PIK3C3, Atg14/ATG14L, Atg6/Beclin-1, and Vps15/PI3R4. Formation of PI3P on the ER is required for recruitment of WIPI2 (Atg18 together with Atg2 in

yeast). This leads to the conjugation of ATG8 to phosphatidylethanolamine (PE) in the isolation membrane with the help of the ATG12 conjugation complex composed of ATG12, ATG5, and ATG16L1.

Selective autophagy pathway requires another step in which selectivity of sorting the cargo into the autophagosome is mediated by several autophagy receptors. The first selective autophagy receptor discovered was the mammalian protein p62 (SQSTM1) (Bjorkoy et al., 2005) (Pankiv et al., 2007) which was shown to be responsible for the degradation of ubiquitylated proteins. This receptor contains a LC3-interaction region (LIR) that mediates the interaction with the nascent autophagosome. Many other autophagy receptors contain the LIR region and their ability to bind to LC3 is fundamental for the selectivity of the process and to link cargo and the autophagosome. For example, mammals have six Atg8 family members known as the light chain 3 (LC3) or γ-aminobutyric acid (GABA) receptor-associated proteins (GABARAP) (Slobodkin & Elazar, 2013) (Weidberg et al., 2010). However, the mechanistic details on how these different LC3 variants contribute to the specificity of selective autophagy were not yet revealed.

CMA involves the identification of misfolded proteins that show a five-amino-acid motif with the consensus sequence KFERQ (Lys-Phe-Glu-Arg-Gln). Approximately one-third of soluble cytosolic proteins have this motif which is hidden in natively-folded proteins and exposed when they are misfolded. Here, the exposed KFERQ-like motifs are recognized by the chaperone Hsc70. The substrate protein is then unfolded and translocated across the lysosome membrane by Hsc70. This process requires Hsc70 on both sides of the lysosomal membrane and the binding of the substrate protein to LAMP-2a (lysosome-associated membrane protein 2a). This binding leads to the multimerization of LAMP-2a facilitating the translocation of the protein substrate across the lysosomal membrane (Cuervo, 2010). The difference between macroautophagy and CMA is that the former does not need to first unfold the substrates with chaperones in order to send them to lysosome. For this reason macroautophagy can complement proteasome and CMA by degrading proteins and aggregates that resist unfolding by chaperone activities.

The Unfolded Protein Response (UPR)

The Unfolded Protein Response (UPR) is activated upon the accumulation of unfolded or misfolded proteins in the ER. The purpose of the UPR is to restore ER homeostasis by reducing unfolded protein load through promotion of protein folding and/or degradation (Fig.1). The ER-stress induced response was first discovered in yeast (Kozutsumi, Segal, Normington, Gething, & Sambrook, 1988) (Mori, Ma, Gething, & Sambrook, 1993) (Cox & Walter, 1996). In the 1990s, the discovery of the three main mammalian UPR sensors inositol-requiring enzyme 1α (IRE1α) (Tirasophon, Welihinda, & Kaufman,

1998), PRK-like ER kinase (PERK) (Haze, Yoshida, Yanagi, Yura, & Mori, 1999) and activating transcription factor ATF6α (J. Shen, Chen, Hendershot, & Prywes, 2002) represented a big achievement in the field. Detection of misfolded proteins in the lumen of the ER by these sensors leads to activation of downstream signaling pathways. The detection is partly dependent on the ER chaperone BiP/GRP78, which binds the three main players of this pathway: ATF6, IRE1 and PERK (Bertolotti et al., 2000) (Saibil, 2008) (Behnke et al., 2015). In the BiP-bound state, these sensors are inactive, but an increase of unfolded proteins titrates BiP away leading to activation of these sensors and induction of the UPR (Marquardt & Helenius, 1992) (J. Shen, Snapp, Lippincott-Schwartz, & Prywes, 2005) (Okamura, Kimata, Higashio, Tsuru, & Kohno, 2000) (Carrara, Prischi, Nowak, Kopp, & Ali, 2015). The ability of BiP to bind the UPR sensors appears to be independent of its chaperon activity, suggesting an allosteric regulation (Scheuner et al., 2001).

Generally, all three ER stress sensors (PERK, IRE1α, ATF6) activate signaling events that increase protein-folding capacity and reduce protein load on the ER. Active PERK phosphorylates the translation initiation factor eIF2α, leading to attenuation of global protein synthesis (Harding et al., 2000) (Harding et al., 2003). Inhibition of eIF2α leads to a selective translation of ATF4, a transcription factor regulating genes involved in many cellular processes such as protein folding, autophagy and apoptosis (J. Ye & Koumenis, 2009) (Ron & Walter, 2007). ATF6α is a transmembrane protein that translocates to the Golgi upon ER stress (J. Shen et al., 2002) where it encounters the proteases S1P and S2P (Yamamoto et al., 2007). The ATF6α cytosolic domain (ATF6f) translocates to the nucleus where it acts as transcription factor leading to upregulation of ERAD components (X. Shen et al., 2001) as well as regulators of membrane trafficking such as ERGIC-53 (Nyfeler, Nufer, Matsui, Mori, & Hauri, 2003). Finally, the last arm of the UPR is represented by ER transmembrane protein IRE1α, which has kinase and endoribonuclease (RNase) activities associated with its cytoplasmic tail. Once unbound to BiP, IRE1α dimerizes and trans-autophosphorylates, inducing a conformational change that activates the RNase domain which catalyzes the excision of a 26-nucleotide intron within the XBP1 mRNA, shifting the reading frame resulting in a translation of a transcription factor known as XBP1s (Yoshida, Matsui, Yamamoto, Okada, & Mori, 2001) (K. Lee et al., 2002) (Calfon et al., 2002) (Hetz, Martinon, Rodriguez, & Glimcher, 2011). XBP1s regulates genes involved in protein folding, secretion, ERAD and lipid synthesis (Acosta-Alvear et al., 2007) (A. H. Lee, Iwakoshi, & Glimcher, 2003) (Shoulders et al., 2013). XBP1s was also shown to heterodimerize with ATF6f to regulate gene expression (Maurel, Chevet, Tavernier, & Gerlo, 2014). IRE1α activity is also involved in the degradation of many RNAs (known as Regulated IRE1-dependent decay or RIDD (Ghosh et al., 2014)). The ability for IRE1α to switch from XBP1 splicing to RIDD is by its oligomeric state (Tam, Koong, & Niwa, 2014). Indeed, when IRE1α exists in dimer and tetramer complexes, its RNase activity is largely restricted to XBP1 splicing. Instead, under high or chronic ER stress, IRE1α forms higher oligomers leading to an expanded RNase activity to many

ER-localized mRNAs through RIDD. Contrary to this, another study suggests that XBP1 splicing requires obligate IRE1α oligomers, but that IRE1α dimers suffice for RIDD (Rubio et al., 2011). So far, there are evidences of a correlation between IRE1α RNase activity and its phosphorylation status (Chawla, Chakrabarti, Ghosh, & Niwa, 2011) (Prischi, Nowak, Carrara, & Ali, 2014) although many identified phosphorylation sites have not yet been functionally tested. Many studies have proposed the tRNA ligase RtcB to be involved in ligating the spliced XBP1 mRNA, thereby generating XBP1s (Jurkin et al., 2014) (Kosmaczewski et al., 2014) (Y. Lu, Liang, & Wang, 2014) (Ray, Zhang, Rentas, Caldwell, & Caldwell, 2014).

Activation of the three arms of the UPR aims to reduce the misfolding burden on the ER thereby allowing the organelle to recover homeostasis through an attenuation of translation of newly synthesized proteins entering the ER. However, when UPR fails in restoring ER proteostasis, a terminal UPR is activated leading to apoptosis (Shore, Papa, & Oakes, 2011). Under chronic ER stress, activated PERK upregulates the transcription factor CHOP/GADD153, leading to inhibition of the expression of the anti-apoptotic BCL-2 to promote cell death (McCullough, Martindale, Klotz, Aw, & Holbrook, 2001) (Marciniak et al., 2004). Moreover, CHOP and ATF4 can cooperate to regulate factors that enhance protein synthesis, and contribute to cell death through ROS production and ATP depletion (Han et al., 2013). Recently it has been shown that PERK activation increases expression of death receptor 5 (DR5) to trigger caspase-8 induced cell death (M. Lu et al., 2014). As already stated, chronic ER stress causes IRE1α to transition to higher oligomeric structures and this switch seems to activate its apoptotic program (Tam et al., 2014). In this situation, the RNase activity of IRE1α decreases the levels of several mRNAs and microRNAs that normally suppress pro-apoptotic targets such as pro-oxidant protein TXNIP (thioredoxin-interacting protein) and caspase-2, leading to their upregulation (Lerner et al., 2012). Moreover, IRE1α assembles into an activation platform for apoptosis signal-regulating kinase 1 (ASK1) and its target c-Jun NH_2-terminal kinase (JNK) (Urano et al., 2000) (Nishitoh et al., 2002). Despite all this advance, we still do not perfectly understand the conditions under which chronic ER stress induces cells death and the timing at which each of the aforementioned mechanisms is involved. For instance, the conversion of B-lymphocytes to plasma cells also leads to chronic ER Stress, without immediately killing the cells. Many cancer cells, exhibit chronic ER stress, which appears to increase their fitness. Thus, more work needs to be done to understand how much ER stress is needed and for how long in order to promote apoptosis versus cell survival.

Although UPR regulators are generally considered to positively modulate secretion, they were also shown to have a proteostatic effect by inhibiting secretion in special cases. Secretion of the amyloidogenic protein transthyretin (TTR) results in formation of extracellular toxic aggregates. Activation of ATF6 was reported to inhibit secretion of disease-associated TTR variants (J. J. Chen et al., 2014). This effect was due to increased targeting of mutant TTR to ERAD. A similar effect was

also reported for XBP1s that prevented secretion of amyloidogenic variant of immunoglobulin light chain (ALLC) associated with light chain amyloidosis by targeting it for ERAD (Cooley et al., 2014).

Thus, UPR regulators can exert their proteostatic effect by promoting degradation and thereby preventing secretion of potentially harmful proteins species. This mechanism was termed "Secretory Proteostasis" by the Wiseman group (Plate & Wiseman, 2017).

Export from the ER

Soluble, GPI-anchored and transmembrane secretory proteins that have reached properly folded and assembled conformations are then ready to be exported from the ER (Fig.1). These proteins are packed into coat protein complex type II (COPII) vesicles which form on ER exit sites (ERES). Vesicles traffic to the ER-Golgi intermediate compartment (ERGIC) and subsequently, to the Golgi apparatus where proteins are then sorted into carriers to reach their final destination. Transmembrane proteins with cytosolic domains are capable of binding directly to the COPII coat. However, GPI- anchored and soluble proteins have to be captured by transmembrane cargo receptor proteins, which mediate concentration of the cargo in ERES and its incorporation into COPII carriers. However, the number of cargo receptors that have been identified so far is rather small number compared to the thousands of cargo molecules that are transported from the ER. This means that either more cargo receptors await to be discovered, or that cargo receptors exhibit a high degree of promiscuity (i.e. a cargo receptor binds to several cargos) or that soluble cargo exits the ER in a manner independent of cargo receptors. This latter mechanism is referred to as bulk flow. According to this model, soluble cargo passively distribute between the ER and the transport vesicles, resulting in equal cargo concentration within these two compartments. They move out of the ER as part of the bulk fluid or membrane (Barlowe & Helenius, 2016). One problem with the bulk flow model is that there is not enough reliable data proving whether the efficiency of bulk flow from the ER is good enough to support the rates observed in protein export. The first study that introduced bulk flow as a potential mechanism for selective ER export was conducted in 1987 (Wieland, Gleason, Serafini, & Rothman, 1987). Bulk fluid flow was measured by adding to cells, membrane-permeable, iodinated acyl- tripeptides that contained the acceptor sequence for *N*-linked glycosylation. Through this approach, the secretion of the *N*-glycosylated peptide was measured over time. It turned out that the peptide was not efficiently secreted because only a small fraction proceeded through the Golgi complex. A more recent pulse-chase study introduced a new method to analyze bulk fluid flow in tissue culture cells. A virus-encoded cytosolic protein tracer was used in Chinese hamster ovary (CHO) and Madin-Darby canine kidney (MDCK) type II cells (Thor, Gautschi, Geiger, & Helenius, 2009). It was shown that the first labeled tracer was detected in the extracellular fluid within 12 min after synthesis, and the $t1/2$ of secretion was 40 min that means that an amount of fluid equivalent to half of the ER volume is

transported out of the ER every 40 min. The problem with this paper is that it relies on a viral protein as a tracer and it is conceivable that this protein has evolved to highjack the secretory machinery.

A third transport mechanism of protein export was shown to be relevant for cargo membrane proteins. This model involves partitioning within the lipid bilayer (Lippincott-Schwartz & Phair, 2010) (Hanulova & Weiss, 2012). Transport by partitioning is different from both bulk flow because by partitioning cargo can be concentrated in transport carriers but it does not depend on specific cargo receptors as for the receptor-mediated transport mode.

In yeast, COPII vesicles are supposed to fuse with the Golgi. However, in mammals, the ERGIC the acceptor compartment for COPII carriers. It was proposed that COPII vesicles may either undergo homotypic fusion or may hetertypically fuse with a preexisting ERGIC (D. Xu & Hay, 2004). The homotypic fusion of COPII vesicles was shown to depend on the tethering complex TRAPPI that interacts with Sec23 (Cai et al., 2007). Recent work suggests that COPII carrier tethering is mediated by Trk-fused gene (TFG) (Hanna et al., 2017) (Johnson et al., 2015) (Witte et al., 2011). Depletion of TFG was shown to lead to the accumulation of COPII carriers in the cytoplasm, and no longer restricted to the ER/ERGIC interface (Hanna et al., 2017) (Johnson et al., 2015). TFG binds directly to Sec23 with high affinity (Johnson et al., 2015) suggesting its potential role in tethering of COPII-coated transport intermediates at the ER/ERGIC interface with specificity. Beyond binding to Sec23 and tethering COPII carriers, TFG was also shown to interact with ALG-2 in a calcium-dependent manner (Kanadome, Shibata, Kuwata, Takahara, & Maki, 2017).

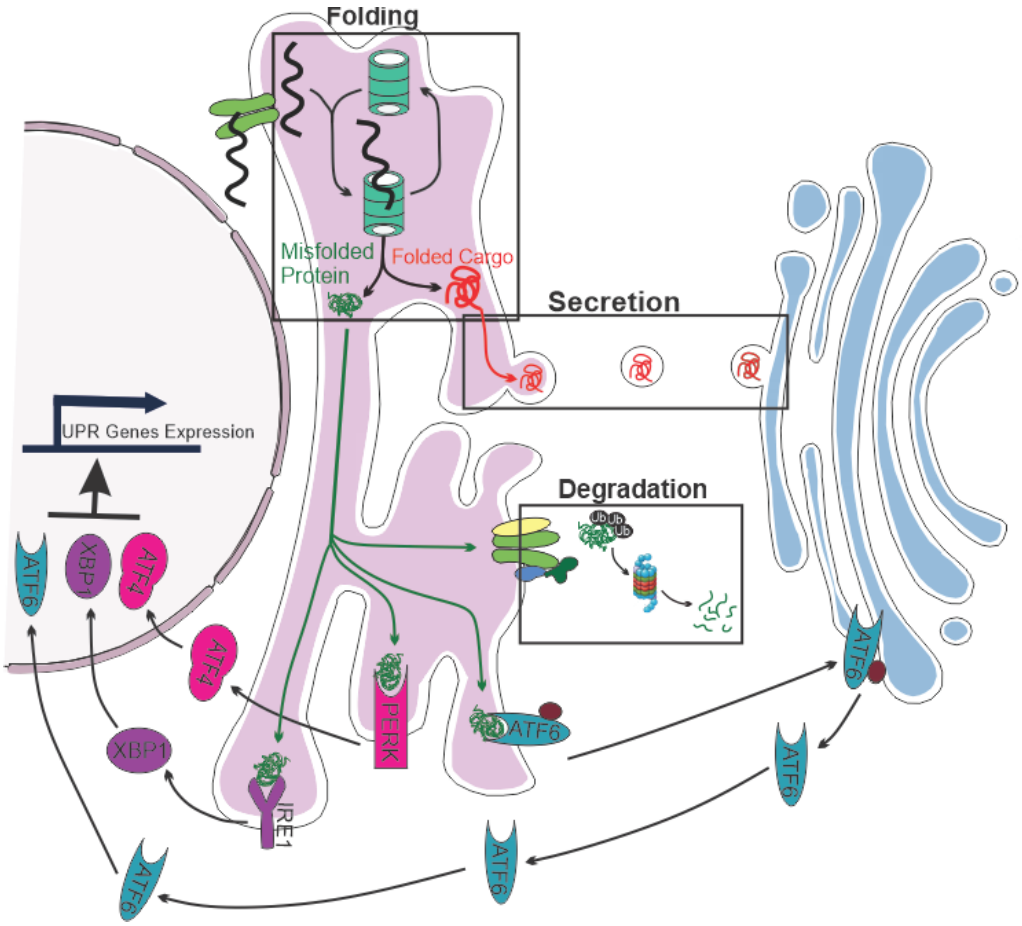


Figure1: ***Proteostasis is the result of a balance between protein folding, trafficking and degradation:*** *Newly synthesized proteins enter the ER in non-native conformations and engage ER-localized chaperones and folding factors that promote their folding. Once properly folded, these proteins are packaged into vesicles for trafficking to their final destination. Proteins that fail to fold in the ER are directed towards degradation pathways such as ER-associated degradation where they are retrotranslocated from the ER to the cytosol and degraded by the ubiquitin-proteasome pathway. The signaling pathways activated downstream of the three UPR sensors IRE1α, PERK and ATF6α restore ER homeostasis by reducing unfolded protein load through transcriptional remodeling of ER proteostasis pathways involved in protein folding, degradation and trafficking.*

COPII machinery

The COPII coat complex consists of a set of cytosolic proteins form a two-layered coat capable of deforming highly ER membranes to generate vesicles or other type of curved membrane carriers. Assembly of COPII complexes is initiated by activation of Sar1, a small GTPase, which is mediated by the transmembrane guanine nucleotide exchange factor (GEF) Sec12 (Nakano & Muramatsu, 1989) (Barlowe & Schekman, 1993) (Weissman, Plutner, & Balch, 2001) (Fig.2). In mammals, there are two Sar1 isoforms: Sar1A and Sar1B. Although in vitro studies, the two isoforms do not show much difference in terms of their activity, in vivo studies have suggested them to play different roles. Sar1B and nor Sar1A, was shown to be linked to ER export of chylomicrons, suggesting that Sar1B is involved in the formation of large COPII-coated transport carriers (Jones et al., 2003) (Fromme et al., 2007) (Fryer et al., 2014). Mutation of threonine 39 to asparagine generates a dominant negative isoform, which is constitutively bound to GDP and blocks COPII carrier formation (Aridor, Bannykh, Rowe, & Balch, 1995). Once GDP is exchanged to GTP, Sar1 undergoes a conformational change that exposes an N-terminal amphipathic α-helix allowing it to insert into the outer leaflet of the ER membrane (Bielli et al., 2005). It has been shown, both in vivo and in vitro, that the amino-terminal region of Sar1 plays an essential role in membrane deformation leading to generation of COPII-coated transport carriers (Bielli et al., 2005) (M. C. Lee et al., 2005) (Long et al., 2010). Sar1 appears to be the curvature-sensing component of the COPII coat complex and was shown to bind with higher affinity to membranes of high curvature (Hanna et al., 2016). In addition, active Sar1 forms dimers and to thereby contribute to the formation of constrictive membrane curvature to promote fission of the COPII carrier (Hariri, Bhattacharya, Johnson, Noble, & Stagg, 2014).

Active, membrane-bound Sar1 recruits the heterodimeric complex of Sec23–Sec24 to the ER, forming the pre-budding complex (Fig.2). Crystallographic and electron microscopy-based analysis in yeast have revealed that the membrane proximal region of Sec23-Sec24 is concave with a positively charged surface that may stabilize curvature of the underlying membrane (Bi, Corpina, & Goldberg, 2002). Sec23 plays a key role in promoting GTP hydrolysis on Sar1 thereby serving as a guanine nucleotide activating protein (GAP) (Bi et al., 2002) (Yoshihisa, Barlowe, & Schekman, 1993). Thus, recruitment of the inner layer of the COPII coat leads to subsequent GTP hydrolysis on Sar1, promoting disassembly from the membrane surface. Mammalian Sec23 exists in two isoforms. Although mutations in Sec23B have been linked to anemia and several types of cancer, and the Sec23A mutations to craniolenticulosutural dysplasia, the two isoforms appear to be very similar in terms of function (Khoriaty et al., 2018) (Schwarz et al., 2009) (Yehia et al., 2015) (Boyadjiev et al., 2006). Both isoforms interact with any of the four mammalian Sec24 isoforms (A-D).

Sec24 functions as a cargo adaptor that is responsible for capturing cargo and incorporating them into the COPII vesicles (Fig.2). In yeast Sec24 has two homologs: Lst1 (Roberg, Crotwell, Espenshade,

Gimeno, & Kaiser, 1999) and Iss1 (Kurihara et al., 2000), but only Sec24 is essential. In mammals, 4 isoforms exist Sec24A-D and have been shown to confer cargo specificity of the COPII coat. Combining genetics and structure biology, it was shown that yeast Sec24 has several binding sites for cargos with different ER-export motifs (Miller et al., 2003). The A-site and B-site recognize two different motifs in Sed5p. In addition, the B-site also binds to the v-Snare Bet1p and the Golgi protein Sys1p. The C-site recognizes a motif in Sec22 (Miller et al., 2003) (Mossessova, Bickford, & Goldberg, 2003). Later work, has identified cargo binding sites on mammalian Sec24 isoforms. The first cargo binding site in mammalian Sec24 was found in Sec24D isoform, where a DD motif binds to an arginine residue in the C-terminus of the GABA transporter 1 (Farhan et al., 2007). In a later study, X-ray crystallographic and biochemical analysis were combined to investigate molecular mechanisms for cargo discrimination by human Sec24 isoforms. Further binding motifs were identified. A conserved IXM motif was shown to bind on a specific site in Sec24C and Sec24D, but this site was shown to be occluded in the Sec24A and Sec24B subunits. Instead, LXXLE and the DXE motifs of VSV glycoprotein were shown to selectively bind Sec24A and Sec24B subunits (Mancias & Goldberg, 2008). Generally, Sec24A&B are considered more similar to each other and are often opposed to Sec24C&D. For example, the cargo receptor ERGIC-53 was shown to use a di-phenylalanine motif in its C-terminus to bind to Sec24A&B and accordingly depletion of these isoforms perturbs trafficking of ERGIC-53 (Wendeler, Paccaud, & Hauri, 2007). Interestingly mutating the FF-motif to either di-isoleucine or a single valine created an ERGIC-53 variant dependent on Sec24C&D (Wendeler et al., 2007). However, the notion that Sec24A&B carry out redundant functions as do Sec24C&D, is certainly an oversimplification. A strong body of literature documents non-overlapping function of the different Sec24 isoforms. For instance, a knockout of Sec24D in mice results is early embryonic lethality (Baines, Adams, Zhang, & Ginsburg, 2013), and Sec24A deficiency results in hypocholesterolemia (X. W. Chen et al., 2013). Only Sec24C is responsible for transport of serotonine transporter (SERT) (Sucic et al., 2011). Knockout of Sec24C in mice could only be partially rescued by Sec24D, indicating non-overlapping functions (B. Wang et al., 2018).

The next step in the assembly cascade is the recruitment of the heterotetramer Sec13–Sec31 complex which forms the outer layer of COPII coat (Fig.2). Interestingly, recruitment of Sec13-Sec31 top the pre-budding complex resulted in a 10fold increase in the GTPase activity of Sar1, an event that also appeared to trigger vesicle fission (Antonny, Madden, Hamamoto, Orci, & Schekman, 2001). The hypothesis that the Sec31-Sec13 complex is essential for vesicle scission is supported by a finding from patients with Craniolenticulo-sutural dysplasia (CLSD). This disease is caused by a point mutation on Sec23A (Boyadjiev et al., 2006). Mutant Sec23A was shown to fail to recruit Sec31, thus failing to increase the GAP activity of Sec23 (Bi, Mancias, & Goldberg, 2007) (Fromme et al., 2007). In mammals, two Sec31 isoforms have been identified and only one for Sec13. Sec13 was shown to be

a dual function protein. Outside being a core component of the COPII coat, Sec13 was shown to be part of the nuclear pore complex (NPC) which facilitates nucleo-cytoplasmic traffic (Enninga, Levay, & Fontoura, 2003). Whether and how these two pools of Sec13 communicate is currently unclear. The Sec31 binding partner ALG-2 (also known as PDCD6) has also been proposed to modulate COPII assembly, in particular by promoting an interaction between the outer COPII cage and the inner coat. Specifically, ALG-2 interacts with Sec31 and alters its conformation to increase its affinity for Sec23 (la Cour, Schindler, Berchtold, & Schekman, 2013). In this way, it was suggested that ALG-2 is capable of negatively regulating GTP hydrolysis on Sar1 until COPII assembly can be completed resulting in a decreased carrier scission. ALG-2 harbors a calcium binding motif (Yamasaki, Tani, Yamamoto, Kitamura, & Komada, 2006) and its interaction with Sec31 is calcium dependent, suggesting that cytosolic calcium levels can regulate COPII carrier formation (Shibata et al., 2015).

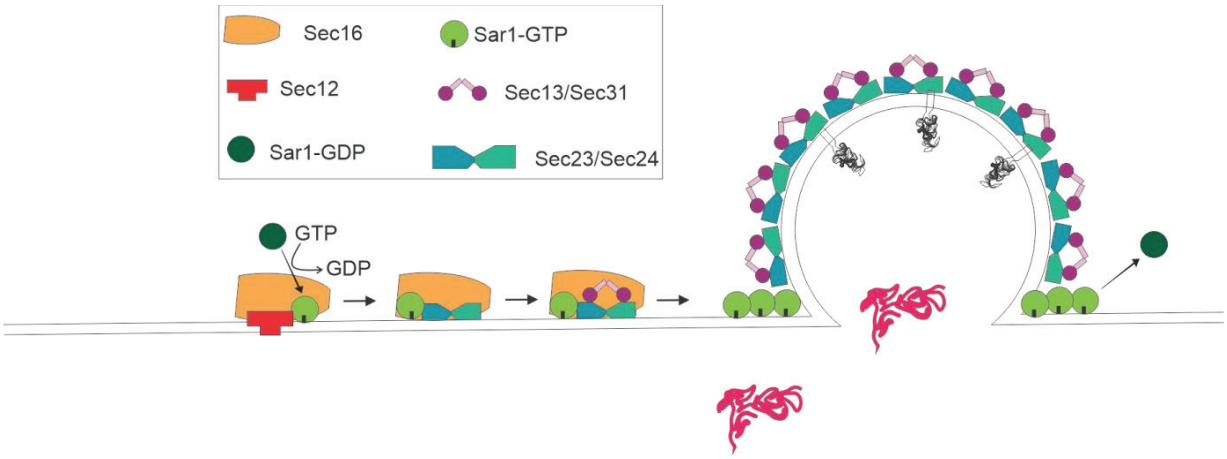


Figure 2: ***COPII assembly:*** *At ERES Sec16 binds the transmembrane GEF protein Sec12, which promotes the GDP–GTP exchange for the cytosolic GTPase Sar1. Active Sar1–GTP then is recruited to the ER membrane. This leads to the recruitment of the inner COPII components Sec23/24. Sec24 binds cargo proteins from the ER lumen. Finally, Sec13/Sec31 are recruited by binding to the inner coat complex and form the outer layer of COPII coat. Sar1–GTP which accumulates at the base of the forming COPII vesicle, undergoes GTP-hydrolysis induced by Sec23–Sec24 together with Sec13–Sec31, resulting in vesicle scission.*

ER export via cargo receptors

In contrast to transmembrane proteins, soluble secretory cargos which are located in the lumen of the ER, do not have direct access to the cytosolic COPII machinery. Therefore, cargo receptors mediate the sorting of these cargos to COPII through recognition of export signals.

The best-characterized mammalian ER export receptor is ERGIC-53, a 53kDa type I transmembrane protein, which was originally discovered in a monoclonal antibody screen for organelle marker proteins (Schweizer, Fransen, Bachi, Ginsel, & Hauri, 1988). ERGIC-53 serves as a marker of the ER-

to-Golgi intermediate compartment (ERGIC) but it is also present in the ER and in the first fenestrated cisterna in the cis-Golgi (Chavrier, Parton, Hauri, Simons, & Zerial, 1990) (Nufer, Kappeler, Guldbrandsen, & Hauri, 2003). Directly after synthesis, ERGIC-53 forms a trimer of homodimers (i.e. a hexamer). Two luminal cysteine residues Cys466 and Cys475 form disulfide-bridges and thereby are important for oligomerization. Indeed, mutation of one of the cysteines abolished hexamere formation, mutation of both abolished the dimer forms as well (Kappeler, Klopfenstein, Foguet, Paccaud, & Hauri, 1997). Efficient transport of ERGIC-53 is dependent on ER export motifs in its cytoplasmic portion. The diphenylalanine (FF) motifs interact with COPII and mediate the export from the ER (Wendeler et al., 2007). As ERGIC-53 cycles between the ER and the ERGIC, a di-lysine motif mediates its retrograde transport via COPI vesicles. ERGIC-53 binds to high mannose glycans in a Calcium dependent manner through the conserved Asp121 and Asn156 (Itin, Roche, Monsigny, & Hauri, 1996). ERGIC-53 is responsible for the transport of several glycoproteins. The first glycoprotein found to interact with this receptor is Cathepsin Z related protein (CatZr). It was shown that a carbohydrate binding-deficient ERGIC-53 mutant is not capable anymore to interact with the cargo protein (Appenzeller, Andersson, Kappeler, & Hauri, 1999). Alpha1-antitrypsin was identified as well as client of ERGIC-53 (Nyfeler et al., 2008). ERGIC-53 is also able to interact with members of the folding machinery as the chaperone ERp44 and together they are involved in the quality control of the oligomerization of IgM and its export (Anelli et al., 2007). After ERGIC-53 has released its cargos, it can recycle back to the ER for another round of transport. ERGL, VIPL, and VIP36 are related lectins with distinct intracellular distributions within the secretory pathway but that also interact with glycoproteins in a calcium and sugar-dependent manner (Kamiya et al., 2008). Their biology is far less understood.

Mammals require a set of transmembrane accessory factors that are involved in the transport of bulky cargos such as fibrillar procollagens through COPII vesicles. TANGO1 and cTAGE5 were shown to play roles in driving the formation of large COPII-coated transport intermediates. TANGO1 is not itself captured into vesicles (Malhotra & Erlmann, 2011) (Malhotra & Erlmann, 2015), making it different from classical cargo receptors, which is why TANGO1 is referred to as a “cargo loader”. TANGO1 interacts with collagen via an SH3 domain facilitating the loading of this bulky cargo into large COPII carriers (K. Saito et al., 2009) (K. Saito et al., 2011). However, it has been suggested that this binding does not happen directly. Instead, Hsp47 seems to mediate the interaction between TANGO1 and many different types of collagens (Ishikawa, Ito, Nagata, Sakai, & Bachinger, 2016). cTAGE5 was shown to interact with Tango1 and with Sec12 as well, facilitating Sec12 accumulation at COPII budding sites, but without affecting GEF activity (K. Saito et al., 2011) (K. Saito et al., 2014). Because of this interaction, TANGO1/cTAGE5 was suggested to modulate the recruitment of COPII coat enough to generate large carriers for procollagen export. Moreover, it was shown that TANGO1/cTAGE5 also binds to the inner COPII coat protein Sec23 (Tanabe, Maeda, Saito, &

Katada, 2016) and that this complex can recruit multiple Sec23 molecules, likely in complex with Sec24, as a transport carrier begins to assemble (Ma & Goldberg, 2016). In the same study it was shown that Sec31 also contains the same motif that in TANGO1/cTAGE5 is responsible for its interaction with Sec23. In this way, Sec31 also binds Sec23 and compete with TANGO1/cTAGE5. Therefore, when Sec31 is recruited to the site of vesicle formation, it displaces TANGO1/cTAGE5 that has delivered the cargo, which is captured in the nascent vesicle. Another type of regulation was identified to modulating COPII assembly during procollagen export. Ubiquitination of Sec31 was shown to drive the assembly of large COPII coats and play a role in collagen export (Jin et al., 2012). Super resolution imaging studies have suggested that TANGO1 assemble into ring structures that encircle COPII carriers providing a scaffold where ERGIC membranes could be recruited (Santos, Raote, Scarpa, Brouwers, & Malhotra, 2015) (Raote et al., 2017) (Raote et al., 2018). However, another model suggests that TANGO1, together with Hsp47, cTAGE5 and Sec12, actually enter the large COPII carriers that enable collagen export from the ER (Yuan, Kenny, Hemmati, Xu, & Schekman, 2018). These factors are subsequently retrieved from ERGIC and Golgi membranes via the action of retrograde COPI transport, while collagen continues on its journey through the secretory pathway toward the cell surface. According to this model of large COPII carrier formation, TANGO1/cTAGE5 family members would promote continual Sec12 GEF activity and maintain Sar1 in a GTP bound state, allowing the growth of the membrane carrier to a size beyond a regular COPII vesicle.

SFT-4, which is a C. elegans orthologue of cargo receptors of the Erv29p family, was recently reported to play a role in the ER export of certain soluble proteins in intestinal cells (Saegusa, Sato, Morooka, Hara, & Sato, 2018). First evidence supporting SFT-4 (SURF4 in mammals) comes from studies conducted in yeast where Erv29p was identified to play role in packaging of glycosylated pro-alpha-factor into COPII vesicles (Belden & Barlowe, 2001). Later, SFT-4 was identified in genome-wide RNAi screening and knockdown of this gene was shown to affect VIT-2–GFP trafficking, However, its role in this process was not studied in detail (Balklava, Pant, Fares, & Grant, 2007). SFT-4 depletion was shown to inhibit the export of certain soluble proteins from the ER resulting in the accumulation of these proteins in granular structures in the ER lumen of intestinal cells. Depletion of SURF4, similarly, was reported to inhibit ER export of ApoB100 in human hepatic cell line HepG2 cells and to lead to a decrease of ERES. Finally, SFT-4/Surf4 family proteins were suggested to regulate the export of soluble proteins such as lipoproteins from the ER and contribute to the biogenesis of ERES in animals (Saegusa et al., 2018).

ER exit sites (ERES)

The organization of COPII dependent budding is different between different species. Pioneering work conducted in mammalian cells (Palade, 1975), showed that, secretory proteins access to specific sub-domains of the ER, right after they get translated and before they leave the ER to the Golgi complex. The term "transitional ER" was given to these sub-domains which represent regions of rough ER lacking of ribosomes, where COPII budding process happen (Orci et al., 1991). Later, thanks to studies on localization and dynamics of COPII, the term ER exit site (ERES) was introduced. ERES is more than just a tER because it includes the post-ER structures which likely represent COPII-coated tubulo-vesicular membranes and undergo fusion with the ERGIC. ERES number and organization differ among the species and appear to correlate with the Golgi organization. The budding yeast P. pastoris shows a clear organization of COPII budding at specific ERES, of which on average four are present in every cell (Rossanese et al., 1999). The stacked Golgi apparatus of P. pastoris appears to be associated with ERES forming a secretory unit (Rossanese et al., 1999). A similar organization of ERES is known for Drosophila (Kondylis & Rabouille, 2003). In mammalian cells, the number and organization of ERES largely differ from what observed in other species. Indeed, hundreds of ERES are distributed throughout the cytoplasm and a significant part accumulates in the juxtanuclear region directly adjacent to the Golgi apparatus. However, the reasons for a direct physical proximity between many ERES and Golgi membrane remain unclear and whether and how peripheral and central ERES are different functionally is also poorly understood. It is possible that the direct coupling of tER and Golgi is needed to provide more direct routes for secretory transport between the two secretory stations. ERES approximately have a diameter of ~0.5 μm and they appear to be relatively stable structures as determined in studies using time-lapse imaging (Stephens, Lin-Marq, Pagano, Pepperkok, & Paccaud, 2000) (A. T. Hammond & Glick, 2000). Each ERES in mammalian cells hosts an average of 2 to 6 COPII-coated buds resulting in approximately 250 buds per cell (Bannykh, Rowe, & Balch, 1996) (Aridor et al., 2001). Time-lapse imaging of COPII-coated structures in live mammalian cells reveals that three mechanisms of de novo formation, fusion and fission of ERES cooperate and regulate the size of these sites (Stephens, 2003).

The cytosolic components of COPII dynamically associate with ERES but many resident proteins were identified to stably associate with ERES and to play roles in the organization of these sites. One of those is Sec16, 250 kDa protein that is evolutionarily conserved from yeast to mammals (Supek, Madden, Hamamoto, Orci, & Schekman, 2002) (Iinuma et al., 2007) (Watson, Townley, Koka, Palmer, & Stephens, 2006) (Connerly et al., 2005). In mammals, two isoform have been identified: a longer Sec16A and a shorter Sec16B (Iinuma et al., 2007) (Watson et al., 2006) (Bhattacharyya & Glick, 2007). Most research has concentrated on Sec16A, which seems to be the orthologue having most similarity to Sec16 in other species. Sec16A is a 250 kDa protein showing a typical ER exit site

localization (Iinuma et al., 2007) (Watson et al., 2006) (Bhattacharyya & Glick, 2007). Studies in Drosophila and mammals have shown that Sec16 uses an arginine-rich domain to localize to the ER and mediate biogenesis of ERES (Ivan et al., 2008) (Hughes et al., 2009). Depletion of Sec16A resulted in a disorganization of the ERES and a delay of ER to Golgi transport (Iinuma et al., 2007) (Watson et al., 2006) (Bhattacharyya & Glick, 2007). Sec16 proteins present a conserved central domain (CCD) that interacts with a Sec13 protein to form a structural domain which is very similar to the one found in the Sec13/31 cage (Whittle & Schwartz, 2010). The C-terminus of Sec16 interacts with Sec23 (Gimeno, Espenshade, & Kaiser, 1996) and with Sec12 (Montegna, Bhave, Liu, Bhattacharyya, & Glick, 2012). Additionally, Sec16 binds Sec31 and Sec24 (Yorimitsu & Sato, 2012) (Kung et al., 2012). Sar1 activation was shown to promote the association of Sec16A with membranes in mammals where it associates with cup-shaped membranes that are adjacent from the COPII-coated buds (Hughes et al., 2009). In yeast, Sec16 was shown to play a role in delaying GTP hydrolysis by Sar1 (Kung et al., 2012). The fact the Sec16 is able to interact with the cytosolic COPII components suggests a key involvement of Sec16 in the formation of COPII-coated vesicles. Therefore, ERES appear to include a network of interacting proteins with regulatory capabilities.

In mammals, TANGO1 is expressed in two splice isoforms, TANGO1L and a short isoform, TANGO1S, which lacks the luminal SH3 domain (Maeda, Saito, & Katada, 2016). TANGO1 binds Sec16A and serves as a membrane receptor for Sec16A. TANGO1-Sec16 interactions are required to localize both proteins at ERES (Maeda, Katada, & Saito, 2017). Another accessory protein at ERES is cTAGE5 which forms a complex with TANGO1 and binds Sec12. This interaction aims at concentrating Sar1 activation and thereby coat assembly at ERES.

Signaling to and from the ER

As stated before, the ER is the site in the cell where synthesis, quality control and trafficking of a third of the eukaryotic proteome happen. Importantly, the ER represents the first station of the secretory pathway and ERES are the sites in the ER where secretory proteins are exported via COPII vesicles. Due to their fundamental role in trafficking, ER and ERES must be subject to a fine regulatory program in order to maintain proteostasis. To test the hypothesis of a role of kinase signaling in ER export, purified COPII components in both membrane recruitment and cargo export assays were utilized and treated with the serine/threonine kinase inhibitor H89, an isoquinolinesulfonamide that is frequently used as a selective protein kinase A (PKA) inhibitor. It was demonstrated that Sar1 recruitment to membranes requires ATP and that the kinase inhibitor H89 abolishes membrane recruitment of Sar1, which is the first step in COPII vesicle formation (Aridor & Balch, 2000). Another study showed that Sec13 recruitment is inhibited by H89 at a step independent of the activation of Sar1 leading, again, to the block of ER export (T. H. Lee & Linstedt, 2000). However,

H89 is a specific inhibitor of PKA at nanomolar concentration but in these studies, the concentration of H89 used to inhibit COPII recruitment was typically higher than 50 μM, which is 100-1000 fold higher than the IC50 for PKA. Thus, it is unclear whether H89-sensitive kinase regulates ER export. Few years later, the first example of a kinase regulating ER export was identified and many others followed later, which will be discussed below in greater depth. Depending on whether the ER and ERES are targets or sites of initiation of signaling pathways, signaling can be divided into signaling to the ER (Fig.3) and signaling from the ER (Fig.4). Finally, among the examples of kinase signaling originating from the ER, autoregulatory pathways at the ER are gaining increasing attention. Signaling to and from the ER will be discussed only in the context of the regulation of ER export. However, many other signaling events are known to occur at the ER regulating calcium homeostasis, ATP flux or ER-selective autophagy (ER-phagy) but they will not be discussed here.

Signaling to the ER

Growth factor signaling

The ER is able to respond to exogenous and endogenous stimuli in order to adapt to environmental and intracellular changes. Environmental stimuli include mitogens that can change proteostasis by modulating secretion. Before the first signaling pathway regulating ER export was identified, few examples of kinases and phosphatases have been linked to the regulation of the early secretory pathway (Kapetanovich, Baughman, & Lee, 2005) (K. J. Palmer, Konkel, & Stephens, 2005) (Bejarano, Cabrera, Vega, Hidalgo, & Velasco, 2006). One example is related to the kinase PCTAIRE that was shown to interact with Sec23A and to lead to defects in early secretory pathway, when depleted (K. J. Palmer et al., 2005) (Fig.3). However, no data about the ability for this kinase to phosphorylate Sec23A were provided in this study. First evidence supporting a link between growth factor signaling and ER export was discovered few years later using systems-wide RNAi screening in mammalian cells. Growth factors bind to corresponding cell surface receptors and trigger signal transduction cascades leading to a specific cellular response. In one study using a siRNA screen, all human kinase and phosphatases were depleted in order to explore their potential role as regulators of the early secretory pathway (Farhan et al., 2010). Changes in the distribution of ERGIC-53 were analyzed in order to determine the functional status of the early secretory pathway. The screening revealed that depletion over 60 kinases caused changes in distribution of ERGIC-53. Among the pathways identified, the Raf–MEK–ERK cascade was able to regulate the number of ERES via ERK2, which was shown to phosphorylate Sec16A (Fig.3). Moreover, overexpression of oncogenic Ras which leads to hyperactivation of ERK2 was shown to enhance the phosphorylation of Sec16A, resulting in an increase in number of ERES. This finding positioned Sec16A as an integrator of growth

factor signaling at the ER but it was not yet clear how Sec16A phosphorylation by ERK2 leads to changes in ERES. In a subsequent study it was shown how mitogen signaling changes levels and dynamics of Sec16A at ERES (Tillmann et al., 2015) (Fig.3). Transient growth factor stimulation accelerates the dynamics of Sec16A at ERES, increases number of ERES but does not affect secretion. This rapid increase in ERES number happens at the expense of ERES size in order to deal with an upcoming wave of secretory cargo, which is expected upon growth factor stimulation. Furthermore, mitogenic stimulation for several hours was shown to increase the levels of Sec16A leading to an increased number and appropriate size of ERES. According to this finding, it was suggested that Sec16A acts as a central node in a coherent feed-forward loop (CFFL) that detects changes in secretory flux upon mitogen signaling and thereby integrates growth factor signaling and ER export. According to this CFFL, an input node which is represented by growth factors is capable to trigger a central node (Sec16A) that subsequently triggers an output node, in this case, ERES number. The aim of a CFFL is to ensure that transient stimuli that can potentially trigger the central node, do not affect the output node. So then, only a prolonged stimulus is able to trigger the output node. The connection between input and central node should be faster than between input and output nodes. Here, the connection between growth factors and Sec16A implies a phosphorylation event which is considered a quite fast type of regulation. More recently, the notion of a link between growth factor signaling and ERES was extended to some of the components of the COPII machinery (Scharaw et al., 2016). This study showed that prolonged stimulation with EGF also increases the levels of Sec23B, Sec24B, and Sec24D. This suggests, in line with previous findings, the existence of a CFFL that detects the persistent stimuli leading an increased transport of newly synthesized EGFRs from the endoplasmic reticulum to the plasma membrane in order to restore its surface levels. In support of the notion of a link between growth factor signaling and ER export are the results of a microscopy-based genome-wide RNAi screening, for regulators of the trafficking of the transmembrane cargo VSVG-ts045 (Simpson et al., 2012). In particular, depletion of EGF signaling pathway factors resulted in inhibition of secretion. Conversely, stimulation of cells with EGF resulted in an increase of secretion efficiency.

Another kinase that was shown to target the COPII machinery is the serine/threonine kinase AKT (also known as protein kinase B) which was shown to phosphorylate Sec24C and Sec24D (Fig.3). This phosphorylation modulates the interaction of Sec24 with Sec23 and therefore it was proposed to increase the efficiency of protein transport within the early secretory pathway (Sharpe, Luu, & Brown, 2011). This finding might help explain how AKT regulates ER-export of the sterol regulatory element binding protein 2 (SREBP-2) which plays a key role in cholesterol homeostasis (Du, Kristiana, Wong, & Brown, 2006). It is very important to establish the physiological context of Sec24 phosphorylation by AKT by determining which stimuli triggers this signaling event and how this affects the ER export, so further investigations are needed. Another evidence showing that signaling to the COPII machinery is involved in lipid metabolism is related to the atypical protein kinase C (PKCζ) which

phosphorylates Sar1B (Fig.3). This phosphorylation, rather than stimulating the activity of Sar1B, frees FABP1 from a cytosolic multiprotein complex allowing FABP1 binding to the ER. This results in budding of pre-chylomicron transport vesicles (PCTV) (Siddiqi & Mansbach, 2012).

Nutrient deprivation signaling

Stress conditions such as nutrients starvation can also trigger signaling pathways targeting the ER. Deprivation of nutrients results in a decrease of a biosynthetic rate in cells and thereby a reduction of protein synthesis and ERES, it is expected. As already stated, nutrients starvation is handled by cells through activation of autophagy. In this way, degradation of cellular components via lysosomes allows a recycle of building blocks of macromolecules such as amino acids in order to compensate the lack of nutrients. There notion of a crosstalk between autophagy and the secretory pathway is based on the idea that membranes of the secretory pathway (in particular ERES) serve as sources and platforms for the autophagosome formation. However, many examples of signaling pathways triggered by nutrient starvation show how ERES are regulated in such condition. An RNAi screening in several Drosophila kinases was conducted in order to gain insight on how signalling molecules regulate the organization of the early secretory pathway (Kondylis, Tang, Fuchs, Boutros, & Rabouille, 2011). This screen led to the identification of the atypical Mitotic-Associated Protein Kinase (MAPK) Extracellularly regulated kinase 7 (ERK7) as a new modulator (Fig.3). However, the overlap between this screen and the previously conducted in human cells (Farhan et al., 2010) was small. This can be explained by the variation in regulation of the secretory pathway from organism to organism, despite the very conserved factors of the machinery. Later, it was shown that ERK7 negatively regulates secretion in response to serum and amino-acid starvation, in both Drosophila and human cells (Zacharogianni et al., 2011). Under these conditions, ERK7 proteasomal degradation is inhibited resulting in higher levels of the kinase. How ERK7 regulates Sec16 remains unclear, because this kinase did not phosphorylate Sec16. Moreover, the ERES disassembly upon starvation is TOR complex 1 (TORC1) independent and so the identity of the signaling molecule that mediates the effect of ERK7 remains elusive. The same group has later investigated the fate of cytosolic Sec16 that dissociates from ERES in Drosophila. They show that upon nutrients starvation, Drosophila S2 cells form membraneless structures that are enriched in Sec16 which were called “Sec bodies” (Zacharogianni, Aguilera-Gomez, Veenendaal, Smout, & Rabouille, 2014) (Fig.3). Formation of these membraneless structures occurred 3–4 h after amino-acid starvation and was reversible upon refeeding. They were associated with ER membranes but distinct from COPII-coated vesicles and autophagosomes. Also, those structures were forming independently of mTORC1. It was proposed that these structures act as reservoirs for ERES constituents to rebuild a functional ER export once the stress is resolved. However, so far, formation of Sec-bodies has been shown only in Drosophila cells and no similar

process was found in a mammalian system. In fact, it was showed that serum-starvation in mammalian cells resulted in degradation of Sec16 (Tillmann et al., 2015). The kinases ULKI/2 have always been linked to the Initiation of autophagy. However, it was showen that ULK1/2 play a role in ER–Golgi traffic by phosphorylating Sec16A under condition where autophagy was not induced (Fig.3). This suggests a new role of ULK1/2 independent of autophagy (Joo et al., 2016). If autophagy was triggered, ULK1/2 are diverted to the autophagic pathway and their effect towards ER-export is reduced. ULK1 as regulator of the early secretory pathway was already suggested by a previous kinome-wide RNAi screen where this kinase was able to regulate ERES (Farhan et al., 2010). Under autophagy conditions, ULK1 was shown to phosphorylate Sec23A (Fig.3). This phosphorylation resulted in a disrupted interaction of Sec23A with Sec31A leading to morphologic changes of ERES. It was also shown that induction of autophagy leads to phosphorylation of Sec23B, via ULK1 which stabilizes this protein and diverts it towards autophagosome biogenesis (Gan et al., 2017). These findings contribute to the notion that autophagy negatively impacts ER export. Recently a non-canonical autophagy pathway was shown to originate at ERES and it is required for targeting procollagen towards lysosomal degradation (Omari et al., 2018). Another study gave another example of regulation COPII-mediated trafficking by nutrient deprivation in vivo. Starvation of mice led to a reduction of ER export in the liver and feeding these mice again, increased their rate of ER-export in a manner dependent on XBP1s (L. Liu et al., 2019). Using live-cell imaging approaches, it was demonstrated that XBP1s is sufficient to promote COPII-dependent trafficking, mediating the nutrient stimulatory effects. Also, chromatin immunoprecipitation (ChIP) coupled with high-throughput DNA sequencing (ChIP-seq) and RNA- sequencing analyses reveal that nutritional signals induce recruitment of XBP1s to promoters several components of the ER-export machinery, thereby driving COPII-dependent trafficking. These findings suggest a new role for the IRE1α-XBP1s axis as a nutrient-sensing regulatory node that integrates the metabolic status of the cell and the ER export.

Signaling to ERES in mitosis

ERES undergo adaptation in response to endogenous stimuli as well. Indeed, many examples show how during mitosis, ERES are subject to regulation by signaling. The general notion that protein trafficking is negatively regulated during mitosis is supported by several studies showing that in mitotic cells, Sec13 and other COPII coat proteins were dispersed to the cytoplasm (Dudognon, Maeder-Garavaglia, Carpentier, & Paccaud, 2004) (Prescott et al., 2001). It was also shown that during interphase, ERES gradually increase in number but undergo rapid disassembly during mitosis (A. T. Hammond & Glick, 2000). These findings raised the hypothesis of the existence of a signaling event capable of targeting ERES and regulating their assembly/disassembly during mitosis. Interestingly, Sec16A remains associated with the ERES during mitosis, most probably to restore the

assembly of ERES at the end of mitosis, which indicates the existence of signaling events that prevent COPII components from associating with Sec16A during mitosis (Stephens, 2003). First evidence of a kinase that regulate the disassembly of ERES during mitosis, is related to the cell division cycle protein 2 (Cdc2) kinase, which phosphorylates p47, a cofactor of the p97 AAA-ATPase (also known as VCP) (Kano, Tanaka, Yamauchi, Kondo, & Murata, 2004). In this study, YIP1A was used as marker for ERES. Since YIP1A cycles in the early secretory pathway, it cannot be considered a reliable marker for ERES because peripheral ERGIC punctae would appear very similar to ERES. Thus, it would be useful to confirm the findings using more established ERES markers such as Sec16A. Moreover, future investigations are needed in order to elucidate mechanistic details of how Cdc2 induces ERES disassembly. Another mechanism that was proposed was that the COPII component Sec24C is O-glycosylated in interphase, which prevents its phosphorylation (Dudognon et al., 2004). It was suggested that during mitosis this COPII component undergoes phosphorylation on serine and threonine residues that are potentially inaccessible during interphase owing to their O-glycosylation affecting its recruitment to ERES (Fig.3). According to these findings it was hypothesized that these post-translational modifications could contribute to the block of ER-to-Golgi transport during mitosis. However, it remains unclear why the disassembly of ERES is sensitive to phosphorylation of Sec24C, which is only one of four Sec24 isoforms. In addition, Sec24 isoforms play a role as cargo adaptors, but are not known to affect ERES biogenesis per se. In order to gain a deeper understanding of the molecular mechanism of this block, identifying the kinase responsible for Sec24C phosphorylation is required.

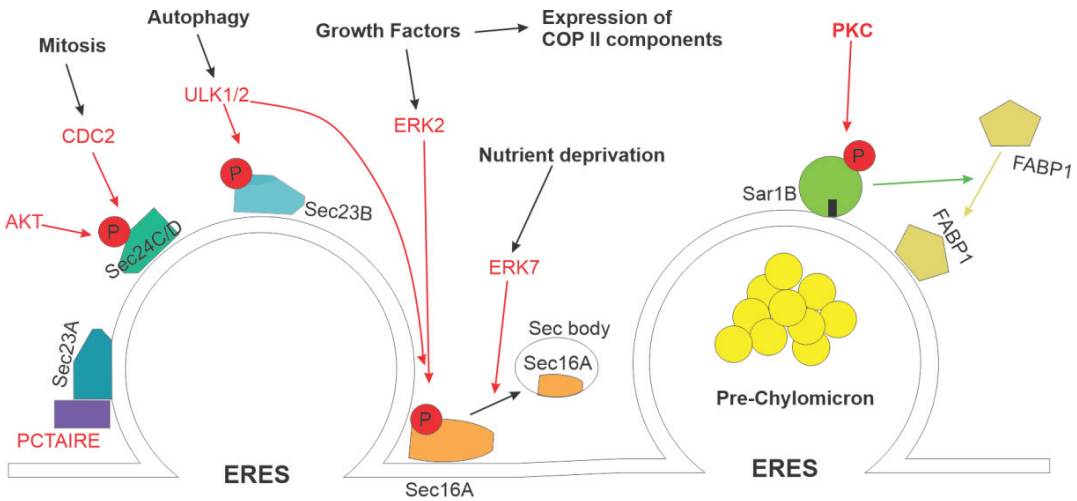


Figure 3: ***Signaling to the ER:*** *Schematic illustration of key signaling events where ERES are targets of signaling pathways.*

Signaling from the ER

Signaling from the ER by mutant proteins

Mutations and gene fusions are known to generate receptor tyrosine kinases (RTKs) that are frequently associated with cancer (Blume-Jensen & Hunter, 2001). Moreover, these mutant RTKs, which normally localize to the plasma membrane, may exhibit abnormal localization, triggering signaling from unusual locations. Most of the aberrant or oncogenic signaling examples available from endomembranes are related to the endosomal compartment (Villarroel-Campos, Schiavo, & Lazo, 2018) (X. Li, Garrity, & Xu, 2013). Far less is known about oncogenic signaling from the ER. First evidence of a receptor tyrosine kinase linked to the ER and myeloid transformation is the kinase KIT. In this study, they attempted to establish a murine model of human KIT carrying a mutation (D816V) associated with acute myeloid leukemia. It was shown that this mutant was blocked in the ER (Xiang, Kreisel, Cain, Colson, & Tomasson, 2007). Interestingly, this block was species specific and only occurred in human cells, but not in murine cells. However, it was not tested whether KIT-D816V is capable of signaling from the ER and whether its signaling is different from plasma membrane KIT. Another example is the receptor tyrosine kinase FLT3 which is expressed on hematopoietic progenitors and regulates early steps of hematopoietic cell proliferation, survival, and differentiation. Constitutive activation of FLT3 is linked to several hematological malignancies such as Acute Myeloid Leukemia (AML) (Gilliland & Griffin, 2002). The most frequent mutation of FLT3 is the internal tandem duplication (ITD) in the juxtamembrane domain that induces not only ligand-independent constitutive activation of the receptor but also impaired trafficking, leading to a mislocalization of the kinase to the ER (D. Schmidt-Arras et al., 2009) (Koch, Jacobi, Ryser, Ehninger, & Thiede, 2008) (Choudhary et al., 2009) (D. E. Schmidt-Arras et al., 2005). Interestingly, the signaling pathways triggered by wild type and mutant FLT3 were different. While the wild type receptor triggered MAPK signaling, FLT3-ITD phosphorylated STAT5 and upregulated its targets, such as Pim-1/2 kinase (D. E. Schmidt-Arras et al., 2005) (Fig.4). This difference in the signaling outcome was not due to the mutation per se, but rather due to the spatial localization. Indeed, targeting the FLT3-ITD to the cell surface rendered it capable of engaging the MAPK signaling pathway. Another example is related to the interleukin 6 (IL-6) and the activation of the IL-6 receptor signalling subunit glycoprotein 130 (gp130). Deletion mutations in gp130 have been identified in inflammatory hepatocellular adenoma. In particular, gp130 deletion mutant was shown to be retained ER most probably because of its prolonged association with the ER quality control component calnexin. From the ER, gp130 initiates mitogenic signaling that is likely contributing to the pathogenesis of the hepatic adenomas (D. Schmidt-Arras et al., 2014).

Another study shows the role of the ER-export regulator TGF-1in oncogenesis. TGF-1 interacts directly with Sec16 and controls the ER export by promoting the co-assembly of Sec16 with COPII

subunits. Moreover, the study shows that oncogenic fusion of TFG-1 and NTRK1 (neutrophic tyrosine kinase receptor type 1) localizes to ERES suggesting the possibility of initiation of an oncogenic signaling cascade, involving components of the ERK1/2 cascade, leading to cell transformation (Witte et al., 2011). Interestingly, the same study showed that fusion of NTRK1 to Sec16 localizes at ERES, independently of TFG-1, promoting transformation.

For a long time, it has been thought that Ras GTPases signal exclusively from the plasma membrane. The idea changed when, using a fluorescent reporter for active Ras, uncovered active Ras from other locations. It was shown that an active form of H-Ras restricted to the ER was capable of signaling through the mitogen-activated protein kinase (MAPK) pathway that promoted cellular transformation (Chiu et al., 2002) (Fig.4). However, in the study, a palmitoylation-deficient version of H-Ras was used and not the naturally oncogenic version that was ER localized. However, in a later study, a version of H-Ras that was engineered to be tethered to the ER was used. It was shown that this ER-tethered H-Ras is activated by RasGRF family exchange factors, thus providing more insights on how endogenous H-Ras is regulated at the ER (Arozarena et al., 2004). Very recently, a systematic analysis of signaling networks regulated by active H-Ras at various subcellular localizations (including the ER) was conducted. This study which combined protein-protein interactions, phosphoproteomics, and transcriptomics gave the most comprehensive attempt to investigate subcellular Ras signaling so far (Santra et al., 2019). As done in the previously conducted studies, subcellular localization was achieved by tethering H-Ras artificially to different compartments. Here, ER-localized H-Ras was involved in controlling cell migration, but had little effects on cell survival. Although all together these reports gave a deeper understanding on ER-localized Ras signaling, it is not yet clear whether endogenous Ras isoforms signal indeed from the ER.

Autoregulation at the ER

When a biological system is perturbed, a response that aims at re-stablishing the normal status, occurs. This response is defined as autoregulation. Many organs are capable of eliminating the perturbing stimulus through autoregulatory systems. For example, in the brain, resistance vessels have an intrinsic capability to dilate or and constrict in response to pressure changes, maintaining cerebral blood flow relatively constant (Strandgaard & Paulson, 1984). As the brain, the ER may also experience many perturbations. Indeed, many genetic and environmental insults challenge the ER quality resulting in accumulation of non-native protein conformations in the ER. This condition is termed ER stress and it has a big impact on the proteostasis network as it challenges the ability of the ER to export properly folded proteins. Furthermore, secretion of non-native protein conformations can disrupt cellular function in downstream secretory environments. In order to resolve ER stress, cells evolved a signaling pathway called the Unfolded Protein Response (UPR). Several can be the cellular

perturbations that can induce ER stress, including hypoxia, nutrient deprivation, mutations in secreted proteins that promote misfolding, imbalances in calcium homeostasis affecting the ER resident calcium-dependent chaperones. The UPR represents the best-understood autoregulatory response of the ER which is activated by the accumulation of unfolded or misfolded proteins and is capable to initiate an adaptive response to restore ER homeostasis by reducing unfolded protein load (Fig.4).

In general, The UPR through signaling to the nucleus, induce the gene expression of chaperones as well as the machinery for vesicle budding, tethering and fusion (Gardner, Pincus, Gotthardt, Gallagher, & Walter, 2013). Recently, a mathematical model of the unfolded protein response was developed in order to determine which sensing and activation strategies are optimally used by the pathway. By comparing a stress-sensing mechanism that responds directly to the level of unfolded protein in the ER to a mechanism that is negatively regulated by unbound chaperones, it was shown that the chaperone- mediated sensors are the most efficient ones in detecting the unfolded proteins. Moreover, it was demonstrated that sensors whose activity is down-regulated by the amount of free chaperone is a more suitable sensory system because of the capability to activate and deactivate at different levels of stress (Stroberg, Aktin, Savir, & Schnell, 2018). Thus, this model shows that the UPR is a typical autoregulatory response that maintains system homeostasis.

The major part of the UPR acts through a broad transcriptional up-regulation which is a relatively slow process. Recently, an example of a rapid, autoregulatory signaling at the ER was identified (Subramanian et al., 2019). Fluctuations in protein synthesis may occur in many cell types could lead to aberrant accumulation of folded and potentially active cargo in the lumen of The ER. Cargo mislocalization and aberrant activation at the ER might lead to diseases such as cancer. Thus, the aim of this study was to investigate the adaptive response that occurs upon production of folded cargo proteins in the ER which is expected to modulate the export process. Thus, a non-toxic and artificial perturbation in the ER was used as stimulus that leads to a synchronous increase of folded cargo in the ER lumen. Then, the detection of cargo increase by a sensor in the ER should activate signaling pathways resulting in acceleration the export process. Temperature-sensitive proteins, such as vesicular stomatitis virus ts045 G protein (VSV-G) and Pro-Collagen I (PC-I) were used as synchronous surge of folded cargo in the ER. By using an antibody array that detects different active kinases, it was shown that the folded cargo stimulus led to activation of the key kinase components of many signaling pathways such as PKA regulatory subunit IIa (PKA-RIIa) and ERK1 or ERK2. The proposed auto-regulatory system at the ER, named AREX (auto-regulation of ER export) engages the COPII subunit Sec24 as a sensor for folded proteins. The cargo-Sec24 complex then binds to Gα12 resulting in the recruitment of this G-protein to the ERES from cytosol. Sec24 acts as a guanine nucleotide exchange factor for Gα12 at ERES that leads to activation and recruitment of other signaling molecules such as PKA. PKA is then able to activate a large number of proteins and promote ER export most probably at late stages (Fig.4). However, the exact mechanism of activation of Gα12

by Sec24 was not defined. Thus, although AREX represents a bona fide autoregulatory response of ERES.

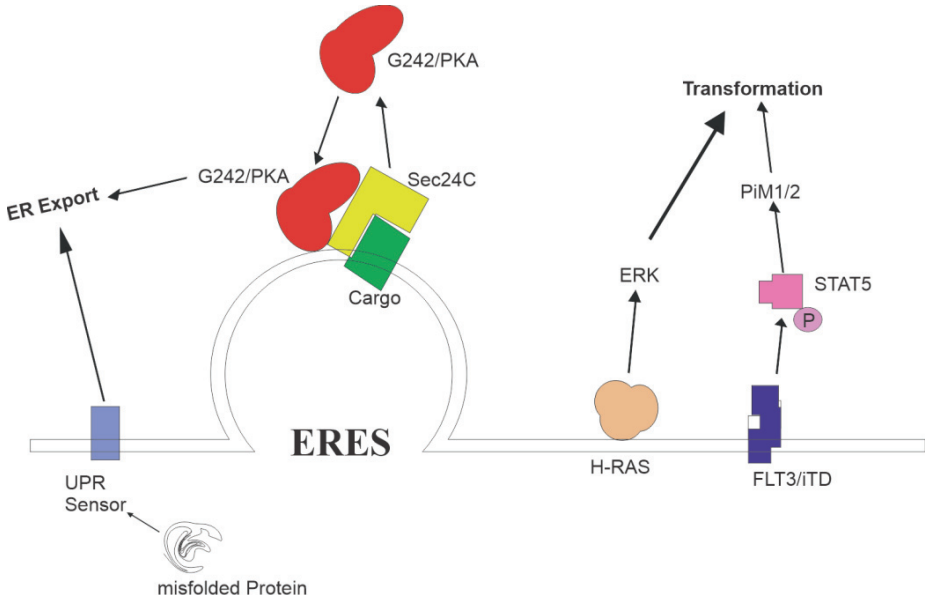


Figure 4: ***Signaling from the ER:*** *Schematic illustration of key signaling events originating from the ER. On the left, the UPR and AREX as the only two known autoregulatory responses at the ER. On the right, examples of mutant variants of kinases or GTPases triggering signaling pathways from the ER.*

Receptor Tyrosine Kinases (RTKs)

Receptors Tyrosine Kinases represent a family of transmembrane receptors that trigger cellular signaling pathways and play important roles in normal development and homeostasis. RTKs are enzymes with tyrosine kinase activity which, catalyze the transfer of a gamma-phosphate group from adenosine triphosphate (ATP) to a tyrosine residue on a substrate protein. In the 1960s, the pioneering discoveries of nerve growth factor and epidermal growth factor (Levi-Montalcini & Booker, 1960) (Cohen, 1962) and their important roles in neuronal differentiation and cell proliferation in vivo and in vitro suggested the idea that these cytokines must bind specifically to cell-surface receptors. By this time, insulin was already discovered as well and used successfully to treat diabetes patients. The first experiments aimed at identifying the related cell-surface receptors were conducted by using homogenous preparations of pure insulin in order to characterize the insulin binding to its receptor on intact cells or to solubilized insulin receptor preparations using radiolabeled insulin (de Meyts, Roth, Neville, Gavin, & Lesniak, 1973). Following studies gave a deeper understanding of the ligand binding characteristics of EGFR as well (Carpenter, Lembach, Morrison, & Cohen, 1975).

In humans, 58 RTKs have been identified to date, which are divided in twenty subfamilies. In general, all RTKs share a molecular architecture which comprises a ligand-binding region in the extracellular domain, a single transmembrane helix, and a cytoplasmic region that contains the protein tyrosine kinase domain (TKD) plus additional domains that are important for protein-protein interactions (Lemmon & Schlessinger, 2010). Several mechanisms of RTK regulation were described through structural and functional studies. A general notion is that growth factor binding promotes dimerization of RTKs which is known to be a pre-requisite for their activation (Ullrich & Schlessinger, 1990). However, RTKs forms oligomers even in the absence of activating ligand. An example is the insulin receptor and the insulin-like growth factor 1 (IGF1)-receptor which are expressed as disulfide-linked $(\alpha\beta)_2$ dimers (C. W. Ward, Lawrence, Streltsov, Adams, & McKern, 2007). The binding of insulin or IGF1, in this case, induces structural changes within the already formed dimeric receptors. This change leads to activation of the TKD. EGF as well, was suggested to bind to pre-existing oligomers of the related receptor (C. W. Ward et al., 2007) (Clayton et al., 2005).

Whether the ligand is required or not for receptor oligomerization, its binding with RTKs is necessary for receptor activation and to stabilize it in an ‘active’ dimer or oligomer. In general, in the ligand-bound receptor, self-association of the extracellular region is followed by a conformational change of the intracellular domain into a dimeric state leading to activation of the TKD. Once the receptor forms a dimer, it is capable to phosphorylate one or more tyrosines in a neighboring RTK, and the phosphorylated receptor then serves as a site for assembly and activation of intracellular signaling proteins (Ullrich & Schlessinger, 1990). When RTKs were discovered, a straightforward mechanism for ligand-induced dimerization was suggested. According to this model, a bivalent ligand interacts

simultaneously with two receptors and this cross-links them into a dimeric complex. Many receptors follow this 'ligand-mediated' mode of dimerization. Few examples are the stem cell factor receptor KIT (H. Liu, Chen, Focia, & He, 2007), the Flt1 vascular endothelial growth factor (VEGF) receptor (Leppanen et al., 2011) (Wiesmann et al., 1997), the nerve growth factor (NGF)/neurotrophin receptor TrkA (Wiesmann, Ultsch, Bass, & de Vos, 1999), Axl (T. Sasaki et al., 2006), Tie2 (Barton et al., 2006), and Eph receptors (Himanen & Nikolov, 2003). By now, other models for receptor dimerization have been proposed, which can be divided into two mechanistic extremes and two intermediate cases. One extreme is represented by the already mentioned "ligand-mediated" mode where the two receptors make no direct contact. This is the case for the TrkA (NGF receptor). Its extracellular region contains two immunoglobulin-like domains (Ig-C1 and Ig-C2) and when NGF binds the receptor, in the induced dimer, the extracellular regions of the two TrkA receptors do not contact each other. Instead, the NGF dimeric ligand contacts the Ig-C2 domain of each receptor, with each Ig-C2 contacting both chains of the NGF dimer (Wiesmann et al., 1999) (Wehrman et al., 2007). According to the other extreme case, dimerization is instead "receptor-mediated". In this case, formation of receptor dimers happens without any direct contribution from the ligand. Indeed, EGFR contains four domains (I-IV) in the extracellular regions and the domains I and III both bind to activating ligands. When the bivalent ligand binds EGFR, it contacts two distinct sites within a single receptor on domains I and III. This promotes substantial conformational changes in the extracellular region of EGFR, which unmask a dimerization arm in Domain II (Burgess et al., 2003). Before the ligand binds, this arm is completely masked by intramolecular interactions with Domain IV. In this state of 'tethered' conformation, both ligand binding and dimerization are autoinhibited (Burgess et al., 2003) (Bouyain, Longo, Li, Ferguson, & Leahy, 2005) (Cho & Leahy, 2002) (Ferguson et al., 2003). Therefore, after ligand binding, the dimerization arm of Domain II is free to interact with a second ligand-bound receptor. Alternatively, dimerization can involve both ligand-mediated and receptor- mediated components. This is the case for KIT and its ligand, the stem cell factor, or SCF. Each SCF molecule binds to one molecule of KIT through contacts with the first three Ig-like domains in the KIT extracellular region (H. Liu et al., 2007) (Yuzawa et al., 2007). This region does not undergo structural changes upon ligand binding. The association with the SCF dimer simply 'cross-links' the two receptors leading to a reorientation of the two Ig-like domains closest to the plasma membrane. These two domains make important homotypic interactions across the dimer interface promoting activation of the KIT molecules Also for Eph receptors, direct interactions between membrane proximal portions appear to be important for their oligomerization and activation (Seiradake, Harlos, Sutton, Aricescu, & Jones, 2010). Overall, dimerization of most RTKs is likely to follow one of the mentioned modes. However, additional dimerization modes might exist among other RTK families.

Once ligand-induced dimerization is accomplished, this needs to lead to the activation of the

intracellular TKD. Here as well, many different activation mechanisms have been identified for many RTKs. In general, all TKDs have an N-lobe and a C-lobe in their TKD. Crystal structures of the activated forms of TKDs revealed a very close similarity (Huse & Kuriyan, 2002). Moreover, the 'activation loop' in the kinase N-lobe adopts a specific configuration in all activated TKDs (Nolen, Taylor, & Ghosh, 2004). The real difference occurs by comparing the structures of inactive TKDs which is most likely the reason for the diversity in their regulatory mechanisms. Indeed, each TKD is cis-autoinhibited by a set of intramolecular interactions specific for its receptor and the release of this autoinhibition is the key event that leads to RTK activation. As for dimerization, many modes of autoinhibition were identified for several RTKs. The first one to be discovered was related to the insulin receptor TKD (Hubbard, 2004). A key tyrosine (Y1162) in the activation loop of the insulin receptor TKD projects into the active site. This stabilizes the activation loop in a configuration that does not allow ATP to access the active site. In this way, the insulin receptor TKD is autoinhibited in cis by its own activation loop. When insulin binds the receptor, Y1162 in one TKD in the dimer becomes phosphorylated by its partner (trans-phosphorylation) and disrupts the cis-autoinhibitory interactions. The activation loop is then in its active state and trigger signaling pathways (Huse & Kuriyan, 2002) (Nolen et al., 2004). Autoinhibition can also occur in other domains of RTKs, outside of the TKD itself. Indeed, many RTKs such as MuSK (Till et al., 2002), Flt3 (Griffith et al., 2004), KIT (Mol et al., 2004) and Eph family RTKs (Wybenga-Groot et al., 2001), show 'juxtamembrane autoinhibition'. In this case, sequences in the juxtamembrane region contact with several parts of the TKD, including the activation loop, and stabilize an autoinhibited conformation. Key tyrosine residues in the juxtamembrane region play central roles in the autoinhibited state. Again, once the receptor dimerized, trans-phosphorylation of these tyrosine residues occurs and this disrupts the cis-autoinhibitory state leading to receptor activation (Hubbard, 2004). Interestingly, the EGFR family does not require trans-phosphorylation of their activation loops for activation (Knowles et al., 2006) (X. Zhang, Gureasko, Shen, Cole, & Kuriyan, 2006). Indeed, an allosteric mechanism of activation was identified for EGFR. The EGFR TKD forms an asymmetric dimer in which the C-lobe of one TKD, called the 'Activator,' makes contacts with the N-lobe of the second TKD, called the 'Receiver.' These contacts induce conformational changes in the N-lobe of the Receiver kinase and disrupt cis-autoinhibitory interactions in this monomer. The active receiver kinase can turn into its active configuration without the need of phosphorylation in the activation loop. The intracellular juxtamembrane region of EGFR also plays a role in promoting the allosteric mechanism of its activation (Jura et al., 2009) (Red Brewer et al., 2009). Part of the juxtamembrane region of the Receiver kinase interacts with the C-lobe of the Activator kinase. This interaction promotes dimerization and allosteric activation of the Receiver.

The first phosphorylation target for RTKs is the receptor itself, which creates a set of phosphotyrosines that form landmarks for the recruitment of downstream signaling molecules in response to ligand stimulation. These cytoplasmic signaling molecules contain domains that recognize

phosphorylated tyrosine residues such as Src homology-2 (SH2) and other phosphotyrosine-binding (PTB) domains. Examples of docking proteins are IRS1 (insulin receptor substrate-1), Gab1 (the Grb2-associated binder) and Shc1 ((Src homology 2 domain containing) transforming protein 1). These proteins contain a membrane targeting site at their N- terminus and a set of tyrosine phosphorylation sites that serve as binding sites for several downstream signaling proteins. The involvement of a broad array of docking proteins allows activated RTKs to recruit a large number of different signaling molecules. Therefore, an activated RTK can be part of a complex signaling network that transmits information from the extracellular environment to the cell.

In light of the wide range of signaling pathways downstream of RTKs, an important question is how the specificity of signaling is conferred. In some cases, as for the EGF and NGF receptors, two different RTKs were shown to engage similar components in the cellular signaling network to elicit quite different cellular responses (proliferation and differentiation, respectively). This diversity in the response might be explained with the concept of the 'bowtie' or 'hourglass' structure of the network (Citri & Yarden, 2006) (Oda, Matsuoka, Funahashi, & Kitano, 2005), where diverse inputs and outputs are linked through a conserved 'processing' core. For example, the four ErbB receptors (EGFR, ErbB2, ErbB3 and ErbB4) are regulated by multiple ligands to bring many signaling inputs into the network. These inputs converge on a limited set of highly conserved 'core processes'. The core processes are then linked to 'output' events that define the cellular response such as proliferation, differentiation or apoptosis. There is a substantial crosstalk between the components of the conserved core processes that contributes to the characteristic feature of robust and complex systems (Kitano, 2004). However, many details of the bowtie model still need to be clarified. It is still unclear which ErbB receptors are activated by each of the several ErbB ligands or how ErbB receptors heterodimerize. Also positive and negative feedback loops, both within and between different levels of the network, play regulatory roles through the bow tie network. In general, through positive feedback the system acquires more sensitivity to signaling inputs by amplifying the stimulus. RTK autophosphorylation is reversed by protein tyrosine phosphatases (PTPs). Thus, RTK activation can be promoted not only by ligand-stimulated kinase activity but also by ligand-inhibited PTP activity, or both. An example of mechanism of positive feedback where PTP is involved is related to EGFR. H_2O_2 and other reactive oxygen species (ROS) are produced upon activation of EGFR through PI-3 kinase and Rac dependent activation of NADPH-oxidase (Bae et al., 1997). These ROS transiently inhibit PTP activity by oxidizing a crucial cysteine in the phosphatase active site and thereby, positively regulates EGFR activation (Tonks, 2006). Negative feedbacks, instead, define the steady-state level of a response and keep it constant over many signaling inputs though many mechanisms. The most obvious example of negative feedback mechanisms is the direct activation of PTPs. The SH2 domain-containing phosphatases Shp1 (PTPN6) and Shp2 (PTPN11) target EGFR and promote its dephosphorylation in a negative feedback loop. Many negative feedback loops can involve receptor-

dependent stimulation of heterologous protein kinases. EGFR stimulation promotes activation of protein-kinase-C (PKC) via PLCγ. In turn, PKC can phosphorylate T654 in the juxtamembrane domain of EGFR. This phosphorylation disrupts affinity binding of EGF to EGFR (Ullrich & Schlessinger, 1990) and thus inhibits EGFR activation in a negative feedback loop.

Another key element of negative feedback in RTK signaling is downregulation of the receptors following their activation. This mode of negative feedback involves ligand-stimulated endocytosis of receptors and subsequent intracellular degradation of both ligand and receptor molecules (Sorkin & Goh, 2009) (von Zastrow & Sorkin, 2007). For example, activated EGFR is internalized primarily by clathrin-mediated endocytosis. EGFR traffics through multi-vesicular bodies (MVBs) into lysosomes where both EGF and EGFR are degraded (Sorkin & Goh, 2009). Indeed, it was for longtime assumed that RTK activation and cell signaling take place primarily at the cell surface, and that its endocytosis occurs exclusively to terminate RTK activation. It is now clear that activated RTKs continue to recruit and activate intracellular signaling pathways even from intracellular vesicles after they are internalized (von Zastrow & Sorkin, 2007) (Di Guglielmo, Baass, Ou, Posner, & Bergeron, 1994) (Miaczynska, Pelkmans, & Zerial, 2004). In these vesicles, they are dephosphorylated, ubiquitylated, and because of the lower endosomal pH, the ligand is dissociated. Moreover, many RTKs can be recycled from endosomes to the plasma membrane or sorted for degradation. Endocytosis can also be a way to prolong the duration of EGFR signaling by targeting the receptor toward a recycling fate, rather than a degradative one (Miaczynska et al., 2004). Also, many studies suggest that the RTKs-mediated signaling at the plasma membrane is different from the one that occur after its internalization. For example, the RTK Met traffics to a perinuclear endosomal compartment and here, appears to be required for tyrosine phosphorylation of STAT3 but not for MAPK activation (Kermorgant & Parker, 2008). I have already alluded above to the different signaling property of mutant, ER-localized FLT3 (Choudhary et al., 2009). Thus, spatial localization clearly has an impact on signaling specificity.

ALK/LTK subfamily

The RTKs Leucocyte Tyrosine Kinase (LTK) and the Anaplastic Lymphoma Kinase (ALK) form the ALK/LTK subfamily. ALK was first discovered as a tyrosine kinase in anaplastic large-cell lymphoma (ALCL) cell lines, where ALK were fused with other proteins (NPM–ALK) resulting from chromosomal translocation (Shiota et al., 1994) (Morris et al., 1995). The human ALK gene encodes the full-length ALK protein with 1620 amino acids and is considered a unique RTK member because of its ECD which contains an extracellular domain structure that does not exist in any other RTK member, including mammalian LTK. ALK is expressed during the development of the nervous system (Iwahara et al., 1997) (Vernersson et al., 2006). Indeed, during mouse development, ALK is expressed in the central and peripheral nervous system as well (Morris et al., 1997) (Weiss et al., 2012) but this expression was shown to decrease after mouse birth. In adult mammals as well, a low-expressed ALK

is present in certain regions of a few organs, such as the hippocampus within the brain (Iwahara et al., 1997) (Vernersson et al., 2006) (Weiss et al., 2012) (Bilsland et al., 2008). So far, no direct biological roles of ALK were identified, although it is highly possible that these roles are related to the development and function of the nervous system.

The unique features in ALK structure are related to its ECD which is composed of 1038 amino acid residues. This domain has a low-density lipoprotein receptor class A domain (LDLa) which is surrounded by two MAM domains (meprin/A5-protein/PTPmu). In addition, there is an N-terminal signal peptide and a glycine-rich region. As for all RTKS, ALK ICD comprises a tyrosine kinase domain and the juxtamembrane region. However, the biological roles of the LDLa domain and the MAM domain are not yet clarified. What is known from studies of the MAM domain in other cell surface receptors is that they might be involved in cell–cell interactions through homophilic binding (Beckmann & Bork, 1993) (Zondag et al., 1995) (Cismasiu, Denes, Reilander, Michel, & Szedlacsek, 2004). The role of the glycine-rich region of ALK which contains consecutive glycine residues is not clear as well and by now, the complete structure of ALK is not available. Most of the information on ALK structure is related to its kinase domain. Although many studies gave a deeper understanding of the structure of ALK, the exact activation mechanism of ALK is still not completely understood. Following the general mode of RTKs activation, ALK dimerization may support the trans-phosphorylation of some tyrosine residues (Y1278, Y1282, and Y1283) in the activation loop. Then, other tyrosine residues can be phosphorylated in turn to activate ALK kinase activity. Activated ALK was shown to activate several downstream pathways. For example, the NPM–ALK fusion protein, which is the result of a specific chromosomal translocation that brings the nucleophosmin *(*NPM*)* gene to the anaplastic large cell lymphoma kinase (ALK), was found to activate the RAS/MAPK pathway, the JAK/STAT pathway, the PI3K/Akt pathway, and the PLC (phospholipase C)-γ pathway (Roskoski, 2013) (Donella-Deana et al., 2005) (Tartari et al., 2008) (Chiarle, Voena, Ambrogio, Piva, & Inghirami, 2008) (R. H. Palmer, Vernersson, Grabbe, & Hallberg, 2009). The activation of these pathways by NPM–ALK occurs through the phosphorylation of specific tyrosine residues corresponding to Y1358, Y1507, and Y1604 of the full-length ALK.

Recent research has discovered potential ligands for ALK: the ALKAL1&2 (also referred to as FAM150A&B or AUGα&β). These studies have shown that ALKALs bind to the ALK and LTK ECD and these RTKs (H. Zhang et al., 2014) (Guan et al., 2015) (Reshetnyak et al., 2015) (Mo, Cheng, Reshetnyak, Schlessinger, & Nicoli, 2017) (Fadeev et al., 2018) (Reshetnyak et al., 2018). It was shown through in vitro studies that ALKALs activate ALK kinase activity. Also, conditioned medium containing ALKALs led to activation of ALK in several ALK-expressing cell lines (Guan et al., 2015) (Reshetnyak et al., 2015). Additionally, studies in Drosophila and zebrafish models showed that expression of ALKALs promote activation of wild-type ALK supporting ALKALs as ligands of the ALK/LTK receptor family (Mo et al., 2017) (Fadeev et al., 2018). Heparin was also suggested to

be a putative ligand of mammalian ALK (Murray et al., 2015). A heparin-binding motif was found in the N-terminal region of the ALK ECD and heparins with a relatively long chain were shown to bind to the ALK ECD and activate ALK (Reshetnyak et al., 2015). In Drosophila model, jelly belly (Jeb) was discovered as ligand of ALK. In this model, during fruit fly embryogenesis, dALK seems to play a role in gut development and one of its downstream pathways, such as the ERK pathway are activated (Loren et al., 2003) (Englund et al., 2003) (H. H. Lee, Norris, Weiss, & Frasch, 2003). In addition, dALK and its ligand Jeb were shown to play a critical role in the development of the visual system as well (Bazigou et al., 2007).

Because oncogenic activation of ALK kinase activity is crucial to ALK fusion proteins and ALK gain-of-function point mutants, inhibition of ALK kinase activity is the key to targeting ALK in various cancers. To date, multiple generations of ALK tyrosine kinase inhibitors (TKIs) have been generated and evaluated for clinical use. Many inhibitors for ALK kinase activity (ALK-TKIs) are currently used in cancer treatment (Sakamoto et al., 2011) (Guan et al., 2016) (J. Lu et al., 2017) (Gettinger et al., 2016) (Infarinato et al., 2016) (S. Zhang et al., 2016) (Carneiro et al., 2018) (Friboulet et al., 2014). So far, several highly potent selective ALK–TKIs were shown to inhibit ALK and some of them have already been approved for clinical treatments of specific cancers. Some examples of ALK inhibitors used in clinical application are ceritinib, crizotinib and alectinib.

As stated previously, human ALK has a unique ECD composition due to the presence of two MAM domains, an LDLa domain and a glycine-rich domain. In comparison, the ECD of human LTK is smaller and lacks the LDLa and both MAM domains. Studies conducted in mice lacking ALK showed defects in neurogenesis but are viable, as are ALK/LTK double mutants (Bilsland et al., 2008) (Weiss et al., 2012) (Witek et al., 2015). However, in the Drosophila melanogaster and Caenorhabditis elegans models only a single ALK RTK is present: DAlk and sdc-2, respectively (Loren et al., 2001) (Reiner, Ailion, Thomas, & Meyer, 2008). Instead, in Zebrafish (Danio rerio) two members of the ALK RTK family are present: LTK (DrLtk) and ALK (DrAlk) (Lopes et al., 2008) (S. Yao et al., 2013). In this system model, LTK possesses two MAM domains, while ALK has a smaller ECD that lacks one MAM domain (Lopes et al., 2008). In zebrafish, LTK is expressed in the early neural crest and gradually becomes restricted to iridophores (Lopes et al., 2008). In this model, LTK was shown to play a role in the development of iridophores in pigment pattern formation. Indeed, zebrafish with mutantions in LTK led to a lack of iridophores and display patterning defects (Lopes et al., 2008) (Fadeev, Krauss, Singh, & Nusslein-Volhard, 2016) (Frohnhofer, Krauss, Maischein, & Nusslein-Volhard, 2013) suggesting a role for the RTK in the establishment and proliferation of iridophores and their progenitors from multipotent neural crest cells (Lopes et al., 2008). Other studies, conducted in chicken, revealed that LTK is very similar in structure to ALK, a feature shared for all avian LTK. Here, it was suggested that LTK is required for survival of migrating cranial neural crest cells that was shown to interact with midkine which is produced by the non-neural ectoderm (Vieceli & Bronner,

2018). LTK was first identified in cells of hematopoietic origin such as pre-B and B lymphocytes and in neuronal cells (Ben-Neriah & Bauskin, 1988) (Bernards & de la Monte, 1990). However, one of the most widely expressed murine LTK isoforms was found to reside in the ER where the protein showed potent activation upon treatment of cells with alkylating or thiol-oxidizing agents. Therefore, LTK was proposed to act as a ligand-independent ER-resident kinase which is regulated by redox changes (Bauskin, Alkalay, & Ben-Neriah, 1991). However, the results showing LTK localization to the ER were obtained exclusively with LTK over-expressing transfected cells. Because it seems unusual to think of a receptor tyrosine kinase residing in the ER and not at the cell surface as happens for all other RTKs, a later study attempted to clarify the retention of LTK in the ER (Snijders, Ho, Haase, Pillai, & Bernards, 1997). It was suggested that ER retention was not the normal fate of LTK. Instead, this study proposed that LTK exists in two forms: a form with the kinase domain in the cytosol and an "inverted" form where the kinase domain is luminal. It was proposed that ER retention is only for the inverted form to prevent surface expression of incompletely folded or partially assembled proteins (Snijders et al., 1997). However, these data are not in agreement with any other previous or subsequent paper on LTK and should be considered with care.

LTK has also been linked to diseases such as Systemic lupus erythematosus (SLE) (N. Li et al., 2004). SLE is an autoimmune disease characterized by aberrant proliferation/maturation of self-reactive B cells that lead to production of autoantibodies against several autoantigens and immune complex-type tissue inflammation. In SLE murine models New Zealand Black (NZB) mice, a gain-of-function polymorphism in the LTK kinase domain was identified. The same type of LTK polymorphism was found in SLE patients as well. This study suggested that these polymorphic LTKs cause up-regulation of the PI3K pathway, in line with previous studies showing that the p85 regulatory subunit of PI3K directly binds to tyrosine 753, in the kinase domain of human LTK (Ueno et al., 1997). It was also found that human LTK utilizes two major signaling molecules, Shc, which binds LTK to the tyrosine 862 and IRS-1 which binds to tyrosine 485, leading to initiation of growth signals transmitted through the Ras pathway (Ueno et al., 1995) (Ueno et al., 1996). The observed NZB-type amino acid substitution in the LTK gain of function polymorphism was suggested to enhance ligand-independent autophosphorylation of LTK and subsequent recruitment and activation of the PI3K pathway, resulting in the genetic susceptibility to SLE in altered autoreactive B cells (N. Li et al., 2004). The mechanistic details of this are unclear. In addition, my own results impose a challenge to these data and imply that the beneficial effect of active LTK in SLE might have other reasons that PI3K activation.

Aims of the study

The main aim of the study was to advance our understanding of autoregulation signaling at the ER. For so long, it remained unknown whether the ER harbors resident signaling molecules that respond to local perturbations and re-establish ER homeostasis. While the adaptive responses to misfolded proteins are reasonably well understood, very little is known about equivalent mechanisms that sense the load of folded protein and respond by regulating ERES and thus, ER export. This work has been inspired by several siRNA screens that have been conducted to study the endomembrane system and that led to the identification of a collection of kinases that regulate ERES. The focus of this work is the leukocyte tyrosine kinase (LTK) and its role as regulator of COPII dependent trafficking and ER proteostasis.

Paper 1: LTK is an ER-resident receptor tyrosine kinase that regulates secretion.

The aim of this study was to investigate the role of the ER-resident signaling event triggered by LTK in the regulation of COPII dependent trafficking.

Paper 2: Targeting proteostasis in Multiple Myeloma through inhibition of LTK.

In paper 1, LTK was identified as an ER-resident RTK that regulates secretion through Sec12 phosphorylation and thus, COPII assembly. The aim of this study was to investigate LTK as a druggable regulator of ER export that responds to the load of secretory trafficking for treatment of diseases that are characterized by excessive secretion, such as MM.

Paper 3: LARP1 binding to hepatitis C virus particles is correlated with intracellular retention of viral infectivity.

In this study, depletion of LARP1 results in a decreased extracellular infectivity of HCV and in an increased intracellular infectivity. The aim of my contribution to this work was to test whether the impact of LARP1 depletion on intracellular infectivity was due to its general impact on the secretory pathway of the hepatocyte.

Summary of included papers

Paper 1: LTK is an ER-resident receptor tyrosine kinase that regulates secretion.

Past research on secretory pathway (SP) provided a deep understanding of the core components that control trafficking between organelles as well as their architecture. However, it is becoming increasingly clear that the SP is subject to regulation by signaling. Much is known on how the secretory pathway responds to external stimuli but our understanding of its autoregulation is less developed. This is mostly due to our ignorance of resident signaling molecules that can potentially trigger homeostasis-maintaining responses. So far, the best-understood example for autoregulation of the secretory pathway is the unfolded protein response (UPR) that is induced by an accumulation of unfolded proteins in the ER and results in an increased expression of machinery for protein folding, degradation, vesicle budding, tethering, and fusion (Gardner et al., 2013).

To systematically assess the extent of kinase/phosphatase signaling at the ER-Golgi interface, an siRNA screen of human phosphatome and kinome for regulators of the functional organization of the ER-Golgi system was previously conducted (Farhan et al., 2010). Among the hits, leukocyte tyrosine kinase (LTK) which was previously reported to partially localize to the ER (Bauskin et al., 1991), emerged as a strong potential regulator of the early SP.

Here, we show that LTK, unlike most RTKs, localizes to the ER. Moreover, inhibition of LTK, either by depletion of by drugs reduces the number of ER exit sites (ERES) and negatively affects ER-to-Golgi trafficking. Therefore, LTK potentially represents an example for autochthonous signaling at the ER.

Next, we wanted to explore LTK signaling pathways by using a proteomic approach to map LTK interactome. The interactome contains many cargo receptors such as ERGIC-53 that regulates the transport of glycoproteins out of the ER and Sec12, the exchange factor for Sar1 that initiates COPII coat assembly. We show that overexpression of clients for ERGIC-53 results in activation of LTK, indicating LTK might be a sensor for cargo load. In addition, LTK interacts with and phosphorylates Sec12. Expression of a phosphoablating mutant of Sec12 reduces the efficiency of ER export. Because Sec12 is an exchange factor for Sar1, we investigated the dynamics of this small GTPase. Chemical inhibition of LTK negatively affected the dynamics of Sar1 at ERES, further supporting the notion that LTK signals to Sec12 and thereby regulates COPII and ERES. Thus, LTK-to-Sec12 signaling represents the first example of an ER-resident signaling event.

This work identifies LTK as the first ER-resident receptor tyrosine kinase that regulates COPII-dependent trafficking and thus represents a potential proteostasis regulator.

Paper 2: Targeting proteostasis in Multiple Myeloma through inhibition of LTK.

Multiple myeloma (MM) is a plasma cell malignancy that is characterized by excessive immunoglobulin secretion thus, making cells addicted to mechanisms that maintain proteome homeostasis (proteostasis) (Auner & Cenci, 2015) (Palumbo & Anderson, 2011). Proteostasis is the result of a balance between, on one hand, the synthesis and trafficking of nascent proteins, and on the other hand, the degradation of unfolded/misfolded proteins. The biosynthetic and degradative parts of proteostasis are highly interconnected and form the so called proteostasis network. Several therapeutic approaches that target the degradative part of proteostasis, have been developed as treatment for MM. Indeed, proteasome inhibitors have been clinically approved as first-line therapy for MM (Moreau et al., 2012). However, although hyper-secretion is a crucial phenomenon in MM, only limited attempts have been made to target protein secretion. This is partly due to the fact that secretion has been considered notoriously undruggable.

In this study, we focus on the leukocyte tyrosine kinase (LTK) which is an ER-resident receptor tyrosine kinase that regulates export from the ER (Centonze et al., 2019) and on the potential use of LTK as druggable target of the biosynthetic part of the proteostasis network in MM therapy. We show that LTK is activated by secretory flux and that MM patients exhibit an increase in LTK copy number. Furthermore, these cells are sensitive to inhibition of LTK resulting in ER stress. Our study suggests that LTK helps MM cells to cope with their higher secretory demand. We were able to mimic this situation experimentally by showing that HeLa cells that are not hypersecretory do not respond to LTK inhibition by ER stress or apoptosis. On the contrary, HeLa cells that are hypersecretory were sensitive to LTK inhibition.

We suggest that targeting the biosynthetic arm of proteostasis through LTK might be a new promising strategy in MM treatment. Indeed, inhibition of LTK in MM cell lines, both PI-sensitive and PI-resistant, and in patient-derived myeloma cells results in a clear reduction of cell viability. Furthermore, in vivo treatment with LTK inhibitors leads to a strong growth suppressive effect.

In this work, we identify LTK as a druggable regulator of ER export that responds to the load of secretory trafficking and that can be potentially used in MM therapy.

Paper 3: LARP1 binding to hepatitis C virus particles is correlated with intracellular retention of viral infectivity.

The translation regulator LARP1 was found on Hepatitis C virus (HCV) virions. HCV buds through the ER membrane and it is therefore expected that virion-bound proteins such as LARP1 translocate to the ER lumen prior to their association with the virion.

This study confirms the LARP1-HCV interaction by using post-virion capture, immunoelectron microscopy, and immunoprecipitation in vitro (Huh7.5 liver cells) and in patient-derived HCV

particles. They show that upon HCV infection of Huh7.5 cells, LARP1 is drastically transfered to lipid droplets, inducing colocalization with core proteins. Moreover, depletion of LARP1 results in a decreased extracellular infectivity of HCV and in an increased intracellular infectivity.

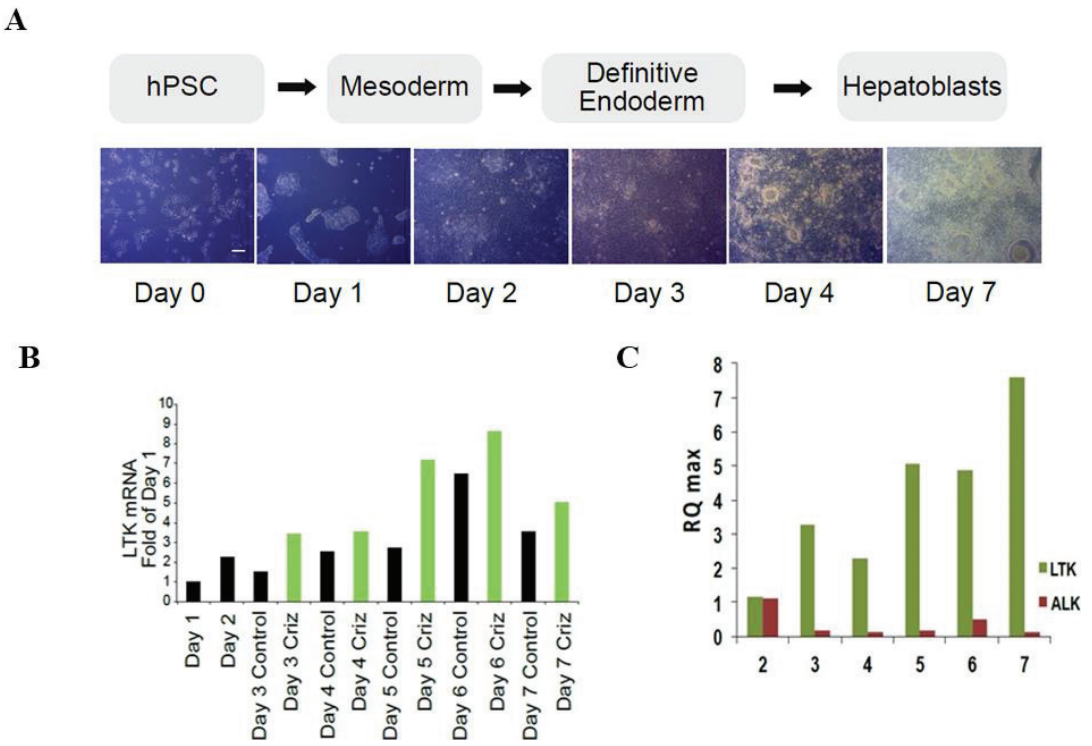
My contribution to this work was to test whether the enhancing impact of LARP1 depletion on HCV intracellular infectivity was due to its general impact on the secretory pathway of the hepatocyte. For this purpose, a RUSH (retention using selective hooks) assay (Boncompain & Perez, 2013) was used. The results reveal that LARP1 depletion does not impair the trafficking of secretory cargoes between the ER and the Golgi apparatus, suggesting that the increased intracellular infectivity is not related to an increased viral output.

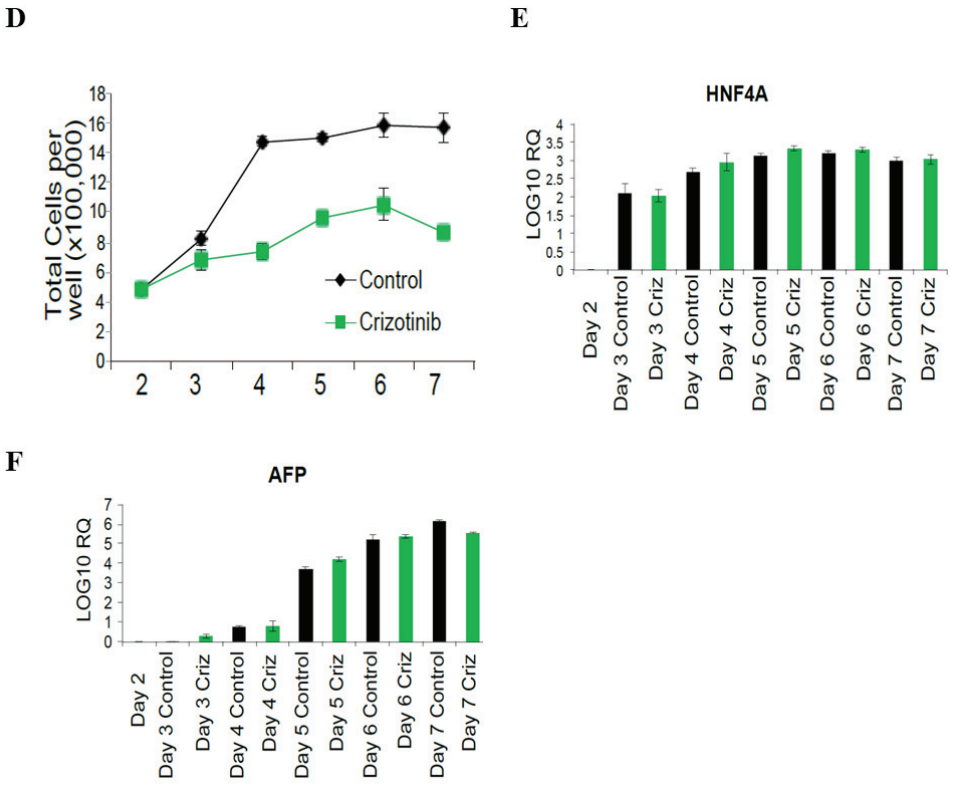
In this study, they identify LARP1 as a new component of a subset of HCV particles and show that it plays a role in the virus life cycle by restricting HCV propagation.

Additional Data

The role of LTK in hepatocyte differentiation

The role of LTK in secretion tempted us to ask whether cellular differentiation towards high secretory cells is accompanied by changes in LTK expression. To test this, we made use of a model that employs a small-molecule driven differentiation of pluripotent stem cells (hPSC) to hepatoblasts over a course of 7 days and further to hepatocytes after 17 days (Siller, Greenhough, Naumovska, & Sullivan, 2015) (Mathapati et al., 2016) (Fig. A). LTK was differentially expressed during the differentiation to hepatocyte like cells, with a peak of activity observed between day 6 and 7 (Fig. B). ALK was barely detectable in these cells with an over 50-fold higher LTK expression over ALK at day 7 (Fig. C). We reasoned that the upregulation of LTK might serve to help cells deal with the higher secretory load, as they become hepatocyte-like cells. In this case, inhibition of LTK is expected to result in cell death. This was the case as shown by the significantly lower cell count in cells treated with crizotinib (Fig. D). We noted a slight upregulation of LTK expression in crizotinib treated cells, which might be due to an attempt to compensate for the loss of LTK function (Fig. B). The lower cell count was not due to a defect in cell differentiation because crizotinib-treated and control cells did not exhibit any differences in the expression of stage specific markers of the differentiation procedure, namely AFP and HNF4A (Fig. E and F).





Figures: A. Schematic representation as well as representative images of the differentiation of iPSC to hepatoblasts. Scale bar= 50 µm. **B.** Quantitative PCR of LTK mRNA levels during different stages of hepatoblast differentiation in conditions treated with 1µM crizotinib (green bars) or with solvent (black bars). Data are from two experiments performed in triplicate. **C.** Comparative plot of quantitative PCR of LTK and ALK mRNA during different stages of hepatoblast differentiation. **D.** Cell count at different stages of the hepatoblast differentiation protocol in cells treated or not with 1µM crizotinib. **E** and **F.** Quantitative PCR of AFP and HNF4A mRNA levels during different stages of hepatoblast differentiation in conditions treated or not with crizotinib.

Discussion

It is very well established that endomembranes are subject to regulation by signaling pathways. This is supported by the fact that many signaling molecules such as kinases and phosphatases were found to localize to all cellular organelles. Organelles are capable to respond to perturbations and reestablish their homeostasis through activation of autoregulatory signaling circuits. Past research on the Golgi identified two types of adaptive responses: a slow mode and a fast mode of adaptation. The slow mode of autoregulation involves transcriptional regulation upon Golgi stress (Oku et al., 2011) (Reiling et al., 2013) to fulfill various roles. While Golgi stress signaling via the transcription factor CREB3 regulates apoptosis, the response via TFE3 regulates glycoprotein transport (K. Sasaki & Yoshida, 2019). The fast autoregulatory response of the Golgi involves enzyme-linked signaling (Pulvirenti et al., 2008) (Cancino et al., 2014). For instance, arrival of a cargo wave at the Golgi results in in local activation of Src family kinases and protein kinase A, resulting in phosphorylation of the transport machinery that regulates intra-Golgi traffic, as well as retrograde traffic to the ER. Those responses are activated by the arrival of KDEL receptor clients at the Golgi, which is a signaling-input to the Golgi that a traffic wave is ongoing. The activation of anterograde traffic through the Golgi as well as retrograde transport to the ER, contributes increase the processivity of the Golgi, as well as to recycle chaperones back to the ER (Cancino et al., 2014; Pulvirenti et al., 2008).

Being a major site for proteostasis, the ER must also be autoregulated. The best-investigated example for autoregulation at the ER is the UPR, which is induced by an imbalance between protein load and chaperones or the accumulation of misfolded proteins. This response activates a transcriptional program that induce the gene expression of chaperones as well as the machinery for vesicle budding, tethering and fusion in order to re-establish the homeostatic balance in the ER (Gardner et al., 2013). A chronic increase in cargo load was shown to result in the biogenesis of ERES in a manner dependent on the UPR sensor IRE1 (Farhan, Weiss, Tani, Kaufman, & Hauri, 2008). Because the UPR is well characterized and understood, all the potential ER-resident adaptive responses should be comparable to it. However, so far, our knowledge on whether the ER is also capable of adapting rapidly to changes in the load of secretory proteins remains superficial. This gap is mainly due to the lack of known signaling molecules that localize to the ER and that modulate from there the function of the organelle itself. Until very recently, the only examples of signaling from the ER were represented by mutant, variants of kinases or GTPases (Choudhary et al., 2009) (Arozarena et al., 2004). In order to overcome this missing piece, several siRNA screens have been conducted in order to find out the mechanisms and players involved in local signaling within the endomembrane system. This led to the identification of a collection of kinases that regulate ERES (Farhan et al., 2010) (Simpson et al., 2012) (Kondylis et al., 2011) (Bard et al., 2006) (Wendler et al., 2010). The two screens conducted in mammalian system (Farhan et al., 2010; Wendler et al., 2010) identified LTK among the top hits that were proposed to regulate ER export.

LTK was already reported to localize (at least partially) to the ER (Bauskin et al., 1991). This group already pointed out the unusual features of LTK in comparison with the other members of RTK family. LTK appeared to have a miniature version of the insulin receptor N-terminal domain. Moreover, it was very surprising to find that LTK localizes to the ER. A problem with this study was that it relied on overexpression of LTK in cells that might not normally express this RTK. Indeed, following studies from the same group, focused on understanding the reason of this LTK ER retention (Snijders et al., 1997). They identified a dual transmembrane topology of LTK in transfected cells that might explain the unusual behavior of LTK to be retained in the ER instead of proceeding to the cell surface. In my thesis, I was able to confirm the observations of LTK localization to the ER in transfected cells supported by glycosylation analysis through PNGaseF or EndoH treatment. Moreover, I showed for the first time that endogenous LTK localizes mostly to general ER and weakly to ERES. Using the RUSH assay, I also tested whether LTK leaves the ER, but I did not observe any evidence that this kinase ever traffics beyond the ER. Moreover, the biochemical analysis of LTK showed that the entire cellular pool was EndoH-sensitive, indicating that LTK resides in a pre-Golgi compartment.

As an ER-resident receptor tyrosine kinase, LTK represents a strong candidate to regulate secretion by local ER-based signaling. Indeed, LTK at the ER regulates general trafficking and ERES number through phosphorylation of Sec12 on Y10. The notion that the effect of LTK on ERES is via Sec12 is supported by the following observations (i) LTK regulates the dynamics of Sar1 on ERES, (ii) LTK regulates the number of Sar1-GTP containing ERES and (iii) a phosphoablating mutant of Sec12 reduces the number of ERGIC structures. I am aware of the fact that this is indirect evidence and that a direct demonstration of an increase of the GEF activity of Sec12 by Y10 phosphorylation is necessary. I attempted at using the tryptophan fluorescence assay (Antonny et al., 2001), and spent 3 weeks in the laboratory of Randy Scheckman, but unfortunately it was not possible for technical reasons. The assay showed a significant sensitivity to some of the reagents that are needed for Sec12 phosphorylation such as ATP. An increased signal of the fluorescence which is the indicator for GTP loading of Sar1, was detectable when Sar1 was incubated with ATP alone (without GTP). Although the reason why this happens it is not clear, it is obvious that the use of ATP is not compatible with this assay. In the future, an alternative approach could be used to overcome this technical difficulty. For example, both LTK kinase domain and Sec12 GEF domain can be co-purified in order to ensure Sec12 phosphorylation. In this way, there is no need to include any ATP in the assay.

Another interesting observation from previous studies on LTK (Bauskin et al., 1991) is that the N-terminal domain of LTK could function as a sensor for the redox balance on the ER. The formation of native disulfide bonds during client protein folding, which also requires reduction of non-native bonds are orchestrated by the protein disulfide isomerase (PDI) family (Appenzeller-Herzog & Ellgaard, 2008). PDIs catalyze oxidation, isomeration and reduction of disulfide bonds, which represent a

limiting step in the process of protein folding (Hatahet & Ruddock, 2007). Alkylating or thiol-oxidizing agents can facilitate the formation of disulfide-linked LTK multimers, possibly through the activity of protein disulfide isomerase, thereby activating the kinase. Because we propose LTK as regulator of protein secretion, which is a crucial process for proteostasis, we do expect LTK to crosstalk with other processes within the proteostasis network. Interestingly, many of the interaction partners of LTK that were identified by our proteomics study, are involved in ERAD or protein folding. For example, protein disulfide isomerase A4 (PDIA4) was one of those and can potentially play a role in redox regulation of LTK. Recently, new functions for members of the ER-resident protein disulfide isomerase family PDIs have been reported in redox regulation of the UPR sensors through thiol–disulfide exchange. One of these studies demonstrated that intramolecular disulfide bridges of the UPR sensor ATF6 have to be cleaved by PDIA5 for transport through the Golgi to its final destination in the nucleus (Higa et al., 2014). This PDI regulation mode cannot be applied for LTK, since we showed that LTK does not leave the ER using the RUSH assay. Another study showed that PDIA6 is able to control inactivation of the UPR sensor IRE1alpha through direct binding via a disulfide bond in the luminal domain (Eletto, Eletto, Dersh, Gidalevitz, & Argon, 2014). Whether LTK and IRE1 compete for binding to PDIs is a hypothesis that could be tested in the future. However, it is also possible that the interaction of LTK with PDIA4 is just a reflection of the normal folding process of LTK. Alternatively, the redox regulation of LTK autophosphorylation may be indirect, possibly through the inhibition of a protein tyrosine phosphatase (PTP). The PTPs are sensitive to inactivation by oxidation (Ostman, Frijhoff, Sandin, & Bohmer, 2011), and therefore it is tempting to speculate that this could be a mechanism how LTK responds to redox changes in the ER.

Understanding how LTK is activated at the ER represents the missing piece and the most challenging aspect of our mechanistic studies on LTK activation model. The main question to answer is: which is/are the stimulus/stimuli for LTK? Few years ago, it was suggested that the secreted proteins ALKAL (previously referred to as FAM150 or AUG) are the ligands for ALK and for LTK as well (Reshetnyak et al., 2015) (H. Zhang et al., 2014) (Fadeev et al., 2018) (Mo et al., 2017) (Guan et al., 2015). It should be pointed out that these studies were either conducted with zebrafish LTK (Fadeev et al., 2018), whose structure is more similar to ALK than human LTK, or they were conducted in cells double-positive for ALK and LTK. In our studies, co-overexpression of ALKAL1 (FAM150A, AUG-beta) and ALK resulted in ALK activation which is in agreement with what was previously showed (Guan et al., 2015; Mo et al., 2017; Reshetnyak et al., 2015; H. Zhang et al., 2014). However, expression of ALKAL1 resulted in LTK activation only when LTK and ALK were co-expressed but not when LTK was expressed alone. We were able to reproduce this outcome using Drosophila eye and where combinatorial expression of ALK and ALKAL1 resulted in a rough eye phenotype which was not the case when ALKAL1 was co-expressed with human LTK. ALKAL1 was also not the stimulus for LTK activation at the ER, because an ER-localized ALKAL1 failed in activating LTK.

However, clarifying the nature and the mechanism of the LTK-ALK crosstalk might be interesting. The fact that ALKAL1-dependent ALK activation at the cell surface leads to LTK activation in the ER might be a bit difficult to explain. It is known that endocytic organelles can play a direct role as intracellular signaling stations in signal propagation and amplification after internalization of some RTKs from the cell surface (Miaczynska et al., 2004). Internalization of ALK, upon ligand-dependent activation might directly or indirectly lead to activation of LTK at the ER membrane. The hypothesis that ALK presence leads to a switch in LTK localization from the ER to the cell surface, can be considered as well. The possibility that ALK "drags" LTK can be tested through confocal microscopy by analyzing their localization in LTK-ALK co-expressed cells and LTK/ALK expressed cells. To note, all human cell lines that were tested for LTK and turned out to be positive, were ALK negative. It seems that, cells that have LTK, do not need ALK. It would be interesting to find out the reason why this happens. A systematic analysis of LTK and ALK expression is required to find out whether there are human cells and tissues that are both LTK and ALK positive and whether these cells have a different LTK localization. Currently, this is pure speculation and more work is needed to illuminate the ALK-LTK crosstalk.

Regardless of the whether ALKAL1 is a ligand for LTK or not, our results propose a different activation mechanism for LTK, namely by a mechanism including cargo receptors such as ERGIC-53, VIP36 and SURF4. Since ERGIC-53 is the best characterized among cargo receptors, we wanted to test whether cargos of ERGIC-53 affect the activation level of LTK. Indeed, overexpression of 4 different ERGIC-53 clients positively regulated LTK activity and knockdown of ERGIC-53, SURF4 or VIP36 reduced LTK activation state. These findings clearly stated the involvement of cargo receptors, in particular ERGIC-53, in LTK activation but the mechanistic insight of the LTK-ERGIC-53 crosstalk is currently unclear. Many hypotheses were tested. The interaction of LTK with ERGIC-53 was not dependent on the ability of this cargo receptor to leave the ER or to form hexamers. In addition, the passage of ERGIC-53 through ERES did not appear to affect the activation state of LTK. We speculate that the ability of ERGIC-53 to form oligomers allows the associated LTK molecules to come in close proximity, thus facilitating trans-autophosphorylation. It was difficult to test this hypothesis, because we did not have a mutant of ERGIC-53 that is monomeric. ERGIC-53 forms a trimer of dimers (i.e. hexamers) when leaving the ER (Neve, Lahtinen, & Pettersson, 2005) and we only have a mutant that does not trimerize, but still forms dimers. We nevertheless observed that cargo-loaded ERGIC-53 associates with a phosphorylated pool of LTK, while a complex of only ERGIC-53, while binding to LTK, appears to contain mainly the inactive form of this kinase. For sure, this proposed model will need further investigation in order to be confirmed. First of all, interaction domains should be mapped to generate mutants of LTK that do not bind ERGIC-53. If our hypothesis is correct, we expect that such a mutant of LTK should be less active. Also, it should be tested whether a monomeric version of ERGIC-53 still associates with an active pool of LTK. Finally, potential

formation of transient clusters in the ER of cargo receptors with LTK could be tracked by live imaging.

LTK was shown to be able to regulate the trafficking of multiple types of cargo. However, if LTK is for instance activated by an ERGIC-53-clients, why should it then upregulate general secretion? Firstly, LTK signals to Sec12 which is the GEF for Sar1 and thus a general regulator of ERES. Secondly, assuming that LTK regulates the ER export of only an ERGIC-53 cargo would imply the existence of ERES dedicated exclusively to these cargos. However, no evidence for ERES that are specialized for a certain type of cargo is available. Thirdly, depletion of VIP36 and SURF4 also led to reduction of LTK activation, implying a broader range of cargos that control LTK activity. One possible model could be that an acute increase in the amount of secretory proteins is likely to include ERGIC-53-dependent glycoprotein cargo (as well as cargos for other receptors such as SURF4). In this scenario, ERGIC-53 and other cargo receptors act as sentinels that sense cargo load and deliver the "message" to LTK, which in turn regulates ERES through Sec12 phosphorylation. The fact that overexpressed wild type AAT, but not ATZ which aggregates in the ER, resulted in LTK activation, indicates that LTK does not respond to a general protein load but to a secretory load, instead. It might therefore be that LTK is part of a folded protein response, in analogy to the UPR. This idea is supported by the fact that LTK shares several features with the IRE1 and PERK sensors of the UPR, which represent the best characterized example of autoregulation at the ER. Like the UPR sensors, LTK is resident to the ER, it is a transmembrane protein and it has a cytosolic signaling competent domain. In addition, once active, LTK regulates a proteostasis-relevant process, very much like IRE1 and PERK. A feature that is different to the UPR is that LTK does not appear to directly sense folded proteins. Rather, this function appears to be taken over by cargo receptors. If LTK were part of a "Folded Protein Response", it would pose that the sensory and signaling module have split, while in the UPR they are united in a single protein. Another common feature between LTK and UPR signaling mediators is that their response is global, while the stimulus might be very restricted. The UPR may be triggered by the overload of a single misfolded protein (Bakunts et al., 2017), but the biologic output is a global increase of the folding and ERAD machinery. Similarly, LTK can be triggered by a single protein (e.g. wild type AAT), but it is capable of regulating the whole secretome. Very recently, another example of autoregulatory circuit operating at the ER was identified (Subramanian et al., 2019). A synchronous surge of folded cargo in the ER was shown to be sensed by the COPII subunit Sec24 which in turn recruits Gα12 and other signaling molecules from the cytosol to ERES. This proposed auto-regulatory system at the ER, named AREX was shown to promote ER export in response to an increased folded cargo load. Unlike LTK, AREX exhibits several differences to the UPR. The main difference is that the signaling module (Gα12) was not resident to the ER and only appeared to localize to ERES very weakly in cells with a traffic wave, but not in steady state. Because Sec24 was suggested to play the role of a "sensor", and because COPII trafficking is constitutive

process, we would expect (at least some) Gα12 to be at ERES. No evidence was provided for constitutive cycling of Gα12 on and off the ER. Again, this would be expected because it was proposed that the Sec24A&B isoforms were the main players involved in AREX, and thus we would expect a wide range of cargos to trigger AREX. Thus, AREX appears to be not relevant for ER export under steady state conditions. This is supported by the observation that inactivating Gα12 had no effect on general secretion. Another difference between AREX and the UPR is that none of the molecules involved in AREX is a transmembrane ER-resident protein. The other signaling mediators of AREX such as PKA and ERK1/2 are largely cytosolic and were barely detectable at ERES. The final and probably most important difference is that the biologic output of AREX is not to globally regulate secretion. Radioactive pulse-chase secretome analyses under depletion or inhibition of AREX signaling components, showed that the secretome was affected. Taken all these aspects together, LTK-mediated folded protein response seems to share more similarities with the UPR. Conversely, AREX represents a specific response of the ER export machinery to a selected set of cargos. How this can be reconciled with an autoregulation has to be carefully investigated in the future. Nevertheless, both AREX and LTK represent the first evidences of autoregulation at the ER in response to folded secretory protein.

Because of the role of LTK in protein secretion and its close similarity to the UPR, we needed to more deeply understand the potential role that LTK can play in a broader context within the proteostasis network. In my thesis, I collaborated with the group of Santiago Schnell to build a mathematical model of proteostasis that includes LTK. This was based on a previously established mathematical model of the UPR (Stroberg et al., 2018). LTK was positioned in the model such that it is activated by secretory cargo, as suggested in our study and thereby positively regulates the ER export machinery. As observed in previous work (Farhan et al., 2008), increasing cargo load induces UPR. The model predicts that when LTK is depleted, this leads to a higher grade of UPR. This prediction was then validated experimentally, by using a HeLa cell line where expression of IgM can be induced to high levels (Bakunts et al., 2017). Interestingly, induction of IgM expression for 24 h resulted in a mild induction of UPR, which was determined by XBP1s levels. When LTK expression was depleted, cells responded with higher levels of UPR upon induction of IgM expression, thus confirming the results or our mathematical model. This finding supports the hypothesis that LTK is a regulatory node of the proteostasis network. Investigating the LTK-UPR crosstalk, surely represents an exciting area for future studies. Since overexpression of folded proteins can trigger UPR, it is necessary to use an approach that activates the UPR or LTK selectively and in the absence of natural triggers. To test whether and how LTK regulates the UPR, LTK activation should be triggered in the absence of folded proteins. Many systems for inducible dimerization of LTK or inducible expression of constitutively active LTK could be used. The converse question should also be tested, namely. Does the UPR affect LTK? In this case, we showed that the misfolded ATZ does not activate LTK. However, it is well

known that this mutant does not active the UPR. In the future, expression of misfolded proteins that trigger the UPR should be performed (e.g. the NHK-mutant of alpha1-antitrypsin. My preliminary data indicate that this mutant is capable of inducing ER stress but does not activate LTK (data not shown). Another stimulus that can be used to trigger UPR can be induced in HeLa cells that express inducible immunoglobulin heavy chain (Ig-HC) that cannot exit the ER without the light chain and thereby, accumulates at the ER (Bakunts et al., 2017). The potential effect of the UPR on LTK can be measured by looking at its gene expression, ubiquitinylation and phosphorylation. Effects on LTK activation should be tested both directly (by determining autophosphorylation) as well as by determining the downstream outcomes such as Sec12 phosphorylation, ERES biogenesis and ER-to-Golgi trafficking. Also, the three arms of the UPR might differently contribute to the crosstalk with LTK. Thus, experiments upon differential inhibition or depletion of the three sensors should be included. Much work still needs to be done, in order to properly understand how LTK acts within the proteostasis network and crosstalk with its components in order to ensure protein homeostasis. However, our work contributes to place LTK as a positive regulator of ER export of folded cargos which can fine-tunes the PN through a crosstalk with the UPR.

If we take a look at LTK from the evolutionary point of view and in comparison to ALK, we see that these two RTKs exhibit the greatest difference in mammals. The main difference between LTK and ALK structure is related to their extracellular (or luminal) domain. Mammalian LTK has lost the two MAM domains which remain in mammalian ALK and the two kinases show different subcellular localization. ALK, as most of the RTKs, localizes at the plasma membrane where it was shown to bind to a set of secreted proteins (ALKALs) and from there to trigger several canonical signaling pathways (Fadeev et al., 2018; Guan et al., 2015; Mo et al., 2017; Reshetnyak et al., 2015). It can be possible that the loss of two MAM domains is responsible for the “unusual” localization of LTK. However, this is pure speculation and no evidences were collected to support this claim. A way to test this hypothesis is by generating an ALK mutant with no MAM domains or an LTK with MAM domains and look for their localization. Also, chimeric LTK/ALK can be used as a result of a fusion of LTK kinase domain and ALK extracellular domain and vice versa. Their localization and their ability to signal to Sec12 (phosphorylation) could be tested in the future. ALK received much more attention in clinical research than LTK. Indeed, many gain of functions mutations of ALK were linked to several types of cancer and several ALK inhibitors are currently in use for treatment of ALK-related diseases (Huang, 2018). My findings that LTK contributes to maintaining proteostasis by regulating the ER export has a strong translational potential. ER export is a fundamental and constitutive process in cells. Indeed, the components of the COPII machinery are highly conserved from yeast to human and cell-type independently expressed. This has led to the notion that ER-export is undruggable. LTK could now be used as a druggable target to inhibit ER export in the future and I will discuss this in the context of myeloma therapy below.

The ER export regulator LTK seems to be mammals-specific and seems to be expressed only in certain types of cells. For example, in our study different cell lines such as HEK, HeLa and HepG2 resulted to be negative, low-positive and high-positive for LTK expression, respectively. How can we explain this difference in expression? LTK is a modulator (or fine-tuner) of ER export but not essential for this process. So some cell types might need LTK more than some others. This difference in the need for LTK can be dependent on their secretory phenotype. Indeed, the fact that HepG2 cells are very LTK-positive can be because those are human liver carcinoma cells and thus, very secretory cells. HEK293 cells, instead, are LTK-negative and the fact that these cells operate without LTK, indicates that their ER export machinery can manage to meet the secretory demand. Thus, they might survive without LTK and still efficiently ER export. This suggests that LTK might be a helper to higher secretory cells to cope with their cargo loads. This is supported by the observation that inhibition of LTK in normal secretory cells had no impact on cell fitness (while inhibiting ER export), but inhibition of LTK in highly secretory cells led to higher apoptosis. Moreover, one possibility explaining why LTK is mammals-specific is that LTK might help in the biogenesis of tissues that are specific to mammals like the mammary gland during lactation or the placenta, which possess high secretory features. Indeed, according to human protein atlas, LTK expression is very high in placenta.

The hypothesis that LTK in mammals has evolved as a regulator of proteostasis and therefore might be involved in the acquisition of a secretory phenotype is supported by the following observations: (i) LTK is up-regulated during the differentiation from pluripotent stem cells to hepatoblasts and in cells with 2 weeks of IgM induction, (ii) cells with high secretory load are more sensitive to LTK inhibition than cells without secretory overload and (iii) a gain of function mutation in LTK has been observed in patients with SLE. Very likely, the upregulation of LTK serves to help cells deal with the higher secretory load, as they become hepatocyte-like cells or upon a prolonged secretory burden (2 weeks IgM-induction). Thus, inhibition of LTK results in reduced cell fitness under conditions of higher proteostatic stress and this is unlikely due to the alteration of the transcriptional program, because LTK inhibition had no effect on expression of markers of liver cell differentiation. SLE is characterized by aberrant proliferation/maturation of self-reactive B cells that lead to overproduction of autoantibodies, resulting in a hypersecretory phenotype (Kaul et al., 2016). Therefore, it might be that this gain of function mutation in LTK confers a selective advantage to autoimmune plasma cells as it allows them to cope with a higher secretory load.

Cancer cells constantly deal with many kinds of stress such as hypoxia, nutrient deprivation, oxidative stress, lactic acidosis and also ER stress that is due to a higher basal rate of protein translation. These environmental insults might even exacerbate ER stress for instance by causing redox imbalances of the ER. Therefore, cancer cells are considered to be addicted to secretion due to a high proteostatic challenge (Urra, Dufey, Avril, Chevet, & Hetz, 2016) (Dejeans et al., 2014). Multiple Myeloma (MM) is composed of malignant plasma cells that are highly secretory cells and is therefore particularly

reliant on mechanisms that maintain proteostasis (Palumbo & Anderson, 2011) (Auner & Cenci, 2015). Our study suggests that LTK helps MM cells to cope with higher secretory demand, which is supported by the fact that: (i) MM patients exhibit an increase in LTK copy number that is accompanied by an increase in the levels of the ER chaperone BiP (indicative of ER stress), (ii) MM cells are sensitive to inhibition of LTK, (iii) inhibition of LTK in MM cells induces ER stress. A possible reason for LTK amplification in MM is that these cells need to cope with a chronic secretory stress. This is supported by the observation that chronic increase of secretory load, increases LTK expression. The mechanism how cells upregulate their LTK levels during chronic adaptation was not revealed, but my preliminary data indicate that ER stress induction does not increase LTK mRNA, making the UPR an unlikely candidate to induce LTK expression under secretory cargo overload (although this has to be rigorously tested). Future work needs to test the possibilities whether transcription factors, increased splicing or mRNA stabilization account for the increase of LTK expression.

Bortezomib (Velcade) is clinically approved as first-line therapy for myeloma. Treatment with bortezomib, inhibits the proteasome leads to myeloma cell death by preventing misfolded proteins to be targeted for degradation through the ERAD and thereby triggering ER stress-induced apoptosis. Unfortunately, the biggest issue with Bortezomib treatment is the resistance that MM patients develop during the therapy (Moreau et al., 2012). So far, the first line treatment for MM is about targeting the degradative part of proteostasis and no attempts have been made to target other branches of proteostasis. Inhibition of LTK has a negative effect on protein secretion. Thus, targeting LTK represents the first attempt to target the biosynthetic branch of proteostasis in MM. Targeting the biosynthetic arm of proteostasis through LTK represents a new promising strategy in MM treatment. This is supported by the fact that: (i) targeting LTK in MM cell lines and in patient-derived myeloma cells showed a clear reduction of cell viability and (ii) in vivo treatment with LTK inhibitors led to a strong growth suppressive effect. LTK targets a different part of the proteostasis network than bortezomib, which also explains that MM cell lines resitant to this proteasome inhibitor are sensitive to LTK inhibition. Thus, LTK inhibitors could in the future be either combined with bortezomib, or be used on patients that developed resistance. Secretion has always been considered undruggable because it is a constitutive biological process. Thus, targeting modulators of ER export in MM would be a more suitable strategy than targeting core components of the COPII coat or the machinery for biogenesis of ER exit sites. As already discussed, LTK is a “helper” for protein secretion but not essential for this process. Another important fact is that all small molecules we used to target LTK are clinically approved drugs, which will facilitate entering clinical trials and make them available for MM patients in the near future.

In order to inhibit LTK, several established ALK inhibitors were used in our study. This was possible due to the high degree of high sequence homology of these two RTKs. These ALK inhibitors are

currently used to treat non-small-cell lung carcinoma (NSCLC). Only a small fraction of NSCLC patients is currently treated with ALK inhibitors (approx. 5%), as only patients with ALK mutations are thought to benefit from these inhibitors. As stated above, many cancers are addicted to mechanisms that maintain proteostasis. This opens an exciting possibility to test whether LTK is expressed in lung cancer patients and whether it could be used as a therapeutic target. This would expand the pool of patients that benefit from the already existing ALK inhibitors. This is clinically desirable, because these drugs have milder side effects compared to cytostatic drugs.

In our study, crizotinib was used to target LTK in the majority of the conducted experiments. Ceritinib and Alectinib were used as well. All three drugs were found to exert growth suppressive effects on L363cells and their bortezomib-resistant version. Moreover, it was showed that crizotinib, alectinib and ceritinib inhibit LTK, reduce the number of ERES and inhibit ER-to-Golgi trafficking. Off-target effects on ALK were ruled out because all experiments were conducted on LTK-positive but ALK-negative cell lines. Also, the cohort of MM patients that was analyzed in our studies, exhibited a very positive mean expression level of LTK, while the one of ALK was almost negative. However, in order to confirm that the effects seen upon ALK inhibitors treatment are LTK-dependent, LTK mutants which are resistant to inhibitors could be used in the future. These mutants could be constructed in analogy to already known ALK-mutations that render the kinase domain resistant to this inhibitor (Katayama et al., 2012). These mutations maintain a normal kinase activity, but do not bind the drugs. These LTK mutants should be expressed in an LTK-knockout background and we predict that such cells would become resistant to a certain LTK inhibitor (to which the kinase is resistant), but not to all inhibitors. By proposing LTK as target for a new therapeutic approach in MM treatment, we support the idea that targeting the part of the proteostasis network that handles folded proteins, might represent a good strategy to target the high secretory burden in cancer and neurodegenerative diseases. Many studies identified a wide range of kinases and phosphatases as regulator of the export from the ER (Farhan et al., 2010; Simpson et al., 2012) and thereby, might represent potential targets in treatment of hyper-secretory like pathologies.

Since we showed that LTK inhibition leads to an increased apoptotic activity leading to a decreased viability in MM cells, further investigation is needed to clarify how this happens. LTK inhibited cells exhibited higher XBP1 splicing which is an indicator of a stronger IRE1 activity. Whether this inhibition affects IRE1 phosphorylation should be tested. Since we proposed a crosstalk between LTK and UPR, we speculate that IRE1-dependent activation of JNK2 might be responsible for activation of apoptosis. Treatment of MM cells with JNK inhibitors would reveal whether the apoptotic effect of LTK inhibition is mediated by JNK. Furthermore, phosphorylation deficient mutants of IRE1 can be used to test whether its kinase activity is relevant for the apoptosis-inducing effect of LTK inhibition.

An important open question is whether LTK targets other substrates than Sec12 at the ER. Besides doing phosphoproteomics in the future, this question can be answered by looking at the top hits from

the LTK interactome. Interestingly, VCP seems to interact with LTK. This AAA+ ATPase was shown to play many roles within the proteostasis network such as in protein degradation through ERAD (Meyer & Weihl, 2014). Therefore, VCP might be phosphorylated by LTK and thereby contributes to the regulation of proteostasis by promoting proteasomal degradation of polyubiquitinated substrates. Future work could test whether LTK regulates VCP function through phosphorylation. If this is the case, exciting new avenues could be opened to test whether VCP-dependent biologic processes are sensitive to LTK inhibition such as dendritic spine formation in neurons (Shih & Hsueh, 2016) or autophagy (Meyer & Weihl, 2014). Another open question is how is LTK signaling terminated? The most obvious hypothesis is that LTK is deactivated through dephosphorylation by one or more phosphatases. Among the candidates there are three phosphatases which were described to localize to the ER (TC48 (Gupta & Swarup, 2006), PTPIP51 (Gomez-Suaga et al., 2019) and PTP1B (Haj, Verveer, Squire, Neel, & Bastiaens, 2002)). Others were described in previously conducted systematic screening to associate with LTK (DUSP6 (Z. Yao et al., 2017)) and two were identified in our own LTK interactome (DUSP9 and PTPN6). Future work will focus on testing whether depletion of these phosphatases increases LTK activity, and thereby affects its outcomes such as the number of ERES and the efficiency of ER-to-Golgi transport. Another possibility for termination of LTK signaling could be by proteosomal degradation. If this is the case, inhibition of the proteasome would affect LTK protein levels, which could be detected by CHX-chase experiments. Frequently, members of the RTKs family are sorted to the lysosome for degradation. This suggests the exciting hypothesis that LTK is terminated through ER-phagy. Upon an increased cargo load, it is known the ER size expands and LTK is activated. When the secretory demand drops, the ER size is reduced through ER-phagy (Fumagalli et al., 2016). ER-phagy would then also eliminate LTK and contribute to termination of its signaling. It might be possible that cells use all three ways to terminate LTK signaling without any preference or that, in specific situations, one way is preferred to the others. For example, in case of oxidative stress in the ER, phosphatases are inhibited and they might not be capable anymore to dephosphorylate LTK. Thus, the other two potential LTK termination mechanisms can take over. In order to test ER-phagy as termination mechanism for LTK, ER-phagy receptors such as RTN3 or FAM134B could be depleted to see whether this affects the level of LTK and its phosphorylation state. The same factors can also be overexpressed to determine whether induction of ER-phagy leads to LTK localization to the lysosome. The discovery that LTK signals locally at the ER to regulate ER export, suggests this RTK as regulator of the branch of the proteostasis network that handles folded secretory proteins. Moreover, through a cross-talk with the UPR, LTK is involved to a more complete and integrative regulation of proteostasis. The strong preliminary data establishing a role for LTK in MM set the basis for the potential of LTK as a drug target. Blocking ER export, by targeting LTK, could represent a new valid strategy in MM therapy. This will hopefully inspire future clinical research to focus on druggable modulators of ER export.

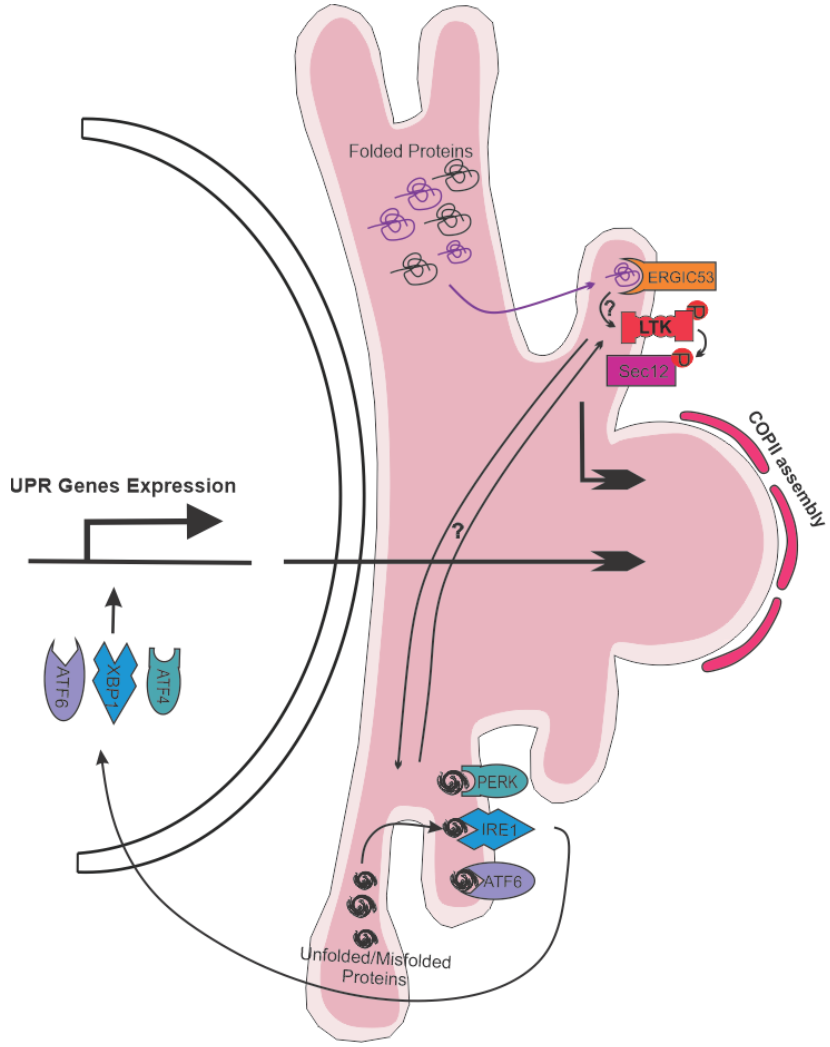


Figure 5: ***LTK as part of the "Folded Protein Response":*** *Detection of misfolded or unfolded proteins in the lumen of the ER by the three sensors of the Unfolded Protein Response (UPR) (IRE1, PERK and ATF6) leads to activation of signaling to the nucleus that induces the gene expression of chaperones as well as the machinery for vesicle budding, tethering and fusion. In analogy to the UPR, LTK is activated by folded proteins in the lumen of ER through its interaction with cargo-loaded cargo receptors such as ERGIC-53. Once active, LTK phosphorylates Sec12 and promotes COPII assembly and thus, ER export. Future work is needed to clarify how cargo-loaded ERGIC-53 leads to LTK activation and how LTK regulates proteostasis through a crosstalk with the UPR.*

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Original publications

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REPORT

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 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

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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 Golgi apparatus. LTK was completely sensitive to EndoH treatment, indicating that it resides in a preGolgi compartment (Fig. 1 F). On the other hand, only 60% of the ALK pool was sensitive to EndoH (Fig. 1 F). Available antibodies do not detect endogenous LTK by immunoblotting, preventing us from performing the same analysis with endogenous LTK.

To rule out that the absence of staining of LTK at the cell surface is due to fixation artifacts, we tagged LTK with GFP and performed live imaging. LTK localization was similar as in fixed cells, and was reminiscent of the ER (Fig. 1 G). Finally, we wanted to directly test whether LTK leaves the ER using the retention using selective hooks (RUSH) assay (Boncompain et al., 2012). The RUSH assay 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). We confirmed that crizotinib and alectinib inhibited LTK autophosphorylation in our experimental system (Fig. 2 D). We also tested the effect on crizotinib in live imaging and found that the onset of ERES reduction is after ∼10 min of treatment (Fig. 2 E). 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

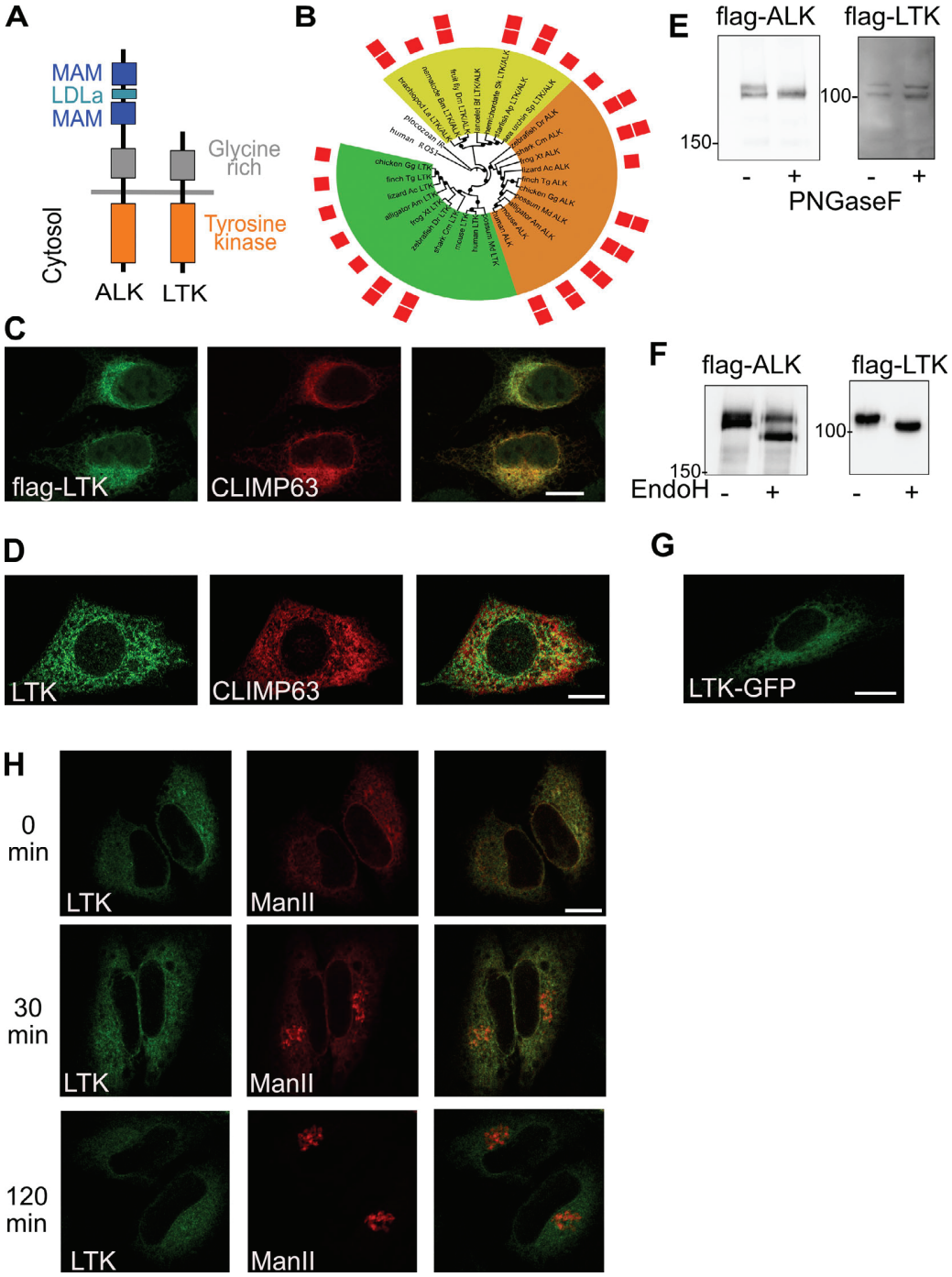

Figure 1. **Subcellular localization of LTK. (A)** Schematic illustrating the domains of LTK and ALK. LDLa, low-density lipoprotein (LDL) receptor class A repeat. **(B)** Phylogenetic tree of ALK and LTK kinase domains. Color ranges highlight invertebrate LTK/ALK-like proteins (yellow), vertebrate ALK 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.

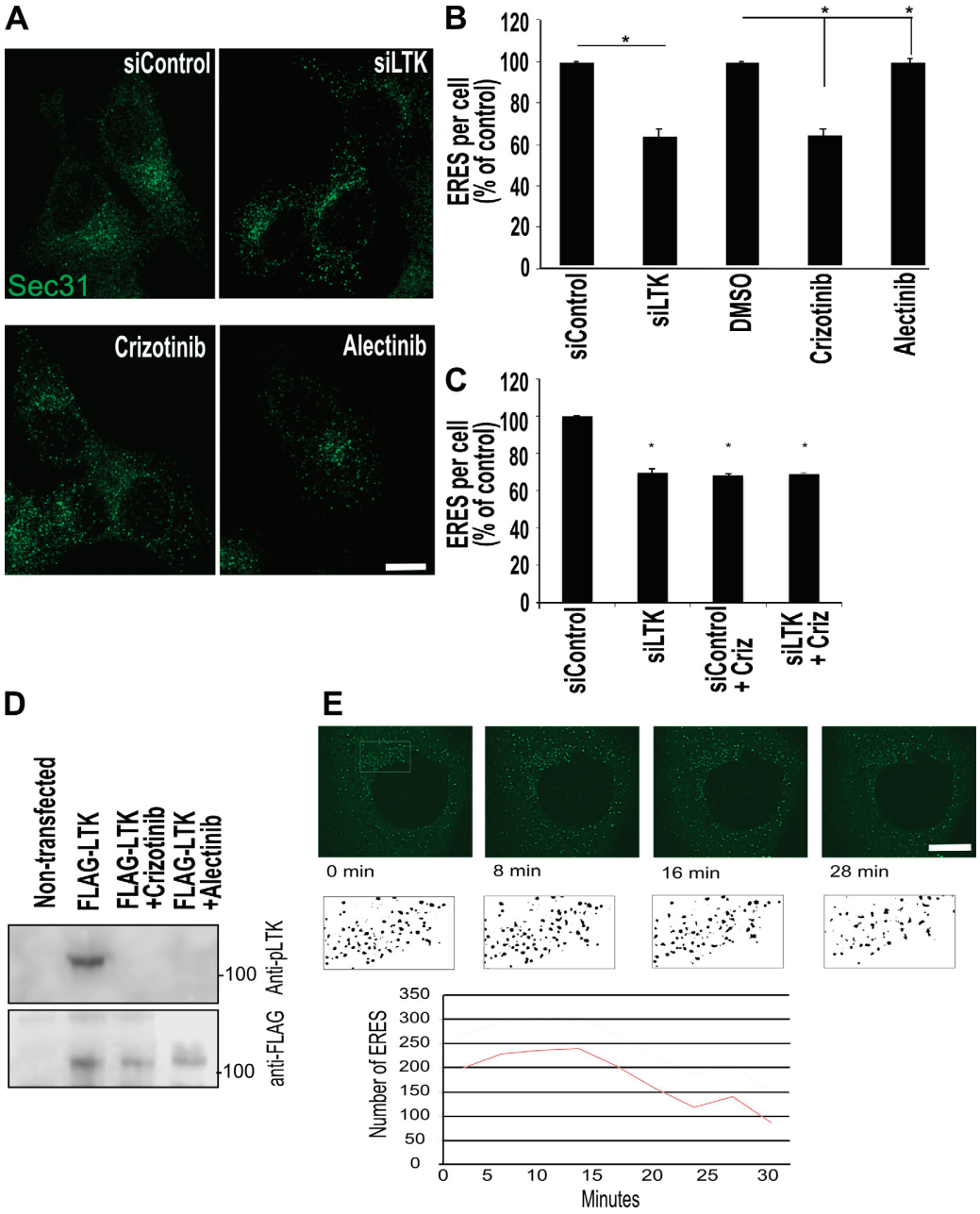

Figure 2. **LTK regulates ER export and ERESs. (A)** HepG2 cells were either subjected to LTK silencing with siRNA followed by fixation and staining after 72 h, or treated with crizotinib and alectinib (1 µM) for 30 min before fixation and immunostaining against Sec31 to label ERESs. **(B)** Quantification 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.

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 C and Fig. S3 A). Co-expression with LTK resulted in an increase in tyrosine phosphorylation of Sec12,

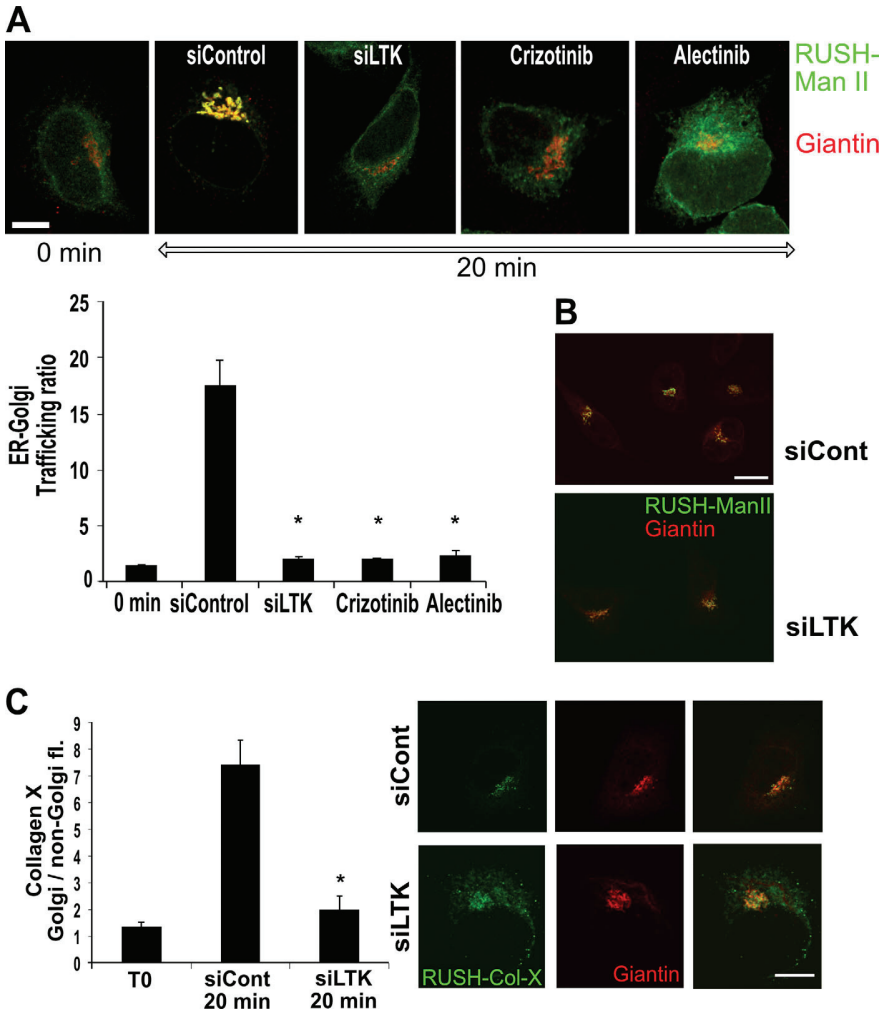

Figure 3. **LTK regulates ER export. (A)** Representative images of HeLa cells stably expressing the GFP-RUSH-Man-II construct (Str-KDEL-Man-II-EGFP) under different conditions: 0 min, cells not treated with biotin; 20 min, cells fixed 20 min after biotin treatment; Cont, control siRNA transfected; siLTK, LTK silenced; crizotinib and alectinib indicate cells treated with 1 µM 30 min before biotin addition. Bar graph shows quantification from three independent experiments. Asterisk indicates statistically significant differences at $P < 0.001$. Scale bars in this figure are 15 µm. **(B)** Representative images of HeLa cells stably expressing the GFP-RUSH-Man-II construct (Str-KDEL-Man-II-EGFP) imaged 2 h after release of the reporter from the ER. Two conditions are depicted, control and LTK knockdown cells. **(C)** HeLa cells expressing the RUSH-Collagen-X construct were transfected with control or LTK siRNA. After 72 h, cells were treated with biotin and fixed immediately (T0) or after 20 min. Cells were immunostained against Giantin to label the Golgi. The increase in green fluorescence in the Golgi region relative to outside the Golgi region was measured using ImageJ. The bar graph represents the mean of four independent experiments.

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.

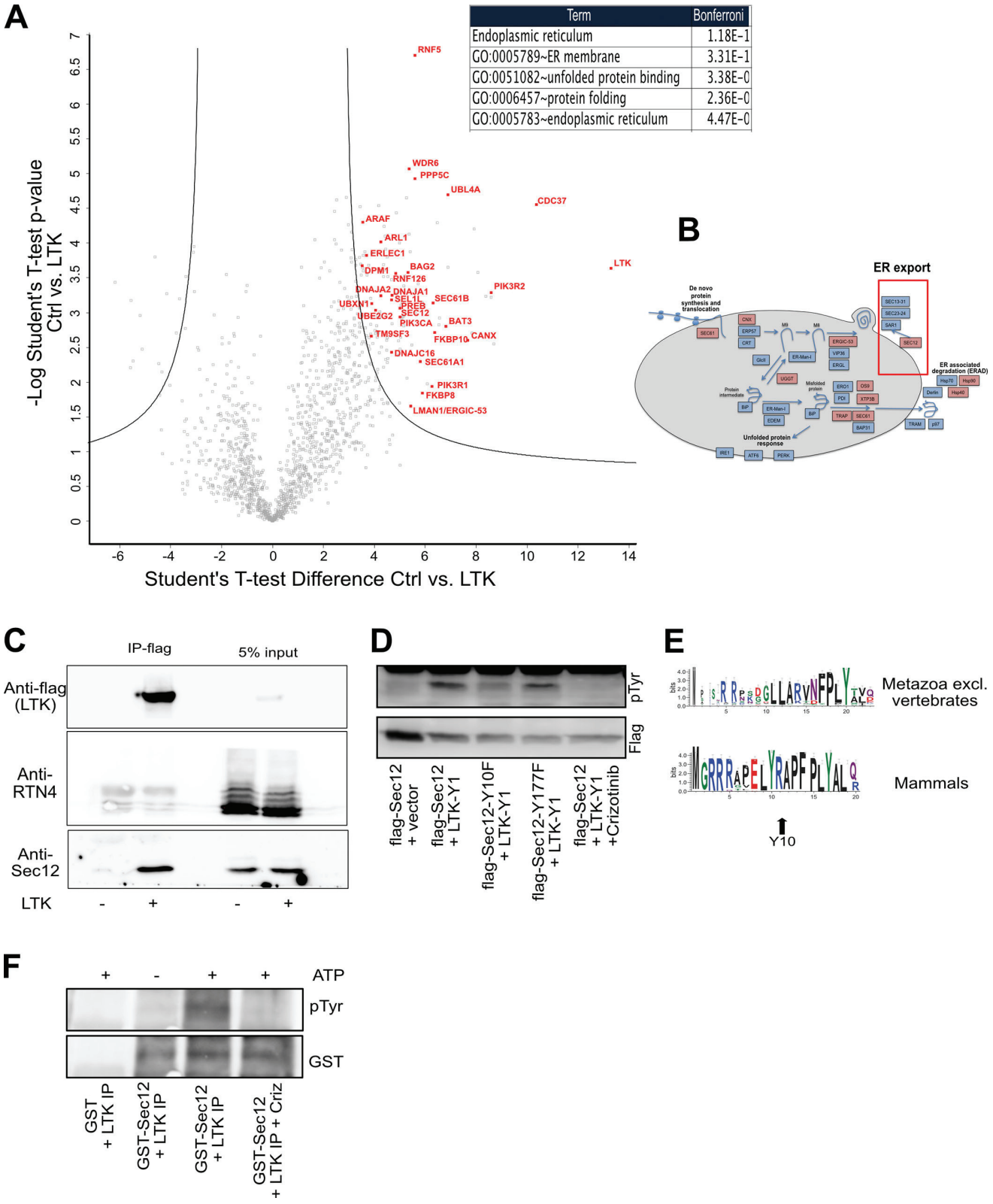

Figure 4. **LTK interacts with and phosphorylates Sec12. (A)** Volcano plot of the interactome of HA-tagged LTK revealed by immunoprecipitation MS from HEK293 cells. The red labeled candidate interacting proteins are related to receptor tyrosine kinase signaling or ER-associated processes. The table 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)** HeLa cells were transfected with vectors encoding flag-tagged Sec12 or its mutants together with an empty vector or with YFP1-tagged LTK (LTK-Y1). In the last lane are lysates from cells pretreated with 1 µM crizotinib for 30 min. **(E)** Sequence logo to demonstrate the conservation of amino acids in Sec12 in mammals or in metazoan excluding vertebrates. **(F)** Purified GST-tagged cytosolic domain of Sec12 or GST were incubated a flag-LTK immunoprecipitate from HEK293 cells (LTK immunoprecipitation) in the presence or absence of ATP or crizotinib.

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

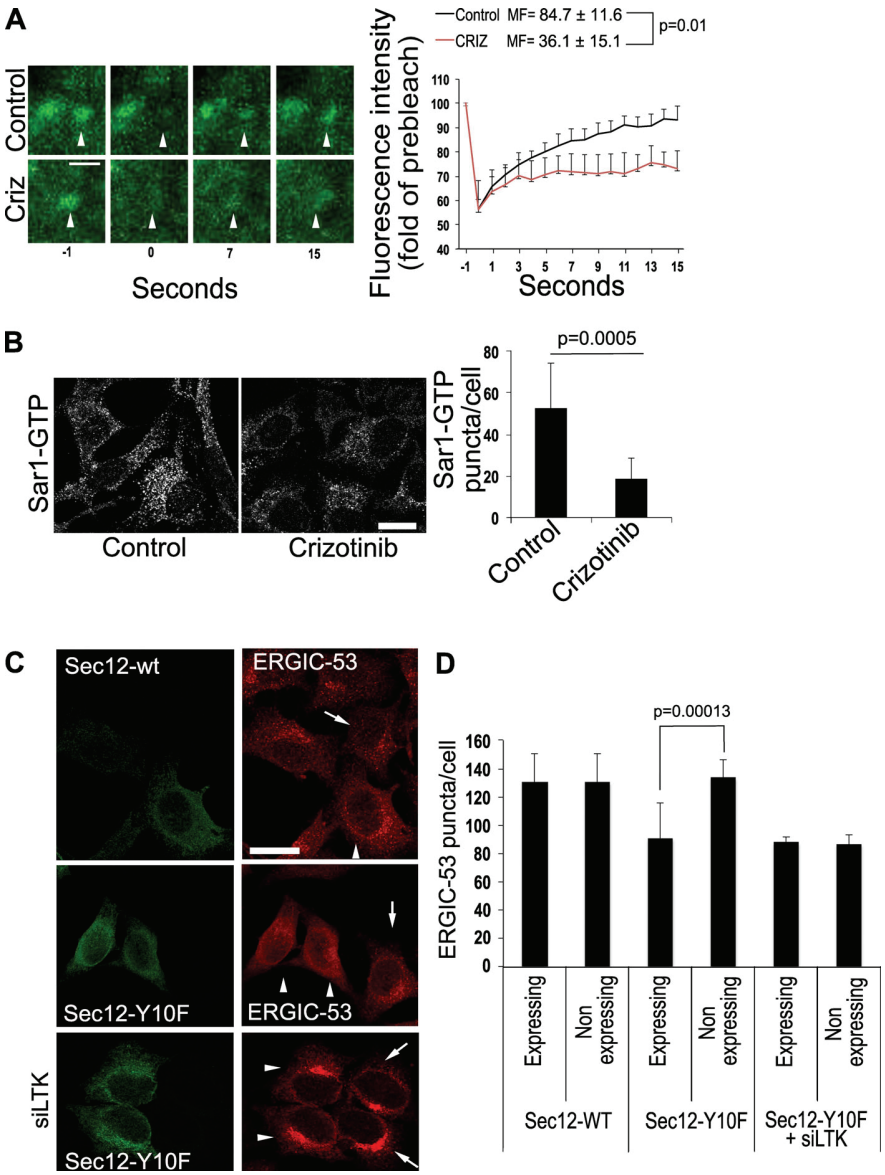

Figure 5. **LTK regulates Sec12 function. (A)** FRAP assay of HepG2 cells expressing YFP-tagged Sar1A. Images on the left side show magnified single ERES at different time points before (−1) and directly after (0) bleaching as well as at the indicated time points after bleaching. Graph shows an evaluation of nine FRAP curves for each condition from three experiments. MF, mobile fraction. Scale bar, 1 µm. Criz indicates a condition where cells were treated with 1 µM of crizotinib for 20 min before the FRAP assay. **(B)** HeLa cells were treated with solvent (Control) or 1 µM crizotinib for 30 min before fixation and immunostaining against Sar1-GTP. The number of Sar1-GTP puncta was counted using ImageJ and is displayed in the bar graph on the right side of the panel. Results represent the average number of puncta per cell obtained from 100–150 cells. Statistical significance was tested using unpaired, two-tailed *t* test. **(C)** Wild-type Sec12 or its mutant Sec12-Y10F were expressed in HeLa cells immunostained for ERGIC-53 and flag. siLTK, expression of Sec12 in LTK-depleted cells. Arrows indicate nontransfected cells. Arrowheads indicate cells expressing Sec12. Scale bars, 30 µm. **(D)** Bar graph showing the quantification of the number of ERGIC-53 puncta per cell from three independent experiments. The graph compares cells expressing the Sec12 construct to directly adjacent nontransfected cells.

(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 Gα12 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 is 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, VIP36, 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 XBP1s 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 3e6 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 x*g* 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 semidry 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 $MgCl_2$, 10% glycerol, 0.5% NP-40, and n-dodecyl-B-D-maltoside).

PGNase F digestion and EndoH

For PGNase F digestion, 3.2×10^5 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 PNGase F (P0704S; NEB) for 6 h. Subsequently, cells were lysed as described above.

For EndoH digestion, 2×10^6 cells were plated into in 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 $MF = (F_\infty - F_0)/(F_i - F_0)$, where F∞ is fluorescence in the bleached region after recovery, Fi is the fluorescence in the bleached region before bleaching, and F0 is the fluorescence in the bleached region directly after bleaching.

ERESs 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 anatine*; 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, *Callorhinchus 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 x*g* 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 $MgCl_2$, and 10% glycerol). Typically, 5×10^6 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.

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 costaining of endogenous LTK and Sec31 in HepG2 cells. Fig. S2 (related to Fig. 1 and Fig. 2) shows qPCR data of LTK and ALK expression in HepG2 and HeLa cells. In addition, it shows a test of knockdown efficiency of three different LTK siRNAs and their effects on ERES numbers. Fig. S3 (related to Fig. 4) shows a coimmunoprecipitation between LTK and Sec12 as well as a test of LTK phosphorylation in Src-inhibited cells. In addition, it shows a FRAP of general ER in crizotinib-treated cells as well as XBP1s levels in crizotinib-treated cells. Table S1 shows the results of the MS experiment of the LTK interactome (related to Fig. 4). Table S2 shows a list of all antibodies used in this work. 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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The authors declare no competing financial interests.

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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Supplemental material

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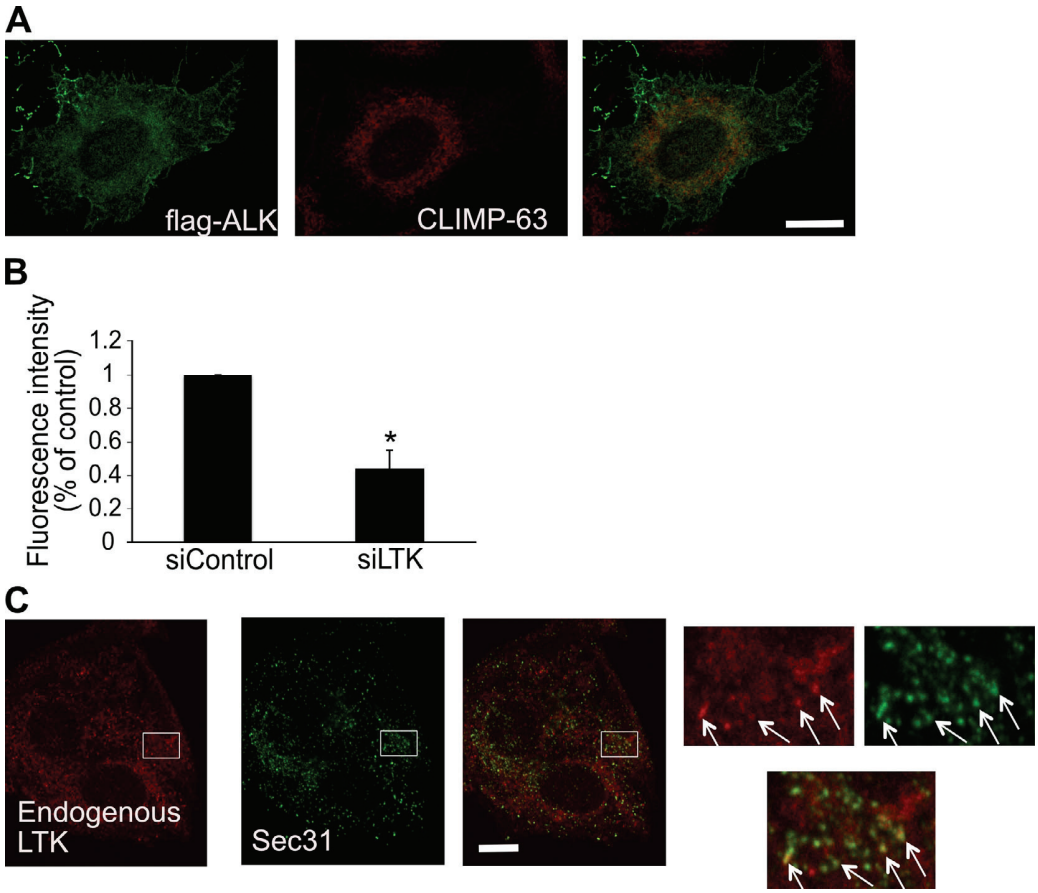


Figure S1. **Subcellular localization of ALK and LTK. (A)** HeLa cells expressing flag-tagged ALK were fixed and immunostained for flag to visualize ALK and CLIMP63 to detect the ER. **(B)** Measurement of fluorescence intensity of HepG2 cells immunostained for endogenous LTK to demonstrate antibody specificity. Asterisk indicates statistically significant difference at $P < 0.05$ (*t* test). **(C)** Immunofluorescence against endogenous LTK and the ERES marker Sec31 in HepG2 cells. Region in white box is magnified, and the positions of colocalization events between LTK and Sec31 are highlighted by arrows. Scale bars in this figure are 15 µm.

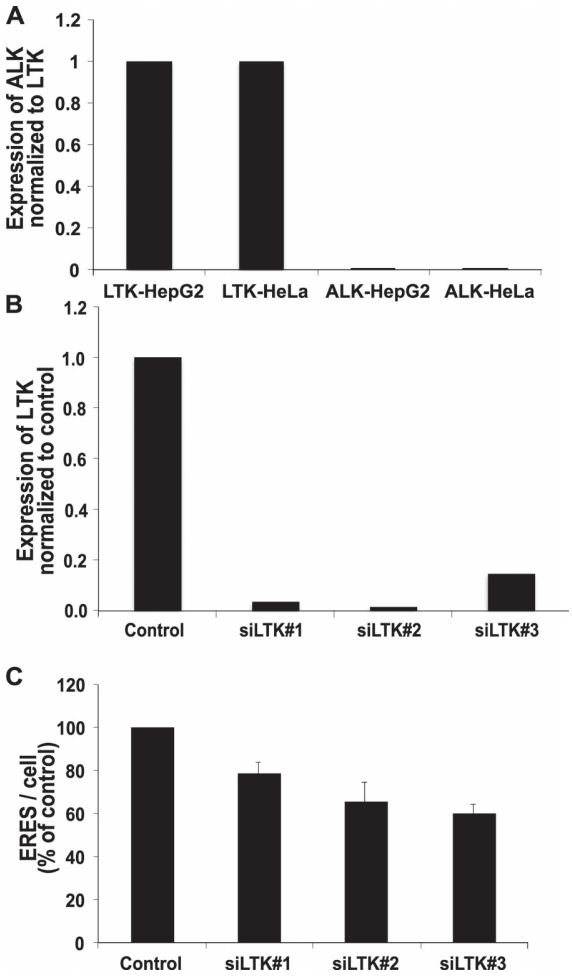


Figure S2. **Expression of ALK and LTK and effect of LTK depletion of ERESs. (A)** Expression of LTK and ALK mRNAs in HepG2 and HeLa cells assessed by qPCR. **(B)** Expression of LTK mRNA 72 h after knockdown with three different siRNAs targeting LTK from two independent experiments. **(C)** HeLa cells were transfected with the indicated siRNA and fixed after 72 h followed by immunofluorescence staining of Sec31 to label ERESs. Error bars represent SD.

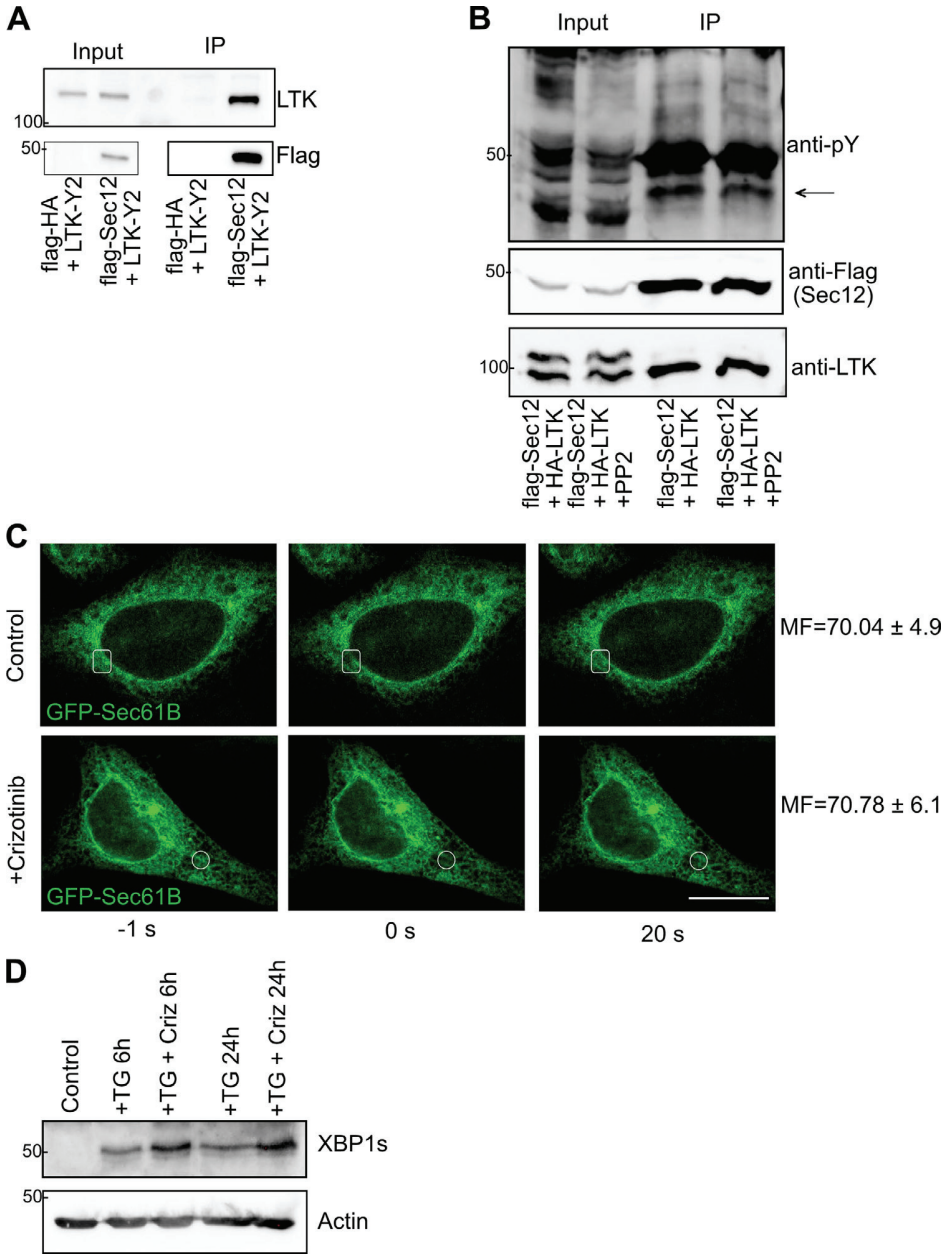


Figure S3. **Interaction of LTK with Sec12 as well as effect of LTK inhibition on ER homeostasis. (A)** HeLa cells expressing Y2-tagged LTK together with an empty flag vector or flag-tagged Sec12. Cells were lysed and subjected to anti-flag immunoprecipitation followed by immunoblotting as indicated. **(B)** HeLa cells expressing flag-tagged Sec12 and HA-tagged LTK were treated with solvent or with PP2 for 30 min before lysis and immunoprecipitation against flag. Immunoblotting was performed as indicated. Arrow indicates the position of phosphorylated Sec12 in the anti-phosphotyrosine blot. **(C)** FRAP microscopy of HeLa cells expressing the ER marker GFP-Sec61A. Cells were treated with solvent or with 1 µM crizotinib before the experiment. Bleaching was performed in the boxed region. MF, calculation of the mobile fractions from three independent experiments with at least six cells per experiment. Scale bar, 10 µm. **(D)** HeLa were treated with thapsigargin (1 µM) for the indicated time points, with or without 1 µM crizotinib. Cells were lysed and subjected to immunoblotting against spliced XBP1 (XBP1s) and β-actin to ensure equal loading. Control, cells treated with solvent.

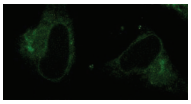
Table S1 is provided online as an Excel document and shows the results of the MS experiment of the LTK interactome.

Table S2. **List of antibodies used in this study**

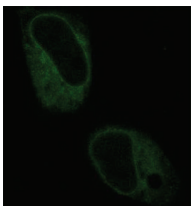
Antigen	Catalog no./source
FLAG-M2	F1804/Sigma-Aldrich
Climp-63	Rabbit/own
LTK	ap7658a/Abgent
Sec31	612351/BD Biosciences
FLAG-M2 HRP	A-8592/Sigma-Aldrich
P-tyrosine	Mouse/own
Giantin	ab80864/Abcam
Phospho-LTK	D59G10/Cell Signaling Technology
ERGIC-53	Rabbit/own
GFP (YFP2)	13026100/Roche
YFP1	ab32146/Abcam
GST	sc-138/Santa Cruz Biotechnology
Alexa Fluor 568 anti-mouse	A11004/Invitrogen
Alexa Fluor 568 anti-rabbit	A11011/Invitrogen
Alexa Fluor 488 anti-mouse	A11001/Invitrogen
Alexa Fluor 488 anti-rabbit	A11008/Invitrogen
Alexa Fluor 647 anti-mouse	A21235/Invitrogen
HRP rabbit	111035144/Jackson ImmunoResearch
HRP mouse	115035003/Jackson ImmunoResearch

Table S3. **List of PCR primers used in this study**

Gene	Sequence or catalog no.
ERGIC-53_C466Afw	5′-GTGGTAGTTCTGGGGCTTTCGGCTTTTCATTTGATGGCATATT-3′
ERGIC-53_C466Arev	5′-AATATGCCATCAAATGAAAAGCCGAAAGCCCCAGAACTACCAC-3′
ERGIC-53_C475Afw	5′-GAAGTGGACCGTAGACAAAGCTGATGGAAATGGTGGTAGT-3′
ERGIC-53_C475Arev	5′-ACTACCACCATTTCCATCAGCTTTGTCTACGGTCCACTTC-3′
SEC12Y10Ffw	5′-GAGCCCGGAACAGCTCTGGCGCCC-3′
SEC12Y10Frev	5′-GGGCGCCAGAGCTGTTCCGGGCTC-3′
SEC12Y177Ffw	5′-CTGGAGGAACAGATGGCTTCGTCCGTGTC-3′
SEC12Y177Frev	5′-GACACGGACGAAGCCATCTGTTCCTCCAG-3′
GST-SEC12fw	5′-ATTCTCGAGCATGGGCCGGCGCCGGGCG-3′
GST-SEC12rev	5′-AATTGCGGCCGCTCATTCATGGGACCCAAGGAG-3′
GAPDHfw	5′-ACAGTTGCCATGTAGACC-3′
GAPDHrev	5′-TTTTTGGTTGAGCACAGG-3′
LTK	QT00219877/Qiagen
ALK	QT00028847/Qiagen



Video 1. **Live imaging of a RUSH experiment in control and crizotinib-treated cells.** HeLa cells stably expressing the GFP-RUSH-Man-II construct (Str-KDEL-Man-II-EGFP). Live imaging was started immediately after addition of biotin. An image was acquired every 30 s, and cells were imaged for 20 min.



Video 2. **Live imaging of a RUSH experiment in control and crizotinib-treated cells.** HeLa cells stably expressing the GFP-RUSH-Man-II construct (Str-KDEL-Man-II-EGFP) were treated with 1 µM crizotinib for 30 min prior to addition of biotin. Imaging was started immediately after biotin addition. An image was acquired every 30 s, and cells were imaged for 20 min.

III

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LARP1 binding to hepatitis C virus particles is correlated with intracellular retention of viral infectivity

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Virions

ABSTRACT

Hepatitis C virus (HCV) virions contain a subset of host liver cells proteome often composed of interesting virus-interacting factors. A proteomic analysis performed on double gradient-purified clinical HCV highlighted the translation regulator LARP1 on these virions. This finding was validated using post-virion capture and immunoelectron microscopy, as well as immunoprecipitation applied to *in vitro* (Huh7.5 liver cells) grown (Gt2a, JFH1 strain) and patient-derived (Gt1a) HCV particles. Upon HCV infection of Huh7.5 cells, we observed a drastic transfer of LARP1 to lipid droplets, inducing colocalization with core proteins. RNAi-mediated depletion of LARP1 using the C911 control approach decreased extracellular infectivity of HCV Gt1a (H77), Gt2a (JFH1), and Gt3a (S52 chimeric strain), yet increased their intracellular infectivity. This latter effect was unrelated to changes in the hepatocyte secretory pathway, as evidenced using a functional RUSH assay. These results indicate that LARP1 binds to HCV, an event associated with retention of intracellular infectivity.

1. Introduction

Viruses and primate cells have coexisted for several million years. Host cells have evolved to eliminate most replicating viruses according to paleovirology studies (Patel et al., 2011). Therefore virion-bound host proteins (VBPs) may be considered to provide viral species with important components for virus persistence and/or propagation through their implication in replication, egress or entry steps of the viral cycle (Arthur et al., 1992; Garrus et al., 2001). Assuming that VBPs are likely to play a more important role in the viral life cycle than the rest of the cell proteome, the aim of our study was to validate the presence of some VBPs on hepatitis C virus (HCV) virions and to unravel a potential role for these VBPs at the entry or egress levels.

HCV is an enveloped positive-strand RNA virus and belongs to the genus *Hepacivirus* in the *Flaviviridae* family. HCV often establishes persistent infection in humans, which may lead to chronic liver disease, cirrhosis, and hepatocellular carcinoma, the third most common cause of cancer-related death (El-Serag, 2012). HCV infects hepatocytes and induces extensive remodeling of endoplasmic reticulum (ER)-derived membranes into a so-called “membranous web” (Egger et al., 2002). This web is composed of double membrane vesicles located in close proximity to lipid droplets (LDs) and serves as the site of viral genome replication and particle assembly (Aizaki et al., 2004), prior to release *via*the secretory pathway, though the precise mechanisms underlying this export process are not fully understood.

HCV comprises both an abundant amount and a broad variety of VBPs. Indeed, HCV virions incorporate not only viral but also host proteins, many of which, notably apolipoproteins B and E, have been shown to be functionally implicated in the viral life cycle by modulating cellular processes involved in lipid metabolism (Chang et al., 2007) (Huang et al., 2007; Meunier et al., 2008). In addition, HCV VBPs implicated in protein folding, *e.g.*HSC70 (Parent et al., 2009), as well as others functions (Benga et al., 2010; Cottarel et al., 2016) have been identified.

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The La-Related Protein 1 (LARP1) is a highly evolutionarily conserved translation regulating and RNA-binding protein (RBP) of the LARP family, each member of which carries a conserved La domain and an RNA-binding region. Following a recent upsurge in studies focusing on LARP1 in human biology, after its initial investigation in plants (Merret et al., 2013), this RBP was identified as a regulator of both mRNA stability and translation (Gentilella et al., 2017; Hong et al., 2017; Lahr et al., 2017), especially with respect to transcripts implicated in cell proliferation and cell survival (Stavraka and Blagden, 2015). Interestingly, LARP1 is overexpressed in hepatocellular, lung and ovarian cancers, where it is an independent predictor of adverse prognosis (Xie et al., 2013) (Hopkins et al., 2016). Moreover, the level of LARP1 is elevated in squamous cervical cancer, where it promotes cell motility and invasion, and binds an mRNA interactome enriched in oncogenic transcripts (Mura et al., 2015). In this study, we identify LARP1 as a novel component of at least a subset of HCV particles and show that it plays a role in the virus life cycle, predominantly by restricting the release of three epidemiologically important HCV genotypes.

2. Results

In order to identify host cell factors that associate with circulating HCV virions, we performed a proteomic analysis of HCV particles isolated from the plasma of two viremic patients. Plasma from an aviremic subject served as a control. After initial pelleting, HCV particles were sedimented on two sequential iodixanol gradients *via*isopycnic centrifugation as described previously (Cottarel et al., 2016; Parent et al., 2009). By monitoring HCV RNA in the collected fractions, we identified a peak of viral RNA at 1.12 g/mL of iodixanol (Fig. 1). To further decrease the level of non-specific co-sedimented background material, we subjected our virus-containing fractions to a second iodixanol gradient-based purification step, together with a naïve plasma sample (Parent et al., 2009). Following HPLC/MS analysis of the virus-containing fractions as described before (Cottarel et al., 2016) (Parent et al., 2009), we detected LARP1 in these fractions, but not in the corresponding aviremic control.

In order to confirm the presence of LARP1 on the particles, we immunoprecipitated LARP1 from JFH1 virus released from Gt2a HCVcc-infected Huh7.5 cells (Delgrange et al., 2007) with anti-LARP1 or anti−HCV E2 (CBH5) (Keck et al., 2005). Material was then

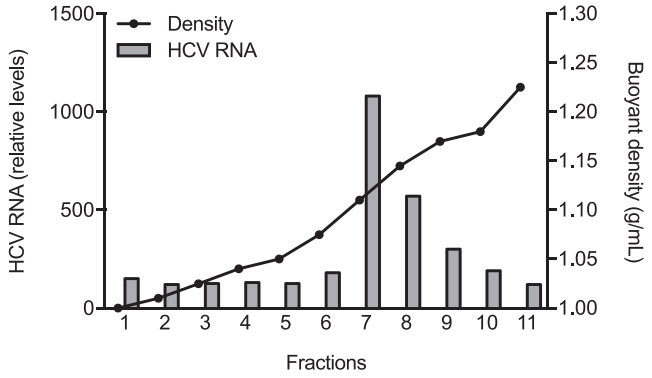


Fig. 1. Double gradient-based enrichment of HCV particles harvested from clinical material. Clarified and 20% sucrose cushion-pelleted plasmas were subjected to isopycnic fractionation using two sequential 10% to 60% linear iodixanol gradients. HCV RNA levels were determined in each fraction after the first run and fractions bearing the highest viral signal by qPCR were loaded atop the second gradient prior to a second round of qPCR. The density of each fraction was determined using a refractometer. The graph is representative of two independent fractionation processes performed on two distinct patient plasmas. Plasma from an aviremic subject served as co-purification / mass spectrometry negative control and was processed in parallel.

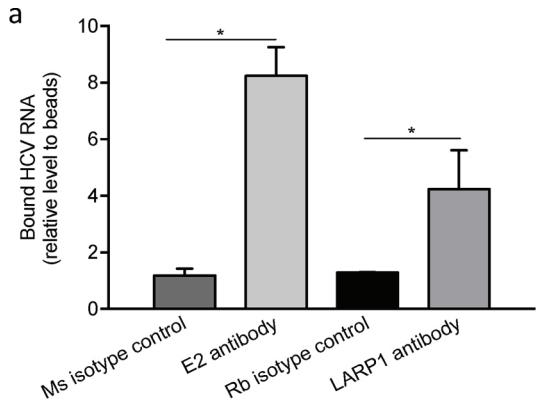
subjected to silica beads-based RNA extraction and RT-qPCR using HCV primers. As expected, our positive control, the human anti-E2 CBH5 monoclonal antibody displayed the highest enrichment ratio (8-fold) when compared to an isotype control. RNA of *in vitro* produced HCV could be enriched 4-fold using an anti-LARP1 Ig compared to its isotypic control (Fig. 2a). Delipidated particles using NP40 (0.1%, 4 °C, overnight) did not immunoprecipitate (not shown) using anti-LARP1 antibodies, suggesting that LARP1 associates with HCV externally rather than being encapsidated.

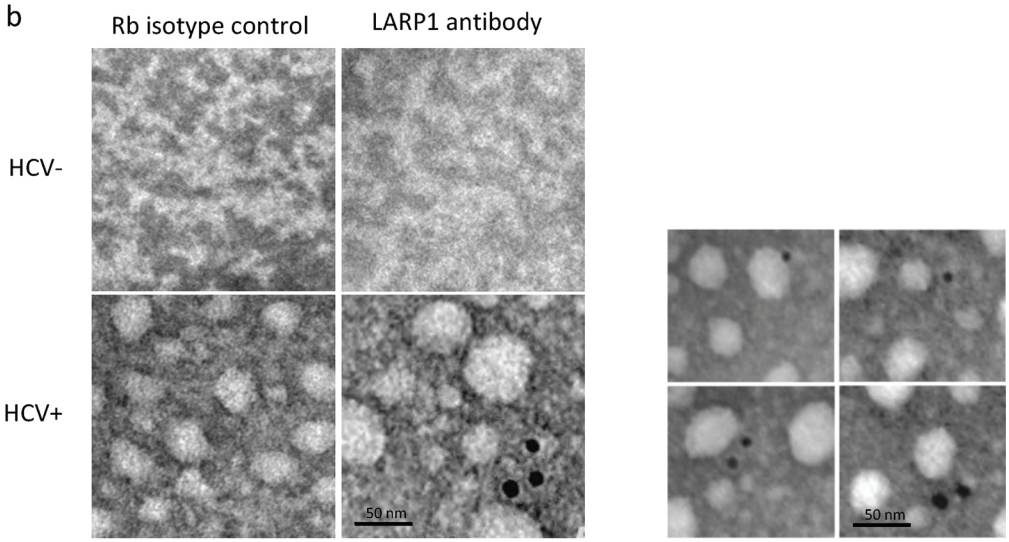
To seek further evidence that LARP1 is associated with HCV virions, we performed immunogold electron microscopy (IEM) on supernatants of infected Huh7.5 cells, and also used immunocapture (Piver et al., 2017) as an alternative and independent EM-related approach. Viral suspensions were generated from the supernatants of JFH1-infected Huh7.5 cells which were clarified and concentrated on sucrose cushions (Parent et al., 2009) or from patient plasma as previously described (Piver et al., 2017). Suspensions were adsorbed on grids and processed (Cottarel et al., 2016). As shown in Fig. 2b (left set of images), no virion-like structure was observed in HCV-negative supernatants. No labeling was found for HCV-positive samples stained with secondary antibodies only, ruling out non-specific staining. Although labeling was scarce, an issue commonly encountered in IEM, as illustrated by our previous ApoE staining (Cottarel et al., 2016; Parent et al., 2009), probing HCV-positive supernatants with anti-LARP1 antibodies exposed virions of 30–60 nm in size (Fig. 2b, right set of images), corroborating previously published features of *in vitro*-derived viral particle preparations (Catanese et al., 2013). Gold particles located ≤ 40 nm (corresponding to a single immunoglobulin length) away from the virion were considered to be specifically bound. These data were confirmed using our recently developed immunocapture approach (Piver et al., 2017), implemented here based on anti-E2 and anti-LARP1 antibodies, and which also revealed small-sized virions (Fig. 2c).

Virion-bound host proteins often contribute to viral budding, not least due to their specific intracellular localization, as initially highlighted in the HIV field (Garrus et al., 2001). Using confocal immunofluorescence microscopy, we studied the localization of LARP1 with respect to the infection status of Huh7.5 cells and vicinity of LDs as major sites for HCV morphogenesis (Miyanari et al., 2007), followed by the investigation of several HCV parameters. LARP1 produced a diffuse signal in the cytosol of uninfected cells, while it relocated to the immediate proximity of LDs upon infection, in most cases exhibiting near total colocalization with the typical LD-associated HCV core protein (Fig. 3a). These data were quantitatively verified using Pearson, morphometric and Li correlation coefficient approaches (Fig. 3b-d). Despite several attempts, co-immunoprecipitation assays between HCV core and LARP1 remained unsuccessful, suggesting a labile interaction between both proteins at this level. These results indicate that an important fraction of the LARP1 cytosolic pool accumulates around core-decorated ER/LD structures as previously documented (Miyanari et al., 2007) in an HCV-positive cell-specific manner. No concomitant increase in LARP1 levels upon infection could be consistently observed (Suppl. Fig. 1). Altogether such data argue for a role for LARP1 in viral assembly processes.

To test this hypothesis, we then modulated LARP1 expression and tested its effect on HCV replication and infectivity. LARP1 levels were transiently modulated by the infection. Huh7.5 cells were transfected with non targeting siRNAs, LARP1 C911-mutated LARP1 siRNAs (Buehler et al., 2012) as a control for excluding off-target effects, or with wt LARP1 siRNAs. Knockdown efficiency was verified by RT-qPCR and Western blotting (Fig. 4a,b). LARP1 depletion-mediated toxicity was ruled out after performing Sulforhodamine B (SRB) (Vichai and Kirtikara, 2006) and Neutral Red (NR) (Repetto et al., 2008) assays (Fig. 4c,d).

The virological consequences of LARP1 depletion were then addressed, by initially considering the highly propagative HCV JFH1 (Gt2a) strain. As shown in Fig. 5a,b, LARP1 depletion weakly decreased





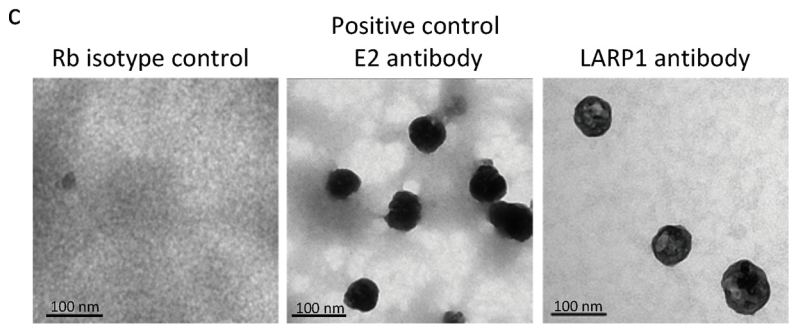


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Fig. 2. LARP1 is a hepatitis C virus particle-bound host factor. (a) LARP1-mediated immunoprecipitation of HCV RNA. Supernatants harvested from HCVcc-infected Huh7.5 cells (3 days post-infection (p.i.)) were subjected to clarification and immunoprecipitation using the indicated antibodies. HCV RNA was extracted and subsequently analyzed by RT-qPCR. Mann-Whitney test, $P < 0.05$ (*). (b) Association of LARP1 with cell culture-derived HCVcc evidenced by IEM. Concentrated supernatants of infected Huh7.5 cells were deposited onto EM grids and processed for immunogold labeling using the indicated antibodies. Bound anti-LARP1 antibodies were detected using secondary Igs conjugated to 10 nm gold particles. Pictures are representative of two labeling procedures. (c) Association of LARP1 with cell culture-derived HCV particles evidenced by immunocapture EM. Grids previously coated with control Igs, anti − HCV E2 (clone #AR3A) or anti-LARP1 were then incubated with supernatants of HCV-infected Huh7.5 cells and visualized under TEM. (n = 3 +/- s.d.). Mann-Whitney, $P < 0.05$ (*). For clarity, only highest significance is shown.

(< 2-fold) intracellular HCV RNA Gt2a levels, though no effect was detected extracellularly. This depletion was correlated with a strong increase in intracellular infectivity (up to 3.5-fold, see Materials and methods section) (Fig. 5c). Intriguingly, no significant effect could be observed on extracellular infectivity (Fig. 5d). The same approach was implemented to determine relative infectivity levels, by measuring $TCID_{50}$/HCV RNA ratios, thus evaluating the intracellular RNA-to-particle conversion yield. Similarly to global infectivity levels, RNAi depletion of LARP1 increased the relative intracellular infectivity up to 3-fold (Fig. 5e). Interestingly, a weak (< 2-fold) reduction in the relative extracellular (particle to RNA copies) infectivity levels could be observed (Fig. 5f), which were confirmed through secreted HCV core antigen (Ag) levels (Fig. 5g).

Finally, we evaluated the validity of these results across other HCV genotypes. We therefore tested the consequences of LARP1 depletion on the H77 (Gt1a) (Blight et al., 2003; Yanagi et al., 1997) and Gt3a-bearing S52 chimeric (Gottwein et al., 2011a) strains after electroporation. Post-electroporation viability and proliferative capacity of the cells were similar in all instances (Fig. 6a-b-e-f). While no difference could be observed at the HCV RNA level irrespective of LARP1 conditions (Fig. 6c-g), significant inhibition (> 2-fold and 10-fold for Gt1a and Gt3a, respectively) of secreted HCV core Ag levels was observed upon LARP1 depletion (Fig. 6d-h). Lack of HCV RNA reduction following siRNA transfection was probably due to the presence in excess of *in vitro* transcribed RNA copies, while absence of detection of intracellular infectivity reflects the poor, if any, propagation rate of non JFH1 strains *in vitro*. Nevertheless, these findings suggest trans-genotypic validity of our results.

To verify whether the enhancing impact of LARP1 depletion on HCV intracellular infectivity was due to its general impact on the secretory pathway of the hepatocyte, we conducted a RUSH (retention using selective hooks) assay (Boncompain and Perez, 2013). Since LARP1 depletion did not impair the trafficking of secretory cargoes between the ER and the Golgi apparatus (Suppl. Fig. 2), *i.e.*increased infectivity was not due to increased viral output, we postulate that LARP1 is involved in restricting viral infectivity downstream of the replication phase. These results may also indicate that the enhanced intracellular infectivity occasioned by LARP1 depletion is reversed and/or compensated by other structural components during the secretory process prior to the extracellular particle release.

3. Discussion

A virus may contain host proteins for the following reasons: the host protein is present at the site of assembly, the protein interacts with a viral protein and is swept up into the virion during budding, or its incorporation is needed to perform a specific function for the virus. Taken together, derived from MS as well as two methodologically-unrelated approaches, our data identify LARP1 as a component of at least a subset of *in vitro*-grown (Gt2a) and clinical (Gt1a) HCV virions. These data also suggest that some LARP1 regions are exposed on the surface of the secreted viral particle since the protein is accessible for antibody binding, though the mechanisms underlying this exposure should be further ascertained. Indeed, HCV buds through the ER membrane and it is therefore expected that virion-bound proteins such as LARP1 that are accessible to antibody binding in IP/RT-qPCR and $TCID_{50}$ assays translocate to the ER lumen prior to their association with the virion. LARP1 rapidly colocalizes with the peri-droplet HCV core signals but not with the envelope E2 glycoprotein. The association of LARP1 with nascent virions may therefore consist in its intercalation either (i) between the ER-derived membrane and the capsid or (ii) as a virion membrane embedded protein. Since none of the LARP1 primary sequence features encode for a signal peptide, as evidenced by its analysis using Predisi or SignalP 4.0 (Petersen et al., 2011) software, non-canonical translocation processes (Giuliani et al., 2011; Nickel and Rabouille, 2009; Nickel and Seedorf, 2008) may thus arise.

These antibody-based results finally indicate that LARP1 plays a role in early interactions of at least a subset of particles with their target cells, as observed for other VBPs (Chang et al., 2007) (Parent et al., 2009). Since HCV displays a high level of association with non viral-encoded host material (Chang et al., 2007) (Huang et al., 2007; Meunier et al., 2008), and, in this study, LARP1 inferring intracellular retention of infectivity across three genotypes, this implies that such material must therefore be of specific structural importance for the life cycle of this pathogen (Lavie and Dubuisson, 2017).

LARP1 was characterized relatively recently and has proven of interest in the field of the Dengue virus (Suzuki et al., 2016), another *Flaviviridae* member. LARP1 is overexpressed in HCC and that it is associated with poorer prognosis (Xie et al., 2013). While our study specifically focused on basic virology of HCV, it may prove pertinent to consider a potential association between intrahepatic LARP1 levels and HCV levels at the cirrhotic stage, the most exposed condition for HCC onset in patients. The fact that LARP1 may restrict HCV propagation deserves further investigation in light of its HCC-related protein status and the decreased viremia observed in HCC in the clinic (Reid et al., 1999).

4. Materials and methods

4.1. Purification of HCV virions

Infected plasma was obtained from three HCV-positive patients and an aviremic control and processed after approval of the French IRB (CPP South-East II, agreement #2010-08-AM2). Plasmas were stabilized with 10 mM Hepes (Gibco), antiproteases (Roche), centrifuged at 8000 g for 15 min at 4 °C, filtered through 0.45 µm membranes, layered onto a 20% sucrose cushion in TNE (10 mM Tris, 150 mM NaCl, 2 mM EDTA) and ultracentrifuged at 27,000 rpm for 4 h at 4 °C. Pellets were then resuspended in 1 mL of TNE, layered on top of 15–40% iodixanol gradients, and submitted to isopycnic ultracentrifugation for 16 h at 31,200 rpm at 4 °C. Fractions were then harvested from the top of the gradient. The amount of HCV RNA in each fraction was determined by real-time quantitative polymerase chain reaction (RT-qPCR). The fractions with the highest RNA content and the corresponding fractions from the uninfected control were pooled and dialyzed against TNE overnight at 4 °C. Fractions were then concentrated 10- to 20-fold in YM-3 concentration devices (Centricon; Millipore, Billerica, MA), subjected to a second ultracentrifugation step as described above and processed for mass spectrometry.

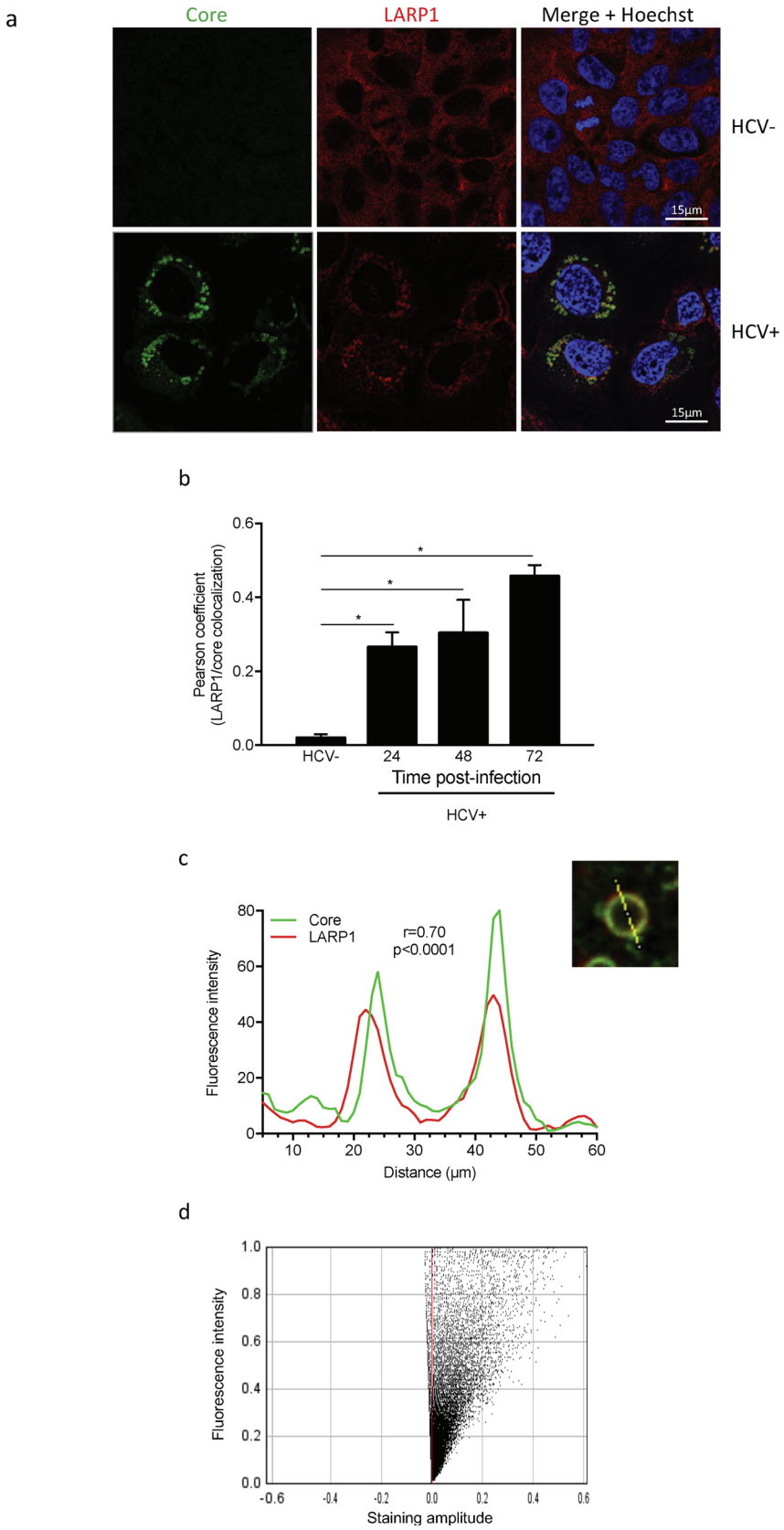

Fig. 3. HCV infection triggers LARP1 accumulation around lipid droplets. (a) LARP1 and core co-localization. HCVcc-infected Huh7.5 cells (3 days p.i.) were fixed, permeabilized and stained for HCV core proteins and LARP1 prior to incubation with Alexa 488 (HCV core signal) and Alexa-594 (LARP1 signal)-conjugated secondary antibodies. Cells were counterstained with Hoechst 33,358. Merged images were obtained using the ImageJ software. (b) Pearson co-localization coefficient. Coefficient was calculated using the ImageJ software and plotted against the indicated time points. (c) Morphometric assessment of co-localization. Green (HCV core) and red (LARP1) fluorescence intensities were plotted for each pixel across a representative lipid droplet image as shown (dashed line) using the Plot Profile function of the ImageJ software. Correlation coefficients for the selected couples of intensity values (core/LARP1) are shown. (d) Further statistical assessment of this co-localization dataset implemented using the Li coefficient. Profile of positive staining amplitude values confirm near total co-localization (Bolte and Cordelieres, 2006). Data are representative of six independent experiments.

4.2. Electron microscopy

Viral suspensions were generated from infected cell supernatants or patient plasma which was clarified and then concentrated on a 20% sucrose cushion as described (Parent et al., 2009). Suspensions were adsorbed on 200 mesh Nickel grids coated with formvar-C for 2 min at room temperature (RT). Immunogold labeling was performed by floating the grids on droplets of reactive medium. Grids were blocked in

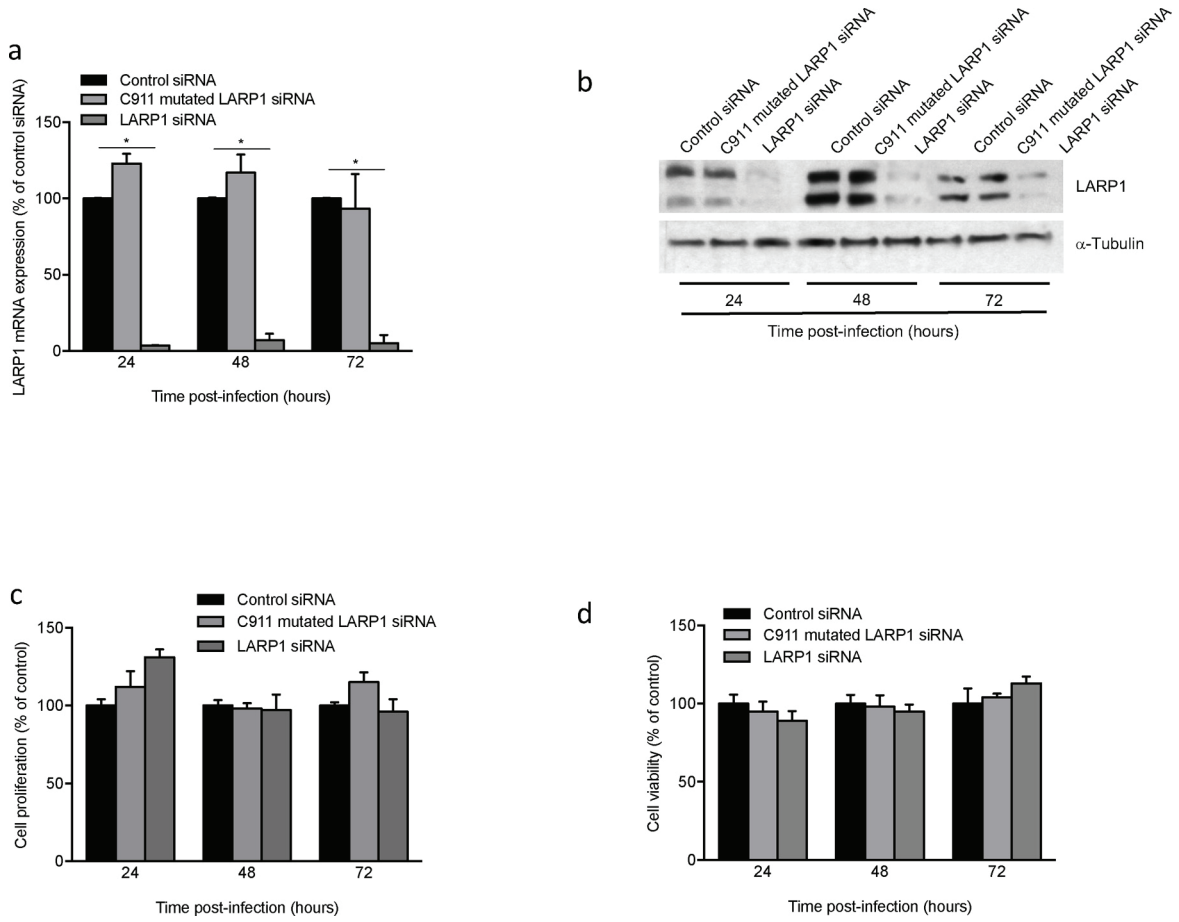

Fig. 4. RNAi-based depletion of LARP1 expression. (a,b) Cells transfected with siRNAs were subsequently cultured for 24–72 h prior to RNA extraction (a) or immunobloting (b). Anti-LARP1-specific primers or anti-LARP1 antibodies, respectively were used. Homogenous loading and blotting were previously assessed by Ponceau Red staining (not shown) and using anti-tubulin antibodies. (c,d) Related cell toxicity assays. The same cultures as **a** were tested for cell proliferation and viability using the SRB and NRA assays, respectively. n = 3, Mann-Whitney test: ns, non-significant.

1% BSA / 1% normal goat serum / 50 mM Tris − HCl, pH 7.4 for 10 min at RT. Incubation with anti-LARP1 primary antibodies (40 µg/ml) was carried out in a wet chamber for 2 h at RT. Following successive washes in 50 mM Tris − HCl, pH 7.4 and pH 8.2 at RT, grids were first incubated in 1% BSA / 50 mM Tris − HCl, pH 8.2 in a wet chamber for 10 min at RT and then labeled with 10 nm gold-conjugated IgG (Aurion) diluted 1/80 in 1% BSA / 50 mM Tris − HCl pH 8.2 for 45 min. Grids were then subjected to two washes in 50 mM Tris − HCl pH 8.2 and pH 7.4 and finally rinsed in distilled water. Following a 2 min fixation with 4% glutaraldehyde, grids were stained with 2% phosphotungstic acid for 2 min and then analyzed using a transmission electron microscope (Jeol 1400 JEM, Tokyo, Japan) equipped with a Gatan camera (Orius 600) and a Digital Micrograph Software.

4.3. Immunocapture

The formvar-carbon EM grids (S162, Oxford Instruments) were initially incubated with 0.01% poly-L lysine for 30 min at RT and then with selected antibodies (20 µg/mL) for 1 h at RT. Grids were washed in PBS and incubated with biological samples containing or not viral particles, for 2 h at RT. EM grids were washed in PBS and incubated for 20 min in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Particles trapped on grids were stained with 0.5% uranyl acetate for examination under a JEOL 1230 transmission electron microscope (Piver et al., 2017).

4.4. Cell culture and HCV infection / electroporation

The human hepatoma cell line Huh7.5 was cultured in Dulbecco's minimal essential medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Thermo Scientific) and 1% penicillin-streptomycin (Life Technologies). Viral stocks (Gt2a) or experiments (Gt1a and Gt3a) were generated *via*transfection of *in vitro* transcripts encoding the JFH1 genotype 2a-derived strain (Delgrange et al., 2007) or H77 (Yanagi et al., 1997) and S52 (Gottwein et al., 2010, 2011b) strains. 2×10^4 cells/cm^2 were infected with HCV JFH1 at an MOI of 0.1.

4.5. siRNA-mediated knockdown

Twenty thousand cells per square centimeter were transfected with 33 nM final concentration of non-targeting control siRNAs, C911-mutated LARP1 siRNAs (Buehler et al., 2012) or wt LARP1 siRNAs (Sigma-Aldrich, sense strand, 5′-3′: GGUGACUUUGGAGAUGCAAUC, antisense strand, 5′-3′: GAUUGCAUCUCCAAAGUCACC) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The target sequence of LARP1, 5′-3′: GGTGACTTTGGAGATGCAATC corresponds to the GenBank Acc.# NM_015315.

4.6. Immunofluorescence

siRNA-transfected Huh7.5 cells were fixed in 2% paraformaldehyde, permeabilized and blocked with 0.1% triton X-100 / 3% BSA in PBS at

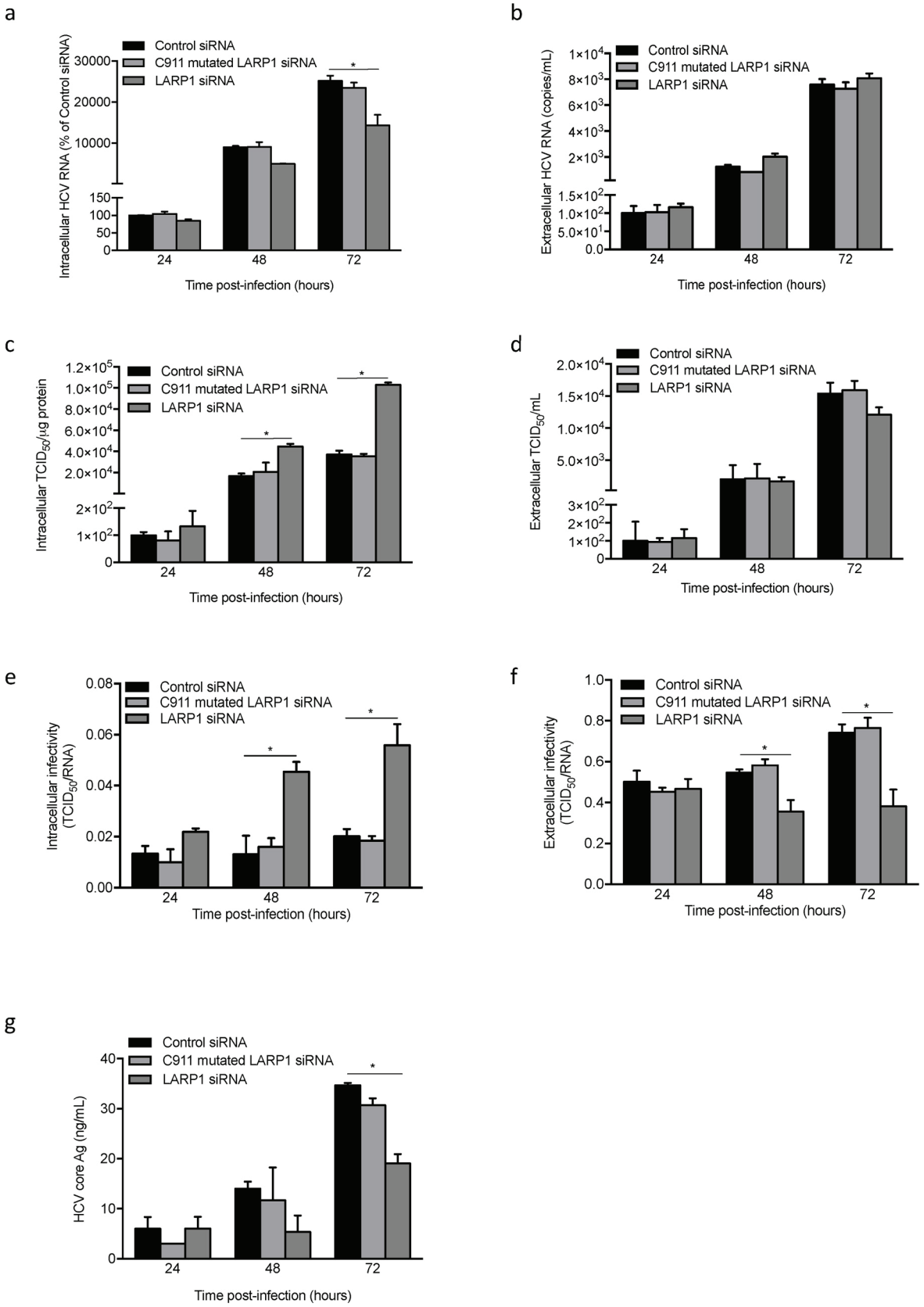

Fig. 5. Virological consequences of LARP1 depletion. HCVcc-infected cells were transfected with siRNAs and subsequently cultured for 24–72 h prior to: intracellular RNA extraction followed by RT-qPCR using HCV and GUS primers (a), extracellular RNA extraction followed by RT-qPCR using HCV primers (b), intracellular or extracellular $TCID_{50}$ quantification followed by protein normalization (c–d) or by HCV RNA normalization (e–f), and finally (g) secreted HCV core Ag quantification by Elisa (n = 3 +/- s.d.). Mann-Whitney, $P < 0.05$ (*).

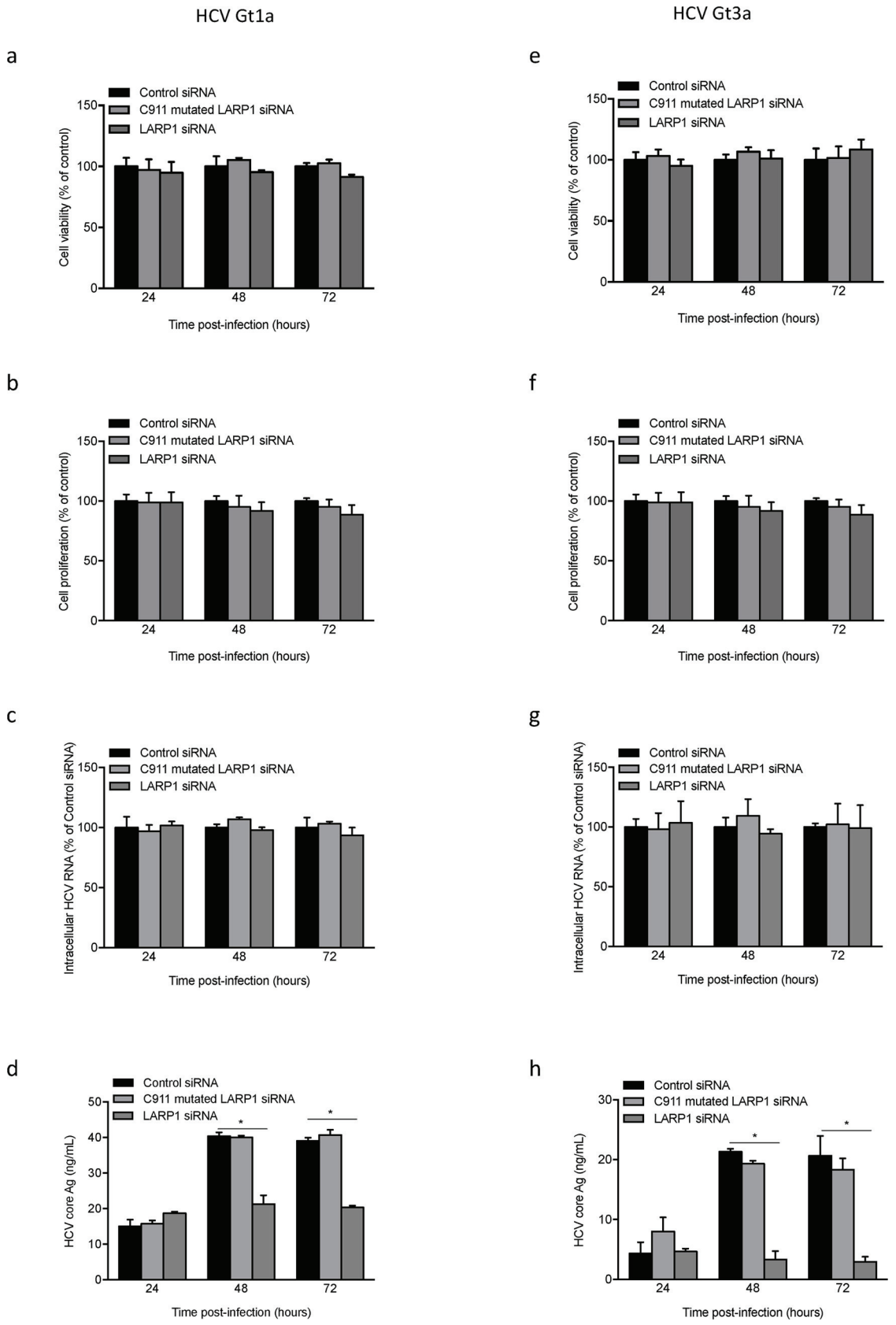

Fig. 6. Validation of virological consequences of LARP1 depletion in HCV Gt1a and Gt3a (chimeric) strains. (a-b-e-f). Evaluation of cell viability post − HCV Gt1a (H77) and Gt3a (S52) electroporation using the NRA (a–e) and SRB (b–f) assays. Intracellular RNA extraction followed by RT-qPCR using HCV RC1-RC21 and GUS primers (c–g). Extracellular quantification of secreted HCV core Ag levels (d–h).

RT, then stained with the primary antibodies overnight at 4 °C (anti-LARP1 from Novus #NBP1-19128, anti − HCV core clone #C7/50 from Santa Cruz, 2 μg/mL) and finally incubated with Alexa–conjugated secondary antibodies (1 μg/mL). Cell nuclei were counterstained with Hoechst 33,358 (0.025 μg/mL in PBS) and visualized under a Leica SP5 confocal microscope. Overlaid images were obtained using the ImageJ software.

4.7. Immunoblotting

Immunoblotting was performed using 30 μg of RIPA-resuspended Huh7.5 cell lysates, then resolved on 10% SDS-PAGE, blotted onto nitrocellulose membranes (Amersham Biosciences), blocked using 5% low fat dried milk in PBS for 1 h at RT and probed overnight at 4 °C with antibodies raised against LARP1 (1/1,000; Novus Biologicals, cat. #NBP1-19128) and tubulin (1/10,000; Sigma-Aldrich, cat. #T5168).

4.8. Immunoprecipitation and neutralization assays

Supernatants from infected cells were harvested 4 days post infection, cleared by centrifugation (8000 g, 15 min. 4 °C) and then supplemented with 10 mM HEPES and protease inhibitors. Immunoprecipitation of secreted virions with antibodies coupled to protein G magnetic beads (Pierce, 2 μg/IP) was carried out as described previously (Jammart et al., 2013). Material was then subjected to RNA extraction (Qiagen) and RT-qPCR.

4.9. HCV $TCID_{50}$ infectivity assay

Cells were seeded onto 96-well plates (6400 cells/well) the day before infection. Cells were then inoculated with 10-fold serial dilutions of the supernatants of interest. 96 h post-infection, cells were washed in PBS, fixed for 10 min in methanol/acetone and blocked for 30 min in 1X PBS / 5% BSA at RT. Cells were then probed with in-house HCV antiserum (#1804; 1/500) in 1X PBS / 3% BSA for 1 h at RT. After three washes in 1X PBS / 3% BSA, bound primary antibodies were probed with 1 μg/mL goat anti-human Alexa Fluor 488 secondary antibodies (Life Technologies) for 1 h at RT and visualized by epifluorescence (Nikon TE2000E). Viral titers were determined using the adapted Reed & Münch method (Lindenbach, 2009).

4.10. Neutral red assay

The Neutral Red (NR) assay was conducted as described by Repetto (Repetto et al., 2008). Briefly, the NR stock solution (40 mg NR dye in 10 mL PBS) was diluted in culture medium to a final concentration of 4 mg/mL and then centrifuged at 600 gfor 10 min to remove any precipitated dye crystals. Cells were then incubated with 100 μL of NR medium for 1 h. NR medium was removed and the cells washed with PBS. Plates were incubated for 10 min under shaking with 150 μL/well of NR destain solution (50% ethanol 96%, 49% deionized water, 1% glacial acetic acid). OD was measured at 540 nm in a microplate spectrophotometer.

4.11. Sulforhodamine B assay

Cells were incubated with 100 μL of 0.057% Sulforhodamine B (SRB) at RT for 30 min and then rinsed four times with 1% acetic acid, followed by four washes with distilled water. Plates were left to dry at RT and then incubated in 200 μL of 10 mM Tris pH 10.5. Plates were placed on an orbital shaker for 5 min and OD measured at 510 nm in a microplate reader.

4.12. Quantitative RT-PCR

Total RNA was extracted using trizol (Invitrogen). 1 μg of RNA was DNAse I-digested (Promega) and then reverse transcribed using MMLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed on a LightCycler 480 device (Roche) using the iQ SYBR Green Supermix (Bio-Rad). PCR primers sequences (5′-3′) and qPCR conditions were defined as follows: GUS-F: CGTGGTTGGAGAGCTCATTTGGAA, GUS-R: ATTCCCCAGCACT CTCGTCGGT, HCV RC1: GTCTAGCCATGGCGTTAGTA, HCV RC21: CTCCCGGGGCACTCGCAAGC, LARP1-F: TCAAACTTTCGGTAGCCAA ACT, and LARP1-R: GCCTGGCAACCAGAGATCAAA. Annealing temparature was 55 °C in all instances. Primer specificity was assessed by melting curves and agarose gel electrophoresis.

4.13. HCV core Elisa assay

The supernatants (100 μl) of HCV Gt1a-, Gt2a- and Gt3a-infected cells were spun down (8000 g, 5 min, 4 °C) prior to Elisa processing using the Quick titer HCV core Antigen Elisa kit (Cell Biolabs Inc) according to the manufacturer's instructions.

Author statement

MLP conceived and implemented experiments, analyzed the data and wrote the paper. JC, EP, MK, SP, HF, JCM and RP conceived and implemented experiments as well as analyzed the data. FZ co-obtained funding and edited the paper. RP obtained funding and wrote the paper.

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Ethical statement

The study complies with the COPE guidelines. Patient samples were obtained and processed after approval of the French IRB (CPP South-East II, agreement #2010-08-AM2).

Declaration of Competing Interest

We have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197679.

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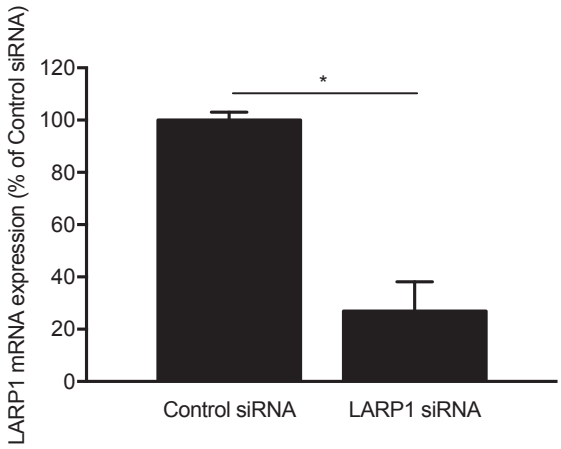
Supplementary Fig. 1

a



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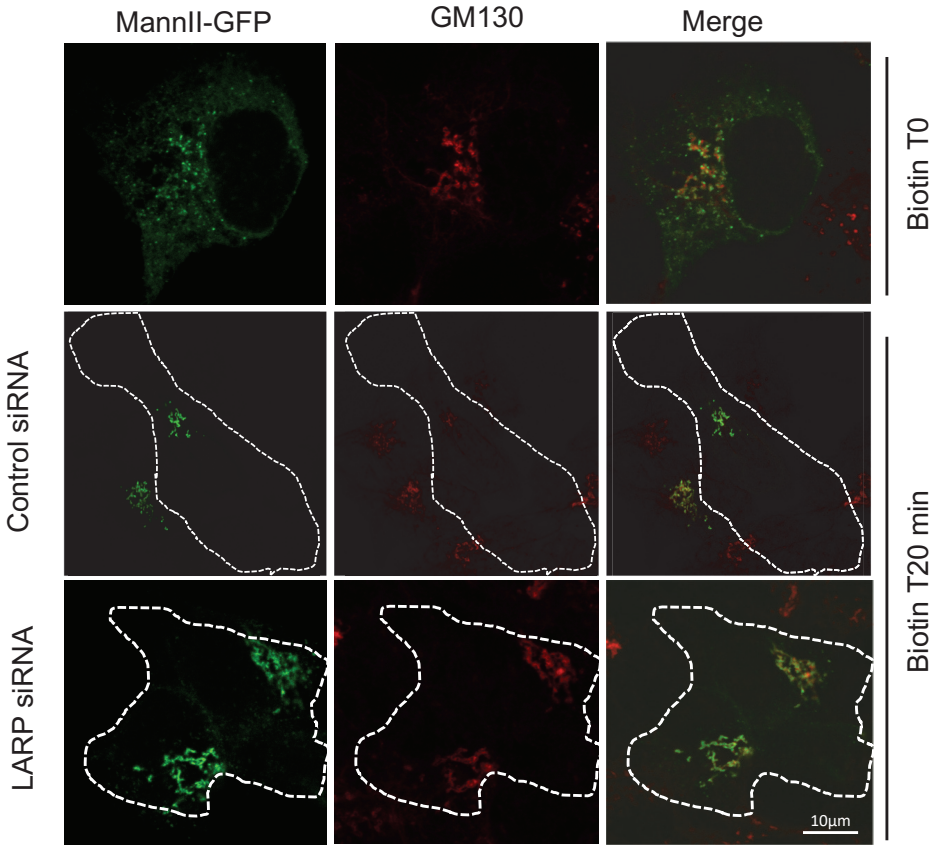

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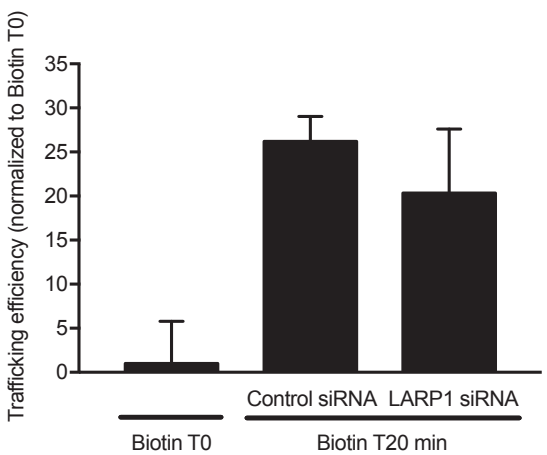

Supplementary Fig. 2