Crosstalk of endoplasmic reticulum exit sites and cellular signaling

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(Received 7 June 2019, revised 28 July 2019, accepted 29 July 2019, available online 12 August 2019)
doi:10.1002/1873-3468.13569

Edited by Felix Wieland

The synthesis, quality control, and trafficking of a third of the eukaryotic proteome takes place at the endoplasmic reticulum (ER), which is the largest cellular organelle. Thus, biosynthetic trafficking from the ER, although constitutive, has to be tightly controlled. Increasing evidence indicates that the ER acts as a platform that initiates signaling events. In this review, we focus on signaling pathways that target components of the ER export machinery to regulate protein export. In addition, we discuss how signaling generated at the ER regulates various homeostatic cellular processes such as cell growth and proliferation, and how the deregulation thereof is involved in disease.

Keywords: COPII; endoplasmic reticulum; ER exit sites; signaling

The endoplasmic reticulum (ER) is an organelle involved in the synthesis, folding, quality control, and transport of one third of all proteins. The ER is the first station of the secretory pathway, which is an array of organelles that communicate via membrane-bound carriers, which deliver proteins and lipids to their final destination. The secretory pathway comprises the rough ER, ER exit sites, the ER-to-Golgi intermediate compartment (ERGIC), the Golgi complex, and post-Golgi carriers. After translocation into the ER, secretory proteins must be correctly folded and modified by ER-resident chaperones and enzymes in order to acquire a properly folded and functional conformation [1]. Many proteins are not fully stable after synthesis and continue to require chaperones, due to the presence of intrinsically disordered regions [2]. Thus, protein misfolding occurs continuously, which is why the ER has developed a quality control system that senses and deals with misfolded proteins. Terminally misfolded secretory proteins are eliminated through a process called ER-associated degradation (ERAD) [3]. Misfolded proteins also trigger a response referred to as the unfolded protein response (UPR), which increases the capacity of the ER for folding, trafficking, and ERAD [4]. Correctly folded and assembled secretory proteins are sorted to ribosome-free regions of the rough ER called ER exit sites (ERES), which are also referred to as transitional ER [5]. ERES are the sites where export of secretory proteins leave the ER in COPII-coated vesicles [6,7]. Assembly of the COPII coat begins with activation of the small GTPase Sar1 by its guanine exchange factor Sec12. Active Sar1 then binds to the ER membrane via its N terminus, which further plays a role in membrane deformation [8,9]. In turn, active Sar1 recruits the inner COPII coat consisting of the heterodimer Sec24-Sec23 followed by the outer coat consisting of the heterotetrameric complex Sec13-Sec31. In addition, the large hydrophilic protein Sec16 of 250 kDa plays an important role in ERES homeostasis by acting as a scaffold that binds to and organizes COPII subunits [10–14]. Indeed, Sec16 is believed to be one of the

Abbreviations
Cdc2, division cycle protein 2; CFFL, coherent feed-forward loop; EGF, epidermal growth factor; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERES, endoplasmic reticulum exit sites; ERGIC, ER-to-Golgi intermediate compartment; ITD, internal tandem duplication; PKA, protein kinase A; UPR, unfolded protein response.
major organizers and determinants of the biogenesis and maintenance of ERES. After budding from the ER, COPII vesicles may undergo homotypic fusion [15] and typically ferry their cargo to the ERGIC [16]. Whether fused COPII carriers fuse with the ERGIC or whether the fused COPII carriers generate the ERGIC remains an open question. Subsequently, cargo proteins are transported to the Golgi complex where they undergo further modifications and are sorted to their final destination. The ERGIC appears to be a compartment that is unique to mammalian cells. In yeast, transport of proteins from the ER to the Golgi was suggested to occur in the absence of vesicular carriers, but rather by transient “hug-and-kiss” events of the cis-Golgi with the ERES [17]. However, no evidence for such a trafficking mode has been reported in other species and still needs to be demonstrated.

Regardless of the mode of transport, the ER and ERES play an fundamental role in membrane trafficking and protein homeostasis. This means that these endomembranes must be subject to a fine regulatory program. First evidence supporting a potential involvement of kinase signaling in regulating ER export came from the observation that treatment with the Protein kinase A (PKA) inhibitor H89 negatively affects COPII assembly and ER export [18,19]. However, the concentrations used to block ER export were 100-fold higher than the IC50 for PKA. Thus, it was unclear whether kinase signaling regulates ER export and which kinase is involved. It was only a decade after this initial report that the first protein kinase regulating ER export was fully characterized. In this review, examples of specific signaling pathways regulating ER homeostasis and ERES will be summarized and discussed. We will discuss signaling that targets the ER as well as signaling emerging from this location. Finally, we will discuss possible roles of aberrant signaling in disease.

**Signaling to the ER**

Alterations in protein synthesis, folding, trafficking, and degradation in the ER lead to disruption of proteinostasis which plays a pathophysiological role in several diseases. Therefore, regulation of those processes is required in order to maintain a balanced proteome. Very importantly, the ER needs to adapt to environmental and intracellular changes and thereby respond to different exogenous and endogenous stimuli. It appears reasonable then to assume that the ER is subject to signaling. In this section, we will give examples on how signaling generated by environmental stimuli such as mitogens change ER homeostasis by modulating secretion. We will also discuss how the ER deals and adapts to stress conditions such as nutrients starvation. Adaptation of ERES during mitosis will also be discussed as best known example of how the secretory pathway responds to endogenous stimuli.

**Growth factor signaling**

Growth factors are sensed by the cell through binding to corresponding cell surface receptors which results in the activation of signal transduction cascades leading to a specific cellular response. So far, many examples of kinases targeting ERES and regulating intracellular trafficking have been collected. First, evidence supporting a link between growth factor signaling and ER export was obtained through systems-wide screening approaches in mammalian cells. In one siRNA screen, all human kinase and phosphatases were depleted and the functional organization of the secretory pathway was determined by determining changes in the distribution of ERGIC-53 [20]. Over 60 kinases were identified, the depletion thereof causes a redistribution of ERGIC-53 to the ER. More specifically, among the pathways identified, the Raf-MEK-ERK cascade was identified to control the number of ERES via ERK2, which was shown to phosphorylate Sec16. Overexpression of oncogenic Ras led to hyperactivation of ERK2 and enhanced the phosphorylation of Sec16, leading to an increase in the number of ERES (Fig. 1). This finding provided for the first time a link between mitogen signaling and ERES and positioned Sec16A as an integrator of growth factor signaling at the ER. Further mechanistic insight into how phosphorylation of Sec16A leads to changes in ERES was obtained later. Using a combination of mathematical modeling and live cell imaging techniques, it was shown that the increase in ERES upon mitogenic stimulation occurred within a few minutes and resulted in accelerated dynamics of Sec16 at ERES. This response allows cells to generate new ERES and to thereby prepare the ER export system to deal with a prospective higher load of secretory cargo, which is expected upon growth factor stimulation. Furthermore, mitogenic stimulation for several hours was shown to increase the levels of Sec16 leading to an increased number of ERES. According to this finding, it was suggested that Sec16 acts as a central node in a coherent feed-forward loop (CFFL) which integrates growth factor signaling and ER export [21]. More recently, it was shown that prolonged stimulation with epidermal growth factor (EGF) also induces the gene expression of several COPII components such as Sec23B, Sec24B, and Sec24D [22] (Fig. 1). In line with the previously
described finding, this suggests the existence of a CFFL that detects the persistent stimuli leading to trafficking of the EGF receptor to restore its surface levels. CFFLs are often part of persistence detector systems that ensure that a given biologic process is only turned on when the stimulus is persistent. The notion of a strong link between growth factor signaling and ER export was further substantiated by results from another siRNA screen that targeted the entire genome and monitored trafficking of a secretory cargo from the ER [23].

Another kinase that was shown to target the COPII machinery is AKT (also known as protein kinase B), which was shown to phosphorylate Sec24C and Sec24D (Fig. 1), thereby increasing the association of SREBP and, therefore, in cholesterol homeostasis, but this needs to be tested experimentally in future work. Further evidence that signaling to the ER export machinery is involved in lipid metabolism comes from the observation that the atypical protein kinase C (PKCζ) phosphorylates Sar1B and thereby stimulates budding of prechylomicron transport vesicles [25]. Rather than stimulating the activity of Sar1b, the phosphorylation appeared to release fatty acid-binding protein 1 from a cytosolic complex allowing it to bind the ER and stimulate lipoprotein particle budding.

**Nutrient deprivation signaling**

The availability of nutrients allows cells to increase their biosynthetic rate, including that of protein synthesis. However, deprivation of nutrients is expected to reduce protein synthesis, thus reducing the need for active ERES. A major response to nutrient starvation is activation of autophagy, an evolutionarily conserved homeostatic process that degrades cellular components by targeting them for lysosomal degradation [26]. There is extensive crosstalk between autophagy and the secretory pathway that has been reviewed elsewhere [27,28]. However, this crosstalk has been mainly viewed in the context of how the secretory pathway (in particular ERES) contributes to autophagosome biogenesis [29]. However, less attention has been devoted to the question of whether and how ERES are regulated.

Among the first examples of regulation of ER export by nutrient deprivation was the demonstration that unconventional mitogen-activated protein kinase ERK7 (also known as MAPK15) mediates ERES disassembly upon depletion of amino acids [30]. Active ERK7 led to the dissociation of Sec16 from ERES (Fig. 1), but the mechanism behind this is unclear because Sec16 is not phosphorylated by ERK7. The same group has later investigated the fate of cytosolic Sec16 that dissociates from ERES in Drosophila. They show that Drosophila S2 cells form membrane-less structures that are enriched in Sec16 and thus they were called “Sec bodies” [31]. The formation of these membrane-less structures occurred 3–4 h after amino acid starvation and was reversible upon refeeding. They were associated with ER membranes and seem to be distinct from COPII-coated vesicles or autophagosomes and are independent of mTORC1. It was proposed that these structures act as reservoirs of ERES to rebuild a functional ER export once stress is resolved. It should be stressed that the formation of Sec bodies has so far been only shown in Drosophila cells and no mammalian counterpart could be found. In fact, starvation of mammalian cells resulted in degradation of Sec16 [21].

Initiation of autophagy is known to be dependent on the kinases ULK1/2. However, the Kundu group showed that ULK1/2 play a role in ER–Golgi traffic by phosphorylating Sec16A [32]. Interestingly, Sec16A phosphorylation by ULK1/2 occurred under condition where autophagy was not induced, suggesting a novel function of these kinases, which is not related to autophagy. Under autophagic conditions, ULK1/2 are diverted to the autophagic pathway, thus reducing their effect toward ER export. This result is in line with earlier observations from a kinome-wide RNAi screen where ULK1 was identified as a potential regulator of ERES [20]. Contrary to its effect on Sec16A, ULK1 was shown also to phosphorylate Sec23A mainly under conditions of active autophagy [33]. This resulted in a morphologic change of ERES under amino acid starvation leading to a disrupted interaction of Sec23A with Sec31A and inhibition of protein transport. Others have shown that the induction of
autophagy results in ULK1-mediated phosphorylation of Sec23B, which stabilizes this protein and diverts it toward autophagosome biogenesis [32] (Fig. 1). Another paper also showed that starvation-induced phosphorylation of yeast Sec24 by the Hrr25 kinase diverts the action of this COPII subunit toward autophagosome biogenesis. The notion that autophagy negatively impacts ER export gained further support from a recent report showing that a noncanonical autophagy pathway originated at ERES is required for disposing procollagen toward lysosomal degradation [34].

The question of whether nutrient signaling might affect COPII trafficking independently of autophagy has recently been addressed in vivo [35]. Starvation of mice resulted in reduced COPII-mediated trafficking in the liver, and feeding these mice again, increased their rate of ER export. The restoration of trafficking seemed to be dependent on XBP1s, a transcription factor normally found under conditions of ER stress such as when misfolded proteins accumulate (see section Autoregulatory signaling at the ER for an introduction to the UPR). Using Chip-seq and RNA sequencing techniques revealed that nutritional signals induce recruitment of XBPs to promoters of several components of the ER export machinery, thereby driving COPII-dependent trafficking. XBPs is normally generated under conditions of ER stress through splicing of XBP1 mRNA. It is currently unclear how nutrient deprivation induces XBP1 splicing and whether this is the same type of ER stress response as that triggered by misfolded proteins. Alternatively, starvation could trigger protein misfolding in the ER that leads to ER stress and thereby XBP1 splicing. Finally, it might be that this is a new function for XBPs, which acts as a nutrient sensor that integrates ER export with the metabolic status of the cell. How exactly this ‘sensing’ of nutrient is achieved remains elusive.

Signaling to ERES during mitosis

We have discussed so far how ERES respond to external stimuli, but internal stimuli might also trigger or necessitate a response by the ER export machinery such as the need to enter mitosis. 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 [36,37]. This is in line with another observation that the number of ERES increases gradually during interphase but undergo rapid disassembly during mitosis [38]. This raised the question of whether there is a landmark molecule that defines sites in the ER for reinitiation of ERES assembly. The Stephens group showed that Sec16A remains associated with the ERES during mitosis, most probably to restore the ERES and thereby the secretory pathway at the end of mitosis [39]. Taken together, those results suggest the existence of a regulation event at ERES leading to their disassembly during mitosis. It was shown that disassembly of ERES during mitosis is mediated by cell the division cycle protein 2 (Cdc2) kinase, which phosphorylates p47, a cofactor of the p97 AAA-ATPase (also known as VCP) [40]. In this paper, YIP1A was used as a marker for ERES. Since YIP1A cycles in the early secretory pathway, it cannot be considered a reliable marker for 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. Upon entry into mitosis, Sec24C undergoes deglycosylation and is phosphorylated, leading to its dissociation from ERES [36]. While this finding is interesting, 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 for any effects in ERES biogenesis per se. In addition, the identity of the kinase responsible for Sec24C phosphorylation remains unknown.

Signaling from the ER

All endomembranes house signaling molecules such as kinases, phosphatases, and GTPases, and this spatial organization has been shown to modulate signaling cascades and to generate alternate biologic outcomes. However, in addition to modulating signaling cascades, the endomembrane system was also shown to be the source of many signaling events. In this section, we will discuss signaling events originating from the ER and how these contribute not only to ER homeostasis but also to global cellular homeostasis as well.

Autoregulatory signaling at the ER

Autoregulation is defined as a response of a biologic system that aims at re-establishing the status of the system prior to the occurrence of the perturbation. Very often, the autoregulatory response directly eliminates or ablates the perturbing stimulus. Autoregulation is a feature of many essential biologic systems
(e.g., cerebral blood flow [41]) and is necessary to make these systems independent of fluctuations. It is conceivable that the ER is autoregulated, being the largest cellular organelle and a hotspot for proteostasis. So far, the best-understood autoregulatory response of the ER is the UPR, is activated by the accumulation of unfolded or misfolded proteins and mounts a response to help the ER eliminate these toxic protein species. The UPR is dependent on three transmembrane ER proteins: IRE1, which harbors nuclease and kinase activities, ATF6 which is cleaved to become a transcription factor and the Ser/Thr kinase PERK. Being a truly autoregulatory response, the activation of the UPR by unfolded proteins mounts a response that aims at abolishing the stimulus. This happens through signaling to the nucleus to induce the gene expression of chaperones as well as the machinery for vesicle budding, tethering and fusion [42]. The fundamental design principles of a sensory system for unfolded proteins has been recently modelled mathematically and shows that the UPR is a typical autoregulatory response that maintains system homeostasis [43]. Because it is so well characterized, all future autoregulatory responses of the ER will have to be compared to it.

The major part of the UPR represents a transcriptional response and is thus relatively slow. It was unclear whether fast autoregulatory signaling at the ER exists, but two recent reports provided evidence that export from the ER is an autoregulated process. The Luini group showed that engagement of the COPII subunit Sec24 with folded proteins renders it capable of acting as a guanine nucleotide exchange factor for G α12 at ERES [44]. How exactly Sec24 activates G α12 was not elucidated. In addition, because COPII assembly is an abundant and constitutive process, we would expect high levels of G α12 at ERES. This is not the case, and the vast majority of G α12 is either cytosolic or at the plasma membrane. The authors named this new response AREX (autoregulation of ER export) and also showed that it includes other components triggered downstream of G α12 including adenylate cyclase 7 and PKA (Fig. 2). They also showed that inhibition or depletion of these signaling proteins inhibits ER export. Of note, none of these proteins is resident to the ER, as is the case for the UPR sensors. Another difference is that the UPR sensors are transmembrane proteins that sense misfolded proteins in the ER, while AREX is composed of only cytosolic proteins. Thus, although AREX follows the same biologic principle of the UPR, it appears to be constructed differently.

The aforementioned differences between AREX and the UPR leads us to the question of whether ER-resident proteins might exist that act as potential autoregulators. Our group has recently provided the first evidence that the receptor tyrosine kinase LTK is an ER-resident kinase that regulates secretion [45]. LTK was previously identified as an ERES regulating hit in two RNAi screening approaches [20,23]. Moreover, it was proposed almost three decades ago to respond to the redox balance of the ER [46]. Many questions remained open such as whether LTK resides in the ER, and what its function in the compartment is, and if different subcellular pools of LTK exist, what the function of these might be. Thus, LTK remained an enigmatic receptor tyrosine kinase. The ER localization of LTK was recently questioned by reports showing that it responds to extracellular ligands [47,48]. We showed that endogenous as well as overexpressed LTK localize to the ER and we did not detect any evidence that LTK ever leaves the ER [45]. We showed that LTK regulates ER export by phosphorylating Sec12 (Fig. 2), the exchange factor for the small GTPase Sar1. Many questions remain open such as what is the stimulus that activates LTK? The interactome of LTK contained several cargo receptors, which allows us to speculate whether LTK responds to these cargo receptors and thus to cargo load. This exciting hypothesis needs to be investigated in the future. Further questions are, how is LTK signaling terminated or whether it targets other substrates than Sec12 at the ER. Clearly, the work on AREX and on LTK represent the initiating sparks that will fuel further research to provide us with a more profound understanding of autoregulation of the ER.

**Mutant proteins signaling from the ER**

Oncogenic mutations in signaling molecules have been described to affect numerous pathways and types of proteins such as mutations in the ERBB family of
receptor tyrosine kinases or in the Ras family of small GTPases. However, these signaling molecules typically reside either at the plasma membrane or are downstream of signaling events initiated at the cell surface. Most evidence for aberrant or oncogenic signaling from endomembranes is available for the endosomal compartment [49,50]. Far less is known about oncogenic signaling from the ER. A mutant of the receptor tyrosine kinase KIT (D816V) that causes acute myeloid leukemia was shown to be blocked in the ER [51]. Interestingly, this block was species specific and only occurred in human cells, but not in murine cells. The mechanism behind this remains elusive. What is also unclear is whether KIT-D816V is capable of signaling from the ER and whether it triggers similar signaling pathways as KIT from the plasma membrane. Another example is the receptor tyrosine kinase FLT3. Activating mutations of FLT3 are a major cause for acute myeloid leukemia [52]. The most frequent mutation is the internal tandem duplication (ITD) in the juxtamembrane domain. This FLT3-ITD was shown to mislocalize to the ER [53–56]. Interestingly, the signaling pathways triggered by wild-type and mutant FLT3 were different. While the wild-type receptor activated MAPK signaling, FLT3-ITD triggered mainly activation of STAT5 and Pim1/2 [55]. This was not due to the mutation per se, but rather due to the spatial localization, because targeting the FLT3-ITD to the cell surface rendered it capable of engaging the MAPK signaling pathway. This provides strong evidence for disease relevance of aberrant spatial signaling from the ER. Another example is a deletion mutation of gp130 (a subunit of the IL6 receptor) that was shown to be associated with inflammatory hepatocellular adenoma. The gp130 deletion mutant was shown to localize to the ER from where it initiates mitogenic signaling that is likely contributing to the pathogenesis of the hepatic adenomas [57].

Another example that links ER-based signaling to pathological is the oncogenic fusion of TFG-1 and NTRK1 (neutrophic tyrosine kinase receptor type 1) that was shown to localize to ERES suggesting the possibility of initiation of an oncogenic signaling cascade, involving components of the ERK1/2 cascade, leading to cell transformation [58]. 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 regulation of signaling pathways by Ras GTPase is an event that exclusively happens at the plasma membrane. The idea changed when it was shown that an active form of H-Ras restricted to the ER was capable of triggering downstream signaling that promoted cellular transformation [59]. However, it should be noted that it was not the naturally occurring oncogenic H-Ras mutation that was ER localized, but only a palmitoylation-deficient version thereof. Further insight into Ras signaling at the ER came later from a study that used a version of H-Ras that was engineered to be tethered to the ER. It was shown that this ER-tethered H-Ras is activated by RasGRF family exchange factors, thus opening the possibility that endogenous H-Ras might also engage activators at the ER [60]. Very recently, a systematic analysis of signaling networks regulated by active H-Ras at various subcellular localizations (including the ER) was conducted. This analysis, that combined protein–protein interactions, phosphoproteomics, and transcriptomics, is the most comprehensive effort for subcellular Ras signaling so far [61]. Again, subcellular localization was achieved by tethering H-Ras artificially to different compartments. An interesting finding was that ER-localized H-Ras was involved in controlling cell migration, but had little effects on cell survival. Although all the aforementioned reports on ER-localized Ras signaling are interesting, they do not address the important question of whether endogenous Ras isoforms signal indeed from the ER. Thus, more future work is needed to clarify the essential question.

**Outlook and future perspectives**

In this review, we focused mainly on signaling events that target the ER and regulate export at ERES. However, the field of signaling to and from the ER is vast and includes research areas such as the regulation of ATP levels in the ER or calcium signaling, in particular at ER–mitochondria contact sites. With respect to the regulation of ER export by signaling, future efforts should use systematic approaches to identify regulators of this early step of secretion. A first step toward this has been achieved through previous siRNA screens [20,23]. However, it is clear that the majority of the hits in these screens are indirect regulators, thus calling for additional efforts. The development of proximity labelling proteomic techniques such as APEX or BioID will allow researchers in the future to use these methods to identify spatial signaling complexes operating at the ER. This could be combined with the RUSH method that allows synchronous release of cargo [62], to test how such spatial signaling complexes regulate, or are regulated by secretory traffic.

Because secretion is part of the proteostasis network, future research has to take into account that signaling pathways that regulate ER export, are part of...
this network. Only recently were modelling approaches introduced to model small parts of the proteostasis network [43]. A challenge for the future will be to expand the use of mathematical modelling, and combine this with the aforementioned systems biology experiments to obtain a full understanding of signaling to and from the ER and its integration in general cellular homeostasis.

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