REVIEW

National Science Open
1: 20220018, 2022
https://doi.org/10.1360/nso/20220018

Life Sciences and Medicine

Diverse cellular strategies for the export of leaderless proteins

Jianfei Zheng & Liang Ge*

The State Key Laboratory of Membrane Biology, Tsinghua University-Peking University Joint Center for Life Sciences, School of Life Sciences, Tsinghua University, Beijing 100084, China

*Corresponding author (email: liangge@mail.tsinghua.edu.cn)

Received 12 April 2022; Revised 26 April 2022; Accepted 29 April 2022; Published online 11 August 2022

Abstract: Unconventional protein export/secretion (UPE/UPS), in contrast to the classical ER-Golgi-dependent export/secretion of proteins with a leader sequence (signal peptide), employs multiple means to release leaderless cargoes (and in some special cases, cargoes with a leader sequence) to the extracellular space. By far, two major types of UPE have been classified, vesicle-independent UPE and vesicle-dependent UPE. In the former, UPE cargoes can directly translocate across the plasma membrane from the cytoplasm without the assistance of a vesicle carrier. In the latter, UPE cargoes translocate into the lumen of a vesicle which then delivers them out of the cell through membrane trafficking. Both types of UPE require multiple unconventional solutions to complete secretion. Here, we briefly discuss the multiple strategies for a UPE cargo release, focusing on two key steps of leaderless cargoes release in UPE: protein translocation and membrane trafficking.

Keywords: secretion, unconventional protein secretion, translocation, ERGIC, autophagosome, multi-vesicular body, lysosome, vesicle transport, FGF2, GSDMD, IL-1

Introduction

Protein secretion is a key cellular process in almost every organism that delivers soluble and integral proteins to the extracellular milieu or plasma membrane. In eukaryotes, most secretory proteins contain a hydrophobic sequence at the NH2-terminus, termed the signal peptide or leader sequence [1]. The signal peptide, which also exists in many transmembrane proteins, directs the entry of secretory proteins into the lumen of the endoplasmic reticulum (ER) via the signal peptide recognition particle [2]. From there, the membrane translocation process is completed by the SEC61 translocon, a protein channel located on the rough ER [3]. Once the secretory protein has entered the ER lumen or inserted into the ER membrane, it is exported out of the cell via membrane trafficking from the ER to the Golgi. Vehicles like COPII- and COPI-coated vesicles assist the transport of secretory cargoes between membrane compartments (Figure 1) [4]. The signal peptide-directed ER-Golgi trafficking route, coined the conventional secretion pathway, has been extensively described since the 1950s [5].

In 1990, interleukin-1β (IL-1β) was identified as a secretory factor lacking a signal peptide in monocytes [6]. In the past decades, it has been found that many cytosolic proteins lacking a signal peptide (leaderless cargoes) can be released, including cytokines (IL-1s and FGF2), nuclear factors (HMGB1), carbohydrate- or phosholipid-binding proteins (galectins or annexins), aggregation-prone proteins (Tau and α-synuclein), and

chaperone proteins (heat shock proteins (HSPs)) [7–9]. Expanding evidence indicates that these secretory proteins function in diverse biological processes, including inflammation, development, immunity, and lipid metabolism. In addition, their abnormal release has been associated with human diseases, such as neurodegeneration (e.g., Tau and α-synuclein) and cancer (e.g., HSPs and FGF2) [10], and therefore they are potential drug targets or markers for diagnosis. Leaderless cargoes do not employ the ER-Golgi trafficking itinerary for secretion, instead they must rely on alternative means to exit the cell, which are collectively termed as unconventional protein secretion (UPS) (Figure 1) [9,11]. In addition, the trafficking of membrane proteins to the post-Golgi stations (e.g., the plasma membrane) independent of the Golgi is also included in UPS. Because the ubiquitin-proteasome system is also abbreviated as UPS, we modify unconventional protein secretion to unconventional protein export (UPE) to avoid possible confusion from readers outside of the unconventional secretion field.

The necessity of certain secretory cargoes being released by unconventional means is currently still being explored, although several explanations have been suggested. Some secretory proteins may be de-activated by glycol-modification in the ER-Golgi secretory pathway, and thus cannot utilize the conventional pathway.

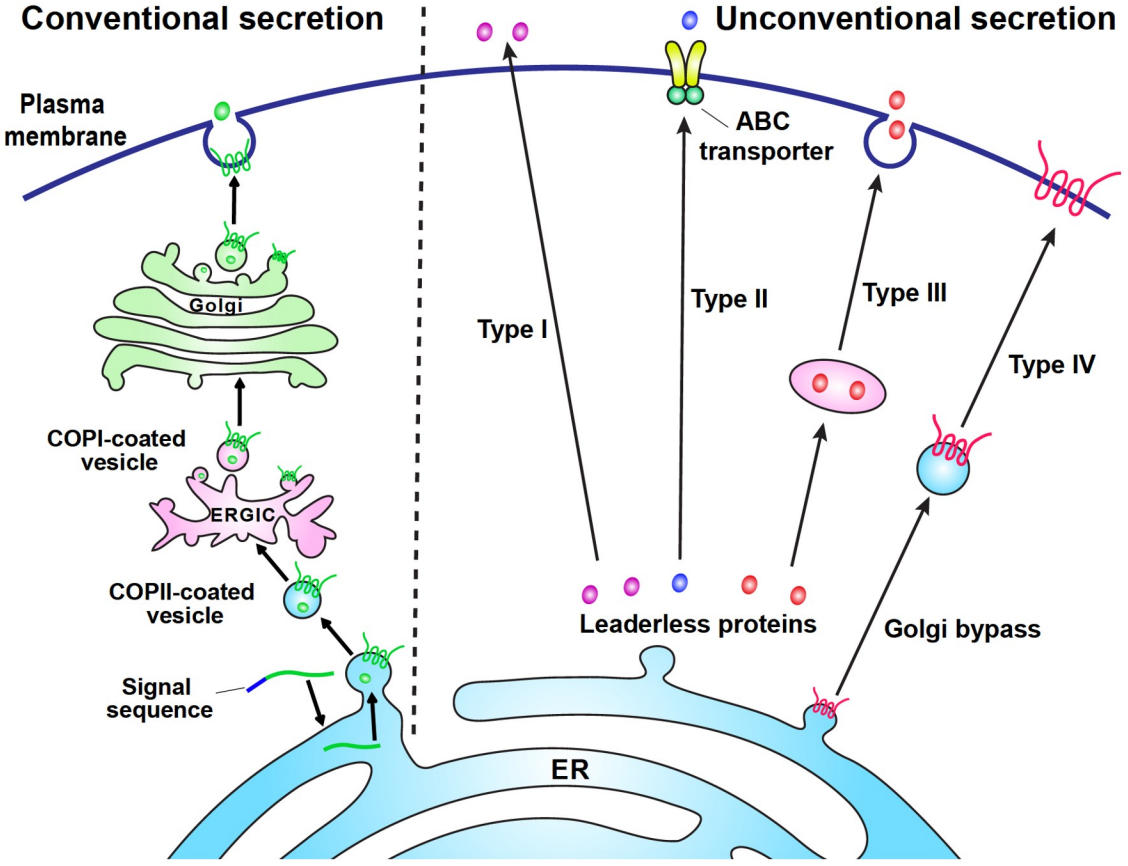

Figure 1 Conventional and unconventional secretory pathways. In conventional secretion (left), cargoes with a signal peptide enter the ER with the signal peptide removed. The secretory cargoes are then delivered out of the cell through vesicle trafficking via the ERGIC and Golgi. In unconventional secretion (right), four types of pathways have been classified. The leaderless cargoes can directly translocate across the plasma membrane (type I) or via ABC transporters (type II). The cargoes can also enter into a membrane-bound organelle which ultimately reaches the plasma membrane through membrane trafficking (type III). In addition, the transport of transmembrane proteins towards the plasma membrane or post-Golgi compartments can occur independently of the Golgi (type IV).

For example, FGF2 loses its function when it is artificially fused with a signal peptide due to undesired glycoslyation [12]. In line with the notion of de-activation, the luminal side of the compartments in the ER-Golgi pathway contains higher concentrations of calcium and a lower pH. This environment may lead to improper protein folding of some UPE cargoes that are normally synthesized in the cytosol. In addition, multiple UPE cargoes have dual functions inside and outside of the cell. For example, annexins are calcium-dependent phospholipid binding proteins that regulate multiple membrane-related processes in the cell, while, outside of the cell, they act as receptors for serum proteases, as well as regulators of cell migration and blood coagulation [13]. Finally, as the release of a majority of UPE cargoes is induced by stress, it is likely that some UPE pathways are generated during evolution for the purpose of anti-stress. One such example of stress-induced UPE cargo release is the release of IL-1β by pathogen infection [14,15].

Until now, four major types of UPE (i.e., type I-IV) have been classified. In type I UPE (vesicle-independent UPE), the protein cargoes directly translocate across the plasma membrane from the cytoplasm to the extracellular space. In this type of secretion, the cargoes can either self-translocate through the plasma membrane via oligomerization (e.g., FGF2 or HIV-TAT) or exit via Gasdermin D (GSDMD) pore formation (e.g., IL-1β or HMGB1) [9,16]. Type II UPE refers to the export of lipidated proteins dependent on ABC transporters and limited studies have been performed regarding the molecular mechanisms driving this activity [11]; therefore, type II UPE will not be the focus of this review. Type III UPE (vesicle-dependent UPE) is the umbrella term for all UPE cargoes that require membrane trafficking to complete secretion and multiple membrane compartments have been reported as stations for cargo transition, including secretory autophagosomes, multivesicular bodies (MVBs), endolysosomes, and a recently identified compartment in the ER-Golgi system known as the ER-Golgi intermediate compartment (ERGIC) [17–19]. Finally, the type IV UPE does not include a soluble cargo but defines the trafficking of a subset of integral membrane proteins from the ER to the plasma membrane in a Golgi-independent manner [20]. It is worth noting that UPE cargoes like IL-1β and Tau can employ different pathways (vesicle-dependent and independent) to facilitate release based on cell type and stress conditions [11,14]. Broadly speaking, another form of protein export, the nanotube formation between cells, can also be included as a means of UPE. However, in this case, the proteins are transmitted between cells instead of reaching the cell's exterior [21]. In this review, we briefly summarize the up-to-date knowledge regarding multiple strategies employed by soluble leaderless cargoes to complete secretion, with a focus on two key steps, membrane translocation and vesicle delivery.

Unconventional solutions to cross the phospholipid bilayer

A key step in releasing a secretory protein into an extracellular space is crossing the border of the phospholipid bilayer. In vesicle-independent UPE, the cytosolic cargoes need to directly cross the plasma membrane. Whereas in vesicle-dependent UPE, secretory cargoes must pass through the border of intracellular membrane compartments into the lumen for subsequent vesicle delivery. Multiple mechanisms regarding cargo transition from the cytosol to the exterior space or the lumen of compartments have been described. Cytosolic cargoes can either be taken up by MVBs, engulfed by the autophagosomes, or incorporated into vesicles shedding from the plasma membrane, including migrasomes [9,22,23]. These pathways will lead to the initial release of cargos in extracellular vesicles and, therefore, not be the focus of

our discussion. Below we focus on illustrating membrane translocation as a mechanism in both vesicle-dependent and independent UPE for transporting cargo across the phospholipid bilayer, which ultimately leads to the release of free proteins.

Direct translocation across the plasma membrane

A simple solution for UPE cargoes to exit the cell in a vesicle-independent manner is through direct translocation across the plasma membrane. Until now, two types of membrane translocation have been identified. One strategy is to form a lipidic membrane pore for self-translocation. The other is to penetrate the membrane with assistance from GSDMD pore formation [15].

FGF2 is the best defined cargo utilizing self-translocation. FGF2 is synthesized in the cytosol and binds to phosphoinositide $PI(4,5)P_2$, an acidic phospholipid enriched in the inner leaflet of the plasma membrane. The interaction between FGF2 and $PI(4,5)P_2$ triggers the oligomerization of FGF2, which then forms a lipidic membrane pore with a toroidal architecture across the plasma membrane. Once FGF2 is located at the outermost portion before reaching the outside of the cell, it binds to acidic heparan sulfate chains of proteoglycans on the surface to complete its translocation to the exterior [15,16,24]. A recent work identified Glypican-1 as a major contributor providing heparan sulfate chains to drive FGF2 translocation [25]. The self-translocation of FGF2 could be reconstituted in an inside-out GUV (giant unilamellar vesicle) system with only $PI(4,5)P_2$ and heparan sulfates located on different layers of the GUV membrane, suggesting that the minimal requirements for a functional translocation system are $PI(4,5)P_2$ and heparan sulfates [26]. In addition, Tec kinase and the Na,K-ATPase have been shown to modulate the translocation process [27,28]. A requirement of $PI(4,5)P_2$ in vesicle-independent UPE has been reported in other cargoes, including HIV-Tat [29], Tau [30], and EN2 [31], among which Tau also requires heparan sulfates for secretion [32]. Whether these cargoes also follow a similar release mechanism remains to be verified (Figure 2A).

The self-translocation model contains several challenges. The first challenge is to ensure directional transport from the plasma membrane's inner leaflet to the outer leaflet. This can be achieved by FGF2's higher binding affinity to heparan sulfates (Kd~100 nM (1 M=1 mol L^{-1}) [33]; outside) than to $PI(4,5)P_2$ (Kd~5 μM [26]; inside). The second challenge is to maintain cargo activity. In this case, protein unfolding should be avoided because the extracellular space lacks the chaperone systems needed for protein refolding. Therefore, FGF2 translocation occurs while FGF2 is in its folded state [34]. The third challenge is the prevention of membrane rupture mediated by pore formation. This problem can be addressed using at least two different strategies. Real time imaging found that FGF2 translocation takes a short amount of time to occur (~200 ms) [35], which minimizes damage to the plasma membrane. In addition, it has been shown that FGF2 translocation occurs in liquid-ordered microdomains, likely to be lipid rafts, where the rigidity of the microdomains can account for the resistance to membrane leakage [36]. Interestingly, different from the requirement of ordered phospholipid microdomains for FGF2 translocation, another kind of vesicle-independent UPE cargo, annexins, require phospholipid remodeling to complete a flip-flop style of translocation [37]. This indicates that translocation of annexins occurs in a liquid disordered region of the plasma membrane. It has been unclear how plasma membrane integrity is maintained during this flip-flop translocation.

It can be imagined that the most straightforward way to release cytosolic proteins is to form a protein pore

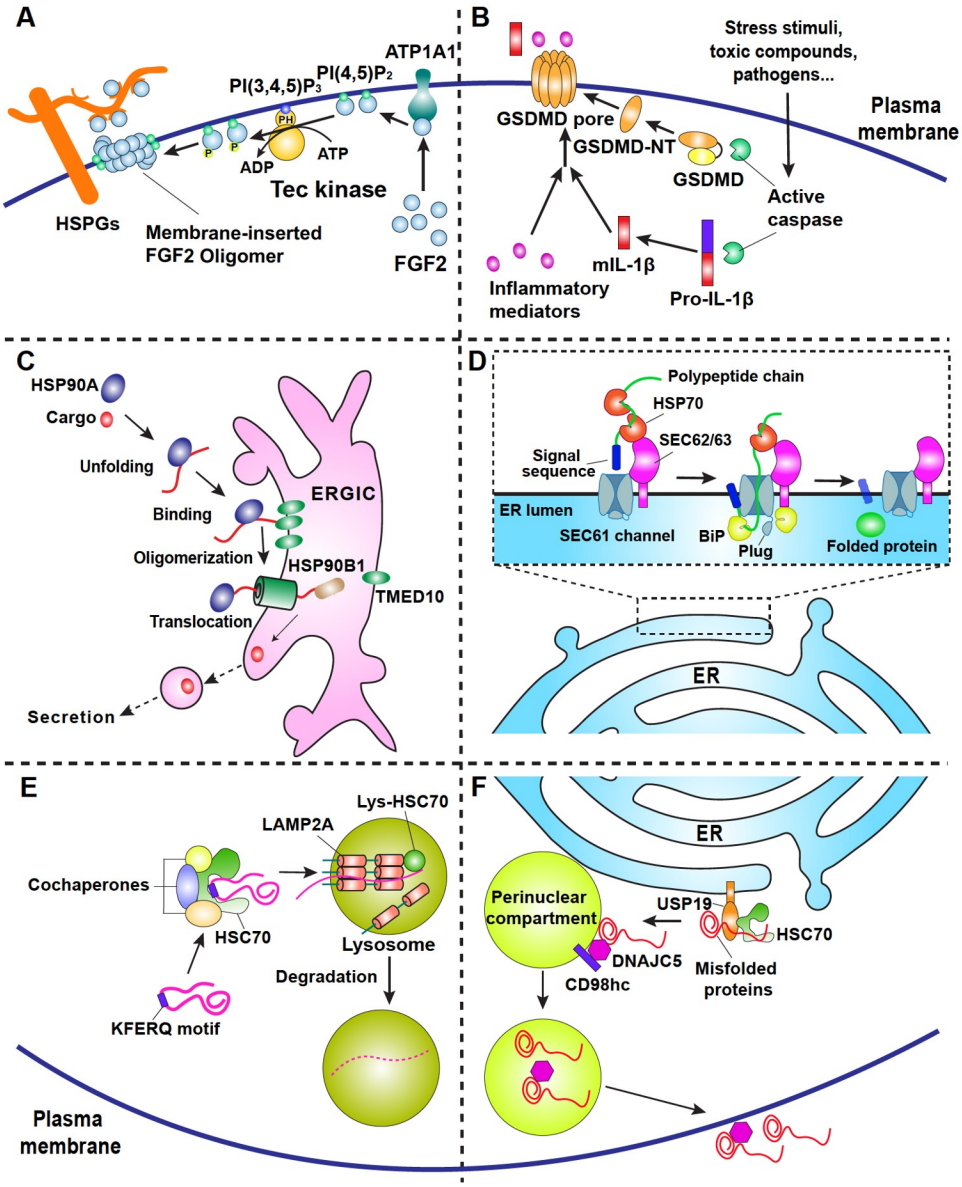

Figure 2 Cargo translocation pathways in conventional and unconventional secretion as well as CMA. (A) FGF2 self-translocation across the plasma membrane. With the assistance of ATP1A1, FGF2 binds to $PI(4,5)P_2$ on the plasma membrane and is phosphorylated by the Tec kinase. FGF2 then oligomerizes to form a toroidal pore and translocates across the plasma membrane. The inside-out direction of translocation is achieved by FGF2 higher binding affinity to heparan sulfate proteoglycan (HSPG) which locates on the outside. (B) GSDMD-mediated pore formation and UPE cargo release. In response to stress signals, toxic compounds, or invasive pathogens, GSDMD is activated by proteolytic cleavage by the active caspase, leading to the release of N-terminal (NT) fragment and subsequently formation of large GSDMD pores. Cytokines and other inflammatory mediators are released through GSDMD pores. (C) The TMED10-channelled unconventional protein secretion (THU). The cytoplasmic HSP90A assists the unfolding of leaderless cargoes which bind to TMED10 on the ERGIC and induce the oligomerization of TMED10 to form a protein channel. With the help of HSP90B1, the cargoes are translocated into the lumen of the ERGIC through the TMED10 channel and are ready to be secreted via membrane trafficking. (D) The SEC61-mediated post-translational translocation in conventional secretion. The process begins with the targeting of polypeptide chain to the SEC61 channel, which is mediated by the chaperone HSP70 and its membrane binding partner (SEC62/63 complex). With the aid of ER-luminal BiP ATPase, the polypeptide is translocated unidirectionally from the cytosol into the ER lumen through the SEC61 channel. (E) Protein translocation in CMA. In CMA, the substrate bearing KFERQ motif is recognized by cytosolic chaperone HSC70 and cochaperones, and then translocated into the lysosome for degradation with the help of LAMP2A channel and the lysosomal HSC70 (lys-HSC70). (F) Protein translocation in MAPS. MAPS involves the selective release of misfolded cytosolic proteins delivered to the perinuclear compartment via the aid of ER-located USP19, chaperone HSC70, DNAJC5 and its regulator CD98hc. The detailed mechanism by which misfolded cargoes are translocated into the perinuclear compartment and subsequently release remains unclear.

on the plasma membrane. The study of GSDMD provides a good example of this. It has been shown that the full length GSDMD contains an active N-terminal domain and a C-terminal domain of self-inhibition [38]. Upon certain cellular stresses (e.g., inflammation), intracellular proteases (such as caspases) are activated to cleave GSDMD, leading to the release of the N-terminal domain (GSDMD-N) [39,40]. GSDMD-N binds to $PI(4,5)P_2$ and oligomerizes to form a protein pore with a diameter of ~20 nm [41]. The protein pore allows for the release of small cytosolic proteins, including IL-1β, IL-18, and HMGB1 [42]. In addition to having a size control of the released proteins, the negative charge of the pore conduit confers a preference towards proteins with more positive charges, which is the case for the specific release of mature IL-1β instead of its precursor [43]. Plasma membrane damage caused by moderate pore formation can be repaired using the ESCRT system [44] or possibly vesicle shedding [39]. If pore formation occurs extensively, it can lead to pyroptosis and the massive release of cellular contents [40,45] (Figure 2B).

Transmembrane P24 trafficking protein 10 (TMED10)-mediated translocation at the ERGIC

While it has been established that the SRP-SEC61 translocation system recognizes secretory proteins with a signal peptide in conventional secretion, it remains unknown how leaderless proteins enter into membrane trafficking systems in vesicle-dependent UPE. Early studies on IL-1β secretion suggested that membrane translocation is required for the incorporation of IL-1β into a membrane compartment [6,46]. A later study confirmed this notion by showing that IL-1β entry into a vesicle depends on protein unfolding assisted by HSP90s [47]. Nonetheless, it has been unclear what transporter is involved in the translocation process.

Using a DHFR-aminopterin system to trap IL-1β in an intermediate state on the membrane, in combination with crosslinking and mass spectrometry, a single membrane protein TMED10 was identified as a possible transporter for IL-1β entry into the membrane compartment during secretion. In this model, TMED10 localizes to the ERGIC and forms a protein channel via homo-oligomerization to translocate unfolded UPE cargoes across the membrane of the ERGIC. At least two HSP90s facilitate this translocation process. HSP90AB1 is located in the cytosol and may assist the unfolding of cargoes or stabilize the unfolding state. In the lumen of the ERGIC, HSP90B1(GRP94) likely retains the unfolded cargo in the ERGIC to ensure unidirectional transport. The process is termed TMED10-channelled unconventional protein secretion (THU) and multiple UPE cargoes are likely regulated by this pathway, including the IL-1 family members, HSPB5, Galectin-1/3, Tau, and Annexin A1. Sequence alignment identified a motif of 14 aa (motif-1) existing in these cargoes and, in the case of IL-1β, the motif-1 is both necessary and sufficient to drive the secretion of cytosolic proteins through THU, indicating that motif-1 may be a UPE signal peptide [17] (Figure 2C).

TMED10 belongs to the P24 protein family, the members of which have been shown to regulate ER-Golgi trafficking via sorting specific cargoes (e.g., GPI-anchored proteins) to the cell surface and assisting vesicle carrier (COPI and COPII vesicles) formation in the ER-Golgi system [48,49]. The P24 protein family is classified into four subfamilies, α, β, δ and γ. As the only member of the δ subfamily in most vertebrates, TMED10 cooperates with other subfamily members in conventional cargo transport [50,51]. How does TMED10 coordinate its functions in both conventional and unconventional secretion? It is likely that different types of oligomers may account for the dual function of TMED10. In the case of ER-Golgi trafficking, TMED10 forms hetero-oligomers with other TMEDs to act as cargo receptors, whereas in THU TMED10 homo-oligomerizes to form a putative protein channel for leaderless cargo translocation [17,50].

The switch of different oligomer formations may be controlled by leaderless cargo abundance because overexpression of IL-1β, as well as several other leaderless cargoes, increased the formation of TMED10 homo-oligomer [17]. Notably, TMED family members share homologous domain distributions. Therefore, one obvious question is whether the TMED family proteins could act as unconventional secretion regulators similarly to TMED10.

The above findings imply that two protein translocation systems may act in parallel for conventional and unconventional secretion. Functionally, THU resembles the SEC61 translocation system for post-translational insertion of substrates into the ER in conventional secretion by harboring functional equivalents. Here, HSP90AB1, TMED10, and HSP90B1 correspond to HSP70, SEC61, and BIP, respectively. However, there are mechanistic differences. For example, SEC61 contains three subunits encompassing a core ten-pass transmembrane SEC61α with two regulatory SEC61β and SEC61γ, whereas TMED10 is a single transmembrane protein that requires cargo-induced homo-oligomerization to form a putative protein channel. In addition, it appears that the location of translocation is different, in which the SEC61 translocon resides on the rough-ER while TMED10 localizes on the ERGIC. Lastly, the SEC62/SEC63 complex associates with SEC61 which assists the docking of substrates in conventional export whereas it is pending to identify more auxiliary factors for TMED10 in THU [3,17,52,53] (Figure 2C and 2D).

It is worth mentioning that another translocation system termed chaperone-mediated autophagy (CMA) mechanistically works similarly to THU. The purpose of CMA is to degrade single proteins in the lysosome. Similar to TMED10, in CMA a single transmembrane protein, LAMP2A, forms a protein channel on the lysosome via oligomerization, which allows unfolded proteins to translocate across the limiting membrane of the lysosome [54,55]. Two HSC70s act in the cytosol and in the lumen of the lysosome to facilitate cargo recognition, unfolding, and translocation [55]. A key feature of the proteins degraded by CMA is that they harbor KFERQ motifs which can be recognized by cytosolic HSC70 [55] (Figure 2E). In THU, two KFERQ-like motifs in IL-1β have been found. Instead of binding to HSC70, these motifs are essential for HSP90AB1 association and IL-1β release [47]. However, it is unknown if other THU cargoes contain KFERQ-like motifs for HSP90AB1 binding, as well as how motif-1 coordinates with KFERQ-like motifs to determine UPE cargo targeting to the THU.

Misfolding-associated protein secretion

It has been shown that misfolded proteins are usually cleared by the ubiquitin-proteasome pathway or the autophagy pathway. In addition to clearance, it was found that cells can also dispose of misfolded proteins via secretion, termed misfolding-associated protein secretion (MAPS) [56]. In this pathway, misfolded proteins are recognized and deubiquitinated by a dual function protein, UPS19, which has both chaperone and deubiquitylase activities. The ER-located UPS19 acts together with two other chaperones, HSC70 and DNAJC5 (CSPα), to tether late endosomes to the ER and promote the translocation of misfolded proteins into the endolysosome and a perinuclear compartment which lacks LAMP1 labeling. Of the two compartments, misfolded protein entry into the endolysosome belongs to the ESCRT-dependent microautophagy which leads to the degradation of the cargoes. Interestingly, it is the perinuclear compartment that facilitates the release of misfolded cargoes. CD98hc cooperates with DNAJC5 to promote the cargo entry into the perinuclear compartment for subsequent release [56–58] (Figure 2F). The cellular function of MAPS is proposed

to act as another layer of quality control to maintain intracellular homeostasis. In addition, one could speculate that this system may contribute to misfolded toxic protein transmission in the state of neurodegeneration, though evidence supporting this hypothesis is still pending. In this pathway, it is unknown whether a protein channel exists on the late endosome for cargo transport. Although LAMP2A, the protein channel for CMA, modulates MAPS, it is not essential in this pathway, suggesting that LAMP2A is not the protein channel for MAPS [59]. Importantly, protein unfolding is not required for MAPS [59].

Multiple vesicular stations on the path to the plasma membrane

In vesicle-dependent UPE, cargoes transit through different membrane stations that are functionally repurposed for UPE. For example, the ERGIC separates from its function in conventional secretion to cope with the need of the UPE. For example, a compartment around the ER-exit site (ERES) called the compartment for unconventional protein secretion (CUPS), which is positive for Grh1 (the yeast homologue of mammalian GRASP), is generated for the UPE of the Acetyl CoA binding protein Acb1 in yeast *S. cerevisiae* [60], and the autophagosome and the endolysosome, usually for the purpose of degradation, are re-routed towards the plasma membrane for secretion [61]. Below we briefly discuss the functional change and remodeling of the endomembrane compartments during UPE.

ER-associated membrane compartments

As is mentioned above, the ERGIC acts as an initial station for multiple UPE cargoes entry into the membrane in THU. The ERGIC-localized protein channel TMED10 can translocate UPE cargoes into the lumen of the ERGIC [17]. Unlike the ER, the ERGIC lacks glycosyltransferase used to initiate protein glycosylation. Therefore, cargo translocation in the ERGIC avoids undesirable glyco-modifications that may cause cargo deactivation. After entering the ERGIC, vesicles may be generated to deliver cargo to the plasma membrane. These vesicles bypass the Golgi station because the ARF1 inhibitor Brefeldin A does not affect UPE. The ERGIC was initially identified as a cycling station between the ER and Golgi for conventional secretion [62] and it is still being characterized how the ERGIC differentiates its dual function in conventional and unconventional secretion, as well as how the ERGIC produces vesicles that can avoid the Golgi. One possible explanation is the inhibition of conventional secretion under stress conditions that activate the UPE. Therefore, stress-related molecular machineries may account for the functional switch of the ERGIC. Furthermore, the ERGIC has been shown to respond to starvation by forming a contact with ERES [63] and generating a special class of COPII vesicles (ERGIC-COPII) which merge with the pathway of autophagosome formation [64–66]. The scenario of ERGIC-autophagosome crosstalk in autophagy may be applied to UPE because cargoes, such as IL-1β and galectins, require both THU and secretory autophagosomes (shown below) to release [67,68]. Therefore, the switch of the ERGIC connection from the Golgi to the autophagosome under stress provides a solution for ERGIC functional conversion and Golgi bypass (Figure 3).

In yeast *S. cerevisiae*, a distinct ERGIC compartment is lacking. Nonetheless, a novel ERGIC-like compartment, termed CUPS, is generated under stress conditions for the release of UPE cargoes Acb1 and SOD1

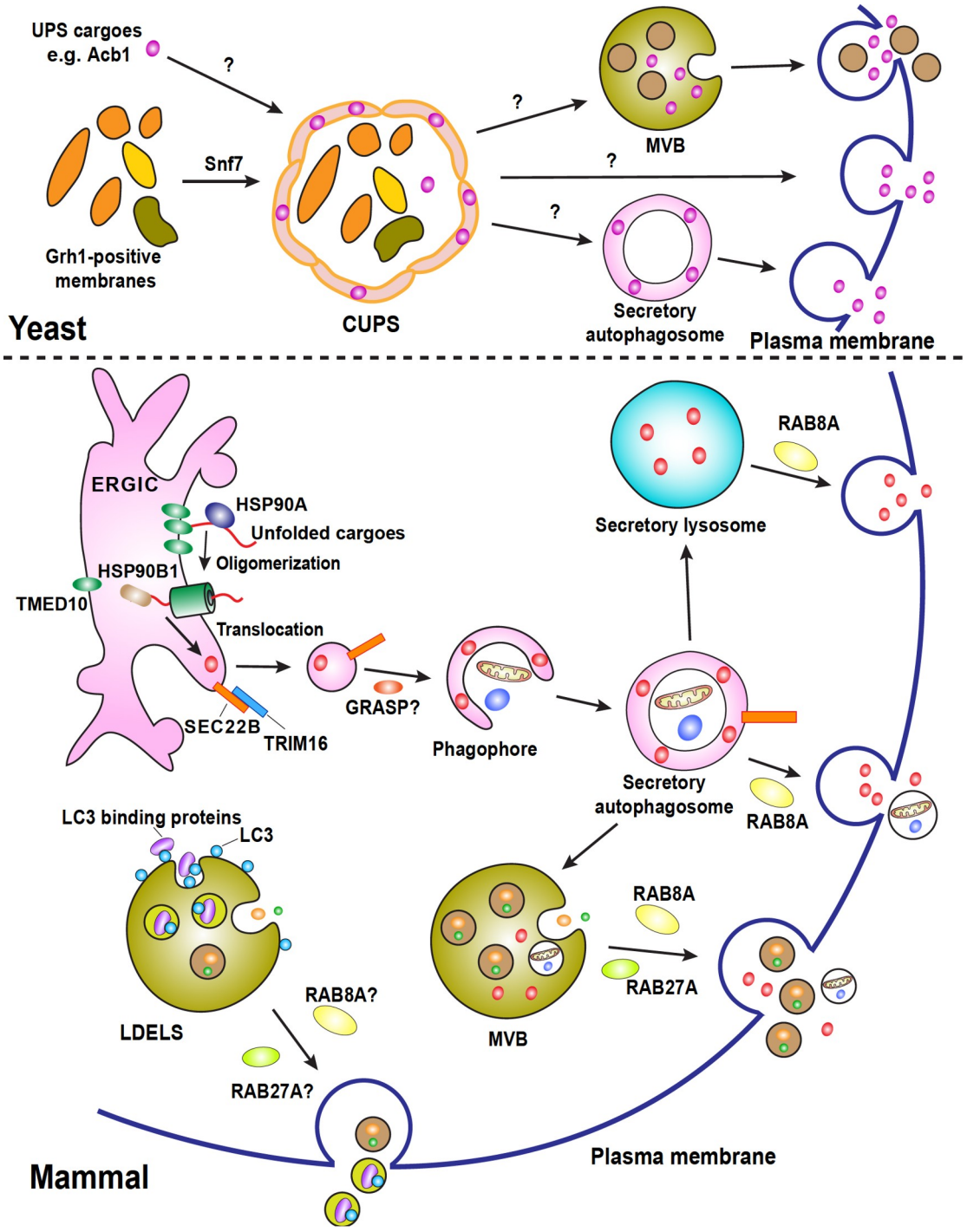

Figure 3 Endomembrane compartments for vesicle-dependent UPE. In yeast (up), an ERGIC-like structure, called CUPS, is generated near the ERES for the secretion of UPE cargoes like Acb1. The cargoes may enter into the CUPS and transport out of the cell through a yet-unknown itinerary likely involving MVBs or secretory autophagosomes. In mammals (down), after the translocation of UPE cargoes into the ERGIC, multiple membrane compartments downstream of the ERGIC may be involved in the delivery of cargoes out of the cell, including secretory autophagosomes, MVBs, and secretory lysosomes. Distinct SNARE and RAB proteins are involved in the selectivity of degradative and secretory autophagy (e.g., SEC22B, RAB8A, RAB27A). GRASP and TRIM16 may be involved in Golgi bypass and SEC22B loading to UPE vehicles. In addition to the ERGIC as a station for cargo loading, MVB invagination and autophagosome engulfment, as well as a hybrid pathway the LC3-dependent EV loading and secretion (LDELS) pathway, also participate in the loading and secretion of UPE cargoes, especially RNA granules and organelles.

[60,69]. Like ERGIC, CUPS localizes adjacent to the ERES and contains properties of the pre-Golgi, and has a tubule-vesicular structure [60,70]. In addition, CUPS is functionally connected with the secretory autophagosome [18,71]. Regarding the function, CUPS is also proposed as the initial site for vesicle-dependent UPE. However, CUPS also harbors its unique features, for example, it is positive for Grh1 [60], and components of the ESCRT complex Snf7 were shown to transiently associate with and maintain the structure of CUPS [72] (Figure 3). It is unclear how UPE cargoes enter into the lumen of the compartments at CUPS. One possibility is through an uncharacterized protein channel. The other possibility is via ESCRT-mediated membrane invagination.

Secretory autophagosome and endolysosomes

Autophagy was initially identified as a lysosome-dependent bulk degradation pathway through the formation of double-membrane autophagosomes [73]. Interestingly, studies in lower organisms have found that autophagy can mediate unconventional secretion of the cytosolic Acb1 in yeast *S. cerevisiae* [74] and AcbA in the slime mold *Dictyostelium* [75]. The involvement of autophagy in UPE was further revealed in mammalian cells when assessing the UPE of IL-1β [67,68]. Secretory autophagy, as well as its vesicle carrier secretory autophagosome (as opposed to degradative autophagy and autophagosome), has become an area of interest in the field of UPE study.

Until now, several types of UPE cargoes have been found to be delivered for secretion by the secretory autophagosome, including soluble proteins (e.g., IL-1β), organelles (e.g., mitochondria), and protein aggregates [76]. At least two pathways have been proposed to explain cargo entry into the secretory autophagosome. One pathway deals with soluble proteins like IL-1β. The proteins are firstly translocated into the ERGIC where vesicles are generated with IL-1β in the lumen and these vesicles subsequently merge into the autophagosome biogenesis pathway. In this case, the topological location of the cargo is the intermembrane space of the double-membrane autophagosome [17,47]. Fusion of the outer membrane of the autophagosome with the plasma membrane leads to the release of contents from the intermembrane space in the form of free proteins and, therefore, enables immediate cytokine function in the case of IL-1β secretion. The other pathway copes with the release of organelles and protein aggregates via secretory autophagy [19,77]. In this pathway, it is highly likely that these cargoes are engulfed by the phagophore (the cup shape precursor of the autophagosome) and localize to the inner membrane of the autophagosome. It is conceivable that these cargoes are released in the form of extracellular vesicles (Figure 3).

A key question relating to the autophagy system is how secretion and degradation are balanced. Early studies found that blockage of degradative autophagy by bafilomycin A1 inhibition of lysosome degradation increased autophagy-mediated secretion [78], suggesting that this double function of autophagy may employ similar early autophagic factors and the molecular machinery diverged in the late step before fusion with the plasma membrane (secretion) or the lysosome (degradation). This notion was also confirmed by a recent study [79]. Later, it was shown that the molecular machinery involved in differentiating secretory autophagy from degradative autophagy contains RAB GTPases. RAB8 and RAB27A have been shown to regulate secretory autophagy, whereas RAB7 directs autophagosomes to lysosomes for degradation [67,78,80] (Figure 3). In addition, it was found that the two types of the autophagosomes contain different SNARE proteins, in which the secretory autophagosomes contain SEC22B, which can pair with the plasma membrane

SNAREs STX3/STX4, SNAP23, or SNAP29 [68], but the degradative autophagosome has Syntaxin 17, which pairs with the lysosome SNARE VAMP8 [81] (Figure 3). Even though we know that different SNAREs and RABs determine the function of autophagosomes, it is unknown how secretory or degradative cargoes determine the loading of specific membrane targeting factors, such as RABs and SNAREs.

It is worth noting that the Golgi protein GRASP, which was initially identified as a regulator of Golgi stacking factor [82,83], has been shown to act as an upstream regulator of the secretory autophagosome in multiple studies, indicating that GRASP may be a determinant of the secretory autophagosome [75,84,85]. However, a recent study also indicated a role for GRASP in the process of degradative autophagy [86]. Therefore, GRASP has multiple functions depending on cellular contexts. One potential regulatory control of GRASP function is post-translactional modification, in which phosphorylation and O-GlcNAcylation regulate the distribution of GRASP55 between the Golgi and the autophagosome [84,86]. Nonetheless, it is still unclear how GRASP differentiates between the secretory and the degradative autophagosome. Another UPE regulatory candidate, TRIM16, was proposed to link cargoes to SEC22B-tagged vesicles for secretory autophagy-mediated UPE trafficking [68]. However, TRIM16 has also been shown to regulate the selective clearance of autophagic cargoes, including aggregates and damaged endomembranes [87,88]. Therefore, it is pending to define how GRASP and TRIM16 specifically control the two types of autophagy. Furthermore, it is necessary to identify additional layers of regulation for the separation of secretory and degradative autophagy.

Studies on degradative autophagy have shown that autophagosome can fuse with endosomes to form an amphisome before fusion with the lysosome. Similarly, the secretory autophagosome can crosstalk with compartments in the endolysosomal system. For example, autophagy-mediated release of Annexin A2 has been shown to associate with MVBs [89]. Consistently, the release of IL-1β was reported to be dependent on both autophagic and ESCRT factors [14]. Furthermore, a recent study found that LC3 lipidation, a marker for the autophagosome, can occur on the MVB to directly mediate the sorting of UPE cargoes (here RNA binding proteins) into the MVB in a mechanism similar to autophagosome engulfment, termed LC3-dependent EV loading and secretion (LDELS) (Figure 3) [90]. In addition to MVBs, secretory lysosomes have also been reported to facilitate UPE in a calcium and pH-dependent manner [91]. Two populations of lysosomes with different pHs have been identified, indicating differential function of lysosomes in the cell [92]. It is likely that a population of high pH lysosomes may cooperate with secretory autophagosomes to complete UPE.

Concluding remarks

Unconventional secretion has been investigated for more than 30 years. Extensive studies have revealed multiple facets of UPE. Many UPE cargoes have been identified and their roles in human health and disease are still being described. It has been difficult to unify all pathways of UPE, and it is increasingly recognized that cells employ different strategies to release UPE cargoes depending on physiological and pathological contents. Multiple types of translocation pathways and membrane trafficking routes have been characterized. However, several major questions still remain, for example:

(1) What makes a cytosolic protein an unconventional secretory protein? Although certain properties for

specific proteins have been proposed, including the $PI(4,5)P_2$ and heparan sulfate binding of FGF2, the diacidic motif of SOD1 [15,17,69], and the motif-1 regarding THU, it has been unclear if these properties are limited to a specific class of cargoes or could be applied to general roles. Therefore, there is a need to systematically identify cargoes undergoing each type of UPE and characterize their common features to provide further clues as to what makes a cytosolic protein an unconventional secretory protein.

(2) Regarding the vesicle-independent UPE, it has been suggested that several other cargoes (e.g., HIV-TAT, Tau) share a similar requirements for $PI(4,5)P_2$ and heparan sulfate for secretion. In addition, HIV-TAT has also been indicated to form membrane pores for translocation [93]. Is formation of a lipidic pore for self-translocation across the plasma membrane a common mechanism for vesicle-independent UPE? If so, how is plasma membrane rupture prevented during translocation?

(3) In the case of THU, TMED10 is sufficient to drive cargo translocation across the membrane and protein unfolding is required. However, it is still unclear if TMED10 forms a protein channel without structural dissection of molecular details. It is not clear if cargo translocates in a totally linearized form or partially unfolded state.

(4) In vesicle-mediated UPE, especially the autophagosome stage, do secretory and degradative autophagosomes share the same autophagosome carriers or are they separated? In the latter case, what is the initial trigger for the formation of a secretory or a degradative autophagosome?

(5) Many UPE cargoes have been shown to undergo multiple pathways of UPE, for example, IL-1β. What determines the selection of a UPE cargo towards a certain pathway to release?

Answering these questions will not only deepen our understanding of perplexing UPE pathways but will also lead to the comprehensive identification of new UPE cargoes and, therefore, shed light on diagnosis and treatment of human diseases related to UPE dysfunction.

Funding

The work was supported by the National Natural Science Foundation of China (32130023, 91854114, and 32061143009), the Ministry of Science and Technology of the People's Republic of China (2019YFA0508602 and 2021YFA0804802), and the Beijing Natural Science Foundation (JQ20028).

Author contributions

J.Z. drafted the manuscript and drew the figures. L.G. organized the contents and finalized the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest to disclose.

References

1 Blobel G, Dobberstein B. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J Cell Biol* 1975; **67**: 835–851.

2 Walter P, Ibrahimi I, Blobel G. Translocation of proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to *in-vitro*-assembled polysomes synthesizing secretory protein. *J Cell Biol* 1981; **91**: 545–550.

3 Rapoport TA, Li L, Park E. Structural and mechanistic insights into protein translocation. *Annu Rev Cell Dev Biol* 2017;

33: 369–390.

4 Lee MCS, Miller EA, Goldberg J, *et al.* Bi-directional protein transport between the ER and Golgi. *Annu Rev Cell Dev Biol* 2004; **20**: 87–123.

5 Schekman RW. George E. Palade (1912-2008). *Science* 2008; **322**: 695.

6 Rubartelli A, Cozzolino F, Talio M, *et al.* A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J* 1990; **9**: 1503–1510.

7 Zhang M, Schekman R. Unconventional secretion, unconventional solutions. *Science* 2013; **340**: 559–561.

8 Cohen MJ, Chirico WJ, Lipke PN. Through the back door: Unconventional protein secretion. *Cell Surf* 2020; **6**: 100045.

9 Rabouille C. Pathways of unconventional protein secretion. *Trends Cell Biol* 2017; **27**: 230–240.

10 Kim J, Gee HY, Lee MG. Unconventional protein secretion—new insights into the pathogenesis and therapeutic targets of human diseases. *J Cell Sci* 2018; **131**: jcs213686.

11 Dimou E, Nickel W. Unconventional mechanisms of eukaryotic protein secretion. *Curr Biol* 2018; **28**: R406–R410.

12 Wegehingel S, Zehe C, Nickel W. Rerouting of fibroblast growth factor 2 to the classical secretory pathway results in post-translational modifications that block binding to heparan sulfate proteoglycans. *FEBS Lett* 2008; **582**: 2387–2392.

13 Popa SJ, Stewart SE, Moreau K. Unconventional secretion of annexins and galectins. *Semin Cell Dev Biol* 2018; **83**: 42–50.

14 Sitia R, Rubartelli A. The unconventional secretion of IL-1β: Handling a dangerous weapon to optimize inflammatory responses. *Semin Cell Dev Biol* 2018; **83**: 12–21.

15 Pallotta MT, Nickel W. FGF2 and IL-1β—explorers of unconventional secretory pathways at a glance. *J Cell Sci* 2020; **133**: jcs250449.

16 Brough D, Pelegrin P, Nickel W. An emerging case for membrane pore formation as a common mechanism for the unconventional secretion of FGF2 and IL-1β. *J Cell Sci* 2017; **130**: 3197.

17 Zhang M, Liu L, Lin X, *et al.* A translocation pathway for vesicle-mediated unconventional protein secretion. *Cell* 2020; **181**: 637–652.e15.

18 Padmanabhan S, Manjithaya R. Facets of autophagy based unconventional protein secretion—the road less traveled. *Front Mol Biosci* 2020; **7**: 586483.

19 Claude-Taupin A, Jia J, Mudd M, *et al.* Autophagy's secret life: Secretion instead of degradation. *Essays Biochem* 2017; **61**: 637–647.

20 Gee HY, Kim J, Lee MG. Unconventional secretion of transmembrane proteins. *Semin Cell Dev Biol* 2018; **83**: 59–66.

21 Zurzolo C. Tunneling nanotubes: Reshaping connectivity. *Curr Opin Cell Biol* 2021; **71**: 139–147.

22 Ma L, Li Y, Peng J, *et al.* Discovery of the migrasome, an organelle mediating release of cytoplasmic contents during cell migration. *Cell Res* 2015; **25**: 24–38.

23 Jiao H, Jiang D, Hu X, *et al.* Mitocytosis, a migrasome-mediated mitochondrial quality-control process. *Cell* 2021; **184**: 2896–2910.e13.

24 Steringer JP, Nickel W. A direct gateway into the extracellular space: Unconventional secretion of FGF2 through self-sustained plasma membrane pores. *Semin Cell Dev Biol* 2018; **83**: 3–7.

25 Sparn C, Dimou E, Meyer A, *et al.* Glypican-1 drives unconventional secretion of fibroblast growth factor 2. *eLife* 2022; **11**: e75545.

26 Steringer JP, Lange S, Čujová S, *et al.* Key steps in unconventional secretion of fibroblast growth factor 2 reconstituted with purified components. *eLife* 2017; **6**: e28985.

27 Ebert AD, Laussmann M, Wegehingel S, *et al.* Tec-kinase-mediated phosphorylation of fibroblast growth factor 2 is essential for unconventional secretion. *Traffic* 2010; **11**: 813–826.

28 Legrand C, Saleppico R, Sticht J, *et al.* The Na,K-ATPase acts upstream of phosphoinositide PI(4,5)P_2 facilitating unconventional secretion of Fibroblast Growth Factor 2. *Commun Biol* 2020; **3**: 141.

29 Rayne F, Debaisieux S, Yezid H, *et al.* Phosphatidylinositol-(4,5)-bisphosphate enables efficient secretion of HIV-1 Tat by infected T-cells. *EMBO J* 2010; **29**: 1348–1362.

30 Katsinelos T, Zeitler M, Dimou E, *et al.* Unconventional secretion mediates the trans-cellular spreading of Tau. *Cell Rep* 2018; **23**: 2039–2055.

31 Amblard I, Dupont E, Alves I, *et al.* Bidirectional transfer of Engrailed homeoprotein across the plasma membrane requires PIP2. *J Cell Sci* 2020; **133**: jcs244327.

32 Merezhko M, Brunello CA, Yan X, *et al.* Secretion of Tau via an unconventional non-vesicular mechanism. *Cell Rep* 2018; **25**: 2027–2035.e4.

33 Faham S, Hileman RE, Fromm JR, *et al.* Heparin structure and interactions with basic fibroblast growth factor. *Science* 1996; **271**: 1116–1120.

34 Torrado ĹĆ, Temmerman K, Müller HM, *et al.* An intrinsic quality-control mechanism ensures unconventional secretion of fibroblast growth factor 2 in a folded conformation. *J Cell Sci* 2009; **122**: 3322–3329.

35 Dimou E, Cosentino K, Platonova E, *et al.* Single event visualization of unconventional secretion of FGF2. *J Cell Biol* 2019; **218**: 683–699.

36 Lolicato, F, Saleppico, R, Griffo, A, *et al*. Cholesterol promotes both head group visibility and clustering of $PI(4,5)P_2$ driving unconventional secretion of Fibroblast Growth Factor 2. bioRxiv: 2021.04.16.440132.

37 Stewart SE, Ashkenazi A, Williamson A, *et al.* Transbilayer phospholipid movement facilitates annexin translocation across membranes. *J Cell Sci* 2018; **131**: jcs217034.

38 Ding J, Wang K, Liu W, *et al.* Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature* 2016; **535**: 111–116.

39 Liu X, Xia S, Zhang Z, *et al.* Channelling inflammation: gasdermins in physiology and disease. *Nat Rev Drug Discov* 2021; **20**: 384–405.

40 Kayagaki N, Stowe IB, Lee BL, *et al.* Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* 2015; **526**: 666–671.

41 Mulvihill E, Sborgi L, Mari SA, *et al.* Mechanism of membrane pore formation by human gasdermin-D. *EMBO J* 2018; **37**: e98321.

42 Evavold CL, Ruan J, Tan Y, *et al.* The pore-forming protein gasdermin D regulates interleukin-1 secretion from living macrophages. *Immunity* 2018; **48**: 35–44.e6.

43 Xia S, Zhang Z, Magupalli VG, *et al.* Gasdermin D pore structure reveals preferential release of mature interleukin-1. *Nature* 2021; **593**: 607–611.

44 Rühl S, Shkarina K, Demarco B, *et al.* ESCRT-dependent membrane repair negatively regulates pyroptosis downstream of GSDMD activation. *Science* 2018; **362**: 956–960.

45 Shi J, Zhao Y, Wang K, *et al.* Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* 2015; **526**: 660–665.

46 Andrei C, Dazzi C, Lotti L, *et al.* The secretory route of the leaderless protein interleukin 1β involves exocytosis of endolysosome-related vesicles. *Mol Biol Cell* 1999; **10**: 1463–1475.

47 Zhang M, Kenny SJ, Ge L, *et al.* Translocation of interleukin-1β into a vesicle intermediate in autophagy-mediated secretion. *eLife* 2015; **4**: e11205.

48 Strating JRPM, Martens GJM. The p24 family and selective transport processes at the ER-Golgi interface. *Biol Cell* 2009; **101**: 495–509.

49 Aber R, Chan W, Mugisha S, *et al.* Transmembrane emp24 domain proteins in development and disease. *Genet Res* 2019; **101**: e14.

50 Pastor-Cantizano N, Montesinos JC, Bernat-Silvestre C, *et al.* p24 family proteins: key players in the regulation of trafficking along the secretory pathway. *Protoplasma* 2016; **253**: 967–985.

51 Nagae M, Hirata T, Morita-Matsumoto K, *et al.* 3D structure and interaction of p24β and p24δ golgi dynamics domains: Implication for p24 complex formation and cargo transport. *J Mol Biol* 2016; **428**: 4087–4099.

52 Park E, Rapoport TA. Mechanisms of Sec61/SecY-mediated protein translocation across membranes. *Annu Rev Biophys* 2012; **41**: 21–40.

53 Wu X, Cabanos C, Rapoport TA. Structure of the post-translational protein translocation machinery of the ER membrane. *Nature* 2019; **566**: 136–139.

54 Cuervo AM, Dice JF. A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* 1996; **273**: 501–503.

55 Kaushik S, Cuervo AM. The coming of age of chaperone-mediated autophagy. *Nat Rev Mol Cell Biol* 2018; **19**: 365–381.

56 Lee JG, Takahama S, Zhang G, *et al.* Unconventional secretion of misfolded proteins promotes adaptation to proteasome dysfunction in mammalian cells. *Nat Cell Biol* 2016; **18**: 765–776.

57 Xu Y, Cui L, Dibello A, *et al.* DNAJC5 facilitates USP19-dependent unconventional secretion of misfolded cytosolic proteins. *Cell Discov* 2018; **4**: 11.

58 Lee J, Xu Y, Saidi L, *et al.* Abnormal triaging of misfolded proteins by adult neuronal ceroid lipofuscinosis-associated DNAJC5/CSPα mutants causes lipofuscin accumulation. *Autophagy* 2022; 1–20, https://doi/org/10.1080/15548627.2022.2065618.

59 Lee J, Xu Y, Zhang T, *et al.* Secretion of misfolded cytosolic proteins from mammalian cells is independent of chaperone-mediated autophagy. *J Biol Chem* 2018; **293**: 14359–14370.

60 Bruns C, McCaffery JM, Curwin AJ, *et al.* Biogenesis of a novel compartment for autophagosome-mediated unconventional protein secretion. *J Cell Biol* 2011; **195**: 979–992.

61 Filaquier A, Marin P, Parmentier ML, *et al.* Roads and hubs of unconventional protein secretion. *Curr Opin Cell Biol* 2022; **75**: 102072.

62 Saraste J, Marie M. Intermediate compartment (IC): From pre-Golgi vacuoles to a semi-autonomous membrane system. *Histochem Cell Biol* 2018; **150**: 407–430.

63 Li S, Yan R, Xu J, *et al.* A new type of ERGIC-ERES membrane contact mediated by TMED9 and SEC12 is required for autophagosome biogenesis. *Cell Res* 2022; **32**: 119–138.

64 Ge L, Melville D, Zhang M, *et al.* The ER-Golgi intermediate compartment is a key membrane source for the LC3 lipidation step of autophagosome biogenesis. *eLife* 2013; **2**: e00947.

65 Ge L, Zhang M, Schekman R. Phosphatidylinositol 3-kinase and COPII generate LC3 lipidation vesicles from the ER-Golgi intermediate compartment. *eLife* 2014; **3**: e04135.

66 Ge L, Zhang M, Kenny SJ, *et al.* Remodeling of ER-exit sites initiates a membrane supply pathway for autophagosome biogenesis. *EMBO Rep* 2017; **18**: 1586–1603.

67 Dupont N, Jiang S, Pilli M, *et al.* Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1β. *EMBO J* 2011; **30**: 4701–4711.

68 Kimura T, Jia J, Kumar S, *et al.* Dedicated SNAREs and specialized TRIM cargo receptors mediate secretory autophagy. *EMBO J* 2017; **36**: 42–60.

69 Cruz-Garcia D, Brouwers N, Duran JM, *et al.* A diacidic motif determines unconventional secretion of wild-type and ALS-linked mutant SOD1. *J Cell Biol* 2017; **216**: 2691–2700.

70 Cruz-Garcia D, Curwin AJ, Popoff JF, *et al.* Remodeling of secretory compartments creates CUPS during nutrient starvation. *J Cell Biol* 2014; **207**: 695–703.

71 Cruz-Garcia D, Malhotra V, Curwin AJ. Unconventional protein secretion triggered by nutrient starvation. *Semin Cell Dev Biol* 2018; **83**: 22–28.

72 Curwin AJ, Brouwers N, Alonso Y, *et al.* ESCRT-III drives the final stages of CUPS maturation for unconventional protein secretion. *eLife* 2016; **5**: e16299.

73 Nakatogawa H. Mechanisms governing autophagosome biogenesis. *Nat Rev Mol Cell Biol* 2020; **21**: 439–458.

74 Duran JM, Anjard C, Stefan C, *et al.* Unconventional secretion of Acb1 is mediated by autophagosomes. *J Cell Biol* 2010; **188**: 527–536.

75 Manjithaya R, Anjard C, Loomis WF, *et al.* Unconventional secretion of Pichia pastoris Acb1 is dependent on GRASP protein, peroxisomal functions, and autophagosome formation. *J Cell Biol* 2010; **188**: 537–546.

76 Gonzalez CD, Resnik R, Vaccaro MI. Secretory autophagy and its relevance in metabolic and degenerative disease. *Front Endocrinol* 2020; **11**: 266.

77 Ponpuak M, Mandell MA, Kimura T, *et al.* Secretory autophagy. *Curr Opin Cell Biol* 2015; **35**: 106–116.

78 Ejlerskov P, Rasmussen I, Nielsen TT, *et al.* Tubulin polymerization-promoting protein (TPPP/p25α) promotes unconventional secretion of α-synuclein through exophagy by impairing autophagosome-lysosome fusion. *J Biol Chem* 2013; **288**: 17313–17335.

79 Solvik TA, Nguyen TA, Tony Lin YH, *et al.* Secretory autophagy maintains proteostasis upon lysosome inhibition. *J Cell Biol* 2022; **221**: e202110151.

80 Hyttinen JMT, Niittykoski M, Salminen A, *et al.* Maturation of autophagosomes and endosomes: A key role for Rab7. *Biochim Biophys Acta (BBA)-Mol Cell Res* 2013; **1833**: 503–510.

81 Itakura E, Kishi-Itakura C, Mizushima N. The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. *Cell* 2012; **151**: 1256–1269.

82 Shorter J, Watson R, Giannakou ME, *et al.* GRASP55, a second mammalian GRASP protein involved in the stacking of Golgi cisternae in a cell-free system. *EMBO J* 1999; **18**: 4949–4960.

83 Barr FA, Puype M, Vandekerckhove J, *et al.* GRASP65, a protein involved in the stacking of golgi cisternae. *Cell* 1997; **91**: 253–262.

84 Nüchel J, Tauber M, Nolte JL, *et al.* An mTORC1-GRASP55 signaling axis controls unconventional secretion to reshape the extracellular proteome upon stress. *Mol Cell* 2021; **81**: 3275–3293.e12.

85 Kinseth MA, Anjard C, Fuller D, *et al.* The golgi-associated protein GRASP is required for unconventional protein secretion during development. *Cell* 2007; **130**: 524–534.

86 Zhang X, Wang L, Lak B, *et al.* GRASP55 senses glucose deprivation through O-GlcNAcylation to promote autophagosome-lysosome fusion. *Dev Cell* 2018; **45**: 245–261.e6.

87 Jena KK, Kolapalli SP, Mehto S, *et al.* TRIM16 controls assembly and degradation of protein aggregates by modulating the p62-NRF2 axis and autophagy. *EMBO J* 2018; **37**: e98358.

88 Chauhan S, Kumar S, Jain A, *et al.* TRIMs and galectins globally cooperate and TRIM16 and galectin-3 co-direct autophagy in endomembrane damage homeostasis. *Dev Cell* 2016; **39**: 13–27.

89 Chen YD, Fang YT, Cheng YL, *et al.* Exophagy of annexin A2 via RAB11, RAB8A and RAB27A in IFN-γ-stimulated lung epithelial cells. *Sci Rep* 2017; **7**: 5676.

90 Leidal AM, Huang HH, Marsh T, *et al.* The LC3-conjugation machinery specifies the loading of RNA-binding proteins into extracellular vesicles. *Nat Cell Biol* 2020; **22**: 187–199.

91 Lee J, Ye Y. The roles of endo-lysosomes in unconventional protein secretion. *Cells* 2018; **7**: 198.

92 Johnson DE, Ostrowski P, Jaumouillé V, *et al.* The position of lysosomes within the cell determines their luminal pH. *J Cell Biol* 2016; **212**: 677–692.

93 Zeitler M, Steringer JP, Müller HM, *et al.* HIV-Tat protein forms phosphoinositide-dependent membrane pores implicated in unconventional protein secretion. *J Biol Chem* 2015; **290**: 21976–21984.