Adaptor functions of the Ca^{2+}-binding protein ALG-2 in protein transport from the endoplasmic reticulum.

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Apoptosis-linked gene 2 (ALG-2) is a Ca^{2+}-binding protein with five repetitive EF-hand motifs, named penta-EF-hand (PEF) domain. It interacts with various target proteins and functions as a Ca^{2+}-dependent adaptor in diverse cellular activities. In the cytoplasm, ALG-2 is predominantly localized to a specialized region of the endoplasmic reticulum (ER), called the ER exit site (ERES), through its interaction with Sec31A. Sec31A is an outer coat protein of coat protein complex II (COPII) and is recruited from the cytosol to the ERES to form COPII-coated transport vesicles. I will overview current knowledge of the physiological significance of ALG-2 in regulating ERES localization of Sec31A and the following adaptor functions of ALG-2, including bridging Sec31A and annexin A11 to stabilize Sec31A at the ERES, polymerizing the Trk-fused gene (*TFG*) product, and linking MAPK1-interacting and spindle stabilizing (MISS)-like (MISSL) and microtubule-associated protein 1B (MAP1B) to promote anterograde transport from the ER.

Keywords: adaptor protein; calcium-binding protein; COPII; endoplasmic reticulum exit site; secretory pathway

Introduction

Apoptosis-linked gene 2 (ALG-2), a product of the *PDCD6* gene, is a 22-kDa penta-EF-hand (PEF) type Ca^{2+}-binding protein (UniProt ID: O75340) [1,2]. There is confusion caused by the use of the same or similar name for different proteins and genes; for example, the product of the *ALG2* gene (without hyphen between "ALG" and "2") is termed ALG2 as an α-1,3/1,6-mannosyltransferase (UniProt ID: Q9H553) and the gene *alg-2* (*Argonaute-like gene 2)* in *C. elegans* encodes a member of the highly conserved eukaryotic RDE-1/AGO1/PIWI family of proteins (UniProt ID: O16720). Here, the term "ALG-2" is used to refer to a product of the *PDCD6* gene.

A cDNA encoding murine *Pdcd6* was first isolated by the death-trap method, in which a cDNA library constructed in a mammalian expression vector was transiently transfected into mouse T cell hybridoma 3DO cells, apoptosis was then induced with T cell receptor stimulation, and the plasmids were recovered from surviving cells [3]. The obtained plasmids containing the *Pdcd6* insert all encoded anti-sense transcripts. The resultant reduced expression of ALG-2 protein conferred resistance to cell death induced by several stimuli, including glucocorticoids, T cell receptors and Fas triggering. Thus, it is conceivable that ALG-2 functions as a proapoptotic protein [4]. However, an unexpected finding is that *Pdcd6*-deficient mice generated by gene targeting exhibit no abnormality in the immune system and that both immature thymocytes and mature T cells from the *Pdcd6*-deficient mouse retain their susceptibility to apoptotic stimuli [5]. Nonetheless, potential roles of ALG-2 in promoting cell death induced by ER stress [6], tumor necrosis factor receptor 1 [7], and DNA damage [8] have been reported. On the other hand, ALG-2 has been reported to be up-regulated in a variety of tumors [9-12]. ALG-2 overexpression is an independent poor prognostic factor in early stage lung adenocarcinoma [11], whereas lower expression levels of *PDCD6* mRNA are correlated significantly with poor overall survival of gastric cancer patients who have received chemotherapy [12]. Thus, ALG-2 may be an important modulator involved in the cellular decision between cell proliferation and cell death [13]. However, the molecular mechanisms of proapoptotic and survival functions of ALG-2 are not yet fully understood.

To elucidate the molecular functions of ALG-2 and its role in Ca^{2+} signaling, searching for interacting proteins of ALG-2 has been conducted by several groups using yeast two-hybrid screening and mass-spectrometric analyses of immunoprecipitates of endogenous or tagged proteins or of pulldown products of recombinant ALG-2

expressed in *E. coli*. Evaluation of different *in vitro* and/or *in vivo* methods has been carried out to validate the direct interactions of ALG-2 with candidate proteins and their Ca^{2+}-dependency [14,15]. Until now, more than 20 proteins have been identified as Ca^{2+}-dependent interacting proteins for ALG-2 [15]. The target proteins of ALG-2 are distributed in various intracellular compartments such as the endoplasmic reticulum [Sec31A [16-18], Scotin [19], Trk-fused gene (TFG) [20] and MAPK1-interacting and spindle-stabilizing (MISS)-like (MISSL) [21]], endocytic organelles [ALG-2 interacting protein X (ALIX) [22,23], tumor susceptibility gene 101 (TSG101) [24], vacuolar protein sorting 37C (VPS37C) [25], IST1 [26], and Mucolipin-1/TRPML1 [27]] and nucleus [RBM22 [28] and calcium homeostasis endoplasmic reticulum protein (CHERP) [29]]. Multifaceted roles of ALG-2 in these compartments were reviewed recently [15]. Here, some of the more recent advances in understanding adaptor functions of ALG-2 in protein transport from the endoplasmic reticulum (ER) are reviewed.

1. ALG-2 is predominantly localized to the Sec31A-positive ERES.

Approximately one-third of all proteins in eukaryotic cells are estimated to translocate into the ER during their synthesis on membrane-bound ribosomes. Only properly folded proteins are packaged into coat protein complex II (COPII)-coated transport vesicles to be dispatched to their final destinations via the Golgi apparatus. Five evolutionally conserved proteins, namely a small GTPase Sar1, inner coat subunits Sec23 and Sec24, and outer coat subunits Sec13 and Sec31, constitute the minimal machinery for generating COPII vesicles [30] (Figure 1A). They are sequentially recruited from the cytosol to a discrete sub-region of the ER called the ER exit site (ERES) or transitional ER (tER) as follows: 1) exchange of GDP for GTP in Sar1 by the guanine nucleotide exchange factor Sec12 leads to exposure of N-terminal

amphipathic α-helix of Sar1, which is inserted into the cytoplasmic leaflet of the ERES membrane, 2) the active form of Sar1 recruits the Sec23-Sec24 heterodimer from the cytosol to the ERES, where Sec24 recognizes and binds exit motifs in the cytosolic region of cargo proteins or cargo receptors to form a pre-budding complex, and 3) the pre-budding complex in turn recruits the Sec13-Sec31 heterotetramer, which forms the outer layer of the COPII coat [31-34].

In contrast to the reticular pattern of the ER, the ERES in mammalian cells shows a small punctate structure distributed throughout the cytoplasm but usually concentrated in the juxtanuclear region detected by the indirect immunofluorescent method (Figure 1B). Three-dimensional immuno-electron tomography of the ERES revealed that COPII components exist on ER-associated buds as well as on vesicles free from the ER and tubules adjacent to the ER [35]. Therefore, in images obtained by the indirect immunofluorescence method using conventional confocal microscopy, each fluorescence spot labeled with an antibody against the COPII component should correspond to one ERES containing a cluster of nascent buds and vesicles and tubules after budding off.

Several independent groups including ours reported that ALG-2 interacts Ca^{2+}-dependently with Sec31A and distributes predominantly to the Sec31A-positive ERES in the cytoplasm [16,17] (Figure 1B). Live-cell imaging of fluorescent protein-fused ALG-2 revealed that ALG-2 translocates from the cytosol to the Sec31A-positive ERES following exposure of cells to Ca^{2+}-mobilizing agents [18,36]. A mutant with glutamate residues substituted for alanine in Ca^{2+}-coordinating positions of EF1 and EF3 (ALG-$2^{E47A/E114A}$) does not interact with Sec31A, and overexpressed ALG-$2^{E47A/E114A}$ is seen distributed diffusely to the cytoplasm and nucleus with no co-localization with ERES marker proteins [16,17]. In addition, the cytoplasmic punctate localization pattern of

ALG-2 is lost to a great extent in Sec31A knockdown cells [16]. Therefore, Ca^{2+}-dependent binding of ALG-2 with Sec31A is essential for the ERES localization of ALG-2.

2. ALG-2 binding site in Sec31A

ALG-2 has five repetitive EF-hand motifs in its C-terminus (Figure 2A), but, like calmodulin, it lacks catalytic activities and is thought to perform its biological functions by interacting with target proteins [15]. Two short peptide motifs that are recognized by ALG-2 have been identified and named ABM-1 (ALG-2 binding motif type-1) of PPYP(*X*)*n*YP (*X*, variable amino acids, n = 3-6) [37] and ABM-2 of [PΦ]P*X*[PΦ]G[FW]Ω ([PΦ], Pro or hydrophobic; [FW], Phe or Trp; Ω, large side chain; *X*, variable) [38].

Murine *Pdcd6* encodes two alternatively spliced transcripts, and the short isoform lacks 6 base pairs; therefore, the corresponding protein lacks Gly121 and Phe122 [39] (Figure 2A). Here, the long and short isoforms are denoted by ALG-2^{WT} and ALG-$2^{\Delta GF122}$, respectively (Figure 2A). ALG-2^{WT} binds both target proteins having ABM-1 or ABM-2. In contrast, ALG-$2^{\Delta GF122}$ has no binding avidity to proteins with ABM-1 or ABM-1-like motifs, such as ALIX [39], TSG101 [24], Scotin [19], annexin A7 and annexin A11 [40]. On the other hand, ALG-$2^{\Delta GF122}$ shows binding to proteins with ABM-2, including Sec31A [17], PLSCR3 [40] and TFG [20]. An RNase protection assay revealed that both the long and short RNAs are transcribed in a molar ratio of 2:1 in the brain, ovary and kidney in mice [39]. In addition to mice, both isoforms of ALG-2 are deposited in human, rat, chicken and frog NCBI protein databases under the following accession numbers: *Homo sapiens*, NP_037364.1 and NP_001254485.1; *Rattus norvegicus*, NP_001100922.1 and XP_006227846.1; *Gallus gallus*, XP_004935237.1 and XP_419075.2; *Xenopus tropicalis*, NP_001008004.1 and

XP_012820018.1. While ALG-2$^{\Delta GF122}$ has a lower Ca^{2+} affinity than that of ALG-2^{WT} [39], they have comparable Mg^{2+}-binding signatures [41], which contribute to dimer stability of ALG-2 proteins [42]. However, it has remained unclear how the expression ratio between isoforms is regulated and whether there are differences between cells in the expression ratio of the two isoforms at protein levels.

To elucidate the biological significance of the interaction between ALG-2 and Sec31A, we first identified the ALG-2 binding site in human Sec31A. An *in vitro* overlay assay using biotin-labeled ALG-2 revealed that a sequence (839-PPPGFIMHGNVNP-851) in the Pro-rich region of Sec31A is necessary and sufficient for binding to ALG-2 [36]. In vertebrates, there are two paralogous genes of Sec31: *SEC31A* and *SEC31B*. Sec31A is expressed abundantly and ubiquitously, whereas Sec31B is enriched particularly in the testis [43-45]. The two proteins share a high degree of amino acid similarity (92% in humans) at their N-terminal WD40 repeat domain. Both proteins have a Pro-rich region at their C-terminus with 70% amino acid similarity. Sec31B, however, has no sequence similar to the ALG-2 binding site of Sec31A. Although there is the possibility that ALG-2 could bind Sec31B through an unidentified motif sequence, the two Sec31 proteins might play distinct roles in the COPII-mediated secretory pathway by interacting with different proteins through their Pro-rich regions. The ALG-2 binding site of human Sec31A is only conserved in homologues of mammals and avians, not in other vertebrates. It is interesting, however, that *Saccharomyces cerevisiae* Pef1p, a unique PEF protein, binds Sec13p-Sec31p in the absence of Ca^{2+}, not in the presence of Ca^{2+} [46]. The opposite dependency of Ca^{2+} in interaction between Pef1p and Sec13p-Sec31p may be related to differences in Ca^{2+} requirement for the ER-to-Golgi transport between mammals and yeast.

We have co-crystallized a complex of an N-terminally deleted ALG-2 (des3-20ALG-2) with a Sec31A peptide (837-NPPPPGFIMHGN-848) in the presence of Zn^{2+} and refined the crystal structure at 2.4 Å resolution [38] (Figure 2B). Like the crystal structure of des3-20ALG-2/the ALIX peptide [37], two ALG-2 molecules are contained as a dimer in an asymmetric unit. The ALIX peptide binds hydrophobic pockets, named Pocket 1 and Pocket 2, whereas the Sec31A peptide is accommodated in Pocket 3 of each ALG-2 molecule, in which the N-terminal Pro residues (838-PPPP-841) form left-handed type II polyproline helices (Figure 2B). Pocket 3 consists of 19 residues that are present in EF1, EF2, EF3, EF4 and the loop connecting EF1 and EF2 [38]. Thus, two distinct ALG-2 binding motifs bind different hydrophobic pockets of ALG-2; therefore, the ALG-2 dimer has at least four binding surfaces and is proposed to function as a Ca^{2+}-dependent adaptor bridging two (or more) target proteins (Figure 2C).

3. ALG-2 stabilizes Sec31A to the ERES.

Biochemical reconstitution studies of purified yeast Sec13p-Sec31p complex demonstrated that this heterotetramer is recruited to the budding site by the active form of Sar1p and the Sec23p-Sec24p complex [30]. Structurally, Sec23p and Sec24p form a bow-tie-shaped dimer with a positively charged concave surface that can make contact with acidic membrane phospholipids to drive curvature [47]. The Sec13p-Sec31p heterotetramer forms an elongated rod-like structure [48] that assembles *in vitro* into polyhedral cages with flexibility in size to accommodate cargoes of various shapes and sizes [49]. Enzymatically, Sec23p functions as a GTPase-activating protein (GAP) for Sar1p [50], and the outer complex further stimulates GAP activity [51]. Hydrolysis of GTP by Sar1p is required for scission of vesicles [31]. It also promotes disassembly of coat components from the membrane [51]; however, loading of cargoes has been shown to delay the uncoating process [52,53]. A peptide sequence corresponding to residues

899-947 located in the middle of the Pro-rich region of yeast Sec31p was identified as an active fragment responsible for the GAP-stimulating activity [54]. The active fragment binds as an extended polypeptide across the composite surface of Sec23p and Sar1p in a crystal structure of the ternary complex. Four critical residues in the active fragment to exert the activity are conserved in vertebrate homologs of Sec31p, while the ALG-2-binding site in human Sec31A is not found in yeast Sec31p as mentioned above. Since the ALG-2-binding site is separated from the active fragment by about 140 amino acids [36], it was thought that Sec31A could bind simultaneously Sec23 and ALG-2 in the presence of Ca^{2+}.

Several independent studies demonstrated that stable localization of mammalian Sec31A to the ERES is required for Ca^{2+} and ALG-2. First, treatment of cells with a membrane-permeable Ca^{2+}-chelator, BAMTA-AM, resulted in removal of Sec31A from the ERES [16,17]. In cells suppressing ALG-2 expression, Sec31A proteins are mainly present in the cytosolic fraction [16]. Second, by using purified recombinant proteins, la Cour et al. found that recruitment of both an inner coat, Sec23-Sec24, and an outer coat, Sec13-Sec31A, to artificial liposomes is increased by the presence of ALG-2 and Ca^{2+} [55]. In addition, they demonstrated that Sec23A is sufficient for the ALG-2/Ca^{2+}-mediated recruitment of Sec13-Sec31A. Mechanistically, it is postulated that binding of ALG-2 to Sec31A endows Sec31A with the ability to robustly bind to Sec23A. Finally, the molecular dynamics of Sec31A on and off the ERES membrane in live cells has been demonstrated by fluorescence recovery after photobleaching (FRAP) analysis in cells expressing green fluorescent protein (GFP)-fused Sec31A. In this analysis, deletion of the ALG-2 binding region in Sec31A reduced the high-affinity population of Sec31A to the ERES to nearly half in HeLa cells, indicating that the region constitutes a high affinity binding site for the ERES [36]. In human fibrosarcoma HT1080 cells, there

is a population of Sec31A stably associated with the ERES membrane [56]. ALG-2 knockdown results in a reduction in the stable immobile population and an increase in the fast mobile population. Thus, ALG-2 stabilizes Sec31A to the ERES in HT1080 cells. It is interesting that expression of ALG-2^{WT}, but not expression of ALG-$2^{\Delta GF122}$, rescues the stable population of Sec31A, even though both isoforms of ALG-2 can interact with Sec31A [56]. The reason why only ALG-2^{WT} is related to stabilization of Sec31A to the ERES is described below.

4. Effects of ALG-2 knockdown on ER-to-Golgi transport.

To monitor transport of newly synthesized proteins from the ER, a temperature-sensitive variant of vesicular stomatitis virus glycoprotein (tsO45 VSV-G) is routinely used as a cargo protein. This variant is a type I transmembrane protein having an F204S substitution in the lumenal/extracellular domain [57]. At a restrictive temperature, the mutant protein is synthesized but misfolded, which allows it to remain in the ER. After shifting from a restrictive temperature to a permissive temperature, the protein is folded correctly to form a trimer and then the proteins are synchronously transported from the ER in a COPII-dependent fashion [58]. To investigate the role of ALG-2 in the ER-to-Golgi transport, the effects of ALG-2 knockdown on the tsO45 VSV-G transport have been monitored by three independent laboratories and inconsistent data were reported. In the first report by Yamasaki et al. [16], it was stated that there was no detectable difference in the rate of ER-to-Golgi transport of tsO45 VSV-G in the ALG-2-depleted HeLa cells. We established human fibrosarcoma HT1080 cells stably expressing GFP-fused tsO45 VSV-G and demonstrated that ALG-2 knockdown enhanced the rate of ER-to-Golgi transport of the GFP-fused protein, suggesting that ALG-2 functions as a negative regulator [56]. This is consistent with the results of an *in vitro* study by la Cour et al. [55] showing that Ca^{2+}-dependent binding of ALG-2 to Sec31A attenuates

budding of COPII. Recently, Sec31A has been reported to be modified by *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) on Ser964 in the Pro-rich region. Although the modification occurs outside the ALG-2 binding sequence, the *O*-GlcNAc-modified Sec31A has reduced ability to bind ALG-2 and therefore accelerates the ER-to-Golgi transport [59]. In contrast, Helm et al. [60] showed delay of the transport of tsO45 VSV-G by ALG-2 knockdown in normal rat kidney (NRK) cells. A later study from the same laboratory showed that knockdown of peflin, a heterodimer partner of ALG-2, enhances the rate of transport of tsO45 VSV-G by increased localization of ALG-2 to the ERES in NRK cells, supporting the role of ALG-2 as a positive regulator [61]. A possible explanation for the discrepancies in these results of ALG-2 knockdown could be the different cell lines used. ALG-2 may play both roles – a positive regulator and a negative regulator – in the ER-to-Golgi transport by selectively interacting with its various target proteins. Different expression levels of peflin and ERES-located target proteins of ALG-2 from cell to cell might provide an enormous combinatorial potential for generating apparent differences in the functional role of ALG-2 in ER-to-Golgi transport.

Large cargoes, such as procollagen fibers and chylomicron particles, are newly synthesized in the ER with sizes that are too large to be incorporated into typical COPII vesicles (60 – 90 nm), but their export from the ER is essentially dependent on COPII components. Missense mutations in the *SAR1B* gene have been shown to be associated with chylomicron retention disease (CMRD) [62], whereas an F382L mutation in the *SEC23A* gene causes cranio-lenticulo-sutural dysplasia (CLSD), an autosomal recessive syndrome characterized by facial dysmorphism, late-closing fontanels, cataracts, and skeletal defects [63,64]. Fibroblasts isolated from patients exhibit diffuse cytoplasmic mislocalization of Sec31 proteins and accumulation of procollagen I within the ER [63].

Structurally, F382 in Sec23A is located on the binding surface for the active fragment of Sec31A in its Pro-rich region as described above [54]. The F382L mutation causes failure to recruit the outer coat Sec13-Sec31A, thereby inhibiting vesicle formation [65]. Recently, molecular machineries required for export of large cargoes from the ER have been identified [66,67] (Figure 3). Transport and Golgi organization protein 1 (TANGO1, a product of the *MIA3* gene) and cutaneous T-Cell lymphoma-associated antigen 5 (cTAGE5) are transmembrane proteins localized to the ERES, where they form a complex [68]. The SH3 domain of TANGO1 faces into the lumen of the ER and directly binds procollagen VII [69] or indirectly binds other procollagens through interaction with HSP47, a collagen-specific molecular chaperone [70], to function as a procollagen receptor. cTAGE5 binds and concentrates Sec12 at the ERES, which is required for efficient export of procollagen VII [71]. Furthermore, recruitment of Sedlin, also known as TRAPPC2, by TANGO1 facilitates efficient GDP-GTP cycling of Sar1 [72]. Both TANGO1 and cTAGE5 bind Sec23-Sec24 via their Pro-rich regions in the cytoplasm, and this binding is thought to stall recruitment of the outer Sec13-Sec31 complex, thereby enabling the COPII vesicle to grow [69,73,74]. Another key molecule is Kelch-like protein 12 (KLHL12), a KLHL family protein that forms a CUL3-based ubiquitin ligase complex, referred to as $CUL3^{KHLH12}$ hereafter. Although KLHL12 interacts directly with Sec31A [75], McGourty et al. [76] reported that the ALG-2-peflin heterodimer functions as a Ca^{2+}-dependent adaptor to bridge $CUL3^{KLHL12}$ and Sec31A for facilitating mono (or multi)-ubiquitylation of Sec31A (Table 1). Recently, Gorur et al. [77] reported large Sec31A-positive structures that encapsulate procollagen I in human fibrosarcoma K16 cells, in which pro-α1(I) collagen and KLHL12 are overexpressed, as well as in human osteosarcoma SaOS-2 cells. In SaOS-2 cells, knockdown of either ALG-2 or peflin resulted in retention of procollagen I within

the ER [76]. Although such large Sec31A-positive structures have not been observed in human IMR-90 fibroblasts, which secrete high levels of procollagen I, we presented evidence showing that ALG-2 knockdown leads to a delayed exit of procollagen I from the ER in IMR-90 cells [21]. Thus, both ALG-2 and Sec31A contribute to efficient export of procollagen from the ER, but it remains uncertain whether large Sec31A-positive-structures mediated by KLHL12 are essential for procollagen secretion.

5. Adaptor functions of ALG-2 in the ERES.

The initial evidence for ALG-2 as a Ca^{2+}-dependent adaptor protein came from biochemical studies on endosomal complex required for transport (ESCRT) machineries and their associated proteins in mammalian cells. ALIX is the first identified protein that interacts Ca^{2+}-dependently with ALG-2 [22,23]. In the ESCRT-mediated membrane remodelling, ALIX binds and recruits ESCRT-III protein CHMP4 through its N-terminal BRO1 domain [78-80], which promotes the formation of a membrane-attached ESCRT-III filament [81,82]. The ESCRT-I protein TSG101 was reported to bind its binding sequence (717-PSAP-720 in human ALIX) in the C-terminal Pro-rich region [79,80]. Therefore, ALIX has been believed to link between ESCRT-I and ESCRT-III. However, in our biochemical analysis using HEK293T cells, the interaction between ALIX and TSG101 was detected only in the presence of Ca^{2+}. Then, we found that ALG-2 homodimer mediates the Ca^{2+}-dependent interaction between ALIX and TSG101 [83]. In the ESCRT-I components, VPS37B and VPS37C have shown to be stronger interacting proteins for ALG-2 than TSG101 [25]. The interaction of ALG-2 and these VPS37 isoforms contributes to stabilization of the Ca^{2+}-sensitive complex containing ALG-2, ALIX and ESCRT-I (Table 1).

Since ALG-2 predominantly distributes to the ERES in the cytoplasm, it was conceivable that ALG-2 could function as an adaptor, not only in the ESCRT system,

but also in the early secretory pathway. Screening of a bridging partner for Sec31A mediated by ALG-2 and searching for novel ALG-2-interacting proteins have unveiled pleiotropic roles of ALG-2 in the ER-to-Golgi transport through its adaptor functions to bridge between different combinations of target proteins as described below (Table 1) (Figure 3).

5.1. Sec31A and annexin A11

Annexins are a family of Ca^{2+}-dependent membrane lipid-binding proteins with a highly conserved C-terminal core domain comprising four or eight annexin repeats, each of which is about 70 amino acids in length [84]. In contrast, N-terminal head regions are variable in length and sequence. Annexin A11 has the longest N-terminal head of the 12 human annexins and provides binding sites for three EF-hand proteins, including calcyclin/S100A6 [85], sorcin [86], and ALG-2 [87,88]. The ALG-2-binding property of anneixn A11 is not similar to that of Sec31A: ALG-2^{WT} binds both proteins, but ALG-$2^{\Delta GF122}$ fails to bind annexin A11. As described above, a reduced stable population of Sec31A at the ERES in ALG-2 knockdown cells was rescued by overexpression of ALG-2^{WT}, but not by overexpression of ALG-$2^{\Delta GF122}$, raising the possibility that ALG^{WT} recruits a specific ALG-2-interacting protein(s). In pulldown and immunoprecipitation analyses, we identified annexin A11 as a bridging partner for Sec31A mediated by ALG-2 [56]. Although only a subpopulation of annexin A11 localizes to the Sec31A-positive ERES, knockdown of annexin A11 in HT1080 cells causes phenotypes similar to those of ALG-2: a decrease in the Sec31A population that is stably associated with the ERES, a scattering of juxtanuclear ERES to the cell periphery, and an acceleration of transport of VSV-G tsO45 from the ER to the Golgi apparatus. From these observations, we propose that ALG-2 and annexin A11 function in the same process by forming a ternary complex with Sec31A [56].

One important question is how annexin A11 regulates Sec31A dynamics at the ERES. Annexins bind negatively charged phospholipids in a calcium-dependent manner and can organize membrane domains that function as platforms for many membrane-related events including membrane trafficking and membrane-cytoskeleton linkages [84,89]. The specific protein-lipid interaction in combination with protein-protein interactions might enable annexin A11 to form a structural prerequisite for stable localization of the COPII coat component at the ERES. In an *in vitro* reconstitution experiment, it was found that acidic phospholipids, particularly phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-4-phosphate, are actually required for efficient recruitment of the COPII component to lipid bilayers in an active Sar1-dependent manner [30]. Annexin A11 assembly at the Sec31A-positive ERES might induce segregation of membrane lipids, with certain acidic phospholipids accumulating underneath annexin A11 clusters. Annexin A11 induces the formation of lens structures in the lipid bilayer *in vitro* [90]. Such an activity could be responsible for organization of membrane domains at the ERES, which may influence the post-translational modification status of Sec31A, including ubiquitylation [75], phosphorylation [91], and *O*-GlcNAcylation [59], and provide a link between calcium signaling and COPII function. Recently, ALS-associated annexin A11 mutations have been found to affect binding to S100A6/calcyclin [92]. Although there is no effect of annexin A11 mutations on binding to ALG-2, annexin A11 mutations have been shown to sequester the wild-type annexin A11 into aggregates with the mutant protein. Therefore, ALS-associated annexin A11 could behave in a dominant negative-manner, which may in turn cause perturbation in protein transport from the ER.

5.2. Polymerization of TFG

Proteins that interact Ca^{2+}-dependently with the ALG-2 homodimer and the ALG-2-peflin heterodimer continue to be identified. Although a protein that interacts Ca^{2+}-dependently with the heterodimer has not been found, we recently identified the *TRK-fused gene* (*TFG*) product as an uncharacterized protein interacting with the ALG-2 homodimer [20]. Since TFG had been shown to localize to the ERES [93], it was predicted that ALG-2 could bridge between Sec31A and TFG, similar to Sec31A and annexin A11. However, Sec31A was not detected in the immunoprecipitate of TFG in the presence of Ca^{2+}, even though overexpressed ALG-2 was efficiently precipitated with TFG [20]. In addition, TFG has been shown to bind Sec23A, resulting in outcompeting Sec31A for binding to Sec23A [94]. Thus, it is less likely that TFG associates and cooperates with Sec31A in regulation of transport from the ER. It is possible that ALG-2 has diverse functions through its mutually exclusive interaction with different proteins, including Sec31A, TFG and MISSL (as described below).

TFG is a ubiquitously expressed gene [95] that encodes a protein with a Phox and Bem1p (PB1) domain at the N-terminus, followed by a coiled-coil domain and a C-terminal Pro and Gln (P/Q)-rich region. The protein oligomerizes to form octamers via its N-terminal region including the PB1 and coiled-coil domains *in vitro*. The oligomerization capability is necessary for transforming activity of the *TRK-T3* gene product, which is expressed as a fusion protein of an N-terminal region of TFG and a tyrosine-kinase domain of NTRK1, a receptor for nerve growth factor [96]. A mutant protein of TFG in autosomal recessive hereditary spastic paraplegia (SPG57), in which Arg106 in the coiled-coil region is replaced by Cys, p.R106C), causes a defect in normal oligomerization [97]. Interestingly, the R106C mutant is defective in ability to bind to ALG-2 [20], suggesting that the incapability of TFG for self-assembly and for

binding to ALG-2 can lead to the development of neurodegenerative disorders. Furthermore, TFG has an ABM2-like sequence in its C-terminal P/Q-rich region that is necessary for efficient binding to ALG-2 [20,98].

Hanna et al. [94,99] recently proposed a phase separation model for regulation of TFG in the anterograde transport of COPII-coated vesicles, in which TFG at an elevated concentration potentially undergoes liquid-liquid phase separation at the interface between the ERES and ER-Golgi intermediate compartment (ERGIC). Actually, they showed that the C-terminal P/Q-rich region of TFG tethers COPII vesicles via interaction of the region with an inner coat component, Sec23A or Sec23B [94]. Double depletion of Sec23A and Sec23B causes TFG to become spread diffusely throughout the cytoplasm and to fail to accumulate near the Sec16A-positive ERES [94]. Although depletion of ALG-2 has no effect on the distribution pattern of TFG, overexpression of ALG-2 and Ca^{2+}-mobilization lead to elevated levels of TFG protein at the ERES [20]. TFG oligomers have been shown to self-associate into larger polymers *in vitro* [100]. The polymerization of TFG oligomers is promoted by ALG-2 in the presence of Ca^{2+} [20]. Therefore, in close juxtaposition of ERGIC, ALG-2 may function as a Ca^{2+} sensor to concentrate TFG, leading to its phase separation (Figure 3). The ALG-2-TFG complex might regulate dissociation of the outer coat and restriction of diffusion of COPII vesicles coated with the inner coat, leading to disassembly of the inner coat to fuse homotypically or heterotypically with ERGIC membranes.

5.3. MISSL and MAP1B

MAPK1-interacting and spindle-stabilizing (MISS)-like (MISSL) is a Pro-rich protein of 245 amino acids containing 83 prolines (33.8%) and two PYP(*X*)nYP motifs that are similar to the ALG-2 binding region in ALIX. Results of our binding analysis suggested that ALG-2 recognizes multiple regions in MISSL [21]. MISSL was named

after the homology to mouse MISS protein [101], but the C-terminal functional region in MISS, including the MAPK-docking site, is lacking in MISSL. When MISSL was identified as an ALG-2-interacting protein with ABM-1 motifs [98], it was initially an uncharacterized protein with no known function. Live-cell imaging of GFP-fused MISSL (GFP-MISSL) and indirect immunofluorescence analyses revealed translocation of MISSL from the cytosol to the ERES in response to Ca^{2+}-mobilization [21]. ALG-2 is necessary for the ERES translocation of MISSL. As in the case of TFG, MISSL did not associate with Sec31A in our binding experiments. Knockdown of MISSL resulted in dispersed localization of Sec31A and Sec16A (ERES markers), ERGIC-53 (an ERGIC marker) and GM130 (a *cis*-Golgi marker). These results indicate that MISSL is recruited to the ERES and has a role in proper positioning of organelles of the early secretory pathway [21].

To understand the molecular mechanism underlying MISSL function in the early secretory pathway, we searched for MISSL-interacting proteins using HeLa cells stably expressing GFP-MISSL and we identified microtubule-associated protein 1B (MAP1B) in the immunoprecipitate of GFP-MISSL from the cell lysate [21]. Further analyses revealed that ALG-2 directly interacts with MAP1B and that ALG-2 mediates indirect association between MISSL and MAP1B in a Ca^{2+}-dependent fashion [21,102]. When secretion of secreted alkaline phosphatase (SEAP) as a model protein from HeLa cells stably expressing SEAP [91] was monitored, it was found that knockdown of either MISSL or ALG-2 resulted in a reduction of the secretion of SEAP [21]. Although knockdown of MAP1B has little effect on secretion of SEAP, it reverts the reduced secretion of SEAP by knockdown of ALG-2 or MISSL. Taken together with previous findings that MAP1B outcompetes dynein for binding to microtubules [103] and that association of inner coat components of COPII with dynactin, an essential cofactor for

dynein, appears to be required for directional transport from the ER [104], it is possible that MAP1B is sequestered by MISSL and ALG-2 away from microtubules (Figure 3), thereby allowing dynein/dynactin to bind to microtubules to promote protein transport from the ER to the Golgi. Since the expression level of MAP1B in cells affects the amount of ALG-2 in the ERES [102], it would be interesting to investigate the involvement of MISSL, ALG-2 and MAP1B in protein transport in neurons, in which MAP1B is highly expressed.

Although ALG-2 directly binds to MAP1B, no sequence similar to ABM-1 and ABM-2 was found in MAP1B. We recently narrowed down the region of mouse MAP1B that is responsible for ALG-2 binding to 36 amino acids, in which the 1825-PYGPR-1829 motif is necessary [102]. In the Catalogue of Somatic Mutations in Cancer (COSMIC) database, there are two mutations, P1829L (mouse P1825L) and R1833G (mouse R1829G), in human MAP1B. These cancer-associated mutations result in an impairment of binding to ALG-2 [102]. Since MAP1B knockout HeLa cells display enhanced recruitment of ALG-2 to the Sec31A-positive ERES [102], the loss of binding of MAP1B to ALG-2 might cause accumulation of ALG-2 at the ERES, which might contribute to a high incidence as well as rapid progression and poor survival in cancer patients.

6. Conclusions and future directions

ALG-2 binds multiple target proteins in response to Ca^{2+}-mobilization and acts as a multifunctional adaptor by utilizing distinct combinations of these targets to deliver different outputs in the early secretory pathway, including intracellular distribution of the ERES, budding of COPII vesicles, and directional transport along microtubules. It will be important to explore in more detail the extent to which ALG-2 bridges distinct combinations of target proteins in different cells. Further studies will also be needed to

elucidate structural features in ALG-2 that govern the specificity of bridging partners. Recently, ALG-2 has been reported to work in repair of the plasma membrane and lysosomal membrane by sensing Ca^{2+} leaked from the site of injury [105,106]. If multiple membrane sites are damaged in severe cases, most of ALG-2 will be recruited to injury sites and the amount of ALG-2 in the ERES will be reduced, thereby affecting protein transport from the ER. Thus, it will be intriguing to see whether ALG-2 acts as a signalling molecule in response to local elevation of intracellular Ca^{2+}, which contributes to communication between the ERES and other spatially separated organelles.

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Disclosure statement

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Table 1. Combinations of proteins bridged by ALG-2 homodimer and ALG-2-peflin heterodimer.

Figure 1. ERES localization of ALG-2. (A) Schematic representation of COPII vesicle formation in the ERES. (B) Representative micrograph showing the ERES localization of ALG-2. HeLa cells were fixed with 4% paraformaldehyde in phosphate buffer, permeabilized with 0.1 % Triton X-100, and then triple stained with antibodies against an ER marker, calnexin (a rabbit polyclonal antibody, Enzo Life Sciences), an ERES marker, Sec31A (a mouse monoclonal antibody, BD Biosciences) and ALG-2 (a goat polyclonal antibody) [98]. The images are superimpositions of serial optical sections taken by a confocal laser-scanning microscope through the whole thickness of the cell. The right panel shows a merged image with the pseudocolors as follows: green (ALG-2) and magenta (Sec31A).

Figure 2. Structures of ALG-2. (A) Schematic representation of primary structure of ALG-2. ALG-2 has an N-terminal extension rich in Ala, Gly and Pro, and a C-terminal penta-EF-hand (PEF) domain. Calcium ions (Ca^{2+}) bind to EF1 and EF3 under physiological conditions. ALG-$2^{\Delta GF122}$ is an alternatively spliced variant of ALG-2 lacking two amino acids, Gly121 and Phe122. (B) Co-crystal structure of the complex between ALG-2 and Sec31A peptide (PDB code, 3WXA). Chain A and B (ALG-2 molecule A and B) are shown by cartoon (green, EF1 and EF3; dark green, EF5, dark grey, EF2 and EF4) and surface representation (blue, pocket 1; light blue, pocket 2; green, pocket 3), respectively, using PyMOL software. Chain C and D (Sec31A peptides) are shown by stick in magenta. Grey spheres indicate zinc ions (Zn^{2+}). (C) Schematic representation of the ALG-2 dimer-mediated physical association between two target proteins. The ALG-2 dimer couples two target proteins in a Ca^{2+}-dependent manner.

Figure 3. Schematic representation of a potential model for adaptor functions of ALG-2 in the ERES. The ALG-2 homodimer couples following combinations of target proteins: 1) Sec31A and annexin A11, 2) two TFG octamers, and 3) MISSL and MAP1B, whereas 4) the ALG-2-peflin heterodimer bridges between Sec31A and $CUL3^{KLHL12}$ in a Ca^{2+}-dependent manner.

Table 1. Combinations of proteins bridged by ALG-2 homodimer and ALG-2-peflin heterodimer.

Adaptor	Target A	Target B	Function	Refs.
ALG-2 homodimer	ALIX	ESCRT-I	Membrane repair	[25,83,105,106]
ALG-2 homodimer	Sec31A	Annexin A11	Stabilization of Sec31A at the ERES	[56]
ALG-2 homodimer	TFG	TFG	Polymerization of TFG	[20]
ALG-2 homodimer	MISSL	MAP1B	Promotion of anterograde transport along microtubules	[21]
ALG-2-peflin heterodimer	Sec31A (ALG-2)	KLHL12 (peflin)	Monoubiquitylation of Sec31A	[76]

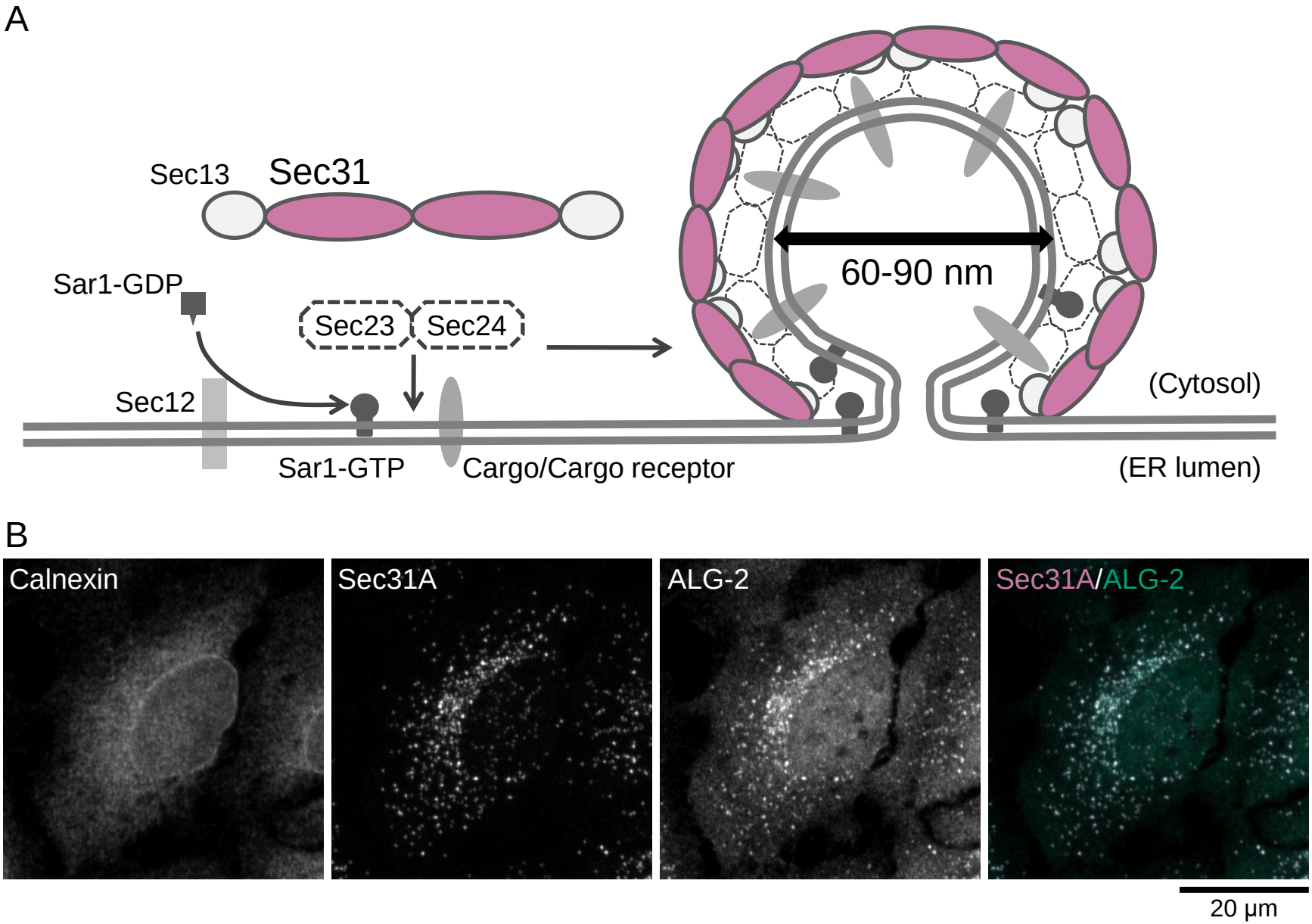
A
Sec13
Sec31
Sar1-GDP
Sec23
Sec24
Sec12
60-90 nm
(Cytosol)
Sar1-GTP
Cargo/Cargo receptor
(ER lumen)
B
Calnexin
Sec31A
ALG-2
Sec31A/ALG-2
20 μm

Figure 1

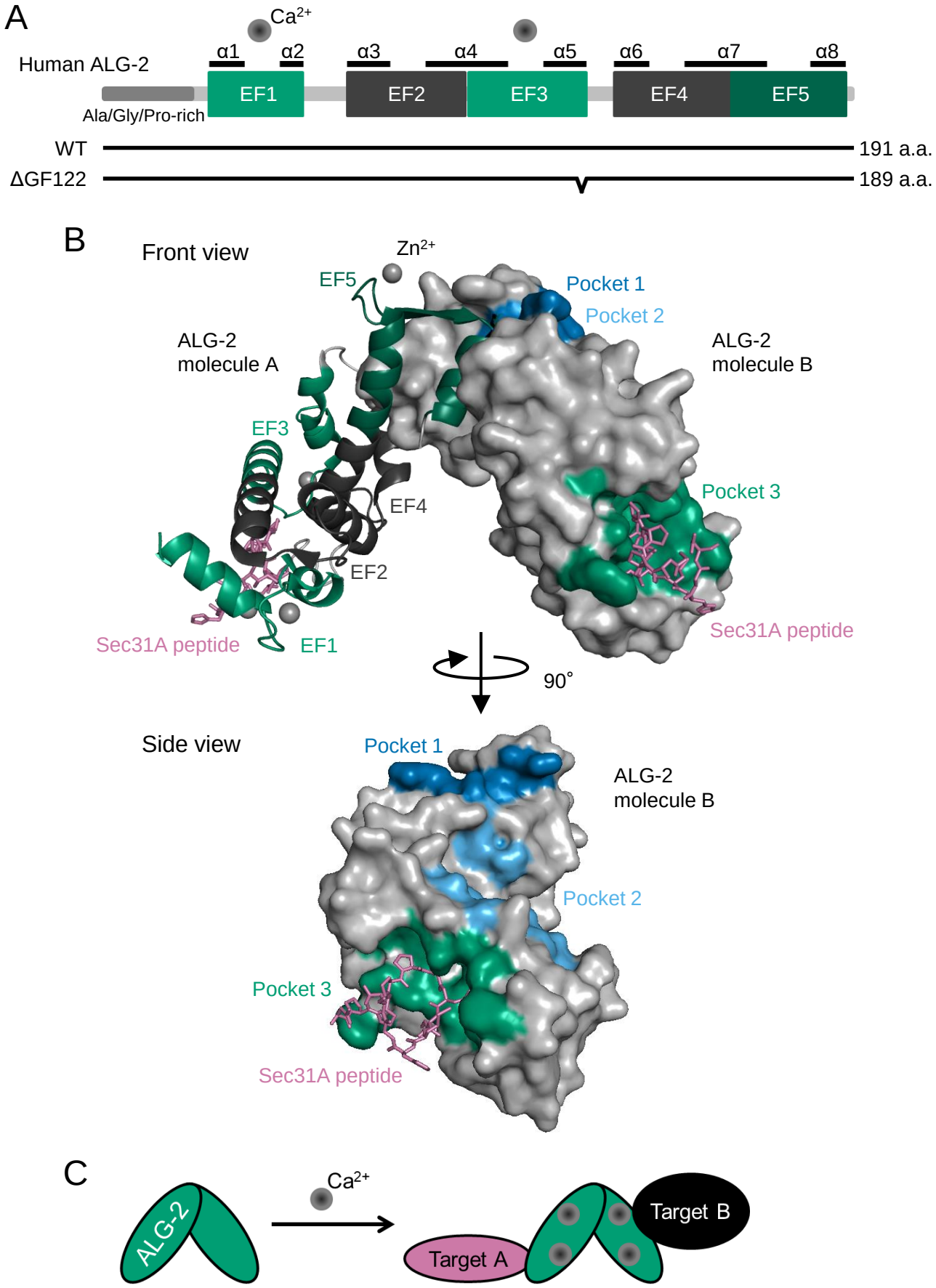
A
Ca2+
α1
α2
α3
α4
α5
α6
α7
α8
Human ALG-2
EF1
EF2
EF3
EF4
EF5
Ala/Gly/Pro-rich
WT
191 a.a.
ΔGF122
189 a.a.
B
Front view
Zn2+
EF5
Pocket 1
Pocket 2
ALG-2
molecule A
ALG-2
molecule B
EF3
Pocket 3
EF4
EF2
Sec31A peptide
EF1
Sec31A peptide
90˚
Side view
Pocket 1
ALG-2
molecule B
Pocket 2
Pocket 3
Sec31A peptide
C
ALG-2
Ca2+
Target B
Target A

Figure 2

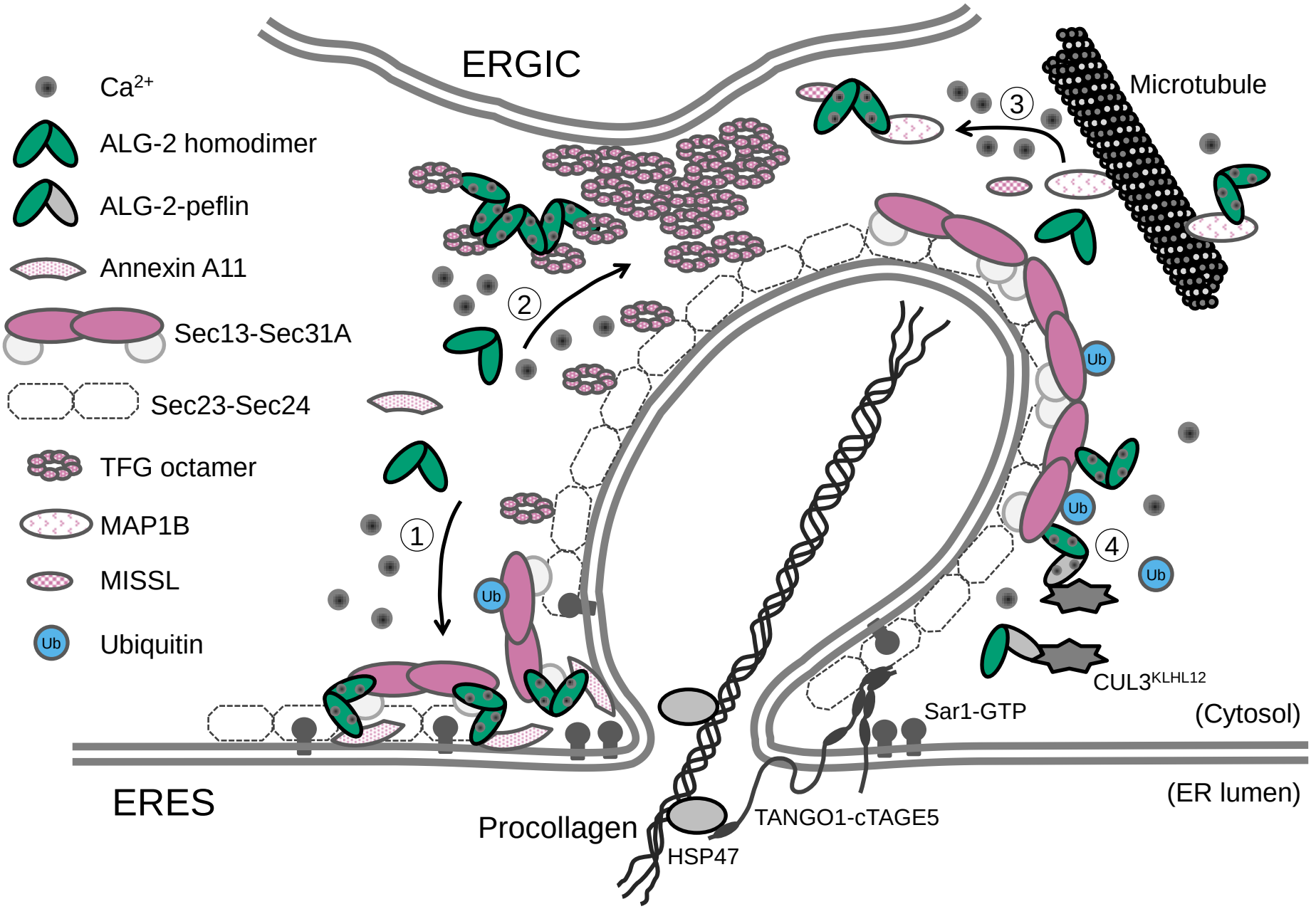
ERGIC
Microtubule
Ca2+
ALG-2 homodimer
ALG-2-peflin
Annexin A11
Sec13-Sec31A
Sec23-Sec24
TFG octamer
MAP1B
MISSL
Ubiquitin
1
2
3
4
Ub
CUL3KLHL12
Sar1-GTP
(Cytosol)
(ER lumen)
ERES
Procollagen
TANGO1-cTAGE5
HSP47

Figure 3