Running Title: ALG-2 and calpain-7

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Review

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Structures and functions of penta-EF-hand calcium-binding proteins and their interacting partners: Enigmatic relationships between ALG-2 and calpain-7 *

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Abstract

The penta-EF-hand (PEF) protein family includes ALG-2 (gene name, *PDCD6*) and its paralogs as well as classical calpain family members. ALG-2 is a prototypic PEF protein that is widely distributed in eukaryotes and interacts with a variety of proteins in a Ca^{2+}-dependent manner. Mammalian ALG-2 and its interacting partners have various modulatory roles including roles in cell death, signal transduction, membrane repair, ER-to-Golgi vesicular transport and RNA processing. Some ALG-2-interacting proteins are key factors that function in the endosomal sorting complex required for transport (ESCRT) system. On the other hand, mammalian calpain-7 (CAPN7) lacks the PEF domain but contains two microtubule-interacting and trafficking (MIT) domains in tandem. CAPN7 interacts with a subset of ESCRT-III proteins through the MIT domains and regulates EGF receptor downregulation. Structures and functions of ALG-2 and those of its interacting partners as well as relationships with the calpain family are reviewed in this article.

Keyword: calcium-binding protein, calpain, ESCRT, penta-EF-hand, protease

(Introduction)

Calcium is a major essential mineral for animals and taken into the body from foods and milk. The total amount of calcium in the adult human body is as high as 1000-1200 grams. Over 99% of calcium is present in the form of hydroxyapatite in bones and teeth, which provides strength of the hard tissues. The remaining calcium is present as cations in blood and body fluids inside and outside cells, and these cations play important roles in mediating vascular contraction, vasodilatation, muscle function, intracellular signaling, nerve transmission and hormonal secretion [1]. Calcium ions (Ca^{2+}) are buffered with small organic compounds and with a variety of low-affinity Ca^{2+}-binding proteins. Concentrations of Ca^{2+} in milk greatly differ among mammal species, but they

are closely correlated with concentrations of casein, the major acidic milk protein [2]. While the concentration of Ca^{2+} in extracellular fluid is 1-2 mM, it is maintained at an extremely low level (as low as 100 nM or less) in the cytosol. Thus, there is a more than 10,000-fold difference between concentrations of Ca^{2+} outside and inside the cells. This feature is in marked contrast to Mg^{2+}, the concentrations which are similar at the millimolar (mM) order on both sides of the cell membrane [3].

Biological effects of Ca^{2+} are mediated through various types of Ca^{2+}-binding proteins, which have structural motifs such as an EF-hand (helix-loop-helix), a C2 domain, an endonexin fold (annexin domain) and acidic clusters [4]. Binding of Ca^{2+} to these motifs stabilizes protein structures, induces conformational changes to activate enzymatic activities, triggers interaction with target factors (proteins and phospholipids), and keeps the free Ca^{2+} concentrations at fixed levels by buffering actions. The EF-hand proteins are the most extensively studied Ca^{2+}-binding proteins [5]. Calmodulin (CaM) is characterized by four EF-hands with sterically separated N- and C-terminal lobes, which contain two paired EF-hands, respectively. CaM is the best known signal transducer that plays important roles in eukaryotic cells and has been well reviewed in the literature [6-8]. Calpain was originally discovered as a Ca^{2+}-dependent cysteine protease present in animal tissues, and it was found to possess a CaM-like Ca^{2+}-binding domain in each large subunit and small subunit based on the primary structure [9,10]. However, 3D-structure analysis of the Ca^{2+}-binding region revealed a novel domain structure with five EF-hand modules [11,12], which was later named penta-EF-hand (PEF) [13]. The PEF domain is also found in non-calpain proteins including ALG-2 [14], which is widely distributed in eukaryotes and regarded as a prototypic penta-EF-hand protein [15]. On the other hand, structurally related homologs of calpain that lack the PEF domain but retain the protease domain (designated non-classical calpains or atypical calpains) have been identified in a wide range of eukaryotes [16-19]. Amino acid sequences related to the cysteine protease domain of calpain have also been reported in bacteria [17,18]. In this review, the author focuses on physiological

relationships between ALG-2 and the non-classical calpain designated calpain-7 (CAPN7) from the viewpoint of interacting partners.

Partial overlap of the PEF family and the calpain family

Ubiquitously expressed conventional calpains designated μ-calpain and m-calpain (~110 kDa) are heterodimers of the large subunit (~80 kDa, CAPN1 or CAPN2) and the common regulatory small subunit (~30 kDa, CAPNS1). The required Ca^{2+} concentrations for protease activation in *in vitro* assays are at micromolar (μM) and millimolar (mM) levels for μ-calpain and m-calpain, respectively [20]. Calcium sensitivity increases by binding to phospholipids, N-terminal processing of subunits and phosphorylation [21-23]. Skeletal muscle-specific p94/calpain-3 (CAPN3) requires Na^{+} instead of Ca^{2+} for its rapid and exhaustive auto-degradation [24], suggesting a structural role of the PEF domain in CAPN3. Calpains have a cysteine protease core domain (CysPc; divided into the two subdomains PC1 and PC2), a calpain type β-sandwich domain (CBSW) (previously called domain III or C2-like domain) and a PEF domain (Figure 1, calpain family) [17,19]. In the human genome, fifteen genes encode the calpain protease domain-containing sequences designated CAPN1-CAPN16, whereas CAPN4, encoding the non-catalytic small subunit, has been renamed and replaced with CAPNS1. While nine calpain paralogs (CAPN1, 2, 3, 8, 9, 11, 12, 13, and 14) contain PEF domains and are called classical or typical calpains, six calpain paralogs lack PEF domains and are called non-classical or atypical calpains [19]. CAPN7 contains additional domains in place of the PEF domain: two microtubule-interacting and trafficking (MIT) domains in tandem and an additional CBSW domain. CAPN16 (also called androglobin; expressed in mammalian testes) has a CAPN7-like protease domain without readily recognizable catalytic His/Asn residues in the corresponding PC2-like subdomain (PC2') [25].

In addition to the two calpain small subunit genes (*CAPNS1* and intron-less *CAPNS2*), four genes for PEF proteins (22~30 kDa) that lack catalytic domains are

known (Figure 1, PEF protein family). While ALG-2 (gene name, *PDCD6*) has the shortest non-PEF N-terminal sequence (23 amino acid residues rich in Pro/Gly/Ala), the closest paralog named peflin (gene name, *PEF1*) has the longest non-PEF sequence with 113 amino acids containing nine repeats of a nonapeptide (A/PPGGPYGGP) sequence [15,26]. Sorcin and grancalcin also contain Gly/Pro-rich sequences at the N-terminal regions [15].

Evolutionary features of ALG-2 and CAPN7

Sequence comparison of the PEF proteins has revealed that the calpain PEF domains are evolutionarily closer to the sorcin/grancalcin subfamily than to the ALG-2/peflin subfamily [15]. As shown in Table 1, ALG-2 and its orthologs are the most widely distributed PEF proteins in eukaryotes ranging from protists to mammals [27]. PEF domain-containing calpains and other PEF proteins are found in higher animals, but their presence depends on classes in other eukaryotes. Interestingly, the fly does not have genes for CAPN7 (ortholog of fungal *PalB* and yeast *Rim13*) nor those for calpain small subunits but possesses a PEF-containing classical calpain. Protists and plants have other types of non-classical calpain homologs [17,18].

ALG-2-interacting proteins and binding motifs

Although ALG-2 was originally identified as a pro-apoptotic factor (*apoptosis-linked gene 2*) [14], roles of ALG-2 in cell death remain unclear. ALG-2 interacts in a Ca^{2+}-dependent manner with a variety of proteins that function in (i) the endosomal sorting complex required for transport (ESCRT) system, (ii) regulation of endoplasmic reticulum (ER)-to-Golgi vesicular transport, (iii) RNA processing, (iv) protein phosphorylation, and (v) other cellular processes (Figure 2) (See Ref. 28 therein and Refs. 29-33 for new reports.). Binding of these proteins with ALG-2 may indirectly promote the cell death pathway at multiple steps. Importantly, the reported ALG-2-interacting partners are mutually physically associated or in close contact at

specific subcellular localizations, e.g., ESCRT system components (ALIX, HD-PTP, TSG101, VPS37B/C, IST1) [34-38], ER exit sites (Sec31A, annexin A11, and TFG) [30,39], and the ER-to-Golgi pathway (MISSL and MAP1B) [31,32].

In vitro binding assays of various deletion and amino acid-substituted mutants enabled narrowing down of the major binding regions for ALG-2: ALIX [40,41], TSG101 [35], Scotin [42], PLSCR3 [43], MCOLN1 (mucolipin-1) [44], Sec31A [45], PATL1 [46], IST1 [38], CHERP [47], MAP1B [32], and SARAF [33,48]. Not all but the majority of ALG-2-interacting proteins possess Pro-rich regions (PRRs) (Figure 2). Comparison of the multiple predicted ALG-2-binding sequences has revealed at least three types of ALG-2-binding motifs (ABMs) that are rich in prolines (Figure 3(a)). X-ray crystal structure analyses of human recombinant ALG-2 proteins in complex with an ALIX peptide [49] and with a Sec31A peptide [48] further defined the motifs and clarified the nature of interactions. Unlike CaM, ALG-2 does not drastically change its conformation to bind its target [49]. Interestingly, ALIX and Sec31A bind ALG-2 at different hydrophobic pockets (Figure 3(b,c)). Although binding sites in some proteins have not been clarified yet, incomplete resemblance to the type 1 ABM (ABM-1) sequence (annexin A7, annexin A11, TSG101, VPS37B, VPS37C, MISSL and Scotin) or to the ABM-2 sequence (PLSCR3 and TFG) suggests that the binding strength of a suboptimal motif is augmented by surrounding sequences [48] or by oligomerization/polymerization [30]. ALIX and IST1 contain Met-Pro (MP) repeat sequences, designated ABM-3. While the ABM-3 sequence in IST1 is essential for interaction with ALG-2 [38], this short motif sequence found in ALIX may be subsidiary to the main ABM-1 sequence. The ALG-2 binding sites described above are all located in the predicted intrinsically disordered regions, which have advantages in protein-protein interactions [50,51]. However, ALG-2 also binds the structurally stable region of HEBP2 [29,52], indicating the presence of diversity in the binding modes.

Calpastatin, an endogenous calpain inhibitor protein, has four repeated inhibitory domains [53-55]. Each inhibitory domain is comprised of three conserved regions,

among which region B is the inhibitory center [56,57], and both regions A and C augment inhibitory potency (Figure 3(d)) [58,59]. Regions A and C are similar in amino acid sequences (Figure 3(e)) but specifically bind PEF domains of the classical calpain large subunit (L-PEF) and the small subunit (S-PEF), respectively, in a Ca^{2+}-dependent manner [57,60]. The peptides of regions A and C form acidic amphiphilic helices (Figure 3(f)) [61-63]. They bind each calpain PEF domain in the hydrophobic cavity similarly found as Pocket 3 in ALG-2. However, the modes of interactions analyzed in the 3D structures of complexes of respective peptides with ALG-2, sorcin and calpain are different and seem to have diverged during evolution of the PEF proteins [64].

ALG-2 functions as a Ca^{2+}-dependent adaptor protein

PEF proteins form homodimers or heterodimers by pairing the fifth EF-hands (EF5s) as revealed by X-ray crystal structure analyses [11,12,65-69]. The presence of one or two ligand binding sites per one monomeric PEF molecule suggests a di- or multi-valent mode of interactions for dimeric PEF proteins. The Ca^{2+}-dependent adaptor function of ALG-2 was first demonstrated for ALG-2 to bridge ALIX and TSG101 [70]. Results of *in vitro* multi-complex formation experiments using either mammalian cell expression constructs or recombinant proteins of ALG-2, ALIX, and ESCRT-I complex (TSG101, VPS28, MVB12A, and one of the VPS37 isoforms A/B/C/D) further indicated that VPS37 isoforms with no or different ALG-2-binding capacities differentially modulate the ternary complex formation of ALG-2, ALIX, and ESCRT-I [37]. Since ALIX contains a PSAP motif and weakly interacts with TSG101 [71], a role of ALG-2 appears to be stabilization of the complex between ALIX and ESCRT-I [28,72]. In the ESCRT system, ESCRT-III plays a key role in membrane deformation by spiral polymerization of CHMP (CHarged Multivesicular body Protein) subunit proteins on the membrane [73]. In mammals, twelve CHMP paralogs are known (CHMP1A/1B/2A/2B/3/4A/4B/4C/5/6/7 and IST1), among which CHMP4s (yeast Snf7 orthologs) are major subunits of ESCRT-III and interact with the Bro1 domain of ALIX

[34,73]. Thus, ALIX is recognized as an ESCRT-III-recruiting adaptor and bridges ESCRT-I and -III, which is essential for membrane deformation of HIV-1 budding, abscission of cells at the final stage of cytokinesis, and membrane repair [73,74]. For multivesicular body (MVB) biogenesis, ALIX and the ALIX paralog HD-PTP differentially activate ESCRT-III depending on cargoes [75]. A complex of Hrs and STAM1/2 recruits ESCRT-I in MVB biogenesis and is sometimes called ESCRT-0 [73]. However, upstream factors that recruit ESCRT-I are different among the ESCRT systems employed in cellular functions: i.e., Gag in retrovirus budding, CEP55 in cytokinesis, and Arrestin-Domain Containing Protein 1 (ARRDC1) in extracellular release of microvesicles [73,74,76-78]. ALG-2 functions as an upstream factor of ESCRT-III or an initiator in membrane repair upon a plasma membrane lesion, where ESCRT-0 (Hrs-STAM1/2), ESCRT-I except for TSG101, and ESCRT-II are not recruited [79]. Injury of the plasma membrane causes Ca^{2+} flux into the cytoplasm and recruitment of the Ca^{2+}-dependent phospholipid binding protein annexin A7, which forms a complex with ALG-2 [80,81], to facilitate proper recruitment of ALIX to the damaged membrane [82]. The ESCRT system is also involved in lysosomal membrane repair and precedes engulfment of unrepairable lysosomes by the autophagic membrane (lysophagy) [83,84]. Requirement of ALG-2 remains to be established in this case.

The Ca^{2+}-dependent adaptor function of ALG-2 has also been demonstrated in proteins involved in regulation of the ER-to-Golgi vesicular transport system [85]: Sec31A-annexin A11 complex formation [39], MISSL-MAP1B complex formation [31,32], and TFG polymerization [30]. Peflin acts as a negative regulator in this transport system [86]. However, positive or negative effects of PEF proteins may depend on cargoes that are to be analyzed. An ALG-2/peflin heterodimeric complex plays a role as a co-adaptor to bridge ubiquitin ligase CUL3 and its substrate adaptor protein KLHL12 for mono-ubiquitination of Sec31A to form larger coat protein complex II (COPII) coats and promote collagen secretion from the ER exit sites [87]. CaM, which has two independently-folded Ca^{2+} binding N- and C-lobes that interact

differentially with target proteins, has also recently been reported to act as a Ca^{2+}-dependent adaptor [88]. It remains unknown whether the presence of two separated binding sites in one ALG-2 molecule for different types of binding motifs (Pocket 1/2 and Pocket 3 for ABM-1 and ABM-2, respectively) enables monomeric ALG-2 to serve as a link to target proteins.

Activation of calpain-7 in the ESCRT system

An oligomeric complex of VPS4 (occasionally called ESCRT-IV), a member of meiotic clade AAA type ATPases (ATPases associated with diverse cellular activities), disassembles ESCRT-III complexes at the last stage of membrane remodeling and abscission [73,89]. The microtubule-interacting and trafficking (MIT) domain of VPS4 binds the C-terminal regions of ESCRT-III proteins by recognizing MIT-interacting motifs (MIMs) [73,89]. Human CAPN7, a non-classical calpain, lacks the PEF domain but possesses a tandem repeat of the MIT domains located at the N-terminus (Figure 1). CAPN7 physically interacts with a subset of CHMP proteins (ESCRT-III subunit paralogs) through the MIT domains such as CHMP1A, CHMP1B, CHMP4B and IST1 [90,91]. Orthologs of CAPN7 in yeast (Rim13) and *Aspergillus* (PalB) have been shown to be activated on the ESCRT-III platform and to play essential roles in alkaline adaptation by limited proteolysis of transcription factors [92]. These eukaryotic microbial CAPN7 orthologs have different preferences for interactions with ESCRT-III proteins, i.e., Rim13 to Snf7 (CHMP4) and PalB to Vps24 (CHMP3) [93,94]. Rim13 does not contain any discernable MIT domain, and PalB has only one MIT domain in contrast to two MIT domains in mammalian CAPN7. The variation or lack of the MIT domains might have allowed the protease activation mechanism to evolve differently in the detailed process.

Although physiological substrates have not been identified yet, human CAPN7 has been shown to possess both catalytic activities for autolysis of the monomeric green fluorescent protein (mGFP)-fused protease and those for processing of artificially

designed non-physiological substrates [91,95]. These proteolytic activities were abrogated by substitution of the active site Cys-290 with Ser (C290S) and were enhanced with different degrees of efficiency by overexpression of ESCRT proteins in HEK293 cells [95]. IST1 activated CAPN7 in an *in vitro* proteolytic assay [91] but showed little effects in transfected cells probably due to a sufficient basal level of the protein [95]. CAPN7 may form a ternary complex with IST1 and with CHMP1B [96]. Fluorescence microscopic analysis of autolysis-defective mGFP-CAPN7^{C290S} revealed time-dependent transient accumulation of CAPN7 at epidermal growth factor (EGF) receptor (EGFR)-positive endosomes after stimulation of HeLa cells with EGF [97]. Knockdown of IST1 by the RNA interference method decreased the rate of subcellular localization of CAPN7 in the EGFR-positive endosomes. Knockdown of CAPN7 caused a decrease in the rate of EGF-stimulated EGFR degradation in HeLa cells. Similarly, mouse embryonic fibroblast (MEF) cells derived from CAPN7 knockout (*Capn7*$^{-/-}$) mice showed a reduced rate of EGFR degradation compared with that of wild-type MEF cells [97]. The rate of EGFR degradation was recovered by exogenous expression of wild-type CAPN7 but not by expression of the CAPN7^{C290S} mutant. These experimental data clearly indicate that CAPN7 plays roles in degradation of endocytosed EGFR. However, the physiological substrate of CAPN7 has not been identified yet. CAPN7 may accelerate multivesicular body (MVB) sorting by cleaving unknown factors that are involved in the pathway from endocytosis to lysosomal degradation (Figure 4).

Perspective

ALIX is a scaffold protein that binds and recruits multiple proteins by using different domains: the Bro1 domain (CHMP4), the V domain (LYPXnL motif-containing proteins including HIV-1 Gag p6, PAR1, and Syntenin), and the Pro-rich region (TSG101, ALG-2, endophilin, and CIN85) (See Refs. 28,76,98, and references therein.). Yeast and fungal substrates of CAPN7 orthologs (yeast Rim13, Rim101; fungal PalB, PacC) bind

the V domain of ALIX homologs (yeast Rim20; fungal PalA) that recognize the YPXL/I motif [99]. During evolution of eukaryotes, a prototype of CAPN7 might have acquired a primitive MIT domain to localize the protease by interaction with ESCRT-III proteins and to increase efficiency of encountering substrates that are recruited by ESCRT-III-interacting adaptor proteins (ALIX homologs). Knockdown of ALG-2 retards EGFR degradation, suggesting inhibition of ALIX activation by ALG-2 [100]. Interestingly, IST1 has a Met-Pro repeat sequence that serves as a binding site for ALG-2 [38]. Moreover, ALG-2 interacts with ESCRT-I proteins (TSG101, VPS37B and VPS37C) [35,37,70]. Thus, ALG-2 seems to play diverse modulatory roles in the MVB sorting pathway including the ESCRT system and CAPN7. It is intriguing to speculate that ALG-2 interacts with CAPN7 substrates directly or indirectly through either the V domain of ALIX (or HD-PTP) or IST1 in a Ca^{2+}-dependent fashion and recruits them to the ESCRT platform on the endosomal membrane. Moreover, classical calpains might have evolved by acquiring PEF domains to change the Ca^{2+}-dependent activation locality from the ESCRT platform to the phospholipid membranes. The author hopes that these hypothetical ideas will be verified in the future by finding endogenous substrates of CAPN7 and elucidating its proteolytic activation mechanism.

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Figure legends

Figure 1. Relationship between the penta-EF-hand (PEF) protein family and the calpain family in mammals. Classical (typical) calpain sequences contain the PEF domain and the calpain type β-sandwich domain (CBSW) in addition to the cysteine protease core domain (CysPc), which is further divided into two subdomains named PC1 (containing a catalytic Cys residue) and PC2 (containing catalytic His and Asn residues). Conventional calpains (μ-calpain and m-calpain) are comprised of each catalytic large subunit (designated CAPN1 for μ-calpain or CAPN2 for m-calpain) and a common regulatory small subunit (CAPNS1). Non-classical (atypical) calpain sequences lack the PEF domain but contain additional domains or motifs [calcium-binding C2 domain, microtubule-interacting and trafficking (MIT) domain, Zinc finger (ZnF), SOL-homology domain (SOH), and circularly permutated globin domain (cpGB) split by the calmodulin-binding IQ motif]. Calpain-3 (CAPN3), specifically expressed in skeletal muscles, has distinct sequences: N-terminal sequence (NS), insertion sequence 1 (IS1), and insertion sequence 2 (IS2). Calpain-7 (CAPN7) is an ortholog of fungal PalB. PEF proteins are classified into two groups based on similarity of the first EF-hand (EF1) sequences [15].

Figure 2. Schematic structures of ALG-2-interacting proteins reported in the literature. The human or murine ALG-2-interacting proteins are classified into five groups for convenience sake based on functional properties: (a) ESCRT system, (b) ER-to-Golgi vesicular transport, (c) RNA processing, (d) protein kinases, and (e) miscellaneous. Underlined proteins have been studied in the author’s group. Red boxes and thick violet bars indicate Pro-rich regions (PRRs) and determined ALG-2-binding regions, respectively. PTP, phosphotyrosine phosphatase; UEV, ubiquitin E2 variant; CC, coiled-coil; SB, steadiness box; LC1, light chain 1; CID, C-terminal domain (CTD)-interacting domain; ZnF, zinc finger; RRM, RNA recognition motif; Ig-like C2,

immunoglobulin-like constant domain type 2; ANK, ankyrin; TM, transmembrane; C2, Protein Kinase C C2-domain-like Ca^{2+}-binding domain; vWFA, von Willebrand factor A.

Figure 3. PEF-binding motifs and 3D structures. (a) Three types of Pro-rich ALG-2-binding motifs (ABMs). Residues conserved among the identified ALG-2-interacting proteins in each type of ABM are indicated in red, and residues compatible with the type 2 motif at the Ω position are indicated in violet. [PΦ], Pro or hydrophobic; [FW], Phe or Trp; Ω, large side chain; *x*, variable. (b) Overall 3D structure of the complex between ALG-2 (homodimer) and ALIX peptides (indicated by magenta arrows) is shown by a cartoon in rainbow colors (from blue in the N-terminal region to red in the C-terminal region) using the 3D presentation software PyMOL and Protein Data Bank (PDB) code 2ZNE. (c) Overall 3D structure of the complex between ALG-2 and Sec31A peptides. PDB code 3WXA. A side view (left panel) and a 90°-rotated bottom view (right panel). (d) Schematic representation of a three-binding-site model of calpain inhibition by calpastatin. Among the three conserved regions of the four repeated domains of calpastatin, region B binds the protease domain and inhibits the proteolytic activity of calpain. Regions A and C bind the PEF domains of the large subunit (L-PEF) and the small subunit (S-PEF), respectively. (e) Amino acid sequences of regions A and C of human calpastatin. Conserved (identical or similar) residues are highlighted in light green for region A and in cyan for region C. Conserved residues between the two regions are marked with asterisks, where high conservation is indicated by bold face. (f) Overall 3D structure of the complex between rat m-calpain and calpastatin domain 1 (PDB code 3DF0). The PEF domains and the calpastatin peptide are shown by cartoon models in rainbow colors and in magenta, respectively. Other calpain domains are shown by surface representation in pale colors.

Figure 4. Schematic diagram of calpain-7 actions on ESCRT-mediated EGF receptor

downregulation in the endosome-to-lysosome pathway. Calpain-7 (CAPN7) is recruited to endosomes after stimulation of cells with epidermal growth factor (EGF) and regulates downregulation of the ubiquitinated and endocytosed EGF receptor (EGFR). Calpain-7 interacts via the tandemly repeated microtubule-interacting and trafficking (MIT) domains with a subset of ESCRT-III subunits (CHMP proteins) and related proteins that contain MIT-interacting motifs (MIMs). ALG-2 interacts with IST1, a CHMP-like protein, in a Ca^{2+}-dependent manner at the Met-Pro repeat (MP) region. Endogenous substrates of calpain-7 have not been identified yet. Fungal and yeast orthologs of calpain-7 cleave ALIX-homolog-interacting transcription factors in association with ESCRT-III proteins. VPS4 (isoforms A and B) and spastin, meiotic clade AAA ATPases containing MIT domains, disassemble ESCRT-III polymers and microtubules, respectively [101,102]. CHMP, charged multivesicular body protein; MTBD, microtubule binding domain; MVB, multivesicular body; Ub, ubiquitin.

Table 1. Distribution of penta-EF-hand (PEF) proteins, classical calpains and calpain-7 orthologs in eukaryotes

PEF proteins and calpains	Protist	Plant	Yeast	Fungus	Nematode	Fly	Mammal
PEF proteins							
ALG-2/PEF [1)]	+	+	+	+	+	+	+
peflin	-	-	-	-	-	+	+
classical calpains							
Large subunit	-	-	-	-	-	+	+
Small subunit	-	-	-	-	-	-	+
sorcin	-	-	-	-	-	-	+
grancalcin	-	-	-	-	-	-	+
CAPN7							
calpain-7/PalB/Rim13 [2)]	-	-	+	+	+	-	+

[1)] Names of homologs are different among organisms, and functions of mammalian ALG-2 are different from those of lower eukaryotic homologs.

[2)] PalB (fungus) and Rim13 (yeast) have similar functions of processing transcription factors involved in alkaline adaptation system, but the physiological substrate of mammalian CAPN7 (calpain-7) has not been identified yet.

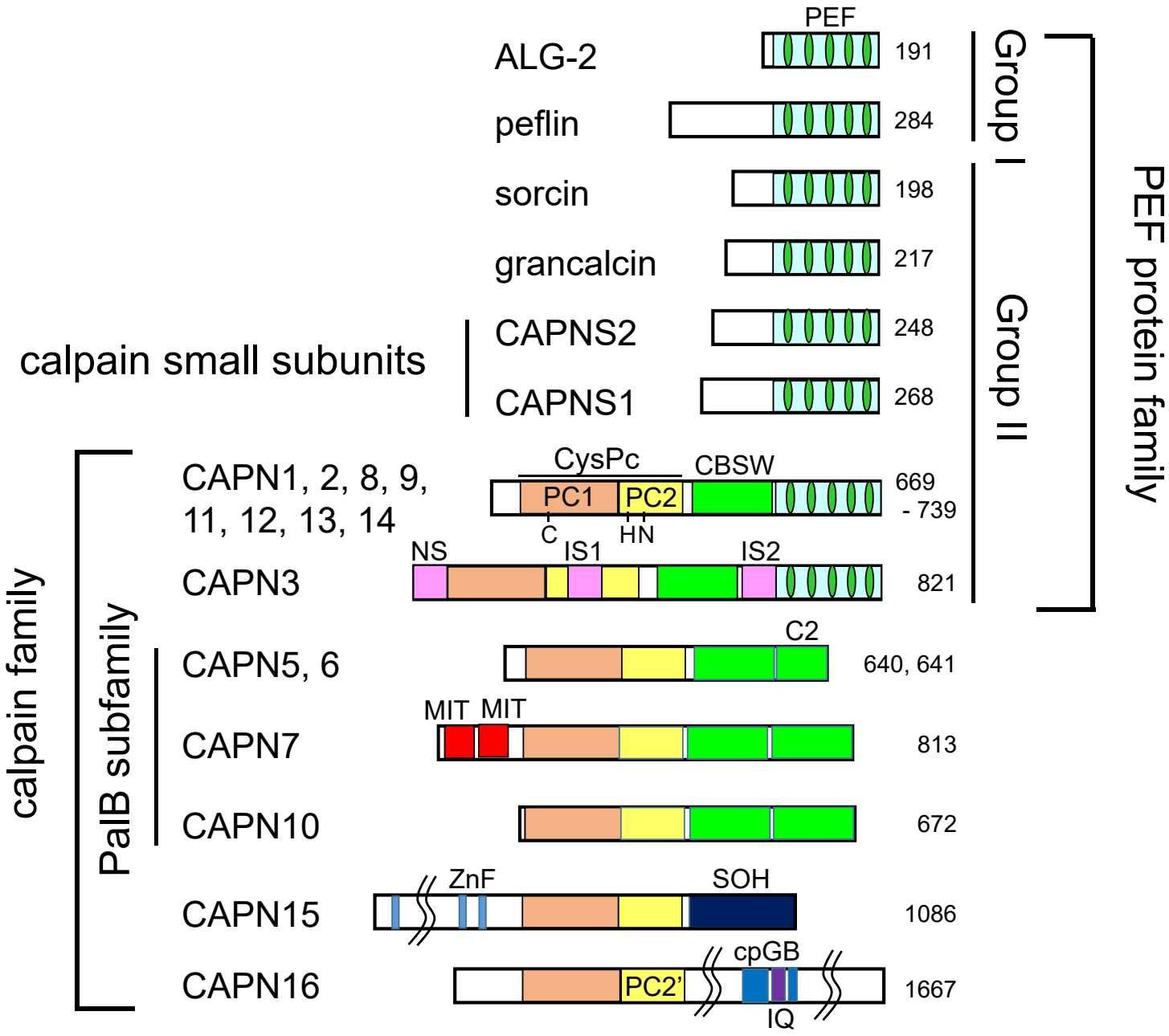
PEF
ALG-2
191
peflin
284
Group I
sorcin
198
grancalcin
217
CAPNS2
248
CAPNS1
268
Group II
PEF protein family
calpain small subunits
CysPc
CBSW
CAPN1, 2, 8, 9,
11, 12, 13, 14
PC1
PC2
669
- 739
C
HN
NS
IS1
IS2
CAPN3
821
C2
CAPN5, 6
640, 641
MIT MIT
CAPN7
813
CAPN10
672
ZnF
SOH
CAPN15
1086
cpGB
CAPN16
PC2'
1667
IQ
calpain family
PalB subfamily

Figure 1

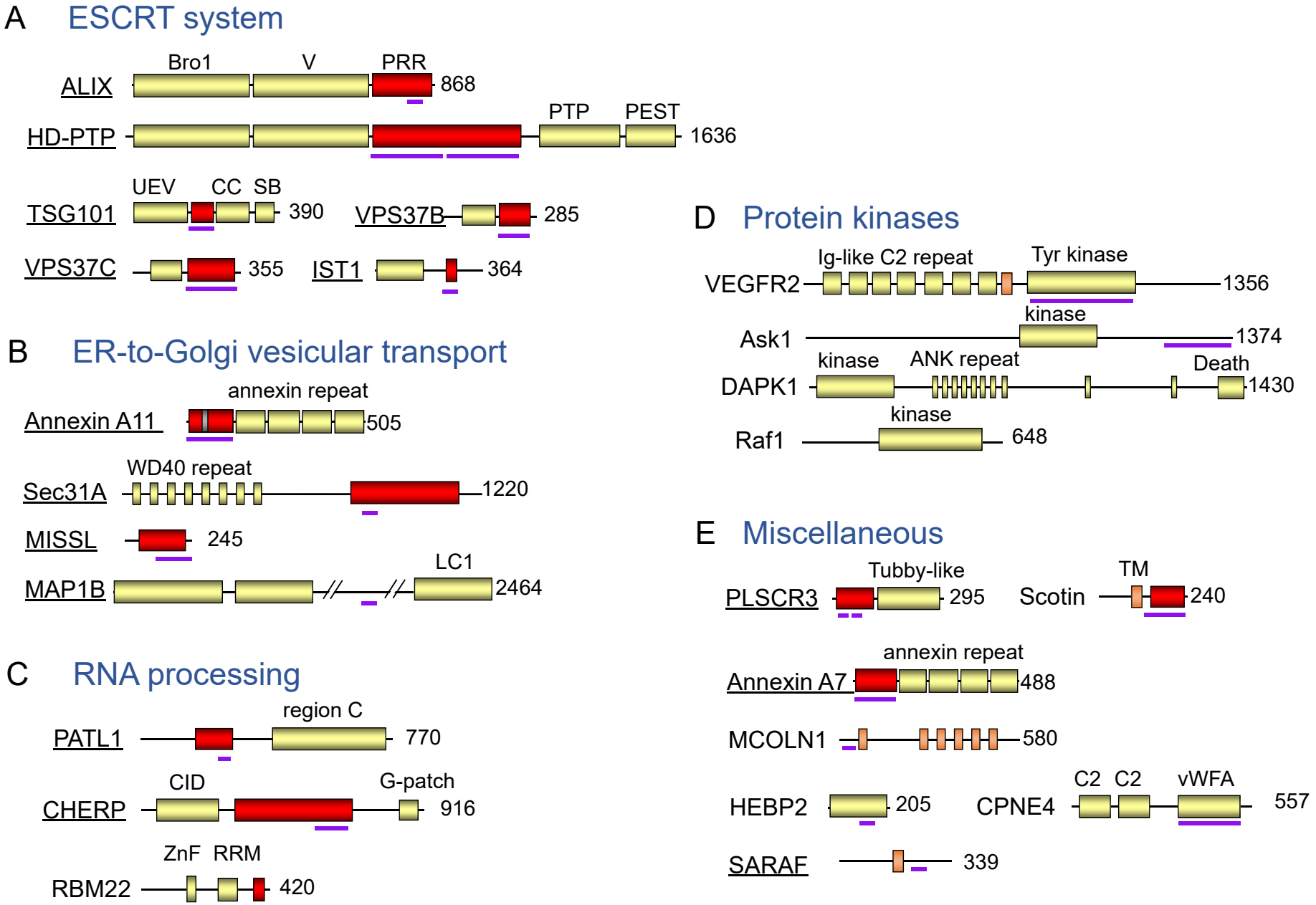
A ESCRT system
Bro1
V
PRR
ALIX
868
PTP
PEST
HD-PTP
1636
UEV
CC
SB
TSG101
390
VPS37B
285
VPS37C
355
IST1
364
B ER-to-Golgi vesicular transport
annexin repeat
Annexin A11
505
WD40 repeat
Sec31A
1220
MISSL
245
LC1
MAP1B
2464
C RNA processing
region C
PATL1
770
CID
G-patch
CHERP
916
ZnF
RRM
RBM22
420
D Protein kinases
Ig-like C2 repeat
Tyr kinase
VEGFR2
1356
kinase
Ask1
1374
kinase
ANK repeat
Death
DAPK1
1430
kinase
Raf1
648
E Miscellaneous
Tubby-like
PLSCR3
295
TM
Scotin
240
annexin repeat
Annexin A7
488
MCOLN1
580
HEBP2
205
C2
C2
vWFA
CPNE4
557
SARAF
339

Figure 2

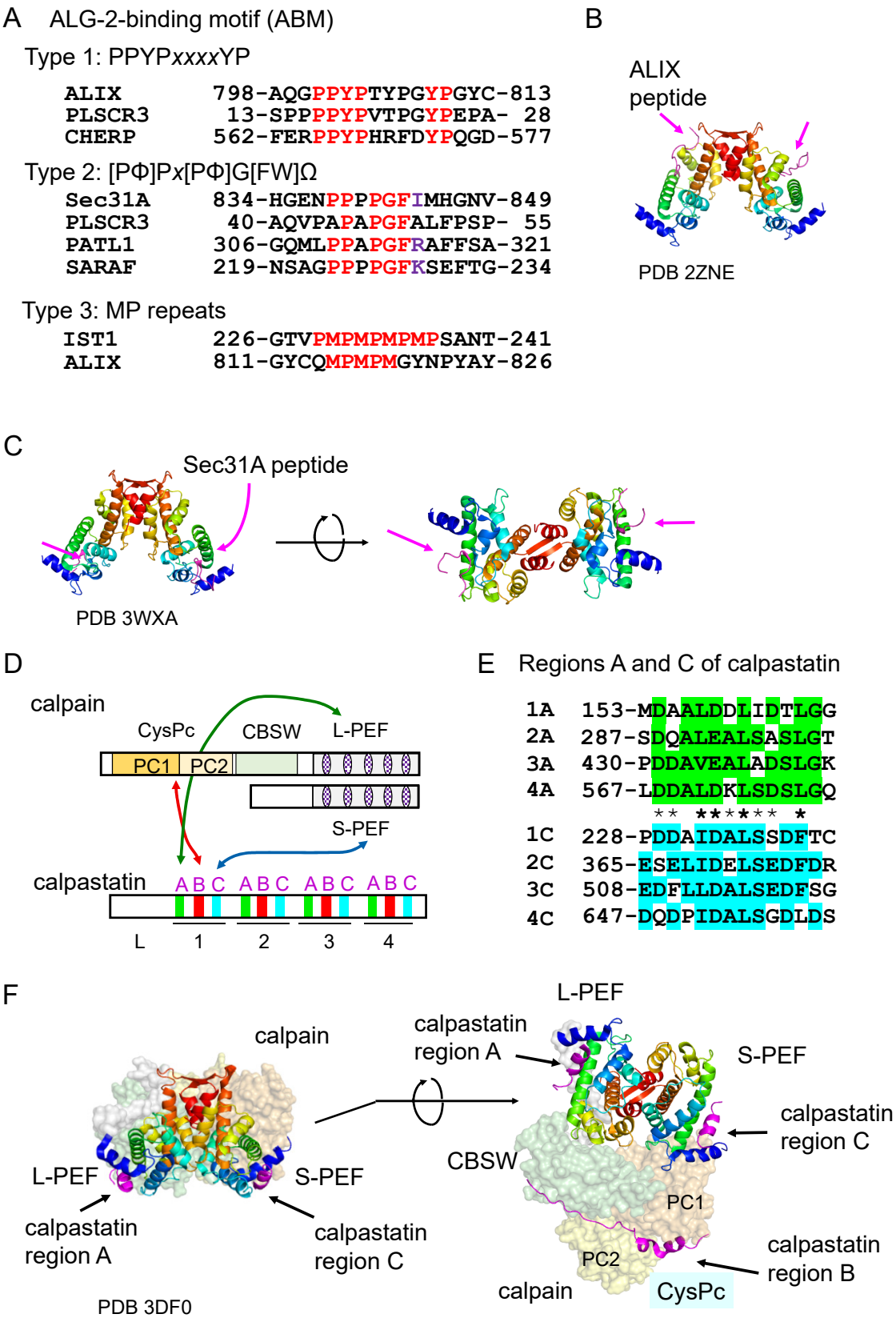
A
ALG-2-binding motif (ABM)
Type 1: PPYPxxxxYP
ALIX 798-AQGPPYPTYPGYPGYC-813
PLSCR3 13-SPPPPYPVTPGYPEPA- 28
CHERP 562-FERPPYPHRFDYPQGD-577
Type 2: [PΦ]Px[PΦ]G[FW]Ω
Sec31A 834-HGENPPPPGFIMHGNV-849
PLSCR3 40-AQVPAPAPGFALFPSP- 55
PATL1 306-GQMLPPAPGFRAFFSA-321
SARAF 219-NSAGPPPPGFKSEFTG-234
Type 3: MP repeats
IST1 226-GTVPMPMPMPMPSANT-241
ALIX 811-GYCQMPMPMGYNPYAY-826
B
ALIX peptide
PDB 2ZNE
C
Sec31A peptide
PDB 3WXA
D
calpain
CysPc
CBSW
L-PEF
PC1
PC2
S-PEF
calpastatin
A B C A B C A B C A B C
L 1 2 3 4
E
Regions A and C of calpastatin
1A 153-MDAALDDLIDTLGG
2A 287-SDQALEALSASLGT
3A 430-PDDAVEALADSLGK
4A 567-LDDALDKLSDSLGQ
1C 228-PDDAIDALSSDFTC
2C 365-ESELIDELSEDFDR
3C 508-EDFLLDALSEDFSG
4C 647-DQDPIDALSGDLDS
F
calpain
L-PEF
S-PEF
calpastatin region A
calpastatin region C
PDB 3DF0
L-PEF
calpastatin region A
S-PEF
calpastatin region C
CBSW
PC1
PC2
calpastatin region B
calpain
CysPc

Figure 3

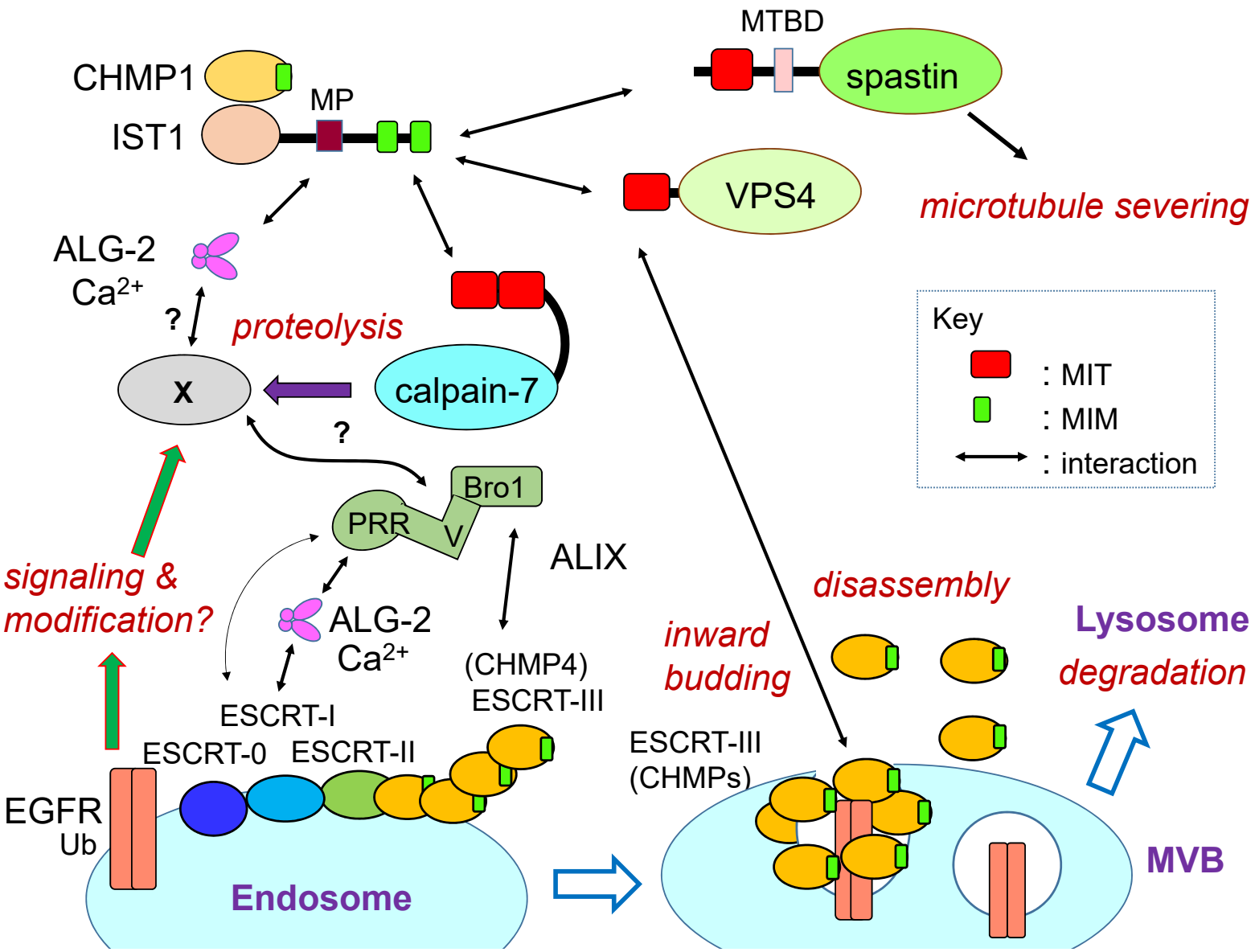
MTBD
spastin
CHMP1
MP
IST1
VPS4
microtubule severing
ALG-2
Ca2+
?
proteolysis
X
calpain-7
?
Key
: MIT
: MIM
: interaction
Bro1
PRR
V
ALIX
signaling &
modification?
ALG-2
Ca2+
disassembly
inward
budding
Lysosome
degradation
(CHMP4)
ESCRT-III
ESCRT-I
ESCRT-0
ESCRT-II
ESCRT-III
(CHMPs)
EGFR
Ub
Endosome
MVB

Figure 4