Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 350 (2006) 82–90
BBRC
www.elsevier.com/locate/ybbrc

Monoubiquitylation of GGA3 by hVPS18 regulates its ubiquitin-binding ability

Satomi Yogosawa [a,b], Masato Kawasaki [c], Soichi Wakatsuki [c], Eiki Kominami [d], Yoko Shiba [e], Kazuhisa Nakayama [e], Shinichi Kohsaka [a,*], Chihiro Akazawa [a]

[a] *Department of Neurochemistry, National Institute of Neuroscience, NCNP, Kodaira, Tokyo 187-8502, Japan*
[b] *Division of Molecular Biology, Research Institute for Biological Sciences, Tokyo University of Science, Noda, Chiba 278-0022, Japan*
[c] *Structural Biology Research Center, Photon Factory, Institute of Materials Structure Science, High Energy Accelerator Research Organization (KEK), Tsukuba, Ibaraki 305-0801, Japan*
[d] *Department of Biochemistry, Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113-8421, Japan*
[e] *Graduate School of Pharmaceutical Science, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*

Received 25 August 2006
Available online 15 September 2006

Abstract

GGAs (Golgi-localizing, γ-adaptin ear domain homology, ADP-ribosylation factor (ARF)-binding proteins), constitute a family of monomeric adaptor proteins and are associated with protein trafficking from the *trans*-Golgi network to endosomes. Here, we show that GGA3 is monoubiquitylated by a RING-H2 type-ubiquitin ligase hVPS18 (human homologue of *vacuolar protein sorting* 18). By *in vitro* ubiquitylation assays, we have identified lysine 258 in the GAT domain as a major ubiquitylation site that resides adjacent to the ubiquitin-binding site. The ubiquitylation is abolished by a mutation in either the GAT domain or ubiquitin that disrupts the GAT-ubiquitin interaction, indicating that the ubiquitin binding is a prerequisite for the ubiquitylation. Furthermore, the GAT domain ubiquitylated by hVPS18 no longer binds to ubiquitin, indicating that ubiquitylation negatively regulates the ubiquitin-binding ability of the GAT domain. These results suggest that the ubiquitin binding and ubiquitylation of GGA3-GAT domain are mutually inseparable through a ubiquitin ligase activity of hVPS18.

Keywords: GGA; GAT-domain; hVPS18; RING-H2 domain; Ubiquitin ligase; Monoubiquitylation; Ubiquitin-binding

In eukaryotic cells, vesicular trafficking of proteins between intracellular compartments, such as the Golgi complex and the multivesicular body (MVB)/lysosome, plays an important role in cellular functions. GGAs (Golgi-localizing, γ-adaptin ear domain homology, ADP-ribosylation factor (ARF)-binding proteins) are a family of monomeric adaptor proteins that regulate delivery of clathrin-coated vesicles from the TGN to endosomes [1–3]. In mammals, there are three GGAs (GGA1, GGA2, and GGA3) that share four functional domains, named VHS (Vps27/Hrs/Stam), GAT (GGA and Tom1 (target of Myb 1)), hinge, and GAE (γ-adaptin ear) [4–7]. The N-terminal VHS domain directly binds to acidic cluster-dileucine motifs found in the cytoplasmic domains of transmembrane cargo proteins [8–10]. The GAT domain is responsible for association of GGAs with the TGN membrane through interacting with activated ARF (a GTP-bound form) [11]. The proline-rich hinge region, the most variable among the GGA isoforms, mediates recruitment of clathrin [12]. The C-terminal GAE domain associates with various accessory proteins that modulate vesicle transport [1,3].

In the past few years, a number of proteins that are involved in membrane trafficking, especially in endocytosis and degradation in lysosomes, have been shown to be

* Corresponding author. Fax: +81 42 346 1751.
E-mail address: kohsaka@ncnp.go.jp (S. Kohsaka).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.09.013

functionally modulated by their ubiquitin binding and ubiquitylation [13–16]. Recently, it has also been shown that ubiquitin binding and ubiquitylation play an important role in selective transport of proteins from the TGN. The GAT domains of GGAs bind to ubiquitin and/or ubiquitylated proteins and undergo monoubiquitylation in the cell [17–20]. The interaction between the GAT domain and ubiquitin may endow GGAs with the ability to sort ubiquitylated transmembrane proteins at both the TGN and endosomes. Several ubiquitin ligases (E3) have been shown to be involved in monoubiquitylation of membrane-associated proteins and to participate in the endocytic and degradation pathways [21]. For example, c-Cbl, a RING-type ligase, ubiquitylates epidermal growth factor receptor (EGFR) depending on ligand-stimulated phosphorylation of the receptor [22], and Nedd4, a HECT-type ligase, is required for monoubiquitylation of eps15 and Hrs [23,24].

The Class C Vps (vacuolar protein sorting) complex is required for vesicle transport from the late endosome to vacuole in yeast [25,26]. In mammals, VPS proteins also appear to control the fusion events of late endosomes and lysosomes [27,28]. In humans, four Class C VPS proteins, hVPS11, hVPS16, hVPS18, and hVPS33, constitute a large hetero-oligomeric complex that interacts with syntaxin 7 at late endosomes/lysosomes [29]. Moreover, they also exist as a large detergent-insoluble HOPS (homotypic fusion and vacuole protein sorting) complex that contains additional components, hVPS39/Vam6, and hVPS41/Vam2 [27]. Overexpression of VPS39/Vam6S alters the late endosome function in mammalian cells [30]. Thus, it is necessary to discuss specific functions of Class C VPS proteins based on the biochemical properties.

In this report, we have demonstrated that GGAs (GGA1 and GGA3 but not GGA2) are monoubiquitylated by hVPS18, a RING-H2 type ubiquitin ligase, and identified a lysine residue in the C-terminal subdomain of the GAT domain as a major ubiquitylation site. Furthermore, we have shown that the monoubiquitylation negatively regulates the ubiquitin-binding ability of GGA itself. These observations shed light on the regulatory mechanisms of GGA to participate in membrane trafficking through the association of hVPS18.

Materials and methods

Plasmid construction. Full-length and various truncated human GGA cDNAs, as described previous, [17], were subcloned into the following vector: the pGEX-4T2 (Amersham Biosciences) prokaryotic expression vector for the production of GST-tagged fusion proteins. Myc-tagged hVPS18 and hVPS11 mammalian expression vectors were prepared as described previously [29]. The full-length hVPS18, hVPS11, and hVPS16 were subcloned into pFastBacHTb insect expression vector (Invitrogen) to generate His_6-tagged fusion proteins.

Expression and preparation of recombinant proteins. E2 ubiquitin-conjugating enzyme, human Ubc4, was produced in *Escherichia coli* strain BL21-AI (Invitrogen). GST-GGA (wild-type and truncated mutants) and GST-ubiquitin (Ub) (wild-type and mutants) were expressed in *E. coli* strain BL21-AI and the recombinant proteins were purified by using glutathione–Sepharose 4B beads (Amersham Biosciences) in PBS containing 1 mM PMSF, Complete protease inhibitor mixture, and 1% Triton X-100. His_6-tagged hVPS18, hVPS11, and hVPS16 were expressed in Sf-9 insect cells using baculovirus protein expression system (Invitrogen) and the recombinant proteins were purified under the denatured condition (8 M urea), and followed by using TALON metal affinity beads (BD Biosciences), and renatured in PBS. Circular dichroism spectra of the wild-type and mutant proteins were recorded on a Jasco J-820 spectropolarimeter at 25 °C using a cuvette with 1 mm path length with the protein concentration of 30 μM in PBS.

Antibody. Polyclonal antibody against human GGA1, GGA2, and GGA3 was raised in rats by immunization with purified full-length GGA1, GGA2, and GGA3, respectively. These antibodies were affinity-purified on HiTrap NHS-activated columns (Amersham Biosciences) conjugated with immunogens. Monoclonal GGA3 antibody was purchased from BD Transduction Laboratories. Anti-hVPS18, hVPS11, and hVPS16 antibodies were prepared as described previously [29].

Immunocytochemistry. HeLa cells were immunostained with mouse anti-GGA3 antibody and rabbit anti-hVPS18 as described previously [29]. To confirm intracellular colocalization, immunoreactivities were analyzed by the sectioning microscope (Delta Vision) calibrated by using fluorescent beads (TetraSpeck Fluorescent Microsphere Standards, 0.1 μm, Invitrogen).

Immunoprecipitation. Immunoprecipitation was performed as described previously [29]. Briefly, the cells were transfected with various plasmids by Fugene 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. At 24 h after transfection, total cell lysates were incubated with 4 μg of anti-FLAG antibodies (monoclonal M2, Sigma) at 4 °C overnight. Immunoprecipitation of the antigen-antibody complex was accomplished by adding 40 μl of protein G–Sepharose for 1 h at 4 °C. Sepharose bound proteins were subjected to SDS–PAGE and detected by Western blot analyses with anti-Myc antibody (Roche Molecular Biochemicals) or anti-FLAG M2 antibody, respectively.

In vitro pull-down assay. In vitro pull-down assay was performed as described previously [31]. Briefly, GST-GGA-GAT proteins were immobilized on glutathione–Sepharose 4B beads and incubated with His_6-hVPS18 at 25 °C for 30 min. The resin was washed, subjected to SDS–PAGE, and detected by Western blot analyses with either anti-His_6 antibody (Santa Cruz Biotechnology) or anti-GST antibody.

We performed assays of GGA3 binding to ubiquitin as described previous [17]. Briefly, various GGA3 proteins were incubated with Ub-(10 μl) or protein A–agarose (15 μl) beads (Sigma) for 1 h at room temperature. The beads were washed, subjected to SDS–PAGE, and detected by Western blot analyses using anti-GST antibody.

To prepare ubiquitylated GGA3 C-GAT proteins, GGA3 C-GAT proteins were subjected to *in vitro* ubiquitylation assay and immobilized on glutathione–Sepharose 4B beads. The beads were then washed with a buffer (25 mM Hepes, pH 7.4, 0.1% Nonidet P-40, 0.5 M NaCl, and 50% ethylene glycol) and eluted with 25 mM Hepes, pH 7.4, 20 mM reduced glutathione.

In vitro ubiquitylation assay. An *in vitro* ubiquitylation assay was performed as described previously [29]. Briefly, GST-GGA proteins were mixed with yeast E1 (500 ng) (Boston Biochem), human Ubc4, ubiquitin (Boston Biochem) or GST-Ub (10 or 5 μg, respectively), and His_6-tagged hVPS18 (or hVPS11 or hVPS16). The mixture was incubated at 25 °C for 30 or 60 min in the presence of 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 4 mM ATP in a 20 μl volume. After incubation, the mixtures were immobilized on glutathione–Sepharose 4B beads and washed with wash buffer for three times. The resin was subjected to SDS–PAGE and detected by Western blot analyses using anti-GST antibody or anti-His_6 antibody or specific antibody.

Results and discussion

Direct interaction with GGAs and hVPS18

Recent studies identified the GAT domain as a ubiquitin-binding module [17–20]. Furthermore, our recent yeast

two-hybrid screening using hVPS18 as bait identified a partial fragment of GGA3 [31]. We first confirmed the molecular interaction by an *in vitro* pull-down assay using recombinant GGAs and hVPS18. As shown in Fig. 1A, His_6-hVPS18 was pulled down with the GST fusion of the GAT domain of GGA1, GGA2 or GGA3. We next analyzed the *in vivo* interaction by a co-immunoprecipitation experiment using lysates of cells cotransfected with FLAG-GGAs and either Myc-hVPS18 or Myc-hVPS11. As shown in Fig. 1B, all GGAs co-immunoprecipitated hVPS18. By contrast, either GGA could not interact with hVPS11, another Class C component having a RING-H2

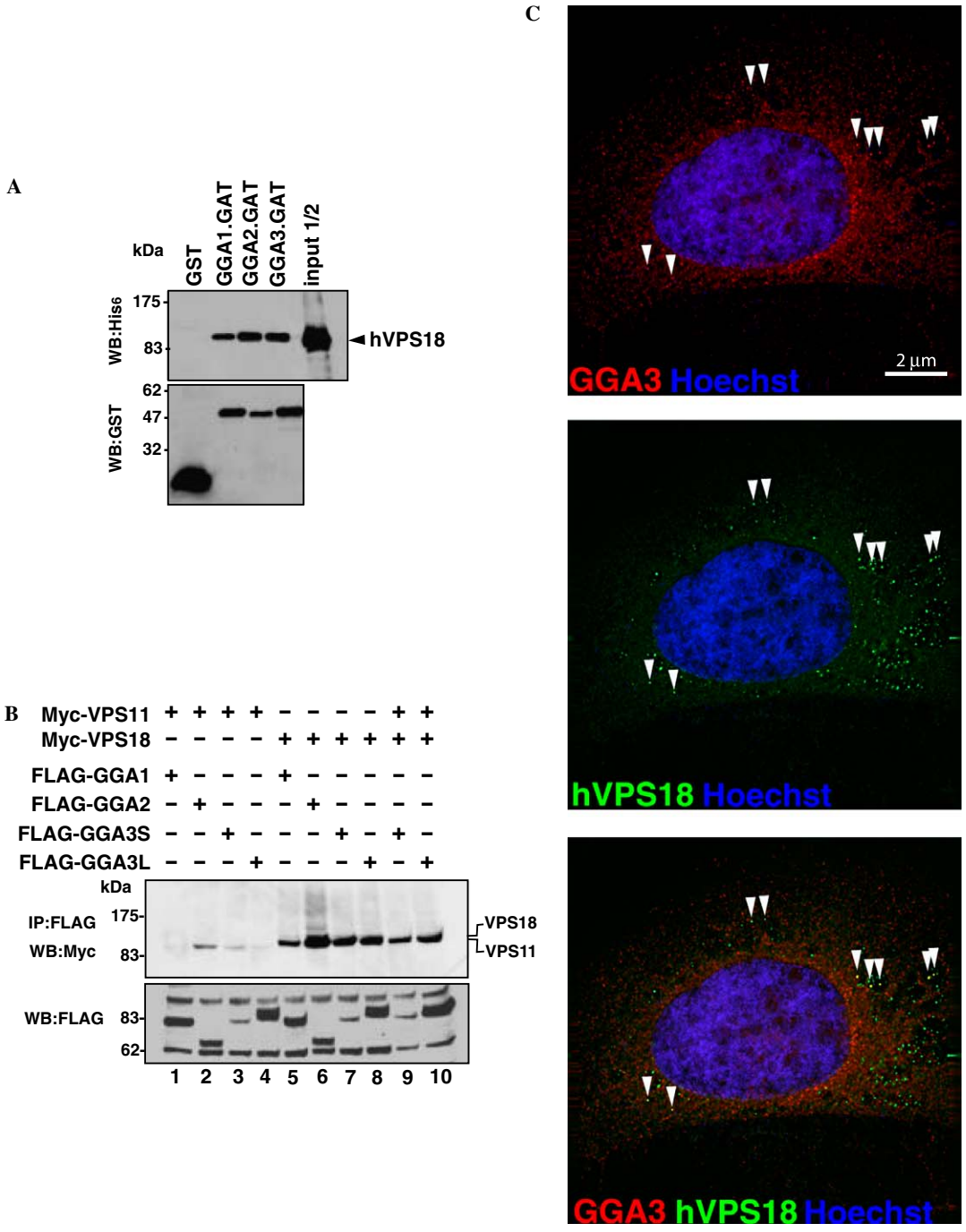

Fig. 1. GGA-GAT domains interact with hVPS18. (A) His_6-hVPS18 bound to three GST-GGA-GAT proteins was detected by Western blot analyses using anti-GST antibody. (B) Cos7 cells were co-transfected with His_6-FLAG-GGAs and Myc-hVPS11 or hVPS18. At 24 h post-transfection, whole-cell lysates were co-immunoprecipitated using anti-FLAG M2 antibody. Immunoprecipitates were resolved by SDS–PAGE and detected by Western blot analyses using indicated antibody. (C) The interaction of endogenous GGA3 (*Red*) and hVPS18 (*Green*) in HeLa cells was detected by immunocytochemistry. Colocalizing profiles are pointed out with *arrows*. *Bar*, 2 μm.

domain. Considering the molecular interaction between GGAs and hVPS18, a major issue that arises is the intracellular localization of these proteins. GGAs have been characterized as TGN-associated clathrin adaptors, whereas the Class C VPS complex has been proposed to function in endosomal/lysosomal compartments. We therefore analyzed the precise intracellular localization of endogenous GGA3 and hVPS18 in HeLa cells by the sectioning microscopy. The optical pathways were calibrated using fluorescent labeled beads (0.1 μm diameter). The thirty series of sections covering 5 μm thickness were captured and deconvoluted images were analyzed. As shown in a representative image Fig. 1C, GGA3 immunoreactivities (colored in *Red*) and hVPS18 immunoreactivities (colored in *Green*) were often colocalized on punctuates (colored in *Yellows*) of perinuclear structures (indicated by arrowheads).

Monoubiquitylation of GGAs by hVPS18, RING-H2 type ubiquitin ligase

Our recent study, showed that the RING-H2 domain of hVPS18 displays a E3 ubiquitin ligase activity [31]. To examine whether GGAs are ubiquitylated by the hVPS18, we performed an *in vitro* ubiquitylation assay using full-length GGAs as substrates. As shown in Fig. 2A, the molecular weight of GGA1 and GGA3 was shifted by ~8 kDa only in the simultaneous presence of ubiquitin, E1, E2 (UbcH4), and hVPS18 (lanes 2 and 6, respectively). By contrast, the shift was not observed in the case of GGA2 (lane 4), being compatible with our previous study showing that GGA2 is not able to interact with ubiquitin nor ubiquitylated in the cell. We next examined whether the *in vitro* modification represents monoubiquitylation/multiubiquitylation or not. In this experiment, we used wild-type (WT) ubiquitin fused to GST, and its K48R and KO (all lysine residues were replaced with arginine) mutants, since these mutants are not conjugated to conventional polyubiquitin chains. As shown in Fig. 2B, when GST-ubiquitin WT was used, the molecular weight of the GGA3-GAT domain was shifted by 35 kDa, which corresponds to the size of GST-ubiquitin. Essentially the same band shift was observed using the K48R and KO mutants, indicating that GGA3 is mainly monoubiquitylated by hVPS18.

Since hVPS18 forms a complex with other Class C VPS components [29], we then examined whether hVPS11 and hVPS16 were also involved in the monoubiquitylation of GGA3. Although hVPS11 also has a RING-H2 domain and shows a ubiquitin ligase activity (data not shown), it did not ubiquitylate the GGA3-VHS+GAT domain irrespective of the presence of hVPS16 (Fig. 2C lanes 2 and 3). However, the monoubiquitylation of GGA3 by hVPS18 was extremely enhanced in the presence of hVPS11 and hVPS16 (compare lane 5 with lanes 6 and 7). This result makes it likely that hVPS18 is involved in the monoubiquitylation of GGA3 as a Class C VPS complex.

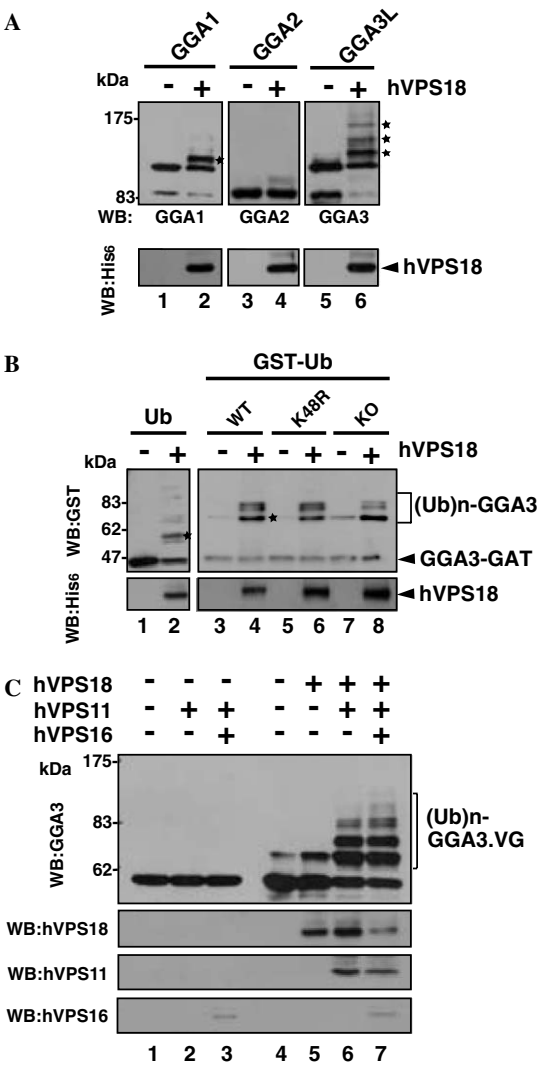

Fig. 2. GGAs are monoubiquitylated by hVPS18. (A) The *in vitro* ubiquitylation of GST-GGAs wild-type by His_6-hVPS18. The sample was detected by Western blot analyses using anti-GGA1, GGA2, or GGA3 (upper panel). Purified His_6-hVPS18 protein was detected by Western blot analyses using anti-His_6 antibody (lower panel). Asterisks represent ubiquitylated form of GGAs. (B) The *in vitro* ubiquitylation of GST-GGA3-GAT protein in the presence of no-tagged ubiquitin (Ub) or GST-fused ubiquitin (GST-Ub); wild-type (WT), K48R, or all lysines mutated to arginines (KO). The sample was detected by Western blot analyses using anti-GST or anti-His_6 antibody. Asterisks represent ubiquitylated forms of GGA3 that conjugate to Ub (left panel) or GST-Ub (right panel). (C) The *in vitro* ubiquitylation of GST-GGA3-VG (VHS+GAT) by hVPS11 or hVPS18 in the presence or absence of hVPS16. Purified His_6-tagged hVPS18 (or hVPS11 or hVPS16) protein was detected by Western blot analyses using specific antibodies.

Ubiquitin binding-dependent monoubiquitylation of GGA3-GAT

Identification of the ubiquitylation site is of great significance to discuss the molecular mechanism underlying the GGA ubiquitylation. We have recently shown that GGA3 is ubiquitylated in the C-terminal subdomain of its GAT domain (C-GAT) *in vivo* [17]. We therefore,

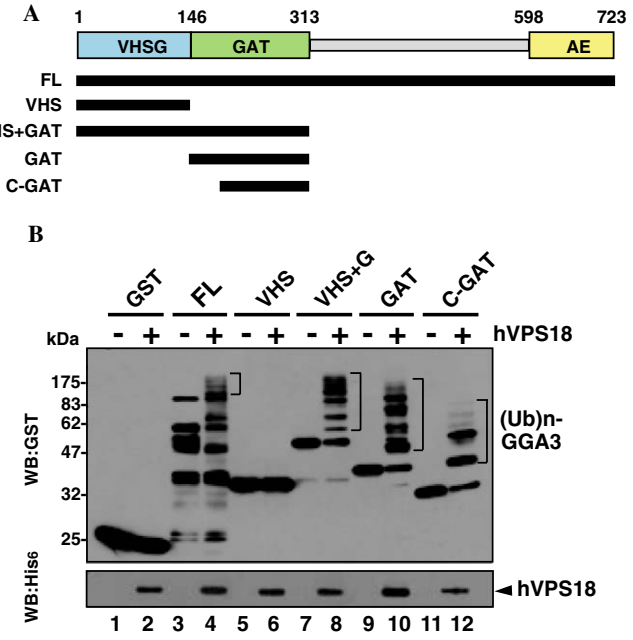

Fig. 3. GGA3 is ubiquitylated in the C-GAT domain. (A) Schematic representation of GGA3. (B) The *in vitro* ubiquitylation of GST-GGA3 full-length or truncated mutants. The sample was detected by Western blot analyses using anti-GST or anti-His_6 antibody. The positions of ubiquitylated GGA3 are indicated by a bracket.

performed an *in vitro* ubiquitylation assay using various truncation mutants of GGA3 (Fig. 3A). As shown in Fig. 3B, ubiquitylation occurred in the GGA3 fragments covering the C-GAT subdomain (lanes 4, 8, 10, and 12) but not in the GST protein (lane 2) nor the VHS domain alone (lane 6), in agreement with our previous ubiquitylation data in the cell [17] .

Recent studies have shown, that many proteins containing ubiquitin-binding modules undergo monoubiquitylation and more importantly, their ubiquitin-binding ability is required for their own monoubuquitylation [23,32,33]. Therefore, we tested *in vitro* whether various GGA3 mutants that lack ubiquitin-binding ability were monoubiquitylated by hVPS18. As shown in Supplementary Fig. 1, the GGA3-GAT helix α3 mutant (L280R or D284G) defective in ubiquitin binding was not monoubiquitylated (compare lane 2 with lanes 6 and 8). Essentially the same result was obtained with a GGA3-GAT helix α2/α3 double mutant, E250N/D284G. These results indicate that binding to ubiquitin is a prerequisite for the GGA3 ubiquitylation. Next we constructed various truncated mutants of the GGA3-GAT domain (Supplementary Fig. 2A) and compared their ubiquitin binding (in Supplementary Fig. 2B) and ubiquitylation by hVPS18 (Supplementary Fig. 2C). Remarkably, all of the GAT fragments that retained ubiquitin-binding ability were monoubiquitylated by hVPS18, whereas the fragments lacking the ability were not monoubiquitylated. These data indicate that binding to ubiquitin and ubiquitylation of the GAT domain are intimately coupled events.

Lys258 is the major ubiquitylation site

In the C-GAT subdomain of GGA3, there are six lysine residues that can be conjugated to ubiquitin. To determine which lysine residue(s) was ubiquitylated, we systematically replaced the lysine residues with arginines (Fig. 4A) and examined binding to ubiquitin and monoubiquitylation of these lysine mutants. As shown in Fig. 4B, all of the C-GAT mutants examined retained their ubiquitin-binding ability. In striking contrast, they were variable in the ubiquitylation efficiency (Fig. 4C). Namely, (i) a mutant, 5KR(258), in which all the lysine residues except for K258 were replaced with arginines (Fig. 4A), underwent monoubiquitylation (lane 10) at comparable efficiency to that of the WT C-GAT subdomain (lane 2); (ii) the 5KR(249), 5KR(264), and 5KR(294) mutants underwent ubiquitylation at extremely low efficiency (lanes 8, 12, and 14); and (iii) ubiquitylation of 5KR(210), 5KR(213), and 6KR mutants was under the detection level (lanes 4, 6, and 16). As shown in Supplemental Figure 4, the circular dichroism spectra of WT and 5KR(258) were almost identical, suggesting that the mutations did not significantly affect the overall conformation of the C-GAT subdomain. Taken together, we conclude that lysine 258 is the major site ubiquitylated by hVPS18, although other lysine residues at positions 249, 264, and 294 were also ubiquitylated to some extent.

Model of GGA3-GAT domain ubiquitylated at Lys258

Previously, we determined the crystal structure of the complex between GGA3 C-GAT and ubiquitin, and showed primarily hydrophobic interactions in which the site 1 in C-CAT constitutes the binding site with three times higher affinity than the site 2 [34]. To understand the molecular basis for the coupling of ubiquitin binding and ubiquitylation of the GGA3-GAT domain, we mapped the positions of lysine residues of GGA3 C-GAT in the complex structure (Fig. 5). Among the six candidate lysine residues, lysine 258 is the closest to the C-terminus of ubiquitin (Fig. 5, Table 1), suggesting that this lysine is most susceptible to ubiquitin conjugation. This model also suggests that no major structural rearrangement between ubiquitin and the GGA3 C-GAT domain is required for the ubiquitylation of lysine 258.

Ubiquitylated GGA3 C-GAT loses its ubiquitin-binding ability

Hicke et al. have proposed a possibility that ubiquitylation of ubiquitin-binding proteins might generally have a regulatory function by affecting association of their ubiquitin-binding modules with either free ubiquitin or ubiquitylated proteins [13]. To address this possibility, we performed *in vitro* pull-down assay using reaction products of the *in vitro* ubiquitylation, in which both ubiquitylated and non-ubiquitylated C-GAT proteins were included. As

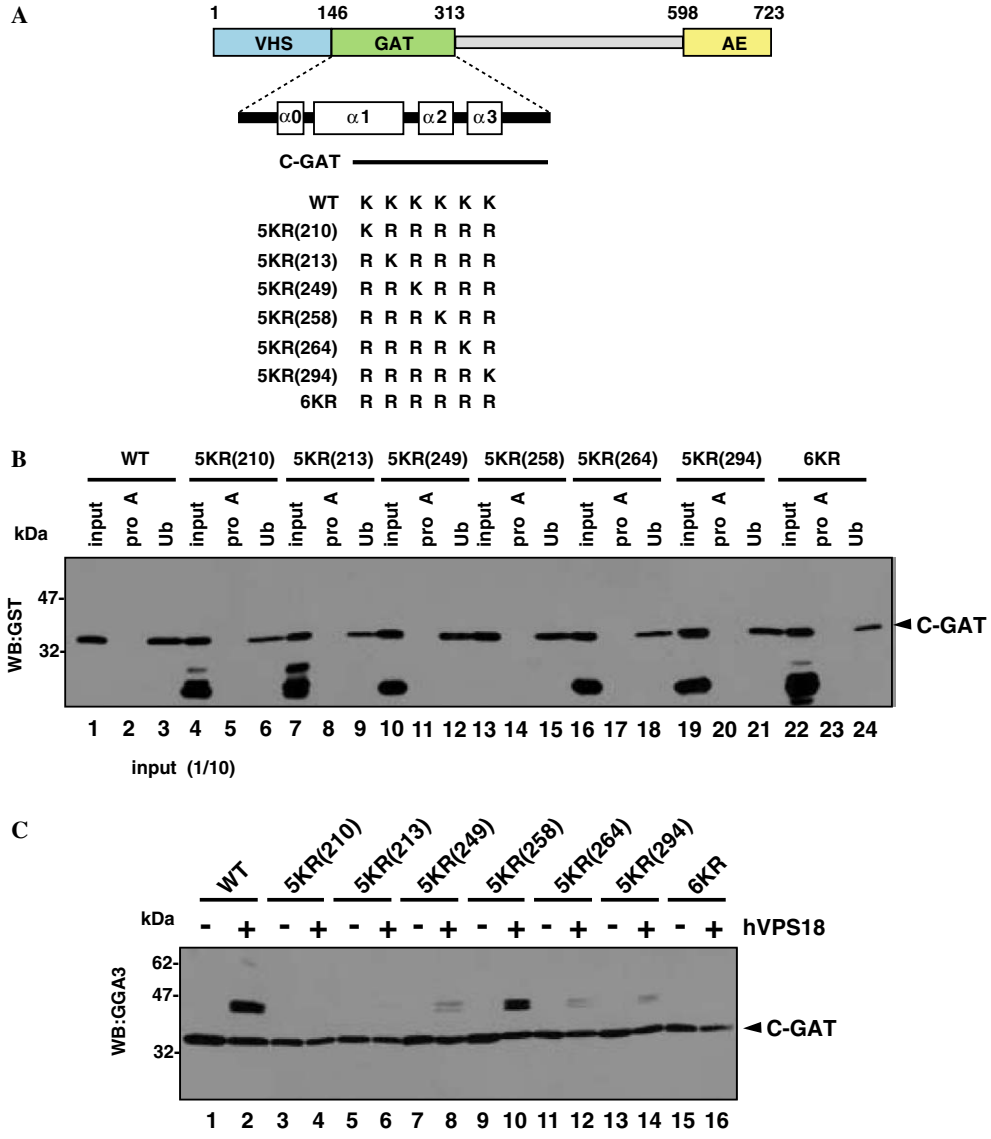

Fig. 4. GGA3 is mainly ubiquitylated at lysine 258 that resides in the two ubiquitin-binding sites. (A) Schematic representation of GGA3-GAT. (B) The *in vitro* pull-down assay of GGA3 C-GAT. Equal amounts of purified GGA3 C-GAT proteins were incubated with Ub- or Protein-A–agarose. The resin was washed, subjected to SDS–PAGE, and detected by Western blot analyses with anti-GST antibody. Ten percent of input samples were loaded on *input lanes*. (C) The *in vitro* ubiquitylation of GST-GGA3 C-GAT proteins. The sample was detected by Western blot analyses using anti-GGA3 antibody.

shown in Fig. 6, the ubiquitylated form of C-GAT WT or its 5KR(258) mutant was not pulled down with ubiquitin-agarose beads (lanes 3 and 7, indicated by asterisks), whereas their non-ubiquitylated forms were pulled down well. This result indicates that covalent modification by ubiquitin at lysine 258 makes C-GAT inaccessible to ubiquitin.

Recent advances have uncovered that several membrane-trafficking events are mediated by ubiquitin binding and monoubiquitylation, including changes in subcellular localization, protein conformation, activity, and protein-protein interaction. [16,35,36]. GGA might have evolved to allow a wide variety of proteins to interact directly with ubiquitin or ubiquitylated proteins during various cellular processes [1,20,37,38]. In this study, we first identified that hVPS18 acts as a genuine ubiquitin ligase of GGAs (Fig. 2A). The modification by hVPS18 slightly differs among GGAs; the monoubiquitylation occurs in one or multiple lysine sites of GGA1 and GGA3 but not of GGA2 (Figs. 2A and B). We then focused on GGA3 and tried to identify the responsible lysine. By taking an advantage of E3 identification, we utilized various KR mutants (Fig. 4). Finally, we identified the lysine 258 is the main target lysine for the ubiquitylation by hVPS18. If we closely look at the results of co-crystallization of GGA3 C-GAT and ubiquitin, the lysine 258 is located at the closest position to the C-terminus of ubiquitin, suggesting that the ubiquitin binding is necessary for ubiquitylation.

It has been recently shown that a free ubiquitin or ubiquitylated proteins are recognized by small (20–150 amino

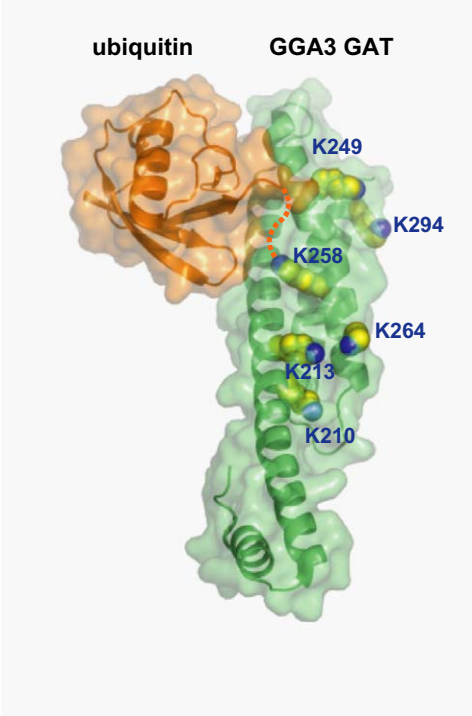

Fig. 5. Molecular surface representation of GGA3 C-GAT. The model was built by combining the crystal structures of the complex between ubiquitin and GGA3 C-GAT subdomain [34] and the entire GGA1 GAT domain [44]. Ubiquitin and GGA3 GAT domain are shown as ribbon diagrams with transparent surface representations (ubiquitin, *Orange*; GGA3 GAT, *Green*). Six lysine residues of the GGA3 C-GAT subdomain are shown with space-filling atoms (carbon atoms, *Yellow*; nitrogen atoms, blue). The C-terminal of ubiquitin (The last visible residue in the crystal, Leu73) and the side chain of Lys258 of GGA3 GAT are connected by an orange dotted line. Figure was drawn using PyMOL (http://pymol.sourceforge.net).

Table 1
Distances between the nitrogen atoms of the lysine side chains of C-GAT and the α carbon atom of Leu73 of ubiquitin, in the model structure in Fig. 5

Lysine	*210*	*213*	*249*	*258*	*264*	*294*
Distance (Å)	32.0	24.1	14.3	12.7	24.5	21.5

Leu73 of ubiquitin is the C-terminal residue visible in the crystal.

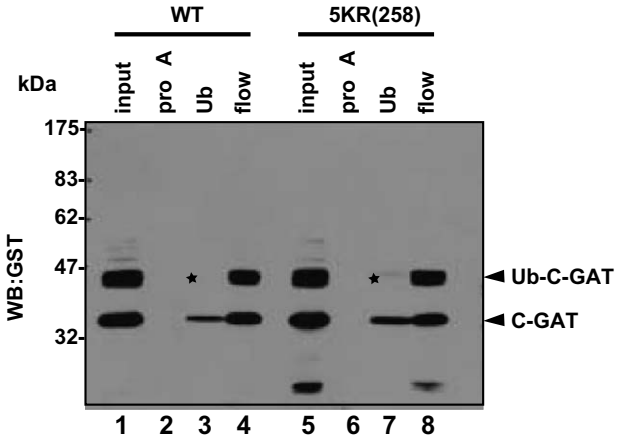

Fig. 6. Ubiquitylated GGA3 prevents further attachment to ubiquitin. The *in vitro* pull-down assay of ubiquitylated GGA3-GAT proteins. Equal amounts of ubiquitylated GGA3-GAT proteins were incubated with Ub- or Protein-A–agarose. The resin was washed, subjected to SDS–PAGE, and detected by Western blot analyses with anti-GST antibody. The supernatants were subjected to incubate with glutathione–Sepharose 4B (lanes 4 and 8). The 25% of input samples were loaded on *input lanes* (lane 1 and 5). Asterisks indicate that ubiquitylated forms of GGA3-GAT could not bind to Ub-agarose.

acids), independently folded motifs; ubiquitin-interacting motif (UIM), ubiquitin-associated (UBA), ubiquitin-conjugating enzyme-like (UBC)/ubiquitin E2 variant (UEV), or Cue1-homologous (CUE) domains [39]. These domains are also referred to as ubiquitin receptors [40]. However, in most cases, little is understood how biochemical interactions are transferred to downstream signals by these ubiquitin-binding proteins. Immediately after ubiquitin-binding abilities were reported, it was generally accepted that ubiquitin receptors are themselves ubiquitylated. Interestingly, the ubiquitylation of ubiquitin receptors requires ubiquitin binding. From our results, all of the GAT mutants that lack ubiquitin binding also inhibit ubiquitylation (Fig. 6). Conversely, a ubiquitin mutant (Ile44Ala) that cannot bind ubiquitin receptors per se cannot be conjugated to GAT domain. Previous reports described that the GAT domain contains two binding sites for ubiquitin [34]. The site 1 centers on leucine 227 and the site 2 centers on leucine 276. The site 1 has a higher affinity for ubiquitin than does site 2. When a ubiquitin was conjugated to the lysine 258 that is close to the site 1, no more ubiquitin can bind to the GGA3 C-GAT. This type of autoinhibition is reminiscent of intramolecular SH2-domain-phosphotyrosine interaction [41]. There has been a strong link between the presence of ubiquitin binding in a protein and its ubiquitylation. Ubiquitin binding and ubiquitylation of ubiquitin receptors are closely coupled and mutually inseparable [42,43]. But so far no ubiquitylation of lysine in the UBD has been reported. This is the first example demonstrating the ubiquitylated lysine resides in the UBD and consequently inhibit further ubiquitin binding. hVPS18 is one of the responsible E3 ubiquitin ligases that directly regulate the ubiquitin binding by conjugating ubiquitin in UBD of GGA3. A major question that has not been answered is to test how E3 hVPS18 is recruited to ubiquitin-binding proteins.

The ubiquitin ligase E3 functions as a monomer or a complex with other cofactors, such as SCF (Skp1/cullin/F-box protein) and the anaphase-promoting complex or cyclosome (APC/C) [35]. But none of the ligase activity has been observed when the catalytic subunit was solely added. Our study showed hVPS18, in the presence of E1 and E2, is sufficient for the *in vitro* ubiquitylation of GGA3, but the modification was significantly enhanced when equal molar of hVPS11 and hVPS16 are mixed. Previous report demonstrated that hVPS18, hVPS11, and hVPS16 constitute a hetero-oligomeric complex in the cytosolic membrane of endosome/lysosome [29]. The aug-

mented ligase-activity implies that the complex formation plays the functionally significant role both *in vivo* and *in vitro*.

In this report, we identified hVPS18 as a ubiquitin ligase (E3) for the monoubiquitylation of GGAs through their ability of ubiquitin-interaction. A number of molecules have been reported to interact with GGA, such as ARF, Rabaptin-5, clathrin, AP-1, and γ-synergin [1,3]. It will be a next issue to address whether these interactions are regulated via monoubiquitylation by hVPS18.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.09.013.

References

[1] J.S. Bonifacino, The GGA proteins: adaptors on the move, Nat. Rev. Mol. Cell. Biol. 5 (2004) 23–32.
[2] I. Hinners, S.A. Tooze, Changing directions: clathrin-mediated transport between the Golgi and endosomes, J. Cell Sci. 116 (2003) 763–771.
[3] K. Nakayama, S. Wakatsuki, The structure and function of GGAs, the traffic controllers at the TGN sorting crossroads, Cell Struct. Funct. 28 (2003) 431–442.
[4] E.C. Dell'Angelica, R. Puertollano, C. Mullins, R.C. Aguilar, J.D. Vargas, L.M. Hartnell, J.S. Bonifacino, GGAs: a family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex, J. Cell Biol. 149 (2000) 81–94.
[5] H. Takatsu, K. Yoshino, K. Nakayama, Adaptor gamma ear homology domain conserved in gamma-adaptin and GGA proteins that interact with gamma-synergin, Biochem. Biophys. Res. Commun. 271 (2000) 719–725.
[6] J. Hirst, W.W. Lui, N.A. Bright, N. Totty, M.N. Seaman, M.S. Robinson, A family of proteins with gamma-adaptin and VHS domains that facilitate trafficking between the trans-Golgi network and the vacuole/lysosome, J. Cell Biol. 149 (2000) 67–80.
[7] M. Kawasaki, K. Nakayama, S. Wakatsuki, Membrane recruitment of effector proteins by Arf and Rab GTPases, Curr. Opin. Struct. Biol. (2005).
[8] R. Puertollano, R.C. Aguilar, I. Gorshkova, R.J. Crouch, J.S. Bonifacino, Sorting of mannose 6-phosphate receptors mediated by the GGAs, Science 292 (2001) 1712–1716.
[9] Y. Zhu, B. Doray, A. Poussu, V.P. Lehto, S. Kornfeld, Binding of GGA2 to the lysosomal enzyme sorting motif of the mannose 6-phosphate receptor, Science 292 (2001) 1716–1718.
[10] H. Takatsu, Y. Katoh, Y. Shiba, K. Nakayama, Golgi-localizing, gamma-adaptin ear homology domain, ADP-ribosylation factor-binding (GGA) proteins interact with acidic dileucine sequences within the cytoplasmic domains of sorting receptors through their Vps27p/Hrs/STAM (VHS) domains, J. Biol. Chem. 276 (2001) 28541–28545.
[11] H. Takatsu, K. Yoshino, K. Toda, K. Nakayama, GGA proteins associate with Golgi membranes through interaction between their GGAH domains and ADP-ribosylation factors, Biochem. J. 365 (2002) 369–378.
[12] R. Puertollano, P.A. Randazzo, J.F. Presley, L.M. Hartnell, J.S. Bonifacino, The GGAs promote ARF-dependent recruitment of clathrin to the TGN, Cell 105 (2001) 93–102.
[13] L. Hicke, H.L. Schubert, C.P. Hill, Ubiquitin-binding domains, Nat. Rev. Mol. Cell. Biol. 6 (2005) 610–621.
[14] M.D. Marmor, Y. Yarden, Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases, Oncogene 23 (2004) 2057–2070.
[15] P.P. Di Fiore, S. Polo, K. Hofmann, When ubiquitin meets ubiquitin receptors: a signalling connection, Nat. Rev. Mol. Cell. Biol. 4 (2003) 491–497.
[16] D.J. Katzmann, G. Odorizzi, S.D. Emr, Receptor downregulation and multivesicular-body sorting, Nat. Rev. Mol. Cell. Biol. 3 (2002) 893–905.
[17] Y. Shiba, Y. Katoh, T. Shiba, K. Yoshino, H. Takatsu, H. Kobayashi, H.W. Shin, S. Wakatsuki, K. Nakayama, GAT (GGA and Tom1) domain responsible for ubiquitin binding and ubiquitination, J. Biol. Chem. 279 (2004) 7105–7111.
[18] R. Puertollano, J.S. Bonifacino, Interactions of GGA3 with the ubiquitin sorting machinery, Nat. Cell Biol. 6 (2004) 244–251.
[19] P.M. Scott, P.S. Bilodeau, O. Zhdankina, S.C. Winistorfer, M.J. Hauglund, M.M. Allaman, W.R. Kearney, A.D. Robertson, A.L. Boman, R.C. Piper, GGA proteins bind ubiquitin to facilitate sorting at the trans-Golgi network, Nat. Cell Biol. 6 (2004) 252–259.
[20] R. Mattera, R. Puertollano, W.J. Smith, J.S. Bonifacino, The trihelical bundle subdomain of the GGA proteins interacts with multiple partners through overlapping but distinct sites, J. Biol. Chem. 279 (2004) 31409–31418.
[21] A. d'Azzo, A. Bongiovanni, T. Nastasi, E3 ubiquitin ligases as regulators of membrane protein trafficking and degradation, Traffic 6 (2005) 429–441.
[22] A.A. de Melker, G. van der Horst, J. Calafat, H. Jansen, J. Borst, c-Cbl ubiquitinates the EGF receptor at the plasma membrane and remains receptor associated throughout the endocytic route, J. Cell Sci. 114 (2001) 2167–2178.
[23] S. Polo, S. Sigismund, M. Faretta, M. Guidi, M.R. Capua, G. Bossi, H. Chen, P. De Camilli, P.P. Di Fiore, A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins, Nature 416 (2002) 451–455.
[24] M. Katz, K. Shtiegman, P. Tal-Or, L. Yakir, Y. Mosesson, D. Harari, Y. Machluf, H. Asao, T. Jovin, K. Sugamura, Y. Yarden, Ligand-independent degradation of epidermal growth factor receptor involves receptor ubiquitylation and Hgs, an adaptor whose ubiquitin-interacting motif targets ubiquitylation by Nedd4, Traffic 3 (2002) 740–751.
[25] T.K. Sato, P. Rehling, M.R. Peterson, S.D. Emr, Class C Vps protein complex regulates vacuolar SNARE pairing and is required for vesicle docking/fusion, Mol. Cell 6 (2000) 661–671.
[26] M.R. Peterson, S.D. Emr, The class C Vps complex functions at multiple stages of the vacuolar transport pathway, Traffic 2 (2001) 476–486.
[27] V. Poupon, A. Stewart, S.R. Gray, R.C. Piper, J.P. Luzio, The role of mVps18p in clustering, fusion, and intracellular localization of late endocytic prganelles, Mol. Bio. Cell 14 (2003) 4015–4027.
[28] S.C. Richardson, S.C. Winistorfer, V. Poupon, J.P. Luzio, R.C. Piper, Mammalian late vacuole protein sorting orthologues participate in early endosomal fusion and interact with the cytoskeleton, Mol. Biol. Cell 15 (2004) 1197–1210.
[29] B.Y. Kim, H. Kramer, A. Yamamoto, E. Kominami, S. Kohsaka, C. Akazawa, Molecular characterization of mammalian homologues of class C Vps proteins that interact with syntaxin-7, J. Biol. Chem. 276 (2001) 29393–29402.
[30] S. Caplan, L.M. Hartnell, R.C. Aguilar, N. Naslavsky, J.S. Bonifacino, Human Vam6p promotes lysosome clustering and fusion in vivo, J. Cell Biol. 154 (2001) 109–122.
[31] S. Yogosawa, S. Hatakeyama, K.I. Nakayama, H. Miyoshi, S. Kohsaka, C. Akazawa, Ubiquitylation and degradation of polo-like kinase SNK by hVPS18, a RING-H2 type ubiquitin ligase, J. Biol. Chem. 280 (2005) 41619–41627.
[32] S.L. Miller, E. Malotky, J.P. O'Bryan, Analysis of the role of ubiquitin-interacting motifs in ubiquitin binding and ubiquitylation, J. Biol. Chem. 279 (2004) 33528–33537.

[33] S.C. Shih, G. Prag, S.A. Francis, M.A. Sutanto, J.H. Hurley, L. Hicke, A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain, EMBO J. 22 (2003) 1273–1281.
[34] M. Kawasaki, T. Shiba, Y. Shiba, Y. Yamaguchi, N. Matsugaki, N. Igarashi, M. Suzuki, R. Kato, K. Kato, K. Nakayama, S. Wakatsuki, Molecular mechanism of ubiquitin recognition by GGA3 GAT domain, Genes Cells 10 (2005) 639–654.
[35] A.M. Weissman, Themes and variations on ubiquitylation, Nat. Rev. Mol. Cell. Biol. 2 (2001) 169–178.
[36] R.C. Aguilar, B. Wendland, Ubiquitin: not just for proteasomes anymore, Curr. Opin. Cell Biol. 15 (2003) 184–190.
[37] Y. Katoh, Y. Shiba, H. Mitsuhashi, Y. Yanagida, H. Takatsu, K. Nakayama, Tollip and Tom1 form a complex and recruit ubiquitin-conjugated proteins onto early endosomes, J. Biol. Chem. 279 (2004) 24435–24443.
[38] M. Yamakami, T. Yoshimori, H. Yokosawa, Tom1, a VHS domain-containing protein, interacts with tollip, ubiquitin, and clathrin, J. Biol. Chem. 278 (2003) 52865–52872.
[39] K. Haglund, I. Dikic, Ubiquitylation and cell signaling, EMBO J. 24 (2005) 3353–3359.
[40] K. Haglund, P.P. Di Fiore, I. Dikic, Distinct monoubiquitin signals in receptor endocytosis, Trends Biochem. Sci. 28 (2003) 598–603.
[41] J. Kuriyan, D. Cowburn, Modular peptide recognition domains in eukaryotic signaling, Annu. Rev. Biophys. Biomol. Struct. 26 (1997) 259–288.
[42] M. Bienko, C.M. Green, N. Crosetto, F. Rudolf, G. Zapart, B. Coull, P. Kannouche, G. Wider, M. Peter, A.R. Lehmann, K. Hofmann, I. Dikic, Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis, Science 310 (2005) 1821–1824.
[43] D. Hoeller, N. Crosetto, B. Blagoev, C. Raiborg, R. Tikkanen, S. Wagner, K. Kowanetz, R. Breitling, M. Mann, H. Stenmark, I. Dikic, Regulation of ubiquitin-binding proteins by monoubiquitination, Nat. Cell Biol. 8 (2006) 163–169.
[44] T. Shiba, M. Kawasaki, H. Takatsu, T. Nogi, N. Matsugaki, N. Igarashi, M. Suzuki, R. Kato, K. Nakayama, S. Wakatsuki, Molecular mechanism of membrane recruitment of GGA by ARF in lysosomal protein transport, Nat. Struct. Biol. 10 (2003) 386–393.