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# VCIP135 acts as a deubiquitinating enzyme during p97–p47-mediated reassembly of mitotic Golgi fragments

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The AAA-ATPase p97/Cdc48 functions in different cellular pathways using distinct sets of adapters and other cofactors. Together with its adaptor Ufd1–Npl4, it extracts ubiquitylated substrates from the membrane for subsequent delivery to the proteasome during ER-associated degradation. Together with its adaptor p47, on the other hand, it regulates several membrane fusion events, including reassembly of Golgi cisternae after mitosis. The finding of a ubiquitin-binding domain in p47 raises the question as to whether the ubiquitin–proteasome system is also involved in membrane fusion events. Here, we show that p97–p47-mediated reassembly of Golgi cisternae requires ubiquitin, but is not dependent on proteasome-mediated proteolysis. Instead, it requires the deubiquitinating activity of one of its cofactors, VCIP135, which reverses a ubiquitylation event that occurs during mitotic disassembly. Together, these data reveal a cycle of ubiquitylation and deubiquitination that regulates Golgi membrane dynamics during mitosis. Furthermore, they represent the first evidence for a proteasome-independent function of p97/Cdc48.

## Introduction

p97/Cdc48 (in mammals also called VCP for valosin-containing protein) is an essential AAA-ATPase that participates in a wide variety of cellular pathways using distinct adapters and other cofactors (Woodman, 2003). These pathways include ubiquitin- and proteasome-dependent processing and degradation processes that are essential for homeostasis and cell cycle progression (Weissman, 2001). Its role is perhaps best illustrated during ER-associated degradation, where, together with its adaptor Ufd1–Npl4, it mediates the mobilization of ubiquitylated substrates into the cytosol for delivery to the proteasome (Bays et al., 2001; Ye et al., 2001; Braun et al., 2002; Jarosch et al., 2002). Although mobilization or unfolding activity is likely involved in all other p97/Cdc48-mediated processes, the extent to which they involve ubiquitin and the proteasome is still unclear.

This question is particularly relevant to p97/Cdc48-regulated membrane fusion events that reassemble fragmented organelles after mitosis, including the nuclear envelope, the ER, and the Golgi apparatus (Woodman, 2003). The best characterized is reassembly of Golgi cisternae from dispersed components at the end of mitosis. At least two pathways are involved, one catalyzed by p97, the other by another AAA-ATPase, N-ethylmaleimide–sensitive factor (NSF) (Shorter and Warren, 2002). NSF uses the energy of ATP hydrolysis to separate SNARE (soluble NSF attachment protein receptor) complexes that form during membrane fusion, thereby reactivating them for another round of fusion (Rothman, 1994; Jahn et al., 2003). However, NSF already completes this process during mitotic fragmentation of the Golgi apparatus, resulting in separated SNARE complexes on mitotic membranes (Muller et al., 2002). This in turn indicates the existence of additional regulatory mechanisms that are needed during the reassembly process.

In the case of NSF-mediated fusion, this mechanism is independent of ATP hydrolysis by NSF and involves the ubiquitin-like protein GATE-16, which regulates SNARE pairing (Muller et al., 2002). The functioning of p97, on the other hand, depends on its adaptor p47, which links it to the SNARE, syntaxin-5 (Rabouille et al., 1998). How this interaction regulates the formation of SNARE complexes that lead to membrane fusion has, however, remained elusive.

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Abbreviations used in this paper: MGF, mitotic Golgi fragment; MVB, multivesicular body; NSF, N-ethylmaleimide–sensitive factor; SNARE, soluble NSF attachment protein receptor; VCIP135, VCP(p97)/p47 complex–interacting protein of 135 kD.


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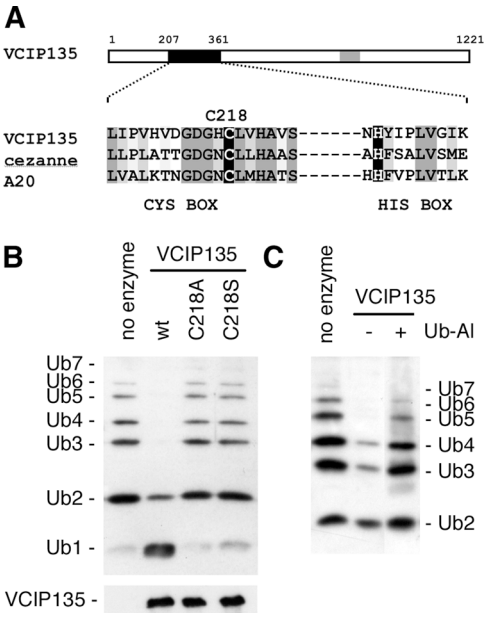


Figure 1. **VCIP135 is a deubiquitinating enzyme.** (A) The peptide sequence of VCIP135 contains a region with homology to the catalytic domains of the deubiquitinating enzymes, cezanne and A20 (filled box). The highly conserved CYS and HIS boxes are aligned, and the catalytic cysteine (position 218 in VCIP135) and histidine residues are highlighted. The position of a ubiquitin homology domain in VCIP135 is also indicated (gray). (B) Recombinant wild-type (wt) VCIP135 and two variants in which the catalytic cysteine is changed to either alanine or serine (C218A and C218S, respectively) were generated in bacteria. A mixture of in vitro–synthesized ubiquitin chains with lengths of 2–7 units (Ub2–7) was incubated alone, with wt, or with mutant VCIP135, and then analyzed by Western blotting with antibodies against ubiquitin (top) and VCIP135 (bottom). (C) The reaction was performed as in B with wt VCIP135 in the absence (−) or presence (+) of 5 μM ubiquitin-aldehyde (Ub-Al), an inhibitor of deubiquitinating enzymes.

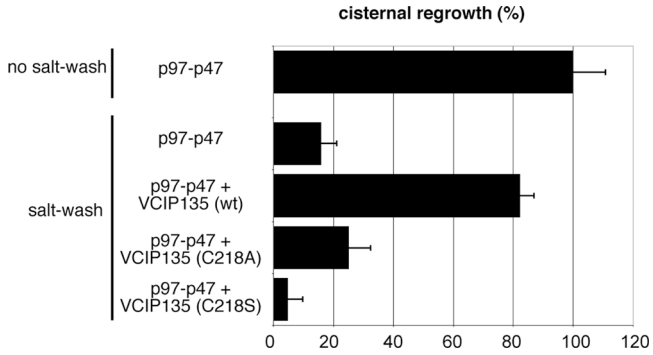


Figure 2. **The deubiquitinating activity of VCIP135 is required for p97–p47-mediated cisternal regrowth.** Rat liver Golgi membranes were fragmented by treatment with mitotic HeLa cytosol, and MGFs were reisolated and washed with low or high salt (no salt wash or salt wash, respectively). MGFs were incubated with p97–p47 alone or in the presence of VCIP135 or its inactive mutants, and cisternal regrowth was quantitated. Results are presented as the mean percentage of membranes in cisternae (±SEM), where 0% represents incubations with buffer alone (26.7 ± 3.1% of membrane in cisternae), and 100% represents MGFs incubated with p97–p47 (56.3 ± 3.2% of membrane in cisternae). Note that wt, but not mutant, VCIP135 could restore fusion activity after salt wash.

p47 also contains a ubiquitin-associated domain (UBA domain), which binds ubiquitin and is required for its activity in Golgi reassembly (Meyer et al., 2002). This in turn suggests (but does not prove) that ubiquitin is involved in p97-mediated reformation of Golgi cisternae and raises questions as to the role of ubiquitin in the process and whether this entails proteasome-dependent proteolysis.

A second cofactor, VCIP135 (VCP[p97]/p47 complex–interacting protein of 135 kD), was recently identified and shown to be needed for p97–p47-mediated reassembly in vitro and in vivo (Uchiyama et al., 2002). Both VCIP135 and p47 have ubiquitin homology domains that mediate binding to p97. VCIP135 binds to the p97–p47–syntaxin-5 complex and can dissociate p97 and p47 from syntaxin-5 (Uchiyama et al., 2002). Although this observation clearly suggests a very direct functional interaction between the three proteins, it does not provide an explanation for the role of VCIP135 in membrane fusion.

## Results and discussion

Using database searches, we identified a region in the peptide sequence of VCIP135 with homology to the catalytic domains of two previously identified deubiquitinating enzymes, cezanne and A20 (Evans et al., 2003; Fig. 1 A). Importantly, the catalytic cysteine residue is conserved at position 218 in VCIP135. To test whether VCIP135, too, is a deubiquitinating enzyme, we expressed it in bacteria along with two variants that contained substitutions of the catalytic cysteine with either alanine or serine (C218A and C218S, respectively). We incubated VCIP135 and its variants with a mixture of in vitro–synthesized oligo-ubiquitin chains (2–7 units, Ub2–Ub7). These chains serve as a substrate for deubiquitinating enzymes that specifically cleave the isopeptide bond between the units. Analysis of the chains after incubation revealed that recombinant VCIP135 disassembled ubiquitin chains, thereby generating monomeric ubiquitin (Fig. 1 B, Ub1). The two mutants, on the other hand, had no detectable activity. The addition of ubiquitin-aldehyde, an inhibitor of deubiquitinating enzymes, significantly reduced chain disassembly by wt VCIP135, indicating that its isopeptidase activity is indeed specific for ubiquitin (Fig. 1 C).

Next, we tested the relevance of this activity in p97–p47-mediated Golgi membrane fusion. The role of VCIP135 can be monitored in vitro (Uchiyama et al., 2002). When mitotic Golgi fragments (MGFs) were salt treated, p97–p47 was no longer sufficient to promote fusion. Addition of exogenous VCIP135 restored this activity, indicating that endogenous VCIP135 had been removed by the salt wash (Uchiyama et al., 2002). We were able to reproduce this published result in that salt treatment of MGFs reduced p97–p47-mediated cisternal regrowth by >80%, and readdition of recombinant VCIP135 restored fusion activity back to ~80% of the value reached without salt treatment (Fig. 2). The catalytically inactive mutants C218A and C218S, on the other hand, were not able to restore the fusion activity. C218S even inhibited compared with p97–p47 alone. These data show that the isopeptidase activity of VCIP135 is required for its function in membrane fusion

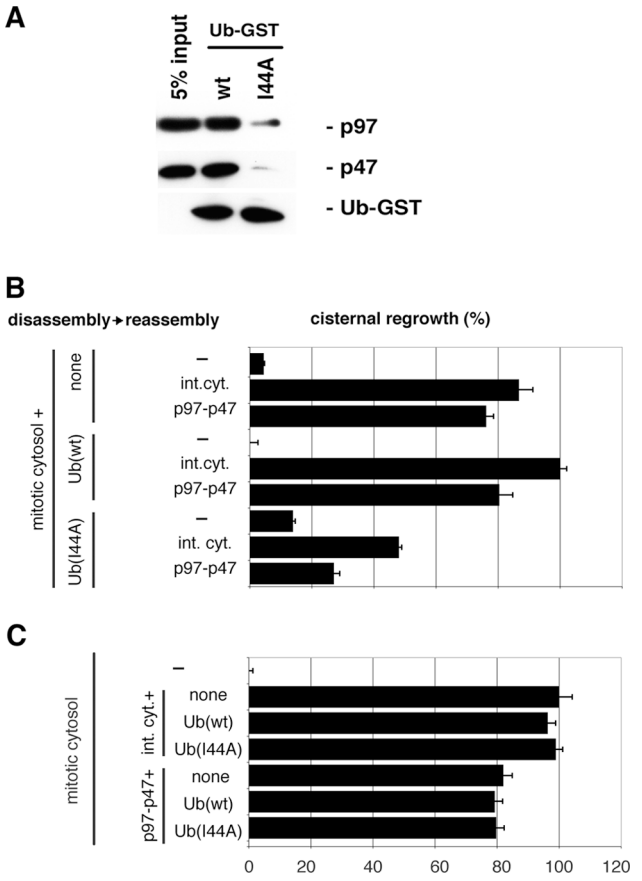


Figure 3. **Ubiquitylation during mitotic Golgi fragmentation is required for post-mitotic reformation of Golgi cisternae.** (A) A ubiquitin-GST fusion protein (Ub-GST, wt) and its isoleucine-44 to alanine variant (I44A) were immobilized and incubated with recombinant p97–p47 complex. The beads were isolated, and bound protein was analyzed by Western blotting together with 5% of the input p97–p47. (B) Golgi membranes were fragmented in the absence or presence of 20 μM exogenous recombinant ubiquitin (Ub[wt]) or the binding-deficient mutant thereof (Ub[I44A]). Membranes were then reisolated and either fixed (−) or incubated with either interphase cytosol (int. cyt.) or p97–p47, and cisternal regrowth was determined. Results are presented as mean percentage (±SEM), where 0% represents mitotic Golgi fragments that were treated with mitotic cytosol plus Ub (wt) and then fixed (25.0 ± 1.0% of membrane in cisternae), and 100% the same membranes reassembled in interphase cytosol (63.7 ± 0.8% of membrane in cisternae). (C) Golgi membranes were fragmented without exogenous ubiquitin. Membranes were reisolated, split into different reactions, and either fixed (−) or reassembled with either interphase cytosol or p97–p47, in the absence (none) or presence of exogenous Ub(wt) or Ub(I44A). The extent of cisternal regrowth was determined as in B.

and suggest that the role of VCIP135 in the reformation of Golgi cisternae from mitotic fragments is to remove ubiquitin from a putative substrate in the membrane.

One can envision two ways as to how this might work. VCIP135 could act antagonistically to a degradation process. This has been described for deubiquitinating enzymes such as HAUSP, which rescues the tumor suppressor p53 by removing ubiquitin moieties that would otherwise target it for degradation by the proteasome (Li et al., 2002; Wing, 2003). Alternatively, ubiquitin could represent a regulatory signal for membrane fusion that needs to be removed by VCIP135 at a specific point during the reaction. In this case, the process would resemble ubiquitin-dependent membrane transport processes in the endocytic or multivesicular body (MVB) pathways (Hicke and Dunn, 2003). Here, mono-ubiquitylation of substrates constitutes a sorting signal that marks cargo for delivery to its final destination. Transport is then mediated through interaction with a series of ubiquitin-binding proteins. In the MVB pathway, the final and obligatory step, however, involves the removal of ubiquitin from the substrate by the deubiquitinating enzyme Doa4p (Amerik et al., 2000; Dupre and Haguenauer-Tsapis, 2001; Katzmann et al., 2001; Losko et al., 2001), a process that triggers sorting to the internal vesicles of the MVBs through a mechanism that is still unclear.

In the case of Golgi reassembly, the results to date only suggest a role for ubiquitin, as does the presence of a ubiquitin-binding domain in p47. We therefore conducted experiments to provide direct evidence. We reasoned that a mutant of ubiquitin that could still be conjugated to substrate proteins, but could not be recognized by p97–p47, would act in a dominant-negative manner since it would be linked to a putative substrate but then block processing by p97 and its accessory factors. These properties have been described in other pathways for a ubiquitin variant with a substitution of isoleucine-44 to alanine (I44A) (Beal et al., 1996; Sloper-Mould et al., 2001). We first confirmed that binding of p97–p47 to ubiquitin is indeed abolished by this mutation. Recombinant p97–p47 complex was incubated with immobilized GST fused to ubiquitin that either represented the wt or the I44A form. Analysis of proteins bound to the two variants revealed that the I44A substitution almost completely abolished binding of p97–p47 to ubiquitin (Fig. 3 A).

We then studied the effect of the I44A ubiquitin variant on the Golgi system in two ways. First, we added wt or I44A ubiquitin to the mitotic cytosol that was used for the disassembly of the membranes, reisolated the membranes, and then performed the reassembly in the absence of exogenous ubiquitin with either interphase cytosol or pure p97–p47. In a second approach, we performed the disassembly under normal conditions and added the exogenous ubiquitin variants during the reassembly step, to either interphase cytosol or p97–p47. In both cases, the extent of Golgi disassembly and subsequent reassembly of cisternae was determined by stereological analysis of EM images. In these experiments, addition of exogenous VCIP135 was not required because the membranes were not salt washed. Furthermore, since the assay relied on physical removal of soluble mitotic regulators, such as cyclin B, that need to be degraded in vivo, this allowed us to look exclusively at the processes occurring on the membrane.

The addition of mutant ubiquitin had a dramatic effect but only when added during disassembly, not reassembly. The ability of interphase cytosol to stimulate reassembly decreased by ~50%, and the activity of p97–p47 was reduced by ~75%, compared with membranes fragmented in the presence of wt ubiquitin (Fig. 3 B). The fragmentation itself was only slightly affected. On the other hand, the addition of either the wt or the I44A variant to the reassembly reac-

tion of normally fragmented mitotic membranes did not affect cisternal regrowth (Fig. 3 C).

We next explored the role of the proteasome in the process. We first asked whether ubiquitin chains with the lysine-48 linkage that direct substrates to the proteasome were involved. We fragmented Golgi membranes in the presence of I44A or ubiquitin variants that had specific lysine-to-arginine mutations (K48R or K63R) to inhibit chain extension. Whereas the I44A mutant again inhibited subsequent Golgi reformation, neither K48R nor even K63R, which inhibits lysine-63 chains involved in certain proteasome-independent pathways, had any effect (Fig. 4 A). We then performed a series of experiments in the presence or absence of the proteasome inhibitors epoxomicin (Fig. 4 B) or MG132 (not depicted) during disassembly and/or reassembly with either interphase cytosol or p97–p47. The solvent used for these inhibitors, DMSO, was used as the control. Neither inhibitor had any effect on any step of the reaction, showing that mitotic Golgi disassembly–reassembly was proteasome independent. Lastly, we wanted to clarify the function of the deubiquitinating activity of VCIP135 in relation to the proteasome. If VCIP135 acted in removing ubiquitin from a crucial factor in order to rescue it from degradation by the proteasome, it would only be needed if the proteasome was functional. VCIP135 was, therefore, removed from MGFs by salt washing (swMGFs), and the effect of proteasome inhibitors was then tested. As shown in Fig. 4 C, the addition of proteasome inhibitors in the absence of VCIP135 did not restore cisternal regrowth. VCIP135 was essential and unaffected by the presence of either inhibitor. This shows that the function of VCIP135 cannot be bypassed by inhibiting the proteasome. VCIP135, therefore, plays an integral role in p97–p47-mediated reassembly.

These data directly show that ubiquitin is required for the mitotic Golgi cycle but in a proteasome-independent manner. Furthermore, they indicate that a critical ubiquitylation step occurs during mitotic fragmentation of the Golgi apparatus. This has important implications for our understanding of the dynamics. It means that the ubiquitin conjugate that is generated during fragmentation remains stable throughout mitosis until it is specifically recognized and processed by p97–p47 and VCIP135. This is best explained by the fact that the activity of p97–p47 is mitotically regulated (Uchiyama et al., 2003). Cdk1-mediated phosphorylation of p47 at the onset of mitosis removes the complex from the membrane. Dephosphorylation in telophase then allows rebinding and consequent processing of a substrate on the membrane. This temporal separation of ubiquitylation and processing is different from the mobilization step that is mediated by p97 and its adaptor Ufd1–Npl4 during ER-associated degradation. Here, ubiquitin modification of the substrate and its mobilization are directly coupled, since efficient ubiquitylation is dependent on the ATPase activity of p97 (Ye et al., 2003).

Together, our findings show that the reaction mediated by p97 and its adaptor p47 is very different from that mediated by Ufd1–Npl4. The temporal separation of ubiquitylation and processing, and the fact that the deubiquitinating activity of VCIP135 is required, but not the proteasome, indicate

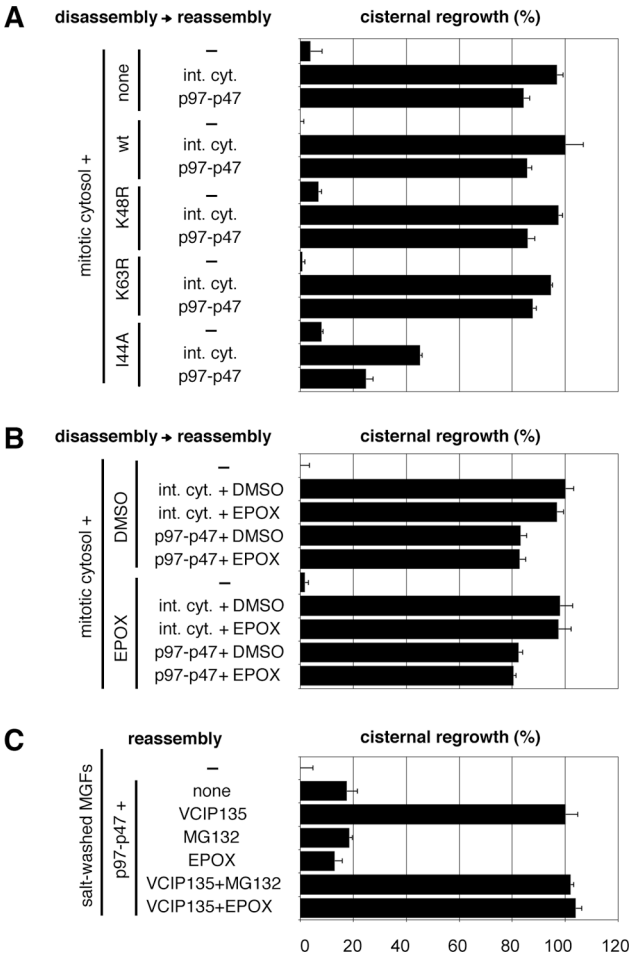


Figure 4. **The mitotic Golgi disassembly–reassembly cycle is proteasome independent.** (A) Golgi membranes were disassembled with mitotic cytosol as in Fig. 3 B in the absence or presence of the ubiquitin variants wt, K48R, K63R, or I44A. They were then reisolated and either fixed (−) or reassembled with either interphase cytosol (int. cyt.) or p97–p47 as indicated. Cisternal regrowth is presented as mean percentage of membranes in cisternae (0% represents 24.7 ± 0.5%, 100% represents 60.7 ± 2.5% in cisternae). Note that reassembly was reduced only when I44A was added to the disassembly incubation. (B) Golgi membranes were disassembled with mitotic cytosol in the presence of the proteasome inhibitor epoxomicin (EPOX, 10 μM) or its solvent, DMSO, as a control. Isolated mitotic membranes were then reassembled with either interphase cytosol (int. cyt.) or p97–p47, again in the presence or absence of EPOX (0% represents 27.0 ± 1.2%, 100% represents 61.7 ± 1.8% of membranes in cisternae). (C) MGFs were salt washed (swMGFs) to remove VCIP135 as in Fig. 2 and then either fixed (−) or reassembled with p97–p47. Reassembly was performed with either p97–p47 alone (none) or together with VCIP135, MG132, EPOX, or combinations as indicated (0% represents swMGFs with 25.3 ± 1.3% membranes in cisternae, 100% represents swMGF reassembled by p97–p47–VCIP135 with 53.5 ± 1.3% in cisternae). Note that the proteasome inhibitors did not substitute for the activity of VCIP135.

that the ubiquitin modification in this pathway is not just an intermediate on the way to the proteasome, but constitutes a regulatory signal as in ubiquitin-dependent membrane transport and other nondegradative pathways (Schnell and Hicke, 2003). This is supported by our previous observation that p47 preferably binds mono-ubiquitin, which usually serves as a regulatory signal, whereas Ufd1–Npl4 binds

lysine-48 linked poly-ubiquitin chains, which direct proteins to the proteasome (Meyer et al., 2002; Ye et al., 2003).

Exactly how ubiquitylation and deubiquitination regulate Golgi dynamics remains to be shown. The intimate interaction of the three factors, however, suggests a mechanism that is dependent on the recognition and processing of a ubiquitin conjugate by p97–p47 and subsequent deubiquitination by VCIP135. It is tempting to speculate that the target in this pathway is part of the fusion machinery that includes SNAREs and SNARE binding proteins. Ubiquitylation of one or more of these proteins could serve as a recruitment signal for p97–p47 that is then removed by VCIP135, leaving the p97–p47 complex to act on the SNAREs. Alternatively, ubiquitin could act, directly or indirectly, in inhibiting SNARE function. The crucial step would then be its removal by VCIP135, a process that may require the assistance of p97–p47. The exact mechanism, however, can only be clarified once the ubiquitylated targets are identified.

## Materials and methods

### Cloning, expression, and purification of VCIP135

VCIP135 was identified as a protein in rat liver cytosol that bound specifically to p97 and comigrated with Ufd2 (Meyer et al., 2000). It was cloned based on data obtained by mass spectrometry (GenBank/EMBL/DDBJ accession no. AF289091). The open reading frame was inserted into pTrcHis (Invitrogen) to generate a prokaryotic expression construct for 6xHis-tagged VCIP135, equivalent to the one used by Uchiyama et al. (2002). Mutations were introduced by changing the codon cysteine-218 to alanine or serine (C218A and C218S, respectively) using the Stratagene Quickchange method followed by confirmation of the entire sequence. Wt and variants were expressed in *Escherichia coli* and purified in parallel as follows. Cells were lysed in buffer A (300 mM KCl, 50 mM Tris, pH 7.4, 2 mM 2-mercaptoethanol, 5% glycerol, 20 mM imidazole, 0.1 mM PMSF). The proteins were isolated using Ni-agarose (QIAGEN) and desalted into buffer B (150 mM KCl, 50 mM Hepes, pH 7.4, 1 mM DTT, 5% glycerol). For the Golgi fusion assay, proteins were further purified by gel filtration (Superdex 200; Amersham Biosciences) in buffer B. Aliquots were frozen in liquid nitrogen and stored at −80°C.

### Expression and purification of other proteins

p97 was purified from rat liver cytosol. Recombinant p97, p47, and Ub-GST were generated in bacteria, and binding experiments were performed as previously described (Meyer et al., 2002). The mutations in ubiquitin were introduced by the Quickchange method and the sequence confirmed. Recombinant ubiquitin and variants were expressed in the *E. coli* strain BL21(DE3) codon+RIL (Stratagene). Cleared lysates were adjusted to 3.5% perchloric acid, and precipitated protein was removed by centrifugation. Ubiquitin was isolated from neutralized supernatants by cation-exchange chromatography.

### Deubiquitination assay

VCIP135 or its variants (2 μg each) were incubated with 0.5 μg of a mixture of oligo-ubiquitin chains (Affiniti UW8860) in 20 μl buffer (150 mM KCl, 50 mM Hepes, pH 7.4, 10 mM DTT, 5% glycerol, 0.01% Triton X-100) for 60 min at 30°C. Aliquots of the reaction were analyzed by Western blotting using an anti-ubiquitin antibody (Zymed Laboratories) and an anti-VCIP135 serum raised in rabbits against GST-VCIP135 (amino acids 870–1221). Ubiquitin-aldehyde was purchased from Calbiochem.

### Golgi reassembly assay

The Golgi reassembly assay was performed as described previously (Rabouille et al., 1995). In brief, rat liver Golgi was fragmented using cytosol from mitotic HeLa cells. MGFs were isolated and soluble factors removed by centrifugation through a 0.4 M sucrose cushion. When salt washes were applied, MGFs were incubated for 30 min in 1 M KCl and then recovered by centrifugation. For the reassembly step, membranes were incubated for 60 min at 37°C with either HeLa interphase cytosol or preformed complexes of p97 (100 ng/μl) and p47 (25 ng/μl). VCIP135 or mutants thereof were added at 30 ng/μl where indicated. Samples were fixed and processed for EM, and cisternal regrowth was quantitated as previously described (Rabouille et al., 1995). Recombinant ubiquitin variants were added at 20 μM where indicated. MG132 and epoxomicin were from Calbiochem and used at 100 and 10 μM, respectively.

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