Published in final edited form as:

J Mol Biol. 2009 January 23; 385(3): 718–725. doi:10.1016/j.jmb.2008.11.018.

Identification of lysines within membrane-anchored Mga2p120 that are targets of Rsp5p ubiquitination and mediate mobilization of tethered Mga2p90

Sabyasachi Bhattacharya^{1,*}, Natalia Shcherbik^{1,*}, Julian Vasilescu², Jeffrey C. Smith², Daniel Figeys², and Dale S. Haines^{1,3}

¹ Fels Institute for Cancer Research and Molecular Biology, Ottawa, Ontario, K1H 8M5, Canada

² Ottawa Institute of Systems Biology, Ottawa, Ontario, K1H 8M5, Canada

³ Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania, 19140, USA

Summary

Mga2p90 is an endoplasmic reticulum (ER) localized transcription factor that is released from the ER membrane by a unique ubiquitin-dependent mechanism. Mga2p90 mobilization requires poly-ubiquitination of its associating membrane-bound Mga2p120 anchor and subsequent Mga2p120-Mga2p90 complex disassembly that is mediated by ATPase Cdc48p and its heteromeric ubiquitin-binding adaptor Npl4p-Ufd1p. Although previous studies have identified the ubiquitin ligase (i.e. Rsp5p) and ligase binding site on Mga2p120 that play a role in this process, the amino acids of Mga2p120 that are targets of ubiquitination and promote Mga2p90 mobilization are unknown. We have identified using mass spectrometry analysis of *in vitro* ubiquitinated Mga2p120-Mga2p90 complex that lysines 983 and 985 contained within the carboxy-terminal domain of Mga2p120 are Rsp5p-directed ubiquitin conjugation sites. Mutation of these residues as well as proximally located lysine 980 results in suppression of Mga2p120 ubiquitination *in vitro* and *in vivo*, inefficient liberation of Mga2p90 by Cdc48p^{Npl4p/Ufd1p} *in vitro*, and ER retention of Mga2p in cells. Moreover, *mga2A/spt23ts* harboring Rsp5p binding and conjugation *mga2* mutants express low *OLE1* (an Mga2p90 target gene) transcripts and display reduced growth. We conclude that residues 980, 983 and 985 are targets of Rsp5p-induced poly-ubiquitination and mediate Cdc48p^{Npl4p/Ufd1p}-dependent Mga2p90-Mga2p120 separation and Mga2p90 mobilization.

Ubiquitination is a post-translation modification process where a 76 amino acid polypeptide called ubiquitin is covalently attached to a lysine residue of substrate (1,2). This process requires the coordinated activities of an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and an ubiquitin ligase (E3). While an attachment of a single ubiquitin moiety can occur, generally, this reaction occurs repeatedly, leading to the attachment of ubiquitin to internal lysines of ubiquitin and/or proximal lysine residues on the substrate. The best understood consequence of substrate ubiquitination is targeting the protein for destruction by the 26S proteasome (3). It is becoming increasingly apparent however that an ubiquitin

Address correspondence to Dale S. Haines, PhD. Temple University School of Medicine, 3307 North Broad Street, Philadelphia PA 19140. dhaines@temple.edu).

*Equal contributors

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

signal can regulate protein function by non-degradative mechanisms, including modulating protein-protein interactions (4).

Ubiquitination plays unique roles in regulating the expression and localization of the Mga2p transcriptional regulator. Mga2p is one of two (Spt23p being the other) homologous endoplasmic reticulum (ER) localized transcription factors in *Saccharomyces cerevisiae* (5). Full-length Mga2p is approximately 120 kDa in size and harbors a carboxy-terminal localized transmembrane domain. It is retained at the ER after synthesis and undergoes homo-dimerization, which is mediated by the centrally localized IPT (Ig-like/Plexin/Transcription factor) domain (6,7). After dimerization, ubiquitination of one of the 120 kDa monomers recruits the proteasome, resulting in endoproteolytic cleavage and bi-directional degradation of one of the monomers (8,9). While this process destroys the carboxy-terminal domain of the monomer, the proteasome is unable to degrade the central and amino-terminal domains of Mga2p. A 90 kDa Mga2p fragment (termed Mga2p90) remains tethered to the membrane via an interaction with its 120 kDa unprocessed anchor (termed Mga2p120) (6,7).

In addition to facilitating Mga2p90 generation, ubiquitination plays an unusual role in promoting release the processed product from the ER. This ubiquitin-directed event does not involve the proteasome, but is dependent on the Cdc48p and its ubiquitin-binding adaptor comprised of Npl4 and Ufd1p. Cdc48p (also called p97 or VCP) is a highly conserved member of the AAA (ATPase Associated with diverse cellular Activities) ATPase family of molecular chaperones. This protein, in collaboration with specific adaptor proteins that bind to ubiquitinated substrates, participates in numerous ubiquitin selective processes such as ubiquitination, deubiquitination, and ubiquitin-dependent protein complex disassembly (10). It is believed that Cdc48p applies mechanical force onto substrates upon ATP hydrolysis, leading to p97-dependent changes in their structure and subsequent biochemical events (11). Ubiquitination of Mga2p120 by the Rsp5p E3 ligase recruits Cdc48p^{Npl4p/Ufd1p} to an Mga2p120-Mga2p90 complex. This interaction results in Cdc48p^{Npl4p/Ufd1p}-mediated Mga2p120-Mga2p90 complex disassembly and release of Mga2p90 from the membrane (12). Upon release, Mga2p90 is capable of migrating to the nucleus where it induces transcription of *OLE1* and other genes involved in lipid metabolism (5,13).

Previous studies have identified the ubiquitin ligase and ligase binding site on Mga2p120 that play a role in Mga2p90 release (12,14). However, the amino acids of Mga2p120 that are targets of ubiquitination and mediate mobilization have yet to be identified. We employed mass spectrometry analysis with *in vitro* ubiquitinated product to identify lysine residues of Mga2p120 that are ubiquitinated by Rsp5p. To generate the substrate, microsomes were first prepared from yeast harboring an amino terminal FLAG-tagged *MGA2* expression construct. Mga2p120-Mga2p90 complexes were immunopurified from microsomes using anti-FLAG antibody conjugated agarose. Bead-bound complexes were then placed in ubiquitination reactions containing E1, E2 and Rsp5p. After termination of these reactions, complexes were washed, resuspended in SDS-PAGE loading buffer, boiled, and resolved by SDS-PAGE. After staining the gel with coomassie blue (Fig 1A) a region containing high molecular weight proteins was excised, subjected to in-gel tryptic digestion and the resulting peptides were analyzed by LC-MS/MS. The analysis successfully identified a peptide matching to Mga2p120 with two ubiquitin conjugation sites at lysine residues 983 and 985 (Fig 1B). This ubiquitinated peptide was assigned a Mascot score of 53, which indicated a high-confidence identification (15).

Based on this information, site directed mutagenesis was performed to generate an *mga2* expression construct encoding FLAG-tagged protein harboring arginine substitutions at residues 983 and 985 (termed Mga2p-2K). Mga2p120-Mga2p90 complexes were purified from cells expressing this mutant and subjected to *in vitro* ubiquitination by Rsp5p. In initial

experiments, as well as the one presented in Fig 2A, mutation of these two amino acids leads to suppression of Rsp5p-induced Mga2p120 ubiquitination. This is evidenced by a reduction in high molecular weight proteins measured by western blotting using anti-FLAG or anti-Ubiquitin (Ub) antibodies. However, high molecular weight Mga2p120 products were evident in reactions harboring this mutant when westerns were probed with the more sensitive anti-Ub antibody, suggesting the presence of additional Rsp5p-induced ubiquitin conjugation sites. Considering that Mga2p harbors another lysine residue at 980 that is in close proximity to 983 and 985 and a peptide fragment containing this amino acid was not covered by the LC-MS/MS analysis (unpublished data), we generated a *mga2* construct encoding a Mga2p120 mutant containing arginine substitutions at 980, 983 and 985 (termed Mga2p-3K). Complexes were again purified from cells harboring this triple lysine mutant as well as those from yeast harboring *mga2-2k* or *mga2 Δ lpky*. *mga2 Δ lpky* encodes a mutant protein lacking the Rsp5p binding site (14) on Mga2p120 and serves as an important control for this and subsequent experiments. Mga2p120-Mga2p90 complexes were then placed in ubiquitination reactions with varying amounts of Rsp5p. As shown in Fig 2A, the Mga2p-3K and Mga2p Δ LPKY mutants are more resistant to Rsp5p-mediated ubiquitination when compared to Mga2p-2K. It also deserves to be mentioned that mutation of any one of these amino acids individually has no detectable effect on Rsp5p ubiquitination (data not shown). These results suggest that lysine residues 980, 983 and 985 can serve as potential acceptor sites for Rsp5p-induced Mga2p120 ubiquitination *in vitro* and that mutation of at least two of these sites are need to suppress Rsp5p-induced ubiquitination.

To determine if mutation of these sites affects Mga2p120 ubiquitination in cells, we measured the amount of ubiquitin modified protein directly after immunopurification of wild-type and mutant proteins with anti-FLAG antibody. Since the amount of ubiquitinated Mga2p120 represents only a small fraction of total Mga2p120 after immunopurification, a greater amount of cells were used for immunopurification. Also, we analyzed increased amounts of immunopurified protein when compared to the inputs for the Rsp5p dependent ubiquitination reactions presented in Fig 1A. Consistent with the *in vitro* ubiquitination data, the Mga2p120 mutant harboring mutations in 983 and 985 displayed reduced ubiquitination (Fig 2B). This figure also shows that elimination of all three sites results in a further reduction in the amount of ubiquitinated Mga2p120 and the amount of ubiquitin modified Mga2p120 immunoprecipitated from cells harboring the *mga2-3k* mutant was similar to that immunopurified from cells harboring the Rsp5p binding deficient *mga2 Δ lpky* mutant. These results suggest that lysine residues 980, 983 and 985 can also serve as targets of the ubiquitination machinery *in vivo*.

One trivial explanation for the observed results is that the engineered mutations abrogate an Mga2p120-Rsp5p interaction. To investigate this possibility, we performed *in vitro* binding assays with recombinant GST-tagged Rsp5p and immunopurified FLAG-tagged Mga2p120-Mga2p90 complexes. As shown in the right panel of Fig 2C, Rsp5p interacts with Mga2p120-Mga2p90 complexes immunopurified from cells harboring *MGA2* or *mga2-3k*, but not *mga2 Δ lpky*. Consistent with this *in vitro* binding data, we also found that Rsp5p bound Mga2p120-Mga2p complex in cells containing *mga2-3k* as assessed by co-immunoprecipitation analysis (Fig 2D).

Rsp5p promotes mobilization of Mga2p90 from the ER membrane by promoting poly-ubiquitination of its interacting membrane bound Mga2p120 anchor. This Rsp5p dependent activity is dependent on Cdc48p^{Npl4p/Ufd1p}, which recognizes the poly-ubiquitinated signal on Mga2p120 and facilitates segregation of Mga2p90 from Mga2p120 (12). Thus, we next wanted to determine how mutation of the identified carboxy-terminal localized Mga2p120 conjugation sites affects Cdc48p^{Npl4p/Ufd1p}-dependent Mga2p90 mobilization *in vitro*. For these and subsequent experiments, we used only the Mga2p-3K Rsp5p ubiquitin conjugation defective

mutant. Microsomes were prepared from cells expressing amino-terminal FLAG-tagged Mga2p products. Microsomes were placed into reactions containing ATP, an ATP regeneration system and recombinant Cdc48p or preformed recombinant Cdc48p^{Npl4p/Ufd1p} complex (left panel of Fig 3A). The ATP depleting enzyme Apyrase was also included in a set of reactions containing Cdc48p^{Npl4p/Ufd1p} to verify that mobilization is ATP dependent. Reactions were subjected to ultracentrifugation and the amount of Mga2p90 and Mga2p120 in the soluble and membrane fractions were measured by western blotting using anti-FLAG antibody. As shown in the right panel of Fig 3A, the greatest amount of soluble Mga2p90 was detected in reactions containing microsomes prepared from cells harboring *MGA2* and recombinant Cdc48p^{Npl4p/Ufd1p} complex. Only background Mga2p90 release was detected in the other reactions, including those containing Cdc48p^{Npl4p/Ufd1p} and microsomes derived from yeast expressing the Rsp5p binding deficient mutant Mga2p Δ LPKY or the Rsp5p conjugation mutant Mga2p-3K (Fig 3A).

In addition to this *in vitro* approach, we also determined the localization of Mga2p in cells containing *MGA2*, *mga2 Δ lpky*, and *mga2-3k* by indirect immunofluorescence using the anti-FLAG antibody, which recognizes both Mga2p120 and Mga2p90. As shown in Fig 3B, Mga2p displays a mix of perinuclear and nuclear staining in cells harboring *MGA2*. In contrast, cells expressing the Rsp5p binding or lysine conjugation mutants show anti-FLAG positive “half-moon structures”, which is a marker of Mga2p90 ER sequestration that has been reported previously (7,8). The ER sequestration is also confirmed by co-localization with the ER-localized protein Kar2p (Fig 3B). These experiments suggest that these lysine residues on membrane-bound Mga2p120 mediate the release of the tethered Mga2p90 product.

We next evaluated the biological consequences of mutating these residues on Mga2p90 transcriptional regulation function *in vivo*. We first generated low copy *MGA2*, *mga2 Δ lpky* and *mga2-3k* expression constructs under the control of the native *MGA2* promoter. We also generated expression constructs that harbored single mutations at each of the lysine residues. These constructs, alone with an empty vector control, were transformed into *mga2 Δ spt23ts* cells. These cells are not viable at elevated temperatures due to lack of both Mga2p and Spt23p, which perform redundant functions inducing the expression of the essential gene, *OLE1* (5). Their growth defects can be suppressed however by the addition of oleic acid to the growth media (5). After transformation with the indicated constructs, cells were grown at 25°C and then placed at the non-permissive temperature of 34°C. Cells were harvested, RNA prepared and RNA levels of the Mga2p90 target gene *OLE1* were measured by northern blotting. As shown in Fig 4A, cells harboring *mga2 Δ lpky* or *mga2-3k* express lower *OLE1* RNA amounts when compared to those with *MGA2*. *OLE1* levels are however higher in *mga2 Δ lpky* or *mga2-3k* cells when compared to the vector alone control (Fig 4A). Cells containing the single lysine *mga2* mutants did not display reduced *OLE1* levels.

We also assessed the proliferative capacity of *mga2 Δ spt23ts* cells transformed with these constructs when grown at non-permissive temperature, both in the absence and presence of oleic acid. As shown in Fig 4B, cells harboring the *mga2 Δ lpky* and *mga2-3k* constructs do not proliferate to the same extent as those containing *MGA2*, *mga2-k980r*, *mga2-k983r* or *mga2-k985r* in the absence of oleic acid, but do grow better than cells containing the empty vector control. There was not a noticeable difference in the growth of any of these transformants when cells were plated on oleic acid containing media (Fig 4B). These results suggest that elimination of the Rsp5p binding and ubiquitin conjugation sites on Mga2p120 suppresses, but does not eliminate, its *OLE1*-inducing function when cells are grown at elevated temperature.

The conjugation of ubiquitin to lysine residues on a substrate by the ubiquitination machinery is generally promiscuous in nature. This lack of ubiquitin conjugation specificity has made it very difficult to identify specific residues on proteins that mediate ubiquitin controlled

signaling events. Taking Rsp5p as an example, numerous substrates for this ligase have been identified (16,17). However, ubiquitin conjugation sites for the vast majority of Rsp5p substrates have yet to be identified. We set out to identify lysine residues of Mga2p120 that are targets for Rsp5p-induced ubiquitination and provide evidence here that three lysine residues, 980, 983, and 985, all located carboxy-terminal to the LPKY motif, can serve as sites for Rsp5p-induced ubiquitin attachment both *in vitro* and *in vivo*. Moreover, mutation of these residues abrogates Cdc48p^{Npl4p/Ufd1p}-mediated mobilization of Mga2p90 from microsomes *in vitro* and ER sequestration of Mga2p90 in cells. Considering that these residues are contained within the membrane-bound Mga2p120 anchor and not in tethered Mga2p90, these findings further support the model (12) where Cdc48p^{Npl4p/Ufd1p}-mediated segregation of Mga2p120-Mga2p90 complexes is mediated by an ubiquitin signal present on Mga2p120. Although all three sites can serve as conjugation sites, it remains unclear if there is a preference for a specific residue or residues under conditions where all three sites are available.

As stated in the introduction, *SPT23* is a homologue of *MGA2* and their encoded protein p90 products undergo Rsp5p dependent release and possess redundant *OLE1* transactivation function (5). We have also attempted to identify Rsp5p-directed ubiquitin conjugation sites on Spt23p120. Unfortunately, using a similar approach described here for Mga2p120, we have not been able to identify Rsp5p-induced ubiquitin conjugation sites on Spt23p120. Moreover, we have mutated 11 lysine residues (alone and in combination) in the carboxy-terminal region of Spt23p120 extending from amino acid 900 to the transmembrane domain and have seen no affect on Spt23p120 ubiquitination, processing or mobilization (SB, NS, and DSH, unpublished data). These lysine residues include five that are amino-terminal (901, 903, 908, 914, and 917) and six that are carboxy-terminal (947, 948, 950, 957, 996, and 997) to the Rsp5p binding LPKY site (938–941). It is conceivable that the Rsp5p-induced ubiquitin signal is placed on lysine residues present in a different locale and there are important functional consequences for this differential placement. Alternatively, the location of ubiquitin conjugation on Spt23p120 may not be as stringent when compared to Mga2p120 and this is preventing us from detecting the true ligation sites, which is contained within the carboxy-terminus of Spt23p120.

Although Rsp5p is largely dispensable for Mga2p120 processing, it has been shown previously by us that it mediates release of Mga2p90 from the ER (12,14). We were thus somewhat surprised by the rescue data presented here as we were expecting that both the Rsp5p binding and ubiquitin conjugation mutants would be unable to rescue the growth defects of *mga2Δspt23ts*. While *mga2Δspt23ts* cells transformed with *mga2Δlpky* and *mga2-3k* do not grow as well when compared to those containing *MGA2*, they proliferate better than the vector alone control. It is possible based on these results that multiple ligases promote Mga2p90 release and Rsp5p plays a contributing rather than an obligatory role. Consistent with this idea, we have found that despite displaying reduced ubiquitination, ubiquitin modified forms of Mga2pΔLPKY and Mga2p-3k can be detected after immunopurification of these mutant proteins from cells (Fig 2B and our unpublished overexposed blots). It is also interesting to note that deletion of the Rsp5p binding and conjugation sites on Mga2p also has a slight effects on Mga2p processing as the ratio of Mga2p90 to Mga2p120 is reduced in cells harboring these mutants. Thus, it is possible that Rsp5p and yet to be identified ligase(s) play roles in providing both the processing and release signals. However, in the absence of knowing ligase(s) that performs such functions, we can't rule out the possibility that Rsp5p-independent Mga2p processing and release is mediated by signals other than ubiquitination. Notwithstanding, it will be interesting to elucidate Rsp5p independent processes that contribute to Mga2p120 processing and/or inducing release of processed Mga2p90 from the ER.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work is supported by NIH grant GM070769 to DSH. We thank David Garfinkel for the *mga2Δspt23ts* strain.

References

1. Hochstrasser M. Ubiquitin-dependent protein degradation. *Annu Rev Genet* 1996;30:405–439. [PubMed: 8982460]
2. Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem* 1998;67:425–479. [PubMed: 9759494]
3. Chau V, Tobias JW, Bachmair A, Marriott D, Ecker DJ, Gonda DK, Varshavsky A. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* 1989;243:1576–1583. [PubMed: 2538923]
4. Haglund K, Dikic I. Ubiquitylation and cell signaling. *EMBO J* 2005;24:3353–3359. [PubMed: 16148945]
5. Zhang S, Skalsky Y, Garfinkel DJ. MGA2 or SPT23 is required for transcription of the delta9 fatty acid desaturase gene, OLE1, and nuclear membrane integrity in *Saccharomyces cerevisiae*. *Genetics* 1999;151:473–483. [PubMed: 9927444]
6. Rape M, Hoppe T, Gorr I, Kalocay M, Richly H, Jentsch S. Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48(UFD1/NPL4), a ubiquitin-selective chaperone. *Cell* 2001;107:667–677. [PubMed: 11733065]
7. Shcherbik N, Zoladek T, Nickels JT, Haines DS. Rsp5p is required for ER bound Mga2p120 polyubiquitination and release of the processed/tethered transactivator Mga2p90. *Curr Biol* 2003;13:1227–1233. [PubMed: 12867034]
8. Hoppe T, Matuschewski K, Rape M, Schlenker S, Ulrich HD, Jentsch S. Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell* 2000;102:577–586. [PubMed: 11007476]
9. Piwko W, Jentsch S. Proteasome-mediated protein processing by bidirectional degradation initiated from an internal site. *Nat Struct Mol Biol* 2006;13:691–697. [PubMed: 16845392]
10. Schubert C, Buchberger A. UBX domain proteins: major regulators of the AAA ATPase Cdc48/p97. *Cell Mol Life Sci*. 2008 Apr 26;Epub ahead of print
11. Halawani D, Latterich M. p97: The cell's molecular purgatory? *Mol Cell* 2006;22:713–717. [PubMed: 16793541]
12. Shcherbik N, Haines DS. Cdc48p(Npl4p/Ufd1p) binds and segregates membrane-anchored/tethered complexes via a polyubiquitin signal present on the anchors. *Mol Cell* 2007;25:385–397. [PubMed: 17289586]
13. Auld KL, Brown CR, Casolari JM, Komili S, Silver PA. Genomic association of the proteasome demonstrates overlapping gene regulatory activity with transcription factor substrates. *Mol Cell* 2006;21:861–871. [PubMed: 16543154]
14. Shcherbik N, Kee Y, Lyon N, Huibregtse JM, Haines DS. A single PXY motif located within the carboxyl terminus of Spt23p and Mga2p mediates a physical and functional interaction with ubiquitin ligase Rsp5p. *J Biol Chem* 2004;279:53892–53898. [PubMed: 15466864]
15. Vasilescu J, Smith JC, Zweitzig DR, Denis NJ, Haines DS, Figeys D. The proteomic reactor facilitates the analysis of affinity-purified proteins by mass spectrometry: application for identifying ubiquitinated proteins in human cells. *J Mass Spectrom* 2008;43:296–304. [PubMed: 17957819]
16. Gupta R, Kus B, Fladd C, Wasmuth J, Tonikian R, Sidhu S, Krogan NJ, Parkinson J, Rotin D. Ubiquitination screen using protein microarrays for comprehensive identification of Rsp5 substrates in yeast. *Mol Syst Biol* 2007;3:116. [PubMed: 17551511]

17. Kus B, Gajadhar A, Stanger K, Cho R, Sun W, Rouleau N, Lee T, Chan D, Wolting C, Edwards A, Bosse R, Rotin D. A high throughput screen to identify substrates for the ubiquitin ligase Rsp5. *J Biol Chem* 2005;280:29470–29478. [PubMed: 15955809]

Figure 1. Mga2p120 is ubiquitinated on lysine 983 and 985 by Rsp5p

(A) Microsomes from cells expressing FLAG-tagged Mga2p120-Mga2p90 complex were collected and the proteins from the microsomes were eluted and subjected to immunoprecipitation with anti-FLAG antibodies. *In vitro* ubiquitination assays were performed using the immunoprecipitated complexes and recombinant E1, E2 and E3 (Rsp5p) as described in supplemental information. Proteins were separated by SDS-PAGE and the gel was stained with coomassie blue. (B) Model depicting Mga2p120 (unprocessed membrane-bound form) and Mga2p90 (processed membrane-tethered form) with noted IPT domain, 2 ankyrin (Anks) repeats, and transmembrane domain. Also shown is the Rsp5p interaction motif LPKY, the ubiquitinated lysine residues that were identified (983 and 985) (blue) by MS/MS analysis (described in detail in supplemental information), and the lysine residue in proximity to the identified residues (980) (green) that was not covered by the MS/MS analysis.

Figure 2. Mutations at lysine 980, 983 and 985 of Mga2p120 abolish Rsp5p mediated poly-ubiquitination

(A) Mga2p120-Mga2p90 complexes were immunoprecipitated from cells harboring galactose inducible *FLAGMGA2*, *FLAGmga2Δlpky*, *FLAGmga2-2k* or *FLAGMga2-3k* as described in supplemental information. The immunoprecipitated proteins were subjected to an *in vitro* ubiquitination assay using recombinant Rsp5p. Modified proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and western blotting was performed with anti-Ubiquitin and anti-FLAG antibodies. (B) Lysates from cells harboring the indicated expression constructs were subjected to immunoprecipitation with anti-FLAG antibodies. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and proteins were detected by western blotting with anti-Ubiquitin and anti-FLAG antibodies. (C) Immunoprecipitated Mga2p120-Mga2p90 complexes from cells harboring *FLAGMGA2*, *FLAGmga2Δlpky*, *FLAGmga2-2k* or *FLAGMga2-3k* were liberated from the beads with FLAG peptide and used in *in vitro* pull down assay with immobilized GST-Rsp5p. Bound material was resolved by SDS-PAGE, transferred to a nitrocellulose membrane and detected by western blotting with anti-FLAG antibodies. The amount of GST-Rsp5p used in the pull down assays was determined by Ponceau S staining of the blot prior to transfer. The left panel shows the input for each Mga2p protein and * denotes an alternatively processed Mga2p product. (D) Cells expressing HA-tagged Rsp5p and/or FLAG-tagged Mga2p proteins were lysed and immunoprecipitations were performed with anti-FLAG antibody. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose

membranes and the membranes were probed with either anti-HA or anti-FLAG antibodies. The left panel shows input levels of Mga2p and Rsp5p proteins in the extracts as determined by western blotting. These and all other experiments presented here were repeated three times and similar results were obtained.

Figure 3. Lysine 980, 983 and 985 of Mga2p120 mediate mobilization of Mga2p90 from membranes *in vitro* and *in vivo*

(A) (*left panel*) Recombinant Cdc48p and Cdc48p^{Npl4p/Ufd1p} complex was prepared, resolved by SDS-PAGE gel and visualized by coomassie blue staining to assess purity as described in supplemental information. (*right panel*) Microsomes were isolated from cells containing *FLAGMGA2*, *FLAGmga2p $\Delta lpky$* , or *FLAGmga2p-3k*, and an *in vitro* mobilization assays were performed as described previously (12) with BSA, Cdc48p or Cdc48p^{Npl4p/Ufd1p} complex with or without Apyrase. Following completion of the assay the soluble and insoluble fractions were separated by ultracentrifugation and amounts of Mga2p products in the two fractions were determined by western blotting with anti-FLAG antibodies. (B) Cells harboring *FLAGMGA2*, *FLAGmga2p $\Delta lpky$* , or *FLAGmga2p-3k* were fixed and localization of Mga2p in these cells was determined by indirect immunofluorescence as described in supplemental information using anti-FLAG mouse monoclonal antibody. Cells were also co-stained with rabbit anti-Kar2p polyclonal antibodies. FLAG tagged proteins were visualized by an Alexa fluor 647 (red)-conjugated anti-mouse secondary antibody while Kar2p was visualized by FITC (green)-conjugated anti-rabbit secondary antibody. N=nuclear.

Figure 4. Lysine 980, 983 and 985 of Mga2p120 negatively affect its function *in vivo*
 (A) *mga2Δspt23-ts* cells were transformed with plasmids harboring *MGA2*, *mga2-k980r*, *mga2-k983r* or *mga2-k985r*, *mga2Δlpky* and *mga2-3k*. Cells were grown up at 25°C and then switch to 34°C for 16 hrs. Cells were harvested, RNA was prepared and northern blotting was carried-out with radiolabeled *OLE1*. RNA loading was assessed by ethidium bromide staining of gel prior to transfer and levels of 28s and 18s rRNA are depicted in the bottom panel. (B) Cells transformed with the indicated constructs were grown-up at 25°C, serially diluted and plated on synthetic dropout media with or without oleic acid. Plates were incubated at 34°C for 2–3 days.