

Rsp5p Is Required for ER Bound Mga2p120 Polyubiquitination and Release of the Processed/Tethered Transactivator Mga2p90

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Summary

A number of eukaryotic transcription factors are held in a latent state by being embedded in, or tethered to, cellular membranes. Mga2p of *Saccharomyces cerevisiae* is an endoplasmic reticulum (ER)-localized transcription factor that plays an overlapping role with homologous Spt23p in upregulating expression of *OLE1*, a gene required for the synthesis of essential oleic acid [1]. Previous studies have documented that proteasome-dependent processing of ER bound 120 kDa Mga2p and Spt23p proteins generates transcriptionally competent 90 kDa polypeptides [2]. In the case of Spt23p90, it is held at the membrane prior to release via a self-interaction with the unprocessed Spt23p120 anchor [3]. It is currently thought that the highly conserved Rsp5p ubiquitin ligase provides the signal for partial degradation of both proteins. Cells lacking Rsp5p function require oleic acid for growth, and Spt23p processing is suppressed in *rsp5Δ* cells and in wild-type *RSP5* cells upon expression of Rsp5p dominant-negative mutants [2, 4]. We report here that Rsp5p is dispensable for Mga2p90 generation but not for release of the processed product from the ER. In addition, we demonstrate that polyubiquitinated Mga2p120 accumulates in cells lacking Npl4p or proteasome function and Rsp5p is required for Mga2p120 polyubiquitination. Finally, we provide evidence that Mga2p90 and Mga2p120 dimerize and that Rsp5p binds heterodimeric Mga2p complexes both in vitro and in vivo. In light of these experiments, we propose that Rsp5p facilitates Mga2p90 release from the ER by promoting polyubiquitination and Npl4p-proteasome-mediated degradation of the interacting Mga2p120 ER bound anchor.

Results

Rsp5p Is Dispensable for Processing but Not ER Release of Mga2p

Prior studies suggest that transcriptionally active Spt23p90 and Mga2p90 are generated from larger-

molecular-weight 120 kDa proteins by a limited endo-proteolytic proteasome degradation mechanism [2]. As mentioned in the Summary, Rsp5p's essential function within the *OLE1* transactivation pathway has been attributed to providing the partial degradation signal for both proteins. To ascertain that Rsp5p promotes Mga2p processing, we first determined if ectopic expression of an Rsp5p dominant-negative mutant (i.e., ^{HA}Rsp5pΔC) suppresses Mga2p90 production. *mga2Δ* cells were transformed with a plasmid containing epitope-tagged *MGA2* (positioned at the 5' end of the coding sequence) under the transcriptional control of its native promoter [2]. Placing the tag at the amino-terminus of Mga2p allows for the detection of unprocessed and processed proteins with an anti-MYC antibody [2]. ^{MYC}*MGA2* cells were then transformed with plasmid bearing a galactose-inducible ^{HA}*rsp5Δc*. *spt23Δ* cells transformed with plasmids harboring ^{FLAG}*SPT23* and ^{HA}*rsp5Δc* were included as a control. Surprisingly, ^{HA}Rsp5pΔC inhibited Spt23p but not Mga2p processing (Figure 1A). The inability to detect a defect in Mga2p processing in cells expressing ^{HA}Rsp5pΔC is unlikely to be due to absence of activity (i.e., inhibiting endogenous Rsp5p function) because ^{HA}Rsp5pΔC suppresses ^{MYC}Mga2p-induced *OLE1* expression and ^{MYC}*MGA2* cells expressing the mutant require oleic acid for growth (see Figure S1 in the Supplemental Data available with this article online). To determine if Rsp5p is dispensable for Mga2p processing by alternative methods, we tested if Mga2p90 is produced in *rsp5-1* cells at the nonpermissive temperature and in *rsp5Δ* cells. The *MGA2* and *SPT23* plasmids used for these experiments harbor tags on both the amino and carboxy-terminal ends of the encoded proteins (see picture in Figure 1E). This allows for the detection of unprocessed (membrane bound) and processed (tethered) polypeptides with an anti-FLAG antibody or the detection of only unprocessed proteins with the anti-HA antibody [2]. As shown in Figure 1B, there was no obvious difference in Mga2p90 levels in *rsp5-1* cells versus wild-type cells at the nonpermissive temperature (i.e., 37°C), whereas less Spt23p90 was observed in *rsp5-1* cells at 37°C (Figure 1B). Also, ^{FLAG}Mga2p90 was produced in *rsp5Δ* cells, and this protein contained the amino-terminal region and lacked carboxy-terminal amino acids (as determined by positive anti-FLAG and negative anti-HA reactivity; Figure 1C). Consistent with previous studies [2], Spt23p90 expression was dramatically lower, if not detectable, in *rsp5Δ* cells. We conclude from these studies that Rsp5p activates Mga2p's *OLE1* transactivation function by a mechanism that is independent of promoting proteasome-dependent processing.

We next examined if Rsp5p functions up- or downstream of Mga2p90 release from the ER. Indirect immunofluorescence was performed with ^{MYC}*MGA2* cells containing an empty vector control (V) or expressing the Rsp5p dominant-negative mutant. As shown in Figure 1D, ^{MYC}Mga2p exhibited ER, perinuclear, and nuclear staining in mock-induced cells, and this result is consistent with previous studies [5]. In contrast, predominant ER staining of ^{MYC}Mga2p was evident in cells expressing

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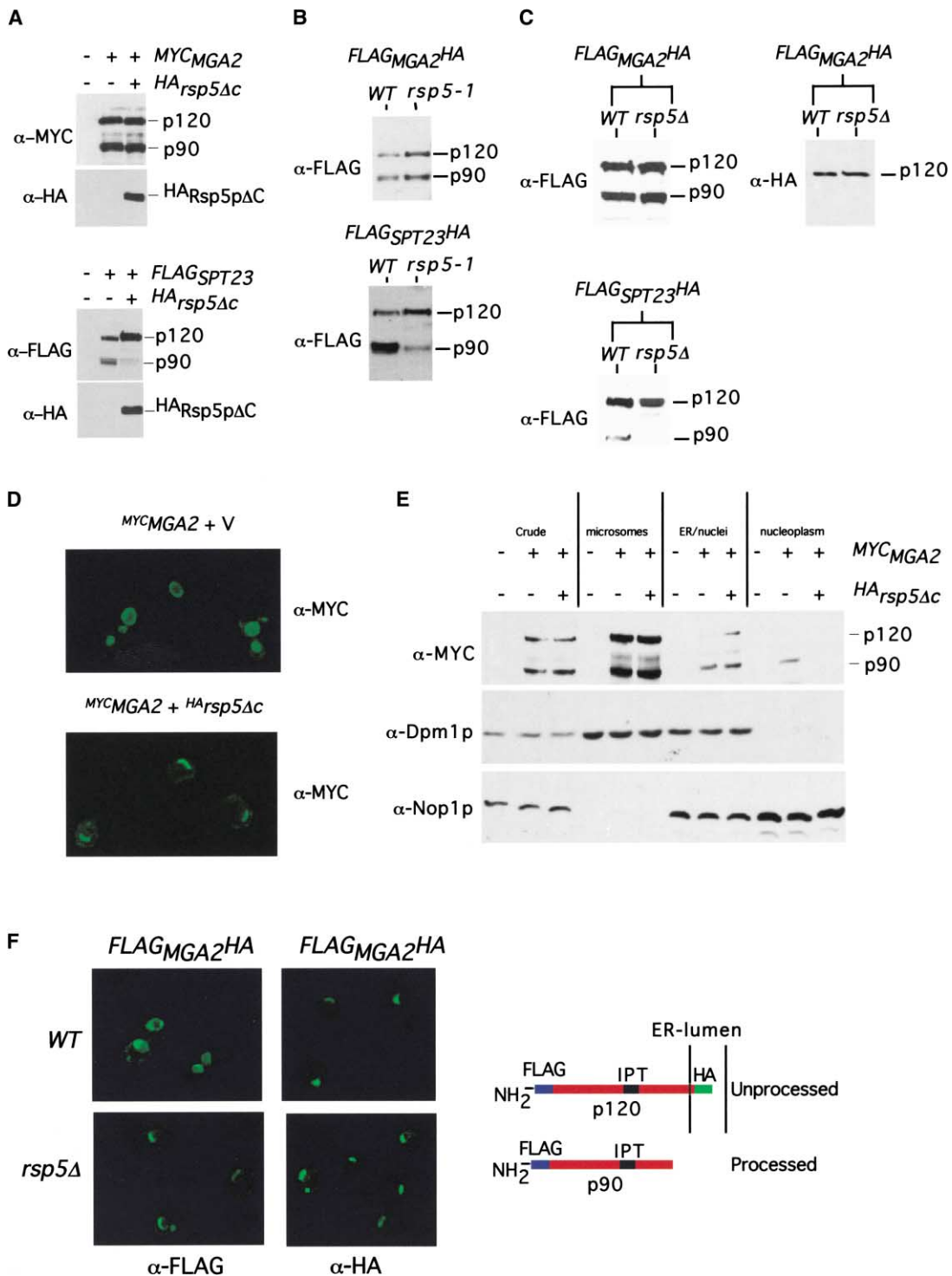


Figure 1. Rsp5p Is Dispensable for Processing but Not ER Release of Mga2p

(A) Yeast harboring the indicated plasmids were grown in glucose media and incubated in galactose media to induce expression of *HA*_{rsp5ΔC}. Cells were harvested, and Western blotting was carried out with the appropriate antibodies.

(B) Wild-type and *rsp5-1* cells were transformed with galactose-inducible *FLAG*_{MGA2HA} or *FLAG*_{SPT23HA}. Transformants were grown in glucose media, pelleted, and resuspended in galactose media. Cells were placed at 37°C. Cells were harvested, and the amount of epitope-tagged protein was determined by a Western blot with an anti-FLAG antibody.

(C) *rsp5Δ* and wild-type cells containing galactose-inducible *FLAG*_{MGA2HA} or *FLAG*_{SPT23HA} were grown in glucose media containing oleic acid. Cells were pelleted and resuspended in galactose media supplemented with oleic acid. Cells were harvested, and Western blotting was performed with the indicated antibodies.

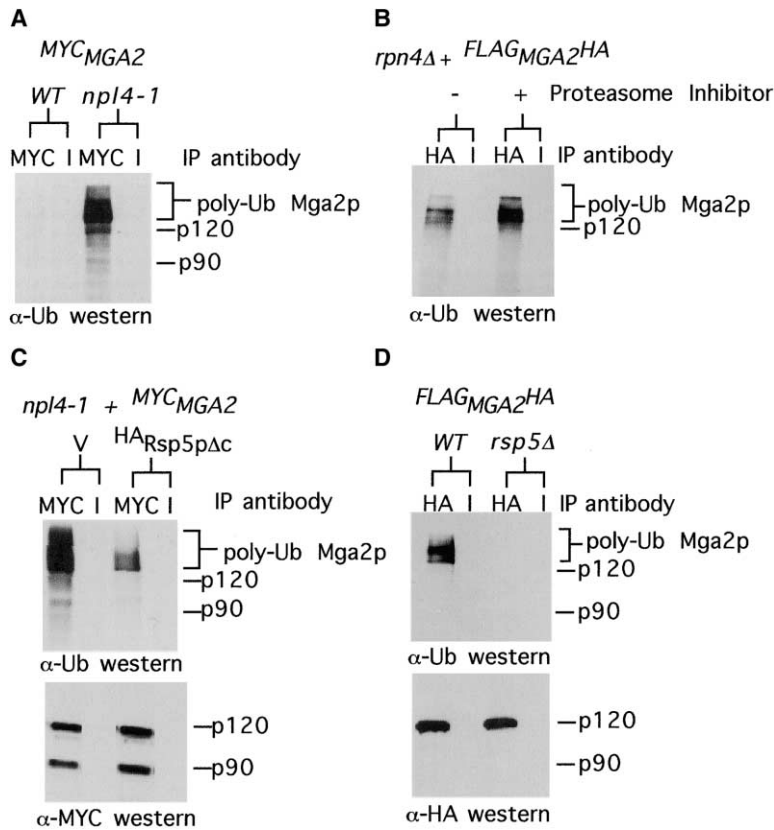


Figure 2. Polyubiquitinated Mga2p120 Accumulates in Cells Lacking Npl4p or Proteasome Function, and Rsp5p Is Required for Mga2p120 Polyubiquitination

(A) *npl4-1* and wild-type cells containing *MYC_{MGA2}* were grown in media at 25°C and placed at the nonpermissive temperature of 30°C. Cells were harvested, and immunoprecipitations were carried out with an anti-Myc or isotype control (I) antibody. Immunoprecipitated proteins were resolved by SDS-PAGE, and Western blotting was performed with an anti-Ub antibody.

(B) *rpn4Δ* cells expressing *FLAG_{MGA2}HA* were incubated for 2 hr in the absence (-) or presence (+) of the proteasome inhibitor Z-Leu-Leu-Leu-B(OH)₂. Immunoprecipitation and Western blotting were performed with the indicated antibodies.

(C) *npl4-1* cells harboring *MYC_{MGA2}* were transformed with pYes-*HA_{Rsp5pΔC}* or with an empty vector control (V). Transformants were grown in glucose media and then incubated in galactose media. Cells were harvested, immunoprecipitations were performed with an anti-MYC or isotype control (I) antibody, and blots of immunoprecipitated proteins were probed with the indicated antibodies.

(D) *rsp5Δ* and wild-type cells containing galactose-inducible *FLAG_{MGA2}HA* were grown in glucose media in the presence of oleic acid and then placed in oleic acid-supplemented galactose media. Cells were harvested and immunoprecipitations and Western blotting were performed with the indicated antibodies.

HA_{Rsp5pΔC}. We performed fractionation experiments to verify that nuclear mobilization of *MYC_{Mga2p90}* is suppressed in cells expressing *HA_{Rsp5pΔC}*. Extracts enriched for microsomal, ER/nuclei, and nucleoplasm proteins were generated. Western blotting was performed with anti-MYC, anti-Dpm1p (Dpm1p is ER-localized), and anti-Nop1p (Nop1p is nucleoplasm localized) antibodies. In mock-induced cells, unprocessed *MYC_{Mga2p120}* was found predominantly in crude and microsomal preparations, whereas *MYC_{Mga2p90}* was detected in all fractions analyzed (Figure 1E). In cells expressing *HA_{Rsp5pΔC}*, *MYC_{Mga2p120}* and *MYC_{Mga2p90}* were detected in microsomal and ER/nuclear fractions, whereas no *MYC_{Mga2p90}* was measured in the nucleoplasm. Because there is no detectable *MYC_{Mga2p120}* or *MYC_{Mga2p90}* in the nucleoplasm in cells expressing *HA_{Rsp5pΔC}*, the greater amount of *MYC_{Mga2p120}* and *MYC_{Mga2p90}* in the ER/nuclear extracts prepared from these cells could be due to *HA_{Rsp5pΔC}*-induced stabilization of ER-localized *MYC_{Mga2p120}* and *MYC_{Mga2p90}*. To gain further evidence that Rsp5p is required for ER release of Mga2p90, we compared the localization of

dual epitope-tagged Mga2p in *rsp5Δ* cells versus wild-type cells. ER, perinuclear, and nuclear localization were detected with the anti-FLAG antibody, whereas ER staining was seen with the anti-HA antibody in cells containing wild-type *RSP5* (Figure 1F). In contrast, epitope-tagged Mga2p in *rsp5Δ* cells was found to be localized predominantly to the ER, regardless of whether the anti-FLAG or anti-HA antibody was used. These results suggest that Rsp5p is required for ER release of Mga2p90.

Polyubiquitinated Mga2p120 Accumulates in Cells Lacking Npl4p or Proteasome Function and Rsp5p Is Required for Mga2p120 Polyubiquitination

Rsp5p has recently been shown to ubiquitinate ER-localized proteins [6]. Thus, we next tested if Rsp5p-induced mobilization of Mga2p90 is linked to Rsp5p-dependent ubiquitination of ER bound Mga2p120 or tethered Mga2p90. Unfortunately, we have found it difficult to detect ubiquitinated forms of Mga2p in *MYC_{MGA2}* cells, which is likely due in part to the insensitive nature

(D) *MYC_{MGA2}* cells were transformed with pYes-*HA_{Rsp5pΔC}* or an empty vector (V) control. Cells were grown and induced as described above, and immunofluorescence was performed with an anti-MYC antibody.

(E) Extracts enriched for microsomal, ER/nuclei, and nucleoplasm proteins were generated, and Western blotting was performed with anti-MYC, anti-Dpm1p, and anti-Nop1p antibodies.

(F) *rsp5Δ* and wild-type cells containing galactose-inducible *FLAG_{MGA2}HA* were grown and induced as described above, and the localization of epitope-tagged Mga2p was determined by immunofluorescence with the indicated antibodies.

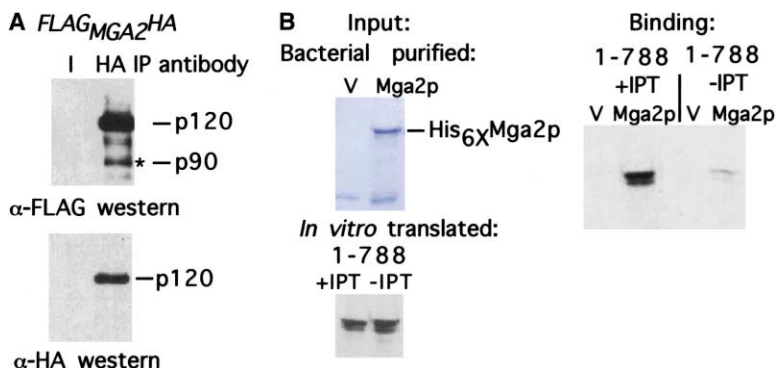


Figure 3. Mga2p90 and Mga2p120 Form Heterodimers

(A) *mga2Δ* harboring pYes-^{FLAG}MGA2^{HA} were grown in glucose media and incubated in galactose media. Cells were pelleted and lysed, and immunoprecipitations were carried out with an anti-HA or isotype control (I) antibody. Immunoprecipitated material was resolved by SDS-PAGE, and Western blots were performed with the indicated antibodies. An asterisk denotes ^{FLAG}Mga2p90.

(B) His_{6x}-tagged Mga2p was purified from bacteria using Ni²⁺ agarose beads and incubated with [³⁵S] labeled Mga2p 1-788 or Mga2p1-788ΔIPT. Protein complexes were pelleted, washed and eluted by re-sus-

pending beads in SDS-PAGE loading buffer and boiling. Proteins were resolved by SDS-PAGE and dried gels were subjected to fluorography. V denotes binding reactions containing Ni²⁺ agarose beads that had been incubated with bacteria containing the empty His_{6x} vector. The left panel depicts recombinant (detected by Coomassie staining) and in vitro translated (detected by fluorography) Mga2p placed in the binding assay.

of anti-Ubiquitin (Ub) antibodies. This has prevented us from determining if ectopic expression of ^{HA}Rsp5pΔC suppresses ^{MYC}Mga2p ubiquitination under normal growth conditions. We have found, however, that polyubiquitinated ^{MYC}Mga2p accumulates to easily measurable levels in *npl4-1* mutant cells at the nonpermissive temperature and, curiously, that the majority of ubiquitinated ^{MYC}Mga2p possesses a molecular weight greater than 120 kDa (Figure 2A). Also, we have found that similar to Spt23p processing [3], ^{MYC}Mga2p processing is unaffected, and both Mga2p120 and Mga2p90 are ER localized in *npl4-1* mutant cells at nonpermissive temperatures (see Figure S2 in the Supplemental Data). Npl4p is an essential member of the Cdc48p-Ufd1p protein chaperone complex, and it has recently been implicated in binding polyubiquitinated ER-localized proteins, separating them from nonmodified partners, and presenting them to the proteasome for degradation (reviewed in [7]). Considering the emerging role of this complex in the degradation of poly-ubiquitinated ER-localized proteins, it is likely that ER bound ^{MYC}Mga2p120 is a substrate of the ubiquitination machinery and that Npl4p is required for proteasome-dependent degradation of polyubiquitinated Mga2p120 and release of tethered Mga2p90. Consistent with the idea that polyubiquitinated Mga2p120 is a substrate of the proteasome, these forms were found to accumulate in *rpn4Δ* cells (these cells display an increased sensitivity to proteasome inhibitors [8]) treated with the proteasome inhibitor Z-Leu-Leu-Leu-B(OH)₂ (Figure 2B).

We next determined if Rsp5p is required for Mga2p120 polyubiquitination. We accomplished this by measuring the amount of polyubiquitinated Mga2p120 in *npl4-1* mutant cells at the nonpermissive temperature with or without expression of ^{HA}Rsp5pΔC as well as in wild-type *RSP5* versus *rsp5Δ* cells. The galactose-inducible ^{FLAG}MGA2^{HA} plasmid was used for the later studies because it allows for a high level of Mga2p expression. Also, it contains a carboxy-terminal HA epitope tag, and this allows for preferential immunopurification of Mga2p120 with the anti-HA antibody. As depicted in Figure 2C, we measured less ubiquitinated ^{MYC}Mga2p in *npl4-1* mutant cells expressing ^{HA}Rsp5pΔC. Figure 2D shows the presence of polyubiquitinated

^{FLAG}Mga2p120^{HA} in cells containing *RSP5* but not in *rsp5Δ* cells. These results suggest that Rsp5p is required for Mga2p120 polyubiquitination. We should point out that we have not observed convincing differences in the stability (as measured by pulse-chase with radioactively labeled methionine) of ^{MYC}Mga2p120 in cells deficient in Rsp5p or Npl4p. This result is not surprising; this assay is insensitive, and loss of Rsp5p or Npl4p function does not lead to an obvious increase in total Mga2p120. Furthermore, the inability to detect labeled Mga2p in fractions enriched for ER and nuclear protein in wild-type cells has prevented us from determining if increased amounts of Mga2p120 and Mga2p90 detected in this particular fraction in cells expressing ^{HA}Rsp5pΔC are associated with enhanced stability of these ER-sequestered proteins. The simplest interpretation of the data is that there is only a minor sub-pool of ER-localized Mga2p120 that has the capacity to be poly-ubiquitinated by Rsp5p and degraded via an Npl4p-proteasome-dependent mechanism under the conditions used here.

Mga2p90 and Mga2p120 Form Heterodimers, and the Mga2p90-Mga2p120 Complex Interacts with Rsp5p

One question that arises from these studies is how does Rsp5p-dependent Mga2p120 polyubiquitination lead to Rsp5p-dependent release of Mga2p90? When one considers that homologous Spt23p90 and Spt23p120 heterodimerize via the IPT domain [3] and Mga2p harbors the same motif, one plausible explanation is that Mga2p90 and Mga2p120 interact and that Rsp5p binding to the complex results in Mga2p120 polyubiquitination and degradation with release of heterodimeric Mga2p90. To determine if epitope-tagged Mga2p90 and Mga2p120 heterodimerize, we performed coimmunoprecipitation studies with cells expressing ^{FLAG}Mga2p^{HA}. Figure 3A shows ^{FLAG}Mga2p90 in immunoprecipitations with the anti-HA antibody and not with the isotype control (I) antibody, indicating that Mga2p90 and Mga2p120 interact. Because the IPT domain is required for Spt23p90 and Mga2p90 production ([3] and data not shown), we were unable to test by coimmunoprecipitation if the Mga2p90-Mga2p120 interaction depends on the IPT domain. Therefore, we tested for dimeric interac-

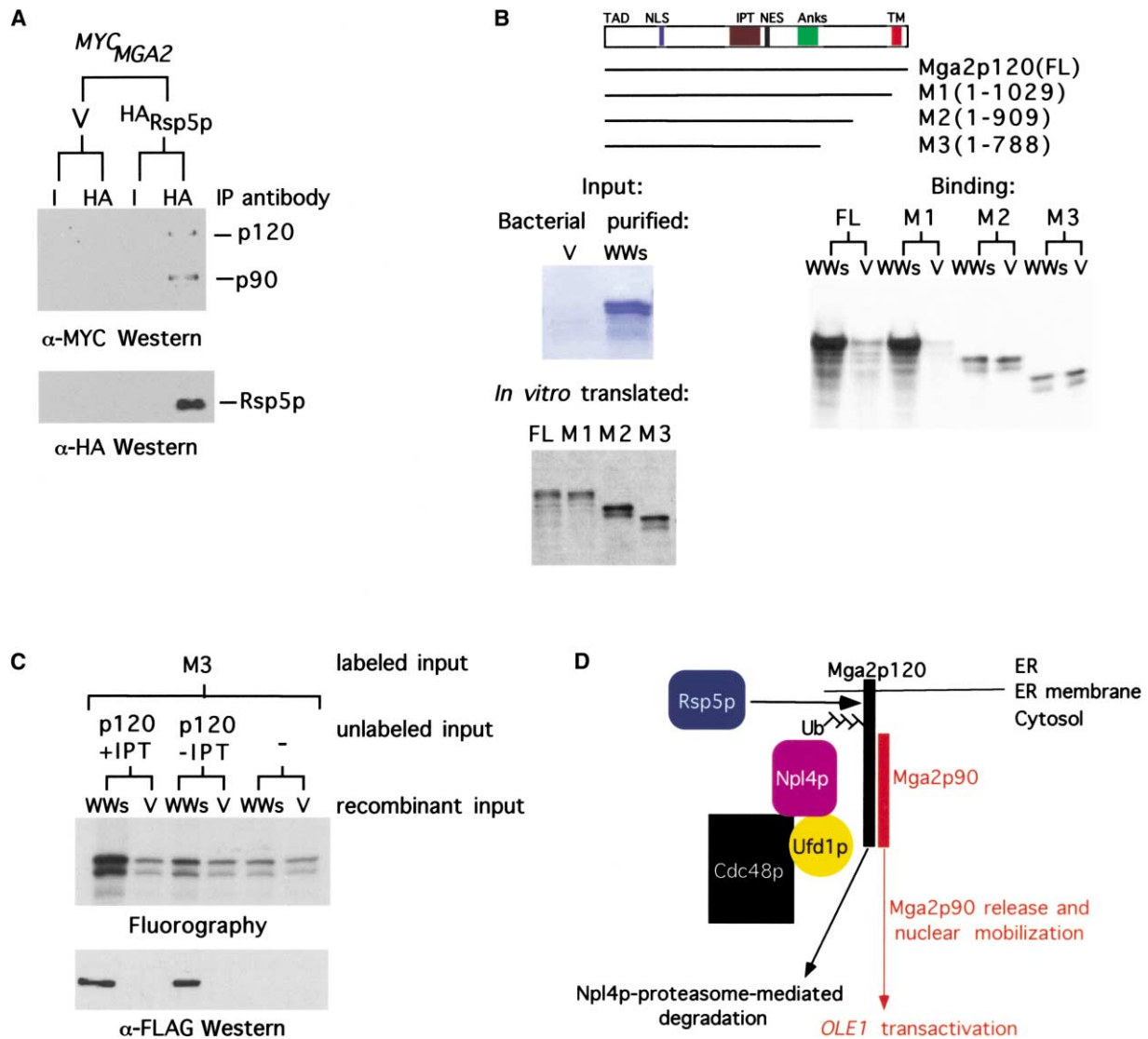


Figure 4. Rsp5p Binds a Mga2p90-Mga2p120 Complex

(A) *MYC*⁺*MGA2* cells were transformed with plasmid encoding galactose-inducible ^{HA}Rsp5p or with an empty vector control (V). Single colonies were grown in glucose-containing media and then incubated in galactose media. Cells were pelleted and lysed, and immunoprecipitation and Western blotting were carried out with the indicated antibodies.

(B) Abbreviations for depicted structural domains are described in the Supplemental Data. His_{6x}-tagged recombinant Rsp5p polypeptide was purified from bacteria via Ni²⁺ agarose beads and incubated with labeled Mga2p120 or Mga2p carboxy-terminal mutants. Protein complexes were processed as described in Figure 3B. The left panel depicts recombinant Rsp5p (detected by Coomassie staining) and in vitro-translated Mga2p (detected by fluorography) placed in the binding assay.

(C) Binding assays with His_{6x}-tagged Rsp5p WW domains and the labeled M3 Mga2p mutant were performed as described above, except that binding reactions included either no Mga2p (-) or, alternatively, unlabeled in vitro-translated Mga2p120 or Mga2p120ΔIPT containing reticulocyte lysate. Bound Mga2p was measured by fluorography for labeled M3 or by Western blotting for unlabeled Mga2p120.

(D) Proposed model of Mga2p activation by Rsp5p. Sites of ubiquitin conjugation remain to be identified.

tions by using an in vitro pull-down assay with recombinant His_{6x}-Mga2p and in vitro-translated Mga2p. As shown in Figure 3B, we found that Mga2p forms dimers and that dimerization is dependent on the IPT domain.

To determine if Rsp5p interacts with Mga2p120-Mga2p90 complexes, extracts were prepared from cells expressing *MYC*⁺Mga2p and ^{HA}Rsp5p or *MYC*⁺Mga2p alone, and immunoprecipitations were performed. As shown in Figure 4A, *MYC*⁺Mga2p90 and *MYC*⁺Mga2p120 were coimmunoprecipitated with an antibody recognizing ^{HA}Rsp5p

in extracts containing ^{HA}Rsp5p (Figure 4A). These proteins were not present in control immunoprecipitations or in cells lacking ^{HA}Rsp5p. To further investigate the Rsp5p-Mga2p interaction, we generated a series of carboxy-terminal Mga2p deletion mutants. These mutants, as well as full-length Mga2p, were tested for Rsp5p binding. An in vitro association assay was chosen to avoid interpretation difficulties arising from likely differential expression, localization, and activity of the mutants in cells. The source of Rsp5p for these binding

studies was recombinant His_{6x}-tagged Rsp5p containing the substrate binding domains of the ligase (i.e., WW domains 1 through 3), whereas Mga2p proteins were generated in rabbit reticulocytes. Rsp5p was found to bind robustly with full-length Mga2p and the M1 mutant (Figure 4B). Only background binding was detected between Rsp5p and the M2 and M3 mutants. These results indicate that the Rsp5p binding domain is located within the carboxyl terminus of Mga2p120, and Mga2p90 interacts with Rsp5p via Mga2p120. We next performed a Rsp5p binding assay with the radioactively labeled M3 mutant in the presence of unlabeled Mga2p120 or Mga2p120ΔIPT. As shown in Figure 4C, we detected a greater amount of the M3 mutant in pull-downs containing His_{6x}-tagged Rsp5p and Mga2p120 in the binding reaction than we did in those containing His_{6x}-tagged Rsp5p and Mga2p120ΔIPT. Similar amounts of Mga2p120 and Mga2p120ΔIPT were present in these Rsp5p pull-downs, whereas only background binding was detected in reactions containing only His_{6x}-tagged Rsp5p and the M3 mutant. We conclude from these experiments that Rsp5p interacts with dimeric Mga2p120-Mga2p90 via a domain that is contained only within Mga2p120.

Discussion

Results presented here suggest that Rsp5p is dispensable for Mga2p120 processing. Although mechanisms involved in Mga2p processing remain uncharacterized, our unpublished studies have revealed that Mga2p90 generation does not require ER retention or a region required for Rsp5p binding *in vitro*. Also, we have found that processing does require the IPT dimerization domain and is suppressed in proteasome-deficient strains. Thus, it is possible that Mga2p90 is generated by a mechanism similar to that of its distant cousin, the p50 subunits of the NF-κB complex [9]. Whether this requires ubiquitination is unclear, although it is curious to note that we were unable to detect ubiquitinated forms of the protein in *rsp5Δ* cells.

Previous experiments have indicated that Rsp5p is essential for the *OLE1* transactivation function of Mga2p [2], and results presented here suggest that it is required for release of Mga2p90 from the ER. Our ubiquitination and interaction studies indicate that Rsp5p induces liberation by binding to the Mga2p120-Mga2p90 complex and promoting polyubiquitination of membrane bound Mga2p120. We propose that Mga2p120 polyubiquitination provides the signal for Npl4p-mediated complex segregation, proteasome-dependent degradation of Mga2p120, and release of transcriptionally competent Mga2p90 (see Figure 4D). However, our current studies do not allow us to exclude alternative mechanisms of Rsp5p-induced Mga2p90 release, including Rsp5p-dependent effects on other proteins (e.g., Spt23p120) promoting Mga2p90 release or less obvious activities (e.g., monoubiquitination) on Mga2p90 that may promote nuclear trafficking.

When one considers that Mga2p120 and Mga2p90 interact with Rsp5p, a specificity determinant for preferential Rsp5p-induced Mga2p120 polyubiquitination

needs to be defined. We have found by using an *in vitro* binding assay that the Rsp5p binding site on Mga2p is located within a region (between or perhaps surrounding amino acids 910–1029) that is probably present only with the membrane bound anchor. This interaction specificity could be important for dictating Rsp5p-dependent polyubiquitination of the membrane bound anchor while sparing the transcriptionally competent form from this potentially deleterious modification process. Interestingly, there is only one known putative WW binding site (i.e., L₉₆₇PKY₉₇₁) within this region, and such a motif has been shown previously to interact with Group I WW domains 2 and 3 of Rsp5p [10]. It should also be noted that besides phosphorylation-dependent (Sp/Tp)P sites, the LPKY sequence is the only known WW binding site within the protein. Curiously, this site and surrounding sequences are remarkably conserved in Spt23p. Thus, it is possible that there is another WW domain containing ligase responsible for Spt23p120 polyubiquitination and release of tethered Spt23p90 or that Rsp5p plays dual roles in Spt23p activation, perhaps by binding to distinct sites. Alternatively, Rsp5p may activate Mga2p and Spt23p function by a similar mechanism. Suppression of Spt23p processing in cells expressing ligase-defective Rsp5p mutants could be due to nonspecific binding effects of the mutants on the processing reaction or on loss of other Rsp5p-regulated processes. In *rsp5Δ* cells, partial degradation may be affected by the presence of high amounts of unsaturated fatty acids or may be due to the loss of other Rsp5p-dependent activities. Future studies are needed to test the model proposed here and to determine if it applies to other eukaryotic transcription factors that are embedded/tethered at the ER or other cellular membranes.

Supplemental Data

Supplemental Data including Experimental Procedures and yeast strains are available with this article online at <http://www.current-biology.com/cgi/content/full/13/14/1227/DC1/>.

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