Yeast ubiquitin ligase Rsp5 contains nuclear localization and export signals

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Short title: signals for Rsp5 nuclear-cytoplasmic distribution

Abbreviations: a.a., amino acids; β-gal, β-galactosidase; LMB, leptomycin B; MVB, multivesicular bodies; NES, nuclear export signal; NLS, nuclear localization signal;

Abstract

The Rsp5 ubiquitin ligase regulates numerous cellular processes. Rsp5 is mainly localized to the cytoplasm but nuclear localization was also reported. A potential nuclear export signal was tested for activity by using a GFP_2 reporter. The 687-LIGGIAEIDI-696 sequence located in the Hect domain was identified as a nuclear export signal active in a Crm1-dependent manner, and its importance for the localization of Rsp5 was documented by using fluorescence microscopy and a *lacZ*-based reporter system. Analysis of the cellular location of other Rsp5 fragments fused with GFP_2 indicated two independent potential nuclear localization signals, both located in the Hect domain. We also uncovered Rsp5 fragments that are important to targeting/tethering Rsp5 to various regions in the cytoplasm. The presented data indicate that Rsp5 ligase is a shuttling protein whose distribution within the cytoplasm and partitioning between cytoplasmic and nuclear locations is determined by a balance between the actions of several targeting sequences and domains.

Keywords: yeast; Rsp5 ubiquitin ligase; nuclear-cytoplasmic transport

Introduction

Cells respond to environmental changes by rearrangement of plasma membrane composition, modulation of transcription, and reorganization of other processes. Unnecessary plasma membrane transporters and activated receptors are removed primarily via the endocytic pathway which directs proteins to multivesicular bodies (MVB) and to the vacuole for degradation. Additionally, many endocytic proteins, from both internalization and MVB machineries, are transported to the nucleus and play important roles in transcription and chromatin modification [reviewed in (Pilecka et al., 2007)]. Endocytic proteins shuttling to the nucleus include mammalian Eps15 (Pilecka et al., 2007), RNF13 (Bocock et al., 2010) and yeast Scd15 (Chang et al., 2006), Sla1 (Gardiner et al., 2007) and Pan1 (Kaminska et al., 2007).

The yeast Rsp5 protein is a member of the Nedd4 family of ubiquitin ligases which displays a C2-WW-Hect structure with lipid-binding, protein-binding and catalytic domains, respectively (Ingham et al., 2004). Rsp5 recognizes and ubiquitinates endocytic cargos such as transporters and receptors (Estrella et al., 2008; Hatakeyama et al., 2010; Rotin et al., 2000). Moreover, Rsp5 affects the endocytic machinery (Dunn and Hicke, 2001; Gajewska et al., 2001) and the actin cytoskeleton - an important element of this machinery (Kaminska et al., 2002). Rsp5 is also involved in MVB (Katzmann et al., 2004; Stawiecka-Mirota et al., 2007) protein sorting [reviewed in (Belgareh-Touze et al., 2008)]. Thus, the Rsp5 protein must contain localization signals required for cell surface and endomembrane recruitment. Previous reports indicated that the C2 domain is a targeting module for membrane phospholipids that facilitates the localization of Rsp5 to endosomal membranes (Dunn et al., 2004) and WW domains bind adaptor proteins that dock Rsp5 in endocytic compartments (Leon et al., 2008; Lin et al., 2008; Nikko et al., 2008).

Interestingly, Rsp5 was also shown to ubiquitinate substrates localized in the nucleus. The best studied example is Rpb1, the largest subunit of RNA polymerase II (RNAPII) (Beaudenon et al., 1999). Ubiquitination of Rpb1 by Rsp5 (Harreman et al., 2009; Somesh et al., 2007) suggests that there may be a nuclear pool of Rsp5. Moreover, several human Nedd4 family members, including Smurf1 (Tajima et al., 2003), WWP1 (Flasza et al., 2006) and Nedd4 (Hamilton et al., 2001), were shown to have a potential to shuttle between the nucleus and cytosol. Notably, Nedd4 is an E3 ligase that associates with and ubiquitinates RNAPII in human cell lines (Anindya et al., 2007). Studies of subcellular distribution of Rsp5 showed that Rsp5 is located in the cytoplasm (Gajewska et al., 2001; Katzmann et al., 2004; Wang et al., 2001) or in the nucleus and cytoplasm (Neumann et al., 2003). The results regarding Rsp5 location and indications that it may function in the nucleus led to our studies to address whether Rsp5 might be another endocytic protein and Nedd4 family member that shuttles between the nucleus and cytoplasm.

The process of nuclear-cytosolic transport of proteins is now well established (Cook et al., 2007). In most cases nuclear proteins require active transport and contain transferable classical basic nuclear localization signals (NLS) which are bound by import receptor importin β or a heterodimer consisting of importin β and the adaptor protein importin α (Lange et al., 2007), but other signals recognized by other importins also exist (Lange et al., 2008; McLane and Corbett, 2009; Suel et al., 2008). Proteins that have capability to exit the nucleus possess nuclear export signals (NES). Co-existence of both NLS and NES motifs in a protein allows it to shuttle between the nucleus and cytoplasm.

In this study we examined several short Rsp5 sequences for NES or NLS activity by using GFP_2-based reporters and tested Rsp5 large N-terminal, central and C-terminal fragments for the ability to direct the GFP_2 reporter to cytoplasmic or nuclear locations. We also used fluorescence microscopy and a lacZ-based plasmid reporter to study the nuclear pool of full-length Rsp5. Our data show that Rsp5 steady state levels are cytoplasmic and nuclear and that Rsp5 possesses nucleus and cytoplasm shuttling ability.

Materials and methods

Strains, media and growth conditions

Saccharomyces cerevisiae strains used are listed in Table 1. Yeast strains growth and transformations followed standard procedures (Sherman, 2002). YPD (1% yeast extract, 1% peptone, 2% glucose), SD+cas-ura (0.68% yeast nitrogen base without amino acids, 0.1% casamino acids, 20 mg/l of adenine and tryptophan) with either 2% glucose, 2% raffinose or 2% galactose as a carbon source, SD-his-ura (0.68% yeast nitrogen base without amino acids, 20-40 mg/l of required amino acids and adenine) and sporulation medium (Sherman, 2002) and SD+5-fluororotic acid (5-FOA) medium were used.

PC1, an *RSP5*/*rsp5Δ* strain was constructed by mating of MHY500 and MHY501 and transformation with EcoRI-KpnI fragment of pBG61 plasmid bearing *RSP5* deletion cassette (Gajewska et al., 2001). The PC1 strain was used to test the complementation of *rsp5Δ* by *LG-RSP5* and *LG-rsp5-nes* fusion alleles. To do that, it was transformed with PA.A.05-RSP5 or PA.A.05-rsp5-nes plasmids, resulting strains (PC27 and PC29, respectively) were sporulated, and two spore clones were chosen for further analysis.

To construct the PC32 strain containing an integrated *rsp5-nes* allele the *RSP5* gene was replaced in the MHY501 strain by transformation with PstI-linearized YIpHA-rsp5-nes plasmid. Integrants were selected on SD+cas-ura plates and then incubated on SD+5-FOA plates to select for cells that had lost the *URA3* marker. Cells were also selected for untagged version of *rsp5-nes* allele after PCR analysis and sequencing.

Plasmids and plasmid constructions

Plasmids used in this study are: pIGinA and pIGoutA (Butterfield-Gerson et al., 2006), pBG61 and YCpHArsp5-w1w2w3 (Gajewska et al., 2001), YIpHA-RSP5 (Kwapisz et al., 2005), PA.A.05 and p80lacZ (Godon et al., 2005), pUC-KK (Gajewska et al., 2001), p416-SNA3-GFP (Reggiori and Pelham, 2001) and pRS414-P_{GAL1}GFP-HA-RSP5 (Wang et al., 2001). Plasmids constructed in this study are listed in Table S1. The sequences of gene-specific primers used to amplify *RSP5* fragments are available upon request. Fragments of *RSP5* with mutant WW domains were amplified using YCpHArsp5-w1w2w3 plasmid as the template. Mutations in region encoding the NES sequence were introduced by PCR mutagenesis of pUC19-KK plasmid bearing the KpnI DNA fragment containing part of *RSP5*, and subsequently confirmed by BbvC1 restriction enzyme digestion and sequencing. YIpHArsp5-nes was constructed by substituting the AgeI-MunI fragment of the YIpHA-RSP5 plasmid by a fragment bearing *rsp5-nes* mutations. pRS414-P_{GAL1}GFP-HA-rsp5-nes was constructed by substituting the KpnI-NotI fragment of pRS414-P_{GAL1}GFP-HA-RSP5 by fragment bearing *rsp5-nes* mutation obtained by PCR amplification using YIpHA-rsp5-nes as a template. To construct plasmids bearing GFP_2 fusions various amplified *RSP5* fragments were cloned individually into the EcoRI-BamHI or EcoRI-SmaI sites of pIGinA or pIGoutA plasmids. The PA.A.05-RSP5 plasmid was constructed as follows: NotI and XhoI sites were introduced by PCR site-directed mutagenesis after the *RSP5* start and stop codons, respectively, to generate pPC36. Subsequently, the NotI-XhoI fragment of pPC36 was cloned

into PA.A.05. To create the PA.A.05-rsp5-nes the AgeI-MunI fragment of pPC36 was substituted by a fragment bearing the *rsp5-nes* mutation, and the NotI-XhoI fragment was cloned into PA.A.05. All PCR-amplified fragments were confirmed by sequencing.

Total protein extracts and Western blot analysis

Protein extracts were prepared as described previously (Kaminska et al., 2002). Samples were analyzed by Western blot using anti-GFP (Roche), anti-Nedd4 WW2 domain (Millipore), anti-Rpb1 4H8 antibody [kind gift of J. Svejstrup (Harreman et al., 2009)] or anti-Pgk1 (Molecular Probes) primary antibodies and secondary anti-rabbit or anti-mouse HRP-conjugated antibodies (DACO), followed by ECL (Amersham or Millipore).

Fluorescence Microscopy

For GFP fluorescence cells were grown at 23°C to logarithmic phase in SD+cas-ura with 2% raffinose as a carbon source. Expression of GFP fusions from *GAL1* promoter was induced for 4-6 hours by addition of galactose to a final concentration of 2%. DNA was stained with Hoechst 33342 (final concentration 0.9 μg/ml, Invitrogen) for 10 min. Cells were placed on ice and treated with NaN_3 (10 mM final) for 10 min and mounted on a slide in 1.67% low-melt agarose. For analysis of Crm1-dependence of NES_{RSP5} in the MNY8 strain, the expression of GFP fusions was induced for 1-2 hours and cells were treated either with ethanol or Leptomycin B (final concentration 100 ng/ml, LC Laboratories) for 30 min. Cells were viewed with an Eclipse E800 (Nikon) fluorescence microscope equipped with a DS-5Mc (Nikon) camera. Images were collected using Lucia General 5.1 software (Laboratory Imaging Ltd.). Cells producing Sna3-GFP were viewed by a confocal laser scanning microscope EZ-C1 (Nicon) Eclipse TE2000-E equipped with a Plan Apo 60X objective (NA 1.4). Images were collected with EZ-C1 confocal V. 3.6 program (Nikon) and processed with EC1 Viewer 3.6 and Adobe Photoshop 8.0 in the Laboratory of Confocal and Fluorescence Microscopy, Institute of Biochemistry and Biophysics PAS.

Activity of β-galactosidase

The β-gal filter assay was performed as described (Miller, 1972). The β-gal activity was quantitatively assayed spectrophotomerically at 420 nm by using *o*-nitrophenyl-β-D-galactopyranoside (Sigma) as a substrate (Guarente, 1983) in cells grown to the OD~1. Three independent experiments were performed. The average activity was expressed in nanomol of *o*-nitrophenol/minutes/μg protein.

Analysis of UV-treated cells

Cells were grown in YPD at 28°C to the log-phase, medium was removed and cells suspended in water (3 OD units in 5 ml) were irradated in culture dish in Vilberlourmet crosslinker with a dose of 250 J/m2. Fresh medium was added to the cells which were then allowed to recover for 1-3 hours.

Results

Identification of a NES sequence in Rsp5

Shuttling proteins contain both functional NLS and NES signals that allow them to travel between the nucleus and cytoplasm. One type of NES sequence consists of hydrophobic residues, preferably leucine or isoleucine (la Cour et al., 2004). To search for such a motif in Rsp5 (NES_{RSP5}) we used the netNES online tool (la Cour et al., 2004). The studies uncovered the sequence 687-LIGGIAEIDI-696 as a potential NES_{RSP5} signal. To test whether this potential NES sequence is functional it was inserted in-frame between the NLS sequence

from histone H2B (NLS_{H2B}) and two tandem GFP proteins (GFP_2) using a reported plasmid (Butterfield-Gerson et al., 2006). We compared the localization of the NLS_{H2B}-NES_{RSP5}-GFP_2 fusion protein containing Rsp5 amino acids (a.a.) 665-706 (Figure 1A) with those of GFP_2, NLS_{H2B}-GFP_2, and the mutant protein NLS_{H2B}-nes_{RSP5}-GFP_2, in which two isoleucines of NES_{RSP5} were changed to alanines (I694A, I696A). As shown in Figure 1B, all fusion proteins analyzed were of the proper size and were expressed at similar cellular levels. In agreement with our prediction, the putative NES_{RSP5} was able to export the NLS_{H2B}-NES_{RSP5}-GFP_2 fusion protein from the nucleus and prominent nuclear exclusion was observed (Figure 1C). Substitution of the two isoleucines with alanines abolished the functionality of NES_{RSP5} and the NLS_{H2B}-nes_{RSP5}-GFP_2 fusion protein accumulated in the nucleus, similarly to the NLS_{H2B}-GFP_2 control (Figure 1C). The data indicated that the NLS_{H2B}-NES_{RSP5}-GFP_2 protein was absent from the nucleus not due to a failure to undergo nuclear entry, but rather due to the nuclear export activity of Rsp5 a.a. 678-696.

The classical leucine-rich NES motif usually utilizes the Crm1-dependent protein export pathway (Fornerod et al., 1997). To test the hypothesis that the nuclear export of the identified NES_{RSP5} is Crm1-dependent, we used the MNY8 strain bearing the *CRM1-T539C* allele which encodes a Crm1 mutant protein sensitive to leptomycin B (LMB) (Neville and Rosbash, 1999; Stade et al., 1997). MNY8 cells were transformed with plasmids encoding NLS_{H2B}-NES_{RSP5}-GFP_2, GFP_2, or NLS_{H2B}-GFP_2 proteins. The fusion proteins were of the proper size and their stabilities were not affected in LMB-treated cells (Figure 2A). NLS_{H2B}-NES_{RSP5}-GFP_2 was cytosolic when LMB was not present in the medium (Figure 2B). Addition of LMB caused nuclear accumulation of NLS_{H2B}-NES_{RSP5}-GFP_2 (Figure 2B), indicating that the NES motif of Rsp5 is Crm1-dependent. Localization of the GFP_2 and NLS_{H2B}-GFP_2 proteins was not affected by LMB, as expected (Figure 2B).

Subcellular localization of N-terminal, central and C-terminal fragments of Rsp5

To identify Rsp5 sequences that might serve as NLS we search the sequence for basic-rich signals and tested these for the ability to locate GFP_2 to the nucleus; however, none of these could direct GFP_2 to the nucleus (not shown). Therefore we initiated a comprehensive approach to assess Rsp5 sequences in its N-terminal (F1), central (F2) and C-terminal (F3) regions for NLS activity. These Rsp5 fragments were fused in-frame with GFP_2 (Figure S1A and 3A). The cellular localizations of the respective protein fusions were compared with the localization of GFP_2 alone and with that of NLS_{H2B}-GFP_2. We also constructed plasmids encoding F1, F2 and F3 GFP_2-fusions containing the NLS_{H2B} signal, to test whether these fragments can undergo nuclear entry or whether they might contain cytoplasmic docking sequences. Moreover, to prevent potential docking of F1 and F2 fragments to cytosolic structures by the action of the three WW domains responsible for substrate protein binding, we introduced a.a. substitutions which had previously been shown to inactivate the WW domains (mutations *rsp5-w123*) (Gajewska et al., 2001). Amino acid substitutions were also introduced to inactivate the NES_{RSP5} sequence present in the C-terminal F3 fragment since the NES activity could potentially mask the action of a weak NLS. Western blot analysis was performed to determine the size of the fusion proteins (Figure S1B and 3B). All fusion proteins were of the expected size except for F1w123-GFP_2 and NLS_{H2B}-F1w123-GFP_2 that underwent partial degradation (Figure S1B).

The F1-GFP_2 fusion proteins demonstrated polarized cytoplasmic localization resembling the pattern of actin cytoskeleton structures, the actin cytokinetic ring and polarized actin patches [reviewed in (Moseley and Goode, 2006)], whereas F2-GFP_2 was localized in 1-5 cytoplasmic foci which were not polarized (Figure S1B). The cytoplasmic location of F2-GFP2 protein

appeared not to depend on the WW domains while F1-GFP2 was WW-dependent. F1Δw-GFP2 protein devoid of WW domains was localized in the cytoplasm and plasma membrane and not polarized. The data are in agreement with the ability of the C2 domain to bind phospholipids (Dunn et al., 2004). When NLS_{H2B} was fused to either F1-GFP_2 or to F2-GFP_2 neither fusion protein demonstrated nuclear location (Figure S1C). Thus, Rsp5 possesses sequences that direct/tether this protein to cytoplasmic areas and these sequences are likely in competition with other sequences involved in Rsp5's dynamics between the nucleus and the cytoplasm.

In contrast to F1-GFP_2 and F2-GFP_2, the F3-GFP_2 protein containing almost the entire Hect domain was evenly distributed throughout the cytoplasm and nucleus, with some cells showing additionally a single dot adjacent to the nucleus and/or the vacuole (Figure 3C). This localization was not changed even when the strong NLS_{H2B} was attached to the F3-GFP_2, indicating that the NES_{RSP5} identified in the Hect domain redistributes the potential nuclear pool to the cytoplasm or the fusion protein is tethered in the cytoplasm. We favor the first interpretation because nuclear localization was apparent in most cells when the NES_{RSP5} sequence was inactivated by a.a. substitutions (Figure 3C; see $F3nes_{RSP5}$-GFP_2 and NLS_{H2B}-$F3nes_{RSP5}$-GFP_2). The results indicate that the Rsp5 Hect domain contains both nuclear export and nuclear import targeting information.

To locate potential NLS sequences within the Hect domain, plasmids encoding GFP_2-fused sub-fragments of $F3nes_{RSP5}$, $C1nes_{RSP5}$ (a.a. 584-706), $C2nes_{RSP5}$ (a.a. 665-809) or nes_{RSP5} (a.a. 665-706) were constructed (Figure 3A). All fusion proteins were of expected size (Figure 3B). Microscopic analysis of their subcellular localization showed that both $C1nes_{RSP5}$ and $C2nes_{RSP5}$ were able to direct GFP_2 to the nucleus (Figure 3C). Moreover, the mutant nes_{RSP5}-GFP_2 was localized exactly as GFP_2 alone (Figure 3C), excluding the possibility that an NLS is located in the $C1nes_{RSP5}$-$C2nes_{RSP5}$ overlapping fragment. These results indicate that there are two potential NLS signals in the Hect domain, one in the C1 and the second in the C2 fragment. Both, however, seem to be weaker than NLS_{H2B} since the cytoplasmic fluorescence of $C1nes_{RSP5}$-GFP_2 and $C2nes_{RSP5}$-GFP_2 was more intense than that of NLS_{H2B}-GFP_2.

Rsp5 has nuclear pool in vivo and NES_{RSP5} inactivation results in its increase and defects in Rsp5 cytoplasmic function

Functionality of the NES signal identified by using reporter system was verified by analysis of their importance for subcellular localization and function of the full-length Rsp5 protein. The cellular localization of GFP-HA-rsp5-nes fusion protein was compared with localization of GFP-HA-Rsp5 expressed from plasmid in wild type strain (MNY7). GFP-HA-Rsp5 was diffused in the cytoplasm and located in cytoplasmic foci corresponding to endocytic compartments as previously reported (Wang et al., 2001) but in 4.3% of cells (total 257 scored in two experiments) a nucleus-enriched pool was observed (Figure 4A). The nuclear pool of GFP-HA-rsp5-nes was clearly increased since the nuclear signal was stronger than the cytoplasmic signal in 52.2% of cells (249 scored) showing that NES signal affects nuclear-cytoplasmic distribution of full- length Rsp5.

To assess the nuclear pool of Rsp5 we also employed a two-plasmid reporter system shown to be more sensitive than classical microscopic assays (Godon et al., 2005). This system consists of the p80lacZ reporter plasmid bearing a P_{lexA}-*lacZ* fusion encoding β-galactosidase (β-gal) and a test plasmid encoding LexA-Gal4 (LG) fused to the protein of interest. LG contains the LexA DNA-binding domain and the Gal4 activation domain, and does not contain an NLS. When a tested protein contains an NLS the fusion protein is transported to the nucleus and activates P_{lexA}-*lacZ* expression. The resulting β-gal activity is therefore a measure of the

nuclear pool of the protein of interest. Plasmids encoding full-length Rsp5 (wild type or mutant) with the N-terminus fused to LG were constructed and the functionality of the fusions was tested by complementation of the *rsp5*Δ mutation. Since *RSP5* is an essential gene a diploid *rsp5*Δ/*RSP5* strain (PC1) was transformed with plasmids expressing *LG-RSP5* or *LG-rsp5-nes*, sporulated, and the resulting tetrads were dissected. Spores *rsp5*Δ [*LG-RSP5*] and *rsp5*Δ [*LG-rsp5-nes*] were viable, indicating complementation of *rsp5*Δ. Growth of spore clones *rsp5*Δ [*LG-RSP5*] and *rsp5*Δ [*LG-rsp5-nes*] additionally transformed with the p80lacZ reporter plasmid in comparison with respective transformants of the parental strain (MHY501) is shown in Figure 4B. Both fusion proteins are functional since each supported growth of *rsp5*Δ strain at 28°C, although they grew slower than the wild type strain.

Wild type strain co-transformed with plasmids encoding LG-Rsp5 or LG-Rsp5-nes fusions together with a plasmid containing the P_{lexA}-*lacZ* reporter were tested by filter assay and quantitatively for β-gal activity. Transformants bearing the plasmid encoding LG only were white and did not show β-gal activity, as expected. Cells producing LG-Rsp5 or LG-Rsp5-nes fusion proteins were blue due to β-gal activity, indicating that these fusions are able to enter the nucleus and have a nuclear pool (Figure 4C). Moreover, the highest β-gal activity was observed in the strain producing the LG-Rsp5-nes protein, indicating that inactivation of NES_{RSP5} caused the nuclear pool of the fusion protein to increase. Hence, NES_{RSP5} is indeed responsible for Rsp5 nuclear export and its inactivation results in nuclear accumulation of Rsp5.

To gain insight into physiological consequences of NES inactivation the *rsp5-nes* strain bearing genomic mutations in NES_{RSP5} was tested for growth; it was temperature sensitive (Figure 5A). The observed growth defect at elevated temperature may be due to instability of the Rsp5-nes protein since Western blot analysis of *rsp5-nes* cells grown at 28°C and then shifted to 37°C showed that Rsp5-nes was less abundant than Rsp5 (Figure 5A).

Increasing the nuclear pool of Rsp5 could deplete its cytoplasmic pool resulting in defects in MVB protein sorting. Sna3 is one of the membrane proteins transported by the vacuolar protein sorting pathway from Golgi apparatus via MVB to the vacuole for degradation, in an Rsp5-dependent manner (Belgareh-Touze et al., 2008; Stawiecka-Mirota et al., 2007). Therefore, the cellular localization of Sna3-GFP in the *rsp5-nes* mutant was analyzed by confocal microscopy and compared with the wild type strain. When expressed in wild type cells Sna3-GFP was delivered to the vacuolar lumen resulting in the GFP fluorescence in vacuolar interior. In addition, a few small fluorescing dots were observed, possibly MVB, as shown before [Figure 5B and (Stawiecka-Mirota et al., 2007)]. In contrast, *rsp5-nes* vacuoles did not possess GFP signal, rather Sna3-GFP was located in large and small dots indicating that the transport of Sna3-GFP is blocked at the step before MVB sorting (Figure 5B) similarly as in *rsp5/npi1* mutant producing very small amounts of Rsp5 (Stawiecka-Mirota et al., 2007). This conclusion was further confirmed by Western blot analysis; in extracts prepared from *rsp5-nes* cells grown at 28°C the vacuolar degradation product of Sna3-GFP (GFP) was less abundant than in extracts prepared from wild type cells (Figure 5B).

We also tested the effect of *rsp5-nes* on nuclear target. It was shown that UV irradiation treatment induces the degradation of Rpb1, the largest subunit of RNA polymerase II, and that Rsp5 mediates this effect (Beaudenon et al., 1999; Harreman et al., 2009). This can be observed as a temporary decrease of the Rpb1 in wild type cells upon high dose of UV-irradiation which is not seen in *rsp5-1* cells when shifted to elevated temperature to deactivate Rsp5-1 (Beaudenon et al., 1999). The *rsp5-nes* strain showed no defect in this process (Figure

5C) at the temperature of 28°C when Rsp5-nes is stable suggesting that degradation of RNA polymerase II subunit occurs normally and nuclear activity of Rsp5 is probably not impaired.

In summary we demonstrated that inactivation of NES in the Rsp5 protein results in an increase of the Rsp5 nuclear pool observed by fluorescence microscopy and plasmid-based reporter system. Consistent with that, we also showed that the cytoplasmic function, but not nuclear function, is impaired in the *rsp5-nes* mutant probably due to depletion of the Rsp5 cytoplasmic pool.

Discussion

The Rsp5 ubiqutin ligase controls many cellular processes by regulation of protein substrates localized to distinct cellular compartments. Rsp5 has been described as cytoplasmic, located to the plasma membrane, endosomal, or nuclear. In this study we attempted to identify localization determinants of Rsp5 protein, focusing on nuclear import/export signals.

Our systematic analysis uncovered sequences important for the Rsp5 presence in specific cytoplasmic structures. An N-terminal (F1) fragment comprising the C2 domain and three WW domains (a.a. 2-479) targeted Rsp5 to polarized patches and the cytokinetic ring, resembling actin cytoskeleton structures. This polarized localization of F1-GFP_2 required a region containing WW domains since a shorter N-terminal fragment (a.a. 2-231) containing the C2 domain was in the cytoplasm and was directed to the plasma membrane. Likewise, the region containing WW domains was not sufficient to mediate polar localization since F2-GFP_2 was observed in cytosolic foci located in the vicinity of the vacuole and/or nucleus. Thus, these data suggest that the polarized localization requires both, the C2 domain and the region containing WW domains. The C2 domain of Rsp5 which binds phospholipids (Dunn et al., 2004) most probably mediates association of Rsp5 with the plasma membrane and contributes to its polarized localization by enriching Rsp5 pool close to endocytic sites. Polarized localization was also observed previously for a full-length GFP-Rsp5 fusion in the study of Neumann et al. (Neumann et al., 2003), but not in other studies (Gajewska et al., 2001; Katzmann et al., 2004; Leon et al., 2008; Wang et al., 2001). This localization is in agreement with the Rsp5 function in endocytosis which takes place in sites co-localizing with actin patches (Smythe and Ayscough, 2006). Several endocytic and actin cytoskeleton associated proteins, such as Rvs167, Sla1, Sla2 and Lsb1, are substrates of and bind Rsp5 (Gupta et al., 2007; Lu et al., 2008; Stamenova et al., 2004) and thus could potentially dock Rsp5 in this location. WW domains can also dock Rsp5 to its PY-containing protein substrates (Hesselberth et al., 2006) or adaptors, and several of the latter were shown recently to bind Rsp5 and function in endocytosis or MVB sorting (Leon et al., 2008; Lin et al., 2008; Nikko et al., 2008). This scenario is supported by the observation that overexpression of the Ear1 endocytic adaptor protein results in accumulation of Rsp5 in endosomes (Hiraki and Abe, 2010; Leon et al., 2008).

Our efforts to characterize sequences of Rsp5 which could be responsible for its nuclear shuttling uncovered the presence of an active NES_{RSP5} sequence (687-LIGGIAEIDI-696) located in the Hect domain. This sequence conforms to the classical leucine-rich NES sequences and it is dependent on Crm1, a nuclear exportin. Hect domains of ubiquitin ligases (E3) possess a characteristic two-lobed structure with N-lobe binding a conjugating enzyme (E2) and C-lobe which includes the ubiqiutin acceptor cysteine (Verdecia et al., 2003). N- and C-lobes are connected with a flexible hinge linker in which the NES_{RSP5} motif is located (Figure 6). Notably, a corresponding region of Smurf1, a human homologue of Rsp5, has also

been shown to function as a Crm1-dependent NES signal (Tajima et al., 2003). Mutation of the identified NES_{RSP5} did result in increased nuclear localization of Rsp5 fragment (F3, a.a. 484-809) comprising the Hect domain fused to GFP_2 and of full-length GFP-HA-Rsp5-nes detected by fluorescence microscopy. Application of a sensitive two-plasmid reporter system (Godon et al., 2005) also indicated the presence of a nuclear pool of Rsp5 protein which was increased by mutation of the NES. Moreover, at the permissive temperature of 28°C the *rsp5-nes* mutant strain showed defects in Sna3-GFP sorting in MVB and transport to the vacuole, indication for the depletion of cytoplasmic pool of Rsp5. However, this phenotype could also be explained as a result of lower catalytic activity of mutant Rsp5-nes. Although we cannot exclude this possibility, the observation that nuclear degradation of Rpb1, the largest subunit of RNA polymerase II, upon UV treatment was normal argue that the nuclear activity of Rsp5 is rather not diminished. Along the same line, computational modeling suggests that the structure of Rsp5 active center and flexibility of hinge linker are not changed (our unpublished results). Thus, Rsp5 nuclear export seems to be important for Rsp5 function in yeast cells.

We also identified two independent regions which might be responsible for nuclear localization of Rsp5, both located in the highly conserved Hect domain. Each nuclear targeting signal appeared to be weaker than the NES_{RSP5} since their activity was observed only when NES_{RSP5} was inactivated. This finding is in agreement with predominantly cytoplasmic location of Rsp5 observed in most studies. Interestingly, signals directing Smurf1 to the nucleus are proposed to be located within its C-terminal region, as a deletion mutant of Smurf1 lacking this fragment fails to accumulate in the nuclei of LMB-treated cells (Tajima et al., 2003). Likewise WWP1, another human homologue of Rsp5, is not capable of entering the nucleus when the C-terminal part of its Hect domain is missing (Flasza et al., 2006). These data suggest that signals directing Rsp5 to the nucleus may be conserved. Our efforts to identify a classical basic NLS [reviewed in (Lange et al., 2007)] or non-classical NLS (McLane and Corbett, 2009) were unsuccessful; none of small basic motifs tested was sufficient to direct GFP_2 reporter to the nucleus. In some proteins larger or distant motifs are required to bind the respective importin and support nuclear import (Streckfuss-Bomeke et al., 2009; Suel et al., 2008). Thus, two NLS-containing regions of the Hect domain, each of about 100 a.a. long, were indicated but the minimal sequence of NLSs and a.a. important for their function remain to be identified.

In summary, we have shown that the Rsp5 ligase contains one NES and two potential NLS signals in the Hect domain. Inactivation of NES results in an increased nuclear pool of Rsp5 and the defect in cytoplasmic but not in nuclear function. The results are consistent with the model that Rsp5 shuttles between the nucleus and cytoplasm and that this mechanism is important for its function.

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Figure legends

Figure 1. Predicted NES directs NLS_{H2B}-GFP_2 to the cytoplasm and substitution of IDI with ADA a.a. prevents this localization. **A,** Schematic representation of Rsp5 domain structure and localization of the sequence tested for NES activity. Amino acids conforming to the NES consensus are underlined and a.a. substitutions are shown. C2 domain is in *black*, WW domains are in *grey* and Hect domain is in *whit*e, NES_{RSP5} is in *dark gray*. **B,** Western blot analysis of a wild type strain (MHY501) transformed with plasmid encoding GFP_2, NLS_{H2B}-GFP_2, NLS_{H2B}-NES_{RSP5}-GFP_2, or mutant NLS_{H2B}-nes_{RSP5}-GFP_2. **C,** Fluorescence microscopy of the same strains as in B. Cells were stained with Hoechst to visualize the nucleus.

Figure 2. NLS_{RSP5} is Crm1-dependent. **A,** MNY8 strain producing LMB-sensitive Crm1 protein was transformed with plasmids encoding GFP_2, NLS_{H2B}-GFP_2 or NLS_{H2B}-NES_{RSP5}-GFP_2 and grown in SD+cas-ura at 23°C and treated either with ethanol or LMB (100 ng/ml) for 30 min. Western blot of cell extracts was performed. **B,** Cells of the same strains as in A were stained with Hoechst and viewed by fluorescence microscopy.

Figure 3. C-terminal part of Rsp5 comprising Hect domain directs GFP_2 to the nucleus by two independent NLS sequences when NES is inactive. **A,** Domain structure of F3, $C1nes_{RSP5}$ and $C2nes_{RSP5}$ fragments of Rsp5. Domains as in Figure 1. Stars represent a.a. substitutions. **B**, Western blot of cell extracts from strain (MHY501) bearing plasmids encoding F3-GFP_2, $F3nes_{RSP5}$-GFP_2, NLS_{H2B}-F3-GFP_2, NLS_{H2B}-$F3nes_{RSP5}$-GFP_2, $C1nes_{RSP5}$-GFP_2, nes_{RSP5}-GFP_2 or $C2nes_{RSP5}$-GFP_2. **C**, Fluorescence of the same set of cells as in B. Arrows point to the nucleus.

Figure 4. Characterization of Rsp5-nes protein nuclear pool. **A,** Localization of GFP-HA-Rsp5-nes protein. MNY7 strain was transformed with plasmids expressing P_{GAL1}*GFP-HA-RSP5* or P_{GAL1}*GFP-HA-rsp5-nes;* transformants were grow in SC-ura-trp containing raffinose at 28°C and expression of the gene fusion was induced for 4 hours by galacose addition. Cells were stained with Hoechst and viewed by epifluorescence. Bar represents 5 µm. **B,** LG-Rsp5 and LG-Rsp5-nes fusion proteins are functional. Growth of transformants of parental strain and *rsp5*Δ spore clones expressing *LG-RSP5* or *LG-rsp5-nes* and of parental strain (MHY501) transformed with empty vector, all transformed also with p80lacZ reporter plasmid, grown on SD-his-ura for 4 days at 28°C. **C,** Assays for β-gal activity indicates nuclear pool of Rsp5 which is increased when the NES is inactivated. Wild type transformants bearing empty vector or plasmids encoding LG-Rsp5 or LG-Rsp5-nes proteins and p80lacZ reporter plasmid were grown, replicated on a filter and β-gal activity filter assay was performed. β-gal activity was also measured in cell extracts and is given in nmol/minutes/µg of protein ± standard errors from three independent experiments.

Figure 5. Phenotypic analysis of *rsp5-nes* mutant. **A,** The *rsp5-nes* mutant is temperature sensitive. Growth of wild type and *rsp5-nes* strains on YPD plates at 28°C or 37°C for 2 days (left panel). Rsp5-nes is less stable than Rsp5 at elevated temperature (right panel). Western blot with anti-Nedd4 WW2 antibody (recognizing Rsp5) of cell extracts from mutant strains *rsp5*Δ, *rsp5-nes,* and their respective wild type controls grown in YPD at 28°C and shifted to 37°C for 0-2 hours, as indicated. Pgk1 is a loading control. The level of Rsp5 relative to wild type time 0 is given below the blot. **B**, Targeting of Sna3-GFP to the vacuole is defective in

rsp5-nes mutant cells. Isogenic wild type and *rsp5-nes* strains transformed with plasmid expressing Sna3-GFP from P_{TPI1} promoter, were grown at 28°C to logarithmic phase. Confocal microscopic images of cells producing Sna3-GFP are shown on the left. Western blot from total extracts performed by using anti-GFP antibody is shown on the right. **C**, Degradation of Rpb1 upon UV-irradiation occurs normally in *rsp5-nes*. Western blot of total Rpb1 after irradiation (250 J/m2) in the indicated strains. 0, no irradiation; 1-3, hours of recovery after irradiation. Pgk1 is a loading control. The level of Rpb1 relative to wild type time 0 is given below the blot.

Figure 6. Ribbon representation of the Rsp5 model structure. NES region is highlighted in *red* with hinge fragment indicated. C777 in catalytic center is presented in *yellow*, I694 and I696 in *red* as sticks.

Supporting information

Table S1. Plasmids constructed in this study.

Figure S1. Localization of N-terminal and central fragments Rsp5. **A,** Domain structure of F1 and F2 fragments of Rsp5. Domains as in Figure 1. Stars represent a.a. substitutions. **B**, Western blot of cells extracts from strain (MHY501) bearing plasmid encoding GFP_2, F1-GFP_2, F1w123-GFP_2, F1Δw-GFP_2, F2-GFP_2, F2w123-GFP_2, NLS_{H2B}-GFP_2, NLS_{H2B}-F1-GFP_2, or NLS_{H2B}-F2-GFP_2. Irrelevant lines were removed. **C**, Fluorescence of the same set of cells as in B. DNA was stained by Hoechst.