REVIEW

RNA Biology 12:12, 1265–1274; December 2015; © 2015 Taylor & Francis Group, LLC

Role of Rsp5 ubiquitin ligase in biogenesis of rRNA, mRNA and tRNA in yeast

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Keywords: mRNA, RNA nuclear/cytoplasmic transport, RNA processing, rRNA, Rsp5 ubiquitin ligase, tRNA, ubiquitination

Abbreviations: Ub, ubiquitin; DUBs, deubiquitinating enzymes; HECT, homologous to E6AP C-terminus; ARTs, arrestin-related trafficking adaptors; pre-rRNA, precursor rRNA; MVB, multivesicular body; Nups, nucleoporins; RNAPII, RNA polymerase II; TC-NER, transcription-coupled nucleotide excision repair; CTD, C-terminal domain; rRNA, ribosomal RNA; NPCs, nuclear pore complexes; pre-mRNAs, mRNA precursors; mRNPs, messenger ribonucleoprotein particles; TREX, transcription/export complex; HSE, heat shock elements; STRE, stress response element; UBA, ubiquitin - associated domain; tRNAs, transfer RNAs; pre-tRNA, precursor tRNA; tDNAs, tRNA genes.

Rsp5 ubiquitin ligase is required for ubiquitination of a wide variety of proteins involved in essential processes. Rsp5 was shown to be involved in regulation of lipid biosynthesis, intracellular trafficking of proteins, response to various stresses, and many other processes. In this article, we provide a comprehensive review of the nuclear and cytoplasmic functions of Rsp5 with a focus on biogenesis of different RNAs. We also briefly describe the participation of Rsp5 in the regulation of the RNA polymerase II complex, and its potential role in the regulation of other RNA polymerases. Moreover, we emphasize the function of Rsp5 in the coordination of the different steps of rRNA, mRNA and tRNA metabolism in the context of protein biosynthesis. Finally, we highlight the involvement of Rsp5 in controlling diverse cellular mechanisms at multiple levels and in adaptation of the cell to changing growth conditions.

Introduction

Ubiquitination is the process of conjugating the small peptide ubiquitin (Ub) to lysine residue of the substrate protein. It can be reversed by the removal of Ub by deubiquitinating enzymes (DUBs) which are ubiquitin-specific proteases. Ubiquitination can regulate diverse properties of a protein such as its half-life, activity, subcellular localization, or ability to form complexes. A protein can be modified by a single Ub (monoubiquitination), a chain of Ubs formed by attachment of consecutive Ub molecules to lysines (K6, K11, K27, K29, K33, K48, or K63) present in Ub itself (polyubiquitination), and by attachement of several Ub molecules individually to several lysine residues of the substrate protein (multiubiquitination). Ub chains linked via K48 or K63 are the most common. Mono- and multiubiquitination can act as signals for endocytosis, histone regulation, DNA repair, or nuclear export. However, substrates modified by K48-linked polyubiquitin chains composed of at least 4 Ub moieties are recognized by the proteasome and directed for proteasomal degradation.[1] Attachment of Ub chains linked through lysines other than K48 can regulate cellular processes in both proteasomal-dependent[2,3] as well as an independent manner, e.g., endocytosis.[4]

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Submitted: 08/07/2015; Revised: 09/03/2015; Accepted: 09/07/2015
http://dx.doi.org/10.1080/15476286.2015.1094604

Ubiquitination is accomplished by a cascade of enzymatic reactions consisting of 3 steps. First, Ub is activated by the E1 enzyme and then, accepted by the conjugating enzyme E2. In the third step, Ub is transferred from E2 to the substrate protein in the presence of ubiquitin ligase E3 (reviewed in ref.[5]). One of the major subfamilies of E3 ligases is the homologous to E6AP C-terminus (HECT) domain-containing family of proteins.[6] The HECT ligases actively participate in the ubiquitination by forming a thioester intermediate with Ub prior to attaching it to the substrate. The best studied subgroup of HECT E3s is the Nedd4-like family of ubiquitin ligases which exhibits a characteristic C2-WW-HECT modular structure (reviewed in ref.[7]).

The *Saccharomyces cerevisiae* Nedd4 homolog, Rsp5, is an essential yeast ubiquitin ligase. It is composed of an N-terminal C2 domain, 3 WW domains, and a C-terminal catalytic HECT domain (**Fig. 1**). The C2 domain is responsible for binding lipids[8] and proteins. WW domains are involved in diverse protein-protein interactions (to date, 124 such interactions have been reported) and recognize proline-rich sequences called PY motifs[9] in Rsp5 substrates or Rsp5 adaptor proteins, such as the arrestin-related trafficking adaptors (ARTs) that mediate Rsp5 - substrate binding[10] (reviewed in ref.[11]). Therefore, Rsp5 recognizes a wide variety of substrates and is a key protein implicated in various signaling pathways. Consequently, regulation of Rsp5 will affect numerous cellular processes. Thus, Rsp5 can coordinate the processes inside the cell with changing conditions and provide response of the cell at multiple levels.[12] The HECT domain is essential for Rsp5 activity in ubiquitination[13] and its structural flexibility underlies the ability to adapt to different substrates and modify them by attachment of ubiquitin moieties.[14] Rsp5 preferentially forms K63-linked Ub chains.[15,16]

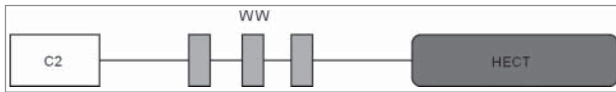

Figure 1. Domain structure of Rsp5 ubiquitin ligase. C2 domain at the N-terminal region binds lipids and proteins; WW domains are responsible for protein-protein interactions; HECT is a catalytic domain.

Rsp5 is localized to the cytoplasm, in cortical patches, perivacuolar endosomal structures, and has also been reported to have a nuclear pool. Cholbinski et al.[17] showed that Rsp5 contains one nuclear export signal (NES) and 2 nuclear localization signals (NLS), both located in the HECT domain and therefore, the protein is able to shuttle between the nucleus and cytoplasm. Rsp5 nuclear export is Crm1-dependent[17] and intriguingly, Crm1 is mainly involved in the export of precursor rRNA (pre-rRNA).[18] The many processes in which Rsp5 plays a key role are highly diverse, including biosynthesis of unsaturated fatty acids and other lipids (reviewed in ref.[19]), endocytosis, multivesicular body (MVB) sorting, lysosomal degradation of plasma membrane proteins (reviewed in ref.[20]), and actin cytoskeleton organization and function.[21,22] Recently, Lu et al.[23] have shown that Rsp5 is also involved in degradation of protein aggregates in the autophagy pathway.

Rsp5 can affect both cytoplasmic and nuclear processes and coordinate many of them with each other to ensure efficient response of the cell to changing conditions. This review will focus on the function of Rsp5 ubiquitin ligase in RNA biology. We present Rsp5 involvement in regulation of Rpb1, the largest subunit of RNA polymerase II (RNAPII) and the novel insights gained from the *in vivo, in vitro* and microarray studies on the role of Rsp5 in rRNA, mRNA and tRNA biogenesis. The article describes Rsp5 requirement in transcription, processing and transport of different RNA species, placing this enzyme as an important regulator of RNA biology.

Rsp5 is involved in regulation of RNAPII

Transcription elongation by RNAPII is not a continuous process and the polymerase is often stalled leading to transcriptional arrest (reviewed in ref.[24]). RNAPII stalling can be caused by numerous factors, e.g., difficult to transcribe sequences,[25] chromatin structure[26] or DNA adducts[27] (reviewed in ref.[28]). Upon DNA damage the RNAPII complex does not dissociate from the site of damage and remains attached to the template, blocking access of repair factors to the lesion. On the other hand, the high stability of the complex is crucial for transcription fidelity.[29] When the transcription-coupled nucleotide excision repair (TC-NER) pathway is unable to remove stalled RNAPII, its largest subunit Rpb1 is polyubiquitinated and degraded.[30,31] Interestingly, this event is induced not only by DNA damage, but it also occurs in different situations leading to transcriptional stalling,[32,33] e.g., a mutation of the gene encoding transcript cleavage factor TFIIS.[34]

RNAPII ubiquitination and degradation is a regulated multistep process. In the first step, Rpb1 is ubiquitinated by Rsp5[30,31] (**Table 1**), which associates with the C-terminal domain (CTD) of Rpb1 via its WW domain.[35] Although Rpb1 is not ubiquitinated and degraded upon Rsp5 inactivation,[31,36] Rsp5 catalyzes only monoubiquitination or K63-linked polyubiquitination of Rbp1,[37] which usually do not direct proteins for proteasomal degradation. In the case of polyubiquitinated Rpb1 with Ubs linked via K63, the Rsp5-associated DUB, Ubp2 trims the Ub chains and leaves a single ubiquitin moiety.[15] Rpb1 is then polyubiquitinated with K48-linked Ubs by the Elc1-Ela1-Cul3-Rbx1 complex.[38,39] Intriguingly, only the pre-monoubiquitinated Rpb1, but not the unmodified protein, can be polyubiquitinated.[36] Moreover, Ubp3 DUB is able to completely remove K48-linked polyubiquitin chains and rescue Rpb1 from degradation. Thus, Ubp3 provides a proof-reading role and can prevent unnecessary degradation.[40] Finally, the ATPase Cdc48-Ubx5 and 26S proteasome are recruited to the polyubiquitinated RNAPII. The polymerase complex disassembles from DNA,

Table 1. Polymerase I, II and III subunits and nucleoporins ubiquitinated by Rsp5

Protein	Process	Method	Reference
Pol I complex			
Rrn3, Rrn5, Rrn6, Rrn7, Rrn9, Rrn10, Rrn11	transcription	*in vitro*	42
Pol II complex			
Rpb1	transcription	*in vitro, in vivo*	30,31
Rpb2, Rpb3, Rpb5, Rpb7, Rpb8, Rpb9, Rpb10, Rpb11		*in vitro*	42
Pol III complex			
Bdp1, Brf1, Maf1, Pzf1, Ret1, Rpc11, Rpc17, Rpc34, Rpc37, Rpc82, Sfp1, Tfc1, Tfc3, Tfc4, Tfc6, Tfc7, Tfc8	transcription	*in vitro*	42
Rpc25		*in vitro*, microarray	42,43
Nucleoporins			
Nup1, Nup2, **Nup42**, **Nup49**, Nup57, Nup82, Nup84, **Nup85**, Nup116, Nup120,Nup133,Nup145, Nup157, Nup159, **Nup188**	mRNA export	*in vitro*	42
Nup53		*in vitro*, microarray	42,43
Nup60	nucleocytoplasmic transport	*in vitro*	42
Nup192	nuclear pore organization		

In bold are Nups which were shown not to be ubiquitinated *in vivo*.[95]
Underlined is Nup159 which was described to be ubiquitinated *in vivo* by Cdc34/SCF.[95]

ubiquitinated Rpb1 dissociates from RNAPII and is degraded in the proteasome while other subunits are released.[41] Taken together, Rsp5 plays an important role in the RNAPII transcription by removing stalled RNAPII complexes and allowing repair of lesions. Moreover, some subunits of RNA polymerase I (RNAPI) or RNA polymerase III (RNAPIII) have also been reported to be Rsp5 substrates, e.g., Rrn subunits (Rrn3, Rrn5, Rrn6, Rrn9, Rrn10 and Rrn11) of RNAPI[42] or different RNAPIII subunits[42,43] (**Table 1**). Therefore, future research is required to provide information whether Rsp5 regulates transcription by affecting all 3 RNA polymerases.

Rsp5 affects rRNA processing and transport

Synthesis of eukaryotic ribosomes is a complex but crucial process for growing cells. Ribosomes are composed of a 60S large subunit containing 3 ribosomal RNA (rRNA) species: 25S, 5.8S, and 5S rRNAs, and a 40S small subunit with 18S rRNA. A large polycistronic transcript containing 18S, 5.8S and 25S rRNAs, generated by RNAPI, undergoes endo- and exonucleolytic processing to yield the mature rRNAs (reviewed in ref.[44,45]). However, 5S rRNA is transcribed independently by RNAPIII.[46]

Export of precursor 40S (pre-40S) and pre-60S ribosomal subunits through nuclear pore complexes (NPCs) in yeast and higher eukaryotes requires Crm1 exportin (also known as Xpo1), which is a member of the β-importin family.[18] The only known adaptor between Crm1 and pre-60S is Nmd3 protein,[47] while the Crm1 adaptor for pre-40S subunit has not been identified yet.[48] Mex67/Mtr2 (mRNA heterodimeric export receptor) and Arx1 proteins also participate in the export of pre-60S subunit.[49] Furthermore, other proteins have been suggested to function in the export of pre-60S subunits, e.g., the mRNA export factor Npl3[50] and Exp-5, the vertebrate ortholog of yeast Msn5 protein that is involved in tRNA transport.[51] Thus, ribosome nuclear export is likely connected to the export of mRNAs (**Fig. 2**) and tRNAs.

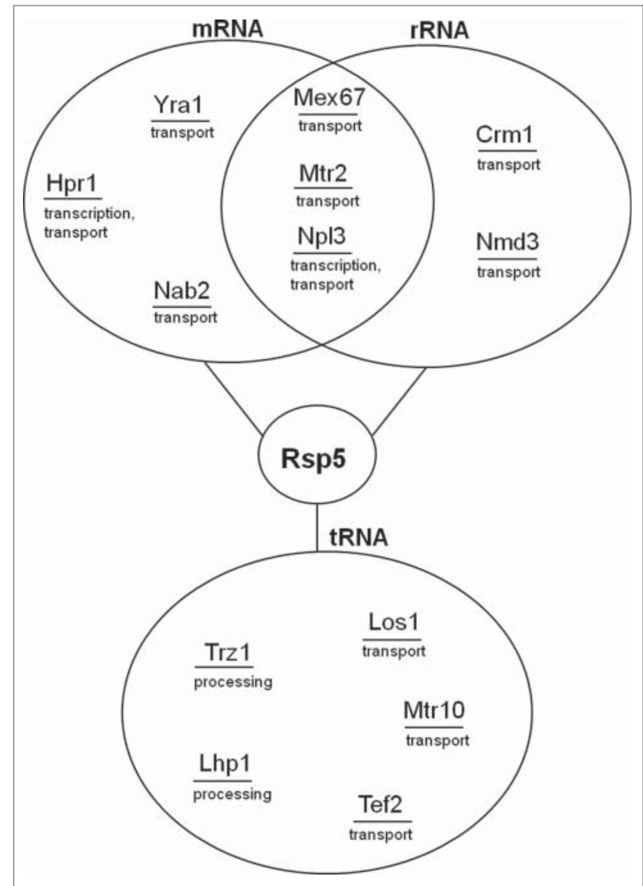

Figure 2. Proteins identified as Rsp5 substrates and involved in biogenesis of rRNA, mRNA and tRNA. The same proteins can participate in biology of various RNAs thus providing a link between different processes.

Several links between Rsp5 and the protein biosynthesis machinery have been reported. Neumann et al.[52] showed that Rsp5 influences pre-rRNA maturation and its transport from the nucleus to the cytoplasm. Nuclear export of the pre-60S subunit was inhibited in *rsp5-3* mutant at the restrictive temperature[52] (**Table 2** and **Table 3**). Thus, Rsp5 is involved in nuclear export of pre-rRNA. Moreover, pre-rRNA analysis showed that 35S pre-rRNA accumulated in the *rsp5-3* mutant and the products of 35S processing (20S and 27S pre-rRNAs) were strongly depleted at the elevated temperature (**Table 2**). These results show that the early pre-rRNA cleavages at some sites can be inhibited in the *rsp5-3* strain. Consistent with the pre-rRNA processing defects, the level of mature 18S, 5.8S and 25S rRNAs was reduced in *rsp5-3* cells[52] (**Table 2**).

A significant depletion of 18S and 25S rRNA was also reported for cells harboring the *rsp5-19* mutation (**Table 3**) after a shift to the restrictive temperature[53] (**Table 2**). Furthermore, a smear of rRNA-derived decay products was clearly visible. This rRNA destabilization was not an effect of the elevated temperature itself because the rRNA level remained unchanged in other temperature-sensitive strains tested.[53] It was reported that other mutations in *RSP5*, namely *rsp5-1* and *rsp5-3*, also led to rRNA degradation[53] (**Table 2** and **Table 3**). Moreover, ribosomes were not degraded in *rsp5-19* cells transformed with plasmid encoding *RSP5*. Based on these results, Shcherbik and Pestov[53] concluded that Rsp5 is important for maintaining rRNA stability in yeast. Noteworthy, Rsp5 affects multiple RNAs,[52] but mature rRNAs are apparently more vulnerable to degradation than other stable RNA species. Consistent with the decrease in the level of mature rRNA observed in *rsp5-19* cells, also the total rate of ribosomes was diminished and the levels of polysomes and 80S ribosomes were strongly reduced in this mutant[53] (**Table 2**) which is in agreement with the studies showing that in yeast rRNA degradation is complex and regulated process.[54]

To verify whether *rsp5* mutations could enhance general autophagy, which directs cytoplasmic ribosomes to destruction in the vacuole,[55] or induce ribophagy,[56] a ribosome-specific autophagy pathway, deletions of key autophagy genes were introduced into the *rsp5-19* strain. Those mutations, however, did not suppress the rRNA degradation caused by the *rsp5* defect. Thus, the enhanced degradation of ribosomes caused by a lack of functional Rsp5 in rich medium utilizes a mechanism different rather than ribophagy.[53] The importance of Rsp5 for ribosome stability

Table 2. Rsp5 alleles and their phenotypes

Mutant	Phenotype			Reference
	rRNA	mRNA	tRNA	
rsp5-1	degradation of 25S rRNA			53
		accumulation of poly(A)$^+$ RNA in the nucleus		71
rsp5-19	degradation of 18S and 25S rRNA (Northern and FISH analysis)			53
	decreased total rate of ribosomes			53
	reduced level of polysomes and 80S ribosomes			53
		accumulation of poly(A)$^+$ RNA in the nucleus		90
			accumulation of tRNA^{Leu} precursors (Northern analysis)	53
			accumulation of tRNA^{Tyr} and tRNA^{Met} in the nucleus (FISH analysis)	90
rsp5-3	inhibition of nuclear export of the pre-60S subunit			52
	accumulation of 35S pre-rRNA			52
	decreased level of 20S and 27S pre-rRNAs			52
	reduced level of mature 18S, 5.8S and 25S rRNAs			52
	degradation of 18S and 25S rRNA			53
		accumulation of poly(A)$^+$ RNA in the nucleus		52
			accumulation of tRNA^{Leu} and tRNA^{Tyr} in the nucleus (FISH analysis)	52
			accumulation of primary transcripts for tRNA^{Leu} and tRNA$^{Arg\text{-}Asp}$ (Northern analysis)	52
rsp5-ww2		accumulation of poly(A)$^+$ RNA in the nucleus		71
rsp5-ww3		accumulation of poly(A)$^+$ RNA in the nucleus		71
rsp5-A401E		accumulation of HSF1 and MSN2/4 mRNAs in the nucleus		72
rsp5Δ		accumulation of poly(A)$^+$ RNA in the nucleus		71
		accumulation of mRNAs encoding Pgk1, Ssa1, Ssa2 and Ssa3 in the nucleus		71

Table 3. *RSP5* mutants

Mutant	Description of mutation	Reference
rsp5-1	L733S substitution in HECT domain	13
rsp5-19	P418L substitution in WW3 domain	90
rsp5-3	T104A substitution between C2 and WW1 domain, E673G and Q716P substitutions in HECT domain	52
rsp5-ww2	W359F and P362A substitutions in WW2 domain	71
rsp5-ww3	W415F and P418A substitutions in WW3 domain	71
rsp5-A401E	A401E substitution in WW3 domain	72
rsp5Δ	deletion of *RSP5* gene	71

is well proven since rRNA degradation was observed in different *rsp5* temperature sensitive mutants and could be prevented by expression of wild-type *RSP5*.

In conclusion, there are considerable data supporting the role of Rsp5 in ribosome assembly, nuclear export and cytoplasmic stability. Moreover, some proteins involved in pre-rRNA nuclear export, e.g., exportin Crm1 and its adaptor, Nmd3, have been shown *in vitro* to be Rsp5 substrates[42] (**Table 4** and **Fig. 2**). Thus, Rsp5 could influence rRNA transport via ubiquitination of export proteins. Intriguingly, Bai et al.[57] demonstrated that rRNA export affects rRNA synthesis. In HeLa cells nucleolar localization of Crm1 and Nmd3 was increased by inhibition of RNAPI. In addition, inhibition of Crm1 and depletion of Nmd3 reduced the level of 47S rRNA precursor, and inhibition of Crm1 compromised the processing of 28S rRNA. The data indicate that Crm1 and Nmd3 function in pathways that couple rRNA synthesis, processing and nuclear transport, and that RNA export machinery modulates rRNA synthesis probably through some yet unidentified signaling pathways. Altogether, a lack of ubiquitination of these proteins could lead to defects in rRNA processing and transport in *rsp5* mutants. Therefore, *in vivo* studies on ubiquitination of Crm1 and Nmd3 proteins by Rsp5 could facilitate our understanding of the role of this modification in rRNA biology. Finally, it is also possible that changes in pre-rRNA processing, transport and the level of ribosomes in *rsp5* cells could be caused by the action of signaling pathways which could be affected in these mutants.

Rsp5 regulates mRNA nuclear-cytoplasmic trafficking

It is well established that mRNA synthesis and processing are coupled to its nuclear export, which requires that the newly synthesized mRNA precursor (pre-mRNA) undergoes several processing steps, including 5′ capping, splicing, 3′ end cleavage and polyadenylation. During transcription nascent mRNA interacts with mRNA-binding proteins and noncoding RNAs leading to the formation of messenger ribonucleoprotein particles (mRNPs) that are transported to the cytoplasm (reviewed in ref.[58]). Various steps leading to mRNP formation are linked and mediated by interactions with the RNAPII elongation complex and the processing machinery. The coupling of different steps of mRNA biogenesis could be a part of quality control mechanisms ensuring that only mature and export-competent transcripts are transported to the cytoplasm.

Nuclear export of mRNA requires the heterodimeric export receptor Mex67/Mtr2, a transporter that is distinct from β-importins.[59] Mex67 interacts with mRNPs while Mtr2 promotes Mex67 interaction with NPCs.[60] As Mex67 exhibits low affinity for RNA, its interaction with mRNA is mediated by adaptor proteins, for example Yra1.[61] The recruitment of Yra1 and its partner Sub2 occurs co-transcriptionally and is splicing-independent.[62,63] Yra1 and Sub2 proteins are associated with the THO complex, implicated in transcription elongation and mRNA export. In yeast, the THO complex and the mRNA export factors Sub2 and Yra1 are components of the multi-subunit transcription/export (TREX) complex engaged in coupling transcription to mRNA export.[64,65] TREX is recruited to an active gene during transcription elongation. It has been proposed that the binding of Mex67 to Yra1 at a later step displaces Sub2 from Yra1 before mRNA export.[63] Furthermore, additional adaptors are implicated in mRNA export since Yra1 does not associate with all yeast transcripts and is dispensable for mRNA export in *Caenorhabditis elegans* and Drosophila melanogaster.[66-68] Interestingly, 2 identified adaptors of Mex67, Npl3 and Nab2 are regulated by ubiquitination (reviewed in ref.[69]).

The involvement of Rsp5 and the ubiquitin/proteasome system in mRNA export is well characterized. Initial evidence for the role of the ubiquitin pathway in mRNA nuclear export was provided by studies performed using a *Schizosaccharomyces pombe* temperature-sensitive ubiquitin-activating E1 enzyme (Ptr3) mutant, in which the mRNA export was defective.[70] To determine the role of the ubiquitin pathway in mRNA export, the function of each ubiquitin-protein ligase from the HECT family was analyzed in *S. cerevisiae*. The temperature sensitive *rsp5-1* mutant accumulated poly(A)$^+$ RNA in the nucleus only at the restrictive temperature, whereas *rsp5*Δ^{71} cells (**Table 3**) also at normal temperature, even when the essential role of Rsp5 in unsaturated fatty acid biosynthesis was bypassed by adding oleic acid to the medium[71] (**Table 2**). Thus, the nuclear mRNA accumulation caused by *RSP5* mutations is not due to a shortage of unsaturated fatty acids. It was indicated using *rsp5-1, rsp5-ww2* and *rsp5-ww3* mutants (**Table 3**) that 3 domains of Rsp5 are implicated in the export of mRNA from the nucleus to the cytoplasm: WW2, WW3 and HECT.[71] Strong nuclear accumulation of poly(A)$^+$ RNA was also observed in the *rsp5-3* strain[52] (**Table 2**).

The role of Rsp5 in the export of specific mRNAs has also been investigated. For example, Rodriguez et al.[71] demonstrated that a lack of functional Rsp5 leads to defect in the nuclear export of mRNA encoding the glycolytic enzyme Pgk1 as well as

Table 4. Proteins involved in RNA biogenesis and ubiquitinated by Rsp5

Protein	Process	Method	Reference
rRNA biogenesis			
Crm1	export	*in vitro*	42
Mex67			
Mtr2			
Nmd3			
Npl3		*in vitro*; microarray	42,43,9
mRNA biogenesis			
Hpr1	transcription, export	*in vivo, in vitro*	73
Mex67	export	*in vitro*	42
Mtr2			
Nab2			
Yra1			
Npl3	transcription, export	*in vitro*; microarray	9,42,43
tRNA biogenesis			
Lhp1	processing	microarray	43,9
Los1	transport	*in vitro*	42
Mtr10			
Tef2	transport	*in vitro*, microarray	42,43
Trz1	processing	microarray	43

mRNAs encoding Ssa heat shock proteins (Ssa1, Ssa2 and Ssa3) (**Table 2**). In addition, other authors have shown that in response to environmental stresses, Rsp5 regulates at the post-transcriptional level the expression of genes for 2 major transcription factors, Hsf1 and Msn2/4 which are responsible for stress-induced gene expression. Hsf1 binds to heat shock element (HSE) whereas Msn2/4 binds to the stress response element (STRE) in the promoters of many stress protein genes.[72] In the *rsp5-A401E* mutant (**Table 3**) that is hypersensitive to various stresses, including high temperature, transcription of stress protein genes is defective. Although mRNA levels of *HSF1* and *MSN2/4* in *rsp5-A401E* are only slightly lower than in wild type cells, the respective protein levels are remarkably decreased in this mutant because *HSF1* and *MSN2/4* mRNAs accumulate in the nucleus of *rsp5-A401E* cells (**Table 2**). Thus, by modulating nuclear mRNA export Rsp5 regulates the expression of transcription factors required for stress responses.[72]

Rsp5 ubiquitin ligase activity is required for mRNA export, suggesting that Rsp5 substrates might include mRNA nuclear export factors. Indeed, Rsp5-dependent regulation of a component of THO complex, Hpr1, was shown.[73] Degradation of Hpr1 is enhanced at high temperature and is linked to on-going RNAPII-mediated transcription while the stability of other components of THO complex is not affected under these conditions suggesting that Hpr1 turnover could control the formation of the TREX complex and mRNA export. Thus, Hpr1 appears to be a key factor of the THO complex and its stability controls the activity of the whole complex. Hpr1 is polyubiquitinated by Rsp5 in conjugation with E1 and Ubc4 as an E2 and degraded in the proteasome both *in vivo* and in vitro[73] (**Fig. 2**). Although the data demonstrate that interactions within the enzyme-substrate complex do not require any adaptor *in vitro*, it cannot be excluded that ubiquitination of Hpr1 can be regulated by an additional ubiquitin ligase or other proteins *in vivo*, as it is for Rpb1 protein. It is known that ubiquitination of proteins with K63-linked Ub chains usually does not lead to their degradation in the proteasome. Nevertheless, it is possible that K63-linked Ub chains could serve as a proteasome targeting signal in vivo.[3] Therefore, further analysis of Hpr1 ubiquitination *in vivo* is required to decipher fully its degradation mechanism. The ubiquitin - associated (UBA) domain of Mex67 binds the Hpr1-attached polyubiquitin chain and transiently protects the protein from degradation.[74] Probably, upon mRNA 3′ end formation, Mex67-Mtr2 is transferred onto nascent mRNA exposing the ubiquitinated Hpr1 to the proteasome. Degradation of Hpr1 releases mRNA and causes disassembly of the THO complex which can then participate in a next transcription event (reviewed in ref.[69]). The Mex67-Hpr1 interaction coordinates transcription with recruitment of the mRNA export machinery.[74] Noteworthy, it has been shown that the human ortholog of Hpr1, Thoc1, is polyubiquitinated by Nedd4 and then, degraded by the proteasome, suggesting that regulation of THO activity by HECT ligases and the ubiquitin-proteasome pathway is evolutionarily conserved between yeast and mammals.[75]

The export receptor Mex67-Mtr2 utilizes 2 adaptor proteins regulated by the ubiquitin pathway. One of them, Npl3, a shuttling protein implicated in coordinating transcription and mRNA export,[76] has been proposed to be an Rsp5 substrate[9,42,43] (**Table 4** and **Fig. 2**). In addition, Npl3 can be phosphorylated and dephosphorylated which influences its interaction with Mex67 and has an impact on mRNA export. It is not clear how Rsp5 affects Npl3 and what the biological function of this interaction is; so further studies are required. However, the data provide evidence that post-translational modifications are important to coordinate recruitment of Mex67 with transcription and export. Also another Mex67 adaptor, a shuttling mRNA binding protein Nab2,[77] which stabilizes the adaptor-receptor interaction by directly binding Mex67 and Yra1, was shown *in vitro* to be an Rsp5 substrate[42] (**Table 4** and **Fig. 2**).

The above examples exemplify Rsp5 contribution to mRNA transport. In addition to Hpr1 or Npl3, other proteins involved in mRNA export have been shown to be Rsp5 substrates. *In vitro* studies have indicated that Mex67, Mtr2 and Yra1 can be ubiquitinated by Rsp5[42] (**Table 4** and **Fig. 2**). The question that remains to be resolved concerns how their modifications could affect mRNA biology. Noteworthy, no defects have been observed in the splicing of the *ACT1* mRNA or pre-mRNA-like intron-containing U3 precursor. According to Neumann et al.[52] Rsp5 seems not to be involved in mRNA processing. However, little is known about the involvement of Rsp5 in mRNA maturation and further studies are necessary to fully understand its role in this process.

Rsp5 modulates tRNA processing and transport

Transfer RNAs (tRNAs) are crucial in protein synthesis and take part in other processes. They deliver amino acids to the ribosome for incorporation into a polypeptide chain and participate in signaling pathways. tRNA biogenesis in yeast involves the synthesis of initial transcript by RNAPIII followed by post-transcriptional alterations to generate mature tRNA. The post-transcriptional steps comprise the removal of the 5′ leader, trimming the 3′ trailer, addition of CCA to the 3′ terminus, removal of introns (from tRNAs encoded by intron-containing genes), modification of multiple nucleoside residues, and export to the cytoplasm where the tRNA functions in translation (reviewed in ref.[78]). In fact, the subcellular traffic of tRNA is bidirectional and occurs in 3 steps. First, the newly synthesized and end-processed tRNAs are exported from the nucleus to the cytoplasm in the primary tRNA export process. Then, spliced tRNAs are imported back to the nucleus via retrograde tRNA nuclear import, likely to be repaired or degraded.[79-83] Finally, mature tRNAs that were imported to the nucleus are re-exported to the cytoplasm.[84]

Twenty percent of yeast tRNAs are transcribed with an intron. In yeast, splicing of tRNA introns occurs on the cytoplasmic surface of mitochondria where the tRNA splicing endonuclease complex resides,[85-87] so end-processed intron-containing tRNAs have to be exported from the nucleus to undergo splicing in the cytoplasm. The primary export of intron-containing precursor tRNA (pre-tRNA), involves the β-importin family member, Los1.[79,88,89] Because *LOS1* is non-essential gene in yeast, there is at least one additional unidentified nuclear exporter for intron-containing pre-tRNAs. Mtr10, a member of the yeast β-importin

family, is implicated in the retrograde nuclear import but whether Mtr10 directly interacts with tRNA remains unknown (reviewed in ref.[78]). tRNA re-export is mediated in yeast by both Los1 and another β-importin family member, Msn5.[79] Again, at least one unidentified transporter involved in the re-export process must exist because *msn5*Δ *los1*Δ double mutants are viable.[79,83] Thus, tRNA biogenesis is highly complex and only partially understood.

Rsp5 is involved in tRNA biology. Neumann et al.[52] reported nuclear accumulation of $tRNA^{Leu}$ and $tRNA^{Tyr}$, encoded by intron-containing genes, as well as $tRNA^{Glu}$ and $tRNA^{Gly}$, encoded by intron-less genes, in *rsp5-3* cells at the nonpermissive temperature (**Table 2**). To determine whether the nuclear accumulation of tRNA in the mutant was a consequence of defects in RNA maturation, tRNA processing was investigated. The analyses revealed that the primary transcripts for $tRNA^{Leu}$ and $tRNA^{Arg\text{-}Asp}$ accumulated in the *rsp5-3* mutant at the elevated temperature[52] (**Table 2**). In addition, Shcherbik and Pestov[53] showed that $tRNA^{Leu}$ precursors accumulated also in *rsp5-19* mutant (**Table 2**). Moreover, nuclear accumulation of $tRNA^{Tyr}$, encoded by intron-containing genes, and $tRNA^{Met}$, encoded by intron-lacking genes, was observed in this mutant after a temperature up-shift[90] (**Table 2**). Thus, defects in pre-tRNA processing in *rsp5* cells might be the cause of tRNA nuclear accumulation.

Other authors showed that the *rsp5-19* mutation alters cell sensitivity to antibiotics that affect protein synthesis and increases the fidelity of translation.[90] The mutant deficient in Rsp5 ubiquitin protein ligase activity was hypersensitive to anisomycin and cycloheximide that inhibit translation and resistant to paromomycin, which causes ribosomal misreading. The altered sensitivity of the *rsp5-19* strain to some translational antibiotics is in agreement with other data showing that Rsp5 affects translation.[91] The *rsp5-19* strain exhibits about a 70% decrease in the rate of total protein synthesis that could be caused by defects in translation or nuclear accumulation of tRNA.[90] Intriguingly, an additional copy of *TEF2* (**Table 4**), one of 2 genes encoding elongation factor eEF-1A which delivers tRNAs to the ribosome, suppressed the defect in the subcellular distribution of tRNA as well as the growth defects in the presence and in the absence of antibiotics caused by *rsp5-19* mutation.[90] This is consistent with earlier studies showing that eEF-1A and tRNA aminoacylation are required for efficient export of mature tRNAs and suggests a coordination between translation and tRNA transport. Inhibition of tRNA aminoacylation by amino acid starvation, lack of CCA addition at the 3′ end of tRNAs or specific inhibition of aminoacyl - tRNA synthetase caused nuclear accumulation of tRNA.[92] Finally, tRNAs that fail to be exported, e.g., because they are not aminoacylated, accumulate in the nucleolus where rRNA synthesis and some processing steps occur. Because the transcription and processing machineries of tRNA and rRNA share several components with each other,[93] the presence of tRNA in the nucleolus could affect rRNA biogenesis.[92] Thus, tRNA accumulation could act as a signal for RNAPI leading to cessation of its transcriptional activity and in this way negatively affect rRNA biogenesis. Therefore, cellular distribution of tRNA is important for the communication and signaling between the cytoplasm and the nucleus and modifications of proteins involved in tRNA dynamics could affect various processes.

Diverse approaches have revealed that some proteins involved in tRNA biogenesis are Rsp5 substrates, including those engaged in tRNA 3′ end processing (Trz1 and Lhp1) and tRNA transport (Mtr10)[44] (**Table 4** and **Fig. 2**). There is no clear evidence for Ub modification of Los1 protein. Huang and Hopper[94] did not find by mass spectrometry Ub modifications for Los1 either in fed or amino acid starved cells – however, the protein coverage was not complete (~60%). On the other hand, Lu et al.[44] identified *in vitro* Los1 as Rsp5 substrate (**Table 4** and **Fig. 2**). Taken together, Rsp5 could be involved in both tRNA processing and nuclear transport and studies of these processes will result in understanding how they can be coordinated by this ligase.

Concluding remarks

Different classes of RNA (rRNA, mRNA and tRNA) accumulate in the nucleus in temperature-sensitive *rsp5* cells at the restrictive temperature. Several mechanisms of Rsp5 involvement in RNA biogenesis are possible. Rsp5 may have multiple targets which participate in the processing and/or export of different RNAs. Indeed, as the studies described above have shown, many proteins involved in the biogenesis of RNA can be Rsp5 substrates, e.g., Crm1, Nmd3, Hpr1, Npl3, Mex67, Mtr10 (**Table 4** and **Fig. 2**). Rsp5 could also act at multiple levels and indirectly influence RNA biology, e.g., by modulating nutrient transport and cellular signaling. The defective export of various RNAs in *rsp5* mutants could also, in principle, be due to Rsp5 influence on the composition of lipids in the nuclear membrane. However, the nuclear membrane organization is apparently unaltered in *rsp5* cells[52] and thus, the role of Rsp5 in the export of various RNAs through its impact on the nuclear membrane can be rather excluded.

Alternatively, Rsp5 could affect nuclear RNA export globally by ubiquitination of the NPC component(s) engaged in all 3 nuclear export systems. Indeed, a systematic analysis showed that more than 50% of nucleoporins (Nups) are ubiquitinated.[95] Prevalence of monoubiquitination and involvement of distinct components of the ubiquitin conjugation machinery suggest that ubiquitination of the Nups is not only connected to proteasome-dependent protein degradation but rather associated with regulation of the NPC function.[95] Some nucleoporins have been postulated to be ubiquitinated by Rsp5 *in vitro*, e.g., Nup2, Nup60, Nup116, Nup192, while the proposed ubiquitination of others was not confirmed *in vivo*, e.g., Nup1, Nup42, Nup49 (**Table 1**). For Nup159, postulated to be ubiquitinated by Rsp5 *in vitro*, another ligase was identified *in vivo* (**Table 1**). However, this does not exclude possibility that investigated Nups are Rsp5 substrates in different conditions. Interestingly, it has been revealed recently that similarly to mRNA, tRNA transcription can be coordinated with its export at NPCs in yeast. Mutants in which tRNA genes (tDNAs) do not establish a contact with NPCs are still viable and therefore the transcription at the NPCs is not essential but rather might facilitate the export of pre-tRNAs and its coordination with transcription.[96] Intriguingly, it has been suggested that tRNA processing might occur at the

NPCs as well. Studies in *Caenorhabditis elegans* have shown that nucleoporins of the NPC can associate with tDNAs to regulate 3′ end processing of nascent tRNA.[97]

Thus, the different steps of tRNA biogenesis could be coordinated with each other and modification of one protein could influence numerous processes. Also, the different steps of biogenesis of mRNA or rRNA known to be coupled with each other could be coordinated at the NPC by modification of a single protein. It will be interesting to decipher the mechanism by which Rsp5 controls the biology of RNA classes at multiple levels. Although the ubiquitination by Rsp5 of many individual components implicated in RNA biogenesis has been suggested or proven, there are still many unknowns. For instance: How does Rsp5 coordinate different steps of biogenesis of an individual RNA? How is the biogenesis of one RNA coordinated with that of others? What environmental signals are important for Rsp5 function? Despite the progress that has been made in the last few years, still much remains to be clarified about the exact role of Rsp5 in RNA biogenesis and further studies are required to determine the full potential of this ligase in RNA biology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are grateful to Anita Hopper, Joanna Kufel and Teresa Zoladek for critical reading of the manuscript.

Funding

This work was supported by Foundation for Polish Science within International PhD Project "Studies of nucleic acids and proteins - from basic to applied research" (MPD/2009-3/2).

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