Chapter 4
mRNA Export

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Abstract Fungi, like all other eukaryotic cells, have a separated place of transcription and translation. Compartmentalization into nucleus and cytoplasm by the nuclear envelope necessitates bidirectional traffic of small molecules, proteins, and large RNA-protein macromolecules, which pass through nuclear pore complexes (NPCs). One major nucleo-cytoplasmic transport process is the mRNA export, not only because of the high amount of permanently generated transcripts, but also because of its enormous size. In contrast to the protein transport and the translocation of small RNAs, such as tRNAs or spliceosomal UsnRNAs, that involve the karyopherins as transport receptors and the Ran GTPase system as the driving force, bulk mRNA export requires other factors. Instead the highly conserved mRNA export receptor heterodimer *Mex67-Mtr2* (*NXF1-NXT1* or TAP-p15 in metazoans) is recruited to the mRNA and contacts the nucleoporins of the NPC to allow transit. Directionality of the transport event is provided by the ATP-dependent remodeling of the RNA/protein complexes by the DEAD-box RNA helicase Dbp5, which acts on the cytoplasmic side of the NPC. mRNA export is tightly coupled to transcription and mRNA maturation and the whole process is surveyed by a nuclear surveillance machinery that prevents immature and false transcripts from slippage into the cytoplasm and their consequent translation. These general processes of transcription, processing, and mRNA export are highly conserved among all eukaryotes, including all members of the fungal kingdom. However, the best-studied organism is the budding yeast *Saccharomyces cerevisiae* and therefore this book chapter will mostly focus on this organism.

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The Early Phase: From Pre-mRNA Transcription and Maturation to Formation of an Export Competent mRNP

All sequential steps in pre-mRNA transcription and processing are interconnected and finally lead to the recruitment of factors that are necessary for proper packaging and export of the mature messenger ribonucleoparticle (mRNP) (Fig. 4.1; see also Chap. 1). During transcript synthesis by RNA polymerase II (RNA pol II), different modifications of its C-terminal domain (CTD) lead to the timely recruitment of mRNA processing factors, such as 5′ capping, splicing, 3′-end cleavage, polyadenylation and export factors, which themselves are influenced in their functions by different modifications (Bentley 2005; Tutucci and Stutz 2011; Hsin and Manley 2012; Heidemann et al. 2013).

Initially, RNA pol II in its unphosphorylated status associates with the promoter region and forms the pre-initiation complex (Buratowski 2009; Bataille et al. 2012; Heidemann et al. 2013). Upon synthesis of the first few nucleotides of the pre-mRNA, serine five phosphorylation of the CTD leads to the recruitment of 5′ RNA capping enzymes that start pre-mRNA processing (Lidschreiber et al. 2013). As a consequence, the 5′-triphosphate end of the pre-mRNA receives a protective 7-methylguanosine cap, which is subsequently recognized and bound by *Cbp80* and *Cbp20* that form the cap binding complex (CBC). This cap structure not only protects the mRNA from degradation but also influences spliceosome assembly and impedes premature transcription termination of cryptic termination sites by preventing the loading of 3′-end processing factors (Colot et al. 1996; Gornemann et al. 2005; Wong et al. 2007).

The shuttling serine/arginine (SR)-rich protein *Npl3* is the first one in the line of several mRNA binding proteins that assemble on the nascent transcript and promote the export of the mature transcript. *Npl3* is transferred to the pre-mRNA upon serine 2 phosphorylation of the CTD of RNA pol II during transcription initiation (Lei et al. 2001; Dermody et al. 2008). Both the phosphorylation status of the CTD and the presence of *Npl3* on the pre-mRNA have an impact on early splicing factor assembly

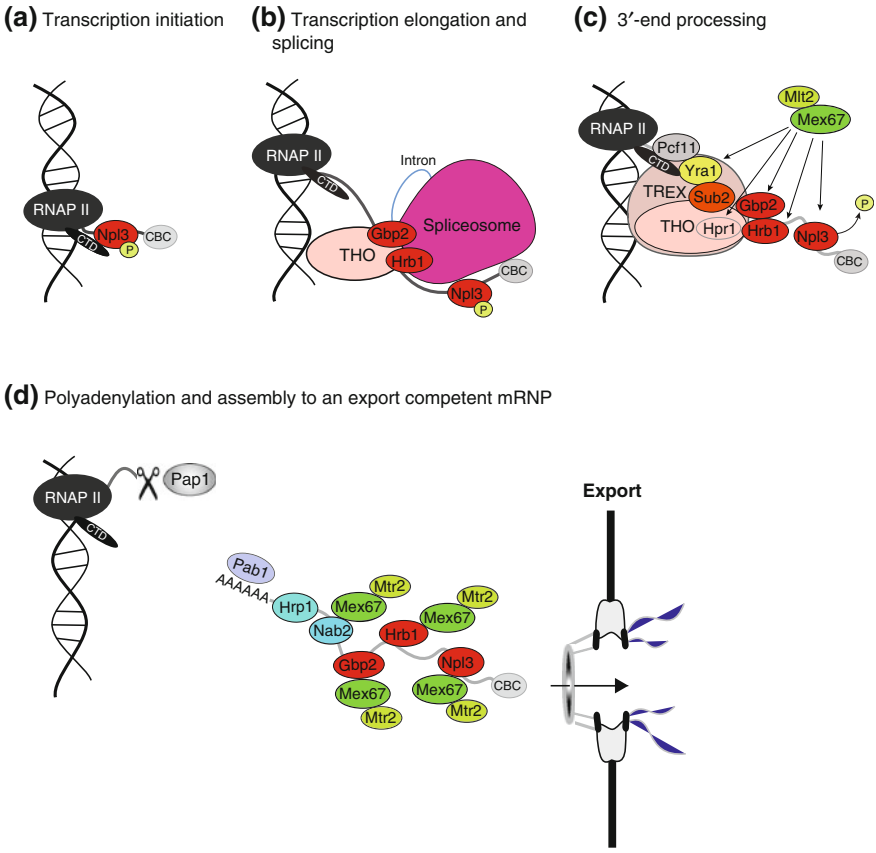

Fig. 4.1 Co-transcriptional processing and assembly of an export competent mRNP. **a** RNA polymerase II (RNAP II) promotes association of mRNA binding proteins such as the CBC and Npl3 during transcription initiation. **b** The THO complex promotes transcription elongation. Splicing occurs co-transcriptionally. *Gbp2* and *Hrb1* bind to the mRNP during splicing. **c** The THO complex interacts with *Sub2* and *Yra1* to form the TREX complex and subsequent 3′-end processing occurs. **d** Polyadenylation and recruitment of *Nab2*, *Hrp1* and *Pab1* takes place. Upon completion of the loading of the export receptor heterodimer *Mex67-Mtr2* onto the mRNP it becomes export competent and can leave the nucleus

and thus proper splicing (Morris and Greenleaf 2000; Kress et al. 2008). On the matured mRNA *Npl3* is one of several proteins that bind the essential mRNA export receptor heterodimer *Mex67-Mtr2* that is required for the passage through the hydrophobic meshwork of the NPC (Lee et al. 1996; Strasser et al. 2000; Gilbert and Guthrie 2004). Consequently, mutations in *NPL3* and *MEX67* or *MTR2* lead to mRNA export defects (Kadowaki et al. 1994; Lee et al. 1996; Hurt et al. 2000).

During transcription elongation, the phosphorylation status of the CTD changes again and RNA pol II recruits another important complex: the heterotetrameric THO complex. THO supports RNA pol II progress by preventing DNA:RNA hybrid formation during transcription elongation (Huertas and Aguilera 2003;

Rondon et al. 2003). Mutations in components of the THO complex do not only impact transcription elongation but also the export of mature mRNAs as it is involved in the recruitment of several proteins to the mRNA (Strasser et al. 2002; Hurt et al. 2004; Gwizdek et al. 2006).

Sub2 and *Yra1* bind to the THO complex on the nascent mRNA to form the *TR*anscription-*EX*port (TREX)-complex (Strasser et al. 2002; Rodriguez-Navarro and Hurt 2011). *Ch*romatin *i*mmuno*p*recipitation experiments (ChIPs) indicated that *Yra1* loading occurs during transcription elongation (Lei et al. 2001; Johnson et al. 2009). In fact, TREX complex formation depends on hyperphosphorylation of the CTD of RNA pol II, as shown by interaction studies with *Yra1* (MacKellar and Greenleaf 2011). Later, *Yra1* and the ubiquitinated THO/TREX component *Hpr1* recruit *Mex67* for proper export (Strasser and Hurt 2000; Gwizdek et al. 2006; Babour et al. 2012; Katahira 2012). Consequently, mutations in the TREX complex components lead to mRNA export defects (Jensen et al. 2001a; Rodriguez-Navarro et al. 2002; Strasser et al. 2002). Interestingly, the Cap structure might support efficient nuclear export of the mature mRNA by proper positioning of the TREX complex (Lewis and Izaurralde 1997; Katahira 2012).

Splicing occurs on intron containing mRNAs co-transcriptionally (see Chap. 2). For more details on the splicing process, some excellent recent reviews are suggested (Meyer and Vilardell 2009; Wahl et al. 2009; Will and Luhrmann 2011; Chen and Cheng 2012). Besides its well-established function in splicing the *Prp19*-complex, which has been identified as a non-snRNP constituent of the spliceosome, was suggested to function additionally in general transcription elongation, because *Prp19* is loaded by the elongating RNA pol II onto intron-containing and intronless transcripts (Tarn et al. 1994; Chanarat et al. 2011). Its proper binding has been shown to be a prerequisite for the TREX complex recruitment (Chanarat et al. 2011, 2012). This reflects the strong interplay between transcription, splicing, and export.

During splicing, two other shuttling SR-proteins are loaded onto the pre-mRNA, termed *Gbp2* and *Hrb1* (Windgassen and Krebber 2003; Hacker and Krebber 2004; Hackmann et al. 2014). In contrast to *Npl3*, *Gbp2* and *Hrb1* are recruited in a THO complex and splicing-dependent manner and interact with *Mex67* (Hacker and Krebber 2004; Hurt et al. 2004; Hackmann et al. 2014).

After capping and splicing, the transcript is finally cleaved and the 3′-end is polyadenylated (Barilla et al. 2001; Ahn et al. 2004). Recruitment of 3′-end processing and polyadenylation factors was shown to require the Ctk1 mediated serine 2 phosphorylation of the RNA pol II CTD (Ahn et al. 2004). Transcription of the polyadenylation signal sequence in the 3′UTR initiates this final pre-mRNA processing step and is recognized by the 3′-end processing machinery (see Chap. 3). This AU-rich sequence is further recognized by *Hrp1*, which is loaded to support the efficiency of the cleavage reaction (Perez-Canadillas 2006; Barnwal et al. 2012). Upon 3′- end processing, *Hrp1* remains bound to the RNA and shuttles with the mature mRNA to the cytoplasm, where it is further involved in the nonsense mediated decay (NMD) of premature stop-codon containing mRNAs (Gonzáles et al. 2000).

Upon cleavage the poly(A) polymerase Pap1 (*Pla1* in *Schizosaccharomyces pombe*) conducts polyadenylation of the upstream cleavage product. Recruitment of the poly(A) binding proteins *Nab2* and *Pab1* leads to a controlled poly(A) tail length (Anderson et al. 1993; Hector et al. 2002; Dunn et al. 2005). This final processing step triggers the displacement of several associated processing factors such as the cleavage and polyadenylation factors and TREX, which leads to the release of the mRNP from the transcription apparatus (Kim et al. 2004; Dunn et al. 2005; Qu et al. 2009). One more time *Mex67-Mtr2* associates with the mRNP by interaction with *Nab2* and the mRNP is now ready for being exported (Green et al. 2002; Batisse et al. 2009). The mature mRNP is now covered with several *Mex67-Mtr2* molecules, which are loaded at different positions, through interaction with *Npl3*, *Gbp2*, *Hrb1*, *Hpr1*, *Yra1*, and *Nab2* (Strasser and Hurt 2000; Green et al. 2002; Gwizdek et al. 2006; Hackmann et al. 2014). These mRNA export adapters for *Mex67-Mtr2* are recruited at individual processing steps and it seems likely that every maturation step is controlled and finally flagged with one or more *Mex67-Mtr2* molecules (Hackmann et al. 2014). Moreover, post-translational modifications such as ubiquitination of *Hpr1* or dephosphorylation of *Npl3* also have an impact on the association of *Mex67-Mtr2* (Strasser and Hurt 2000; Gilbert and Guthrie 2004). This might prevent a too early loading of *Mex67* to immature transcripts. Nuclear RNA surveillance mechanisms during the processing events and at the NPC ensure that only completely processed and correctly assembled mRNPs leave the nucleus while unprocessed or faulty mRNAs are retained and degraded.

The TREX Complex

The co-transcriptionally loaded TREX complex is essential for formation and export of the mRNP. TREX is conserved among eukaryotes and connects transcription elongation with mRNA maturation and export. TREX consists of the heterotetrameric transcription elongation complex THO and the export factors *Sub2* and *Yra1* (Strasser et al. 2002; Rougemaille et al. 2008). The recruitment of the TREX complex involves *Syf1*, which is a component of the *Prp19* splicing complex (Chanarat et al. 2011). THO is composed of *Hpr1*, *Mft1*, *Thp2*, and the eponymous *Tho2*. RNA/DNA-protein crosslinking experiments revealed that THO directly interacts with chromatin and RNA (Jimeno et al. 2002; Pena et al. 2012). The THO complex contributes to transcription elongation and genetic stability by preventing the formation of DNA:RNA hybrids known as R-loops and transcription-associated recombination (Huertas and Aguilera 2003; Jimeno and Aguilera 2010).

Recently, another THO complex component was identified as *Tex1*. It stably associates with the mRNA as an integral part of the THO complex (Jimeno et al. 2002; Hurt et al. 2004; Gewartowski et al. 2012; Pena et al. 2012). However, depletion of *Tex1* has no effect on THO complex assembly and binding to nucleic acids (Pena et al. 2012). Moreover, while the other THO complex components

show hyper-recombination and mRNA export defects when mutated, only mild effects are observed in a *TEX1* deletion strain (Luna et al. 2005).

THO binding to nascent mRNAs leads to the recruitment of *Yra1* and the DEAD-box RNA helicase *Sub2*, which is involved in early and late steps of spliceosome assembly (Jensen et al. 2001b; Strasser and Hurt 2001; Strasser et al. 2002; Hurt et al. 2004). Like observed for components of the THO complex, *Sub2* mutants show an elongation-dependent hyper-recombination phenotype and mRNA export defects (Chavez et al. 2000; Strasser et al. 2002; Garcia-Rubio et al. 2008). In higher eukaryotes, *Sub2* is part of the exon junction complex (EJC) that marks the exon-exon boundaries, however, this complex has not been identified in fungi yet (Abruzzi et al. 2004).

Interestingly, like *Sub2* and *Yra1*, the *Prp19* complex also binds to intronless pre-mRNAs suggesting a function that is not limited to spliceosome assembly and splicing (Lei and Silver 2002; Abruzzi et al. 2004; Chanarat et al. 2011). This is very similar to *Npl3*. This SR protein helps to recruit the splicing machinery onto intron containing transcripts (Kress et al. 2008), but is also present on intron free mRNAs (Lee et al. 1996; Krebber et al. 1999; Kim Guisbert et al. 2005). For *Sub2* an additional function was reported in inhibiting the transcription activity of RNA pol II toward the 3′ end, to prevent premature polyadenylation and mRNP release (Saguez et al. 2008).

A THO complex-dependent recruitment of the shuttling serine-arginine (SR) rich proteins *Gbp2* and *Hrb1* to the pre-mRNA has been shown earlier (Hacker and Krebber 2004; Hurt et al. 2004). Interestingly, their recruitment is further dependent on the splicing machinery, where both proteins play a crucial role in the quality control of splicing. Upon correct completion of splicing, both SR proteins bind to *Mex67* (Hackmann et al. 2014).

Earlier studies suggested that upon loading of *Sub2* onto the pre-mRNP *Yra1* binds and subsequently recruits *Mex67-Mtr2*, which is necessary for the export of the mature mRNP (Zenklusen et al. 2001; Stewart 2010). However, recent studies provide evidence for an alternative model in which the *Yra1* association occurs independently of *Sub2*, but via interaction with the 3′-end processing factor *Pcf11*. The protein binds to the phosphorylated CTD, recruits *Yra1* to the transcription elongation complex and the emerging mRNA (Johnson et al. 2009, 2011). *Yra1* itself contains a phospho-CTD-interacting domain, which also comprises an RNA recognition motif (RRM). Deletion of this domain leads to strong mRNA export defects (Stewart 2010; MacKellar and Greenleaf 2011). Strikingly, *Yra1* unlike the other *Mex67* interacting factors, does not shuttle with the mature mRNP to the cytoplasm, which leaves the question unanswered if *Mex67* upon *Yra1* loading contacts the mRNA directly or if it is transferred to another adapter protein after release of *Yra1*. Moreover, *Sub2* and *Mex67* share the binding site for *Yra1* so that their binding is mutually exclusive. Only upon *Sub2* release, *Mex67* can bind to *Yra1* (Strasser and Hurt 2001; Johnson et al. 2011).

Recently, the ATP-dependent RNA helicase *Dbp2* was introduced as a new mRNP remodeling factor that permits the recruitment of *Yra1* and *Nab2* to prevent premature 3′ end processing (Ma et al. 2013). In vivo crosslinking experiments

have shown that a decreased association of *Yra1*, *Nab2*, and *Mex67* to mRNA is detectable in *dbp2Δ* cells. Moreover, upon *Yra1* binding to the mRNA and physical interaction with *Dbp2*, *Yra1* finally inhibits the *Dbp2* unwinding activity, which might result in the *Dbp2* release from the mRNP. The *Yra1* induced release of *Dbp2* was suggested to represent a quality control step during transcription termination and 3′-end processing (Cloutier et al. 2012; Ma et al. 2013).

The TREX-2 and the SAGA Complexes

The *TREX-2* and the SAGA complexes cooperate to localize the expression of certain genes to the NPC for an efficient coupling of transcription, mRNP assembly and transport through the NPC (Stewart 2010). The *TREX-2* complex is localized in close proximity to the nuclear pore complex and assists the *TREX* complex in mRNA transcription and export. *TREX-2* consists of *Thp1*, *Cdc31*, *Sac3* and *Sus1*. Mutations of *TREX-2* complex factors show similar defects in transcription and mRNA export as observed for mutants of the *TREX* complex (Rodriguez-Navarro et al. 2004; Dieppois et al. 2006; Luthra et al. 2007; Jani et al. 2009). While *Sac3*, *Sus1* and *Cdc31* interact with the nucleoporin *Nup1* to connect the complex with the NPC, a linkage to the transcription process is provided by a physical interaction of *Thp1* and *Sus1* with the *S*pt-*A*da-*G*cn5 *a*cetyltransferase (SAGA) complex. Additionally, the nuclear basket associated protein Mlp1 binds to the promoter of active genes through interactions with SAGA subunits (Dieppois et al. 2006; Luthra et al. 2007).

The SAGA complex functions as a co-activator for transcription initiation by acetylating histones during transcription and thereby supporting the accessibility of transcription complexes to genomic DNA (Rodriguez-Navarro et al. 2004). Sus1 is part of both complexes, the *TREX-2* and the SAGA complex, and co-purifies with RNA pol II and the mRNA export factors *Yra1* and *Mex67* (Rodriguez-Navarro et al. 2004; Pascual-Garcia et al. 2008). *Sus1* might therefore couple transcription of SAGA-bound chromatin to the subsequent export of the transcripts by interaction with the NPC-attached *TREX-2* complex and *Mex67*. This tethering of certain actively transcribed genes (e.g., cell cycle regulated or less abundant transcripts) to the vicinity of the NPC allows an immediate export of such transcripts, which may compete with the export of highly and/or constitutively expressed mRNAs (Rodriguez-Navarro et al. 2004).

mRNA Export Receptors

The only known mRNA export receptors to date are *Mex67-Mtr2* and *Xpo1/Crm1*, although more might exist (Hieronymus and Silver 2003; Rodriguez-Navarro et al. 2004; Carmody and Wente 2009). The key factor for mRNA export is the

heterodimer *Mex67-Mtr2*, which works independently of the Ran GTPase system, required for general protein transport. As *Mex67* has a low affinity for direct binding to mRNA, the interaction seems to be mediated by several mRNA covering adaptor proteins (Strasser et al. 2002; Kohler and Hurt 2007). As already mentioned and to summarize at this point, so far the mRNA binding proteins *Hpr1* (of the THO-complex), *Yra1* (of the TREX-complex), *Npl3*, *Gbp2* and *Hrb1* (of the SR-protein family) and *Nab2* (involved in 3′-end processing) are known to interact with *Mex67* (Zenklusen et al. 2001; Gilbert and Guthrie 2004; Hobeika et al. 2007; Batisse et al. 2009; Iglesias et al. 2010; Hackmann et al. 2014). Interestingly, for the export of the large ribosomal subunit *Mex67* was suggested to directly contact the 5S rRNA as was shown in vitro experiments (Yao et al. 2007). However, adapter proteins might contribute to this binding in vivo.

The interaction domain necessary for the NPC binding of *Mex67* has been mapped to its C-terminus, which also represents the *Mtr2* binding domain. *Mtr2* in turn interacts with the phenylalanine/glycine (FG)-rich repeats of the nucleoporins (Nups). During translocation *Mtr2* exposes its Nup-binding sites, shielding the transport cargo from the hydrophobic meshwork of the inner channel and thereby allowing translocation (Kohler and Hurt 2007).

A second mRNA export receptor is *Crm1*/*Xpo1*, which is involved in the transport of very few mRNAs (Ohno et al. 2000; Kohler and Hurt 2007). *Crm1*/*Xpo1* is a karyopherin and uses the Ran GTPase system for export. It functions in the transport of nuclear export signal (NES) containing cargoes, including NES containing proteins associated with UsnRNAs, pre-ribosomal subunits and mRNAs (Fornerod et al. 1997). In yeast only slight mRNA export defects can be detected when *XPO1* is mutated (Hodge et al. 1999; Neville and Rosbash 1999). One interesting example of an *Xpo1*-transported mRNA is the *YRA1* transcript, which controls its own expression (Dong et al. 2007).

mRNA Export Adapters

mRNA export adapter proteins connect the receptors to the mRNA. While for the *Xpo1*-mediated export these are currently unknown, several factors were identified for *Mex67*. Of those, some adapter interactions are transient while other adapter proteins stay bound for an extended time and accompany the mRNA through the NPC into the cytoplasm where some of these proteins can persist until translation (Windgassen et al. 2004). Those that leave the mRNA already in the nucleus are *Yra1* and *Hpr1*, which interact with *Mex67* (Zenklusen et al. 2001; Hobeika et al. 2007). *Yra1* contacts *Mex67* upon release of *Sub2*, as they share the binding sites (Johnson et al. 2009; Iglesias et al. 2010). It is currently unclear if upon their dissociation *Mex67* binds to the mRNA or is transferred to other proteins.

Those adapter proteins that escort the mature transcript to the cytoplasm are *Nab2* and the three SR-proteins, *Npl3*, *Gbp2* and *Hrb1*. As already pointed out, all three yeast SR-proteins are recruited to the pre-mRNA co-transcriptionally,

however, *Npl3* is recruited early by RNA pol II prior to splicing and *Gbp2* and *Hrb1* to a later time point via the THO complex and in dependence of splicing (Lei et al. 2001; Hacker and Krebber 2004; Hurt et al. 2004; Hackmann et al. 2014). *Npl3* contains two typical RNA recognition motifs (RRM) for RNA binding, an N-terminal domain with several APQE (alanine, proline, glutamine, and glutamate) repeats of unknown function and a C-terminal domain with several SR (serine, arginine) and RGG (arginine, glycine, glycine) repeats, termed the SR-domain. Interestingly, the deletion of *NPL3* in certain backgrounds of *Saccharomyces cerevisiae*, such as *BY4743*, does not cause visible mRNA export defects, revealing that other factors can compensate for the function of *Npl3* in mRNA transport (Hackmann et al. 2011). The growth defects visible in this background rather result from defects in ribosomal subunit joining important for translation initiation (Baierlein et al. 2013). In yeast strains in which *NPL3* is essential such as *S288C* or *W303*, mutations in its gene lead to mRNA export defects (Lee et al. 1996; Krebber et al. 1999). Moreover, mutations in both RRMs of *NPL3* do not only lead to mRNA but also to pre-60S export defects (Stage-Zimmermann et al. 2000; Hackmann et al. 2011). *Npl3* physically interacts with *Mex67* and this interaction is not dependent on RNA (Gilbert and Guthrie 2004; Hackmann et al. 2014). Strikingly, *Npl3* can also interact directly with FG-rich elements of the nucleoporins, which might be important for the nuclear export of large ribosomal subunits, because the association of *Mex67* to pre-60S ribosomal subunits is independent of *Npl3* (Hackmann et al. 2011). However, it might also suggest that the protein is more than just an adapter for *Mex67*, but rather actively contributes to the shielding of the transport particle from the hydrophobic interior of the NPC, which might also be conceivable for the transport of mRNAs.

Two other nuclear functions have been reported for *Npl3*: (a) *Npl3* is involved in the co-transcriptional assembly of the early spliceosome on the nascent pre-mRNAs and it interacts genetically and physically in the presence of RNA with U1 and U2 snRNP splicing factors (Kress et al. 2008). Moreover, deletion of *NPL3* leads to an accumulation of intron containing pre-mRNAs (Kress et al. 2008). (b) Furthermore, *Npl3* acts as an anti-terminator by competing for RNA binding with the 3′-end processing machinery and in this way prevents polyadenylation at weak polyadenylation signal sequences (Bucheli and Buratowski 2005; Bucheli et al. 2007; Dermody et al. 2008). Although *Npl3* has several nuclear functions and most of the protein is localized to the nucleus, an essential cytoplasmic function has been discovered recently in which *Npl3* mediates the joining of the ribosomal subunits during translation initiation for which it needs to form dimers (Baierlein et al. 2013). Furthermore, *Npl3* is the target of several different posttranslational modifications, which have an impact on several functions and protein-protein interactions (Inoue et al. 2000; Gilbert et al. 2001; McBride et al. 2005).

Gbp2 and *Hrb1* are recruited during transcription elongation to pre-mRNAs by the THO complex (Hacker and Krebber 2004; Hurt et al. 2004). Their recruitment further depends on splicing and their initial binding to bulk mRNA is tightened on intron containing transcripts by the splicing machinery (Hackmann et al. 2014). Recently, the Tollervey lab analyzed the composition of different RNP complexes

and found increased interactions of *Gbp2* with spliced transcripts at their 5′-ends (Tuck and Tollervey 2013). In contrast to that, *Mex67* was detected all over the mRNA sequences, revealing binding to the nascent transcript at various points (Tuck and Tollervey 2013). *Gbp2* and *Hrb1* share a significant homology in their amino acid sequence (47 % identity) (Hacker and Krebber 2004). Both contain three RRM motifs and an N-terminal SR/RGG domain. Strikingly, deletions of *Gbp2* and *Hrb1*, show no mRNA export defects, but rather the opposite, an increased slippage of unspliced transcripts into the cytoplasm, which suggests a function in retaining unspliced pre-mRNAs in the nucleus, important for mRNA surveillance (Hackmann et al. 2014). In fact, *Gbp2* and *Hrb1* stabilize the TRAMP complex association with the transcript and channel false transcripts into degradation. Upon TRAMP complex release from correct RNAs, *Gbp2* and *Hrb1* recruit *Mex67* for export (Hackmann et al. 2014).

After 3′-end processing and polyadenylation *Nab2* associates with the nascent poly(A) tail. *Nab2* is required for the trimming of the poly(A) tail and for nuclear mRNA export via association of *Mex67* (Hector et al. 2002). *Yra1* participates in 3′-processing as mutations in *Yra1* that are otherwise lethal, can be suppressed by overexpression of *Mex67* or *Nab2* (Anderson et al. 1993; Hector et al. 2002; Iglesias et al. 2010). The N-terminal domain of Nab2 physically interacts with the nucleoporin Mlp1 (Fasken et al. 2008). Therefore, it was suggested that Nab2 may help to concentrate mature mRNAs at the nuclear face of the NPC for nuclear export (Soucek et al. 2012).

In summary, different mRNA adapter proteins are recruited over the course of mRNA maturation, some of which dissociate prior to export, some of which remain bound to the mRNA during translocation. However, all have in common that they recruit *Mex67-Mtr2*, which might function as flags for the properly matured mRNA that are recognized at the NPC by Mlp1 (Hackmann et al. 2014). During transit through the NPC it covers, likely together with other molecules, the charged backbone of the mRNA from the hydrophobic interior of the NPC.

The Late Phase: The Nuclear Pore Complex and mRNA Translocation

Once the mRNAs are matured and covered with *Mex67-Mtr2* molecules they are ready for their passage through the NPC. These eight-fold symmetrical complexes with a molecular mass of ~50 MDa are highly conserved and comprised of ~30 different proteins, termed nucleoporins (Nups) (Strambio-De-Castillia et al. 2010a, b). NPCs are embedded into the nuclear envelope with two coaxial rings positioned coplanar with the inner and outer membranes (Fig. 4.2). This central structure extends into the cytoplasm with eight cytoplasmic filaments that are connected with the cytoskeleton to alleviate the way toward protein synthesis. The nuclear basket on the other side channels incoming cargo toward the nuclear

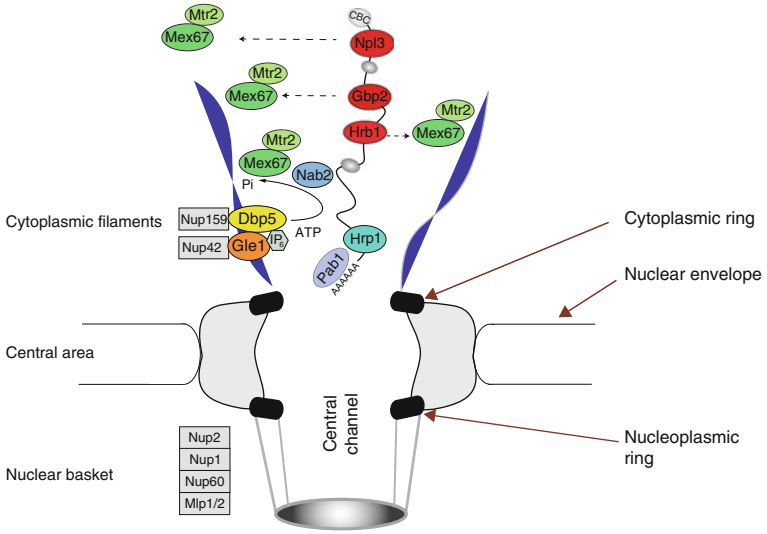

Fig. 4.2 Release of mRNA transport factors from the mRNP after export through the Nuclear Pore Complex (NPC). Upon arrival of the mRNP at the cytoplasmic side of the NPC *Dbp5* displaces *Mex67-Mtr2* and *Nab2* from the mRNP in an ATP-dependent step, leading to the directionality of the transport event

interior (Pante and Aebi 1995; Fahrenkrog and Aebi 2003; Strambio-De-Castillia et al. 2010a, b). Current views reflect the NPCs not as isolated complexes through which transport occurs, but rather as areas for gated, selectively promoted gene expression, platforms for macromolecular assembly and funnels for efficient translation (Strambio-De-Castillia et al. 2010a, b). The central channel has an approximate diameter of 35 nm and is filled with flexible filamentous FG (phenylalanine-glycine) Nups that prevent macromolecules from entering. Cargoes bound to transport receptors can overcome this hydrophobic barrier, which due to its flexible nature, accommodates the transport of differently sized cargoes. FG Nups are characterized by regions of multiple FG-repeats separated by hydrophilic spacer sequences of 5–30 amino acid residues. In *S. cerevisiae* around 160 individual FG Nups edge the transport channel in each NPC (Rout et al. 2000; Alber et al. 2007). The NPC contains a central area, a nuclear basket and cytoplasmic filaments (Fig. 4.2). The central area is composed of central FG Nups, inner ring- and outer ring Nups, linker Nups and transmembrane ring Nups (Strambio-De-Castillia et al. 2010a, b). One of the central FG Nups in *S. cerevisiae* is *Nup116* (a Nup only found in fungi; Sampathkumar et al. 2012), *Nup100*, *Nsp1*, *Nup57* and *Nup49* (Aitchison and Rout 2012). Interestingly, *Nup116* interacts with the NPC-associated protein *Gle2* (Rae1 in *S. pombe)*, which was suggested to play a role in mRNA export as mutants show mRNA export defects and *Gle2* was found in a complex with *Mex67*. Moreover, *Gle2* mutants show defects in the NPC structure (Wente and Blobel 1993; Murphy et al. 1996; Bailer et al. 1998; Pritchard et al. 1999).

The nuclear basket is made of *Nup1*, *Nup2*, and *Nup60* and two myosin-like proteins *Mlp1* and *Mlp2*, which form a filamentous structure that reaches into the nuclear interior (Galy et al. 2004, Strambio-De-Castillia et al. 2010a, b). *Mlp1* and *Mlp2* play a key role in the entry of mRNPs into the NPC (Green et al. 2003; Galy et al. 2004). They interact with the C-terminal part of *Mex67* and *Mex67*-interacting shuttling RNA binding proteins such as *Nab2*, *Npl3*, *Gbp2*, and *Hrb1* (Green et al. 2003; Fasken et al. 2008; Hackmann et al. 2014). As their deletion leads to the slippage of intron-containing transcripts into the cytoplasm, a key role in nuclear quality control had been proposed for *Mlp1* and *Mlp2* (Galy et al. 2004; Hackmann et al. 2014). As nuclear gate keepers, the Mlp proteins might monitor the completed mRNA maturation by checking for proper *Mex67* association (Hackmann et al. 2014). *Sac3* is an NPC associated factor, which is part of the TREX-2 multiprotein complex that interacts with *Nup1*. Its association is stabilized via *Sus1* and *Cdc31* (Garcia-Olivier et al. 2012). *Sac3* supports anchoring of mature *Mex67*-bound mRNPs to the nuclear entry side of the NPC (Lei et al. 2003).

The cytoplasmic filaments of the NPC are composed of *Nup159* and *Nup42* and bind the DEAD-box RNA helicase *Dbp5*, which by its remodeling activity is the driving force of mRNA export and leads to directionality (Snay-Hodge et al. 1998; Tseng et al. 1998; Strambio-De-Castillia et al. 2010a, b; Aitchison and Rout 2012; Tieg and Krebber 2013) (Fig. 4.2). When the transported mRNP reaches the cytoplasmic side of the NPC, it contacts *Dbp5* and its co-factors *Gle1* and inositol hexakisphosphate (IP_6) that stimulate the ATPase activity of *Dbp5* (Alcazar-Roman et al. 2006; Weirich et al. 2006). The consequent *Dbp5* dependent mRNP remodeling was suggested to first link the RNA leading to the displacement of *Mex67* and *Nab2* and to subsequently release of the remaining mRNP into the cytoplasm, preventing a back-slippage of the mRNP into the nucleus (Tseng et al. 1998; Tran et al. 2007) (Fig. 4.2). This whole process occurs in several steps: *Dbp5* in its open conformation binds ATP, leading to a cooperative binding of Gle1-IP_6 and the exported mRNP. Binding causes a conformational change, leading to ATP hydrolysis. The transition to the ADP bound form leads to the specific displacement of mRNP bound proteins and to the dissociation of mRNA, allowing an interaction of *Dbp5* with *Nup159*. This interaction induces a conformational change resulting in the release of the ADP and the rebinding of ATP (Hodge et al. 2011; Noble et al. 2011; Tieg and Krebber 2013).

Interestingly, *Nup159* is specifically required for the export of mRNAs, as temperature sensitive mutants show strong mRNA export defects while the import of NLS containing proteins is unaffected (Gorsch et al. 1995; Del Priore et al. 1997). Another *Dbp5* associated protein of unknown function is *Gfd1* (*g*ood *f*or *Dbp5*), which in high copy suppresses temperature sensitive mutants of *Dbp5* (Zheng et al. 2010). Interestingly, besides their function in mRNA export, *Dbp5* and *Gle1* have been shown to function in translation termination together with the eukaryotic release factor 1 (eRF1) (Bolger et al. 2008). This involvement of mRNA export factors in other essential subsequent processes, such as *Dbp5* in

translation termination, *Npl3* in translation initiation and *Hrp1* in NMD, again emphasizes the intimate coupling of basic functions in gene expression and reveals the high efficiency in nature of using one protein for several cellular functions.

mRNA Surveillance and Quality Control in the Nucleus

The synthesis of pre-mRNAs and their processing to mature mRNPs are not error free processes. Consequently, the cell has evolved different mechanisms to prevent the expression of false transcripts. Similar to the intimate linkage of transcription and export, the nuclear quality control is also tightly connected with pre-mRNA maturation and occurs at several steps. Co-transcriptional degradation processes are mainly executed by the nuclear exosome and co-factors like the Nrd1-Nab3-Sen1 complex for degradation of premature cryptic transcripts, the Ccr4-Not complex that might be a scaffold for the assembly of factors involved in ubiquitination and deadenylation, and the TRAMP complex, which marks RNAs with short oligo(A) tails to initiate their degradation (Fasken and Corbett 2009; Houseley and Tollervey 2009; Collart and Panasenko 2012; Porrua and Libri 2013).

The exosome is an evolutionary conserved nine-subunit complex including Dis3/Rrp44 that exhibits exo- and endoribonuclease activity (see Chap. 7 for more details). The exclusively nuclear exosome component *Rrp6* contains additional exonuclease activity. The main co-factor of the exosome is the *Tr*f-*A*ir-*M*tr4 *p*olyadenylation complex (TRAMP), which exists in two forms that recognizes different RNA substrates (Houseley and Tollervey 2009; Kong et al. 2013; Tuck and Tollervey 2013). TRAMP5 consists of a noncanonical poly(A) polymerase (*Trf5*), a zinc-knuckle RNA binding protein (*Air1*) and the DExH-box RNA helicase *Mtr4* and seems to be mostly restricted to nucleolar rRNA processing. The TRAMP4-complex (*Trf4*, *Air2*, and *Mtr4*) is the major co-factor that mediates the nuclear quality control by recognizing aberrant RNAs and directing them for degradation to the nuclear exosome (San Paolo et al. 2009; Callahan and Butler 2010). The mechanism by which RNAs are recognized as aberrant is not fully understood. However, it has been shown that the zinc-knuckle Air proteins provide the initial contact of the TRAMP complex to the RNA and modulate the Trf4/5 polymerase activity (San Paolo et al. 2009; Hamill et al. 2010). In contrast to the general poly(A) polymerase *Pap1*, which adds 70–90 nucleotides long poly(A) tails in *S. cerevisiae*, the polymerases *Trf4*/*Pap2* and *Trf5* add only short poly(A) tails of 10 or less nucleotides to the 3′-end of an RNA, which could act as a label for degradation. The RNA helicase Mtr4 controls the length of the oligo(A) tail and unwinds secondary structures, leading to an unstructured 3′-end that can enter the exosome (Jia et al. 2011).

As far as it is known, defects in early transcription are monitored and the defective RNAs are eliminated by the immediate recruitment of the exosome (Hilleren et al. 2001; Jensen et al. 2003). How abnormal pre-mRNAs are released from the transcription apparatus and whether co-factors are involved in this

process is currently unclear. However, the SR-protein *Npl3*, which is recruited early during transcription, might be the most 5′ located protein that receives a *Mex67* molecule, which might indicate proper 5′ maturation (Lei et al. 2001; Hackmann et al. 2014). A recent publication in which the *cr*osslinking and *a*nalysis of *c*DNA (CRAC) technique was applied, revealed that the TRAMP4 complex factors *Trf4* and *Mtr4* bind the transcripts in their 5′ regions, close to the transcription start site, which is crucial for an early RNA surveillance (Granneman et al. 2009; Tuck and Tollervey 2013). The experiments further showed a high binding density of these proteins on intron sequences, which are in yeast usually very close to the 5′ end of the transcripts. Moreover, an interaction with several splicing factors was shown for *Trf4* and *Mtr4*, which support the idea of a TRAMP and exosome-dependent surveillance mechanism of pre-mRNA splicing (Kong et al. 2013; Tuck and Tollervey 2013). In fact, key factors in nuclear surveillance of spliced transcripts are the SR proteins *Gbp2* and *Hrb1* because they connect the TRAMP complex to spliced transcripts and if splicing is not properly executed, initiate their degradation. On correct RNAs they instead recruit *Mex67* to signal export competence (Hackmann et al. 2014). Another nuclear quality control protein is the pre-mRNA *re*tention and *s*plicing (RES)-complex protein *Pml1* that was suggested to contact intron containing mRNAs prior to *Mlp1* (Dziembowski et al. 2004; Palancade et al. 2005). However, the function of *Pml1* in this process is rather nebulous.

Along the road, *Yra1* and *Nab2* associate with the transcripts and might control 3′-end processing events. On correctly processed mRNAs the association of *Mex67* molecules close to their 3′ ends mark them for export. Interestingly, the CRAC assay revealed a second place of *Nab2* association close to the 5′ end of transcripts, suggesting that the protein not only acts in 3′-end processing events (Tuck and Tollervey 2013). Additionally, all defects in transcription, splicing and 3′-end processing might lead to a delay or failure of *Pap1* mediated polyadenylation that favours oligo-adenylation by TRAMP and subsequent degradation by the exosome (Tutucci and Stutz 2011).

Prior to export, mRNPs are surveyed by a quality control checkpoint directly situated at the nuclear basket of the NPC. *Mlp1* and *Mlp2*, both anchored by *Nup60* block the export of intron-containing mRNAs and aberrantly assembled mRNPs at the NPC. Upon deletion of their genes all three of them show leakage of unspliced transcripts into the cytoplasm (Galy et al. 2004; Palancade et al. 2005). *Pml39* (*Rsm1* in *S. pombe*) is an NPC-associated factor that contacts *Nup84* and the Mlp proteins that show leakage of unspliced pre-mRNA into the cytoplasm when deleted (Palancade et al. 2005). The integral inner nuclear membrane protein *Esc1* interacts with *Mlp1* and *Mlp2*. *Esc1* functions in maintaining the correct composition of the nuclear basket and therefore might be responsible for proper positioning of the NPC-associated surveillance factors (Lewis et al. 2007; Niepel et al. 2013). The cytoplasmic appearance of unspliced transcripts can also be observed in mutants of *Ulp1*. The SUMO protease *Ulp1* was suggested to contribute to intron-containing mRNA retention at the NPC by de-sumoylation of pre-mRNA associated proteins that were marked for degradation. *Ulp1* is localized to the NPC

by association with *Nup2*, *Esc1*, *Nup60* and *Mlp1* (Lewis et al. 2007). Finally, *Swt1* is an RNA endoribonuclease, which is transiently recruited to NPCs. The inactivation of its endonuclease activity leads to leakage of intron mRNAs to the cytoplasm. Possibly, the endonucleolytic cleavage of false transcripts by *Swt1* is necessary for the degradation of such messages (Skruzny et al. 2009).

An early model of how defective transcripts are detected at the NPC suggests that *Mlp1* might directly contact an intron-associated factor to recognize unspliced mRNAs, as the branch point binding protein *Msl5* was shown to interact with *Mlp1* in an RNA-dependent manner (Galy et al. 2004). On the other side, *Mlp1* also reduces chromatin crowding and might therefore contribute to gene gating (Niepel et al. 2013). However, *Mlp1* might alternatively rather be a detector of proper *Mex67* association. Like a ticket controller, it might survey the transcript for proper *Mex67* coverage (Hackmann et al. 2014). Indeed, the CRAC technique revealed an even distribution of *Mex67* over the entire transcript length (Tuck and Tollervey 2013), supporting the stepwise recruitment of *Mex67* upon completion of individual processing steps.

mRNA Export and its Regulation Via Post-Translational Protein Modifications

Gene expression is regulated by a complex network, which coordinates mRNA synthesis, processing, export, translation, and several layers of quality control. These events are affected by phosphorylation, methylation, and ubiquitination of mRNA-associated factors. Foremost these post-transcriptional modifications influence protein–protein or protein-RNA interactions. Differential phosphorylation of the CTD of the RNA pol II leads to the recruitment of certain proteins at specific time points to guarantee a smooth maturation of the transcripts, but also the loaded proteins are themselves modified. The following examples will describe a few cases in which the impact of the modifications is quite well understood.

The SR protein *Npl3* is phosphorylated and methylated. One phosphorylation site in the SR motif closest to the C-terminus is targeted by the cytoplasmic kinase *Sky1* (Gilbert et al. 2001). Phosphorylated *Npl3* leads to a reduced RNA binding affinity and an increased reimport into the nucleus mediated by the SR protein specific karyopherin *Mtr10* (Gilbert et al. 2001). Interestingly, the reduced RNA binding affinity seems to be important for its nuclear association with the mRNA, rather than for its RNA dissociation in the cytoplasm, and although the deletion strain of the cytoplasmic kinase Sky1 shows an increased poly(A)$^+$RNA binding phenotype of *Npl3*, its dissociation from mRNAs engaged in translation is not affected (Windgassen et al. 2004). It was rather shown that the dissociation of *Npl3* from polysomal mRNAs requires *Mtr10* (Windgassen et al. 2004). Upon loading of *Npl3* onto the emerging pre-mRNA in the nucleus, *Npl3* is phosphorylated during later steps of transcription, which supports the binding of Rna15 to the polyadenylation signal to initiate 3′-end cleavage and polyadenylation.

Finally, *Npl3* is dephosphorylated by the exclusively nuclear phosphatase *Glc7*, which leads to the dissociation of the 3′-end processing factors and importantly promotes loading of the mRNA export receptor *Mex67* (Gilbert and Guthrie 2004).

Additionally, it was shown that several RGG motifs of *Npl3* are methylated by the methyltransferase Hmt1, which decreases the interactions of *Npl3* with the CBC, *Tho2*, and with itself (McBride et al. 2005; Erce et al. 2013). These results provide evidence that upon *Npl3* methylation, interactions with processing and transcription elongation factors are loosened to promote the release of the export competent mRNP from the transcription apparatus, which might support an interaction of *Npl3* with the export receptor *Mex67* (McBride et al. 2005). Interestingly, unmethylated *Npl3* does not seem to shuttle to the cytoplasm anymore, however, a knock-out of *HMT1* is viable and shows no bulk mRNA export defects (Shen et al. 1998). Less is known about the two other SR proteins *Gbp2* and *Hrb1*, but first evidence exists that they might like *Npl3* undergo methylation and phosphorylation (Windgassen and Krebber 2003; Erce et al. 2013).

The THO complex subunit *Hpr1* has been shown to be ubiquitinated in its C-terminal region during transcription elongation (Hobeika et al. 2009; Gewartowski et al. 2012). The ubiquitinated protein is recognized and bound by the C-terminal domain of the export receptor *Mex67*. A block in *Hpr1* ubiquitination results in a decrease of co-transcriptional recruitment of *Mex67* to the mRNA. Structural studies revealed that ubiquitinated *Hpr1* and the NPC subunits may bind to the *Mex67* ubiquitin associated domain in a mutually exclusive manner (Hobeika et al. 2007; Iglesias et al. 2010).

Moreover, *Yra1* is ubiquitinated by the E3 ubiquitin ligase *Tom1*. This ubiquitination leads to the dissociation of *Yra1* from the mRNP and coincides with the delivery of *Mex67* to *Nab2* (Iglesias et al. 2010). Interestingly, *Tom1* interacts genetically with *Rrp6* and *Mlp2* and loss of perinuclear Mlp proteins suppress the growth defects of *Tom1* and *Yra1* ubiquitination mutants, suggesting that *Tom1*-mediated dissociation of *Yra1* from *Nab2*-bound mRNAs is part of a surveillance mechanism at the pore, ensuring the export of matured mRNPs (Iglesias et al. 2010).

mRNA Export During Cellular Stress

Environmental changes like heat shock, osmotic and oxidative stress, or nutrient starvation needs a cellular adaption by a rapid alteration in global gene expression patterns. Under stress conditions, the nuclear export of regular mRNAs is blocked while stress-specific mRNAs are rapidly exported and translated in the cytoplasm (Saavedra et al. 1996, 1997). This nuclear mRNA retention might be caused by the dissociation of *Mex67* adapter proteins such as *Npl3* and *Nab2* from bulk mRNAs (Krebber et al. 1999; Carmody et al. 2010). For *Nab2* it was shown that the MAP kinase *Slt2* phosphorylates *Nab2*, leading to a decreased binding of *Mex67* and an accumulation of *Nab2*, *Yra1* and *Mlp1* in nuclear foci (Carmody et al. 2010). The mRNA export block in the nucleus also coincides with the release of the

NPC-associated *Gle2* into the cytoplasm upon heat and ethanol stress. Furthermore, the 3′-end processing factor *Hrp1* shifts from the nuclear localization at steady state to a cytoplasmic enrichment during osmotic stress, and shifts back to the nucleus upon removal of the stress condition (Henry et al. 2003; Tutucci and Stutz 2011). Interestingly, *Npl3*, *Yra1* and THO complex factor mutants which regularly lead to poly(A)$^+$ RNA export defects under normal conditions do not inhibit the export of heat shock mRNAs, while *Mex67-5* does (Rollenhagen et al. 2007; Gewartowski et al. 2012). It is currently unclear how *Mex67* mediates the transport of stress specific mRNAs and if other proteins are involved.

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