Chapter 2
It's Not the Destination, It's the Journey: Heterogeneity in mRNA Export Mechanisms

Daniel D. Scott, L. Carolina Aguilar, Mathew Kramar, and Marlene Oeffinger

Abstract The process of creating a translation-competent mRNA is highly complex and involves numerous steps including transcription, splicing, addition of modifications, and, finally, export to the cytoplasm. Historically, much of the research on regulation of gene expression at the level of the mRNA has been focused on either the regulation of mRNA synthesis (transcription and splicing) or metabolism (translation and degradation). However, in recent years, the advent of new experimental techniques has revealed the export of mRNA to be a major node in the regulation of gene expression, and numerous large-scale and specific mRNA export pathways have been defined. In this chapter, we will begin by outlining the mechanism by which most mRNAs are homeostatically exported ("bulk mRNA export"), involving the recruitment of the NXF1/TAP export receptor by the Aly/REF and THOC5 components of the TREX complex. We will then examine various mechanisms by which this pathway may be controlled, modified, or bypassed in order to promote the export of subset(s) of cellular mRNAs, which include the use of metazoan-specific

D. D. Scott · M. Kramar
Institut de recherches cliniques de Montréal, Montréal, QC, Canada

Faculty of Medicine, Division of Experimental Medicine, McGill University, Montréal, QC, Canada
e-mail: daniel.scott@ircm.qc.ca; mathew.kramar@mail.mcgill.ca

L. C. Aguilar
Institut de recherches cliniques de Montréal, Montréal, QC, Canada
e-mail: LisbethCarolina.Aguilar@ircm.qc.ca

M. Oeffinger (✉)
Institut de recherches cliniques de Montréal, Montréal, QC, Canada

Faculty of Medicine, Division of Experimental Medicine, McGill University, Montréal, QC, Canada

Faculté de Médecine, Département de Biochimie, Université de Montréal, Montréal, QC, Canada
e-mail: marlene.oeffinger@ircm.qc.ca

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orthologs of bulk mRNA export factors, specific *cis* RNA motifs which recruit mRNA export machinery via specific *trans*-acting-binding factors, posttranscriptional mRNA modifications that act as "inducible" export *cis* elements, the use of the atypical mRNA export receptor, CRM1, and the manipulation or bypass of the nuclear pore itself. Finally, we will discuss major outstanding questions in the field of mRNA export heterogeneity and outline how cutting-edge experimental techniques are providing new insights into and tools for investigating the intriguing field of mRNA export heterogeneity.

Keywords mRNA export · NXF1 · CRM1 · Sequence elements · Nuclear pore complex

2.1 Introduction

The defining feature of eukaryotic organisms is the presence of the nucleus, which compartmentalizes the vast majority of the cell's DNA. While this compartmentalization has numerous advantages for the cell including reducing the DNA mutation rate and allowing more efficient regulation and replication of eukaryotes' large genomes, it has resulted in the physical separation of the cell's mechanisms for mRNA production (transcription, processing, maturation, and packaging into mRNPs) and mRNA metabolism (translation, localization, and degradation) into two compartments, physically separated by the nuclear membrane.

Material entering and departing the nucleus passes through the nuclear pore complex (NPC), a multi-megadalton protein complex which spans the inner and outer nuclear membranes and creates a pore through which molecules can pass between the cytoplasm and the nucleus (Beck and Hurt 2017). While molecules of less than ~30 kDa are capable of diffusing freely through the nuclear pore, nuclear mRNPs require an active and energy-dependent process to facilitate their translocation. This process, termed "mRNA export," involves the sequential loading and remodeling of a series of mRNA export factors onto the mRNP which, collectively, identify mature and correctly processed mRNAs, recruit them to the NPC, and facilitate their translocation through the nuclear pore before releasing them into the cytoplasm (Björk and Wieslander 2017; Folkmann et al. 2011; Oeffinger and Zenklusen 2012; Okamura et al. 2015).

The first investigations into the mechanistic basis of mRNA export occurred in the late 1980s through the observation that nuclear mRNA splicing in *S. cerevisiae* was able to control the subsequent availability of mRNAs to the translation machinery in the cytoplasm (Legrain and Rosbash 1989). However, it was not until the mid-to-late 1990s that work on retroviral RNA dynamics revealed the existence of unique mRNA export pathways and their particular *cis*- and *trans*-acting factors (Cullen 1998; Jarmolowski et al. 1994). Since these early observations, it has become clear that mRNA export, far from being a passive mechanism of bulk transport, is a highly complex system in which numerous overlapping and competing pathways act to

control the translocation across the nuclear pore in response to competing spatial, temporal, and environmental demands (Delaleau and Borden 2015; Wickramasinghe et al. 2014). New mRNA export pathways, regulatory systems, and interfaces with other cellular processes are being reported on a regular basis, with each new discovery adding to our comprehension of how the regulation of mRNA export contributes to cellular homeostasis and disease.

Despite these recent advances, however, much still remains to be discovered in the field, and numerous fundamental questions regarding the machinery and regulation of mRNA export remain unanswered (Okamura et al. 2015). The considerable complexity has made it difficult to dissect the precise molecular biology of mRNA export (Delaleau and Borden 2015; Wickramasinghe et al. 2014), exacerbated by a lack of experimental techniques capable of addressing the complex hypotheses that have been proposed. Historically, the field of mRNA export research has relied heavily on the use of model systems such as *Xenopus* oocytes, in which numerous steps that intimately couple transcription and mRNA processing to mRNA export factor loading and maturation are bypassed by the microinjection of mature, in vitro-transcribed mRNAs. Similarly, a reliance on bulk mRNA-focused quantification techniques, particularly oligo(dT) fluorescence in situ hybridization (FISH), prevents the quantification of mRNA export defects on a transcript-specific basis, resulting in either over- or underreporting of phenotypic severity (Guria et al. 2011; Katahira et al. 2009; Rehwinkel et al. 2004). In recent years, the emergence of new technologies and approaches in microscopy, transcriptomics, and proteomics has finally enabled us to address these limitations; in so doing, these techniques—and others still to come—are altering the paradigms of research in the field, allowing researchers to address new and fundamental questions regarding the mRNA export machinery and its regulation.

In this chapter, we will examine in detail the numerous overlapping and complementary mRNA export pathways that have been described thus far in higher eukaryotes. We will discuss the key unifying principles and unique features of these pathways and will outline important outstanding questions in the field. In so doing, we will take a particular look at how cutting-edge techniques are allowing researchers to dissect these pathways in unprecedented detail and how novel techniques are expected to provide new paradigms for the investigation of mRNA export research. This chapter will focus primarily on work conducted in metazoans, with examples from other eukaryotes drawn upon where relevant.

2.2 The Ground State: Bulk Export Pathways for mRNAs

The most thoroughly explored and described pathway of mRNA export is the "bulk mRNA export" pathway which exports the majority of cellular mRNAs during homeostatic growth conditions (Fig. 2.1). Components of this pathway are highly conserved throughout the eukaryotes and are often—though not always—essential proteins for the growth of the cell (Björk and Wieslander 2017; Okamura et al.

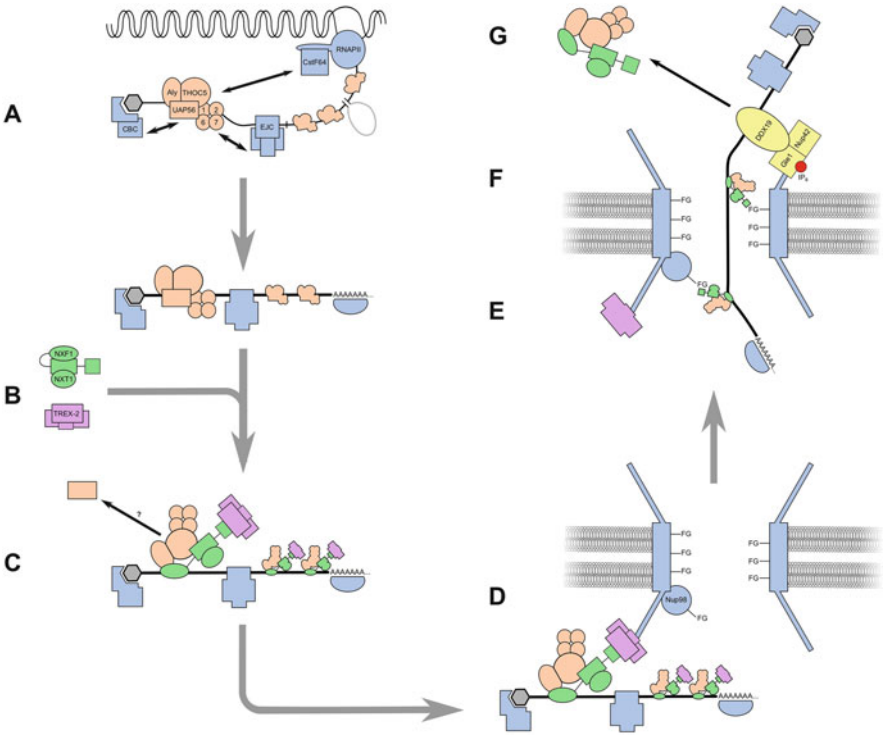

Fig. 2.1 Schematic of the bulk mRNA export pathway in metazoans. Nascent mRNA (**a**) is co-transcriptionally assembled with maturation factors including the cap-binding complex (CBC), exon-exon junction complex (EJC), and the 3′ processing machinery, including the component CstF64 (blue). Each of these components acts to recruit one or more copies of the TREX complex (orange)—including the mRNA export adaptor Aly/REF (*Aly*) and the coadaptor THOC5—which are deposited along the length of the mRNA, ultimately forming a TREX-coated, transcribed mRNA (**b**). Aly/REF and THOC5 bind the export receptor heterodimer NXF1:NXT1 (green), causing a structural rearrangement that exposes NXF1's RNA-binding domain and allows hand-off of RNA from Aly/REF:THOC5 to NXF1:NXT1 (**c**). Upon association with the chaperone complex TREX-2 (purple), the NXF1:NXT1-loaded mRNA is translocated to the nuclear pore via an interaction between TREX-2 and the nuclear basket (**d**). After quality control and/or structural rearrangements, the FG-Nup-binding C-terminal domain of NXF1 is released by TREX-2 and can interact with the proximal FG-Nup NUP98 (**e**) and then other FG-Nups deeper within the nuclear pore channel (**f**). Upon reaching the cytoplasmic surface of the nuclear pore, the NXF1:NXT1:TREX complex is unloaded from the cargo mRNA by the actions of the DDX19:Gle1:Nup42 complex (yellow) bound by IP_6 (red), releasing the mRNA for further cytoplasmic maturation and/or metabolism (**g**). See Sect. 2.2 for a detailed description of this pathway. See text for more details

2015). Instead of relying on specific sequence or structural elements of the mRNA transcript to identify mRNA cargoes, the bulk mRNA export pathway is instead closely interfaced with the co-transcriptional processes of mRNA maturation, thereby (a) preventing the preferential export of particular mRNA species and

(b) ensuring exclusive export of mature, translation-competent mRNAs to the cytoplasm.

The key initiating factor in the bulk mRNA export pathway is the TREX complex, a conserved multiprotein complex composed in humans of the mRNA export adaptor Aly/REF (Yra1 in *S. cerevisiae*), the helicase UAP56/DDX39B (Sub2), CIP29 (Tho1), and the THO subcomplex, composed of the conserved subunits hHpr1/THOC1 (Hpr1), hTho2/THOC2 (Tho2), hTex1 (Tex1), THOC7 (Mft1), and the metazoan-specific THOC5 and THOC6 (Katahira 2012). TREX assembles in a highly cooperative fashion which requires ATP binding of the scaffolding UAP56 helicase (Chi et al. 2013; Dufu et al. 2010) and plays a central role in the coupling of successful mRNA maturation to the subsequent deposition of Aly/REF, an RNA-binding protein whose loading onto mRNA is the initiating step in bulk export and which acts as a key "mRNA export adaptor" for subsequent factors in the pathway (Rodrigues et al. 2001).

In metazoans, the most significant activator of mRNA export is the completion of splicing and, in particular, the deposition of the exon junction complex (EJC) on the maturing mRNA, which carries a checkpoint function for completed splicing (Le et al. 2001; Masuda et al. 2005). The core EJC components interact directly with several TREX components, including UAP56 and Aly/REF, and are required for the recruitment of these proteins and their subsequent loading onto the mRNA (Gerbracht and Gehring 2018; Gromadzka et al. 2016; Le et al. 2000; Viphakone et al. 2018). This loading mechanism distinguishes metazoans from *S. cerevisiae*, wherein the TREX complex becomes associated with mRNA co-transcriptionally *via* THO subcomplex interactions with the phosphorylated C-terminal domain of RNAPII and/or the CTD-loaded Prp19 splicing complex (Heath et al. 2016; Katahira 2012; Masuda et al. 2005; Zenklusen et al. 2002). This transition from transcription- to splicing-dependent loading likely evolved in response to the massive proliferation of splicing in metazoans; however, the observation that Aly/REF interacts with the RNAPII-CTD-binding Iws1:Spt6 complex suggests that at least some of the ancestral co-transcriptional loading machinery may have been retained in higher eukaryotes (Yoh et al. 2007). In addition to being loaded onto mRNA in a splicing-dependent fashion, Aly/REF and/or the TREX complex can be added via an interaction between Aly/REF and the CBP80 component of the cap-binding complex (CBC) once it is bound to a mature 5′ N^7-methylguanosine (m^7G) cap structure (Cheng et al. 2006). Lastly, an interaction between Aly/REF and CstF64, a component of the CstF complex required for mRNA 3′-end processing and polyadenylation, promotes loading of Aly/REF onto nascent mRNA (Shi et al. 2017), possibly in a mechanism similar to the Pcf11-dependent loading of Yra1 in *S. cerevisiae* (Johnson et al. 2009). Collectively, these mechanisms allow the loading of TREX and, as a result, Aly/REF onto the nascent mRNA (Shi et al. 2017; Viphakone et al. 2018) and, furthermore, ensure its coupling to several key steps in the maturation of nascent mRNAs including 5′ capping, splicing, and 3′-end processing/polyadenylation (Fig. 2.1a, b).

Following its deposition onto mRNA, Aly/REF and a component of the THO subcomplex, THOC5, act coordinately as adaptor/coadaptor pair to recruit the key

mRNA export receptor, NXF1/TAP (Mex67 in *S. cerevisiae*), in a heterodimeric complex with its partner, NXT1/p15 (Mtr2) (Stutz et al. 2000). Unlike other nuclear export receptors of the importin-β subfamily, NXF1 does not require the GTP-binding protein Ran for NPC transit; instead, it exhibits a modular domain arrangement with an N-terminal Aly/REF/RNA-binding domain, a central NTF2L-like domain responsible for interactions with THOC5 and NXT1, and a C-terminal domain capable of directly interacting with FG regions within the NPC (Herold et al. 2000). Upon its recruitment to mRNA, NXF1 displaces UAP56 from Aly/REF, likely via steric effects resulting from their closely juxtaposed Aly/REF-binding sites (Hautbergue et al. 2008). Whether this displacement involves eviction of Aly/REF from TREX, or a more subtle rearrangement of TREX interactions, remains unknown (Fig. 2.1b).

In its free state, NXF1:NXT1 exhibits poor affinity for mRNA due to an intramolecular interaction between its N-terminal RNA-binding domain and central NTF2L domain which masks the RNA-binding surface. The binding of Aly/REF and THOC5 to NXF1:NXT1—to NXF1's RNA-binding domain and NTF2L domains, respectively—is able to disrupt this intramolecular interaction, opening up the NXF1 RNA-binding domain (Viphakone et al. 2012). As a consequence, an unusual rearrangement of the Aly/REF:NXF1:RNA complex occurs, in which the bound region of mRNA is released by Aly/REF and "handed off" to NXF1's now-available RNA-binding domain, while Aly/REF binds to a remote surface on NXF1's RNA-binding domain (Hautbergue et al. 2008; see Fig. 2.1c). This hand-off is facilitated by the methylation of key arginine residues in the Aly/REF RNA-binding surface by PRMT1, resulting in a decreased affinity for RNA relative to that for NXF1 (Hung et al. 2010).

Once deposited onto mRNA, NXF1:NXT1 must traverse the nucleoplasm with its mRNA cargo to reach the NPC. Detailed microscopic studies of single RNA molecules in living cells have suggested that mRNA transport through the nucleoplasm is a passive process involving diffusion through channels between chromatin domains (Mor and Shav-Tal 2010). Upon reaching the nuclear rim, NXF1:NXT1 interaction with the nuclear basket—an extended structure associated with the NPC protruding into the nucleoplasmic space and formed by TPR (Mlp1 in *S. cerevisiae*)—is believed to require the activity of chaperones (Wickramasinghe et al. 2010). The most prominent of these is TREX-2, a complex independent of TREX, which contains several subunits including the scaffolding GANP, ENY1, PCID2, DSS1, and several centrin proteins (Jani et al. 2012; Wickramasinghe et al. 2010). Like the TREX complex, TREX-2 is widely conserved but has evolved different functions from its *S. cerevisiae* ancestor, which is required for the tethering of transcriptionally active genes to the NPC (Cabal et al. 2006; Rodríguez-Navarro et al. 2004). In metazoans, TREX-2 loading onto NXF1:NXT1 is mediated by interaction of the NXF1 C-terminal domain with an N-terminal FG-Nup-like region on GANP (Jani et al. 2012; Umlauf et al. 2013; see Fig. 2.1c). It is believed that, following loading onto NXF1:NXT1, TREX-2 is then able to direct its mRNA cargo to the NPC via the interaction of GANP, ENY1, and/or PCID2—and, potentially, other proteins loaded onto the transported mRNP—with the nuclear basket

component TPR (Fasken et al. 2008; Umlauf et al. 2013; Wickramasinghe et al. 2010; see Fig. 2.1d). However, some confusion remains regarding the exact timing and location of the loading of NXF1:NXT1 onto TREX-2, and the mechanism by which TREX-2 is recruited to nascent mRNAs (Jani et al. 2012; Umlauf et al. 2013). In addition to the TREX-2 complex, several other possible NXF1:NXT1 NPC chaperones have been reported, including the WD-repeat protein RAE1 in complex with the nucleoplasmic-mobile FG-Nup NUP98 (Blevins et al. 2003; Pritchard et al. 1999) and the inner nuclear membrane-embedded SUN1 protein (Li and Noegel 2015; Li et al. 2017b). It is important to note that these different chaperones may not be exclusive and may mediate different stages in the chaperoning of NXF1:NXT1-loaded mRNA to and through the NPC.

Interestingly, detailed microscopy studies have suggested that, upon reaching the nuclear basket, mRNPs are frequently returned to the nucleoplasm, with only a minority of mRNAs proceeding from basket binding to transit through the pore (Grünwald and Singer 2010; Ma et al. 2013; Siebrasse et al. 2012). Kinetic analyses of mRNA residency at the NPC in living cells have additionally shown a pause step at the nuclear basket, with mRNAs spending significant time resident on the basket prior to a relatively quick traversal of the nuclear pore (Grünwald and Singer 2010; Siebrasse et al. 2012), possibly due to a requirement for both quality control and remodeling of the mRNPs on the basket prior to mRNP transit through the nuclear pore (Grünwald and Singer 2010; Siebrasse et al. 2012). The precise mechanisms by which mRNAs transit the nuclear pore upon commitment to translocation remain a topic of considerable debate; however, it is generally agreed that successive interactions between the NXF1 C-terminal domain and the exposed tails of FG-Nup proteins throughout the channel mediate an mRNP's entry into and transit through the pore (Oeffinger and Zenklusen 2012; see Fig. 2.1e, f).

Upon reaching the cytoplasm, NXF1:NXT1-loaded mRNPs make contact with a complex of proteins loaded onto the cytoplasmic fibrils of the NPC that includes the helicase DDX19/Dbp5, Gle1, Nup42, and the activating signaling molecule inositol hexaphosphate (IP_6). This complex is responsible for remodeling the mRNP on the cytoplasmic face of the NPC, releasing the cargo mRNA from export factors including NXF1:NXT1, which are recycled back into the nucleus (Adams et al. 2017, 2018; Folkmann et al. 2011; see Fig. 2.1g). An important secondary function of this unloading is to act as a "ratchet" for directional translocation of the mRNA across the NPC; the interaction of NXF1 with FG-Nups has been found not to be inherently directional, allowing NXF1-mRNP complexes to move back and forth within the nuclear pore (Grünwald and Singer 2010). The eviction of NXF1:NXT1 from the mRNA prevents this backwards diffusion and ensures that mRNAs migrate through the NPC in a directional manner (Folkmann et al. 2011). The cytoplasmic mRNA cargo is then free to undergo any cytoplasmic-specific mRNA maturation steps prior to translation.

2.3 Orthology of Bulk mRNA Export Factors

Much of the early work on mRNA export was performed in *S. cerevisiae*, where the bulk export pathway is relatively canonical; the loading of Yra1 onto mRNA via TREX and subsequent recruitment of Mex67 are key steps in the export of most cellular mRNAs in this species, as evidenced by the lethal phenotypes of both *yra1Δ* and *mex67Δ* deletions and the severe mRNA export defects of hypomorphic mutations (Portman et al. 1997; Santos-Rosa et al. 1998; Segref et al. 1997; Zenklusen et al. 2002). While limited evidence has emerged of possible heterogeneity in mRNA export pathways in *S. cerevisiae*, including the description of a nonessential Yra1 homolog, Yra2, that can suppress Yra1 phenotypes when overexpressed, the linear and nonredundant bulk export pathway remains the major means of mRNA export in these cells (Hieronymus and Silver 2003; Okamura et al. 2015; Zenklusen et al. 2001).

In higher eukaryotes, however, several proteome-wide screens for mRNA export factors have demonstrated that while the bulk mRNA export pathway has been conserved from *S. cerevisiae*, metazoans have evolved numerous other mRNA export factors which are able to modify and/or complement this bulk pathway, greatly expanding the mechanistic and regulatory complexity of mRNA export (Delaleau and Borden 2015; Farny et al. 2008; Rehwinkel et al. 2004; Wickramasinghe and Laskey 2015). A major source of this variation has been the proliferation of orthologs of numerous key export factors (Delaleau and Borden 2015; Wickramasinghe and Laskey 2015). These "alternative factors" are able to functionally substitute for their orthologs in a constitutive and/or conditional fashion (Fig. 2.2a). Indeed, the identification of such factors is beginning to explain the long-time contradiction in the field that, while bulk export factors such as Yra1 are essential in *S. cerevisiae*, depletion of their metazoan homologs has only relatively mild effects on mRNA export efficiency in vivo (Gatfield and Izaurralde 2002; Katahira et al. 2009; Longman et al. 2003).

2.3.1 mRNA Export Adaptor Heterogeneity

The relatively mild effects of Aly/REF depletion in metazoans led numerous groups to explore the possibility of alternative mRNA adaptors for the recruitment of NXF1:NXT1 to mRNA. A particularly successful approach to this was taken by Stuart Wilson's group, whose strategy of searching for conserved UAP56-binding motifs (UBMs) has identified several possible functional substitutes for Aly/REF in human cells (Chang et al. 2013; Hautbergue et al. 2009; Viphakone et al. 2015).

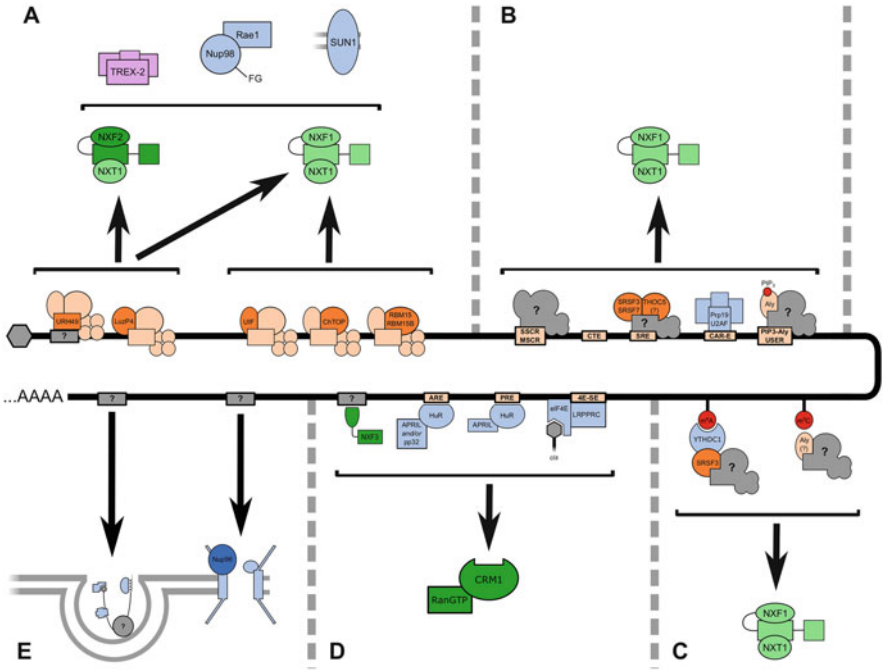

Fig. 2.2 Schematic of characterized selective mRNA export paths in metazoans. Where *cis*- and/or *trans*-acting factors are unknown, they are shown in gray with a question mark. (**a**) Orthologs (dark orange/green) of bulk export pathway factors (light orange/green) may be loaded onto mRNAs and cooperate with bulk export pathway factors to promote selective mRNA export. Several different export receptor chaperones (purple/light blue) may act to direct the export receptor(s) to the nuclear pore. See Sect. 2.3. (**b**) *cis*-acting sequence/structural USER codes (light orange) can recruit *trans*-acting mRNA export adaptors/coadaptors from the bulk (light orange) or selective (dark orange) mRNA export pathways; alternatively, they may rely on repurposing of other cellular RBPs (light blue) or alteration of bulk export pathways by second messengers (red). See Sect. 2.4. (**c**) Posttranscriptionally added mRNA modifications (red) can act as sequence-nonspecific, *cis*-acting USER codes through recruitment of bulk (light orange) or selective (dark orange) export adaptors, with or without the assistance of mRNA modification reader proteins (light blue). See Sect. 2.5. (**d**) A number of *cis*-acting USER codes rely on binding to cellular RBPs (light blue) that lack canonical export adaptor/coadaptor activity, but can recruit an alternate mRNA export adaptor heterodimer, CRM1:RanGTP (dark green), via a leucine-rich nuclear export signal (NES). These include the atypical bulk mRNA export receptor ortholog, NXF3 (dark green). See Sects. 2.4 and 2.6. (**e**) Finally, mRNA export may also be modulated through the activity of specific nuclear pore components (dark blue) or may bypass the nuclear pore entirely through direct budding through the inner and outer nuclear membranes (gray lines). See Sect. 2.7. Given that one or more of these pathways may act in conjunction both with each other and with the bulk export pathway (see Fig. 2.1), they are depicted as being resident on the same mRNA (black line). See text for more details

2.3.1.1 UIF

The first of these to be identified was UIF/FYTTD1, a protein that was evolutionarily unrelated to Aly/REF but shares many of its basic features, including being ubiquitously expressed throughout tissues and, upon tethering to a reporter mRNA, the ability to directly support export of mRNA to the cytoplasm in an NXF1:NXT1-dependent manner (Hautbergue et al. 2009). UIF is able to interact with both UAP56 and NXF1 in a mutually exclusive fashion reminiscent of Aly/REF loading onto TREX/RNA. Indeed, it has been postulated that UIF may be able to functionally substitute for Aly/REF in the bulk mRNA export pathway. Consistent with this hypothesis, UIF depletion resulted in a relatively mild mRNA export phenotype, while co-depletion of Aly/REF and UIF resulted in a severe export phenotype, indicating that these two proteins are able to act redundantly along mRNA export pathways (Hautbergue et al. 2009). The recent identification of two homologs of UIF in *Arabidopsis thaliana*, UIEF1 and UIEF2, both of which are required for mRNA export, suggests that the UIF-dependent mRNA export pathway may be widely conserved among eukaryotes (Ehrnsberger et al. 2019).

The fact that Aly/REF and UIF single depletions exhibited mild export defects suggests that these proteins are not completely redundant, and evidence of possible mechanistic differences has emerged with the observation that the two proteins are likely loaded onto nascent mRNA in different ways, namely, via SPT6/IWS1 and/or CstF64 for Aly/REF and via SSRP1 for UIF (Hautbergue et al. 2009; Shi et al. 2017; Yoh et al. 2007). Interestingly, overexpression of both Aly/REF and UIF also induced mRNA export defects, raising the possibility that the relative stoichiometry of these two proteins is important for mRNA export, though a possible indirect mechanism dependent on sequestration of other export factors such as TREX and/or NXF1:NXT1 cannot be excluded (Hautbergue et al. 2009).

2.3.1.2 Luzp4

A similar UBM-searching strategy as for UIF was used to identify Luzp4/CT-8, a leucine zipper-containing protein (Viphakone et al. 2015). Like Aly/REF, Luzp4 localizes to the nuclear splicing speckles and is capable of interacting with the TREX complex, NXF1 and RNA (Türeci et al. 2002; Viphakone et al. 2015). Luzp4 overexpression was shown to completely rescue a modest mRNA export defect caused by Aly/REF depletion, confirming Luzp4 as a genuine mRNA export adaptor able to act redundantly with Aly/REF (Viphakone et al. 2015); however, it could only partially rescue the more severe defects of Aly/REF and UIF co-depletion, suggesting that it may not share complete functional redundancy with these ubiquitously expressed factors (Viphakone et al. 2015).

One major point of difference is that Luzp4 expression is tightly restricted to the testes during homeostatic growth, and its upregulation in numerous cancers marked it as a member of the cancer-testis antigen family of genes (Türeci et al. 2002;

Viphakone et al. 2015). In line with its testes-restricted expression pattern, Luzp4 was found to interact with NXF2, a testis-specific NXF1 ortholog discussed below, raising the possibility of an alternative, Luzp4-NXF2-based mRNA export pathway specific to the testes.

2.3.1.3 Other Candidate Export Adaptors: SKAR and ZC11A

While UIF and Luzp4 remain the only definitively characterized Aly/REF substitutes in bulk mRNA export, several other export adapter candidates have been identified in other studies. Dufu et al. (2010) identified five previously unreported proteins in a proteomic screen for common interactors of UAP56, THOC5, and CIP29, suggesting that these factors may be novel components of the TREX complex; one of these, SKAR/POLDIP3, shows significant sequence and domain-arrangement homology with Aly/REF, while SKAR and another protein, ZC11A, associate with UAP56 in an ATP-dependent manner and induce an mRNA export defect upon depletion (Folco et al. 2012). While these proteins require further investigation prior to their confirmation as bona fide Aly/REF functional orthologs, their identification raises the possibility that the complete suite of mRNA export adaptors in human cells remains to be fully elucidated.

2.3.2 Export Coadaptor Heterogeneity

While Aly/REF is essential for the recruitment and subsequent binding of NXF1:NXT1 to mRNA, it alone is not sufficient. The disruption of the RNA-binding domain-masking intramolecular interaction of NXF1 requires the combined activity of both an mRNA adaptor, such as Aly/REF, and a coadaptor, THOC5. While THOC5 is reported to interact with RNA less stringently than NXF1:NXT1, biochemical experiments showed its activity to be required for efficient handover of mRNA from Aly/REF to NXF1:NXT1 and consequent mRNA export, marking it as a core component of the bulk mRNA export pathway (Chang et al. 2013; Viphakone et al. 2012).

Despite this central role, however, THOC5 depletion, like that of Aly/REF, causes only modest defects in bulk mRNA export (Chang et al. 2013); indeed, experiments comparing the cytoplasmic versus nuclear distribution of RNAs by microarray found that only 0.7–2.9% of mRNAs showed a significant alteration in nuclear/cytoplasmic ratio following THOC5 knockdown (Guria et al. 2011; Rehwinkel et al. 2004). Furthermore, numerous reports have emerged identifying essential roles for THOC5 in the regulation of particular functional subsets of mRNA, including those involved in heat shock (Katahira et al. 2009), stem cell pluripotency (Ratnadiwakara et al. 2018; Wang et al. 2013), hematopoiesis (Mancini et al. 2010), and adipogenesis (Mancini et al. 2006). Collectively, these results suggest that, far from being a unique regulator of bulk mRNA export, THOC5,

like Aly/REF, may simply be one of a set of mRNA export coadaptors with redundant and/or specialized roles in mRNA export.

2.3.2.1 ChTOP

Like UIF and Luzp4, ChTOP was identified based on a canonical UBM required for interaction with UAP56 in the context of the TREX complex, suggesting a mutually exclusive loading onto TREX with Aly/REF (Chang et al. 2013). In support of an Aly/REF-esque role, ChTOP was shown to localize to splicing speckles, interact with RNA, and undergo a PRMT1 methylation-based hand-off interaction with NXF1:NXT1. However, examination of the interaction between ChTOP and other export components revealed that, unlike Aly/REF, UIF, and Luzp4, ChTOP interacted with the THOC5-binding site on NXF1's central NTF2L domain; furthermore, ChTOP bound NXF1:NXT1 cooperatively, rather than exclusively with Aly/REF, and, in so doing, additively enhanced NXF1's affinity for mRNA, identifying ChTOP as a novel alternative coadaptor for Aly/REF on NXF1 (Chang et al. 2013).

2.3.2.2 RBM15 and RBM15B

RBM15 and its distantly related ortholog, RBM15B/OTT3, are RNA-binding proteins with diverse reported roles within cells, including the regulation of site-specific RNA methylation on N^6-adenosine (m^6A) and the transcriptional regulation of Notch signaling pathways (Ma et al. 2007; Patil et al. 2016). In the context of mRNA export, RBM15 has been reported to recruit the helicase DDX19 to NPC-transiting mRNAs for eviction of NXF1:NXT1 at the cytoplasmic face (Zolotukhin et al. 2009) and is a *trans*-acting factor responsible for the binding of a *cis*-acting mRNA export sequence element, the retroviral transport element (RTE), discussed below (Lindtner et al. 2006).

In addition to these myriad roles, a study by Uranishi et al. (2009) has reported the likely function of both RBM15 and RBM15B as alternative NXF1:NXT1 coadaptors. RBM15B, like RBM15 (Lindtner et al. 2006), can promote the export of tethered RNAs via a direct interaction with NXF1:NXT1. This interaction involved the C-terminal domains of RBM15/RBM15B binding to the central NTF2L domain of NXF1 in a fashion resembling that of THOC5 and ChTOP; the same domain of RBM15 also mediated an interaction with Aly/REF, suggesting that RBM15 is able to assemble a functional Aly/REF:RBM15/B:NXF1:NXT1 adaptor–coadaptor–receptor complex on bound mRNAs (Uranishi et al. 2009). While these observations remain incomplete with regard to a role for RBM15/RBM15B as true coadaptors in bulk mRNA export, and further experiments are essential to dissect the relative overlap and interplay of the multiple reported roles of RBM15/RBM15B, these observations suggest the possibility that RBM15 and/or RBM15B may act as

additional Aly/REF-paired coadaptors, at least on a subset of mRNAs (Chang et al. 2013; Uranishi et al. 2009).

2.3.3 *Additional Heterogeneity Within the TREX Complex: UAP56 and URH49*

In *S. cerevisiae*, deletion of the TREX complex core helicase, Sub2, is lethal, likely due to a near-complete failure of bulk mRNA export (Sträßer et al. 2002). Similarly, depletion of the metazoan Sub2 homolog, UAP56, causes a significant export defect, supporting its identification as the major Sub2 homolog (Sträßer et al. 2002). However, in mammals, sequence analysis revealed that Sub2 in fact possesses two closely related orthologs, UAP56/DDX39B and URH49/DDX39, both of which are capable of partially rescuing a *sub2Δ* deletion phenotype in *S. cerevisiae*, raising the possibility that these two proteins may act in parallel (Kapadia et al. 2006; Pryor et al. 2004). In support of this observation, both UAP56 and URH49 are able to interact with Aly/REF, and their co-depletion results in a severe mRNA export defect greater than loss of either single protein.

However, a major point of difference between the UAP56/URH49 and the redundancy paradigm of Aly/REF and other export adaptors above is that depletions of UAP56 and URH49, while additive, do exhibit significant mRNA export defects by themselves, suggesting these proteins have at least partially independent functions in mRNA export. Indeed, a cytoplasmic RNA microarray analysis of the two proteins found that, while the depletion of each reduced the export of approximately 300 mRNAs, only approximately 60 of these were common between the two proteins (Yamazaki et al. 2010). Furthermore, phenotypic analysis of UAP56 and URH49 depletions revealed that, while they both induced mitotic dysfunction, they did so via different mechanisms, with UAP56 depletion causing premature sister chromatid separation during mitosis, while URH49 depletion prevented efficient chromosome arm resolution and cytokinesis (Yamazaki et al. 2010). It has also been suggested that these two helicases may have different affinities for non-Aly/REF TREX components, in particular the THO subcomplex and CIP29; however, subsequent work has disputed at least some of these findings (Dufu et al. 2010). Finally, URH49, but not UAP56, has been reported to exhibit highly regulated expression, both tissue-specific and growth-regulated, suggesting it may act as a stimulus-responsive complement to the constitutive activity of UAP56 (Pryor et al. 2004). Collectively, these observations suggest that, while UAP56 and URH49 likely play similar mechanistic roles in the regulation of mRNA export, their mRNA targets and the conditions under which they act may vary significantly.

2.3.4 The NXF1 Family of Export Receptors

NXF1 was the first mRNA export factor to be identified through its recruitment to *cis*-acting sequence elements in retroviral transcripts (see below; Grüter et al. 1998). Its clear homology to the essential yeast export receptor Mex67, and the severe export defect resulting from its depletion, has led to its characterization as the sole mRNA export receptor for constitutive bulk mRNA export (Björk and Wieslander 2017; Okamura et al. 2015). While this is likely to be true in the majority of cell types during constitutive growth, it is nonetheless notable that the *NXF1/Mex67* family has undergone significant diversification during eukaryotic evolution. While *S. cerevisiae* only possess one copy of the family (Mex67), there are two members in *C. elegans*, four in *D. melanogaster*, and five (including NXF1) in humans (Herold et al. 2000). In humans, these paralogs, termed NXF1-5, have generally retained the domain arrangement of Mex67/NXF1; however, NXF4 and NXF5 are likely not expressed due to the presence of multiple frameshifts and/or premature stop codons in their coding sequence, while NXF3 contains several truncations that prevent it from replicating the various interactions required for NXF1's export activity (Herold et al. 2000; Yang et al. 2001). Remarkably, NXF3 has in fact been found to be able to promote mRNA export via an entirely novel mechanism involving the export receptor CRM1, which will be discussed below (Yang et al. 2001).

NXF2 is one homolog that has retained a close similarity to NXF1 and the ability to interact with known mRNA export coadaptors, including ChTOP, as well as with mRNA itself (Chang et al. 2013; Herold et al. 2000). Immunofluorescence analyses showed that NXF2 localizes to the nucleoplasm and accumulates at the nuclear rim like NXF1 and is able to promote mRNA export in at least some assays, although the latter has been disputed by other groups working in heterologous systems (Herold et al. 2000; Yang et al. 2001). Interestingly, expression analysis determined that NXF2's expression, like that of LuzP4 and URH49, is tightly constrained to the testes, and reports that NXF2 cooperates with the RNA-binding protein (RBP) FMRP to cooperatively destabilize the *NXF1* mRNA and thus repress NXF1 expression suggest that, at least in the testes, NXF2 may take over as the predominant receptor in bulk mRNA export (Herold et al. 2000; Zhang et al. 2007). In addition to their observations on the NXF1 family, Herold et al. (2000) also noted the existence of a second NXT1 ortholog, termed p15-2. However, beyond identification of a broad expression profile with moderate upregulation in the testes by high-throughput tissue screening, the function of this ortholog has not yet been investigated (Herold et al. 2000).

2.3.5 *Chaperoning of NXF1:NXT1 to the NPC: Pervasive Mechanism or "Fast-Track" Selectivity?*

As mentioned above, the recruitment of mRNA-bound NXF1:NXT1 to the NPC is suggested to be mediated by a number of different chaperones. The best explored of these has been the TREX-2 complex, in which the scaffolding protein GANP binds the NXF1 C-terminal domain and directs NXF1 to the nuclear basket (Jani et al. 2012; Wickramasinghe et al. 2010). While this was assumed to be a universal process in bulk mRNA export, recent work by Wickramasinghe et al. (2014) profiled the mRNA export defect upon GANP depletion and found, surprisingly, that export of only approximately 50% of all NXF1 target mRNAs was negatively affected; furthermore, these 50% were predominantly abundant, short-lived mRNAs and were enriched for gene ontology annotations covering a variety of RNA processing functions. Strikingly, the export of those GANP-dependent RNAs was found to be significantly more rapid than those whose export was unaltered by GANP depletion, raising the possibility that GANP-mediated NXF1:NXT1 chaperoning may not represent a ubiquitous pathway but instead a "fast-track" export mechanism for a specific functional or regulatory subset of mRNAs (Okamoto et al. 2010; Wickramasinghe et al. 2014). It is not known whether GANP-independent mRNAs are dependent on other reported NXF1:NXT1-NPC chaperones, such as RAE1-NUP98 (Blevins et al. 2003; Pritchard et al. 1999) and/or SUN1 (Li and Noegel 2015; Li et al. 2017b), and, if so, what impact these chaperones have on the export rate of their mRNA cargoes; considerable further research is required to dissect if and how the different reported chaperone pathways interact. However, the finding that diffusion of mRNAs through the nucleoplasm and their loading onto the nuclear basket represent rate-limiting steps in mRNA export (Mor and Shav-Tal 2010; Shav-Tal et al. 2004) suggests that chaperoning of NXF1:NXT1 to the NPC may represent a good candidate target for the manipulation of mRNA export rate for subsets of mRNAs.

2.3.6 *Defining the Complete Suite of Bulk mRNA Export Orthologs and Parallel Pathways*

Despite the extensive work described above identifying orthologous bulk mRNA export factors acting on metazoan mRNA, our knowledge of the complete suite of bulk mRNA export orthologs likely remains incomplete, especially with regard to the existence of further tissue- and/or stimulus-specific factors. While biochemical testing of the mRNA export roles of these candidate orthologs remains important, additional alternative approaches are required to identify the complete suite of mRNA export factor orthologs in mammals.

The Wilson group recently used in silico homology screening of the key UBM interaction motif within Aly/REF to identify several novel mRNA export adaptors/

coadaptors including UIF, LuzP4, and ChTOP (Chang et al. 2013; Hautbergue et al. 2009; Viphakone et al. 2015) suggesting that in silico identification represents a viable strategy for the identification of future candidates. Alternatively, the ongoing development of high-content screening microscopy systems, which allow the automated imaging and quantitative analysis of thousands of immunofluorescence samples, enables the use of mRNA export readouts such as nuclear-cytoplasmic poly $(A)^+$ RNA ratios in the screening of large candidate protein classes to identify putative mRNA export factors (Mattiazzi Usaj et al. 2016). The recent use of these systems to generate a large immunofluorescence-based database colocalizing a library of approximately 300 RBPs with a range of different cellular structures emphasizes the viability of such experimental approaches and provides an important comparative database for the immunofluorescent localization of possible mRNA export factors identified by siRNA/overexpression screening as described above (Van Nostrand et al. 2018).

A second major question concerning orthologous bulk mRNA export adaptors/coadaptors is their degree of orthology. The relatively mild mRNA export defects resulting from the individual knockdowns of bulk mRNA export factors such as Aly/REF, UIF, and THOC5 coupled with the synthetic, severe export deficits upon co-depletion of pairs such as Aly/REF:UIF, Aly/REF:THOC5, and Aly/REF:ChTOP argue for near-complete overlap of these factors' export activities (Chang et al. 2013; Gatfield and Izaurralde 2002; Hautbergue et al. 2009; Katahira et al. 2009; Longman et al. 2003). However, several observations suggest that the system may be more complicated than these observations imply. While the pairwise testing of adaptor/coadaptor co-depletions is far from complete, several unexpected observations have emerged, such as that co-depletion of ChTOP:THOC5 causes no additive export defect, despite these two proteins supposedly acting in a redundant and compensatory mechanism as mRNA coadaptors (Chang et al. 2013). Furthermore, the co-depletion of both Aly/REF:THOC5 and Aly/REF:ChTOP resulted in severe export defects, despite the existence in each case of a hypothetically redundant adaptor-coadaptor pair (UIF:ChTOP and UIF:THOC5) (Chang et al. 2013). Other observations have shown that LuzP4 overexpression is able to completely rescue the export defect of Aly/REF depletion but can only partially rescue the defect of Aly/REF:UIF co-depletion, suggesting that UIF (or the combination of Aly/REF and UIF) performs functions that are not redundantly regulated by LuzP4 (Viphakone et al. 2015). Profiling of mRNA transcriptional upregulation upon depletion of particular mRNA export factors has revealed a complex but incomplete network of compensatory regulation in which, for example, depletion of either Aly/REF or UIF upregulates the expression of the other, while knockdown of Aly/REF does not induce expression of LuzP4; similarly, while ChTOP knockdown induces expression of UAP56, it does not alter expression of Aly/REF—despite knockdown of Aly/REF upregulating ChTOP (Chang et al. 2013; Hautbergue et al. 2008; Viphakone et al. 2015). Perhaps one of the most compelling observations speaking to this complexity, as discussed in the sections above, is that, while orthologous adaptors and coadaptors may act redundantly on most mRNAs, specific subsets of mRNAs seem to be regulated exclusively by a particular adaptor-

coadaptor pairing. The prototypical example of this is the export of *HSP70* mRNA which remained unaltered by Aly/REF or THOC5 knockdown under constitutive growth conditions; however, upon induction of heat shock, Aly/REF and THOC5 became essential for its export (Guria et al. 2011; Katahira et al. 2009). Collectively, these observations suggest that, while many of the mRNA export adaptors and coadaptors share at least some redundancy in bulk mRNA export, many of these factors retain at least some partially or wholly unique functions. These observations also raise the possibility of an "adaptor-coadaptor code," in which particular combinations of adaptor(s) and coadaptor(s) govern the export of particular subsets of mRNA ("regulons"; Keene 2007). While this possibility is intriguing, future experiments will be required to test it, possibly in the form of complete pairwise testing of co-depletion or overexpression-rescue phenotypes to determine redundancy as well as the identification of the complete set of mRNAs bound not only to each adaptor and coadaptor (much of the data of which has already been generated from the large-scale ENCORE RBP iCLIP screening program; Van Nostrand et al. 2018) but specifically to each possible adaptor-coadaptor pairing.

2.4 Noncanonical Recruitment of NXF1 to mRNA: USER Codes and mRNA Regulons

While much work in the last 15 years has focused on the mechanisms by which mRNA adaptors and coadaptors can recruit NXF1:NXT1 to mRNA as part of the bulk export pathway, it is interesting to note that NXF1 was first identified by its ability to promote export of unspliced Mason-Pfizer monkey virus RNA via direct interaction, independently of adaptors or coadaptors, with a *cis-acting sequence element, termed the constitutive transport element* (CTE) (Braun et al. 1999; Bray et al. 1994; Grüter et al. 1998). Indeed, the unique requirement of retroviruses for parallel export of both spliced (coding) and unspliced (genomic) RNAs has led to the proliferation of numerous virally encoded sequence elements that coopt cellular mRNA export machinery in order to promote RNA export and/or bypass the mRNA quality control machinery (Cullen 1998; Hammarskjöld 1997). Some of these viral sequence elements, including the CTE itself, have subsequently been co-opted by host mRNAs to promote their specialized export (Li et al. 2006; see Fig. 2.2b).

As research on mRNA export pathways in metazoans progressed, it became clear that the use of *cis*-acting export specificity elements in RNA was not confined to viral RNAs, and that a range of different elements existed within metazoan transcriptomes that allowed coordinated export regulation of an mRNA, or set of mRNAs, through recruitment of mRNA export machinery independently of the bulk export pathway (Delaleau and Borden 2015; Wickramasinghe and Laskey 2015). This concept became formalized through the terminology of "USER" (*u*ntranslated *s*equence *e*lements for *r*egulation) codes, small sequence elements that direct the

coordinated posttranscriptional regulation of a group of mRNAs, termed an "mRNA regulon" (Keene 2007). This section will focus on the discussion of USER codes and regulons that promote export of endogenous mRNAs via NXF1 (Fig. 2.2b) or the importin-β family member CRM1, while other mechanisms of mRNA export by CRM1 will be discussed in Sect. 2.6 (Fig. 2.2d).

2.4.1 USER Codes in the Export of Intronless mRNAs

One major question raised with the discovery of the link between splicing and mRNA export was how intronless mRNAs are exported (Le et al. 2001; Masuda et al. 2005). It was known that the export of such mRNAs was dependent on the bulk mRNA export machinery, including Aly/REF and NXF1 (Sträßer et al. 2002); however, the mechanism by which these factors were deposited on the mRNA was unclear. While subsequent observations on the recruitment of Aly/REF to bulk mRNA via interactions with the CBC (Cheng et al. 2006) and the 3′ processing factor CstF64 (Shi et al. 2017) have provided possible mechanisms for export, a parallel thread of research has revealed the widespread use of coding region-embedded USER codes to recruit the canonical mRNA export machinery to intronless mRNAs.

2.4.1.1 The SRSF Family of Splicing Factors

The first intronless USER code in metazoans was identified through work on the replication-dependent histone mRNAs, a class of intronless mRNAs defined by a unique 3′ structure lacking a poly(A) tail but instead incorporating a conserved stem-loop structure (Dominski and Marzluff 2007). The *H2A* mRNA was found to contain a 22-nucleotide (nt) sequence element within its coding region that was necessary for export of this mRNA to the cytoplasm (Huang and Carmichael 1997; Huang and Steitz 2001). A UV-cross-linking approach was used to show that this sequence element was bound by two members of the SRSF family of splicing factors, SRSF3/SRp20 and SRSF7/9G8, and that these *trans*-acting factors were essential to the export of *H2A* mRNA in a fashion independent of their previously described roles in mRNA splicing (Huang and Steitz 2001). Upon binding to the *H2A* USER code, SRSF3 or SRSF7 is able to interact with NXF1 via its N-terminal domain and to mediate RNA hand-off to NXF1 in a fashion identical to Aly/REF, suggesting that SRSF3 and/or SRSF7 is able to functionally substitute to Aly/REF in NXF1:NXT1 loading of *H2A* mRNA onto NXF1:NXT1 (Hargous et al. 2006; Hautbergue et al. 2008; Huang et al. 2003). Given that both SRSF3 and SRSF7 bind via the Aly/REF-binding site, the identity of the coadaptor, if any, in the export of *H2A* remains unclear; however, the observations that SRSF3 is able to cooperate with coadaptors including THOC5 and YTHDC1 in other export pathways (Ratnadiwakara et al. 2018; Roundtree et al. 2017; Wang et al. 2013) and that the 3′-terminal stem-loop

binding protein of replication-dependent histone mRNAs, SLBP, is also necessary for export (Sullivan et al. 2009) raise several possible candidates. Interestingly, this work also observed that a third SRSF protein, SRSF1/SF2, was able to interact with the NXF1 N-terminal domain redundantly with Aly/REF, raising the possibility that it too may regulate the export of as-yet-undetermined mRNA(s) (Huang et al. 2003; Lai and Tarn 2004; Tintaru et al. 2007).

While for many years *H2A* mRNA was considered to be the only mRNA export target of SRSF3/SRSF7, the recent emergence of high-resolution RNA-protein cross-linking methodologies has allowed the identification of putative RNA targets of export factors with unprecedented sensitivity and has redefined our understanding of SRSF protein-mediated mRNA export. In 2016, Müller-McNicoll et al. (2016) used the individual-nucleotide resolution cross-linking immunoprecipitation (iCLIP) methodology (König et al. 2010) to identify >1000 mRNAs whose export is dependent on SRSF proteins, including several hundred possible targets of SRSF3. These observations chime with earlier reports of SRSF protein being capable of binding and promoting the export of both intronless and spliced mRNAs, suggesting that the SRSF family may represent a large group of previously unknown mRNA export adaptors whose function requires further exploration (Masuyama et al. 2004). In addition, Müller-McNicoll et al. (2016) were able to demonstrate by iCLIP the closely juxtaposed binding of SRSF3/SRSF7 and NXF1 to mRNA targets, confirming an NXF1-dependent export pathway, and to identify sequence-specific binding of SRSF3 and SRSF7 to several degenerate motifs in last exons of mRNAs, suggesting the existence of as-yet-unidentified additional USER codes responsible for the recruitment of SRSF3 and/or SRSF7 to target mRNAs.

Consistent with the redefinition of SRSF3 as a potentially promiscuous USER code-directed mRNA export adaptor, several very recent studies have identified new pathways in which SRSF3 substitutes for Aly/REF in the export of specialized mRNA subsets. Roundtree et al. (2017) reported that SRSF3 cooperates with the N^6-methyladenosine (m^6A) reader protein YTHDC1 to promote the export of mRNAs marked by m^6A modification (see below). In embryonic stem cells, SRSF3 cooperates with the known mRNA export adaptor THOC5 in a novel adaptor/coadaptor pairing to promote the export of key pluripotency maintenance factor mRNAs including *Nanog*, *Sox2*, *Klf4*, and *Esrrb* (Ratnadiwakara et al. 2018; Wang et al. 2013). THOC5 is highly expressed in embryonic stem cells and is downregulated over the course of differentiation; artificial expression of THOC5 is able to sustain stem cell pluripotency, while its depletion prevents reprogramming of somatic cells into induced pluripotent stem cells (iPSCs), suggesting that SRSF3/THOC5-mediated mRNA export regulation may represent an important and previously unknown axis of pluripotency control (Wang et al. 2013). While no USER code has yet been identified for these target mRNAs, it remains likely that such an element is responsible for the temporally and cell type-restricted regulation of these target mRNAs.

2.4.1.2 The Prp19 Complex, U2AF, and the CAR-E Element

In 2011, a second, evolutionarily unrelated coding region-resident USER code, termed the cytoplasmic accumulation region (CAR), was identified in several independent intronless mRNAs, including *HSPB3*, *IFNα1*, and *IFNβ1*, as well as the previously reported *c-Jun* (Guang and Mertz 2005; Lei et al. 2011). The CAR element contains a variable number of copies of a short, 10nt USER code, termed the CAR element (CAR-E) which collectively promote the export of their resident mRNAs via the canonical TREX-NXF1:NXT1 export pathway (Lei et al. 2011, 2013). RNA immunoprecipitation-mass spectrometry (IP-MS) approaches revealed that CAR-E elements were bound by components of the Prp19 complex as well as by U2AF65/U2AF2 (Lei et al. 2013). The Prp19 complex and U2AF65 (as part of the heterodimeric U2AF complex with U2AF35) possess well-described, mRNA export-independent roles in the coordination of transcription with splicing (David et al. 2011); nevertheless, subsequent analyses suggested that the binding of the Prp19 complex and U2AF65 to CAR-E was necessary for the NXF1-mediated export of CAR-E-carrying intronless mRNAs, suggesting possible activity of the Prp19 complex and U2AF65 as export adaptors/coadaptors upstream of TREX (Lei et al. 2013). While a role for these splicing/transcription-regulatory factors in the coordination of specialized mRNA export seems surprising, it is noted that several previous papers have provided confirmatory evidence, reporting that the U2AF complex is able to recruit NXF1 to mRNA in both human cells and *Drosophila* (Blanchette et al. 2004; Zolotukhin et al. 2002), suggesting that this activity may represent a conserved mechanism both of intronless mRNA export and of coupling of export to the processes of splicing and transcription. While the extent of the CAR-E-mediated export pathway remains to be determined, it is notable that the model mRNAs tested here—*HSPB3*, *IFNα1*, *IFNβ1*, and *c-Jun*—derive from different functional classes and are not otherwise known to function as a regulon, supporting the possibility of CAR-E export being a widespread mechanism of intronless mRNA export.

2.4.2 *The Signal Sequence Coding Region as a Dual-Functional Regulatory Element*

mRNAs that encode proteins destined for cellular export or embedding in the cellular membrane encode a short sequence, termed the signal sequence, immediately following the start codon of their ORFs; upon translation, this sequence targets the nascent polypeptide to the endoplasmic reticulum for subsequent membrane embedding or secretion (Aviram and Schuldiner 2017). Recently, it was revealed that, in addition to encoding the ER-targeting signal sequence, the signal sequence coding region (SSCR) is able to act in *cis* as a USER code governing export of mRNAs (Palazzo et al. 2007). The SSCR's nucleotide sequence is optimized not

only to encode the requisite amino acid consensus for a function signal sequence but also to selectively exclude AMP nucleotides, enriching in particular for extended stretches of C/G bases; remarkably, silent coding mutations that introduce AMP residues to the SSCR prevent it from driving export of an unspliced reporter mRNA (Palazzo et al. 2007). Subsequent analysis of similar leader sequences on mRNAs encoding mitochondrially localized proteins, namely, mitochondrial signal coding regions (MSCRs), revealed this sequence is functionally identical to the SSCR in promoting mRNA export (Cenik et al. 2011). While much of the mechanism regulating SSCR/MSCR-dependent mRNA export remains to be characterized, it has been noted that the export activity of an SSCR/MSCR can be blocked by the presence of a 5′-proximal splice site, suggesting that proximity of the SSCR/MSCR to the cap and bound CBC may be important for the export (Cenik et al. 2011). Furthermore, SSCR/MSCR-containing intronless mRNAs have been observed transiting the splicing speckles, suggesting a role for this nuclear subdomain in the regulation and/or licensing of SSCR/MSCR-dependent mRNA export (Akef et al. 2013).

2.4.3 Posttranscriptional Control of USER Code Recognition by Aly/REF

While the examples outlined thus far in this section establish a pattern in which sequence-nonspecific bulk mRNA export factors are recruited by sequence-specific *trans*-acting factors, one recently described pathway has challenged this paradigm and suggested that the bulk mRNA export factors themselves may be modified in order to promote sequence-specific mRNA export.

IPMK is a multifunctional phosphatidylinositol kinase which synthesizes several inositol phosphate products including IP_4, IP_5, IP_6, and the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). IPMK localizes to splicing speckles in humans and has been identified as a regulatory hub for multiple aspects of nuclear RNA metabolism, including mRNA export (Kim et al. 2017; Salamon and Backer 2013). siRNA-mediated knockdown of IPMK resulted in the reduced export of approximately 13% of the mRNAs tested by cytoplasmic RNA microarray, including a significant enrichment for mRNAs that regulate processes including cell cycle progression and DNA double-strand break repair by homologous recombination but not nonhomologous end joining, suggesting function-specific regulons exist within the IPMK-regulated mRNA population (Wickramasinghe et al. 2013). The key regulatory step in this export pathway is the previously reported binding of the IPMK product PIP_3 to Aly/REF via an interaction surface on Aly/REF's RNA-binding domain (Okada et al. 2008). This interaction appears to alter the RNA-binding mode of Aly/REF, resulting in its interaction with a specific, 10nt USER code within the 3′-UTRs of target genes and thereby directing these mRNAs for NXF1:NXT1-dependent export. Supportive of this model, Aly/REF binding to

the identified USER code was inhibited by IPMK but could be rescued by the exogenous addition of PIP_3 (Wickramasinghe et al. 2013).

Additional support for this model came from an independent study of two DNA damage-responsive splicing regulators, BCLAF1 and THRAP3 (Vohhodina et al. 2017). The semi-redundant BCLAF1 and THRAP3 were found to promote the export of a set of mRNAs in response to DNA damage response signaling that closely resembled the IPMK regulon. Crucially, in silico motif analysis of BCLAF1/THRAP3 mRNA export targets returned a highly enriched 3′-UTR motif almost identical to the USER code recognized by PIP_3-bound Aly/REF, suggesting that IPMK-regulated Aly/REF and BCLAF1/THRAP3 act via a common USER code in the 3′-UTRs of their target mRNAs (Vohhodina et al. 2017; Wickramasinghe et al. 2013). While the exact relationship between PIP_3-Aly/REF and BCLAF1/THRAP3 in the regulation of this pathway is not yet clear, the observation that both BCLAF1 and THRAP3 contain WxHD motifs reported to regulate interaction of Aly/REF with the EJC components eIF4A3 (Gromadzka et al. 2016; Vohhodina et al. 2017) and the identification of a BCLAF1-NXF1 interaction in a recent high-throughput proteomics study (Castello et al. 2012) make it tempting to speculate that PIP_3-Aly/REF in concert with BCLAF1 and/or THRAP3 may form an NXF1-regulatory adaptor/coadaptor pair on the 3′-UTR USER code of their target mRNAs. In addition to providing new insights into the mechanisms of USER code recognition in mRNA export, the IPMK-Aly/REF-BCLAF1/THRAP3 pathway also points toward a new layer of mRNA export regulation by second messenger-dependent recognition of cryptic USER codes by mRNA export adaptors.

2.4.4 Trans-*Acting Factors Without a USER Code: Candidate mRNA Regulon Export Factors*

While the above pathways represent examples of cases in which known *cis*-acting USER codes are able to direct the export of their carrier mRNAs, a number of RNA-binding proteins have been identified that are not assignable to known mRNA regulons or USER codes, but which represent candidate mRNA regulon export adaptors/factors that warrant further investigation.

In addition to its other roles in the regulation of mRNA export (discussed in Sects. 2.3.2.2 and 2.5.1), RBM15 and/or RBM15B has been reported to bind specific RNA sequences and mediate their RNA modification and export, including a role directing the m^6A methyltransferase complex to specific sites on the *XIST* lncRNA (Patil et al. 2016), raising the possibility that RBM15/B may also recognize specific USER codes in endogenous metazoan mRNAs to mediate their metabolism and/or export. Moreover, hnRNP L, a multifunctional RNA-binding protein, was found to interact specifically with an RNA element in herpesvirus TK mRNA and promote the export of this mRNA (Liu and Mertz 1995) suggesting that such an action on endogenous mRNAs remains a possibility. Lastly, a more direct coupling of 3′-end processing of

mRNA to mRNA export has been suggested by observation that CFIm68/CPSF6, a component of the CFI complex, is able to shuttle nucleocytoplasmically in a transcription-dependent fashion, bind to NXF1, and cause nuclear accumulation of polyadenylated mRNA upon its depletion (Ruepp et al. 2009). CFIm68 possesses mRNA-binding activity (Dettwiler et al. 2004) and is able to interact directly with THOC5 to regulate alternative polyadenylation (Katahira et al. 2013), raising the possibility that CFIm68 may act as an ortholog of Aly/REF on yet-to-be-identified mRNA targets.

2.4.5 *The AU-Rich Element as a mRNA Export USER Code*

AU-rich elements (AREs) are among the best described of all *cis*-acting mRNA elements. They are bound by a range of different *trans*-acting RBPs, including HuR, TTP, and AUF1 among others, and these *trans*-acting factors control the fate of the mRNA throughout the cell, including at the levels of mRNA processing, stability, translation, subcellular localization, and mRNA export (Garcia-Mauriño et al. 2017; see Fig. 2.2d).

The ARE-binding protein HuR/ELAVL1 is known to stabilize bound mRNAs and promote their translation in the cytoplasm (Wu et al. 2018). In the nucleus, HuR bound to AREs interacts mutually exclusively with two accessory proteins, APRIL/TNFSF13 and pp32/ANP32A, both of which contain leucine-rich NESs and can shuttle nucleocytoplasmically in a manner dependent of the export receptor CRM1 (see Sect. 2.6). Importantly, inhibition of pp32 and/or APRIL shuttling using the CRM1 inhibitor leptomycin B (LMB) results in the accumulation of HuR:pp32, HuR:APRIL, and HuR:RNA complexes in the nucleus, suggesting that APRIL/pp32 are shuttled in the context of an assembled ARE-RNA:HuR:APRIL/pp32 complex (Brennan et al. 2000). While treatment with LMB induced no visible change in total nuclear polyadenylated RNA levels assayed by oligo(dT) FISH, testing of candidate ARE-containing mRNAs such as *c-fos*, *COX-2*, and *tp53* revealed that HuR-mediated shuttling by CRM1 was necessary for the efficient export of these mRNAs (Brennan et al. 2000; Dixon 2004; Jang et al. 2003; Nakamura et al. 2011). While the ubiquity of this export pathway on ARE-containing mRNAs is yet to be examined, the number of ARE-containing targets of HuR, and their myriad roles in the regulation of cellular growth, immune regulation, and cellular mobility, suggests that HuR-mediated mRNA export may represent an important regulator of cellular growth and oncogenesis (Garcia-Mauriño et al. 2017; Wu et al. 2018).

It should be noted that the ARE may not be the only USER code that can target mRNAs for export via HuR. The immune-regulatory *CD83* mRNA has been reported to undergo CRM1-mediated nuclear export in a manner dependent on the presence of HuR and APRIL (Fries et al. 2007; Prechtel et al. 2006). However, in this mRNA the element that recruits HuR was found not to be a 3′-UTR ARE but instead a novel sequence element, termed the posttranscriptional regulatory element (PRE), within the mRNA coding region (Prechtel et al. 2006). Similarly, while the

export of the ARE-containing *IFNα1* mRNA was found to be dependent on CRM1, the ARE was found to be dispensable for this export, suggesting an alternative USER code (Kimura et al. 2004). It is noted that this latter paper did not test whether *IFNα1* mRNA export was dependent on HuR, and that it is not clear how, if at all, this CRM1-dependent export pathway overlaps or interacts with the Prp19:U2AF65-NXF1-dependent export pathway for *IFNα1* discussed above (Sect. 2.4.1.2).

2.5 Posttranslational Formation of USER Codes by mRNA Modification

The modification of mRNAs in order to alter their biochemical and functional properties has been known about since the early days of research on mRNAs. Indeed, several mRNA modifications—the m^7G cap, the poly(A) tail, and the splicing out of introns—are so well established and ubiquitous that they are intricately woven into the cell's definition of functional, translatable mRNAs, and loss of any one of these modifications has drastic effects on the functionality of almost all mRNAs (Chan et al. 2011; Ramanathan et al. 2016; Will and Lührmann 2011). However, these canonical modifications are far from being the only ones that may occur on mRNA; indeed, over 100 nucleotide and/or base modifications have now been identified from cellular mRNA, exhibiting a wide range of frequencies, specificities, and biochemical and/or functional consequences (Boccaletto et al. 2018). Furthermore, some of these modifications are both regulatable and reversible, leading to the concept of "epitranscriptomics" in which a code of mRNA modifications is able to direct the posttranscriptional fate of mRNAs in a manner analogous to epigenetic marks controlling regulation of a gene (Eckmann et al. 2011; Mauer et al. 2016; Trotman and Schoenberg 2019).

While early research on mRNA modifications was limited by the low-resolution and/or piecemeal experimental techniques available, a recent explosion of next-generation sequencing-based techniques has allowed the mapping of particular RNA modifications with unprecedented detail and sensitivity (Li et al. 2016). In so doing, these techniques have revealed a plethora of insights into how mRNA modifications influence numerous posttranscriptional pathways of mRNA regulation, including mRNA export. Specifically, several mRNA modifications have been found to be specifically bound by "reader" proteins that subsequently recruit the mRNA export machinery to direct export of these mRNAs (Roundtree et al. 2017; Yang et al. 2017), suggesting a new regulatory paradigm of "inducible USER codes"—the use of mRNA modifications to posttranscriptionally mark a subset of mRNAs with a *cis*-acting sequence/structural element and thereby allow mRNA export (or other posttranscriptional metabolic processes; see Fig. 2.2c).

It should be noted that mRNA adenosine-inosine deamination editing has been extensively reported to regulate the retention of mRNAs carrying extended double-stranded regions in the subnuclear paraspeckle domains (Chen and Carmichael

2009; Chen et al. 2008; Fox and Lamond 2010; Fox et al. 2018; Prasanth et al. 2005). However, an extended discussion of the mechanics of mRNA nuclear retention is beyond the scope of this chapter, and interested readers are encouraged to consult several excellent recent reviews on the topic (Fox et al. 2018; Palazzo and Lee 2018; Wegener and Müller-McNicoll 2018).

2.5.1 N^6-Methyladenosine in the Regulation of mRNA Export

The addition of a methyl group to the 6-nitrogen (N^6) of adenosine bases in RNA to generate N^6-methyladenose (m^6A) is the most common mRNA modification in human cells outside of the canonical m^7G cap, poly(A) tail, and splicing. The development of sequencing methodologies capable of specifically mapping this methylation event revealed its presence on approximately 30% of the human transcriptome, with a specific deposition on [A/G][A/G]A∗C[A/C/U] consensus motifs predominantly, though not exclusively, found in RNA 3′-UTRs and within long internal exons (Dominissini et al. 2012; Harper et al. 1990; Meyer et al. 2012). This modification is deposited by the m^6A methyltransferase/"writer" complex containing a catalytic heterodimer (METTL3/METTL14), several scaffolding/accessory proteins (ZC3H13, WTAP, and KIAA1429), and, at least in some cases, the RNA-binding proteins RBM15/RBM15B (Duan et al. 2019; Lesbirel and Wilson 2019) and can be removed by one of the two m^6A demethylases/"erasers," FTO (which recognizes cap-proximal m^6A) and ALKBH5 (which acts on the gene body; Hess et al. 2013; Jia et al. 2011; Ke et al. 2017; Mauer et al. 2016; Zheng et al. 2013). The final components of the m^6A regulatory system are the "readers," a set of nuclear (hnRNP A2B1, YTHDC1, YTHDC2, and, indirectly, hnRNP C) and cytoplasmic (YTHDF1,2,3 and eIF3) m^6A-binding proteins that promote different downstream metabolic processes such as splicing, mRNA destabilization, and mRNA translation, as well as the mRNA export discussed herein (Meyer and Jaffrey 2017).

Several early pieces of evidence pointed to a possible role for the regulation of mRNA export by m^6A, including the findings that inhibition of total mRNA methylation reduced mRNA export (Camper et al. 1984). Furthermore, knockdown of the m^6A methyltransferase factor METTL3 slowed the circadian clock due to reduced export of circadian clock-related mRNAs (Fustin et al. 2013), while knockdown of the m^6A "eraser" ALKBH5 caused increased translocation of poly(A)$^+$ RNA to the cytoplasm, suggesting an enhancement of mRNA export activity (Zheng et al. 2013). However, the molecular mechanism by which m^6A regulates mRNA export was not delineated until 2017, when detailed work by Roundtree et al. (2017) demonstrated that, in the nucleus, m^6A sites in mRNA were bound by the nuclear "reader" protein YTHDC1, and that m^6A-bound YTHDC1 is able to recruit the known USER code-interacting mRNA export adaptor SRSF3. SRSF3 loaded onto YTHDC1 subsequently recruits NXF1 to promote export of m^6A-modified mRNAs, though YTHDC1 and NXF1 do not themselves interact; the loss of any one of these

elements was sufficient to inhibit mRNA export of candidate mRNA targets. A combination of sequencing-based RNA-protein interaction mapping techniques was used to identify greater than 700 endogenous mRNAs whose export may be regulated by the YTHDC1-SRSF3-NXF1 pathway, suggesting the existence of a large mRNA regulon targeted by these proteins (Roundtree et al. 2017). Independent work has additionally reported direct interactions between components of the TREX complex and the catalytic METTL3/METTL14 subunits of the m^6A methyltransferase complex (Lesbirel et al. 2018), suggesting that the closely juxtaposed association of TREX and SRSF3:YTHDC1 with mRNA may enhance m^6A-dependent mRNA export; however, the relative contributions and mechanism of cooperation, if any, between these two pathways have yet to be tested.

Interestingly, while the above observations deal with the deposition of m^6A within the mRNA body, a functionally discrete pathway has evolved to regulate mRNAs that initiate transcription with an AMP residue, placing AMP at the N1 position immediately 3′ to the m^7GpppN cap (m^7GpppA). These N1-AMP residues undergo opposed N^6-methylation and demethylation by their own dedicated factors, namely the methyltransferase CAPAM/PCIF1 and the demethylase FTO (Akichika et al. 2019; Boulias et al. 2018; Cowling 2019; Hess et al. 2013; Jia et al. 2011; Mauer et al. 2016; Sendinc et al. 2018; Sun et al. 2019). Since the first two nucleotides 3′ to the cap in almost all mammalian mRNA undergo ribose 2′-OH methylation by CMTR1 and CMTR2, respectively, these modifications result in the establishment of N^6, 2′-*O*-dimethylated AMP (m^6A_m) at the N1 position of a large number of cellular mRNAs (Linder et al. 2015). The predominant biological consequence of m^6A_m formation at the N1 nucleotide is the inhibition of decapping enzyme Dcp2 binding to the m^7G cap, resulting in stabilization of these mRNAs (Mauer et al. 2016). However, early biochemical studies of the canonical CBC found that, while m^7GpppA caps bound CBC approximately 50% less well than m^7GpppG, the addition of an N^6-methyl group on the N1 nucleotide (m^7Gpppm^6A) restored CBC binding to a level comparable to that of m^7GpppG (Worch et al. 2005). While a role for m^6A_m-dependent modulation of CBC-binding efficiency in mRNA export remains purely theoretical at this point, the central role that CBC binding to the m^7GpppN cap plays in the initiation and control of mRNA export (Cheng et al. 2006; Ohno et al. 2002; Shi et al. 2017), as well as the considerable variation in m^6A_m deposition between tissues and transcripts (Kruse et al. 2011), makes this an intriguing hypothesis worthy of further testing.

2.5.2 N^5-Methylcytosine as a Possible Regulator of Aly/REF-Dependent Recruitment to mRNA

Like m^6A, N^5-methylcytosine (m^5C) is a highly abundant RNA modification that has recently been implicated in mRNA export regulation. While m^5C was initially identified only in highly stable ncRNAs such as tRNAs and rRNAs (García-Vílchez

et al. 2019), the development of a range of m^5C-focused sequencing strategies including bisulfite sequencing have allowed the identification of m^5C in mRNA (Schaefer et al. 2009). Aza-IP-Seq (Khoddami and Cairns 2013) and miCLIP (methylation iCLIP) (Hussain et al. 2013) have revealed a distribution of m^5C throughout mRNAs, with a potential enrichment in C/G-rich stretches in the vicinity of the ATG start codon (Amort et al. 2017; Yang et al. 2017). These analyses have also uncovered extensive variation of m^5C site choice and saturation across tissues and developmental stages (Yang et al. 2017). The deposition of m^5C in mRNA is predominantly mediated by the methyltransferase NSUN2, although other NSUN family members are also likely to act on mRNA (García-Vílchez et al. 2019; Yang et al. 2017). However, while m^5C in the context of mRNA has been posited to regulate mRNA stability and/or translation, little hard evidence of a functional role has been forthcoming (García-Vílchez et al. 2019).

Recently, Yang et al. (2017) have reported a potential role for posttranscriptionally deposited m^5C in the regulation of mRNA export. They used a comparative IP-MS protocol with a short RNA probe with or without CMP N^5-methylation to unexpectedly identify Aly/REF as a reader protein for m^5C both in vitro and in vivo. Supporting a role for this interaction in a putative m^5C:Aly-dependent mRNA export pathway, the authors reported that depletion of the m^5C methyltransferase NSUN2 resulted in decreased Aly/REF binding to candidate m^5C target mRNAs, an accumulation of Aly/REF in splicing speckles, and reduced trafficking to the cytoplasm and an accumulation of polyadenylated RNA in the nucleus (Yang et al. 2017). It should be noted that this study omitted several important supporting experiments, including testing for a requirement for NXF1:NXT1 for m^5C-driven mRNA export; also, other groups have disputed the finding that Aly/REF is able to interact directly with m^5C (Lesbirel and Wilson 2019). However, while the proposal that Aly/REF is able to bind directly to m^5C in mRNA is markedly different from the paradigm of reader proteins recruiting downstream mRNA export factors established for m^6A by Roundtree et al. (2017), an earlier observation that Aly/REF is able to modify its RNA-binding specificity in response to binding of intracellular signaling molecules (Wickramasinghe et al. 2013) suggests that the implementation of a specific binding mode of Aly/REF on m^5C is not beyond the realms of possibility.

2.6 Regulation of mRNA Export by the Protein Export Receptor CRM1

CRM1 is a member of the importin-β superfamily of nuclear transport receptors which, like other members of this family but unlike the canonical mRNA export receptor NXF1, relies on successive cycles of GTP hydrolysis by its binding partner Ran to drive its nucleocytoplasmic shuttling through the nuclear pore. CRM1 binds specifically to leucine-rich nuclear export signals (NESs) in proteins to promote their

export from the nucleus; indeed, CRM1:RanGTP is the dominant receptor for leucine-rich NES export from the nucleus (Fornerod et al. 1997; Hutten and Kehlenbach 2007).

In addition to its role in the export of proteins from the nucleus, CRM1:RanGTP is required for the export of a number of highly structured endogenous ncRNAs from the nucleus, including U snRNAs and maturing ribosomal subunits. In order to achieve export of these RNAs, CRM1LRanGTP relies on a mechanism reminiscent of that of NXF1:NXT1 in which RBP adaptor(s) bind directly to the target RNA, then recruit CRM1:RanGTP via one or more canonical leucine-rich NESs to assemble an export-competent RNA:adaptor:receptor complex (Nerurkar et al. 2015; Okamura et al. 2015). Also like NXF1:NXT1, CRM1:RanGTP has been repurposed by viral proteins and/or *cis*-acting sequences to promote export of unspliced mRNAs; the most well-characterized example of this is the export of HIV-1 RNAs in response to the NES-containing viral protein Rev binding to the *cis*-acting Rev response element (RRE) in the viral RNA (Suhasini and Reddy 2009).

Early studies in *S. cerevisiae* and *Xenopus* oocytes concluded that CRM1 was not required for the export of mRNA in eukaryotes (Fischer et al. 1994; Jarmolowski et al. 1994; Neville and Rosbash 1999). However, a range of subsequent studies have refined this view, finding that, while CRM1:RanGTP is indeed dispensable for the constitutive export of bulk mRNA, it is capable of mediating the export of several functional mRNA regulons through the action of discrete mRNA USER codes and *trans*-acting export adaptors (see Fig. 2.2d). Export of ARE-containing mRNAs via HuR:CRM1:RanGTP has been discussed in Sect. 2.4.5.

2.6.1 A Nuclear Role for the Cytoplasmic Cap-Binding Protein eIF4E

eIF4E is an m^7G cap-binding protein that, in complex with the helicase eIF4A and the scaffolding protein eIF4G, assembles the heterotrimeric complex eIF4F. eIF4F is a key activator of translation in the cytoplasm, coordinating the loading of the 40S ribosomal subunit onto mRNAs (Pelletier et al. 2015). Given the importance of this role, it is not surprising that cytoplasmic eIF4E is a major regulatory node in the coordination of mRNA translation and/or metabolism in the cytoplasm and is an important oncogene in numerous cancers (Ho and Lee 2016; Pelletier et al. 2015). While eIF4A and eIF4G are predominantly cytoplasmic proteins, a significant portion of cellular eIF4E localizes to the nucleus, where it is additionally able to regulate nuclear mRNA dynamics, and in particular mRNA export, by a mechanism independent of its cytoplasmic translation-regulation activity (Osborne and Borden 2015; Rousseau et al. 1996). eIF4E's roles in mRNA export regulation contribute to its function as an oncogene, emphasizing the pathological importance of the eIF4E-mediated mRNA export pathway (Osborne and Borden 2015; Pelletier et al. 2015).

mRNA export via eIF4E was found to require two key *cis*-acting elements: the RNA m^7G cap and a structurally but not sequence-conserved USER code within the

3′-UTR of target mRNAs, termed the 4E-sensitivity element (4E-SE) (Culjkovic et al. 2005, 2006). IP-MS analyses have revealed that, while eIF4E predominantly binds the cap structure on its target mRNAs, the 4E-SE is bound by an accessory protein, LRPPRC, which binds mRNA cooperatively with eIF4E and promotes stability of the ternary RNA:eIF4E:LRPPRC complex (Topisirovic et al. 2009). Unlike in the NXF1:NXT1 export pathway, eIF4E:LRPPRC-bound mRNAs are not recruited to the splicing speckles; instead, they accumulate in discrete foci throughout the nuclei enriched in eIF4E, termed "eIF4E granules," which exhibit partial overlap with PML bodies (discussed below; Cohen et al. 2001; Culjkovic et al. 2005; Topisirovic et al. 2002). The export receptor complex CRM1:RanGTP is recruited to the ternary RNA:eIF4E:LRPPRC complex via prototypical leucine-rich NES elements within LRPPRC and mediates translocation of the cargo mRNA through the nuclear pore via an as-yet poorly characterized mechanism. This mechanism culminates in the binding of CRM1:RanGTP to the cytoplasmic fibril protein Nup358/RanBP2 which, with RanGAP, promotes release of cargo mRNA through RanGTP→RanGDP hydrolysis (Culjkovic et al. 2006; Hutten and Kehlenbach 2007; Volpon et al. 2017).

The first identified mRNA target of the eIF4E-dependent export pathways was the key cell cycle-regulatory mRNA *CYCD1* (Rousseau et al. 1996), and it was this RNA on which many of the mechanistic dissections of the eIF4E-dependent export pathway were conducted. However, a subsequent RNA immunoprecipitation (RIP)-differential display study was able to identify hundreds of mRNAs that interacted with eIF4E within the nucleus and therefore represent candidate mRNA export cargoes of eIF4E:LRPPRC (Culjkovic et al. 2006). These mRNAs were markedly enriched for a range of pro-growth factors including cell cycle components and regulators of the pro-growth AKT signaling pathway, among others; the strongly proliferative nature of the mRNAs within the eiF4E regulon provides significant insight in to the mechanisms by which eIF4E-dependent mRNA export can promote oncogenesis (Culjkovic et al. 2006, 2008; Osborne and Borden 2015).

Given the highly proliferative nature of the eIF4E export regulon, it is not surprising that mechanisms have evolved to constrain and regulate its export activity in vivo. It was observed in early studies that a subset of nuclear eIF4E granules overlapped with PML bodies and that eIF4E binding to mRNAs in the nucleus specifically targeted them to eIF4E granules that lacked significant PML staining (Cohen et al. 2001; Culjkovic et al. 2005; Topisirovic et al. 2002). Consistent with this, PML was found to be a negative regulator of eIF4E-driven mRNA export, through a direct interaction with eIF4E that reduced its affinity for the m^7G cap in the nucleus (Cohen et al. 2001; Culjkovic et al. 2006). The PML-eIF4E regulatory axis allows the direct regulation of pro-growth mRNA export by environmental stimuli, as evidenced by the finding that cadmium treatment and IFNγ treatment have positive and negative effects, respectively, on the PML-eIF4E interaction and consequently on the extent of PML-dependent inhibition of eIF4E mRNA export (Topisirovic et al. 2002). Similarly, the myeloid cell line-specific homeobox transcription factor, PRH, is able to bind eIF4E and negatively influence its ability to export mRNAs (Topisirovic et al. 2003). In fact, eIF4E-binding motifs were found to

be conserved across almost 25% of the human cell's 803 homeobox proteins (Topisirovic et al. 2003), suggesting that homeobox proteins may represent a large class of eIF4E modulators; indeed several other homeobox proteins have been found to modulate eIF4E's activity, either at the level of mRNA export (HOXA9; Topisirovic et al. 2005) or translation initiation (Emx2, Otx2, and En-2; Brunet et al. 2005; Nédélec et al. 2004).

A number of questions remain to be answered regarding the mechanism of eIF4E-mediated mRNA export, not least the question of how and when the bulk mRNA cap-binding complex, CBC, is evicted to allow access of eIF4E, and how the large suite of possible eIF4E-regulatory homeobox proteins may contribute to the efficacy of this export path across different tissues and developmental stages (Osborne and Borden 2015).

2.6.2 *Teaching an Old Dog New Tricks: CRM1-Dependent Export Via NXF3*

As discussed in Sect. 2.3.4, the NXF1 family of bulk mRNA export adaptors has diversified from its single *S. cerevisiae* ancestor, Mex67, to include multiple paralogs in metazoans, including five (NXF1-5) in humans and mice (Herold et al. 2000). Of these five members, NXF4 and NXF5 possess corrupted coding sequences and are unlikely to be expressed, while NXF2 bears a close resemblance to NXF1 and appears to duplicate NXF1 function in a tissue-specific fashion, though its functionality in mRNA export has been questioned by other groups (Herold et al. 2000; Yang et al. 2001).

NXF3 represents an anomaly among the NXF1 family in that, while it is an intact ORF and is expressed in humans, albeit in a tissue-restricted fashion (GTEx Consortium 2015), it contains several truncations, including of 32 amino acids in its RNA-binding domain and the loss of its C-terminal FG-Nup-binding domain, that are expected to prevent it acting as a NXF1-like mRNA export receptor (Herold et al. 2000). Despite these observations, tethering of NXF3 to a reporter RNA in human cells (Yang et al. 2001) but not in quail cells (Herold et al. 2000) unexpectedly resulted in the robust export of the reporter mRNA; however, this export, instead of proceeding via a prototypical NXF1-like export pathway, was reliant on the binding of CRM1 to a conserved leucine-rich NES in the NXF3 sequence, suggesting that NXF3 has evolved alternative mechanisms to promote mRNA export of target mRNAs in the absence of the FG-Nup-binding capability typical of its protein family (Yang et al. 2001).

While NXF3 was reported to interact with poly(A)$^+$ mRNA in vivo, the mechanism of this binding, given that NXF3 lacks a significant portion of its RNA-binding domain, remains unclear (Yang et al. 2001). Similarly, unresolved are the identity of NXF3's mRNA export cargo(es), which await a detailed analysis with high-sensitivity cross-linking methods such as iCLIP for identification. Interestingly,

both NXF2 and NXF3 show strong tissue-specific expression in the testes (Herold et al. 2000; Yang et al. 2001) in which NXF1 expression is repressed (Zhang et al. 2007), suggesting that these two NXF1 orthologs may act together or independently to reproduce NXF1's mRNA export activities in this tissue. The recent identification of an NXF3:CRM1-mediated export pathway for snoRNAs in response to stress conditions also suggests that NXF3 may regulate multiple classes of endogenous RNAs in humans (Li et al. 2017a).

2.6.3 CRM1 Versus NXF1 in the Export of mRNAs in Metazoans

Much like the cases of bulk mRNA export orthology and NXF1-dependent USER codes discussed above, the current suite of known CRM1-mediated export pathways is likely to only scratch the surface of the total CRM1-dependent export program in metazoan cells; characterization of this complete program is likely to face many of the same challenges and require many of the same approaches, as have been described in Sect. 2.3.6. Other unresolved points raised by the pathways above are specific to the regulation of CRM1, such as the mechanism by which nuclear eIF4E mediates eviction of the canonical CBC to promote CRM1-mediated export at the likely expense of NXF1-depdendent pathways.

2.7 Roles for the NPC in mRNA Export Heterogeneity

The nuclear pore complex (NPC) is one of the largest protein assemblies in the human cell, containing 8–64 copies of more than 30 different "nucleoporins" in a precisely arranged eightfold-symmetric structure weighing in excess of 100 MDa (Beck and Hurt 2017). The pore contains several regions, including a partially ordered nuclear "basket," a central pore, and an array of cytoplasmic nucleoporins, each of which plays an essential role in the transport of proteins and RNAs between the nucleus and the cytoplasm (Beck and Hurt 2017; Oeffinger and Zenklusen 2012; Okamura et al. 2015). The NPC is the site of a complicated series of mRNP metabolic steps, including RNA quality control and remodeling on the nuclear basket, NXF1:NXT1/CRM1:RanGTP-chaperoned transit through the central pore, and release of the mRNA cargo from its export adaptors on the cytoplasmic side of the NPC (Beck and Hurt 2017; Oeffinger and Zenklusen 2012).

Given its status as the sole conduit for the nuclear export of the vast majority of cellular mRNAs, and the many and varied protein-RNA interactions that are required for an mRNA to transit, the NPC represents an ideal candidate for mRNA export heterogeneity regulation. However, the considerable technical difficulties of investigating a structure of the size and complexity of the NPC, as well as the highly

intertwined interaction networks and rapid, reversible kinetics of mRNA transit through the NPC, have limited our ability to dissect the role of the NPC in specialized and/or regulated mRNA export (Grünwald and Singer 2010; Ma et al. 2013; Saroufim et al. 2015; Shav-Tal et al. 2004; Siebrasse et al. 2012). Nevertheless, one example of Nup-dependent mRNA export heterogeneity has been reported, providing a possible precedent for NPC regulation of mRNA export heterogeneity (Fig. 2.2e).

2.7.1 NUP96 in Cell Cycle and Interferon Regulation

NUP96 is a conserved nucleoporin that is a component of the Y-complex (also termed the Nup107-160 complex) subdomain of the NPC. The Y-complex contains seven different Nups in stoichiometric ratios and is an essential factor in the assembly of functional NPCs. In mature NPCs, the Y-complex forms a double ring at both the nuclear and cytoplasmic surfaces of the central NPC channel, ideally positioning NUP96 as one of the first Nups able to come into contact with mRNPs that have left the basket to transit the central channel (Beck and Hurt 2017). Upon disassembly of the NPC during mitosis, NUP96 is also reported to play an independent role in the regulation of mitotic kinetochore dynamics (Belgareh et al. 2001; Mishra et al. 2010).

Several papers have found *NUP96* to be an essential gene in both *S. cerevisiae* and in mice, consistent with a central role in NPC assembly and transport (Dockendorff et al. 1997; Faria et al. 2006). However, the depletion rather than knockout of NUP96 in heterozygous *Nup96*$^{+/-}$ mouse models was found to have a variable effect of the export of different mRNAs, with a subset of interferon-inducible mRNAs including *MHC-I* and *-II*, *ICAM-1*, and *CD86* showing particularly robust export inhibition (Faria et al. 2006). A separate paper reported that NUP96, unlike other members of the Y-complex, undergoes rapid proteasomal degradation during mitosis, resulting in depletion of NUP96 during the following G_1 phase; this depletion was required for the timely progression of the cell cycle, with G_1-specific overexpression of NUP96 causing a severe cell cycle progression arrest (Chakraborty et al. 2008). This arrest resulted from a NUP96 overexpression-specific inhibition of the export of several cell cycle-regulatory mRNAs including *CYCD1*, *CDK6*, and *IκBα*, suggesting that, contrary to the regulation reported for IFN-regulated transcripts above, NUP96 actually represses the export of these transcripts during G_1 (Chakraborty et al. 2008; Faria et al. 2006).

These observations raise a number of significant questions regarding the mechanism by which NUP96 specifically regulates the export of these target mRNAs, including: how and why is the polarity of NUP96 action on the export of IFN-induced versus cell cycle-regulatory mRNAs reversed? If NUP96 is a core structural component of the NPC, what happens to NPC structures when NUP96 is proteasomally degraded during mitosis? Does NUP96 act within the context of the NPC, or is it a nucleoplasmically mobile Nup like the putative NXF1:NXT1

chaperone NUP98? And lastly, how does NUP96 identify the mRNAs which undergo selective mRNA export modulation?

2.7.2 *Bypassing the NPC: Nuclear Membrane Budding for the Export of Large mRNP Complexes*

A long- and firmly-held belief in the field of mRNA export was that the nuclear pore represents the exclusive port of nuclear egress of exported mRNAs, and that all mRNA export pathways, however divergent, must ultimately end with translocation of their mRNA cargoes through the nuclear pore. However, this creed has recently been challenged by a remarkable pathway recently reported in *Drosophila* muscle cells (Fig. 2.2e).

The Wnt signaling pathways are a class of related signaling cascades that regulate a diverse range of cellular processes including embryonic pattern formation, cellular differentiation, and the formation of synaptic junctions, among other functions (Wiese et al. 2018). Wnt pathway activation at *Drosophila* neuromuscular junctions involves the binding of the protein ligand Wg to the DFz2 receptor; this receptor is then internalized, and the cytoplasmic C-terminal tail (DFz2C) is released by proteolysis to govern downstream signaling pathways throughout the cytoplasm and nucleus (He et al. 2018).

Studies of the DFz2C moiety revealed that, upon Wnt activation, this signaling factor is specifically localized to the nucleus, where it assembles large granules containing numerous mRNAs and a range of mRNA-associated proteins; notably, the size of these granules—on average, 192 nm in diameter, compared to a total diameter of the NPC of ~120 nm—suggests that they are likely to be unable to transport to the cytoplasm via the NPC (Speese et al. 2012). Remarkably, super-resolution microscopy approaches combined with live-cell imaging and rapid fixation/co-staining were able to reveal an export pathway that did not require the NPC, but in which the large DFz2C granules were able to bud directly from the inner nuclear membrane into the perinuclear space. This process required phosphorylation-dependent disruption of the nuclear lamina and was followed by fusion of the nascent vesicle with the outer nuclear membrane to allow release of the contents into the cytoplasm (Speese et al. 2012). mRNAs targeted to the DFz2C granules in the nucleus were found to form a specific mRNA regulon enriched for regulators of synaptic structure and synaptogenesis, consistent with a key role for DFz2C signaling in the formation of functional neuromuscular synapses (He et al. 2018; Speese et al. 2012).

While it appears to be a unique and highly unusual pathway upon first inspection, it should be noted that large RNA-containing granules have been serendipitously observed in the perinuclear space in other organisms, ranging from plants to humans, suggesting that this may in fact represent a conserved pathway for the export of mRNPs above the size exclusion limit of the NPC (Speese et al. 2012). In addition,

this export mechanism bears remarkable resemblance to that used by human pathogenic herpesviruses and, potentially, cytomegaloviruses to export their large DNA-containing capsids from the nucleus to the cytoplasm by cell-internal capsid envelopment/de-envelopment across the nuclear membranes (Buser et al. 2007; Lee and Chen 2010). When first identified, these pathways were thought to be exclusive to the viruses and to be mediated exclusively by viral proteins; however, the observations here suggest that these pathways may instead represent the repurposing of a latent cellular export pathway for large nucleic acid-containing complexes (Buser et al. 2007; Lee and Chen 2010; Speese et al. 2012).

2.8 Outstanding Questions in mRNA Export Heterogeneity

Above we described the overlapping and complementary mechanisms of mRNA export from the nucleus to the cytoplasm. While these studies have provided considerable insights into the guiding principles of mRNA export so far, a number of important questions regarding their function still remain unanswered and will be discussed here.

2.8.1 Stoichiometry of Export Adaptors/Coadaptors on mRNA

One fundamental question is whether mRNAs might contain competing signals that drive export or retention toward a specific export pathway and whether the deposition of a single or multiple export complexes stimulates this process. This can be best illustrated by the question of how many Aly/REF molecules are actually deposited on an mRNA, and whether this number influences export. Early investigations of Aly/REF deposition on mRNA reported noncontradictory interactions between Aly/REF (in the context of TREX) and the CBC-bound 5′ m^7G cap and/or EJCs, leading to the hypothesis that Aly/REF binding (and subsequent NXF1:NXT1 loading) at these site(s) was sufficient for export (Cheng et al. 2006; Masuda et al. 2005). However, subsequent characterization of the DDX19/Gle1/Nup42 complex revealed a mechanism of nuclear pore transit in which DDX19 helicase bound to the NPC's cytoplasmic fibrils evicts the FG-Nup-binding NXF1:NXT1 complex from cargo mRNAs upon transit of the central NPC pore, acting as a "ratchet" to instill directionality to the transit of mRNPs through the NPC (Adams et al. 2017, 2018; Folkmann et al. 2011). A key requirement of this model is that NXF1:NXT1, and therefore Aly/REF, must be deposited throughout the length of the mRNA in order to allow regular interactions of the RNA:NXF1:NXT1 complex with DDX19 during NPC transit; indeed, several cross-linking-immunoprecipitation studies of Aly/REF found binding along the length of mRNA, albeit with enrichment at the 5′- and

3′-ends (Shi et al. 2017; Viphakone et al. 2018). A possible explanation for these apparently contradictory observations emerged from the observation that the core helicase of the TREX complex, UAP56, must bind ATP in order to assemble the TREX complex, and that Aly/REF binding to UAP56 promotes its ATPase activity (Dufu et al. 2010). It was further found that UAP56's ATPase activity was required for deposition of Aly/REF onto mRNA, leading to a compromise model in which TREX is recruited to 5′ caps and/or EJCs and then undergoes successive rounds of ATP hydrolysis to deposit Aly/REF along the target mRNA (Taniguchi and Ohno 2008). While this model satisfies extant observations regarding Aly/REF-binding mechanisms on mRNA, it was complicated by the finding that Aly/REF and THOC5 were coordinately required for the activation of NXF1:NXT1 RNA binding during bulk mRNA export (Viphakone et al. 2012), since under this model THOC5 was expected to comigrate with UAP56 rather than remaining in close coordination with Aly/REF on the mRNA. The model was further complicated by the discovery of numerous orthologous export adaptors/coadaptors that are able to act with and/or substitute for Aly/REF in the bulk export pathway (Chang et al. 2013; Hautbergue et al. 2009; Uranishi et al. 2009; Viphakone et al. 2015). The observation that these proteins typically bind TREX and NXF1:NXT1 in an identical manner to the Aly/REF:THOC5 suggests that they may also be deposited by UAP56, suggesting a possible heterogeneous series of adaptors/coadaptors along the length of mRNAs destined for bulk mRNA export.

Given these contradictory observations and their self-evident relevance to our understanding of the mechanisms and regulation of bulk mRNA export, it is imperative to establish the relative position, order, and stoichiometry of export adaptors and coadaptors on mRNAs in vivo. However, currently available protein-RNA-focused sequencing techniques such as iCLIP are not capable of answering this question due to their generation of population-wide averages of mRNA site binding (van Dijk et al. 2018), and alternative approaches are required to address these questions. One alternative avenue might be the use of sequential IPs of serried adaptor/coadaptor pairs followed by iCLIP analysis to demonstrate the binding patterns of a given factor when co-deposited with another. Other possible approaches could make use of third-generation sequencing technologies which allow the sequencing of entire mRNA molecules without fragmentation (van Dijk et al. 2018). Pairing such an approach with a protein proximity-dependent RNA tagging strategy such as APEX-Seq or, alternatively, fusions of specific proteins with an mRNA-modifying enzyme such as NSUN2 in order to promote the specific modification of mRNAs with diagnostic sequence modifications in the immediate vicinity of an mRNA-bound protein of interest would be particularly powerful (Padròn et al. 2018). Finally, imaging-based approaches that allow the determination of stoichiometry of proteins within complexes using stepwise photobleaching of fluorescently labeled components upon purification and immobilization onto microscopy coverslips might be useful in complementing sequencing-based methods (Jain et al. 2011). Such future investigations will be required to define the binding patterns of export factors on candidate mRNAs and thereby provide new insides into how the stoichiometry of adaptors and coadaptors may coordinate mRNA export.

2.8.2 *Posttranslational Regulation of mRNA Export Pathways in Response to Cellular Stimuli*

In addition to the already formidable complexity of mRNA export engendered by the variety of pathways thus far reported, evidence is emerging that mRNA export pathways are highly sensitive to environmental, tissue-specific, and developmental cues and that their activity can be altered by posttranslational signaling. Early reports regarding PML-mediated suppression of eIF4E:CRM1:RanGTP-mediated export found that this regulatory axis could be manipulated by exposure of cells either to the stressor cadmium or to the signaling molecule IFNγ (Topisirovic et al. 2002), while NXF1:NXT1-dependent export of the *HSP70* mRNA appears to gain an absolute requirement for Aly/REF and THOC5 upon transition from constitutive to heat shock conditions (Guria et al. 2011; Katahira et al. 2009). In addition to its role as a bulk mRNA export coadaptor, THOC5 has been shown to play specific roles in hematopoiesis and adipogenesis in mice (Mancini et al. 2006, 2010). Most strikingly, numerous export factors, including LuzP4, URH49, CIP29, NXF2, and NXF3, all exhibit highly restricted expression in the testes, suggesting the possible existence of an entirely distinct and parallel mRNA export pathway in this tissue (Kapadia et al. 2006; Pryor et al. 2004; Viphakone et al. 2015; Yang et al. 2001; Zhang et al. 2007). The regulation of mRNA export by posttranslational factors is exemplified by the IPMK:Aly/REF export pathway in which the secondary messenger PIP_3 binds to Aly/REF and modulates its mRNA-binding motif (Okada et al. 2008; Wickramasinghe et al. 2013). Interestingly, the binding of PIP_3 to Aly/REF can itself be modulated by AKT-mediated phosphorylation of Aly/REF, suggesting this mechanism may represent a regulatory hub for several signaling pathways (Wickramasinghe et al. 2013). Prior to its identification as an mRNA export factor, THOC5 (a.k.a. FMIP) was found to be phosphorylated by the GM-CSF and PKC signaling pathways, resulting in its partitioning to the cytoplasm (Mancini et al. 2004; Tamura et al. 1999); similarly, the HuR cofactor APRIL can be phosphorylated at a site adjacent to its nuclear localization signal, resulting in accumulation in the cytoplasm (Fries et al. 2007). Lastly, it has been found both SRSF1 and SRSF3 must be in a hypophosphorylated state to participate in their mRNA export activities, possibly as a means of isolating these activities from their other roles in splicing regulation (Lai and Tarn 2004; Roundtree et al. 2017). Collectively, these results underline the highly regulatable activities of the mRNA export machinery. Detailed investigation of the cellular- and tissue-specific expression patterns and PTM-dependent regulatory pathways of mRNA export will require a detailed and interdisciplinary approach; however, it is noted that recent large-scale collaborative projects cataloging proteome-wide cell- and tissue-specific expression profiles represent important first ports of call for investigation of novel or established export factors (GTEx Consortium 2015; Uhlén et al. 2015) while new highly sensitive mass spectrometry-based methods for the profiling of posttranslational modifications are emerging on a regular basis, providing a powerful experimental tool to dissect regulatory mechanisms contacting the mRNA export machinery (Ke et al. 2016).

2.8.3 Cooperation or Competition of Export Pathways on Common mRNAs

Given the remarkable diversity and ubiquity of specific mRNA export pathways described herein, it is almost inevitable that these pathways may intersect on particular mRNAs. Understanding how these different pathways may coordinately or competitively regulate export of a common mRNA target will provide key insights into our understanding of the complex network of mRNA export pathways described above. While a thorough description of the extent and significance of export pathway overlap must await exhaustive characterization of the mRNA targets of all known mRNA export pathways, several instances of pathway interface have already been reported serendipitously. A key feature of the eIF4E:CRM1-mediated export pathway is the binding of eIF4E to the 5′ m^7G cap in concert with LRPPRC; however, this interaction necessitates the eviction of the canonical CBC from the 5′-end of mRNA and its replacement by eIF4E via an as-yet undescribed mechanism (Culjkovic et al. 2006). Given the significant though nonessential role that CBC plays in the promotion of bulk export, it seems likely that partitioning of mRNA into the eIF4E export pathway is likely to abolish or attenuate bulk mRNA export (Cheng et al. 2006). Two different mRNA export pathways have also been found to target the *IFNα1* mRNA which can undergo both Prp19 complex/U2AF65-mediated export via a coding-region USER code, the CAR-E, and can also be exported via an incompletely described pathway dependent on NUP96 (Faria et al. 2006; Lei et al. 2011, 2013). More generally, several distinct mRNA regulons have been reported to contain highly similar functional groups of mRNAs, including the observation that cell cycle-regulatory factors are enriched in the regulons of IPMK:Aly/REF, eIF4E:CRM1, and NUP96-dependent mRNA export pathways (Chakraborty et al. 2008; Culjkovic et al. 2006; Wickramasinghe et al. 2013). Finally, the observation that eIF4E is able to promote the export and expression of key components of the AKT signaling pathway, a key regulator of Aly/REF binding to PIP_3 in the IPMK:Aly/REF export pathway, suggests that distinct export pathways may utilize cellular signaling pathways to balance their activities in vivo (Culjkovic et al. 2008; Okada et al. 2008; Wickramasinghe et al. 2013).

The first and most fundamental step in characterizing cross-talk between mRNA export pathways will be to define the complete set of mRNA targets of all known export pathways; while candidate-specific XL-Seq methodologies such as iCLIP will likely prove useful for this, a major resource is likely to be the database of iCLIP profiles for >300 RBPs generated by the ENCODE consortium (Van Nostrand et al. 2018). Once a common mRNA target of two export pathways is identified, a suite of exploratory techniques will likely be required to define the protein interactome of these mRNAs (Castello et al. 2012; Ramanathan et al. 2018), their behavior within the nucleoplasm and at the NPC, and the relative contributions of the two or more export pathways to the mRNA's export kinetics in living cells (Grünwald and Singer 2010; Heinrich et al. 2017; Ma et al. 2013; Siebrasse et al. 2012). Given the likely number and complexity of interactions between export pathways in metazoan cells,

it is expected that new, network-level experimental techniques will be required to dissect the complex interplay of these pathways.

2.9 Concluding Remarks

Historically, research into the mechanisms of protein expression heterogeneity have focused on transcriptional and translational regulation, control of splicing, and the means of mRNA degradation in the cytoplasm. However, mRNA export is far from being a passive player in this process and is itself a significant source of heterogeneity in the gene expression program and an important regulatory hub. As further details of mRNA export pathways emerge, it is expected that they will provide new insights into the means by which cells regulate their expression program and identify new possible therapeutic angles by which mRNA export may contribute to the pathogenesis and/or treatment of human disease.

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