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SRAG Functions as a New mRNA Export Co-adaptor

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by

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Abstract

DEAD box RNA helicases play important roles in many cellular processes including splicing, mRNA export and translation. The two domains of many DEAD box RNA helicases adopt radically different conformations according to whether they are nucleotide free, ADP or ATP bound. This change in conformation is often harnessed to drive subunit rearrangements in multiprotein complexes. Assembly of the TREX complex which plays a role in mRNA export requires a DEAD box helicase, UAP56 to bind ATP. Here we show that a novel mRNA export co-adaptor, SRAG, binds UAP56 in a mutually exclusive manner with REF, yet both REF and SRAG are found in a fully assembled TREX complex. Interestingly, REF and SRAG stimulate ATP hydrolysis and RNA helicase activity. This implies that UAP56 goes through at least two rounds of ATP hydrolysis to assemble TREX. Within assembled TREX, SRAG functions as an mRNA export co-adaptor and binds synergistically with REF to the TAP mRNA export factor, whose recruitment to TREX triggers UAP56 loss. Depletion of REF or SRAG alone *in vivo* has a modest effect on mRNA export, but their combined knockdown causes a drastic mRNA export block. Interestingly, the TAP:SRAG interaction is dependent on methylation of SRAG. SRAG binds to TAP in a manner which is mutually exclusive with the TREX component THOC5 and yet TAP, SRAG and THOC5 are found in a single complex in vivo. These data indicate that TREX undergoes substantial rearrangements during its assembly and interaction with TAP, and these rearrangements are driven by UAP56 dependent ATP hydrolysis.

Abbreviations

aa	amino acid
Ad5	adenovirus 5
APS	ammonium persulfate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CAT	chloramphenicol acetyl transferase
CSK	cytoskeletal
dCTP	deoxy-cytidine triPhosphate
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DMEM	Dulbecco's modified Eagles' medium
DMSO	dimethyl sulfoxide
DNA	deoxynucleic acid
DNA-AD	DNA-activator domain
DNA-BD	DNA-binding domain
DTT	dithiotreitol
E. coli	*Escherichia coli*
E1B-AP5	early 1B associated protein 5
EDTA	ethylendiaminetetraacetic acid
EJC	exon junction complex
Env	envelope glyco-protein

F.I.S.H	fluorescence *in situ* hybridization
FRAP	fluorescence recovery after photobleaching
g	gram
GAP	guanine activator protein
GDP	guanosine diphosphate
GEF	guanine exchange factor
GFP	green fluorescent protein
GST	gluthathione-S-transferase
GTP	guanosine triphosphate
HIV	human immunodeficiency virus
hnRNP	heterogeneous ribonucleoprotein
HRP	Horse Radish Peroxidase
IP	immuno-precipitation
IPTG	isopropyl-β-D-thiogalactopyranoside
L	liter
MBq	megabecquerel
mg	milligram
min	minute
miRNA	micro RNA
ml	milliliter
mM	millimolar
mRNA	messenger RNA
NBD	nucleotide binding domain
NES	nuclear export signal
ng	nanogram

NLS	nuclear localisation signal
nm	nanometres
NPC	nuclear pore complex
nt	nucleotides
NTF2	nuclear transport factor 2
NTP	nucleoside 5’ triphosphate
Nups	nucleoporins
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonylfluoride
Pol.	polymerase
RNA	ribonucleic acid
RNAi	RNA interference
RNPs	ribonucleoprotein complexes
ROI	region of interest
rpm	revolutions per minute
RT	room temperature
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	seconds
siRNA	small interference RNA
SSC	sodium saline citrate

TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	thin layer chromatography
TRIS	tris (hydroxymethyl)-aminomethane
UTR	untranslated region
UV	ultra-violet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
TB	terrific broth
μg	microgram
μL	microliter

Chapter I

Introduction

The Central Dogma of molecular biology states that DNA is transcribed to RNA, and then translated to protein. In prokaryotic cells, transcription and translation occur in the same cellular space. Unlike prokaryotic cells, eukaryotic cells have a nuclear envelope (NE) which separates transcription and translation. While small molecules and ions may cross the membranes through small pores or ion pumps, macromolecules such as proteins and RNAs are transported via the channel of the nuclear pore complex (NPC) by specific receptors.

1.1 The Nuclear Pore Complex (NPC)

In interphase eukaryotic cells, the double-membraned nuclear envelope (NE) separates the cytoplasm from the cell nucleus. The nucleus and cytoplasm can exchange materials via the nuclear envelope channels called nuclear pore complexes (NPCs) that form aqueous channels inserted in the nuclear membrane (Rabut et al., 2004; Vasu and Forbes, 2001). This ~40nm channel is composed of 8 to 30 copies of ~30 different nucleoporins (Nups) spanning the nuclear envelope in yeast (~50 in vertebrates) (Cronshaw et al., 2002; Fahrenkrog et al., 2001; Rout and Aitchison, 2001; Rout et al., 2000).

1.1.1 The structure of NPCs

In structural terms the Nups can be divided into three groups: The pore membrane proteins which anchor the NPC in the nuclear envelope; The FG (Phenylalanine-glycine) nucleoporins have FG, GLFG or FXFG amino acid repeats (where X is any amino acid),

which interact with transport receptors (e.g. importins, exportins, and TAP) directly (Bednenko et al., 2003; Fabre and Hurt, 1997; Stewart et al., 2001; Tran and Wente, 2006). The third class of Nups supports the structure of the NPC. The central pore is able to dilate to ~40 nm in order to allow molecule passage (Kiseleva et al., 1998).

Small molecules, which like ions, small metabolites and proteins under 40 kDa, can diffuse freely through NPCs (Fried and Kutay, 2003). However, the trafficking of macromolecules (proteins, RNAs, RNPs) is transported through the pore requires binding to specific transport factors (importins and exportins), which can recognize the target signals (nuclear localisation signal (NLS) or nuclear export signal (NES)) and transport cargoes through the NPC (Nakielny and Dreyfuss, 1997; Nakielny and Dreyfuss, 1999).

1.1.2 Cargo translocation

Although it is still largely unknown how translocation of cargo complexes through the NPC occurs, there is emerging evidence that interactions between FG nucleoporins and transport receptors are involved (Rexach and Blobel, 1995; Shah et al., 1998). Crystal structures have been obtained of FG-repeat peptides in complex with karyopherin, the mRNA export receptor TAP/NXF1, and NTF2, which is the important receptor for RanGDP (Bayliss et al., 2002a).

FG repeats are often embedded in larger repeat motifs, such as FXFG repeats and GLFG repeats. Both of them bind to transport receptors (Bayliss et al., 2002b; Grant et al., 2003). However, the FXFG-repeat nucleoporins are exclusively located on the nuclear side of the NPC, whereas the GLFG-repeat nucleoporins have been found on both sides of the NPC (Bayliss et al., 2002b). Based on the types of interactions between transport receptors and FG nucleoporins, different translocation models through the NPC have been proposed.

The affinity-gradient model

The affinity-gradient model predicts that cargo–receptor complexes bind to FG nucleoporins, and then pass through the NPC driven by Brownian motion (Ben-Efraim and Gerace, 2001). However, high-affinity interactions would slow down cargoes through the NPC and perturb the recycling of the receptors. To address this question, kinetic studies have shown that high affinity interactions between transport receptors and nucleoporins are not required for efficient NPC translocation, and that the dissociation rates for receptor–nucleoporin interactions are high, which indicates that nucleoporins are forming an attractive channel more than a proper binding interface to trigger cargos move rapidly through the NPC (Bayliss et al., 1999; Delphin et al., 1997; Grant et al., 2002).

The Brownian affinity-gating model

The Brownian affinity-gating model is based on the diffusion of transport complexes through the NPC. Cargo that is targeted to the NPC through a transport receptor has a higher probability of entering and traversing the central pore than cargo that is not targeted to the NPC. The Brownian affinity-gating model therefore does not require a physical barrier that controls and regulates the translocation of cargo through the central pore (Rout et al., 2000).

The selective phase model

By contrast, the selective-phase model assumes that the FG nucleoporins that line the central pore attract each other through weak hydrophobic interactions and therefore form a meshwork that functions as a physical barrier and only allows the translocation of cargo that can interact with the transport receptors, which are able to associate with the FG repeats (Jaggi et al., 2003; Ribbeck and Gorlich, 2001; Shulga and Goldfarb, 2003).

There are three major classes of transport proteins to facilitate all transport through NPC. They are the importin β-like/exportin transport factors, the small nuclear transport factor (NTF2), and the transporter involved in the export of mRNA molecules TAP. These proteins can be separated into different categories determined by the identity of the cargo they recognise and the directionality of the transport being carried out. Interestingly, even though all three classes carry out the same function they share very little sequence homology (Weis, 2003).

The direction of protein transport through the NPC is generally signal dependent. Proteins to be imported into the nucleus carry defined sequence motifs referred to as nuclear localisation sequences (NLSs). There are two major NLS motifs: a rather basic monopartite motif and a more complicated bipartite motif. The monopartite sequence was originally discovered in simian virus 40 (SV40) large-T antigen and consists of the short peptide sequence PKKKRK (Macara, 2001). In contrast, the sequence signals which promote nuclear export tend to be short motifs (~10 amino acids) with no defined consensus sequence. The nuclear export signals (NESs) are rich in hydrophobic residues and were first found in the cellular protein kinase inhibitor (PKI) and the HIV protein Rev (Hope, 1997). Both NLS and NES act to affect the cellular localisation of the proteins which carry them, by serving as recognition sites for transporter proteins called karyopherins, or exportins.

With the exception of mRNA, cargoes that contain targeting signals are recognized by members of the karyopherin family of transport receptors (also named importins and exportins) (Gorlich and Kutay, 1999; Moore and Blobel, 1995). Besides their ability to bind their respective cargoes as well as the FG-repeats of nucleoporins, the karyopherins are characterized by a conserved N-terminal domain, which interacts with the small GTPase, Ran, to direct nucleo-cytoplasmic transport (Moore and Blobel, 1993). Ran can be considered as a molecular switch which is able to exist in two distinct forms: a GTP bound nuclear form and a GDP-bound cytoplasmic form. These two types of Ran become apparent when the processes of nuclear import and export are examined.

1.2 Ran-dependent Nuclear Export of RNAs

Ran, which is a GTPase and a member of the Ras superfamily, exists in either a GDP or a GTP-bound form to regulate transport factors. When Ran is in the nucleoplasm, the Ran guanine nucleotide exchange factor (RanGEF), which associates with histones H2A and H2B, can convert Ran into the GTP-bound form (Nemergut et al., 2001). In contrast, the Ran GTPase activating protein (RanGAP) is located on the cytoplasmic face of the NPC (Hopper et al., 1990; Mahajan et al., 1998; Matunis et al., 1998). Similarly, the RanGAP activation co-factors, RanBP1 and RanBP2, are located in the cytoplasm, which results in Ran being predominantly in the GDP-bound state in the cytoplasm. (Coutavas et al., 1993; Wu et al., 1995; Yokoyama et al., 1995).

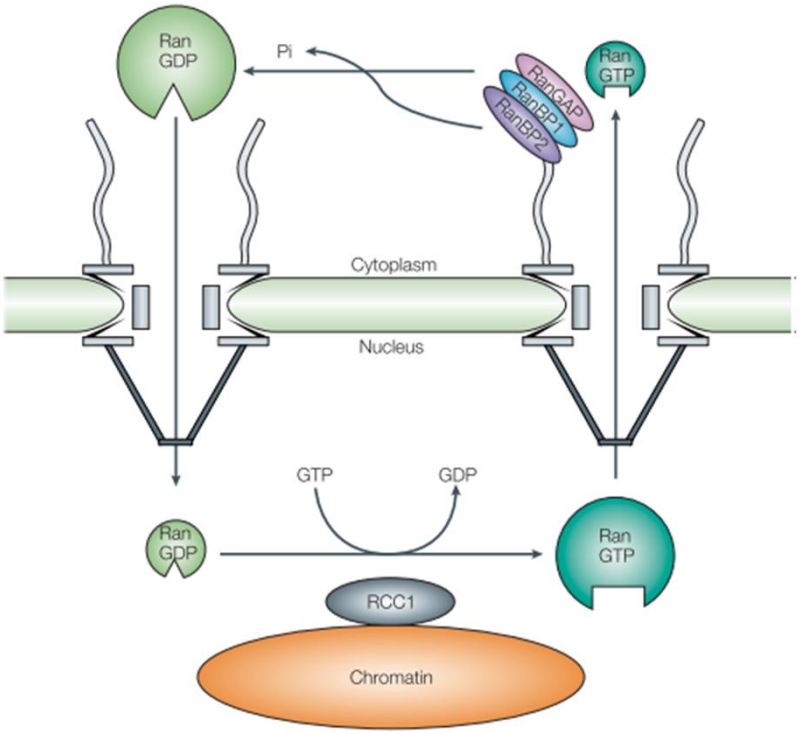
RanGTP would bind export factors in the nucleus. Once in the cytoplasm, RanBP1 and RanGAP can stimulate RanGTP to be converted into RanGDP. This conversion would lead Ran to dissociate from the export factor. The free export factor returns to its original conformation, and then, releases the cargo into the cytoplasm (Cullen, 2003a; Rodriguez et al., 2004a). Finally, NTF2, a RanGDP-binding protein, mediates the nuclear import of Ran in its GDP-bound form (**Figure. 1-1**).

1.2.1 Crm1-dependent nuclear export

Human CRM1 export receptor was identified based on its ability to bind nucleoporins. Adaptor proteins can mediate an interaction between mature RNA and export receptors. Ribosomal RNA (rRNA) and spliceosomal U snRNA utilise the CRM1 export pathway. These two kinds of RNA share the same export receptor known as CRM1, but arerecognized by different adaptor proteins (Allison et al., 2000) (**Figure. 1-2**).

Pre-snRNAs

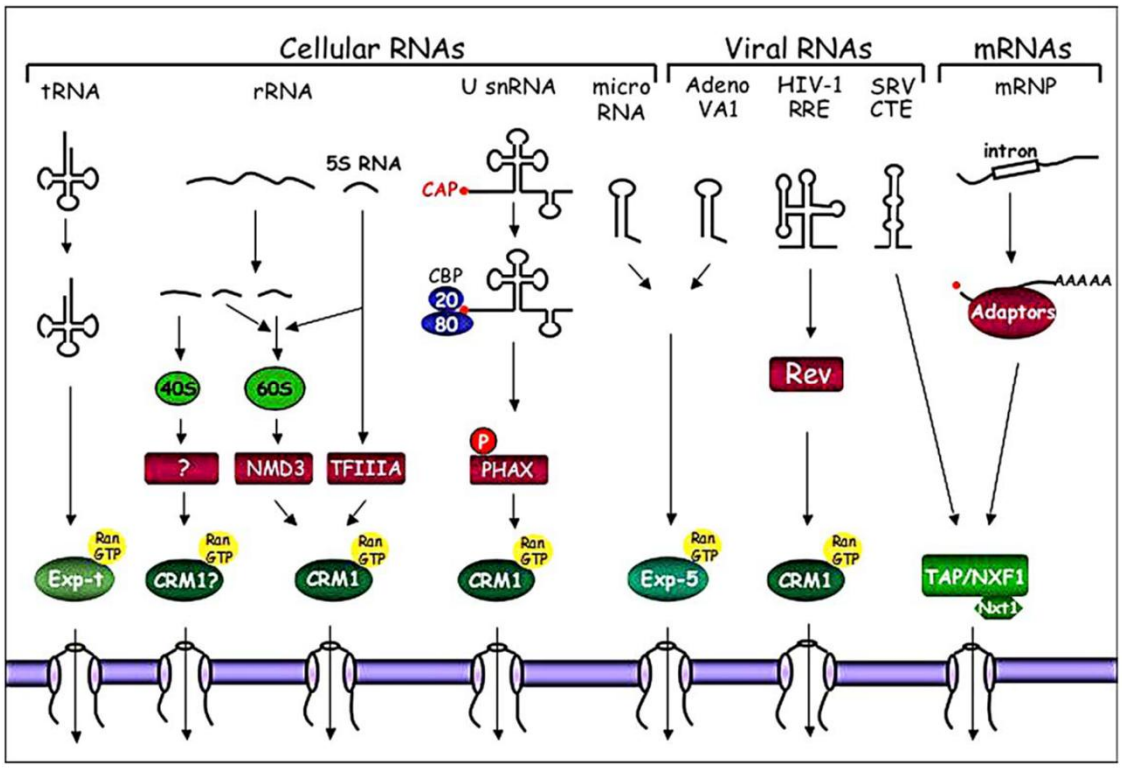
Major spliceosomal U snRNAs are transcribed by RNA polymerase II in the nucleus, but need to be exported to the cytoplasm to associate with Sm proteins and become hypermethylated on their 5’ cap structure. Once mature, functional snRNPs are re-imported back into the nucleus (Johnson et al., 2011; Phelan et al., 2011). Experiments



[Figure. 1-1] Model of Ran-GTPase cycle

The Ran-GTPase cycle regulates the binding and release of the transport cargos from its transport receptor.

(Taken form Fahrenkrog et al., 2003)



[Figure. 1-2] The different RNAs are exported via distinct pathways.

Crm1 interacts with specific adaptors to export U snRNAs and ribosomal subunits. Moreover, Crm1 also interacts with HIV-1 Rev adaptor protein to export viral RNAs containing RRE. Crm1, Exp-t and Exp-5 export their cargoes all via the Ran-GTP pathway. However, mRNA export is a TAP/NXF1-p15/Nxt1 dependent process. TAP also binds to CTE-containing viral RNAs directly to promote their export.

(Taken form Rodriguez et al., 2004)

revealed that CRM1 is responsible for the transport of U snRNAs from the nucleus to the cytoplasm (Allison et al., 2000; O'Connor et al., 2000). However, CRM1 does not directly interact with snRNAs and therefore requires an additional protein, PHAX (phosphorylated adaptor for RNA export), to mediate an interaction between the snRNA and CRM1 (Ohno et al., 2000).

Ribosomal subunits

CRM1 was also described as the export receptor for small and large rRNA subunits in higher eukaryotes. Nmd3p was proposed to act as an adaptor for Crm1p on 60S ribosomal subunits (Hanamura et al., 1998). Nmd3p is a shuttling protein containing a leucine-rich NES and mutations in Crm1p result in the accumulation of Nmd3p in the nucleus. (Caceres et al., 1998; Wang et al., 1998b). In addition, human NMD3 directly interacts with CRM1 in a Ran-GTP dependent manner consistent with its proposed role as an adaptor (Hanamura et al., 1998).

Some viruses also export their RNA through this pathway. For example, HIV can produce an adaptor protein, Rev, to interact with CRM1 and then export HIV-1 RNAs which contain a specific sequence called the REV responsive element (RRE) (O'Connor et al., 2000). However, some types of RNA export do not have adaptors. tRNA can be recognized by the receptor protein (Exportin-t) without adaptors (Arts et al., 1998b; Cullen, 2003a). Micro RNA and adeno virus RNA ,VA1, directly bind to Exportin-5 (Gwizdek et al., 2003).

1.2.2 Exportin-t (Exp-t)

Using the ability of exportins to bind RanGTP in a cargo- dependent manner, a member of this family, exportin-t, was identified as the nuclear export receptor for tRNA in higher eukaryotes (Los1p in yeast). Unlike CRM1-dependent export pathways, tRNAs directly bind exportin-t and don’t require any adaptor. Exportin-t also serves as a quality control checkpoint for tRNAs before their export. Unprocessed or mutant pre-tRNAs are

not exported because of their poor interaction with exportin-t (Arts et al., 1998a; Cullen, 2003b; Kutay et al., 1998; Rodriguez et al., 2004b).

1.2.3 Exportin-5 (Exp-5)

Exportin-5, a member of the karyopherin β family related to the *S. cerevisiae* protein, Msn5p/Kap142p, was identified as the transport receptor for the minihelix-containing RNAs (Kaffman et al., 1998). Interestingly, tRNAs also contain a highly degenerate minihelix motif able to directly interact with exportin-5. Although compared to an optimal minihelix structure it only provided a weak affinity, exportin-5 was proposed to define an alternative tRNA export pathway.

In addition to the minihelix-containing RNAs, exportin-5 has initially been reported to mediate the nuclear export of the interleukin enhancer-binding factor (ILF3) and the eukaryotic elongation factor 1A (eEF1A) (Bohnsack et al., 2002). Also, two groups reported that exportin 5 mediates the nuclear export of short microRNA (miRNA) precursors (pre-miRNAs) (Lund et al., 2004; Yi et al., 2003). Therefore, Exp5, in addition to being an export factor, is central to miRNA biogenesis and might help to coordinate nuclear and cytoplasmic processing steps.

1.3 mRNA Export

In eukaryotes, gene expression starts with DNA transcription in the nucleus and ends with protein synthesis at the cytoplasm. A myriad of different proteins bind mRNA as it progresses to form a mature mRNP in the nucleus (Kelly and Corbett, 2009a; Moore and Proudfoot, 2009). Many of these proteins are associated with steps in the gene expression pathway, such as 5’ capping, splicing, and 3’ polyadenylation, and many are removed before export. There is also a system called nonsense-mediated decay (NMD), which eliminates mRNAs with premature termination codons to control the mRNA quality.

The first step that the nascent pre-mRNA transcript undergoes is 5’ capping. When a transcript reaches about 20-30 nucleotides in length, a 7-methylguanosine cap is

added to the 5' end, which protects the nascent pre-mRNA from degradation (Shatkin and Manley, 2000). The 5' cap is bound by the cap binding complex (CBC) composed of CBP20 and CBP80 (Izaurralde et al., 1995). Next, a transcript undergoes splicing. During this stage, a set of proteins called the exon-exon junction complex (EJC) is simultaneously deposited at the site of exon fusion. Capping and splicing are both important for the recruitment of the transcription-export (TREX) complex. In higher eukaryotes, the TREX complex is poorly recruited to transcripts that lack either the 5' cap or the EJC (Cheng et al., 2006; Masuda et al., 2005; Zhou et al., 2000). The final step of pre-mRNA processing events are 3' end cleavage and polyadenylation. A polyadenylation signal is recognised by cleavage and polyadenylation specific factor (CPSF) in the 3' untranslated region (UTR), resulting in the pre-mRNA polyadenylation site cleavage. Then, the poly(A) tail is added by a poly(A) polymerase and bound by a poly(A) binding protein (Proudfoot, 1994). Through these steps, mRNA is packaged into a messenger ribonucleoparticle (mRNP) and prepared for exported.

Unlike proteins and several RNAs (tRNA, rRNA, and snRNA) which require karyopherins, export of mRNAs is mediated by a conserved heterodimeric receptor (TAP-p15 in metazoans; Mex67p-Mtr2p in yeast) that is structurally unrelated to the karyopherin family.

1.3.1 Bulk mRNA nuclear export is mediated by TAP-p15

Nuclear export of mRNA does not depend on RanGTP (Zenklusen and Stutz, 2001). Instead, the mRNA export receptor is highly conserved and is called TAP-p15 (or NXF1-NXT1) in metazoans and Mex67-Mtr2 in *Saccharomyces cerevisiae* (Herold et al., 2000). Nuclear export factor 1 (NXF1/TAP) is considered to be the major receptor for bulk mRNA exports and transports mRNA through interactions with adaptor RNA-binding proteins (Katahira et al., 1999; Strasser and Hurt, 2000).

TAP contains five domains: the N-terminal domain, RNA recognition motif (RRM), and leucine-rich (LRR) domain, mediate binding to mRNA, whereas the C-terminal region, which contains a nuclear transport factor 2 (NTF2) -like domain and a UBA

domain (homologous to ubiquitin-binding domains), binds FG nucleoporins and facilitates movement through NPCs (Suyama et al., 2000). Through the last two C-terminal domains, TAP can form a heterodimer with p15 as well as associate with nucleoporins of the NPC (Fribourg et al., 2001; Levesque et al., 2001; Strasser et al., 2000b). The phenylalanines of the FG repeat cores bind to shallow hydrophobic cavities on these domains. In metazoans, two separate FG binding domains are required for efficient transport (Braun et al., 2002). Although Mex67–FG nucleoporin interactions facilitate export through NPCs, they do not impose directionality, which instead involves export complex disassembly mediated by Dbp5.

1.3.2 The mRNA export adaptors

Recruitment of TAP-p15 is crucial for generating export-competent mRNPs. However, TAP binds bulk mRNA only weakly, and is thought to be recruited to mRNPs primarily through adaptors (Carmody and Wente, 2009; Iglesias and Stutz, 2008; Kelly and Corbett, 2009b; Köhler and Hurt, 2007).

RNA-binding and export factor (REF)

An intensely studied adaptor is REF (Yra1 in yeast), a TREX complex component that binds mRNA cotranscriptionally and recruits TAP (Hautbergue et al., 2008).

The REF proteins belong to a family of hnRNP-like proteins which contain two major domains with a characteristic arrangement. In the central region is a RNP-motif RNA-binding domain (RRM) while at each terminus is a highly conserved sequence unique to the REF family. These sequences are known as REF-N and REF-C respectively. Between the RRM and the REF-N and REF-C sequences lie variable regions rich in glycine, arginine, and serine residues which are referred to as arginine-rich domains. This structural arrangement allows REF to bind to both RNA and single-stranded DNA and also interact with both TAP and UAP56 (Golovanov et al., 2006; Stutz et al., 2000). Surprisingly, REF contacts RNA only very weakly via their RRM domain with the majority of their RNA-binding activity being conferred by the two arginine-rich regions. The

interaction between REF and TAP is mediated by a combination of the N-terminal domain and the RRM while REF binds UAP56 via the conserved REF-N and REF-C motifs (Golovanov et al., 2006; Hautbergue et al., 2008). Other notable observations regarding REF include its ability to shuttle between the nucleus and the cytoplasm and to co-localise with splicing factors in bodies called nuclear speckles (Zhou et al., 2000).

Yra1 is essential for mRNA export in *Saccharomyces cerevisiae*. Metazoan REF/Aly also mediates mRNA export, but is not essential for bulk mRNA export in *Caenorhabditis elegans, Drosophila melanogaster, Arabidopsis thaliana, Mus musculus,* and *Homo sapiens* (Strasser and Hurt, 2000; Stutz et al, 2000). However, REF/Aly is not the only factor to promote nuclear export of mRNA.

UAP56-interacting factor (UIF)

In metazoan, REF/Aly is not essential for bulk mRNA export (Gatfield and Izaurralde, 2002; Longman et al., 2003). In yeast, Yra1 appears to bind only a subset of the *S. cerevisiae* mRNA (Hieronymus and Silver, 2003). This evidence suggests that there is not only one mRNA adaptor.

In 2009, a novel mRNA export adaptor, UAP56- interacting factor (UIF) was identified (Hautbergue et al., 2009). As with REF, UIF interacts with UAP56 and TAP to deliver mRNA to the NPC. Moreover, UIF is recruited to mRNA via the FACT histone chaperone complex subunit, SSRP1. The finding of UIF supports the view that cells contain more than one adaptor to ensure mRNA export efficiently.

However, adaptor proteins might also need co-adaptor proteins to ensure efficient translocation of the mRNA. For example, TREX component THOC5 binds directly to the middle domain of TAP, which contains an NTF2-like domain. Although not required for bulk mRNA export, THOC5 is crucial for nuclear export of a specific mRNA, HSP70, in conjunction with the adaptor protein REF/Aly (Katahira et al., 2009). This result suggests that not only adaptor proteins, but co-adaptors could also be involved in the export of different classes of mRNA.

1.3.3 THO and TREX complex

THO is a multimeric complex conserved in higher eukaryotes and required for mRNP biogenesis. In yeast, it comprises Tho2, Hpr1, Mft1, and Thp2. In humans and *Drosophila*, THO complexes contain subunits homologous to Tho2 and Hpr1 and three additional subunits THOC5, THOC6, and THOC7.

THO is recruited to chromatin during transcription and needed for transcription elongation (Köhler and Hurt, 2007). Previous work has shown that the THO complex, two mRNA export factors, UAP56 and REF/Aly (known as Sub2 and Yra1 in yeast), as well as a novel protein termed Tex1 are present in the so-called TREX (transcription/export) complex (Chavez et al., 2000; Strasser et al., 2002). The TREX complex is highly conserved from yeast to metzoans. However, recent studies shows that this complex is recruited onto mRNAs during transcription in yeast, whereas it is recruited by the splicing factors during a late step in splicing in mammals (Reed and Cheng, 2005b). TREX complex is required for transcription elongation. In addition, in yeast, Sub2 and Yra1 are co-transcriptionally recruited to nascent mRNA by THO complex. In contrast, human TREX is loaded onto mRNA during splicing.

RNA interference and biochemical studies indicate that TREX complex functions in mRNA export. Katahira et al. reported that TREX component THOC5 binds directly to the NTF2-like doamin of TAP and is crucial for HSP70 mRNA export (Katahira et al., 2009). In 2010, Reed and our lab collaborated to identify a new subunit of hTREX, CIP29, which works with REF in mRNA export (Dufu et al., 2010). Gwizdek et al. were found that Mex67 can also be recruited to mRNPs through interactions between its UBA domain and the TREX component Hpr1, which is ubiquitinylated in a transcription-dependent manner (Gwizdek et al., 2006). The Mex67 UBA domain also binds FG nucleoporins at a site that overlaps with the Hpr1 binding site (Grant et al., 2002; Hobeika et al., 2009). Mex67 cannot bind FG nucleoporins while it is bound to ubiquitinylated Hpr1, and so the Mex67–Hpr1 interaction might contribute to a checkpoint that prevents export until Hpr1 is deubiquitinylated. Although ubiquitinylation appears to participate in the recruitment of Mex67–Mtr2 and other mRNA export machinery components to actively

transcribing genes, the actual transfer of Mex67 to mRNA appears to occur during 3' end processing (Johnson et al., 2009).

1.3.4 Coupling splicing and mRNA export

Besides THO/TREX complex, many other proteins have been found to be involved in mRNA export. For example, several EJC proteins—including Magoh and Upf3—cooperate to recruit TAP. Otherwise, some SR (serine/arginine)-like proteins, members of the splicing factor family, also can directly bind to TAP (Huang et al., 2003; Huang and Steitz, 2001).

Exon junction complex (EJC)

The exon junction complex (EJC) is a set of proteins stably deposited on spliced mRNAs about 20 nucleotides upstream of exon-exon junctions (Le Hir et al., 2000). EJC functions as a signal capable of tagging splice junctions to mark mature mRNAs (Thermann et al., 1998; Zhang et al., 1998). The core proteins of EJC are MLN51, Magoh, Y14 and the DEAD-box protein eIF4AIII (DDX48) (Le Hir and Andersen, 2008). MLN51 associates with RNA directly and also interacts with eIF4AIII and Magoh. Magoh is able to form a heterodimer with Y14 (Bono et al., 2006). It is thought that this core set of proteins acts as a platform for other EJC components which can be loaded during the splicing and mRNA export process.

In higher eukaryotes, the TREX complex is poorly recruited to transcripts that lack either the 5' cap or the EJC, indicating that mRNA export mechanism is linked to splicing and/or capping (Cheng et al., 2006; Zhou et al., 2000).

SR proteins

In addition to the REF/Aly and UIF, SR and SR-like proteins can also function as mRNA export adaptors (Huang and Steitz, 2005). As with REF/Aly, the shuttling SR proteins SRp20, 9G8, and ASF/SF2 can all serve as mRNP binding sites for the general export receptor TAP (Hargous et al., 2006; Tintaru et al., 2007). Intriguingly, TAP preferentially

interacts with the hypophosphorylated form of SR proteins (Huang and Steitz, 2005; Köhler and Hurt, 2007). In contrast, lower eukaryotic organisms contain very few intron-containing genes. This lack of intron-containing transcripts means that the majority of mRNA export is coupled to transcription rather than splicing. However, in *S. cerevisiae*, there are still some SR-like proteins, such as Npl3, which is another mRNA export adaptor and is essential for mRNA export, where it associates with mRNA cotranscriptionally and can recruit Mex67–Mtr2.

Members of the SR protein family contain one or more N-terminal RNA Recognition Motifs (RRM) and a C-terminal domain rich in serines and arginines (RS/SR). The sequences recognized by SR proteins are known as exonic splice enhancer (ESE) (Smith et al., 2006). RRMs can control the recognition of ESEs and the SR domain provides a protein-protein interaction region to assemble the spliceosome (Hertel and Graveley, 2005). SR proteins are generally thought to function by binding to ESE and promoting the recruitment of U1 and U2 snRNPs to alter pre-mRNA splicing (Graveley, 2000; Tacke and Manley, 1999). However, the previous studies reported that this interaction could also regulate mRNA export, and translation initiation (Kress et al., 2008; Sanford et al., 2004).

SR proteins are primarily localized to the nuclear speckles, and a subset of SR proteins shuttle continuously between the nucleus and the cytoplasm (Caceres et al., 1998). Within the cell, the RS domain acts as a nuclear localization signal by mediating the interaction with the SR protein nuclear import receptor transportin-SR and also influences the nucleo-cytoplasmic shuttling of individual SR proteins (Wang et al., 1998a). The shuttling ability of a subset of SR proteins suggested additional roles in mRNA transport, and/or in cytoplasmic events, such as mRNA localization, stability, or regulation of translation (Sanford et al., 2004).

Notable members of the SR protein family are 9G8, SRp20 and SF2/ASF. These proteins are able to shuttle between the nucleus and the cytoplasm and share a similar modular structure. SF2/ASF is made up of two RRM domains and a C-terminal arginine/serine-rich (RS) domain while 9G8 and SRp20 contain only one RRM.

Significantly, it was shown that both 9G8 and SF2/ASF can interact with TAP. 9G8 is able to form an interaction with TAP via a short arginine-rich located at the C-terminus of the RRM domain, while SF2/ASF associates with TAP using the arginine-rich region located between its two RRMs (Hargous et al., 2006; Tintaru et al., 2007). Further evidence implicating 9G8 in mRNA export via TAP has come from microinjection experiments involving *Xenopus* oocytes. These experiments showed that injection of a dominant form of 9G8 blocked mRNA export and that this blockage could be lifted by co-injection of TAP (Huang et al., 2003).

Because most yeast genes are intronless, the widespread belief is that yeast also lacks SR proteins. Yet a recent study reported that Npl3 and Gbp2 have a high structural similarity to canonical SR proteins and directly bind to Mex67 (Gilbert and Guthrie, 2004). Thus, this result suggests that SR proteins also play an important role in mRNA export.

Furthermore, splicing factor 2/alternative splicing factor (SF2/ASF) also stimulates translation. Cytoplasmic SF2/ASF associated with the translation machinery is hypophosphorylated, suggesting that the phosphorylation state of RS domain may influence the role of SF2/ASF in mRNA processing. Indeed, phosphorylation can regulate its ability to bind to mTOR (the eIF4E binding protein kinase) (Michlewski et al., 2008). Although the regulation of the processes of mRNA export remains unclear, these results imply that mRNA export is not only regulated by nuclear processing events, but also coupled to the cytoplasmic events of translation.

1.3.5 Export of intronless transcripts

Although many studies showed that the splicing process is able to enhance mRNA export, some genes don't contain introns and therefore never undergo splicing. However, these transcripts are still exported efficiently. Replication-dependent histones and a natural intronless human gene, c-Jun, both belong to this type of gene.

There are around 65 non-allelic replication-dependent histone genes in the human genome and they encode the four core histones as well as histone H1. The mRNA

transcripts of these genes lack intron and are not polyadenylated. Instead of polyadenylation the mature 3' ends are formed by cleavage after a highly conserved stem loop sequence. This cleavage process requires the presence of the stem loop and a second cis-acting element called the histone downstream element (HDE) as well as two trans-acting factors called stem loop binding protein (SLBP) and U7 snRNP respectively.

Replication-dependent histone transcripts also contain transport elements which recruit export factors (such as 9G8 and SRp20). This export depends on the length of the region upstream of the stem loop structure which provides a platform for export adaptor binding, and then promoting the histone transcript export. Because the length of the transcript was shown to significantly affect the efficiency of export, these adaptor binding sites may be required to ensure efficient export of intronless transcripts.

Another natural intronless gene is a human gene called c-Jun. This transcript contains elements within the protein coding region called c-Jun processing enhancers (CJEs) which enhance RNA stabilisation and 3'-end formation. The CJE has, in addition, been shown to be a transferable element which promotes export by an as yet uncharacterised mechanism (Guang and Mertz, 2005).

Recently, Reed's lab reported that TREX and TAP are required for the export of three naturally intronless mRNAs (HSPB3, IFN-a1, and IFN-b1). These intronless genes contain specific sequences, which allow them to be packed into TREX complex, therefore bypassing the splicing requirement (Lei et al., 2011).

1.3.6 Role of 3' end processing and mRNA export

mRNA 3' polyadenylation processing is necessary for mRNA export. This physical link could be to ensure the termination of transcripts is correct before export. For example, Yra1 preferentially associates with the 3' end of transcripts from intronless genes (Lei and Silver, 2002). However, although Yra1 is recruited to intron-containing transcripts in a splicing-dependent manner, 3' end formation is also required for its recruitment. Thus,

this result indicates that Yra1 is recruited during 3'-end processing (Johnson et al., 2009).

Studies in yeast also showed that Yra1 recruitment appears to be independent of Sub2 (Johnson et al., 2009), and instead requires the CF1A components Rna14, Rna15 and Pcf11. It has been proposed that Pcf11 binds the Sub2/Mex67-interacting regions of Yra1. These interactions are conserved in both yeast and humans, which has led to the proposal that Yra1–Pcf11 binding is an important early step (Johnson et al., 2009). Moreover, the Bentley group reported that depletion of Yra1 affects poly(A) site choice. Yra1 competes with the CF1A subunit Clp1 for binding to Pcf11, and overexpression of Yra1 inhibits 3'-end processing in vitro (Johnson et al., 2011).

However, although Yra1 can be recruited to mRNA via Pcf11, it could also be recruited to Mex67 by other pathways involving, for example, the SR-like protein, Npl3. The poly(A) tail length control factor Nab2 also function in mRNA export in *Saccharomyces cerevisiae* (Green et al., 2002; Viphakone et al., 2008). Similar to yeast, polyadenylation and mRNA export are coupled in higher eukaryotes. Silver's group reported that a drosophila Zn-finger protein, ZC3H3, which interacts with both polyadenylation and mRNA export components, is required for mRNA export (Hurt et al., 2009).

1.3.7 Export of viral RNAs

Although the mechanism of mRNA export functions to ensure that the incompletely spliced transcripts are retained within the nucleus, many viruses are able to hijack the export machinery, bypass normal cellular controls and promote the export of incompletely spliced RNAs to the cytoplasm. There are a variety of different ways to achieve this, including using viral expressed protein factors or incorporating attractive secondary sequence elements (e.g. RRE and CTE) into viral RNA.

Rev/Rev-like protein dependent transport

Some transcripts of retrovirus, such as HIV, are spliced and some are not. The spliced transcripts are able to be exported via the normal pathway. However, the incompletely spliced transcripts are exported by a protein called Rev, which is the product of one of the fully spliced viral transcripts.

Rev is a shuttling protein which consists of two distinct functional domains. It contains an NES motif at the C-terminus; at the N-terminus, there is a specific structure called the Rev response element (RRE), which is present in the incompletely spliced viral transcripts, required for both Rev multimerisation and recognition. Rev is able to bind to any RNAs containing the RRE and promote their export by interacting with the export factor Crm1. Other viruses such as human T-cell leukaemia virus encode a Rev-like protein called Rex which binds to an element in the viral long terminal repeats (Bogerd et al., 1992).

CTE-dependent transport

The constitutive transport element (CTE) is an RNA structure found in the transcripts of many retroviruses. It was originally identified in the Mason-Pfizer monkey virus (MPMV) genome. The CTE functions by binding to TAP directly, allowing viral RNAs to be exported through the mRNA export pathway. Interestingly, CTEs exist in both unspliced and spliced viral RNAs, but are only required for unspliced transcript export (Strasser et al., 2000a). Significantly, the TAP pre-mRNA has also been shown to contain a CTE (Li et al., 2006).

ICP27/ORF57 dependent transport

ICP27 and ORF57 are proteins encoded by many herpesviruses and promote the export of viral RNAs. The viral RNAs are bound by ICP27/ORF57 which in turn binds to the mRNA export adaptor REF. Binding to REF allows the viral RNAs to proceed through the mRNA export pathway. Unlike Rev and CTE-dependent transport, ICP27/ORF57 dependent transport is not used to force export of unspliced viral RNA. Instead, this

method ensures the preferential export of viral intronless RNAs (Koffa et al., 2001; Williams et al., 2005)

1.3.8 Regulation of mRNA export

mRNA export can also be regulated by the modification of proteins. There are many different quality controls of mRNA export. For instance, Tom1, the E3 ubiquitin ligase, associates with the mRNA binding protein Nab2p and blocks mRNP complex export (Duncan et al., 2000). Another example is Rrp6p, which is an exosome specific component in the nucleus. Rrp6p can stimulate mRNP assembly. If the pre-mRNA is not adenylated, Rrp6p will trigger TREX complex disassembly and cause mRNA export to be stopped (Hilleren et al., 2001; Libri et al., 2002).

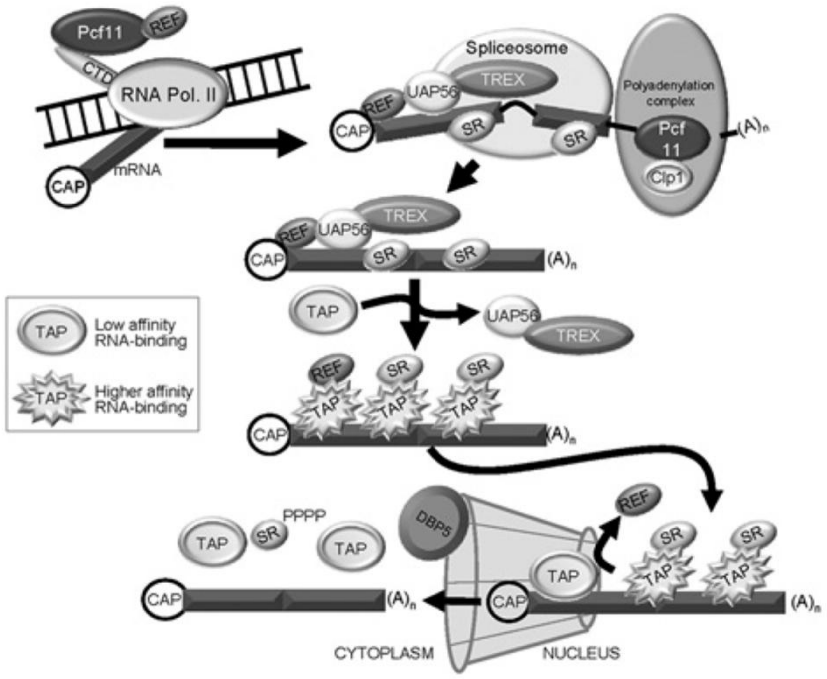
Phosphorylation is another key point of regulation during mRNA export. For example, in yeast, Npl3 can associate with mRNA in its phosphorylated form; however, only in the unphosphorylated form can Npl3 interact with the export receptor (Gilbert et al., 2001; Gilbert and Guthrie, 2004). By contrast, studies in mammals also revealed the regulation between SR proteins and TAP. The hypophorylated SR proteins could associate with mRNA and TAP (Lai and Tarn, 2004). This evidence provides interesting views of the coupling between transcription, splicing, and mRNA export.

In addition, Dufu et al. reported that the novel TREX component, CIP29, assembles into hTREX in an ATP-dependent manner with UAP56. This evidence suggests a new level of mRNA export regulation (Dufu et al., 2010).

1.3.9 Model of mRNA export

It is crucial that only fully processed mRNAs will be exported. To achieve this, the cell has efficient quality control mechanisms for mRNA export. The following discussion presents a summary of the current working model for mRNA export (Walsh et al., 2010) (**Figure. 1-3**).

During transcription the carboxyl terminal domain (CTD) of the large subunit of RNA polymerase II deposits a multitude of protein factors on the nascent transcript. Subsets



[Figure. 1-3] A model for mRNA export

During transcription, Pcf11 is recruited to the CTD of Pol.II together with REF. At the late stage of transcription, Clp1 displaces REF to generate the 3'-end processing complex with Pcf11. REF is then transferred to the cap-binding complex to form the TREX complex *via* UAP56. REF and hypophosphorylated SR proteins recruit and hand the mRNA over to TAP. When the TAP reaches the nuclear pore, Dbp5 displaces TAP and releases the mRNA on the cytoplasmic side.

(Taken form Walsh et al., 2010)

of these proteins are involved in adding the 5' cap to the mRNA and recruiting the TREX complex.

Another group of proteins called the transcription elongation factors promote transcription elongation by RNA polymerase II and also recruit the splicing machinery. The splicing machinery can then act to remove introns from the pre-mRNA and deposit EJC at exon-exon junctions. Disruption of 5' capping or splicing both disrupt normal mRNA export.

In the TREX complex, REF could recruit the essential export factor TAP. Then, UAP56 is displaced by TAP and mRNA is handed over from REF to TAP (Strasser and Hurt, 2001). Although under normal conditions TAP has a low affinity for mRNA, the binding of REF is able to increase this affinity significantly. It is notable that once TAP has been recruited REF remains bound to mRNP via TAP but not via mRNA (Hautbergue et al., 2008). The TAP-mRNA complex can then interact with nucleoporins at the NPC and promote translocation to the cytoplasm; then, Dbp5p, a RNA helicase and also a component of mRNP, triggers the dissociation of mRNP particles and releases mRNA into the cytoplasm (Hodge et al., 1999; Schmitt et al., 1999; Strahm et al., 1999).

1.4 Post-transcriptional control of gene expression

The regulation of gene expression is a fundamental process to allow a cell to respond to both intrinsic and environmental information to ensure the best use of its resources. The most basic form of gene expression regulation is at the transcriptional level where different genes can be selected for transcription by various mechanisms including chromatin-remodeling or by the action of specific transcription factors. However, a more complex form of control is exerted at the post-transcriptional level.

After transcription has occurred, the cell can regulate the fate of the transcripts using a number of processes. In all these different processes, RNA-binding proteins (RBPs) are important players.

1.4.1 Regulation of mRNA turnover/stability

mRNA could decay naturally or be actively targeted for degradation. The natural decay rate of mRNA can be specified by control elements present in the 3' untranslated regions (UTRs) which are recognised by RBPs. The RBPs can act to increase the stability of the transcript or protect it from decay.

Some transcripts can be actively degraded because of sequence error or as a way of regulating transcript levels. In eukaryotes, most cytoplasmic mRNA degradation begins with the shortening of the poly(A) tail by deadenylating proteins. The decapping enzyme Dcp1 and Dcp2 can then remove the 5' cap structure of mRNAs. After that, mRNAs are able to be degraded by the action of an exonuclease called Xrn1p or the cytoplasmic exosome complex. The exosome also contributes to other mRNA degradation processes called nonsense-mediated decay (NMD) and non-stop decay (NSD).

If a transcript contains a premature stop codon, it is identified by the NMD complex and degraded by the exosome. In contrast, if a transcript has no stop codon, NSD complex will target it for degradation. These processes ensure that potentially aberrant protein products are not translated (Hausmann et al., 2000).

1.4.2 Regulation of mRNA translation

The initiation of translation is the rate-limiting step in eukaryotic translation and is the main target for mRNA translation control. There are two categories in translational control: global control and transcript-specific control.

Global control affects the translation of many transcripts at the same time and is usually achieved by changing the phosphorylation state of translation initiation factors or by changing the number of ribosomes present. In contrast, transcript-specific control changes the translation rate of a distinct subset of transcripts by a diverse array of mechanisms. This type of control is particularly important during conditions which require rapid change in proteins levels, such as stress response, apoptosis, and cell development (Stenzl et al., 2000; Strasser et al., 2000c).

As with mRNA decay, this process involves the interaction of RBPs with structural or control elements located in the UTR sequences of target transcripts. The presence of these RBPs can either enhance the translation by promoting its association with the ribosome or decrease by blocking association. A prominent example of this kind of control is the regulation of iron-dependent translation by the protein aconitase (Haile et al., 1997)

1.5 Aims of this Study

As mentioned previously, the human TREX complex is composed of the hTHO sub-complex (hHpr1, THOC2, THOC5, THOC6, and THOC7), Tex1, UAP56, and REF/Aly. TREX complex is required for coupled transcription elongation and nuclear export of mRNAs, and provides an example of an mRNA-specific adaptor (Aguilera, 2005; Köhler and Hurt, 2007; Reed and Cheng, 2005a; Reed and Hurt, 2002). REF/Aly is recruited to mRNA though the interaction with UAP56 and directly interacts with TAP-p15. In *Xenopus* oocytes, REF/Aly was shown to be a limiting factor for nuclear export of mRNA (Zhou et al., 2000). In Drosophila, gene knockdown experiments have shown that only the UAP56 orthologue, but not the other THO/TREX component is required for mRNA export (Farny et al., 2008; Gatfield and Izaurralde, 2002; Gatfield et al., 2001; Herold et al., 2001). It has also been shown that nuclear export of only a subset of mRNAs is affected by depletion of TREX components (Farny et al., 2008). These results suggest the existence of additional adaptors.

Indeed, except REF, there are many different mRNA export adaptor proteins in eukaryotes. As we mentioned before, SR/SR-like proteins also can bind to TAP to enhance mRNA export. Another example is UIF, which also functions as an export adaptor binding TAP and delivering mRNA to the nuclear pore. Both REF and UIF are required for efficient export of mRNA (Hautbergue et al., 2009). These data imply that various nuclear mRNA export pathways, which may be dictated by different adaptor RNA binding proteins, exist in higher eukaryotes.

The mechanism of RNA export remains to be established, there are many details still unknown. Only when we understand all the functions of each RNA export factor, we can view the panorama of RNA export. Thus, in this study, we aim to find new mRNA export factors and through analysing the biological function of a novel protein, SRAG, with focus on a possible role in mRNA export.

Chapter II

Materials and Methods

2.1 Materials

2.1.1 Bacterial Strains

The following bacteria strains were used during the project all from Invitrogen

Strain	Genotype
DH5α	*E.coli sup*E44 Δ*lac* U169 (Ø80 *lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1
BL21 CodonPlus(DE3) RP Stratagene	*E.coli* B F^- *omp*T

Growth Media

All media were sterilised before use by autoclaving at 15 lb/in^2 for 15 minutes and then stored at room temperature until use. All recipes were taken from Sambrook's Molecular Cloning: A Laboratory Guide (Sambrook, 1989).

- **Luria Bertani (LB)**: 10 g/L Bacto-trypton, 10 g/L NaCl, 5 g/L Yeast Extract.
- **LB Agar:** as above, but supplemented with 15 g/L Agar.
- **Terrific Broth (TB):** 12 g/L Trypton, 24 g/L Yeast Extract, 4 mL/L Glycerol, 2.31 g/L KH_2PO_4, 12.54 g/L K_2HPO_4.
- **SOB Medium:** 2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$.

Antibiotics used as selection agents in E. coli

In order to select for bacterial cells carrying particular plasmids, antibiotics were used as selective agents. The concentrations of these antibiotics are outlined in the table below.

Antibiotic	Final Concentration
Ampicillin	100 µg/mL
Kanamycin	50 µg/mL
Chloramphenicol	12.5 µg/mL
Spectinomycin	50 µg/mL

2.1.2 Tissue Culture

Cell lines

The following cell-line strains were used during the project all from ATCC or Invitrogen

- **HEK-293T –** Human embryonic kidney transformed with SV40 which expresses the large T antigen.
- **Flp-In 293 –** These cells contain a single stably integrated FRT site at a transcriptionally active genomic locus.
- **Cos7 –** Vervet Monkey kidney fibroblast
- **HeLa** – Human cervical epithelial carcinoma.

Growth Media

The cells were propagated in Dulbeccos Modified Eagle Media (DMEM) (Sigma D-5796) supplemented with 10% fetal calf serum serum (Life Technologies 10106-169), 1% penicillin-streptomycin (Life Technologies 15070-063) and 2 mM glutamine (Life Technologies 25030-024).

2.1.3 Vectors

Plasmid	Manufacturer	Features/Promoter
p3XFlag-myc-CMV-26	Sigma	Mammalian expression vector, CMV promoter, ampicillin resistance. Allows transient or stable expression of dual tagged N-terminal 3XFLAG and C-terminal *c-myc* fusion proteins.
pCI-neo	Promega	Mammalian expression vector, CMV I/E promoter/enhancer, neomycin resistance.
pEGFP-N1	BD Biosciences	Mammalian expression vector, CMV promoter, neomycin/kanamycin resistance. Allows expression of GFP fusion proteins in mammalian cells.
pET24b	Novagen	Prokaryotic expression vector, T7 promoter, kanamycin resistance, C-terminal His-Tag. Allows expression of 3' His-tagged fusion proteins in *E.coli*.
pcDNA6.2-GWEmGFP-miR	Invitrogen	Mammalian RNAi vector, miR cassette, CMV promoter, Spectinomycin/Blasticidin resistance. Allows knockdown of specific mRNA targets in mammalian cells.
pGEX-6P1	Amersham Bioscience	Prokaryotic expression vector, ampicillin resistance. Allows expression of GST fusions in *E.coli* under control of the *tac* promoter which is induced by IPTG.

2.1.4 Buffers

All buffers which were required to be sterile were sterilised by autoclaving or by being passed through a 0.2 μm filter.

DNA & RNA protocol buffers

- **TE (Tris-EDTA):** 10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0.
- **6x DNA Loading Buffer:** 0.25% (w/v) Xylene Cyanol; 0.25% (w/v) Bromophenol Blue; 30% (v/v) Glycerol.
- **1x TBE:** 90 mM Tris; 90 mM boric acid; 2.5 mM EDTA, pH 8.0.
- **mRNP Capture Assay**

  Lysis Buffer: 50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 2 mM $MgCl_2$; 1 mM EDTA, pH 8.0; 0.5% (v/v) Igepal Ca-360; 0.5% (w/v) Sodium Deoxycholate; 2 mM PMSF.

  2x Binding Buffer: 20 mM Tris-HCl, pH 7.5; 1 M NaCl; 1% (w/v) SDS; 0.2 mM EDTA, pH 8.0.

  Elution Buffer: 10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0; 2 mM PMSF; 2mM RNase A.
- **Hypotonic Buffer:** 10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 M DTT.

SDS-PAGE/Western Buffers

- **4x SDS-PAGE Loading Buffer:** 200 mM Tris-HCl, pH 6.8; 1% (w/v) Bromophenol Blue; 50% (v/v) glycerol; 10% (w/v) SDS.
- **4x SDS-PAGE Stacking Gel Buffer:** 0.5 M Tris-HCl, pH 6.8; 0.15% (w/v) SDS.
- **4x SDS-PAGE Resolving Gel Buffer:** 1.5 M Tris-HCl, pH 8.8; 0.15% (w/v) SDS.
- **SDS-PAGE Running Buffer:** 25 mM Tris; 250 mM glycine; 0.1% (w/v) sodium dodecyl sulphate (SDS).
- **Coomassie Brilliant Blue Stain:** 0.1% (w/v) Coomassie Brilliant Blue R-250, 40% Methanol, 10% Acetic Acid.
- **Destain Solution:** 40% (v/v) Methanol, 10% (v/v) Acetic Acid.

- **Transfer Buffer:** 39 mM Glycine, 48 mM Tris, 0.037% (w/v) SDS, 20% Methanol.

Miscellaneous Buffers

- **1x PBS (phosphate buffered saline):** 137 mM NaCl; 2.7 mM KCl; 4.3 mM NaH_2PO_4; and 1.47 mM KH_2PO_4. pH to 7.4 using HCl. 0.1% (v/v) Tween-20 was added to make into 1x PBS-Tween.
- **1x TBS (Tris buffered saline):** 50 mM Tris-HCl, pH 7.5; 150 mM NaCl. 2% Tween-20 was added to make into 1x TTBS.
- **Transformation buffer:** 10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH to 6.7 with KOH. The $MnCl_2$ was added last, after the pH was altered. Buffer was then filter sterilised.
- **2M Imidazole stock:** 136.2 g/L Imidazole, pH to 7.5 with NaOH.
- **Immunoprecipitation Lysis Buffer:** 50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 2 mM $MgCl_2$; 1 mM EDTA, pH 8.0; 0.5% (v/v) Igepal Ca-630; 0.5% (w/v) Na-deoxycholate; 2 mM PMSF.
- **Immunoprecipitation Wash Buffer:** 50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 2mM $MgCl_2$; 1 mM EDTA, pH 8.0.
- **GSH Elution Buffer:** 100 mM Tris-HCl, pH 7.5; 100 mM NaCl; 100 mM reduced Glutathione.
- **Luciferase Assay Buffer:** 25 mM Gly-Gly, pH 7.8; 15 mM Potassium Phosphate, pH 7.8; 15 mM $MgSO_4$; 4 mM EDTA, pH 8.0; 1 mM DTT. 2 mM fresh ATP was added when ready to use.
- **11.1% Paraformaldehyde:** 24 mL 1x PBS, 2.87 g paraformaldehyde. Dissolve paraformaldehyde by adding a few drops of NaOH and heating to 50°C. Adjust to pH 7.4.
- **3.7% Paraformaldehyde Fixing Solution**: 10 ml 1x PBS, 33.4 ml 11.1% Paraformaldehyde (pH 7.4), 0.6 mL 30% Triton X-100, 56 ml Millipore-filled H_2O.

- **10x Oligo Annealing Buffer:** 100 mM Tris, pH 8.0; 10 mM EDTA, pH 8.0; 1 M NaCl.
- **ECL Solution #1:** 100 mM Tris-HCl, pH 8.5; 2.5mM luminol; 400 µM p-coumaric acid; **ECL Solution #2:** 100 mM Tris-HCl, pH 8.5; 5.3 mM hydrogen peroxide

2.1.5 Molecular Biology Kits

Small scale plasmid DNA purification: QIAGEN Mini spin preparation kit.

Midi scale plasmid DNA purification: QIAGEN Midi spin preparation kit; Promega Midi spin preparation.

DNA extraction from agarose: QIAGEN Gel Extraction Kit.

***In vitro* transcription / translation:** T7 Quick Coupled Transcription / Translation System (Promega).

β-Galactosidase Assay: Clontech Luminescent β-Galactosidase Detection Kit II.

2.2 Methods

2.2.1 Molecular Biology

Phenol Chloroform-Isoamyl Alcohol DNA Purification

One volume of phenol chloroform-isoamyl alcohol (25:24:1, pH 6.6/8) was added to DNA solution. Mixture was vortexed for 1 minute and then centrifuged for 5 minutes at 13,000 rpm. The upper phase was transferred to a fresh tube and 2.5-3 volumes of 100% ethanol were added. 3 M Sodium acetate (pH 5.3) was also added to a final volume of 10%. This mixture was incubated on ice or -20˚C for 20 minutes and then centrifuged for 20 minutes at 13,000 rpm. The supernatant was removed and discarded before 1 mL of 70% ethanol was added. Sample was then centrifuged again for 20 minutes at 13,000 rpm. Supernatant was discarded and the pellet was air-dried. Dried pellet was resuspended in required volume of Millipore-filled H_2O.

Isolation of Plasmid DNA from *E. coli*

For mini-preps, single bacterial colonies were inoculated into 3ml LB supplemented with the appropriate antibiotic (for midi-preps and maxi-preps, 200 mL and 400 mL LB was used respectively). The cultures were incubated overnight at 37˚C with shaking to allow bacterial growth. To purify plasmid DNA the appropriate QIAGEN or Promega kit was used, following the manufacturer's instructions.

DNA restriction digest

Unless otherwise specified, restriction digests were carried out in the optimal buffer supplied by the enzyme manufacturer and with the amount of enzyme never exceeding 10% of the reaction volume (to avoid problems caused by high glycerol concentration). Typically, reactions were incubated at 37˚C for 1-2 hours to allow efficient digestion.

DNA ligation

Approximately 200ng of purified, cut vector (previously treated with 1U of alkaline phosphatase for 15 minutes at 37°C) was incubated with purified DNA insert in the presence of 1 U T4 DNA ligase (Roche) for 2 hours at room temperature. The ligation products were then transformed into competent DH5α *E. coli*.

Making competent cells: chemical method

Overnight cultures were prepared by inoculating 50 mL LB with the appropriate bacterial strain. This overnight culture was used to inoculate SOB medium to an OD_{600} ~ 0.05-0.1 and incubated at 25˚C with shaking. When the OD_{600} reached 0.4-0.5 the cells were placed on ice for 10 minutes. After this incubation the cells were centrifuge at 4000 rpm for 15 minutes at 4˚C in pre-sterilised Beckman JA10 pots. Cell pellets were then resuspended in 160 mL transformation buffer (TB) and left on ice for 10 minutes. Cells were then spun down again at 4000 rpm for 15 minutes at 4˚C. Cells were gently resuspended in 40 mL TB and DMSO was added to a final concentration of 7%. Cell suspensions were left on ice for a further 10 minutes before being aliquoted and frozen in liquid N_2. Aliquots of competent cells were stored at -80˚C until needed.

Transformation of chemically competent bacteria

Competent cells were thawed at room temperature and added to DNA (either half a ligation reaction or ng quantities of plasmid DNA). Mixtures were incubated on ice for 20-30 minutes and then heat-shocked at 42˚C for 30 seconds. LB (800 µL) was then added and the cells were incubated at 37˚C for 1 hour to allow recovery. Cells were then spun down at 7000 rpm for 1 minute, resuspended in a small volume residual LB and spread onto LB plates supplemented with the appropriate antibiotic(s).

Polymerase Chain Reaction (PCR)

PCR reactions of 50 µl were set up as follows: 100 ng DNA template, 10 µM forward primer, 10 µM reverse primer, 1x reaction buffer, 2.5-5 U DNA polymerase, 200 µM dNTPs and 1.5 mM $MgCl_2$. Routinely, 25 cycles were performed using an annealing temperature between 55-65 ˚C and an extension temperature of 72 ˚C.

Molecular cloning

Molecular cloning is a technique which enables the introduction of a known DNA fragment (insert) into a plasmid (vector) with the required genetic properties. The inserts to be used are usually produced by PCR but can also be excised from an existing plasmid and transferred to a new host vector.

Recipient plasmids were prepared for cloning by digestion with the desired restriction enzymes. Usually 5 µg of plasmid is digested for 2 hours at 37˚C with 50 U of enzyme, followed by 30 minutes incubation at 37˚C with 1 µL calf intestinal alkaline phosphatase (CIAP) (1 U/µL). This CIAP treatment prevents the cut vector from re-ligating to itself by removing the 5' phosphate (PO_4). After this treatment the CIAP was inactivated and the plasmid DNA purified by phenol-chloroform extraction followed by ethanol precipitation. If the insert was produced by excision from an existing plasmid, restriction digests of the plasmid were carried out so that ~1 µg of insert DNA is released. The digestion products were separated by agarose gel electrophoresis and the desired DNA fragment was cut from the gel and purified using a QIAGEN Gel Extraction Kit. The DNA obtained was ethanol precipitated and resuspended in an appropriate volume of TE buffer or Millipore-filled H_2O.

In most cases, insert fragments were generated by PCR. The primers required for each cloning were specific for the reaction carried out, but all incorporated the necessary restriction sites flanked by 6 additional 5' bases. These 5' bases were introduced to provide a platform through which the restriction enzyme(s) could bind and digest the DNA efficiently. After amplification, the PCR reaction is gel extracted and digested overnight at 37˚C with 100 U of the appropriate restriction enzyme(s). The

digestion reactions were then phenol-chloroform extracted and ethanol precipitated before being resuspended in an appropriate volume of TE buffer or Millipore-filled H_2O. To determine DNA concentration and whether complete vector linearisation had occurred samples of both the vector and insert DNA were run on an agarose gel. Once DNA concentration is determined 100 ng of linearised vector was added to the remaining insert DNA, along with 10 U of T4 DNA Ligase in T4 DNA Ligase Buffer (1x). This reaction mix was incubated overnight at 16˚C to allow efficient ligation to occur. Half of the ligation mixture was then transformed into competent *E. coli*.

Plasmid construction & primer sequences

- **p3XFLAG-myc-CMV-26 vectors**

The full length SRAG construct was PCR amplified from the I.M.A.G.E. clone (IMG 4450075). This construct was cloned into p3XFLAG-myc-CMV-26.

- **pCI-neo-MS2 vectors**

The pCI-neo-MS2 vector and pCI-neo-MS2-GFP, pCI-neo-MS2-REF, pCI-neo-MS2-TAP were previously created in the laboratory. The full-length SRAG constructs were PCR amplified from the I.M.A.G.E. clone (IMG 4450075) and then sub-cloned into pCI-neo-MS2.

- **pET24b & pET24b-GB1 vectors**

The full-length SRAG construct and various SRAG truncations were amplified from the I.M.A.G.E. clone (IMG 4450075). These constructs were cloned into pET24b or pET24b-GB1 vectors. Mutated version of pET24b or pET24b-GB1 were produced by site-directed mutagenesis.

- **pGEX-6P1 (GST) vectors**

pGEX-6P1 vectors containing various truncations of UAP56, REF, and TAP were previously created in the laboratory. The full-length SRAG construct and various SRAG truncations were amplified from the I.M.A.G.E. clone (IMG 4450075) and then sub-cloned into pGEX-6P1.

Cloning BLOCK-IT RNAi (Invitrogen) vectors

The BLOCK-IT RNAi system is a commercially available cloning system allowing the user to clone pre-miRNA sequences of their choice into linearised pcDNA 6.2-GW/EmGFP-miR vector. These vectors can then be used to "knockdown" the levels of the mRNA for which the pre-miRNA is specific.

DNA sequencing

The fidelity of cloned inserts was determined using the ABI Big Dye 3.1 Terminator system. Various primers were used in the sequencing reactions including standards such as T7/T3 promoters, M13 as well as gene specific primers. Reactions were set up as follows: 1-2 µg DNA template, 1 µL sequencing primer (10 µM), 1 µL Big Dye mix V3.1, 1x sequencing reaction buffer. The reaction mixes were then subject to following PCR parameters:

Cycles	Temperature (˚C)	Time
25	96	30 sec
	55	15 sec
	60	4 min

After PCR the reactions were cleaned up by ethanol precipitation. The DNA pellets were air-dried and then sent to the University of Oxford sequencing facility to be read. The sequences produced were analysed using Chromas II software and the NCBI database.

In vitro site-directed mutagenesis

This is a PCR based method used to introduce nucleotide changes into a wildtype sequence. In each round of PCR, up to 6 closely positioned nucleotides can be altered. Primers are designed for the area which requires mutating. The nucleotides to be altered are positioned in the centre of the primer sequences and are flanked either side by up to 18 nucleotides of wildtype sequence (these ensure correct annealing during PCR). Reactions were set up with 125 ng of each primer, 50-100 ng dsDNA plasmid template, 200 µM of each dNTP, 2.5 U *Pfu Turbo*™ polymerase and 1x *Pfu Turbo*™ polymerase buffer in a total volume of 50 µL. All PCR reactions were then carried out using the parameters outlined in below.

Section	Cycles	Temperature (˚C)	Time (min)
1	1	95	0.5
2	25	95	0.5
		55	1.0
		68	1.0/kb plasmid size

After PCR, reactions were treated with 10 U *Dpn*I for 1 hour at 37˚C. *Dpn*I cuts at 5'-Gm^6ATC-3' and is specific for methylated DNA. Generally, DNA produced by *E. coli* (i.e. the original plasmid template) is *dam* methylated and therefore susceptible to *Dpn*I, while the PCR produced mutant DNA is unmethylated. This difference allows for an easy enrichment of intact mutant plasmid by *Dpn*I digestion. After digestion the reaction was ethanol precipitated and transformed into DH5α, where only the intact mutant plasmid can be propagated. The resultant colonies were then screened for presence of the desired mutation(s) by DNA sequencing.

Total cellular RNA and cytoplasmic RNA extraction using Trizol

To each 100 mg of harvested cells, 1 mL Trizol reagent was added. The mixture was homogenised by pipetting and incubated at room temperature for 5 minutes. After

incubation, 200 µL Chloroform was added and the tubes were shaken vigorously for ~30 seconds. Samples were then centrifuged for 15 minutes at 13,000 rpm and 4˚C. The upper phase was removed and the RNA was precipitated by addition of an equal volume of isopropanol. Samples were centrifuged for 20 minutes at 13,000 rpm and 4˚C. Supernatant was discarded and 1ml of 70% ethanol was added. Samples were centrifuged again for 20 minutes at 13,000 rpm and 4˚C and the supernatant discarded. Pellet was then air-dried and resuspended in the required volume of Millipore-filled H_2O.

To isolate cytoplasmic RNA, 293T cells were first grown in 24-well plates. For each condition 12 wells of cells were trypsinised and pooled before being centrifuged for 5 minutes at 2000 rpm and room temperature. The pelleted cells were then washed once with serum-free DMEM before being resuspended on ice-cold hypotonic buffer and incubated an ice for 15 minutes. The samples were then centrifuged for 8 minutes at 2500 rpm and 4°C to clear nuclear components. The supernatant was transferred to a fresh tube and residual nuclear contaminants were removed by centrifuging for 5 minutes at 6000 rpm and 4°C. Again the supernatant was removed and transferred to a fresh tube before being centrifuged for 1 minute at 16000 rpm and room temperature. The remaining supernatant was brought to 100 mM NaCl, 0.5% NP40, 10 mM EDTA (pH 8.0), 0.5% SDS and phenol-chloroform extracted twice. These extractions were followed by a single chloroform extraction before the RNA was precipitated and resuspended in 44 µL Millipore-filled H_2O and 5 µL DNase I buffer. Each sample was then treated with 2 U RNase-free DNase I for 30 minutes at 37°C. After DNase treatment another phenol-chloroform extraction was carried out, followed by a single chloroform extraction. Finally, the RNA was precipitated again and resuspended in 24 µL Millipore-filled H_2O. The concentration of the RNA was then determined by NanoDrop (Thermo).

Reverse transcription PCR (RT-PCR)

Purified RNA (1-3 µg) was incubated with RNase-free DNase I (4 U) for 30 minutes at 37˚C to remove any contaminating DNA. The DNase was then inactivated by incubation

at 70˚C for 15 minutes. After DNase inactivation the following were added, 1 µL oligo$(dT)_{20}$ primer (50 µM), 4 µL dNTP mix (2.5 µM per oligo) and Millipore-filtered H_2O to a total volume of 13 µL. Reaction mix was then incubated at 65˚C for 5 minutes to denature RNA secondary structure. After this incubation the tubes were immediately put on ice for 1 minute. To the cooled reaction mixes the following were then added, 4 µL first strand synthesis buffer (5x), 1 µL DTT (0.5 M), 1 µL RNase inhibitor and 1µl Superscript III reverse transcriptase (200 U/µL). These reactions were mixed by gentle pipetting and then incubated at 50˚C for 45 minutes. After this incubation the reverse transcriptase was inactivated by incubation at 70˚C for 15 minutes. The cDNA was then ready to be used in subsequent PCR reactions. Typically, 2 µL of cDNA was used per PCR reaction.

Quantitative PCR (qPCR)

Total cellular RNA and cytoplasmic RNA preparations were reverse transcribed as described previously using between 1-2 µg template RNA and the resultant cDNA was diluted two-fold and stored at -20°C until required. Each qPCR reaction contained 1 µL cDNA (1/40), 0.4 µL SYBR green, 5 µL Sensimix SYBR Kit (2x) (Bioline), 1 µL primer mix (100 ng/µL) and 2.6 µL Millipore-filled H_2O. Reactions were then cycled according to the parameters outlined in the table below.

Section	Cycles	Temperature (˚C)	Time (mins:secs)
1	1	95	10:00
2	40-50	95	0:10
		58-60	0:15
		72	0:25

The qPCR analysis of total cellular RNA and cytoplasmic RNA preparations were used for **Figure. 5.8**.

Agarose gel electrophoresis of DNA

DNA can be separated in relation to its size by gel electrophoresis, with smaller fragments migrating faster through the mesh-like agarose. Due to its negatively charged phosphate backbone DNA migrates towards the positively charged anode. Different percentages of agarose can be used to resolve different sizes of DNA fragment.

The appropriate amount of agarose was dissolved in SB (1x) or TBE (1x) and heated in the microwave for 3-5 minutes. During heating the mixture was gently swirled to ensure the agarose dissolved completely.

Agarose Concentration (%)	Efficient Range of Separation of DNA Molecules (kb)
0.5	1-20
0.8	0.8-10
1.0	0.5-7
1.5	0.2-3
2.0	0.1-2

Melted agarose was allowed to cool to around 50˚C before ethidium bromide (10 mg/mL) was added to a final concentration of 10 µg/mL. Agarose was then poured into a mould with a comb inserted and left to set at room temperature. Set gels were placed in the appropriate electrophoresis tank and the tank was filled with either SB (1x) or TBE (1x) until buffer just covered the gel.

1/6th volume of 6 x loading buffer was added to the DNA samples. These samples were then loaded onto the gel along with an appropriate commercial size marker and run at a constant voltage (150 volts for SB; 100 volts for TBE) until the dye front reached the end of the gel. DNA bands were then visualised by fluorescence upon exposure to a UV light using a transiluminator.

Gel extraction of DNA

DNA fragments were resolved by agarose gel electrophoresis, as described previously. Desired DNA fragments were excised from the gel and the purified using a QIAGEN Gel Extraction Kit, following the manufacturer's instructions.

Recombinant protein expression in *E. coli*

Recombinant proteins such as GST fusions were expressed in *E. coli* transformed with the appropriate plasmids. Competent *E. coli* strain BL21 RP was transformed with the required expression vector and plated out on selective media. Single colonies were inoculated with 50 mL overnight culture of TB. This culture was incubated overnight at 37˚C with shaking.

The following day, 2 liter flasks containing 600 mL of TB were inoculated to an OD_{600} = 0.05-0.1. The cultures were then incubated at 37˚C with shaking until the OD_{600} = 0.7. Once this optical density had been reached the cells were induced by addition of IPTG to a final concentration of 200 µM. The cultures were then grown at 37˚C with shaking for a further 1 hour before being switched to 25˚C overnight. After incubation the culture was spun down at 4000 rpm for 15 minutes and the resultant pellet stored at -20˚C until the protein was required.

Purification of affinity tagged recombinant proteins from *E. coli*

For GST proteins the appropriate amount of cell pellet was resuspended in 1x PBS/1% Triton X-100. The mixture was then sonicated to lyse the cells. After sonication the samples were centrifuged at 13000 rpm at 4˚C for 10 minutes to pellet cellular debris and the supernatant was applied to glutathione sepharose resin equilibrated with 1x PBS/1% Triton X-100. The beads were incubated at 4˚C for 1 hour with agitation before the slurry was spun down at 2000 rpm for 2 minutes. The supernatant was removed and the beads were washed three times with 1x PBS/1% Triton X-100. To elute, an equal amount (relative to slurry) of GSH elution buffer was added and the mixtures were briefly vortexed. Samples were then incubated at room temperature for 10 minutes

before being centrifuged at 2000 rpm for 2 minutes. The supernatant containing the eluted protein was carefully removed and transferred to a fresh tube. Eluted protein was then stored at either 4˚C or -20˚C depending on the subsequent application.

His-tagged proteins were purified using immobilised metal affinity chromatography. The appropriate amount of cell pellet was resuspended in Co^{2+} loading buffer (50 mM Tris-HCl, pH 8.0; 1 M NaCl; 0.5% Triton X-100), sonicated and spun down as described previously. The supernatant was then applied to Co^{2+} beads (Geneflow) and incubated at 4°C for 1 hour with agitation. The beads were then washed three times with Co^{2+} wash buffer (50 mM Tris-HCl, pH 8.0; 1 M NaCl; 5 mM Imidazole). The bound proteins were then eluted by addition of Co^{2+} elution buffer (50 mM Tris-HCl, pH 8.0; 100 mM NaCl; 200 mM imidazole) and vortexing. The supernatant containing the eluted protein was carefully removed and transferred to a fresh tube. The protein sample was dialysed against an appropriate buffer to remove any residual imidazole. Samples were then stored at either 4°C or -20°C depending on the subsequent application. The protein samples obtained were of sufficient purity for the required applications.

Western blotting

Western blotting is a technique which involves the transfer of proteins, previously separated by SDS-PAGE, onto nitrocellulose membrane (Whatman Optitran BA-S 83). Protein samples were separated by electrophoresis as described previously with pre-stained protein markers (BIORAD Precision Plus Protein Standards: All Blue) replacing unstained markers. After electrophoresis the contents of the gel were transferred to the nitrocellulose using the semi-dry Biometra western blotting apparatus.

To set up the blot a “sandwich” structure was made consisting of 3 pieces of 3 mm of Whatman paper, the gel to be blotted, a piece of nitrocellulose membrane and a further 3 pieces of 3 mm Whatman paper.

All these components were saturated in transfer buffer prior to assembly. The completed “sandwich” was then placed membrane side down (closest to the positive electrode) on the blotting apparatus and air bubbles were removed by rolling with a

10ml pipette. A constant current of 2 mA/cm^2 was applied in order to transfer the proteins. Blots were carried out for between 45-60 minutes depending on the percentage gel used. Once the blot was completed the gel was discarded and the pre-stained standards were marked on the membrane in pencil.

Identification of immuno-reactive proteins by ECL detection

The membrane produced by western blotting was incubated overnight at 4˚C in blocking solution (4% milk powder in 1x TBS/Tween-20). The membrane was then incubated for 1 hour with shaking in blocking solution containing the required dilution of primary antibody. Three 5 minute washes with 1x TBS/Tween-20 were carried out to remove any unbound primary antibody. The membrane was then incubated for 1 hour with shaking in blocking solution containing the required dilution of secondary antibody. This antibody is conjugated to horse radish peroxidase (HRP). Three 5 minutes washes with 1x TBS/Tween-20 were again carried out to remove any unbound secondary antibody. Once the washes were completed the membrane was ready for ECL detection.

This detection method works on the principle that the HRP molecule conjugated to the secondary antibody can react with a luminal substrate found in the ECL reagents leading to light being emitted. This light can then be detected by exposure to film. To carry out this detection method the ECL reagents were first prepared and then applied to the membrane. The membrane was then transferred to a film cassette and exposed to film for between 10 seconds and 30 minutes, depending on the intensity of the signal. The film was then developed using an automatic film developer and the pre-stained standards were marked.

GST pull-down assay

This assay was used to detect protein-protein interactions between GST fusion proteins and radio-labeled proteins of interest. GST tagged protein was immobilised on GSH sepharose and used as bait for interaction with radio-labeled proteins produced in the T7 Quick Coupled Transcription / Translation system (Promega).

The transcription / translation reactions were set up as follows: 1.5 µL mini-prep DNA; 0.5 µL [^{35}S]-Methionine (18.5 MBq); and 8 µL rabbit reticulocyte lysate master mix. These reactions were mixed by gentle pipetting and then incubated at 30˚C for 90 minutes. During this incubation time the GST proteins were extracted as described previously. For GST extraction 0.1 g of cell pellet was used and for GST fusion protein extraction 0.4 g of cell pellet was used. The supernatant from these extractions was removed and applied to 30 µL Glutathione (GSH) sepharose slurry. The extracts were incubated with the beads for 30 minutes at 4˚C on a rotating wheel to allow binding of the GST protein. After this incubation the beads were washed twice with 1x PBS/0.1% Tween 20. Once the transcription translation reaction was completed, 8 µL of the reaction mix was removed and mixed with 400 µL 1x PBS/0.1% Tween 20. This mixture was then applied to the GSH beads and the tubes were incubated for 30 minutes at 4˚C on a rotating wheel. The remaining reaction mix was kept to be resolved by SDS-PAGE. After this incubation the beads were again washed twice with 1x PBS/0.1% Tween 20. Elution was then carried out by adding 50 µL GSH elution buffer and then vortexing the tubes briefly. The beads were then allowed to settle for 5 minutes before the supernatant was removed. Two 12% SDS-PAGE gels were prepared and the collected samples were resolved on them. On one gel 0.5 µL of each input sample was run and on the second gel 12 µL of each eluted sample was run. The radiolabelled protein was visualized by autoradiography.

His-tagged proteins *in vitro* pull-down assay

This assay was used to detect interactions between purified recombinant proteins. To begin with His-TAP, purified previously using immobilised metal affinity chromatography, was dialysed against RB100 buffer (25 mM HEPES-KOH, pH 7.5; 100 mM potassium acetate; 1 mM EDTA, pH 8.0; 1 mM DTT; 10 mM $MgCl_2$; 10% glycerol; 0.05% Triton X100). GST proteins of interest were then immobilised on GSH resin and washed. The bound proteins were then incubated with the RB100 buffer at 4°C for 1 hour with agitation. After incubation, 50 µg dialysed His-tagged protein was added to

the beads and the mixture was again incubated at 4°C for 1 hour with agitation. The protein-protein complexes were then eluted from the beads by addition of GSH elution buffer and vortexing. Eluted complexes were then resolved by SDS-PAGE and the resultant gels were coomassie-stained.

2.2.2 Biochemistry

RNA UV cross-linking assay

Before the experiment could begin properly a radio-labelled RNA probe was produced by *in vitro* T4 PNK kit (Roche). 0.5 µg short strand of the substrate RNA was labeled at the 5'-end with [γ-^{32}P]ATP using T4 PNK at 37 °C for 1 hr. The reaction mixture was boiled for 5 min to inactivate polynucleotide kinase. The complementary strand was added to reach a molar ratio of 1:1.2 between the labeled and the unlabeled complementary strand. The reaction mixture was boiled for 3 min and cooled to room temperature in 3 hr. Labeled dsRNA was purified using phenol:chloroform extraction to remove excess [γ-^{32}P]ATP.

To set up the cross-linking reactions the required amount of protein was added to a fresh microcentrifuge tube and the NaCl content of each tube was then standardised. To each tube, 5 µL RNA binding buffer (4x) was added and the reactions were then made up to 20 µL with DEPC-d$H_2$0. After this 40 ng radio-labelled RNA probe was added and the mixtures were incubated on ice for 15 minutes. After this incubation the tubes were opened, kept on ice and then cross-linked at 254 nm for 20 minutes using an UVItec CL-508 cross-linker. After cross-linking the samples were incubated with 1 µL RNase A (10 U/µL) at 37˚C for 15 minutes in order to remove all unbound RNA probe. The remaining complexes were then resolved by 12% SDS-PAGE and visualized by autoradiography.

UV cross-linking: trimeric complex assay

This assay was used to determine whether a GST-tagged protein of interest could form a trimeric complex with both TAP-p15 and RNA *in vitro*. Reaction mixes were made up in 1x RNA binding buffer with the required amount of GST-protein and 1 µL radio-labeled

RNA. The mixtures were incubated on ice for 10 minutes and then at room temperature for a further 10 minutes. After incubation the appropriate amount of TAP-p15 was added and the amount of NaCl in each tube was standardised. The reaction mixes were then incubated on ice for 10 minutes and then at room temperature for 10 minutes. The samples were returned to ice and then cross-linked at 254 nm for 20 minutes using an UVItec CL-508 cross-linker. After cross-linking the samples were incubated with 1 µL RNase A (10 U/µL) at 37˚C for 15 minutes in order to remove all unbound RNA probe. During RNase treatment, an appropriate volume of GSH sepharose slurry was washed with 1x RNA binding buffer/50 mM NaCl. The beads were then spun down and resuspended in 500 µL 1x RNA binding buffer/50 mM NaCl before being equally distributed to the RNase A treated samples. These reaction mixes were incubated on ice for 10 minutes with occasional mixing. After incubation the samples were washed three times with 1x RNA binding buffer/50 mM NaCl/0.5% Triton before complexes were eluted in 20 µL GSH elution buffer. The eluted complexes were then resolved by 12% SDS-PAGE and the gel as dried and visualized by autoradiography.

Immunoprecipitation: FLAG-tagged proteins

Flag-tagged proteins were purified from eukaryotic cells by immunoprecipitation using mouse α-FLAG M2 agarose. Tissue used was from 293T cells transfected with flag vector. Cells were lysed with immunoprecipitation lysis buffer (either 48 hours after transfection or 72 hours after plating depending on the cells used) and the extracts were centrifuged at 13,000 rpm for 10 minutes at 4˚C. The supernatant was then removed and transferred to eppendorf tubes containing 30µl mouse α-FLAG M2 agarose slurry. These tubes were then incubated at 4˚C for 3 hours with constant agitation. After incubation the beads were spun down at 2000 rpm for 2 minutes and the supernatant was removed. The beads were then washed twice with 1x PBS and then 4 times with immunoprecipitation wash buffer. Flag-tagged protein was then eluted from the beads by addition of 10 µg 3x FLAG peptide in wash buffer and incubation at 4˚C for 30

minutes with constant agitation. After incubation the beads were again spun down at 2000 rpm for 2 minutes and the supernatant was removed.

mRNP capture assay

This assay was used to determine whether proteins of interest could interact with mRNA *in vivo*. On the day of the experiment, the media was removed and the cells were washed once with 1x PBS. After washing a small amount of 1x PBS is added so that the cells are just covered in liquid. The cells were then exposed to UV light (300 mJ/cm^2) for 2 minutes. After exposure to UV light, 400 µL mRNP capture assay lysis buffer was added and the lysed cells were scraped and transferred to a fresh microcentrifuge tube. Lysates were then spun at 10000 rpm for 10 minutes at 4˚C to pellet the cell debris and the supernatant was transferred to another tube. The supernatant was denatured by addition of 400 µL 2x mRNP capture assay binding buffer. This mixture was then added to 25 µL (bed packed volume) of oligo-d(T) cellulose and the tubes were incubated at 4˚C for 1 hour with rotation. After incubation the cellulose was spun down and the supernatant was discarded. The cellulose was then washed three times with 1x mRNP capture assay binding buffer. Elution then carried out by addition of 400 µL mRNP capture assay elution buffer and 4 µL RNase A (10 mg/mL). The cellulose was incubated with this buffer for 30 minutes at 37˚C with agitation. After incubation with elution buffer, the cellulose was spun down and the supernatant transferred to a fresh tube. The proteins in the solution were precipitated by the addition of 400 µL ice-cold 20% TCA and incubation on ice for 20 minutes. The precipitated proteins were spun down by centrifugation at 10000 rpm for 20 minutes at 4˚C and the resultant pellets were washed with ice-cold acetone. The cleaned pellets were then resuspended in the required volume of dH_20 and resolved by SDS-PAGE. The SDS-PAGE gels were then blotted to nitrocellulose and the membranes were probed for the presence of the FLAG-tagged proteins of interest with α-FLAG primary antibody.

***In vivo* co-immunoprecipitation assay**

293T cells were grown in 24-well plates and each well was transfected with 350 ng plasmids encoding *myc*-tagged target protein and 350 ng plasmid encoding FLAG-SRAG. For each condition, 3 wells were transfected. After 48 hours cells were lysed in 80 µL 1x Reporter Gene Lysis buffer (Roche) and the extract was scraped and transferred to a fresh 1.5 mL eppendorf tube. For each condition, one cell extract was treated with 1 µL RNase A (10 mg/mL) for 30 minutes at 37˚C, while the other was left untreated. This was to ensure any interaction between *myc*-TAP and FLAG-SRAG was direct and not bridged by RNA. After the RNase A treatment the cell extracts were applied to 20 µL pre-washed FLAG agarose slurry and the immunoprecipitation was carried out as described previously.

After immunoprecipitation was completed, the eluted complexes were resolved by SDS-PAGE and the gel was blotted onto nitrocellulose membrane. Two membranes were prepared using the same samples. One membrane was probed with α-*myc* primary antibody and the other was probed with α-FLAG primary antibody.

2.2.3 Cell Biology

Mammalian tissue culture

All cell types were grown as a monolayer in Dulbecco's modified Eagles' medium (DMEM) supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum, 1% (v/v) streptomycin and 1% (v/v) penicillin. Cells were maintained in an incubator set at 37˚C with 5% CO_2.

Cells were passaged 2 to 3 times a week (depending on the cell line). During each passage, the media was removed and cells were washed once with 1x PBS. After washing the cells were detached by incubation with 0.25% trypsin/EDTA solution at 37˚C for 5 minutes. Trypsin was then deactivated by addition of DMEM and the cells were transferred to new culture flasks containing the appropriate volume of DMEM.

Long term storage of cell lines

Tissue culture cell lines can be stored cryogenically until needed. The cells to be stored were spun down at 1000 rpm in a swinging bucket centrifuge and the pellet was gently resuspended in cell freezing media (FCS/10% DMSO) at a concentration of 3 x 10^5 cells/mL. The cell suspension was divided into 1 mL aliquots and transferred to 2 mL cryo-vials. These vials were placed in an insulated box and transferred to -80˚C until fully frozen. Once they were fully frozen the vials were transferred to liquid nitrogen where they were stored indefinitely.

To revive cells from cryogenic storage, they were thawed quickly at 37˚C, washed and gently pelleted in 10 mL of pre-warmed media to removed cytotoxic DMSO. The pellet was then gently resuspended in 5 mL pre-warmed media, transferred to a sterile tissue culture flask and allowed to grow until confluent.

Transient transfection: calcium phosphate method

One day prior to transfection cells were split to a confluency of ~80% in the required culture dish/plate. Transfections were typically carried out on 150 x 15 mm dishes which required between 20-30 µg of DNA per transfection. The following 1 mL reaction mixes were set up: 20-30 µg DNA; 250 mM $CaCl_2$; 1x HBS. The transfection mixes were gently mixed and incubated at room temperature for 20 minutes to allow precipitate to form. After incubation the transfection mixes were gently mixed again and then added drop-wise to the cells. After 12 hours the cells were washed once with 1x PBS and the media was replaced.

Transient transfection: Lipofectamine 2000 method

Lipofectamine 2000 (Life Technologies) is a commercial transfection reagent which contains cationic lipids. One day prior to transfection cells were split to a confluency of ~80% in the required culture dish/plate. On the day of transfection, DNA was aliquoted and then incubated with Optimem (Gibco) and Lipofectamine 2000 reagent (Invitrogen)

(concentrations as described in user's manual) for 20 minutes at room temperature. After incubation the mixture was gently dispensed onto the cells to be transfected.

Generating stable cell lines: p3XFLAG-myc-CMV-26 vectors

To begin with, 30 µg of the appropriate p3XFLAG-myc-CMV-26 vector was digested with 2 µL *Xmn*I (10 U/µL) for 2 hours at 37˚C. The DNA was then phenol-chloroform extracted and precipitated, before the pellet was resuspended in 16 µL Millipore-filled H_2O. This DNA was then transfected into confluent HeLa S3 cells grown in 6cm tissue culture dishes using 20µl Lipofectamine 2000 reagent. After transfection the cells were allowed to grow for 48 hours at 37˚C before being split into 96 well plates containing media supplemented with the selective agent G418 (1 mg/mL). In cells in which the linearised plasmid had been successfully integrated single colonies were present. Once the cells reached the appropriate confluency any colonies produced were transferred to 24 well plates and allowed to grow to confluency. The potential clones were then screened for efficient expression of the desired flag-tagged protein by western analysis of total cell extracts.

Immunostaining

Cells were grown on coverslips for 48 hours at 37°C before the media was removed. The cells were then washed once with 1x PBS to remove residual media and permeabilised in 3.7% paraformaldehyde fixing solution for 15 minutes at room temperature. The cells were then washed three times with 1x PBS and incubated for 1 hour with 1x PBS (1% BSA). After this the cells were incubated for 1 hour with primary antibody diluted in 1x PBS (1% BSA). Following this incubation the cells were washed three times again and then incubated for 30 minutes with secondary antibody (Invitrogen) diluted 1/800 in PBS^+. The cells were then washed again and coverslips were then mounted in Vectashield anti-fade medium (Vector) on glass slides and sealed using nail vanish.

Luciferase assay

This assay was carried out as part of MS2-tethered assay experiments and was used to give a relative estimate of the amount of luciferase protein in the cell cytoplasm by measuring luminescence produced by cell extracts.

Previously transfected 293T were washed with 1x PBS 48 hours after transfection then lysed with 80 µL 1x Reporter Gene lysis buffer for 10 minutes. Cell extracts were then harvested and transferred to microcentrifuge tube. Extracts were then spun down at 10,000 rpm for 3 minutes to pellet cell debris and the supernatant was transferred to a fresh tube. Luciferase activity was measured using a Sirius Luminometer (Berthold Detection Systems). For each sample 10 µL of supernatant was added to a luminometer tube and the tubes were put into the luminometer. To each luminometer tube 200 µL of luciferase assay buffer was added and after 10 seconds the luminescence was recorded. Each experiment was usually carried out in triplicate and an average reading was determined at the end.

Chemi-luminescent β-Galactosidase assay

This assay was used as an indicator of transfection efficiency and various experiments, most notably in the MS2-tethered assay experiments. Previously transfected 293T cells were lysed and harvested as described for the luciferase assay. From each supernatant, 5 µL was taken and diluted 1 in 100 in 500 µL of 1x Reporter Gene lysis buffer. From these dilutions 10µl was taken and mixed with 50 µL β-Galactosidase Detection Kit reaction mix (49 µL buffer; 1 µL reagent) (BD Biosciences) in a luminometer tube. The mixtures were then left to incubate for 1 hour at room temperature. After incubation the β-galactosidase activity was measured using a Sirius Luminometer (Berthold Detection Systems). The luminescence of each sample was recorded after 10 seconds. Each experiment was carried out in triplicate and an average reading was determined at the end.

ATPase assay

ATPase assays were essentially performed as described in Shi et al., 2004. Reactions of 50 µL were stopped by the addition of 10 µL 0.5 M EDTA, pH 8.0 and supplemented with 140 µL Millipore-filtered H_2O before addition of 800 µL of Malachite green-Phosphomolybdenum reagent.

Helicase assay

Unwinding of duplex RNA was monitored by following the displacement of a short ^{32}P-end radiolabeled strand (GCUUUACGGU) from the duplex formed with a long non-labeled strand (AAAAACAAAACAAAACAAAACAAAACUAGCACCGUAAAGC) essentially as described in Chang et al., 2009. Briefly, 2 µg recombinant UAP56-6His synthesised in *E. coli* were incubated in presence or absence of recombinant SRAG, REF-6His, CIP29, or Magoh-6His at a 4:1 molar ratio for 10 minutes at room temperature prior addition of 125 µM duplex RNA and 1 mM ATP. Products of reactions were run on 15% native polyacrylamide gels in TBE buffer before autoradiography.

Chapter III

Discovery of a new mRNA export factor - SRAG

In eukaryotic cells, mRNA has to be transported from the nucleus to the cytoplasm to be translated. Various protein factors are involved in mRNA export including UAP56, REF/Aly, and TAP/NXF1. mRNA export adaptor proteins such as REF, UIF and certain SR proteins serve an essential function in mRNA export. The recruitment of some adaptor proteins to the mRNP are driven by UAP56, which is an RNA helicase required for spliceosome assembly and mRNA export.

During mRNA processing, UAP56 provides a bridge between THO proteins and REF to form the TREX complex, which indirectly couples transcription and export by splicing (Masuda et al, 2005). REF recruits TAP/NXF1 to mRNP and UAP56 is displaced by TAP. REF binds to the N-terminal domain of TAP directly and hands mRNA over to TAP. In turn, TAP associates with the nuclear pore delivering mRNA to the cytoplasm. However, depletion of REF does not block bulk mRNA export, suggesting that other proteins can fulfill this role and that there may be functional redundancy between export adaptors.

3.1 Identification of UAP56-associated proteins by BLAST

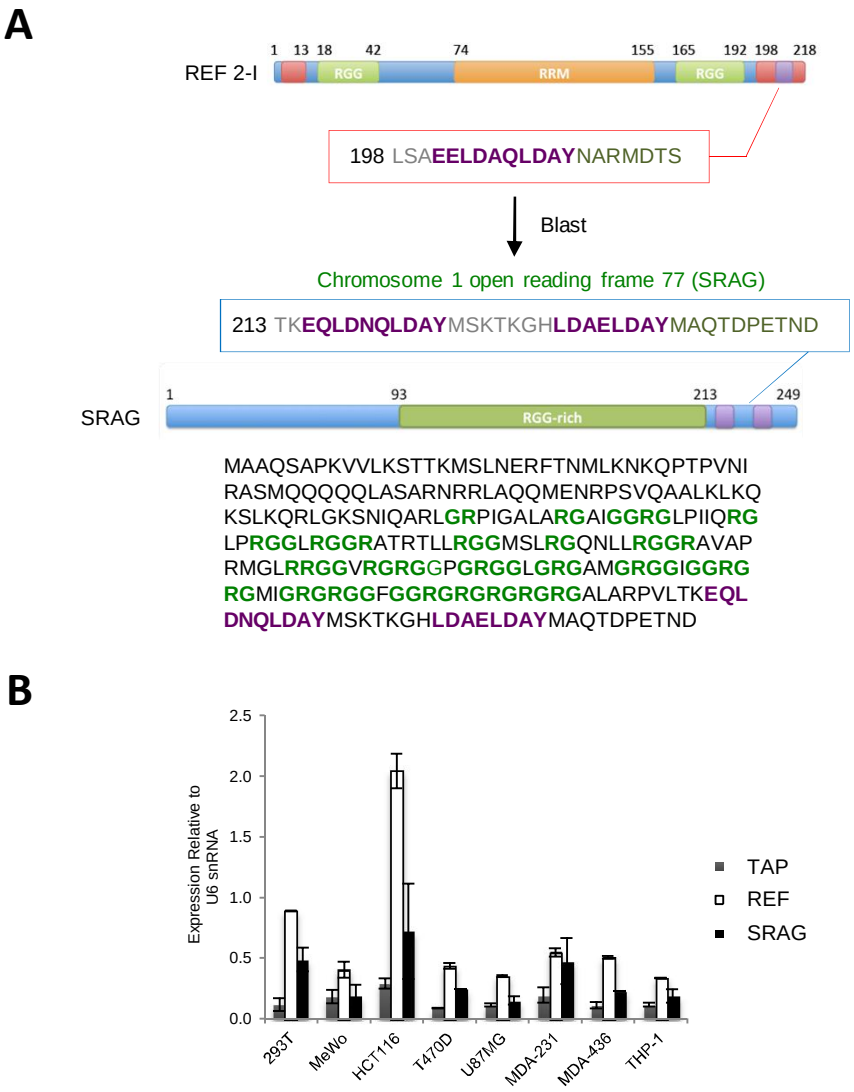
To identify export factors with functions possibly overlapping those of REF, we examined its interaction with UAP56. From a previous study, we knew that a C-terminal peptide of REFc (amino acids 198-218) was involved in binding UAP56. Using basic local alignment search tool (BLAST) database search with the C-terminal REF peptide, we identified an uncharacterized protein named Small protein Rich in Arginine and Glycine (SRAG; also known as FOP or CHTOP), which is identical to the predicted protein product

of the human C1orf77 gene (**Figure. 3-1A**) (Zullo et al., 2009; van Dijk et al., 2010b). SRAG has an expected molecular mass of 27 kDa (248 amino acids) and is highly conserved in all vertebrates (human SRAG: NP_056422, mouse SRAG: BAE31263, Xenopus SRAG: NP_001011271, zebrafish SRAG: NP_955840, and chicken SRAG: XP_424013), while no orthologs could be identified in yeast, worm, or flies. However, we cannot exclude the possibility that there may be highly divergent members of SRAG that nevertheless fulfill a similar function in other species. Secondary structure predictions suggest that SRAG lacks known conserved domains, but its central sequence contains an Arg/Gly-rich region (RGG-rich, amino acids 87-208), while the C-terminal region harbors a duplication of the sequence LDXXLDAY (where X is any amino acid) which is similar to the UAP56 binding peptide of REF.

To date, a small amount of information regarding SRAG has been deposited in the literature. However, in the course of characterizing proteins encoded in the human major histocompatibility complex class III region, yeast-two hybrid analysis detected an interaction between SRAG and UAP56 (Lehner and Sanderson, 2004). These studies imply that SRAG is a potential UAP56 binding protein. We used real time-PCR to detect the amount of endogenous SRAG in different cell-lines. The results show that SRAG is widely expressed in all tested cell-lines (**Figure. 3-1B**).

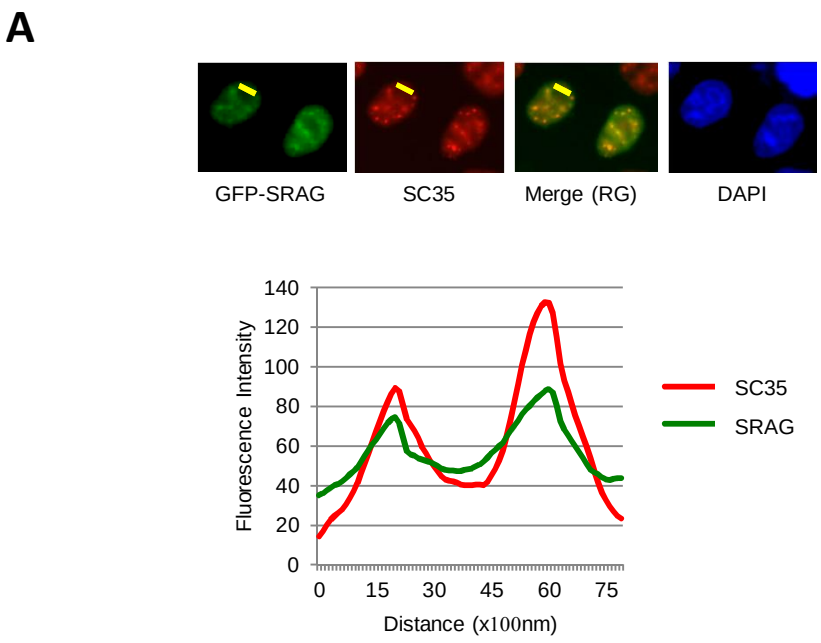
3.2 SRAG is a nuclear protein localised in nuclear speckles

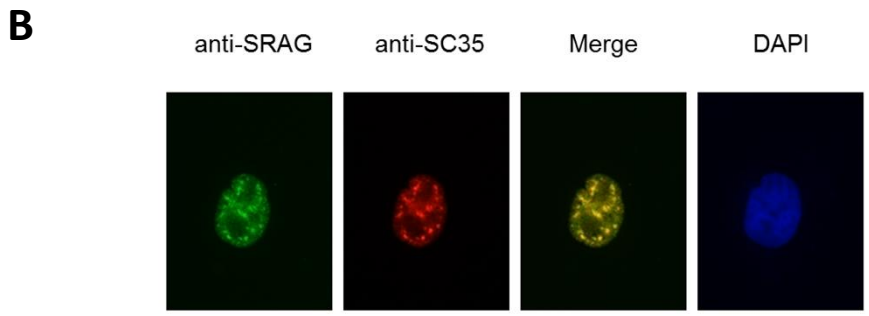
In order to determine whether SRAG had the potential to function as an export factor, initial experiments were to be focused on determining whether SRAG was a nuclear protein. The localisation of SRAG was analysed first by analysis of a GFP-SRAG fusion. As shown in **Figure. 3-2A**, we observed that SRAG (green) displayed a predominately nuclear localisation in HeLa cells. Moreover, SRAG co-localised in nuclear speckles with the splicing factor SC35 (red) where splicing factors are dominant. This distribution pattern was further confirmed by using SRAG antibody to detect endogenous SRAG (**Fig. 3-2B**).



[Figure. 3-1] SRAG has two potential UAP56 binding sites.

(A) To identify export factors with functions possibly overlapping those of REF, we examined its interaction with UAP56. From previous studies, we knew that a C-terminal peptide from REF is involved in binding UAP56. Through basic local alignment search tool (BLAST) database search with the C-terminal REF peptide we identified an uncharacterized protein named small protein rich in Arg and Gly (SRAG). The amino acid sequences of SRAG is shown (green: RGG-rich region; purple: REF similar peptides). **(B)** Quantitative RT-PCR analysis of the SRAG mRNA transcriptional level in different cell-liness. The ratio of cytoplasmic to total RNA normalized to U6 small nuclear RNA (snRNA) with values for control samples set at 100%. Error bars represent standard error of the mean for three or more experiments.





[Figure. 3-2] SRAG localises in nuclear speckles

(A) (Upper panel) HeLa cells were placed onto coverslips and transfected with GFP-SRAG for 48 hours. The fixed cells were then probed with anti-SC35 primary antibody and anti-mouse ALEXA555 (red). The cells were then imaged using fluorescence microscopy. (Lower panel) Using ImageJ to analyse the fluorescence intensity between two speckles (Bars, 7.5 μm). **(B)** Localization of SRAG in HeLa cells. Cells were stained with anti-SRAG and anti-SC35 antibodies.

Nuclear speckles (also known as the SC35 domain) has been traditionally considered as a storage site for splicing factors which are recruited to active genes for co-transcriptional splicing. However, nuclear speckle domains are not only the sites enriched in mRNA splicing machineries, but also contain mRNA export factors (for example, TREX complex) (Zhou et al, 2000). The localization of SRAG implies it might be involved in mRNA processing.

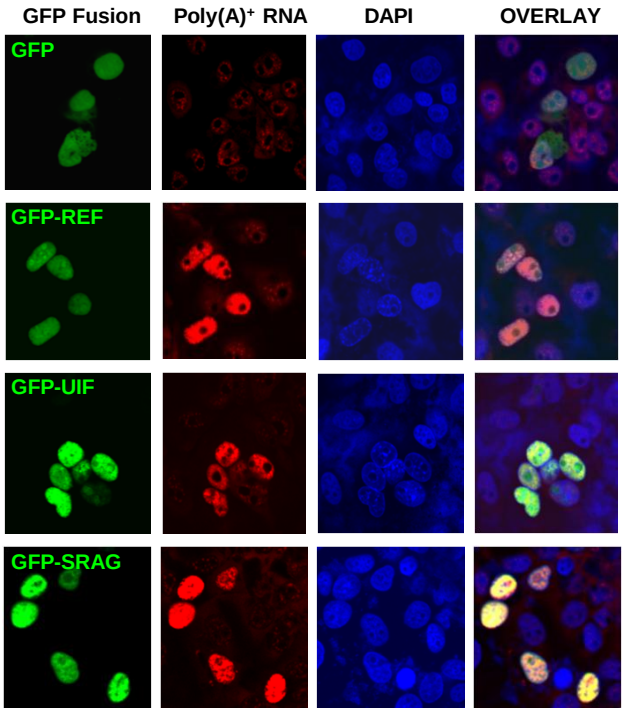
3.3 Overexpression of SRAG causes mRNA accumulation in the nucleus

To examine whether SRAG has an important function in mRNA export, overexpression experiments were performed. In these experiments, we used Fluorescence in situ hybridization (FISH) assays with Cy3-oligo dT probe to detect poly(A) RNA to infer the distribution pattern of mRNA and investigate changes after overexpressing GFP-SRAG in HEK-293T cells. After 48 hours of overexpression of GFP-SRAG, it is possible to observe a dramatic mRNA accumulation inside the nucleus (**Figure. 3-3**). Similarly, overexpression of GFP-REF or GFP-UIF caused an mRNA export block, whereas cells expressing the GFP control showed a normal mRNA distribution.

3.4 Double knockdown of SRAG and REF causes a major mRNA export block

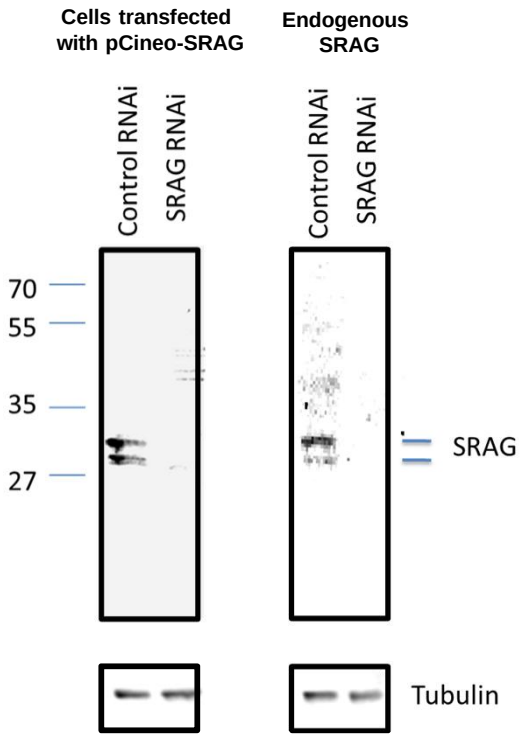
To obtain further evidence for the function of SRAG in mRNA export, development of an RNA interference (RNAi) system was considered necessary. The BLOCK-iT Pol II miR Expression vector system (Invitrogen) was used in this study. A vector specific for SRAG was constructed as detailed in materials and methods.

To test for knockdown of exogenous SRAG, HEK-293T cells were co-transfected with 350ng pCineo-SRAG and 350ng negative control RNAi or SRAG RNAi vector. The cells were then lysed 72 hours after transfection and an equal amount of each total lysate was used in western analysis. The results are shown in **Figure. 3-4**. SRAG was



[Figure. 3-3] Overexpression of SRAG causes mRNA accumulation in the nucleus.

Overexpression of GFP, GFP-REF, GFP-UIF, and GFP-SRAG in COS-7 cells leads to nuclear accumulation of poly(A)$^+$ RNA. Panels are shown at the same exposure. Poly(A)$^+$ RNA was detected via fluorescence in situ hybridization (FISH) with a Cy3-oligo-dT probe.



[Figure. 3-4] Tested of SRAG RNAi vector on exogenous and endogenous SRAG.

Western analysis was used to determine the effectiveness of the RNAi vectors at knocking down both exogenous and endogenous SRAG. For the exogenous experiment, co-transfections were carried out in HEK-293T cells with pCineo-SRAG and the appropriate RNAi vector and the cells were incubated for 72 hours. For the endogenous experiment only the appropriate RNAi vector was transfected and cells were incubated for 72 hours. In each lane an equal amount of total protein extract was loaded as shown by the tubulin loading controls.

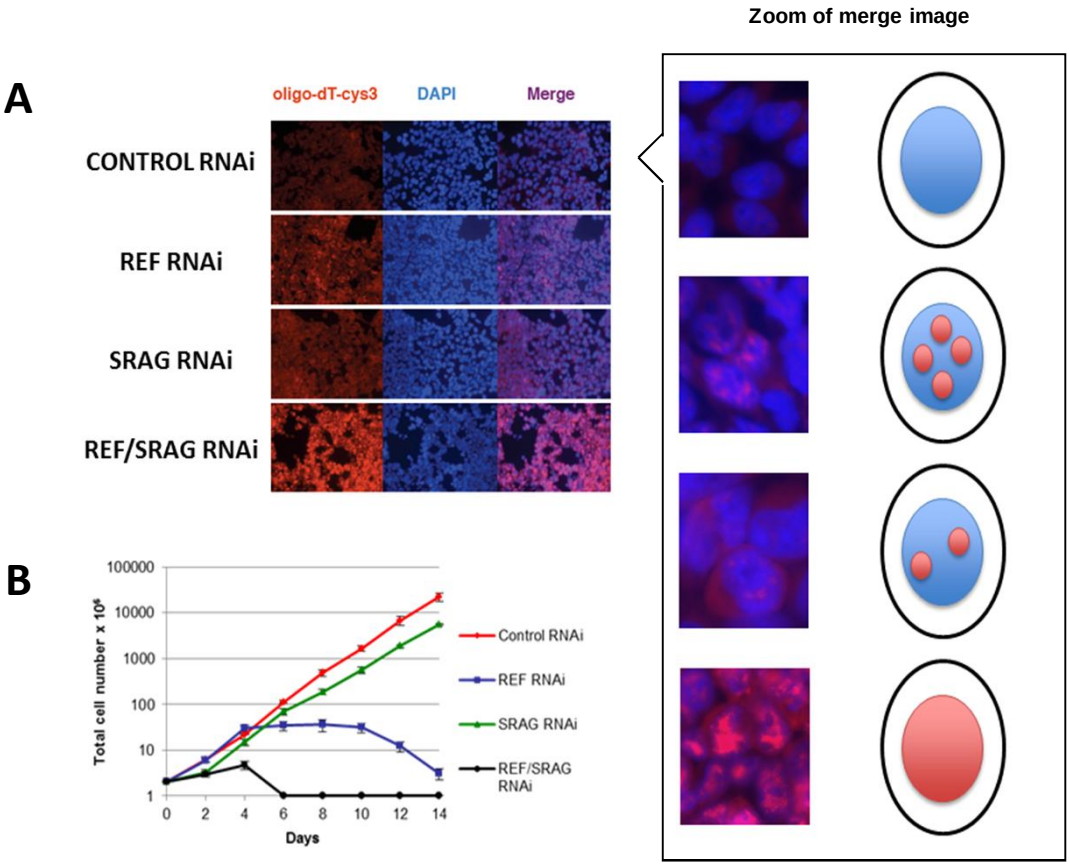
successfully knocked down by the SRAG RNAi vector. The same experiment was also repeated with endogenous SRAG.

After finding the SRAG RNAi target sequence, we constructed control, REF, SRAG, and REF/SRAG double RNAi FLP-IN 293T stable cell-lines. Fluorescence *in situ* hybridization assays were performed on the FLP-IN 293T cells after 96 hours siRNA induction, the results are shown in **Figure. 3-5A**. Cells depleted of REF showed a modest accumulation of poly(A)$^+$ RNA as reported previously (Katahira et al., 2009). In contrast, knockdown of SRAG gave a weak phenotype. However, double knockdown of REF and SRAG resulted in a robust mRNA accumulation in the nucleus, clearly visible in the majority of cells.

The cell growth curves shown in **Figure. 3-5B** are consistent with the overexpression SRAG FISH experiment. Knockdown of both REF and SRAG caused the cells to die after six days. These data indicate that REF and SRAG work together and play a crucial role in nuclear export of poly(A)$^+$ RNA.

3.5 SRAG is involved in mRNA export

Previous results implied that SRAG is involved in mRNA export processing. Therefore, we used a tethered mRNA export assay to test whether SRAG has the ability to export mRNA. In this assay, a reporter construct called Luc6xMS2 was used. This vector consists of a luciferase open reading frame (ORF) within an inefficiently spliced intron derived from the HIV-1 env region, which is constitutively expressed from a CMV promoter. Six MS2 operators are located adjacent to the 3' end of the luciferase ORF. MS2 is a bacteriophage RNA binding protein which binds to stem-loop structures present in RNA called operators (Koning et al. 2003). Under normal circumstances, any intron-containing pre-mRNA molecules would be retained within the nucleus while fully processed, mature mRNA would be exported to the cytoplasm and expressed. Since splicing removes the luciferase ORF, the fully spliced version of the transcript doesn't give rise to luciferase activity in the cytoplasm. However, if a protein actively involved in mRNA export is overexpressed, it may overcome the nuclear retention of the immature



[Figure. 3-5] Double knockdown of SRAG and REF causes blocking in bulk mRNA export.

(A) Localization of poly(A)$^+$ RNA following induction of miRNAs targeting export factors for 96 hr. All equivalent panels are shown at the same exposure. **(B)** Growth of stable cell lines following induction of miRNAs targeting the indicated genes. Error bars represent the standard deviation of three independent experiments.

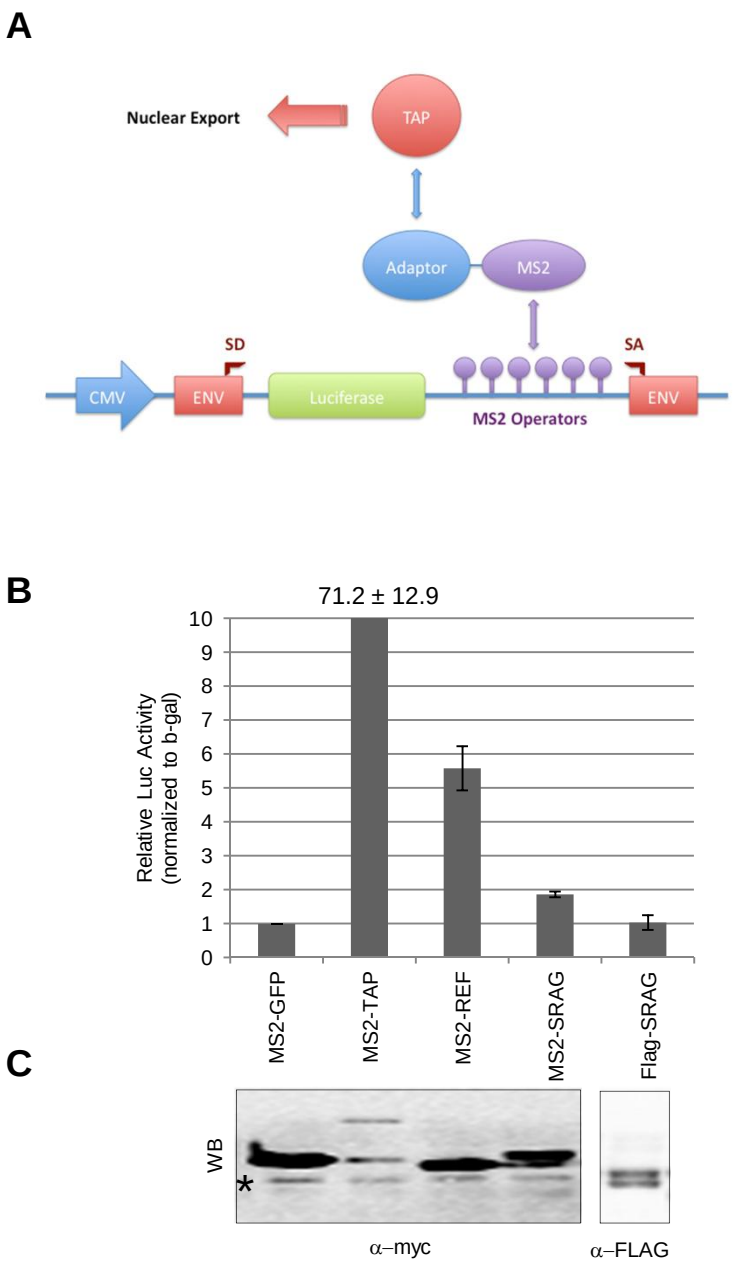
pre-mRNA and lead to its export and subsequent expression of the luciferase ORF. This effect can then be measured by assaying for luciferase activity present in total cell lysate.

Figure. 3-6 shows the results of the MS2 assay. The positive controls MS2-TAP and MS2-REF gave 71 and 5 fold activations respectively, corroborating previously reported results (Hargous et al., 2006). Only low levels of luciferase activity were detected for MS2-SRAG. All proteins were expressed at comparable levels. Compared to MS2-GFP and FLAG-SRAG, MS2-SRAG has weak activity for mRNA export, though it is less active than TAP and REF in this assay. However, the activity of MS2 assay is specific for mRNA export; therefore we conclude that SRAG is involved in mRNA export.

3.6 SRAG interacts with mRNA export factors *in vivo*

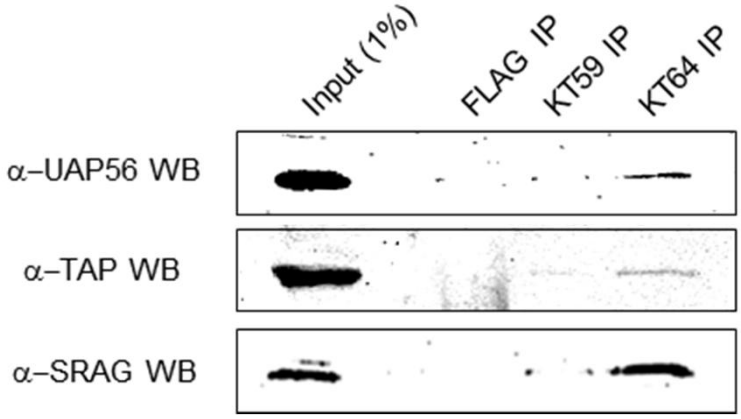
Given the role of SRAG in mRNA export, we next tested whether SRAG can interact with important export factors. We used two different SRAG monoclonal antibodies (KT59 and KT64) to immunoprecipitate endogenous SRAG from 293T cells. As a negative control Flag antibody was used in this experiment. The results showed that SRAG can successfully IP both UAP56 and TAP (**Figure. 3-7**). Because UAP56 and TAP are known to have a crucial function in mRNA export, the interaction between SRAG and the TREX complex components shown here suggest that SRAG may also function in mRNA export.

The ability of SRAG to bind both UAP56 and TAP was investigated via IP assays. To further systematically investigate the network of interactions between SRAG and export factors, co-immunoprecipitation (co-IP) experiments were performed. In these experiments, HEK-293T cells were co-transfected with FLAG-SRAG and 13-myc-tagged fusion proteins and allowed to grow for 48 hours before total cell lysates were obtained. These cell lysates were then applied to M2 flag-agarose in the presence or absence of RNase A. Any bound complexes were then eluted, resolved by SDS-PAGE and then blotted to nitrocellulose. The resultant membranes were then probed for either anti-myc or anti-flag antibodies.



[Figure. 3-6] Weak but specific mRNA export activity of SRAG

(A) Schematic of MS2 aassay system. **(B)** 293T cells were co-transfected with Luc6xMS2 and respective MS2-fusion proteins. Cells were lysed and luciferase assays were carried out 48 hours after transfection. The graph shows an average of three independent experiments carried in triplicate. A value of 1 was assigned to MS2-GFP and all other experiments were normalised to it. **(C)** Western analysis to detect MS2-fusion protein. The MS2-fusions are N-terminally myc tagged and detected using a myc antibody.



[Figure. 3-7] SRAG interacts with UAP56 and TAP *in vivo*.

Immunoprecipitation of SRAG from 293T cells extracts shows that SRAG is in complex with UAP56 and TAP *in vivo* (KT59 and KT64 are two SRAG monoclonal antibodies).

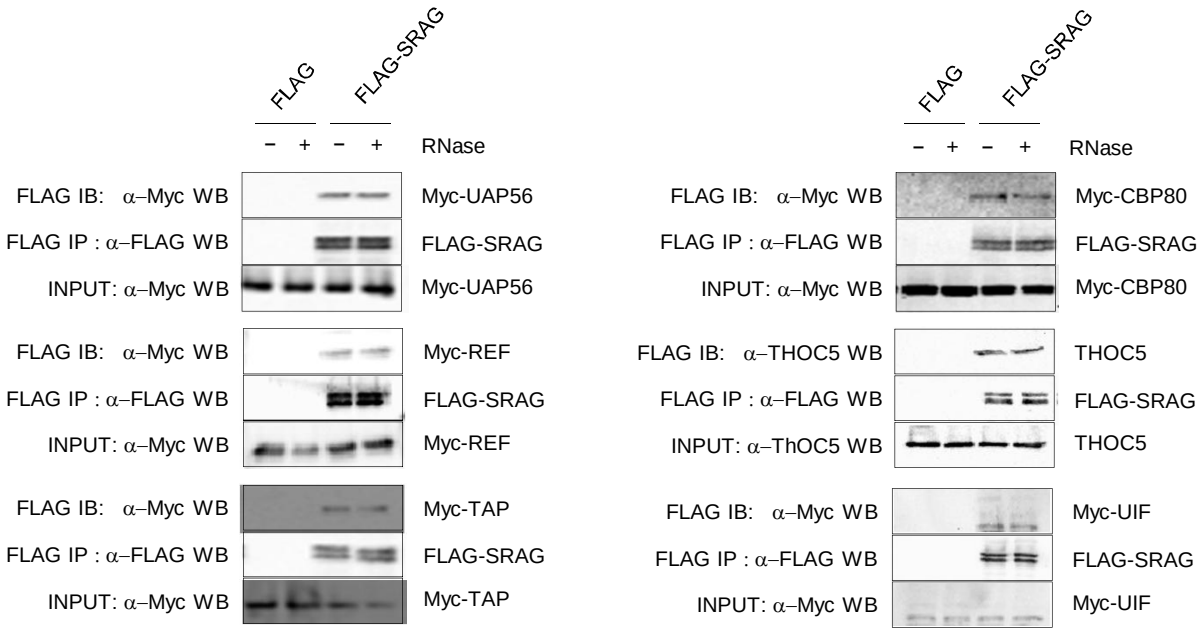
The results obtained from this analysis are shown in **Figure. 3-8**. We observed that SRAG coimmunoprecipitated a lot of mRNA export factors, including UAP56, TAP, REF, UIF, THOC5, and CBP80. These interactions were all insensitive to RNase A treatment. Taken together, SRAG interacts with lots of mRNA export factors *in vivo* consistent with a role for SRAG in mRNA processing and/or export.

3.7 SRAG can be methylated

In coimmunoprecipitation analysis, we noticed that there were two bands present when we overexpressed Flag-SRAG. This result implied that SRAG may be subject to post-translational modification. Since arginine methylation is reported to affect RNA binding affinity of REF (Hung et al., 2010), we investigated whether SRAG can be methylated. To test this possibility, extracts were prepared from HEK-293T cells transfected with FLAG-SRAG expression vector, which had been incubated with/without the methylation inhibitor adenosine dialdehyde (AdOx). Analysis of total extracts revealed a clear electrophoretic mobility shift for SRAG following the inhibition of methylation (**Figure. 3-9A**). We further tested whether SRAG could be phosphorylated, but the level of SRAG was not affected at any time point tested in the presence of the alkaline phosphatase (**Figure. 3-9B**).

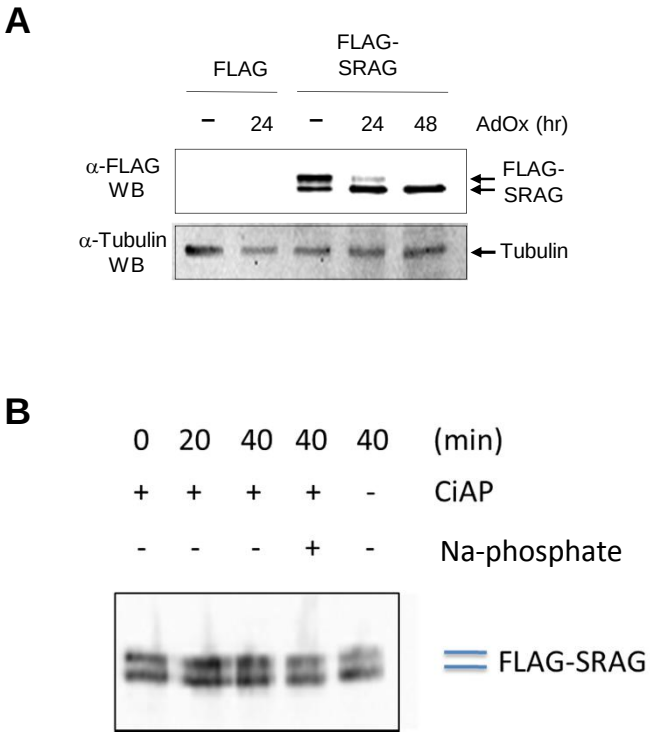
Although we showed that SRAG can be methylated, the unmethylated form of SRAG can only be detected by overexpression or AdOx treatment. However, it does not mean there is no unmethylated form of SRAG in the cells. Unmethylated SRAG may exist transiently so that it is difficult to detect.

Next, we treated 293T cells with cycloheximide for 8 hours to prevent new protein synthesis; after that, we lysed the cells with IP lysis buffer with/without AdOx. When we lysed cells with normal lysis buffer, there was only fully methylated SRAG present in the nucleus. However, if cells were lysed with the lysis buffer containing AdOx, we could successfully detect the unmethylated form of SRAG (**Figure. 3-10**). These results showed that both forms of SRAG exist *in vivo* and unmethylated SRAG is a transient state.



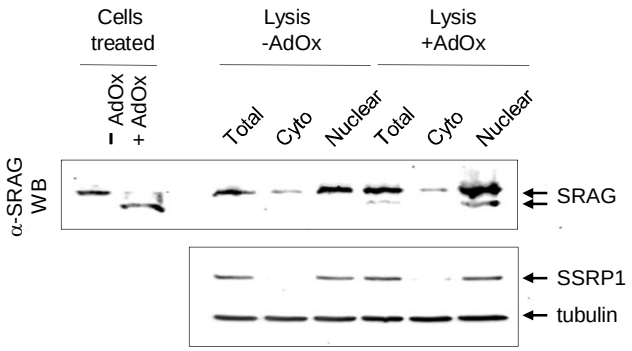
[Figure. 3-8] SRAG interacts with mRNA export factors.

Co-immunoprecipitation of SRAG and FLAG-tagged mRNA export factors under native conditions. Proteins were immunoprecipitated with FLAG antibody. The following abbreviations are used: IP, immunoprecipitation; WB, western blot.



[Figure. 3-9] SRAG can be methylated but not phosphorylated.

(A) FLAG-tagged SRAG was expressed in 293T cells grown in the presence or absence of AdOx for different time. After treatment, 10 μg of total cell extracts were analysed by western blot using anti-FLAG antibody. **(B)** Flag-tagged SRAG was expressed in 293T cells and treated with Ca^{++} inhibitional alkaline phosphatase (CIAP) as indicated. Na-Phosphate is used as a competitive inhibitor of CIAP.



[Figure. 3-10] Endogenous SRAG exists two different forms.

293T cells were incubated with 10 μg/mL cycloheximide for 8 hours. After treatment, Western blot analysis of 293T cell extracts (-/+ Adox, a competitive inhibitor of all S-Adenosyl Methionine dependent methylation) with an antibody (KT64) raised against SRAG.

3.8 SRAG interacts with PRMT1

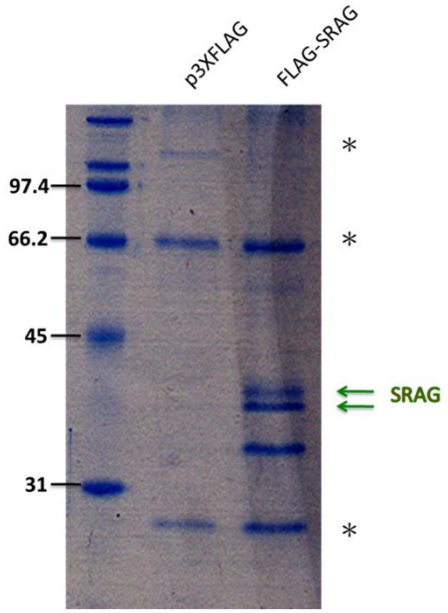
In humans, there are nine different arginine methyltransferases (PRMTs) that have been discovered. PRMTs divide into two groups: PRMT1, 3, 4, 6, and 8 all belong to type I asymmetric arginine methylation enzymes (Lin et al., 1996; Scott et al., 1998; Tang et al., 1998; Zhang et al., 2000; Frankel et al., 2002); whereas PRMT5, 7, and 9 belong to type II symmetric arginine methylation enzymes (Branscombe et al., 2001; Miranda et al., 2004; Cook et al., 2006). However, PRMT2 has not yet been classified (Scott et al., 1998).

To identify which PRMT enzymes can catalyse SRAG methylation, proteomic mass spectrometry was carried out with immunopurified FLAG-SRAG. 293T cells were transfected with FLAG-tagged SRAG for 48 hours, lysed with IP lysis buffer and immunoprecipitated by M2 FLAG-agarose. The eluted protein mixtures were analysed by mass spectrometry. The identification revealed that PRMT1 was present in the immunoprecipitate independently. Van Dijk et al. also showed that SRAG was a substrate for PRMT1 (van Dijk et al., 2010b) (**Figure. 3-11**).

From the mass spectrometry data, many partners of SRAG were defined. UAP56, importin-7, and importin subunit beta-1 are all involved in transport of molecules, however, there are also some factors involved in various other processes. For example, protein SET which is involved in apoptosis, transcription, nucleosome assembly, and histone binding. This evidence implies that SRAG may have multiple functions which could lead to further study of relationships between SRAG and gene regulation.

3.9 Summary

In this chapter, we used BLAST to find a functionally unknown TAP-interacting factor, SRAG. Immune-fluorescence assays showed that SRAG is a nuclear protein and co-localises with SC35. Overexpression of SRAG caused modest mRNA accumulation similar to other mRNA export adaptors. In contrast, when depleting SRAG, the phenotype was far less severe than knockdown of REF. However, knockdown of REF/SRAG showed huge mRNA accumulation in the nucleus, and cell growth curves also supported these results. This implies REF and SRAG work together. MS2 assays demonstrated SRAG has weak but



Strept. PD of FLAG-SRAG		
Name	Unique Peptides	MASCOT score
SRAG	**35**	**530**
Importin subunit beta-1	12	539
GAPDH	10	418
SET	9	296
UAP56	6	295
Importin-7	5	211
PABP1	6	136
PRMT1	**4**	**115**

[Figure. 3-11] Mass spectrometry analysis of the SRAG binding proteins.

Coomassie stained gel of a control (p3XFLAG) or FLAG-SRAG immunoprecipitate. (*: non-specific binding)

specific mRNA export activity. IP and co-IP assays indicated that SRAG interacts with lots of mRNA export factors *in vivo*.

Another interesting observation is that SRAG can be methylated. In 2010, Philipsen's group identified that SRAG can be methylated by PRMT1 (van Dijk et al., 2010b). This result supports our mass spectrometry data. Arginine methylation is a common post-translational modification in mRNA binding proteins. In earlier studies, we showed that arginine methylation of REF reduced its RNA binding affinity (Hung et al., 2010). This modification ensures mRNA can be efficiently displaced by TAP during mRNA export. Moreover, many proteins involved in mRNA export (e.g., hnRNP proteins and Y14) are arginine methylated. A number of these proteins may be regulated by methylation to switch the protein-protein and/or protein-RNA interactions as RNA processing proceeds. To understand whether arginine methylation plays a regulatory role for SRAG, more functional assays are needed.

Collectively, these observations indicate that SRAG is a novel mRNA export factor that functions in REF-mediated mRNA export. However, the biological function of SRAG is still unknown. In the next chapter, we will focus on further characterising SRAG and demonstrating its function.

Chapter IV

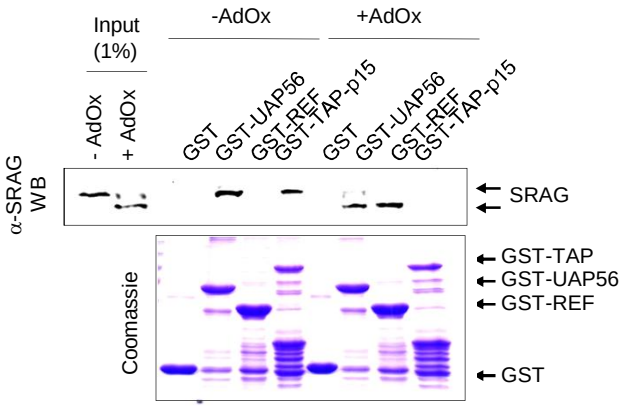
SRAG functions as a new mRNA export co-adaptor

In the previous chapter I showed that SRAG is a novel mRNA export factor and interacts with PRMT1. However, the molecular function of SRAG is still unclear. To further understand the function of SRAG, we continued to work on the relationship between methylation and SRAG.

4.1 Methylation regulates the protein-protein interaction activity of SRAG

To determine the influence of arginine methylation on SRAG, pull-down assays were used to analyse the interaction between SRAG and mRNA export factors. In these assays, GST tagged UAP56, REF, and TAP-p15 were used to pulldown endogenous SRAG from the extracts of HEK-293T cells cultured in the presence or absence AdOx. The results are shown in **Figure. 4-1**. It was noticeable that the SDS-PAGE mobility of SRAG changed in the presence of AdOx. This change in mobility suggests that AdOx treatment was successful in inhibiting arginine methylation. Analysis of pulldown assays indicated GST-UAP56 can interact with both forms of SRAG. Interestingly, GST-TAP can only pulldown methylated SRAG but not the unmethylated one; in contrast, GST-REF cannot pulldown the methylated form of SRAG but interacts with the unmethylated form.

To gain a precise understanding of the interactions between mRNA export factors and SRAG, we decided to purify recombinant SRAG proteins expressed in E.*coli* and GST pulldown assays were performed. In order to characterize whether methylation influenced the interactions between SRAG and mRNA export factors, we decided to



[Figure. 4-1] Methylation affects protein:protein interactions between SRAG and REF and TAP *in vivo*.

GST-UAP56, GST-REF, and GST-TAP were used in pull-down assays with 293T cell extract with/without AdOx in the presence of RNase A. Proteins were detected via Coomassie staining and Western Blot.

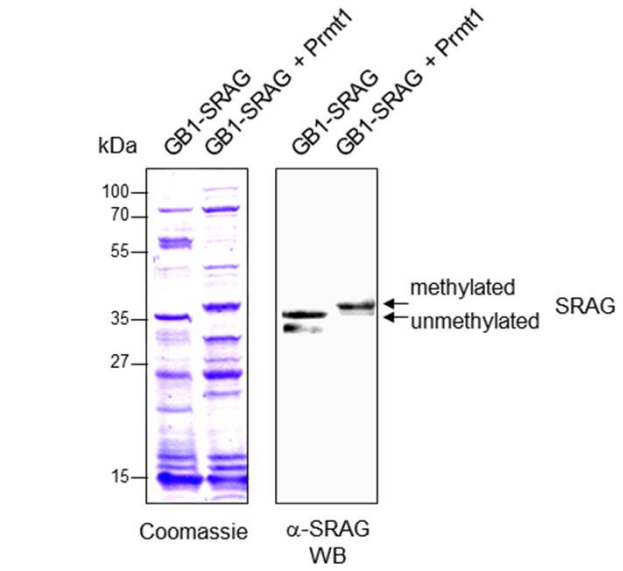
create GB1 tagged SRAG fusion protein. Immunoglobulin-binding domain of streptococcal protein G (GB1; 56 residues) is the solubility-enhancement tag and also increased the sample stability (Huth et al, 1997). We purified GB1-SRAG with/without co-expressed PRMT1 to produce unmethylated or methylated SRAG for use in *in vitro* GST pulldown assays (**Figure. 4-2A**).

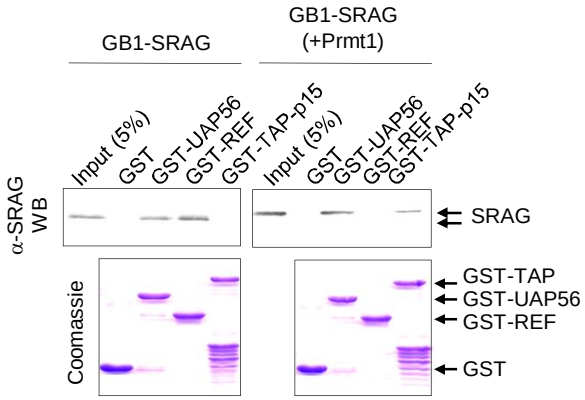
Through this assay, we found SRAG can form a direct interaction with UAP56, REF and TAP. GST-tagged UAP56, REF and TAP were used as the bait while purified methylated/unmethylated GB1-SRAG was used as the prey protein. The results of this experiment are detailed in **Figure. 4-2B**. As in previous experiments, it was observed that UAP56 can pull down both forms of SRAG. However, GST-REF only pulled down unmethylated SRAG and GST-TAP only interacted with methylated SRAG. Collectively, our results indicate that SRAG can directly interact with UAP56, REF and TAP and that the interaction was regulated by arginine methylation. An important implication from these results is that SRAG acts as a regulator molecule. By methylation, it can bridge the interaction between the mRNA export adaptor, REF, and the receptor, TAP.

4.2 Methylation reduced the RNA-binding affinity of SRAG

Having established that SRAG can interact with UAP56, REF and TAP directly, we tested for whether SRAG could interact with RNA *in vivo*. The experimental approach undertaken was the mRNP capture assay. In this assay, in vivo UV cross-linking was carried out on HEK-293T cells. The UV crosslinking step led to formation of direct links between proteins and RNA molecules which were in close enough proximity with each other in the cell (~2-3Å).

To determine the influence of arginine methylation on the ability of SRAG to bind RNA, we took HEK-293T cells, which had been grown for 48 hours with/without AdOX, and crosslinked using UV radiation. Cells were then lysed and the lysates were applied to oligo-d(T) cellulose and allowed to bind under denaturing conditions. The purification enriched for poly(A) RNA and proteins crosslinked to the RNA were eluted by the action of RNase A and then detected by western blots.





[Figure. 4-2] Methylation affects protein:protein interaction between SRAG and REF and TAP *in vitro*.

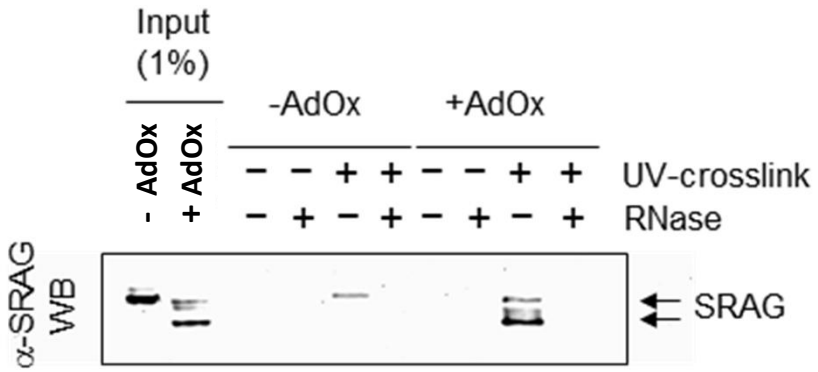
GST-UAP56, GST-REF, and GST-TAP were used in pull-down assays with recombinant SRAG alone or co-expressed with PRMT1 in the presence of RNase A. Proteins were detected via Coomassie staining and Western Blot.

Figure. 4-3 shows the results of the Western analysis. Both forms of SRAG were eluted from the oligo-d(T) cellulose after UV crosslinking, indicating that SRAG could associate directly with poly(A) RNA in the cell. Whilst SRAG showed a clear UV-dependent crosslink with RNA when methylated, the unmethylated SRAG UV cross-linked with RNA much more efficiently. We measured the difference of the yield of crosslinked RNA between two forms of SRAG. The results demonstrated that there was a ~3-fold increase in SRAG crosslinked RNA when cells were AdOx-treated and had reduced arginine methylation. Since the Adox treatment of cells does not block methylation completely and methylated arginine involved in RNA crosslinking can still be detected, it is likely that the significance of methylation on SRAG binding to RNA is greater than the effect observed

4.3 Characterising the binding regions of SRAG

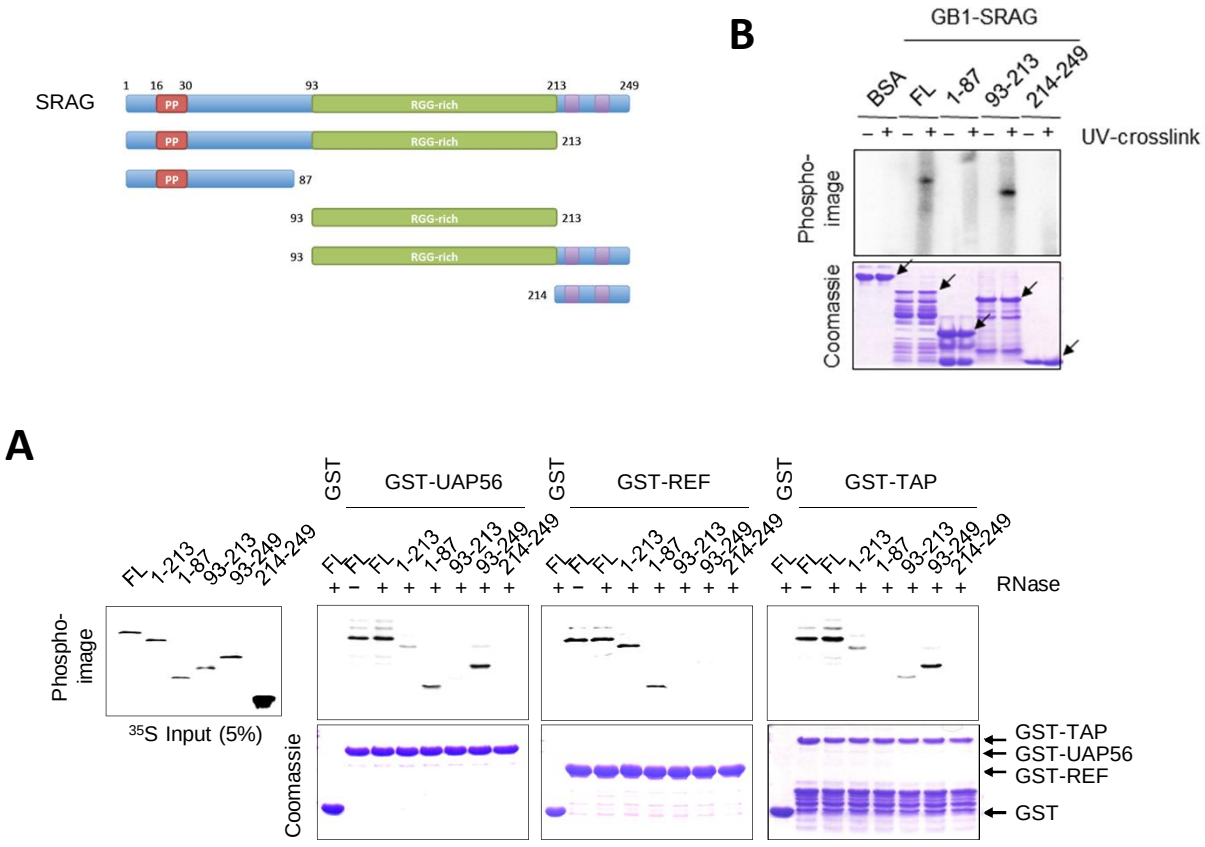
To further characterise which regions of SRAG were required for interaction with mRNA export factors, GST-UAP56, GST-REF, and GST-TAP fusion proteins were used in pulldown assays with various SRAG truncations. To establish which regions of SRAG conferred binding activity, GB1-tagged SRAG recombinant truncation constructs were generated. The plasmids were transformed into competent BL21 RP *E. coli* cells and purified using protein G beads. GST fusion proteins were immobilized on glutathione sepharose (GSH sepharose) and used as bait while various ^{35}S-labeled GB1-SRAG truncations (synthesized in rabbit reticulocytes) were used as the prey proteins.

In **Figure. 4-4A,** we observed that UAP56 interacted with N-terminal and C-terminal fragments (amino acids 1-87 and 93-249) of SRAG, but not with a construct containing its RGG-rich region (amino acids 93-213). Surprisingly, amino acids 214-249 of SRAG contain the regions similar to UAP56 binding region of REF. However, this peptide did not appear to bind UAP56. It is possible that this region (amino acids 214-249) is too small to be able to form the appropriate structure which allows interaction with UAP56. In contrast, TAP can only bind to the RGG-rich region and REF can only interact with the N-terminal but not to other regions of SRAG**.**



[Figure. 4-3] Methylation increases SRAG:RNA interaction

mRNP capture assay. Poly(A)$^+$ RNA from 293T cells -/+ AdOx was purified on oligo-dT beads in denaturing conditions after UV cross-linking (+) or not (–). Total extract (1% of input) and eluted proteins were analysed by western blotting (WB) with α-SRAG antibody.



[Figure. 4-4] Chracterization of the binding regionsof SRAG to UAP56, REF, TAP, and RNA

(A) GST-UAP56, GST-REF, and GST-TAP pulled down ^{35}S-labelled SRAG full-length and truncations. **(B)** GB1-SRAG full-length, aa 1-87, 93-213, and 214-249 UV-crosslinked to ^{32}P-labelled RNA.

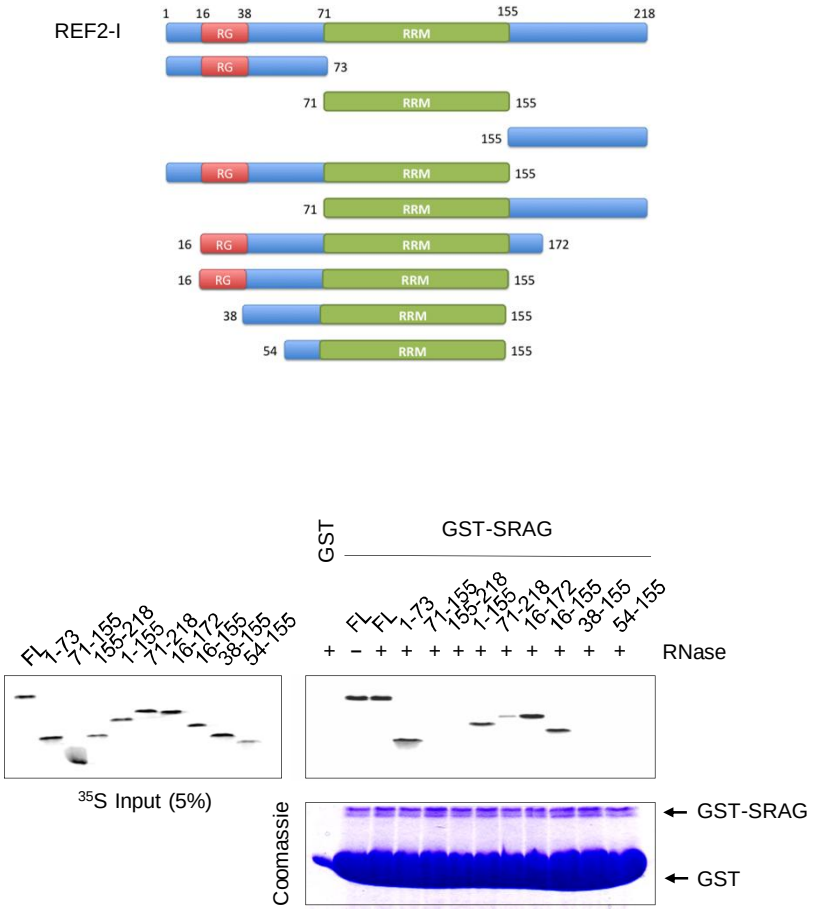
Next, we set out to determine whether SRAG binds to RNA directly and which regions of SRAG were involved in the interaction with RNA. Each binding reaction was set up in duplicate (with/without UV crosslinking) using 1 μL of radio-labeled 15mer RNA (5'-CAGUCGCAUAGUGCA-3'). The mixtures were incubated for 10 min at room temperature. After the incubations, the samples were UV crosslinked and the complexes were resolved by SDS-PAGE before being exposed as phosphoimages.

As shown in **Figure. 4-4B**, the results observed indicate that SRAG could bind to RNA directly through its RGG-rich (amino acids 93-213). Interestingly, the minimum TAP-binding region of SRAG is also the RGG-rich region, implying SRAG may coordinate the mechanism of mRNA handover to TAP through mutually exclusive interactions involving this region. However, in contrast to other *in vivo* and *in vitro* data the ^{35}S-labelled SRAG produced by TNT system (Promega) can bind to both REF and TAP. As we described before, REF and TAP interact with different forms of SRAG. The fact that the TNT-produced SRAG can interact with both proteins in this assay means it is difficult for us to definitively conclude whether the ^{35}S-labelled SRAG is methylated or not.

4.4 SRAG and REF bind to different regions of TAP

After establishing the important mRNA export factor binding regions of SRAG, we focused on determining SRAG binding sites on REF. As previously described, we used GST-SRAG to pulldown ^{35}S-labelled GB1-REF truncations. The results show that SRAG interacts with the RG box (amino acids 16-79) of REF, which is also required for TAP binding (**Figure. 4-5**). To understand the relationships between SRAG, REF, and TAP was the next target.

Because both SRAG and REF bind to TAP, I tried to define which regions of TAP were required for the interaction to determine the functionality of SRAG. In this experiment, TAP was separated into two regions: the N-terminal region (amino acids 1-198) which is known to be involved in adaptor binding (e.g., REF, 9G8, or SRP20) and the C-terminal region (amino acids 204-619) which interacts with p15 and mRNA export co-adaptors (e.g., THOC5 and RBM15B).



[Figure. 4-5] REF binds to SRAG through its N-terminal region

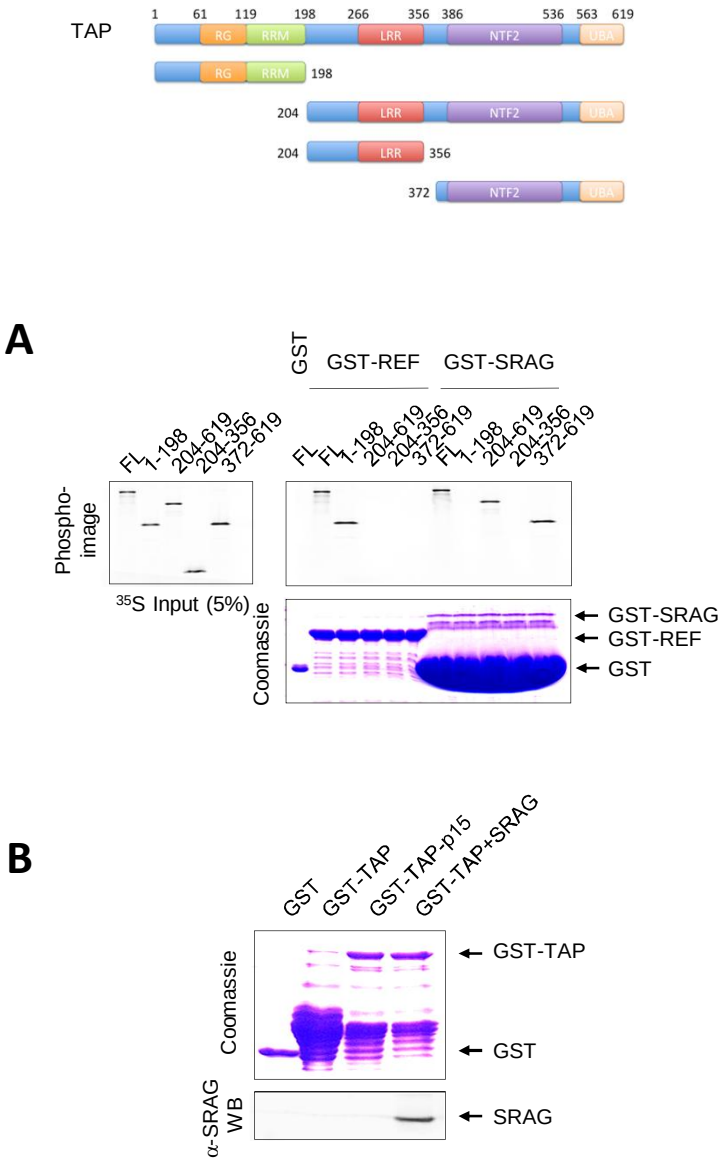
GST-SRAG was able to pull down REF full-length and aa 1-73, 1-155, 71-218, 16-172, and 16-155.

As described before, we analysed the ability of GST-REF and GST-SRAG to pull down ^{35}S-labeled TAP truncations. As expected, GST-REF pulled down the N-terminal part of TAP; however in contrast, we found that SRAG bound to the C-terminal region of TAP (**Figure. 4-6)**. Importantly, these results imply that SRAG is not an mRNA export adaptor but a co-adaptor. Furthermore, the previous tethering assays also supported this conclusion that the mRNA export activity of SRAG is not as efficient as REF (**Figure. 3-6**).

4.5 SRAG and REF bind concomitantly to the TAP-p15 heterodimer

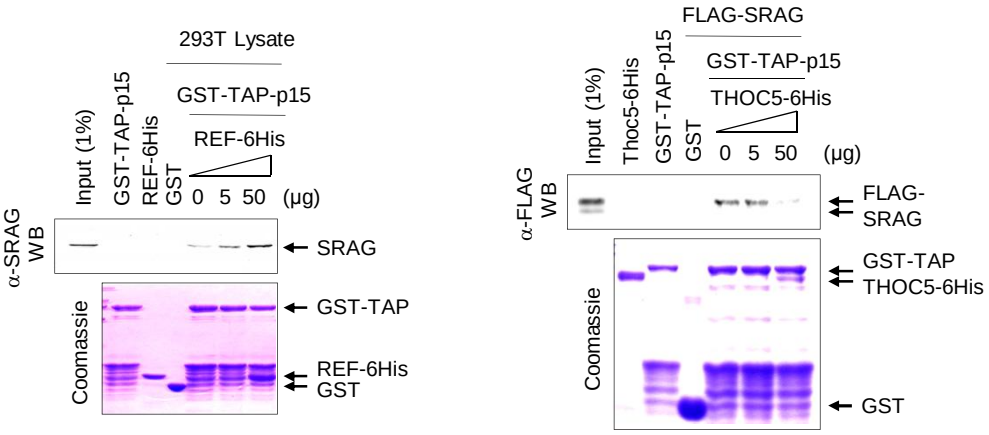
Previous results imply that SRAG plays a co-adaptor role in mRNA export. Although SRAG and REF bind to different domains within TAP-p15, it is possible that they bind concomitantly to the mRNA export receptor. To confirm this suggestion, we used purified REF and THOC5, which is a component of TREX and functions as an mRNA export co-adaptor (Katahira, et al., 2009), to perform the competition assays. In these assays, HEK-293T cell extracts or cell extracts transfected with FLAG-SRAG was pre-incubated with increasing amounts of purified REF-6His or THOC5-6His to compete SRAG, respectively, and added to GST-TAP immobilized on GSH beads.

Figure. 4-7 shows that SRAG was not competed by REF. Moreover, REF could enhance the binding affinity of SRAG to TAP implying that SRAG, REF, and TAP can assemble into a complex and SRAG may work co-operatively with REF; yet, the TAP interaction with SRAG and THOC5 appears to be mutually exclusive, suggesting that the binding surfaces partially overlap. Alternatively, THOC5 may interfere with SRAG binding as the result of steric hindrance. Because SRAG can only be competed by THOC5 but not REF, our observation further demonstrates that SRAG is an mRNA export co-adaptor but not an adaptor.



[Figure. 4-6] SRAG binds to C-terminal region of TAP

(A) GST-REF and GST-SRAG pulled down ^{35}S-labelled TAP full-length, aa 1-198, 204-619, and 372-619. (B) Purified GST-TAP, GST-TAP+p15, and GST-TAP+SRAG from *E. coli*.



[Figure. 4-7] SRAG and THOC5 binds at the same region of TAP

Pull-down competition assay with GST-TAP-p15, 293T lysate (or overexpressed FLAG-SRAG), and increasing amounts of purified GB1-6His-REF2-I (or THOC5-6His) in the presence of RNase. Proteins were detected via Coomassie staining or Western Blot.

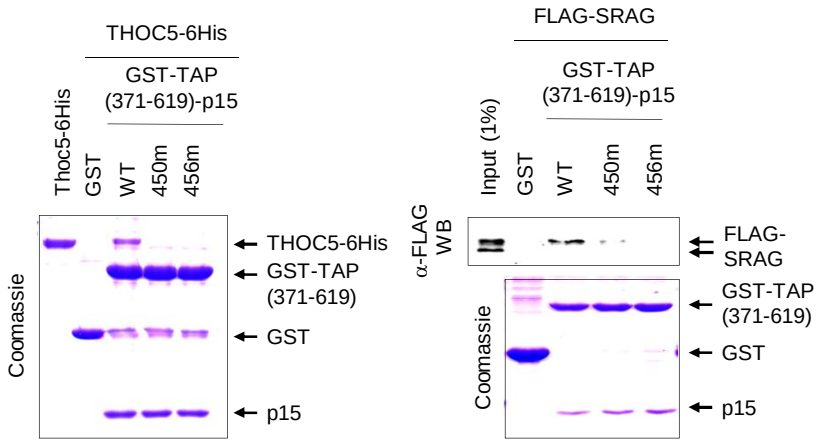
4.6 SRAG and THOC5 bind at the same sites on TAP

To further investigate whether SRAG and THOC5 form mutually exclusive interactions with TAP, we decided make use of the TAP mutations known to prevent interaction with THOC5 and new pulldown assays were carried out. In 2009, Yoneda's group defined four surface residues of TAP involved in the interaction with THOC5. The quadruple alanine substitutions of TAP (450m: aa 450-453; 456m: aa 456-459) effectively disrupted binding to THOC5, but p15 binding was unaffected (Katahira et al., 2009). To determine whether SRAG competes with THOC5 for the same binding surface, we generated these two mutants within a GST-TAP fragment containing the NTF2-like domain (amino acids 371-619) and carried out pulldown assays. Either purified THOC5-6His or HEK-293T cell extracts from cells transfected with FLAG-SRAG in the presence of RNase A was added to examine whether SRAG and THOC5 bind at the same sites on TAP.

In competition assays, the two TAP mutant fragments did not interact with THOC5, as expected. However, SRAG cannot bind to the TAP mutants either (**Figure. 4-8**). Since p15 still binds to the mutant forms of TAP this indicates there has not been a general disruption of the protein fold. Our results suggest that SRAG and THOC5 bind at the same surface on TAP. After combining the results from competition assays, we conclude that SRAG is a new mRNA export co-adaptor.

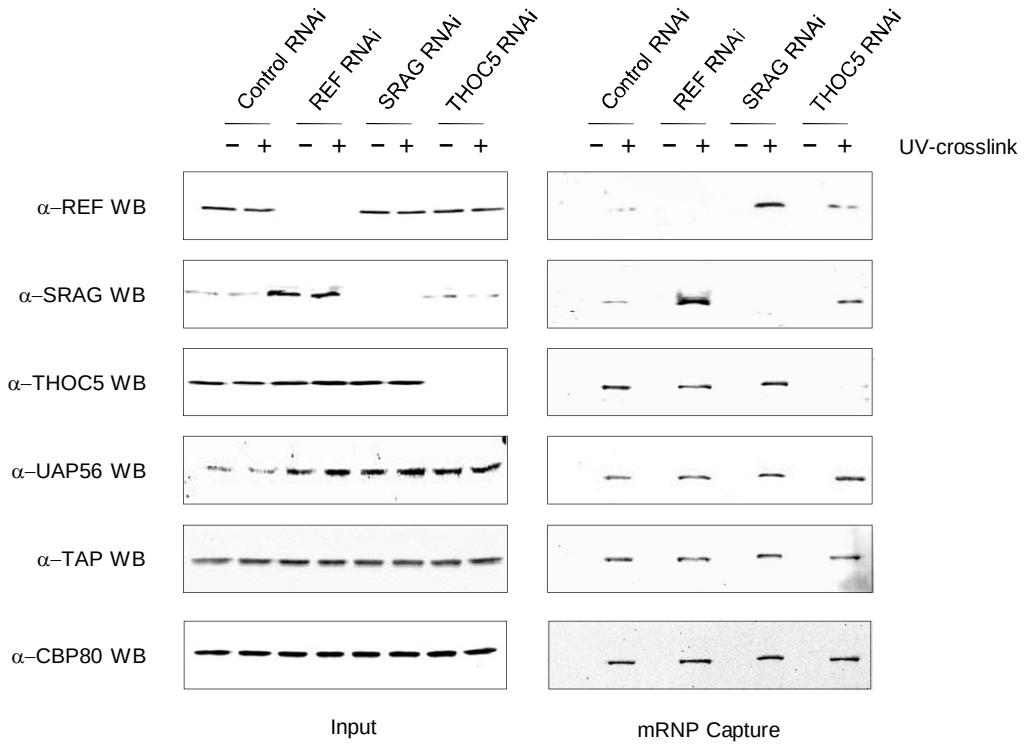
4.7 SRAG stimulates mRNA handover from REF to TAP

After the encouraging results outlined in the previous experiments, the biological function of SRAG was investigated further. In RNAi knockdown assays, we showed that co-depletion of SRAG and REF cause a huge mRNA block in the nucleus. These results imply SRAG and REF may work together. To understand the relationship between SRAG and REF, we tested REF, SRAG, and THOC5 RNAi stable cell-line extracts by western blot. As shown in **Figure. 4-9**, in REF knockdown cell-line, SRAG protein level was higher than other knockdown cells. This result strongly suggests that cells increase SRAG level to compensate REF knockdown. As suspected, UAP56 increased in all knockdown cell-lines. In contrast, the protein level of REF is stable in all knockdown cell-lines. We also



[Figure. 4-8] SRAG and THOC5 bind at the same site on TAP

Pull-down assays using GST-TAP(371-619)-p15 wild type or mutants with purified THOC5-6His or 293T cell extracts from cells transfected with FLAG-SRAG in the presence of RNase A. Proteins were detected by Coomassie staining or Western Blot.



[Figure. 4-9] SRAG and REF works in the same pathway

Poly(A)$^+$ RNA from stable FLP-IN 293T cells, expressing Control RNAi, REF RNAi, SRAG RNAi, or THOC5 RNAi, was purified on oligo-dT beads in denaturing conditions after UV cross-linking (+) or not (–). Total extract (1% of input) and eluted proteins were analysed by Western Blotting (WB) with α-ALY, SRAG, THOC5, UAP56, TAP, or CBP80 antibody.

performed mRNP capture assays in RNAi stable cell-lines. Interestingly, knockdown of SRAG led to an accumulation of REF associated with mRNA *in vivo*. The accumulation of REF on mRNA may result from the failure to transfer RNA from REF to TAP during mRNA export. Consistent with this idea is the observation that SRAG and REF cooperate with each other to interact with TAP. SRAG also accumulated on mRNA in response to REF RNAi (~10.7 fold); however, on the level of SRAG increased in the input samples (~4.3 fold) it is not clear whether the increased levels of SRAG on the mRNA were simply caused by higher levels of SRAG in the cell or by genuine accumulation of SRAG on the mRNP.

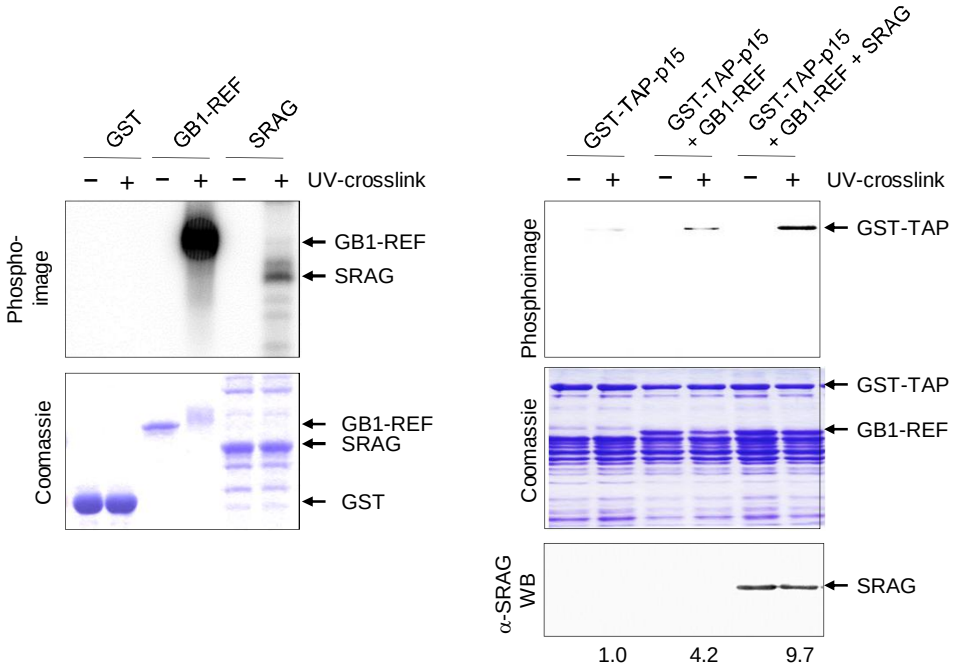
During export, once REF is loaded onto mRNA, it subsequently recruits TAP. mRNA is displaced from REF by TAP, which in turn binds directly to the mRNA. REF bound to TAP also enhances TAP RNA-binding activity (Hautbergue et al., 2008). Since the data presented above shows that SRAG is an mRNA co-adaptor working with REF, to better understand the function of SRAG's contribution to mRNA export, we performed the remodeling assay to test if SRAG can stimulate mRNA handover from REF to TAP.

In this experiment, GB1-REF was first incubated with ^{32}P-radiolabeled RNA; and then, GST-TAP-p15 with/without SRAG was added to the REF-RNA complexes. After protein–RNA complexes were eluted, bound RNA was UV-cross-linked. Eluted complexes were analyzed by Coomassie blue, Western Blot and PhosphorImaging.

The results indicate that TAP-p15 showed a weak cross-link with RNA and adding REF would increase mRNA handover from REF to TAP. Interestingly, when adding SRAG into REF-RNA complex mixed with TAP, we found SRAG increased the amount of TAP RNA UV-crosslinking ~10 fold (**Figure. 4-10**). Taken together, these data show that SRAG and REF synergistically enhance the RNA binding activity of TAP.

4.8 Summary

In this chapter, we showed that arginine methylation not only reduced RNA-binding activity, but affects the protein-protein interactions of SRAG. This observation unravels a novel way to regulate mRNA export factors. Using pulldown assays, we found that



[Figure. 4-10] SRAG enhances mRNA handover from REF to TAP

In vitro remodelling assay. GB1-REF was first incubated with ^{32}P-radiolabeled RNA; then, GST-TAP-p15 with/without SRAG were added to the REF-RNA complexes. After protein–RNA complexes eluted, bound RNA was UV-cross-linked. Eluted complexes were analyzed by Coomassie blue, Western Blot and PhosphorImaging.

SRAG and REF bind in the different regions of TAP. In contrast, SRAG and THOC5 interact with TAP at the same surface. Therefore, we conclude SRAG functions as a new mRNA export co-adaptor but not an adaptor.

The earlier studies on mRNA export co-adaptors did not indicate how co-adaptors help mRNA export processing. To answer this question, we began to study the relationship between adaptor and co-adaptor. Here, we used western blots and mRNP capture assays to examine the protein expression level in different RNAi knockdown stable cell-lines. Through these results, we confirmed that SRAG and REF affect each other and mRNA transfer might be the answer. This is consistent with the earlier FISH assays, double knockdown REF and SRAG cause strong mRNA blocking.

Finally, we used *in vitro* remodeling assay to prove that SRAG can help REF to remodel TAP so that it binds RNA with higher affinity. These results indicate both proteins are required for optimal TAP:mRNA interactions.

Chapter V

TREX assembly is driven by UAP56 dependent ATP hydrolysis

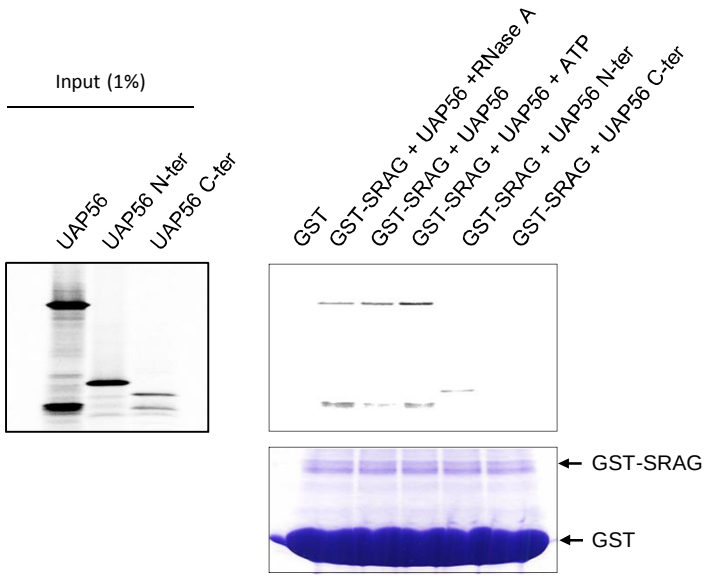
In this chapter, I further investigated the biochemical functions of SRAG, in particular the function relationship between SRAG and UAP56 and the role of SRAG in TREX assembly.

5.1 SRAG and REF cannot interact with UAP56 simultaneously

In the previous chapter it was shown that SRAG can bind to UAP56 directly. The DEXD/H-box helicase UAP56 is essential for the export of the majority of mRNAs from the nucleus to the cytoplasm. REF also interacts with UAP56, we were interested in how SRAG binds to UAP56 and exploring the relationship between SRAG and REF binding to UAP56.

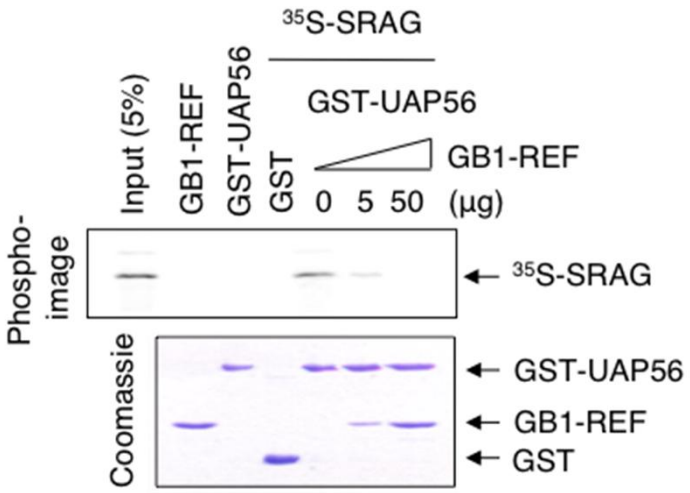
To investigate this further, we analysed which regions of UAP56 were required for the interaction with SRAG. First, we characterized SRAG binding using GST pulldown assays. GST-SRAG was incubated with ^{35}S-labeled UAP56 N-terminal and C-terminal truncations and pulled down by GSH beads. Interestingly, SRAG and REF both bind at the N-terminal region of UAP56 (**Figure. 5-1**). These results suggest that SRAG and REF may not bind to UAP56 at the same time.

To confirm this, competition assays were performed. GST-UAP56 was used to pull down ^{35}S-SRAG before adding increasing amounts of purified GB1-REF to the UAP56:SRAG complex. As shown in **Figure. 5-2**, when increasing the amount of REF, SRAG dissociated from UAP56. These results indicate that SRAG and REF cannot interact with UAP56 at the same time.



[Figure. 5-1] SRAG binds to the N-terminal region of UAP56

GST and GST-SRAG were used in pull-down assays with ^{35}S-labeled UAP56 full-length or truncations. Proteins were detected via Coomassie staining and phosphoimage.



[Figure. 5-2] REF and SRAG can not bind to UAP56 at the same time

Pull-down competition assay with GST-UAP56 complexed with S^{35}-labeled SRAG and increasing amounts of GB1-6His-REF2-I. Proteins were detected via Coomassie staining and phosphoimage.

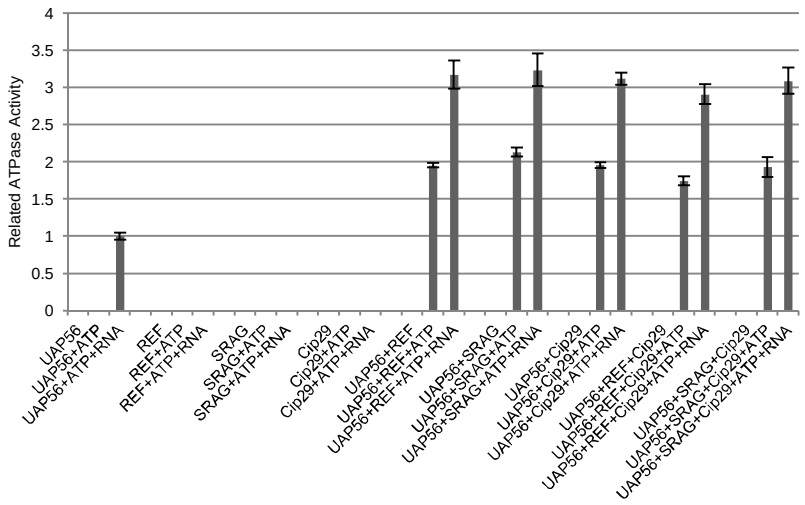
5.2 SRAG stimulates ATPase and helicase activity of UAP56

In isolation, UAP56 has a weak ATPase and helicase activity that is similar to eIF4A alone. In the case of eIF4A, the presence of eIF4B and eIF4H stimulates eIF4A's ATPase (~3-fold) and helicase activity (4–8-fold) (Richter-Cook et al., 1998; Rogers et al., 2001). These stimulations likely contribute to the ability of eIF4A to efficiently unwind secondary structures in the 5'-untranslated region of the mRNA. We evaluated here whether UAP56's ATPase and helicase activity can be affected by protein factors known to interact with UAP56.

To test whether SRAG stimulates ATP hydrolysis, we performed biochemical characterization of UAP56's ATPase and helicase activity. We first examined UAP56's ATPase activity. His-tagged UAP56 was expressed in *E. coli*. UAP56-6His was first purified with Co^{2+} resin and then was further purified using gel filtration chromatography. Purified UAP56-6His was incubated at 37 °C with ATP, in the presence or absence of mRNA, and REF, SRAG, and CIP29. The amount of ATP hydrolyzed was quantified on ELISA plate reader. The results show that UAP56 has an RNA-stimulated ATPase activity and not only SRAG but also REF and CIP29 can robustly activate the ATPase activity of UAP56 (**Figure. 5-3**).

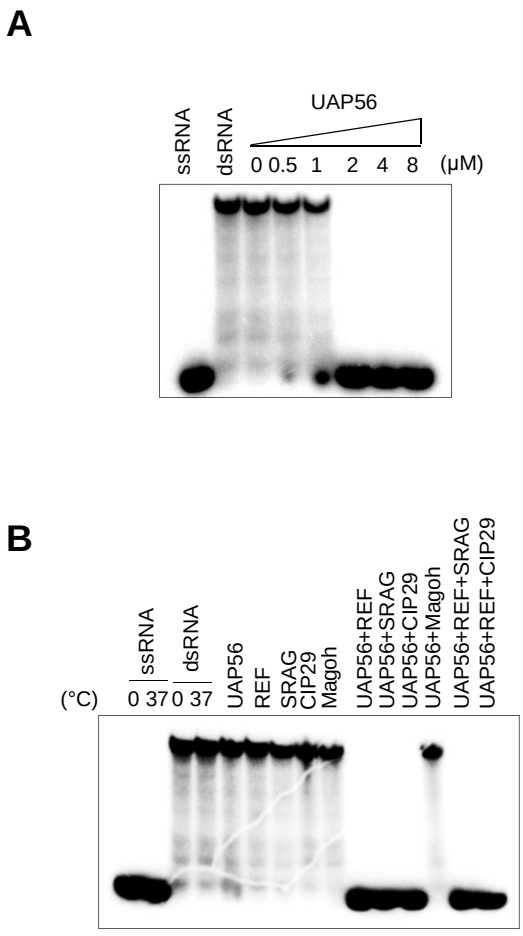
The ATPase assay data for SRAG was surprising because all the UAP56-binding mRNA export factors promote ATP hydrolysis of UAP56. We further examined the helicase activity of UAP56 by using a 15-nt cohesive end RNA duplex. First, we tested the helicase activity of UAP56. The weak helicase activity is common to DEXD/H-box helicases, and large amounts of proteins are routinely used for helicase assays of DEXD/H-box proteins. For example, 0.8 uM protein was used for eIF4A's helicase assay that produces a roughly 35% unwinding of dsRNA with the same substrate (Rogers et al., 2001). UAP56 clearly unwinds this dsRNA in a protein concentration-dependent manner (**Figure. 5-4A**). The helicase activity is weak, and a large amount of UAP56 (0.5 μM or higher) is needed to observe significant unwinding.

To further confirm the relationship between SRAG and UAP56 helicase activity, we performed helicase assays using recombinant UAP56 and purified SRAG, REF, CIP29, and



[Figure. 5-3] SRAG enhances UAP56 ATPase activity

ATPase activities for purified UAP56 added REF, SRAG, and/or CIP29 containing -/+ RNA and/or ATP. Values are the average from 3 independent assays and error bars represent the SD.



[Figure. 5-4] SRAG enhances UAP56 helicase activity

(A) UAP56 unwinds a RNA duplex in a protein concentration-dependent manner. (B) The helicase assay was performed at 37°C for 15min with 3 m M ATP, 100 nM unlabeled complementary strand RNA, 2.5 nM labelled dsRNA substrate, and 1μM UAP56, 2 μM REF, SRAG, CIP29, or Magoh.

Magoh. Interestingly, SRAG, REF, and CIP29 dramatically increase UAP56 helicase activity; in contrast, adding Magoh has no significant effect (**Figure. 5-4B**). Given that REF and CIP29 specifically bind UAP56 in the presence of ATP (Dufu et al., 2010) these results suggest that the interaction of REF, SRAG, and CIP29 with UAP56 might be transient, leading to the stimulation of UAP56 ATPase activity and subsequent dissociation of REF, SRAG, and CIP29 from UAP56.

5.3 UAP56 enhances SRAG loading onto mRNA

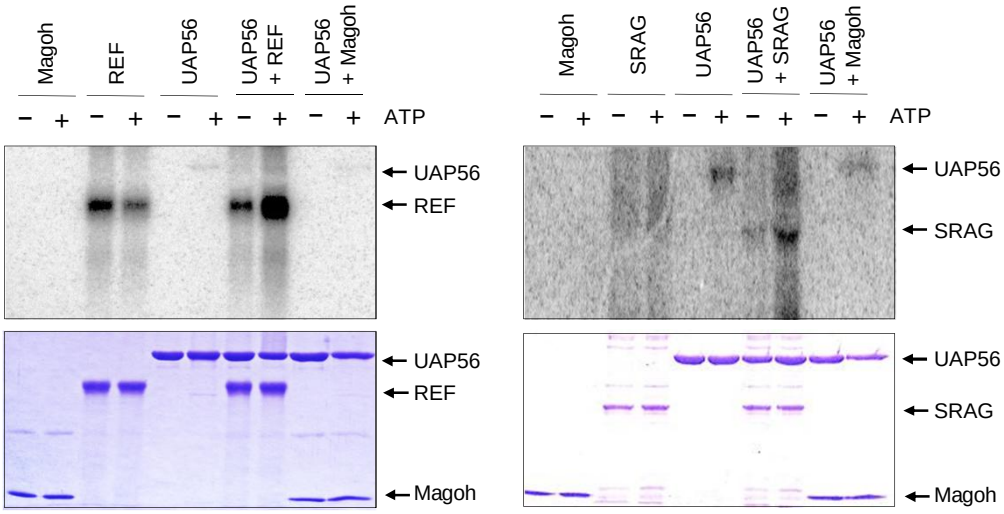
To explore the idea that UAP56 might drive TREX assembly by sequentially loading export factors onto mRNA I investigated what impacts UAP56 had on the RNA binding activities of REF and SRAG.

In the presence of UAP56 and ATP the RNA binding activity of REF and SRAG are enhanced (**Figure. 5-5**). Of note, without ATP, SRAG and REF mRNA binding affinity are not influenced by UAP56. These results indicate that UAP56 can enhance SRAG and REF loading onto mRNA. Furthermore, SRAG and REF cannot bind to UAP56 at the same time, indicating that UAP56 triggers SRAG and REF loading onto mRNA sequentially. However, the requirement of UAP56 ATP hydrolysis still needs to be tested.

5.4 SRAG triggers TAP opening

In **Figure. 4-10**, we revealed that SRAG stimulates mRNA handover from REF to TAP, but the mechanism is still unknown. Recently, our lab reported that TAP uses an intramolecular interaction to silence its own RNA binding activity. Moreover, when the TREX subunits REF and THOC5 make contact with TAP, this drives TAP into an open conformation, exposing its RNA binding domain, allowing efficient mRNA binding (Viphakone et al., *Nature Communications*, in Revision).

Since SRAG and THOC5 bind the NTF2-like domain of TAP, SRAG may also be able to trigger TAP opening. To test this possibility, we used GST-TAP mixed with ^{35}S-labelled TAP to mimic its intramolecular interaction. Consistently, the addition of REF alone does



[Figure. 5-5] UAP56 stimulate REF and SRAG loading onto mRNA

In vitro protein:RNA UV cross-linking assay. Purified GST-UAP56 add GB1-REF (or SRAG) with/without ATP were UV-crosslinked with ^{32}P-radiolabelled RNA oligonucleotide. Resulting complexes were analysed by SDS-PAGE stained with Coomassie blue and Phosphoimage.

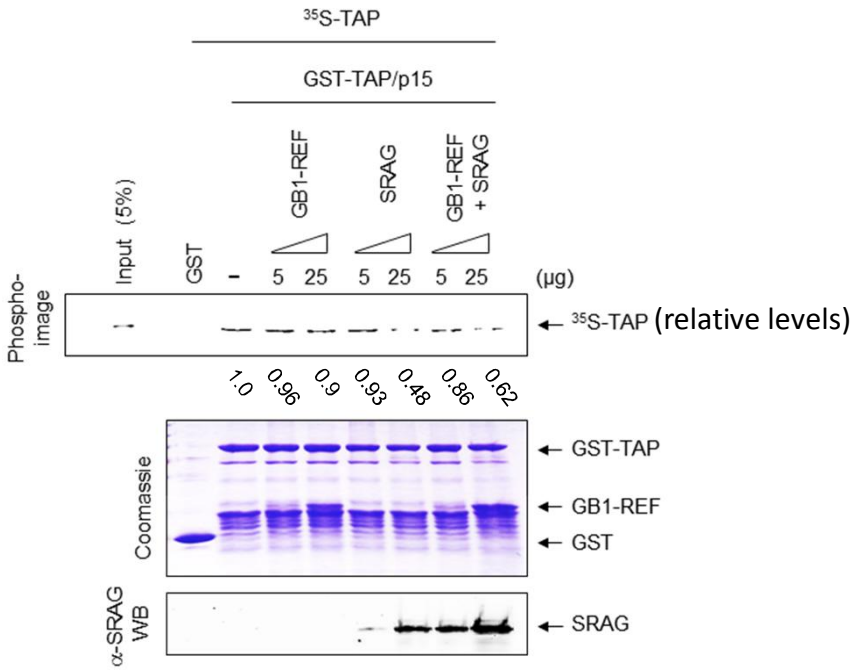
not prevent the interaction between TAP domains. However, unlike THOC5, SRAG appears to be sufficient to trigger TAP:TAP dissociation, as supported by the approximately 2-fold decrease in ^{35}S-TAP retained by GST-TAP in the presence of SRAG (**Figure. 5-6**). These observations lead to the suggestion that REF binds to TAP and makes the NTF2-like domain more accessible to SRAG.

5.5 SRAG and THOC5 are part of the same TAP-containing complex(es) *in vivo*

Since the NTF2-like domain of TAP was used for both SRAG and THOC5 binding, there is potentially functional redundancy between SRAG and THOC5, therefore we considered that SRAG and THOC5 may not be present in the same TREX complex. To explore this, we designed double immunoprecipitations to determine whether SRAG and THOC5 are in the same TAP-containing complexes. In this assay, we immunoprecipitated Flag tagged TAP in 293T cells. After elution, we performed a second immunoprecipitation by using SRAG or THOC5 antibody, and then detected components of each complex by western blotting.

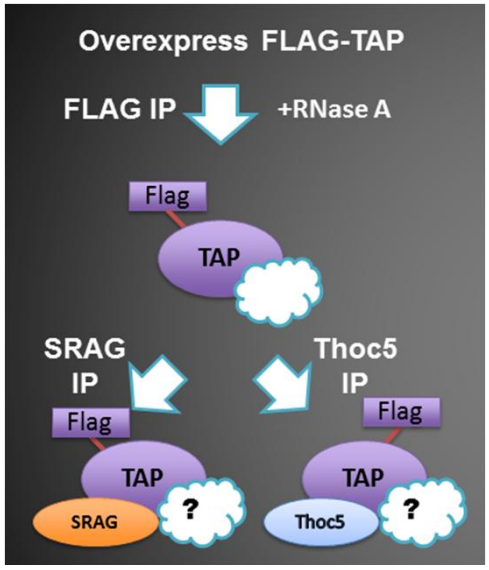
Surprisingly, whilst SRAG and THOC5 bind at the same site of TAP, the results showed they still bound TAP together (**Figure. 5-7**). This finding indicates that SRAG and THOC5 are in a single complex with TAP. Given SRAG and THOC5 bind to TAP in a mutually exclusive manner these data imply there are significant rearrangements within the TREX-TAP complex during assembly of the complex and mRNA export.

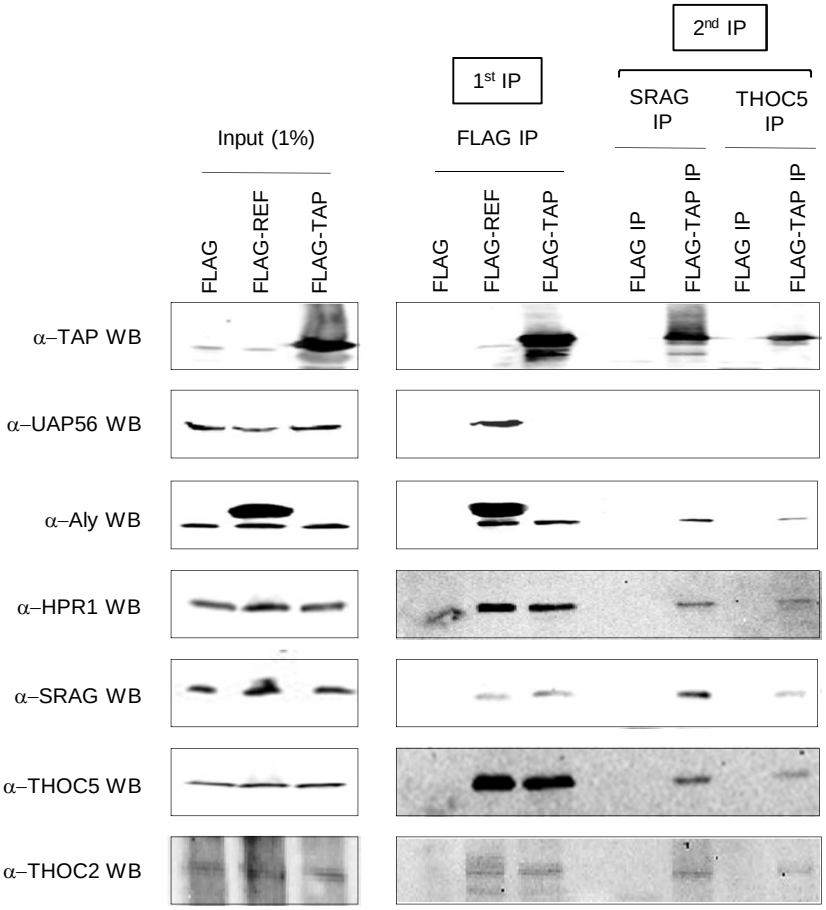
Although the previous results show SRAG and THOC5 are in the same complex, it generates another issue of whether mRNA export depends on the same core set of export factors and occurs in precisely the same manner in every mRNA type. For example, whereas bulk mRNA export occurs via TAP pathway, a subset of endogenous transcripts is exported *via* the karyopherin Crm1 (Cullen et al., 2003a). Crm1 is not an RNA-binding protein, and thus must use different adaptors for the export of endogenous mRNAs. Some possible adaptors have been reported, including HuR for the



[Figure. 5-6] SRAG enhances conformational change of TAP

GST-TAP pulldown ^{35}S-labeled TAP and then using purified 5 or 25 μg GB1-REF, SRAG, or both to compete ^{35}S-labeled TAP.





[Figure. 5-7] Western analysis of Double IP between FLAG-TAP and SRAG (or THOC5)

Immunoprecipitations were carried out with anti-FLAG antibody followed by anti-SRAG or anti-THOC5 antibody. Proteins were detected via Western Blot.

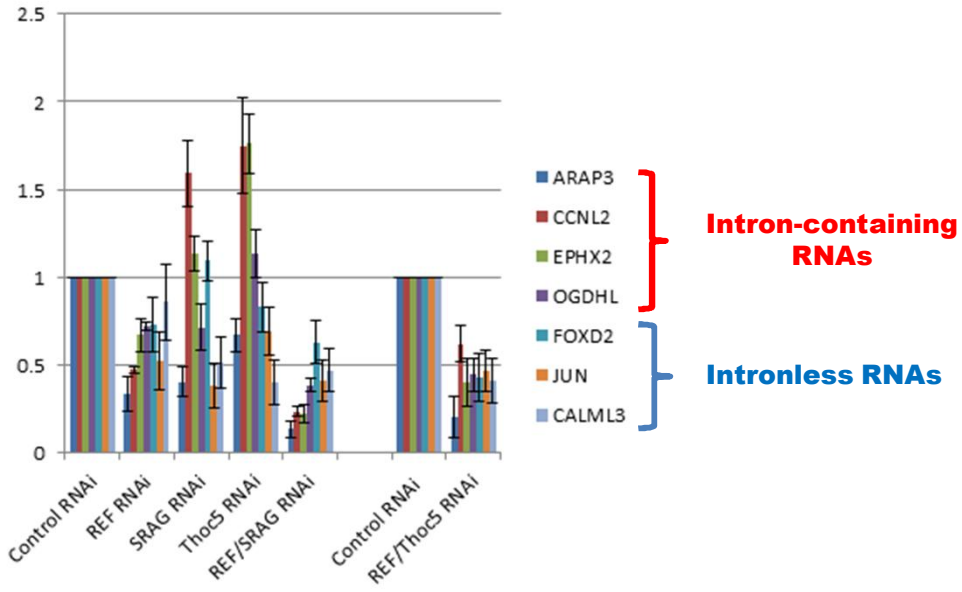
export of *Cd83* and *Fos* mRNAs, and eukaryotic translation initiation factor 4E (eIF4e) for *cyclin D1* mRNA in human cells (Brennan et al., 2000; Culjkovic et al., 2006; Prechtel et al., 2006). Since Katahira et al. reported that THOC5 only enhances intron-less mRNA HSP70 export but not general mRNAs export (Katahira et al., 2009), it is possible that TAP uses different mRNA export co-adaptors to recognize variant mRNAs instead of adaptors.

To test this hypothesis, we used qRT-PCR to test intron-less and intron-containing mRNA export efficiency in SRAG or THOC5 knockdown stable cell-lines. Following RNAi of REF, SRAG, or THOC5 individually there were variable effects on mRNA export (**Figure. 5-8**). Double RNAi of REF/SRAG or REF/THOC5 caused a stronger block in mRNA export, compared with RNAi of the single genes, for all the genes tested.

5.6 Summary

In this chapter, I present further evidence for a role for SRAG in mRNA export. Through pulldown assays, we observed SRAG and REF both interact with the N-terminal domain of UAP56. Moreover, SRAG, REF, and CIP29 all stimulate the ATPase and helicase activity of UAP56. The mRNA binding assays indicate that UAP56 enhances the mRNA binding affinity of SRAG and REF in an ATP-dependent manner. These results suggest that TREX assembly is driven by UAP56 dependent ATPase and helicase activity.

In the previous chapter I found that SRAG and THOC5 bind in a mutually exclusive manner to TAP. In this chapter I have found that both SRAG and THOC5 exist in a complex with TAP in the cell. Moreover, both SRAG and THOC5 are required for optional mRNA export of the same genes, indicating they work in the same export pathway. Together these results indicate that TREX is a dynamic complex, which is likely to undergo significant rearrangements at the point that TAP binds TREX and displaces UAP56 and at subsequent steps in the mRNA export pathway.



[Figure. 5-8] Expression of Various Genes in Several Induced-RNAi Human Cell Lines

Quantitative RT-PCR analysis was used on cyto/total RNA to assess the levels of each gene relative to the U1 snRNA. Efficiency of RNAi was controlled for each RNAi stable cell lines. Error bars represent s.e.m. from 3 experiments. (Intron-containing RNAs: ARAP3, CCNL2, EPHX2, and OGDHL; Intronless RNAs: FOXD2, JUN, and CALML3.)

Chapter VI

Discussion

The mRNA export mechanism is a largely unexplored area. Many questions remain in the field. The precise biochemical determinants of mRNA export factors and whether they are the same for every mRNA are not fully defined. In this study, through discovery and characterization of a novel mRNA export co-adaptor, SRAG, I addressed some outstanding questions in the field of mRNA nuclear export, and a model for the mRNA export mechanism is proposed.

UAP56 recruits TREX components onto mRNA by hydrolyzing ATP

Previous mRNA export models have assumed the formation of TREX complex begins at transcription. During transcription elongation, the nascent mRNA 5' cap is bound by the cap-binding complex (CBC; composed of CBP20 and CBP80) (Izaurralde et al., 1995). Next, a transcript undergoes splicing and the EJC is deposited at the site of exon fusion. Capping and splicing are both important for the recruitment of the TREX complex. The TREX complex is poorly recruited to transcripts that lack either the 5' cap or the EJC, indicating that its mechanism of recruitment is linked to splicing and capping (Cheng et al., 2006; Masuda et al., 2005; Zhou et al., 2000). It should be pointed out that UAP56 is essential for the export of most mRNAs, but this is not the case with REF in *Drosophila melanogaster* and *Caenorhabditis elegans* (Gatfield and Izaurralde, 2002; Longman et al., 2003). Therefore, UAP56 may have additional functions beyond recruiting REF, alternatively other proteins may functionally replace REF in its absence, such as UIF (Hautbergue et al., 2009)

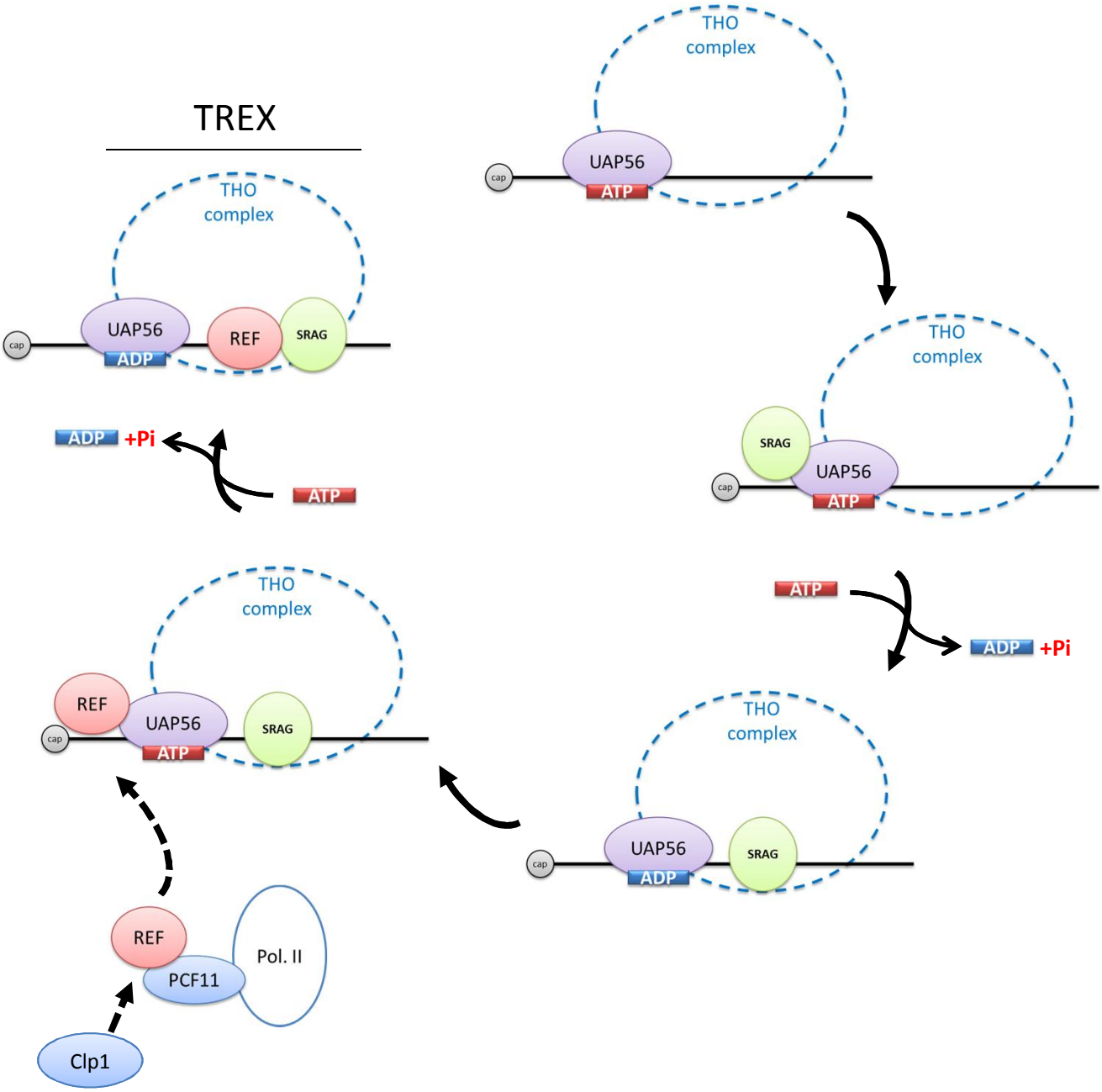
In this study, I demonstrated that TREX components bound to UAP56 can stimulate its ATPase and helicase activity. Moreover, UAP56 drives SRAG and REF loading onto

mRNA by hydrolyzing ATP. These results imply that after TREX components are recruited, UAP56 can hydrolyze ATP and promote TREX components binding to mRNA. Because SRAG and REF both interact with the N-terminal domain of UAP56, it indicates this processing is likely to be a mechanochemical cycle.

Based on the results obtained in this study, I propose a model of how UAP56 may recruit export factors onto mRNAs (**Figure. 6-1**). In the ATP bound state, UAP56 associates with mRNA and provides a site for recruitment of SRAG. SRAG preferentially binds ATP-bound UAP56 (Robin Reed, personal communication). Once SRAG is recruited, this stimulates ATP hydrolysis by UAP56, which results in release of SRAG from UAP56 and its loading onto mRNA. UAP56 is then reloaded with ATP ready to recruit REF. The mechanism by which ADP is exchanged with ATP on UAP56 remains to be determined. In turn REF binds ATP bound UAP56 (Dufu et al., 2010), stimulates ATP hydrolysis and is itself loaded onto the mRNA. This loading cycle may repeat, and multiple proteins may be loaded onto a single RNA molecule. It is not clear on the basis of my data whether SRAG or REF is loaded onto the mRNA first and a number of experiments designed to address this question produced inconclusive results. However, loss of SRAG in cells leads to accumulation of REF on the mRNP (**Chapter IV**). This may arise because SRAG helps the association of REF with TAP which results in the transfer of RNA from REF to TAP and this transfer cannot occur in the absence of SRAG, therefore it is plausible that SRAG associates with TAP first during TREX maturation.

SRAG enhanced handover of mRNA to TAP

TAP binds RNA weakly via a non-canonical arginine rich RNA binding domain (Hautbergue et al., 2008). Because the intrinsic RNA binding activity of TAP was found to be weak *in vitro*, it was proposed that TAP required an adaptor protein (e.g. REF or UIF), which bound mRNA with high affinity, to bridge its interaction with mRNA (Liker et al., 2000). In 2011, our lab reported that THOC5 and REF, which together drive TAP into an open conformation, allow it to stably bind mRNA and trigger its export (Viphakone et al, *Nature Communications*, in Revision).



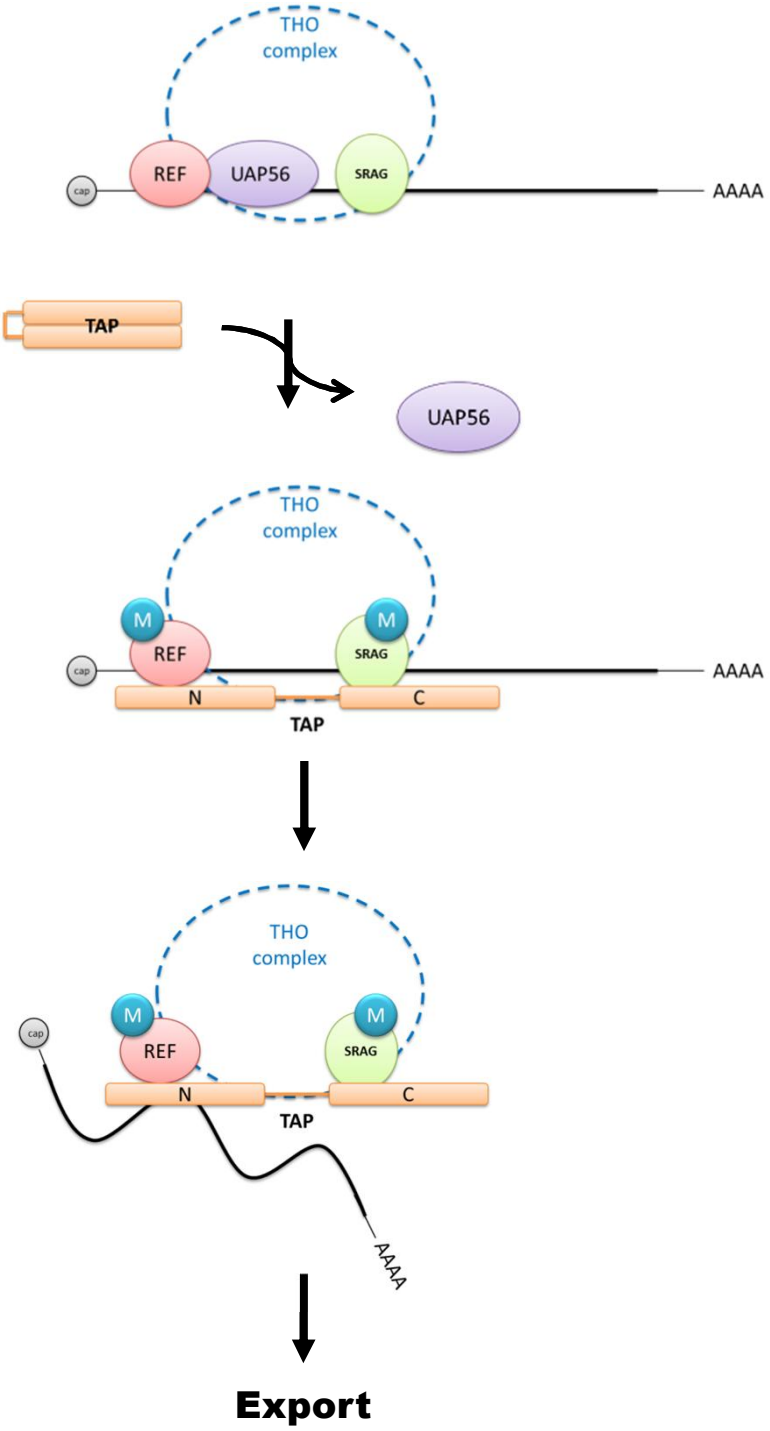
[Figure. 6-1] A model of TREX complex assembly needs ATP hydrolysis of UAP56.
TREX complex assembly involves at least two rounds of ATP hydrolysis by UAP56 triggered by REF and SRAG.

In this study I showed that SRAG also functions as an mRNA export co-adaptor and enhances mRNA transfer from REF to TAP mRNA (**Figure. 6-2**). Depletion of REF or SRAG alone *in vivo* has a modest effect on mRNA export, but their combined knockdown causes a drastic mRNA export block. The ability of the cell to maintain recruitment of TAP to mRNA in the absence of SRAG may be since another mRNA export co-adaptor, THOC5, has been shown to bind the TAP NTF2-like domain and may compensate for the SRAG loss, maintaining TAP in an open conformation able to bind mRNA. However, SRAG binds to TAP in a mutually exclusive manner with the THO complex component THOC5 and yet TAP, SRAG, and THOC5 are found in the same complex *in vivo*. This suggests some rearrangements that must take place during mRNP formation and/or export and indicate that TREX is a dynamic complex undergoing substantial rearrangements during mRNA export.

Arginine methylation regulates mRNA export factors

Protein methylation is a common and stable post-translational modification (PTM) in higher eukaryotes. Protein methylation has been shown to affect several cellular processes, including protein:protein interaction, intracellular localisation, and maturation of heterogeneous ribonuclearproteins (hnRNPs) (Liu and Dreyfuss, 1995; Bedford et al., 2000; Cote et al., 2003). In 2010, our lab reported that a key TAP-binding site of REF is contained within a small arginine-rich region, which can be methylated by PRMT1. Functional studies of REF methylation revealed that it reduces the RNA-binding properties of the protein. Furthermore, the reduced RNA-binding affinity of REF facilitates handover of bound mRNA to TAP during mRNA nuclear export (Hung et al., 2010). This finding indicates the importance of arginine methylation to direct TAP-mRNA interactions.

Here, I reveal that arginine methylation influences not only protein:RNA interaction, but protein:protein interaction. My data provide strong evidence that methylation of SRAG by PRMT1 does occur *in vivo* and plays an important role in facilitating SRAG mRNA export co-adaptor function: (1) REF can only interact with unmethylated SRAG.



[Figure. 6-2] A model of SRAG and REF drive TAP into an open conformation.
SRAG and REF drive TAP into an open conformation allowing handover mRNA to TAP. (M: hypermethylation)

(2) TAP:SRAG interaction is dependent on methylation of SRAG. (3) The Arginine residues that are methylated by PRMT1 are important for the ability of SRAG to bind to TAP and mRNA. (4) Endogenous SRAG contains both forms and the unmethylated form is transient. These evidences point towards another possible regulation mechanism in the mRNA export pathway through arginine methylation. Methylation of SRAG and REF by PRMT1 may be regulated, for example, when they are both recruited into TREX complex and then handed over mRNA to TAP, arginine methylation adopts the appropriate conformation. In support of this idea, the methylation of SRAG regulated its protein:protein interactions and methylated REF has weaker mRNA binding affinity.

The findings with SRAG reveal that arginine methylation is used to fine tune mRNA export and also raises the possibility that combinatorial use of different adaptors and co-adaptors give rise to transcript specific mRNA export.

Future Work

Eukaryotic mRNA export is controlled by multiple mechanisms and regulated by post-translational modification of mRNA export factors. As we delve deeper and wider into the mRNA export mechanism, the emerging landscape becomes ever more complex.

This study demonstrates that arginine methylation takes place in regulation of mRNA export factors. It is worth further examining whether there is an arginine demethylase which interacts with and regulates the arginine modification of mRNA export factors. Until now, there is no convinced evidence to prove an arginine demethylase exists.

Another issue is whether mRNA export depends on the same core set of export factors in every organism, or in every cell type. For example, Mex67 is essential for mRNA export in the budding yeast *S. cerevisiae* (Segref et al., 1997), but not in the fission yeast *Schizosaccharomyces pombe* (Yoon et al., 2000); Yra1 is essential for export in *S. cerevisiae* (Strasser and Hurt, 2000), but not in *Drosophila* or *C. elegans* (Gatfield and Izaurralde, 2002; Longman et al., 2003). In this study, I presented results indicating that SRAG and Thoc5 provide redundant mechanisms. Since SRAG only exists in

vertebrates, it is worth investigating more fully whether SRAG and THOC5 contribute to different kinds of mRNA export to understand these species-specific differences.

The present studies provide compelling evidence that SRAG is an mRNA export co-adaptor; nevertheless, some evidence suggests that SRAG regulates cell proliferation (Zullo et al., 2009). Thus, a critical question is raised: Does SRAG have other functions and play a role in different mechanisms? Since SRAG is an mRNA export factor, I cannot conclude that the inhibition of the cell cycle is an independent mechanism in which SRAG functions, or whether it is a consequence of a primary effect on blocking mRNA export?

However, more and more studies indicate that mRNA export co-adaptor cannot be the only function of SRAG. In 2010, Duensing's group showed that SRAG is involved in centriole length control. Depletion of SRAG is implicated in microtubule anchoring and stability that attenuated daughter centriole elongation (Korzeniewski et al., 2010). Moreover, van Dijk et al. showed that knockdown of SRAG in adult erythroid progenitors strongly induces HbF (van Dijk et al., 2010a). These results conclude that SRAG plays a critical role in fetal globin expression. Therefore, the present studies add to the growing support for the idea that SRAG is a multi-functional protein. Although it remains unclear how SRAG affects both mRNA export and gene regulation, one possibility is that SRAG contributes to target mRNA recognition, thereby increasing target export. However, this cannot be the only function, because knocking down both SRAG and REF causes a huge mRNA block in the nucleus. Although we cannot provide an answer for what this mechanism might be, by studying the function of SRAG, we have a starting point in the linkage between mRNA export mechanisms and gene regulation.

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Appendix

The Manuscript

SRAG mRNA export factor identification reveals dynamic interactions of TREX with UAP56 and NXF1

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Abstract

The TREX complex couples nuclear pre-mRNA processing with mRNA export and contains multiple protein components, including UAP56, ALY, CIP29 and the multi-subunit THO complex. Here we have identified SRAG as a novel TREX component. We show that both SRAG and ALY activate the ATPase and RNA helicase activities of UAP56 and that UAP56 functions to recruit both ALY and SRAG onto mRNA. As observed with the THO complex subunit THOC5, SRAG binds to the NTF2-like domain of NXF1, and this interaction requires arginine methylation of SRAG. Using RNAi, we show that co-knockdown of either ALY and SRAG or ALY and THOC5 results in a potent mRNA export block. Interestingly, SRAG binds to UAP56 in a mutually exclusive manner with ALY, and SRAG binds to NXF1 in a mutually exclusive manner with THOC5. However, SRAG, THOC5 and NXF1 exist in a single complex *in vivo.* Together, our data indicate that TREX and NXF1 undergo dynamic remodeling, driven by the ATPase cycle of UAP56 and post-translational modifications of SRAG.

Keywords: C1ORF77/nuclear export/REF/TAP

Introduction

The TREX complex plays a central role in eukaryotic gene expression, integrating information from nuclear mRNA processing events to ensure the timely export of mRNA to the cytoplasm (Rodriguez-Navarro & Hurt, 2011). The metazoan core TREX complex contains multiple subunits including the RNA helicase UAP56 (Sub2 in yeast), ALY (THOC4/REF/BEF; Yra1 in yeast), CIP29 (Tho1 in yeast), and the THO subcomplex which comprises THOC1 (HPR1), THOC2, THOC3 (TEX1), THOC5, THOC6, THOC7 (Dufu et al, 2010). The recruitment of TREX to mRNA is splicing dependent (Masuda et al, 2005) and results in the loading of TREX near the 5' end of mRNA through an interaction between CBP80 and ALY (Cheng et al, 2006). The assembly of UAP56, CIP29 and ALY within TREX is dependent on UAP56 binding ATP whereas the association of UAP56 with THOC2 is ATP independent (Dufu et al, 2010). The recruitment of metazoan ALY to the TREX complex is probably coupled with 3' end processing since Yra1 is specifically recruited to Sub2 in yeast via the 3' end processing factor Pcf11 and the ALY:PCF11 interaction is conserved (Johnson et al, 2009). Clp1 displaces Yra1 from Pcf11 as does Sub2 when loaded with RNA and ATP (Johnson et al, 2011). Moreover, the recruitment of Clp1 to Pcf11 is an important step in generating an active CF1A complex required for cleavage-polyadenylation (Haddad et al, 2011),(Ghazy et al, 2011). Therefore recruitment of ALY to TREX via PCF11 provides a means to couple 3' end processing with mRNA export.

The mRNA export receptor NXF1 (Mex67 in yeast) functions to translocate mRNPs through the nuclear pore complex and GANP facilitates NXF1 interaction with the nuclear pore (Wickramasinghe et al, 2010). The N-terminus and the NTF2-like domain of NXF1 bind to the TREX components ALY and THOC5, respectively, and the term co-adaptor has been used for THOC5 since it binds to NXF1 simultaneously with the adaptor protein ALY (Katahira et al, 2009). During export, mRNA is handed over from ALY to NXF1 and NXF1 uses an N-terminal arginine-rich peptide to interact directly with RNA (Hautbergue et al, 2008; Walsh et al, 2010). The methylation of arginines in ALY, reduces its ability to bind mRNA and therefore this post-translational modification facilitates hand over of mRNA from ALY to NXF1 (Hung et al, 2010). In yeast, the TREX

subunit Hpr1 binds to the C-terminal UBA domain of Mex67 and recruits Mex67 to actively transcribed genes (Gwizdek et al, 2006).

The loss of THO subunits in yeast leads to a rapid mRNA export block (Strasser et al, 2002) and recent evidence indicates that TREX plays a genome-wide role in mRNP biogenesis in yeast (Gomez-Gonzalez et al, 2011). In contrast knockdown of metazoan THO components leads to a less severe export phenotype (Rehwinkel et al, 2004). Similarly loss of ALY in metazoans results in a modest mRNA export block. This may be partly accounted for by the existence of redundant pathways for mRNA export. For example, loss of ALY triggers increased expression of UIF which can also act as a link between UAP56 and NXF1 (Hautbergue et al, 2009). In yeast, Nab2 also appears to be capable of promoting recruitment of Mex67 to the mRNP (Iglesias et al, 2010). The loss of UAP56 in combination with its paralogue DDX39, leads to a severe mRNA export block in humans (Hautbergue et al, 2009) and this is accompanied by accumulation of mRNA within nuclear speckles (Dias et al, 2010). This export block applies to both spliced and unspliced mRNAs and interestingly unspliced mRNAs require a specific coding region sequence to promote their export (Lei et al, 2011).

Here we describe and characterise a new component of the TREX complex, SRAG. We show that SRAG binds UAP56 and activates its ATPase and RNA helicase activities. SRAG also competes with THOC5 for binding to the NTF2-like domain of NXF1 and yet both are found associated in a single complex *in vivo*. Thus the discovery of SRAG indicates that the TREX complex is likely to undergo substantial rearrangements during mRNP formation. In this respect TREX may well act like other macromolecular machines such as the spliceosome, undergoing numerous conformational and compositional rearrangements in performing its cellular functions.

Results

SRAG binds UAP56 and associates with TREX.

To identify novel UAP56 binding proteins we took the REF2-I (the murine orthologue of ALY) C-terminal UAP56 binding motif (UBM) (Hautbergue et al, 2009) and used this in a

BLAST search which led to the identification of SRAG/FOP/C1ORF77/CHTOP, a nuclear protein previously shown to be a substrate for the arginine methylase PRMT1 and required for ligand-dependent activation of estrogen receptor target genes (van Dijk et al, 2010). SRAG contains two copies of a sequence similar to the REF2-1 UBM located towards the C-terminus. Alignment of metazoan UBMs reveals an invariant subsequence DXXLD (Figure 1A), which forms part of a transient alpha helix in the REF2-1 protein (Golovanov et al, 2006). Since SRAG and ALY use a similar peptide sequence to bind UAP56 we investigated whether they could simultaneously bind UAP56 *in vitro*. Pulldown assays confirmed that SRAG bound GST-UAP56 (Figure 1B). Increasing concentrations of ALY efficiently displaced SRAG from GST-UAP56, indicating that SRAG and ALY bind in a mutually exclusive manner to UAP56. Since UAP56 is a component of the TREX mRNA export complex, we investigated whether SRAG might also associate with TREX components. This analysis confirmed that SRAG co-immunoprecipitated with multiple components of the TREX complex including UAP56 (Figure 1C) and the interaction was not sensitive to the presence of ribonuclease (Figure 1C and Supplementary Figure S1). These data are consistent with the identification of SRAG by mass spectrometry in TREX complexes immunopurified with antibodies to THOC2, CIP29 and UAP56 TREX subunits (Dufu et al, 2010). Moreover, mass spectrometry analysis led to the identification of multiple proteins which co-purify in vivo with biotinylated SRAG. These proteins included multiple TREX components which are shown in Supplementary Table S1 (Fanis, P, Gillemans, N, Pourfarzad, F., Aghajanirefah, A., Demmers, J., Esteghamat, F., Vadlamudi, R.K., Grosveld, F., Philipsen, S and van Dijk, T., manuscript in preparation). In common with several other mRNA export factors, SRAG co-localises with the splicing factor SC35 in nuclear speckles (Supplementary Figure S2).

SRAG, ALY and CIP29 stimulate the ATPase and helicase activities of UAP56

We examined the effects of both ALY and SRAG on the ATPase and helicase activity for UAP56. UAP56 showed minimal ATPase activity in the absence of RNA, but significant activity in its presence, consistent with an earlier report (Figure 2A) (Taniguchi & Ohno,

2008) . SRAG, ALY and CIP29 displayed no ATPase activity even in the presence of RNA. However, ALY, SRAG and CIP29 individually stimulated both the RNA-dependent and RNA-independent ATPase activity of UAP56, although combinations of ALY and CIP29 or SRAG and CIP29 did not further enhance the observed ATPase activity for UAP56. We also found that ALY, SRAG and CIP29 all stimulated the UAP56 helicase activity, whereas the exon junction complex component MAGOH did not (Figure 2B).

UAP56 loads SRAG and ALY onto mRNA.

An earlier study examined how UAP56 and ALY bound RNA using immunoprecipitation (IP) of radiolabelled RNA (Taniguchi & Ohno, 2008). However, this study did not address which proteins were directly bound to RNA within ternary complexes. Therefore to examine how RNA associates with UAP56 and its binding partners we firstly carried out UV cross-linking experiments with proteins in solution (Figure 2C). In isolation, ALY cross-linked with RNA well, irrespective of the presence of ATP whereas UAP56 showed a weak cross-link with RNA but only in the presence of ATP as reported previously (Taniguchi & Ohno, 2008). When ALY and UAP56 were mixed, there was a dramatic stimulation of RNA cross-linked to ALY and this was dependent on ATP (Figure 2C left panel lanes 3,4 &7,8). Similarly for SRAG, there was an extremely weak cross-link with RNA in the absence of UAP56 (Figure 2C right panel lanes 3,4), but in the presence of ATP and UAP56, there was a dramatic increase in the amount of RNA cross-linked to SRAG (Figure 2C right panel lanes 7,8). Together these data indicate that in the presence of UAP56 and ATP, SRAG and ALY are loaded onto mRNA. Given that both ALY and SRAG stimulate the helicase and ATPase activity of UAP56 we also carried out RNA binding studies in the presence of the non-hydrolysable ATP analogue AMP-PNP. For these studies we also ensured that all UAP56 present in the RNA UV cross-linking step was complexed with ALY by isolating a GST-ALY:UAP56 complex by pulldown on glutathione sepharose beads (Figure 2D). Here we observed that UAP56 still cross-linked with RNA in isolation but failed to cross-link with RNA when in complex with GST-ALY. However, GST-ALY cross-linked with RNA in the complex. Therefore the RNA is normally likely to

be handed over from UAP56 to ALY. Since in the presence of the non-hydrolysable AMP-PNP, UAP56 failed to stimulate the loading of RNA onto GST-ALY above that seen with GST-ALY alone, this transfer is normally likely to take place concomitantly with ALY- or SRAG-stimulated ATP hydrolysis (Figure 2D). We were unable to perform a similar experiment with SRAG, since GST-SRAG expressed poorly in *E.coli.* We further investigated the role of UAP56 and its paralogue DDX39, in loading TREX factors onto the mRNP using *in vivo* UV cross-linking in a stable cell line where UAP56 and DDX39 expression can be knocked down by RNAi (Hautbergue et al, 2009) (Figure 2E). These experiments showed that both SRAG and ALY loading onto the mRNP is dependent on UAP56/DDX39. Since both SRAG and ALY are found in the TREX complex and bind UAP56 in a mutually exclusive manner, these data suggest that UAP56 may go through more than one round of ATP hydrolysis during TREX assembly and this ATP hydrolysis drives loading of both TREX components onto the mRNP.

Methylation of SRAG regulates its interactions with ALY, NXF1 and RNA but not UAP56.

To generate SRAG protein for further analysis we constructed a FLAG-tagged SRAG cDNA expression vector and transfected it transiently into 293T cells. Western blot analysis reproducibly generated two discrete bands (Figure 3A). Since SRAG is a binding partner for PRMT1 (van Dijk et al, 2010), we investigated whether the two bands correspond to different methylation states of SRAG. Cells expressing FLAG-SRAG were treated with the methylation inhibitor AdOx for varying times. We found that by 48 hours the slower migrating SRAG band disappeared, suggesting this band corresponded to a hyper-arginine methylated SRAG form, whereas the faster migrating band corresponding to hypo-arginine methylated SRAG remained (Figure 3A). We then investigated the methylation status of endogenous SRAG in both nuclear and cytoplasmic fractions. In whole cell lysates, SRAG was predominantly methylated while growth of cells in AdOx, shifted SRAG predominantly to a faster migrating hypo-methylated state (Figure 3B, lanes 1,2). However, we considered that PRMT1 may

methylate some SRAG post cell lysis and during preparation of subcellular fractions. To prevent this we prepared fractions using a lysis buffer containing AdOx from cells grown in the absence of AdOx. This revealed that a significant proportion of SRAG exists in the nuclear fraction in the hypo-methylated state (Figure 3B, lanes 3-8). We then investigated whether SRAG associated with other mRNA export factors and what impact methylation might have on such interactions using GST-pulldown assays with 293T cell extracts (Figure 3C). GST-UAP56 bound both hyper- and hypo-methylated SRAG, whereas GST-ALY only bound hypo-methylated SRAG. In direct contrast, GST-NXF1 only associated with hyper-methylated forms of SRAG. To confirm that SRAG associated with NXF1 we immunoprecipitated NXF1 from cells and found that it did IP with SRAG but only in the absence of AdOx (Figure 3D). To establish that the interactions between SRAG and NXF1/ALY were direct and regulated by SRAG methylation we generated and purified methylated SRAG in *E.coli* by coexpression with PRMT1 (Supplementary Figure S3). In pulldown assays direct interactions were observed between GST-NXF1, ALY and UAP56. Interactions with GST-UAP56 were not influenced by SRAG methylation, in contrast GST-NXF1 interaction required SRAG methylation whereas GST-ALY only bound hypo-methylated SRAG. (Figure 3E). We further investigated whether methylation of SRAG influenced its ability to bind the mRNP *in vivo* as it does for ALY (Hung et al, 2010) and found that the hypo-methylated form of SRAG cross-links with mRNA more efficiently *in vivo* than the methylated state (Figure 3F). We conclude that SRAG exists in both hypo- and hyper-methylated states in the nucleus and that its methylation status governs its interactions with ALY, NXF1 and mRNA.

SRAG and THOC5 bind NXF1 in a mutually exclusive manner

To examine the interaction of SRAG with NXF1 we mapped which domain of NXF1 was responsible for interaction with SRAG using GST pulldown assays (Figure 4A,B). This analysis revealed that whilst ALY bound amino acids (aa) 1-198 of NXF1, corresponding to the N-terminal RNA binding domain and pseudo-RRM, SRAG bound aa 372-619, encompassing the NTF2-like and UBA domains. We therefore investigated what impact

ALY had on the interaction of SRAG with NXF1 (Figure 4C) and found that increasing amounts of GB1-ALY bound to NXF1, stimulated the interaction of FLAG-SRAG with NXF1. In contrast, FLAG-UIF, which binds aa1-198 of NXF1 was efficiently displaced by GB1-ALY in this assay. Together these data suggest that ALY and SRAG bind cooperatively to NXF1. Since the NTF2-like domain of NXF1 is also known to bind the TREX component THOC5, we examined whether SRAG might recognise a similar surface of the NTF2-like domain of NXF1. Point mutations in the NTF2-like domain which are known to block the NXF1:THOC5 interaction (Katahira et al, 2009), also block the NXF1:SRAG interaction (Figure 4D). To confirm that SRAG and THOC5 utilise a common binding site on NXF1 we examined the effects of increasing amounts of THOC5 on the NXF1:SRAG interaction and found that THOC5 efficiently displaces SRAG from NXF1 (Figure 4E). We conclude that SRAG and THOC5 bind in a mutually exclusive manner to a common binding site on NXF1 which encompasses the NTF2-like domain.

SRAG works with ALY to enhance the RNA binding activity of NXF1

When NXF1 binds to ALY, it hands mRNA over to NXF1 and remodels NXF1 so that it binds RNA with higher affinity (Hautbergue et al, 2008). We therefore investigated whether SRAG might also play a role in remodelling NXF1. We incubated radiolabelled RNA with GB1-ALY and SRAG and then pulled down the protein:RNA complex using GST-NXF1. The complex was then eluted and the RNA was subsequently UV cross-linked to proteins. Since GST-NXF1 produced a number of truncation products which masked where SRAG would migrate on the gel we confirmed the presence of SRAG in the pulldowns by Western blotting (Figure 5A). In this assay GST-NXF1 bound RNA weakly, but showed significantly enhanced RNA cross-linking activity in the presence of ALY, an effect that was further enhanced in the presence of SRAG (Figure 5A). In complex with NXF1, no RNA binding to ALY or SRAG was detected. In the case of ALY which cross-links with RNA efficiently by itself (Figure 5B), it is clear that the RNA is handed over to NXF1. In the case of SRAG it is less clear whether SRAG retains RNA binding activity in the complex with ALY and NXF1 as it binds RNA poorly in isolation (Figure 5B).

SRAG is required for efficient mRNA export

To investigate the role of SRAG *in vivo* we generated stable HEK 293 cell lines which expressed inducible miRNAs targeting either SRAG, ALY or SRAG in combination with ALY and examined the impact of RNAi of these export factors on the protein levels of other export factors by Western blotting (Figure 6A). The levels of UAP56 were increased following RNAi of each export factor, with a particularly dramatic increase in the cell line where both ALY and SRAG were depleted. The levels of ALY did not increase in the SRAG RNAi cell line, whereas the levels of SRAG increased significantly following ALY RNAi. Together these data indicate that the cell mounts a compensatory response to loss of specific export factors by increasing the levels of other export factors in the same pathway. This response has previously been observed in both *Drosophila* cells (Herold et al, 2003) and human cells where UIF levels increase following ALY RNAi (Hautbergue et al, 2009). We also investigated the effects of SRAG RNAi on cell growth (Figure 6B) and found that SRAG RNAi had no significant impact whereas ALY RNAi led to a significant growth defect. Strikingly, the combined RNAi of ALY and SRAG led to cell death within 6 days. We investigated the impact of SRAG RNAi on mRNA export using fluorescence in situ hybridisation with oligo(dT) (Figure 6C). As reported previously ALY RNAi led to a small but detectable mRNA export block which was most apparent at 96 hours post-induction of the ALY miRNA (Hautbergue et al, 2009). In contrast there was no discernable mRNA export block in cells following SRAG RNAi. However, the combined RNAi of ALY and SRAG led to a very strong mRNA export block which was visible as early as 48 hours post miRNA induction and was very severe by 72 hours. This strong export block probably accounts for the death of the SRAG/ALY RNAi cell line by 6 days (Figure 6B). Together these data indicate that SRAG cooperates with ALY to ensure efficient mRNA export *in vivo*. We further investigated the impact of ALY and SRAG RNAi on the ability of mRNA export factors to directly associate with the mRNP using *in vivo* UV crosslinking assays in denaturing conditions (Figure 6D). Strikingly SRAG RNAi led to a significant accumulation of ALY on mRNA, despite ALY levels remaining constant in the cell. This suggests that ALY displacement from RNA which is normally accompanied by

NXF1 binding to ALY (Hautbergue et al, 2008) is disrupted in these cells. We also observed increased levels of SRAG associated with the mRNP following ALY RNAi, however, the levels of SRAG were also significantly increased in the cell, so it is not clear from these data whether SRAG is specifically blocked on the mRNP in these cells. Depletion of ALY or SRAG had no impact on UAP56 association with the mRNP, consistent with it lying upstream of these proteins in the mRNA export pathway.

SRAG, THOC5 and NXF1 exist in the same complex and are all required for export of mRNAs.

We have established that SRAG and ALY bind to different regions of NXF1 and that the proteins appear to cooperate to ensure efficient mRNA export. Moreover SRAG and THOC5 binding to NXF1 are mutually exclusive. This raises the possibility that different combinations of ALY with SRAG or ALY with THOC5 might promote export of different groups of mRNAs in the cell. Alternatively but not mutually exclusively, THOC5, SRAG and ALY might exist in a single dynamic complex in which NXF1 might exchange binding partners during mRNA export. To address this we immunoprecipitated FLAG-NXF1 and FLAG-ALY from cells and gently eluted the IP from beads using FLAG peptide. A proportion of the IP was subjected to Western analysis for TREX components (Figure 7A). In the FLAG-ALY IP we detected multiple TREX components including UAP56. FLAG-NXF1 associated with multiple TREX components except UAP56, as expected since UAP56 is displaced from ALY by NXF1 (Hautbergue et al, 2008). We then took the eluate from the FLAG-NXF1 IP and subjected it to a second round of IP with either SRAG or THOC5 antibodies and again analysed these IPs for TREX components. In both cases we identified multiple TREX components. These data indicate that NXF1, SRAG and THOC5 exist in a single complex in cells. To examine whether loss of SRAG and THOC5 affected export of common mRNAs we analysed the export of specific spliced and intronless mRNAs in cell lines depleted for ALY, SRAG, THOC5 and combinations (Figure 7B). ALY RNAi blocked export of all mRNAs tested with variable efficiencies ranging from 15-70% inhibition. SRAG and THOC5 caused a much less severe block of export for the mRNAs

tested. In fact several mRNAs showed no export block at all and for the spliced mRNAs CCNL2 and EPHX2, and we even observed increased cytoplasmic RNA levels. The combined RNAi of ALY/SRAG (40-90% export inhibition) or ALY/THOC5 (40-80% export inhibition) gave a robust mRNA export block for all mRNAs tested. Together these data indicate that SRAG and THOC5 exist in the same TREX complex and act in the same pathway to export mRNAs from the nucleus to the cytoplasm.

Discussion

We have defined SRAG as a new component of the TREX complex which interacts with both UAP56 and NXF1. SRAG harbours a short peptide motif found in other UAP56 binding proteins and competes with ALY for association with UAP56, though both SRAG and ALY are found in a single complex with NXF1 in the cell. ALY, SRAG and CIP29 are able to activate the ATPase and helicase activity of UAP56 and this in turn stimulates loading of SRAG and ALY onto mRNA. This suggests a model in which UAP56 acts as an assembly factor for TREX, recruiting subunits in a sequential manner and loading them into an assembling TREX complex on mRNA (Figure 7). Since CIP29 can bind UAP56 simultaneously with ALY it may well be loaded into TREX in combination with ALY or may remain bound to UAP56 during subsequent ATPase cycles.

It is not uncommon for RNA helicases to drive assembly and conformational changes in protein complexes and associate with specific proteins at different stages in the ATPase cycle. For example, the translation initiation factor eIF4A associates with eIF4G heat repeat domain 1, EIF4H and RNA in an ATP dependent manner and in the nucleotide free state associates with eIF4G heat repeat domain 2 (Marintchev et al, 2009). Similarly the DBP5 RNA helicase, which is involved in a terminal step in mRNA export makes interactions with other proteins and RNA which are coupled with the ATPase cycle (Folkmann et al, 2011). Gle1 together with IP_6 stimulate ATP binding by DBP5 which reciprocally stimulates Gle1 and IP_6 binding to DBP5 and in this respect the action of ATP parallels that observed previously for ALY and CIP29 interacting with UAP56 (Dufu et al, 2010) . The ATP bound DBP5 is then thought to associate with RNA

and similarly UAP56 requires ATP to bind efficiently to RNA. Gle1 and IP_6 stimulate ATP hydrolysis by DBP5, which also mirrors what we observe with SRAG and ALY. Subsequently, NUP159 promotes ADP release from DBP5 and ATP hydrolysis, together with ADP release, reduces the affinity of DBP5 for RNA. Whether UAP56 also requires an ADP release factor remains to be determined. UAP56 associates with the THO complex and components of this complex may be involved in such an activity. Ultimately the DBP5 ATPase cycle is coupled with displacement of mRNA export factors such as Mex67 from the mRNP on the cytoplasmic face of the nuclear pore (Lund & Guthrie, 2005). Thus, whilst there are certainly apparent similarities between UAP56 and DBP5 in their ATPase cycles, UAP56 appears to drive assembly of a protein complex whereas DBP5 appears to be disassembling complexes, therefore the details of how these two RNA helicases work may have important differences.

SRAG is a substrate for the arginine methylase PRMT1 (van Dijk et al, 2010) and here we have shown that arginine methylation enhances the ability of SRAG to bind NXF1 and reduces its ability to bind mRNA. Interestingly, ALY is also arginine methylated by PRMT1 and this promotes the handover of mRNA from ALY to NXF1 during mRNA export (Hung et al, 2010). Moreover ALY and SRAG appear to bind synergistically to NXF1 (Figure 4C) and loss of SRAG leads to accumulation of ALY on mRNA *in vivo* (Figure 6D), suggesting it may be incapable of recruiting NXF1 efficiently and handing mRNA over to it. Therefore the combined methylation of SRAG and ALY is likely to be an important control step in the recruitment of NXF1 to mRNA. However, ALY only binds hypomethylated SRAG *in vitro* (Figure 3C). Therefore SRAG and ALY may directly interact at an early stage in TREX assembly prior to arginine methylation which promotes their association with NXF1. The observed synergy of binding to NXF1 may result from the altered conformation of NXF1 on binding ALY and SRAG rather than a direct association of the two proteins on binding NXF1. The use of methylation in the regulation of mRNA export is conserved since in yeast the methylation of mRNA export factors by the PRMT1 orthologue Hmt1 also alters their interaction with other export factors. Moreover Hmt1 is recruited to genes during transcription (Yu et al, 2004), therefore

methylation may provide an additional transcription coupled trigger for TREX assembly, together with splicing (Masuda et al, 2005) and polyadenylation (Johnson et al, 2011).

SRAG and THOC5 bind in a mutually exclusive manner to the NTF2-like domain of NXF1 (Figure 4E). However, SRAG and THOC5 are found in a single complex with NXF1 *in vivo*. Together these data suggest that the NTF2–like domain of NXF1 exchanges binding partners within TREX during mRNA export (Figure 7C), though the order of binding and stages of mRNA export where partner exchange occurs remains to be determined. Consistent with the NTF2-like domain dynamically interacting with TREX components and other export factors, RBM15 and the related protein RBM15B also interact with the NTF2-like domain of NXF1 (Uranishi et al, 2009). RBM15 preferentially associates with the nuclear pore and promotes the association DBP5 with NXF1-mRNP complexes (Zolotukhin et al, 2009). Therefore RBM15 may represent the terminal partner for the NXF1 NTF2-like domain during mRNA export. One explanation for why proteins maintain the interaction with the NTF2-like domain of NXF1 maybe that this, together with ALY bound to the N-terminus, enhances the NXF1 RNA binding activity, which is essential for NXF1 mRNA export activity (Hautbergue et al, 2008). Consistent with this idea, we found that SRAG works synergistically with ALY to enhance the NXF1 RNA binding activity (Figure 5A). In yeast, the loss of Yra1 (ALY in metazoans) from the mRNP through ubiquitinylation by Tom1 occurs on the nuclear side of the nuclear pore complex (Iglesias et al, 2010). Such a mechanism, if conserved in metazoans would also destabilise the association of SRAG and THOC5 which bind synergistically with ALY to NXF1 (Katahira et al, 2009). Loss of both ALY and SRAG/THOC5 from NXF1 would be predicted to dramatically reduce its RNA binding activity and this together with the action of DBP5 on the cytoplasmic side of the nuclear pore is predicted to trigger dissociation of NXF1 from the mRNP.

In addition to SRAG, five other putative new components of TREX have recently been identified, though none of these have UBMs which might promote direct interaction with UAP56. Therefore, assembly of these other components into TREX may be driven by interaction with UAP56 via an alternative interaction mechanism, as is the

case for CIP29, or indirectly by binding ALY or SRAG or via interactions with other TREX components. However, the role of these additional TREX components in mRNA export remains unclear. The regulation of the UAP56 ATPase cycle by TREX components and establishing how UAP56 is first recruited to mRNA remain important questions for the future.

Materials and Methods

Plasmid, Antibodies, and Cell cultures

FLAG-NXF1, GST-NXF1 and 6His-ALY full-length and truncations were described previously (Hautbergue et al, 2008). The open reading frame of SRAG and THOC5 were cloned into pGEX-6P1, pET24b, and pET24b-GB1 vectors. Mutations in NXF1 and THOC5 were generated by Quickchange mutagenesis (Stratagene). Human inducible FLP-In T-REX 293 RNAi cell lines were constructed as described previously (Hautbergue et al, 2009) using the following target sequences: SRAG (ATATGCATCCAATTGGTTGTC) and ALY (CCGATATTCAGGAACTCTTTG). miRNA expression was induced with 1 µg/ml tetracycline (Sigma). The NXF1, CBP80 and Hpr1 antibodies were from Abcam. The ALY and FLAG monoclonal antibodies, anti-6His antibody, FLAG-agarose and FLAG peptide were from Sigma. The SRAG monoclonal antibody (KT64) was described previously (van Dijk et al, 2010). Antibodies to THOC5 (Hautbergue et al, 2009) and THOC2 (Masuda et al, 2005) were described previously.

GST-pulldown experiments and immunoprecipitations

The indicated GST-fusion constructs were expressed and purified essentially as described previously (Hautbergue et al, 2009). The binding reactions were performed in 1 ml of either PBST buffer (1X PBS, 0.1% Tween) for GST-fusion proteins interaction or RB100 buffer (25 mM HEPES-KOH pH 7.5, 100 mM KOAc, 10 mM $MgCl_2$, 1 mM DTT, 0.05 % Triton X-100, 10 % Glycerol) when purified proteins were present. Both buffers contained RNase A (10 µg/ml). The bound proteins were then washed with RB100 buffer, and then eluted with 50 mM Tris-HCl (pH 8.2), 40 mM reduced glutathione, 100

mM KOAc. Endogeneous SRAG was immunoprecipitated using 2 µg KT64 SRAG monoclonal antibody bound to 40 µl Protein G sepharose. FLAG-SRAG was immunoprecipitated using 30 µl FLAG-agarose (Sigma). RNAse A was used in immunoprecipitations where indicated at 10 µg/ml.

Sequential immunoprecipitations

18 µg of FLAG or FLAG-NXF1 was transfected into a 10-cm dish of 293T cells. After 48 hours, each dish was lysed in 1 ml of IP lysis buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 % Triton X-100, 10% glycerol) with 10µg/ml RNase A. The supernatants of cell extracts were incubated with 30 µl of FLAG-agarose beads (1% BSA blocked overnight) for 1 hour. The beads were then washed with 1 ml of IP lysis buffer 3 times and the bound proteins were subsequently eluted in 1 ml of IP lysis buffer with 100 ng/µl FLAG peptide. 2 µg of anti-SRAG or 50 µL of anti-THOC5 antibody was bound to 30 µL of Protein G-Sepharose. The eluate from the first IP was incubated with anti-SRAG or anti-THOC5 beads for 1 hour. The beads were then washed with 900 µL of IP lysis buffer 3 times. The bound proteins were finally eluted from the protein G-Sepharose with 50 µl of (0.2 M glycine pH 2.8, 1mM EDTA), and analysed by 12% SDS-PAGE and Western Blot with the indicated antibodies.

mRNP capture assay

A 15-cm dish of 293T cells was UV-crosslinked in 1 mL PBS with 300 mJ/cm^2 and then lysed in 1 ml IP lysis buffer described above. The extracts were cleared by centrifugation at 16100 g for 5 minutes 2 mg of total protein was denatured in Binding Buffer (10 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.5 % SDS, 0.1 mM EDTA) then incubated with 25 µl (bed volume) of oligo(dT)-cellulose beads (Sigma) for 1 hour at room temperature. The beads were then washed with 900 µl of Binding Buffer 3 times. The mRNPs were finally eluted for 30 min in Elution Buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 µg/ml RNase A) and analysed by 12% SDS-PAGE and Western Blot with the indicated antibodies.

UV-crosslinking experiments

A 5'-radiolabeled 15-mer RNA (5'-CAGUCGCAUAGUGCA-3') described previously (Hung et al, 2010) was incubated with the indicated proteins for 15min on ice in 20µL of RNA binding buffer (15 mM HEPES pH 7.9, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.05 % Tween 20, 10 % Glycerol, 100 mM NaCl). The reactions were then UV-irradiated for 15 minutes on ice and then analysed by SDS-PAGE. The gels were dried and the results were visualized by Phosphorimaging.

Human SRAG expression and purification

Full length SRAG was subcloned into a modified pFASTBAC (Invitrogen) vector. The corresponding baculoviruses were made in SF9 cells according to the instructions of the Bac-to-Bac Baculovirus Expression System from Invitrogen. Full length SRAG was purified on cobalt beads (TALON) from a 400 ml roller bottle culture of SF9 cells three days after infection. Purified SRAG was used in the ATPase, helicase, and RNA remodeling experiments.

NXF1 Remodeling assay

The RNA remodeling assay was described previously (Hautbergue et al, 2008). Purified GB1-ALY was first incubated with ^{32}P-radiolabeled RNA; then, GST-NXF1-p15 with/without SRAG were added to the ALY:RNA complexes and complexes purified on glutathione sepahrose. After protein:RNA complexes were eluted using reduced glutathione, bound RNA was UV-cross-linked. Eluted complexes were analyzed by Coomassie blue staining Western Blotting and PhosphorImaging.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization using Cy3 labelled oligo(dT) was carried out as described previously (Hautbergue et al, 2008).

ATPase assay

ATPase assays were essentially performed as described in (Cruz-Migoni et al, 2011). Reactions of 50 µl were stopped by the addition of 10 µl 0.5 M EDTA and supplemented with 140 µl H_20 before addition of 800 µl of Malachite green-Phosphomolybdenum reagent.

Helicase assay

The helicase assay was described in (Cruz-Migoni et al, 2011). Briefly, 2 µg recombinant UAP56-6His synthesised in *E. coli* were incubated in the presence or absence of recombinant GB1-ALY, SRAG, or MAGOH-6His at a 2:1 molar ratio for 15 minutes at room temperature prior addition of duplex RNA and ATP. Products of reactions were run on 15% native polyacrylamide gels in TBE buffer before Phosphorimaging.

Quantitative Analysis of Total and Cytoplasmic mRNA Levels

Total and cytoplasmic RNA were extracted from indicated RNAi stable cell lines as described (Hautbergue et al, 2009). Dried RNA pellets were resuspended in H_2O and 2 µg RNA was used for cDNA synthesis using Poly$(dN)_6$ random priming as described by the manufacturer (Bioscript kit from Bioline). 35 µl H_2O were added to 20 µl cDNA reactions and 1 µl diluted cDNA with 5 ng/µl primers were used in 10 µl quantitative PCRs (Quantace) run on a Rotorgene 6000 (Qiagen).

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Author Contributions

CC, MW, GH, NV and TvD carried out the experiments and helped with data analysis. SP and SW designed the experiments, helped analyse the data and wrote the manuscript.

Conflict of Interest

None to declare.

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Figure Legends

Figure 1. SRAG interacts with UAP56 and TREX. (A) Alignment of the UAP56-binding motif (UBM) from REF2-1 and UIF with SRAG proteins. (B) Pull-down competition assay with GST-UAP56 complexed with ^{35}S-labeled SRAG and increasing amounts of GB1-ALY. Proteins were detected by Coomassie staining and Phosphorimaging.(C) Coimmunoprecipitation of SRAG with TREX subunits using 293T cell extract. SRAG was immunoprecipitated using anti-SRAG monoclonal antibody and TREX subunits were detected by Western blotting with the indicated antibodies. Monoclonal FLAG antibody was used as a control for the immunoprecipitations.

Figure 2. SRAG activates the ATPase and helicase activity of UAP56. (A) ATPase activities for purified UAP56 in the presence of ALY, SRAG, CIP29, RNA and ATP as indicated. Values are the average from 3 independent assays and error bars represent the SD. Values are shown relative to the values observed for UAP56 + ATP + RNA. (B) The

helicase assay was performed at 37°C for 15 min with 1 mM UAP56, 2 mM ALY, SRAG, CIP29 or MAGOH. (C) *In vitro* protein:RNA UV cross-linking assay. Purified GST-UAP56 and GB1-ALY, SRAG, or MAGOH with/without ATP were UV-crosslinked with ^{32}P-radiolabelled RNA oligonucleotide. Resulting complexes were analysed by SDS-PAGE stained with Coomassie blue and Phosphorimaging. (D) *In vitro* reconstitution of UAP56-RNA-ALY complexes. Purified UAP56 expressed in *E. coli* was first incubated with continuously ^{32}P-radiolabelled RNA and non-hydrolysable ATP. Recombinant GST or GST-ALY were added to the reactions when indicated. Bound RNA was cross-linked (+) or not (-) to the proteins by UV irradiation, treated with RNAse A, and the resulting RNA-ALY protein complexes were purified using glutathione sepahrose. Eluted complexes were analysed on SDS-PAGE by Coomassie blue (left panel) and Phosphorimaging (right panel).(E) mRNP capture assay. Poly(A)$^+$ RNA from stable FLP-In 293 cells, expressing Control or UAP56/DDX39 miRNAs was purified on oligo-dT beads in denaturing conditions after UV cross-linking (+) or not (–). Total extract (1% of input) and eluted proteins were analysed by Western Blotting with SRAG antibody.

Figure 3. Arginine methylation of SRAG regulates its interaction with export factors. (A) FLAG-tagged SRAG was expressed in 293T cells grown in the presence or absence of AdOx for different time. After treatment, 10µg of total cell extracts were analysed by Western Blot using anti-FLAG antibody. (B) Western Blot analysis of 293T cells incubated with 10 mg/ml cycloheximide for 8 hours. Whole cell extracts of cells grown +/- AdOx were analysed (lanes 1,2). Additionally, untreated cells were lysed in a buffer +/- AdOx and nuclear and cytoplasmic fractions were analysed with the indicated antibodies (lanes 3-8). SSRP1 Western blotting was used to confirm that the cytoplasmic fractions were not contaminated with nuclear material. (C) GST-UAP56, GST-ALY, and GST-NXF1 were used in pull-down assays with 293T cell extract with/without AdOx in the presence of RNase. Proteins were detected via Coomassie staining and Western Blot. (D) Immunoprecipitation of NXF1 from 293T cells treated with AdOx as indicated. Immunoprecipitations were carried out in the presence of

RNase A. Proteins were detected by Western Blotting. (E) GST-UAP56, GST-ALY, and GST-NXF1 were used in pull-down assays with recombinant SRAG alone or co-expressed with PRMT1 in the presence of RNase A. Proteins were detected via Coomassie staining and Western Blot.(F) mRNP capture assay. Poly(A)$^+$ RNA from 293T cells +/– AdOx was purified on oligo-(dT) beads in denaturing conditions after UV cross-linking (+) or not (–). Total extract (1% of input) and eluted proteins were analysed by Western Blotting with SRAG antibody.

Figure 4. Mutually exclusive binding of THOC5 and SRAG to NXF1. (A) Schematic representation of NXF1 truncations used in this study. (B) GST-ALY and GST-SRAG pulled down ^{35}S-labelled NXF1 full-length and truncations. (C) Pull-down competition assay with GST-NXF1-p15, 293T overexpressed FLAG-SRAG or FLAG-UIF, and increasing amounts of purified GB1-ALY in the presence of RNase A. Proteins were detected by Coomassie staining or Western Blot. (D) Pull-down assays using GST-NXF1(aa 371-619)-p15 wild type or mutants with 293T cell extracts from cells transfected with FLAG-SRAG in the presence of RNase A. Proteins were detected by Coomassie staining or Western Blot. (E) Pull-down competition assay with GST-NXF1-p15, with 293T cell extracts from cells overexpressed FLAG-SRAG and increasing amounts of purified THOC5-6His in the presence of RNase. Proteins were detected via Coomassie staining or Western Blot.

Figure 5. SRAG and ALY modulate the RNA binding activity of NXF1. (A) GB1-ALY was first incubated with ^{32}P-radiolabeled RNA. Then GST-NXF1-p15 with/without SRAG were added to the ALY:RNA complexes. After protein:RNA complexes were purified using glutathione sepharose and subsequently eluted with reduced glutathione, bound RNA was UV-cross-linked. Eluted complexes were analyzed by Coomassie blue, Western Blot and PhosphorImaging. (B) Purified GST, GB1-ALY, or SRAG was incubated with ^{32}P-radiolabeled RNA with/without UV-crosslinking. Eluted protein:RNA complexes were analyzed by Coomassie blue and PhosphorImaging.

Figure 6. SRAG is required for efficient mRNA export *in vivo*. (A) Total cell extracts from stable FLP-In 293 cells expressing Control RNAi, ALY RNAi, SRAG RNAi and ALY/SRAG were analysed by Western Blot.(B) Growth of stable cell lines following induction of miRNAs targeting the indicated genes. Error bars represent the standard deviation of three independent experiments.(C) Localization of Poly(A)$^+$ RNA following induction of miRNAs targeting export factors for 48, 72, and 96 hr. All equivalent panels are shown at the same exposure.(D) Poly(A)$^+$ RNA from stable FLP-In 293 cells, expressing Control RNAi, ALY RNAi, or SRAG RNAi, was purified on oligo-(dT) beads in denaturing conditions after UV cross-linking (+) or not (–). Total extract (1% of input) and eluted proteins were analysed by Western Blotting with ALY, SRAG, UAP56, or CBP80 antibodies.

Figure 7. SRAG, THOC5 and ALY function in the same mRNA export pathway. (A) Immunoprecipitations were carried out with anti-FLAG antibody followed by anti-SRAG or anti-THOC5 antibody. Proteins were detected by Western Blot. (B) Quantitative RT-PCR analysis was used on cytoplasmic and total mRNA to assess the levels of each gene relative to the U1 snRNA. mRNA levels for the cytoplasmic/total ratio are expressed relative to the values seen in the control RNAi which was set at 1.0. Error bars represent s.e.m. from 3 experiments. (C) A model for assembly and maturation of the TREX mRNA export complex. Me = arginine methylation.

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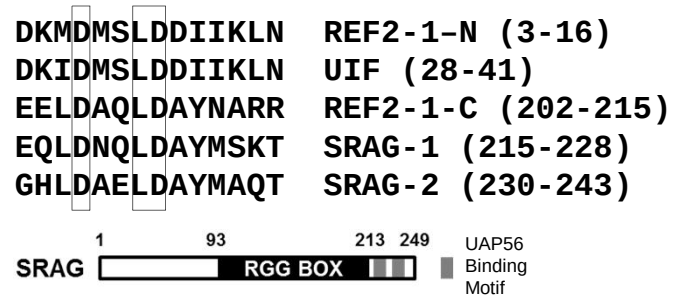
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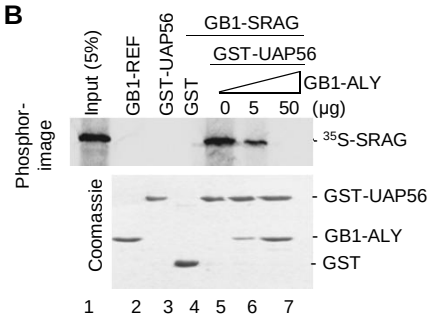
DKIDMSLDDIIKLN UIF (28-41)
EELDAQLDAYNARR REF2-1-C (202-215)
EQLDNQLDAYMSKT SRAG-1 (215-228)
GHLDAELDAYMAQT SRAG-2 (230-243)
1
93
213
249
SRAG
RGG BOX
UAP56
Binding
Motif

B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GST-UAP56
GST
GB1-SRAG
GST-UAP56
GB1-ALY
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Coomassie
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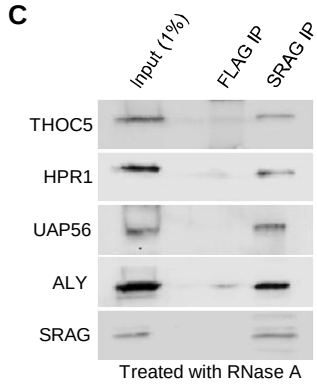
GB1-ALY
GST
1 2 3 4 5 6 7

C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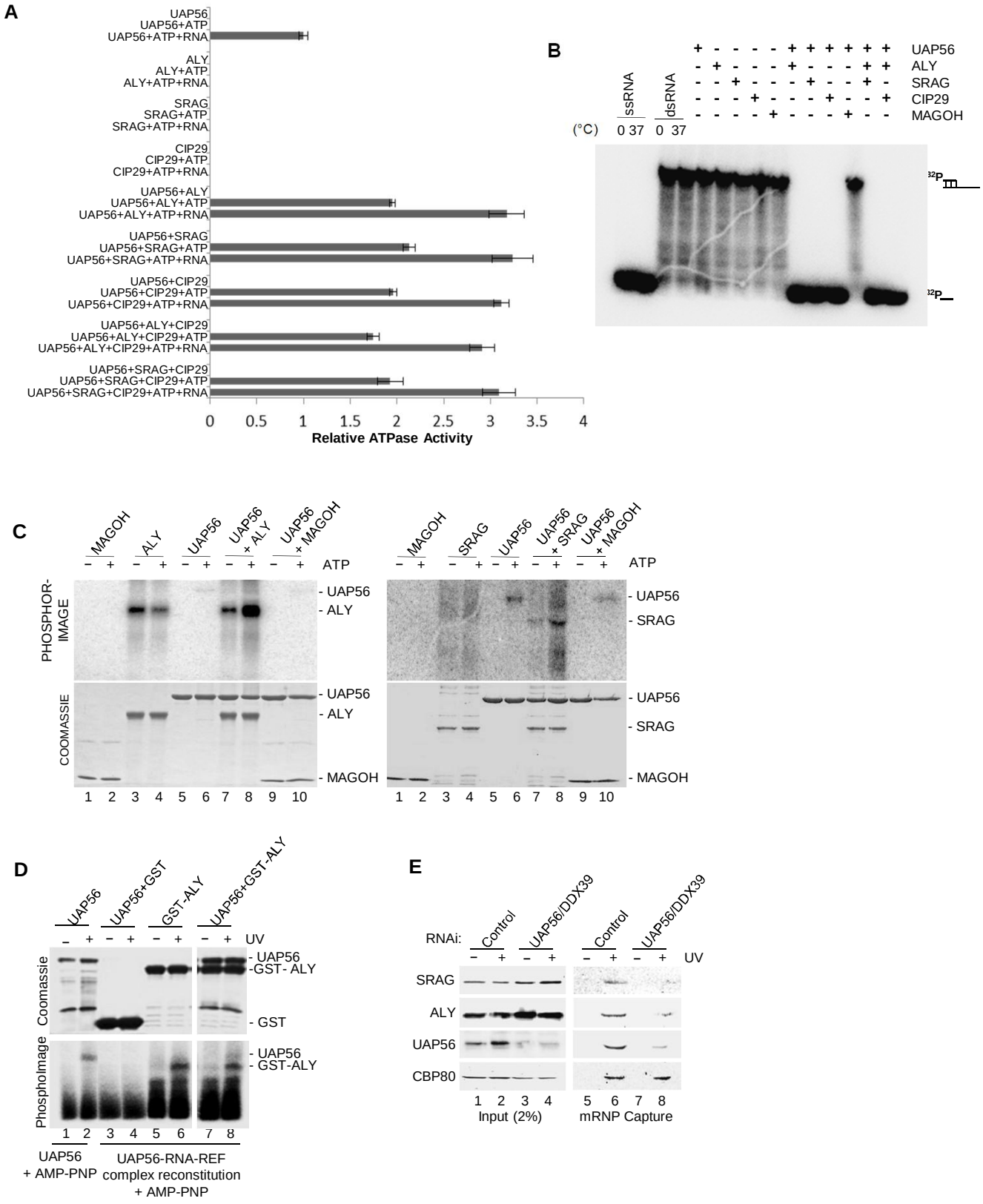
THOC5
HPR1
UAP56
ALY
SRAG
Treated with RNase A

Figure 1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UAP56+ATP+RNA
ALY
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ALY+ATP+RNA
SRAG
SRAG+ATP
SRAG+ATP+RNA
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CIP29+ATP
CIP29+ATP+RNA
UAP56+ALY
UAP56+ALY+ATP
UAP56+ALY+ATP+RNA
UAP56+SRAG
UAP56+SRAG+ATP
UAP56+SRAG+ATP+RNA
UAP56+CIP29
UAP56+CIP29+ATP
UAP56+CIP29+ATP+RNA
UAP56+ALY+CIP29
UAP56+ALY+CIP29+ATP
UAP56+ALY+CIP29+ATP+RNA
UAP56+SRAG+CIP29
UAP56+SRAG+CIP29+ATP
UAP56+SRAG+CIP29+ATP+RNA
0 0.5 1 1.5 2 2.5 3 3.5 4
Relative ATPase Activity
B
UAP56
ALY
SRAG
CIP29
MAGOH
ssRNA
dsRNA
(°C) 0 37 0 37
32P
32P
C
MAGOH
ALY
UAP56
UAP56 + ALY
UAP56 + MAGOH
ATP
MAGOH
SRAG
UAP56
UAP56 + SRAG
UAP56 + MAGOH
ATP
PHOSPHOR-IMAGE
COOMASSIE
- UAP56
- ALY
- SRAG
- MAGOH
1 2 3 4 5 6 7 8 9 10
D
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GST-ALY
UAP56+GST-ALY
UV
Coomassie
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- GST
- UAP56
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UAP56 + AMP-PNP
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UAP56/DDX39
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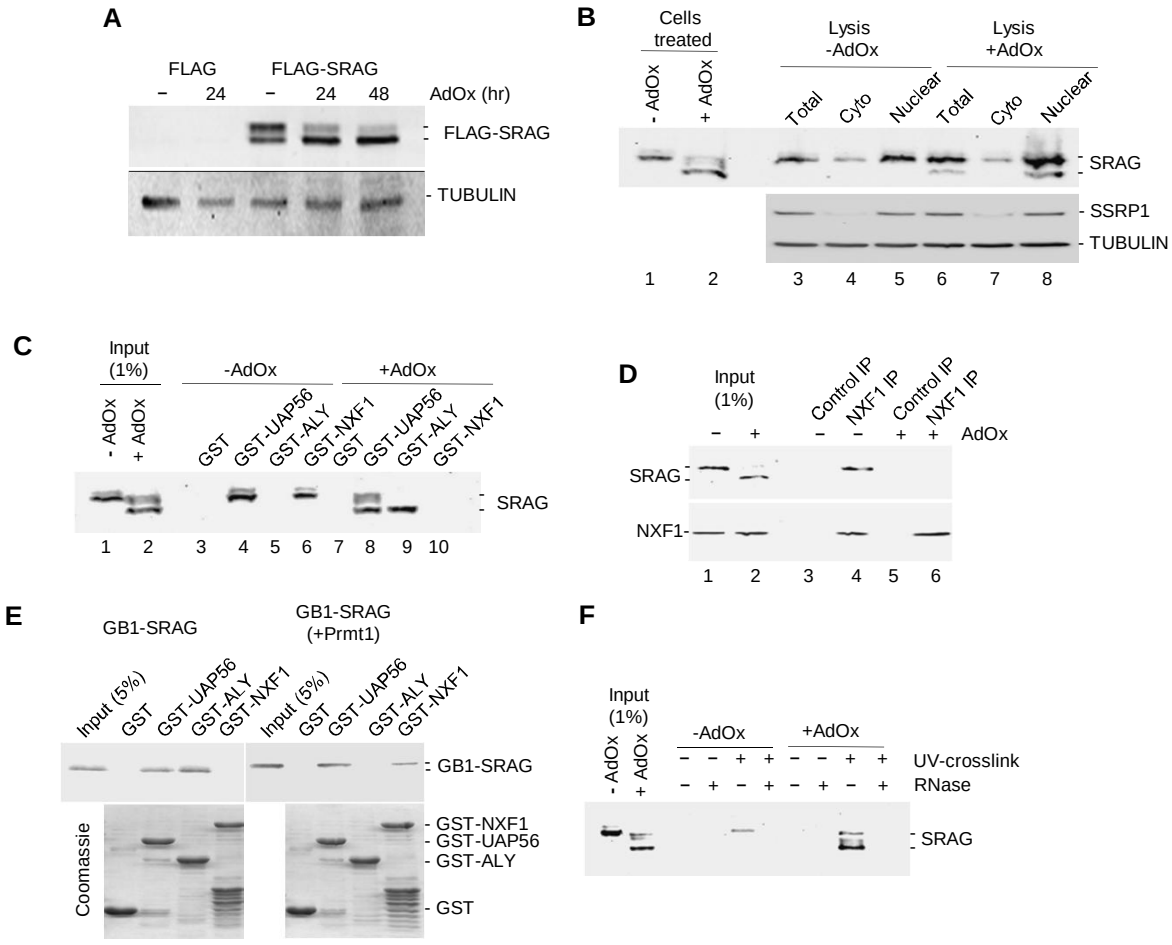
mRNP Capture

Figure 2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FLAG-SRAG
TUBULIN
B
Cells treated
- AdOx
+ AdOx
Lysis -AdOx
Lysis +AdOx
Total
Cyto
Nuclear
Total
Cyto
Nuclear
SRAG
SSRP1
TUBULIN
1 2 3 4 5 6 7 8
C
Input (1%)
-AdOx
+AdOx
- AdOx
+ AdOx
GST
GST-UAP56
GST-ALY
GST-NXF1
GST
GST-UAP56
GST-ALY
GST-NXF1
SRAG
1 2 3 4 5 6 7 8 9 10
D
Input (1%)
Control IP
NXF1 IP
Control IP
NXF1 IP
– + – – + + AdOx
SRAG
NXF1
1 2 3 4 5 6
E
GB1-SRAG
GB1-SRAG (+Prmt1)
Input (5%)
GST
GST-UAP56
GST-ALY
GST-NXF1
Input (5%)
GST
GST-UAP56
GST-ALY
GST-NXF1
GB1-SRAG
Coomassie
GST-NXF1
GST-UAP56
GST-ALY
GST
F
Input (1%)
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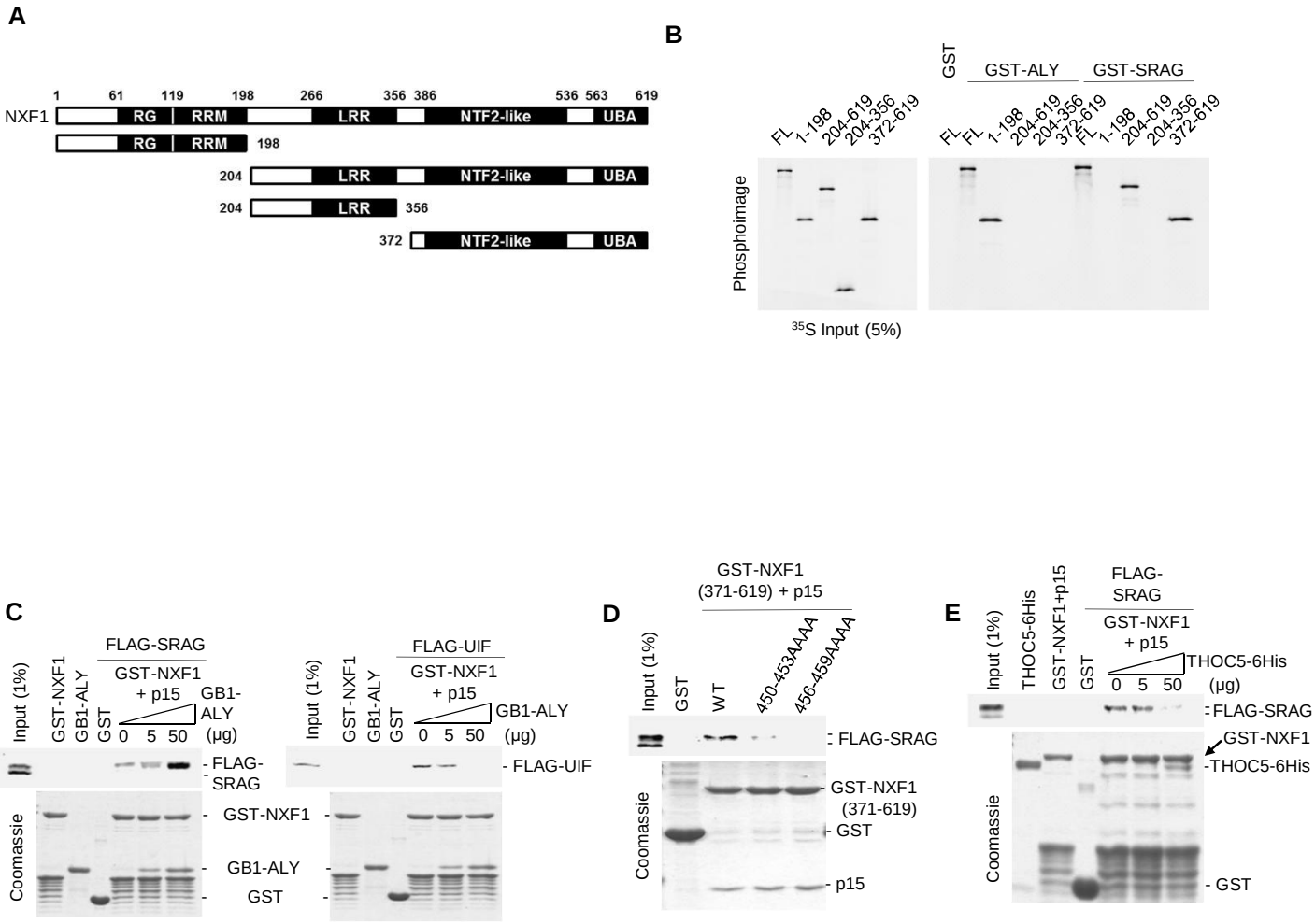
+AdOx
- AdOx
+ AdOx
– – + + – – + + UV-crosslink
– + – + – + – + RNase
SRAG

Figure 3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372-619
Phosphoimage
35S Input (5%)
C
FLAG-SRAG
FLAG-UIF
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GB1-ALY
Input (1%)
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GB1-ALY
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0 5 50 (µg)
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GB1-ALY
GST
Coomassie
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GST
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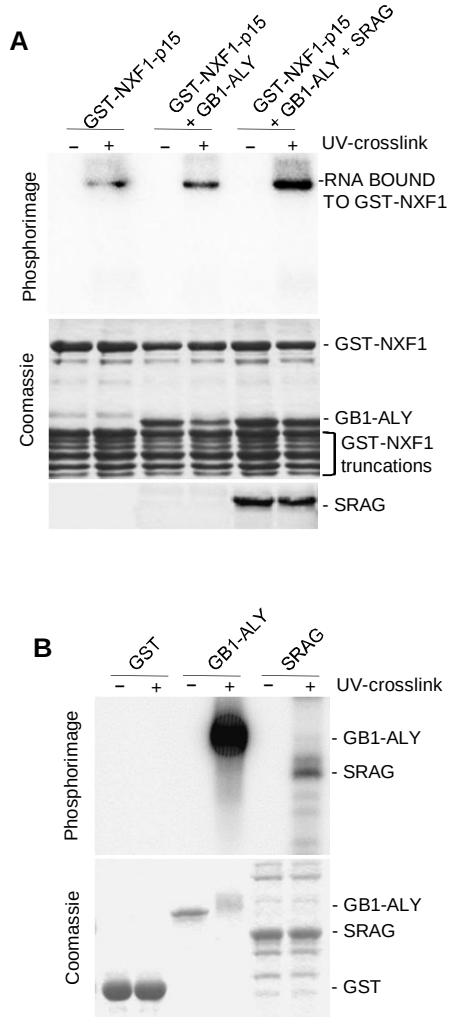
Figure 4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GST-NXF1-p15
GST-NXF1-p15 + GB1-ALY
GST-NXF1-p15 + GB1-ALY + SRAG
UV-crosslink
– + – + – +
Phosphorimage
-RNA BOUND TO GST-NXF1
Coomassie
- GST-NXF1
- GB1-ALY
GST-NXF1 truncations
- SRAG
B
GST
GB1-ALY
SRAG
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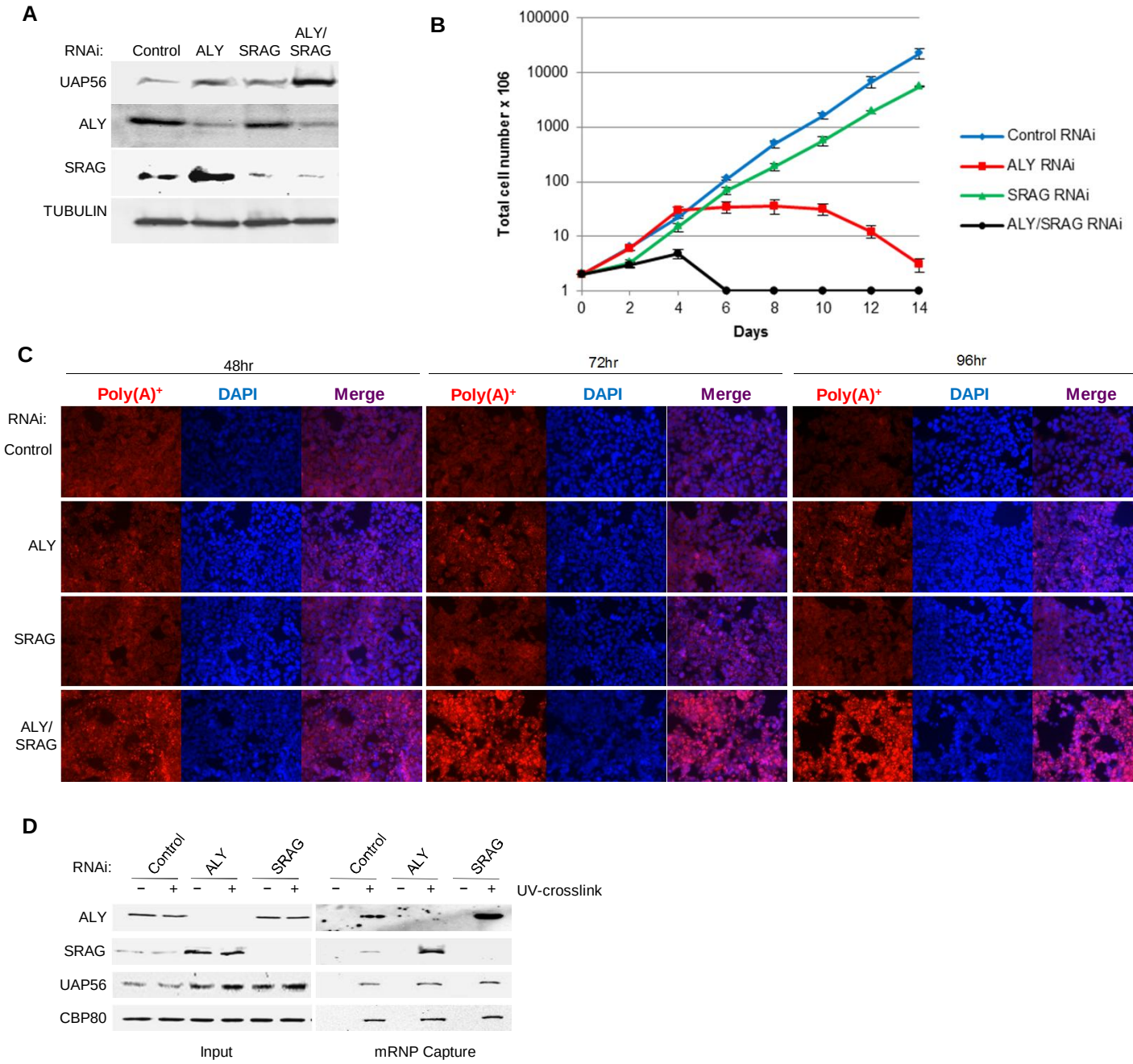
- GST

Figure 5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1000
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10
1
0
2
4
6
8
10
12
14
Days
Control RNAi
ALY RNAi
SRAG RNAi
ALY/SRAG RNAi
C
48hr
72hr
96hr
Poly(A)+
DAPI
Merge
RNAi:
Control
ALY
SRAG
ALY/ SRAG
D
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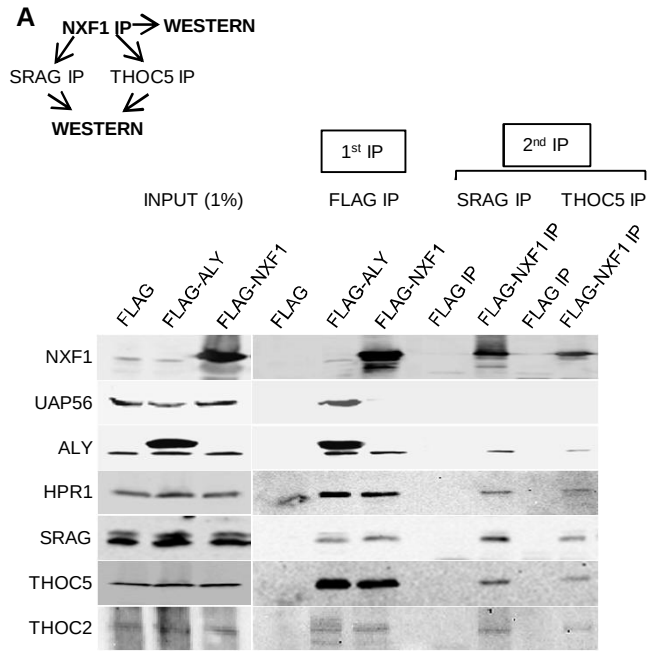
ALY
SRAG
UV-crosslink
ALY
SRAG
UAP56
CBP80
Input
mRNP Capture

Figure 6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NXF1 IP
WESTERN
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FLAG-ALY
FLAG-NXF1
FLAG
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FLAG IP
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FLAG IP
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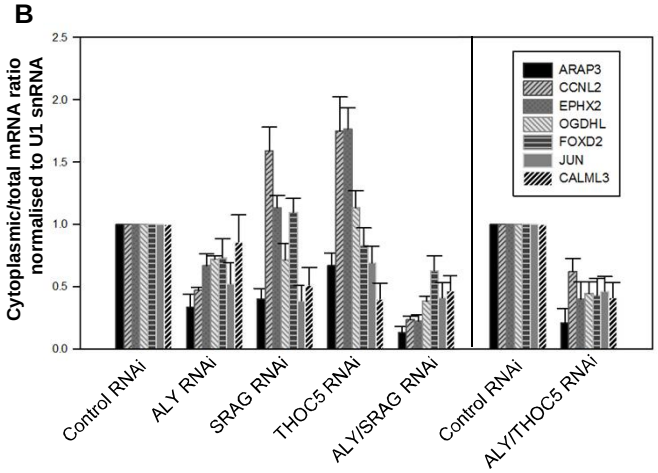
NXF1
UAP56
ALY
HPR1
SRAG
THOC5
THOC2


B
Cytoplasmic/total mRNA ratio normalised to U1 snRNA
2.5
2.0
1.5
1.0
0.5
0.0
ARAP3
CCNL2
EPHX2
OGDHL
FOXD2
JUN
CALML3
Control RNAi
ALY RNAi
SRAG RNAi
THOC5 RNAi
ALY/SRAG RNAi
Control RNAi
ALY/THOC5 RNAi

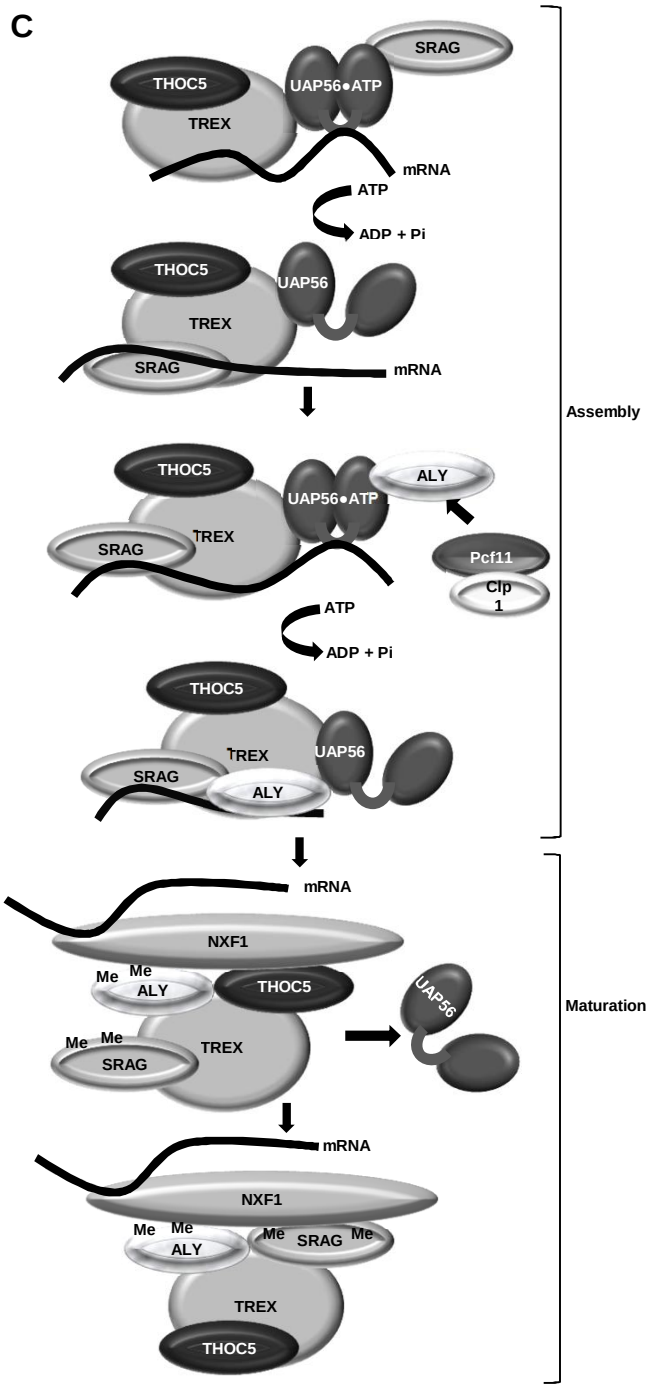

C
SRAG
THOC5
UAP56•ATP
TREX
mRNA
ATP
ADP + Pi
THOC5
UAP56
TREX
SRAG
mRNA
THOC5
UAP56•ATP
ALY
TREX
SRAG
Pcf11
Clp 1
ATP
ADP + Pi
THOC5
TREX
UAP56
SRAG
ALY
Assembly
mRNA
NXF1
Me
Me
ALY
THOC5
UAP56
Me
Me
SRAG
TREX
mRNA
NXF1
Me
Me
ALY
Me
SRAG
Me
TREX
THOC5
Maturation

Figure 7

Supplementary data

Table S1

Strept. PD of Bio-SRAG				
Name	Aliases	IPI	Unique Peptides	MASCOT score
SRAG	FOP, CHTOP	IPI00469107	4	340
DDX39	DDX39a	IPI00123878	4	159
UAP56	BAT1, DDX39b	IPI00409462	4	266
HPR1	THOC1, p84	IPI00153778	3	191
THOC2	Tho2	IPI00664886	7	345
ALY	REF, THOC4	IPI00114407	4	343
THOC5	fSAP79, Fmip	IPI00222687	2	156
THOC6	fSAP35, WDR58	IPI00123949	2	92
UIF	FYTTD1	IPI00462979	4	372

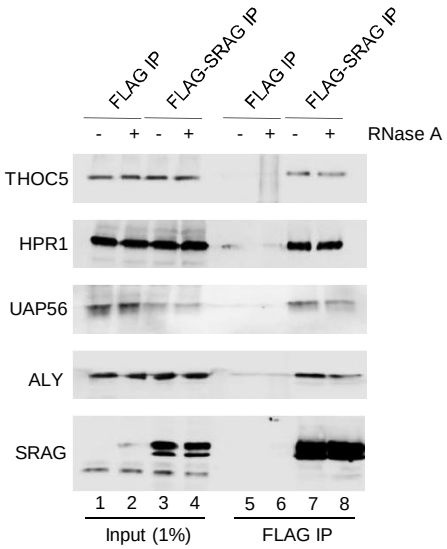

FLAG IP
FLAG-SRAG IP
FLAG IP
FLAG-SRAG IP
- + - + - + - +
RNase A
THOC5
HPR1
UAP56
ALY
SRAG
1 2 3 4 5 6 7 8
Input (1%)
FLAG IP

Figure S1.

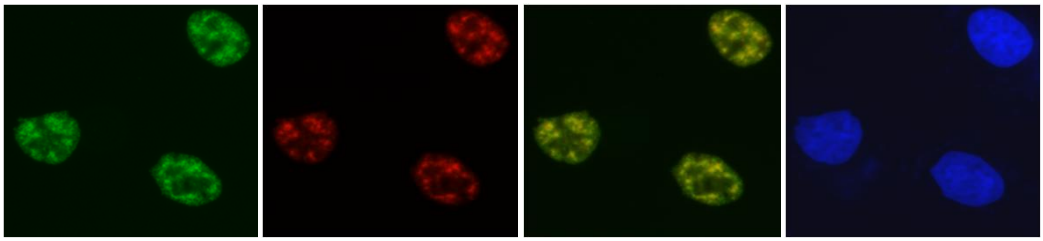


Figure S2. Localization of SRAG in HeLa cells. Cells were stained with antibodies to SRAG and SC35 and DNA was stained with DAPI.

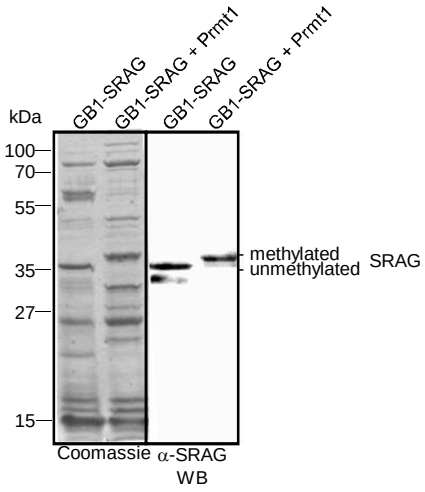


Figure S3
Purified GB1-SRAG alone or co-expressed with PRMT1 from *E.coli*. Proteins were detected via Coomassie staining and Western Blot.