Université de Montréal

A mechanism for co-transcriptional recruitment of mRNA localization factor on nascent mRNAs in budding yeast

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Cette thèse intitulée :

A mechanism for co-transcriptional recruitment of mRNA localization factor on nascent mRNAs in budding yeast

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Résumé

Le transport et la localisation des ARN messagers permettent de réguler l'expression spatiale et temporelle de facteurs spécifiques impliqués dans la détermination du destin cellulaire, la plasticité synaptique, la polarité cellulaire et la division asymétrique des cellules. Chez *S.cerevisiæ*, plus de trente transcrits sont transportés activement vers le bourgeon cellulaire. Parmi ces transcrits, l'ARNm *ASH1* (asymetric synthesis of HO) est localisé à l'extrémité du bourgeon pendant l'anaphase. Ce processus va entrainer une localisation asymétrique de la protéine Ash1p, qui sera importée uniquement dans le noyau de la cellule fille, où elle entraine le changement de type sexuel. La localisation asymétrique de l'ARNm *ASH1*, et donc de Ash1p, implique la présence de différents facteurs de localisation. Parmi ces facteurs, les protéines She (She1p/Myo4p, She2p et She3p) et les répresseurs traductionnels (Puf6p, Loc1p et Khd1p) participent à ce mécanisme. La protéine navette She2p est capable de lier l'ARNm *ASH1* et va entrainer le ciblage de cet ARNm vers l'extrémité du bourgeon en recrutant le complexe She3p-Myo4p. Des répresseurs traductionnels régulent la traduction de cet ARNm et évitent l'expression ectopique de la protéine Ash1p pendant son transport. Alors que la fonction cytoplasmique de She2p sur la localisation des ARNm est connue, sa fonction nucléaire est encore inconnue.

Nous avons montré que She2p contient une séquence de localisation nucléaire non classique qui est essentielle à son import nucléaire médié par l'importine α (Srp1p). L'exclusion de She2p du noyau par mutation de son NLS empêche la liaison de Loc1p et Puf6p sur l'ARNm *ASH1*, entrainant un défaut de localisation de l'ARNm et de la protéine. Pour étudier plus en détail l'assemblage de la machinerie de localisation des ARNm dans le noyau, nous avons utilisé des techniques d'immunoprécipitation de chromatine afin de suivre le recrutement des facteurs de localisation et des répresseurs traductionnels sur les ARNm naissants. Nous avons montré que She2p est recruté sur le gène *ASH1* pendant sa transcription, via son interaction avec l'ARNm *ASH1* naissant. Puf6p est également recruté sur *ASH1*, mais d'une manière dépendante de la présence de She2p. De façon intéressante,

nous avons détecté une interaction entre She2p et la plus grande sous-unité de l'ARN polymérase II (Rpb1p). Cette interaction est détectée avec la forme active en élongation de l'ARN polymérase II. Nous avons également démontré que She2p interagit avec le complexe d'élongation de la transcription Spt4p/Spt5p. Une délétion de *SPT4* ou une mutation dans *SPT5* (Ts *spt5*) à température restrictive empêche l'interaction entre She2p et Rpb1p, et diminue le recrutement de She2p au gène *ASH1*, entrainant un défaut de localisation de l'ARNm et un défaut de localisation asymétrique de la protéine Ash1p. De manière globale, nos résultats montrent que les facteurs impliqués dans la localisation cytoplasmique des ARNm et dans leur contrôle traductionnel sont recrutés de façon co-transcriptionnelle sur les ARNm naissants via leur interaction avec la machinerie de transcription, suggèrant un rôle important de la machinerie transcriptionelle dans la localisation des ARNm.

Mots-clés : localisation des ARNm, ARNm ASH1, séquence de localisation nucléaire (NLS), She2p, recrutement co-transcriptionnel, ARN polymérase II, complexe Spt4-Spt5p, levure.

Abstract

Cytoplasmic transport and localization of messenger RNAs allows temporal and spatial expression of specific factors involved in cell fate determination, synaptic plasticity, cellular polarity or asymmetric cell division. In *S. cerevisiae*, over thirty transcripts are actively transported and localized to the bud tip of budding yeast. One of them, the *ASH1* mRNA (for Asymmetric Synthesis of HO), is localized at the bud tip in late anaphase cells. This allows Ash1p, a transcriptional repressor of the HO endonuclease, to be sorted exclusively to the daughter cell nucleus, where it prevents mating type switching. Proper *ASH1* mRNA localization and Ash1p asymmetric expression involve localization factors, which are part of the She-proteins (She1p/Myo4p, She2p and She3p), and translational repressors (the proteins Puf6, Loc1 and Khd1). The nucleo-cytoplasmic shuttling protein She2p binds the *ASH1* mRNA and targets it for localization at the bud tip by recruiting the She3p-Myo4p complex. Translational repressors regulate the translation of *ASH1* mRNA and avoid ectopic expression of the Ash1 protein during the transport of its transcript. While the cytoplasmic role of She2p in mRNA localization is known, its nuclear function is still unclear.

We now show that She2p contains a non-classical nuclear localization signal sequence (NLS) which is essential for its nuclear import via the importin α Srp1p. Exclusion of She2p from the nucleus by mutagenesis of its NLS disrupt the binding of Loc1p and Puf6p to the *ASH1* mRNA, leading to defective mRNA localization and Ash1p sorting. To further investigate the assembly of the mRNA localization machinery in the nucleus, we used chromatin immunoprecipitation (ChIP) to follow the recruitment of localization factors and translational repressors on nascent localized mRNAs. We found that She2p is recruited on the *ASH1* gene during transcription, via its interaction with the nascent *ASH1* mRNA. Puf6p is also recruited on the *ASH1* gene, but in a She2p-dependent manner. Interestingly, we detected an interaction between She2p and Rpb1p, the largest

subunit of RNA polymerase II in vivo. This interaction is independent of the RNA-binding properties of She2p, and involves the elongating form of the RNA polymerase II. We also found that She2p interacts with both members of the elongation factors Spt4p /Spt5p; Deletion of SPT4 or Ts *spt5* mutants at restrictive temperature disrupted the interaction between She2p and Rpb1p, and then reduced the recruitment of She2p on the ASH1 gene, resulting in *ASH1* mRNA delocalization and defective Ash1p sorting. Altogether, our results show that factors involved in cytoplasmic mRNA localization and translational control are recruited co-transcriptionally on nascent mRNAs via interation with the transcription machinery, pointing toward a role of the transcription machinery in the mRNA localization process.

Keywords: mRNA localization, *ASH1* mRNA, nuclear localization signal (NLS), She2p, Co-transcriptional recruitment, RNA polymerase II, Spt4-Spt5 complex, yeast.

Table of Contents

List of tables

Chapter I

Chapter II

Chapter III

List of figures

Chapter I

Chapter II

Chapter III

Abbreviations

Amp	Ampicillin
ASH	asymmetric synthesis of HO
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
°C	Grade Celsius
CTD	Carboxy terminal domain
CTR	Carboxy terminal region
Da	Dalton
DAPI	diamidino-2-phenylindol dihydrochloride
DEPC	Diethylpyrocarbonate
DIC	Differential interference contrast
DNA	deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Dideoxynucleotides
DTT	Dithiotreitol
E. coli	Escherichia coli
E1-3	*ASH1* mRNA localization element
ECL	Enhanced chemoluminiscence
EDTA	Ethylenediaminotetraaceticacid
eIF	Eukaryotic (translation-) initiation factor
ER	endoplasmatic reticulum
et al.	et alii (from Latin, “and others”)
FISH	Fluorescence in situ hybridization
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GTP	Guanosine triphosphate

HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hnRNP	Heterogeneous nuclear ribonucleoprotein
HO endonuclease	Homothallic switching endonuclease
Ig	Immunoglobulin
kb	Kilo bases
l	Litre
LB-Medium	Liquid-Broth-Medium
M	Molar
mA	Milliampere
MDa	Megadalton
mg	Milligramm
µg	Microgramm
µl	Mikroliter
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
NES	Nuclear export signal
NLS	Nuclear localization signal
NP-40	Nonidet P-40 (Igepal-CA-630)
NPC	Nuclear pore complex
nt	Nucleotide
OD	Optical density
oligo	Oligonucleotide
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

pH	Potential of Hydrogen
Pol II	Polymerase II
RBP	RNA-binding domain
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rpm	Rounds per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Svedberg unit
S. cerevisiae	Saccharomyces cerevisiae
SDS	Sodium dodecyl sulfate
SHE	Swi5p-dependent HO expression
TAE	Tris-acetate-EDTA buffer
TAP	Tandem affinity purification
TBS	Tris-buffered saline
TEMED	N, N, N', N'-Tetramethylethylenediamine
Tris	Trishydroxymethylaminomethane
tRNA	Transfer ribonucleic acid
UTR	Untranslated region
V	Volt
wt	Wild type
YEP	Yeast Extract Peptone

Amino acid codes

Amino acid	Three letter code	One letter code
alanine	ala	A
arginine	arg	R
asparagine	asn	N
aspartic acid	asp	D
cysteine	cys	C
glutamic acid	glu	E
glutamine	gln	Q
glycine	gly	G
histidine	his	H
isoleucine	ile	I
leucine	leu	L
lysine	lys	K
methionine	met	M
phenylalanine	phe	F
proline	pro	P
serine	ser	S
threonine	thr	T
tryptophan	try	W
tyrosine	tyr	Y
valine	val	V

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To

my mother

And in memory of my father

Chapter I

Introduction

Following transcription in the nucleus, most RNAs are exported through the nuclear pores into the cytoplasm, where they are translated into a distinct set of proteins. For specific intracellular compartments and their localized distributions, most proteins are targeted to their destination on the basis of signals in the peptide sequence. In contrast to these post-translational event, messenger RNA localization has classically been considered to be a mechanism used to spatially and temporally restrict gene expression to specific regions within the cell or embryo. This mechanism has obviously different advantages. For instance, it can rapidly generate large amounts of proteins locally, offers the potential for a local regulation of protein expression and protects the rest of the cell from proteins that might be toxic or deleterious in other cellular compartments.

1.1 The intracellular transport and localization of mRNAs in eukaryotes

Twenty-seven years ago, beta-Actin mRNA that accumulates in the myoplasm of Ascidian eggs was discovered as the first localized mRNA (Jeffery et al. 1983). Since then, examples of localized transcripts dramatically increase year by year, encoding for either cytoplasmic, nuclear, secreted, membrane-associated or cytoskeletal proteins from yeasts to fruit flies, frogs, zebrafish and mammals (Bashirullah et al., 1998). The localization of specific mRNAs has been shown to play an important role during the development of multicelluar organisms and also in processes like synaptic transmission, cell motility and asymmetric cell division (Du et al., 2007; Kiebler et al., 2000; Kislaukis et al., 1997; Jan et al., 1998).

1.1.1 Roles of localized mRNAs in development

The localization of specific mRNA has been shown to be essential for the establishment of the antero-posterior axis of *Drosophila* and animal-vegetal axis of *Xenopus* (Lasko 1999; Kloc et al., 2001). The disruption of their localization machineries results in gross abnormalities during the development of these organisms. In Drosophila, embryonic axis specification is determined by two localized mRNAs: *bicoid*, which is localized to the anterior pole, and *nanos*, which is localized to posterior pole (Figure 1A) (Van Eeden et al. 1999). These localized RNAs set up opposing protein gradients that define the anterior and posterior axes of the embryo.

In addition to *bicoid* and *nanos* mRNA, at least four other types of localized mRNA are required for the determination of the abdomen and the pole cells, the founders of germline lineage (St Johnston 1993). All of them are localized to the posterior region of the egg. The first mRNA to reach the posterior of the oocyte is *oskar* mRNA, which is sufficient to define the site of pole plasm formation. Unlike Bicoid and Nanos, however, Oskar protein does not seem to play a direct role in determining cell fates in the embryo and acts instead to nucleate the polar granules of the pole plasm by recruiting other mRNAs and proteins to the posterior pole (Breitweiser et al. 1996). One of the most important mRNA recruited to the posterior of the egg is *nanos* mRNA (Wang & Lehmann 1991). The second one is germ cell-less (*gcl*) mRNA that localizes to the posterior of the oocyte after stage 11 of oogenesis. The role of Gcl is unclear, but seems to function in formation of ectopic polar buds (Jongens et al. 1994). The third mRNA is the non-coding transcript *Pgc*, which localizes to the posterior at around stage 11 and is a component of the polar granules (Nakamura et al. 1996). The fourth class of localized RNAs specifically required for pole cell formation are the large and small ribosomal RNAs encoded by the mitochondrial genome (mt rRNA). In a recent striking study involving high-throughput and high-

resolution in situ hybridizations of over 3000 transcripts in Drosophila embryos, 71% were found to be expressed in spatially distinct patterns (Lecuyer et al., 2007), suggesting that the role of localization of mRNAs in cellular development may be much more prevalent than previously thought.

As in Drosophila, similar processes occur in oocytes of the frog *Xenopus*, where the mRNA encoding the T-box transcription factor VegT localizes to the vegetal pole and induces endodermal and mesodermal cell fates in the embryo (King et al., 2005). In addition, homologues of the localized maternal mRNAs that play a role in pole cell development in *Drosophila* have been implicated in the determination of the primordial germ cells (PGCs) in some other organisms. For instance, three Nanos homologues have been identified in the nematode *Caenorhabditis elegans*, and one of them has been shown to be a component of the P-granules, which segregate into primordial germ cells (PGCs), suggesting the existence of a conserved pathway for specifying the germline lineage (Subramaniam & Seydoux 1999, Schisa et al. 2001).

1.1.2 The localization and transport of mRNA in neurons

There is increasing evidence that an important aspect of gene expression in neurons involves the targeting of certain mRNAs to particular subcellular domains. It is now well known that localized mRNAs in dendrites encode proteins of different functional types. For example, the high molecular weight microtubule-associated protein (MAP2) mRNA and an activity-regulated cytoskeleton-associated protein (ARC) mRNA both encode certain cytoskeletal proteins; CaMKII α mRNA encode the α subunit of calcium/calmodulin-dependent protein kinase II that has a role in synaptic plasticity (Steward et al. 2001). During brain development, localization of mRNAs in axonal growth cones allows neurons to respond to local environmental cues as the distal axonal processes navigate toward their

synaptic partners (Lin and Holt, 2007). More recent studies indicate that hundreds of mRNAs are present in neuronal processes, where they encode diverse functions, suggesting that neurons may use the general mechanism of RNA targeting for different purposes at different times in their life time (Eberwine et al., 2002; Martin and Zukin, 2006).

Some evidences suggest that localized mRNAs contain a localization element in the 3' untranslated region (UTR) required for sufficient localization in neurons. For example, MBP mRNA contains an A2RE localization element in its 3' UTR, recognized by Heterogeneous nuclear ribonucleoprotein (hnRNP) A2 (Ainger et al., 1997). The A2RE has been shown to be sufficient to transport mRNAs into neuronal dendrites (Shan et al., 2003). The hnRNP E1, another RNA-binding protein that regulates translation of specific mRNAs, is recruited to A2RE RNA granules by binding to hnRNP A2. This recruitment inhibits translation of A2RE RNA during granule transport (Linda et al. 2006). Similar studies show that CamKIIα mRNA contains a 94 nucleotide-long element in its 3'UTR, required for dendritic localization in neurons. Deletion mutants of the 3'UTR of CaMKII mRNA drastically reduce the amount of transcript in mice dendrites (Mori et al. 2000; Ouyang et al. 1999). Interestingly, recent works show that microtubules play a critical role in the dendritic localization of RNA granules in neurons (Kiebler et al., 1999; Tang et al., 2001; Kanai et al., 2004). The purified large RNA granules from mouse brain that associate with the tail of the kinesin motor protein KIF5 contain localized mRNAs (like CamKIIa mRNA) and mRNA binding proteins (like Staufen and FMRP). This result further confirmed the importance of this mechanism in neurons (Kanai et al., 2004).

1.1.3 Localized mRNAs and cell motility

Localization of β-actin messenger RNA to sites of active actin polymerization modulates cell motility during embryogenesis and differentiation (Condeelis et al. 2005).

The localization of β-actin mRNA requires a conserved 54-nucleotide cis-acting element located in the 3' UTR of the mRNA, called the "zipcode" (Condeelis et al. 2005). In chicken developing neurons, a protein of 68 kDa zipcode binding protein 1 (ZBP1) associates with the zipcode to regulate the localization of β-actin mRNA at the leading edge of fibroblasts and growth cones for enrichment of ß-actin protein and forward movement of growth cones (Figure 1B). Moreover, a mutated zipcode unable to localize was unable to bind ZBP1 (Zhang et al 2001; Condeelis et al. 2005). More recent evidence shows that β-actin translation can be regulated by the phosphorylation of ZBP1 through Src kinase (Huttelmaier et al. 2005). In Xenopus, the homolog of ZBP1, Vg1RBP (also known as Vera), is required for the motility of neural crest cells. This protein binds β-actin mRNA and controls its localization in retinal growth cones. During this process, Netrin-1 induces the movement of Vg1RBP granules into filopodia and plays an important role in the localization and translation of β-actin mRNAs in growth cones (Piper et al; Yisraeli et al. 2005; Leung et al. 2006). In cell movement, site-directed actin polymerization is induced at their surface, via the activation of the actin-related protein 2/3 (Arp2/3) complex by the WASp (Wiskott-Aldrich Syndrome protein) family proteins (Machesky et al. 1999). A recent study shows that the Arp2/3 complex required for actin polymerization is localized to the leading protrusions of migrating cells, further supporting that the Arp2/3 complex is targeted to its site of function by mRNA localization (Mingle et al. 2005).

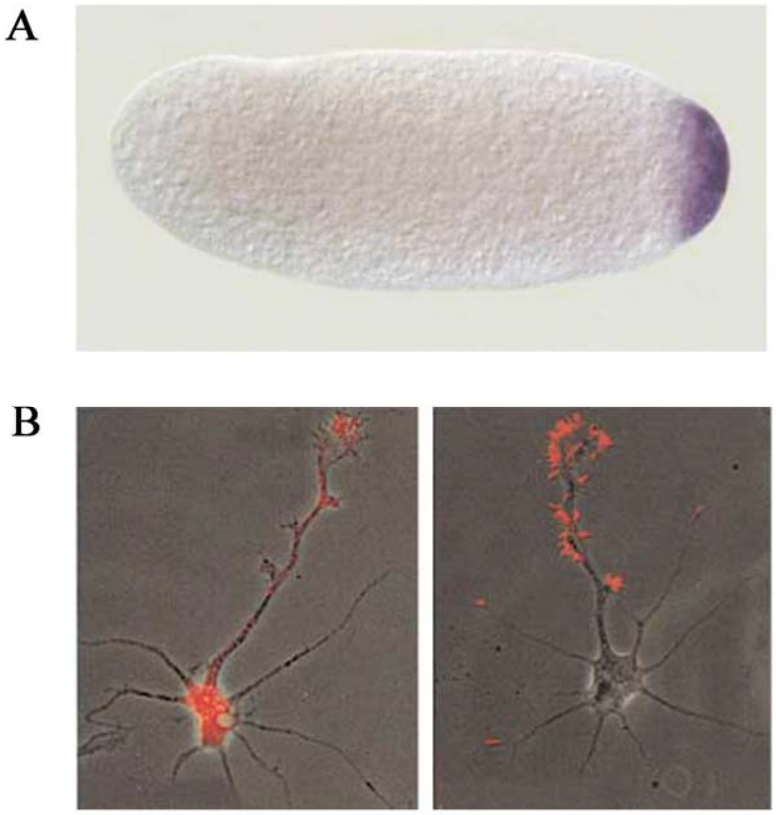

Figure 1. Examples of localized RNAs in different organisms and cell types
(A) The localization of maternal *nanos* mRNA is shown in purple at the posterior of an activated, unfertilized Drosophila egg (Lipshitz et al., 2000). (B) β-actin mRNA (Red) localization in the neurite and growth cone (left) and β-actin protein highly enriched in growth cone and filopodia (right) for forward movement of growth cones (Bassell et al., 2001).

1.1.4 Localized mRNAs and Asymmetric Cell Division

Asymmetric cell division gives rise to two daughter cells with different cell fates which are partly generated by active localization of the cell fate determinant mRNA at only one daughter cell during mitosis (Bernardoni et al. 1999). The examples of asymmetric segregation of mRNAs during cell division can be found in Drosophila, Yeast and other

higher eukaryotes. The asymmetric division of *Drosophila* neuroglioblasts provides a well characterized example where mRNA localization is responsible for determining different sibling cell fates (Akiyama-Oda et al. 1999, Bernardoni et al. 1999). In *Drosophila* embryos, Neuroblasts usually divide into an apical ganglion mother cell (GMC) and a new neuroblast daughter at the basal. One important cell fate determinant is prospero mRNA, which is localized to the cell cortex of the basal side of the GMC (Broadus et al., 1998). In the budding yeast *Saccharomyces cerevisiae*, specific mRNAs are localized to daughter cells to asymmetrically segregate cell-fate determinants during development (Paquin and Chartrand. 2008). Interestingly, in a mollusc embryo, *IoEve* mRNAs, encode a protein involved in anterior–posterior axis specification, associate with centrosomes in the cell and are also subsequently distributed asymmetrically to the pericentriolar matrix in a microtubule-dependent manner during division (Lambert and Nagy, 2002).

1.1.5 Mechanisms of mRNA localization

Studies in diverse organisms have demonstrated the existence of several potential mechanisms by which mRNAs can be localized, like the active directional transport of RNA along cytoskeletal elements or random cytoplasmic diffusion and trapping. However, the most common mechanism invoked for mRNA localization involves the active transport of the mRNA along the cytoskeleton (Bashirullah et al. 1998; St Johnston et al. 1995; Lipshitz et al. 2000). The observation that most localized mRNAs get delocalized in the presence of microtubule depolymerizing drugs supports the active transport model (St Johnston et al.1995). For some transcripts, actin filaments must remain intact in order to maintain a proper localization (Long et al. 1997; Sundell et al. 1991). Localized mRNAs are recognized by their localization machinery via specific cis-acting localization elements or “zipcode” present within the transcript sequence (Bashirullah et al. 1998). For most localized mRNA, these zipcode have been found in the 3’ UTR, while a few, like the yeast

ASH1 mRNA or the *Drosophila gurken* and *bitesize* mRNAs, also contain additional zipcodes in the coding sequence or 5'UTR, suggesting that localization elements may be quite variable between mRNAs, both in term of sequence and secondary structure (Chartrand et al. 1999; Gonzalez et al. 1999; Serano et al.2003; Thio et al. 2000).

During the transport process, localized mRNA is first packaged into a "locasome", which contains the mRNA and several trans-acting factors, such as RNA-binding proteins and molecular motors (Oleynikov et al. 1998). Several localized transcripts have been shown to form large particles or granules when microinjected or expressed *in vivo* (Ainger et al. 1993; Ferrandon et al. 1994; Bertrand et al. 1998). While this observation has been frequently reported, the composition and function of these particles are still mostly unknown. Currently, a few RNA-binding protein have been reported to directly bind the zipcode region of a localized mRNA, such as ZBP1 binding to the chicken β-actin mRNA, Vera binding to the *Xenopus Vg1* mRNA, hnRNP2 binding to *MBP* mRNA in oligodendrocytes, staufen and VPS36 binding to the *Drosophila bicoid* mRNA or She2p to the yeast *ASH1* mRNA (Kelsey et al. 2009).

Recent evidences suggest that several molecular motors are involved in the transport of localized mRNAs in different organisms. The type V myosin Myo4p has been shown to actively transport the *ASH1* mRNA along the actin cytoskeleton of budding yeast (Bertrand et al. 1998). More recently, microtubule-associated motors like dynein and kinesin were shown to be involved in the sorting of the *Drosophila bicoid* and *oskar* mRNA, respectively (Schnorrer et al. 1999; Brendza et al. 2000). Adaptor proteins which associate with both mRNA and molecular motor, like She3p in yeast and Swallow in *Drosophila,* have also been identified (Schnorrer et al. 1999; Takizawa et al. 2000).

1.1.6 Translational regulation and mRNA localization

Translational control is essential for the localization of zipcode-containing mRNAs and can be regulated by their localization machinery (Lipshitz et al. 2000). This mechanism prevents the untimely translation of mRNAs, which could have deleterious effects on cellular function. Several localized mRNAs are not translated unless they are properly localized. For instance, in *Drosophila* oocytes, the translation of unlocalized *oskar* mRNA is repressed by the binding of the Bruno protein on its 3'UTR (Kim-Ha et al. 1993). The unlocalized nanos mRNA is also translationally repressed by the binding of Smaug protein on its 3'UTR (Smibert et al. 1996; Crucs et al. 2000). In both case, the proper localization of these mRNAs results in the activation of their translation (Lipshitz et al. 2000). The mechanism by which some of these proteins repress translation has been recently identified. This mechanism reveals that eIF4E-binding proteins compete for the eIF4G binding site on mRNA, disrupting the eIF4E- eIF4G interaction that is essential for the recruitment of the 40S ribosomal subunit to the mRNA (Gingras et al. 1999). An example is the Drosophila Cup protein, an eIF4E-binding protein that blocks the interaction between eIF4E and eIF4G. Cup interacts with Smaug to repress the translation of unlocalized *nanos* mRNA, and with Bruno to repress the translation of the *oskar* mRNA (Nelson et al. 2004; Nakamura et al. 2004).

1.2 The yeast *S. cerevisiae* as model organism to study localized mRNA

Saccharomyces cerevisiae, the budding yeast, is the common yeast used in baking and brewing industry. Yeast was the first eukaryotic organism whose complete genomic sequence was established (Dujon 1996; Goffeau et al., 1996). With its 12.8 Mb, the yeast genome is divided up into 16 chromosomes ranging in size between 250 kb and >2500 kb

and about 200 times smaller than the human genome, but less than four times bigger than that of *E.coli.* The complete genome sequence now defines some 6000 open reading frames (ORFs), most of which are likely to encode specific proteins.

In biomedical science, the buding yeast is a popular "model" organism in laboratories because it is a unicellular eukaryote whose many essential cellular processes are conserved between yeast and other higher eukaryotes. Among all eukaryotic model organisms, *S. cerevisiae* combines several advantages, for instance, it is an unicellular organism and has a short generation time, so they can be easily cultivated and grown on defined media. In addition, its entire genome is known and it can be easily transformed with genes from other sources. Importantly, in yeast, cell architecture and fundamental cellular mechanisms can be successfully investigated and many sophisticated genetic tools and biochemical approaches have been developped, making it a convenient and powerful model system to study eukaryotic cellular processes.

1.2.1 The yeast life cycle

Budding yeast can live with either two genomes (diploid) or one (haploid). In either case, it divides by forming buds, the production of a small outgrowth from the parent cell. In nature, and when nutrients are available, yeast reproduces asexually mainly in the diploid stage. Budding starts at late G1-phase. At the end of M-Phase, the emerged daughter bud has reached the size of the mother cell. The subsequent cell division results in two cells, termed “mother cell” and “daughter cell”. Under conditions of nutrient deprivation, diploid cells may undergo meiosis and revert to the haploid stage by sporulation. After meiosis, the formed tetrade consists of usually four ascospores, two of which with the mating type a and two with mating type α. When nutrients are available, the spores germinate and the resulting cells may either multiply asexually as haploids or may serve as a gamete (Figure 2). In yeast, this sexual process is termed “mating” and occurs

when two haploid cells with different mating types fuse to form a diploid (a/α) zygote. Cells from each haploid type produce a secreted mating-pheromone. These mating type-specific pheromones, termed a- and α-factor, act to synchronize the cell cycle of the mating partners and to prepare cells for mating (Herskowitz, 1988).

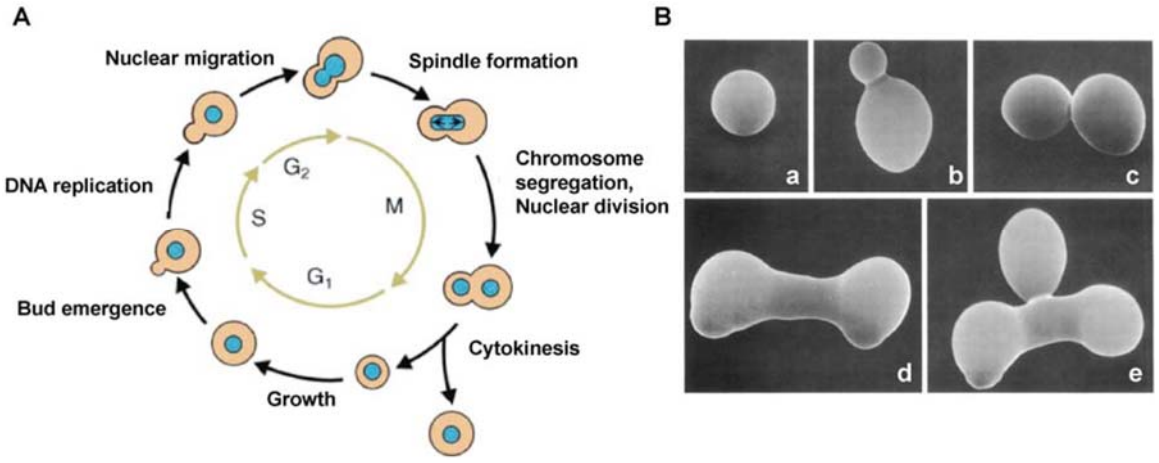

Figure 2: The yeast life cycle.
(A) The cell cycle of *S. cerevisiae* (Source: Lodish et al., 1999). Yeast cells multiply asexually by budding. At the end of G1, a bud emerges from the mother cell. Prior to cytokinesis, the daughter bud has reached size of the mother cell. After cell division, the resulting cells grow in G1 until reaching the appropriate size for bud formation. (B) Morphology of *S. cerevisiae* cells (Source: Herskowitz, et al., 1988). Upper panel shows an unbudded cell in G1 (a) and cells with different bud sizes S (b), G2 (c). Mating of a- and α-haploids leads to formation of a diploid (a/α) zygote (d). The zygote is able to produce diploid (a/α) daughter cells by budding. Bud emerges often at the neck (e).

1.2.2 Mating type switching

The cell mating type is determined by the *MAT* locus. After cytokinesis of a haploid cell, the interconversion between *MATa* and *MATα* is due to the protein product of the *HO*

gene, which is an endonuclease that promotes mating-type switching in *S. cerevisiae* (Herskowitz, 1988; Nasmyth, 1982). Mating type switching occurs only in mother cells but not in daughter cells during late G1.

Switching to the opposite mating type occurs by gene conversion at the active *MAT* locus. The template used for this conversion is issued from the silenced *HMLa* or *HMRa* locus of the opposite type. In haploid cells, expression of one of the two alleles leads to cells with either mating type a or α, whereas diploid cells express both alleles (Mating type a/α). *MATα* codes for two proteins termed α1p and α2p. The transcription factors Mcm1p and α1p are responsible for the activation of α-specific genes (Shore et al. 1995). In contrast, α2p and Mcm1p serve to repress a-specific genes (Wolberger, 1998). The *MATa*-locus codes for two proteins, of which only A1p is known to have a biological function. A1p and α2p form a heterodimer, which is required to repress haploid-specific genes (Li et al., 1995). Consequently, there is no expression of α-specific genes in a-cells because α1p and α2p are missing, whereas through the activation by Mcm1p, a-specific genes are expressed (Bruhn et al. 1994).

Mating type switching occurs when either *HMLa* or *HMLα* is recombined into the transcriptionally active *MAT*-locus by gene conversion (Hicks et al. 1977; Strathern et al.1982). Thus, the *MAT*-locus is replaced by the genetic information of the opposite mating type. This recombination event is initiated by a double-strand break, catalyzed by the haploid-specific *Ho* endonuclease. In diploid cells, binding of the heterodimer A1p/α2p inhibits *HO* expression (Herskowitz, 1992). Yeast strains used for biological studies in laboratories have lost their ability to change mating types due to a point mutation in the *HO* gene. These strains are called heterothallic and are more accessible to genetic manipulations because of a stable haploid phase.

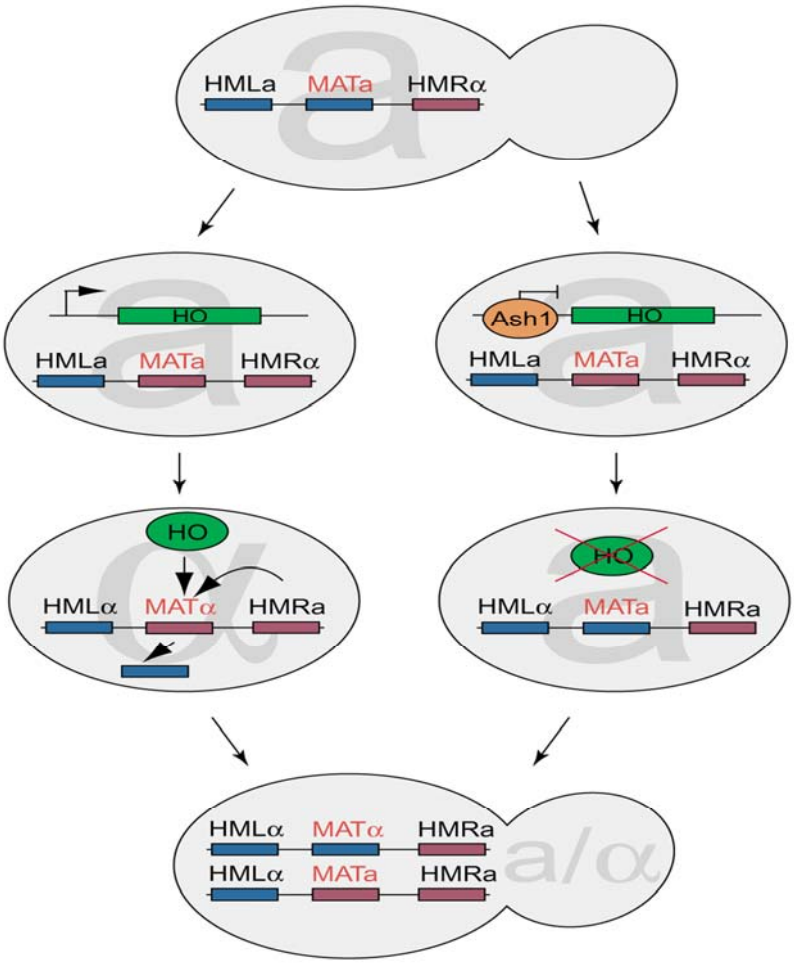

Figure 3: Mating type switching. After division of a haploid cell, only the mother but not the daughter cell can switch the mating type. Switching to the opposite mating type occurs by gene conversion at the active MATlocus. Ho endonuclease cuts the active MAT locus, initiating this replacement. In daughter cells, the transcriptional repressor Ash1p inhibits the expression of HO, thereby repressing mating type switching (Source: Darzacq et al., 2003).

1.2.3 Control of HO expression

The *HO* gene product of *Saccharomyces cerevisiae* is a site-specific endonuclease that initiates mating type interconversion during late G1 in mother cells. The transcription activation program of *HO* is cell cycle regulated. The expression occurs only transiently and starts during late mitosis, when Cdk1p is inactive and ends during late G1-phase, when Cdk1p is reactivated (Nasmyth, 1993). The *HO* promoter can be divided in two regions: a distant upstream region called URS1 ("Upstream Regulatory Sequence"), which regulates mother cell expression specificity, and a proximal region called URS2 that controls *HO* cell-cycle regulation (Nasmyth, 1993).The late-G1 specificity of *HO* transcription depends on a heteromeric factor, SBF, which is composed of the Swi4 and Swi6 proteins. Mother-cell specificity involves a second site-specific DNA-binding factor, Swi5, which is synthesized in the G2 and M phases and only enters the nucleus at the end of mitosis. Swi5 enters mother and daughter nuclei and binds to two sites of URS1 regions within the *HO* promoter in equal amounts. This event triggers the recruitment of the SWI/SNF chromatin-remodelling complex to URS1 and URS2, resulting in recruitment of RNA polymerase lI and the general factors TFIIB/TFIIH for transcription initiation (Bhoite et al., 2001; Cosma et al., 1999; Krebs et al., 1999). However, the highly concerted recruitment of all these transcription factors does not occur in daughter cell nuclei.

The *ASH1* gene (Asymmetric synthesis of *HO*) was identified through the isolation of mutants in which the daughter cells were defective in *HO* repression and so were able to switch mating type. Ash1p is a 66-kDa zinc-finger transcriptional repressor that inhibits *HO* transcription through its asymmetric accumulation in the daughter nucleus in late anaphase (Bobola et al, 1996; Sil et al. 1996). Ash1p contains a domain which is highly homologous to the zinc-finger domain of the erythroid cell nuclear protein GATA-1 (Bobola et al., 1996; Sil et al. 1996). All GATA-like factors bind to GATA motifs, which leads to either transcriptional activation or repression. Recently, the Ash1-binding

consensus sequence, YTGAT, was identified within the *HO* promoter (Maxon et al. 2001). This consensus, which is related to the canonical (A/T)GATA(A/G) sequence bound by most GATA factors, is present at least 20 times within the upstream repression sequence 1 (URS1) region of the *HO* promoter (Maxon et al. 2001). Ash1p has two principal domains: the C-terminal DNA-binding domain, which binds to the YTGAT consensus within URS1 of *HO*; and the amino-terminal domain, which is devoted to the repression of *HO* transcription (Maxon et al. 2001). The asymmetric control of *HO* expression, which is caused by the sorting of Ash1p to daughter cells, explains why only haploid mother cells can undergo mating type switching. Moreover, a recent study show that Ash1p also involved in daughter cell size control using quantitative time-lapse microscopy (Di Talia et al., 2009).

1.2.4 *ASH1* mRNA in yeast *S. cerevisiae*

The Ash1p localizes to the daughter cell nucleus in late anaphase where it prevents mating type switching. The asymmetric accumulation of this cell fate determinant in only daughter cell nuclei is mediated by the products of five genes, termed *SHE1–SHE5* (Swi5p-dependent HO Expression), each of which has a specific function (Jansen et al, 1996). They were identified in a genetic screen for factors that are required for asymmetric HO-expression in yeast cells (Jansen et al., 1996). *SHE* mutants fail to restrict Ash1p to the daughter cells nucleus, therefore, HO transcription is repressed in both mother and daughter cells, resulting in none of cells switching. Interestingly, except for Ash1p sorting, *SHE* genes have no function in *SWI* regulation.

Some of the She proteins have been found to be localized asymmetrically to a crescent at the cortex of the daughter bud. Ash1p is unlikely to be directly targeted by She-proteins transport because Ash1p appears in nuclei of daughter cells at much later stages of the cell cycle, at a moment when the She-proteins are no longer localized (Chang et al. 1996). Thus, it soon became clear that asymmetric localization of Ash1p originates from

the asymmetric localization of the *ASH1* mRNA (Figure 4) (Long et al., 1997). Each of the *SHE* gene products is essential for targeting the *ASH1* mRNA to the bud tip (Long et al., 1997; Takizawa et al., 1997) (see section 1.3.2.).

The post-transcriptional level of regulation of gene transcription through the asymmetric localization of mRNA is well known in different organisms (Kloc et al, 2002). The key elements of *ASH1* mRNA localization to the bud tip of daughter cell have been identified as follows: the cis-acting sequences within *ASH1* mRNA; the secondary structure of the *ASH1* mRNA; the cytoskeleton; a macromolecular complex that includes the She1–3 proteins, some factors that are required for the recognition of *ASH1* mRNA in the nucleus and translational repression before proper bud tip localization (Paquin et al., 2008).

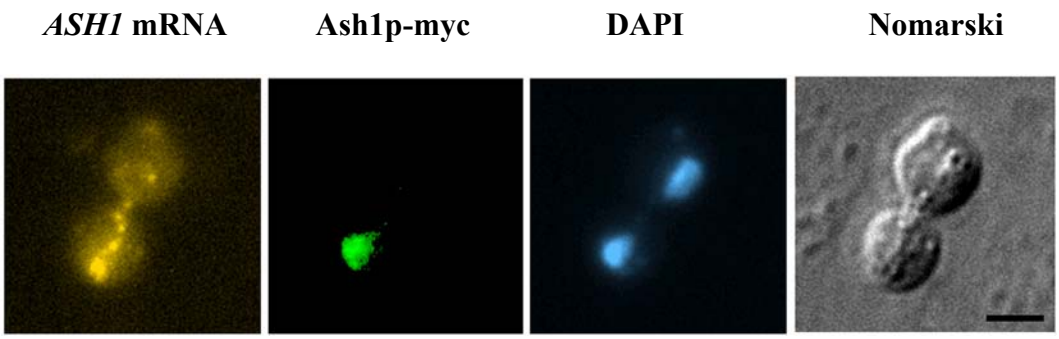

Figure 4: Localization of *ASH1* mRNA and Ash1p to daughter cells of budding yeast.
ASH1 mRNA (light yellow) is actively transported to the bud tip of daughter cells where it is translated into Ash1p (green), the repressor for *HO* expression. Ash1p is exclusively sorted to daughter cell nucleus to prevent mating type switching (Long et al., 1997).

1.2.5 Other bud-localized mRNAs in *S. cerevisiae*

In *Saccharomyces cerevisiae*, *ASH1* mRNA is localized to bud tip of daughter cell where it is translated into Ash1p, a cell-specific transcription factor (Long et al. 1997; Takizawa et al. 1997). This represented the first description of RNA localization in a single-cell eukaryote. A second localized mRNA was also found, the *IST2* mRNA, whose localization to the cortex of daughter cells creates a higher concentration of Ist2 protein in the bud, and this asymmetry is maintained by a septin-mediated membrane diffusion barrier at the mother-bud neck (Takizawa et al. 2000). A microarray-based screen identified a set of 22 additional mRNAs, all of which become localized to bud tip in a She-dependent manner (Shepard et al., 2003). These messages encode a wide variety of proteins, including several involved in stress responses, cell wall maintenance and membrane proteins. And recent studies show that over thirty mRNA are localized to the bud of daughter cells (Table 1) (Aronov et al. 2007; Oeffinger et al., 2007; Shepard et al. 2003). Among these bud-localized mRNAs, including *ASH1* mRNA, some were found to cofractionate with ER microsomes in a She2-dependent manner. Thus, asymmetric mRNA transport and cortical ER inheritance are connected processes in yeast (Aronov et al. 2007; Schmid et al. 2006). However, the biological significance of localizing these RNAs remains to be elucidated, since asymmetric distribution of several of these proteins also occurs in the absence of mRNA transport (Takizawa et al. 2000).

Table1: The list of bud localized mRNA in yeast *S. cerevisiae*

Gene	Function
ASH1	M Transcription
BRO1	Stress transduction
CLB2	Cyclin B
CPS1	Carboxypeptidase
DNM1	Mitochondrial fission
EGT2	Cellulase
ERG2	Sterol isomerase
IST2	Tranporter
MID2	Membrane receptor
MMR1	Unknown
SRL1	Unknown
TPO1	Polyamine transport
WSC2	Membrane receptor
TAM41	Import into the mitochondrial matrix
TCB3	Lipid-binding protein
EAR1	Unknown
TCB2	Membrane trafficking
KSS1	Mitogen-activated protein kinase
LCB1	Endoplasmic reticulum, lipid synthesis
MET4	Transcription
MTL1	MID2-like
CDC42	Small rho-like GTPase
RHO3	Cell polarity
YPT1	ER-to-Golgi step of the secretory pathway
SEC1	Exocytose
SEC3	Exocytose
EXO84	Exocytose
SRO7	Exocytose
SRO77	Exocytose
SEC4	Exocytose
BUD8	Bud-site selection
CIS3	Glycoprotein constituent of the cell wall

Source : Aronov et al., 2007 ; Oeffinger et al., 2007; Shepard et al., 2003

1.3. Localized *ASH1* mRNA as a model system to study localization mechanism in eukaryotyes

Data so far have shown that more than thirty mRNAs are localized to the bud of daughter cells in budding yeast *Saccharomyces cerevisiae*, among these localized mRNAs, the *ASH1* mRNA is the best characterized example. Because mRNA transport and localization in yeast shares many features with RNA localization in higher eukaryotes, including cis-acting sequences within localized mRNA, formation of a large ribonucleoprotein (RNP) localization complex, cell cytoskeleton and molecular motors, some factors that are required for mRNA recognition in the nucleus, thus, the mechanism of *ASH1* mRNA localization can be used as a paradigm to explore the molecular basis of this process.

1.3.1 The localization elements in *ASH1* mRNA

To address the cis-acting sequences required for the localization of the *ASH1* mRNA, different fragments of *ASH1* were inserted into a reporter mRNA and the cytoplasmic distribution of these chimeric mRNAs was determined by in situ hybridization (Chartrand et al., 1999; Gonzalez et al., 1999). These studies have shown that there are four minimal localization sequences within the *ASH1* mRNA. Among these elements, three are located within the coding region of the *ASH1* mRNA, and they have been named as E1 (spanning between nucleotides 598 and 750), E2A (between 1044 and 1196) and E2B (between 1175 and 1447), whereas the remaining element (E3), which is located primarily within the *ASH1* 3'UTR, spans seven nucleotides before and 67 after the stop codon (Figure 5A). Each single element is sufficient to localize mRNA at the bud of yeast cells (Chartrand, et al. 1999; Gonzalez, et al. 1999).

These localization elements are predicted to form RNA secondary structures containing stem-loops (Figure 5B) (Chartrand, et al. 1999; Chartrand, et al. 2001; Gonzalez, et al. 1999), and this secondary structure is crucial for *ASH1* mRNA localization (Bertrand et al, 1998). For example, mutations in the stem loop of the E3 element can affect the localization of *ASH1* mRNA (Chartrand et al, 1999). Recent work shows that the *ASH1* mRNA contains a similar loop-stem-loop structure with a highly conserved CGA triplet in one loop and a single conserved cytosine in the other loop. Mutating these conserved nucleotides, or the stem separating them, resulted in the delocalization of a reporter mRNA (Olivier, et al. 2005). In addition, in an independent approach, a predicted single-stranded core CG dinucleotide appears to be an important component of the RNA-protein interface, although other nucleotides contribute in a context-dependent manner (Jambhekar et al., 2005).

1.3.2 The machinery of bud localized *ASH1* mRNA

SHE1–SHE5, at beginning, were identified in a genetic screen for factors required for asymmetric HO-expression in yeast cells (Jansen et al., 1996). After that, it is soon clear that each of the *SHE* gene products was essential for targeting the *ASH1* mRNA to the bud tip (Long et al., 1997; Takizawa et al., 1997). So far, several trans-acting factors involved in the localization of the *ASH1* mRNA in yeast have been identified (Table 2). In budding yeast*, ASH1* mRNA association with these trans-acting factors triggers the packaging into a locasome (Figure 6), which is actively transported to the bud in late anaphase, ensuring the exclusive translation of Ash1p in the daughter cell (Long et al, 1997; Takizawa et al, 1997). During this process, *ASH1* mRNA localization is mediated by four cis-elements within the mRNA and each of them can be recognized by She2p, an RNA-binding protein (Long, et al. 2000; Bohl, et al. 2000). The link between the RNA-binding protein She2p and the motor She1p/Myo4p is mediated by She3p (Long, et al. 2000; Takizawa, et al. 2000). These studies lead to the development of a working model for this pathway (Figure 7) (Chartrand,

et al. 2001). In this model, the *ASH1* mRNA is first recognized in the nucleus by proteins which act as "tags" for the localization machinery. Once in the cytoplasm, the localization machinery assembles on the tagged mRNA as a complex called "locasome", the locasome is transported along the actin cytoskeleton to the bud tip where it becomes anchored. Once localized, the mRNA is translated and Ash1p appears at the bud tip and diffuses back to the daughter cell nucleus.

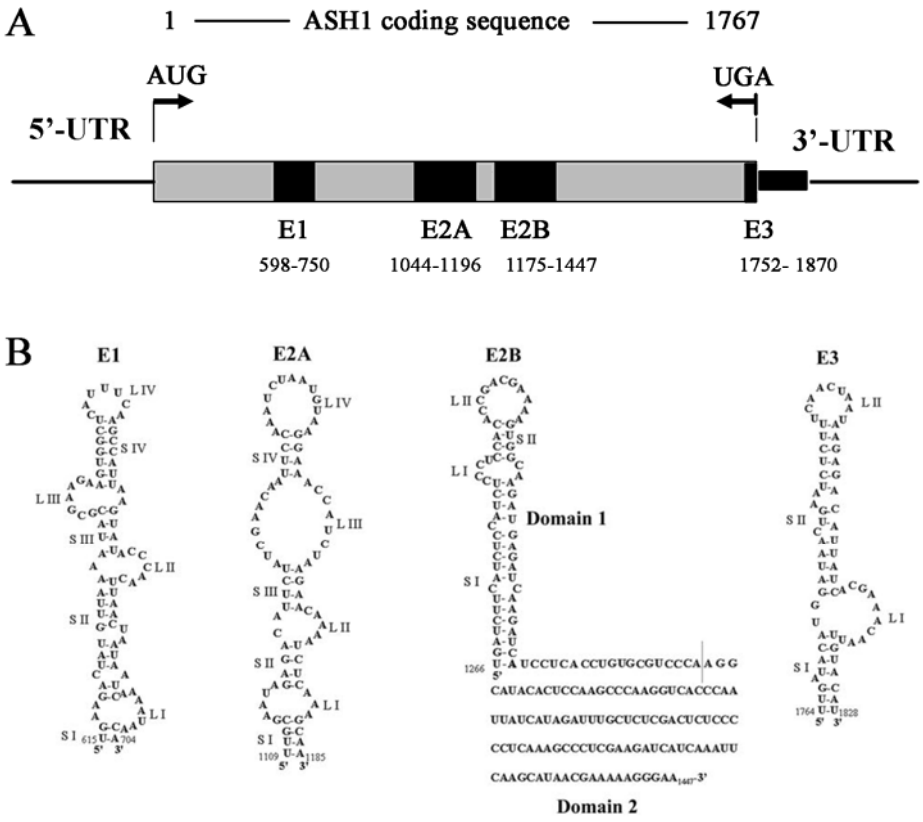

Figure 5: Localization elements of the *ASH1* mRNA and its second structure.
(A) The position of localization elements in *ASH1* mRNA. (B) Three of the localization elements, namely E1, E2A and E2B, are located within the ASH1 coding sequence. The E3 element spans the stop codon and the first 100 nucleotides of the 3' untranslated region (UTR) (Source: Chartrand et al., 2002; Olivier et al., 2005).

1.3.3 She1/Myo4p- a yeast class V myosin motor

She1p, also called Myo4p, is the motor protein required for active transport of the *ASH1* mRNA locasome along the actin cytoskeleton to the bud tip of daughter cell (Bertrand et al., 1998; Haarer et al., 1994; Jansen et al., 1996; Münchow et al., 1999). In budding yeast, there are two class V unconventional myosins, Myo4p and Myo2p (Titus, 1997). Both of them non-processively localize to the bud tip of daughter cells (Karpova et al., 2000; Lillie et al. 1994; Schott et al.1999). Myo2p is required to set up the orientation of the mitotic spindle (Yin et al., 2000), and has a role in the polarized transport of secretory vesicles (Govindan et al., 1995; Johnston et al., 1991; Lillie, et al. 1994; Pruyne et al., 1998; Schott et al., 1999), inheritance of the vacuole and the Golgi apparatus (Catlett et al., 2000; Catlett, et al, 1998; Rossanese et al., 2001). Myo4p, a single-headed and nonprocessive class V myosin in budding yeast, transports >20 different mRNAs asymmetrically to the bud (Long et al., 1997; Reck-Peterson et al., 2000). Since Myo4p does not contain intrinsic RNA-binding activity, accessory proteins are necessary to interface the myosin with *ASH1* mRNA localization elements, some evidence show that Myo4p associates with *ASH1* mRNA, which is dependent on She2p and She3p (Münchow et al. 1999, Takizawa et al. 2000). Furthermore, it was observed that Myo4p colocalizes with *ASH1* mRNA-containing particles (Bertrand et al. 1998, Takizawa et al. 2000), and in living yeast cells, Myo4p directly transports *ASH1* mRNA to daughter cells (Bertrand et al. 1998, Beach et al. 1999). While type V myosins are thought to require dimerization for processive movement, Myo4p is strictly monomeric at physiologic concentrations (Heuck et al., 2007). A recent study shows that the rod region of Myo4p contains the primary binding site for She3p and is essential for correct localization of *ASH1* mRNA (Bookwalter et al., 2009). Interestingly, Myo4p is also involved in the inheritance of cortical ER (Estrada et al., 2003), and recent works suggested that both Myo4p-dependent processes (mRNA localization and cortical ER inheritance) are tightly coordinated (Aronov et al., 2007; Schmid et al., 2006).

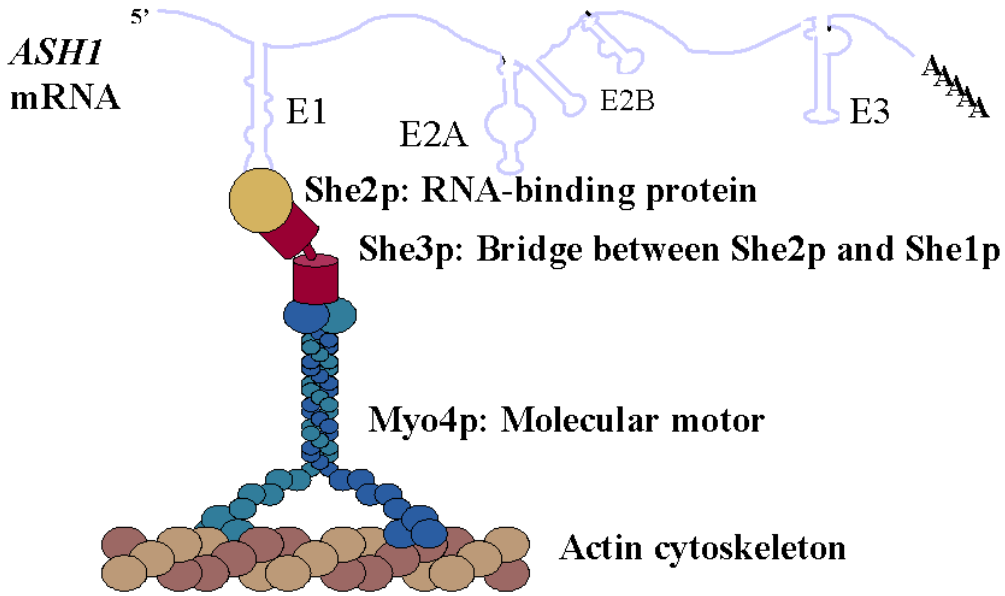

Figure 6: The *ASH1* mRNA locasome

ASH1 mRNA contains four localization elements within its sequence and each of them can be recognized by She2p, an RNA-binding protein, containing a basic helical hairpin motif for RNA binding activity. The link between the RNA-binding protein She2p and the motor She1p/Myo4p is mediated by She3p (Chartrand et al., 2001).

Table 2 *Trans*-acting factors involved in *ASH1* mRNA localization

Name	Function	Role in *ASH1* localization	Interactions	Location in the cell
She1p/ Myo4p	Type V unconventional myosin	Molecular motor	She3p	Bud tip
She2p	RNA-binding protein	Binds *ASH1* mRNA localization elements	She3p	Cytoplasm/ bud tip
She3p	Myosin-binding protein	Bridges She2p with the the myosin She1p	She1p She2p	Bud tip
She4	Unknown	Stabilizes the actin cytoskeleton (?)	Unknown	Cytoplasm
She5p/ Bni1p	Scaffold protein	Involved in the polarization of the actin cytoskeleton	Cdc42p, Bud6p EF1α, Pfy1p	Bud tip
Loc1p	RNA-binding protein	Binds *ASH1* localization elements; efficiency factor	Unknown	Nucleolus/ Nucleoplasm
KHD1	RNA -binding protein	Translational control	eIF4G1	cytoplasm
PUF6	RNA-binding protein	Translational control	?	Nucleolus/ Nucleoplasm

1.3.4 The adapter protein She3p

She3p is a novel protein with no significant homology to any known proteins. Some studies have shown that She3p colocalizes with Myo4p and *ASH1* mRNA at the bud tip and plays an important role in the association of Myo4 motor protein with *ASH1* mRNA-She2p complex (Kruse et al., 2002; Münchow et al., 1999; Takizawa et al. 2000). Furthermore, by two-hybrid, three-hybrid, and coimmunoprecipitation experiments, the N-terminal half of the She3p was found to interact with the C-terminal tail of Myo4p (Münchow, et al. 1999; Böhl et al. 2000; Long et al. 2000). Moreover, sucrose density gradients demonstrated a cosedimentation of Myo4p together with She3p, suggesting a tight and permanent association of both proteins (Böhl et al., 2000). A recent study show that She3p binds to the rod of yeast Myosin V and prevents it from dimerizing, forming a single-headed motor

complex (Hodges et al., 2008). The C-terminus of She3p provides the binding to She2p. Thus, She3p serves as an adapter that docks the myosin motor onto an *ASH1* mRNA–She2p ribonucleoprotein (RNP) complex. Interestingly, She3p might have an influence on *ASH1* mRNA- She2p interaction, the evidence comes from binding of She2p to *ASH1* mRNA zipcode was enhanced in the presence of She3p by a gel-shift assays (Böhl et al., 2000). She3p association with *ASH1* mRNA was observed to be dependent on She2p, suggesting that the Myo4p-She3p complex interacts with the *ASH1* mRNA cis-acting localization elements through She2p (Münchow, et al. 1999; Böhl et al. 2000; Long et al. 2000). In addition, She3p also possesses a novel activity required for *ASH1* mRNA localization since some mutants of She3p are defective for *ASH1* mRNA localization and retain the ability to associate with Myo4p and She2p (Landers et al., 2009). Importantly, this novel function for She3p could be negatively regulated by phosphorylation (Landers et al., 2009).

1.3.5 She2p- the mRNA binding protein

She2p is also a novel protein with no homology to known proteins in higher eukaryotes. As a small 28 kDa RNA binding protein, She2p is the key component in the assembly of the *ASH1* mRNA locasome (Long et al, 1997; Takizawa et al, 1997).Yeast two-hybrid analysis and experiments using recombinant She2p and She3p demonstrated that She2p directly interacts with She3p through a domain in the C terminus of She3p (Böhl et al. 2000, Long et al. 2000), suggesting that She2p is required to link the Myo4p-She3p complex to *ASH1* mRNA. Disruption of *SHE2* abolishes Myo4p's association with the mRNA (Jansen et al., 1996; Münchow et al., 1999). She2p is also required to mediate the association of She3p to *ASH1* mRNA (Böhl et al., 2000). These results suggest that She2p is the factor acting directly on *ASH1* mRNA, independent of She3p and Myo4p.

Although no classical RNA-binding motifs can be identified in the She2 protein, it directly and specifically interacts *in vitro* and *in vivo* with each of the *ASH1* mRNA cis-acting elements, but with apparently weak affinity (Böhl et al., 2000; Darzacq et al., 2003; Long et al., 2000). This was well demonstrated by electrophoretic gel mobility shift assays (Böhl et al., 2000), and further confirmed using filter binding experiments (Niessing et al., 2004), where purified recombinant She2p displayed specific binding to *ASH1* mRNA cis-acting localization elements.

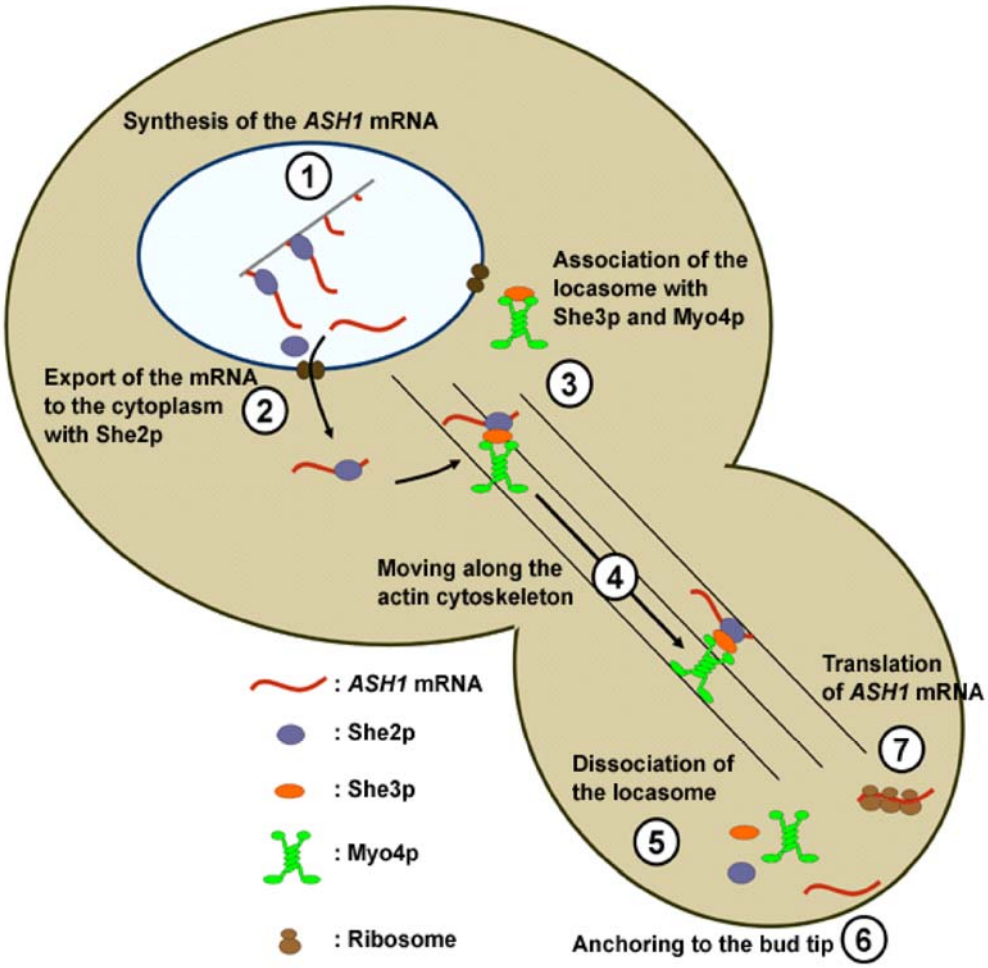

Figure 7: Model of *ASH1* mRNA localization.

(1) Synthesis of the *ASH1* transcription in late anaphase, association of *ASH1* mRNA with She2p, interaction with Loc1p in the nucleus. (2) Export of *ASH1* mRNA-She2p RNP from nucleus. (3) Assembly of the locasome by association of the newly exported RNP with She3p and She1p/Myo4p. (4) Locasome atively transported to bud tip of the daughter cells along the actin cytoskeleton. (5) Dissociation of the locasome after proper localization at the bud. (6)- (7) Anchoring and translation. *ASH1* mRNA was translated into Ash1p after it was accurately anchored at the bud, then Ash1p enters the daughter cell nucleus where prevent mating type switching (Source: Chartrand et al., 2001).

The first 70 amino acids residues of the She2p are apparently essential for RNA-binding activity, since a deletion of this region abolishes the ability of She2p to associate with *ASH1* mRNA, resulting in an accumulation of this mutant in the nucleus (Kruse et al., 2002). This was the first evidence indicating that She2p's nuclear export is dependent on RNA binding. A cluster of arginine residues within this region (R43, R44, R52 and R63) as well as asparagine residue 36 (N36) have been found to play a key role in She2p RNA-binding activity (Gonsalvez et al., 2003). Interestingly, classical RNA-binding motifs, like the RNA recoginition motif (RRM), the arginine rich motif (ARM), the RGG box and the double-stranded RNA-binding motif (dsRBD), all of them contain arginine residues critical for RNA binding activity. However, so far, no evidence shows that any of the arginine residues in She2p directly contact *ASH1* mRNA or they play a more indirect role in mRNA association (Gonsalvez et al., 2003). Mutations of N36S, R43A, R44A, R52A, R52K, R63A, and R63K lead to a loss of RNA binding and consequently, to defective mRNA localization (Gonsalvez et al., 2003; Niessing et al., 2004). Moreover, block of mRNA export caused the accumulation of She2 in the nucleus as well (Kruse et al., 2002), and more recent evidence show that She2 accumulates in nucleoli during mRNA export block (Du, et al., 2008). This indicates that She2 can enter the nucleus for the binding of its RNA target and thus is able to shuttle between the nucleus and cytoplasm. However, other experiments using She2p mutants N36S and R63K, which are specifically defective for *ASH1* mRNA association, do not support the assertion that nuclear export of She2p is dependent of both mRNA transport and the ability of She2 to bind mRNA (Gonsalvez et al., 2003). While She2p is not excluded from the nucleus and hence could bind localization substrates in the nucleus prior to export to the cytoplasm, resolution of these apparently contradictory results will require further investigation into the nuclear function of She2p nucleo-cytoplasmic shuttling.

The crystal structure of She2p was recently determined and revealed that it is an almost exclusively α-helical protein unrelated to any previously described RNA-binding

protein (Niessing et al., 2004). Moreover, She2p forms symmetric homodimers that contains basic helical hairpin motif for RNA binding (Figure 8). Mutations in the motif result in loss of mRNA binding in vitro and defective mRNA transport in vivo (Niessing et al., 2004; Gonsalvez et al., 2003). A possible explanation for the need of She2p dimerization is that additional surface regions from both subunits are required for efficient RNA binding by She2p. However, these additional surface regions seem not enough for its RNA-binding functions. By using analytical ultracentrifugation, Müller and colleagues found that She2p adopts a tetrameric structure at physiological concentrations, which is required for RNA binding, efficient mRNP assembly, and mRNA localization in vivo (Müller, et al. 2009).

Indeed, besides *ASH1* mRNA, She2p are also essential for some of other localized mRNAs to the bud of daughter cells (Takizawa, et al. 2000; Shepard et al., 2003; Oeffinger et al., 2007; Aronov et al., 2007). Meanwhile, recent evidence indicates that She2p is required for the localized messenger ribonucleoprotein (mRNP) particles that comigrate with tubular endoplamic reticulum (ER) structures to the bud. Moreover, She2p associates with cortical ER independently of polysomes and She3p-Myo4p complex, suggesting that asymmetric mRNA transport and cortical ER inheritance are connected processes in yeast (Schmid et al., 2006; Aronov et al., 2007). However, it is still unknown how She2p asscociates with ER.

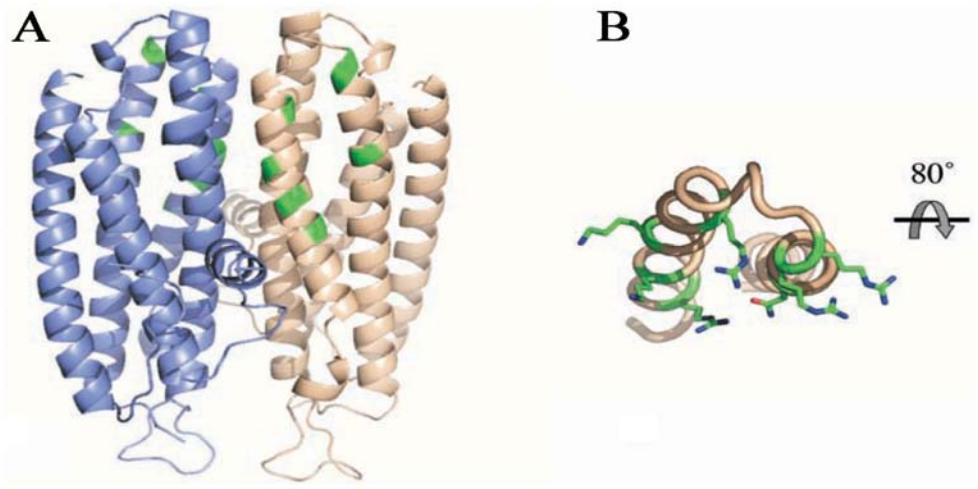

Figure 8: Schematic view of the She2p homodimer with each monomer in blue and grey. (A) She2p forms a stable symmetric homodimer. Each monomer consists of five α helices. Amino acids and the basic helical hairpin RNA-binding motif are highlighted in green. (B) View of the basic helical hairpin motif of the gray monomer. Amino acids of the basic helical hairpin motif required for RNA binding are highlighted in green with their side chains shown (Source: Müller et al., 2007).

1.3.6 Translational regulators of *ASH1* mRNA

The *ASH1* mRNA needs to be translationally repressed to be efficiently localized to the bud tip of daughter cells, and this requires the presence of translational regulators. In the past years, several regulators have been identified and characterized, giving rise to the evidence that it requires more than just a functional motor complex to target a transcript effectively. Among them, Khd1p, Loc1p and Puf6p, which bind directly to the *ASH1* mRNA, are clearly required to control *ASH1* mRNA translation during its localization to the yeast bud tip (Irie et al., 2002; Long et al., 2001; Gu et al., 2004; Paquin et al., 2007; Komili et al., 2007).

1.3.6.1 Khd1p

Khd1p, a protein with three RNA binding hnRNP K homology (KH) domains, has been previously reported to be required for efficient localization of *ASH1* mRNA. Khd1p interacts with the *ASH1* mRNA and colocalizes with *ASH1* mRNA at the bud tip of daughter cells (Irie et al., 2002). Khd1p also interacts with hundreds of transcripts in yeast, with a bias for localized mRNAs (Hogan et al., 2008; Hasegawa et al., 2008). The first 800 nucleotides of the *ASH1* mRNA coding sequence has been identified to be responsible for the association with Khd1p *in vivo* (Irie et al., 2002). In a recent study, our lab has shown that Khd1p interacts with localization element E1 in the *ASH1* mRNA (Paquin et al., 2007). Overexpression of Khd1p lowers the expression of Ash1p, and *ASH1* mRNA was not efficiently localized compared to wild type, suggesting a role in anchoring and/or translational control through its interaction with *ASH1* mRNA (Irie, et al., 2002). Alternatively, Khd1p over-expression may sequester factors that positively affect translation of *ASH1* mRNA. *khd1* mutant cells are defective for anchoring of *ASH1* mRNA (Irie et al., 2002). Moreover, block of mRNA export caused the accumulation of Khd1p in the nucleus as well (Du, et al., 2008), suggesting that Khd1p needs to shuttle between the two compartments in order to function in cytoplasmic translational control. However, deletion of *KHD1* had only little effect on HO expression, and frequency of mating-type switching was the same as in wild type strain (Irie et al., 2002). Consistent with its role as a translational regulator, Khd1p has recently been reported to interact with the C-terminal domain of the translation-initiation factor eIF4G1 to regulate the translation of *ASH1* mRNA (Paquin et al., 2007). Interestingly, disruption of this interaction leads to increased translation of an *ASH1* reporter mRNA *in vivo*, suggesting that Khd1p may act to reduce translation initiation during transport to the bud. Furthermore, Khd1p interacts with Yck1p (yeast casein kinase) at the plasma membrane. The phosphorylation by this kinase results in the release of Khd1p from *ASH1* mRNA, which subsequently activates the translation of this transcript when it reachs its proper target site (Paquin et al., 2007).

1.3.6.2 Loc1p

Loc1p, an exclusively nuclear protein enriched in the nucleolus, was identified as an RNA-binding protein required for efficient *ASH1* mRNA localization and Ash1p sorting (Huh et al., 2003; Long et al., 2001). Using a yeast three-hybrid screen, band mobility shift assays and immunoprecipitation followed by RT-PCR of myc-tagged Loc1p, this protein was found to bind *in vitro and in vivo* to the localization element E3 within *ASH1* mRNA. Deletion of *LOC1* resembles the phenotype of a *she*-mutant, indicating a defective targeting of *ASH1* mRNAs to the bud and, consequently, ubiquitous distribution of Ash1p (Long et al., 2001; Komili, et al., 2007). One possibility why this protein was not identified in the original *SHE*-screen may be due to its location near the centromer. Thus, cloning a fragment containing a centromeric region is difficult since plasmids with two centromers are highly unstable when transformed into yeast cells. A recent study using a live-cell mRNA reporter system analyzed *ASH1* expression levels in wild-type and *loc1Δ* cells and confirmed a role for Loc1p in the translational regulation of *ASH1* mRNA (Komili, et al., 2007). The requirement for Loc1p in translational regulation of *ASH1* mRNA localization to the bud tip may be related to its role in ribosomal assembly. Loc1p was shown to be associated with 66S pre-ribosome complex and is required for efficient 60S ribosome biogenesis (Harnpicharnchai et al., 2001). Cells from loc1Δ strain apparently contain increased levels of large subunit precursor rRNAs as examined by a functional genomics approach analyzing non-coding RNA processing (Peng et al., 2003). Moreover, high-throughput immunoprecipitations and sucrose gradient analysis identified ribosomal proteins associated with Loc1p (Collins et al., 2007; Komili, et al., 2007), suggesting that Loc1p may play a direct role in ribosome biogenesis. Taken together, the data so far suggest that the effect of Loc1p on *ASH1* mRNA localization may be a consequence of its role in ribosomal assembly (Kruse et al., 2002; Komili, et al., 2007). However, whether RNA localization and ribosomal biogenesis are really linked is still largely unknown.

1.3.6.3 Puf6p

Puf6p, a novel member of the PUF (Pumilio/FBF) family with highly conserved RNA-binding proteins such as Pumilio in *Drosophila*, co-purifies with She2p and plays a role in *ASH1* mRNA localization and translational repression (Gu et al., 2004). Puf proteins contain several repeats of the Pumilio homology domain (Pum-HD), which confers RNA binding activity (Wang et al., 2002). In general, Puf proteins have been reported to bind to sequences within the 3'-UTR encompassing a so-called UGUR tetranucleotide motif and thereby repress gene expression by affecting mRNA translation or stability (Gu et al., 2004). Thus, all known Puf-proteins (Puf1-Puf6p) in yeast are involved in posttranscriptional regulation of mRNAs (Gerber et al., 2004). While predominantly a nuclear protein, a fraction of Puf6p co-localizes with the *ASH1* mRNA at the distal bud tip (Gu et al., 2004). Consistent with this observation, Puf6p associates with *ASH1* mRNA *in vivo* and *in vitro,* depending on a UUGU sequence element present in the E3 localization element. Moreover, *puf6Δ* cells or mutations of UUGU elements show a dramatic decrease in Ash1p sorting as well as *ASH1* mRNA localization. In the opposite way, overexpression of Puf6p resulted in a reduced amount of Ash1p. The function of Puf6p in *ASH1* mRNA localization may be related to its ability to partially repress translation of *ASH1* mRNA. Puf6p binds to eIF5B and inhibits translation. This repression is relieved by phosphorylation of Puf6p by the casein kinase CK II (Deng et al., 2008). Interestingly, Puf6p, like Loc1p, has been implicated in ribosomal assembly. The evidences come from several observations. First, Puf6p has been identified among the proteins that sediment in the 60S preribosomal fraction and is involved in the 60S ribosomal subunit biogenesis (Nissan, et al., 2002; Lee, et al., 2007). These results suggest that translational regulators, like Loc1p and Puf6p, may affect *ASH1* mRNA localization to the bud tip via their effect on the ribosome biogenesis. Second, although Puf6p is a predominantly nuclear protein, it has been found to colocalize with the *ASH1* mRNP *in vivo*, suggesting that this protein shuttles between the nucleus and the cytoplasm to achieve translational regulation.

1.4 Nucleo-cytoplasmic shuttling of proteins

The Myo4p/She3p/She2p heterotrimeric complex is essential for *ASH1* mRNA trafficking to the bud tip. These localization factors are thought to constitute the cytoplasmic core RNP, also termed 'locasome', that is actively transported along actin cytoskeleton to the bud tip of daughter cell (Beach, et al., 2001; Bertrand et al., 1998; Darzacq et al., 2003). The *ASH1* mRNA is translationally repressed before it gets properly localized. This process requires translational regulators that have been identified and characterized in the past several years. Among these localization factors and translational repressors, some of them (like She2p, Khd1p and Puf6p) shuttle between the nucleus and the cytoplasm. However, the mechanism and the importance of this shuttling in *ASH1* mRNAs localization to the bud tip are still largely unknown.

Shuttling proteins (cargoes) in eukaryotic cells are transported through the nuclear pore complexes (NPCs) accross the nuclear membrane (Rout, et al., 2000). NPC consists of about 30 different proteins and forms a ring channel, which allows for passive as well as active transport of cargoes. Ions, as well as small neutral proteins that do not bind to nucleoporins, run passively through the nuclear pore complex due to diffusion (Gorlich et al., 1999). Proteins and nucleic acids larger than 40 kDa are actively transported through NPC along the pore axis (Simos et al., 1995). Factors that are important for active nuclear transport can be divided into four categories: the proteins of the NPC (nucleoporins), the Ran GTPase, transport receptors called karyopherins (or importins/exportins) that recognize cargoes for transport, and specialized factors that promote transport of some protein/RNA complexes. Cargoes transport in or out of the nucleus generally begins with recognition of the transported cargo by a receptor, followed by interaction with and through NPC. The Ran GTPase and its regulators associated with NPC, resulting in the loading and unloading of cargoes.

1.4.1 Signals for nucleo-cytoplasmic shuttling and transport factors

Each eukaryotic cell must control the rapid and vectorial transport of thousands of proteins and RNAs into and out of the nucleus. Most nucleo-cytoplasmic transport pathways are mediated by a large, evolutionarily conserved family of transport factors, the karyopherin-β family (karyopherins). Most of these mediate either nuclear import (also called importins) or nuclear export (also called exportins), and all karyopherins interact directly with their cargoes, although some also use adapter proteins. The best-characterized adapter protein is the evolutionarily conserved protein, karyopherin-α (also called importin-α, Srp1p in yeast). In human cells there are at least 20 karyopherins, and in yeast at least 14 (Mosammaparas, et al., 2004; Pemberton, et al., 2005). Studies have shown that proteins undergoing nuclear import or export generally contain a nuclear localization signal (NLS) or nuclear export signal (NES), respectively (Fried, et al., 2003; Weis, et al., 2003).

1.4.1.1 Nuclear localization signals and importin–α

Protein transport to the nucleus was first described for nucleoplasmin and the virus SV40 large T antigen. The nucleoplasmin NLS consists of two clusters of positively charged residues separated by a spacer (KR-10aa-$KKKL_{171}$), and the large T antigen NLS is a repeat of positively charged residues (PKKKRKV_{132}) (Jans, et al., 2000). NLSs of such type have been shown to be very frequent and conserved in several proteins. They are called classical or basic nuclear localization signals (cNLS). Classical NLS are recognized by importin-α. Human cells contain at least 6 homologous members of this family compared to only one, called Srp1p in yeast. Nevertheless, import signals unrelated to the basic NLS exist; indeed, the characterization of most NLSs is still in its infancy.

Numerous studies have provided a detailed molecular understanding of how the import receptor (importin-α) recognizes cNLS-containing cargoes (Conti et al. 1998; Kobe 1999). Importin-α consists of three functional domains in the coding sequence. The N-terminal region contains an importin-β binding (IBB) required for interaction with importin-β (Görlich et al. 1996; Weis et al. 1996). Some evidence show the IBB domain also contains an auto-inhibitory motif required for regulation in cNLS cargo binding in the cytoplasm and release in the nucleus (Kobe 1999; Harreman et al. 2003). The central region of importin-α consists of 10 armadillo repeat motifs (ARM) that form the pocket involved in NLS binding (Conti et al. 1998; Conti, et al., 2000; Fontes et al. 2000). The C-terminal domain of importin-α contains a binding site for the export receptor, Cse1/CAS (Hood, et al., 1998; Solsbacher et al. 1998; Schroeder et al. 1999), which is required for recycling importin-α back to the cytoplasm after the cNLS cargo is released in the nucleus (Gilchrist et al. 2002; Gilchrist, et al 2003; Matsuura, et al., 2004).

1.4.1.2 Nuclear localization signals and importins.

While a set of evidences clearly showed that importin-α, as an adaptor, interacts with NLS within its cargoes, most importins bind directly to cargoes independently of importin-α (Fried, et al., 2003; Weis, et al., 2003). Usually, it is very hard to identify nuclear localization signals recognized by importins. The NLSs recognized by these importins have often proved to be variable and hard to define. For instance, NLS identified in core histones (Muhlhausse, et al., 2002; Muhlhausse, et al., 2001), ribosomal proteins (Jakel, et al., 1998) and some RNA-binding proteins (Senger, et al., 1998; Leslie, et al., 2004) contains several basic amino acids. In another case, the M9 NLS consists of 38 amino acids that is glycine rich and deficient in basic amino acids (Pollard, et al., 1996). Moreover, in a multifunctional RNA-binding protein, a very large portion has been identified as its NLS, suggesting that the three-dimensional structure of the protein is

critical (Rosenblum, et al., 1998). Recent studies also suggest that there are considerable conformational variabilities between individual importins bound to different substrates, and between different importins (Fukuhara, et al., 2004). This can explain how a limited number of importins can transport a vast number of various substrates that frequently have no similarity in amino acid sequences in their NLS.

1.4.1.3 Nuclear export signals and exportins.

The nucleo-cytoplasmic shuttling proteins and RNAs are actively exported to the cytoplasm by exportins. Exportins recognize specific signals, for example, nuclear export signals (NESs) in proteins (Fried, et al., 2003; Weis, et al., 2003). Several of these proteins, like many transcription factors, cell-cycle regulators, as well as the viral HIV Rev protein and the protein kinase A inhibitor contain an hydrophobic leucine-rich NES (la Cour, et al., 2003; Fischer, et al., 1995; Wen, et al.,1995). It is a nonconserved motif with 3 or 4 hydrophobic residues, like $LPPLERLTL_{83}$ in protein HIV Rev. These NES are recognized by the exportin Crm1p. Similarly to importin-β1, Crm1p can bind a number of substrates with or without adapter proteins (Johnson , et al., 2002; Ohno, et al., 2000 ; Paraskeva, et al., 1999).

However, proteins that lack hydrophobic NES can also be exported into the cytoplasm. Their export depend on a specific exportin, Msn5p (Mosammaparast, et al., 2004). This exportin functions both in nuclear import and export (Kaffman, et al., 1998; Yoshida, et al., 2001). Interestingly, phosphorylation regulation is involved in most Msn5p export substrates, suggesting that the phosphosite is either part of the NES, or phosphorylation indirectly affects NES recognition by Msn5p (Mosammaparast, et al., 2004). Apart Msn5p, several other similar exportins have been characterized, including CAS (Cse1 in yeast), which exports importin-α from the nucleus, thereby performing a

critical recycling function (Kutay, et al., 1997; Solsbacher, et al., 1998; Matsuura, et al., 2004).

Compared to protein cargoes, several types of RNA constitute a diverse group of export cargoes (Lei, et al., 2002). To date, two exportins, exportin-t and exportin 5, have been identified to bind RNA directly (Kutay,et al., 1998; Arts, et al., 1998; Kim, et al., 2004). Exportin-t mediates the export of tRNAs into cytoplasm through recognition of an export signal within the RNA sequence (Kutay, et al., 1998; Arts, et al., 1998). Relatively, exportin 5 exports microRNA precursors from the nucleus by binding an RNA hairpin structure (Kim, et al., 2004; Zeng, et al., 2004). Export of mRNA does not appear to be mediated by any member of the karyopherin family, but this process requires the Mex67/Mtr2 heterodimeric receptor, which is part of a basic transport machinery conserved from yeast to humans (Segref et al., 1997; Tan et al., 2000; Gallouzi and Steitz, 2001). Moreover, evidence has been accumulated that mRNAs export to the cytoplasm is coupled to upstream steps in gene expression, such as pre-mRNA splicing, and to down-stream events, including nonsense-mediated decay (see section 1.5.5).

1.4.2 Mechanism of receptor mediated nucleo-cytoplamic shuttling

1.4.2.1 Nuclear pore complex (NPC)

The nuclear envelope, which contains a double membrane, surrounds the nucleus. Its outer nuclear membrane is continuous with the endoplasmic reticulum, whereas the inner nuclear membrane contains a unique set of proteins — some of which interact with chromatin. The two membranes are joined at the nuclear pores (Figure. 9). Movement of macromolecules between the nucleus and the cytoplasm occurs via aqueous channels that are formed by the nuclear pore complexes (NPCs) in the nuclear envelope (Figure. 9). The molecules — or cargo — that are transported through these channels include proteins and

RNAs that move in and out of the nucleus as individual entities, or as part of larger complexes, such as the ribosome subunits. Proteomic studies of yeast NPCs showed that an NPC consists of about 30 different nucleoporin proteins covering several families. Among them, FG-nucleoporins have FG repeats (GLFG, FXFG, or FG) and hydrophobic linkers. Some of them are only on the nuclear or cytoplasmic face of the NPC (Rout, et al., 2000). Thus, due to distribution difference in the NPC, FG-nucleoporins contribute a higher affinity nuclear face to importins, and are cytoplasmic face to exportins (Shah, et al., 1998).

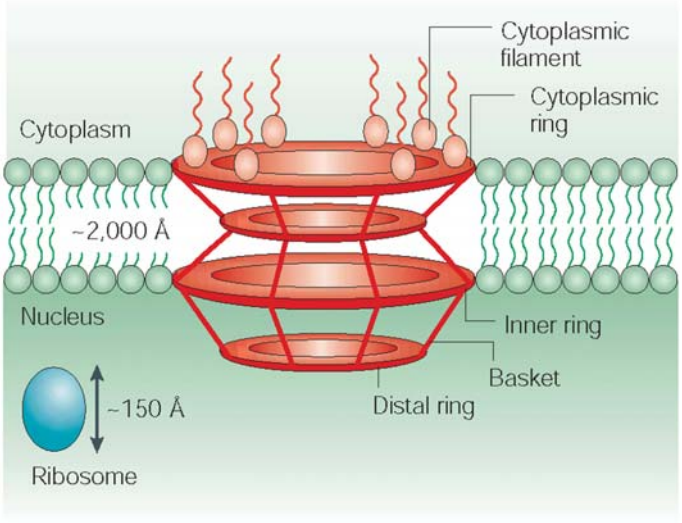

Figure 9: Schematic view of the nuclear pore complex.

The nuclear-pore complex, which is ~200 nm in length, is made up of nucleoporins. Cytoplasmic filaments extend into the cytoplasm and are anchored to a cytoplasmic ring at the plasma membrane. The pore's inner ring is part of the pore basket-like structure, which extends into the nucleus. The ribosome, which is ~150 Å, is shown for size comparison (Source: Tweeny et al., 2004).

1.4.2.2 RanGTP-RanGDP recycling

Nucleo-cytoplasmic shuttling is usually monitored by the small Ras family Ran GTPase, which plays a key role in the formation of diverse transport complexes. Like other GTPase, Ran cycles between a GTP- and a GDP- bound state (Bourne et al., 1990). Importins have a relatively high affinity for RanGTP, an abundant preferably nuclear protein, and RanGTP binding leads to the dissociation of their import cargoes. However, exportins form a trimeric complex with RanGTP and cargo have a relatively low affinity for Ran in the absence of cargo (Weis et al., 2003). In the cytoplasm, RanGTP is hydrolyzed to RanGDP, dependent on RanGAP (a protein that stimulates the GTPase activity of Ran) and RanBP1 (promotes RanGTP release from the karyopherin) (Görlich et al., 1999; Bischoff et al., 1994). Ran import into the nucleus is mediated by NTF2, a homodimeric protein originally purified as a factor that stimulates NLS-dependent import (Ribbeck, et al., 1998). NTF2 interacts with the RanGDP, which is predominantly in the cytoplasm. RanGDP imports into the nucleus by NTF2 association with FG- nucleoporins (Bayliss, et al., 2002). After delivering RanGDP into the nucleoplasm, NTF2 returns to the cytoplasm to mediate another round of Ran import. The conversion of RanGDP to RanGTP in the nucleus relies on the guanine nucleotide exchange factor RanGEF, also known as RCC1. RCC1 is a chromatin-associated protein which is present at approximately one copy per nucleosome, where it binds directly to the core histones H2A and H2B (Ohtsubo, et al., 1987; Bischoff, et al., 1991).

1.4.2.3 Model of nuclo-cytoplasmic shuttling

Taking all the data so far, the classical import pathway consists of four key steps (Stewart 2007). First, the trimeric import complex consisting of importin-β, importin-α, and the NLS containing cargo is assembled in the cytoplasm where the cargo is recognized by importin-α. Second, the import complex is targeted to the nuclear pore by importin-β

association with nucleoporins, and the trimeric complex is translocated through the NPC (Bayliss et al. 2000; Liu, et al., 2005). Third, the complex is disassembled in the nucleus following binding of the small GTPase Ran in its GTP-bound form to importin-β, which triggers the release of the cNLS cargo and delivery into the nucleus (Vetter et al. 1999; Lee et al. 2005). Finally, the import receptors are recycled to the cytoplasm for another round of protein import with importin-α exported to the cytoplasm by Cse1/CAS in complex with RanGTP (Figure 10) (Matsuura, et al., 2004).

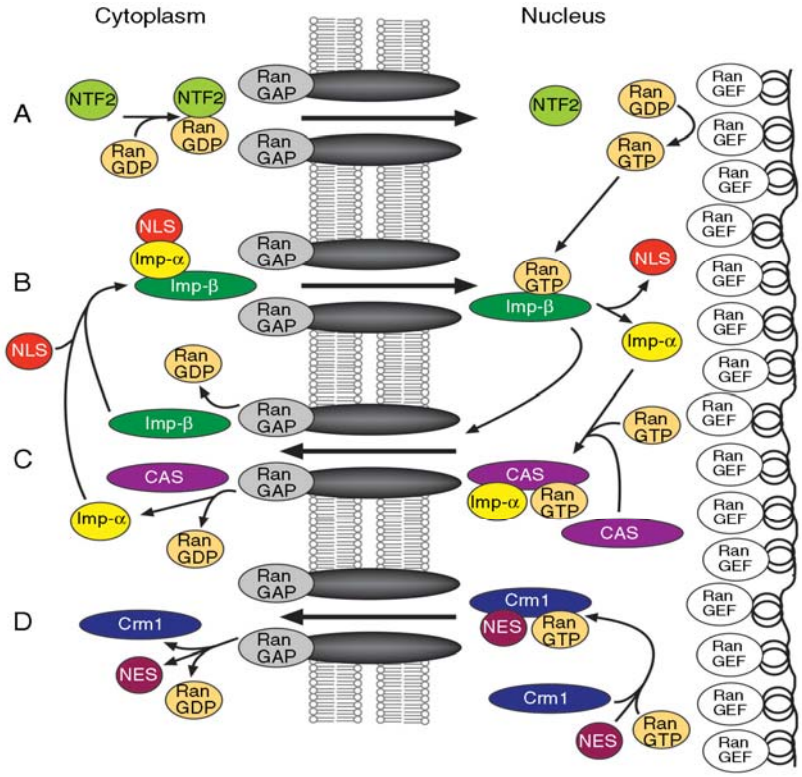

Figure 10: Overview of some of the major nuclear transport pathways in eukaryotic cells. (A) Nuclear import of RanGDP mediated by NTF2. (B) Nuclear import of nuclear localization sequence (NLS) cargo mediated by the karyopherin-α: importin-β1

heterodimer (abbreviated Imp-α and Imp-β). Nuclear import of NLS cargo mediated by direct binding to importin- β1 and other karyopherin β1- family members is not shown. (C) Nuclear export pathways that mediate recycling of importin -β1 and karyopherin-α; the latter requires CAS as an export receptor. (D) Nuclear export of the cargo containing NES mediated by Crm1p. RanGAP is anchored to the cytoplasmic side of the NPC and RanGEF (RCC1) is shown bound to chromatin (Source: Lucy et al., 2005).

1.5 Coupling between transcription and mRNA maturation

Eukaryotic pre-mRNAs undergo several processing events before maturation, including addition of a cap structure to the 5′ terminus of nascent transcripts, removal of introns by splicing, and formation of the polyA tail at the 3′ end. The mature mRNA is then exported through nuclear pore complexes (NPCs) to the cytoplasm for localization and translation (Figure 11). During this process, mRNAs are bound by numerous distinct factors and assemble in differently functional mRNP. These factors include cap binding complex (CBC) (Listerman et al., 2006; Visa et al., 1996), hnRNPs (Daneholt, 2001), SR proteins (Long, et al., 2009), the exon junction complex (EJC) (Custodio et al., 2004), and zipcode binding proteins (ZBP) (Pan et al., 2007). Moreover, recent studies suggest that these proteins profoundly influence pre-mRNA processing as well as export, localization, translation and stability (Perales et al., 2009). Although distinct and complex cellular machineries carry out each of these steps in the gene-expression process, increasing evidence now suggests that the transcription and mRNA maturation processing are functionally integrated in a reciprocal fashion such that individual cotranscriptional processing events also influence transcription at different phases (Figure 12) (Perales, et al., 2009; Iglesias, et al, 2008; Schmid, et al, 2008; Shatakshi et al., 2008).

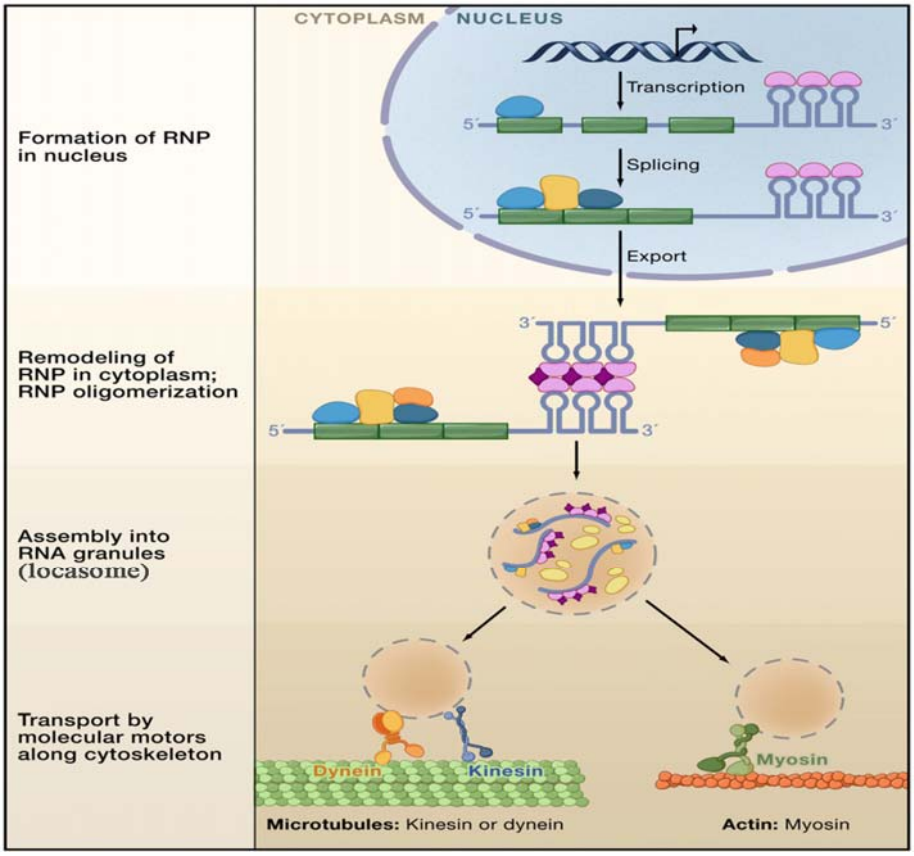

Figure 11: mRNA localization is a multistep process

The pre-mRNA (exons in green; introns in grey) has cis-acting localization elements in its primary sequence. RNA-binding proteins (blue and purple) bind the pre-mRNA. During splicing, additional RNA-binding proteins (golden and dark blue) are added to form a ribonucleoprotein (RNP) complex. Following export into the cytoplasm, the RNP is remodeled and additional proteins (orange, dark purple) are added. In the cytoplasm, RNPs are assembled into RNA granules (locasome) that are likely to be a heterogeneous population of structures containing diverse RNAs, ribosomal subunits (yellow), as well as many factors involved in translational regulation (Kelsey et al., 2009).

1.5.1 Transcription and the elongation factor Spt4-Spt5 complex in yeast

RNA polymerase II (Pol II) is the enzyme that transcribes DNA sequences into pre-mRNAs to carry out the first step of gene expression in eukaryotic cells (Roeder 1996). Pol II is a dodecameric protein complex assembled from a tightly associated 10-subunit core and a heterodimeric subcomplex of subunits Rpb4 and Rpb7 (Edwards, et al., 1991). Although the 10-subunit core contributes the central transesterase activity that catalyzes RNA-chain polymerization, the Rpb4/7 heterodimer enables promoter- dependent initiation by the polymerase and supports yeast growth under stress conditions (Woychik, et al., 1989; Rosenheck, et al., 1998).

The largest subunit of eukaryotic DNA-dependent RNA polymerase II, Rpb1, has a specific additional C-terminal domain (CTD), which includes a varying number of tandem heptad repeats with the canonical sequence YSPTSPS. The CTD size varies from about 15 (malaria plasmodium) and 26 (yeasts) to 52 (mammals) heptad repeats and correlates with the organism complexity (Stiller, et al., 2002; Stiller, et al., 2004). Post-translational modification on serine residues in the heptad repeats play major role in RNA Pol II. Phosphorylation regulation at Ser2, Ser5 or Ser7 is the most common. Phosphorylation of Ser5 by the TFIIH-associated kinase Cdk7, or Kin 28 in yeast, occurs first at initiation, whereas Ser2 phosphorylation by Cdk9/ PTEFb (positive transcription elongation factor b), or Ctk1 and Bur1 in yeast, occurs later during elongation (Komarnitsky, et al., 2000; Phatnani, et al., 2006, Mosley, et al., 2009). In fact, the major function of the Pol II CTD is to act as a platform for the direct binding of factors involved in pre-mRNA capping, 3'end processing, transcription elongation, termination, and chromatin modification (Phatnani et al., 2006).

The mRNA-transcription cycle can be divided into three stages: initiation, elongation and termination. During elongation, Pol II moves along a DNA template and synthesizes a complementary RNA chain in a processive manner. However, the

understanding of the elongation phase of transcription is incomplete. *In vitro*, two general transcription elongation factors, TFIIF and TFIIS, are sufficient to stimulate rates of elongation on naked DNA templates (Izban, et al., 1992). In contrast, elongation on nucleosome-bound templates is inefficient, even in the presence of TFIIF and TFIIS, suggesting the requirement of other factors (Chang, et al., 1997; Izban, et al., 1992; Izban, et al., 1991). Several factors have been implicated in the regulation of transcription elongation through chromatin. Among these factors are the polymerase-associated factor (PAF) complex, which associates with RNAPII, the Spt4-Spt5 complex, and several cyclin-dependent kinases, including P-TEFb in humans and BUR kinase (Bur1–Bur2) and C-terminal domain (CTD) kinase 1 (CTDK1) in *Saccharomyces cerevisiae*. One well characterized Pol II transcription elongation factor in yeast is a complex of two proteins, Spt4-Spt5.

Spt4-Spt5 is highly conserved among eukaryotes, also known as DSIF (DRB sensitivity-inducing factor) which plays a role in Pol II transcription elongation in mammalian cells (Wada et al., 1998). In budding yeast, the *SPT4* and *SPT5* genes were among many genes isolated for their ability to suppress transcription defects caused by insertions of the retrotransposon *Tyl* in the 5' noncoding regions of yeast genes (Winston, et al., 1984). Spt4-Spt5 associates with Pol II and the general elongation factors TFIIF and TFIIS (Lindstrom, et al., 2003). Recent observations suggest that SPT4-SPT5 display an extensive set of genetic interactions with the CTD and enzymes that modify the CTD's phosphorylation status, including protein kinases similar to P-TEFb (Lindstrom, et al., 2001; Murray, et al., 2001). Interestingly, Spt5p coimmunopurifies with the yeast capping enzyme and cap methyltransferase. Moreover, *spt4* and *spt5* mutations cause splicing defects in yeast (Lindstrom, et al., 2003), suggesting important roles for Spt4-Spt5 in pre-mRNA processing.

In *metazoan*, P-TEFb is responsible for phosphorylating both Pol II on Serine 2 of the CTD and the elongation factors Spt4-Spt5 (Peterlin, et al., 2006). Phosphorylation of

Spt4-Spt5 on threonine2 of the Spt5p C-terminal region (CTR) converts this complex from a negatively acting elongation factor to a positively acting one (Yamada T, et al. 2006). A more recent work suggests that BUR kinase is also involved in the phosphorylation of Spt5 CTR, and further data show that phosphorylated Spt5 CTR is needed for the recruitment of the PAF complex to transcribed regions (Zhou, et al., 2009). Since the CTR of yeast Spt5 contains 15 repeats of a hexapeptide (consensus sequence is S [T/A] WGG [Q/A]), these results suggest that the Spt5 CTR, like the CTD of the Rpb1 subunit of Pol II, is likely to be a platform for the association of regulatory factors that promote both transcription elongation and histone modification in transcribed regions (Zhou, et al., 2009).

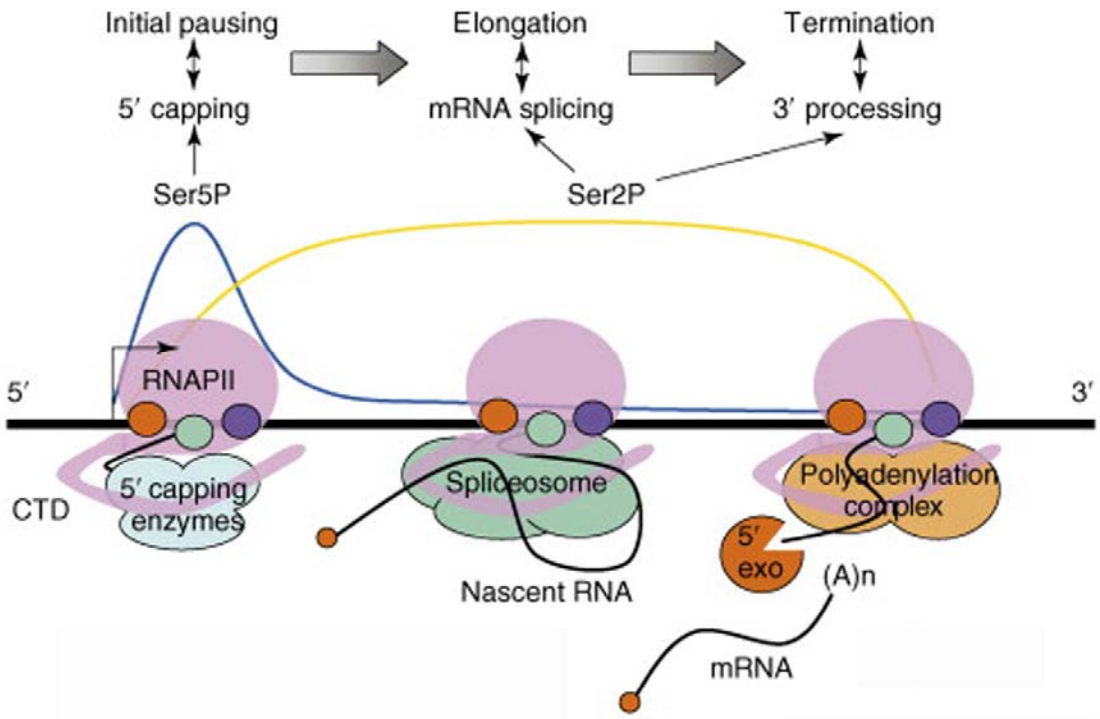

Figure 12: Coupling between transcription and pre-mRNA processing

The RNA polymerase II (RNAPII) is modified on its CTD with Ser5 phosphorylation predominately at the beginning of the gene (blue line) and Ser2 phosphorylation in the middle and end of the gene (yellow line). 5′-Capping enzymes are recruited through direct interactions with Ser5 phosphorylated CTD to catalyze the cotranscriptional capping reaction. Various splicing factors are recruited during the elongation phase of transcription, most of which in a CTD Ser2 phosphorylation-dependent manner, to facilitate cotranscriptional splicing. The 3′-end formation is functionally tied to transcription termination (Source: Shatakshi et al., 2008).

1.5.2 Coupling transcription with mRNA capping

The 5'cap structure is a critical feature of an mRNA required not only for protection against exonucleolysis but also for transcript processing, export, and translation (Varani 1997). On pre-mRNAs is added a 7-methyl G5'ppp5'N cap at their 5' end when the transcript is only 25–50 bases long (Shuman et al., 2001). Capping is a three-step process that requires specific enzymes. The RNA triphosphatase removes the gamma-phosphate of the first nucleotide, and then GMP is added by RNA guanylyltransferase. Finally, the guanine is methylated at N7 by a methyltransferase. However, the recognition by these enzymes does not require specific sequences in the RNA. Both capping enzyme and the methyltransferase bind directly and specifically to the phosphorylated CTD of Rpb1p (Shuman 2001). When transcription initiates, phosphorylation of the CTD on Ser5 residues allows loading of capping enzyme onto the transcription elongation complex (TEC), resulting in allosteric activation of the guanylyltransferase (Ho et al., 1999).

Although capping is not regarded as a regulated step in gene expression, capping enzymes recruitment could be regulated by the elongation factor Spt5p or Pol II CTD. Some evidence has illustrated the fact that capping enzymes associate with the CTD, resulting in an enhanced capping, and stimulates or inhibits transcription initiation and/or early elongation (Mandal et al., 2004; Myers et al., 2002; Schroeder et al., 2004). In metazoan, RNA polymerase complexes paused at 5' ends plays an important role for cotranscriptional capping. For example, Spt5p is involved in the regulation of 5' pausing and allosterically activates the cap guanylyltransferase (Wen et al.,1999). Inhibition of the capping enzyme recruitment has been reported at the yeast silent mating type loci (Gao et al., 2008). Interestingly, human capping enzymes are found at 5'end and throughout genes, including 3' flanking regions even more than a kilobase downstream of the polyA site (Glover-Cutter et al., 2008). These results suggest that the influence of capping enzymes on mRNA production is not limited to 5' ends, but probably to elongation, termination, and

3'end processing. Moreover, evidences also show that capping enzymes are involved in R loop formation, suggesting a role in transcription elongation (Kaneko et al., 2007).

1.5.3 Coupling transcription with mRNA splicing

In general, the splicing of pre-mRNA is mediated by components of the spliceosome (Dziembowski et al. 2004). The spliceosome is an excellent example of ordered self assembly, the evidence coming from ChIP analysis of cotranscriptional splicing on yeast genes (Gornemann et al., 2005; Lacadie, et al., 2005). Previous studies showed that splicing of synthetic pre-mRNAs injected in Xenopus oocytes is less efficient than splicing coupled to transcription in the same cells, consistent with stimulation of splicing *in vivo* by the phosphorylated CTD (Bird et al., 2004; Hirose, et al., 2000). In addition, some evidence suggest that there are different effects on splicing between cotranscription and uncoupled transcription, since exons are tethered to the polymerase in the former (Dye et al., 2006). A set of studies have shown that there are some intriguing connections between the splicing machinery and TEC (Transcription Elongation Complex) -associated proteins. For instance, the yeast U1 snRNP protein Prp40 can bind directly to the phospho-CTD (Phatnani, et al., 2006), although the functional significance of this interaction remains to be established. Interestingly, human U1 snRNP also coimmunoprecipitates with Pol II (Das et al., 2007). Moreover, U1 snRNP actively recruits Pol II at a 5'splice site and general transcription factors to the promoter independently of splicing (Damgaard et al., 2008). These results suggest that the U1 snRNP has a special relationship with Pol II TECs.

Coupling mRNA splicing with transcription can be well illustrated by the study of the Serine/arginine-rich (SR) family of splicing factors. SR family proteins regulate spliceosome assembly and coimmunoprecipitate with Pol II (Long, et al., 2009; Lin et al., 2008). The SC35 family member (the prototypical SR protein) has been implicated in

stimulating transcriptional elongation through its interaction with P-TEFb (Lin et al., 2008). A more recent study showed that SR proteins associate with TECs exclusively through the nascent mRNA, rather than through protein interaction with Pol II (Sapra et al., 2009). SRp20, another SRs family member, is required for regulation of alternative splicing in a Pol II CTD dependent manner (de la Mata, et al., 2006), suggesting a functional link between SRs and transcription. Interestingly, in an *in vitro* system in which transcripts made by Pol II are selectively stabilized and spliced compared to T7 transcripts, SRs have also been identified to couple transcription with splicing (Hicks et al., 2006). One understanding of this coupling is that RNA-binding proteins (RBPs), including SR proteins, bind to nascent transcripts to protect them from degradation and enhance spliceosome assembly (Das et al., 2007).

1.5.4 Coupling transcription with 3'end processing and polyadenylation

The understanding of the coupling between transcription and 3'end processing was made possible by our increasing knowledge of the components of the core cleavage/polyadenylation machinery. Several complexes act in concert to recognize and process the pre-mRNA 3′ end (Edmonds, 2002; Shatkin, et al., 2000). In *S. cerevisiae*, accurate cleavage of pre-mRNA requires cleavage factor I (CFI) and cleavage/polyadenylation factor (CPF), whereas polyA addition requires CFI, CPF, and the polyA binding protein (Pab1) (Edmonds, 2002). CFI and CPF complexes have been identified in higher eukaryotes, suggesting a strong conservation of this essential process (Edmonds, 2002). 3'end processing of most mRNAs is a two-step reaction comprising endonucleolytic cleavage after the AAUAAA sequence, followed by polyadenylation of the exposed 3'OH (Edmonds, 2002). Previous results suggested that cleavage and early polyadenylation occur at the site of transcription (Bauren et al., 1998), consistent with the

fact that cleavage/polyadenylation factors are found at transcribed genes (Ahn et al., 2004; Gall et al., 1999; Glover- Cutter et al., 2008; Licatalosi et al., 2002).

3'end-processing factors associate with the Pol II CTD, which stimulates cleavage/polyadenylation *in vivo* and *in vitro* (Hirose, et al., 1998; McCracken et al., 1997), and recent evidence show that some subunits of these factors bind directly the CTD (Meinhart et al., 2005; Phatnani, et al., 2006; Kim et al., 2004). Ser2 phosphorylation of the CTD plays an essential role in 3'end processing, and one evidence comes from the cleavage/polyadenylation factor Pcf11 specific binding to this modification (Ahn, et al., 2004; Licatalosi et al., 2002; Meinhart, et la., 2004). Unexpectedly, the yeast CF1A subunits and human CPSF are found at 5' ends, suggesting the fact that 3'end-processing factors are not confined to the 3' ends of pre-mRNAs (Dantonel et al., 1997; Glover-Cutter et al., 2008; Licatalosi et al., 2002). Interestingly, the yeast RNA-binding proteins Npl3p and Rna15p, a CF1A subunit, both load onto the TEC before it reaches the 3' end of the gene, and compete for binding to similar sites on the nascent RNA. These results suggest that this competition enhances the accuracy of 3'end processing by preventing Rna15p from recognizing polyA sites within genes (Bucheli et al., 2007).

1.5.5 Coupling transcription with mRNA export

Nuclear export of mRNAs is a complex and evolutionarily conserved process, essential for gene expression in all eukaryotic cells. To date, it is generally agreed that the export machinery recognizes signals within proteins of these complexes rather than in the mRNA itself. From yeast to human, mature and well-processed mRNPs are exported to the cytoplasm by Mex67/TAP and the adaptor proteins Yra1 and Sub2 in yeast. It has been suggested that Yra1p and Sub2p are loaded onto all yeast mRNAs via the THO complex (complex of Tho2p, Hpr1p, Mft1p, and Thp2p) previously implicated in transcriptional

elongation (Chavez et al., 2000). This THO-Sub2p-Yra1p complex, collectively called the TREX (transcription and/or export) complex, is specifically recruited to activated genes during transcription and travels with the polymerase (Strasser, et al., 2002). Recent works have suggested that Mex67 is recruited on mRNPs during the transcription process, mainly through its short C-terminal UBA (ubiquitin-associated) domain (Reed, et al., 2002; Gwizdek, et al., 2006; Dieppois, et al., 2006). Interestingly, another supramolecular complex, called TREX-2, also tightens the connection between transcription and mRNA export (Fischer, et al., 2002; Rodriguez-Navarro, et al., 2004).

Cotranscriptional mRNP assembly plays an important role in preparation for nuclear export. Nascent transcripts are packaged for export by loading adaptor proteins that associate with the export receptors Mex67/Mtr2 in yeast and TAP/p15 in metazoans (Iglesias, et al., 2008). Only fully processed mRNPs can be properly exported from the nucleus due to the sequential recruitment of the export adaptors Yra1p and REF/Aly to the nascent transcript (Lei et al., 2001; Schmid, et al, 2008). Yra1p binds Sub2p, a DEAD-box RNA helicase that also associates with the THO complex on the TEC (Strasser et al., 2002). Moreover, Yra1p recruitment to the phosphorylated CTD is dependent on Pcf11, a 3'end-processing factor (Johnson et al., 2009). The mechanism by which Yra1p loads on TEC illustrates the exclusive order of assembly of mRNPs that can be determined by CTD phosphorylation. Pcf11 specifically interacts with Ser2 phosphorylated CTD, thus resulting in indirect recruitment of the export factor Yra1p to transcription sites at later stage. Following initial Yra1p loading by interaction with Pcf11, Sub2p associates with Yra1p and then facilitates a transfer to the mRNA export receptor Mex67/Mtr2 complex. Due to the localization of all the players, Pcf11, Yra1p, Sub2p, and Mex67/Mtr2, at transcription sites, these reactions have been suggested to occur at the TEC (Figure 13) (Iglesias, et al., 2008).

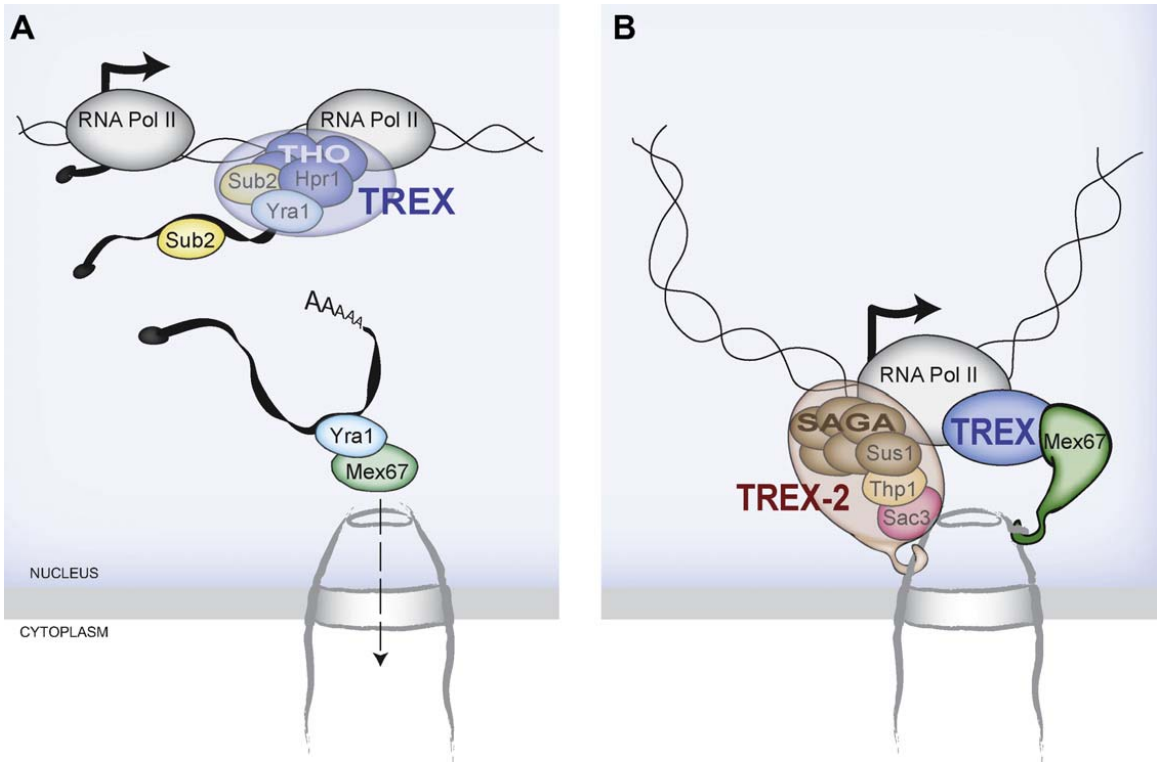

Figure 13 (A) Simplified model of mRNA export. The export receptor Mex67p binds mature mRNPs through adaptor proteins such as Yra1p and promotes their export through nuclear pore complexes. Yra1p and its partner Sub2p are part of the TREX complex recruited to active genes through an interaction of THO with the transcription machinery (B) TREX-2 links transcription initiation to NPC-bound mRNA export factors. Both TREX-2 and Mex67p participate in the anchoring of activated genes to nuclear pore complexes (Source: Iglesias et al., 2008).

1.5.6 Cytoplasmic mRNA Localization is initiated in the Nucleus

While mRNA localization is a cytoplasmic process, recent studies suggest that nuclear proteins or nucleo-cytoplasmic shuttling proteins are required for localizated mRNAs to their proper destination (Czaplinski, et al., 2006; Giorgi, et al., 2007). In

Drosophila, HRP48, a hnRNPA/B family member, binds to three sites in the *oskar* mRNA 3'UTR co-transcriptionally and regulates its translation (Huynh, et al., 2004; Yano, et al., 2004). Moreover, the exon junction complex (EJC) is essential for localizing *oskar* mRNA to the posterior pole of oocytes (Hachet, et al., 2004). During pre-mRNA splicing in the nucleoplasm, the EJC assembles on the mRNA at a precise position upstream of newly formed exon-exon boundary and follow the mRNA until the first round of translation (Giorgi, et al., 2007; Lejeune, et al., 2005). The cytoplasmic protein Barentsz (BTZ), required for *oskar* mRNA localization, is recruited by the EJC component eIF4AIII as the mRNA is exported from the nucleus (Palacios, et al., 2004; Van Eeden, et al. 2001). Finally, Staufen is thought to associate with *oskar* mRNA in the nurse cell cytoplasm and is then involved in the transport of *oskar* mRNA into the oocyte (Kim-Ha, et al., 1991; St Johnston, et al., 1991; St Johnston, et al.,1992), where these factors direct its localization to the posterior pole (Figure 14). The *oskar* mRNA localization complex therefore seems to assemble in a stepwise manner, in which factors that associate with the mRNA in the nucleus are essential for the recruitment of other essential localization factors in the cytoplasm.

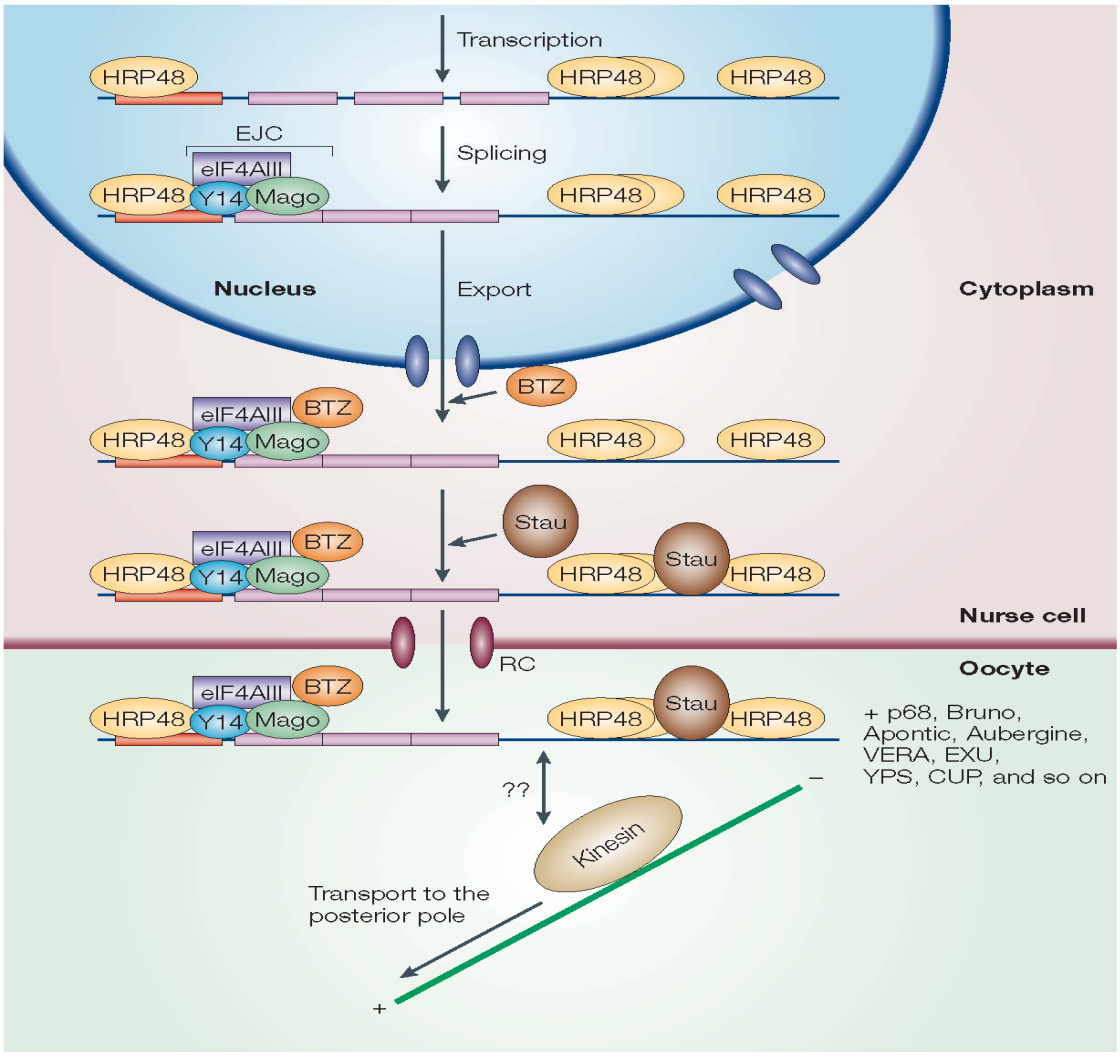

Figure 14 *oskar* mRNA localization starts in the neucleus.

HRP48 binds to *oskar* mRNA co-transcriptionally, whereas the exon junction complex (EJC) binds during splicing, upstream of where each intron has been removed. Barentsz (BTZ) protein is then recruited by the EJC component eIF4AIII, as the mRNA is exported from the nucleus. Finally, Staufen is thought to associate with oskar mRNA in the nurse cell cytoplasm.The mRNA is then transported from nurse cells into the oocyte, where these factors direct its localization to the posterior pole (Source: Daniel et al., 2005).

The cytoplasmic localization of several other mRNAs also depends on the binding of proteins in the nucleus. Vg1 and VegT mRNAs are localized to the vegetal pole of oocytes of the claw frog Xenopus laevis (King, et al., 2005). Vg1RBP60/hnRNPI and VERA/Vg1RBP have been shown to associate with both mRNAs in the nucleus and in the cytoplasm (Kress, et al., 2004). Recent studies suggest that a core mRNP containing Vg1 or VegT mRNA assembles in the nucleus and is subsequently exported to the cytoplasm, where remodeling and association with other factors occurs (Kress, et al., 2004). An homolog of Vg1RBP/VERA, called zip-code binding protein 1 (ZBP1), mediates asymmetric localization and translational silencing of β-actin mRNA in various vertebrate cell types (Condeelis, et al., 2005; Hüttelmaier, et al., 2005; Oleynikov, et al., 2003). ZBP1 binds specifically to the β-actin mRNA localization element and accumulates at sites of β-actin mRNA transcription in the nucleus, although it is predominantly cytoplasmic (Condeelis, et al., 2005; Hüttelmaier, et al., 2005; Oleynikov, et al., 2003). Whereas ZBP2, a shuttling heterogeneous nuclear ribonucleoprotein (hnRNP) that is predominantly nuclear, was shown to bind β-actin mRNA co-transcriptionally to facilitate subsequent recruitment of ZBP1 (Pan, et al., 2007), resulting in β-actin mRNA proper export from the nucleus and localization in the cytoplasm. From the discussion above, these studies favour the idea that nuclear and cytoplasmic steps in the RNA transport pathway are intimately linked.

1.6 Research objectives of this work

Evidence have been accumulating that the nuclear history of an mRNA has an important role in the determination of its fate in the cytoplasm (Farina et al., 2002; Giorgi et al., 2007; St Johnston 2005). In budding yeast, previous studies have shown that nuclear factors play an important role in yeast mRNA localization. Puf6p and Loc1p are primarily nucleolar proteins and are involved in the translational repression of the *ASH1* mRNA (Gu et al., 2004; Komili et al., 2007). Knockouts of these genes disrupt *ASH1* mRNA localization and Ash1p sorting to the daughter cell (Long et al., 2001; Gu et al. 2004).

She2p is shuttling between the cytoplasm and the nucleus (Kruse et al., 2002), and recent evidence suggest that nuclear transit of She2p is involved in the translational regulation of *ASH1* mRNA (Du et al., 2008). However, the mechanism by which She2p interacts with the *ASH1* mRNA in the nucleus in order to control its cytoplasmic localization and translation is still unknown.

Our major hypothesis is that the co-transcriptional recruitment of She2p to nascent *ASH1* mRNA by interaction with RNA Pol II enhances the bud targeting of this transcript in yeast. To address this issue, we first explored the role of She2p in the nucleus. We detected an interaction between She2p and Srp1p by molecular genetics and biochemical assays. Using immunofluorescence and fluorescence *in situ* hybridization, we also investigated the nuclear import of She2p, *ASH1* mRNA localization and Ash1p sorting in a *she2*Δ strain transformed with She2p mutants, which failed to interact with Srp1p. We further identified the nuclear role of She2p in the recruitment of translational regulators Loc1p and Puf6p to *ASH1* mRNA. Moreover, to understand the assembly of the mRNA localization machinery in the nucleus, we investigated the interaction between She2p and the nascent *ASH1* transcript using chromatin immunoprecipitation (ChIP). We identified an interaction bewteen She2p and both initiating and elongating forms of Pol II. We further studied the role of elongation complex Spt4-Spt5 in the association of She2p with Pol II. Knowledge resulting from thes studies will contribute to a better understanding of the nuclear role of the nucleo-cytoplasmic shuttling She2p in mRNA localization to the bud of daughter cells.

Results

Chapter II: Nuclear shuttling of She2p couples *ASH1* mRNA localization to its translational repression by recruiting Loc1p and Puf6p

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Contribution of authors

Zhifa Shen:	FISH, immunofluorescence, GST-pull down assay, yeast two hybrid, IP, IP &RT-PCR, genetic assay
Nicolas Paquin:	Figure 1A, She2p deletion plasmids construction
Amélie Forget:	Protein expression level assay between She2p and its mutants
Pascal Chartrand:	General supervisor and writing

Nuclear shuttling of She2p couples *ASH1* mRNA localization to its translational repression by recruiting Loc1p and Puf6p

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Running title: She2p links Puf6p and Loc1p to *ASH1* mRNA

Abbreviations: NLS: nuclear localization sequence.

ABSTRACT

The transport and localization of mRNAs results in the asymmetric synthesis of specific proteins. In yeast, the nucleo-cytoplasmic shuttling protein She2 binds the *ASH1* mRNA and targets it for localization at the bud tip by recruiting the She3p-Myo4p complex. While the cytoplasmic role of She2p in mRNA localization is well characterized, its nuclear function is still unclear. Here, we show that She2p contains a non-classical nuclear localization signal (NLS) which is essential for its nuclear import via the importin α Srp1p. Exclusion of She2p from the nucleus by mutagenesis of its NLS leads to defective *ASH1* mRNA localization and Ash1p sorting. Interestingly, these phenotypes mimic knockouts of *LOC1* and *PUF6*, which encode for nuclear RNA-binding proteins that bind the *ASH1* mRNA and control its translation. We find that She2p interacts with both Loc1p and Puf6p, and that excluding She2p from the nucleus decreases this interaction. Absence of nuclear She2p disrupts the binding of Loc1p and Puf6p to the *ASH1* mRNA, suggesting that nuclear import of She2p is necessary to recruit both factors to the *ASH1* transcript. This study reveals that a direct coupling between localization and translation regulation factors in the nucleus is required for proper cytoplasmic localization of mRNAs.

INTRODUCTION

The cytoplasmic transport and localization of mRNAs is used by several eukaryotic organisms to control, in space and time, the expression of proteins involved in cell fate determination, cellular polarity or asymmetric cell division (St Johnston, 2005; Du *et al.*, 2007). With over 30 mRNAs localized at the bud tip, the budding yeast *S. cerevisiae* is an excellent model system for studying the mechanisms behind mRNA transport and localization (Chartrand *et al.*, 2001; Darzacq *et al.*, 2003). One of these transcripts is the *ASH1* mRNA, which is localized at the distal tip of daughter cells during late-anaphase (Long *et al.*, 1997; Takizawa, 1997). This localization promotes the asymmetric sorting of Ash1p to the daughter cell nucleus and results in the inhibition of mating-type switching in the daughter cell (Jansen, 1996; Sil, 1996). The RNA-binding protein She2p is responsible for the recognition of bud-localized mRNAs, via its interaction with specific localization elements in these transcripts (Olivier *et al.*, 2005). She2p also interacts with the transport machinery, constituted of the type V myosin Myo4p, via the bridging protein She3p (Bohl *et al.*, 2000; Long *et al.*, 2000; Takizawa and Vale, 2000). During its transport to the bud tip, the *ASH1* mRNA is translationally repressed by the RNA-binding proteins Khd1p (Irie *et al.*, 2002) and Puf6p (Gu *et al.*, 2004), and phosphorylation of these factors at the bud tip promotes the local synthesis of the Ash1 protein (Paquin *et al.*, 2007; Deng *et al.*, 2008).

Previous studies have shown that nuclear factors play an important role in yeast mRNA localization. Puf6p and Loc1p are predominantly nucleolar RNA-binding proteins which bind directly the 3'UTR of *ASH1* mRNA *in vitro* and are involved in the

translational repression of this transcript (Long *et al.*, 2001; Gu *et al.*, 2004; Komili *et al.*, 2007). Knockouts of these genes disrupt *ASH1* mRNA localization and Ash1p sorting to the daughter cell (Long *et al.*, 2001; Gu *et al.*, 2004). She2p shuttles between the cytoplasm and the nucleus (Kruse *et al.*, 2002), and recent evidence suggests that the nuclear transit of She2p is involved in the translational regulation of *ASH1* mRNA (Du, 2008). However, the specific role of She2p in the nucleus is still unclear.

In this study, we show that She2p contains a non-classical nuclear localization signal (NLS) which is essential for its nuclear import by the importin α Srp1p. Exclusion of She2p from the nucleus by mutagenesis of its NLS leads to defective mRNA localization and Ash1p sorting. We find that nuclear She2p is associated with Puf6p and Loc1p independently of their interaction with RNA. Exclusion of She2p from the nucleus decreases its interaction with Loc1p and Puf6p, and disrupts the binding of these factors to the *ASH1* mRNA. This study leads us to suggest a mechanism where She2p interacts with the translation regulation factors Puf6p and Loc1p in the nucleus, and recruits these factors to the *ASH1* mRNA, thereby promoting the localization of this transcript at the bud tip. This coordinated recruitment of a localization factor and translational repressors to *ASH1* transcripts in the nucleus suggests that mRNA transport and translational control machineries are coupled, and that this coupling in the nucleus is required for proper cytoplasmic localization and local translation of this transcript.

MATERIAL AND METHODS

Growth media and yeast strains

Yeast cells were grown in either synthetic growth media lacking the nutrients indicated or rich media (Rose, 1990). Transformation was performed according to the protocol of Gietz and Schiestl (Schiestl, 1989). Yeast gene disruption cassette was created by PCR amplification of the *loxP*-KAN-*loxP* construct in plasmid pUG6 and primers specifics for the gene of interest (Guldener *et al.*, 1996). Specific disruption was confirmed by PCR analysis of genomic DNA. Yeasts strains and plasmids used in this study are described in the Supplementary Tables 1 and 2, respectively.

Immunoprecipitation and reverse transcription-PCR.

50 mL of yeast cells were grown to early log phase (OD_{600} around 1) at 30°C in the appropriate medium. Formaldehyde was added to a final concentration of 1% and cells were incubated at room temperature (RT) for 20 min. Glycine was added to a final concentration of 300mM. Cells were washed twice in 1×PBS, harvested by centrifugation and resuspended at an OD_{600} of 100 in the extraction buffer (25 mM HEPES-KOH, pH 7.5, 150 mM KCl, 2 mM $MgCl_2$, 0.1% IGEPALCA-630, 1 mM dithiothreitol, 87.5 µg/ml phenylmethylsulfonyl fluoride, 0.5µg/ml pepstatin, 0.5 µg/ml leupeptin, 0.5 µg/ml aprotinin; and 23 U/ml of RNAguard). The cells were broken with glass beads, vortexed 5 times for 30 sec, with 1 min pause on ice between each vortex. The supernatant was used for immunoprecipitation and Western blot. For the immunoprecipitation of myc-tagged She2p, 10µg of anti-myc antibody (9E10) was added to 500 µl of supernatant and

incubated at 4°C with agitation for 1 h; 50 µl of protein A-Sepharose beads was then added and the incubation at 4°C was continued for 2 h. For immunoprecipitation of TAP-tagged proteins, 50 µl of IgG-agarose beads was added to 500 µl of supernatant. The beads were washed four times for 3 min at 4°C with a wash buffer (25 mM HEPES-KOH, pH 7.5, 150 mM KCl, 2 mM $MgCl_2$). The RNA was eluted from the beads with 200 µl of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA and 1% sodium dodecyl sulfate by incubating 10 min at 65°C, followed by a phenol-chloroform extraction and ethanol precipitation. For the reverse transcription, 2 µl of RNA was incubated at 70°C for 5 min in the presence of 0,5 µg of pd(N)6 and quickly chilled on ice. The reverse transcription reaction was performed according to indications in a 1× buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 10 mM dithiothreitol) containing 10 mM dNTPs and 20 U of RNAguard, with 100U reverse transcriptase for 1 h at 42°C. The cDNAs were then amplified by PCR using primers in the *ASH1* sequence.

Fluorescence in situ hybridization and immunofluorescence

Yeast cells were processed for fluorescent in-situ hybridization and immunofluorescence according to the protocols described in (Chartrand, 2000). For in-situ hybridization, yeast spheroplasts were hybridized with a pool of Cy3-conjugated *ASH1* DNA oligonucleotide probes. For immunofluorescence, a 1:50 dilution of a mouse anti-myc 9E10 antibody (Oncogene Science) was used as primary antibody. For the secondary antibody, a 1:1000 dilution of a anti-Mouse Cy3-conjugated antibody (Jackson Laboratories) was used.

Protein expression and purification

Recombinant protein GST-She2, GST-She2-M2 and GST-Srp1 were overproduced in *Escherichia coli* BL21 transformed with pGEX-6P1-She2, pGEX-6P1-She2-M2 and pGEX-5X3-Srp1. The cells were harvested 3 hours after induction with 1mM IPTG at 30°C, resuspended in PBS-TritonX100 0,1%, 1M NaCl, 1mg/ml lysozyme and antiproteases cocktail (PMSF + pepstatine + leupeptin + aprotinine) for 30 min on ice and sonicated. The lysate was cleared by centrifugation for 15 min at 15000g, 4°C, to yield the supernatant with the overexpressed soluble protein. The GST fusion proteins were purified by affinity chromatography with Glutathione-Sepharose 4B (GE Healthcare) and eluted with 10mM reduced glutathione in PBS. The recombinant protein fractions were dialysed overnight in PBS and concentrated using a 10-kDa molecular weight cut-off filter unit (Centricon-Millipore). For the elution of Srp1p, the GST tag was cleaved with Factor Xa overnight at room temperature.

GST pull-down assays

For the recombinant protein interactions of Srp1p with She2p and She2-M2, purified Srp1p was incubated with 5 µg of GST-She2p (wild type or mutant) bound to glutathione-Sepharose 4B (GE Healthcare). The binding was performed at room temperature for 3h in 500 µL of binding buffer (50 mM HEPES-KOH (pH 7.3), 20 mM potassium acetate, 2 mM EDTA, 0.1% Triton X-100, and 5% glycerol). The matrix was recovered by centrifugation and washed four times with 500 µL of binding buffer. The bound proteins were eluted by boiling in Laemmli buffer and separated on a 10% SDS-PAGE. For the interactions

between recombinant GST-Srp1p and endogenous She2p-myc, She2-M2-myc and She2-M5A-myc, 5 µg of recombinant GST-Srp1p was bound to glutathione-Sepharose 4B and incubated with yeast extract for 2.5 h at 18°C. The matrix was recovered by centrifugation and washed four times with 500 µL of binding buffer. The bound proteins were eluted with preheated SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA and 0.02 % bromophenol blue). Eluted proteins were analyzed by western-blot.

RESULTS

Monomeric She2p interacts directly with the importin α Srp1p in order to enter the nucleus.

Previous work has shown that She2p transits through the nucleus (Kruse *et al.*, 2002; Du, 2008). Due to its size (26 kDa), which is below the nuclear pore diffusion limit (40 kDa in yeast), a She2p monomer may enter passively through the nuclear pores. However, since the functional structure of She2p is that of a homodimer of over 50 kDa (Niessing *et al.*, 2004), it is possible that active nuclear import is required. To determine if She2p shuttles between the nucleus and the cytoplasm actively or passively, a yeast genetic assay was used (Rhee *et al.*, 2000). In this assay, the protein of interest is fused to a chimera made of a modified LexA protein (mLexA), containing a disrupted nuclear localization signal (NLS), and of the GAL4 activation domain (mLexA-GAL4AD). If the protein of interest contains a NLS, it promotes the nuclear import of the mLexA-GAL4AD

chimera, which activates the expression of reporter genes (*LacZ* and *HIS3*). In this assay, a fusion of She2p with the mLexA-GAL4AD resulted in the activation of *LacZ*, while the mLexA-GAL4AD itself induced little β-galactosidase activity, suggesting that She2p promotes the nuclear import of the fusion protein (Figure 1A). It was possible that the She2p-mLexA-GAL4AD fusion protein could diffuse passively through the nuclear pores. To eliminate this possibility, the 70 kDa protein VirE2, from *Agrobacterium tumefaciens*, was added to the She2p-mLexA-GAL4AD in order to increase the size of the fusion protein (Rhee *et al.*, 2000). Even this large fusion protein was actively imported in the nucleus in a She2p-dependent manner (Figure 1A), suggesting that She2p contains an active NLS.

The main nuclear import pathway in yeast depends on the importin α Srp1p (Lange *et al.*, 2007). A previous large scale two-hybrid screen in yeast found that Srp1p interacts with She2p (Ito *et al.*, 2001), suggesting that nuclear import of She2p may depend on Srp1p. To explore this possibility, the interaction between She2p and Srp1p was tested in a yeast two-hybrid assay. However, no interaction between these two proteins could be detected in this assay (Figure 1B). While She2p forms a homodimer, and dimerization is important for its RNA-binding capacity (Niessing *et al.*, 2004), it is possible that Srp1p interacts only with the She2p monomer. Two mutants of She2p that have been shown to disrupt She2p dimerization, She2p-M1 and She2p-M2, containing the mutations Cys68→Tyr and Ser120→Tyr respectively, were generated (Niessing et al. 2004; and data not shown). When these monomeric mutants were tested in the yeast two-hybrid assay, both interacted strongly with Srp1p (Figure 1B), suggesting that it is indeed the She2p

monomer which interacts with Srp1p. To confirm these results, recombinant Srp1p, GST-She2p and GST-She2p-M2 were purified from bacteria and the direct interaction between Srp1p and the She2p variants was tested by GST pull-down. As shown in Figure 1C, while the GST-She2 protein was unable to pull-down Srp1p, GST-She2p-M2 and Srp1p did bind in this assay, suggesting a direct interaction between Srp1p and a She2p monomer.

The observation that only monomeric She2p interacted with Srp1p raised the possibility that a fraction of endogenous yeast She2p may be able to interact with Srp1p. To investigate this question, wild-type She2p and mutant M2 tagged with 9xMyc were expressed at endogenous levels in a *she2* yeast strain. Recombinant GST-Srp1p was used to pull-down the She2p-myc variants from yeast protein extracts. Interestingly, using this GST pull-down assay, both wild-type She2p-myc and She2p-M2-myc from yeast extracts interacted with GST-Srp1p at similar levels (Figure 1D). While one would have expected that more She2p-M2-myc than WT She2p-myc would be pulled-down by GST-Srp1p, equal amount of both proteins were repeatedly pulled-down. This is possibly due to the saturation of GST-Srp1p by the numerous NLS-containing proteins in the extract, so that only a small but equal amount of She2p WT and M2 mutant may be pulled-down by GST-Srp1p. Nevertheless, these results suggest that a significant fraction of She2p-myc can interact with Srp1p *in vivo*.

Since the monomeric She2p interacts with Srp1p better than the She2p dimer, this raises the possibility that the monomeric protein may accumulate in the nucleus. The myc-tagged She2 wild-type, M1 and M2 proteins were expressed at endogenous levels in a *she2*

yeast strain and their intracellular distribution was determined by immunofluorescence. As shown in Figure 2A, while the wild-type She2p-myc was present in both cytoplasm and nucleus of yeast cells, She2p-M1-myc and She2p-M2-myc accumulated only in the nucleus. To determine if this nuclear accumulation of the monomeric She2p depends on Srp1p, She2p-M2-myc was expressed in a temperature sensitive mutant strain of *SRP1*, the *srp1-31* strain (Loeb *et al.*, 1995), and its distribution at permissive (25°C) and restrictive (37°C) temperatures was measured. While She2p-M2-myc was mostly nuclear in the *srp1-31* strain at 25°C, its nuclear import was impaired when the strain was shifted to non-permissive temperature for 2 hrs (Figure 2B). Altogether, these data suggest that the nuclear import of She2p depends on Srp1p.

A non-classical NLS promotes the nuclear import of She2p

As mentioned previously, no NLS had yet been identified in She2p. To map this NLS, we used the genetic nuclear import and yeast two-hybrid assays described above. Five different deletions were generated in She2p (She2p-H1 to H5, Figure 3A), fused to the mLexA-GAL4AD protein and tested in the nuclear import assay. As shown in Figure 3A, only the deletions containing the last 61 amino acids of She2p (She2p-H2 and H4) were able to activate the expression of *LacZ*. In the yeast two-hybrid assay using Srp1p as bait, only the deletion that contains the C-terminal end of She2p (She2p-H2) interacted with Srp1p (Figure 3B), confirming the results from the nuclear import assay. In order to define the She2p NLS more precisely, successive deletions of 16 amino acids were performed

from the C-terminal end of the She2p-M2 protein (She2p-Δ16 to Δ64) and tested for their interaction with Srp1p in the yeast two-hybrid assay. As shown in Figure 3C, while a deletion of the last 16 amino acids of She2p still interacted with Srp1p (She2-Δ16), deletion of the last 32 amino acids completely disrupted this interaction (mutant She2-Δ32), suggesting that part of the NLS lies between amino acids 214 and 230 of She2p.

Surprisingly, the amino acid sequence of this region of She2p is very poor in basic amino acids (arginine and lysine), which are commonly found in classical NLS sequences (Lange *et al.*, 2007). An alignment of amino acids 200 to 230 of She2p from *Saccharomyces sensu stricto* species showed the presence of only one highly conserved lysine (position 222) and no arginine (Figure 4A). To determine if this region of She2p can independently act as a NLS, a 30 amino acid peptide encompassing positions 200 to 230 of She2p was fused at the amino-terminal end of GFP (She2NLS-GFP) and the distribution of this fusion protein was determined by epifluorescence microscopy. As shown in Figure 4B, GFP alone was distributed in both the cytoplasm and the nucleus of yeast cells, as a She2p-GFP fusion protein (which shuttles between the nucleus and cytoplasm). However, She2NLS-GFP accumulated in the nucleus of yeasts. This She2NLS peptide was also found to interact with Srp1p in the yeast two hybrid assay (Figure 4C), suggesting that this peptide has the properties of a true NLS.

To better define the NLS in this peptide, the K_{222} residue and four other highly conserved amino acids surrounding this lysine (W_{215}, I_{219}, L_{220} and L_{223}) were mutated to alanine in She2p to generate the mutant She2-M5A (Figure 4A). When tested in the

nuclear import assay, the She2-M5A protein failed to promote the nuclear accumulation of the mLexA-GAL4AD fusion protein and to activate the expression of *LacZ* (Figure 5A). A myc-tagged She2-M5A protein was expressed at endogenous levels in a *she2* strain and its interaction with Srp1p was tested using a GST pull-down assay. Mutation of these five residues in the NLS disrupted the interaction between She2p and GST-Srp1p (Figure 5B). To eliminate the possibility that the M5A mutation favors the dimeric conformation of She2p over the monomeric form and therefore inhibits its interaction with Srp1p, the Ser120→Tyr mutation, which produces a She2p monomer, was introduced in She2p-M5A. The resulting protein, She2p-M2-M5A-myc, was still unable to bind GST-Srp1p (Figure 5B), suggesting that the M5A mutation disrupts the binding interface between She2p and Srp1p. Finally, the distribution of this mutant She2p was determined by immunofluorescence. As shown in Figure 5C, while the wild-type She2p-myc was found in both cytoplasm and nucleus, the She2-M5A-myc protein was excluded from the yeast nucleus. Altogether, these results show that the NLS of She2p is present between amino acids 214-222 at the C-terminal end of this protein, and that mutation of five specific residues in this NLS disrupts the nuclear targeting of She2p.

Nuclear import of She2p is required for proper localization of the ASH1 mRNA at the bud tip and for the sorting of Ash1p.

The generation of a mutant She2 protein that cannot be targeted to the nucleus opened the possibility of exploring the nuclear function of She2p in terms of *ASH1* mRNA

localization and Ash1p asymmetric distribution. First, the She2p-M5A mutant was tested to determine if the point mutations in the NLS had any effect on its RNA-binding capacity or its interaction with She3p. Expression levels of She2p-myc and She2p-M5A-myc in yeast were similar, as shown by Western blot (Figure 6A). Immunoprecipitation of the myc-tagged She2p variants, followed by RT-PCR amplification of the *ASH1* mRNA, showed no significant difference in *ASH1* mRNA binding between the wild-type and the M5A mutant *in vivo* (Figure 6B). Since only a small amount of *ASH1* mRNA is present in the nucleus, this result shows that even when She2p is excluded from the nucleus, it can still efficiently interact with this transcript in the cytoplasm. This was confirmed in a yeast three-hybrid assay that measures the interaction between She2p and the *ASH1* mRNA zipcodes (Long *et al.*, 2000; Olivier *et al.*, 2005). In this assay, one of the *ASH1* zipcode (the domain 1 of element E2B or D1), was used as bait for the interaction with She2p. No difference between wild-type She2 and She2-M5A proteins was observed in binding of this *ASH1* localization element (Figure 6C). Finally, to determine if this mutation cause any disruption in the binding of She2p with She3p, which bridges She2p to the myosin Myo4p, a yeast two-hybrid assay was used (Long *et al.*, 2000). In this assay, She2p-M5A showed no defect in its interaction with the C-terminal domain of She3p compared to wild-type She2p (Figure 6D).

To determine the effect of the nuclear exclusion of She2p on *ASH1* mRNA localization, myc-tagged She2p or She2p-M5A were expressed in a *she2* yeast strain and the localization of *ASH1* mRNA in these strains was visualized by fluorescent *in situ*

hybridization (FISH). As shown in Figure 7A, while the wild-type She2p promoted the localization of the *ASH1* mRNA at the bud tip of cells in anaphase, the She2p-M5A had a reduced efficiency in *ASH1* mRNA localization, as it accumulated in the whole bud of the cells. Indeed, while over 90% of late-anaphase cells expressing wild-type She2p had bud tip localization of the *ASH1* mRNA, this percentage dropped to below 10% in yeasts expressing She2p-M5A (Figure 7B). The effect of the mutation of the She2p NLS on the asymmetric distribution of the Ash1 protein and *HO* promoter activity was determined using the yeast strain K5547, in which the *ADE2* gene is under the control of the *HO* promoter and which contains a deletion of the *SHE2* gene (Jansen, 1996). In this strain, symmetric distribution of Ash1p leads to repression of the *ADE2* gene and absence of growth on plate lacking adenine (-Ade). If She2p function is restored, Ash1p accumulates in the daughter cell, so expression of the *ADE2* gene in the mother cell allows growth on -Ade plates. When transformed with a plasmid expressing the wild-type She2p, the K5547 strain grew on -Ade plates, while the same strain transformed with the empty vector did not grow (Figure 7C). When transformed with a plasmid expressing She2p-M5A, a slower growth on -Ade plates was observed compared to the strain expressing wild-type She2p, suggesting that the M5A mutation partially disrupt the asymmetric distribution of Ash1p. However, expression of a She2-M5A protein containing the SV40 NLS (She2p-M5A+SV40NLS), which restores the nuclear localization of She2p-M5A and maintains its interaction with *ASH1* mRNA *in vivo* (Figure 6B), resulted in the same growth on -Ade plates as the strain expressing wild-type She2p (Figure 7C). This suggests that it was the

defective nuclear import of She2p-M5A that disrupted the asymmetric localization of Ash1p. Altogether, these results show that the nuclear import of She2p is required for the localization of the *ASH1* mRNA at the bud tip and the sorting of the Ash1 protein to the daughter cell nucleus.

Nuclear import of She2p is essential for the recruitment of Loc1p and Puf6p to the ASH1 mRNA

The work presented so far shows that the nuclear import of She2p is important for its function in cytoplasmic mRNA localization, but the reason is not clear. Two other nuclear RNA-binding proteins are known to play a role in *ASH1* mRNA localization and Ash1p sorting: Puf6p and Loc1p. Puf6p is predominantly nucleolar, binds the localization element E3 in the 3'UTR of *ASH1* and is involved in the translational repression of this transcript (Gu *et al.*, 2004; Deng *et al.*, 2008). Its deletion results in defects in both *ASH1* mRNA localization and Ash1p asymmetric distribution. Loc1p is also a nucleolar protein, and it binds the 3'UTR of the *ASH1* mRNA (Long *et al.*, 2001). Its deletion disrupts *ASH1* mRNA localization and recent data suggest that it is also involved in the translational regulation of this transcript (Long *et al.*, 2001; Komili *et al.*, 2007). Since the She2p-M5A mutant displayed phenotypes similar to the *PUF6* and *LOC1* deletions (whole bud accumulation of *ASH1* mRNA, partial asymmetric distribution of Ash1p), we raised the hypothesis that nuclear She2p might be involved in the binding of Puf6p and Loc1p to the *ASH1* mRNA. Hence, the absence of She2p in the nucleus might disrupt the recruitment of

Puf6p and Loc1p to the *ASH1* mRNA, leading to phenotypes similar to *PUF6* and *LOC1* knockouts.

To explore this possibility, She2p and She2p-M5A were expressed at endogenous levels in strains deleted of the endogenous *SHE2* gene and containing a TAP-tag integration at the C-terminus of either *PUF6* or *LOC1* open reading frames. Expression of She2p-M5A had no effect on Loc1p-TAP and Puf6p-TAP expression levels (data not shown). The interaction between the *ASH1* mRNA and the Puf6-TAP and Loc1-TAP proteins *in vivo* was determined by RNA immunoprecipitation (RIP). In this assay, the RNP complexes were cross-linked *in vivo* with formaldehyde, followed by immunoprecipitation of the Puf6-TAP and Loc1-TAP proteins. After decrosslinking, the associated mRNAs were purified, reverse transcribed and the *ASH1* cDNA was detected by PCR amplification. In a yeast strain expressing the wild-type She2p, both Puf6p-TAP and Loc1p-TAP interacted with the *ASH1* mRNA *in vivo* (Figure 8A), but not with *ACT1* mRNA (Figure 8B). However, when She2p-M5A was expressed, no *ASH1* mRNA was found associated with Puf6p-TAP and very little to Loc1p-TAP (Figure 8A), suggesting that the presence of She2p in the nucleus is essential for Puf6p and Loc1p to bind the *ASH1* mRNA *in vivo*.

These results raise the possibility that She2p interacts with Puf6p and Loc1p, and recruit them to the *ASH1* mRNA. Therefore, interaction between She2p, Puf6p and Loc1p *in vivo* was explored using co-immunoprecipitation. Pull-down of both TAP-tagged Puf6p and Loc1p resulted in the co-immunoprecipitation of She2p-myc (Figure 8C), suggesting an interaction between these factors *in vivo*. This interaction was independent of RNA

since treatment of yeast extracts with RNAse A prior to immunoprecipitation still resulted in an efficient pull-down of She2p-myc by both Puf6p-TAP and Loc1p-TAP (Figure 8C). Finally, to determine if the presence of She2p in the nucleus is important for its interaction with Puf6p and Loc1p, the co-immunoprecipitation was repeated with She2p-M5A-myc mutant. In this experiment, cross-linking of protein complexes with formaldehyde before immunoprecipitation was performed in order to avoid reconstitution of complexes in the yeast extract after breaking the cells. As shown in Figure 8D, a clear reduction in the amount of She2p-M5A-myc that co-immunoprecipitated with either Puf6p-TAP or Loc1p-TAP was observed compared to wild type She2p-myc. Wild-type levels of interaction were recovered when the SV40 NLS was fused to the She2p-M5A protein (Figure 8D), suggesting that nuclear import of She2p is required for its interaction with Loc1p and Puf6p. Altogether, these results suggest that nuclear import of She2p is required for its interaction with Puf6p and Loc1p, and for their recruitment to the *ASH1* mRNA.

DISCUSSION

A non-classical NLS promotes the nuclear import of She2p by binding importin α

In this work, we show that She2p is actively imported into the nucleus via its interaction with the importin α Srp1p. Our data suggest that She2p is not imported as a native dimer, which is the conformation that binds RNA (Niessing *et al.*, 2004), since the She2p dimer was unable to interact with Srp1p *in vitro*. She2p from yeast extracts was able to interact with Srp1p, suggesting that a fraction of She2p *in vivo* may adopt a conformation

that is different from the native dimer. However, it is still unclear if this population of She2p corresponds to monomers. A possibility is that this population of She2p may contain post-translational modifications. Phosphorylation is known to regulate the nuclear import of proteins, like Gln3p in yeast for instance (Carvalho *et al.*, 2001), and She2p has been reported to be a phosphoprotein *in vivo* (Gonsalvez *et al.*, 2003). Once in the nucleus, She2p adopts a dimeric conformation since it can bind RNA. Intriguingly, wild-type She2p-myc was not excluded from the nucleus of the *srp1-31* strain at non-permissive temperature (data not shown). This is possibly due to the rapid shutdown of transcription when this strain is shifted at 37ºC (Liu *et al.*, 1999). Since She2p nuclear export depends on the nuclear export of newly synthesized mRNAs (Kruse *et al.*, 2002), the shutdown of transcription would explain the absence of nuclear depletion of wild-type She2p in the *srp1-31* strain.

Using the interaction between Srp1p and She2p, a 30 amino acid sequence with NLS properties was identified. This NLS promotes the nuclear import of GFP and interacts with Srp1p. Interestingly, its sequence is very divergent from classical monopartite and bi-partite NLS since it contains only one lysine and is rich in hydrophobic residues. To our knowledge, all the currently reported NLS that bind directly importin α contain at least two essential basic amino acids (Chen *et al.*, 2005; Lange *et al.*, 2007), suggesting that the repertoire of nuclear localization signals may be larger than suggested. Mutation of five conserved residues in this NLS disrupted the nuclear targeting of She2p and its interaction with Srp1p, confirming its role as a nuclear localization sequence. The defective *ASH1*

mRNA localization and poor asymmetric sorting of Ash1p seen in the She2p-M5A mutant strain seem to result from the nuclear exclusion of this protein and not from secondary effects of this mutation. Indeed, the RNA-binding capacity of the NLS-mutated She2p was similar to wild type She2p, as was its interaction with She3p. More important, adding an heterologous classical NLS (like the SV40 NLS) to the She2p-M5A completely restored the function of this protein *in vivo*.

Nuclear She2p couples mRNA localization and translational repression

Disrupting the nuclear import of She2p affects the localization of the *ASH1* transcript and the asymmetric distribution of Ash1p. We provide evidence that this localization defect is linked to the disrupted interaction between Puf6p and Loc1p with the *ASH1* mRNA since: 1) She2p interacts *in vivo* with both Puf6p and Loc1p; 2) the presence of She2p in the nucleus is important for this interaction; 3) exclusion of She2p from the nucleus disrupts the binding of Loc1p and Puf6p to the *ASH1* mRNA, and 4) nuclear exclusion of She2p phenocopies the knockouts of *LOC1* and *PUF6* in term of *ASH1* mRNA localization and Ash1p distribution.

Altogether, these data suggest a direct coupling between the mRNA transport and translational control machineries. A role of nuclear She2p in translational control is indeed supported by recent data from the Jansen lab, which showed that nuclear exclusion of She2p accelerates Ash1p synthesis (Du, 2008). Intriguingly, Du et al. did not report any defect in *ASH1* mRNA localization when She2p was excluded from the nucleus, unlike

what we observed (see Figure 7). They used a She2 protein fused to the Myo4p-binding domain of She3p, which resulted in a fusion protein that remained anchored on the actin cytoskeleton via its binding to Myo4p and is excluded from the nucleus. However, it is possible that such tight association of She2p, and of the *ASH1* mRNA, to the localization machinery and to the actin cytoskeleton suppresses the localization defects caused by the nuclear exclusion of She2p.

Since She2p binds several localization elements within the coding sequence of the *ASH1* mRNA (Chartrand, 2002), this coupling may reduce the possibility that elongating ribosomes could displace She2p from this transcript. Such coupling is supported by the finding that She2p co-immunoprecipitates with Puf6p and Loc1p, suggesting an interaction between these proteins *in vivo*. The mechanism by which She2p promotes the recruitment of Loc1p and Puf6p on the *ASH1* mRNA *in vivo* is not known, since all three proteins can bind the 3'UTR of this transcript independently *in vitro* (Bohl *et al.*, 2000; Long *et al.*, 2001; Gu *et al.*, 2004). One possibility is that, being in the nucleolus, Puf6p and Loc1p are spatially restricted from polyA$^+$ mRNAs. Since She2p has been recently shown to transit through the nucleolus (Du, 2008), it may either bring the *ASH1* mRNA in the nucleolus, where Puf6p and Loc1p can bind this transcript, or She2p may recruit these two factors in the nucleolus and bring them to the *ASH1* mRNA in the nucleoplasm.

Roles of nuclear proteins in cytoplasmic mRNA localization

The importance of nuclear events in cytoplasmic mRNA localization is a well described phenomenon. Several RNA-binding proteins implicated in cytoplasmic mRNA localization are known to be exclusive residents of the nucleus or to shuttle between the cytoplasm and the nucleus (Farina and Singer, 2002). Reports from several model systems have shown that mRNA processing in the nucleus affects its cytoplasmic fate. For instance, proper splicing of the *oskar* mRNA is required for its localization at the posterior pole of the *Drosophila* embryo (Hachet and Ephrussi, 2004). In this case, members of the exon junction complex (EJC), like Y14-Mago and eIFIIIA, are assembled on the *oskar* mRNA in the nucleus and are involved in the cytoplasmic localization of this transcript. In *Xenopus* oocyte, the nucleo-cytoplasmic shuttling proteins hnRNP I and Vg1RBP/Vera initiate a localization complex with the Vg1 mRNA in the nucleus (Cote *et al.*, 1999; Kress *et al.*, 2004). Remodeling of this ribonucleoprotein complex has been shown to occur after its nuclear export (Kress *et al.*, 2004). In fibroblasts, both ZBP1 and ZBP2/KSRP proteins can bind the β-actin mRNA in the nucleus (Gu *et al.*, 2002; Oleynikov and Singer, 2003). A handover mechanism has been proposed where the predominantly nuclear ZBP2 binds the nascent β-actin transcript and facilitates the subsequent recruitment of ZBP1, the factor involved in the cytoplasmic localization of the β-actin mRNA (Pan *et al.*, 2007).

Our study reveals another function for the nucleo-cytoplasmic shuttling of RNA-binding proteins involved in mRNA localization. By promoting the recruitment of Puf6p and Loc1p on the nuclear *ASH1* mRNA, She2p initiates the translational repression of the

localized mRNA before its export in the cytoplasm and prevents premature translation of this transcript. Coupling mRNA localization and translational control constitutes an efficient way to ensure that the translation of transcripts targeted for localization will be properly regulated. This raises the possibility that other transcripts that are localized at the bud tip of yeasts may also be translationally repressed by Puf6p and/or Loc1p via their recruitment with She2p.

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FIGURES

Figure 1: Monomeric She2p interacts directly with the importin α Srp1p and is actively imported into the nucleus. (**A**) Yeast nuclear import assay. Plasmids expressing the chimeric protein mLexA/GAL4AD alone, or in fusion with She2p, VirE2, or She2p+VirE2 were transformed in L40 yeast strain, and their nuclear import efficiency was determined by measuring β-galactosidase activity. (**B**) Yeast two-hybrid assay. Srp1p was used as bait, and co-expressed with She2 WT or the mutants She2-M1 and She2-M2 in pJ69-4A strain. Protein interactions were determined by measuring β-galactosidase activity. (**C**) GST pull-down assay to detect interaction between Srp1p and She2p *in vitro*. Recombinant proteins GST, Srp1p, GST-She2p, GST-She2-M2 expressed and purified from bacteria were loaded on gel for input levels. Equal amount of Srp1p were loaded on glutathion beads bound with GST, GST-She2p or GST-She2-M2. After washes, proteins on the beads were eluted by boiling, loaded on SDS-PAGE gel and detected by Coomassie Blue staining. (**D**) GST pull-down assay to detect interaction between Srp1p and She2p-myc or She2p-M2-myc from yeast extracts. Input: total She2p-myc or She2p-M2-myc in yeast extracts. GST: She2p from yeast extracts interacting with GST alone. GST-Srp1p: She2p from yeast extracts interacting with GST-Srp1p.

Figure 2: Nuclear import of monomeric She2p depends on Srp1p. (**A**) Immunofluorescence detection of wild-type She2p-myc (WT) and mutants She2p-M1-myc (*SHE2-M1*) and She2p-M2-myc (*SHE2-M2*) in yeast cells. Percentage of cells with the displayed phenotype is indicated. Scale = 2μm. (**B**) Immunofluorescence on She2p-M2-myc in *srp1-31* strain at permissive (top panels) or restrictive (bottom panels) temperature. Percentage of cells with the displayed phenotype is indicated. Scale = 2μm.

Figure 3: Identification of a NLS at the C-terminal end of She2p. (**A**) Yeast nuclear import assay. Diagram of the She2p deletions used in this assay (H1 to H5). She2p deletions were fused to the LexA/GAL4AD chimera and their nuclear import efficiency was determined by measuring β-galactosidase activity. Vector: plasmid expressing LexA/GAL4AD chimera alone. (**B**) Yeast two-hybrid assay between Srp1p and She2p-M2 deletions. Diagram of the She2p-M2 deletions used in this assay (H1 to H3). Interaction between Srp1p and a She2p-M2 deletion was determined by measuring β-galactosidase activity. AD: plasmid expressing Gal4 activation domain only. (**C**) Yeast two-hybrid assay between Srp1p and She2p-M2 deletions at its C-terminus. Diagram of the She2p-M2 deletions used in this assay (Δ16 to Δ64). Interaction between Srp1p and a She2p-M2 deletion was determined by measuring β-galactosidase activity. AD: plasmid expressing Gal4 activation domain only.

Figure 4: A non-classical NLS mediates nuclear import of She2p. (**A**) Sequence alignment of a 30 amino acid peptide containing the NLS of She2p. Sequences from She2p homologues from *S. cerevisiae* (Scer), *S. paradoxus* (Spar), *S. mikatae* (Smik), *S. bayanus* (Sbay), *S. kuderii* (Skud) and *S. casei* (Scas) are shown. Amino acids mutated are underlined in grey. **(B)** Epifluorescence microscopy on yeast cells expressing She2-GFP (top panels), GFP alone (bottom panels) or GFP fused to a 30 amino acid NLS of She2p (middle panels). Scale = 2μm. (**C**) Srp1p interacts with the 30 amino acid NLS peptide from She2p in a yeast two hybrid assay. Interaction between Srp1p and She2p-M2 protein or the She2NLS peptide was determined by measuring β-galactosidase activity. AD: plasmid expressing Gal4 activation domain only.

Figure 5: Mutations in NLS of She2p impair interaction with Srp1p and nuclear import of this factor. (**A**) Yeast nuclear import assay. She2p WT or NLS mutant (She2p-M5A) were fused to the LexA/GAL4AD chimera and their nuclear import efficiency was determined by measuring β-galactosidase activity. Vector: plasmid expressing LexA/GAL4AD chimera alone. (**B**) GST pull-down assay to detect interaction between Srp1p and She2p-myc, She2p-M2-myc, She2p-M5A-myc or She2p-M2-M5A-myc from yeast extracts. Input: total She2p-myc, She2p-M2-myc, She2p-M5A-myc or She2p-M2-M5A-myc in yeast extracts. GST: She2p from yeast extracts interacting with GST alone. GST-Srp1p: She2p from yeast extracts interacting with GST-Srp1p. (**C**) Immunofluorescence on yeast cells expressing wild-type She2p-myc (top panels) or She2p-M5A-myc (bottom panels).

White arrows point to low She2p-myc level in the nuclei. Numbers reflect extent of the phenotype observed. Scale = 2μm.

Figure 6: The NLS-mutated She2 protein is as functional as the wild-type She2p. (**A**) Expression levels of wild-type She2p-myc and She2p-M5A-myc proteins determined by Western blot. Expression levels were normalized with Pgk1 protein. (**B**) Immunoprecipitation of She2p-myc wild-type (WT), M5A mutant (M5A) or M5A mutant+SV40NLS, followed by reverse transcription and PCR amplification of endogenous *ASH1* mRNA. Input: *ASH1* mRNA from total yeast extract. IP+RT-PCR: *ASH1* mRNA from immunoprecipitate, reverse transcribed and amplified by PCR. IP + PCR (-RT): *ASH1* mRNA from immunoprecipitate, amplified by PCR without reverse transcriptase. (**C**) Three-hybrid assay using yeast strain YBZ1 *she2* transformed with plasmid expressing either wild-type (She2WT) or NLS mutant (She2M5A) of She2p. This strain also expressed the GAL4 activation domain fused with the C-terminal domain of She3p, along with a plasmid expressing a chimeric RNA containing the localization E2B-D1 fused to the MS2 stem-loop (D1). Controls: empty vector (pIIIA/MS2-2) and an inactive fragment of the localization element E2B (D2) (Olivier *et al.*, 2005). (**D**) Interaction between the C-terminal domain of She3p and wild-type (She2WT) or NLS mutated (She2M5A) She2 proteins in a yeast two hybrid assay. AD: plasmid expressing Gal4 activation domain only.

Figure 7: Nuclear import of She2p is required for proper *ASH1* mRNA localization and Ash1p sorting. (**A**) Fluorescent in situ hybridization on *ASH1* mRNA in yeast cells expressing either wild-type She2p-myc (top panels) or She2p-M5A-myc (bottom panels). Scale = 2μm. (**B**) Scores on mRNA localization phenotypes from (**A**). (**C**) Yeast genetic assay for Ash1p asymmetric distribution. Tenfold dilutions of exponentially growing K5547 (*HO-ADE2*, *she2*) transformed either with the empty YCPlac22 plasmid (vector), YCP22-She2-myc (*SHE2*), YCP22-She2-M5A+SV40NLS or YCP22-She2-M5A-myc were spotted on plates lacking tryptophan (-Trp) or lacking tryptophan and adenine (-Trp – Ade), and incubated at 30ºC.

Figure 8: Nuclear She2p recruits Puf6p and Loc1p on the *ASH1* mRNA. (**A**) Immunoprecipitation of Loc1p-TAP and Puf6p-TAP from strains expressing either She2p-myc wild-type (WT) or She2-M5A-myc (M5A), followed by RNA purification and RT-PCR amplification of *ASH1* mRNA. Input: *ASH1* mRNA from total yeast extract. IP+RT-PCR: *ASH1* mRNA from immunoprecipitate, reverse transcribed and amplified by PCR. IP + PCR (-RT): *ASH1* mRNA from immunoprecipitate, amplified by PCR without reverse transcriptase. Beads: mRNA from yeast extract incubated with Protein A-Sepharose beads, without antibody. (**B**) Detection of *ACT1* mRNA from immunoprecipitated Puf6p-TAP and Loc1p-TAP. Input: *ACT1* mRNA from total yeast extract. IP+RT-PCR: *ACT1* mRNA from immunoprecipitate, reverse transcribed and amplified by PCR. These results are representative of three independent experiments. (**C**) She2p-myc co-

immunoprecipitates with Loc1p-TAP and Puf6p-TAP. No TAP: yeast strain without TAP-tagged Loc1p or Puf6p. Input: She2p-myc from total yeast extract. IP: immunoprecipitated She2p-myc. IP+RNAse: immunoprecipitated She2p-myc after treatment with RNAse A. The asterisk corresponds to the IgG heavy chain. (**D**) Nuclear import of She2p is required for its interaction with Loc1p and Puf6p. Immunoprecipitation of Loc1p-TAP and Puf6p-TAP from strains expressing either wild-type She2p-myc (WT), She2-M5A-myc (M5A) or She2-M5A+SV40NLS (M5A+SV40NLS). Input: wild-type She2p-myc from total yeast extract. IP: immunoprecipitation. The asterisk corresponds to the IgG heavy chain.

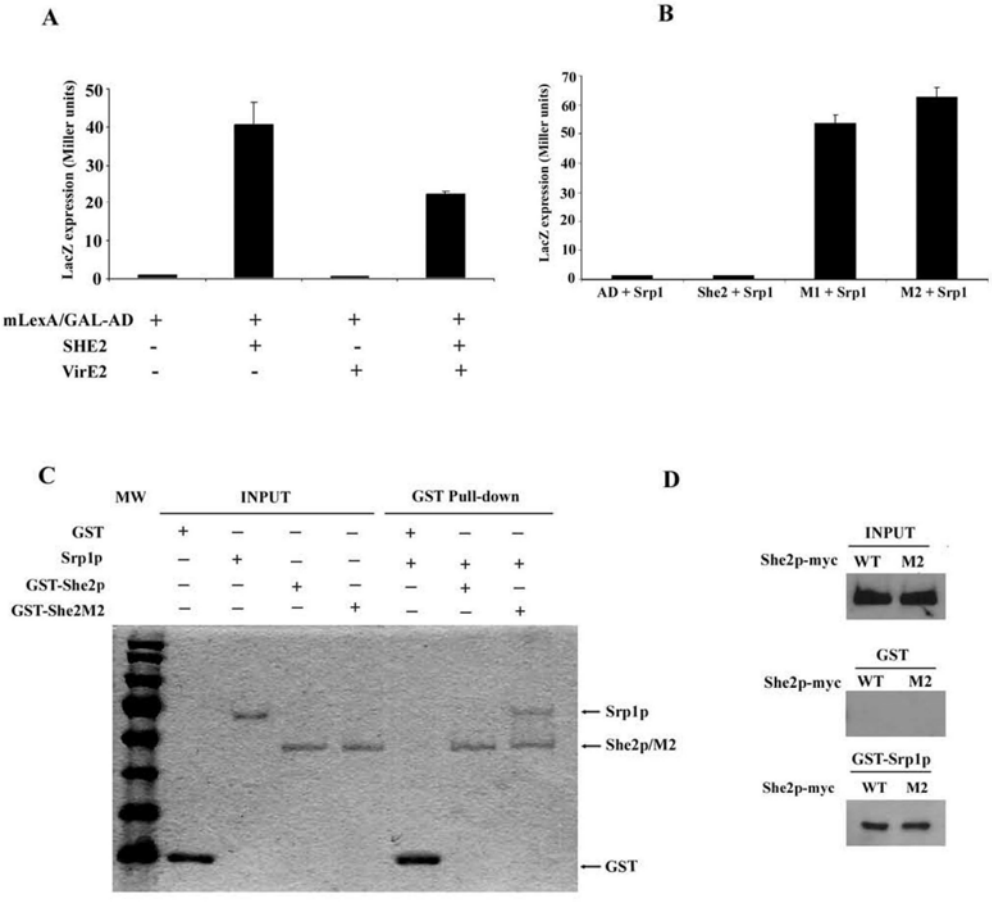

Figure 1: Monomeric She2p interacts directly with the importin α Srp1p and is actively imported into the nucleus.

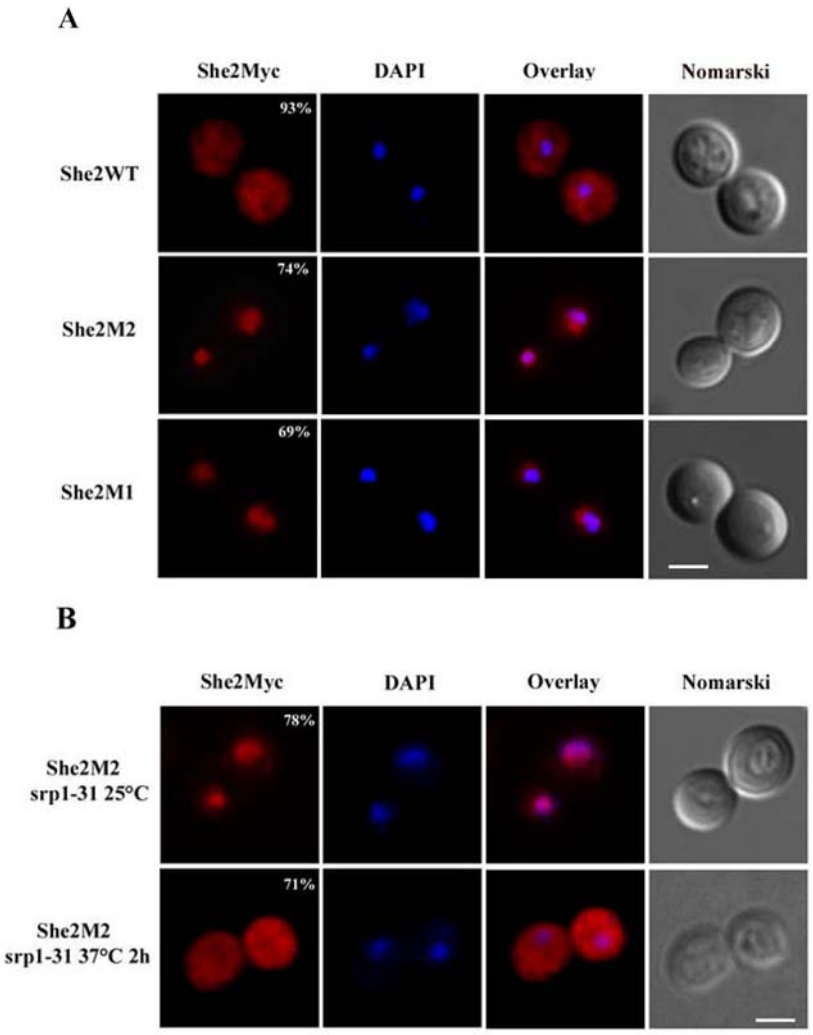

Figure 2: Nuclear import of monomeric She2p depends on Srp1p.

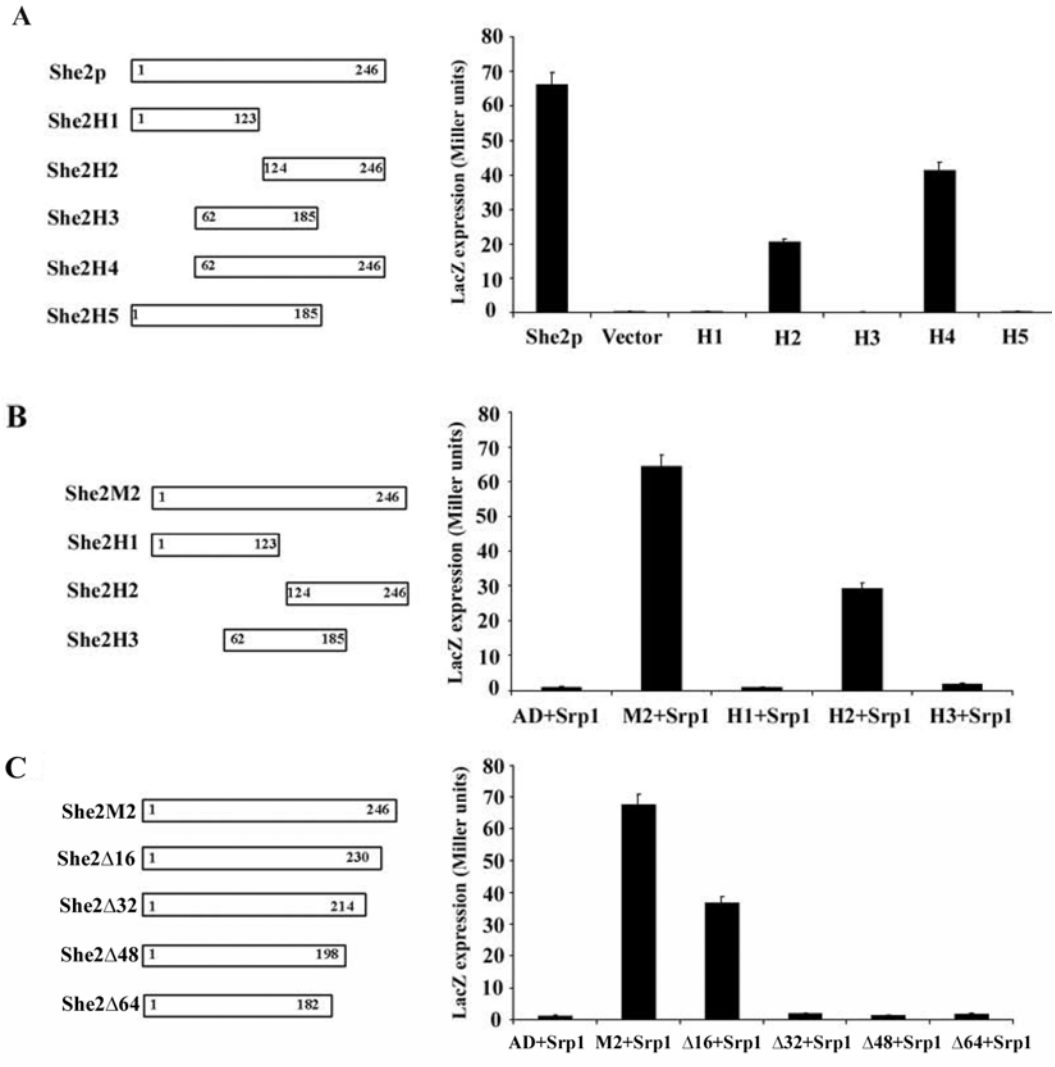

Figure 3: Identification of a NLS at the C-terminal end of She2p.

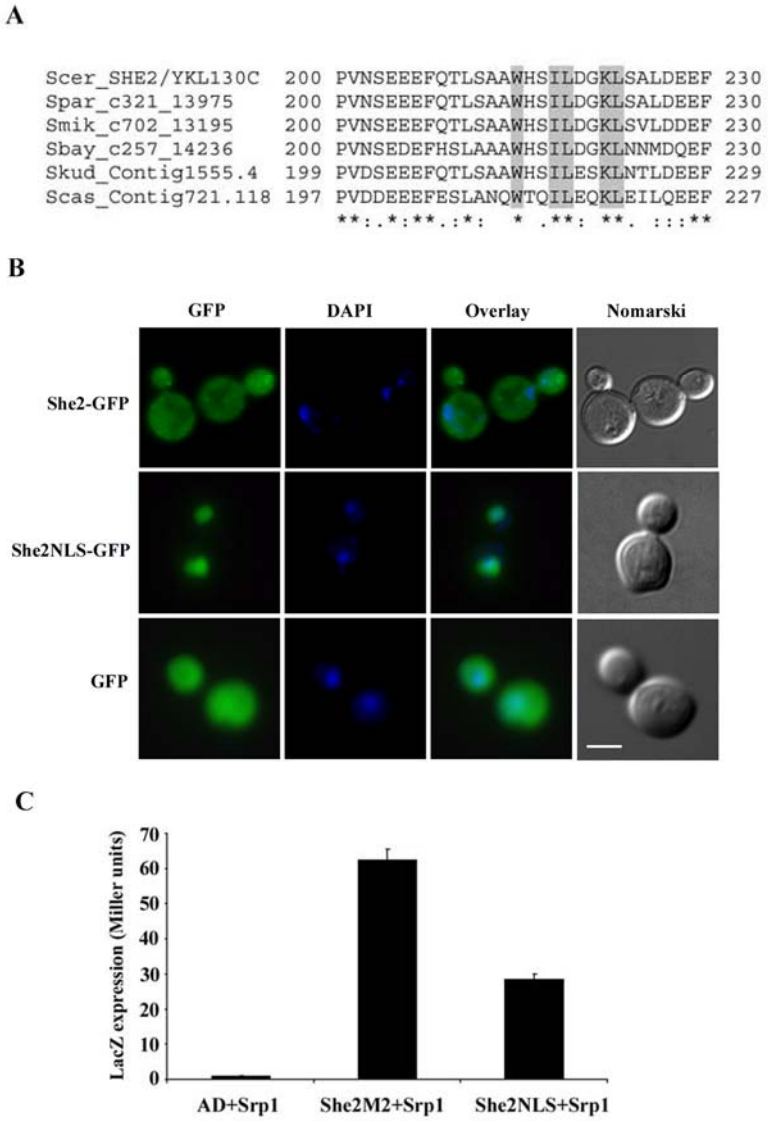

Figure 4: A non-classical NLS mediates nuclear import of She2p.

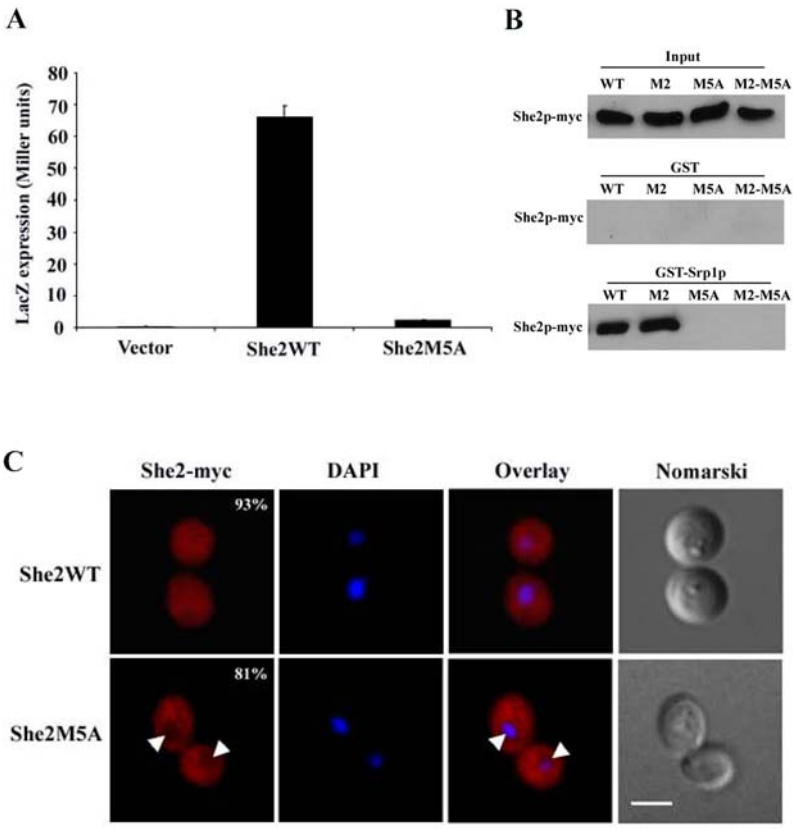

Figure 5: Mutations in NLS of She2p impair interaction with Srp1p and nuclear import of this factor.

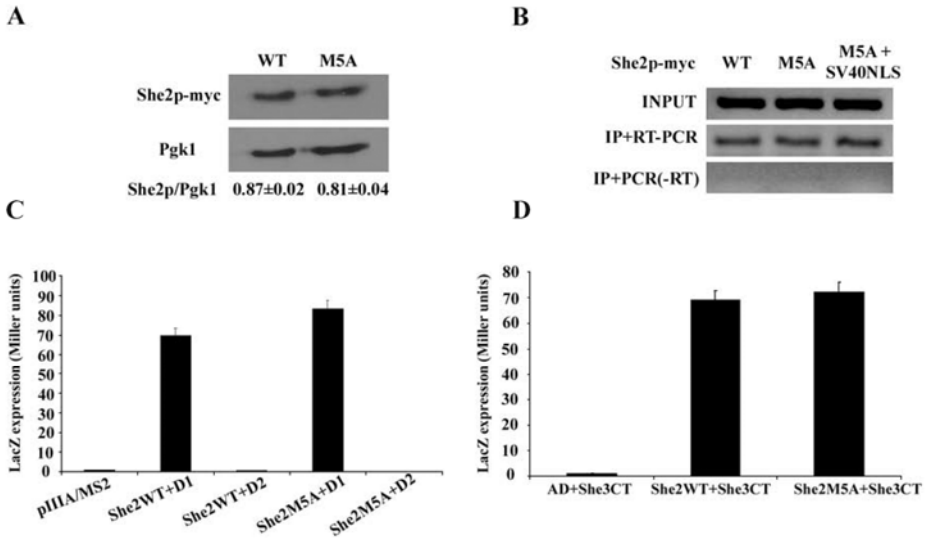

Figure 6: The NLS-mutated She2 protein is as functional as the wild-type She2p.

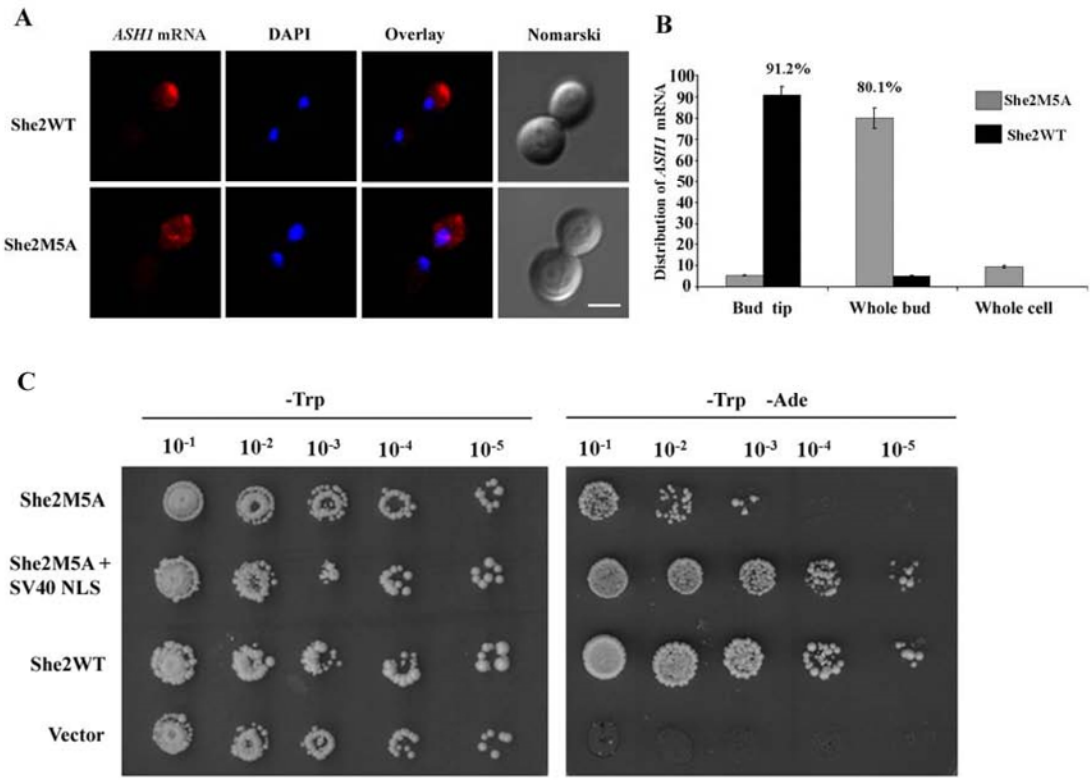

Figure 7: Nuclear import of She2p is required for proper *ASH1* mRNA localization and Ash1p sorting.

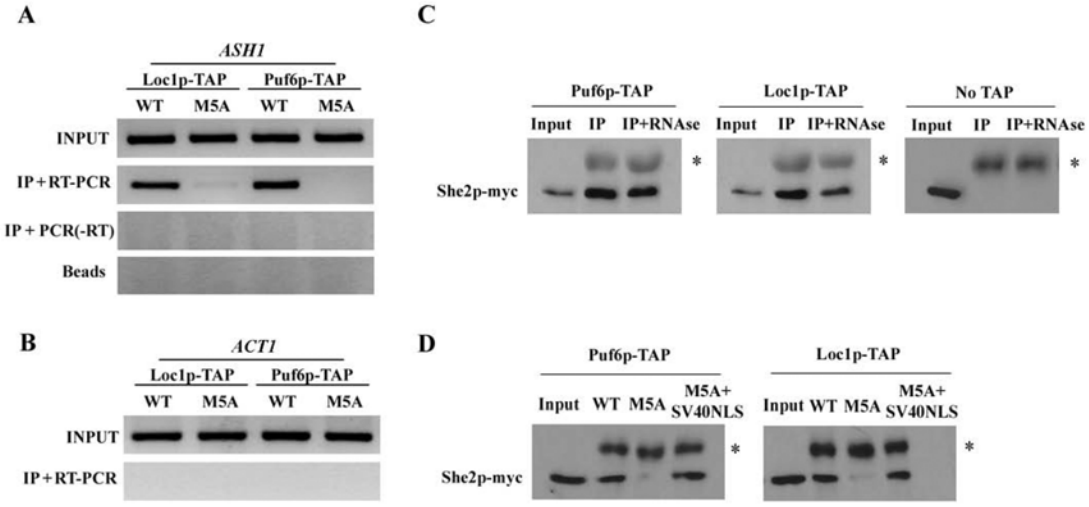

Figure 8: Nuclear She2p recruits Puf6p and Loc1p on the *ASH1* mRNA.

SUPPLEMENTARY DATA

MATERIAL AND METHODS

Yeast three-hybrid assay

The strain YBZ1 was used in the three-hybrid assay (Hook et al. 2005). This strain was transformed with the appropriate plasmids and grown in synthetic media lacking uracil and leucine. Dilution assays were made on plates lacking uracil, leucine and histidine, with or without 3-amino-1,2,4-triazole (3-AT). The β-galactosidase expression level was measured quantitatively using O-nitrophenyl-β-D-galactopyranoside (ONPG) according to the protocol provided by Clontech. The reported β-galactosidase expression levels represent the average of at least three independent experiments.

Plasmid constructions.

All the mutants of She2p were generated by PCR using site-directed mutagenesis. She2p constructs used in the yeast one and two-hybrid assays were amplified by PCR from YCP22 plasmids containing the SHE2 gene and cloned in the XmaI site of plasmid pNIAVirE2, and EcoRI/BamHI sites of plasmid pGADT7. The SV40-NLS (PKKKRKV) was inserted in the C terminus of She2p in a BamH1 site in the middle of the 9xmyc tags of the YCP22-She2-myc plasmid. She2-GFP and She2NLS-GFP were generated by introducing the She2 and She2NLS cDNA into BamHI /NotI sites of pG14-MS2/GFP plasmid. A linker (AEAAAKEAAAKA) was introduced between She2p and GFP. GST-

She2WT, and the M1 and M2 mutants were generated by PCR from YCP22-She2-myc and cloned in the EcoRI/NotI sites of plasmid pGEX-6P-1; GST-Srp1 was generated by PCR and cloned in the BamHI/NotI site of plasmid pGEX-5X-3.

SUPPLEMENTARY TABLE 1: Yeast strains used in this study.

strain	genotype	source
K699	*Mat a, ura3-1, leu2-3, his3-11, trp1-1, ade2-1*	(Jansen 1996)
K699-*she2*	K699, *she2::KAN*	this study
K5547	*Mat a, his3, leu2, ade2, trp1, ura3, HO-ADE2 HO-CAN1 she2::URA3*	(Jansen 1996)
L5850	*Mat, leu2-3,112, ura3-1, ade2-1, his3-11,15, can1-100, trp1-63, srp1-31*	(Loeb et al. 1995)
L40	*MATa, trp1, leu2, his3, LYS2::lexA-HIS3, URA3::lexA-LacZ*	(Hollenberg et al. 1995)
YBZ1	*MATa, ura3-52, leu2-3,112, his3-200, trp1-1, ade2, LYS2:(LexAop)-lacZ, LexA-MS2-MS2 coat (N55K)*	(Hook et al. 2005)
YCO1	YBZ1 *she2::KAN*	(Olivier et al. 2005)
pJ69-4A	*Mata, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ gal80Δ, LYS2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ*	(James et al. 1996)
BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Open Biosystems
LOC1-TAP	BY4741 *LOC1-TAP::HIS3*	Open Biosystems
LOC1-TAP *she2*	BY4741 *LOC1-TAP::HIS3, she2::KAN*	this study

PUF6-TAP	BY4741 *PUF6-TAP::HIS3*	Open Biosystems
PUF6-TAP *she2*	BY4741 *PUF6-TAP::HIS3, she2::KAN*	this study

SUPPLEMENTARY TABLE 2: Plasmids used in this study.

Plasmid	Features	Source
pAF20	pGBDU-C1 with *SRP1*	(Shirley 2002)
C3319	YEPlac181 with *ASH1* under endogenous promoter	(Jansen 1996)
pGEX-6P1	Vector for GST fusion proteins expression	Amersham
YCPlac22	Single copy yeast vector with *TRP1* selectable marker	(Gietz 1988)
YIPlac128	Yeast integrative vector with LEU2 selectable marker	(Gietz 1988)
pNIA	Yeast 2μ, *TPR1* vector expressing mLexA-Gal4 AD fusion protein	(Rhee et al. 2000)
pNIAVirE2	Yeast 2μ, *TPR1* vector expressing mLexA-Gal4 AD-VirE2 fusion protein	(Rhee et al. 2000)
pGADT7	Yeast two-hybrid vector expressing protein fused to the Gal4p activation domain	Amersham
pG14	Yeast 2μ, *LEU2* vector for protein expression from *GPD* promoter	J. Warner
pGEX-SHE2	pGEX-6P1 vector for expression of GST-She2	this study
pGEX-SHE2-	pGEX-6P1 vector for expression of GST-She2	this study

M2	mutant M2	
pGEX-SRP1	pGEX-6P1 vector for expression of GST-Srp1	this study
YCP22-SHE2-MYC	YCPlac22 vector for expression of She2p-myc from endogenous promoter	(Long et al. 2000)
YCP22-SHE2-M1-MYC	YCPlac22 vector for expression of myc-tagged She2p-M1 mutant	this study
YCP22-SHE2-M2-MYC	YCPlac22 vector for expression of myc-tagged She2p-M2 mutant	this study
YCP22-SHE2-M5A-MYC	YCPlac22 vector for expression of myc-tagged She2p-M5A mutant	this study
YCP22-SHE2-M2-M5A-MYC	YCPlac22 vector for expression of myc-tagged She2p-M2-M5A mutant	this study
YCP22-SHE2-M5A-MYC+SV40NLS	YCPlac22 vector for expression of myc-tagged She2p-M5A mutant with the SV40 NLS	this study
pNIA-SHE2	pNIA vector fused with wild-type *SHE2*	this study
pNIAVirE2-SHE2	pNIAVirE2 vector fused with wild-type *SHE2*	this study
pNIAVirE2-SHE2 H1	pNIAVirE2 vector fused with amino acids 1 to 123 of She2p	this study
pNIAVirE2-SHE2 H2	pNIAVirE2 vector fused with amino acids 124 to 246 of She2p	this study
pNIAVirE2-SHE2 H3	pNIAVirE2 vector fused with amino acids 62 to 185 of She2p	this study
pNIAVirE2-SHE2 H4	pNIAVirE2 vector fused with amino acids 62 to 246 of She2p	this study
pNIAVirE2-SHE2 H5	pNIAVirE2 vector fused with amino acids 1 to 185 of She2p	this study
pGAD-SHE2	pGADT7 vector expressing wild-type She2p fused to Gal4p activation domain	this study

pGAD-SHE2-M2	pGADT7 vector expressing the mutant She2p-M2 fused to Gal4p activation domain	this study
pGAD-SHE2-H1	pGADT7 vector expressing the amino acids 1 to 123 of She2p fused to Gal4p activation domain	this study
pGAD-SHE2-H2	pGADT7 vector expressing the amino acids 124 to 246 of She2p fused to Gal4p activation domain	this study
pGAD-SHE2-H3	pGADT7 vector expressing the amino acids 62 to 185 of She2p fused to Gal4p activation domain	this study
pGAD-SHE2-Δ16	pGADT7 vector expressing the amino acids 1 to 230 of She2p fused to Gal4p activation domain	this study
pGAD-SHE2-Δ32	pGADT7 vector expressing the amino acids 1 to 214 of She2p fused to Gal4p activation domain	this study
pGAD-SHE2-Δ48	pGADT7 vector expressing the amino acids 1 to 198 of She2p fused to Gal4p activation domain	this study
pGAD-SHE2-Δ64	pGADT7 vector expressing the amino acids 1 to 182 of She2p fused to Gal4p activation domain	this study
pIIIA/MS2-2	Yeast three-hybrid vector for the expression of MS2-RNA fusion	M. Wickens
pIIIA-D1	pIIIA/MS2 plasmid expressing the MS2-D1 fusion RNA	(Olivier et al. 2005)

pIIIA-D2	pIIIA/MS2 plasmid expressing the MS2-D2 fusion RNA	(Olivier et al. 2005)
pG14-She2-GFP	pG14 plasmid expressing the She2-GFP fusion protein	this study
pG14-GFP	pG14 plasmid expressing the GFP protein	this study
pG14-She2NLS-GFP	pG14 plasmid expressing GFP fused to the NLS of She2p	this study

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Chapter III: Cotranscriptional recruitment of She2p by RNA pol II elongation factor Spt4-Spt5 promotes mRNA localization to yeast bud

Manuscript to be submitted to *Genes & Development*

Cotranscriptional recruitment of She2p by RNA pol II elongation factor Spt4-Spt5 promotes mRNA localization to yeast bud

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Running title: Spt5 links transcription to mRNA localization

ABSTRACT

Nuclear steps in mRNA biogenesis are coupled with transcription. How the transcription machinery directly affects the cytoplasmic fate of a transcript, like its intracellular localization, is still unclear. In yeast, the RNA-binding protein She2p binds and targets several mRNAs for localization at the bud. One of these transcripts, the *ASH1* mRNA, is localized at the bud tip in late anaphase cells. Here, we report that She2p is recruited cotranscriptionally to the *ASH1* gene, via interaction with the nascent mRNA. She2p interacts *in vivo* with the elongating form of RNA polymerase II and this interaction occurs via the transcription elongation factor Spt4-Spt5. Mutations in either *SPT4* or *SPT5* reduce the cotranscriptional recruitment of She2p on the *ASH1* gene, disrupt the proper localization of *ASH1* mRNA at the bud tip and Ash1p sorting to the daughter cell nucleus. We propose that She2p is recruited by the RNA pol II machinery prior to its transfer to nascent bud-localized mRNAs. Indeed, chromatin immunoprecipitation shows that She2p is associated with genes coding for both non-localized and bud-localized mRNAs, but this association is RNA-dependent only for the later group. This study reveals a mechanism for the cotranscriptional assembly of mRNP competent for localization in the cytoplasm.

INTRODUCTION

Cytoplasmic localization of mRNAs is used by several eukaryotic organisms to control, in space and time, the expression of proteins involved in cell fate determination, cellular polarity or asymmetric cell division (St Johnston 2005; Du et al. 2007). With over 30 mRNAs localized at the bud tip, the budding yeast *Saccharomyces cerevisiae* is an excellent model system for studying the mechanisms behind mRNA transport and localization (Chartrand et al. 2001; Darzacq et al. 2003). The RNA-binding protein She2p is involved in this localization process, as it binds to specific localization elements within bud-localized transcripts (Olivier et al. 2005). She2p promotes the formation of an mRNA localization complex or "locasome" by recruiting the type V myosin Myo4p, via the bridging protein She3p (Bohl et al. 2000; Long et al. 2000; Takizawa and Vale 2000).

One of the localized transcripts is the *ASH1* mRNA, which accumulates at the bud tip of daughter cells during anaphase (Long et al. 1997; Takizawa et al. 1997). This asymmetric localization results in the specific sorting of the Ash1 protein to the nucleus of the daughter cell, where it controls mating-type switching and cell size of the daughter (Jansen et al. 1996; Sil and Herskowitz 1996; Di Talia et al. 2009). During its transport to the bud tip, the *ASH1* mRNA is translationally repressed by the RNA-binding proteins Khd1p (Irie et al. 2002; Paquin et al. 2007) and Puf6p (Gu et al. 2004), and phosphorylation of these factors at the bud tip promotes the local synthesis of the Ash1 protein (Paquin et al. 2007; Deng et al. 2008). Recent evidence has shown that nuclear events are important for the proper localization and translational repression of the *ASH1* transcript. Indeed, She2p actively shuttles between the cytoplasm and the nucleus, and this

nuclear transit is important for the recruitment of the translational repressor Puf6p on the *ASH1* mRNA (Du et al. 2008; Shen et al. 2009). However, the mechanism by which She2p is recruited to the *ASH1* mRNA in the nucleus in order to promote its cytoplasmic localization is still unknown.

A possibility is that She2p may be recruited during transcription, to the nascent *ASH1* mRNA. Indeed, transcription by the RNA polymerase II (RNA pol II) is coupled to several steps in pre-mRNA processing, like capping, splicing, 3'end processing and nuclear export of the transcripts (Moore and Proudfoot 2009). These steps in mRNP biogenesis are coordinated, especially via interactions between factors involved in these processes and the RNA pol II transcription elongation complex (TEC) (Perales and Bentley 2009). The large subunit of RNA pol II, Rpb1p, is one of the main actors in this coupling. The C-terminal domain (CTD) of Rpb1p is constituted of heptads repeats (26 in yeast, 52 in mammals) with the consensus sequence $YS_2PTS_5PS_7$, which are differentially phosphorylated during transcription (Phatnani and Greenleaf 2006; Buratowski 2009). Phosphorylation of serine 5 (Ser5) in the heptad sequence by the TFIIH associated kinase Cdk7 (Kin28 in yeast) occurs first during transcription initiation (Komarnitsky 2000). Serine 2 (Ser2) phosphorylation by the kinase Cdk9/PTEFb (Ctk1 and Bur1 in yeast) occurs later during elongation (Peterlin and Price 2006; Qiu et al. 2009). This differential phosphorylation of serine 5 and serine 2 residues during the transcription cycle allows the recruitment of RNA processing factors by the CTD at specific moments on the nascent mRNA. Early step in mRNA processing, like capping, require Ser5 phosphorylation of the CTD, as the capping enzyme and guanylyltransferase bind the Ser5 phosphorylated CTD during transcription initiation

(Ho and Shuman 1999). Factors involved in 3'end processing, like the cleavage/polyadenylation factor Pcf11 bind the Ser2 phosphorylated CTD, which occur later during transcription elongation (Licatalosi et al. 2002).

Another member of the TEC involved in cotranscriptional RNA processing is the Spt4-Spt5 transcription elongation factor, also known as DSIF (Wada et al. 1998). Spt4 and Spt5 form an heterodimeric complex which regulates the processivity of RNA pol II (Hartzog et al. 1998). Beside its role in RNA pol II elongation, Spt5 is also implicated in mRNA capping, as it interacts and activates the cap guanylyltransferase (Wen and Shatkin 1999), and evidence suggest a role in mRNA splicing (Lindstrom et al. 2003). While the coupling between transcription and pre-mRNA processing involves steps in mRNP biogenesis that occur prior to nuclear export (from capping to mRNP export complex assembly), it is not clear yet if the cytoplasmic fate of an mRNA; like its translational regulation, cytoplasmic localization or degradation, is directly modulated by the transcription machinery via interactions between the TEC and factors involved in these cytoplasmic processes.

In this study, we used chromatin immunoprecipitation to show that She2p interacts cotranscriptionally with the nascent *ASH1* mRNA. She2p was found associated with the elongating form of RNA polymerase II *in vivo*, and this interaction depends on the transcription elongation factor Spt4-Spt5. Mutations in either *SPT4* or *SPT5* reduced the cotranscriptional recruitment of She2p on the *ASH1* gene, and resulted in an increased delocalization of both *ASH1* mRNA and Ash1p. We hypothesize that She2p is recruited by the RNA pol II machinery prior to its transfer to nascent bud-localized mRNAs. Consistent

with this model, chromatin immunoprecipitation shows that She2p is associated with genes coding for both non-localized and bud-localized mRNAs, but this association is RNA-dependent only for the latest group. Altogether, these data reveal a mechanism for the cotranscriptional assembly of mRNP competent for localization in the cytoplasm.

RESULTS

She2p is recruited cotranscriptionally at the ASH1 and IST2 genes

Previous results have shown that She2p actively shuttles between the nucleus and the cytoplasm, and nuclear She2p promotes the binding of the nuclear proteins Loc1 and Puf6 to the *ASH1* mRNA *in vivo* (Shen et al. 2009). This raises questions about when She2p binds to the *ASH1* mRNA and how this binding is organized. One hypothesis is that She2p may be recruited early, to the nascent *ASH1* mRNA, where it promotes the formation of the RNP complex before nuclear export. To explore this possibility, we used chromatin immunoprecipitation (ChIP) to determine if She2p is associated cotranscriptionally with the *ASH1* gene. A *she2* yeast strain transformed with a plasmid expressing endogenous levels of She2p-myc was fixed with formaldehyde, the chromatin was extracted and sonicated. An anti-Myc antibody was used to immunoprecipitate She2p-myc and the associated chromatin was purified by treatment with protease, phenol extraction and ethanol precipitation. After ChIP, PCR amplification of specific regions of the endogenous *ASH1* gene was performed to determine if She2p-myc was associated with the *ASH1* locus *in vivo* (Figure 1A). As shown in Figure 1B, PCR amplification of regions surrounding the localization elements E1, E2A and E3 (which are bound by She2p) were enriched after

ChIP, while amplicons from the *ASH1* promoter, the N-terminus of the *ASH1* coding sequence or the *ACT1* gene (the *ACT1* mRNA does not interact with She2p) were poorly amplified, suggesting that She2p-myc is associated with the *ASH1* gene *in vivo*. To determine if the interaction between She2p and the *ASH1* gene depends on the interaction between She2p and the nascent *ASH1* transcript, the ChIP was repeated in the presence of RNAse. A threefold reduction in chromatin enrichment was observed for the E1, E2A and E3 amplicons after RNAse treatment (Figure 1B), suggesting that the interaction between She2p and the *ASH1* gene is in part RNA-dependent.

To further confirm that the interaction between She2p-myc and the *ASH1* gene depends on transcription, a galactose-inducible *ASH1* gene was integrated in the genome of yeast strain deleted of its endogenous *ASH1* gene, and ChIP of She2p-myc was performed in the presence of glucose (repressed transcription) or galactose (active transcription). PCR amplification in regions of the *ASH1* gene containing the localization elements E1 and E2A were positive in the presence of galactose, while no PCR amplification was detected when yeasts were grown in glucose (Figure 1C). No PCR amplification was detected from the *ASH1* gene when IgG antibodies were used for the immunoprecipitation or with anti-Myc antibody when She2p was expressed without a myc tag (data not shown). To confirm that the interaction between She2p-myc and the *ASH1* gene is RNA-dependent, RNAse A treatment of the chromatin was performed before immunoprecipitation of She2p-myc. While in galactose-induced cells, She2p-myc ChIP of the *ASH1* gene was positive, pre-treatment with RNAse A eliminated this ChIP (Figure 1C). Altogether, these results

suggest that She2p is recruited during the transcription of the nascent *ASH1* mRNA at the *ASH1* locus.

She2p is known to be involved in the localization of over thirty mRNAs at the bud of yeast cells, beside *ASH1* (Shepard et al. 2003; Aronov et al. 2007). To determine if She2p was also associated cotranscriptionally with another bud-localized mRNA, PCR amplification of a region of the endogenous *IST2* gene, near the localization element of the *IST2* mRNA (Olivier et al. 2005), was performed after immunoprecipitation of She2p-myc. Indeed, an amplicon in the *IST2* gene near the localization element was detected after ChIP (Figure 1D). Pre-treatment with RNAse strongly reduced this PCR amplification, suggesting an RNA-dependent interaction. These results show that She2p is recruited cotranscriptionally to bud localized mRNAs like *ASH1* and *IST2*.

She2p interacts with the elongating form of RNA polymerase II in vivo

The cotranscriptional interaction between She2p and the *ASH1* mRNA raised questions on how this RNA-binding protein gets recruited on the nascent transcript. One possibility is that it interacts with the RNA polymerase II transcription machinery. To test this hypothesis, co-immunoprecipitation between She2p-myc and the large subunit of RNA polymerase II (Rpb1p) was performed. Immunoprecipitation of She2p-myc expressed at endogenous levels using anti-myc antibody resulted in the pull-down of Rpb1p (Figure 2A). This interaction between She2p and Rpb1p was confirmed by performing the reverse co-IP, in which Rpb1p was immunoprecipitated using the 8WG16 antibody (Figure 2B). Interestingly, this interaction was independent of RNA since RNAse treatment did not

affect the co-IP between She2p-myc and Rpb1p (Figure 2A, B). To confirm that this interaction is RNA-independent, the co-IP was repeated using a mutant of She2p that cannot bind RNA, She2R63K (Gonsalvez et al. 2003). The co-IP between She2R63K-myc and Rpb1p confirmed that She2p interacts with the RNA polymerase II via protein-protein interactions (Figure 2C).

RNA polymerase II is phosphorylated during transcription, especially on the C-terminal domain (CTD) of Rpb1p, which consists of 26 repeats of the heptad consensus sequence YSPTSPS in yeast. Phosphorylation of serine 5 (Ser5) and serine 2 (Ser2) of the heptad sequence occur during transcription initiation and elongation, respectively (Phatnani and Greenleaf 2006). Antibodies that recognize these phospho-residues were used to determine if She2p is associated with the active, elongating form of RNA polymerase II. Immunoprecipitation of She2p-myc was followed by detection of unphosphorylated Rpb1p (8WG16 antibody), serine 5 phosphorylated Rpb1p (H5 antibody) or serine 2 phosphorylated Rpb1p (H14 antibody) by western blot. As shown in Figure 2D, all the phosphorylated forms of Rpb1p co-immunoprecipitated with She2p-myc. Treatment with RNAse prior to immunoprecipitation had no effect on these interactions. To confirm these interactions, the reverse co-immunoprecipitations were performed, using the anti-Ser5 (H5) or anti-Ser2 (H14) antibodies for immunoprecipitation of Rpb1p, followed by detection of She2p-myc by western blot. For both antibodies, She2p-myc was detected in the co-immunoprecipitate, even after treatment with RNAse (Figure 2E, F). Altogether, these results show that She2p interacts with the active, elongating RNA polymerase II, independently of its binding to RNA.

The interaction between She2p and RNA polymerase II occurs via the transcription elongation factor Spt4-Spt5

To better understand the biological relevance of the interaction between She2p and the RNA polymerase II, we decided to identify the binding site of She2p on the RNA polymerase II machinery. Since the C-terminal domain (CTD) of Rpb1p is a major binding site for RNA processing factors (Perales and Bentley 2009), a possible interaction between She2p and Pol II CTD was explored. However, no interaction between She2p-myc and the non-phosphorylated or phosphorylated CTD has been detected using pull-down experiments (data not shown). Therefore, we decided to focus on transcription factors which have the same pattern of interaction with RNA pol II as She2p, i.e which are associated with the phosphorylated forms of RNA pol II. Using co-immunoprecipitation, we measured the interaction between She2p and Rpb1p in strains carrying mutations or deletions of genes coding for the transcription elongation factors (i) Rad6p and Bre1p, which are required for histone H2B ubiquitylation and H3 methylation; (ii) Paf1p and Rtf1p, which are part of the PAF1 complex that promotes histone methylation; and (iii) Spt4p and Spt5p, which regulate RNA pol II processivity. For each mutant strain and its isogenic wild-type strain, a 13xmyc tag was integrated at the C-terminal end of the coding sequence in the *SHE2* gene, and co-immunoprecipitation of Rpb1p was performed. None of these mutants had an effect on She2p-myc expression levels (data not shown). Of the 7 deletion mutants (*rad6*, *bre1*, *paf1*, *spt4* and *rtf1*) or temperature sensitive mutants (*spt5-4*, *spt5-194*), only the *SPT4* and *SPT5* mutants had an impact on the interaction between

She2p-myc and Rpb1p (Figure 3A, B and data not shown). Indeed, deletion of *SPT4* strongly reduced the interaction between She2p-myc and Rpb1p (Figure 3A), while deletion of *PAF1* had no effect. For *SPT5*, two temperature sensitive mutants were tested: *spt5-4* and *spt5-194* (Hartzog et al. 1998). Both mutants displayed a reduced interaction between She2p-myc and Rpb1p when shifted to non-permissive temperature (Figure 3B).

Spt4-Spt5 form a complex called DSIF, which is highly conserved from yeast to humans (Wada et al. 1998). Since mutations in both members of the DSIF complex affect the interaction between She2p and Rpb1p, it strongly suggests that Spt4-Spt5 participates in the recruitments of She2p to the RNA pol II. To determine if Spt4 and Spt5 interact with She2p, pull-down experiments were performed using TAP-tagged Spt4 and Spt5, and myc-tagged She2p, all expressed from their chromosomal locus. As shown in Figure 3C, both Spt4-TAP and Spt5-TAP were able to pull down She2p-myc. Altogether, these results suggest that She2p interacts with the transcription elongation factor Spt4-Spt5 *in vivo* and this interaction is important for the association between She2p and the RNA polymerase II.

Mutations in SPT4-SPT5 disrupt the cotranscriptional recruitment of She2p to the ASH1 gene and affect ASH1 mRNA localization

Now that specific mutants that affect the interaction between She2p and Rpb1p have been identified, we explored the role of the Spt4-Spt5 complex in the cotranscriptional recruitment of She2p at the *ASH1* gene. We repeated the chromatin immunoprecipitation of She2p-myc in the *spt4*, *spt5-4* and *spt5-194* strains and measured the enrichment of the *ASH1* gene using qPCR. To control for the possibility that disruption of a transcription

elongation factor may affect the transcription level of the *ASH1* gene, we tested in parallel another elongation factor, Paf1p, which is part of the PAF1C transcription elongation complex, and depends on Spt4p for its association with RNA pol II (Qiu et al. 2006). *ASH1* mRNA expression levels were measured in all mutants strains using quantitative RT-PCR, and revealed no significant decrease in the *spt4, paf1*, and for the *spt5-4* and *spt5-194* strains at non-permissive temperature. (Supplementary Figure 1). ChIP was performed using She2p-myc, and enrichment of the N-terminus (Nt) and E3 amplicons of the *ASH1* gene was determined by qPCR. As shown in Figure 4A, deletion of *SPT4* resulted in a two-fold reduction of the *ASH1* E3 amplicon compared to the isogenic wild type or *paf1* strains. Similar results were observed in the *spt5-4* and *spt5-194* strains at non-permissive temperature, where a 2 to 2.5 fold decrease in the enrichment of *ASH1* E3 amplicon was also measured (Figure 4B). Interestingly, enrichment of the *ASH1* Nt amplicon was also significantly reduced in the *spt4*, *spt5-4* and *spt5-194* strains. These results suggest that the Spt4-Spt5 complex is important for the cotranscriptional recruitment of She2p to the *ASH1* gene.

The effect of the *spt4* and *spt5* mutations on *ASH1* mRNA localization was then determined using fluorescent in situ hybridization (FISH) against the *ASH1* transcript. Wild type and mutants strains were scored for either bud tip localization of *ASH1* mRNA (localized), full bud localization (bud localized) or delocalization of this mRNA (Figure 5A). While 80% of wild type and *paf1* late-anaphase cells displayed localization of the *ASH1* mRNA at their bud tip, this percentage dropped to 20% in *spt4* cells (Figure 5B). A similar effect was observed in *spt5-4* and *spt5-194* strains when they were shifted to the

non-permissive temperature, as over 50% of late-anaphase cells had delocalized *ASH1* mRNA (Figure 5C). The effect of the disruption of the Spt4-Spt5 complex on the asymmetric distribution of the Ash1 protein and *HO* promoter activity was also determined using a genetic assay in which the *ADE2* gene is under the control of the *HO* promoter (Jansen et al. 1996). In this assay, asymmetric distribution of Ash1p results in the expression of the *HO-ADE2* gene and growth on plate lacking adenine (-Ade), while symmetric Ash1p represses *HO-ADE2* expression and cell growth in this medium. Disruption in *ASH1* mRNA localization and Ash1p sorting to the daughter cell nucleus (like a *SHE2* deletion) resulted in growth defect on -Ade plate (Figure 5D). Deletion of *SPT4* in this genetic background also led to a partial growth defect on -Ade plate (Figure 5D), suggesting that the defect in *ASH1* mRNA localization observed in this mutant resulted in an incomplete sorting of the Ash1 protein. Altogether, these results provide evidence that the cotranscriptional recruitment of She2p on the *ASH1* gene via the Spt4-Spt5 elongation complex is important for cytoplasmic *ASH1* mRNA localization.

She2p is associated with genes coding for both bud-localized and non-localized mRNAs.

The results presented above show that She2p interacts with Rpb1p via the Spt4-Spt5 transcription elongation complex, and this interaction is independent of the RNA-binding property of She2p. From these results, a possible model for the recruitment of She2p on bud-localized mRNAs could be proposed: She2p interacts first with the transcription elongation complex (TEC), via the Spt4-Spt5 complex, and it is then transferred to the nascent mRNA when a localization element RNA motif emerges from the elongating RNA

polymerase. This model predicts that She2p would be associated with genes coding for both bud-localized and non-localized mRNAs, via its interaction with the TEC. However, this association would be in part RNA-dependent only with genes coding for bud-localized mRNAs. Indeed, the ChIP data in Figure 1 show that, even after RNAse treatment, there was still a significant association of She2p-myc with the *ASH1* and *IST2* genes. Therefore, for these two genes, part of the She2p-myc ChIP may reflect the interaction between She2p and the nascent mRNA (which is RNAse sensitive) and some of the association may reflect the direct interaction between She2p and the TEC (which is RNAse insensitive).

To explore this hypothesis, we repeated the She2p-myc chromatin immunoprecipitation on genes whose mRNAs are not bound by She2p (Shepard et al. 2003; Oeffinger et al. 2007). In parallel, we performed ChIP on the same genes using Rpb1p, to estimate their level of transcription. Both She2p-myc and Rpb1p ChIPs were treated or not with RNAse to determine if the association is RNA-dependent or not. We focused on five genes: *ACT1*, *PMA1*, *FBA1*, *PGK1* and *ASC1* which have been previously characterized by ChIP (Abruzzi et al. 2004), and which do not encode for known bud-localized/She2p-associated mRNAs. These five genes were compared with *ASH1, IST2* and *EAR1*, as genes coding for bud-localized mRNAs. As negative controls, we used genes transcribed by RNA pol III, coding for $tRNA^{CUU}$, $tRNA^{GUC}$ and U6 snRNA. These three RNA pol III genes showed no association with Rpb1p or She2p-myc by ChIP (Figure 6B). Both Rpb1p and She2p-myc ChIPs on *ASH1, IST2* and *EAR1* genes were positive (Figure 6C). While treatment of chromatin with RNAse prior to immunoprecipitation had no effect on the Rpb1p ChIP, it resulted in a decreased level of She2p-myc ChIP for all three genes,

suggesting that part of the association of She2p-myc with these genes is RNA-dependent (Figure 6C).

Interestingly, She2p-myc ChIP revealed that the *ACT1*, *PMA1*, *FBA1*, *PGK1* and *ASC1* genes were all associated with this RNA-binding protein (Figure 6D). Their enrichment in She2p-myc ChIP followed the same pattern as for the Rpb1p ChIP, suggesting a relationship between She2p interaction and the transcription level of these genes. As predicted in our hypothesis, RNAse treatment had no effect on the association of She2p-myc with the *ACT1*, *PMA1*, *FBA1*, *PGK1* and *ASC1* by ChIP (Figure 6D), suggesting an RNA-independent interaction. To further confirm these results, we performed chromatin immunoprecipitation using the RNA-binding mutant She2R63K, and measured the effect of this mutation on the association between She2p-myc and the abovementioned genes. As shown in Figure 6E, while the R63K mutation reduced (but did not abolished) She2p-myc ChIP on the *EAR1*, *ASH1* and *IST2* genes, it had no effect on the association between She2p-myc and the *FBA1*, *PGK1* and *ASC1* genes. Altogether, these results show that She2p is associated with genes transcribed by RNA pol II independently of their nascent mRNAs. However, RNA-dependent interaction of She2p occurs only on genes whose mRNAs contain localization elements.

DISCUSSION

Recent literature shows that most processes related to mRNA maturation (capping, splicing, processing) and mRNA export, which all occur in the nucleus, are initiated on nascent transcripts (Komili and Silver 2008). Co-transcriptional recruitment of mRNA

processing, splicing and mRNA export factors has been well documented and several of these factors interact with the transcription elongation complex (TEC) (Perales and Bentley 2009). Since several RNA-binding proteins involved in cytoplasmic mRNA localization are known to be exclusive residents of the nucleus or to shuttle between the cytoplasm and the nucleus (Farina and Singer 2002), it raises the possibility that some of these factors may also be recruited cotranscriptionally to the nascent mRNAs.

In this work, we show that the mRNA localization factor She2p interacts cotranscriptionally with the nascent *ASH1*, *IST2* and *EAR1* mRNAs. While She2p was already known to be associated with mRNAs in the nucleus (Kruse et al. 2002), how it is recruited on bud-localized transcripts was not yet clear. Chromatin immunoprecipitation revealed that She2p is associated with the *ASH1*, *IST2* and *EAR1* genes, that this association is in part RNA-dependent and occurs only during transcription of the *ASH1* gene. These data support evidence from other model organism that the mRNA localization process is initiated on nascent mRNAs. For instance, in chicken fibroblasts, the RNA-binding protein ZBP1, which is involved in β-actin mRNA localization at the leading edge, is recruited on nascent β-actin mRNA (Oleynikov and Singer 2003; Pan et al. 2007). Localization of the *oskar* mRNA at the posterior pole of the *Drosophila* embryo requires members of the exon junction complex (EJC), like Y14-Mago and eIFIIIA (Hachet and Ephrussi 2004). Assembly of the EJC was previously shown to occur cotranscriptionally, at least in mammalian cells (Custodio 2004). In both cases, it is still unclear if this recruitment is coupled to the transcription machinery, as it was shown for nuclear pre-mRNA processing pathways.

In this work, we also found that She2p is associated with the phosphorylated, elongating form of RNA polymerase II *in vivo*, via an interaction with the transcription elongation factor Spt4-Spt5. Mutations in either *SPT4* or *SPT5* reduced the cotranscriptional recruitment of She2p on the *ASH1* gene, pointing toward a role of the TEC in the recruitment of She2p on the nascent *ASH1* transcript. These results reveal a novel function for the Spt4-Spt5 complex, beside its roles in RNA pol II elongation, mRNA capping and splicing (Hartzog et al. 1998; Wen and Shatkin 1999; Lindstrom et al. 2003). Spt4-Spt5 interacts with RNA pol II throughout most of the gene, as it gets recruited early on initiating and elongating RNA pol II (Pokholok et al. 2002). Since most of the known RNA localization motifs in yeast have been found within the coding sequence of the localized mRNAs, as close as 600 nucleotides from the start codon in the case of the E1element of *ASH1* mRNA (Chartrand et al. 1999; Jambhekar et al. 2005; Olivier et al. 2005), the early recruitment of She2p on the TEC via Spt4-Spt5 may ensure that She2p will be present when a localization element emerges from the RNA polymerase.

More important, mutations in *SPT4* and *SPT5* significantly reduced the localization of the *ASH1* mRNA at the bud tip and disrupted the asymmetric sorting of Ash1p. None of these mutants had an effect on *ASH1* mRNA expression level, suggesting a post-transcriptional role of Spt4-Spt5 on *ASH1* mRNA localization. Altogether, these results suggest that the cotranscriptional recruitment of She2p on the *ASH1* gene is important for the proper cytoplasmic localization of this mRNA. By binding directly on the nascent mRNA, She2p makes this mRNP competent for localization and defines the fate of the transcript before its export to the cytoplasm. Moreover, She2p has to compete with more

abundant non-specific nuclear RNA-binding proteins for interaction with the nascent transcript, like mRNA export factors for instance. Since several of these mRNA export factors are known to interact with the TEC and are recruited cotranscriptionally to mRNAs (Perales and Bentley 2009), interaction between She2p and the TEC may enable this factor to compete with these RNA-binding proteins. In the *SPT4* and *SPT5* mutants, She2p may still have opportunities to interact with *ASH1* mRNA in the nucleus or the cytoplasm, but less efficiently.

Based on our observation that She2p interacted with RNA pol II independently of its RNA-binding function, we hypothesize that She2p is recruited by the TEC prior to its transfer to nascent bud-localized mRNAs. Consistent with this model, chromatin immunoprecipitation shows that She2p is associated with genes coding for both non-localized and bud-localized mRNAs, but this association is RNA-dependent only for the latest group. Altogether, these data reveal a mechanism for the cotranscriptional assembly of mRNP competent for localization in the cytoplasm (Figure 7). Since the Spt4-Spt5 complex, also known as DSIF in metazoan, is highly conserved from yeast to humans (Wada et al. 1998), it may play a similar role in promoting the assembly of mRNA localization complex in these organisms.

MATERIAL AND METHODS

Growth media and yeast strains

Yeast cells were grown in either synthetic growth media lacking the nutrients indicated or rich media (Rose 1990). Transformation was performed according to the

protocol of Gietz and Schiestl (Schiestl 1989). Yeast gene disruption cassette was created by PCR amplification of the *loxP*-KAN-*loxP* construct in plasmid pUG6 and primers specifics for the gene of interest (Guldener et al. 1996). Specific disruption was confirmed by PCR analysis of genomic DNA. Yeasts strains and plasmids used in this study are described in the Supplementary Tables 1 and 2, respectively.

Chromatin immunoprecipitation

Chromatin IPs were performed essentially as described (Ezhkova 2006; Gilbert 2006). Briefly, 100 mL of cells were grown to early log phase (OD_{600} around 1) at 30°C in YPD or the appropriate medium. For galactose induction, galactose was added to cultures to a final concentration of 3% or 2% glucose for the negative control. To increase the number of cells with *ASH1* transcription, yeasts were treated 2 hrs with nocodazole (at a final concentration of 15µg/ml), then washed once with YPD. Formaldehyde was added to a final concentration of 1% and cells were incubated at room temperature for 20 min. The crosslinking time was reduced from 20 to 5 min when RNAse treatment was performed. Glycine was added to a final concentration of 300mM. Cells were washed twice in 1× PBS and lysed with glass beads in FA lysis buffer (50mM HEPES/KOH pH 7.5, 140mM NaCl, 1mM EDTA, 1% Triton, 0.1% sodium deoxycholate, 40U RNasin/mL (Promega), 88µg/mL PMSF, 10µg/mL Leupeptin, 10µg/mL pepstatin and 5µg/mL Aprotinin). Cells were lysed by vortexing 5 times for 30 sec, with a pause of l min on ice between each vortex. A hole was punctured at the bottom of the tube with a hot 21G needle and cross-linked chromatin was collected by centrifugation for 5 min at top-speed in a benchtop centrifuge. Pellets were washed twice in FA buffer and resuspended in 600µL FA buffer.

Samples were sonicated for three cycles of a 20 s pulse followed by a 60 s pause on ice using a 100 Sonic Dismembranator (Fisher Scientific). Chromatin was sonicated to yield an average size of 300-500 base pairs (bp) DNA fragments.

Immunoprecipitations were performed as followed. Purified 9E10 antibody (Oncogene Science) against myc, or monoclonal antibodies 8WG16 against total RNA Pol II (Abcam) was coupled to protein A/G-sepharose beads, respectively. After 3hrs incubation at 4 °C on a rotator, beads were washed successively with 0.7 mL FA lysis buffer, then 1 mL of FA 500 buffer (50mM HEPES/KOH pH 7.5, 500mM NaCl, 1mM EDTA, 1% Triton, 0.1% sodium deoxycholate, 40U RNasin/mL), 0.7mL of LiCl wash (10mM Tris.Cl, pH 8.0, 250mM LiCl, 0.5%(v/v) NP-40, 0.1% sodium deoxycholate, 1mM EDTA) and finally 0.7mL of TE/100mM NaCl. When an RNAse treatment step was added to the ChIP protocol, chromatin from the same experiment was treated with 4 Kunitz units of RNase A (Sigma). After incubating at room temperature for 45 min, immunoprecipitations were performed as described above. Immunoprecipitated material was eluted from the beads by heating for 10 min at 65 °C in 400 µl of 25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS. To reverse crosslinks, samples were treated with 1 mg/ mL of Proteinase K for 2 hrs at 37 °C and overnight at 65 °C. After extraction with phenol/chloroform, DNA was ethanol-precipitated 3 hrs in dry-ice ethanol bath in the presence of 20 µg of glycogen, and resuspended in 40 µl TE buffer.

The amount of DNA in the ‘input’ and ‘IP’ samples was quantified using real-time PCR (LightCycler 480, Caliper Life Sciences and Roche Applied Science). Real-time PCR reaction (20µl) contained 2µl sample DNA, 10µl SYBR green I master mix (Roche), 0.25

mM each primer. PCR was performed under the following conditions: Pre-incubation for 5 mins at 95°C, followed by 45 cycles of 10 s at 94°C, 15 s at 55°C and 10 s at 72°C. Each 'input' and 'IP' sample was analyzed in triplicate using real-time PCR, and the resulting values were averaged to determine the concentration of each sample. The primers used in this study are described in the Supplementary Table 3. The *PMA1*, *FBA1*, *PGK1*, *ASC1*, $tRNA^{GUC}$, $tRNA^{CUU}$ and intergenic primers (IntergenicV-1 and V-2) have been previously described (Zenklusen et al. 2002; Abruzzi et al. 2004). The calculations and normalizations for each ChIP were carried out as followed. Briefly, after completing PCR cycles, for each reaction, determine the cycle threshold (C). Average the cycle threshold for each triplicate set of reactions. The enrichment of the protein of interest (i.e., She2p, Rpb1p) above background was calculated by dividing (the relative IP signal for gene-specific primer pair) by the (the relative input signal for gene-specific primer pair). The 'intergenic' primer pair is in a nontranscribed region of chromosome IV and should account for any nonspecific DNA binding. A fold enrichment of 1 indicates that the amount of DNA immunoprecipitated by the protein of interest is that same as from nonspecific DNA binding.

Fluorescence in situ hybridization (FISH)

FISH experiments using oligonucleotide DNA probes have been described previously (Chartrand et al, 2000). Following fixation of yeasts, the cells were rehydrated with two washes of 2xSSC for 5 min at room temperature. The coverslips were incubated with 2xSSC 40% formamide for 5 min at room temperature. In parallel, 10 µl of a mix of

Cy3-conjugated *ASH1* oligonucleotide probes (1 ng/µl) was mixed with 4 µl of a 5 mg/ml solution of 1:1 sonicated salmon sperm/E. coli tRNA (Sigma). The mix was lyophilized in a speed vacuum. The pellet was resuspended in 12 µl of 80% formamide and 10mM sodium phosphate (pH 7.0). The 12 µl probe solution was heated at 95°C for 5 min and 12 µl of 4xSSC, 20mM VRC, 4 mg/ml BSA and 50U of RNA guard was added. The probe preparation was finally dropped on a parafilm sheet and the coverslips were placed on the drop face-down. Hybridization was then carried out overnight at 37°C. Following hybridization, the cells were washed sequentially twice with 2xSSC 40% formamide for 15 min at 37°C, once with 2XSSC 0.1% Triton X-100 and twice with 1XSSC. The coverslips were incubated in 1xPBS containing diamidino phenylindole (DAPI) for 2 min and mounted on the microscope slides with mounting medium (86% glycerol, 1mg p-phenylene diamine, 1xPBS).

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FIGURES

Figure 1: She2p-myc interacts cotranscriptionally with bud-localized mRNAs. (**A**) Diagram of *ASH1*, *ACT1* and *IST2* genes, and the relative position of the amplicons used for each gene. Dark boxes in *ASH1* and *IST2* correspond to localization elements. Gray box in *ACT1* correspond to the intron. In *ASH1* gene, amplicon Pro spans a region 500 bp upstream of the start codon, while the amplicon Nt is upstream of the start codon. Amplicons E1, E2A and E3 overlap with 5' half of the corresponding localization element. (**B**) She2p-myc immunoprecipitates the endogenous *ASH1* coding sequence near localization elements in an RNA-dependent manner. She2p-myc ChIP was performed using anti-myc antibody, followed by PCR amplification of amplicons specific to endogenous *ASH1* (Pro, Nt, E1, E2A, E3) or *ACT1* gene. Chromatin was treated (+RNAse) or not with ribonuclease A prior immunoprecipitation. (**C**) Transcription-dependent interaction between She2p-myc and a galactose-inducible *ASH1* gene. She2p-myc ChIP was performed using anti-myc antibody, followed by PCR amplification of amplicons specific to galactose-induced (galactose) or repressed (glucose) *ASH1* gene (E1, E2A) or endogenous *ACT1* gene. Chromatin was treated (+RNAse) or not with ribonuclease A prior immunoprecipitation. (**D**) She2p-myc immunoprecipitates the *IST2* coding sequence near localization element. She2p-myc ChIP was performed using anti-myc antibody, followed

by PCR amplification of amplicon specific to endogenous *IST2* (Nt, Ct) or *ACT1* gene. Chromatin was treated (+RNAse) or not with ribonuclease A prior immunoprecipitation. The data are presented as the mean ± SEM (N≥3).

Figure 2: She2p-myc interacts with the elongating form of RNA polymerase II *in vivo* (**A**) Rpb1p, the large subunit of RNA pol II, co-immunoprecipitates with She2p-myc. She2p-myc was immunoprecipitated with anti-myc antibody (9E10), followed by detection of Rpb1p by western blot using 8WG16 antibody. Input: total yeast extract; IP: immunoprecipitate; IP+RNAse: yeast extract treated with RNAse prior immunoprecipitation; CTL: strain K699 with untagged She2p. Pkg1 was used as negative control. (**B**) She2p-myc co-immunoprecipitates with Rpb1p. Rpb1p was immunoprecipitated with 8WG16 antibody, followed by detection of She2p-myc by western blot using 9E10 antibody. Input: total yeast extract; IP: immunoprecipitate; IP+RNAse: yeast extract treated with RNAse prior immunoprecipitation; CTL: strain K699 with untagged She2p. Pkg1 was used as negative control. (**C**) Rpb1p interacts with She2R63K-myc, a mutant defective in RNA binding. She263K-myc was immunoprecipitated with anti-myc antibody (9E10), followed by detection of Rpb1p by western blot using 8WG16 antibody. Input: total yeast extract; IP: immunoprecipitate; CTL: strain K699 with untagged She2R63K. Pkg1 was used as negative control. (**D**) The elongating form of Rpb1p co-immunoprecipitates with She2p-myc. She2p-myc was immunoprecipitated with anti-myc antibody (9E10), followed by detection of unphosphorylated (8WG16), Ser-5 phosphorylated (H5) or Ser-2 phosphorylated (H14)

Rpb1p b western blot. Input: total yeast extract; IP: immunoprecipitate; IP+RNAse: yeast extract treated with RNAse prior immunoprecipitation; CTL: strain K699 with untagged She2p. Pkg1 was used as negative control. (**E**) She2p-myc co-immunoprecipitates with Ser-5 phosphorylated Rpb1p. Rpb1p was immunoprecipitated with H5 antibody, followed by detection of She2p-myc by western blot using 9E10 antibody. Input: total yeast extract; IP: immunoprecipitate; IP+RNAse: yeast extract treated with RNAse prior immunoprecipitation; CTL: strain K699 with untagged She2p. Pkg1 was used as negative control. (**F**) She2p-myc co-immunoprecipitates with Ser-2 phosphorylated Rpb1p. Rpb1p was immunoprecipitated with H14 antibody, followed by detection of She2p-myc by western blot using 9E10 antibody. Input: total yeast extract; IP: immunoprecipitate; IP+RNAse: yeast extract treated with RNAse prior immunoprecipitation; CTL: strain K699 with untagged She2p. Pkg1 was used as negative control.

Figure 3: She2p-myc interacts with RNA pol II via the transcription elongation factor Spt4-Spt5. (**A**) Deletion of *SPT4* decreases the interaction between She2p-myc and Rpb1p *in vivo*. Rpb1p was immunoprecipitated with 8WG16 antibody, followed by detection of She2p-myc by western blot using 9E10 antibody. Input: total yeast extract; IP: immunoprecipitate; CTL: yeast extract treated with beads only (no antibody). The ratio She2p/Rpb1p reflects the results from three independent experiments. (**B**) Temperature-sensitive mutations in *SPT5* decrease the interaction between She2p-myc and Rpb1p *in vivo*. Rpb1p was immunoprecipitated with 8WG16 antibody, followed by detection of She2p-myc by western blot using 9E10 antibody. Input: total yeast extract; IP:

immunoprecipitate; CTL: yeast extract treated with beads only (no antibody). The ratio She2p/Rpb1p reflects the results from three independent experiments. (**C**) She2p-myc co-immunoprecipitates with Spt4-TAP and Spt5-TAP. Spt4-TAP or Spt5-TAP was immunoprecipitated with IgG, followed by detection of She2p-myc by western blot using 9E10 antibody. Input: total yeast extract; IP: immunoprecipitate; CTL: yeast extract treated with beads only (no antibody). Pkg1 was used as negative control.

Figure 4: She2p-myc co-transcriptional interaction with *ASH1* depends on the transcription elongation factor Spt4-Spt5. (**A**) Deletion of *SPT4* decreases She2p-myc ChIP of the endogenous *ASH1* gene. She2p-myc ChIP was performed using anti-myc antibody, followed by PCR amplification of amplicons specific to endogenous *ASH1* (Nt, E3) in wild type (WT), *paf1* and *spt4* strains. (**B**) Temperature-sensitive mutants of *SPT5* decrease She2p-myc ChIP of the endogenous *ASH1* gene. She2p-myc ChIP was performed using anti-myc antibody, followed by PCR amplification of amplicons specific to endogenous *ASH1* (Nt, E3) in wild type (WT), *spt5-4* and *spt5-194* mutant strains at 25°C or 37°C. The data are presented as the mean ± SEM (N=3).

Figure 5: Spt4-Spt5 is required for proper *ASH1* mRNA localization and Ash1p sorting. (**A**) Phenotypes of *ASH1* mRNA localization as detected by Fluorescent in situ hybridization. Localized: *ASH1* mRNA localized at bud tip; Bud localized: *ASH1* mRNA filling the entire bud; Delocalized: *ASH1* mRNA in both bud and mother cell. Scale = 2μm. (**B**) Scores on *ASH1* mRNA localization phenotypes from wild type (WT), *paf1* and *spt4*

strains. (**C**) Scores on *ASH1* mRNA localization phenotypes from wild type (WT), *spt5-4* and *spt5-194* mutant strains at 25°C or 37°C. (**D**) Yeast genetic assay for Ash1p asymmetric distribution. Tenfold dilutions of exponentially growing K5547 (*HO-ADE2*, *she2*) transformed either with YCP22-She2-myc (*SHE2/SPT4*), YCP22 empty (*she2/SPT4*) or YCP22-She2-myc transformed in K5547 *spt4::KAN* (*SHE2/spt4*) were spotted on plates lacking tryptophan (-Trp) or lacking tryptophan and adenine (-Trp -Ade), and incubated at 30ºC.

Figure 6: She2p-myc interacts cotranscriptionally with genes coding for both non-localized and bud-localized mRNAs. (**A**) Diagram of *EAR1*, *PGK1, PMA1, ASC1* and *FBA1* genes, and the relative position of the amplicons used for each gene. Dark boxes in *EAR1* correspond to localization element. Gray box in *ASC1* correspond to the intron. For all ChIP experiments, RNA pol II (Rpb1p) and She2p-myc ChIPs were performed using 8WG16 and anti-myc antibodies, respectively. Quantitative PCR (qPCR) amplification of amplicons specific to endogenous genes was performed on immunoprecipitated chromatin. Chromatin was treated (+RNAse) or not with ribonuclease A prior immunoprecipitation. **(B)** She2p-myc is not associated with genes coding for $tRNA^{CUU}$, $tRNA^{GUC}$ and U6 snRNA, which are transcribed by RNA pol III. (**C**) She2p-myc immunoprecipitates the *ASH1, IST2* and *EAR1* genes, which encode for bud-localized mRNAs (* $P < 0.001$). (**D**) She2p-myc immunoprecipitates the *ACT1, PMA1, FBA1, PGK1* and *ASC1* genes, which do not encode for bud-localized/She2p-associated mRNAs. **(E)** An RNA-binding defective

mutant of She2p, She2R63K, still associates with Pol II transcribed genes by ChIP. The data are presented as the mean ± SEM (N=3).

Figure 7: Model for cotranscriptional recruitment of localization factor She2p. She2p is recruited to the transcription machinery via its interaction with Spt4-Spt5 complex in the preinitiation complex and then transferred to the zipcode in the nascent transcript.

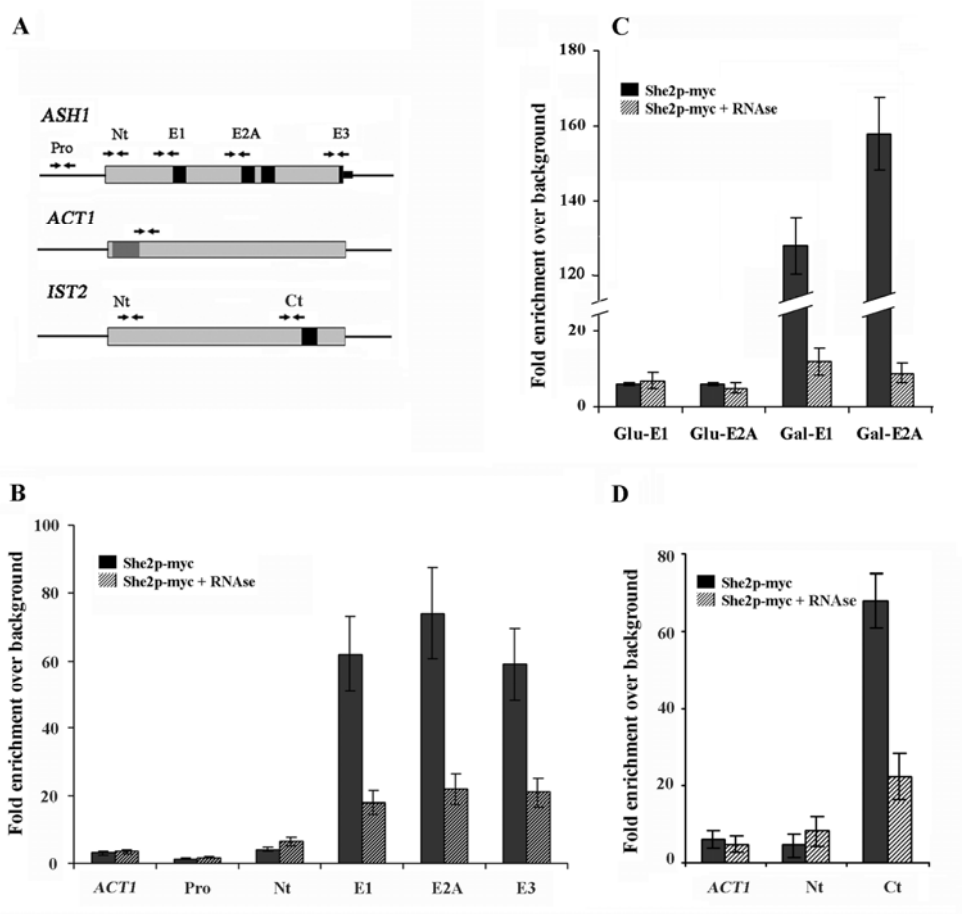

Figure 1: She2p-myc interacts cotranscriptionally with bud-localized mRNAs.

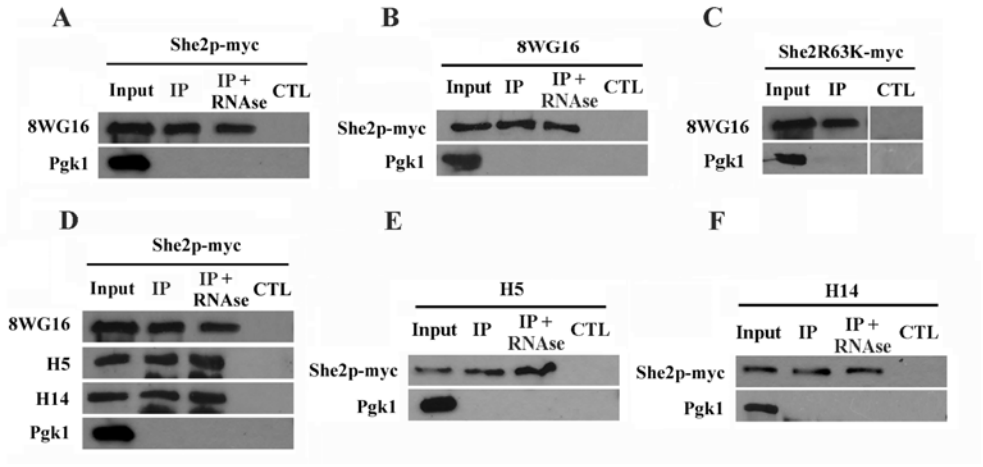

Figure 2: She2p-myc interacts with the elongating form of RNA polymerase II *in vivo*

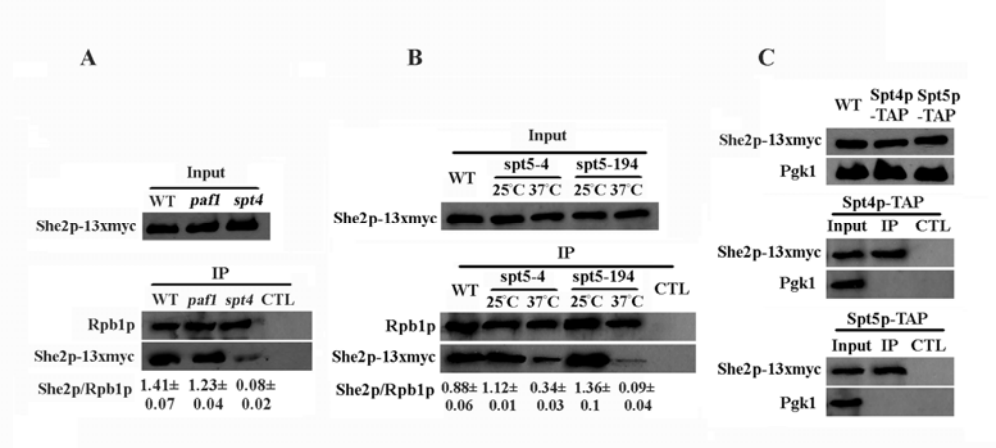

Figure 3: She2p-myc interacts with RNA pol II via the transcription elongation factor Spt4-Spt5.

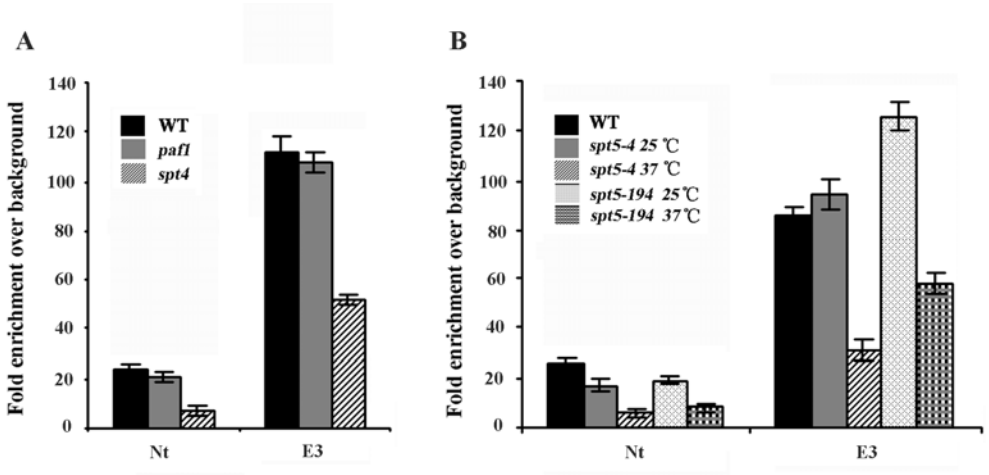

Figure 4: She2p-myc co-transcriptional interaction with *ASH1* depends on the transcription elongation factor Spt4-Spt5.

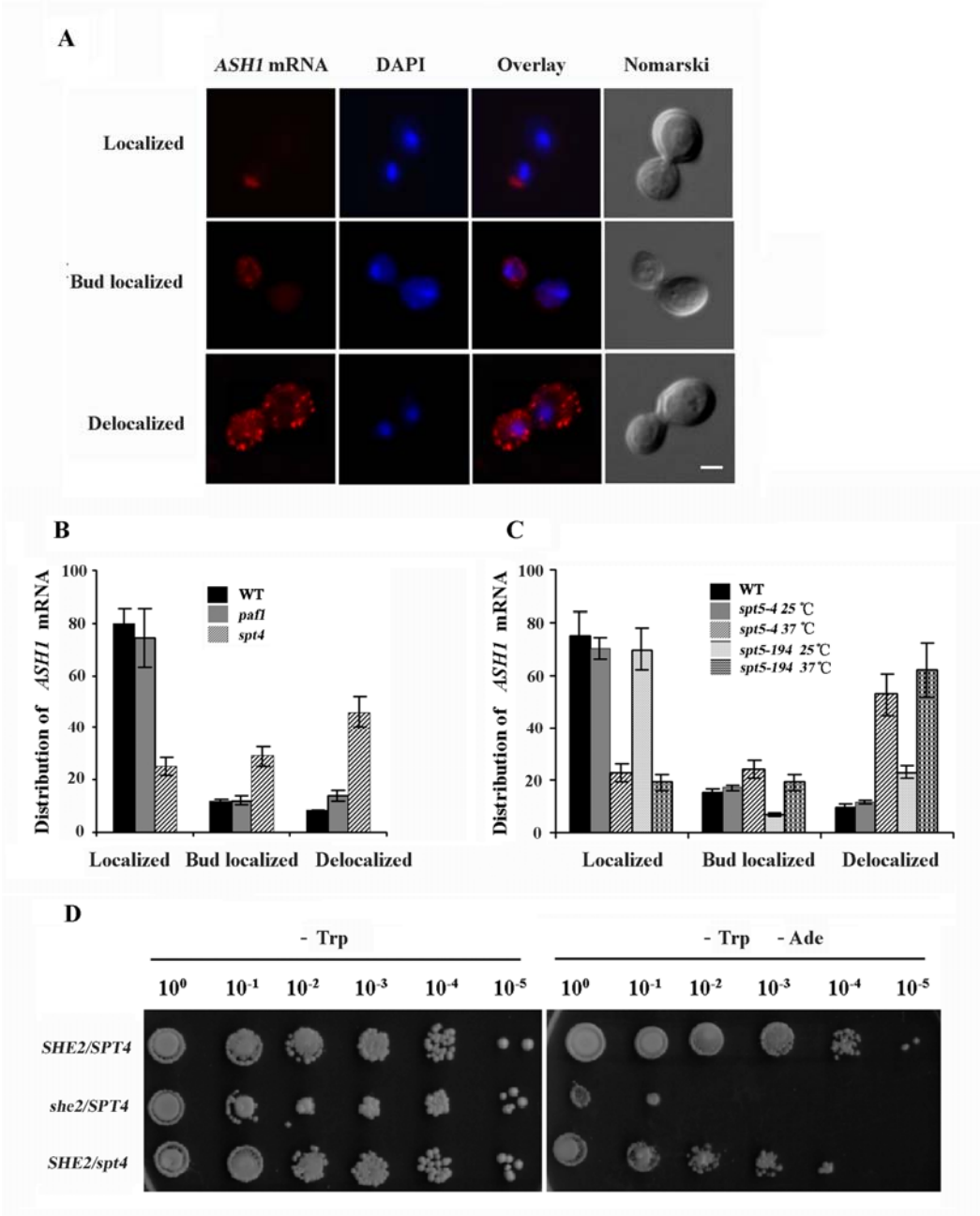

Figure 5: Spt4-Spt5 is required for proper *ASH1* mRNA localization and Ash1p sorting.

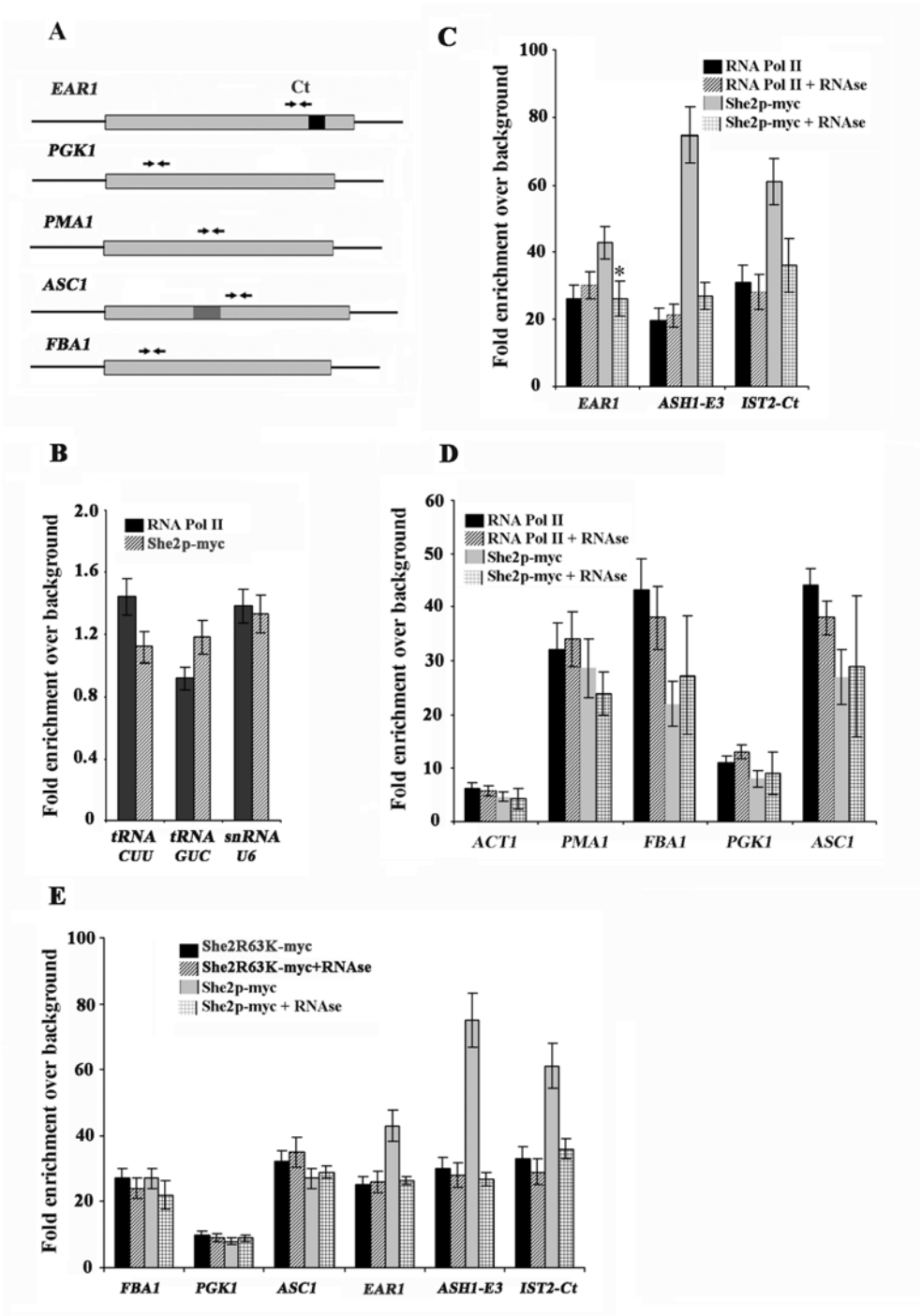

Figure 6: She2p-myc interacts cotranscriptionally with genes coding for both non-localized and bud-localized mRNAs.

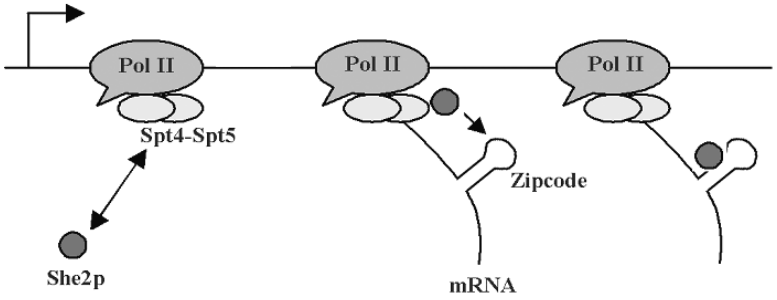

Figure 7: Model for cotranscriptional recruitment of localization factor She2p.

SUPPLEMENTARY DATA

SUPPLEMENTARY FIGURES

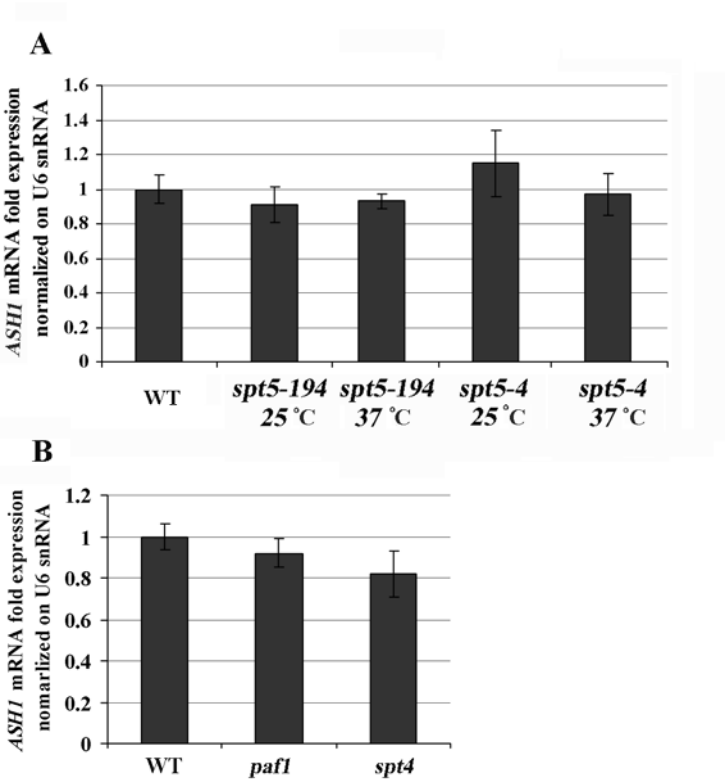

Figure 1: Expression level of *ASH1* mRNA in the *SPT4* and *SPT5* mutant strains. **(A)** Quantitative RT-PCR analysis of *ASH1* mRNA expression in wild type (FY119) and *SPT5* temperature sensitive mutants *spt5-4* and *spt5-194* at permissive (25°C) and restrictive (37°C) temperature. **(B)** Quantitative RT-PCR analysis of *ASH1* mRNA expression in wild type (BY4741), *spt4* and *paf1* strains.

MATERIAL AND METHODS

Co-Immunoprecipitation

Cells were grown to mid-log phase in yeast extract-peptone-dextrose (YPD) unless otherwise noted. Cells were harvested by centrifugation and resuspended at an OD600 of 100 in the extraction buffer (25 mM HEPES-KOH, pH 7.5, 150 mM KCl, 2 mM MgCl2, 0.1% IGEPAL CA-630, 1 mM dithiothreitol, 87.5 µg/ml phenylmethylsulfonyl fluoride, 0.5 µg/ml pepstatin, 0.5 µg/ml leupeptin, 0.5 µg/ml aprotinin; and 23 U/ml of RNAguard). The yeast cells were broken with glass beads and the supernatant was used for immunoprecipitation and Western blot. For the immunoprecipitation, either anti-Myc antibody (9E10), anti-TAP antibody (IgG), or anti-Rpb1p antibody (8WG16) was added to 800 µl of supernatant and incubated at 4°C with agitation for 2 h, respectively; 40 µl of protein A/G-Sepharose beads was then added and the incubation at 4°C was continued for 3 h. The beads were washed four times for 3 min at 4°C with a wash buffer (25 mM HEPES-KOH, pH 7.5, 150 mM KCl, 2 mM MgCl2). Immunoprecipitated material was eluted from the beads by heating for 10 min at 95 °C in 600 µl of 25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS.

Quantitative RT–PCR analysis

Total RNA was extracted from yeast strains using the yeast RNA miniprep protocol as described previously (Schmitt et al. 1990). A 2 µg portion of total RNA was reverse-transcribed using U6 snRNA- and *ASH1* RNA-specific primers. Total RNA was resuspended in DEPC-treated water containing 2 pmol of gene specific primers and 1 µl of

25mM dNTP to 12 µl. The samples were heated for 5 min at 65°C and chilled on ice rapidly. A 4 µl volume of 5x RT–PCR buffer (250mM Tris–HCl (pH 8.3), 250mM KCl, 20mM $MgCl_2$, 50mM DTT), 2 µl of 1M DTT and 1 µl of RNA guard were added to the reaction. The samples were preheated for 2 min at 42°C and 1 µl (200U) of Revert-aid M-MuLV-RT was added to start the reaction. The samples were reverse-transcribed for 50 min at 42°C. The expression level of *ASH1* mRNA was quantified using real-time PCR (LightCycler 480, Caliper Life Sciences and Roche Applied Science). Each 20µl real-time PCR reaction contained 2µl cDNA samples (50 ng), 10µl SYBR green I master mix (Roche), 0.25 mM each primer. PCR was performed under the following conditions: Pre-incubation for 5 mins at 95°C, followed by 45 cycles of 10 s at 94°C, 15 s at 55°C and 10 s at 72°C. All the reactions were run in triplicate and the average values were used for quantification. The RNA pol III transcribed U6 snRNA was used as an endogenous control. The relative quantification of target genes was determined using the rCT method.

SUPPLEMENTARY TABLE 1: Yeast strains used in this study.

strain	genotype	source
K699	*Mat a, ura3-1, leu2-3, his3-11, trp1-1, ade2-1*	Ralf-Peter Jansen
K699-*she2*	K699, *she2::KAN*	this study
K699-*GAL-ASH1*	*Mat a, ura3-1, leu2-3, his3-11, trp1-1, ade2-1*	this study
W303	*Mat a*, *ura3-52 trp1Δ2 leu2-3_112 his3-11 ade2-1 can1-100*	Ralf-Peter Jansen
W303 *SHE2* 13x MYC	*Mat a*, *ura3-52 trp1Δ2 leu2-3_112 his3-1 ade2-1 can1-100, SHE2-13xMYC::KAN*	this study
K5547	*Mat a, his3, leu2, ade2, trp1, ura3, HO-ADE2, HO-CAN1 she2::URA3*	Ralf-Peter Jansen
BY4741	*Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Open Biosystems
BY4741 *SHE2*-13xMYC	BY4741 *SHE2-13xMYC::KAN*	this study
SPT4-TAP	BY4741 *SPT4-TAP::HIS3*	Open Biosystems
SPT4-TAP *SHE2*-13x- MYC	BY4741 *SPT4-TAP::HIS3, SHE2-13xMYC::KAN*	this study
SPT5-TAP	BY4741 *SPT5-TAP::HIS3*	Open Biosystems
SPT5-TAP *SHE2*-13x- MYC	BY4741 *SPT5-TAP::HIS3, SHE2-13xMYC::KAN*	this study
BY4741-*spt4*	BY4741 *SPT4::KAN*	Open Biosystems
BY4741-*spt4 SHE213x MYC*	BY4741 *SPT4::KAN, SHE2-13xMYC::HIS3*	this study

BY4741-*paf1*	BY4741 *PAF1::KAN*	Open Biosystems
BY4741-*paf1 SHE213x MYC*	BY4741 *SPT41::KAN SHE2-13xMYC::HIS3*	this study
GHY13	*Mat a his4-912δ lys2-12δ leu2*Δ1 *ura3-52, trp1*Δ*63 spt5-194*	Grant Hartzog
GHY13 *SHE2-13xMYC*	GHY13 *SHE2-13xMYC::TRP1*	this study
GHY611	*Mat a his4-912δ lys2-128δ leu2*Δ*1 ura3-52, trp1*Δ*63 SPT5-MYC*	Grant Hartzog
GHY1073	*Mat α his4-912 δ lys2-128 δ trp1*Δ63 *spt5-4*	Grant Hartzog
GHY1073 *SHE2-13xMYC*	GHY1073 *SHE2-13xMYC::TRP1*	this study
FY119	*Mat α his4-912 δ lys2-128 δ leu2*Δ*1 ura3-52, trp1*Δ*63*	Grant Hartzog
FY119 *SHE2-13xMYC*	FY119 *SHE2-13xMYC::TRP1*	this study

SUPPLEMENTARY TABLE 2: Plasmids used in this study.

Plasmid	Features	Source
YCPlac22	Single copy yeast vector with *TRP1* selectable marker	Gietz lab
YIPlac128	Yeast integrative vector with LEU2 selectable	Gietz lab
YIPlac128 GAL1-*ASH1*	YIPlac128 vector for expression of *ASH1* mRNA from GAL promoter	(Chartrand 2002)
YCP22-SHE2-MYC	YCPlac22 vector for expression of She2p-myc from endogenous promoter	(Long et al. 2000)
YCP22-SHE2R63K-MYC	YCPlac22 vector for expression of myc-tagged She2R63K mutant	this study

SUPPLEMENTARY TABLE 3:

PCR primers used for chromatin immunoprecipitation.

Primer	Sequence
ASH1-pro-for	5'-CACATCTAACTGATTAGTTTTCCGT-3'
ASH1-pro-rev	5'- CAATCCACGTAAGGAAAATGATCAG-3'
ASH1-E1-for	5'- TGGTGTAAGGATACAAACTATCAA-3'
ASH1-E1-rev	5'- TTTTGATTATTAGTTAAGTTGGGTATAC-3'
ASH1-E2A-for	*5*'- TTCAAGAAGTTAAACATCAAAG-3'
ASH1-E2A-rev	5'- TTTGCAAAGCTTTGAAACTGTT-3'
ASH1-E3-for	5'- AGATCCCCACA AAGGGTGAAATAAACA-3'
ASH1-E3-rev	5'- ATTACAAAATAAGCAACGGTACCCTTCAAT-3'
ACT1-for	5'- GTTGCTGCTTTGGTTATTGA-3'
ACT1-rev	5'- CCATGA TACCTTGGTGTCTT-3'
IST2-Nt-for	5'- CTGGCCATGATCTGCAAACAAGCTA-3'
IST2-Nt-rev	5'- GGAATCAACCATTTTATATAGTTCTG-3'
IST2-Ct-for	5'- AGTGGCTACTG AACAAACAAAA-3'
IST2-Ct-rev	5'- GCATCACGATGGC GGTG GTGGTGAT-3'
PGK1-for	5'- TTCCAGAAAGGTCGATGGTC-3'
PGK1-rev	5'- GTCTGGTTGGGTCGATGGTC-3'
RPL28-for	5'- TTATCGTCAAAGCTAGATTCGCTC-3'
RPL28-rev	5'-ATCTGCCACTGGTAACTTCAAAATA-3'
ASC1-for	5'- TTCATCGGTCACAACTCCAA-3'
ASC1-rev	5'- GCAGCAGCCAACCAGTATCT-3'
tRNA GUC-for	5'- CACCACAAATGGAAAAGCGACTTTC-3'
tRNA GUC-rev	5'- CCTGTTATTTCCAAAGAACTGGGTTC-3'
tRNA CUU-for	5'- GCACTAGTTGATTCTTGTTCCAACAG-3'
tRNA CUU-rev	5'- CCGTTTTTCCCCAGAGCACTTTTA-3'
CLB2-for	5'-CTTGCCAAATTCTTATTAGAG-3'
CLB2-rev	5'- CACTAGATAATCCATTATCATGTG-3'
YMR171C-for	5'- GATTCCTTACCTATGCTACCACCA-3'
YMR171C-rev	5'- TGCATCCTGCTTTCGTAATCATC-3'
U6 snRNA-for	5'- GTTCGCGAAGTAACCCTT-3'
U6 snRNA-rev	5'- AAAAGCAAATAAATCTC-3'
OFS710	5'- CGCATTACCAGACGGAGATGT-3'
OFS711	5'- CAAGCAAGCCTTGTGCATAAGA-3'

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Chartrand, P., Meng, X., Huttelmaier, S., Donato, D., and Singer,R.H. 2002. Asymmetric sorting of Ash1p in yeast results from inhibition of translation by localization elements in the mRNA. *Mol Cell* **10**: 1319-1330.

Long, R.M., Gu, W., Lorimer, E., Singer, R.H., and Chartrand, P. 2000. She2p is a novel RNA-binding protein that recruits the Myo4p-She3p complex to ASH1 mRNA. *EMBO J* **19**(23): 6592-6601.

Schmitt, M.E., Brown, T.A., and Trumpower, B.L. 1990. A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. *Nucl Acids Res* **18**(10): 3091-3092.

Chapter IV

Discussion

4.1 Nuclear factors are required for cytoplasmic mRNA localization

The importance of nuclear events in cytoplasmic mRNA localization is a well described phenomenon. In the past years, a large number of trans-acting factors involved in mRNA localization have been characterized. Interestingly, many of them are either predominantly nuclear proteins or nucleo-cytoplasmic shuttling proteins (Perales, et al., 2009). This suggests that mRNA processing in the nucleus affects its cytoplasmic fate. This mechanism becomes more and more evident as coupling between transrcription and mRNA maturation is described (Perales, et al., 2009; Iglesias, et al, 2008; Schmid, et al, 2008; Shatakshi et al., 2008). For instance, proper splicing of the *oskar* mRNA is required for its localization at the posterior pole of *Drosophila* embryo (Hachet and Ephrussi 2004). In this case, Y14-Mago and the translation initiation factor eIFIIIA (an RNA DEAD box helicase), as members of the exon junction complex (EJC), are predominantly nuclear proteins and involved in the localization of this transcript (Palacios et al., 2004). Remodeling of the ribonucleoprotein complex (RNP) responsible for the localization of the Vg1 mRNA in *Xenopus* oocyte has been shown to occur prior to export of this mRNA (Kress et al. 2004). Moreover, several RNA-binding proteins involved in cytoplasmic mRNA localization are known to be exclusive residents of the nucleus or to shuttle between the cytoplasm and the nucleus (Farina and Singer 2002), like ZBP1, ZBP2 in fibroblasts (Oleynikov et al., 2003; Pan, et al., 2007), and hnRNP I in *Xenopus* (Cote et al. 1999). In general, many factors involved in the localization of a transcript either have an additional nuclear function such as splicing or are already recruited in the nucleus.

In yeast, studies have shown that two out of four *ASH1* mRNA binding proteins, Loc1 and Puf6, are predominantly nuclear. Loc1p is therefore often regarded as the nuclear component of the localization machinery that is likely needed to mark the *ASH1* mRNA for cytoplasmic RNA transport (Long et al., 2001; Urbinati et al., 2006; Du et al., 2008). Previous studies showed that Loc1p is required for efficient *ASH1* mRNA localization and

Ash1p sorting (Huh et al., 2003; Long et al., 2001). Moreover, Loc1p is also involved in translational regulation of *ASH1* mRNA (Brodsky et al., 2002; Komili, et al., 2007). Interestingly, this nuclear protein is a constituent of pre-60S ribosomes and is required for the assembly and export of the 60S ribosomal subunit (Urbinati et al., 2006). Puf6p is a member of the PUF family, and has been proposed to function in *ASH1* mRNA localization and translational repression (Gu et al., 2004). Although being predominantly a nuclear protein, a fraction of Puf6p co-localizes with the *ASH1* mRNA at the distal bud tip. To date, there is no evidence showing that Puf6p has a nuclear function other than translational regulation in mRNA localization. Khd1p interacts with localization element E1 of *ASH1* mRNA and is required for efficient translational control of *ASH1* mRNA (Paquin et al., 2007; Irie et al., 2002). Block of mRNA export caused the accumulation of Khd1p in the nucleus as well (Du, et al., 2008), suggesting that Khd1p needs to shuttle between the two compartments in order to function in cytoplasmic mRNA localization.

She2p is the key component in the assembly of the *ASH1* mRNA locasome which is actively transported along actin filaments to bud tip of daughter cells (Long et al, 1997; Takizawa et al, 1997). Mutants of She2p fail to localize *ASH1* mRNA and restrict Ash1p to the daughters (Jansen et al, 1996). Previous evidence suggested that She2p shuttles between the nucleus and cytoplasm in an RNA-dependent manner (Kruse et al., 2002; Du, et al., 2008). However, the mechanism by which She2p shuttles is not clear yet. In this thesis, we show that She2p is actively imported into the nucleus via its interaction with the importin α Srp1p. Our data suggest that She2p is not imported as a native dimer or tetramer, which is the conformation that binds RNA (Niessing et al., 2004; Müller, et al., 2009). One reason is that Srp1p interacts only with monomeric She2p, but not dimeric She2p *in vitro*. Interestingly, using GST-pull down assay, our results show that Srp1p interacts with She2p from yeast extracts, suggesting that a fraction of She2p *in vivo* possibly adopts a conformation different from the native dimer or tetramer. However, the possibility that this population of She2p corresponds to monomers remains elusive. One possibility is that this population of She2p may contain post-translational modifications,

like ubiquitination and phosphorylation. Previous studies have shown that some nuclear import factors can be regulated by phosphorylation, like Gln3p and Npl3p in yeast (Carvalho et al. 2001; Krebber et al., 1999), and phosporylated She2p has been reported *in vivo,* with an uncertain function (Gonsalvez et al. 2003). In this thesis, to verify that She2p is a phosphoprotein, recombinant GST-She2p and GST alone were incubated with yeast extract and γ-P^{32}-labeled ATP, followed by immunoprecipitation using an anti-GST antibody. As shown in Chapter IV, Figure 1A, P^{32}-labeled GST-She2p was detected by autoradiography, which shows that GST-She2p can be indeed phosphorylated. In fact, the interaction between Srp1p and She2p was lost when the yeast extracts was treated with phosphatase using GST-pull down assay (Chapter IV, Figure 1B). This result futher confirms that She2p is a phosphoprotein *in vivo*, and suggests that phosphorylation may promote nuclear import of She2p. Once in the nucleus, She2p may adopt a dimeric or tetrameric conformation for its interaction with target mRNAs. Intriguingly, wild-type She2p-myc was still present in the nucleus of the *srp1-31* strain at non-permissive temperature (data not shown). One possibility is from the rapid shutdown of transcription when this strain is shifted at 37ºC (Liu et al. 1999). Since She2p nuclear export depends on the export of newly synthesized mRNAs (Kruse et al. 2002; Du et al., 2008), the shutdown of transcription would explain the presence of She2p in nucleus of the *srp1-31* strain.

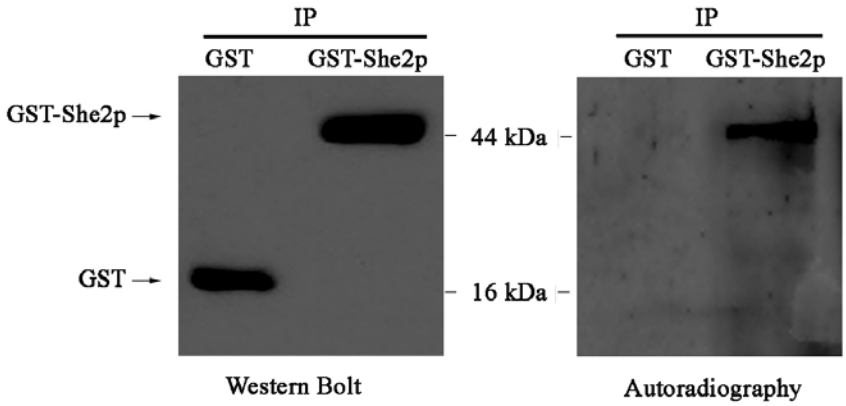

Figure 1A: She2p is a phosphoprotein. Immunopurification of recombinant GST or GST-She2p, followed by treatment with yeast extract and γ-P^{32}-labeled ATP. Left panel, western blot using anti-GST antibody. Right panel, autoradiography.

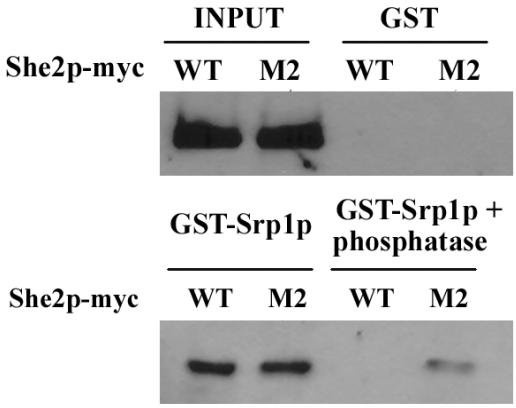

Figure 1B: GST pull-down assay to detect interaction between Srp1p and She2p-myc or She2p-M2-myc from yeast extracts. Input: total She2p-myc or She2p-M2-myc from yeast extracts. GST: She2p from yeast extracts interacting with GST alone. GST-Srp1p: She2p from yeast extracts interacting with GST-Srp1p. GST-Srp1p+Phosphatase: She2p from yeast extracts interacting with GST-Srp1p after phosphatase treatment.

Unfortunately, no classical NLS has been identified in She2p so far. To map this NLS, a genetic nuclear import and yeast two-hybrid assays were used. A 30 amino acids sequence at the C-terminus of She2p with NLS properties was identified. This NLS promotes the nuclear import of GFP and interacts with Srp1p. Surprisingly, the amino acid sequence of this region of She2p contains only one lysine and is rich in hydrophobic residues. The data suggest that She2p NLS is very different from the classical NLS which is rich in basic amino acids (arginine and lysine). Chen et al. have also characterized a non-classical NLS in the phospholipids scramblase 1 (PLSCR1), which lacks a contiguous stretch of positively charged residues and is enriched in hydrophobic residues. The PLSCR1 NLS was mapped to the C-terminus region and is sufficient for nuclear import of bovine serum albumin via Srp1p (Chen et al., 2005). In fact, in the set of 224 yeast proteins that interact with Srp1p, according to the BioGRID database, 49 of them (21.9%) contain a predicted bipartite cNLS and 61 of them (27.2%) contain a predicted monopartite cNLS, meaning that about one-half contain non-classical NLSs (Lange et al., 2007). However, these nuclear import signals are largely unknown, suggesting that the characterization of most NLSs is still in its infancy.

To better define the NLS in this peptide, the K_{222} residue and four other highly conserved amino acids surrounding this lysine (W_{215}, I_{219}, L_{220} and L_{223}) were mutated to alanine in She2p to generate the mutant She2-M5A (Chapter II, Figure 4A). Mutagenesis of these five residues in the NLS disrupted the interaction between She2p and GST-Srp1p, and the She2-M5A-myc protein was excluded from the yeast nucleus (Chapter II, Figure 5B; Figure 5C). The phenotype resulting from the mutations in the NLS of She2p seems to primarily result from the nuclear exclusion of this protein and not from secondary effects of these mutations. Indeed, our results also showed that the RNA-binding capacity of the NLS-mutated She2p was similar to wild-type She2p, as its interaction with She3p. More interestingly, adding a heterologous SV40 NLS to the NLS-mutated She2p completely restored the activity of this protein *in vivo*, like its recruitment on nascent *ASH1* mRNA and proper Ash1p asymmetric distribution (see discussion latter). Altogether, these results show

that non-classical NLS of She2p is present between the amino acids 214-222 at the C-terminal end of this protein, and that mutation of five specific residues in this NLS disrupts the nuclear targeting of She2p

The generation of a She2M5A protein that cannot be targeted to the nucleus raised an interesting question: what is the nuclear function of She2p in terms of A*SH1* mRNA localization and Ash1p asymmetric distribution. To determine the effect of the nuclear exclusion of She2p on *ASH1* mRNA localization, myc-tagged She2p or She2p-M5A was expressed in a *she2* yeast strain and the localization of *ASH1* mRNA in these strains was visualized by fluorescent *in situ* hybridization (FISH). As shown in Chapter II, Figure7A, while the wild-type She2p promoted the localization of the *ASH1* mRNA at the bud tip of cells in anaphase, the She2p-M5A had a reduced efficiency in *ASH1* mRNA localization, as it accumulated in the whole bud of the cells. However, Du et al. did not report any defect in *ASH1* mRNA localization when She2p was excluded from the nucleus, unlike what we observed (Chapter II, Figure 7). They constructed a fusion of She2 protein with the Myo4p-binding domain of She3p, which is excluded from the nucleus by the association of Myo4p with the actin cytoskeleton (Du et al., 2008). One possibilty is that such tight association of She2p, and of the *ASH1* mRNA, to the localization machinery and actin cytoskeleton suppresses the localization defects caused by the nuclear exclusion of She2p.

The effect of the mutation of the She2p NLS on the asymmetric distribution of the Ash1 protein and *HO* promoter activity was also determined. The results suggested that the M5A mutation partially disrupts the asymmetric distribution of Ash1p. However, expression of a She2-M5A protein containing the SV40 NLS (She2M5A+SV40NLS), which restores the nuclear localization of She2p-M5A and maintains its interaction with *ASH1* mRNA *in vivo* (Chapter II, Figure 6B), complemented the M5A mutations (Chapter II, Figure 7C). These data suggested that the nuclear function of the nucleo-cytoplamic shuttling She2p is required for *ASH1* mRNA localization and Ash1p asymmetric distribution in the daughter cells.

In this thesis, our results have illustrated that the presence of She2p in the nucleus is essential for Puf6p and Loc1p to bind the *ASH1* mRNA. In fact, these results are consistent with previous data, as the She2p-M5A mutant displays phenotypes similar to the *PUF6* and *LOC1* deletions (whole bud accumulation of *ASH1* mRNA, partial asymmetric distribution of Ash1p) (Gu, et al., 2004; Huh et al., 2003; Long et al., 2001). Moreover, pull-down of both TAP-tagged Puf6p and Loc1p resulted in the co-immunoprecipitation of She2p-myc (Chapter II, Figure 8C), suggesting an interaction between these factors *in vivo*. This interaction was independent of RNA since treatment of yeast extracts with RNAse A prior to immunoprecipitation still resulted in an efficient pull-down of She2p-myc by both Puf6p-TAP and Loc1p-TAP (Chapter II, Figure 8C). More interestingly, a clear reduction in the amount of She2p-M5A-myc that co-immunoprecipitated with either Puf6p-TAP or Loc1p-TAP was observed compared to wild type She2p-myc. Wild-type levels of interaction were recovered when the SV40 NLS was fused to the She2p-M5A protein (Chapter II, Figure 8D). Altogether, these results suggest that nuclear import of She2p is required for its interaction with Puf6p and Loc1p, and for their recruitment to the *ASH1* mRNA.

Since all three proteins bind independently to the 3'UTR of *ASH1* mRNA *in vitro* (Bohl *et al.*, 2000; Long *et al.*, 2001; Gu *et al.*, 2004), the mechanism by which She2p promotes the recruitment of Loc1p and Puf6p on the *ASH1* mRNA is not clear yet. One possibility is that, being in the nucleolus, Puf6p and Loc1p are spatially restricted from polyA$^+$ mRNAs. Since She2p has been recently shown to transit through the nucleolus (Du, 2008), it may either bring the *ASH1* mRNA in the nucleolus, where Puf6p and Loc1p can bind this transcript, or She2p may recruit these two factors in the nucleolus and bring them to the *ASH1* mRNA in the nucleoplasm. A role of nuclear She2p in translational control is indeed supported by recent data from the Jansen lab, which showed that the nuclear exclusion of She2p accelerates Ash1p synthesis (Du, 2008). Since She2p binds several localization elements within the coding sequence of the *ASH1* mRNA (Chartrand, 2002), this mechanism may reduce the possibility that elongating ribosomes could displace She2p

from this transcript. Such coupling is supported by the finding that She2p co-immunoprecipitates with Puf6p and Loc1p, suggesting an interaction between these proteins *in vivo*.

Our work suggest that the nuclear interaction between the She2 protein and the *ASH1* mRNA is important for recruiting the translation repressors Puf6p and Loc1p, which are responsible for the asymmetric translation of Ash1p after cytokinesis. By this mechanism, She2p may initiate the translational repression of the localized mRNA before its export into the cytoplasm and prevent premature translation of this transcript.

4.2 Coupling transcription with *ASH1* mRNA localization

As mentioned previously, during expression of protein-coding genes, pre-mRNAs are transcribed in the nucleus and undergo several processing steps, including capping, splicing, 3′-end processing and polyadenylation. The mature mRNA is then exported through the nuclear pore to the cytoplasm for translation. While distinct and highly complex cellular machines carry out each of these steps in the gene-expression process, evidence has been accumulating for the existence of gene-expression factories in which individual machines are functionally coupled (Perales, et al., 2009; Iglesias, et al, 2008; Schmid, et al, 2008; Shatakshi et al., 2008). Thus, coupling is probably involved in the entire process of gene expression *in vivo*. The following discussion will focus on the work described in this thesis and other advances done in the past years in elucidating the mechanisms of coupling between transcriptional machinery and cytoplamic mRNA localization.

4.2.1 She2p binds co-transcriptionally to nascent *ASH1* mRNA

Recent studies have shown that most processes related to mRNA maturation (capping, splicing, processing) and mRNA export, which all occur in the nucleus, are

initiated on nascent transcripts (Komili and Silver 2008). Co-transcriptional recruitment of mRNA processing, splicing and mRNA export factors has been well documented and several of these factors interact with RNA polymerase II (Pandit et al. 2008). However, do cytoplasmic processes, like mRNA localization and translational regulation, also require co-transcriptional association with nascent transcripts? Recent studies in fibroblasts have shown that ZBP-1, which is involved in β-actin mRNA localization at the leading edge, is recruited on nascent β-actin mRNA (Oleynikov and Singer 2003; Pan et al. 2007), but the importance of this recruitment in mRNA localization is not clear yet.

In this thesis, we found that She2p is associated to the vicinity of transcribed *ASH1* gene by chromatin immunoprecipitation. We chose to analyze the *ASH1* gene promoter and the region of the localization elements within this transcript, as well as *ACT1* gene and a nontranscribed intergenic region on a different chromosome. As shown in Chapter III, Figure 1, we were able to see a strong association between She2p and regions surrounding the localization elements E1, E2A and E3 (which are bound by She2p), compared to the regions from the *ASH1* promoter and N-terminus, and the *ACT1* gene (the *ACT1* mRNA does not interact with She2p). We also determine that the interaction between She2p-myc and the *ASH1* gene depends on transcription, using a galactose-inducible *ASH1* gene integrated in the genome of a yeast strain deprived of its endogenous *ASH1* gene. ChIP of She2p-myc was performed in the presence of glucose (repressed transcription) or galactose (active transcription) showed a 100-fold increase at regions of the *ASH1* gene containing the localization elements E1 and E2A after galactose induction compared to non-inducing conditions, indicating that She2p is associated with the *ASH1* gene in a transcription dependent manner. We confirmed that mRNA was in part necessary for She2p recruitment to *ASH1* gene, since the association of She2p with E1, E2A and E3 regions were decreased when chromatin immunoprecipitation was performed after extensive RNase A treatment. It is possible that She2p association with the *ASH1* gene relies on contacts that are mediated by large RNA/protein complexes. Altogether, our results illustrate that She2p is recruited to localization elements during the transcription of the nascent *ASH1* mRNA at the *ASH1*

locus. Interestingly, we found that She2p is also recruited co-transcriptionally to another bud-localized *IST2* mRNA, other than non-bud localized like *ACT1*, suggesting that cotransciptional association of She2p with bud localized mRNA genes may initiates the assembly of cytoplamic mRNAs localization machinery.

We looked if the translational regulators Puf6p and Loc1p are also recruited to the *ASH1* gene using chromatin immunoprecipitation. We chose to analyze the *ASH1* gene promoter and the region of the localization elements within its transcript. The region of the element E3 is enriched in Puf6p or Loc1p chromation immunoprecipitation compared to other regions of the *ASH1* gene, suggesting that Loc1p and Puf6p are also associated with *ASH1* gene (Chapter IV, Figure 2). These results are consistent with previous data that have shown that Puf6p and Loc1p bind the localization element E3 in the 3'UTR of *ASH1* (Long et al. 2001; Gu et al., 2004). Importantly, these translational repressors are recruited to the *ASH1* gene in a She2p-dependent manner, as a 4-fold decrease of the region of *ASH1* gene containing the element E3 was immunoprecipitated in the strain expressing She2-M5A compared to She2p (Chapter IV, Figure 2B and 2C). Moreover, immunoprecipitation of Puf6p-TAP in a *loc1* strain resulted a 3.5-fold decrease of the element E3 domain (Chapter IV, Figure 2E), suggesting that the association of Puf6p with *ASH1* gene is Loc1p-dependent. However, we did not detect any difference in Loc1p chromatin immunoprecipitation at the region of element E3 in a *puf6* strain, suggesting that the association of Loc1p with *ASH1* gene is Puf6p-independent (Chapter IV, Figure 2D).

Our data suggest a stepwise assembly of localization factor (She2p) and translational repressors (Puf6p and Loc1p) on nascent *ASH1* mRNA. This mechanism may explain early observations in which ß-actin mRNA was properly localized to the leading edge of chicken embryo fibroblasts (CEFs) and growth cones of developing neurons by the sequential binding of the two zipcode binding proteins ZBP2 and ZBP1 to the zipcode in its 3'-UTR (Farina et al., 2003; Pan et al., 2007). ZBP1 colocalizes with nascent ß-actin mRNA in the nucleus but is predominantly a cytoplasmic protein. ZBP2, in contrast, is

predominantly nuclear. ZBP2, a homologue of the splicing factor KSRP, binds initially to nascent ß-actin transcripts and facilitates the subsequent binding of the shuttling ZBP1. ZBP1 then associates with the RNA throughout the nuclear export and cytoplasmic localization process. Loss of ZBP2 function significantly reduced the efficiency of binding of ZBP1 to the ß-actin transcripts (Pan et al., 2007). Altogether, these results indicate that the cooperation of trans-factors on nascent mRNA regulates the formation of localizable mRNPs.

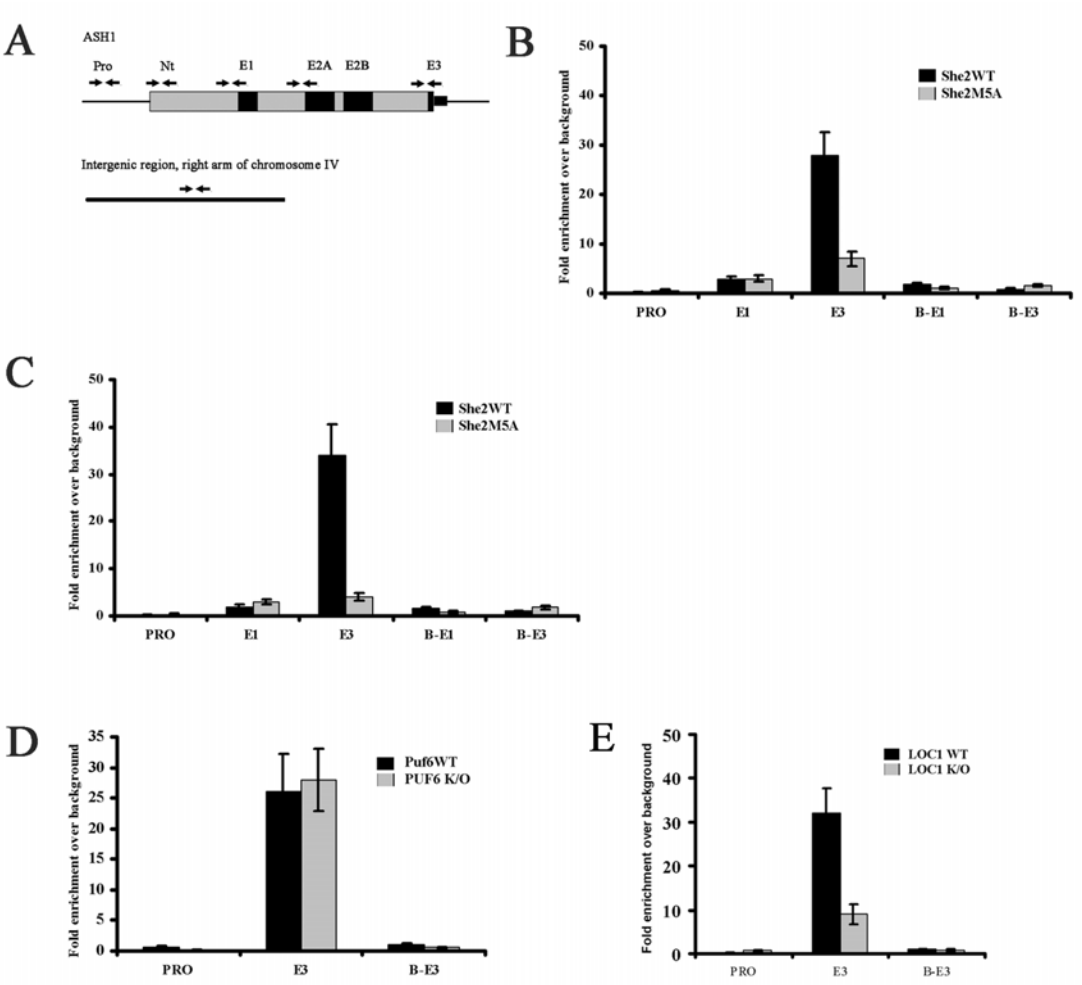

Figure 2: Puf6p, Loc1p are recruited to *ASH1* gene around the region of element E3.

The ChIP assay was performed with a yeast strain in which either Puf6p or Loc1p was epitope-tagged with TAP tag. After immunoprecipitation with the IgG antibody and reversal of crosslinks, co-precipitating DNAs were detected by Q-PCR. (A) Diagram of *ASH1*. Primer set Pro spans the promoter region. Primer set Nt spans the 5' region of the coding sequence. Primer sets E1, E2 and E3 spans the region of element E1, E2A and E3, respectively. Fold enrichment values were obtained by normalizing to non-transcribed region of chromosome IV. (B) Loc1p immunoprecipitates the region of element E3 of *ASH1* in She2p-dependent manner. (C) Puf6p immunoprecipitates the region of element E3

of ASH1 in a She2p-dependent manner. (D) Loc1p is recruited to *ASH1* in a Puf6p independent manner. (E) Puf6p is recruited to *ASH1* in a Loc1p dependent manner.

4.2.2 Association of She2p with Pol II via the Spt4-Spt5 complex is essential for the cytoplasmic fate of *ASH1* mRNA

Previous data showed that Pol II undergoes cycles of phosphorylation and dephosphorylation at its CTD, and this cycle coincides with Pol II activity (Hirose and Manley 2000). Pol II is hypophosphorylated while engaging in initiation and hyperphosphorylated during elongation (Hirose and Manley 2000). Furthermore, multiple elongation and mRNA processing factors are recruited by the Pol II CTD in a manner that is dependent on the state of CTD phosphorylation (Phatnani et al., 2006). Since She2p is associated with the transcribed *ASH1* gene, it seems likely that She2p might interact with RNA polymerase II either directly or indirectly during transcription. To test this possibility, we analyzed the association of She2p with RNA polymerase II using co-immunoprecipitation assay. Our results show that She2p interacted with Rpb1p, the largest subunit of RNA polymerase II using an antibody that recognizes the unphosphorylated CTD (8WG16). Interestingly, we also detected the interaction of She2p with the elongating form of the RNA polymerase II using lower affinity antibodies directed to two different phosphorylation sites in the CTD (H14 and H5). Our results are consistent with the possibility that She2p may be recruited by unphosphorylated Pol II in the preinitiation complex and transferred to the nascent RNA at the start of elongation. However, She2p may also be recruited by phosphorylated Pol II in initiation and elongation complex (Chapter III, Figure 2D and 2E). Therefore, She2p may be recruited to elongating polymerase soon after transcription initiation and then transferred to RNA. Moreover, She2p may be further recruited by cooperative self-association or by the RNA itself.

The association of She2p with Pol II occurs in a RNA-independent manner, as we did not lose this interaction after treatment with RNAse A prior to immunoprecipitation or

from mutant of She2p that failed to bind RNA (She2R63K), suggesting the presence of protein-protein interactions between She2p and Pol II. However, we cannot rule out the possibility that RNA might be playing an important role to maintain this association during transcriptional elongation. Moreover, we did not detect a direct interaction between She2p with either phosphorylated or unphosphorylated CTD of Pol II in *vitro* (data not shown). These results indicate that the association of She2p with Pol II may require additional factors.

Previous data have shown that efficient elongation of a transcript by RNA polymerase II (Pol II) requires several accessory factors to facilitate its movement along chromatin-assembled genes. The *Saccharomyces cerevisiae* Paf1 complex (Paf1C) colocalizes with Pol II during transcription elongation and is required for the normal expression of a subset of genes (Krogan et al., 2002; Simic et al., 2003). The Paf1 complex contains five subunits, Paf1, Ctr9, Cdc73, Rtf1, and Leo1, and physically associates with Pol II (Krogan et al., 2002; Squazzo et al., 2002). The association of Spt4-Spt5 with Pol II, the PAF1 complex, and the general elongation factors TFIIF and TFIIS has been reported previously (Lindstrom et al., 2003; Krogan et al., 2002; Squazzo et al., 2002). Proper elongation of a transcript by Pol II requires efficient navigation in the chromatin template. Histones are subject to a set of post-translational modifications, like phosphorylation, ubiquitylation, and sumoylation (Shilatifard 2006). The regulated placement and removal of these modifications control chromatin structure and influence transcription (Lieb et al., 2005). The ubiquitin-conjugating enzyme Rad6p and the ubiquitin protein ligase Bre1 are required for ubiquitylation of histone H2B (Xiao et al., 2005). H2B ubiquitylation is enriched in the coding regions of active genes (Xiao et al., 2005). Rad6p is recruited to open reading frames (ORFs) coincident with gene activation and modify histones during transcription (Xiao et al., 2005).

To identify factors that may mediate the interaction between She2p and Rpb1p, we looked at the transcriptional elongation factors Paf1C, Rad6p, Bre1p, and the Spt4-Spt5

complex, which have a similar binding pattern to Rpb1p as She2p. We did not detect any difference in the interaction of She2p with Rpb1p using co-immunoprecipitation in strains of *paf1, bre1, rad6*, and *rtf1*, suggesting that these transcription elongation factors are not required for the interaction between She2p and Rpb1p (data not shown). Interestingly, we found that the Spt4-Spt5 complex interacts with She2p *in vivo*, and is required for the association of She2p with RNA Pol II. Deletion of *SPT4* or Ts^- mutants *spt5* at restrictive temperature disrupt the association of She2p with Pol II, and affect its recruitment to the *ASH1* gene, leading to the delocalization of *ASH1* mRNA and the failure of Ash1p sorting to daughter cells (Chapter III, Figure 5B and 5C).

To account for these new observations, we suggest a model for *ASH1* mRNA cytoplasmic localization (Chapter IV, Figure 3): She2p, a nucleo-cytoplasmic shuttling protein, is actively imported into nucleus, and then associates with RNA Pol II by its interaction with Spt4-Spt5 complex, resulting in co-transcriptional binding of She2p to *ASH1* mRNA to facilitate subsequent recruitment of translational repressors Loc1p and Puf6p on nascent *ASH1* mRNA. In the nucleus, Loc1p and Puf6p initiate the translational repression of the localized mRNA before its export in the cytoplasm and prevent premature translation of this transcript. This model proposes that an important early step is She2p binding to the Spt4-Spt5 complex. Both *SPT4* and *SPT5* display an extensive set of genetic interactions with the CTD and the general elongation factors TFIIF and TFIIS, enzymes that modify the phosphorylation status of the CTD (Lindstrom et al., 2003; Lindstrom et al., 2001; Murray et al., 2001). Therefore this interaction can explain how She2p binds the Pol II independently of its CTD. Following association of She2p with Pol II via the Spt4-Spt5 complex, we suggest that She2p is recruited to nascent *ASH1* mRNA on the localization elements as they emerge from the elongating polymerase (Chapter III, Figure 3).

Since She2p interacts with the RNA Pol II via the Spt4-Spt5 complex, in an RNA-independent manner, it raises the possibility that this factor may be associated with other

RNA pol II-transcribed genes, beside those coding for bud-localized mRNAs like *ASH1* and *IST2*. To address this question, we used ChIP to analyze the association of She2p with the *ACT1*, *FBA1* and *PMA1* genes, whose transcripts are not known to be bound by She2p (Oeffinger et al. 2007, Shepard et al, 2003). Interestingly, ChIP revelead an association between She2p and these three genes (Chapter III, Figure 6). Unlike with *ASH1*, the association of She2p with *ACT1*, *PMA1* and *FBA1* is insensitive to RNAse treatment, suggesting that She2p is recruited to RNA pol II transcribed genes coding for both bud-localized and non-localized mRNAs. Altogether, these results show that She2p can be recruited to genes via the transcription machinery independently of the nascent mRNA. Therefore, transcription-coupled binding of She2p to nascent mRNAs is distinct from transcription-coupled recruitment of She2p to the Pol II elongation complex; the former requires a zipcode within mRNA sequence and RNA binding domain within She2p, and the later requires the Spt4-Spt5 complex.

The requirement for the elongation factor Spt4-Spt5 complex for She2p loading may provide an opportunity to exert quality control. In the event that a fully functional transcription elongation complex associated Spt4-Spt5 complex is not assembled, then She2p loading would be limited thereby, preventing formation of a localization competent mRNP. Similarly, She2p loading onto the nascent mRNA would be limited if the Spt4-Spt5 elongation complex assembly was defective. However, whether the Spt4-Spt5 complex interacts with She2p before association with Pol II or is recruited to Pol II before association with She2p remains unclear. While our experiments establish a Spt4-Spt5 complex dependent mechanism of She2p recruitment, they do not exclude the possibility of additional mechanisms that could also contribute to She2p recruitment at transcribed genes. This model is indeed supported by recent data from the Hartzog lab, which used mass spectrometry to show that Spt5p coimmunopurifies with translational repressors Loc1p and Puf6p (Lindstrom et al., 2003). Interestingly, among the proteins that coimmunopurify with Spt5p, Lindstrom et al did not detected She2p, probably due to its small size or higher-salt conditions which disrupted its interaction with Spt5p. Moreover, a recent study showed that

RAD6, which encodes a E2 ubiquitin-conjugating enzyme required for post-replicational DNA repair, transcriptional activation and repression, protein degradation, and sporulation (Prakash et al., 2005; Jentsch et al., 1987), genetically interacts with *SHE2* (Pan et al., 2006). These results are consistent with an interaction between She2p and the transcription machinery.

In summary, we have identified a new connection between cytoplasmic mRNA localization and the transcriptional machinery mediated by an interaction between the localization factor She2p and transcriptional elongation factor Spt4-Spt5 complex. By physically linking the recruitment of She2p with Spt4-Spt5 complex, this mechanism coordinates the assembly of mRNA localization RNP with transcription. Since several RNA-binding proteins involved in mRNA localization have a nuclear residency, this mechanism may represent a universal processing event among localized mRNAs in eukaryotes. Although our results demonstrate a link between mRNA localization and transcription elongation factors, the details of how mRNA localization is coordinated with transcriptional machinery are still unclear. In the future, it will be interesting to elucidate how the interaction between She2p and Spt4-Spt5 complex affects transcription and assembly of the localization competent mRNP.

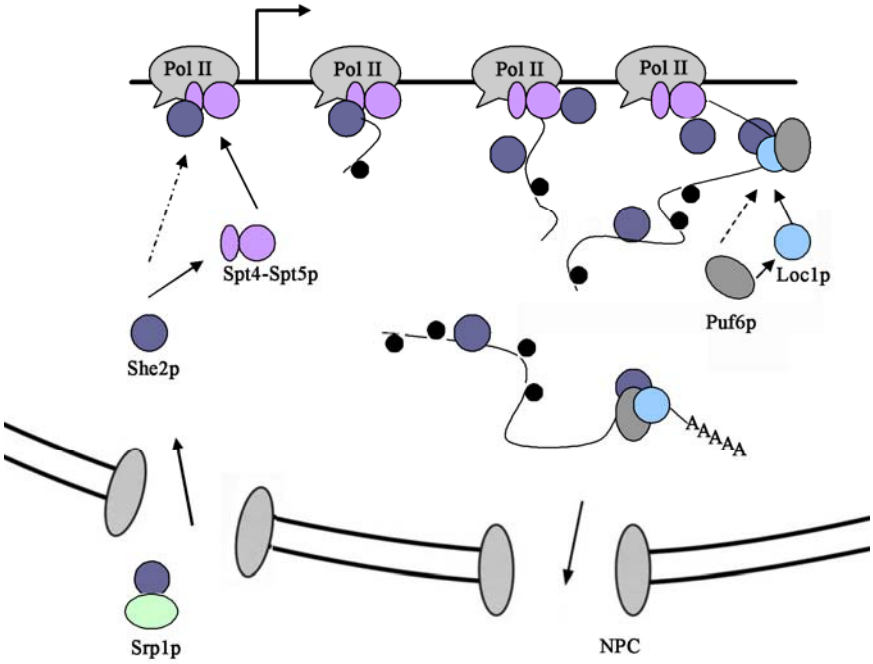

Figure 3: Model for cotranscriptional recruitment of *ASH1*mRNA trans-acting factors. She2p is actively imported into nucleus by Srp1p, and it gets recruited to the transcription machinery by its interaction with the Spt4-Spt5 complex in the preinitiation complex and/or elongating Pol II. She2p is then transferred to the zipcode as it appeared in the nascent transcript. Further recruitment of She2p continues as elongation proceeds for next zipcode. Loc1p and Puf6p are also recruited to the localization element E3 of the nascent *ASH1* transcript, possibly dependent on She2p. Other RNA-binding proteins are indicated (black). Cotranscriptional recruitment of these factors promotes efficient cytoplasmic *ASH1* mRNP localization to the bud tip of daughter cells.

Conclusion

1) She2p contains a non-classical nuclear localization signal sequence (NLS) which is essential for its nuclear import via the importin-α Srp1p. Exclusion of She2p from the nucleus by mutagenesis of its NLS disrupt the binding of Loc1p and Puf6p to the *ASH1* mRNA, leading to defective mRNA localization and Ash1p sorting.

2) She2p interacts with the elongating RNA pol II and is associated with genes coding for both localized and non-localized mRNAs. Our results showed that RNA Pol II may be used as a plateform by She2p to promote its recruitment on nascent transcripts. Association of She2p with Pol II by its interaction with Spt4-Spt5 complex resulted in this localization factor cotransciptional binding to the nascent *ASH1* mRNA. Moreover, Puf6p is also recruited on the *ASH1* gene, but in a She2p-dependent manner.

3) Our results suggest that stepwise assembly of localization factor (She2p) and translational repressors (Puf6p and Loc1p) on nascent *ASH1* mRNA is important for the cytoplasmic fate of this transcript. More importantly, we confirm a new connection between cytoplasmic mRNA localization and transcriptional machinery mediated by a contact between the localization factor She2p and elongation complex Spt4-Spt5. The mechanism of coupling beween cytoplasmic localication of mRNA and transcription machinery may represent a universal processing event among localized mRNAs.

In the future, it will be interesting to elucidate the role of post-translational modification in the regulation of the nuclear import of She2p, the interaction of She2p with Loc1p, Puf6p, and Spt4-Spt5 complex. As well as how these interactions affect transcription and assembly of the localization competent mRNP for their cytoplasmic fate.

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