Université de Montréal

Phosphorylation of the RNA-binding protein She2 and its impact on mRNA localization in yeast

Par
Nastaran Farajzadeh

Département de Biochimie et Médecine Moléculaire
Programme de biochimie Faculté de médecine

Thèse présentée à la Faculté des études supérieures
En vue de l'obtention du grade de *Philosophiae Doctor (Ph.D)*
En biochimie

Novembre 2022

Université de Montréal

Faculté des études supérieures

Cette thèse intitulée

Phosphorylation of the RNA-binding protein She2 and its impact on mRNA localization in yeast

Présentée par

Nastaran Farajzadeh

A été évaluée par un jury composé des personnes suivantes

Marlene Oeffinger
Président-rapporteur

Pascal Chartrand
Directeur de recherche

Serguei Chteinberg
Membre du jury

Maria Vera Ugalde
Examinateur externe

Hugo Wurtele
Représentant du doyen de la FES

RÉSUMÉ

La localisation de l'ARNm est un mécanisme post-transcriptionnel régulant l'expression des gènes qui donne un contrôle précis sur la production spatiale et temporelle des protéines. Des milliers de transcrits dans un large éventail d'organismes ou de types cellulaires se sont avérés localisés dans un compartiment sous-cellulaire spécifique. La levure bourgeonnante *Saccharomyces cerevisiae* est l'un des organismes modèle les plus étudiés pour comprendre le processus de localisation de l'ARNm. Plus de trente ARNm sont activement transportés et localisés à l'extrémité du bourgeon de la levure bourgeonnante. Dans cet organisme, la localisation des transcrits à l'extrémité du bourgeon, tels que l'ARNm *ASH1*, dépend de la protéine de liaison à l'ARN She2, qui interagit directement avec les éléments de localisation dans ces ARNm durant leur transcription. She2 est une protéine liant l'ARN non-canonique, qui s'assemble en tétramère pour pouvoir lier l'ARN. Lorsque le complexe ARNm-She2 est exporté vers le cytoplasme, celui-ci interagit avec la protéine She3 et la myosine Myo4, qui transportent le complexe vers le bourgeon. Une fois qu'un ARNm est correctement localisé, sa traduction est activée pour permettre la synthèse locale de sa protéine. Les mécanismes régulant la localisation des ARNm sont encore très peu connus. Cependant, plusieurs évidences suggèrent que la machinerie de localisation peut être régulée par des modifications post-traductionnelles. Dans notre étude, en utilisant une colonne de purification de phosphoprotéines, nous avons constaté que She2 est une phosphoprotéine. Nous avons utilisé une approche de phosphoprotéomique pour identifier les résidus phosphorylés dans She2 *in vivo*. Nous avons identifié plusieurs nouveaux phosphosites qui affectent la capacité de She2 à favoriser l'accumulation asymétrique de la protéine Ash1. Fait intéressant, plusieurs phosphosites sont présents aux interfaces de dimérisation et de tétramérisation de She2. En nous concentrant sur la position T109, nous montrons qu'un mutant phosphomimétique T109D inhibe l'interaction She2-She2 et diminue l'interaction de She2 avec ses cofacteurs Srp1, She3 et l'ARNm *ASH1*. Fait intéressant, la mutation T109D réduit considérablement l'expression de She2 et perturbe la localisation de l'ARNm *ASH1*. Nos résultats montrent que le contrôle de l'oligomérisation de She2 par phosphorylation représente un mécanisme qui régule la localisation de l'ARNm dans la levure bourgeonnante.

Dans le but d'identifier la ou les kinases impliquées dans la phosphorylation de She2, nous avons recherché des motifs de reconnaissance de kinases connues parmi les phosphosites que nous avons identifiés. Nous avons trouvé que les résidus T109, S217 et S224 font partie de sites putatifs de la Caséine kinase II (CKII), suggérant que ces positions seraient susceptibles d'être phosphorylés par cette kinase. Un essai de phosphorylation *in vitro* a révélé que She2 est phosphorylée par CKII au niveau des résidus S217 et S224, mais pas au résidu T109. Nous avons montré que la phosphorylation de la forme monomérique de She2 par CKII *in vitro* est augmentée par rapport à la forme sauvage tétramérique. De plus, nous avons observé que le domaine C-terminal de She2, qui contient sa séquence de localisation nucléaire (NLS) est phosphorylé par CKII. Cependant, le rôle de la phosphorylation dans le NLS de She2 demeure inconnu. Dans l'ensemble, nos résultats montrent que les modifications post-traductionnelles sur She2 régulent la localisation de l'ARNm chez la levure. Cette étude permettra d'élucider les mécanismes de contrôle de la localisation de l'ARNm chez la levure et comment des modifications post-traductionnelles sur She2 régulent ce processus.

Mots-clés: localisation de l'ARNm, ARNm *ASH1*, modifications post-traductionnelles, phosphorylation de She2, signal de localisation nucléaire (NLS), caséine kinase II (CKII), levure.

ABSTRACT

mRNA localization is a post-transcriptional mechanism regulating gene expression that gives precise control over the spatial and temporal production of proteins. Thousands of transcripts in a wide array of organisms or cell types were shown to localize to specific subcellular compartments. The budding yeast *Saccharomyces cerevisiae* serves as one of the best model organisms to study the mechanisms of mRNA localization. Over thirty transcripts are actively transported and localized at the bud tip of the budding yeast. In this organism, localization of transcripts to the bud tip, such as the *ASH1* mRNA, depends on the RNA-binding protein She2, which is responsible for recognizing localization elements in these mRNAs during transcription. She2 is a non-canonical RNA-binding protein which assembles as a tetramer in order to bind RNA. When the mRNA-She2 complex is exported to the cytoplasm, the protein She3 and myosin Myo4 join the complex to transport it to the bud. Once an mRNA is properly localized, its translation is generally activated to allow the local synthesis of its protein.

The mechanisms regulating the localization of mRNAs are still poorly known. Still, several pieces of evidence suggest that post-translational modifications may regulate the localization machinery. Using a phosphoprotein purification column, we found that She2 is a phosphoprotein. We used a phosphoproteomic analysis to identify the phosphorylated residues in She2 *in vivo*. We identified several new phosphosites that impact the capacity of She2 to promote the asymmetric accumulation of Ash1. Interestingly, several of these phosphosites are present at the dimerization and tetramerization interfaces of She2. Focusing on T109, we show that a phosphomimetic mutant T109D inhibits She2-She2 interaction and decreases the interaction of She2 with its co-interactors Srp1, She3 and *ASH1* mRNA. Interestingly, the T109D mutation significantly reduces the expression of She2 and disrupts *ASH1* mRNA localization. Altogether, our results show that the control of She2 oligomerization by phosphorylation represents a mechanism that regulates mRNA localization in budding yeast.

In order to identify which kinase(s) are involved in She2 phosphorylation, we searched for known kinases recognition motifs among the identified phosphosites. We found that T109, S217 and S224 are putative Casein kinase II (CKII) sites, suggesting that this kinase may phosphorylate these residues. Indeed, an *in vitro* phosphorylation assay revealed that She2 is phosphorylated by CKII

at S217 and S224 but not at T109. We found that the phosphorylation of a monomeric She2 mutant by CKII *in vitro* is increased compared to the wild-type tetrameric protein. Furthermore, we found that the C-terminal domain of She2, which contains its nuclear localization signal (NLS), is phosphorylated by CKII. However, the biological function of the phosphorylation in the NLS is still unknown. Altogether, our results show that post-translational modifications in She2 regulate mRNA localization in yeast. This study will help elucidate the mechanisms that control mRNA localization in yeast and how post-translational modifications in She2 regulate this process.

Keywords: mRNA localization, *ASH1* mRNA, post-translational modifications, She2 phosphorylation, nuclear localization signal (NLS), casein kinase II (CKII), yeast.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

Abbreviations

RNA	Ribonucleic acid
ASH	asymmetric synthesis of HO
ATP	Adenosine triphosphate
hnRNP	Heterogeneous nuclear ribonucleoprotein
HO endonuclease	Homothallic switching endonuclease
NLS	Nuclear localization signal
NPC	Nuclear pore complex
S. cerevisiae	Saccharomyces cerevisiae
SHE	Swi5p-dependent HO expression
UTR	Untranslated region
lncRNA	Long non-coding RNAs
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green fluorescent protein
YFP	Yellow fluorescent protein
siRNA	Small interfering RNA
Kd	kilodalton
tRNA	Transfer ribonucleic acid
ORF	Open reading frame
et al.	et al.ii (from Latin, “and others”)
DNA	deoxyribonucleic acid

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

Chapter 1

Introduction

1.1 Post-transcriptional gene regulation

Post-transcriptional gene regulation is required for cellular metabolism, maturation, transport, stability, and degradation of all RNA types. Cells regulate many stages in the RNA-to-protein route to control gene expression [1]. Most genes are assumed to be regulated at several levels, but regulation of transcriptional initiation often predominates. On the other hand, some genes are transcribed at a steady rate and rolled by posttranscriptional regulatory mechanisms. Following transcription within the nucleus, a series of conserved processing steps, known as post-transcriptional regulation, are required to produce a mature, functioning mRNA molecule capable of translation in the cytoplasm. These processes include: (1) termination of the mRNA transcription, (2) alternative mRNA splicing, (3) control of 3 ′-end formation by cleavage and poly-A addition, (4) RNA editing, (5) control of mRNA transport from the nucleus to the cytosol, (6) regulated mRNA degradation, (7) control of mRNA translation initiation [2, 3]. Once in the cytoplasm, mRNA can be localized to particular regions of the cell, stored in cytoplasmic bodies for future translation or targeted for decay. In this thesis, we focus on the mechanisms of mRNA localization and localized translation, which represent crucial steps in post-transcriptional gene regulation and protein synthesis.

1.1.1 mRNA localization

mRNA localization is a post-transcriptional mechanism regulating gene expression that gives precise control over the spatial and temporal production of proteins. Beta-actin mRNA was the first localized transcript discovered in the myoplasm of Ascidian eggs in the 1980s [4]. Since recent high-throughput technologies have exposed the extent of this phenomenon and revealed that it is much more prevalent than previously assumed. Thousands of transcripts were shown to localize to specific subcellular compartments in a wide array of organisms or cell types [5, 6]. Analysis of thousands of genes has shown that 71% of mRNAs are targeted to a specific subcellular location in Drosophila oocytes [7]. Also, a combination of biochemical cell fractionation with RNA-sequencing (CeFra-seq) analysis revealed that more than 80% of transcripts are

asymmetrically localized in human and Drosophila cells [8]. New technologies such as APEX-RIP, an alternate high-throughput method relying on two combined technologies, APEX (engineered ascorbate peroxidase-catalyzed proximity biotinylation of proteins and RNA) and Immunoprecipitation, allow the detection of localized RNAs in a variety of subcellular compartments [9]. Recently, computational predictions of mRNA sub-cellular localization based on sequence features, along with microscopy-based approaches, have paved the way to characterize mRNA localization for an extensive range of RNAs and conditions [10].

Restriction of protein expression to a specific subcellular region plays a crucial role during developmental processes such as asymmetric cell division, cell migration, neuronal maturation and embryonic patterning (Figure 1-1) [11]. Cytoplasmic mRNA transport and localization have been studied in various organisms, ranging from yeast to highly polarized cells like neurons. They have even been identified in simple systems such as bacteria (Figure 1-1) [12]. Different transcripts in these various cells recruit machinery that shares common characteristics. Typically, these transcripts contain a cis-acting element(s) in their sequence, known as zipcode or localization elements, which are recognized by specific trans-acting elements or RNA-binding proteins [3, 13].

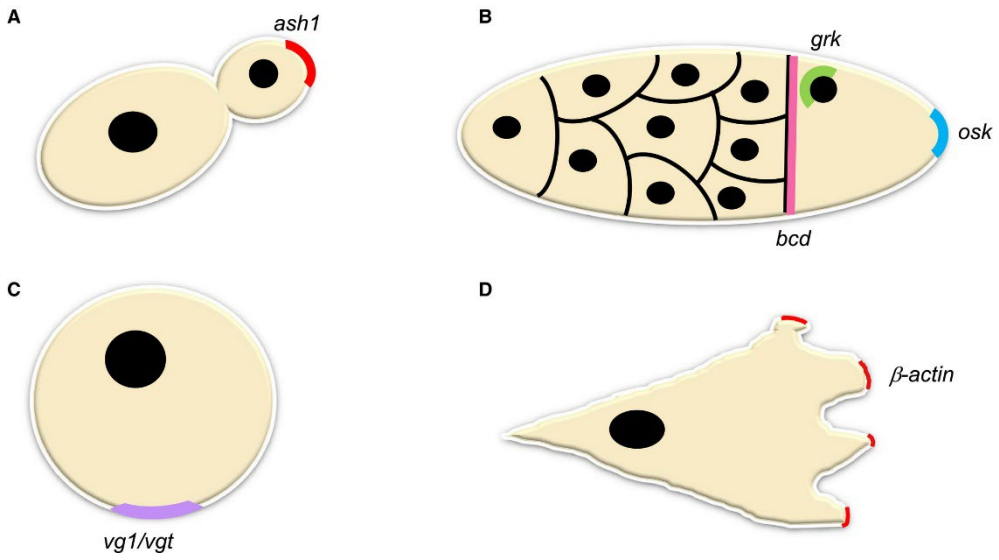


FIGURE 1-1 MRNA LOCALIZATION HAS BEEN FOUND IN A VARIETY OF SPECIES AND CELL TYPES.

(A) *ASH1* MRNA LOCALIZATION TO *SACCHAROMYCES CEREVISIAE* BUD TIP. (B) OSK, GRK, AND BCD MRNA LOCALIZATION IN THE DROSOPHILA OOCYTE. (C) VG1/VGT MRNA LOCALIZATION IN THE XENOPUS OOCYTE. (D) Β-ACTIN MRNA LOCALIZATION TO THE CELL LEADING EDGE OF CHICKEN FIBROBLASTS. (SOURCE: BOVAIRD ET AL., 2018)

1.1.1.1 Biological functions of mRNA Localization

Subcellular localization of maternal transcripts has been reported in various organisms and cells, such as in *Drosophila melanogaster*, *Xenopus laevis,* and mammalian cells, which are crucial to determine cell fate. During oogenesis in the fruit fly Drosophila, *oskar* and *nanos* mRNAs are restricted to the posterior pole of the oocyte and are responsible for the posterior development of the early embryo [14]. Similarly, proper localization of *bicoid* mRNA to the anterior pole is essential for the anterior development of the embryo [15]. Restriction of several transcripts to the vegetal pole of *Xenopus* oocyte, such as *Xcat2/Nanos1*, *Xdazl*, *Vg1* and *VegT*, is necessary for mesoderm and endoderm induction and patterning [11]. In addition to the role of localized mRNA in embryonic patterning, observation of intracellular trafficking of mRNA in a variety of somatic

cells, such as fibroblasts [16], oligodendrocytes [17] and neurons [18], suggests the broader biological importance of mRNA localization. In several cell types, *β-actin* mRNA undergoes localization to the cell protrusions, where actin is actively polymerized at the leading edge of migratory cells. Chicken fibroblast *β*-actin mRNA was the first examined transcript in differentiated cell types and was shown to be essential for cell motility [19] and in the motility of neuron growth cones [20].

In the central nervous system, mRNA coding for the microtubule-associated protein tau, which is responsible for the stabilization of microtubules in neurons, and myelin basic protein (MBP) in oligodendrocytes are localized in axons [21-23]. A number of transcripts are asymmetrically distributed in the dendrites of mature neurons, like mRNAs encoding the α-subunit of calcium-calmodulin kinase II (CaMKII), Arc protein, MAP2, and the α-subunit of the glycine receptor [18, 24-26]. In neural cells, mRNA localization regulates synaptic plasticity. The activity-regulated cytoskeletal (Arc) gene encodes a protein that mediates memory consolidation and is involved in synaptic strength and plasticity. Removal of Arc in knockout (KO) animals affects long-term learning [27].

Messenger RNA localization is not only confined to animal cells, but it has also been documented in bacteria, algae, and plants [28, 29]. Interestingly, mRNA localization occurs within plant cells[30], like rice seed storage protein localization to the specific region of the endoplasmic reticulum or in several chloroplast proteins that are targeted to a specific compartment of chloroplasts in the green alga *Chlamydomonas*. In addition, a growing number of mRNA in plants undergo extracellular trafficking through plasmodesmata into the plant vascular system. Ham and his colleagues revealed that *GAIP* mRNA undergoes long-distance extracellular trafficking within the phloem stream of pumpkin [31]. In the following sections, we will review the currently known biological functions of mRNA localization.

1.1.1.1.1 Localized mRNAs and cell motility

Cell motility is a key biological process involved in tissue formation and homeostasis. Extending protrusions at a migrating cell's leading front is a crucial step in directed cell migration. Signaling and cytoskeletal proteins were discovered to be asymmetrically distributed at the leading protrusions, where they play critical roles in cell polarity and motility [32]. A large number of protrusion-localized mRNAs have been detected in cultured neurons, cancer cells, fibroblasts, and astrocytes using a modified Boyden chamber method to separate cell pseudopodia from the cell body [33].

One of the best-studied mRNAs targeted to cell protrusions is the *β-actin* mRNA, which localizes to the protrusion of migrating fibroblasts, myoblasts and in the growth cone of neurons. *β-actin* mRNA localization requires a conserved 54 nucleotide sequence acting as a zipcode in its 3' UTR and a combination of microtubule and/or actin-dependent transport, depending on the cell type [4, 19]. Early research revealed that chemoattractant signals, like lysophosphatidic acid (LPA) and serum, induce β-actin transcript to rapidly polarize towards the leading edge of motile cells. Translationally silent *β-actin* mRNA is transported to its destination by the Rho-mediated pathway and the subsequent activity of the motor protein myosin IIB [34]. *β-actin* mRNA interacts with the translation elongation factor 1α (EF1α) once reaching its final destination, and this interaction is important for anchoring transcripts to the cytoskeleton (Figure 1-2) [35]. Interestingly, delocalization of β-actin mRNA from the leading-edge results in loss of cell polarity and alters the cell migration ability [36]. The Zipcode-binding protein 1 (ZBP1) is an RNA-binding protein with two KH domains that bind to the *β-actin* mRNA zipcode sequence in the nucleus, guiding it through the nucleus in a translationally repressed form to the cell edge [37]. The Src kinase phosphorylates ZBP1 at the cell edge in response to an extracellular signal, leading to the dissociation of ZBP1 from *β-actin* mRNA and translation (Figure 1-2) [38]. In addition to being abundant at the leading edge of migratory fibroblasts, *β-actin* mRNA is also concentrated at focal adhesions (FAs) and enhances the adhesive capabilities of migrating fibroblasts and epithelial cells [39].

The tumour suppressor protein adenomatous polyposis coli (APC), which has been less investigated than ZBP1, is another trans-acting factor that plays a key role in mRNA localization

during cellular migration. Interaction of APC with the plus end of microtubules growing in protrusion areas increases the stability of microtubules and cell motility [40]. Mechano-transduction signals followed by substrate stiffness and microtubule detyrosination promote APC-dependent RNA localization at cell protrusions [41]. The RNA-binding protein FUS (Fused in Sarcoma) is a component of APC-RNPs at cell protrusions and is required for the regulation of APC-transported transcripts but not for APC-RNP localization [42]. Mutations and inclusions in FUS observed in amyotrophic lateral sclerosis (ALS) patients disrupt microtubule detyrosination and prevent transcript targeting through APC, suggesting a direct correlation between mRNA localization mechanisms and disease (Figure 1-2) [33].

The actin polymerization nucleator ARP2/3 complex is also localized at the leading edge during cell migration and plays a critical role in site-directed actin polymerization [43]. All of the seven transcripts encoding Arp2/3 complex subunits are localized at the protrusions of primary chicken embryo fibroblasts and primary human foreskin fibroblasts [43]. Like *β-actin* mRNA, the localization of Arp2/3 complex subunits is dependent on the activation of Rho and myosin II. Mis-localization of *Arp2* mRNA alone results in a decrease in Arp2/3 complex formation as well as in narrow protrusions, increased random cell migration, and a lack of directionality in cell movement [44]. In addition to the *β-actin* and *Arp2/3* mRNAs, the Cofilin1 transcript (*CFL1* mRNA), which encodes for a regulator of actin dynamics by enhancing actin treadmilling, promoting membrane protrusion and directed cell motility, is also targeted to the cellular front of motile cells [45, 46]. CFL1 deficiency causes distinct changes in cell migration, focal adhesion turnover, and the production of abnormal actin structures [47]. The activity of cofilin is regulated not only by post-translational processes but also by the localization of its mRNA by VICKZ. The VICKZ family of RNA-binding proteins (which includes ZBP1) associates with the cofilin mRNA 3′UTR and mediates its localization.

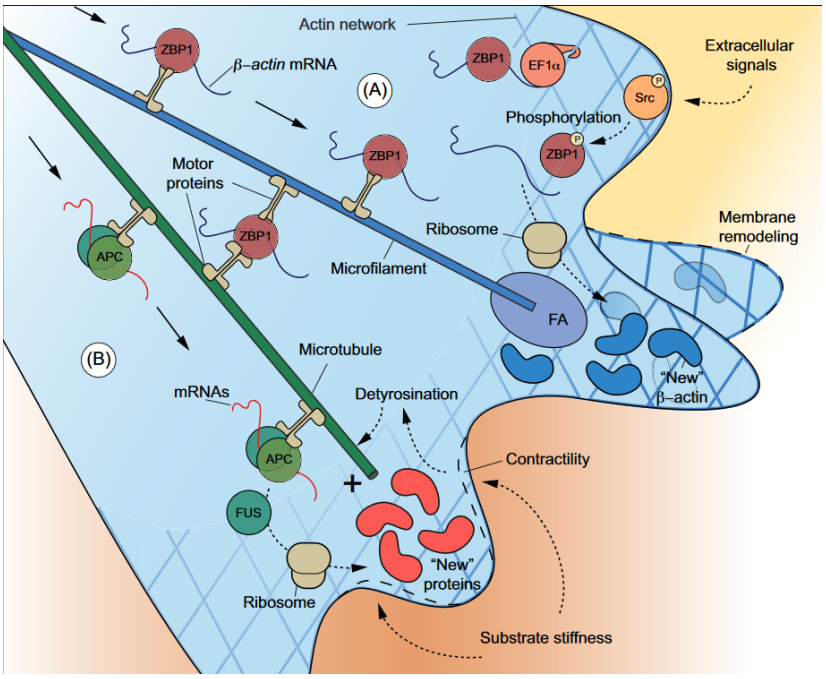


FIGURE 1-2 TWO MAIN PROCESSES COORDINATE MRNA LOCALIZATION TO CELLULAR PROTRUSIONS.

(A) ZBP1 TRANSPORTS *β-ACTIN* MRNA IN A TRANSLATIONALLY SUPPRESSED STATE ALONG MICROFILAMENTS AND MICROTUBULES. (B) APC GUIDES MRNAS TO THE POSITIVE (+) END OF DETYROSINATED MICROTUBULES. THE RNA-BINDING PROTEIN FUS REGULATES THE TRANSLATION OF LOCALIZED MRNAS IN APC-RNP GRANULES. FA: FOCAL ADHESIONS (SOURCE: HERBERT ET AL., 2019)

1.1.1.1.2 Localized mRNAs and Asymmetric Cell Division

Asymmetric cell division is a conserved mechanism that controls cell fate and gives rise to cell diversity. Asymmetric mRNA segregation can occur during cell division and participates in asymmetric cell division in Drosophila, yeast and other eukaryotes cell types. For instance, localization of the glial determinant-encoding *glide/gcm* mRNA in early Drosophila embryos results in the asymmetric division of neuroglioblasts and determines the cell fate of the different siblings [48]. The asymmetric localization of cell fate determinants does not always depend on mRNA localization. While localization of *prospero* mRNA is not essential for Prospero

asymmetric localization in Drosophila neuroblasts, proper asymmetric location of Ash1 to the daughter cell nucleus relies on asymmetric *ASH1* mRNA localization in budding yeast [49].

Interestingly, Spiralian has provided excellent model organisms to study the association between the localization of mRNAs and the asymmetric distribution of their protein products. For example, centrosomal localization of mRNAs from several patterning genes, such as Eve, DPP and Tld, allows their segregation into one daughter cell during cellular division [50]. Widespread analysis of mRNA localization revealed that centrosomal localization of mRNAs is found in oocytes of the surf clam (*Spisula solidissima*) and in *D. melanogaster* embryos [7, 51]. Messenger RNA localization to centromeres during embryonic patterning has been frequently observed, which suggests that this mechanism is part of the developmental process [52]. A significant proportion of mRNAs are localized to one of the two centrosomes and are asymmetrically inherited throughout the early cleavage cycles of the *Ilyanassa* mollusk embryo (Figure 1-3A). The majority of these mRNAs encode for proteins involved in developmental patterning and regulate cell fate in these animals, suggesting that their differential segregation regulates cell fate determination [50, 53]

The transcription factor *Not* mRNA is transferred to the cytoplasm of the future mesoderm-forming pole in mesendoderm cells of Halocynthia ascidian embryo and is asymmetrically targeted to the mesoderm daughter cell by cytokinesis (Figure 1-3B). *Not* mRNA promotes mesoderm destiny and inhibits endoderm fate, implying that asymmetric mRNA inheritance is responsible for the segregation of germ layer fates [54]. Furthermore, in Drosophila embryos, neural precursors (neuroblasts) usually divide asymmetrically to form an apical ganglion mother cell (GMC) and a neuroblast daughter. The *prospero* mRNA is localized to the basal side of neuroblasts and encodes for Prospero, a transcription factor critical for the activation of GMC-specific gene expression. The adaptor protein Inscuteable is targeted apically during anaphase in Drosophila neuroblasts and promotes coupling of cell division direction and asymmetric mRNA localization. Notably, mislocalization of the *inscuteable* mRNA reduces the apical concentration of Inscuteable protein and is related to mitotic spindle misorientation. (Figure 1-3C) [11].

Besides mRNAs, long noncoding RNAs (lncRNAs) are also submitted to asymmetric subcellular localization. For instance, the long non-coding RNA *cherub* segregates into the differentiating daughter cell in mitotic larval neuroblasts. *Cherub* lncRNA relies on Staufen for its localization and function as a molecular link between RNA-binding proteins Staufen and the late temporal identity factor Syncrip [55]. Surprisingly, the *cherub* is not required for the normal development of neuroblasts but is indispensable for brain tumour development in Drosophila. The long non-coding RNA lincGET (Gm45011) plays a key role in cell fate establishment in early mouse embryos. LincGET is asymmetrically distributed in two- to four-cell blastomeres and interacts physically with coactivator-associated arginine methyltransferase 1 (CARM1), which promotes its nuclear localization. LincGET/CARM1 biases early blastomeres towards an inner cell mass (ICM) fate by activating inner cell mass genes [56].

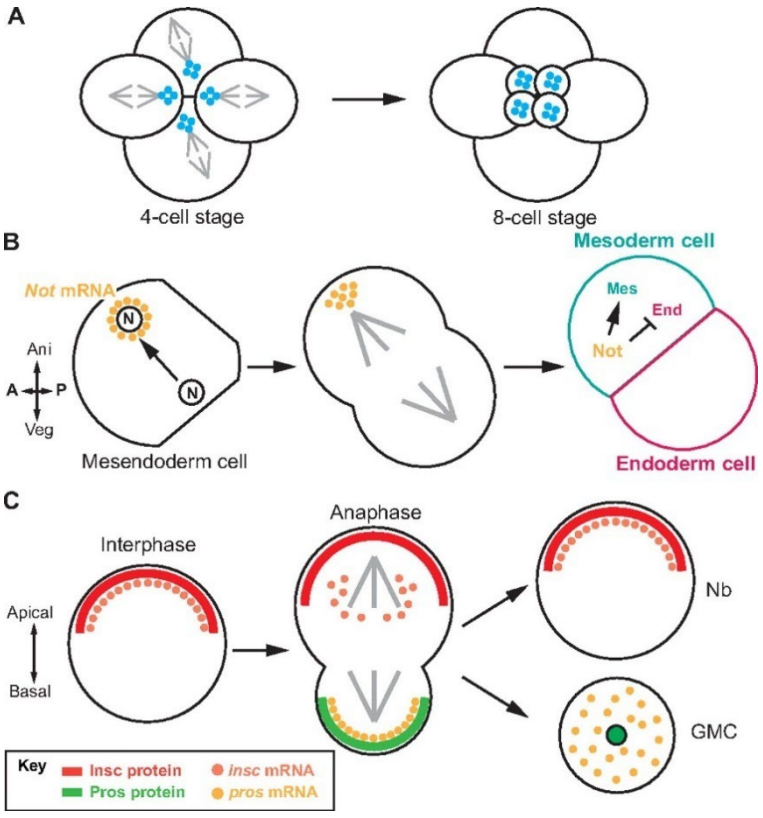


FIGURE 1-3 MESSENGER RNA LOCALIZATION IN DIVIDING CELLS

(A) AT THE 4-CELL STAGE IN ILYANASSA EMBRYOS, DISTINCT MRNAS (BLUE) LOCALIZE TO ONE OF THE TWO CENTROSOMES OF METAPHASIC CELLS (LEFT). THESE MRNAS ARE INHERITED DIFFERENTLY BY DAUGHTER CELLS DURING DIVISION (RIGHT). (B) NUCLEAR MIGRATION DELIVERS *NOT* MRNA (YELLOW) TO ONE SIDE OF A HALOCYNTHIA EMBRYO MESENDODERM CELL. (C) NEUROBLASTS (NB) FROM DROSOPHILA EMBRYOS DIVIDE ASYMMETRICALLY TO REPRODUCE A NEUROBLAST AND GENERATE A SMALLER CELL, THE GANGLION MOTHER CELL (GMC). *PROSPERO* (*PROS*) MRNA (YELLOW) AND PROS PROTEIN (GREEN) LOCALIZE BASALLY DURING ANAPHASE, WHEREAS *INSCUTEABLE* (*INSC*) MRNA (ORANGE) AND INSC PROTEIN (RED) ARE LOCATED TO THE APICAL SIDE OF NEUROBLASTS, ENSURING THAT THE TWO COMPONENTS ARE INHERITED DIFFERENTLY. A: ANTERIOR; ANI: ANIMAL; P: POSTERIOR; VEG: VEGETAL. (SOURCE: MEDIONI ET AL., 2012).

1.1.1.1.3 mRNA localization and embryonic patterning

In many organisms, including Drosophila, the asymmetric distribution of mRNA molecules in oocytes and eggs regulates early development that determines embryonic axes and cell fates [11]. The Drosophila oocyte is an established model to study not only mRNA localization but also translational regulation. Based on morphological criteria, the development of the egg chamber during *Drosophila* oogenesis is composed of 14 defined stages. A set of 16 germline cells, which are enveloped by somatically derived follicular epithelial cells, form an egg chamber. Only one of these germline cells gives rise to the future oocyte, whereas the remaining 15 cells develop into nurse cells that synthesize maternal mRNAs and cytoplasmic components needed for oocyte specification and growth [57]. Anterior-posterior axis specification of the Drosophila embryo is one of the most intensively studied developmental processes. Four maternal mRNAs: *gurken* (*grk*), *bicoid* (*bcd*), *oskar* (*osk*) and *nanos* (*nos*) are, playing key roles in the development of the embryonic body axes in the Drosophila oocyte (Figure 1-4A) [58]. During anterior-posterior patterning events, *bicoid* and *oskar* mRNAs are transcribed in the nurse cells and move to the oocyte through cytoplasmic bridges termed ring canals. *Oskar* mRNA localizes to the posterior of the oocyte during mid-oogenesis and is translated immediately to produce the Oskar protein, which nucleates the polar granules [59]. Polar granules contain posterior and germ cell determinants such as Nos, leading to posterior patterning and primordial germ cell specification in the embryo (Figure 1-4B) [60, 61]. The establishment of both anterior-posterior and dorsal-ventral axes during oogenesis is achieved by Gurken (Grk) protein, an epidermal growth factor receptor (EGFR) ligand. The RNA-binding protein Orb/CPEB activates *oskar* and *gurken* mRNA translation by

promoting their cytoplasmic polyadenylation, which increases mRNA stability and translation [62, 63]. The Orb protein is phosphorylated by Casein Kinase 2, and Orb phosphorylation is required for its proper function, including *oskar* and *gurken* mRNAs translational control [62].

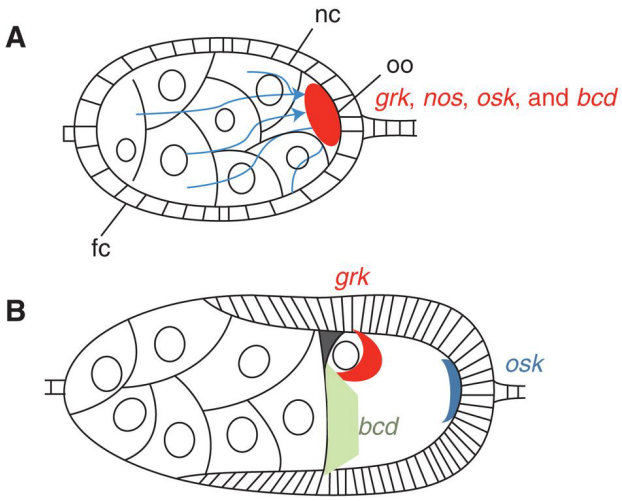


FIGURE 1-4 LOCALIZATION OF PATTERNING MRNAS DURING DROSOPHILA OOGENESIS

(A) IN EARLY OOGENESIS, SEVERAL MRNAS, INCLUDING *GRK*, *NOS*, *OSK*, AND *BCD*, ARE TRANSPORTED FROM THE NURSE CELLS THROUGH CYTOPLASMIC BRIDGES CALLED RING CANALS INTO THE OOCYTE. ABBREVIATIONS: NC, NURSE CELLS, FC, FOLLICLE CELLS, OO, OOCYTE. *(B)* IN MID-OOGENESIS, *OSK* MRNA LOCALIZES TO THE POSTERIOR OF THE OOCYTE, *GRK* MRNA LOCALIZES TO THE ANTERODORSAL CORNER IN CLOSE ASSOCIATION WITH THE OOCYTE NUCLEUS, AND *BCD* MRNA LOCALIZES TO THE ANTERIOR POLE. (SOURCE: LASKO, 2012).

1.1.1.1.4 Localization and transport of mRNAs in neurons

Neurons are highly polarized cells with particular dendritic and axonal compartments with unique functions that rely on their specific morphologies. In neurons, mRNA localization is required for establishing and maintaining such polarity [64]. Messenger RNA localization plays a key role in both central and peripheral nervous systems like neuronal cell division and neuroblast

maintenance, neuron migration, and synaptic activity (Figure 1-5). Fast Spatio-temporal regulation of protein synthesis required for long-term synaptic plasticity, the cellular basis for learning and memory, relies on mRNA localization [65]. Several mRNAs have been found to localize to the dendrites, among which Camk2a, Map2, Arc and Insp3r1 mRNAs. Recently, thousands of mRNAs localized to dendrites have been identified from the dendrite-enriched layer (SR) in the rodent hippocampus following a breakthrough approach in deep sequencing technology [66].

The α subunit of Calcium/calmodulin-dependent protein kinase II (CaMKII) is one of the well-studied dendritically localized mRNA at sites receiving high-frequency stimulation [67]. NMDA-induced neuronal activation promotes the localization of *Calmodulin-3* mRNA into dendrites in rat cortical neurons [68]. *β-actin* mRNA and its associated proteins are among transcripts localized in dendritic spines upon neuronal stimulation. Live-cell imaging of a GFP-tagged version of the *β-actin* mRNA binding protein ZBP1 in hippocampal neurons revealed the movement of the ZBP1 protein from the cell body to the base and spines of dendrites in response to KCl-induced depolarization [69]. Some studies suggest that proper subcellular localization of mRNAs in neurons is associated with their 3′ untranslated regions (UTR) [70, 71]. For instance, MBP mRNA contains cis-regulatory elements in the 3′ UTR, which are sufficient for mRNA localization to myelin sheaths in living zebrafish [72]. Despite the fact that some studies determined several 3′ UTR cis-acting elements, attempts to identify a universal “zipcode” sequence conducting mRNA localization have failed [72]. Microtubule filaments are essential to maintain directed mRNA transport in neurons. Two major motor proteins move cargoes in a plus-end or minus-end direction, namely kinesin and dynein, respectively [73]. Also, disruption of the actin filaments results in slightly affected cue-stimulated increases in axonal mRNA localization, suggesting that myosin-driven motor transport might be involved in short-range axonal mRNA transport and axonal cargo sorting [74].

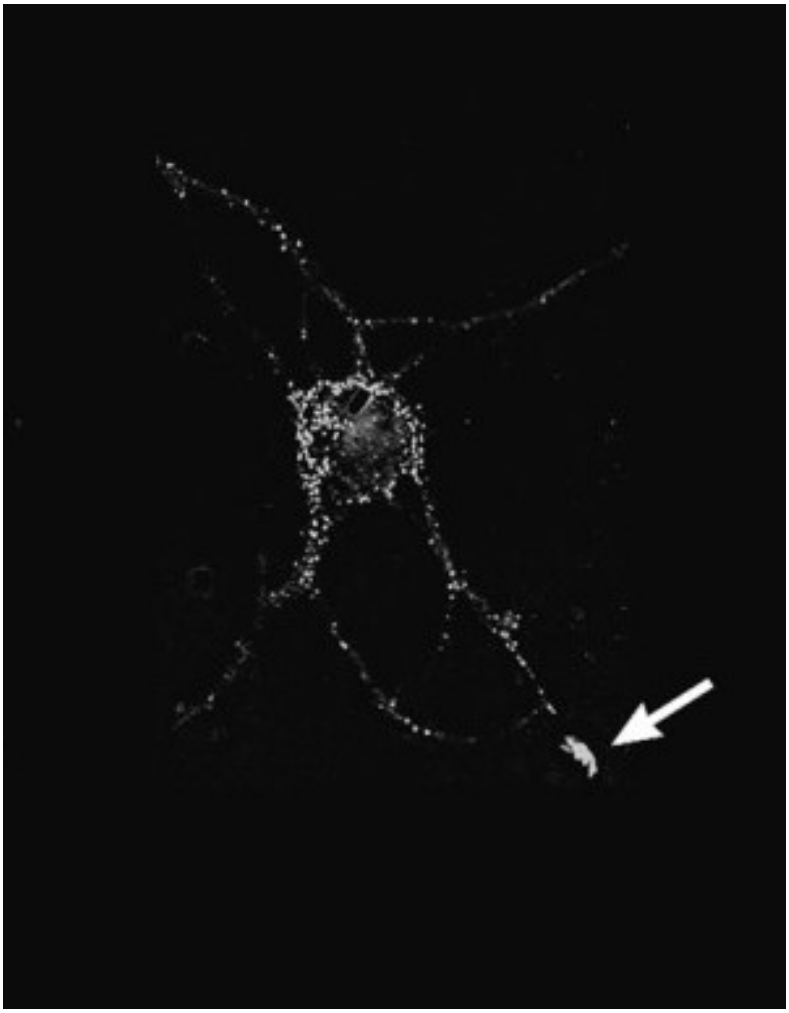


FIGURE 1-5 *β-ACTIN* MRNA LOCALIZATION IN THE AXON OF CULTURED HIPPOCAMPAL NEURONS**.**

ARROW INDICATES THE ACCUMULATION OF MRNA IN THE GROWTH CONES OF AXONS. (SOURCE: BASSELL ET AL, 1998).

1.1.1.2 Mechanisms of mRNA localization

After transcription, most mRNAs leave the nucleus to the cytoplasm, where they are translated. However, an increasing number of mRNAs have been found to be targeted to distinct regions within the cells. Studies in various systems revealed that three primary mechanisms had been documented for mRNA localization. One mechanism consists of the diffusion of the transcript

in the cytoplasm and its anchoring to a membrane [75]. Another mechanism of mRNA localization is by protecting the transcript from degradation in a particular subcellular compartment via selective stabilization while the mRNA is rapidly degraded elsewhere in the cell [76]. Active transport of transcripts, which depends on molecular motors, appears to be the most predominant mechanism of localization used by the majority of cell types. These mechanisms of mRNA localization are not mutually exclusive, and a combination of mechanisms may be involved in the localization of a single transcript [77].

Several molecular motors, like dynein and kinesin, are implicated in the active transport of transcripts along microtubules, mostly for transport over long distances [78, 79]. However, non-canonical type V myosin usually carries mRNA over shorter distances [80]. Most localized mRNAs are delocalized in the presence of microtubule or actin-depolymerizing drugs, such as cytochalasin and nocodazole, to block the polymerization of actin and microtubules, respectively [81]. This supports the importance of active transport mechanisms [82-84]. The sequence of localized mRNAs contains specific signals required for reaching their destination. These signals are referred to as localization elements (LEs) or zipcodes [85].

Over the last decade, a large number of RNA-binding proteins have been characterized with a direct role in the transport of mRNA to their final destination [86]. An RNA-binding protein can interact with multiple transcripts, like the Fragile X mental retardation protein (FMRP), which is present in multiple mRNPs and interacts with mRNAs such as Arc, CaMKIIα, and PSD-95 [87]. Messenger RNAs, after being recognized by their corresponding RNA-binding proteins, are carried to their destination in the form of large RNA granules or particles [88]. The radius of RNA granules has been measured to be around 0.7 μm [89], suggesting that these particles can accommodate a large amount of RNA-binding proteins and mRNAs. Characterization of several granules in neurons demonstrated that such macromolecular complexes contain different RNA-binding proteins, localized mRNAs and molecular motors [90, 91].

Another newly described localization mechanism involves mRNA aggregation through phase separation and biomolecular condensates [92]. In this mechanism, mRNAs come together

into membrane-less organelles using liquid-liquid phase separation (LLPS) to form liquid-like RNP granules. The formation and dissociation of granules are under the control of post-translational regulation. Weak and multivalent interactions between RNA-binding proteins (RBPs) and RNAs are responsible for LLPS and the formation of RNP droplets [92]. Depending on the cellular environment, specific RNAs are enriched within biomolecular condensates in response to cellular cues. For instance, in order to reduce energy expenditure during damage repair, mRNAs from house-keeping genes, such as β-actin and GAPDH, are concentrated and restricted from translation in stress granules, while mRNAs implicated in stress-related repairs, such as heat shock proteins, are excluded from stress granules [93]. Despite limited knowledge about the mechanisms of mRNA incorporation into these structures, mRNAs enriched in biomolecular condensates play several roles during their formation, such as buffering phase separation or regulating the physical state of the condensates [94].

1.1.1.2.1 Localization elements (LEs) or zipcodes

Cis-acting elements act as a postal address, or zipcode, which contain the information necessary for the final sub-cellular destination of a specific mRNA. LEs are mostly present in the 3' untranslated region (UTR) of the mRNA but can also be found in the coding region, like in the yeast *ASH1* mRNA or *Drosophila gurken* and *bitesize* mRNAs [95, 96]. Zipcodes range in length from a very short localization element in myelin basic protein mRNA (MBP), which contains only 11 nucleotides, to several hundred nucleotides [97], or a series of short sequences, as in the case of *β-actin* or *Vg1* mRNAs [98]. Each of these elements can lead to the localization of a heterologous reporter mRNA molecule (e.g., GFP, YFP, etc.) when fused downstream to the reporter. Studies revealed that when localization elements were removed from their original parental transcript, they functioned as a separate functional entity [5]. Experiments may not always confirm the precise binding process of RNA-binding proteins to zipcodes, perhaps because of various binding preferences. The recognition and stabilization of the RBP-RNA interaction by certain RBPs are purely dependent on the well-defined sequence of their target RNA, while in other cases, the structural characteristics of their target RNA are essential [99].

Zipcodes can form different secondary and tertiary structures, such as stem-loops, at the RNA level to form the binding site for specific *trans*-acting factor(s). Eventually, localized mRNAs can have several zipcodes containing a number of sequentially acting or repeating signals. Several maternal mRNAs are localized in Drosophila and Xenopus oocytes through sequential targeting elements in their 3′UTR [100]. The bicoid localization element (BLE) in the *bicoid* mRNA 3′ UTR, with its modular architecture, is a well-known example of a zipcode containing several overlapping localization elements involved in sequential transport steps [98]. The whole 625-nucleotide of the *bicoid* mRNA 3′ UTR is required for proper localization of *bcd* mRNA to the anterior pole of the Drosophila oocyte. This includes a 50-nucleotide localization element called BLE1 (bcd localization element 1), which is essential to direct *bcd* mRNA from nurse cells into the oocyte and also for the early accumulation of this transcript at the anterior oocyte border [101]. Two stem-loop structures (stems IV and V) along with the BLE1 are involved in the early and later stages of mRNA localization, while anchoring the mRNA to the anterior pole of the Drosophila embryo requires the stem III [102].

The prevalent belief that zipcodes necessary for mRNA localization are only found in the 3′ UTR has been called into question since localization elements can be found in the 5′ untranslated regions (5′ UTR) or even in the coding sequence in yeast and Drosophila mRNAs [81]. For instance, *gurken* mRNA zipcodes, which are directed to the anterior–dorsal pole of Drosophila oocytes, are dispersed in both 5' and 3' regions of the transcript. Sequences in the 5' noncoding region enable the transcript to localize in the oocyte during the initial localization steps, while signals in the coding region and the 3'UTR are involved in later stages of *gurken* mRNA localization in egg chambers [103]. Another example of the transcript with localization signals embedded in its coding sequence is the *ASH1* mRNA in budding yeast (see Section 1.3.2).

1.1.1.2.2 Active mRNA Transport

There is abundant evidence implicating the active transport of mRNAs along cytoskeletal filaments as the most prevalent localization mechanism. Cytoskeletal-associated mRNA localization has been reported in neurons, where long-distance transport has been observed, but also in smaller cells such as budding yeast and in *Drosophila* embryos. The role of the cytoskeleton

in mRNA transport was revealed by the use of tubulin or actin-depolymerizing drugs, such as nocodazole or cytochalasin A, which inhibit mRNA transport and localization [104, 105]. Active mRNA transport is driven by motor proteins from all three main classes of motor proteins, which include kinesins, dyneins, and myosins, which carry cargoes along the cytoskeleton throughout the cell. These motors create force by hydrolyzing adenosine triphosphate (ATP), which causes conformational changes and allows active movement of the protein [74].

Myosins move on actin filaments, while kinesin and dynein motors use microtubules to deliver their cargo. While kinesins are responsible for plus-end directed transport (or anterograde transport) on microtubules, dyneins are primarily moving toward minus-end (or retrograde transport), although they are also able of bidirectional mobility [106-108]. These motor proteins can bind to different cargoes, such as cytoplasmic organelles or protein complexes, and then transport them to various intracellular regions either directly or through adaptor proteins [109]. One example of how motor-based transport is crucial for the localization of RNAs is the kinesin-based transport of *β-actin* mRNA to growth cones in developing neurons [110]. In addition, kinesin motors have been implicated in targeting the *Vg1* mRNA to the vegetal cortex of Xenopus oocytes. This process requires the coordinated activity of the microtubule-associated Kinesin1 and Kinesin2 motor proteins [111]. Active transport of *oskar* and *bicoid* mRNAs in *Drosophila melanogaster* from nurse cells into the oocyte is dependent on dynein to facilitate movement through polarized microtubule arrays [112].

Egalitarian (Egl) is an RNA-binding adaptor protein for the dynein motor and binds directly to mRNA and BicaudalD (BicD), the factor responsible for linking the Egl/mRNA complex with the dynein motor. Interaction between the Egl/BicD complex and dynein activates mRNA localization [113]. Interestingly, Egalitarian (Egl) is required for correct spatial localization of *oskar*, *bicoid* and *gurken* mRNAs in *Drosophila* oocytes and embryos [114]. A recent study identified PAT1 as a kinesin adapter which mediates *β-actin* mRNA localization to dendrites during neural development in mice. PAT1 was discovered to associate directly with ZBP1, and it also binds to KLC, a cargo-binding component of kinesin-I. Depletion of PAT1 by siRNAs disrupts the morphology of dendritic protrusions and neuronal growth cones [115]. The most well-studied

example of myosin-dependent mRNA localization is the budding yeast mRNA localization pathway to the bud tip, where several transcripts are actively transported along actin filaments via the type V myosin motor Myo4 [116] (see Section 1.3.3.3).

1.1.2 mRNA localization and translational control

Messenger RNA targeting combined with localized translation has been recognized as a key mechanism to restrict the spatio-temporal expression of proteins in cells. The ability to precisely control protein synthesis is particularly relevant for proteins that might be harmful to the cell when produced ectopically [117] and enhances interaction fidelity and signalling sensitivity [118]. The identification of RNA cis-regulatory elements by trans-acting factors and the subsequent formation of RNP complexes with distinctive structures and compositions allows for such accurate sorting. These complexes begin to form in the nucleus, sometimes in a co-transcriptional manner, and undergo dynamic remodelling at various stages [119]. As these mRNAs are exported to the cytoplasm, they must be isolated from the translational machinery until they reach their final destination. One solution to this problem involves packing these mRNAs into ribonucleoprotein complexes which include translational repressors to inhibit translation at various steps. Conserved RNA-binding proteins, such as ZBP1, have been found to mediate both mRNA targeting and translational repression, establishing a mechanistic connection between the two processes [120].

1.1.2.1 Mechanisms of translational control of localized mRNA

One mechanism to control the translation of localized mRNAs is through the inhibition of translation initiation, which is a highly regulated step in translation. Blocking the eIF4E–eIF4G interaction by recruiting an eIF4E-binding protein (eIF4E-BP) to the mRNAs is frequently used to repress translation during mRNA transport. For instance, in Drosophila oogenesis, translational repression of the *oskar* mRNA is achieved through the interaction of the Cup protein, which is an eIF4E-BP, and the RNA-binding protein Bruno, which binds the *oskar* mRNA 3′ UTR [121], Cup is recruited by 3′ UTR-associated repressors Smaug and inhibits translation of non-localized nanos mRNA in early Drosophila embryos [122]. The translational repressor Fragile X mental retardation protein (FMRP) inhibits translation initiation by recruiting the eIF4E-BP CYFIP1 (Cytoplasmic FMR-Interacting Protein-1) and binds several localized mRNAs in mammalian neurons [123].

Similarly, the eIF4E–eIF4G interaction is blocked when 3′UTR-bound CPEB RNA-binding protein (Cytoplasmic Polyadenylation Element-Binding protein) interacts with either the eIF4E-BPs maskin or neuroguidin [124].

Other steps of the translation initiation process can be targeted to inhibit the translation of localized mRNAs. Mammalian non-coding RNA BC1 is enriched in dendrites and synapses and may prevent translation initiation of its neuron-associated mRNAs by blocking the activity of the eIF4A helicase and the consequent recruitment of the 40S ribosomal subunit [125]. Translational repression during mRNA transport can also occur by blocking 60S ribosomal subunit recruitment. For example, the pumilio-homology domain family-6 (Puf6) protein blocks the recruitment of the 60S subunit and interferes with 80S assembly on the *ASH1* mRNA by interacting with eIF5B/Fun12 in budding yeast [126]. In the same way, the RNA-binding protein ZBP1 impairs the formation of 80S ribosomal complexes on the *β-actin* mRNA, which results in decreased translation initiation [120]. Short poly(A) tails have been linked to a repressed state, whereas long poly(A) tails enhance translation by recruiting the poly(A)-binding protein PABPC1. Translational activation of transported mRNAs is linked to poly(A) tail elongation. The opposing activities of poly(A) polymerase and the deadenylation complex regulate the length of poly(A) tails, and various translational repressors have been shown to affect this equilibrium [127]. By interacting directly with one of the subunits of the CCR4–NOT deadenylation complex, Smaug promotes the shortening of the poly(A) tail of unlocalized *nanos* mRNA [128].

1.1.2.2 Coupling translation with mRNA localization

Many mRNAs initiate translation as soon as they reach their final destination. This is often accomplished by post-translational modification of the translation repressors in a spatially confined manner or by competitive binding of pre-localized proteins, resulting in a reduction in their affinity for target mRNAs. After localization of the *β-actin* mRNA, the ZBP1 repressor is inactivated to allow the local synthesis of β-actin. Phosphorylation of ZBP1 by the kinase Src has been shown to reduce ZBP1 binding affinity for the *β-actin* transcript. Interestingly, the Src–ZBP1 interaction appears to be confined to β-actin translation sites [120]. Another mechanism of activating the local

translation of mRNAs can occur through the degradation of a translational repressor. For instance, degradation of FMRP is required for translational activation of its target mRNAs. In this case, phosphorylated FMRP binds ribosomes and inhibits the translation of several mRNAs localized to dendrites [129]. Dephosphorylation of FMRP mediated by the PP2A phosphatase after dendritic stimulation leads to its degradation by the ubiquitin-proteasome system [130]. The release of translational repressors could be the result of competitive binding between a translational repressor and locally produced proteins. For instance, Oskar promotes *nanos* mRNA translation by blocking the activity of Smaug/CCR4-NOT deadenylase in Drosophila embryos [128].

1.1.3 mRNA localization to sites of polarized growth in Saccharomyces cerevisiae

Studies in *Saccharomyces cerevisiae* revealed that this fungus is an excellent model organism to get mechanistic insights that broaden our understanding of mRNA localization pathways [131, 132]. Several dozen mRNAs are localized to sites of polarized growth in budding yeast, i.e. the bud tip. Among them is the *ASH1* mRNA, which was the first asymmetrically localized transcript identified in single-cell eukaryotes (Figure 1-6) [84, 133, 134]. In-depth analysis of the mechanism of *ASH1* mRNA localization led to the characterization of the She2-She3-Myo4 localization complex (see section 1.3.3 below). Asymmetric localization of *ASH1* mRNA is evolutionary conserved, as the *Candida albicans ASH1* mRNA is localized to the hyphae in a She3-dependent manner in this organism [135]. Using RNA immunoprecipitation combined with DNA microarray analysis, a set of 22 additional She2-She3-Myo4-associated mRNAs were identified [133]. These transcripts encode for a wide variety of proteins, including several involved in stress responses and cell wall maintenance, and the majority of these mRNAs are localized to the bud (Table 1-1).

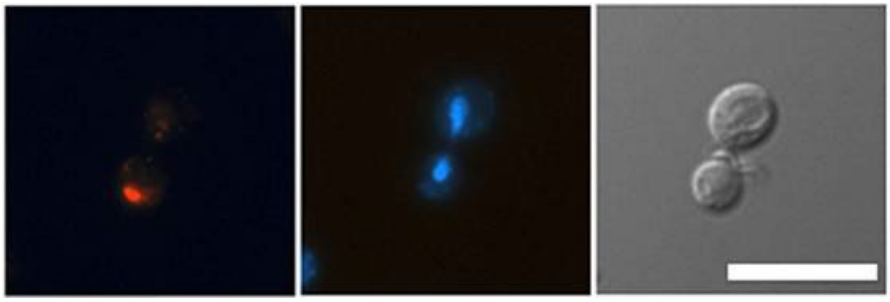


FIGURE 1-6 *ASH1* MRNA LOCALIZATION AT THE BUD TIP IN *SACCHAROMYCES CEREVISIAE*.

FLUORESCENT IN SITU HYBRIDIZATION (FISH) ON *ASH1* MRNA (LEFT PANEL). NUCLEAR DNA IS STAINED WITH DAPI (MIDDLE PANEL). DIFFERENTIAL INTERFERENCE CONTRAST SHOWS THE SHAPE OF THE YEAST CELL IN LATE ANAPHASE (RIGHT PANEL). SCALE BAR: 10μM. (SOURCE: CHARTRAND ET AL, 2001).

Among these transcripts, the *IST2* mRNA was the second bud-localized mRNA characterized in yeast [136]. The Ist2 protein is a cortical ER protein involved in ER-plasma membrane tethering. Localization of the *IST2* mRNA to the bud is required for the accumulation of Ist2 protein at the bud plasma membrane. However, Ist2 diffuses rapidly within the plasma membrane, and the asymmetric distribution of Ist2 protein in the bud is maintained by the septin-mediated membrane diffusion barrier at the mother-bud neck. Another study used microarray analyses to globally identify the RNAs associated with the RNA-binding protein She2 and identified 41 mRNAs associated with She2 [3]. While this study validated several of the mRNAs previously found associated with She2, it also identified several new mRNAs. Interestingly, some of these transcripts code for proteins localized to the vacuole, endoplasmic reticulum and mitochondria [3]. What emerged from these studies is that specific families of mRNAs are targeted to the bud. Some of these mRNAs encode for cell cycle regulators, such as *ASH1* and *CLB2* mRNA. The *CLB2* mRNA, which encodes a mitotic regulator B-type cyclin, is localized and translated at the bud in *S. cerevisiae* during the G2/M phase [116]. The *CLB2* mRNA is carried to the yeast bud by the She2-She3-Myo4 complex via a conserved mRNA zipcode located in its coding sequence. Remarkably, whereas the *CLB2* mRNA is segregated at the bud tip, the Clb2 protein diffuses back to the mother cell nucleus, indicating a rare example of uncoupling between mRNA and protein

localization. *CLB2* mRNA localization provides a novel function for mRNA localization, possibly by regulating cell growth and cell cycle progression by monitoring bud translation capacity through *CLB2* mRNA transport and local translation [137].

Transcripts coding for proteins involved in the regulation of cell polarity also localizes to the bud in a She2-She3 dependent manner [138]. For instance, mRNAs coding for the Rho-like GTPases Cdc42 and Rho3; the Sec1, Sro7 and Sro77 SNARE regulators; the Sec3 and Exo84 exocysts components, and the GTPase Ypt1 localize totally or partially to the bud [139]. Finally, transcripts coding for proteins involved in cell wall integrity and stress response, such as *STL2*, *MID2* and *WSC2* mRNAs, are also localized to the emerging bud [133]. Furthermore, mRNA localization plays a significant role in lipid biogenesis in yeast. For instance, the *ERG2* mRNA, coding for a sterol isomerase, localizes to the endoplasmic reticulum membrane. *LCB1* mRNA is another ER-localized transcript which is implicated in sphingolipid synthesis [133].

Under stress conditions, translationally repressed mRNAs can be localized in RNA processing bodies (P-bodies) and stress granules, which are phase-separated cytoplasmic bodies involved in mRNA degradation and storage, respectively [140, 141]. Recently, novel RNA granules termed “Translation factors mRNA granules” have been identified in budding yeast. These granules include mRNAs coding for translation initiation factors (eIF4G, eIF4A1, eIF4E) and are translationally active under growth conditions. The translation factors mRNA granules are targeted to the developing daughter cell in a She2/She3-dependent manner, where a higher protein production rate is highly demanded [142].

TABLE 1-1 EXAMPLES OF MRNA LOCALIZATION AND THEIR BIOLOGICAL FUNCTION IN BUDDING YEAST

Localized mRNAs	Protein function/localization
ASH1, CLB2, CDC42	cell cycle regulation/mating-type switching
IST2, WSC2, MID2	Membrane proteins
PMT2, ERG2	ER proteins
SUN4, YSP2	Mitochondrial protein
ENO2, PDC1	Glycolysis
BRO1, *KSS1*	stress response
ERG2, *LCB1*	Lipid synthesis

1.1.3.1 ASH1 mRNA localization and mating type switching

The Ash1 protein is a transcriptional repressor segregated to the daughter cell nucleus in late anaphase, where it inhibits mating type switching. Mating type switching is a reversible, programmed DNA rearrangement process which regulates the cell differentiation process in yeast [143]. The budding yeast *S. cerevisiae* exists either in a diploid state or, under conditions of nutrient

deprivation, sporulates and germinates in a haploid state. Two haploid cells of the opposite mating type (‘a’ or ‘α’) have the ability to fuse to form a diploid cell. Mating-type switching occurs only in the mother cell but not in daughter cells and gives rise to two cells with opposite mating types, which allows mating and the formation of a diploid. The sexual behaviour of both haploid and diploid cells is determined by two different alleles of the mating-type (*MAT*) locus: *MATa* and *MATα.*

The endonuclease HO is a key actor in the mating-type switching process and is required for gene conversion within the MAT locus. The HO endonuclease is responsible for mating-type switching by promoting the conversion of an ‘a’ cell to an ‘α’ (or in reverse) by initiating a genomic rearrangement of the MAT locus [144]. This site-specific endonuclease cleaves a degenerated 24 base-pair site at *MAT*, making a double-stranded break in DNA during late G1 in mother cells to initiate switching [145]. Asymmetric expression of the HO endonuclease, which is restricted in the mother cells, results in mating-type switching only in the mother cells [146]. This asymmetric expression of HO depends on the asymmetric localization of the Ash1 protein in the nucleus of the daughter cell [147]. The 66-KDa Ash1 protein is a GATA-family zinc finger protein and a key determinant of asymmetric cell fate as it binds at multiple sites in the *HO* promoter and represses its expression in the daughter cell [148].

In an attempt to identify factors that are required for asymmetric HO synthesis, a genetic screen revealed a group of proteins that were identified as HO endonuclease expression regulators *SHE1-SHE5* (for Swi5-dependent HO expression) that are major mRNA localization factors in yeast and in control of HO synthesis through *ASH1* mRNA localization [146]. In addition, defective Ash1 protein localization in *she* mutants highlighted the importance of the *SHE* genes for the localization of *ASH1* mRNA to the incipient daughter cell [84].

1.1.3.2 Localization elements involved in mRNA localization to the bud of yeast

As for other localized mRNAs, localization of transcripts to the bud tip requires the presence of cis-acting localization elements. In the *ASH1* mRNA, four cis-acting elements (E1, E2A, E2B, and E3) direct the targeted localization of this transcript from the mother cell to the bud

tip of the daughter cell [149]. While the E1, E2A, and E2B elements are found in the *ASH1* coding sequence, the E3 element is located in the 3′-UTR, next to the stop codon [95, 150]. Importantly, each of these zipcodes can individually target a heterologous reporter mRNA to the bud tip [150]. Bioinformatic analyses revealed little similarities in sequence homology or secondary structure between these four zipcodes sequences [151]. Interestingly, a common three-dimensional motif has been suggested in *ASH1* localization elements, which is essential for She2 recognition: the four zipcode elements have a consensus sequence motif consisting of a single-stranded CGA-base triplet and a single cytosine separated by six bases at either the 3′ or 5′ ends from the CGA triplet [149]. This short motif is present in all four elements of the *ASH1* transcript, as well as in other localized mRNAs such as *IST2* and *EAR1*. It is also found in a zipcode in the coding sequence of the *CLB2* mRNA [137].

1.1.3.3 Trans-acting factors involved in mRNA localization to the bud

1.1.3.3.1 She2 –The key component in mRNA localization in budding yeast

She2 is a small 28 KDa RNA-binding protein and the essential element in the assembly of the budding yeast mRNA locasome, i.e. the mRNA localization complex in yeast [84, 152]. She2 is a non-canonical RNA-binding protein which displays no similarity to previously known nucleic acid-binding proteins [153]. She2 shares structural homology with the central domain of fumarase/aspartase superfamily members, although these proteins bear no evident evolutionary conservation at the sequence level or functional homology. Therefore, She2 is a novel protein fold of distinct evolutionary origin [154].

The main function of She2 is to bind the localization elements of bud-localized mRNAs and establish the assembly of these mRNPs [95, 153, 155, 156]. Deletion of *SHE2* eliminates the association of Myo4 with the *ASH1* mRNA [157]. Furthermore, the interaction of She3 with the *ASH1* mRNA depends on She2, suggesting that She2 binds to the *ASH1* mRNA independently of She3 and Myo4 [153]. She2 is a key component of the mRNA localization machinery as it interacts directly and specifically with each of the mRNA localization elements. Indeed, on *ASH1* mRNA, She2 binds the localization elements E1, E2A, E2B and E3 *in vitro* and *in vivo* [95, 153, 155, 156]. Structural studies of She2 in solution revealed an extended conformation of two She2 dimers in a

head-to-head arrangement, resulting in an elongated tetramer with two continuous RNA-binding sites (Figure 1-7). The assembly of She2 in tetramer structure is required for the recognition of localization element RNAs [158]. Besides *ASH1* mRNA, She2 associates with more than 50 additional bud-localized transcripts [3, 133]. The majority of She2-associated mRNAs encode for membrane and secreted proteins that are targeted to the endoplasmic reticulum (ER). She2 also interacts directly with liposomes, suggesting a membrane-binding activity for this protein [159]. Sucrose gradient experiments showed that She2 cofractionates with ER, independently from She3 and Myo4, indicating that mRNA localization and cortical ER inheritance may be coupled in budding yeast [159-161].

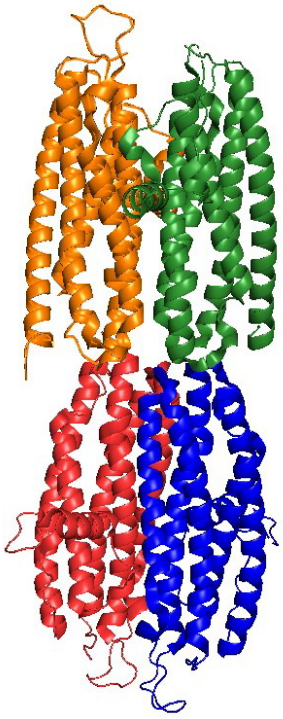


FIGURE 1-7 SHE2 ASSEMBLES AS A TETRAMER AT PHYSIOLOGICAL CONCENTRATION.

She2 dimers interact in a head-to-head fashion, resulting in an elongated tetramer with two continuous RNA-binding sites required for the recognition of localization element RNAs. (Source: Müller et al., 2009)

1.1.3.3.2 The adapter protein She3

She3 is a novel protein present in *Saccharomyce*s, with a homologue in various *Candida* subspecies [135]. She3 is part of the mRNA localization machinery and acts as a protein adapter between She2, Myo4 and bud-localized mRNAs. Indeed, She3 interacts directly with Myo4 and plays a key role in the association of the Myo4 motor protein with the *ASH1* mRNA-She2 complex [162]. The N-terminus of She3 forms a coiled-coil domain that interacts with the C-terminal tail of Myo4 [163]. She3-Myo4 interaction prevents the dimerization of Myo4, creating a single-headed motor [164]. Structural insights from X-ray crystallography showed that a truncated dimeric fragment of She3 exposes hydrophobic residues for binding to the globular tail domain of Myo4 [165]. She3 also forms a synergistic complex with She2 associated with any of the four *ASH1* mRNA localization elements. Surprisingly, She2 has a low binding affinity for RNA, with an equilibrium-dissociation constant (Kd) in the low micromolar range for the *ASH1* mRNA localization elements. She2 has the strongest binding affinity for the E3 RNA localization element (Kd = 0.10 μM) and the weakest binding affinity for E2B (Kd= 0.31 μM) [158], which suggests that other factors must be present *in vivo* to reach the high selective transport of transcripts.

This question was addressed by studies on She3's RNA-binding characteristics, as well as its synergistic interaction with She2. Direct interaction of She2 with the C-terminal domain of She3 is required for the association of the Myo4-She3 complex to the *ASH1* mRNA [153, 156]. Indeed, the She2-She3 complex has a 60-fold higher affinity for zipcode-containing RNAs compared to She2 alone [151, 166]. A C-terminus fragment of She3 (331–405) binds to She2 and forms a ternary complex with an *ASH1* mRNA zipcode. She3 interacts with She2 through two distinct sites: the P site, which associates entirely with She2, and the R site, which binds both She2 and the RNA, suggesting that not only She2 but also She3 participates in RNA recognition and binding (see section **1.3.3.4**) [167].

1.1.3.3.3 She1/Myo4- a non-conventional type V myosin motor

Budding yeast possesses two classes of type V myosins, which mediate the transport of cargoes on actin filaments, unlike higher eukaryotes, where long-range transport is mediated by microtubules and kinesin/dynein motors [168]. The two budding yeast type V myosins, Myo4 and

Myo2, are unconventional myosins because they form dimers, unlike canonical myosins. Both Myo4 and Myo2 accumulate at the bud tip of daughter cells [169-172]. Myo2 and Myo4 display 71% sequence homology in their motor domain but only 18% sequence similarity in their globular tail domain [173]. They transport distinct sets of cargoes, perhaps due to the low level of similarity between their tail domains [173]. Myo2 is involved in the segregation and inheritance of mitochondria, vacuole, peroxisomes and late Golgi compartments to the daughter cell during cell division [170, 171, 174-179]. Myo2 is also essential for setting up the alignment of the mitotic spindle [180]. She1/Myo4 provides the required motor activity for the transport of the mRNA locasome along the actin cytoskeleton to the bud tip of daughter cells [80, 157, 175, 181]. Myo4 is also involved in ER inheritance in *Saccharomyces cerevisiae* [182]. Furthermore, a recent study showed that the unidirectional movement of P-bodies from the mother to the daughter cell during mitosis relies on the She2/She3/Myo4 transport machinery [183].

Type V Myosins were known to be necessary dimers, which allow for processive movement along the actin filaments [184]. Surprisingly, Myo4 was found to act as a monomer and in a non-processive manner [185, 186]. Indeed, She3 can dimerize via its N-terminus, and a She3 dimer interacts constitutively with a single Myo4 motor in order to form a stable complex [187]. The RNA-binding protein She2 recruits Myo4-She3 to form a motile active transport complex via its direct interaction with She3 [156, 163, 166, 188]. Assembly of a stable She2/She3/Myo4 complex occurs in the absence of RNA at physiological conditions and even at salt concentrations above cellular levels [189]. Myo4-She3-She2 complex with the myosin chaperonin She4 and myosin light chains are adequate to support the processive transport of *ASH1* mRNA along the actin filaments [187, 189, 190]. Interestingly, in the transport of pheromone-induced mRNAs, the interaction of Myo4 with the RNA-binding protein Scp160 (analogous to She2) is essential for the localization of these mRNAs at the schmoo during polarized growth of mating yeast cells [191].

1.1.3.3.4 Structural studies of She2 and its complex with She3 and Myo4

She2 is an RNA-binding protein with a unique protein fold of distinct evolutionary origin. She2's three-dimensional structure has been determined at 1.95 Å resolution and demonstrated that this protein folds into a single globular domain with five anti-parallel α helix bundle that forms a

symmetric homodimer required for RNA binding [154]. She2 displays no resemblance to known nucleic acid-binding proteins when compared to structures already reported in the PDB database. However, the central domain of She2 resembles those of fumarase/aspartase homologous superfamily members [154], as they share the general structural arrangement of their respective five helix cores, with no evolutionary conservation or functional similarity observed [154]. Moreover, She2 does not display any clearly defined RNA-binding domain but contains two unusual basic helical hairpin-type RNA-binding domains. Mutations in residues of this hairpin domain (N36S, R43A, R44A, R52A, R52K, R63A, and R63K) have been found to disrupt the interaction between She2 and RNA and consequently abolish *ASH1* mRNA localization [154, 192]. Interestingly, several RNA-binding motifs, including the RNA recognition motif (RRM), the arginine-rich motif (ARM), the RGG box, and the double-strand RNA-binding motif (dsRBD) contain conserved arginine residues essential for RNA binding [192].

Analytical ultracentrifugation analyses further revealed that two dimers of She2 come together in a head-to-head fashion to form a tetrameric complex, which generates a large complex with two RNA-binding sites [158]. Cross-linking analyses of She2-RNA complexes revealed that one peptide of She2 was cross-linked to the *ASH1* mRNA E3 localization element. This peptide is part of a short helix that protrudes at a right angle from the She2 core and plays a key role in binding to the localization elements and interaction with She3 [151]. Crystallography and small angle X-ray scattering (SAXS) investigation of the *ASH1* E3 element alone and in association with its partners She2 and She3 provides significant information regarding the molecular aspects of the E3-She2-She3 complex interaction (Figure 1-8A).

Interestingly, the crystal structure of the She2-E3 complex showed significant rearrangements in the bulged segment of E3 after binding to She2, going from an elongated state to a kinked state. As a result, this E3 RNA element is organized in an L-shaped conformation along the She2 tetramer. Importantly, She3 interaction with the She2-E3 complex depends on the kinked E3 RNA three-dimensional structure instead of any particular RNA sequence (Figure 1-8B). The nucleotides C1779 and C1813 are two essential residues in the E3 RNA element that makes specific interaction with N36, R52 and R63 amino acids of She2 (Figure 1-8B). She2 mutations at N36, R52 or R63

each result in a complete loss of function. Furthermore, following the co-complex formation of the She2-E3-She3, the uracil in position 1780 of E3 is displaced to make space for She3. This rotation in the E3 RNA stem is required for the rearrangement of the uracil residue, which promotes She3 interaction and further maintains the She2-E3-She3 complex [167].

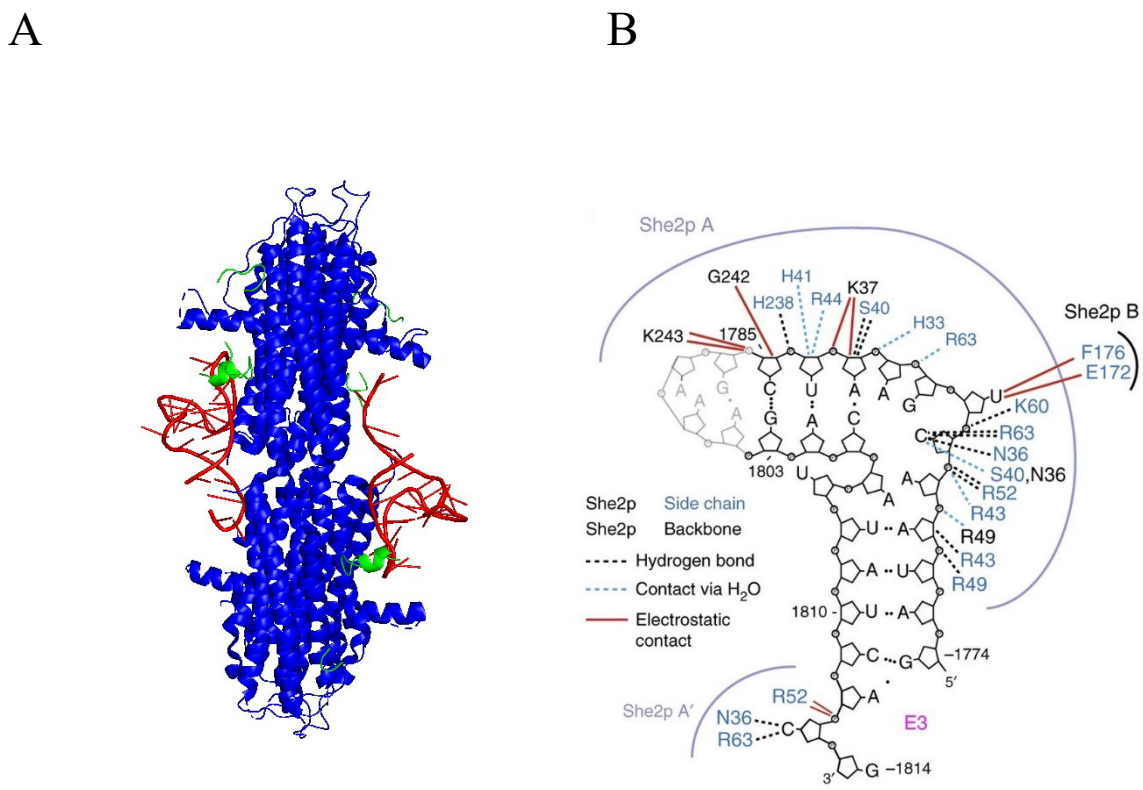


FIGURE 1-8 CRYSTAL STRUCTURE OF THE COMPLEX BETWEEN SHE2 AND THE *ASH1* MRNA E3 LOCALIZATION ELEMENT.

(A) **CRYSTAL STRUCTURE OF SHE2 (AMINO ACIDS 6-246, C-S) FUSED TO SHE3 (AMINO ACIDS 331–405) IN COMPLEX WITH *ASH1* E3 (28-NT LOOP) RNA AT 2.80-Å RESOLUTION. SHE2, SHE3 AND *ASH1* E3 ARE DEPICTED IN BLUE, GREEN AND RED, RESPECTIVELY.** (B) **SCHEMATIC DRAWING OF PROTEIN CONTACTS WITH THE E3 RNA. EACH OF THE TWO RNA CHAINS CONTACTS THREE SHE2 SUBUNITS. THE NUCLEOTIDES C1779 AND C1813 ARE TWO ESSENTIAL RESIDUES IN THE E3 RNA ELEMENT THAT MAKES SPECIFIC INTERACTION WITH N36, R52 AND R63 AMINO ACIDS OF THE SHE2 TETRAMER. (SOURCE: EDELMANN ET AL., 2017).**

Furthermore, a crystal structure of a She2 tetramer bound in complex with a 30 amino acids peptide of She3 C-terminus revealed that a highly conserved LPGV motif in the She3 peptide folds into a hook-like shape. Each hook binds to a shallow hydrophobic pocket on the surface of one of the She2 tetramer subunits, forming a 4:4 stoichiometric She2-She3 complex [193]. The binding of the LPGV motif is accompanied by local conformational changes in the She2 tetramer around the She3 hook binding regions. A highly conserved motif containing around 20 residues positioned N-terminal to the LPGV hook is thought to contribute to the She3 RNA binding site because of its basic and aromatic residues. This region of the She3 peptide is aligned with the RNA binding site in the waist region of the She2 tetramer, which results in a high-affinity RNA binding site (Figure 1-9) [193].

Structural information is also available for the She3-Myo4 complex. Determination of a crystal structure of Myo4 and She3 revealed that Myo4 and She3 form a 1:2 heterotrimer in solution. Also, the claw-like C-terminal domain (CTD) of Myo4 attaches to a highly conserved pseudocoiled-coil region of She3 to produce a stable complex of [Myo4-She3-She2-RNA] in a 2:4:4:1 stoichiometry (Figure 1-9. A) C) and two Myo4-She3 heterotrimers with a single She2 tetramer and at least one RNA zipcode is required [165]. The x-ray structure of the Myo4 globular tail was also characterized, and a conserved surface patch containing W1325 and Y1329 is required for binding She3 [194]. Furthermore, deletion studies and point mutations in the protease-sensitive linker region and globular tail of Myo4 revealed that both regions are required for efficient She3 binding and *ASH1* mRNA localization.

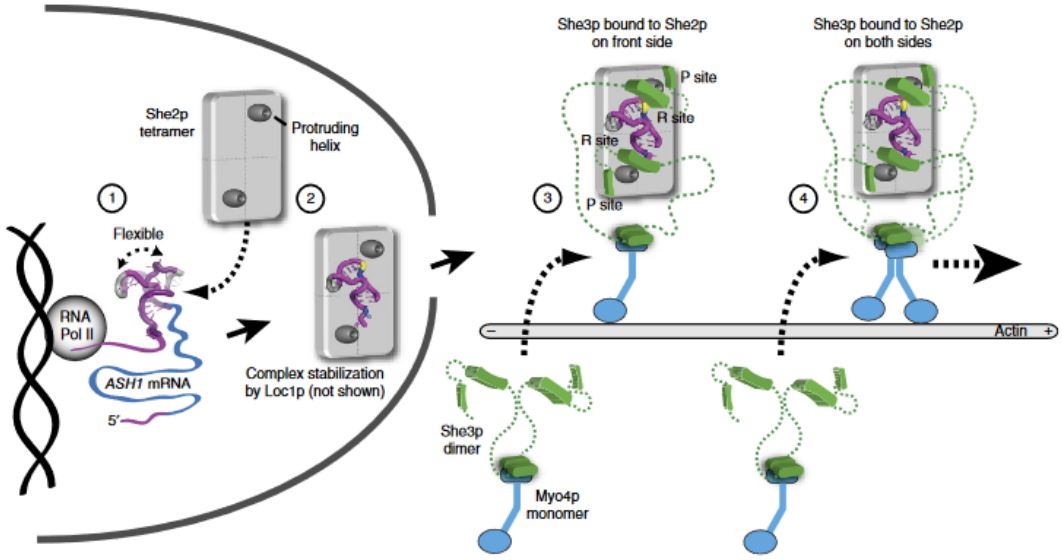


FIGURE 1-9 SCHEMATIC MODEL FOR SPECIFIC *ASH1* MRNA RECOGNITION AND TRANSPORT BY SHE2, SHE3 AND MYO4.

STEP-BY-STEP ASSEMBLY OF THE SYNERGISTIC TERNARY COMPLEX USING A STRUCTURE-BASED APPROACH. STEP 1: **THE *ASH1* E3 RNA FORMS A STEM-LOOP WITH A DYNAMIC BULGE REGION IN ITS STEM THAT SERVES AS A FLEXIBLE HINGE.** STEP 2: **AFTER BINDING TO SHE2, THE E3 RNA UNDERGOES SIGNIFICANT REARRANGEMENTS IN THE BULGE AREA OF THE STEM AND FORMS AN L-LIKE SHAPE ALONG THE SHE2 TETRAMER.** STEPS 3 AND 4: **SHE3 JOINS THE SHE2-RNA COMPLEX ON BOTH SIDES OF THE SHE2 TETRAMER. SHE3 INTERACTS WITH SHE2 THROUGH TWO DISTINCT SITES: THE P SITE, WHICH ASSOCIATES ENTIRELY WITH SHE2, AND THE R SITE, WHICH BINDS BOTH SHE2 AND THE RNA. SHE3 INCREASES AFFINITY FOR THE RNA BY CREATING STERIC RESTRICTIONS AND BRINGS A MYO4 MONOMER PER SHE3 DIMER. (SOURCE: EDELMANN ET AL., 2017)**

1.1.3.4 Translational regulators of localized mRNAs in yeast

1.1.3.4.1 The nuclear RNA-binding protein Loc1

Loc1 is a strictly nuclear protein which was identified as an RNA-binding protein required for the proper targeting of *ASH1* mRNA to the bud tip of daughter cells [166, 195]. Loc1 binds both localization elements E1 and E3 within the *ASH1* mRNA. However, Loc1 possesses a lower affinity for the E1 localization element compared to the E3 element [195, 196]. Deletion of *LOC1* results in less efficient *ASH1* mRNA localization and up-regulation of cytoplasmic *ASH1* mRNA

translation, suggesting that this protein is required for translational repression during localization [195, 197]. Apparently, translational repression of *ASH1* mRNA by Loc1 is mediated only by the localization element E3 in the 3'UTR of this transcript [197]. Loc1 was shown to bind both She2 and Puf6 and to promote the co-transcriptional loading of Puf6 on the 3'UTR of *ASH1* mRNA [193]. Despite the fact that Loc1 is important but not essential for proper *ASH1* mRNA localization, *loc1*Δ cells display a slow-growing phenotype [198]. Indeed, Loc1 is also involved in 60S ribosomal subunit biogenesis since in the absence of Loc1 protein, levels of mature 25S rRNA are dramatically reduced, and the levels of large subunit precursor rRNAs (35S pre-RNA), as well as abnormal 23S RNA, were increased [197, 199]. Besides that, 60S nuclear export is reduced in *loc1*Δ cells [198]. A recent study indicates that Loc1 and Puf6 interact co-transcriptionally with Rpl43 (ribosomal protein large subunit 43) and form a ternary complex in yeast and the Rpl43 protein is less stable in the *loc1* and *puf6* mutants [200]. The proper biogenesis of the 60S subunit depends on the formation of Loc1, Puf6 and Rpl43 ternary complex, which is loaded onto pre-60S [201].

1.1.3.4.2 The translational repressor Puf6

Puf6 is a specific RNA-binding protein which belongs to the highly conserved pumilio/fem-3 domain family (PUF), which usually contains eight conserved PUM (pumilio) repeats of ~36 amino acids required for RNA-binding [202-207]. Unlike classical PUF proteins, which usually have eight PUF repeats that recognize specific RNA sequences, crystal structure analyses of human Puf-A and budding yeast Puf6 proteins reveal a novel class of PUF family with 11 PUM repeats in an L-like shape [208]. Each of the eight RNA-recognition repeats of the PUF domain interacts with a single nucleotide in the target mRNA 3'UTR [202, 206]. While the PUF domain binds to a conserved UGUR tetranucleotide motif, additional sequences in the 3' UTR are required to determine the specificity of PUF–mRNA interactions [209]. Yeast has six PUFs, Drosophila two, vertebrates three, *C. elegans* 12 and *Arabidopsis* 26 [209]. Each of the six PUF family members in yeast binds to a particular set of mRNAs [210]. Inactivated *puf* mutants display distinctive phenotypes, suggesting that they have unique non-redundant functions [211-213]. On the other hand, phenotypic analysis of the *puf* mutants and the existence of common mRNA targets in PUFs imply that yeast PUF proteins also act in combination with each other [210, 214, 215].

Puf6 and its human ortholog Puf-A (also known as KIAA0020) possess different mRNA regulatory functions from the other PUF family members [208]. For instance, in contrast to the cytoplasmic localization of PUF proteins, Puf-A and Puf6 are localized to the nucleolus. Furthermore, yeast Puf6 co-sediments with the 60S ribosomal subunit and is involved in 60S ribosome biogenesis [216, 217]. Puf6 and its human homolog Puf-A bind to single or double-stranded RNA with no apparent sequence selectivity, and the majority of their PUM repetitions lack the distinctive RNA base recognition residues observed in classical PUF proteins [218]. However, conserved basic residues in Puf6 N-terminus are required for nucleic acid interaction, pre-rRNA processing, and *ASH1* mRNA localization [208]. Puf6 binds directly to the *ASH1* mRNA 3′ untranslated region (UTR) and plays an important role in the translational repression of this transcript [195, 197, 219]. Puf6 inhibits the translation of the *ASH1* mRNA via its interaction with the translation initiation factor eIF5B (Fun12 in *S. cerevisiae*) [126]. Elongating ribosomes and She2 compete for access to the E1, E2A and E2B localization elements in the *ASH1* mRNA open reading frame [150]. Puf6 promotes *ASH1* mRNA localization by inhibiting its translation and therefore restricts the competition between translating ribosomes and She2 for binding the *ASH1* ORF [220, 221]. Phosphorylation of the N-terminal region of Puf6 by the protein kinase CK2 (casein kinase II) induces the release of Puf6-mediated translational repression on *ASH1* mRNA when the complex reaches the bud tip [126].

1.1.3.4.3 The translational repressor Khd1/Hek2

Khd1 belongs to the family of RNA-binding proteins that contains three heterologous nuclear ribonucleoprotein K (hnRNP K)-like homology (KH) domains [222]. Khd1 homologues in humans are the polyC-binding proteins (PCBP) 1 to 4 and hnRNP K, which also contain three KH domains. These homologues regulate mRNA translation and stability [223]. PCBP1, one of the Khd1 homologues, is also involved in regulating the translation of localized mRNAs in neural cells [224]. Khd1 was first found to repress *ASH1* mRNA translation during its transport to the bud tip [225]. Two elegant studies revealed that Khd1 is associated with hundreds of mRNAs, corresponding to 20% of the yeast transcriptome [226, 227]. Hasegawa and co-workers reported that Khd1 binds to a potential recognition motif-containing CNN repeats present 5’ of the E1 element in *ASH1* mRNA [226]. Khd1 represses *ASH1* mRNA translation by binding near the

localization element E1 in the *ASH1* coding sequence and the C-terminal domain of the translation initiation factor elF4G [225], reducing translation initiation on this mRNA. Phosphorylation of Khd1 by the membrane-associated casein kinase Yck1, the yeast homolog of casein kinase 1 (CK1), is responsible for decreasing the affinity of Khd1 for RNA, and relieves the translational blockage on the *ASH1* mRNA, leading to the local expression of the Ash1 protein at the bud tip.

Through its repression of *ASH1* mRNA, Khd1 indirectly regulates filamentous growth by regulating the *FLO11* transcript. *ASH1* is involved in the regulation of yeast filamentous growth by activating *FLO11* expression, which encodes a cell wall protein required for the filamentous growth form. While the repression of *FLO11* is *ASH1*-dependent, Khd1 also represses Flo11 expression independent of *ASH1* [228]. Khd1 also positively regulates the expression of *MTL1* mRNA, which encodes a membrane sensor of the cell wall integrity pathway. Khd1 affects the stability of the *MTL1* mRNA and prevents its degradation by binding to cis-acting elements in the coding region of the mRNA. Under glucose deprivation conditions, Khd1 colocalizes with the decapping enzymes in processing bodies (P-bodies) involved in mRNA degradation [229]. This suggests that Khd1 is involved in preventing the degradation of *MTL1* mRNA in P-bodies [230]. Despite the fact that Khd1 binds to numerous mRNAs that encode cell wall and membrane proteins, deletion of the *KHD1* gene has no detectable phenotype on cell wall synthesis or function. However, *KHD1* deletion causes severe cell lysis along with the deletion of the *CCR4* gene, which encodes for a cytoplasmic deadenylase, suggesting a possible role for Khd1 in the cell wall integrity pathway in yeast [231].

1.1.3.4.4 The DEAD-box RNA helicase Dhh1

Control of mRNA translation and degradation is an important aspect of eukaryotic gene expression regulation. Deadenylation of the 3′ poly(A) tail, followed by cleavage of the 5′ m7GpppN caps (decapping), and finally, 5′ to 3′ exonucleolytic degradations, is a general mechanism for mRNA decay in eukaryotes [232]. Decapping is a key step in the decay process since it allows the degradation of the mRNA body and is a site for many regulatory inputs. The two processes of translation and mRNA decapping are linked. For instance, decreasing translation initiation increases the rate of decapping. Inversely, the rate of decapping is greatly slowed when

translation elongation is suppressed [233]. The Dhh1 protein, which is a member of the yeast DEAD-box RNA helicase family, is involved in triggering mRNA decapping in the mRNA decay pathway and is a key component of P-bodies (processing bodies) in yeast [234]. Dhh1 is involved in the regulation of *ASH1* mRNA translation after its proper localization. Dhh1 is associated with the 5'-UTR of untranslated *ASH1* mRNA, possibly in the cytoplasm, and its co-precipitation with Puf6 suggests that a portion of *ASH1* mRNAs could remain translationally repressed after localization. The loss of the *DHH1* gene causes *ASH1* mRNA to diffuse in the bud and affects its translation [235]. Dhh1 has been thoroughly investigated as a translational repressor, but emerging evidence suggests that it also acts as an activator of translation [236, 237]. Dhh1 stimulates *STE12* mRNA translation, which is a major transcriptional activator of roughly 200 mating-specific genes in yeast [236]. Dhh1 is functionally connected with Loc1 and Puf6 in order to regulate *STE12* mRNA translation. Notably, Ste12 expression and the Dhh1-Puf6 association were significantly reduced when Thr16 in the N-terminus of Dhh1 was phosphorylated [238], suggesting that post-translational modifications play a crucial role in the repression mechanisms that are mediated by Dhh1.

1.1.3.5 Proposed mechanism of *ASH1* mRNA localization and translational repression

From the data presented above, a global mechanism of mRNA localization to the bud tip can be proposed in (Figure 1-10). The RNA-binding protein She2 is actively imported into the nucleus (see section 1.4.2 below). In the nucleus, She2 interacts with the elongating RNA polymerase II machinery via the transcription elongation factor Spt4–Spt5 and binds the nascent *ASH1* mRNA [239]. She2 is required for the co-transcriptional recruitment of Loc1 and Puf6 to the *ASH1* mRNA 3'UTR and, thereby, the proper localization of this mRNA at the bud tip of the daughter cell [196]. Besides Loc1 and Puf6, the translational repressor Khd1 also binds to the *ASH1* transcript in the nucleus. The particular activity of the nucleoporin Nup60 is required for efficient nuclear export of bud-localized mRNAs [240]. Once in the cytoplasm, She3 competes with Loc1 to bind the *ASH1*-She2 complex and increases the affinity of She2 for the RNA localization elements, leading to a reorganization of the *ASH1* mRNP [166]. She3 is associated with Myo4 and forms the transport complex termed "locasome." Through the actin filaments, the locasome transports silent *ASH1* mRNPs to the bud. Puf6 and Khd1 prevent mRNA translation during this

transport. While Puf6 interacts with eIF5B, preventing the 60S ribosomal subunit from being recruited, Khd1 binds eIF4G and blocks the recruitment of the 40S ribosomal subunit to the mRNA. Once the complex is localized at the bud tip, membrane-associated kinases CK2 and Yck1 phosphorylate Puf6 and Khd1, respectively, and allow translation of the *ASH1* mRNA at the bud.

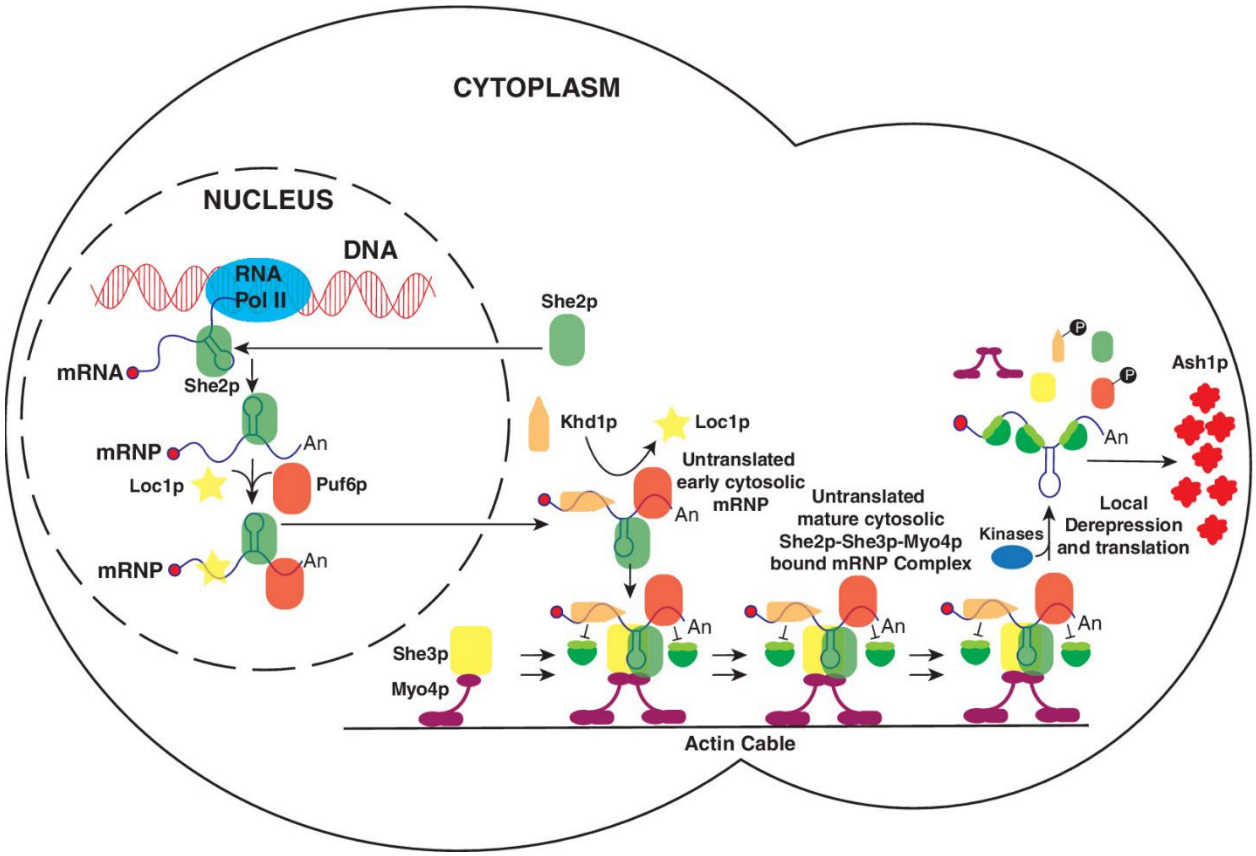


FIGURE 1-10 SCHEMATIC DIAGRAM OF MOLECULAR EVENTS INVOLVED IN *ASH1* MRNA LOCALIZATION TO THE BUD TIP IN *SACCHAROMYCES CEREVISIAE*.

MOLECULAR COMPONENTS/PROTEIN FACTORS INVOLVED IN THE NUCLEAR BIOGENESIS, MATURATION AND LOCALIZATION OF THE *ASH1* MRNA ARE INDICATED BY VARIOUS COLOUR-CODED SYMBOLS. EVENTS INVOLVED IN SPECIFIC PROCESSES ARE DESCRIBED IN THE MAIN TEXT. (SOURCE: CHAUDHURI A, ET AL., 2020)

1.1.3.6 Regulation of the mRNA localization machinery in budding yeast

Asymmetric sorting of mRNA to its ultimate destination in *Saccharomyces cerevisiae* is mediated by several steps, including RNA binding in the nucleus and nuclear export, assembly of transport-competent RNPs, recruitment of molecular motors, mRNA anchoring and local translation. Messenger RNA localization is a strictly regulated process, and so far, most research on the role of post-translational modifications, such as phosphorylation or ubiquitination, has focused on the activation or repression of mRNA translation. For instance, the kinases Yck1 and Ck2 phosphorylate Khd1 and Puf6, respectively, leading to their release from the *ASH1* mRNP complex and translational activation. However, the regulation of mRNA localization factors, such as She2, She3 and Myo4, still remains elusive. Some components of the *ASH1* mRNA-transport complex are known to be phosphorylated, and their phosphorylation impacts mRNA segregation. For example, She3 came out in several large-scale phosphoproteomics analyses, which identified up to nine distinct phosphorylated residues. Interestingly, She3 phosphomimetic mutants at S348E or S343E S361E are both deficient in *ASH1* mRNA localization [241]. While these mutants maintain the capacity of She3 to bind Myo4 and She2, they reduce the interaction between She3 and *ASH1* mRNA, suggesting that She3 phosphorylation may negatively regulate the association of the She2-She3 complex with the *ASH1* mRNA [241]. The kinases that mediate these phosphorylation events in She3 are still unknown. Myo4 is another phosphorylated protein, but the impact of these phosphosites remains unclear. Interestingly, the serine/threonine phosphatase Ptc1 regulates the distribution of Myo4 cargoes and is required for the proper localization of *ASH1* mRNA at the bud tip, suggesting that Ptc1, and specific phosphorylation events, regulate the assembly of Myo4-specific cargoes [242].

Ubiquitination is another post-translational modification that affects nearly every aspect of eukaryotic biology, with different types of ubiquitylation resulting in different functional outcomes, such as DNA repair, protein turnover, stress response, protein binding, subcellular localization, and membrane protein trafficking [243]. She3 undergoes ubiquitination by the SCF/Grr1 ubiquitin ligase, and its degradation is required for optimal cell growth, but it is not required for regulating *ASH1* mRNA asymmetric localization. Yet, the role of She3 ubiquitination and degradation in the daughter-cell localization of other mRNAs remains unknown [244]. A

proteomic study used the expression of lysine-less ubiquitin (K0 Ub), and screening for mono-ubiquitinated proteins revealed that She2 is ubiquitinated *in vivo* [245]. She2 ubiquitination favours its interaction with the Cdc48 AAA ATPase via its Ubx domain adaptors, which bind ubiquitinated proteins in a large protein complex [246].

1.1.4 Nuclear factors regulating cytoplasmic mRNA localization

1.1.4.1 Nuclear RNA-binding proteins are required for proper mRNA localization in the cytoplasm

Nuclear binding by trans-acting localization factors and nuclear processing events play crucial roles in determining the cytoplasmic fate of localized mRNAs [247, 248]. For example, several RNA-binding proteins that shuttle between the nucleus and the cytoplasm are required for *oskar* mRNA localization to the posterior pole of the Drosophila oocyte. One of these RBPs is the Drosophila hnRNPA/B homolog Hrp48 which binds co-transcriptionally to three regions in the *oskar* mRNA 3'UTR and represses *oskar* mRNA translation during transport [249]. Furthermore, the multi-protein exon junction complex (EJC), with Y14, Mago, eIF4AIII and Barentsz proteins at its core, assembles on mRNA exon-exon junctions upon splicing and follows spliced mRNAs until their translation in the cytoplasm. During mRNA nuclear export, the EJC component eIF4AIII recruits the cytoplasmic protein Barentsz (BTZ), which is needed for *oskar* mRNA localization [250].

RNA-binding proteins in the nucleus are also required for the proper cytoplasmic localization of mRNAs in vertebrates. For example, the restriction of Vg1 expression to the vegetal hemisphere of the Xenopus embryo is essential for the establishment of the animal-vegetal axis [251]. Vegetal localization of *Vg1* mRNA is initiated with the formation of an mRNP complex in the nucleus. In the oocyte nucleus, both PTB/hnRNP I and Vg1-RBP/Vera interact with *Vg1* mRNA. Both proteins play key roles in *Vg1* RNP remodelling and mRNA localization [252]. The *Vg1* mRNP undergoes remodelling during its cytoplasmic transport, which triggers distinct steps in the localization pathway. Importantly, PTB/hnRNPI is essential for the remodelling of the *Vg1* mRNA-Vg1RBP/Vera interaction and, eventually, Vg1 localization [252]. The homolog of

Vg1RBP/Vera in higher vertebrates, the zipcode binding protein 1 (ZBP1), regulates asymmetric localization and translational suppression of the *β-actin* mRNA [253]. Although ZBP1 is mainly cytoplasmic, it binds selectively to the *β-actin* mRNA zipcode and is detected at β-actin transcription sites in the nucleus [254]. Another RNA-binding protein called ZBP2, a shuttling heterogeneous nuclear ribonucleoprotein (hnRNP), is mainly nuclear and binds to the nascent *β–actin* mRNA co-transcriptionally, allowing ZBP1 to be recruited to the transcription sites, where it interacts with the RNA and forms an mRNP complex required for cytoplasmic localization [255]. Altogether, these data show that the nuclear history of an mRNA affects its cytoplasmic fate.

1.1.4.2 She2 shuttles between the nucleus and the cytoplasm

Several studies have shown that She2 shuttles actively between the nucleus and the cytoplasm [220, 256]. She2 is more abundant in the cytoplasm, although there is a pool of She2, which is nuclear, and She2 is never excluded from the nucleus [153]. Either inhibition of mRNA export, such as in a *mex67-5* mutant, or deletion of the first 70 amino acids residues of She2 results in the nuclear accumulation of this protein, suggesting that She2 shuttles between the nucleus and cytoplasm and its nuclear export depend on its capacity to bind RNA [188]. Proteins with a size below the nuclear pore diffusion limit (40 KDa) can passively transit to the nucleus in yeast [257]. Although She2 molecular weight is 28 KDa and is small enough to enter passively through the nuclear pore, the active form of She2 is a symmetrical homo-tetramer of more than 110 KDa [154, 167]. Yeast genetic nuclear import assay and two-hybrid assay identified a 30-amino acid sequence in She2, which is able to interact with the importin α Srp1 and contains the nuclear localization signal (NLS) of She2 [220]. The thirty amino acid sequence at the C-terminus of She2 includes only one basic residue and several hydrophobic residues, which is very different from classical NLS (see section 1.5.2 below). In addition, mutation of five conserved residues in this non-classical NLS impaired the interaction between She2 and Srp1, as well as *ASH1* mRNA localization.

The presence of She2 in the nucleus is essential for the recruitment and proper localization of bud-localized transcripts in yeast. In the nucleus, She2 interacts co-transcriptionally with the nascent *ASH1* mRNA. This interaction is promoted by the association of She2 with the elongating form of RNA polymerase II via its interaction with the transcription elongation factor Spt4-Spt5.

Mutations in *SPT4* or *SPT5* significantly decreased the association of She2 with the RNA pol II complex and *ASH1* mRNA localization at the bud tip [258]. Furthermore, the nuclear import of She2 is crucial for the translational repressor Puf6, which is primarily a nucleolar protein, to interact with *ASH1* mRNA [220] and for the translational repression of this mRNA [256]. Both Puf6 and Loc1 are associated with the nascent *ASH1* mRNA during its transcription, and their association depends on She2 [259], suggesting that the translational regulation of *ASH1* mRNA is dependent on the nuclear targeting of She2.

Finally, a study showed that the nuclear export pathway affects downstream *ASH1* and *IST2* mRNAs localization to the bud tip. Interestingly, the nuclear pore protein Nup60 is required for the formation of a functional locasome complex, which occurs in the nucleus. Also, Nup60 may be involved in the quality control of complex cargoes, such as the nonsense-mediated decay of premature termination codon-containing mRNAs and has been suggested to be implicated in a quality-control step for *ASH1* mRNA located at the bud tip. Additionally, Nup60 might link transcription to nuclear export by binding the *ASH1* gene locus to the nuclear periphery [240].

1.1.5 Mechanisms of nuclear import of proteins

1.1.5.1 The Nuclear pore complex (NPC)

NUCLEOCYTOPLASMIC TRANSPORT OCCURS THROUGH THE NUCLEAR PORE COMPLEX (NPC) THAT SPANS THE NUCLEAR ENVELOPE, WHICH IS THE DOUBLE MEMBRANE THAT ENCOMPASSES THE CELL NUCLEUS [260]. THE NPC IS A LARGE, MULTIMERIC AND SIEVE-LIKE STRUCTURE THAT ALLOWS FOR THE PASSIVE DIFFUSION OF SMALL MOLECULES WHILE RESTRICTING THE TRANSPORT OF CARGOES LARGER THAN THE TYPICALLY REPORTED THRESHOLD, OF 40 KDA [261]. THE PROTEINS THAT COMPOSE THE NUCLEAR PORE COMPLEX ARE A FAMILY OF AROUND 30 DIFFERENT PROTEINS KNOWN AS NUCLEOPORINS (NUPS) [262]. APPROXIMATELY ONE-THIRD OF ALL NUCLEOPORINS CONTAIN HIGHLY CONSERVED TANDEMS OF PHENYLALANINE-GLYCINE REPEATS (FG REPEATS) THAT BLOCK THE FREE NUCLEOCYTOPLASMIC EXCHANGE OF PARTICLES LARGER THAN APPROXIMATELY 5 NM, WHICH FORMS A DIFFUSION BARRIER [263, 264]. NUCLEAR TRANSPORT RECEPTORS (NTRS) THAT BIND BOTH THE FG REPEATS AND CARGOES CAN OVERCOME THE NPC PERMEABILITY BARRIER BY INTERACTING WITH THE FG REPEATS [263, 265]. NPC CENTRAL REGION HAS AN EIGHT-FOLD ROTATIONAL SYMMETRY ACROSS ITS NUCLEO-CYTOPLASMIC AXIS, COMPOSED OF THE CYTOPLASMIC, CENTRAL SPOKE, AND NUCLEAR RING STRUCTURES (

Figure 1-11) [266, 267]. Proteinaceous filaments, which extend to the cytoplasm and nucleoplasm, are attached to the cytoplasmic and nuclear ring, respectively, with the nuclear filament forming the nuclear basket [268-271]. Although overall NPCs architectural features are conserved during evolution, the size of the NPC differs from 66 MD in yeast to 125 MD in vertebrates [272, 273]. Recent studies showed that yeast NPC harbours only 16 copies of most Nups, while human NPC contains twice or more the amount of Nups compared to yeast [274, 275].

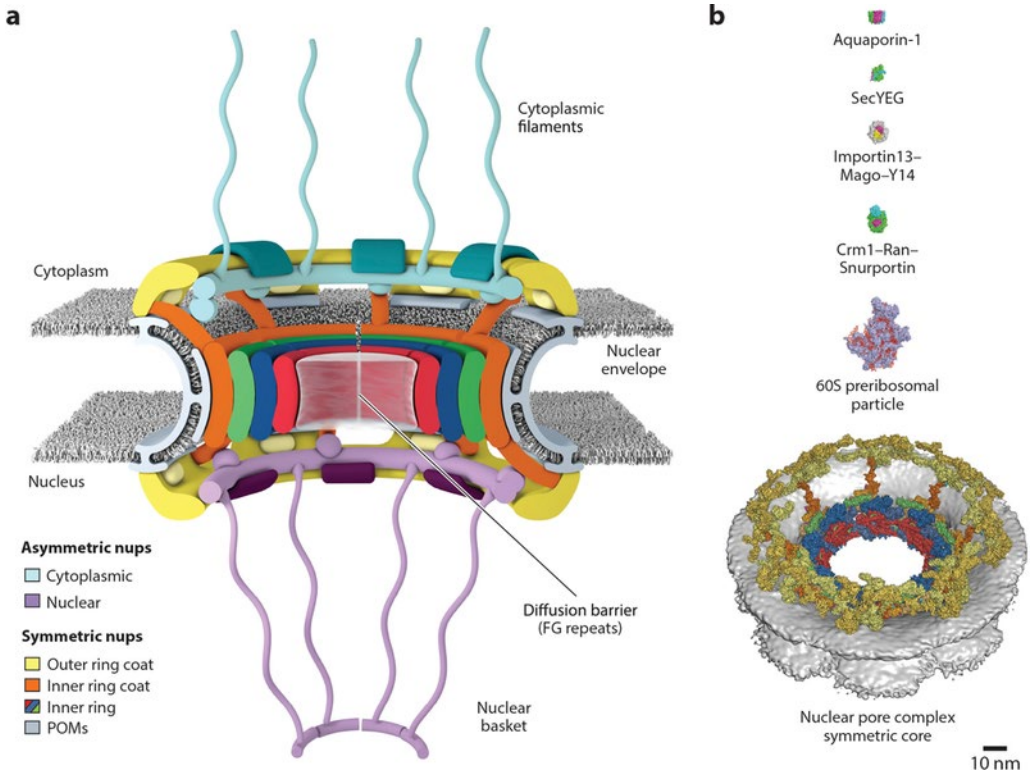


FIGURE 1-11 THE STRUCTURE OF THE NUCLEAR PORE COMPLEX.

(A) **SCHEMATIC REPRESENTATION OF THE NPC ARCHITECTURE. A CUTAWAY VIEW DEPICTING HALF OF AN NPC IS SHOWN.** (B) **COMPARISON OF SIZES OF THE NPC WITH OTHER MEMBRANE TRANSPORTERS AND EXAMPLES OF NUCLEOCYTOPLASMIC TRANSPORT CARGOES. SURFACE REPRESENTATIONS OF HUMAN AQUAPORIN-1 (PDB 1FQY), BACTERIAL SECYEG (PDB 1RHZ), *DROSOPHILA MELANOGASTER* IMPORTIN 13–MAGO–Y14 (PDB 2X1G), MAMMALIAN CRM1–RAN(GTP)–SNURPORTIN (PDB 3GJX), AND A *SACCHAROMYCES CEREVISIAE* 60S PRERIBOSOMAL PARTICLE (PDB 3JCT) ARE SHOWN TO SCALE WITH THE HUMAN NPC SYMMETRIC CORE. (SOURCE: DH LIN ET AL., 2019)**

1.1.5.2 Nuclear localization signals (NLS)

Nuclear localization signals (NLS) are often short peptides that function as signal segment that mediates protein trafficking from the cytoplasm into the nucleus. Classical NLS (cNLS) sequences are the most well-studied transport signals, which consist of either one (monopartite) or two (bipartite) clusters of basic amino acids [276-278]. SV40 large T antigen NLS Pro-Lys-Lys-Lys-Arg-Lys-Val (PKKKRKV), with five consecutive positively charged amino acids, serves as a typical example of monopartite cNLSs [279]. Monopartite cNLS (P_{640}KLKRQ_{646}) in the vasopressin/cullin5-activated calcium mobilization protein (VACM1/CUL5) is required for localization and cell growth inhibitory effects. This sequence begins with a proline (P) and is followed by an amino acid sequence containing three of the four basic radicals (PKLKR)[280]. A similar NLS (RPRK) modulates the nuclear translocation of CXCR4 [281].

On the other hand, the bipartite cNLSs is distinguished by two clusters of 2–3 positively charged amino acids connected by a 9–12 amino-acid linker region that includes few proline (P) residues [282]. The sequence (K/R)(K/R)$X_{10–12}$ $(K/R)_{3/5}$ has been characterized as a putative bipartite consensus sequence, where $(K/R)_{3/5}$ represents at least three lysine or arginine residues out of five amino acids in a row [283]. Interestingly, the two clusters of amino acids in the bipartite cNLSs are mutually dependent and essential as they work together to achieve protein localization to the nucleus. Bipartite cNLSs are exemplified by the nucleoplasmin NLS ($\underline{K}_{155}\underline{R}$PAATKKAGQA$\underline{KKKK}_{170}$) that promotes nuclear accumulation [276, 277].

Classical NLS is recognized by importin α, which is an adaptor protein linking cargo to the importin β. Importin β mediates cargo translocation into the nucleus via its interaction with the nuclear pores (see section 1.5.3 below) [284, 285]. Although cNLS motifs promote the majority of nuclear protein import, there are many additional nuclear import events mediated by non-classical NLS motifs, which are neither canonical nor rich in arginine or lysine residues [286]. Most non-classical NLS are directly recognized by importin β, independently of the importin α [287]. PY-NLS, a non-classical (ncNLS) from the "proline-tyrosine" category, is the most thoroughly studied. PY-NLS is distinguished by 20–30 amino acids with a disordered structure, which contains N-terminal hydrophobic or basic motifs and a C-terminal R/K/H$(X)_{2-5}$PY motif (where X_{2-5} is any

sequence of 2–5 residues) [288]. The N-terminal motif of two subclasses, hPY-NLS and bPY-NLS, were used to classify them. The φG/A/Sφφ motif (where φ is a hydrophobic residue) is found in hPY-NLS, whereas basic residues are found in bPY-NLS [27]. In general, PY-NLS consensus is [basic/hydrophobic]-Xn- [R/H/K]-(X) $_{2–5}$-PY [38], with X being any residue [289]. Human heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) NLS is characterized as an hPY-NLS because of its sequence F_{263}GNYNNQSSNFGPMKGGNFGGRSSGPY$_{289}$, which contains a hydrophobic region (F_{273}GPM$_{276}$) necessary for nuclear localization. The NLS of heterogeneous nuclear ribonucleoprotein 1 (Hrp1) is quite similar to that of bPY-NLS. Nuclear import, receptor binding, and protein function are all dependent on the basic residues (H_{511}RR$_{513}$) and the C-terminal R_{525} (X)$_{2-5}$PY motif [286].

1.1.5.3 Regulation of NLS activity by phosphorylation

Post-translational modifications (PTM) at or near nuclear localization signals (NLS) can help modulate the capacity of an NLS to promote the nuclear import of a protein. Phosphorylation of NLSs can increase or decrease their ability to bind the nuclear transport receptors, adding another level of control over nuclear import. An example in budding yeast is the Cbk1-mediated phosphorylation of the nucleocytoplasmic shuttling protein Ssd1 within its N-terminal NLS. Ssd1 is an RNA-binding protein associated with nearly 50 mRNAs encoding for cell wall proteins and targeting them to sites of polarized growth. Ssd1 phosphorylation by the kinase Cbk1 promotes its nuclear import and the cortical localization of Ssd1-associated mRNAs [290]. Also, in budding yeast, the nuclear import of the transcription factor Pho4 is regulated by phosphorylation. Pho4 regulates phosphate homeostasis, and upon phosphate starvation, dephosphorylated Pho4 is transported to the nucleus to activate genes that respond to the levels of cellular phosphate**.** On the other hand, under circumstances when phosphate is supplied, Pho4 is depleted from the nucleus by the nuclear export factor Msn5 after being phosphorylated by the cyclin-dependent kinase (CDK) complex Pho80-Pho85 [291]. Finally, the cell cycle-dependent phosphorylation of serine 160 of the transcription factor Swi6, located immediately upstream of the NLS, prevents the nuclear localization of Swi6 [292]. Phosphorylation of Swi6 by Clb6/Cdc28 leads to its cytoplasmic localization and downregulation of its target genes. Conversely, dephosphorylation of serine 160 correlates with Swi6 nuclear accumulation [293].

1.1.5.4 A family of protein transport receptors: The karyopherins

In vitro transport assay, together with biochemical purifications, have led to the identification of soluble factors required for efficient nuclear transport, called karyopherins [294, 295]. Importin α, also known as karyopherin α, has three functionally distinct domains, including the N-terminal importin β-binding (IBB) domain, the armadillo (Arm) repeats that serve as internal cargo cNLS-binding sites, and a C-terminal region that interacts with the nuclear export factor of importin α CAS in conjunction with RanGTP [296-298]. Whereas budding yeast contains only one importin α, called Srp1, mice and humans encode six and seven subtypes, respectively [299-301]. The central region of importin α comprised ten tandem arrays of armadillo motifs of about 42–43 amino acids. The helical ARM repeats fold into a twisted slug-like structure, whose belly functions as the groove that binds to the cNLS.The helical surface groove of Arm repeats can bind classical nuclear signals in two NLS-binding sites, known as major (Arm repeats 2-4) and minor (Arm repeats 6-8). SV40 large T antigen NLS, as typical monopartite NLS, binds to the major binding site, whereas bipartite cNLS, like the nucleoplasmin NLS, binds to both the major and minor binding sites [302].

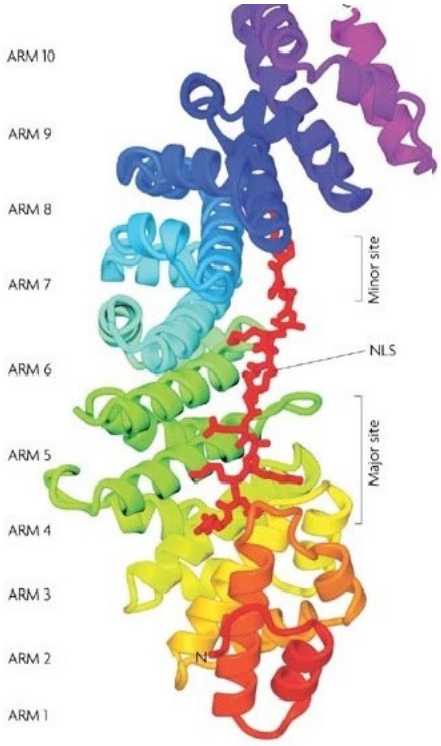


FIGURE 1-12 STRUCTURE OF THE NLS (RED) BOUND TO IMPORTIN-A.

THE 10 DIFFERENT ARMADILLO (ARM) REPEATS OF IMPORTIN-A ARE SHOWN IN DIFFERENT COLOURS. THE NLS-BINDING SITES ARE LOCATED IN A GROOVE ON THE INNER SURFACE OF IMPORTIN-A: THE MAJOR BINDING SITE SPANS ARM REPEATS 1–4, WHEREAS THE MINOR SITE SPANS ARM REPEATS 6–8. (SOURCE: STEWART, 2007).

Importin-β is another member of the karyopherin family, which, either directly or via binding to importin α, can interact with cargoes and mediate their nuclear import. Importin β is part of a large family with 14 members in the yeast *Saccharomyces cerevisiae* and at least 20 members in humans, that play key roles in the nuclear import and export of proteins and RNAs [303]. Interestingly, importin β1 is the only member of the Importin β family that needs the adaptor protein importin α to bind cargoes [304]. Importin β family can undergo nuclear transport in the absence of importin α through direct binding to the nuclear pore FG repeats, possibly due to the HEAT repeats, which contain multiple flexible helices [305-307].

1.1.5.5 Ran GTPase: A key to transport directionality

Ran guanine triphosphatase (GTPase) belongs to the Ras superfamily of GTPase. Ran GTPase is a highly abundant small protein located mainly in the nucleus. Several cellular functions, including nucleo-cytoplasmic transport, cell-cycle progression, and nuclear envelope assembly, are accomplished by Ran GTPase [308-310]. Like other small GTPase, Ran functions as a molecular switch which cycles between inactive GDP-bound (RanGDP) and active, GTP-bound states that are asymmetrically distributed in the nucleus and cytoplasm, respectively [311-313]. Conversion between these two forms is driven by a guanine nucleotide exchange factor (RanGEF), also known as RCC1. As chromatin-associated RCC1 is a nuclear protein, Ran in the nucleus is mainly in the GTP-bound state [314, 315]. A conformational change induces upon the interaction of importin β family molecules with RanGTPase results in the release of both importin-α and its cargo protein [302, 316]. On the other hand, although Ran has intrinsic GTPase activity, the cytoplasmic hydrolysis of RanGTP to RanGDP is greatly stimulated by the GTPase-activating protein RanGAP1, coupled with Ran-binding protein 1 (RanBP1) and/or Ran-binding protein 2 (RanBP2, also called Nup358) [317, 318]. The cytoplasmic compartmentalization of Ran GTPase regulators generates a relatively high concentration of RanGTP in the nucleus and a lower concentration of RanGDP in the cytoplasm. The steep gradient of RanGTP/GDP between the nucleus and cytoplasm determines the direction of nuclear transport [311]. In addition, even during mitosis, the steep gradient in RanGTP/GDP ratio is sustained, which plays a critical role in regulating spindle assembly [319, 320].

1.1.5.6 Molecular mechanism of classical nuclear protein import

The first step in the protein nuclear import cycle is the recognition of the cNLS-containing cargo protein by an adapter molecule, importin α, and subsequently, the interaction of importin α with importin β to form a stable complex called nuclear pore-targeting complex (PTAC)[321]. The nuclear pore-targeting complex is targeted to the nuclear pore via the association of importin β with nucleoporins, where it translocates through the nuclear pore. In the nucleus, the high concentration of the small GTPase Ran in its GTP-bound form binds to importin β, which triggers the disassembly of the complex, resulting in the release of the cNLS-containing cargo in the nucleus. Other molecules that associate with importin α, like the nucleoporin Npap60 or RBBP4 (Retinoblastoma

binding protein 4, also refers as RbAp48), are probably involved in the release of importin α/importin β1/NLS-cargo complex in the nucleus. Npap60 is an FG repeat-containing nucleoporin which directly binds to importin α and promotes the dissociation of the cNLS-cargo from importin α [322-324]. RBBP4 could accelerate the dissociation of importin β from importin α by binding to the IBB domain of importin α [325].

After releasing its cargo in the presence of RanGTP, importin α is exported to the cytoplasm by binding to the karyopherin-β family member called CAS/CSE1L as a ternary complex [296, 297]. Importin β1 also returns to the cytoplasm in the RanGTP-dependent fashion [298]. After passing through NPCs, the dissociation of the RanGTP/importin β1 export complex and RanGTP/CAS/importin α export complex is made by hydrolysis of RanGTP to RanGDP by RanGAP1, together with accessory proteins RanBP1 and/or RanBP2 in the cytoplasm [326, 327]. Like importin α and importin β, Ran recycling between the nucleus and cytoplasm is needed for the next round of transport. The Nuclear transport factor-2 (NTF2) acts as a transport receptor for Ran, binding especially to RanGDP and allows its rapid entry in the nucleus [328, 329]. Furthermore, during Ran nuclear import, NTF2 functions as a RanGDP dissociation inhibitor (RanGDI) and maintains the GDP-bound state during Ran recycling in the nucleus [330].

1.1.6 Hypothesis and research objectives of this project

Messenger RNA localization has been studied in several organisms, and thousands of transcripts have been found to be distributed asymmetrically within cells [116]. Still, in most cases, the molecular pathways responsible for the sorting of these mRNAs have not been identified, and for just a small number, the regulation of their localization process has been explored [331]. So far, most studies have focused on the roles of post-translational modifications in the translational regulation of localized mRNAs. For instance, in yeast, the kinases Yck1 and CK2 phosphorylate Khd1 and Puf6, respectively, triggering their release from the *ASH1* mRNP complex and promoting the translation of *ASH1* mRNA at the bud tip [126, 225]. How post-translational regulation may affect the mRNA localization machinery itself is not clear. In budding yeast, among the mRNA localization factors She2, She3 and Myo4, only She3 post-translational modifications have been studied so far, but their role(s) remain unclear [332-334].

While She2 is the key RNA-binding protein that plays a critical role in mRNA localization at the bud tip, its regulation is still poorly understood. So far, large-scale phosphoproteomic investigations have identified only one phosphorylated residue in She2 at S166 [335], and there is evidence that She2 is ubiquitinated [245]. How and when post-translational modifications on She2 may regulate the mRNA localization pathway remains unknown. *Our main hypothesis is that phosphorylation regulates the activity of She2 and mRNA localization in yeast*. We propose the following objectives to explore this question:

1) Perform a phosphoproteomic analysis of She2 to identify novel phosphorylated residues
2) Determine the role of key phosphosites in the regulation of She2 activity
3) Identify which kinases(s) are involved in these phosphorylation events

In chapter 2, we determined that She2 is a phosphoprotein and employed mass spectrometry analysis to identify phosphorylated residues in this protein *in vivo*. We identified several phosphosites in She2, including at the oligomerization interfaces of She2 monomers. We uncovered a role for T109 phosphorylation in the regulation of She2 dimerization, interaction with She3 and binding to *ASH1* mRNA. These findings suggest that phosphorylation regulates She2 oligomerization, which emerges as a key regulatory step in mRNA localization at the bud tip.

In chapter 3, we aimed to identify the kinases(s) involved in T109 phosphorylation. Although T109 is present within a putative casein kinase 2 (CK2) recognition motif, we demonstrated that T109 phosphorylation is not dependent on CK2. Instead, we found that She2 monomers are preferentially phosphorylated by CK2 at S217 and S224 *in vitro*. Since these two amino acids are in the C-terminal NLS of She2, we explored the putative role of these two phosphosites in the regulation of She2 interaction with the importin-α Srp1 and its nuclear import.

Chapter 2

2 Phosphorylation controls the oligomeric state of She2 and mRNA localization in yeast

Karen Shahbabian*, Nastaran Farajzadeh*, Yani Bouaziz, Emmanuelle Querido and Pascal Chartrand

Department of Biochemistry and Molecular Medicine, Université de Montréal

Montréal, Qc H3C 3J7 Canada

* These authors contributed equally

Corresponding author: p.chartrand@umontreal.ca

Manuscript to be submitted

2.1 AUTHORS' CONTRIBUTION

Karen Shahbabian She2 purification and mass spectrometry analysis, Yeast two-hybrid assay, RNA immunoprecipitation and RT-qPCR analysis

conceived the project, performed genetic assays, analyzed the data and wrote the first draft of the manuscript

2.2 ABSTRACT

Messenger RNA (mRNA) localization is an important mechanism for controlling local protein synthesis. In budding yeast, localization of transcripts to the bud tip, such as the *ASH1* mRNA, depends on the RNA-binding protein She2. Despite our understanding of this process in yeast, how the mRNA localization machinery is regulated still remains unknown. Herein, we performed a phosphoproteomic analysis of She2 *in vivo* and identified new phosphosites in this protein. Interestingly, several of these phosphorylated residues are located at the dimerization or tetramerization interfaces of She2. Phosphomimetic mutations at these residues disrupt the capacity of She2 to promote Ash1 asymmetric accumulation. A detailed analysis of one of these residues, T109, shows that a T109D mutation inhibits She2 oligomerization and its interaction with She3. More important, this mutation strongly impairs the capacity of She2 to bind RNA and disrupts *ASH1* mRNA localization. These results show that control of She2 oligomerization by phosphorylation constitutes an important regulatory step in the mRNA localization pathway.

2.3 INTRODUCTION

Messenger RNA (mRNA) localization contributes to the post-transcriptional regulation of gene expression by controlling the synthesis, in space and time, of specific proteins [336]. This process has been observed in several organisms, tissues and cell types and can involve a significant proportion of the transcriptome in some organisms [336, 337]. The budding yeast *Saccharomyces cerevisiae* is an established model organism to study this question since dozens of transcripts are transported from the mother cell and localized to the budding daughter cell [133, 338-340]. One of these transcripts, *ASH1* mRNA, is transported to the bud during late anaphase and has been extensively studied [84, 341]. The journey of *ASH1* mRNA to the bud tip starts with the co-transcriptional interaction of She2, the main RNA-binding protein involved in the bud-localization of mRNAs, with four *cis*-acting elements (or zipcodes) along the mRNA sequence [239, 342]. She2 helps recruit the translational repressor Puf6 on *ASH1* via a common partner, Loc1 [196]. Khd1, another translational repressor, is also loaded on this transcript in the nucleus [343]. After transcription, the *ASH1* mRNP complex is exported to the cytoplasm, where She3 replaces Loc1 to assemble a stable mRNP complex competent for localization [344, 345]. Transport of the mRNP requires the type V myosin Myo4 and the actin cytoskeleton until the complex reaches the bud tip, where the local translation of the transcript occurs [346, 347].

She2 is the key RNA-binding protein (RBP) responsible for the localization of transcripts at the bud tip [348, 349]. She2 is a non-canonical RBP which assembles into a tetramer and contains two basic-rich RNA-binding domains that can bind two independent RNA localization elements [350]. Oligomerization of She2 is also essential for its interaction with Loc1 and She3, which help stabilize the RNA-protein complex in the nucleus and cytoplasm, respectively [345, 351]. On the

other hand, She2 oligomerization decreases its capacity to bind the importin α Srp1, which promotes the nuclear import of She2 via its interaction with a non-canonical nuclear localization sequence at the C-terminus [352].

So far, studies exploring the regulation of mRNA localization and local translation have mostly focused on the post-translational mechanisms regulating translational repression [353]. In yeast, the translational repressors Khd1 and Puf6 are bound to the *ASH1* mRNA and repress eIF4G-40S and eIF5B-60S interactions, respectively [126, 354]. At the bud tip, the kinases Yck1 and CK2 phosphorylate Khd1 and Puf6, respectively, leading to the release of translational repression on *ASH1* and local Ash1 protein synthesis [126, 354]. Still, how the localization machinery itself is regulated remains unknown. Both She3 and Myo4 are phosphoproteins, and phosphorylation of She3 at S343, S348 and S361 inhibits its capacity to bind RNA [355]. However, the biological function(s) of these phosphorylation events remain unknown. More important, little is known about the regulation of the key RNA-binding protein She2. While She2 has been reported to be phosphorylated *in vivo* [356], only one phosphorylated residue (S166) has been identified so far in a large-scale phosphoproteomic study [357].

Here, we used phosphoproteomic analysis to identify the phosphorylated residues in She2 *in vivo*. We identified several phosphosites that impact the capacity of She2 to promote the asymmetric accumulation of Ash1. Interestingly, some of these phosphosites are present at the dimerization and tetramerization interfaces of She2. Focusing on T109, we show that a phosphomimetic mutant T109D inhibits She2-She2 interaction and decreases the interaction of She2 with its partners Srp1, She3 and *ASH1* mRNA. Interestingly, the T109D mutation significantly reduces the expression of the She2 protein. Altogether, our results show that the control of She2 oligomerization by phosphorylation represents a mechanism regulating mRNA localization in budding yeast.

2.4 RESULTS

2.4.1 Identification of phosphorylated amino acid residues in She2

To determine if She2 is a phosphoprotein, we used the phosphoprotein enrichment Pro-Q Diamond column, which binds specifically to phosphoproteins [358], to detect the presence of She2 in the phosphorylated fraction. Passage of a yeast extract on a Pro-Q column, followed by washes and elution, revealed the presence of She2-myc in the eluate (Figure 2-1A), suggesting that a fraction of She2 may be phosphorylated *in vivo*. As a positive control, the phospho-S6 protein was also detected in the eluate (Figure 2-1A). To validate the specificity of the Pro-Q column for phosphorylated binding She2, yeast extracts were treated with phosphatase prior to loading to the column. Compared to She2-myc from untreated yeast extract, which binds to the column, She2-myc from phosphatase-treated yeast extract was not retained on the Pro-Q column (Figure 2-1B), suggesting that phosphorylation of She2 is required for its interaction with this column. Still, it remains possible that She2 interaction with a phosphorylated protein may explain its binding to this column [358]. Indeed, She2 interacts with She3, which is a known phosphoprotein [355]. To eliminate this possibility, yeast extracts were treated with urea to denature proteins prior to loading on the Pro-Q column. As shown in (Figure 2-1C), urea treatment did not impact the binding of She2-myc to the column, suggesting that She2 directly interacts with the Pro-Q column. Altogether, these results suggest that a fraction of She2 is phosphorylated in budding yeast.

To identify phosphorylated residues in She2, a yeast strain expressing a GST-She2 fusion protein under the galactose-inducible *GAL1* promoter was generated. This strain allowed sufficient induction of GST-She2 for subsequent purification of the full-length protein using glutathione beads, SDS/PAGE and LC/MS-MS analysis. Three independent phosphopeptide analyses revealed the presence of several phosphosites in She2 (Figure 2-2A and Supplementary Tables 2-1 to 2-3). Interestingly, some phosphosites are present at the dimerization interface between two She2 monomers, such as Y65, S91, S101 and T109 (Figure 2-2A and B). Another phosphosite identified (T47) is present at the interface between two She2 dimers (Figure 2-2B) [359]. Finally, two phosphorylated residues are in the nuclear localization sequence (NLS) of She2 (S217 and S224).

2.4.2 Phosphorylation of She2 impacts Ash1 asymmetric distribution

In order to identify phosphorylated residues that regulate the activity of She2, we generated phospho-null (S/T to A; Y to F) and phospho-mimetic (S/T to D) mutations at specific

phosphoresidues on a myc-tagged She2 protein expressed from its endogenous promoter on a centromeric plasmid. The activity of the mutated She2 proteins was tested in a yeast genetic assay to assess the asymmetric distribution of the Ash1 protein and its capacity to repress the *HO* promoter. Using the strain K5547, in which the *ADE2* gene is under the control of the *HO* promoter and which contains a deletion of the *SHE2* gene, symmetric distribution of Ash1 between mother and daughter cells (due to defective *ASH1* mRNA localization) leads to repression of the *ADE2* gene and poor growth on plate lacking adenine (-Ade). Expression of WT She2 or a functional She2 mutant in this strain would rescue *ASH1* mRNA localization and accumulation of Ash1 in the daughter cell, so the expression of the *ADE2* gene in the mother cell allows growth on -Ade plates [175].

Using serial dilutions and spot assays, the expression of wild-type She2 rescued the growth defect of the K5547 strain on a -TRP -ADE medium, while the expression of the She2 M5A mutant, which disrupts the NLS of She2 [352], or the empty vector YCPlac22 did not rescue cell growth on –TRP –ADE medium (Figure 2-3A). The impact of mutations at phosphorylated residues S2, T47, Y65, S91, S101, T109, Y148, S217 and S224 on Ash1 asymmetric localization was tested in this genetic assay. As shown in (Figure 2-3B), most mutations at phosphosites did not affect the capacity of She2 to promote Ash1 asymmetric distribution and growth on –ADE –TRP medium. However, the expression of a T109A mutant only partially rescued the growth defect, while the expression of the phosphomimetic T109D mutant did not rescue growth (Figure 2-3A-B). Interestingly, besides T109, phosphomimetic mutations at T47 (T47D) or S101 (S101D) did not rescue yeast growth on the selection medium, while the phospho-null mutant at T47 (T47A) or S101 (S101A) did, suggesting that phosphorylation of these residues also disrupt She2 activity (Figure 2-3B, Supplementary Figure 1).

Expression of the wild-type and T109 mutated She2-myc proteins was measured by western blot, which revealed a twofold decrease in the expression of the She2 T109D mutant compared to the wild-type She2 protein (Figure 3-3C-D). RT-qPCR analysis of *SHE2* mRNA levels showed that the T109D mutation did not affect *SHE2* mRNA expression (Figure 2-3E), suggesting that a phosphomimetic mutation at T109 significantly reduces the accumulation of the She2 protein in

budding yeast. Unlike T109D, expression of the S101D and T47D mutants of She2 was similar to the wild-type protein, even if these mutants also disrupt Ash1 protein asymmetric localization (Supplementary Figure 1).

Since phosphomimetic mutations at S101 and T109 strongly impact the function of She2, and both residues are located at the dimerization interface between She2 monomers (Figure 2-2B), we also tested a phosphomimetic mutant at S166, which is also located at the dimerization interface (Figure 2-2A) and was previously identified in a large scale phosphoproteomic study [357]. Unlike S101D and T109D, expression of She2 S166D fully complemented the *SHE2* knockout, and these cells were able to grow on –ADE –TRP medium, like the cells expressing She2 WT (Figure 2-3B).

Finally, double phospho-null or phosphomimetic mutants were also tested to explore the possible contribution of multiple phosphorylation events on the activity of She2. Double phospho-null mutations at Y65 Y148 or S217 S224 reproduced the same phenotype has a single mutation at these residues (Figure 2-3B). Similarly, a double phosphomimetic mutant at S217 S224 showed the same growth as She2 WT. On the other hand, the double mutation S101A T109A reproduced the same phenotype as a single T109A mutation (Figure 2-3B), suggesting that these mutations do not have an additive impact on the function of She2.

2.4.3 Phosphomimetic mutation at Threonine 109 disrupts She2 dimerization and its interactions with Srp1 and She3

Our phosphoproteomics analysis revealed that phosphorylation at threonine 109 (T109) is the most frequently found phosphorylated residue on She2. Indeed, it was the only phosphosite found in all three independent experiments, with a total of 30 peptides containing this phosphorylated amino acid (Supplementary Tables 2-1 to 2-3). Since a phosphomimetic T109 mutant in She2 disrupts Ash1 protein asymmetric distribution (Figure 2-3), the impact of T109 phosphorylation on the function of She2 was explored. First, the impact of the mutations T109A or T109D on She2-She2 interaction was assessed in a yeast two-hybrid assay. The assay was performed in a *she2Δ* background to eliminate the potential effect of an endogenous wild-type She2 on the two-hybrid interaction. As shown in (Figure 2-4A), the presence of a T109A mutation on

She2 in both bait and prey leads to a 3-fold reduction in β-galactosidase activity compared to the wild-type She2-She2 interaction. The impact of the T109D mutation on She2-She2 interaction is even more important, with a nearly 20-fold decrease in interaction for the homodimer She2-T109D: She2-T109D or for the formation of the heterodimer She2 WT: She2-T109D (Figure 2-4A). These results suggest that phosphorylation at T109 inhibits the dimerization and subsequent oligomerization of She2.

Since She2 oligomerization is important for its interaction with She3 [359] and modulates its binding to Srp1 [352], the impact of phospho-null or phosphomimetic mutations at T109 on She3 and Srp1 binding was also tested in a yeast two-hybrid assay, as previously described [352]. As shown in (Figure 2-4B), the phosphomimetic T109D mutation decreased by twofold the formation of the She2-She3 complex, while the T109A mutant had no impact on She2 interaction with She3. Another key interactor of She2 is the importin-α Srp1, which mediates the nuclear import of She2 via its interaction with an NLS at the C-terminus of She2 [352]. In a yeast two-hybrid assay to detect the interaction between She2 and Srp1 [352], a six-fold decrease in the interaction between She2 T109D and Srp1 was observed compared to She2 WT or She2 T109A (Figure 2-4C). Altogether, these results suggest that phosphorylation at T109 inhibits the capacity of She2 to interact with its protein cofactors.

2.4.4 Phosphomimetic mutation at Threonine 109 inhibits She2 interaction with ASH1 mRNA and localization of this transcript

A recent structural study of She2 bound to the *ASH1* mRNA localization element E3 revealed that the tetrameric structure of She2 is essential for binding this RNA [350]. Therefore, disruption of She2 dimerization should affect its capacity to bind RNA. The impact of phospho-null or phosphomimetic mutations at T109 on She2 interaction with the *ASH1* mRNA was tested using immunoprecipitation and RT-qPCR. As shown in (Figure 2-5A), a 40% reduction in *ASH1* mRNA binding was observed with the T109A mutation. On the other hand, the T109D mutation had a greater effect, with a reduction of 80% in *ASH1* mRNA binding. These results show that a phosphomimetic mutation at T109 strongly decreases the capacity of She2 to bind RNA. Even the phospho-null mutation T109A significantly impacts the interaction between She2 and *ASH1*

mRNA, which fit previous results showing that mutations in the dimerization interface of She2 decrease its capacity to bind RNA [154].

To determine the impact of a phosphomimetic mutation at T109 on the capacity of She2 to promote mRNA localization at the bud tip, single-molecule RNA FISH (smFISH) was performed on *ASH1* mRNA in strains expressing She2 WT or She2 T109D. As shown in (Figure 2-5B), the reduced interaction between She2 T109D and *ASH1* mRNA results in the delocalization of this transcript compared to cells expressing wild-type She2. Altogether, these results suggest that phosphorylation at T109 inhibits the capacity of She2 to bind *ASH1* mRNA and to promote its localization at the bud tip.

2.5 DISCUSSION

In this work, we report that the RNA-binding protein She2 is phosphorylated *in vivo*, and we identified several novels phosphorylated amino acids in this protein. While a previous article mentioned that She2 is a phosphoprotein, experimental evidences were not provided [356]. Furthermore, with the exception of one study[357], global phosphoproteomics analyses in yeast have not identified phosphopeptides from this protein so far. We used two complementary assays to explore the phosphorylation status of She2. First, using a phosphoprotein enrichment column combined with phosphatase treatment, we showed that a fraction of She2 is phosphorylated *in vivo* and can be enriched using this column. Second, we expressed and purified a GST-tagged version of She2 from yeast, which was submitted to LC/MS-MS analysis to identify phosphopeptides. From three independent experiments, we identified 13 phosphorylated residues: S2, S29, T47, Y65, S91, S101, T109, T147, Y148, S155, S217, S224 and T235. With the exception of T147, Y148 and S155, all these residues are located on the outside of She2 and are solvent-accessible in the properly folded monomeric protein. Surprisingly, five of these phosphosites are present at the dimerization or tetramerization interfaces of She2: Y65, S91, S101 and T109 are at the interface between two She2 monomers, while T47 is at the interface between two She2 dimers. Interestingly, the only other reported phosphosite in She2, S166, is also at the dimerization interface between She2 monomers [357], suggesting that She2 oligomerization interfaces may constitute important sites of regulation for this protein.

These observations raised questions about the role(s) of these phosphorylation events in the regulation of She2 and mRNA localization in budding yeast. A review of published mutagenesis studies on She2 revealed that mutagenesis of T235 to tyrosine (T235Y) has no impact on the capacity of She2 to bind RNA (Niessing et al., 2004). On the other hand, mutation of T47 to tyrosine (T47Y) leads to decreased RNA binding (Niessing et al., 2004), suggesting that some of these phosphosites may have regulatory functions. To identify these residues, we performed site-directed mutagenesis at phosphorylated amino acids to identify key residues that impact She2 capacity to promote the asymmetric accumulation of Ash1, which was assessed in a genetic assay. With the exception of T109, phospho-null substitutions at positions S2, T47, Y65, S91, S101, Y148, S217 and S224 retained proper asymmetric localization of Ash1, suggesting that phosphorylation at these residues is not essential for *ASH1* mRNA localization. Regarding T109, a T109A mutation partially disrupts yeast growth in the genetic assay. This phenotype can be explained by a reduction in She2-She2 and She2-*ASH1* mRNA interactions, as shown in yeast two-hybrid and co-immunoprecipitation assays, respectively. It is possible that the hydroxyl group of T109 participates in important hydrogen bond interactions at the binding interface between She2 monomers, interactions that would be disrupted with the alanine mutant. Therefore, we think that phosphorylation at T109 is not essential for *ASH1* mRNA localization.

A similar analysis was performed with phosphomimetic mutations, which revealed that the substitution of S2, S91, S217 or S224 with aspartic acid did not impact the function of She2. On the other hand, phosphomimetic mutations at T47, S101 and T109 disrupt the asymmetric accumulation of Ash1. Interestingly, all these amino acids are at the interface between two She2 monomers or dimers. Still, not all residues at these interfaces are essential for the function of She2 since phosphomimetic mutations at S91 and S166 remained fully functional. However, we cannot eliminate the possibility that these phosphosites are involved in other regulatory functions that are not essential for the capacity of She2 to promote mRNA localization.

Threonine 109 is the most frequently phosphorylated residue in She2, as it was detected in all three LC/MS-MS analyses, with a total of 30 peptides identified with this modification. Functional analysis of the phosphomimetic mutation at T109 revealed that this mutant strongly

inhibits She2-She2 interaction, including heterologous interaction between She2 WT and She2 T109D, suggesting that this mutant is possibly monomeric. The T109D mutation also disrupted the interaction between She2 and the importin α Srp1, which is surprising since previous results showed that monomeric She2 interacts more strongly with Srp1 compared to the She2 tetramer [352]. Consequential with the strong impact of T109D on She2 oligomerization, this mutant displays strongly reduced interaction with both She3 and *ASH1* mRNA, leading to the delocalization of this transcript from the bud tip.

Since the T109D mutant has such a negative effect on the function of She2, what could be the biological function of this phosphosite? The observation that the T109D mutant results in a twofold decrease in She2 levels raise the possibility that this phosphorylation may lead to the destabilization of the protein. Since She2 expression levels are regulated by ubiquitination[360], it will be interesting to explore the link between She2 phosphorylation and its ubiquitination.

Previous biophysical and structural studies on recombinant She2 revealed that this protein assembles as a tetramer at physiological concentration [359]. This tetrameric structure is essential for the interaction of She2 with both RNA and She3 [350]. Recent observations from in-depth phosphoproteomic analyses in budding yeast suggest that phosphorylation at protein-protein interfaces constitutes an important regulatory mechanism [361]. Our data support these observations and suggest that phosphorylation controls the assembly of the She2 tetramer and constitutes an important regulatory mechanism for She2 activity *in vivo*. How She2 can be phosphorylated at these interfaces remains unclear. One possibility is that only newly synthesized monomers of She2 are phosphorylated. It is also possible that phosphorylation occurs after the disassembly of the She2 tetramer and inhibits its reassembly by blocking interactions between She2 monomers or dimers. Dephosphorylation of She2 by phosphatase(s) may allow the reassembly of the tetramer at some point. Which kinases are responsible for these phosphorylation events is still unknown.

Besides She2, other components of the budding yeast mRNA localization machinery are also regulated by post-translational modifications. Indeed, She3 is negatively regulated by

phosphorylation at S343, S348 and S361, which inhibits its capacity to bind RNA but still maintains its interaction with She2 and Myo4[355]. These phosphorylation events have been linked to the stress response, such as osmotic stress or exposure to the reducing agent dithiothreitol (DTT)[362, 363]. This suggests that, in the presence of stress, the mRNA localization pathway may be inhibited, in part via She3 phosphorylation. Some of the phosphorylation events on She2 could play a similar role and may be part of a mechanism that inhibits the mRNA localization pathway in specific conditions.

2.6 ACKNOWLEDGMENTS

We thank the Proteomic Core facilities at the Institute for Research on Immunology and Cancer (Université de Montréal) and the Montreal Clinical Research Institute (IRCM). This project was funded by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC RGPIN-2019-05023) to PC.

2.7 METHODS

2.7.1 Yeast strains and DNA manipulation

Yeast growth was performed in YPD or synthetic selective media at 30˚C. PCR-based gene disruption was performed as described previously[364]. Gene disruptions were all confirmed by PCR analysis of genomic DNA. The strains used are listed in Supplementary (Table 2-2).

2.7.2 Plasmids construction

To generate She2 mutants, YCP22-She2-myc was PCR amplified using primers containing the appropriate mutation and cloned into PstI/KpnI sites of YCPlac22. The She2 mutants T109A and T109D were also cloned in pGADT7 and pGBKT7 plasmids for yeast two-hybrid assay. All constructions were confirmed by Sanger sequencing. The plasmids used are listed in Supplementary (Table 2-1).

2.7.3 Pro-Q® Diamond phosphoprotein enrichment columns

Yeast strain K699 *she2* + YCP22-SHE2-WT was grown in 500 ml of selection media until OD_{600} of 0.8. After centrifugation to pellet the cells, the pellet was resuspended in 5 ml of ice-cold lysis buffer (50 mM HEPES pH 7.5, 10 % Glycerol, 150 mM NaCl, 0.1 % NP-40, 1µM okadaic acid, protease inhibitors and RNAsin) and incubated 30 minutes on ice. Using tissue lyser II with a pre-cooled adapter set and glass beads, cells were lysed for 2 min at 20 Hz and centrifuged at 14000xg for 30 min at 4°C. The supernatant was collected, and protein concentration was measured with Bradford assay. The lysate was diluted with the wash buffer provided with the Pro-Q® Diamond phosphoprotein enrichment kit to a final concentration of 0.1 mg/ml. Pro-Q columns were prepared according to the manufacturer and equilibrated with 2x 1 mL of wash buffer. The diluted lysate was applied on the column, 1 mL at a time, followed by 3 washes with 1 ml of wash buffer. Flow-through and the three washes were conserved for western blot analysis. Elution of the phosphoproteins from the column was performed with 5x 250 µL of elution buffer (provided with the kit). Eluates were pooled and concentrated to a volume of 50 µL with 25mM Tris pH 7.5, 0,25% CHAPS using a Vivaspin® filtration concentrator. For phosphatase treatment, 10 µL of fastAP Thermosensitive Alkaline phosphatase (1U/ul) was added to a solution of 0.1mg/ml of yeast protein extract in 1x reaction buffer (10 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 0.02 % Triton X-100 and 0.1 mg/ml BSA). The extract was incubated at 37°C for 3 hours before loading on the Pro-Q column.For urea-mediated protein denaturation, the yeast lysate was diluted 10 times with the Pro-Q Diamond phosphoprotein Enrichment Kit Wash buffer containing 8M urea and run through the column. The column was washed 3 times with the same wash buffer containing 8 M urea before elution with the elution buffer.

2.7.4 She2 purification and Mass spectrometry analysis

Yeast strain YS1052 GST-SHE2 with *GAL1$_{prom}$-GST-SHE2* was grown in YEP + 2% raffinose until OD_{600} 0.2, where GST-She2 expression was induced with 3% galactose. At OD_{600} 0.8, total proteins were extracted using glass beads in lysis buffer (20mM HEPES pH. 7.5, 20% glycerol, 250mM NaCl, 0,05% NP-40, 50x protease inhibitors + PMSF, 5mM Na_3VO_4 and 25mM Na_3F). GST-She2 was purified using glutathione beads and eluted with 25mM glutathione in 1xPBS. Following elution from the beads, GST-She2 was further purified on an SDS-PAGE gel, stained with Coomassie blue and GST-She2 bands were cut from the gel. Destaining was

performed in 50% MeOH. Bands were shrunk in 50% ACN, reconstituted in 50 mM ammonium bicarbonate with 10 mM TCEP and vortexed for 1 h at 37°C. Chloroacetamide was added for alkylation to a final concentration of 55 mM. Samples were vortexed for another hour at 37°C. 1 µg of trypsin was added, and the digestion was performed for 8 h at 37°C. Peptide extraction was conducted with 90% ACN. Extracted peptide samples were dried down and solubilized in ACN 5% formic acid (FA) 0.2%. Samples were loaded on a homemade C18 pre-column (0.3 mm i.d. x 5 mm) connected directly to the switching valve and separated on a homemade reversed-phase column (150 µm i.d. x 150 mm) with a 56-min gradient from 10–30% acetonitrile (0.2% FA) and a 600 nl/min flow rate on an Ultimate 3000 nano-LC (Dionex) connected to a Q-Exactive Plus (Thermo Fisher Scientific). Each full MS spectrum acquired with a 70,000 resolution was followed by 12 MS/MS spectra, where the 12 most abundant multiply charged ions were selected for MS/MS sequencing. Tandem MS experiments were performed using HCD at a collision energy of 25%. The data were processed using PEAKS 7.0 (Bio-informatics Solutions) and the *Saccharomyces cerevisiae* Uniprot database. Tolerances on precursors and fragments were 10 ppm and 0.01 Da, respectively. Variable selected post-translational modifications were carbamidomethyl (C), oxidation (M), deamidation (NQ), and Phosphorylation (STY). The identification of phosphopeptides was further validated using Mascot. Cutoff for phosphopeptide identification probability was established at 70%. Three independent mass spectrometry analyses were performed, with peptide coverage of She2 at 76%, 86% and 99%, respectively.

2.7.5 Yeast two-hybrid assay

Yeast two-hybrid assay was performed as previously[349, 352]. Plasmids pGADT7 or pGBKT7 expressing She2, She2T109A and She2T109D were transformed in pJ69-4A *she2Δ* strain with the appropriate bait. Expression of the fusion proteins was confirmed by western blot. β-galactosidase activity was measured in solution using ONPG (o-nitrophenyl-D-galactopyranoside) as previously described[352], from at least three independent yeast cultures.

2.7.6 RNA immunoprecipitation and RT-qPCR analysis

Yeast cells were grown at 30°C in 50 ml culture of –TRP + 2% glucose to an OD_{600} ~1.0. Cells were harvested by centrifugation and resuspended in 900 µl of lysis buffer (25mM HEPES

pH 7.5, 2mM $MgCl_2$, 0.1% IGEPAL, 150mM KCl, 1mM DTT, protease inhibitors, 2mM VRC and 100U/ml of RNAsin). The cells were broken with glass beads by vortexing 6x 1 minute, with 1 minute pause on ice between each vortex. The lysate was centrifuged 10 minutes at 6500 RPM for 10 minutes, and the supernatant was recovered. From the supernatant, 600 µl was used for immunoprecipitation, and 300 µl was kept as input. Immunoprecipitation of She2-myc was performed using 10 µg of 9E10 mouse anti-myc antibody added to the supernatant and incubated overnight at 4°C. The day after, the solution was incubated for 4 hours with 30 µl of Protein G Sepharose at 4°C. The beads were washed once with the lysis buffer for 5 minutes, followed by three washes of 5 minutes with the wash buffer (25 mM HEPES pH 7.5, 150 mM KCl, 2 mM $MgCl_2$). Elution was performed by incubation of the beads with 200 µl of 20mM Tris pH 8.0, 100mM NaCl, 2mM EDTA, 5% SDS for 10 minutes at 65°C. From the eluate, 35 µl was used for western blot to detect She2-myc.With the remaining volume of eluate, phenol/chloroform and ethanol precipitation were performed to recover the RNA. Reverse transcription was performed using half of the purified RNA with 0.2 µg of $pd(N)_6$ oligos, incubated at 70°C for 5 minutes and quickly chilled on ice. A control without $pd(N)_6$ oligos was prepared with the remaining RNA. Reverse transcription was performed on samples in the Reverse transcriptase buffer with 100U/ml of RNAsin, 10 mM dNTPs and 100U of reverse transcriptase for 1 hour at 42°C. The enzyme was inactivated by incubation at 70°C for 15 minutes. Quantitative real-time PCR on cDNA was performed as previously [196], using primers on *ASH1* and *ACT1* mRNA. Cycle thresholds (Ct) for each triplicate of sample and input were averaged, and immunoprecipitation enrichment was calculated by dividing the amount of IP over input using the $2^{-\Delta CT}$ formula.

2.7.7 Fluorescence in situ hybridization on *ASH1* mRNA

Yeast strain K699 *she2* + C3319 expressing YCP22-SHE2 WT or T109D was grown in 50 ml of selection media until OD_{600} 0.6-0.8. Cells were fixed for 45 minutes with 4% paraformaldehyde and harvested by centrifugation at 2500g for 4 minutes at 4°C. The cell pellets were washed three times using ice-cold 1X buffer B (1.2M Sorbitol, 0.1M potassium phosphate at pH 7.5), with 4 minutes of spins at 2500g at 4°C between each wash. The pellets were resuspended in 1ml of buffer B containing 20 mM vanadyl ribonucleoside (VRC), 28 mM β-mercaptoethanol,

0.06 mg/ml phenylmethylsulfonyl fluoride (PMSF) and 120 U/ml of RNAse inhibitor. Cells were transferred into a tube containing 250 units of lyticase (Sigma-Aldrich) and incubated for 5-10 minutes at 30°C until the cell wall was fully digested. The spheroplasts were pelleted by centrifugation for 4 minutes at 2500g at 4°C, and washed with 1 ml of ice-cold 1xbuffer B. The pellets were resuspended in 750 µl of 1xbuffer B, and 100 µl of spheroplast suspension was spotted per poly-L-lysine coated coverslip. The coverslips were stored at 4°C for 30 minutes. The spheroplasts were dehydrated by adding 5 ml of 70% ethanol and incubated for at least 20 minutes at -20°C before performing the in-situ hybridization.

A set of 18 *ASH1* smiFISH probes was designed using Oligostan R[365], and diluted to a concentration of 0.833 µM in Tris-EDTA pH 8.0 (TE) buffer. The diluted probe-set was hybridized with 50 µM of a Cy3-labelled FLAP oligo in a PCR machine. Coverslips were rinsed once with 1xPBS and incubated in 15% formamide freshly prepared in 1xSSC for 15 minutes at room temperature. The hybridization *Mix 1* (2xSSC, 34 µg of E. coli tRNA, 30% formamide and FLAP-bound *ASH1* probe-set) and *Mix 2* (20 µg of RNAse-free BSA, 4 mM VRC and 21% dextran sulphate) were put together and spotted on the coverslips. The coverslips were incubated in an airtight hybridization chamber at 37°C overnight[366]. After being placed in a 6-well plate, the coverslips were washed twice for 30 minutes with freshly prepared 15% formamide/1xSSC solution at 37°C and rinsed twice in 1xPBS before mounting. The coverslips were placed on slides with Vectashield antifade mounting medium containing DAPI (VECTOR Laboratories). Imaging was performed using a Zeiss Axio-Imager Z2 upright microscope.

2.8 FIGURES LEGENDS

Figure 2-1: She2 is a phosphoprotein. (**A**) Binding of She2-myc on the Pro-Q phosphoprotein enrichment column. Detection of She2-myc by western blot from the whole lysate (I: input), flow-through (FT), first wash (W1), third wash (W3) and eluate (E) from the Pro-Q column. Phosphorylated ribosomal protein S6 (phospho-S6) was used as the positive control (bottom). (**B**) Binding of She2 to the Pro-Q column depends on its phosphorylation state. Yeast extracts were treated with Fast phosphatase (+ phosphatase) or incubated without phosphatase (- phosphatase) prior to binding to the Pro-Q column. Detection of She2-myc was performed as in A. (**C**)

Denaturation of She2 with urea does not reduce its binding to the Pro-Q column. Yeast extracts were treated with 8M urea (+ urea) or not (- urea) prior binding to the Pro-Q column. Detection of She2-myc was performed as in A.

Figure 2-2: Identification of phosphorylated residues in She2. (**A**) Sequence of She2 with phosphorylated amino acids identified by LC-MS/MS colored in red. Amino acids in the dimerization interface of She2 are underlined. Amino acids in the dimer-dimer interface of She2 are in bold. Amino acid in blue are phosphosites identified in previous studies. (**B**) Structure of the She2 tetramer. The location of phosphorylated residues T47, S91, S101 and T109 in the 3D structure is highlighted. She2 3D structure was generated with PyMOL software using the 5MOJ structure from PDB database.

Figure 2-3: Specific phosphoresidues modulate the capacity of She2 to promote the asymmetric distribution of Ash1. (**A**) Genetic assay to assess Ash1 asymmetric distribution in She2 mutants. Serial dilutions of K5547+YCP22-She2-myc, K5547+YCP22-She2-M5A-myc, K5547+YCP22-She2-T109A-myc, K5547+YCP22-She2-T109D-myc and K5547+YCP22 empty. (**B**) Impact of various phospho-null and phosphomimetic mutants of She2 on the growth of K5547 on –ADE –TRP medium. (**C**) Western blot analysis of She2-myc WT, T109A and T109D expression in the K5547 strain. YCP: K5547+YCPlac22 empty. Tubulin was used as loading control. (**D**) Quantification of She2 WT, She2 T109A and She2 T109D expression in K5547 strain. *** $p< 0.005$. N=3. (**E**) Relative expression of *SHE2* mRNA in K5547 strain expressing YCPlac22 (YCP), She2 WT, She2 T109A or She2 T109D proteins. N=2.

Figure 2-4: Phosphomimetic mutation at T109 inhibits the oligomerization of She2 and its interaction with cofactors Srp1 and She3

(**A**) Yeast two-hybrid assay to detect She2-She2 interaction. Homo- or hetero-oligomerization of wild-type She2 (WT), She2 T109A (T109A) or She2 T109D (T109D) was quantified by measuring β-galactosidase activity (N=3). ** $p<0.01$; *** $p<0.005$. (**B**) Yeast two-hybrid assay to detect interaction between She3 and She2 mutants at T109. Interaction between She3 and wild-type She2

(WT), She2 T109A (T109A) or She2 T109D (T109D) was quantified by measuring β-galactosidase activity (N=3). ** $p<0.01$. (**C**) Yeast two-hybrid assay to detect interaction between Srp1 and She2 mutants at T109. Interaction between Srp1 and wild-type She2 (WT), She2 T109A (T109A) or She2 T109D (T109D) was quantified by measuring β-galactosidase activity (N=3). **** $p<0.001$.

Figure 2-5: Mutation at T109 disrupts She2 interaction with *ASH1* mRNA and its localization at the bud tip.

(A) RNA immunoprecipitation of *ASH1* mRNA by She2 wild-type or mutants. Myc-tagged wild-type She2 (WT), She2 T109A (T109A) or She2 T109D (T109D) were immunoprecipitated using anti-myc antibody, followed by RNA purification and RT-qPCR analysis. Left panel: Enrichment of *ASH1* mRNA following immunoprecipitation is reported as a ratio immunoprecipitate versus input (IP/INPUT), with wild-type She2 set as 1.0. Right panel: Western blot of Myc-tagged wild-type She2 (WT), She2 T109A (T109A) or She2 T109D (T109D) from input or immunoprecipitate (IP). (N=3). * $p<0.05$. (**B**) Fluorescent in situ hybridization (FISH) on *ASH1* mRNA in yeast cells expressing wild-type She2 (WT) or She2 T109D (T109D) proteins. Scale bar: 5 μm.

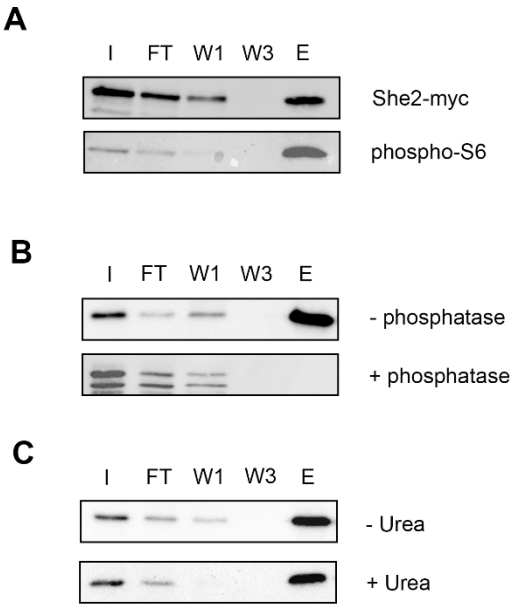


FIGURE 2-1 SHE2 IS A PHOSPHOPROTEIN.

A

```
  1 MSKDKDIKVT PGTCELVEQI LALLSRYLSS YIHVLNKFIS HLRRVATLRF ERTTLIKFVK
 61 KLRFYNDCVL SYNASEFINE GKNELDPEAD SFDKVILPIA SMFVKCVETF DLLNYYLTQS
121 LQKEILSKTL NEDLTLTAES ILAIDDTYNH FVKFSQWMIE SLRIGSNLLD LEVVQFAIKC
181 ADEDGTNIGE TDNIFLQEIL PVNSEEEFQT LSAAWHSILD GKLSALDEEF DVVATKWHDK
241 FGKLKN*
```

B

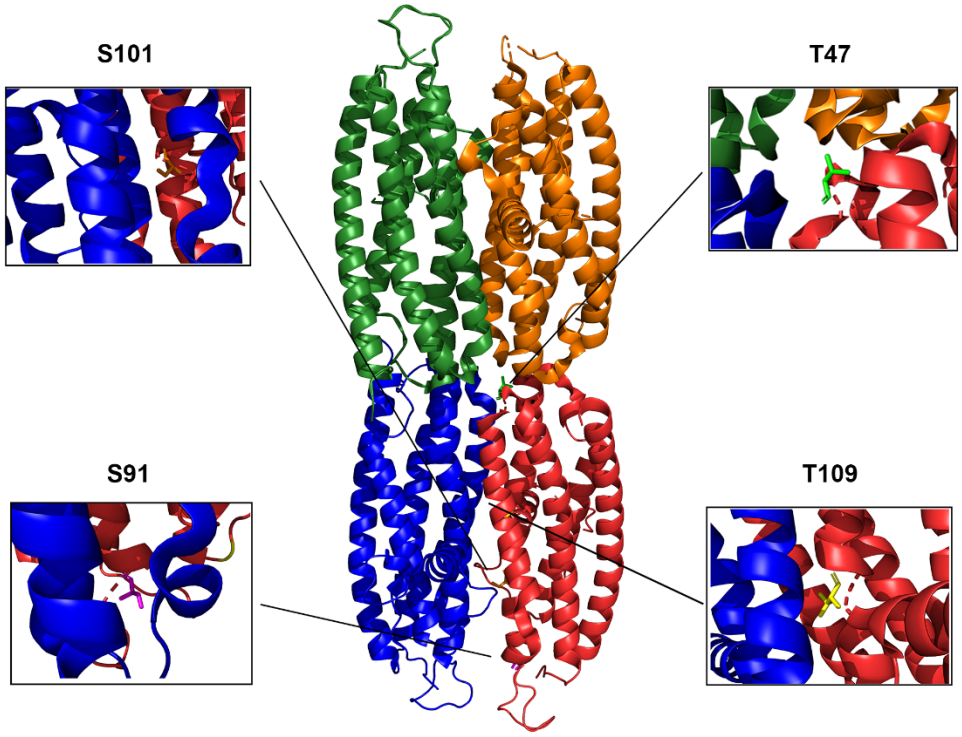
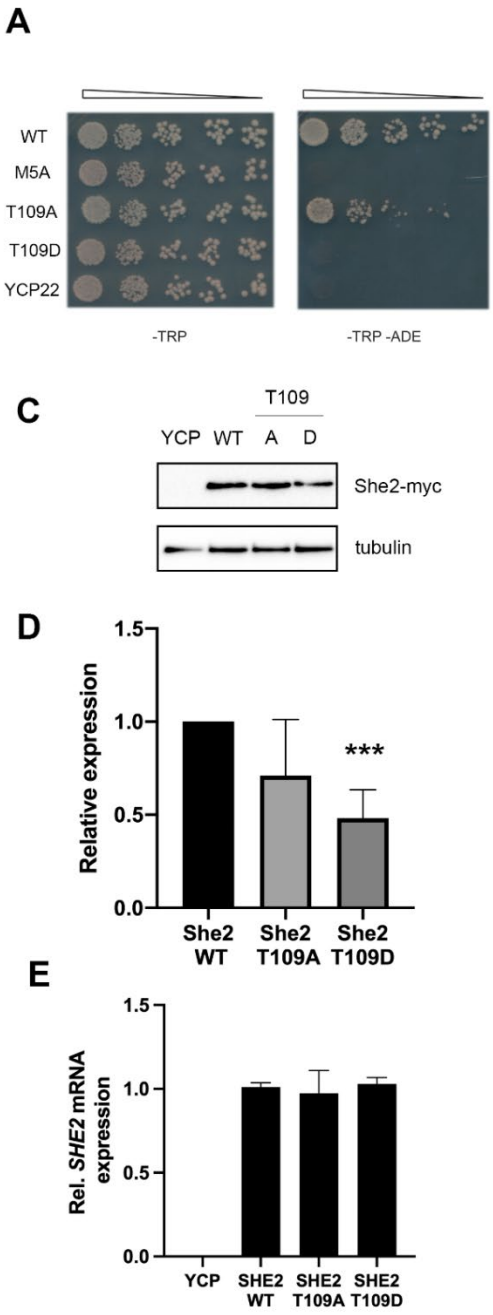


FIGURE 2-2 IDENTIFICATION OF PHOSPHORYLATED RESIDUES IN SHE2.



B

Mutation	Growth on –TRP –ADE medium
S2A	+++++
S2D	+++++
T47A	+++++
T47D	-
Y65F	+++++
S91A	+++++
S91D	+++++
S101A	+++++
S101D	-
T109A	++
T109D	-
Y148F	+++++
S166D	+++++
S217D	+++++
S224A	+++++
S224D	+++++
Y65F Y148F	+++++
S101A T109A	++
S217A S224A	+++++
S217D S224D	+++++

FIGURE 2-3 SPECIFIC PHOSPHORESIDUES MODULATE THE CAPACITY OF SHE2 TO PROMOTE THE ASYMMETRIC DISTRIBUTION OF ASH1.

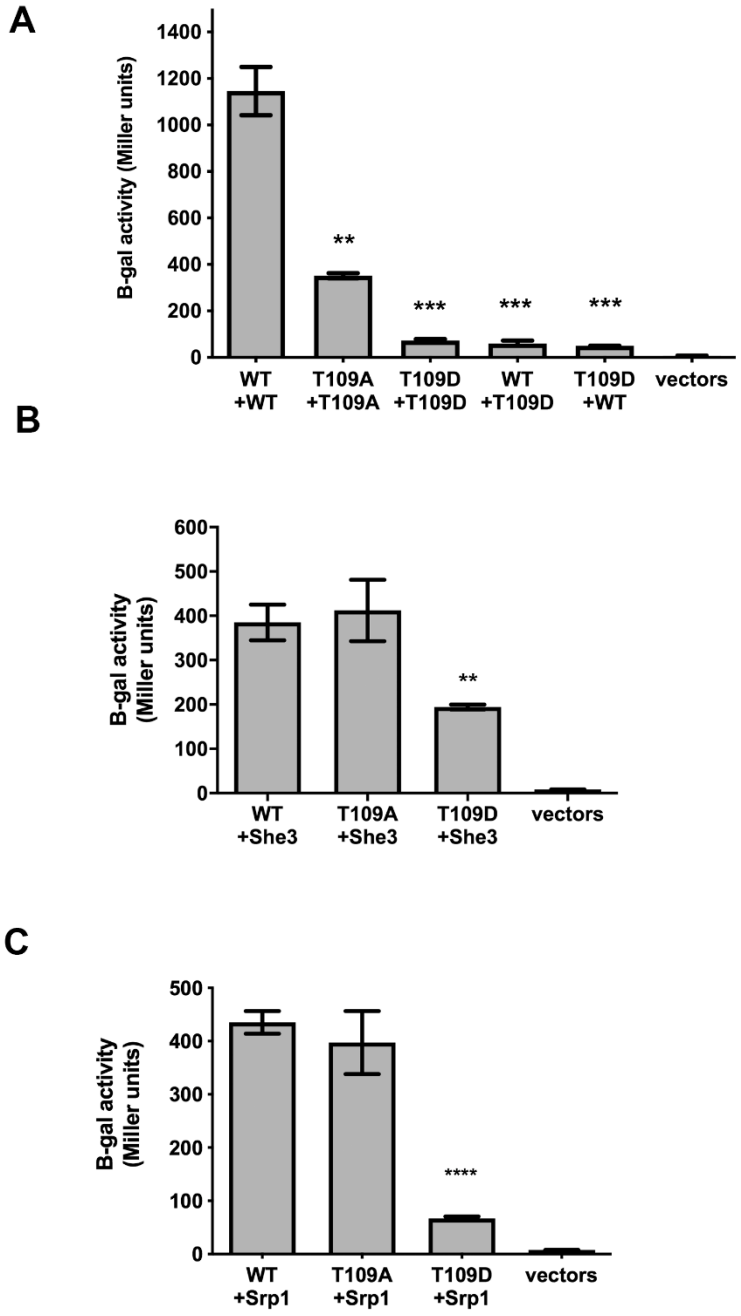


FIGURE 2-4 PHOSPHOMIMETIC MUTATION AT T109 INHIBITS THE OLIGOMERIZATION OF SHE2 AND ITS INTERACTION WITH COFACTORS SRP1 AND SHE3

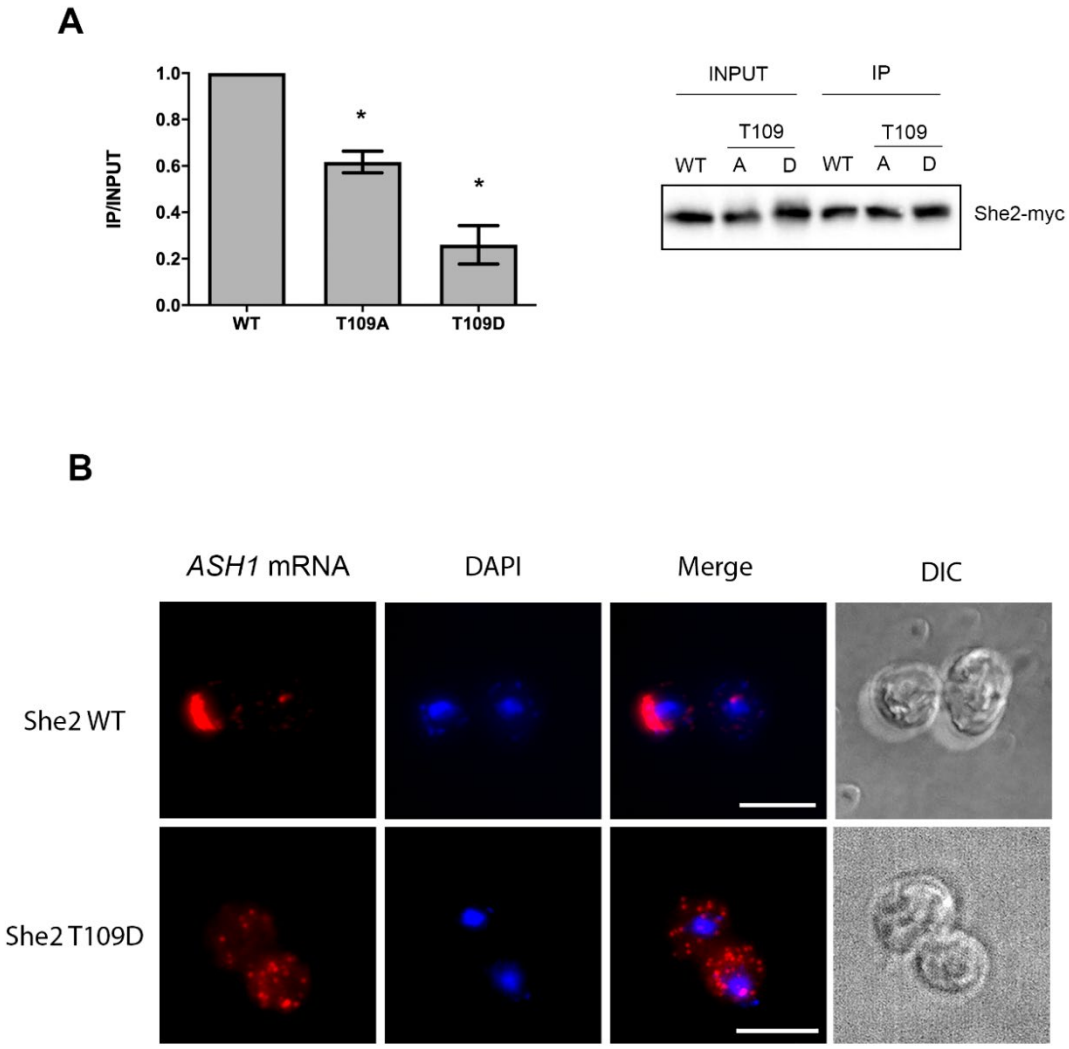
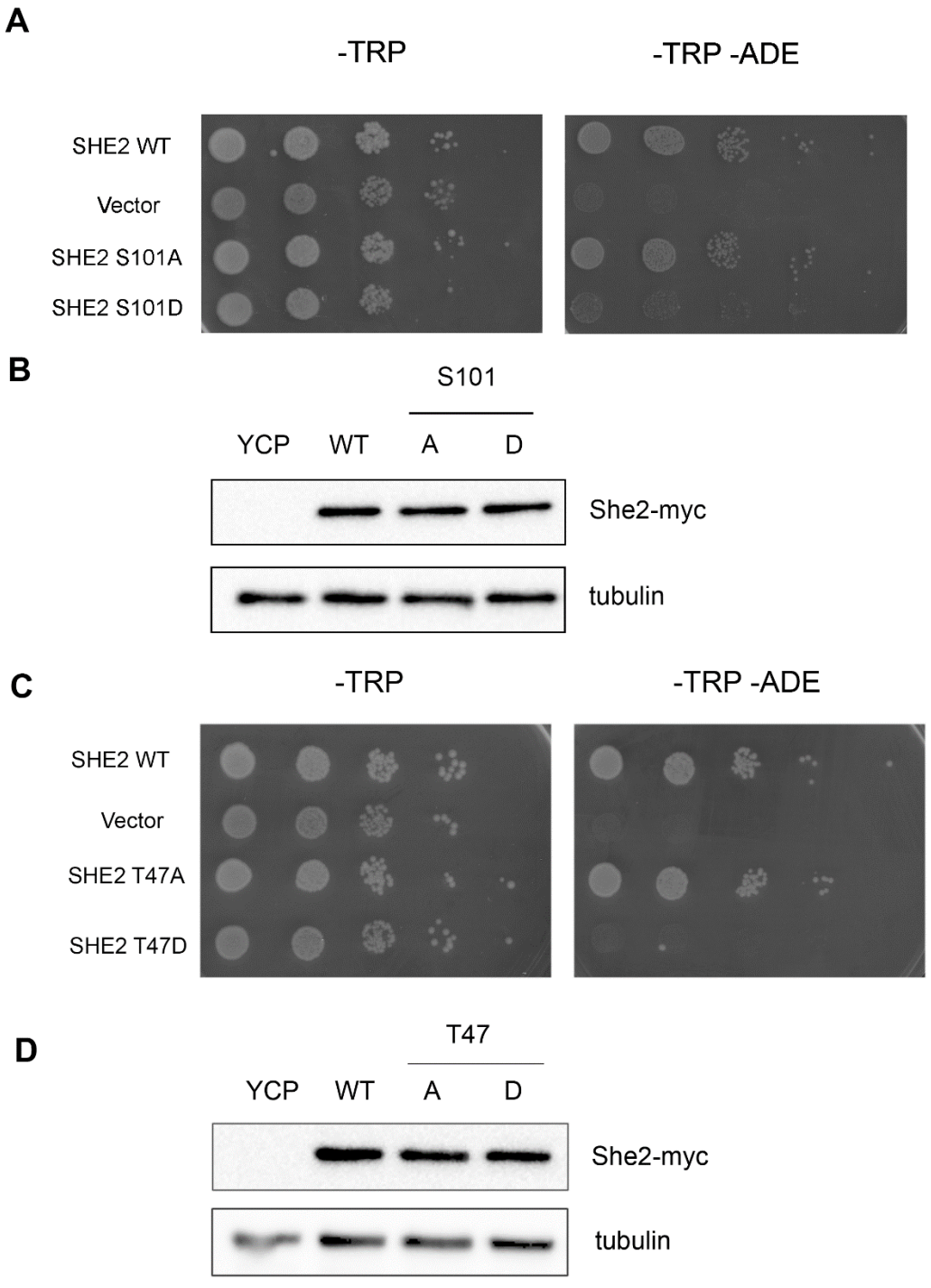


FIGURE 2-5 MUTATION AT T109 DISRUPTS SHE2 INTERACTION WITH ASH1 MRNA AND ITS LOCALIZATION AT THE BUD TIP.

Supplementary Figure 1: Phosphomimetic mutations at She2 S101 and T47 disrupt asymmetric distribution of Ash1. (**A**) Genetic assay to assess Ash1 asymmetric distribution in She2 S101 mutants. Serial dilutions of K5547+YCP22-She2-myc (WT), K5547+YCP22 empty (vector), K5547+YCP22-She2-S101A-myc and K5547+YCP22-She2-S101D-myc. (**B**) Western blot analysis of She2-myc WT, S101A and S101D expression in the K5547 strain. YCP: K5547+YCP22 empty. Tubulin was used as loading control. (**C**) Genetic assay to assess Ash1 asymmetric distribution in She2 T47 mutants. Serial dilutions of K5547+YCP22-She2-myc (WT), K5547+YCP22 empty (vector), K5547+YCP22-She2-T47A-myc and K5547+YCP22-She2-T47D-myc. (**D**) Western blot analysis of She2-myc WT, T47A and T47D expression in the K5547 strain. YCP: K5547+YCP22 empty. Tubulin was used as loading control.



Supplementary Figure 1: Phosphomimetic mutations at She2 S101 and T47 disrupt asymmetric distribution of Ash1.

SUPPLEMENTARY DATA

Tables 2-1 to 2-3 are in the Annex appended to the thesis (Excel files)

TABLE 2-1 PLASMIDS USED IN THIS STUDY

Plasmid	Features	Source
YCPlac22	Single copy yeast vector with *TRP1* selectable marker	Gietz, 1988
YCP22-SHE2MYC	Expresses SHE2-6xMYC from endogenous promoter	Long et al., 2000
YCP22-SHE2-MYC S2A	Expresses SHE2-S2A-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S2D	Expresses SHE2-S2D-MYC from endogenous promoter	This study
YCP22-SHE2-MYC T109A	Expresses SHE2-T109A-MYC from endogenous promoter	This study
YCP22-SHE2-MYC T109D	Expresses SHE2-T109D-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S101A	Expresses SHE2-S101A-MYC from endogenous promoter	This study

YCP22-SHE2-MYC S101D	Expresses SHE2-S101D-MYC from endogenous promoter	This study
YCP22-SHE2-MYC T47A	Expresses SHE2-T47A-MYC from endogenous promoter	This study
YCP22-SHE2-MYC T47D	Expresses SHE2-T47D-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S91A	Expresses SHE2-S91A-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S91D	Expresses SHE2-S91D-MYC from endogenous promoter	This study
YCP22-SHE2-MYC Y65F	Expresses SHE2-Y65F-MYC from endogenous promoter	This study
YCP22-SHE2-MYC Y148F	Expresses SHE2-Y148F-MYC from endogenous promoter	This study
YCP22-SHE2-MYC Y65F Y148F	Expresses SHE2-Y65F Y148F-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S217D	Expresses SHE2-S217D-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S166D	Expresses SHE2-S166D-MYC from endogenous promoter	This study

YCP22-SHE2-MYC S224A	Expresses SHE2-S224A-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S224D	Expresses SHE2-S224D-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S101A T109A	Expresses SHE2-S101A T109A-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S217A S224A	Expresses SHE2-S217A S224A-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S217D S224D	Expresses SHE2-S217D S224D-MYC from endogenous promoter	This study
pGADT7	Yeast two-hybrid vector expressing protein fused to the Gal4 activation domain	Clontech
pGBKT7	Yeast two-hybrid vector expressing protein fused to the Gal4 binding domain	Clontech
pGADT7-She2	plasmid for expression of She2-Gal4AD	Shen et al., 2009
pGBKT7-She2	plasmid for expression of She2-Gal4BD	This study
pGADT7-She2-T109A	plasmid for expression of She2-T109A-Gal4AD	This study
pGADT7-She2-T109D	plasmid for expression of She2-T109D-Gal4AD	This study

pGBKT7-Srp1	plasmid for expression of Srp1-Gal4BD	This study
pGBKT7-She3-CT	plasmid for expression of She3-CT-Gal4BD	This study
C3319	YEPlac181 with *ASH1* under endogenous promoter	Jansen et al., 1996

TABLE 2-2 STRAINS USED IN THIS STUDY

Strain	Genotype	Source
K699	*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1*	Jansen et al., 1996
K699 *she2*	*K699 she2::KAN*	Shen et al., 2009
K5547	*Matα, his3, leu2, ade2, trp1, ura3, HO-ADE2, HO-CAN1, she2::URA3*	R-P Jansen lab
YS1052	*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 cdc28-as1*	S. Michnick lab
YS1052 GST-SHE2	*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 cdc28-as1 GAL1prom-GST-SHE2::KAN*	This study

pJ69-4A	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	S. Field lab
YS1116	*pJ69-4A SHE2::KAN*	This study

Chapter 3

3 CK2-dependent phosphorylation of She2 nuclear localization signal

Nastaran Farajzadeh, Amélie Forget, Zhifa Shen and Pascal Chartrand

Department of Biochemistry and Molecular Medicine, Université de Montréal

Montréal, Qc H3C 3J7 Canada

Corresponding author: p.chartrand@umontreal.ca

Manuscript in preparation

3.1 AUTHORS' CONTRIBUTION

Nastaran Farajzadeh: GST-She2 protein expression and purification, cloning and mutagenesis of She2, *in vitro* phosphorylation of recombinant She2, Yeast two hybrid assays, analyzed the data and wrote the first draft of the manuscript.

Amélie Forget: GST-Srp1 pulldown assay of She2-myc

Zhifa Shen: GST-Srp1 pulldown assay of She2-myc

Pascal Chartrand: conceived the project, analyzed the data and edited the manuscript.

3.2 ABSTRACT

Asymmetric mRNA localization is used by several eukaryotes as a mechanism to control gene expression with precise temporal and spatial regulation. In budding yeast, transport and selective distribution of mRNAs from the mother cells to the bud of the daughter cell are achieved by specific mRNA localization proteins. Among them, the RNA-binding protein She2 is a key player in the bud-localized mRNP and its presence in the nucleus is crucial for the formation of initial nuclear mRNPs. She2 nuclear import relies on the interaction between the importin-α Srp1 and a non-canonical nuclear localization signal (NLS) at the C-terminus of She2. In this study, we show that phosphorylation of She2 is required for its interaction with Srp1. We identified the casein kinase CK2 as the main kinase that phosphorylates She2 at S217 and S224, two residues which are embedded in its NLS. Analysis of mutants at S217 and S224 reveals that these phosphorylation events do not impact She2 interaction with Srp1 or with the adaptor protein She3. So far, the specific role of She2 phosphorylation at S217 and S224 and phosphorylation-mediated regulation of nuclear import of She2 is still unknown.

3.3 INTRODUCTION

mRNA localization is a widespread mechanism that has been identified in a variety of organisms and that gives precise control over spatial and temporal production of proteins. Subcellular localization of mRNA followed by localized protein expression to a specific cell-compartment is associated with many vital cellular processes such as establishment of cell polarity, cell asymmetry, embryonic axis determination, and neuronal plasticity [5]. Unicellular budding yeast *Saccharomyces cerevisiae* is used as a robust model organism to study mRNA localization, in which more than 30 mRNAs localize at its bud tip [3, 133, 367]. Among them, the *ASH1 mRNA* is a well-studied transcript that is localized to the distal tip of daughter cells and is required for mating type-switching inhibition by preventing HO transcription in daughter cells [368].

The RNA-binding protein She2 is the main protein involved in targeting bud-localized transcripts by binding to the *cis*-acting elements (or zipcodes) along the mRNA sequence. In the nucleus, She2 recognizes *ASH1* mRNA co-transcriptionally by binding to an RNA polymerase II-associated protein complex Spt4–Spt5/DSIF to form an initial mRNP complex in the nucleus [239]. She2 also promotes the recruitment of the translational repressor Puf6 on *ASH1* through Loc1, an exclusively nuclear RNA-binding protein [259]. As the *ASH1* mRNA is exported to the cytoplasm, Loc1 is replaced by She3 and forms a stable mRNP complex [166]. She3 recruits the Myo4 myosin motor, which allows this complex to be transported along the actin cytoskeleton and anchored to the bud tip [152]. Once localized at the bud tip, the translational repressors Khd1 and Puf6 are released from *ASH1* mRNA by phosphorylation [225, 369].

Nuclear import of She2 is required for its recruitment on its target mRNAs and for its interaction with the translational repressors Loc1 and Puf6 in the nucleus [220, 256]. The C-terminal domain of She2 is necessary and sufficient for its interaction with the importin-α Srp1 and nuclear import. Surprisingly, only one highly conserved lysine, and no arginine, were found in an alignment of this C-terminal domain of She2 from *Saccharomyces sensu stricto* species, suggesting that She2 contains a non-classical NLS [220]. Furthermore, mutations in five conserved residues in this NLS disrupted the interaction between She2 and Srp1, as well as the localization of *ASH1* mRNA. However, the regulation of nuclear import and nuclear availability of She2 and

its effect on mRNA localization is still unclear. A number of distinct mechanisms controls nuclear transport. Among them, the best-understood process by which protein nuclear import is regulated is by post-translational modification (PTM) at or near the NLS, particularly through phosphorylation [292, 370].

In a recent study, we used mass spectrometry analysis to identify phosphopeptides from the She2 protein *in vivo* and identified several phosphorylated serine and threonine residues [371]. Among these residues, a phosphomimetic mutation at T109 disrupted the dimerization of She2 and its interaction with She3 and *ASH1* mRNA, suggesting a regulatory role for these phosphorylation events. Herein, we aim to identify the kinase(s) responsible for She2 phosphorylation.

3.4 RESULTS

3.4.1 She2 is phosphorylated by CK2 in vitro

We previously performed a phosphoproteomic analysis of She2 using mass spectrometry and identified several new phosphosites in She2 by three separate phosphopeptide analyses [371]. Several phosphosites, such as Y65, S91, S101, and T109 are located at the dimerization interface of She2 monomers. Another phosphosite discovered at T47 is at the interface of two She2 dimers [158]. We found that a phosphomimetic mutation at T109 disrupts She2 dimerization, its interaction with She3 and binding to the *ASH1* mRNA [371], suggesting that this phosphosite has an inhibitory effect on She2 activity. An analysis of the amino acid sequence surrounding T109 revealed that this phosphosite is present within the sequence $E_{108}TFD_{111}$, which shows similarities to the consensus CK2 site: D/E/X-Sp/Tp-D/E/X-D/E/X-D/E-D/E/X (where X is any non-basic amino acid) (Figure 3-1A) [372]. We therefore decided to explore whether She2 is phosphorylated by CK2.

Protein kinase CK2 is a constitutively active serine/threonine kinase found in almost all eukaryotes and is involved in a wide range of cellular functions. In *Saccharomyces cerevisiae*, this tetrameric enzyme is comprised of two catalytic (Cka1 and Cka2) and two regulatory subunits (Ckb1 and Ckb2) [373]. A previous proteomic analysis using TAP-tagged She2 revealed that the Cka2 catalytic subunit is associated with She2-TAP, suggesting that She2 could be a substrate of

this kinase [126]. To explore the possibility that She2 is phosphorylated by CK2, recombinant GST-She2 was purified and tested as substrate in an *in vitro* phosphorylation assay in the presence of [γ-^{32}P] ATP and recombinant CK2, followed by separation on SDS-PAGE and autoradiography. As shown in (Figure 3-1B), our result reveals that She2 is a substrate for CK2 *in vitro*. Since T109 is at the dimerization interface between She2 monomers, we also tested as a substrate the GST-She2-M2 mutant (S120Y), which is a monomeric and properly folded She2 protein [154]. Interestingly, the She2 monomer demonstrates higher level of phosphorylation by CK2 *in vitro* compared to the wild-type She2 (Figure 3-1B), suggesting that the She2 monomer is a better substrate for this kinase.

3.4.2 Mutations at Ser217 and Ser224, but not at T109, inhibit the phosphorylation of She2 by CK2 in vitro

In order to determine if T109 is a putative residue phosphorylated by CK2, we performed an *in vitro* phosphorylation assay with purified GST-tagged She2 containing an alanine mutation at position T109 in order to abolish its phosphorylation. Recombinant GST-She2 and GST-She2-T109A were incubated with CK2 and [γ-32P]-ATP, followed by autoradiography. Surprisingly, the T109A mutation did not abolish the phosphorylation of She2 by CK2 (Figure 3-2A). On the contrary, an increased phosphorylation was observed with this mutant. This could be explained by our previous observation that the T109A mutant of She2 partially disrupts the dimerization of this protein [371], and the monomer is a better CK2 substrate, as we showed with the She2 S120Y mutant (Figure 3-1B).

Still, these results raise questions regarding which other amino acids in She2 may be phosphorylated by CK2. An analysis of the sequence surrounding the known phosphosites in She2 revealed two others putative CK2 sites: Ser217 (sequence $S_{217}ILD_{220}$) and Ser224 (sequence $S_{224}ALD_{227}$) (Figure 3-1A). To determine if these residues are phosphorylated by CK2, we generated a double mutation S217A S224A in GST-She2 and tested this mutant in the *in vitro* CK2 phosphorylation assay. As shown in (Figure 3-2A), the double mutant S217A S224A abolished phosphorylation by CK2, suggesting that Serine 217 and 224 might be the primary CK2 phosphorylation sites in She2.

In order to validate these results, we cloned the C-terminal peptide of She2, which contains the NLS with the two serine at the positions 217 and 224. We generated the phospho-null mutations S217A and S224A in the peptide and expressed this peptide in fusion with GST. We purified recombinant GST-She2 C-terminus WT and 2A (S217A S224A) containing the NLS, followed by *in vitro* phosphorylation with CK2. Our results show that the She2 C-terminus is a good substrate for CK2 (Figure 3-2B). On the other hand, mutation of S217 and S224 to alanine inhibits the phosphorylation of this peptide (Figure 3-2B), which indicates that the She2 NLS is a substrate for the CK2 kinase.

3.4.3 Interaction between She2 and Srp1 depends on phosphorylation

Previous work from our lab has shown that the importin-α Srp1 interacts with a non-canonical NLS at the C-terminus of She2 and this interaction is required for She2 nuclear import [220]. Herein, our data show that She2 is phosphorylated in its NLS at residues Ser217 and Ser224. Phosphorylation represents an important mechanism that regulates the nuclear transport and consequently the function of an NLS cargo protein [374]. To test whether phosphorylation of She2 modulates its interaction with Srp1, we carried out a GST-Srp1 pulldown assay of She2-myc or She2-M2-myc from yeast extracts, with or without phosphatase treatment. Our results reproduced previous data showing that She2 and Srp1 interact in this assay (Figure 3-3A) [220]. Interestingly, treatment of yeast extracts with phosphatase prior to the GST pull-down shows that She2-myc interaction with Srp1 is eliminated, while She2-M2-myc interaction with Srp1 is reduced (Figure 3-3A). Altogether, these results suggest that She2 phosphorylation is required for its interaction with Srp1.

3.4.4 Phosphorylation of She2 monomer at S217 and S224 does not control its interaction with Srp1

Since i) the interaction between She2 and Srp1 depends on phosphorylation, and ii) S217 and S224 in the NLS of She2 are the main sites of CK2-dependent phosphorylation and are solvent-accessible at the surface of the She2 C-terminal helix (Figure 3-3B), this raises the possibility that

phosphorylation of She2 by CK2 regulates its interaction with Srp1 and its nuclear import. To test this possibility, we used a yeast two-hybrid assay to determine whether CK2-dependent phosphorylation at S217 and S224 affects the interaction between She2 and Srp1. We generated the phospho-null S217A S224A or phospho-mimetic S217D S224D mutations in the She2-M2 monomeric protein and expressed these mutants in the yeast two-hybrid strain with Srp1 as bait. We found that both phospho-null or phospho-mimetic mutations at S217 and S224 do not affect the interaction of She2-M2 with Srp1 (Figure 3-3C). These results suggest that phosphorylation at S217 and S224 does not modulate the She2-Srp1 interaction in the context of the monomeric She2 protein.

3.4.5 Impact of mutations at S217 and S224 on the interaction between a minimal She2 NLS and Srp1

Still, these results do not eliminate the possibility that phosphorylation at S217 and S224 may regulate the interaction between She2 and Srp1 in combination with other phosphorylated residues in the She2 protein. Therefore, we decided instead to focus on the nuclear localization signal (NLS) of She2, where the residues S217 and S224 are embedded, and how these phosphosites may affect the interaction between the NLS and Srp1. Our previous work identified a thirty amino acids non-canonical nuclear localization signal at the C-terminus of She2 (amino acids 200-230) [220], but its exact minimal length required for interaction with the importin-α Srp1 has never been defined. Sequence alignment of amino acids 200-230 of She2 from *Saccharomyces* and its close species (*Zygosaccharomyces*, *Candida*, *Kazachstania*) shows that the N-terminal amino acids of this peptide are more conserved than the C-terminus (Figure 3-4A). To better characterize the She2 NLS residues important for Srp1 binding, we generated a series of deletions at the N- and C-terminus of the 30 amino acids NLS, as shown in (Figure 3-4A). Interaction between Srp1 and the She2 NLS deletions were tested in the yeast two-hybrid assay. The experiment was carried out in a *she2Δ* background to avoid the possibility that the endogenous wild-type She2 may impact the two-hybrid interaction. Surprisingly, we observed that all three deletion mutants showed a strong decrease in the interaction with Srp1, suggesting that the 30 amino acids She2 NLS is the minimal size required for its interaction with Srp1 (Figure 3-4B).

We used this minimal NLS to assess the impact of phosphomimetic S217D S224D or phospho-null S217A S224A mutations on the interaction with Srp1 in the yeast two-hybrid assay. As shown in (Figure 3-4C), phosphomimetic mutations at S217 and S224 in the minimal She2 NLS lead to a 1.7x fold increase in Srp1 interaction. However, the difference is not statistically significant. Phospho-null mutations at S217 and S224 lead instead to a decreased interaction with Srp1. Again, this difference is not statistically significant (Figure 3-4C). These results suggest that phosphorylation on Serine 217 and 224 does not play a role in modulating the interaction between the NLS of She2 and Srp1.

3.4.6 Mutations at S217 and S224 on She2 do not affect its interaction with She3

She3 is the protein adaptor between She2 and bud-localized mRNAs, and it is essential for the binding of the Myo4 motor protein to the mRNA-She2 complex [375]. Interestingly, She3 itself is a phosphoprotein and it has been suggested that She3 interaction with RNA could be negatively regulated by phosphorylation [241]. Structural analysis of the She3 C-terminus in complex with She2 and the *ASH1* mRNA localization element E3 revealed that joining She3 to the She2-mRNA complex enhanced the binding of She2 to the *ASH1* mRNA zipcode [167]. She3 binds She2 at two independent sites: a P-site and a R-site [167]. A detailed analysis of She2-She3 interaction revealed that residues A214, W215 and I218 of She2 are present at the P-site and interact with P365 and L364 of She3 [376]. Since the phosphorylation of She2 by CK2 at S217 and S224 occurs near the P-site, this prompted us to explore the possibility that these phosphosites may impact She2 interaction with She3. To address this question, the interaction between She2 WT and She2 mutants S217A S224A (2A) or S217D S224D (2D) with the C-terminal domain of She3 was investigated in a yeast two-hybrid assay. Our results show that a strong interaction was still detected between the She3 C-terminus and She2 with phospho-null or phosphomimetic mutations at S217 and S224, similar to the She2WT (Figure 3-5), suggesting that She2 phosphorylation at S217 and S224 does not affect its interaction with She3.

3.5 DISCUSSION

She2 is the main RNA-binding protein of the RNA localization machinery that promotes the accumulation of mRNAs from the nucleus of the mother cells to the bud of the daughter cell.

Previous studies showed that She2 shuttles between the cytoplasm and the nucleus, and a non-classical nuclear localization signal (NLS) promotes its active nuclear import [220]. In the present study, we determined that She2 is phosphorylated by CK2 *in vitro* and demonstrated that amino acids S217 and S224 in the NLS region of She2 are phosphorylated by this kinase. This result is consistent with our previous study which showed that She2 is a phosphoprotein and identified several phosphorylation sites within She2, including T109, S217 and S224 with the use of LC/MS-MS analysis [371]. However, She2 phosphorylation at S217 and S224 does not affect the interaction of She2 with She3 or the importin-α Srp1. Also, we observed that She2 phosphorylation is required for its interaction with the importin-α Srp1. Our previous study showed that phosphorylation of She2 controls its oligomerization [371]. Since monomeric She2 interacts with Srp1 better than the She2 dimer [220], this suggests that kinase(s) may participate in the modulation of She2 nuclear import by regulating the oligomeric state of She2.

Which kinases may participate in the phosphorylation of She2 besides CK2? Large-scale protein microarrays study showed that She2 interacts with the protein kinases Iks1 and Kkq8 [377]. IKS1 expression is increased in the response to mild heat, copper sulphate, and sorbate stress [378-380]. Iks1 is phosphorylated by CK2 at S295, raising the possibility that She2 could be a substrate of CK2 and Iks1 kinases [381]. Although the function of Kkq8 is unknown, it may be involved in the cellular response to heat due to its interaction with heat shock transcription factors [382].

An alignment of amino acids 200-230 of the She2 NLS from ten *Saccharomyces sensu stricto* species identified 6 highly conserved amino acids, including only one lysine at position 222. Previous research found that mutations at K_{222}, and its adjacent amino acids (W_{215}, I_{219}, L_{220}, and L_{223}) disturbed the interaction between She2 and Srp1 [220]. We sought to determine whether other highly conserved amino acids within the NLS, such as P_{201}, V_{202}, and E_{207}, are required for the interaction with Srp1. We identified a minimal NLS in She2 that is required for its interaction with Srp1, which suggests that these three conserved amino acids are required for the interaction with Srp1. As described in this study, phosphorylation is a key regulatory mechanism for She2 nuclear import. Phosphorylation not only regulates She2 stability via its oligomeric state [371], but it also affects its nuclear availability. We show that She2 NLS could be phosphorylated by casein kinase

2 *in vitro*. However, this phosphorylation does not increases the affinity of She2 for importin-α Srp1, as being demonstrated through yeast two-hybrid assay. Therefore, we cannot conclude that She2 nuclear import is dependent on phosphorylation of its NLS.

Still, we cannot rule out the possibility that phosphorylation at other residues plays a role in the nuclear import of She2. For instance, phosphorylation of histone chaperone Nap1 by CK2 mediates its nuclear import in a NLS-dependent manner. However, the mechanism by which CK2 phosphorylation promotes nuclear import of Nap1 is not clear yet since phosphorylation does not affect the interaction of Nap1 with its known karyopherin Kap114 [383]. Interestingly, Nap1 has been found in two major state: dimer and octamer. Nap1 higher oligomer was identified in the nucleus and phosphorylation was suggested to regulate its dimer/octamer equilibrium and nuclear import [384]. In a previous study, we we identified 13 phosphorylated residues in She2 *in vivo*, including S2, S29, T47, Y65, S91, S101, T109, T147, Y148, S155, S217, S224 and T235. Of which, S217, S224 and T235 are located at or near She2 NLS [371]. Although, we did not detect phosphorylation of T235 by CK2, it remains possible that phosphorylation of this site by another kinase could be necessary for She2 nuclear import.

Importin-α Srp1 interacts with a non-canonical NLS at the C-terminus of She2 and this interaction is required for She2 nuclear import and its proper function [220]. In this study, She2 WT and a monomeric mutant of She2 was used in a pull-down assay with Srp1. The addition of phosphatase before the pull-down assay disrupted completely the interaction between She2 and Srp1, while monomeric mutants of She2 showed a reduced interaction. In agreement with previous study, our data suggest that monomeric She2 interacts more strongly with Srp1 compared to the She2 tetramer. Previously, we determined that phosphorylation controls the oligomeric state of She2 and inhibit its oligomerization [371]. We propose that a fraction of She2 is phosphorylated in the cytoplasm, which inhibits the formation of She2 tetramers and favors the accumulation of She2 monomers. Phosphorylation may lead to a structural rearrangement of She2 that exposes other site(s), possibly on the dimerization or tetramerization interface, for subsequent modification. She2 may undergo another phosphorylation, probably in the NLS domain to increase its capacity to bind the importin-α Srp1. Since direct interaction of Iks1 and She2 has been reported and this kinase is

a substrate for CK2, a cascade of kinases including Iks1 and CK2 may be involved in She2 monomerization and nuclear import.

In conclusion, our data show that phosphorylation of She2 is required for its interaction with Srp1. We identified two residues within the NLS of She2 that are phosphorylated by CK2. However, these phosphorylation events do not modify the capacity of She2 to bind Srp1. It is possible that phosphorylation at S217 and 224 is not sufficient to modulate She2-Srp1 interaction, and other residues within or adjacent to the NLS may also be involved. Further analysis of other phosphosites in She2 will be necessary to identify key phosphorylation events that modulate the nuclear import of this protein.

3.6 MATERIAL AND METHODS

3.6.1 Yeast strains and DNA manipulation

Yeast growth was carried out at 30°C in yeast extract peptone dextrose (YPD) or synthetic selective medium [385]. Yeast transformation was performed using single strand nucleic acids carrier according to Gietz and Schiestl [386]. Gene disruption using PCR was carried out as explained previously [387]. PCR examination of genomic DNA validated all of the gene disruptions. Yeasts strains and plasmids used in this study are described in the Supplementary (Table 3-1 and 2), respectively. The C-terminus of She2, containing amino acids 201 to 235, was cloned in the pGEX-6P1 plasmid.

3.6.2 Protein Expression and purification

Expression of recombinant GST-She2 was achieved in *Escherichia coli* BL21 cells transformed with the pGEX-6P1-She2 plasmid. Protein induction was conducted overnight at 25°C using 1 mM final concentration of Isopropyl beta-D-1-thiogalactopyranoside (IPTG) after cells had grown to the mid-log phase in Luria–Bertani (LB) medium. Cells were extracted and resuspended in Tris 50 mM pH 8, 5 mM EDTA, 1 M NaCl, complete protease inhibitor cocktail (Roche), 1 mg/ml lysozyme, and RNase A at 15 ug/ml final concentration after centrifugation (15 000 g, 15 min, 4°C). The lysate was sonicated after being incubated on ice for 30 minutes. Centrifugation

(16 000 g, 15 min, 4°C) separated the soluble proteins from pellet, and the supernatant was used for affinity chromatography with glutathione sepharose beads (GE healthcare). Beads were washed four times using wash buffer (Tris 50 mM pH 8, 5 mM EDTA, 1.5 M NaCl), and proteins were eluted from beads by using reduced glutathione (Sigma) at a final concentration of 10 mM in PBS. When necessary, the GST tag was cleaved by PreScission protease (GE healthcare).

3.6.3 GST pull-down assay

For the interactions between recombinant GST-Srp1 and endogenous She2-myc or She2-M2-myc, 5 µg of recombinant GST-Srp1 was bound to glutathione-Sepharose 4B and incubated at 18°C for 2.5 h with extract from yeasts expressing She2-myc or She2-M2-myc. For phosphatase treatment, yeast extracts were treated with 5 µl of alkaline phosphatase for 1 hr at 37°C prior pull-down. The matrix was recovered by centrifugation and washed four times with 500 µl of binding buffer. The bound proteins were eluted with pre-heated 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.

3.6.4 In vitro phosphorylation of recombinant She2

GST-tagged She2 WT, She2-M2, She2-T109A, She2-S117A S224A, She2-Cterminus or GST proteins were purified from *E. coli* using glutathione beads. The recombinant proteins (each 4 µg/reaction) were incubated in kinase reaction buffer [50 mM Tris-HCl. pH7.5, 200 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT, 100 µM ATP] with 100 U casein kinase II (P6010, New England Biolabs, Ipswich, MA, US) in the presence or absence of 100 µCi of [γ-32P] ATP for 30 min at 30°C. The reaction was terminated by adding SDS sample loading buffer (50 mM Tris–Cl. pH 6.8, 2% SDS, 10% glycerol, 0.2% bromophenol blue dye, 4% β-mercaptoethanol). After separation on SDS-PAGE, the gel was transferred into a PVDF membrane and visualized by direct exposure of the membrane to X-ray film.

3.6.5 Yeast two-hybrid assay

Yeast two-hybrid assay was carried out as previously described [388]. Yeast strain pJ69-4A *she2Δ* was transformed with the plasmids pGADT7 expressing She2 WT or mutants, as well as pGBKT7 expressing suitable baits (Srp1 or She3 C-terminus). *O*-nitrophenyl-β-**d**-O-nitrophenyl-d-galactopyranoside (ONPG) was used to assess β-galactosidase activity from at least three different yeast cultures, as described before [388]. β-Galactosidase activity is expressed as nmole/minute/mg protein.

3.7 FIGURES LEGENDS

Figure 3-1: She2 is phosphorylated by CK2 *in vitro*

(A) Sequence of She2 with phosphorylated amino acids identified by LC-MS/MS colored in red. Amino acids in the dimerization interface of She2 are underlined. Amino acids in the dimer-dimer interface of She2 are in bold. Amino acid in blue are phosphosites identified in previous studies. **(B)** *In vitro* phosphorylation of recombinant wild-type GST-She2 (WT) or GST-She2 M2 mutant (M2) by CK2. Arrowhead: phosphorylated full-length GST-She2; *: phosphorylated truncated She2 fragments

Figure 3-2: Mutations at Ser217 and Ser224 suppress the phosphorylation of She2 by CK2 *in vitro*

(A) *In vitro* phosphorylation of recombinant wild-type GST-She2 (WT), GST-She2 T109A mutant (T109A) or GST-She2 S217A S224A mutant (S217A S224A) by CK2. **(B)** *In vitro* phosphorylation of recombinant wild-type GST-She2 C-terminus (WT), GST-She2 C-terminus S217A S224A mutant (2A) or GST alone by CK2. Arrowhead: phosphorylated full-length GST-She2; *: phosphorylated truncated She2 fragments

Figure 3-3: Interaction between She2 and Srp1 depends on She2 phosphorylation

(A) GST pulldown assay to detect the interaction between Srp1 and She2-myc or She2-M2-myc from yeast extracts. Input: total She2-myc or She2-M2-myc in yeast extracts; GST-Srp1: She2 from yeast extracts interacting with GST-Srp1; GST-Srp1+ Phos: She2 from yeast extracts pre-treated with phosphatase interacting with GST-Srp1. **(B)** Zoomed view on the 3D structure of She2 C-terminal helix with highlighted amino acids S217 (green) and S224 (red). **(C)** Yeast two-hybrid assay using pGBK-Srp1 and pGAD-She2-M2 with mutations S217A S224A (M2-2A) or S217D S224D (M2-2D). Interaction between Srp1 and She2 was determined by measuring β-galactosidase activity. pGAD: plasmid expressing Gal4 activation domain only. N=4. ns: non-significant.

Figure 3-4: Mutations at S217 and S224 of She2 NLS do not impact its interaction with Srp1

(A) Mapping the minimal NLS of She2 required for its interaction with Srp1. Sequence alignment of a 30 amino acid peptide containing the NLS of She2 in various fungi. Deletion fragments of NLS: fragment Δ1, fragment Δ2, fragment Δ3. **(B)** Yeast two-hybrid assay between Srp1 and She2 minimal NLS or NLS fragments Δ1, Δ2 or Δ3. Interaction between Srp1 and She2 NLS was determined by measuring β-galactosidase activity. pGAD: plasmid expressing Gal4 activation domain only. **(C)** Yeast two-hybrid assay between Srp1 and She2 minimal NLS or NLS with mutations S217A S224A (NLS-2A) or S217D S224D (NLS-2D). Interaction between Srp1 and She2 NLS was determined by measuring β-galactosidase activity. pGAD: plasmid expressing Gal4 activation domain only. N=3. ns: non-significant.

Figure 3-5: Mutations at S217 and S224 on She2 do not affect its interaction with She3

Yeast two-hybrid assay between She3 C-terminal domain and She2 wild-type (WT) or with mutations S217A S224A (2A) or S217D S224D (2D). Interaction between She3 and She2 was determined by measuring β-galactosidase activity. pGAD: plasmid expressing Gal4 activation domain only. N=4. ns: non-significant.

A

```
  1 MSKDKDIKVT PGTCELVEQI LALLSRYLSS YIHVLNKFIS HLRRVATLRF ERTTLIKFVK
 61 KLRFYNDCVL SYNASEFINE GKNELDPEAD SFDKVILPIA SMFVKCVETF DLLNYYLTQS
121 LQKEILSKTL NEDLTLTAES ILAIDDTYNH FVKFSQWMIE SLRIGSNLLD LEVVQFAIKC
181 ADEDGTNIGE TDNIFLQEIL PVNSEEEFQT LSAAWHSILD GKLSALDEEF DVVATKWHDK
241 FGKLKN*
```

B

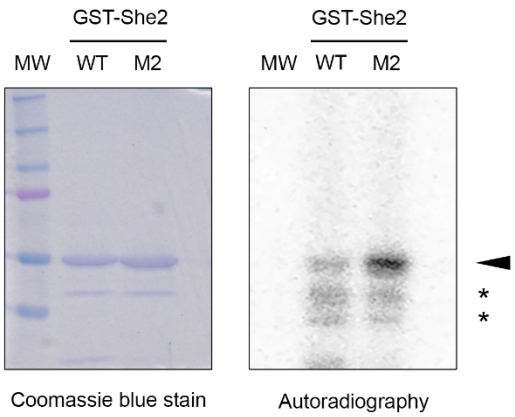


FIGURE 3-1 SHE2 IS PHOSPHORYLATED BY CK2 IN VITRO

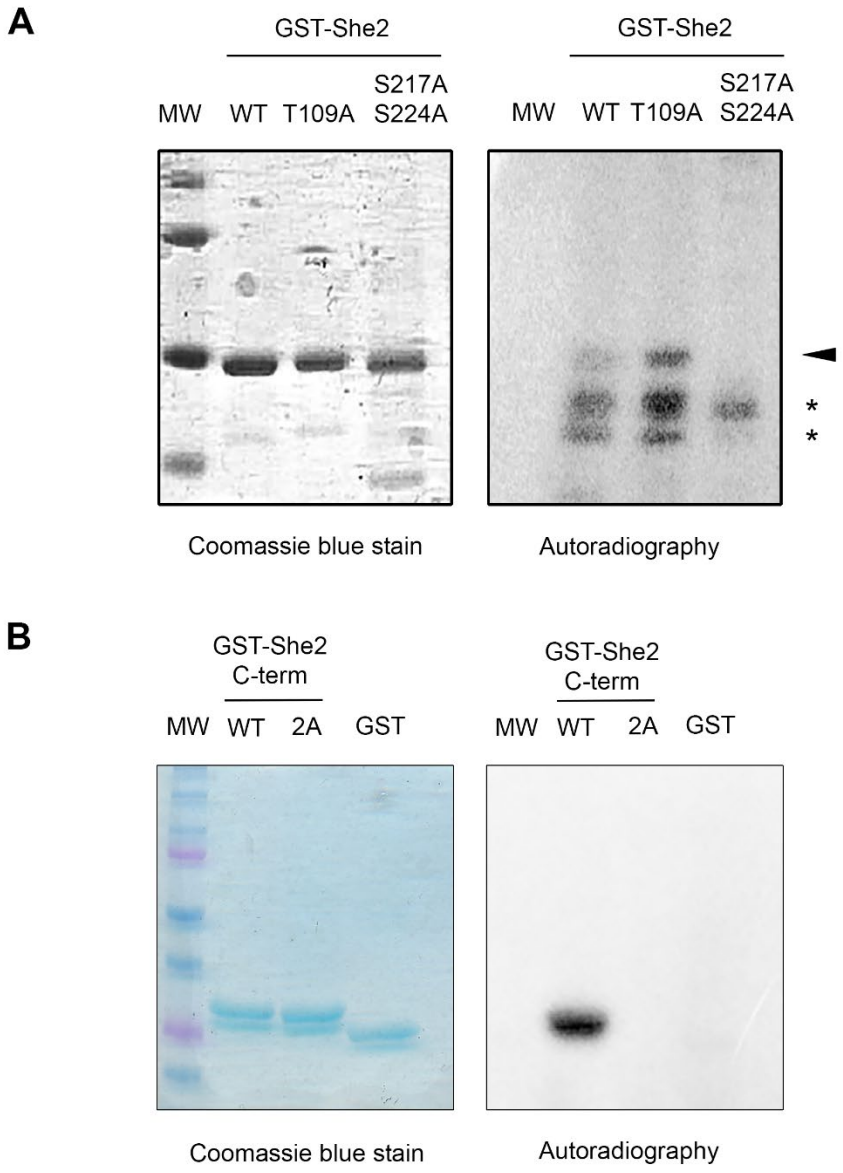


FIGURE 3-2 MUTATIONS AT SER217 AND SER224 SUPPRESS THE PHOSPHORYLATION OF SHE2 BY CK2 IN VITRO

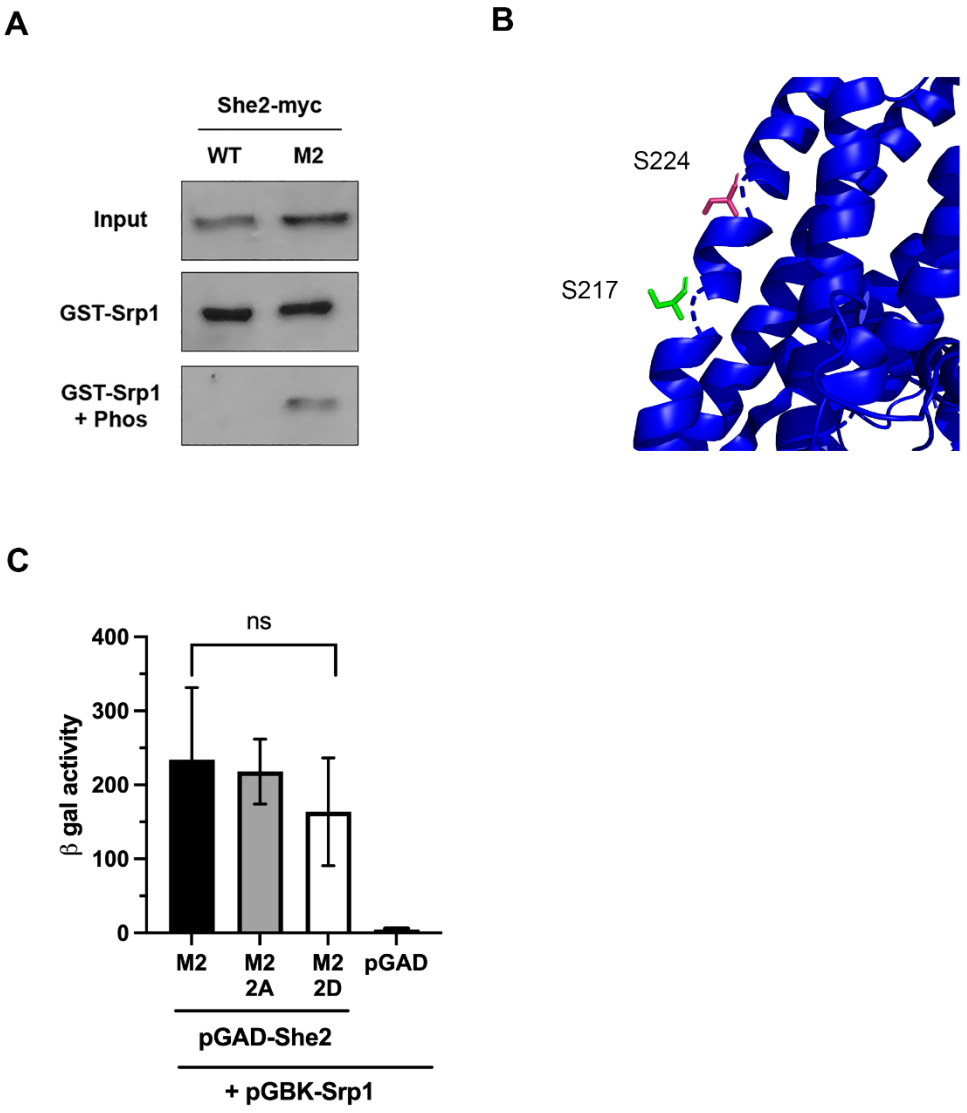


FIGURE 3-3 INTERACTION BETWEEN SHE2 AND SRP1 DEPENDS ON SHE2 PHOSPHORYLATION

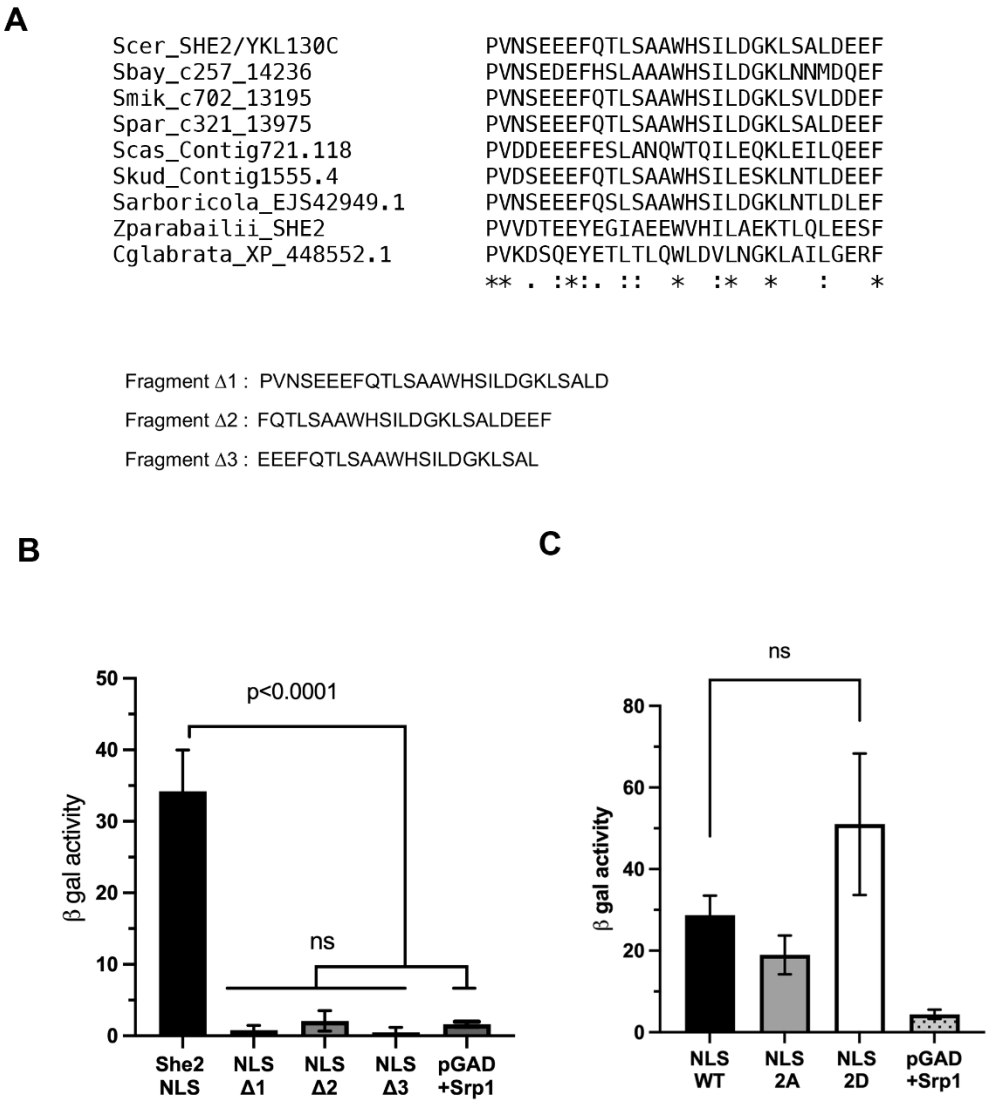


FIGURE 3-4 MUTATIONS AT S217 AND S224 OF SHE2 NLS DO NOT IMPACT ITS INTERACTION WITH SRP1

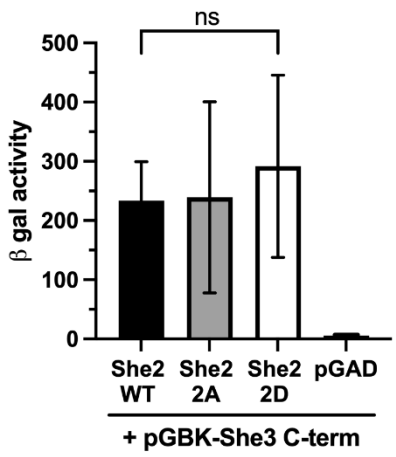


FIGURE 3-5 MUTATIONS AT S217 AND S224 ON SHE2 DO NOT AFFECT ITS INTERACTION WITH SHE3

3.8 SUPPLEMENTARY DATA

TABLE 3-1 PLASMIDS USED IN THIS STUDY

Plasmid	Features	Source
pGEX-6P1	Vector for the expression of GST fused proteinswith PreScission cleavage site	GE healthcare
pGEX-6P1-SHE2	Vector for expression of She2-GST	Shen Z, 2010
pGEX-SHE2-M2	pGEX-6P1 vector for expression of GST-She2 mutant M2	Shen Z, 2010
pGEX-SRP1	pGEX-6P1 vector for expression of GST-Srp1	Shen Z, 2010
YCPlac22	Single copy yeast vector with *TRP1* selectable marker	Gietz, 1988
YCP22-SHE2MYC	Expresses SHE2-6xMYC from endogenous promoter	Long et al., 2000
YCP22-SHE2-MYC T109A	Expresses SHE2-T109A-MYC from endogenous promoter	This study

YCP22-SHE2-MYC T109D	Expresses SHE2-T109D-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S217A	Expresses SHE2-S101A-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S217D	Expresses SHE2-S217D-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S224A	Expresses SHE2-S224A-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S224D	Expresses SHE2-S224D-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S101A T109A	Expresses SHE2-S101A T109A-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S217A S224A	Expresses SHE2-S217A S224A-MYC from endogenous promoter	This study
YCP22-SHE2-MYC S217D S224D	Expresses SHE2-S217D S224D-MYC from endogenous promoter	This study
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 Gal4 activation domain	Clontech
pGBKT7	Yeast two-hybrid vector expressing protein fused to the Gal4 binding domain	Clontech
pGADT7-She2	plasmid for expression of She2-Gal4AD	Shen et al., 2009
pGBKT7-She2	plasmid for expression of She2-Gal4BD	This study
pGBKT7-Srp1	plasmid for expression of Srp1-Gal4BD	This study
pGBKT7-She3-CT	plasmid for expression of She3-CT-Gal4BD	This study

TABLE 3-2 STRAINS USED IN THIS STUDY

Strain	Genotype	Source
K699	*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1*	Jansen et al., 1996
K699 *she2*	*K699 she2::KAN*	Shen et al., 2009
K5547	*Matα, his3, leu2, ade2, trp1, ura3, HO-ADE2, HO-CAN1, she2::URA3*	R-P Jansen lab
YS1052	*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 cdc28-as1*	S. Michnick lab
YS1052 GST-SHE2	*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 cdc28-as1 GAL1prom-GST-SHE2::KAN*	This study
pJ69-4A	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	S. Field lab
YBZ1	*MATa, ura3-52, leu2-3,112, his3-200, trp1-1, ade2,LYS2:(LexAop)-lacZ, LexA-MS2-MS2 coat (N55K)*	(Hook et al. 2005)

Chapter 4

4 Discussion

4.1 Mapping She2 phosphorylation sites reveals phosphorylation events that regulate mRNA localization.

Although mRNA localization is a widespread phenomenon, it is still being determined how the mRNA localization machinery is regulated. In budding yeast, She2 is the main RNA-binding protein involved in assembling the mRNA locasome, which targets the bud of daughter cells [220]. According to previous structural studies, She2 forms a tetramer with two dimers in a head-to-head conformation required for RNA binding and activity of this protein [167]. The N-terminus of each subunit in the tetrameric state points to the poles of the elongated structure, while the C-terminus points to the waist, where the two opposing dimers meet [193]. She2 oligomerization is required for its interaction with Loc1 and She3, which assist in stabilizing the RNA-protein complex in the nucleus and cytoplasm, respectively [151, 166]. How the She2 function is regulated and how it impacts mRNA localization remains mostly unknown.

In this thesis, we report that She2 is a phosphoprotein *in vivo*. Using a phosphoproteomics approach, we identified novel She2 phosphorylated residues, including 13 phosphorylated amino acids: S2, S29, T47, Y65, S91, S101, T109, T147, Y148, S155, S217, S224 and T235. All of these residues, except for T147, Y148, and S155, are present at the surface of She2 and are solvent-accessible in the properly folded monomeric protein. Five of these phosphosites are found at the dimerization or tetramerization interfaces of She2: Y65, S91, S101, and T109 are at the interface between two She2 monomers, while T47 is at the interface between two She2 dimers. Studies showed that phosphorylation at a protein-protein interface could result in steric or electrostatic clashes predicted to disrupt interaction and potentially play key regulatory roles [332]. Our results show that T109 is a novel and the most abundant phosphosite in She2. A phosphomimetic mutant at T109 inhibits She2-She2 interaction and decreases She2 association with its co-interactors Srp1, She3, and *ASH1* mRNA.

4.2 Functional significance of She2 oligomeric structure

She2 is a homo-oligomeric complex which exists in equilibrium between dimers and tetramers or higher order complex in physiological conditions [154, 389]. The advantages of homo-oligomeric complexes over monomer proteins include increased function diversification, allosteric modulation, resistance to denaturation, and the ability to build oligomers without increasing genome size [390]. In certain instances, phosphorylation may affect transitions between various oligomeric states in homo-oligomers. This might be a key mechanism for controlling the activity of these proteins [391-393]. In this case, phosphosites tend to be located at binding interfaces in protein complexes, and reversible phosphorylation events may regulate the reversible transitions between different oligomeric states in the homo-oligomeric complexes. Substitution of residues located at the interaction interfaces by other amino acids (for example, Ala) can result in significant differences in binding energy and, consequently, in the complex's destabilization [394].

In our study, we found residues Y65, S91, S101 and T109, located at the interface between two She2 monomers, and T47, which is at the interface between two She2 dimers, can be phosphorylated *in vivo*. Phosphorylation of these residues might promote a transition from tetramer to dimer or monomer. All these residues are located at the surface of She2 and are solvent-accessible in the properly folded monomeric protein (Figure 4-1). The She2 protein, in its tetrameric state, has a significantly smaller buried surface interface than the monomer–monomer interface, implying that She2 tetramers potentially disassemble more easily into dimers than She2 dimers into monomers [389]. She2 dimers may require post-translational modification, such as phosphorylation, to change local configuration and be able to favour the monomeric state. Indeed, our data show that the phosphomimetic T109D mutation inhibits She2 oligomerization and its interaction with She3 and *ASH1* mRNA. A phosphomimetic mutation at S101 also disrupts the function of She2, suggesting that phosphorylation at this residue may impede She2 dimerization.

Phosphorylation has been seen predominantly in flexible, disordered and accessible protein regions [395, 396]. However, phosphorylation at a helix's interior or the C-terminal position may disrupt the helical structure [397]. Several phosphorylated residues in She2 are present within α-helices (Figure 4-1), including S29, Y65, S101, T109, T147, Y148, S217 and S224. She2

expression levels are reduced twofold in the T109D mutant. The position of T109 within the helix may explain its significant impact on She2 function, most likely by destabilizing this protein and decreasing its capacity to promote mRNA localization. Phosphomimetic mutations at S101 and T47 do not have the same impact on the stability of She2. On the other hand, phosphomimetic or phospho-null mutations at phosphosites such as Y65, Y148, S217 and S224 did not affect the capacity of She2 to promote Ash1 asymmetric localization. However, we cannot rule out the possibility that these phosphosites are engaged in other regulatory functions and do not directly impact mRNA localization.

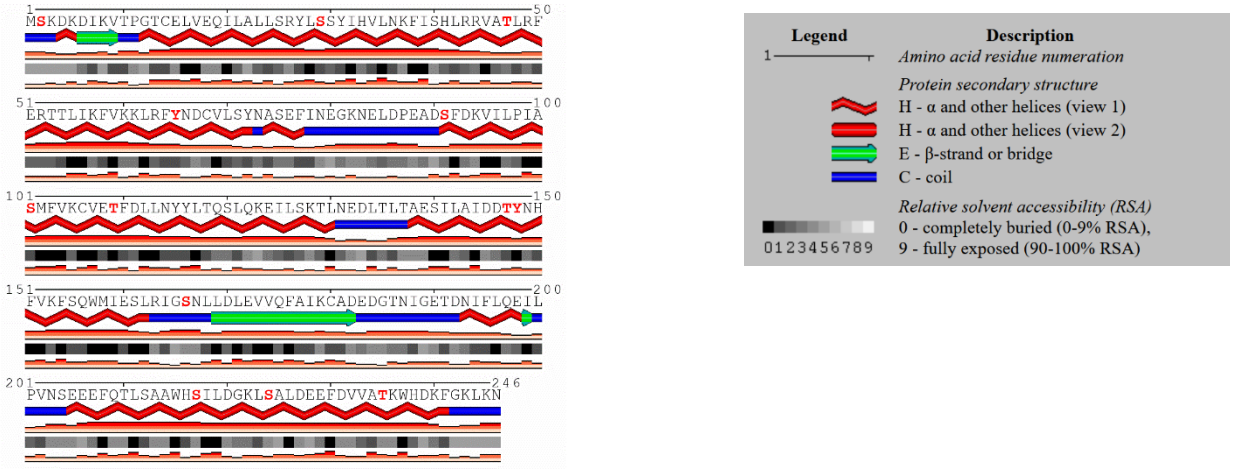


FIGURE 4-1 SHE2 SECONDARY STRUCTURE AND RELATIVE SOLVENT ACCESSIBILITY (CHAIN A)

The location of phosphorylated amino acids identified by LC-MS/MS in this study is highlighted in red. She2 secondary structure was visualized with POLYVIEW-2D software using the SABLE prediction structure [398].

Oligomeric proteins degrade at a slower pace than monomeric proteins, and oligomerization hampers degradation [399]. This impact appears to be mediated, at least partially, by burying ubiquitination sites and internal disordered segments, which would allow the protein to escape from proteasomal detection and engagement [400]. Phosphorylation of She2 at T109 may

lead to the exposure of ubiquitination sites or disordered segments and affect protein degradation rates (Figure 4-3) [399].

A phosphosite at S166, also located at the dimerization interface between two She2 monomers, was previously found in a whole-yeast proteome phosphoproteomics study, indicating phosphorylation of this residue may also play a role in regulating She2 oligomerization and its function in mRNA localization [335]. We also identified phosphorylation sites at T235 and T47, with T47 at the interface of interaction between two She2 dimers (Figure 4-3). A previous mutagenesis study revealed that mutation of T235 to tyrosine does not affect the ability of She2 to bind RNA. Interestingly, the T47 mutation to tyrosine resulted in decreased RNA binding, suggesting that phosphorylation may interfere with She2 tetramerization and its RNA binding activity [154]. Indeed, the tetrameric structure of She2 is essential for binding to the *ASH1* mRNA [167].

She2 is not only an RNA-binding protein, but it also binds to ER-derived membranes [401]. She2 does not contain any known membrane-binding domain. Therefore, the association of She2 with ER is less likely to be dependent on phosphorylation. Importantly, the association of She2 to the ER membrane depends on its oligomerization but not its RNA-binding activity. The fraction of She2 that comigrates with the ER in sucrose gradients is reduced in a mutant carrying the L130Y or S120Y mutations, which impair the tetramerization of She2 [401]. She2 tetramerization seems to affect the capacity of She2 to bind membranes, probably by broadening the interaction interface of She2 with components of the cytoplasmic ER membrane [401]. Since some of the She2 phosphosites, including S91, S101, T109 and T47, are located at the interfaces between two monomers or dimers, it is probable that phosphorylation may also regulate She2-ER association.

4.3 Possible cross-talk between different protein PTMs in mRNA localization

The presence of one post-translational modification can influence the appearance of others. For instance, phosphorylation can promote subsequent ubiquitylation in one substrate, in which phosphorylation more frequently precedes ubiquitylation [402]. Moreover, ubiquitination can also turn on/off the kinase activity on a protein. The coordinated targeting of a substrate by several

modification types is likely best demonstrated by phosphodegrons, in which one or more phosphorylation site(s) function in-cis to promote subsequent ubiquitylation of a substrate. She2 was found to be ubiquitylated in a large-scale proteomic study, with a ubiquitination site mapped at the N-terminus M_1SKDKDIK$_8$ peptide [245]. Also, the ubiquitination of She2 is regulated by Cdc48 and its UBX domain adaptors, which may be linked to ER-associated degradation [403]. In this thesis, we found that the T109D mutant causes a two-fold reduction in She2 expression. Because She2 ubiquitination regulates its expression level, it is possible that T109 phosphorylation may cause protein instability and degradation via ubiquitination. In addition, S91 is another phosphosite that was discovered in our study and is located at the She2 dimerization interface. This residue is in a potential PEST motif (K_{82}NELDPEADSFDK$_{94}$) predicted using ePESTfind [404]. Future investigations will be needed to shed light on the link between She2 phosphorylation and its ubiquitination.

Post-translational modifications control She2 and other members of the mRNA localization machinery in budding yeast. Phosphorylation of She3 at S343, S348 and S361 inhibits its ability to bind RNA while maintaining its interaction with She2 and Myo4 [241]. The stress response, including osmotic stress and exposure to the reducing chemical dithiothreitol (DTT), has been connected to these phosphorylation events [333]. This shows that She3 phosphorylation may play a role in the stress-induced regulation of the mRNA localization pathway. Additionally, the She complex targets are species-specific. Since *Candida albicans* lack a She2 orthologue, it is possible that She3 is directly interacting with the mRNA-protein complex or that this interaction is maintained by an unidentified RNA-binding protein. *C. albicans* contains two orthologues of *SHE3* [405], one of which is necessary for hyphal development and for the transport of *ASH1* and other mRNAs to the hyphal tip [135]. The *CHT2* mRNA and other cell wall-encoding mRNAs are among the targets of the She machinery in *C. albicans*, and the She complex is necessary for chitin remodelling in response to low pH [406]. Consequently, the She complex is necessary for correct cell wall biosynthesis in response to environmental stress.

4.4 Prediction of potential kinases involved in She2 phosphorylation

More than 129 Ser/Thr protein kinases expressed in *Saccharomyces cerevisiae* have been categorized into seven groups [407]. The majority of human protein kinases, such as protein kinase A (PKA), protein kinase C (PKC), Akt (PKB), calcium/calmodulin-dependent kinase type II (CaMK2), 5'-AMP-activated kinase (AMPK), cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), casein kinase II (CK2), with the exception of protein tyrosine kinases, have yeast orthologues [408]. Tyrosine can be phosphorylated by yeast dual-function kinases, usually simultaneously with serine/threonine residues, such as kinases involved in MAPK pathways and the protein kinase Swe1 [409]. *Saccharomyces cerevisiae* possesses five MAPK pathways, which are mediated by Fus3 and Kss1, Hog1, Slt2 (also named Mpk1), and the non-conventional Smk1 MAPK [410, 411]. In our first manuscript, we identified 13 phosphorylated residues in She2: S2, S29, T47, Y65, S91, S101, T109, T147, Y148, S155, S217, S224 and T235 from three independent mass spectrometry analyses. We also showed that She2 is phosphorylated by CK2 at S217 and S224 *in vitro.* However, the kinases that are responsible for the phosphorylation of the other residues are still unknown. Identifying these kinases will allow us to better understand the function of phosphorylation events that regulate the function of She2.

To determine which kinase could phosphorylate She2 phosphosites, we used the NetPhosK and KinasePhos prediction tools [412]. One of the phosphorylated residues identified in our study is T109, which disrupts She2 dimerization and regulates mRNA localization. An analysis of the amino acid sequence surrounding T109 revealed that this phosphosite is present within the sequence $E_{108}TFD_{111}$, which shows similarities to the consensus CK2 site: D/E/X-Sp/Tp-D/E/X-D/E/X-D/E-D/E/X (where X is any non-basic amino acid). Interestingly, the NetPhosYeast software predicts that T109 could be phosphorylated by CK2 (Figure 4-2). NetPhosYeast also predicts that S91 is a target of CK2 (Figure 4-2). To determine if T109 is a putative residue phosphorylated by CK2, we carried out an *in vitro* phosphorylation assay with purified GST-tagged She2 containing an alanine mutation at position T109 in order to abolish its phosphorylation. Surprisingly, the T109A mutation did not abolish the phosphorylation of She2 by CK2. We showed in this work that other She2 phosphosites, namely S217 and S224, are phosphorylated by CK2 *in vitro*. Both phosphorylation sites have been identified in our phosphoproteomic analysis, and while

NetPhosYeast predicted these phosphosites with high probability, it did not assign CK2 as their potential kinase.

NetPhos-3.1 Server Output - DTU Health Tech

```
>Sequence       246 amino acids
#
# netphos-3.1b prediction results
#
# Sequence                # x   Context     Score   Kinase    Answer
# ----------------------------------------------------------------------
# Sequence                2 S   ---MSKDKD   0.488   CKI         .
# Sequence               10 T   DIKVTPGTC   0.472   GSK3        .
# Sequence               13 T   VTPGTCELV   0.472   GSK3        .
# Sequence               25 S   LALLSRYLS   0.477   cdc2        .
# Sequence               27 Y   LLSRYLSSY   0.376   INSR        .
# Sequence               29 S   SRYLSSYIH   0.879   unsp       YES
# Sequence               30 S   RYLSSYIHV   0.780   PKC        YES
# Sequence               31 Y   YLSSYIHVL   0.521   INSR       YES
# Sequence               40 S   NKFISHLRR   0.531   cdc2       YES
# Sequence               47 T   RRVATLRFE   0.961   unsp       YES
# Sequence               53 T   RFERTTLIK   0.436   GSK3        .
# Sequence               54 T   FERTTLIKF   0.835   unsp       YES
# Sequence               65 Y   KLRFYNDCV   0.385   INSR        .
# Sequence               71 S   DCVLSYNAS   0.498   CKII        .
# Sequence               72 Y   CVLSYNASE   0.392   EGFR        .
# Sequence               75 S   SYNASEFIN   0.452   CKII        .
# Sequence               91 S   PEADSFDKV   0.504   CKII       YES
# Sequence              101 S   LPIASMFVK   0.451   GSK3        .
# Sequence              109 T   KCVETFDLL   0.530   CKII       YES
# Sequence              115 Y   DLLNYYLTQ   0.425   INSR        .
# Sequence              116 Y   LLNYYLTQS   0.390   INSR        .
# Sequence              118 T   NYYLTQSLQ   0.534   DNAPK      YES
# Sequence              120 S   YLTQSLQKE   0.488   DNAPK       .
# Sequence              127 S   KEILSKTLN   0.542   cdc2       YES
# Sequence              129 T   ILSKTLNED   0.504   CKII       YES
# Sequence              135 T   NEDLTLTAE   0.447   CKII        .
# Sequence              137 T   DLTLTAESI   0.449   GSK3        .
# Sequence              140 S   LTAESILAI   0.623   unsp       YES
# Sequence              147 T   AIDDTYNHF   0.444   GSK3        .
# Sequence              148 Y   IDDTYNHFV   0.474   SRC         .
# Sequence              155 S   FVKFSQWMI   0.635   ATM        YES
# Sequence              161 S   WMIESLRIG   0.479   DNAPK       .
# Sequence              166 S   LRIGSNLLD   0.795   PKA        YES
# Sequence              186 T   DEDGTNIGE   0.529   CKII       YES
# Sequence              191 T   NIGETDNIF   0.443   cdc2        .
# Sequence              204 S   LPVNSEEEF   0.986   unsp       YES
# Sequence              210 T   EEFQTLSAA   0.613   unsp       YES
# Sequence              212 S   FQTLSAAWH   0.559   PKC        YES
# Sequence              217 S   AAWHSILDG   0.940   unsp       YES
# Sequence              224 S   DGKLSALDE   0.958   unsp       YES
# Sequence              235 T   DVVATKWHD   0.634   PKC        YES
#
```

FIGURE 4-2 NETPHOS SERVER OUTPUT FOR SHE2 PHOSPHORYLATION SITES AND THEIR PUTATIVE KINASES

We also found that T47 is phosphorylated in She2, where a phosphomimetic mutation at this residue interferes with the asymmetric accumulation of Ash1. T47 is present within a putative protein kinase C (Pkc1) consensus sequence that contains positively charged arginine residues

adjacent to the site of phosphorylation (RXXS/T, where X indicates any amino acid). She2 is also phosphorylated at T235, which is not found in a Pkc1 consensus motif but is predicted to be phosphorylated by this kinase. In budding yeast, the Pkc1 kinase plays a key role in the highly-conserved cell wall integrity signalling pathway, which regulates functions essential for growth and the integrity of proliferating cells [413]. Pkc1 kinase regulates a MAPK pathway in response to cell wall stress, which is triggered by five plasma membrane proteins that act as sensors to activate the cell wall integrity pathway, including Wsc1, Wsc2, Wsc3, Mid2, and Mtl1 [414]. Interestingly, *MID2* and *WSC2* mRNAs interact with She2 [3]. Furthermore, Puf6 interacts with several transcripts of the cell wall integrity pathway, and its co-transcriptional recruitment to these mRNAs depends on She2 [259]. Altogether, that could point toward additional roles for She2 in the cell wall integrity pathway.

In addition, we identified S2 as a phosphorylated amino acid in She2. Intriguingly, the NetPhosYeast software predicts that S2 could be phosphorylated by Casein kinase I (CK1). Furthermore, She3 was predicted to be phosphorylated by CK1 on both S199 and S202. Mutation on S199 and S202 increases She3 stability and its interaction with the E3 ubiquitin ligase F-box protein Grr1, which promotes She3 degradation [415]. It is possible that phosphorylation of S2 on She2 may play the same role as in She3 since the She2 ubiquitination site mapped to the M_1SKDKDIK$_8$ peptide that contains the S2 phosphosite. Three protein kinases involved in the DNA repair pathway, including DNAPK, ATM and PKA, were predicted to phosphorylate S155 on She2.

Interestingly, She2 was found to interact with the putative serine/threonine kinases Kkq8 and Iks1 in a large-scale protein microarray analysis [377]. Iks1 not only physically interacts with She2 but also genetically interacts with this protein [416], suggesting that Iks1 may be a strong candidate kinase that could phosphorylate She2 [416]. Iks1 expression is increased in response to mild heat, copper sulphate and sorbate stress, but its function and specificity remain unknown [378-380]. Although the function of Kkq8 is not known yet, it may be implicated in the cellular response to heat due to its interaction with two heat shock transcription factors [382].

4.5 Coupling She2 oligomerization and its nuclear import

She2 has been reported to shuttle actively between the cytoplasm and the nucleus. A non-canonical nuclear localization signal (NLS) promotes its active nuclear import via a direct interaction with the importin-α Srp1 [220]. In this work, we observed that phosphorylation is required for the interaction of She2 with the importin-α Srp1 since treatment of yeast extract with phosphatase inhibits the pull-down of She2-myc by GST-Srp1. We show that the NLS region of She2 is phosphorylated by CK2 at S217 and S224 *in vitro.* Still, She2 phosphorylation at S217 and S224 did not affect the binding of She2 with the importin-α Srp1 or with She3, suggesting phosphorylation of She2 at S217 and S224 may be implicated in another role of She2, such as RNA binding or association with membranes. Although our data do not support a role for S217 and S224 in modulating the interaction with Srp1 [372], it remains possible that other phosphosites in She2, such as T235, which is located at the She2 NLS, may also play a role in this interaction (Figure 4-3).

Since monomeric She2 interacts better with Srp1 than the She2 dimer [220], this raises the possibility that phosphorylation may play a key role in the modulation of She2 nuclear import by regulating the oligomeric state of the protein. Therefore, it is possible that phosphorylation at S91, Y65, S101 or T47, which disrupt the She2 dimer or tetramer, may be implicated in promoting the interaction with Srp1 and She2 nuclear import. On the other hand, we observed that a phosphomimetic mutation at T109 decreases the interaction between She2 and Srp1, probably by destabilizing the She2 protein. We speculate that the She2 nuclear localization signal (NLS) may be less accessible in the She2 tetramer, while in the monomeric state, the NLS of She2 is conformationally more accessible to interact with Srp1 (Figure 4-3). This model also fits with our observation that S217 and S224 in the NLS of the She2 monomer are more accessible for CK2 phosphorylation *in vitro* compared to the NLS of the wild-type She2 protein. Our data suggest that the transition of She2 tetramer to monomer is essential to increase the accessibility of the NLS prior to Srp1 binding and CK2 phosphorylation.

One example of coupling between phosphorylation and nuclear import is the phosphorylation of the histone chaperone Nap1 by CK2, which mediates its nuclear import in an NLS-dependent manner. The mechanism by which CK2 phosphorylation promotes the nuclear import of Nap1 is not clear yet since phosphorylation does not affect the interaction of Nap1 and

its known karyopherin Kap114 [383]. Interestingly, Nap1 has been found in two major states: dimer and octamer. The Nap1 oligomer was identified in the nucleus and, phosphorylation was suggested to regulate its dimer/octamer equilibrium and nuclear import [384]. How the non-canonical NLS of She2 is recognized by Srp1 remains unclear. An alignment of amino acids 200-230 of the She2 NLS from ten *Saccharomyces sensu stricto* species identified 6 highly conserved amino acids, including only one lysine at position 222. Previous research found that mutations at K_{222} and its adjacent amino acids (W_{215}, I_{219}, L_{220}, and L_{223}) disrupt the interaction between She2 and Srp1 [220]. We sought to determine whether other highly conserved amino acids within the NLS, such as P_{201}, V_{202}, and E_{207}, are required for the interaction with Srp1. We found that a minimal NLS of She2 must include these three amino acids, which suggests that all three conserved amino acids may be required for the interaction with Srp1.

Additional work will be needed in order to understand the role of She2 phosphorylation by CK2. As we showed that phosphorylation is required for She2-Srp1 interaction, it will be interesting to elucidate the role of CK2 phosphorylation in the regulation of She2 nuclear import by treating yeast extract with CK2 kinase inhibitor (CX-4945) prior to pull-down of She2 with GST-Srp1. Additionally, it will be useful to examine the interaction between She2 and Srp1 in $cka2^{ts}$ cells at non-permissive temperatures.

4.6 Limitations of the approaches for mapping and analyzing phosphorylation sites

The most frequent post-translational modification that occurs within proteins is phosphorylation. Phosphorylation is involved in the regulation of most of the major functions that occur within eukaryotic organisms and governs a variety of physiological functions, including gene expression and metabolism [417]. However, only around 5% of the thousands of newly found phosphosites in yeast have been functionally annotated [418]. Recent studies determined that more than 75% of yeast proteins are phosphorylated [419]. Although protein phosphorylation has been investigated for decades, the identification and validation of phosphorylation sites within proteins and their related function is historically a challenge. In this study, we used three LC/MS-MS analyses to identify novel phosphopeptides in She2, with a peptide coverage at 76%, 86% and 99%, respectively. While we performed three independent LC/MS-MS analyses to provide more reliable

phosphopeptide detection, we used a single sequence-specific protease (trypsin) for each analysis. This may affect the overall sequence coverage because not every phosphorylated residue is located in areas that will produce MS-friendly peptides after being digested by a single protease [420].

On the other hand, the stoichiometry of the phosphorylation site, also known as its occupancy, cannot be determined by means of standard mass spectrometry. This is due to the fact that phosphorylated and unphosphorylated peptides act differently during mass spectrometry because they are chemically distinct. Therefore, comparing the ratios of phosphorylated and unphosphorylated peptides does not provide enough data to determine the stoichiometry of phosphorylation [421].

We analyzed the sites identified by mass spectrometry and generated phosphomimetic mutations to determine their biological significance and to study the constitutively phosphorylated state. In this method, serine or threonine are mutated to aspartic acid residues. This approach has its drawback. While the Asp carboxylate might roughly reflect a single negative charge linked to phosphate-mediated electrostatic effects, the carboxylate moiety possesses only one negative charge that does not fully match the dianionic phosphate monoesters of a phosphorylated residue at physiological pH. Additionally, phospho-Ser and phospho-Thr have distinct geometries and are larger than Asp residue, which is critical, especially in mediating adaptor interactions [422]. As a result of these limitations, the behaviour of some phosphomimetic mutations may be difficult to comprehend. A combination of *in vivo* and *in vitro* research may be required to confirm the role of phosphorylation in order to overcome this limitation.

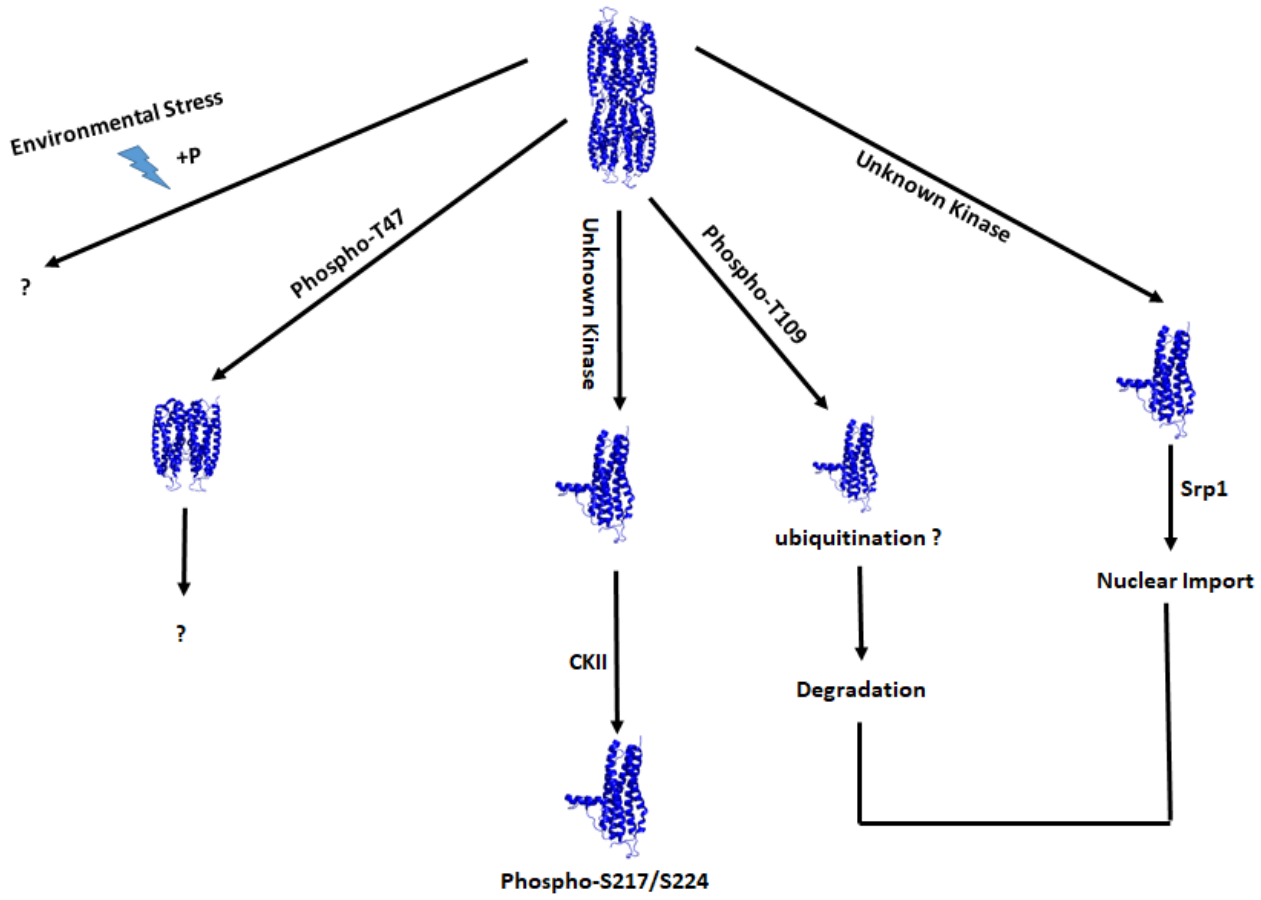


FIGURE 4-3 THE PHOSPHORYLATION OF SHE2 AFFECTS ITS FUNCTION BY CONTROLLING ITS OLIGOMERIZATION STATE.

4.7 CONCLUSION

1) We report that the RNA-binding protein She2 is phosphorylated *in vivo* and identified 13 novel phosphorylated residues in this protein. Although a prior study indicated that She2 is a phosphoprotein, no experimental support was provided. Moreover, with the exception of one study, no phosphopeptides from this protein have been detected in global phosphoproteomics investigations in yeast.

2) Phosphorylation state of She2 was investigated using two distinct assays. First, we demonstrate that a fraction of She2 is phosphorylated *in vivo* and can be isolated using a phosphoprotein enrichment column in combination with phosphatase treatment. Second, we purified a GST-tagged version of She2 from yeasts, which was then analyzed using LC/MS-MS to identify phosphopeptides.

3) In the present study, our attention was mainly focused on phospho-Thr109 and phospho-S217 and S224. Threonine 109 is a major phosphosite in She2, and its phosphorylation disrupts She2 oligomerization, interaction with She3 and binding to *ASH1* mRNA. S217 and S224 are located in the NLS in the C-terminal domain of She2. Although casein kinase CK2 phosphorylates She2 at S217 and S224, these phosphorylation events do not affect She2 interaction with Srp1 or the adaptor protein She3. Identifying the specific role of She2 phosphorylation at S217 and S224 would be an interesting future study.

4) Our results suggest that phosphorylation of She2 is required for its interaction with the importin-α Srp1. Treatment of yeast extracts with phosphatase prior to the GST-Srp1 pull-down shows that She2-myc interaction with Srp1 is eliminated in such conditions, while She2-M2-myc interaction with Srp1 is only reduced.

5) We identified a minimal She2 NLS required for interaction with Srp1. A 30 amino acids peptide NLS is the minimal size required for interaction with Srp1. This peptide includes highly conserved amino acids within the NLS, such as P_{201}, V_{202}, and E_{207}.

6) In the future, it will be interesting to investigate the kinase (s) that phosphorylate T109 and also to identify clearly the biological function of phosphorylation at S217 and S224 in She2. As She2 plays a key role in mRNA localization in yeast, the present study will help us better understand how mRNA localization machinery are regulated in yeast and other organisms.

4.8 REFERENCES

1. Halbeisen, R.E., et al., *Post-transcriptional gene regulation: From genome-wide studies to principles.* Cellular and Molecular Life Sciences, 2007. **65**(5): p. 798.
2. Keene, J.D., *RNA regulons: coordination of post-transcriptional events.* Nature Reviews Genetics, 2007. **8**(7): p. 533-543.
3. Oeffinger, M., et al., *Comprehensive analysis of diverse ribonucleoprotein complexes.* Nat Methods, 2007. **4**(11): p. 951-6.
4. Jeffery, W.R., C.R. Tomlinson, and R.D. Brodeur, *Localization of actin messenger RNA during early ascidian development.* Dev Biol, 1983. **99**(2): p. 408-17.
5. Chaudhuri, A., S. Das, and B. Das, *Localization elements and zip codes in the intracellular transport and localization of messenger RNAs in Saccharomyces cerevisiae.* Wiley Interdiscip Rev RNA, 2020. **11**(4): p. e1591.
6. Martin, K.C. and A. Ephrussi, *mRNA localization: gene expression in the spatial dimension.* Cell, 2009. **136**(4): p. 719-730.
7. Lécuyer, E., et al., *Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function.* Cell, 2007. **131**(1): p. 174-87.
8. Benoit Bouvrette, L.P., et al., *CeFra-seq reveals broad asymmetric mRNA and noncoding RNA distribution profiles in Drosophila and human cells.* Rna, 2018. **24**(1): p. 98-113.
9. Kaewsapsak, P., et al., *Live-cell mapping of organelle-associated RNAs via proximity biotinylation combined with protein-RNA crosslinking.* Elife, 2017. **6**.
10. Savulescu, A.F., et al., *Prediction of RNA subcellular localization: Learning from heterogeneous data sources.* iScience, 2021. **24**(11): p. 103298.
11. Medioni, C., K. Mowry, and F. Besse, *Principles and roles of mRNA localization in animal development.* Development (Cambridge, England), 2012. **139**(18): p. 3263-3276.
12. Du, T.G., M. Schmid, and R.P. Jansen, *Why cells move messages: the biological functions of mRNA localization.* Semin Cell Dev Biol, 2007. **18**(2): p. 171-7.
13. Bergalet, J. and E. Lécuyer, *The functions and regulatory principles of mRNA intracellular trafficking.* Adv Exp Med Biol, 2014. **825**: p. 57-96.
14. Wilhelm, J.E. and C.A. Smibert, *Mechanisms of translational regulation in Drosophila.* Biol Cell, 2005. **97**(4): p. 235-52.
15. Johnstone, O. and P. Lasko, *Translational regulation and RNA localization in Drosophila oocytes and embryos.* Annu Rev Genet, 2001. **35**: p. 365-406.
16. Lawrence, J.B. and R.H. Singer, *Intracellular localization of messenger RNAs for cytoskeletal proteins.* Cell, 1986. **45**(3): p. 407-15.
17. Trapp, B.D., et al., *Differentiation and death of premyelinating oligodendrocytes in developing rodent brain.* J Cell Biol, 1997. **137**(2): p. 459-68.
18. Garner, C.C., R.P. Tucker, and A. Matus, *Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites.* Nature, 1988. **336**(6200): p. 674-7.

19. Condeelis, J. and R.H. Singer, *How and why does beta-actin mRNA target?* Biol Cell, 2005. **97**(1): p. 97-110.
20. Zhang, H.L., et al., *Neurotrophin-induced transport of a beta-actin mRNP complex increases beta-actin levels and stimulates growth cone motility.* Neuron, 2001. **31**(2): p. 261-75.
21. Behar, L., et al., *cis-acting signals and trans-acting proteins are involved in tau mRNA targeting into neurites of differentiating neuronal cells.* Int J Dev Neurosci, 1995. **13**(2): p. 113-27.
22. Ainger, K., et al., *Transport and localization elements in myelin basic protein mRNA.* J Cell Biol, 1997. **138**(5): p. 1077-87.
23. Kleiman, R., G. Banker, and O. Steward, *Development of subcellular mRNA compartmentation in hippocampal neurons in culture.* J Neurosci, 1994. **14**(3 Pt 1): p. 1130-40.
24. Burgin, K.E., et al., *In situ hybridization histochemistry of Ca2+/calmodulin-dependent protein kinase in developing rat brain.* J Neurosci, 1990. **10**(6): p. 1788-98.
25. Kleiman, R., G. Banker, and O. Steward, *Differential subcellular localization of particular mRNAs in hippocampal neurons in culture.* Neuron, 1990. **5**(6): p. 821-30.
26. Link, W., et al., *Somatodendritic expression of an immediate early gene is regulated by synaptic activity.* Proc Natl Acad Sci U S A, 1995. **92**(12): p. 5734-8.
27. Korb, E. and S. Finkbeiner, *Arc in synaptic plasticity: from gene to behavior.* Trends Neurosci, 2011. **34**(11): p. 591-8.
28. Colon-Ramos, D.A., et al., *Asymmetric distribution of nuclear pore complexes and the cytoplasmic localization of beta2-tubulin mRNA in Chlamydomonas reinhardtii.* Dev Cell, 2003. **4**(6): p. 941-52.
29. Nevo-Dinur, K., et al., *Translation-independent localization of mRNA in E. coli.* Science, 2011. **331**(6020): p. 1081-4.
30. Tian, L., et al., *mRNA Localization in Plant Cells.* Plant physiology, 2020. **182**(1): p. 97-109.
31. Ham, B.K., et al., *A polypyrimidine tract binding protein, pumpkin RBP50, forms the basis of a phloem-mobile ribonucleoprotein complex.* Plant Cell, 2009. **21**(1): p. 197-215.
32. Liao, G., et al., *Control of cell migration through mRNA localization and local translation.* Wiley Interdiscip Rev RNA, 2015. **6**(1): p. 1-15.
33. Herbert, S.P. and G. Costa, *Sending messages in moving cells: mRNA localization and the regulation of cell migration.* Essays Biochem, 2019. **63**(5): p. 595-606.
34. Latham, V.M., et al., *A Rho-dependent signaling pathway operating through myosin localizes beta-actin mRNA in fibroblasts.* Curr Biol, 2001. **11**(13): p. 1010-6.
35. Liu, G., et al., *Interactions of elongation factor 1alpha with F-actin and beta-actin mRNA: implications for anchoring mRNA in cell protrusions.* Molecular biology of the cell, 2002. **13**(2): p. 579-592.
36. Song, T., et al., *Specific interaction of KIF11 with ZBP1 regulates the transport of β-actin mRNA and cell motility.* J Cell Sci, 2015. **128**(5): p. 1001-10.
37. Nicastro, G., et al., *Mechanism of β-actin mRNA Recognition by ZBP1.* Cell Rep, 2017. **18**(5): p. 1187-1199.
38. Wu, B., et al., *Quantifying Protein-mRNA Interactions in Single Live Cells.* Cell, 2015. **162**(1): p. 211-20.
39. Ryder, P.V. and D.A. Lerit, *RNA localization regulates diverse and dynamic cellular processes.* Traffic, 2018. **19**(7): p. 496-502.
40. Harris, E.S. and W.J. Nelson, *Adenomatous polyposis coli regulates endothelial cell migration independent of roles in beta-catenin signaling and cell-cell adhesion.* Mol Biol Cell, 2010. **21**(15): p. 2611-23.

41. Wang, T., et al., *Extracellular matrix stiffness and cell contractility control RNA localization to promote cell migration.* Nat Commun, 2017. **8**(1): p. 896.
42. Yasuda, K., et al., *The RNA-binding protein Fus directs translation of localized mRNAs in APC-RNP granules.* J Cell Biol, 2013. **203**(5): p. 737-46.
43. Mingle, L.A., et al., *Localization of all seven messenger RNAs for the actin-polymerization nucleator Arp2/3 complex in the protrusions of fibroblasts.* J Cell Sci, 2005. **118**(Pt 11): p. 2425-33.
44. Liao, G., B. Simone, and G. Liu, *Mis-localization of Arp2 mRNA impairs persistence of directional cell migration.* Experimental Cell Research, 2011. **317**(6): p. 812-822.
45. Maizels, Y., et al., *Localization of cofilin mRNA to the leading edge of migrating cells promotes directed cell migration.* J Cell Sci, 2015. **128**(10): p. 1922-33.
46. Kanellos, G. and M.C. Frame, *Cellular functions of the ADF/cofilin family at a glance.* J Cell Sci, 2016. **129**(17): p. 3211-8.
47. Tahtamouni, L.H., et al., *Non-overlapping activities of ADF and cofilin-1 during the migration of metastatic breast tumor cells.* BMC Cell Biology, 2013. **14**(1): p. 45.
48. Bernardoni, R., et al., *Gliogenesis depends on glide/gcm through asymmetric division of neuroglioblasts.* Dev Biol, 1999. **216**(1): p. 265-75.
49. Paquin, N. and P. Chartrand, *Local regulation of mRNA translation: new insights from the bud.* Trends Cell Biol, 2008. **18**(3): p. 105-11.
50. Lambert, J.D. and L.M. Nagy, *Asymmetric inheritance of centrosomally localized mRNAs during embryonic cleavages.* Nature, 2002. **420**(6916): p. 682-6.
51. Alliegro, M.C., M.A. Alliegro, and R.E. Palazzo, *Centrosome-associated RNA in surf clam oocytes.* Proc Natl Acad Sci U S A, 2006. **103**(24): p. 9034-8.
52. Sunchu, B. and C. Cabernard, *Principles and mechanisms of asymmetric cell division.* Development, 2020. **147**(13).
53. Kingsley, E.P., et al., *Widespread RNA segregation in a spiralian embryo.* 2007. **9**(6): p. 527-539.
54. Takatori, N., et al., *Segregation of Germ Layer Fates by Nuclear Migration-Dependent Localization of Not mRNA.* Developmental Cell, 2010. **19**(4): p. 589-598.
55. Landskron, L., et al., *The asymmetrically segregating lncRNA cherub is required for transforming stem cells into malignant cells.* Elife, 2018. **7**.
56. Wang, J., et al., *Asymmetric Expression of LincGET Biases Cell Fate in Two-Cell Mouse Embryos.* Cell, 2018. **175**(7): p. 1887-1901.e18.
57. Weil, T.T., *mRNA localization in the Drosophila germline.* RNA biology, 2014. **11**(8): p. 1010-1018.
58. Becalska, A.N. and E.R. Gavis, *Lighting up mRNA localization in Drosophila oogenesis.* Development, 2009. **136**(15): p. 2493-503.
59. Lasko, P., *mRNA localization and translational control in Drosophila oogenesis.* Cold Spring Harbor perspectives in biology, 2012. **4**(10): p. a012294.
60. González-Reyes, A., H. Elliott, and D. St Johnston, *Polarization of both major body axes in Drosophila by gurken-torpedo signalling.* Nature, 1995. **375**(6533): p. 654-8.
61. Roth, S., et al., *cornichon and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in Drosophila.* Cell, 1995. **81**(6): p. 967-78.
62. Derrick, C.J. and T.T. Weil, *Translational control of gurken mRNA in Drosophila development.* Cell Cycle, 2017. **16**(1): p. 23-32.
63. Müller-McNicoll, M., et al., *Auto-regulatory feedback by RNA-binding proteins.* Journal of molecular cell biology, 2019. **11**(10): p. 930-939.
64. Dahm, R., M. Kiebler, and P. Macchi, *RNA localisation in the nervous system.* Semin Cell Dev Biol, 2007. **18**(2): p. 216-23.

65. Fonkeu, Y., et al., *How mRNA Localization and Protein Synthesis Sites Influence Dendritic Protein Distribution and Dynamics.* Neuron, 2019. **103**(6): p. 1109-1122.e7.
66. Ohashi, R. and N. Shiina, *Cataloguing and Selection of mRNAs Localized to Dendrites in Neurons and Regulated by RNA-Binding Proteins in RNA Granules.* Biomolecules, 2020. **10**(2): p. 167.
67. Nihonmatsu, I., et al., *Selective targeting of mRNA and the following protein synthesis of CaMKIIα at the long-term potentiation-induced site.* Biol Open, 2020. **9**(1).
68. Sharangdhar, T., et al., *A retained intron in the 3'-UTR of Calm3 mRNA mediates its Staufen2- and activity-dependent localization to neuronal dendrites.* EMBO Rep, 2017. **18**(10): p. 1762-1774.
69. Tiruchinapalli, D.M., et al., *Activity-dependent trafficking and dynamic localization of zipcode binding protein 1 and beta-actin mRNA in dendrites and spines of hippocampal neurons.* J Neurosci, 2003. **23**(8): p. 3251-61.
70. Willis, D.E., et al., *Axonal Localization of transgene mRNA in mature PNS and CNS neurons.* J Neurosci, 2011. **31**(41): p. 14481-7.
71. Andreassi, C. and A. Riccio, *To localize or not to localize: mRNA fate is in 3'UTR ends.* Trends Cell Biol, 2009. **19**(9): p. 465-74.
72. Yergert, K.M., et al., *Identification of 3' UTR motifs required for mRNA localization to myelin sheaths in vivo.* PLoS Biol, 2021. **19**(1): p. e3001053.
73. Rodrigues, E.C., et al., *Mammalian Neuronal mRNA Transport Complexes: The Few Knowns and the Many Unknowns.* Front Integr Neurosci, 2021. **15**: p. 692948.
74. Turner-Bridger, B., C. Caterino, and J.M. Cioni, *Molecular mechanisms behind mRNA localization in axons.* Open Biol, 2020. **10**(9): p. 200177.
75. Tautz, D. and C. Pfeifle, *A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback.* Chromosoma, 1989. **98**(2): p. 81-5.
76. Bashirullah, A., R.L. Cooperstock, and H.D. Lipshitz, *Spatial and temporal control of RNA stability.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7025-8.
77. Farris, S., et al., *Selective localization of arc mRNA in dendrites involves activity- and translation-dependent mRNA degradation.* J Neurosci, 2014. **34**(13): p. 4481-93.
78. Zarnack, K. and M. Feldbrugge, *Microtubule-dependent mRNA transport in fungi.* Eukaryot Cell, 2010. **9**(7): p. 982-90.
79. Bullock, S.L., *Translocation of mRNAs by molecular motors: think complex?* Semin Cell Dev Biol, 2007. **18**(2): p. 194-201.
80. Bertrand, E., et al., *Localization of ASH1 mRNA particles in living yeast.* Mol Cell, 1998. **2**(4): p. 437-45.
81. Engel, K.L., et al., *Mechanisms and consequences of subcellular RNA localization across diverse cell types.* Traffic, 2020. **21**(6): p. 404-418.
82. St Johnston, D., *The intracellular localization of messenger RNAs.* Cell, 1995. **81**(2): p. 161-70.
83. Sundell, C.L. and R.H. Singer, *Requirement of microfilaments in sorting of actin messenger RNA.* Science, 1991. **253**(5025): p. 1275-7.
84. Long, R.M., et al., *Mating type switching in yeast controlled by asymmetric localization of ASH1 mRNA.* Science, 1997. **277**(5324): p. 383-7.
85. Jambhekar, A., et al., *Unbiased selection of localization elements reveals cis-acting determinants of mRNA bud localization in Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 2005. **102**(50): p. 18005-10.
86. Kazan, H. and Q. Morris, *RBPmotif: a web server for the discovery of sequence and structure preferences of RNA-binding proteins.* Nucleic Acids Res, 2013. **41**(Web Server issue): p. W180-6.

87. Darnell, J.C., et al., *FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism.* Cell, 2011. **146**(2): p. 247-61.
88. Macdonald, P.M., *mRNA localization: assembly of transport complexes and their incorporation into particles.* Curr Opin Genet Dev, 2011. **21**(4): p. 407-13.
89. Barbarese, E., et al., *Protein translation components are colocalized in granules in oligodendrocytes.* J Cell Sci, 1995. **108 (Pt 8)**: p. 2781-90.
90. Kanai, Y., N. Dohmae, and N. Hirokawa, *Kinesin transports RNA: isolation and characterization of an RNA-transporting granule.* Neuron, 2004. **43**(4): p. 513-25.
91. Elvira, G., et al., *Characterization of an RNA granule from developing brain.* Mol Cell Proteomics, 2006. **5**(4): p. 635-51.
92. Langdon, E.M. and A.S. Gladfelter, *A New Lens for RNA Localization: Liquid-Liquid Phase Separation.* 2018. **72**(1): p. 255-271.
93. Fay, M.M. and P.J. Anderson, *The Role of RNA in Biological Phase Separations.* Journal of molecular biology, 2018. **430**(23): p. 4685-4701.
94. Cabral, S.E. and K.L. Mowry, *Organizing the oocyte: RNA localization meets phase separation.* Current topics in developmental biology, 2020. **140**: p. 87-118.
95. Chartrand, P., et al., *Structural elements required for the localization of ASH1 mRNA and of a green fluorescent protein reporter particle in vivo.* Curr Biol, 1999. **9**(6): p. 333-6.
96. Serano, J. and G.M. Rubin, *The Drosophila synaptotagmin-like protein bitesize is required for growth and has mRNA localization sequences within its open reading frame.* Proc Natl Acad Sci U S A, 2003. **100**(23): p. 13368-73.
97. Munro, T.P., et al., *Mutational analysis of a heterogeneous nuclear ribonucleoprotein A2 response element for RNA trafficking.* J Biol Chem, 1999. **274**(48): p. 34389-95.
98. Jansen, R.-P., *mRNA localization: message on the move.* Nature Reviews Molecular Cell Biology, 2001. **2**(4): p. 247-256.
99. Kim, H.H., et al., *Different motif requirements for the localization zipcode element of β-actin mRNA binding by HuD and ZBP1.* Nucleic Acids Res, 2015. **43**(15): p. 7432-46.
100. Kloc, M., et al., *The Targeting of Xcat2 mRNA to the Germinal Granules Depends on a cis-Acting Germinal Granule Localization Element within the 3'UTR.* Developmental Biology, 2000. **217**(2): p. 221-229.
101. Macdonald, P.M., et al., *RNA regulatory element BLE1 directs the early steps of bicoid mRNA localization.* Development, 1993. **118**(4): p. 1233-43.
102. Ferrandon, D., et al., *Staufen protein associates with the 3'UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner.* Cell, 1994. **79**(7): p. 1221-32.
103. Thio, G.L., et al., *Localization of gurken RNA in Drosophila oogenesis requires elements in the 5' and 3' regions of the transcript.* Dev Biol, 2000. **221**(2): p. 435-46.
104. Bloom, K. and D.L. Beach, *mRNA localization: motile RNA, asymmetric anchors.* Curr Opin Microbiol, 1999. **2**(6): p. 604-9.
105. Yisraeli, J.K., S. Sokol, and D.A. Melton, *A two-step model for the localization of maternal mRNA in Xenopus oocytes: involvement of microtubules and microfilaments in the translocation and anchoring of Vg1 mRNA.* Development, 1990. **108**(2): p. 289-98.
106. Vale, R.D., *The molecular motor toolbox for intracellular transport.* Cell, 2003. **112**(4): p. 467-80.
107. Holt, C.E. and S.L. Bullock, *Subcellular mRNA localization in animal cells and why it matters.* Science, 2009. **326**(5957): p. 1212-6.
108. Ross, J.L., et al., *Processive bidirectional motion of dynein-dynactin complexes in vitro.* Nat Cell Biol, 2006. **8**(6): p. 562-70.

109. Barlan, K. and V.I. Gelfand, *Microtubule-Based Transport and the Distribution, Tethering, and Organization of Organelles.* Cold Spring Harb Perspect Biol, 2017. **9**(5).
110. Das, S., R.H. Singer, and Y.J. Yoon, *The travels of mRNAs in neurons: do they know where they are going?* Curr Opin Neurobiol, 2019. **57**: p. 110-116.
111. Messitt, T.J., et al., *Multiple kinesin motors coordinate cytoplasmic RNA transport on a subpopulation of microtubules in Xenopus oocytes.* Dev Cell, 2008. **15**(3): p. 426-436.
112. Reck-Peterson, S.L., et al., *The cytoplasmic dynein transport machinery and its many cargoes.* Nat Rev Mol Cell Biol, 2018. **19**(6): p. 382-398.
113. Neiswender, H., et al., *Dynein light chain-dependent dimerization of Egalitarian is essential for maintaining oocyte fate in Drosophila.* Dev Biol, 2021. **478**: p. 76-88.
114. Sanghavi, P., et al., *Multiple Roles for Egalitarian in Polarization of the Drosophila Egg Chamber.* Genetics, 2016. **203**(1): p. 415-32.
115. Wu, H., et al., *A kinesin adapter directly mediates dendritic mRNA localization during neural development in mice.* J Biol Chem, 2020. **295**(19): p. 6605-6628.
116. Das, S., et al., *Intracellular mRNA transport and localized translation.* Nat Rev Mol Cell Biol, 2021. **22**(7): p. 483-504.
117. Kress, T.L., Y.J. Yoon, and K.L. Mowry, *Nuclear RNP complex assembly initiates cytoplasmic RNA localization.* J Cell Biol, 2004. **165**(2): p. 203-11.
118. Weatheritt, R.J., T.J. Gibson, and M.M. Babu, *Asymmetric mRNA localization contributes to fidelity and sensitivity of spatially localized systems.* Nat Struct Mol Biol, 2014. **21**(9): p. 833-9.
119. Lipshitz, H.D., J.M. Claycomb, and C.A. Smibert, *Post-transcriptional regulation of gene expression.* Methods, 2017. **126**: p. 1-2.
120. Hüttelmaier, S., et al., *Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1.* Nature, 2005. **438**(7067): p. 512-5.
121. Teixeira, F.K. and R. Lehmann, *Translational Control during Developmental Transitions.* Cold Spring Harb Perspect Biol, 2019. **11**(6).
122. Nelson, M.R., A.M. Leidal, and C.A. Smibert, *Drosophila Cup is an eIF4E-binding protein that functions in Smaug-mediated translational repression.* Embo j, 2004. **23**(1): p. 150-9.
123. Chalkiadaki, K., et al., *Translational control in neurovascular brain development.* R Soc Open Sci, 2021. **8**(10): p. 211088.
124. Jung, M.Y., L. Lorenz, and J.D. Richter, *Translational control by neuroguidin, a eukaryotic initiation factor 4E and CPEB binding protein.* Mol Cell Biol, 2006. **26**(11): p. 4277-87.
125. Lin, D., et al., *Translational control by a small RNA: dendritic BC1 RNA targets the eukaryotic initiation factor 4A helicase mechanism.* Mol Cell Biol, 2008. **28**(9): p. 3008-19.
126. Deng, Y., R.H. Singer, and W. Gu, *Translation of ASH1 mRNA is repressed by Puf6p-Fun12p/eIF5B interaction and released by CK2 phosphorylation.* Genes & development, 2008. **22**(8): p. 1037-1050.
127. Xiang, K. and D.P. Bartel, *The molecular basis of coupling between poly(A)-tail length and translational efficiency.* eLife, 2021. **10**: p. e66493.
128. Zaessinger, S., I. Busseau, and M. Simonelig, *Oskar allows nanos mRNA translation in Drosophila embryos by preventing its deadenylation by Smaug/CCR4.* Development, 2006. **133**(22): p. 4573-83.
129. Narayanan, U., et al., *FMRP phosphorylation reveals an immediate-early signaling pathway triggered by group I mGluR and mediated by PP2A.* J Neurosci, 2007. **27**(52): p. 14349-57.

130. Nalavadi, V.C., et al., *Dephosphorylation-induced ubiquitination and degradation of FMRP in dendrites: a role in immediate early mGluR-stimulated translation.* J Neurosci, 2012. **32**(8): p. 2582-7.
131. Singer-Krüger, B. and R.P. Jansen, *Here, there, everywhere. mRNA localization in budding yeast.* RNA Biol, 2014. **11**(8): p. 1031-9.
132. Heym, R.G. and D. Niessing, *Principles of mRNA transport in yeast.* Cell Mol Life Sci, 2012. **69**(11): p. 1843-53.
133. Shepard, K.A., et al., *Widespread cytoplasmic mRNA transport in yeast: identification of 22 bud-localized transcripts using DNA microarray analysis.* Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11429-34.
134. Takizawa, P.A., et al., *Actin-dependent localization of an RNA encoding a cell-fate determinant in yeast.* Nature, 1997. **389**(6646): p. 90-3.
135. Elson, S.L., et al., *An RNA transport system in Candida albicans regulates hyphal morphology and invasive growth.* PLoS Genet, 2009. **5**(9): p. e1000664.
136. Takizawa, P.A., et al., *Plasma membrane compartmentalization in yeast by messenger RNA transport and a septin diffusion barrier.* Science, 2000. **290**(5490): p. 341-4.
137. Tutucci, E., et al., *Cyclin CLB2 mRNA localization determines efficient protein synthesis to orchestrate bud growth and cell cycle progression*. 2022, bioRxiv.
138. Aronov, S., et al., *mRNAs Encoding Polarity and Exocytosis Factors Are Cotransported with the Cortical Endoplasmic Reticulum to the Incipient Bud in Saccharomyces cerevisiae.* 2007. **27**(9): p. 3441-3455.
139. Ziman, M., et al., *Subcellular localization of Cdc42p, a Saccharomyces cerevisiae GTP-binding protein involved in the control of cell polarity.* Mol Biol Cell, 1993. **4**(12): p. 1307-16.
140. Balagopal, V. and R. Parker, *Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs.* Curr Opin Cell Biol, 2009. **21**(3): p. 403-8.
141. Buchan, J.R. and R. Parker, *Eukaryotic stress granules: the ins and outs of translation.* Mol Cell, 2009. **36**(6): p. 932-41.
142. Pizzinga, M., et al., *Translation factor mRNA granules direct protein synthetic capacity to regions of polarized growth.* J Cell Biol, 2019. **218**(5): p. 1564-1581.
143. Hanson, S.J. and K.H. Wolfe, *An Evolutionary Perspective on Yeast Mating-Type Switching.* Genetics, 2017. **206**(1): p. 9-32.
144. Haber, J.E., *Mating-type genes and MAT switching in Saccharomyces cerevisiae.* Genetics, 2012. **191**(1): p. 33-64.
145. Nickoloff, J.A., E.Y. Chen, and F. Heffron, *A 24-base-pair DNA sequence from the MAT locus stimulates intergenic recombination in yeast.* Proc Natl Acad Sci U S A, 1986. **83**(20): p. 7831-5.
146. Cosma, M.P., *Daughter-specific repression of Saccharomyces cerevisiae HO: Ash1 is the commander.* EMBO reports, 2004. **5**(10): p. 953-957.
147. Darzacq, X., et al., *RNA asymmetric distribution and daughter/mother differentiation in yeast.* Curr Opin Microbiol, 2003. **6**(6): p. 614-20.
148. Maxon, M.E. and I. Herskowitz, *Ash1p is a site-specific DNA-binding protein that actively represses transcription.* Proc Natl Acad Sci U S A, 2001. **98**(4): p. 1495-500.
149. Olivier, C., et al., *Identification of a conserved RNA motif essential for She2p recognition and mRNA localization to the yeast bud.* Molecular and cellular biology, 2005. **25**(11): p. 4752-4766.
150. Chartrand, P., et al., *Asymmetric sorting of ash1p in yeast results from inhibition of translation by localization elements in the mRNA.* Mol Cell, 2002. **10**(6): p. 1319-30.

151. Müller, M., et al., *A cytoplasmic complex mediates specific mRNA recognition and localization in yeast.* PLoS biology, 2011. **9**(4): p. e1000611-e1000611.
152. Takizawa, P.A. and R.D. Vale, *The myosin motor, Myo4p, binds Ash1 mRNA via the adapter protein, She3p.* Proc Natl Acad Sci U S A, 2000. **97**(10): p. 5273-8.
153. Böhl, F., et al., *She2p, a novel RNA-binding protein tethers ASH1 mRNA to the Myo4p myosin motor via She3p.* Embo j, 2000. **19**(20): p. 5514-24.
154. Niessing, D., et al., *She2p is a novel RNA binding protein with a basic helical hairpin motif.* Cell, 2004. **119**(4): p. 491-502.
155. Gonzalez, I., et al., *ASH1 mRNA localization in yeast involves multiple secondary structural elements and Ash1 protein translation.* Curr Biol, 1999. **9**(6): p. 337-40.
156. Long, R.M., et al., *She2p is a novel RNA-binding protein that recruits the Myo4p-She3p complex to ASH1 mRNA.* Embo j, 2000. **19**(23): p. 6592-601.
157. Münchow, S., C. Sauter, and R.P. Jansen, *Association of the class V myosin Myo4p with a localised messenger RNA in budding yeast depends on She proteins.* J Cell Sci, 1999. **112 (Pt 10)**: p. 1511-8.
158. Müller, M., et al., *Formation of She2p tetramers is required for mRNA binding, mRNP assembly, and localization.* RNA (New York, N.Y.), 2009. **15**(11): p. 2002-2012.
159. Genz, C., et al., *Association of the yeast RNA-binding protein She2p with the tubular endoplasmic reticulum depends on membrane curvature.* J Biol Chem, 2013. **288**(45): p. 32384-93.
160. Schmid, M., et al., *Coordination of endoplasmic reticulum and mRNA localization to the yeast bud.* Curr Biol, 2006. **16**(15): p. 1538-43.
161. Fundakowski, J., O. Hermesh, and R.P. Jansen, *Localization of a subset of yeast mRNAs depends on inheritance of endoplasmic reticulum.* Traffic, 2012. **13**(12): p. 1642-52.
162. Niedner, A., F.T. Edelmann, and D. Niessing, *Of social molecules: The interactive assembly of ASH1 mRNA-transport complexes in yeast.* RNA Biol, 2014. **11**(8): p. 998-1009.
163. Bohl, F., et al., *She2p, a novel RNA-binding protein tethers ASH1 mRNA to the Myo4p myosin motor via She3p.* Embo j, 2000. **19**(20): p. 5514-24.
164. Hodges, A.R., E.B. Krementsova, and K.M. Trybus, *She3p binds to the rod of yeast myosin V and prevents it from dimerizing, forming a single-headed motor complex.* J Biol Chem, 2008. **283**(11): p. 6906-14.
165. Shi, H., et al., *Structure of a myosin•adaptor complex and pairing by cargo.* Proc Natl Acad Sci U S A, 2014. **111**(12): p. E1082-90.
166. Niedner, A., et al., *Role of Loc1p in assembly and reorganization of nuclear ASH1 messenger ribonucleoprotein particles in yeast.* Proc Natl Acad Sci U S A, 2013. **110**(52): p. E5049-58.
167. Edelmann, F.T., et al., *Molecular architecture and dynamics of ASH1 mRNA recognition by its mRNA-transport complex.* Nat Struct Mol Biol, 2017. **24**(2): p. 152-161.
168. Krementsova, E.B., et al., *Two single-headed myosin V motors bound to a tetrameric adapter protein form a processive complex.* J Cell Biol, 2011. **195**(4): p. 631-41.
169. Titus, M.A., *Motor proteins: myosin V--the multi-purpose transport motor.* Curr Biol, 1997. **7**(5): p. R301-4.
170. Lillie, S.H. and S.S. Brown, *Immunofluorescence localization of the unconventional myosin, Myo2p, and the putative kinesin-related protein, Smy1p, to the same regions of polarized growth in Saccharomyces cerevisiae.* J Cell Biol, 1994. **125**(4): p. 825-42.
171. Schott, D., et al., *The COOH-terminal domain of Myo2p, a yeast myosin V, has a direct role in secretory vesicle targeting.* J Cell Biol, 1999. **147**(4): p. 791-808.
172. Karpova, T.S., et al., *Role of actin and Myo2p in polarized secretion and growth of Saccharomyces cerevisiae.* Mol Biol Cell, 2000. **11**(5): p. 1727-37.

173. Reck-Peterson, S.L., et al., *Class V myosins.* Biochim Biophys Acta, 2000. **1496**(1): p. 36-51.
174. Pruyne, D., et al., *Mechanisms of polarized growth and organelle segregation in yeast.* Annu Rev Cell Dev Biol, 2004. **20**: p. 559-91.
175. Jansen, R.P., et al., *Mother cell-specific HO expression in budding yeast depends on the unconventional myosin myo4p and other cytoplasmic proteins.* Cell, 1996. **84**(5): p. 687-97.
176. Govindan, B., R. Bowser, and P. Novick, *The role of Myo2, a yeast class V myosin, in vesicular transport.* J Cell Biol, 1995. **128**(6): p. 1055-68.
177. Catlett, N.L., et al., *Two distinct regions in a yeast myosin-V tail domain are required for the movement of different cargoes.* The Journal of Cell Biology, 2000. **150**(3): p. 513-526.
178. Catlett, N.L. and L.S. Weisman, *The terminal tail region of a yeast myosin-V mediates its attachment to vacuole membranes and sites of polarized growth.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 14799-804.
179. Rossanese, O.W., et al., *A role for actin, Cdc1p, and Myo2p in the inheritance of late Golgi elements in Saccharomyces cerevisiae.* J Cell Biol, 2001. **153**(1): p. 47-62.
180. Yin, H., et al., *Myosin V orientates the mitotic spindle in yeast.* Nature, 2000. **406**(6799): p. 1013-5.
181. Haarer, B.K., et al., *Identification of MYO4, a second class V myosin gene in yeast.* J Cell Sci, 1994. **107 (Pt 4)**: p. 1055-64.
182. Estrada, P., et al., *Myo4p and She3p are required for cortical ER inheritance in Saccharomyces cerevisiae.* J Cell Biol, 2003. **163**(6): p. 1255-66.
183. Garmendia-Torres, C., et al., *Unidirectional P-body transport during the yeast cell cycle.* PLoS One, 2014. **9**(6): p. e99428.
184. Heuck, A., et al., *Monomeric myosin V uses two binding regions for the assembly of stable translocation complexes.* Proc Natl Acad Sci U S A, 2007. **104**(50): p. 19778-83.
185. Dunn, B.D., et al., *Myo4p is a monomeric myosin with motility uniquely adapted to transport mRNA.* J Cell Biol, 2007. **178**(7): p. 1193-206.
186. Reck-Peterson, S.L., et al., *The yeast class V myosins, Myo2p and Myo4p, are nonprocessive actin-based motors.* J Cell Biol, 2001. **153**(5): p. 1121-6.
187. Heym, R.G., et al., *In vitro reconstitution of an mRNA-transport complex reveals mechanisms of assembly and motor activation.* J Cell Biol, 2013. **203**(6): p. 971-84.
188. Kruse, C., et al., *Ribonucleoprotein-dependent localization of the yeast class V myosin Myo4p.* J Cell Biol, 2002. **159**(6): p. 971-82.
189. Edelmann, F.T., A. Niedner, and D. Niessing, *ASH1 mRNP-core factors form stable complexes in absence of cargo RNA at physiological conditions.* RNA Biol, 2015. **12**(3): p. 233-7.
190. Sladewski, T.E., et al., *Single-molecule reconstitution of mRNA transport by a class V myosin.* Nat Struct Mol Biol, 2013. **20**(8): p. 952-7.
191. Gelin-Licht, R., et al., *Scp160-dependent mRNA trafficking mediates pheromone gradient sensing and chemotropism in yeast.* Cell Rep, 2012. **1**(5): p. 483-94.
192. Gonsalvez, G.B., et al., *RNA-protein interactions promote asymmetric sorting of the ASH1 mRNA ribonucleoprotein complex.* Rna, 2003. **9**(11): p. 1383-99.
193. Singh, N., G. Blobel, and H. Shi, *Hooking She3p onto She2p for myosin-mediated cytoplasmic mRNA transport.* 2015. **112**(1): p. 142-147.
194. Heuck, A., et al., *The structure of the Myo4p globular tail and its function in ASH1 mRNA localization.* J Cell Biol, 2010. **189**(3): p. 497-510.
195. Long, R.M., et al., *An exclusively nuclear RNA-binding protein affects asymmetric localization of ASH1 mRNA and Ash1p in yeast.* J Cell Biol, 2001. **153**(2): p. 307-18.

196. Shahbabian, K., et al., *Co-transcriptional recruitment of Puf6 by She2 couples translational repression to mRNA localization.* Nucleic Acids Research, 2014. **42**(13): p. 8692-8704.
197. Komili, S., et al., *Functional specificity among ribosomal proteins regulates gene expression.* Cell, 2007. **131**(3): p. 557-71.
198. Yang, Y.T., et al., *The Roles of Puf6 and Loc1 in 60S Biogenesis Are Interdependent, and Both Are Required for Efficient Accommodation of Rpl43.* J Biol Chem, 2016. **291**(37): p. 19312-23.
199. Harnpicharnchai, P., et al., *Composition and functional characterization of yeast 66S ribosome assembly intermediates.* Mol Cell, 2001. **8**(3): p. 505-15.
200. Liang, K.-J., et al., *Puf6 and Loc1 Are the Dedicated Chaperones of Ribosomal Protein Rpl43 in Saccharomyces cerevisiae.* International journal of molecular sciences, 2019. **20**(23): p. 5941.
201. Liang, K.J., et al., *Puf6 and Loc1 Are the Dedicated Chaperones of Ribosomal Protein Rpl43 in Saccharomyces cerevisiae.* Int J Mol Sci, 2019. **20**(23).
202. Wang, X., et al., *Modular recognition of RNA by a human pumilio-homology domain.* Cell, 2002. **110**(4): p. 501-12.
203. Jenkins, H.T., R. Baker-Wilding, and T.A. Edwards, *Structure and RNA binding of the mouse Pumilio-2 Puf domain.* J Struct Biol, 2009. **167**(3): p. 271-6.
204. Zhu, D., et al., *A 5' cytosine binding pocket in Puf3p specifies regulation of mitochondrial mRNAs.* Proc Natl Acad Sci U S A, 2009. **106**(48): p. 20192-7.
205. Wang, Y., et al., *Structural basis for specific recognition of multiple mRNA targets by a PUF regulatory protein.* Proc Natl Acad Sci U S A, 2009. **106**(48): p. 20186-91.
206. Wang, X., P.D. Zamore, and T.M. Hall, *Crystal structure of a Pumilio homology domain.* Mol Cell, 2001. **7**(4): p. 855-65.
207. Miller, M.T., J.J. Higgin, and T.M. Hall, *Basis of altered RNA-binding specificity by PUF proteins revealed by crystal structures of yeast Puf4p.* Nat Struct Mol Biol, 2008. **15**(4): p. 397-402.
208. Qiu, C., et al., *A divergent Pumilio repeat protein family for pre-rRNA processing and mRNA localization.* Proc Natl Acad Sci U S A, 2014. **111**(52): p. 18554-9.
209. Wickens, M., et al., *A PUF family portrait: 3'UTR regulation as a way of life.* Trends Genet, 2002. **18**(3): p. 150-7.
210. Gerber, A.P., D. Herschlag, and P.O. Brown, *Extensive association of functionally and cytotopically related mRNAs with Puf family RNA-binding proteins in yeast.* PLoS Biol, 2004. **2**(3): p. E79.
211. Traven, A., et al., *The yeast PUF protein Puf5 has Pop2-independent roles in response to DNA replication stress.* PLoS One, 2010. **5**(5): p. e10651.
212. Eliyahu, E., et al., *Tom20 mediates localization of mRNAs to mitochondria in a translation-dependent manner.* Mol Cell Biol, 2010. **30**(1): p. 284-94.
213. García-Rodríguez, L.J., A.C. Gay, and L.A. Pon, *Puf3p, a Pumilio family RNA binding protein, localizes to mitochondria and regulates mitochondrial biogenesis and motility in budding yeast.* J Cell Biol, 2007. **176**(2): p. 197-207.
214. Hook, B.A., et al., *Two yeast PUF proteins negatively regulate a single mRNA.* J Biol Chem, 2007. **282**(21): p. 15430-8.
215. Ulbricht, R.J. and W.M. Olivas, *Puf1p acts in combination with other yeast Puf proteins to control mRNA stability.* Rna, 2008. **14**(2): p. 246-62.
216. Li, Z., et al., *Rational extension of the ribosome biogenesis pathway using network-guided genetics.* PLoS Biol, 2009. **7**(10): p. e1000213.
217. Tafforeau, L., et al., *The complexity of human ribosome biogenesis revealed by systematic nucleolar screening of Pre-rRNA processing factors.* Mol Cell, 2013. **51**(4): p. 539-51.

218. Qiu, C., et al., *Distinct RNA-binding modules in a single PUF protein cooperate to determine RNA specificity.* Nucleic Acids Res, 2019. **47**(16): p. 8770-8784.
219. Gu, W., et al., *A predominantly nuclear protein affecting cytoplasmic localization of beta-actin mRNA in fibroblasts and neurons.* J Cell Biol, 2002. **156**(1): p. 41-51.
220. Shen, Z., et al., *Nuclear shuttling of She2p couples ASH1 mRNA localization to its translational repression by recruiting Loc1p and Puf6p.* Mol Biol Cell, 2009. **20**(8): p. 2265-75.
221. Quenault, T., T. Lithgow, and A. Traven, *PUF proteins: repression, activation and mRNA localization.* Trends Cell Biol, 2011. **21**(2): p. 104-12.
222. Irie, K., et al., *The Khd1 protein, which has three KH RNA-binding motifs, is required for proper localization of ASH1 mRNA in yeast.* EMBO J, 2002. **21**(5): p. 1158-67.
223. Chaudhury, A., P. Chander, and P.H. Howe, *Heterogeneous nuclear ribonucleoproteins (hnRNPs) in cellular processes: Focus on hnRNP E1's multifunctional regulatory roles.* RNA, 2010. **16**(8): p. 1449-62.
224. Kosturko, L.D., et al., *Heterogeneous nuclear ribonucleoprotein (hnRNP) E1 binds to hnRNP A2 and inhibits translation of A2 response element mRNAs.* Mol Biol Cell, 2006. **17**(8): p. 3521-33.
225. Paquin, N., et al., *Local activation of yeast ASH1 mRNA translation through phosphorylation of Khd1p by the casein kinase Yck1p.* Mol Cell, 2007. **26**(6): p. 795-809.
226. Hasegawa, Y., K. Irie, and A.P. Gerber, *Distinct roles for Khd1p in the localization and expression of bud-localized mRNAs in yeast.* RNA, 2008. **14**(11): p. 2333-47.
227. Hogan, D.J., et al., *Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system.* PLoS Biol, 2008. **6**(10): p. e255.
228. Wolf, J.J., et al., *Feed-forward regulation of a cell fate determinant by an RNA-binding protein generates asymmetry in yeast.* Genetics, 2010. **185**(2): p. 513-22.
229. Mitchell, S.F., et al., *Global analysis of yeast mRNPs.* Nat Struct Mol Biol, 2013. **20**(1): p. 127-33.
230. Mauchi, N., Y. Ohtake, and K. Irie, *Stability control of MTL1 mRNA by the RNA-binding protein Khd1p in yeast.* Cell Struct Funct, 2010. **35**(2): p. 95-105.
231. Ito, W., et al., *RNA-binding protein Khd1 and Ccr4 deadenylase play overlapping roles in the cell wall integrity pathway in Saccharomyces cerevisiae.* Eukaryot Cell, 2011. **10**(10): p. 1340-7.
232. and, J.C. and R. Parker, *Eukaryotic mRNA Decapping.* 2004. **73**(1): p. 861-890.
233. Nissan, T., et al., *Decapping Activators in Saccharomyces cerevisiae Act by Multiple Mechanisms.* Molecular Cell, 2010. **39**(5): p. 773-783.
234. Coller, J. and R. Parker, *General Translational Repression by Activators of mRNA Decapping.* Cell, 2005. **122**(6): p. 875-886.
235. Zhang, Q., et al., *Binding of DEAD-box helicase Dhh1 to the 5'-untranslated region of ASH1 mRNA represses localized translation of ASH1 in yeast cells.* J Biol Chem, 2017. **292**(23): p. 9787-9800.
236. Ka, M., Y.U. Park, and J. Kim, *The DEAD-box RNA helicase, Dhh1, functions in mating by regulating Ste12 translation in Saccharomyces cerevisiae.* Biochem Biophys Res Commun, 2008. **367**(3): p. 680-6.
237. Park, Y.U., et al., *Identification of translational regulation target genes during filamentous growth in Saccharomyces cerevisiae: regulatory role of Caf20 and Dhh1.* Eukaryot Cell, 2006. **5**(12): p. 2120-7.
238. Jung, D., et al., *Functional association of Loc1 and Puf6 with RNA helicase Dhh1 in translational regulation of Saccharomyces cerevisiae Ste12.* PLoS One, 2019. **14**(7): p. e0220137.
239. Shen, Z., A. St-Denis, and P. Chartrand, *Cotranscriptional recruitment of She2p by RNA pol II elongation factor Spt4-Spt5/DSIF promotes mRNA localization to the yeast bud.* Genes & development, 2010. **24**(17): p. 1914-1926.

240. Powrie, E.A., D. Zenklusen, and R.H. Singer, *A nucleoporin, Nup60p, affects the nuclear and cytoplasmic localization of ASH1 mRNA in S. cerevisiae.* Rna, 2011. **17**(1): p. 134-44.
241. Landers, S.M., et al., *She3p possesses a novel activity required for ASH1 mRNA localization in Saccharomyces cerevisiae.* Eukaryot Cell, 2009. **8**(7): p. 1072-83.
242. Jin, Y., et al., *PTC1 is required for vacuole inheritance and promotes the association of the myosin-V vacuole-specific receptor complex.* Mol Biol Cell, 2009. **20**(5): p. 1312-23.
243. Hovsepian, J., et al., *Studying Protein Ubiquitylation in Yeast.* Methods Mol Biol, 2016. **1449**: p. 117-42.
244. Wang, R. and M.J. Solomon, *Identification of She3 as an SCF(Grr1) substrate in budding yeast.* PloS one, 2012. **7**(10): p. e48020-e48020.
245. Ziv, I., et al., *A perturbed ubiquitin landscape distinguishes between ubiquitin in trafficking and in proteolysis.* Mol Cell Proteomics, 2011. **10**(5): p. M111.009753.
246. Kolawa, N., et al., *Perturbations to the ubiquitin conjugate proteome in yeast δubx mutants identify Ubx2 as a regulator of membrane lipid composition.* Molecular & cellular proteomics : MCP, 2013. **12**(10): p. 2791-2803.
247. Giorgi, C. and M.J. Moore, *The nuclear nurture and cytoplasmic nature of localized mRNPs.* Seminars in Cell & Developmental Biology, 2007. **18**(2): p. 186-193.
248. Forget, A. and P. Chartrand, *Cotranscriptional assembly of mRNP complexes that determine the cytoplasmic fate of mRNA.* Transcription, 2011. **2**(2): p. 86-90.
249. Yano, T., et al., *Hrp48, a Drosophila hnRNPA/B homolog, binds and regulates translation of oskar mRNA.* Dev Cell, 2004. **6**(5): p. 637-48.
250. Hachet, O. and A. Ephrussi, *Splicing of oskar RNA in the nucleus is coupled to its cytoplasmic localization.* Nature, 2004. **428**(6986): p. 959-63.
251. White, J.A. and J. Heasman, *Maternal control of pattern formation in Xenopus laevis.* J Exp Zool B Mol Dev Evol, 2008. **310**(1): p. 73-84.
252. Lewis, R.A., J.A. Gagnon, and K.L. Mowry, *PTB/hnRNP I is required for RNP remodeling during RNA localization in Xenopus oocytes.* Mol Cell Biol, 2008. **28**(2): p. 678-86.
253. Biswas, J., et al., *Zipcode Binding Protein 1 (ZBP1; IGF2BP1): A Model for Sequence-Specific RNA Regulation.* Cold Spring Harb Symp Quant Biol, 2019. **84**: p. 1-10.
254. Oleynikov, Y. and R.H. Singer, *Real-time visualization of ZBP1 association with beta-actin mRNA during transcription and localization.* Curr Biol, 2003. **13**(3): p. 199-207.
255. Pan, F., et al., *ZBP2 Facilitates Binding of ZBP1 to β-Actin mRNA during Transcription.* 2007. **27**(23): p. 8340-8351.
256. Du, T.G., et al., *Nuclear transit of the RNA-binding protein She2 is required for translational control of localized ASH1 mRNA.* EMBO Rep, 2008. **9**(8): p. 781-7.
257. Wendler, P. and C. Enenkel, *Nuclear Transport of Yeast Proteasomes.* Front Mol Biosci, 2019. **6**: p. 34.
258. Shen, Z., A. St-Denis, and P. Chartrand, *Cotranscriptional recruitment of She2p by RNA pol II elongation factor Spt4-Spt5/DSIF promotes mRNA localization to the yeast bud.* Genes Dev, 2010. **24**(17): p. 1914-26.
259. Shahbabian, K., et al., *Co-transcriptional recruitment of Puf6 by She2 couples translational repression to mRNA localization.* Nucleic Acids Res, 2014. **42**(13): p. 8692-704.
260. Lin, D.H. and A. Hoelz, *The Structure of the Nuclear Pore Complex (An Update).* Annu Rev Biochem, 2019. **88**: p. 725-783.
261. Paci, G., et al., *Molecular determinants of large cargo transport into the nucleus.* Elife, 2020. **9**.

262. Rout, M.P., et al., *The yeast nuclear pore complex: composition, architecture, and transport mechanism.* J Cell Biol, 2000. **148**(4): p. 635-51.
263. Mohr, D., et al., *Characterisation of the passive permeability barrier of nuclear pore complexes.* Embo j, 2009. **28**(17): p. 2541-53.
264. Ribbeck, K. and D. Görlich, *Kinetic analysis of translocation through nuclear pore complexes.* Embo j, 2001. **20**(6): p. 1320-30.
265. Frey, S. and D. Görlich, *A saturated FG-repeat hydrogel can reproduce the permeability properties of nuclear pore complexes.* Cell, 2007. **130**(3): p. 512-23.
266. Unwin, P.N. and R.A. Milligan, *A large particle associated with the perimeter of the nuclear pore complex.* J Cell Biol, 1982. **93**(1): p. 63-75.
267. Hinshaw, J.E., B.O. Carragher, and R.A. Milligan, *Architecture and design of the nuclear pore complex.* Cell, 1992. **69**(7): p. 1133-41.
268. Schwartz, T.U., *The Structure Inventory of the Nuclear Pore Complex.* J Mol Biol, 2016. **428**(10 Pt A): p. 1986-2000.
269. Fahrenkrog, B. and U. Aebi, *The nuclear pore complex: nucleocytoplasmic transport and beyond.* Nat Rev Mol Cell Biol, 2003. **4**(10): p. 757-66.
270. Dickmanns, A., R.H. Kehlenbach, and B. Fahrenkrog, *Nuclear Pore Complexes and Nucleocytoplasmic Transport: From Structure to Function to Disease.* Int Rev Cell Mol Biol, 2015. **320**: p. 171-233.
271. von Appen, A. and M. Beck, *Structure Determination of the Nuclear Pore Complex with Three-Dimensional Cryo electron Microscopy.* J Mol Biol, 2016. **428**(10 Pt A): p. 2001-10.
272. Rout, M.P. and G. Blobel, *Isolation of the yeast nuclear pore complex.* J Cell Biol, 1993. **123**(4): p. 771-83.
273. Reichelt, R., et al., *Correlation between structure and mass distribution of the nuclear pore complex and of distinct pore complex components.* J Cell Biol, 1990. **110**(4): p. 883-94.
274. Rajoo, S., et al., *Stoichiometry and compositional plasticity of the yeast nuclear pore complex revealed by quantitative fluorescence microscopy.* Proc Natl Acad Sci U S A, 2018. **115**(17): p. E3969-e3977.
275. Kim, S.J., et al., *Integrative structure and functional anatomy of a nuclear pore complex.* Nature, 2018. **555**(7697): p. 475-482.
276. Kalderon, D., et al., *Sequence requirements for nuclear location of simian virus 40 large-T antigen.* Nature, 1984. **311**(5981): p. 33-8.
277. Robbins, J., et al., *Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence.* Cell, 1991. **64**(3): p. 615-23.
278. Dingwall, C. and R.A. Laskey, *Nuclear targeting sequences--a consensus?* Trends Biochem Sci, 1991. **16**(12): p. 478-81.
279. Adam, S.A., et al., *Identification of specific binding proteins for a nuclear location sequence.* Nature, 1989. **337**(6204): p. 276-279.
280. Willis, A.N., et al., *Nuclear localization signal sequence is required for VACM-1/CUL5-dependent regulation of cellular growth.* Cell Tissue Res, 2017. **368**(1): p. 105-114.
281. Xu, Z., et al., *NMMHC-IIA-dependent nuclear location of CXCR4 promotes migration and invasion in renal cell carcinoma.* Oncol Rep, 2016. **36**(5): p. 2681-2688.
282. Bradley, K.J., et al., *Parafibromin is a nuclear protein with a functional monopartite nuclear localization signal.* Oncogene, 2007. **26**(8): p. 1213-21.
283. Kosugi, S., et al., *Six classes of nuclear localization signals specific to different binding grooves of importin alpha.* J Biol Chem, 2009. **284**(1): p. 478-485.

284. Marfori, M., et al., *Molecular basis for specificity of nuclear import and prediction of nuclear localization.* Biochim Biophys Acta, 2011. **1813**(9): p. 1562-77.
285. Lange, A., et al., *Classical nuclear localization signals: definition, function, and interaction with importin alpha.* J Biol Chem, 2007. **282**(8): p. 5101-5.
286. Lange, A., et al., *A PY-NLS nuclear targeting signal is required for nuclear localization and function of the Saccharomyces cerevisiae mRNA-binding protein Hrp1.* J Biol Chem, 2008. **283**(19): p. 12926-34.
287. Mattaj, I.W. and L. Englmeier, *Nucleocytoplasmic transport: the soluble phase.* Annu Rev Biochem, 1998. **67**: p. 265-306.
288. Wang, L., et al., *A PY-nuclear localization signal is required for nuclear accumulation of HCMV UL79 protein.* Med Microbiol Immunol, 2012. **201**(3): p. 381-7.
289. Mallet, P.L. and F. Bachand, *A proline-tyrosine nuclear localization signal (PY-NLS) is required for the nuclear import of fission yeast PAB2, but not of human PABPN1.* Traffic, 2013. **14**(3): p. 282-94.
290. Kurischko, C. and J.R. Broach, *Phosphorylation and nuclear transit modulate the balance between normal function and terminal aggregation of the yeast RNA-binding protein Ssd1.* Mol Biol Cell, 2017. **28**(22): p. 3057-3069.
291. Hall, R.A. and E.W.J. Wallace, *Post-transcriptional control of fungal cell wall synthesis.* Cell Surf, 2022. **8**: p. 100074.
292. Nardozzi, J.D., K. Lott, and G. Cingolani, *Phosphorylation meets nuclear import: a review.* Cell Commun Signal, 2010. **8**: p. 32.
293. Belanger, K.D., et al., *Exportin Crm1 is important for Swi6 nuclear shuttling and MBF transcription activation in Saccharomyces cerevisiae.* BMC Mol Cell Biol, 2022. **23**(1): p. 10.
294. Imamoto, N., et al., *In vivo evidence for involvement of a 58 kDa component of nuclear pore-targeting complex in nuclear protein import.* Embo j, 1995. **14**(15): p. 3617-26.
295. Imamoto, N., et al., *The nuclear pore-targeting complex binds to nuclear pores after association with a karyophile.* FEBS Lett, 1995. **368**(3): p. 415-9.
296. Kutay, U., et al., *Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor.* Cell, 1997. **90**(6): p. 1061-71.
297. Herold, A., et al., *Determination of the functional domain organization of the importin alpha nuclear import factor.* J Cell Biol, 1998. **143**(2): p. 309-18.
298. Matsuura, Y. and M. Stewart, *Structural basis for the assembly of a nuclear export complex.* Nature, 2004. **432**(7019): p. 872-7.
299. Yano, R., et al., *Cloning and characterization of SRP1, a suppressor of temperature-sensitive RNA polymerase I mutations, in Saccharomyces cerevisiae.* Mol Cell Biol, 1992. **12**(12): p. 5640-51.
300. Yano, R., et al., *Yeast Srp1p has homology to armadillo/plakoglobin/beta-catenin and participates in apparently multiple nuclear functions including the maintenance of the nucleolar structure.* Proc Natl Acad Sci U S A, 1994. **91**(15): p. 6880-4.
301. Goldfarb, D.S., et al., *Importin alpha: a multipurpose nuclear-transport receptor.* Trends Cell Biol, 2004. **14**(9): p. 505-14.
302. Stewart, M., *Molecular mechanism of the nuclear protein import cycle.* Nat Rev Mol Cell Biol, 2007. **8**(3): p. 195-208.
303. Ström, A.C. and K. Weis, *Importin-beta-like nuclear transport receptors.* Genome Biol, 2001. **2**(6): p. Reviews3008.
304. Oka, M. and Y. Yoneda, *Importin α: functions as a nuclear transport factor and beyond.* Proc Jpn Acad Ser B Phys Biol Sci, 2018. **94**(7): p. 259-274.

305. Yoshimura, S.H. and T. Hirano, *HEAT repeats - versatile arrays of amphiphilic helices working in crowded environments?* J Cell Sci, 2016. **129**(21): p. 3963-3970.
306. Nakielny, S. and G. Dreyfuss, *Import and export of the nuclear protein import receptor transportin by a mechanism independent of GTP hydrolysis.* Curr Biol, 1998. **8**(2): p. 89-95.
307. Kose, S., et al., *Ran-unassisted nuclear migration of a 97-kD component of nuclear pore-targeting complex.* J Cell Biol, 1997. **139**(4): p. 841-9.
308. Sachdev, S., et al., *Nuclear import of IkappaBalpha is accomplished by a ran-independent transport pathway.* Mol Cell Biol, 2000. **20**(5): p. 1571-82.
309. Melchior, F. and L. Gerace, *Two-way trafficking with Ran.* Trends Cell Biol, 1998. **8**(5): p. 175-9.
310. Nakielny, S. and G. Dreyfuss, *Transport of proteins and RNAs in and out of the nucleus.* Cell, 1999. **99**(7): p. 677-90.
311. Görlich, D., et al., *Identification of different roles for RanGDP and RanGTP in nuclear protein import.* Embo j, 1996. **15**(20): p. 5584-94.
312. Weis, K., C. Dingwall, and A.I. Lamond, *Characterization of the nuclear protein import mechanism using Ran mutants with altered nucleotide binding specificities.* Embo j, 1996. **15**(24): p. 7120-8.
313. Lyman, S.K., et al., *Influence of cargo size on Ran and energy requirements for nuclear protein import.* J Cell Biol, 2002. **159**(1): p. 55-67.
314. Ohtsubo, M., H. Okazaki, and T. Nishimoto, *The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA.* J Cell Biol, 1989. **109**(4 Pt 1): p. 1389-97.
315. Bischoff, F.R. and H. Ponstingl, *Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1.* Nature, 1991. **354**(6348): p. 80-2.
316. Catimel, B., et al., *Biophysical characterization of interactions involving importin-alpha during nuclear import.* J Biol Chem, 2001. **276**(36): p. 34189-98.
317. Bischoff, F.R., et al., *RanGAP1 induces GTPase activity of nuclear Ras-related Ran.* Proc Natl Acad Sci U S A, 1994. **91**(7): p. 2587-91.
318. Bischoff, F.R., et al., *Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1.* Embo j, 1995. **14**(4): p. 705-15.
319. Carazo-Salas, R.E., et al., *Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation.* Nature, 1999. **400**(6740): p. 178-81.
320. Kalab, P., R.T. Pu, and M. Dasso, *The ran GTPase regulates mitotic spindle assembly.* Curr Biol, 1999. **9**(9): p. 481-4.
321. Yoneda, Y., *Nuclear pore-targeting complex and its role on nuclear protein transport.* Arch Histol Cytol, 1996. **59**(2): p. 97-107.
322. Matsuura, Y. and M. Stewart, *Nup50/Npap60 function in nuclear protein import complex disassembly and importin recycling.* Embo j, 2005. **24**(21): p. 3681-9.
323. Lindsay, M.E., et al., *Npap60/Nup50 is a tri-stable switch that stimulates importin-alpha:beta-mediated nuclear protein import.* Cell, 2002. **110**(3): p. 349-60.
324. Ogawa, Y., et al., *Two isoforms of Npap60 (Nup50) differentially regulate nuclear protein import.* Mol Biol Cell, 2010. **21**(4): p. 630-8.
325. Tsujii, A., et al., *Retinoblastoma-binding Protein 4-regulated Classical Nuclear Transport Is Involved in Cellular Senescence.* J Biol Chem, 2015. **290**(49): p. 29375-88.
326. Weis, K., *Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle.* Cell, 2003. **112**(4): p. 441-51.
327. Kobe, B., *Autoinhibition by an internal nuclear localization signal revealed by the crystal structure of mammalian importin alpha.* Nat Struct Biol, 1999. **6**(4): p. 388-97.

328. Smith, A., A. Brownawell, and I.G. Macara, *Nuclear import of Ran is mediated by the transport factor NTF2.* Curr Biol, 1998. **8**(25): p. 1403-6.
329. Ribbeck, K., et al., *NTF2 mediates nuclear import of Ran.* Embo j, 1998. **17**(22): p. 6587-98.
330. Yamada, M., et al., *Nuclear transport factor p10/NTF2 functions as a Ran-GDP dissociation inhibitor (Ran-GDI).* Curr Biol, 1998. **8**(24): p. 1339-42.
331. Velázquez-Cruz, A., et al., *Post-translational Control of RNA-Binding Proteins and Disease-Related Dysregulation.* Front Mol Biosci, 2021. **8**: p. 658852.
332. Lanz, M.C., et al., *In-depth and 3-dimensional exploration of the budding yeast phosphoproteome.* EMBO Rep, 2021. **22**(2): p. e51121.
333. MacGilvray, M.E., et al., *Phosphoproteome Response to Dithiothreitol Reveals Unique Versus Shared Features of Saccharomyces cerevisiae Stress Responses.* J Proteome Res, 2020. **19**(8): p. 3405-3417.
334. Swaney, D.L., et al., *Global analysis of phosphorylation and ubiquitylation cross-talk in protein degradation.* Nat Methods, 2013. **10**(7): p. 676-82.
335. Smolka, M.B., et al., *Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases.* Proc Natl Acad Sci U S A, 2007. **104**(25): p. 10364-9.
336. Das, S., et al., *Intracellular mRNA transport and localized translation.* Nature Reviews Molecular Cell Biology, 2021. **22**(7): p. 483-504.
337. Lécuyer, E., et al., *Global Analysis of mRNA Localization Reveals a Prominent Role in Organizing Cellular Architecture and Function.* Cell, 2007. **131**(1): p. 174-187.
338. Oeffinger, M., et al., *Comprehensive analysis of diverse ribonucleoprotein complexes.* Nat Meth, 2007. **4**(11): p. 951-956.
339. Pizzinga, M., et al., *Translation factor mRNA granules direct protein synthetic capacity to regions of polarized growth.* Journal of Cell Biology, 2019. **218**(5): p. 1564-1581.
340. Chaudhuri, A., S. Das, and B. Das, *Localization elements and zip codes in the intracellular transport and localization of messenger RNAs in Saccharomyces cerevisiae.* WIREs RNA, 2020. **11**(4): p. e1591.
341. Takizawa, P.A., et al., *Actin-dependent localization of an mRNA encoding a cell-fate determinant in yeast.* Nature, 1997. **389**: p. 90-93.
342. Chartrand, P., et al., *Structural elements required for the localization of ASH1 mRNA and of a green fluorescent protein reporter particle in vivo.* Current Biology, 1999. **9**(6): p. 333-336.
343. Irie, K., et al., *The Khd1 protein, which has three KH RNA-binding motifs, is required for proper localization of ASH1 mRNA in yeast.* The EMBO journal, 2002. **21**(5): p. 1158-1167.
344. Müller, M., et al., *A cytoplasmic complex mediates specific mRNA recognition and localization in yeast.* PLoS Biol, 2011. **9**(4): p. e1000611.
345. Niedner, A., et al., *Role of Loc1p in assembly and reorganization of nuclear ASH1 messenger ribonucleoprotein particles in yeast.* Proceedings of the National Academy of Sciences, 2013. **110**(52): p. E5049-E5058.
346. Takizawa, P.A. and R.D. Vale, *The myosin motor, Myo4p, binds Ash1 mRNA via the adapter protein, She3p.* Proceedings of the National Academy of Sciences, 2000. **97**(10): p. 5273-5278.
347. Munchow, S., C. Sauter, and R. Jansen, *Association of the class V myosin Myo4p with a localised messenger RNA in budding yeast depends on She proteins.* J Cell Sci, 1999. **112**(10): p. 1511-1518.
348. Bohl, F., et al., *She2p, a novel RNA-binding protein tethers ASH1 mRNA to the Myo4p myosin motor via She3p.* EMBO J., 2000. **19**(20): p. 5514-5524.
349. Long, R.M., et al., *She2p is a novel RNA-binding protein that recruits the Myo4p-She3p complex to ASH1 mRNA.* EMBO J., 2000. **19**(23): p. 6592-6601.

350. Edelmann, F.T., et al., *Molecular architecture and dynamics of ASH1 mRNA recognition by its mRNA-transport complex.* Nature Structural &Molecular Biology, 2017. **24**: p. 152.
351. Müller, M., et al., *A Cytoplasmic Complex Mediates Specific mRNA Recognition and Localization in Yeast.* PLoS Biol, 2011. **9**(4): p. e1000611.
352. Shen, Z., et al., *Nuclear Shuttling of She2p Couples ASH1 mRNA Localization to its Translational Repression by Recruiting Loc1p and Puf6p.* Mol. Biol. Cell, 2009. **20**(8): p. 2265-2275.
353. Shahbabian, K. and P. Chartrand, *Control of cytoplasmic mRNA localization.* Cellular and Molecular Life Science, 2012. **69**: p. 535-552.
354. Paquin, N., et al., *Local Activation of Yeast ASH1 mRNA Translation through Phosphorylation of Khd1p by the Casein Kinase Yck1p.* Molecular Cell, 2007. **26**(6): p. 795-809.
355. Landers, S.M., et al., *She3p possesses a novel activity required for ASH1 mRNA localization in Saccharomyces cerevisiae.* Eukaryotic cell, 2009. **8**(7): p. 1072-1083.
356. Gonsalvez, G.B., et al., *RNA-protein interactions promote asymmetric sorting of the ASH1 mRNA ribonucleoprotein complex.* RNA, 2003. **9**(11): p. 1383-1399.
357. Smolka, M.B., et al., *Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases.* Proceedings of the National Academy of Sciences, 2007. **104**(25): p. 10364-10369.
358. Kristjansdottir, K., et al., *Phosphoprotein Profiling by PA-GeLC–MS/MS.* Journal of Proteome Research, 2008. **7**(7): p. 2812-2824.
359. Müller, M., et al., *Formation of She2p tetramers is required for mRNA binding, mRNP assembly, and localization.* RNA, 2009. **15**(11): p. 2002-2012.
360. Ziv, I., et al., *A Perturbed Ubiquitin Landscape Distinguishes Between Ubiquitin in Trafficking and in Proteolysis.* Molecular & Cellular Proteomics, 2011. **10**(5).
361. Lanz, M.C., et al., *In-depth and 3-dimensional exploration of the budding yeast phosphoproteome.* EMBO reports, 2021. **22**(2): p. e51121.
362. MacGilvray, M.E., et al., *Phosphoproteome Response to Dithiothreitol Reveals Unique Versus Shared Features of Saccharomyces cerevisiae Stress Responses.* Journal of Proteome Research, 2020. **19**(8): p. 3405-3417.
363. Soufi, B., et al., *Global analysis of the yeast osmotic stress response by quantitative proteomics.* Molecular BioSystems, 2009. **5**(11): p. 1337-1346.
364. Guldener, U., et al., *A new efficient gene disruption cassette for repeated use in budding yeast.* Nucl. Acids. Res., 1996. **24**(13): p. 2519-2524.
365. Tsanov, N., et al., *smiFISH and FISH-quant - a flexible single RNA detection approach with super-resolution capability.* Nucleic Acids Res, 2016. **44**(22): p. e165.
366. Querido, E., A. Sfeir, and P. Chartrand, *Imaging of Telomerase RNA by Single-Molecule Inexpensive FISH Combined with Immunofluorescence.* STAR Protoc, 2020. **1**(2): p. 100104.
367. Aronov, S., et al., *mRNAs encoding polarity and exocytosis factors are cotransported with the cortical endoplasmic reticulum to the incipient bud in Saccharomyces cerevisiae.* Mol Cell Biol, 2007. **27**(9): p. 3441-55.
368. Cosma, M.P., *Daughter-specific repression of Saccharomyces cerevisiae HO: Ash1 is the commander.* EMBO Rep, 2004. **5**(10): p. 953-7.
369. Deng, Y., R.H. Singer, and W. Gu, *Translation of ASH1 mRNA is repressed by Puf6p-Fun12p/eIF5B interaction and released by CK2 phosphorylation.* Genes Dev, 2008. **22**(8): p. 1037-50.
370. Bartko, J.C., et al., *Phosphorylation within the bipartite NLS alters the localization and toxicity of the ER stress response factor DDIT3/CHOP.* Cell Signal, 2020. **74**: p. 109713.
371. Karen Shahbabian*, N.F., Yani Bouaziz, Emmanuelle Querido and Pascal Chartrand,

Phosphorylation controls the oligomeric state of She2 and mRNA localization in yeast. 2022.
372. Meggio, F. and L.A. Pinna, *One-thousand-and-one substrates of protein kinase CK2?* Faseb j, 2003. **17**(3): p. 349-68.
373. Glover, C.V., A.P. Bidwai, and J.C. Reed, *Structure and function of Saccharomyces cerevisiae casein kinase II.* Cell Mol Biol Res, 1994. **40**(5-6): p. 481-8.
374. Harreman, M.T., et al., *Regulation of nuclear import by phosphorylation adjacent to nuclear localization signals.* J Biol Chem, 2004. **279**(20): p. 20613-21.
375. Niedner, A., F.T. Edelmann, and D. Niessing, *Of social molecules: The interactive assembly of ASH1 mRNA-transport complexes in yeast.* RNA biology, 2014. **11**(8): p. 998-1009.
376. Singh, N., G. Blobel, and H. Shi, *Hooking She3p onto She2p for myosin-mediated cytoplasmic mRNA transport.* Proc Natl Acad Sci U S A, 2015. **112**(1): p. 142-7.
377. Fasolo, J., et al., *Diverse protein kinase interactions identified by protein microarrays reveal novel connections between cellular processes.* Genes Dev, 2011. **25**(7): p. 767-78.
378. Mollapour, M., et al., *Screening the yeast deletant mutant collection for hypersensitivity and hyper-resistance to sorbate, a weak organic acid food preservative.* Yeast, 2004. **21**(11): p. 927-46.
379. Sakaki, K., et al., *Response of genes associated with mitochondrial function to mild heat stress in yeast Saccharomyces cerevisiae.* J Biochem, 2003. **134**(3): p. 373-84.
380. Ptacek, J., et al., *Global analysis of protein phosphorylation in yeast.* Nature, 2005. **438**(7068): p. 679-84.
381. Helbig, A.O., et al., *Perturbation of the yeast N-acetyltransferase NatB induces elevation of protein phosphorylation levels.* BMC Genomics, 2010. **11**: p. 685.
382. Venters, B.J., et al., *A comprehensive genomic binding map of gene and chromatin regulatory proteins in Saccharomyces.* Mol Cell, 2011. **41**(4): p. 480-92.
383. Calvert, M.E., et al., *Phosphorylation by casein kinase 2 regulates Nap1 localization and function.* Mol Cell Biol, 2008. **28**(4): p. 1313-25.
384. Zlatanova, J., C. Seebart, and M. Tomschik, *Nap1: taking a closer look at a juggler protein of extraordinary skills.* Faseb j, 2007. **21**(7): p. 1294-310.
385. Radford, A., *Methods in Yeast Genetics — A Laboratory Course Manual: by M Rose, F Winston and P Hieter. pp 198. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 1990. $34 ISBN 0-87969-354-1.* Biochemical Education, 1991. **19**(2): p. 101-102.
386. Schiestl, R.H. and R.D. Gietz, *High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier.* Current Genetics, 1989. **16**(5): p. 339-346.
387. Longtine, M.S., et al., *Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae.* Yeast, 1998. **14**(10): p. 953-61.
388. Fields, S. and O. Song, *A novel genetic system to detect protein-protein interactions.* Nature, 1989. **340**(6230): p. 245-6.
389. Müller, M., et al., *Formation of She2p tetramers is required for mRNA binding, mRNP assembly, and localization.* Rna, 2009. **15**(11): p. 2002-12.
390. Nishi, H., et al., *Evolutionary, physicochemical, and functional mechanisms of protein homooligomerization.* Prog Mol Biol Transl Sci, 2013. **117**: p. 3-24.
391. Jia-Lin Ma, N. and D.F. Stern, *Regulation of the Rad53 protein kinase in signal amplification by oligomer assembly and disassembly.* Cell Cycle, 2008. **7**(6): p. 808-17.
392. Nishi, H., A. Shaytan, and A.R. Panchenko, *Physicochemical mechanisms of protein regulation by phosphorylation.* Front Genet, 2014. **5**: p. 270.
393. Hashimoto, K., et al., *Caught in self-interaction: evolutionary and functional mechanisms of protein homooligomerization.* Phys Biol, 2011. **8**(3): p. 035007.

394. Nishi, H., K. Hashimoto, and A.R. Panchenko, *Phosphorylation in protein-protein binding: effect on stability and function.* Structure, 2011. **19**(12): p. 1807-15.
395. Iakoucheva, L.M., et al., *The importance of intrinsic disorder for protein phosphorylation.* Nucleic Acids Res, 2004. **32**(3): p. 1037-49.
396. Jiménez, J.L., et al., *A systematic comparative and structural analysis of protein phosphorylation sites based on the mtcPTM database.* Genome Biol, 2007. **8**(5): p. R90.
397. Andrew, C.D., et al., *Effect of phosphorylation on alpha-helix stability as a function of position.* Biochemistry, 2002. **41**(6): p. 1897-905.
398. Porollo, A.A., R. Adamczak, and J. Meller, *POLYVIEW: a flexible visualization tool for structural and functional annotations of proteins.* Bioinformatics, 2004. **20**(15): p. 2460-2462.
399. Mallik, S. and S. Kundu, *Topology and Oligomerization of Mono- and Oligomeric Proteins Regulate Their Half-Lives in the Cell.* Structure, 2018. **26**(6): p. 869-878.e3.
400. Correa Marrero, M. and I. Barrio-Hernandez, *Toward Understanding the Biochemical Determinants of Protein Degradation Rates.* ACS Omega, 2021. **6**(8): p. 5091-5100.
401. Genz, C., et al., *Association of the yeast RNA-binding protein She2p with the tubular endoplasmic reticulum depends on membrane curvature.* J Biol Chem, 2013. **288**(45): p. 32384-32393.
402. Nguyen, L.K., W. Kolch, and B.N. Kholodenko, *When ubiquitination meets phosphorylation: a systems biology perspective of EGFR/MAPK signalling.* Cell Communication and Signaling, 2013. **11**(1): p. 52.
403. Kolawa, N., et al., *Perturbations to the ubiquitin conjugate proteome in yeast δubx mutants identify Ubx2 as a regulator of membrane lipid composition.* Mol Cell Proteomics, 2013. **12**(10): p. 2791-803.
404. Rogers, S., R. Wells, and M. Rechsteiner, *Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis.* Science, 1986. **234**(4774): p. 364-8.
405. Skrzypek, M.S., et al., *The Candida Genome Database (CGD): incorporation of Assembly 22, systematic identifiers and visualization of high throughput sequencing data.* Nucleic Acids Research, 2016. **45**(D1): p. D592-D596.
406. Sherrington, S.L., et al., *Adaptation of Candida albicans to environmental pH induces cell wall remodelling and enhances innate immune recognition.* PLoS Pathog, 2017. **13**(5): p. e1006403.
407. Breitkreutz, A., et al., *A global protein kinase and phosphatase interaction network in yeast.* Science, 2010. **328**(5981): p. 1043-6.
408. Brinkworth, R.I., A.L. Munn, and B. Kobe, *Protein kinases associated with the yeast phosphoproteome.* BMC Bioinformatics, 2006. **7**: p. 47.
409. Brinkworth, R.I., A.L. Munn, and B. Kobe, *Protein kinases associated with the yeast phosphoproteome.* BMC Bioinformatics, 2006. **7**(1): p. 47.
410. Gnad, F., et al., *High-accuracy identification and bioinformatic analysis of in vivo protein phosphorylation sites in yeast.* Proteomics, 2009. **9**(20): p. 4642-52.
411. González-Rubio, G., et al., *Differential Role of Threonine and Tyrosine Phosphorylation in the Activation and Activity of the Yeast MAPK Slt2.* Int J Mol Sci, 2021. **22**(3).
412. Blom, N., S. Gammeltoft, and S. Brunak, *Sequence and structure-based prediction of eukaryotic protein phosphorylation sites.* J Mol Biol, 1999. **294**(5): p. 1351-62.
413. Sussman, A., et al., *Discovery of cercosporamide, a known antifungal natural product, as a selective Pkc1 kinase inhibitor through high-throughput screening.* Eukaryot Cell, 2004. **3**(4): p. 932-43.
414. Chen, R.E. and J. Thorner, *Function and regulation in MAPK signaling pathways: lessons learned from the yeast Saccharomyces cerevisiae.* Biochim Biophys Acta, 2007. **1773**(8): p. 1311-40.

415. Wang, R. and M.J. Solomon, *Identification of She3 as an SCF(Grr1) substrate in budding yeast.* PLoS One, 2012. **7**(10): p. e48020.
416. Costanzo, M., et al., *A global genetic interaction network maps a wiring diagram of cellular function.* Science, 2016. **353**(6306).
417. Day, E.K., N.G. Sosale, and M.J. Lazzara, *Cell signaling regulation by protein phosphorylation: a multivariate, heterogeneous, and context-dependent process.* Curr Opin Biotechnol, 2016. **40**: p. 185-192.
418. Sadowski, I., et al., *The PhosphoGRID Saccharomyces cerevisiae protein phosphorylation site database: version 2.0 update.* Database (Oxford), 2013. **2013**: p. bat026.
419. Viéitez, C., et al., *High-throughput functional characterization of protein phosphorylation sites in yeast.* Nat Biotechnol, 2022. **40**(3): p. 382-390.
420. Dau, T., et al., *Sequential Digestion with Trypsin and Elastase in Cross-Linking Mass Spectrometry.* Analytical Chemistry, 2019. **91**(7): p. 4472-4478.
421. Dephoure, N., et al., *Mapping and analysis of phosphorylation sites: a quick guide for cell biologists.* Mol Biol Cell, 2013. **24**(5): p. 535-42.
422. Chen, Z. and P.A. Cole, *Synthetic approaches to protein phosphorylation.* Curr Opin Chem Biol, 2015. **28**: p. 115-22.