The role of the budding yeast RNA-binding proteins Khd1p and Loc1p in mRNA targeting

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Abbreviations

aa	aminoacid
ATP	adenosine triphosphate
Arg or R	arginine
Asn or N	asparagine
ß-Me	beta-mercaptoethanol
bp	basepair
°C	degree centigrade
clonNAT	nourseothricin
C-terminal	carboxy terminal
Da	dalton
DEPC	diethylpyrocarbonate
DIC	Differential interference contrast
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleosid triphosphate
DsRed	*Discosoma sp.* red fluorescent protein
DTT	dithiothreitol
ECL	enhanced chemoluminiscence
E. coli	*Escherichia coli*
EDTA	ethylenediaminotetraacetic acid
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
et al.	*et alii* (from Latin, “and others”)
FMR	Fragile X mental retardation
FT	Flow through
g	gram
x g	relative centrifugal force (rcf)
G418	Geniticin
gDNA	genomic DNA
GFP	green fluorescent protein
GST	glutathione *S*-transferase
h	hour
HA	hemagglutinin
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO	endonuclease homothallic switching endonuclease
i.e.	*id est* (Latin: that is; this means)
IP	immunoprecipitation
IPTG	isopropyl-beta-D-thiogalactoside
Ile or I	isoleucine
k	kilo
kb	kilo basepairs
KH-domain	heterogeneous nuclear (hn)RNP K-homology domain
l	litre
LB	Luria Bertani
LE	localization element
LN2	liquid nitrogen

µ	micro
m	milli
M	molar
MAPK	mitogen-activated protein kinase
mCherry	red fluorophore
Met	Methionine
min	minutes
MS2-CP	MS2 coat protein
MS2L	MS2 loops
NP-40	Nonidet P-40 (Igepal-CA-630)
nt	nucleotide
N-terminal	amino terminal
NTP	nucleoside triphosphate
OD	optical density
ORF	open reading frame
P	p-value
PAGE	polyacrylamide gelelectrophoresis
P-body	processing body
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	potential of hydrogen
PVDF	polyvinylidene fluoride (membrane)
qRT-PCR	quantitative real time PCR
RNA	ribonucleic acid
RBP	RNA-binding protein
RNP	ribonucleoprotein
rpm	revolutions per minute
RT	room temperature
RT-PCR	Reverse transcription PCR
SC	synthetic complete
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
sec	second
SHE	Swi5p-dependent HO expression
SLIC	sequence and ligation-independent cloning
SRP	signal recognition particle
TAE	Tris-acetate-EDTA
TCA	trichloroacetic acid
TGF	transforming growth factor
Tris	trishydroxymethylaminomethane
UTR	untranslated region
V	volt
WCE	whole cell extract
wt	wild type
w/v	weight per volume
YEPD	yeast extract peptone with glucose
YEPG	yeast extract peptone with galactose

1. Introduction

1.1. mRNA localization

mRNA localization is a widely used mechanism by cells to attain polarity and cellular asymmetry. In addition to its controlled distribution, such localized mRNAs are subjected to translational regulation during transport and thereby gene expression can be locally regulated at post-transcriptional level. Localization of mRNAs, which has first been described in oocytes of fruit flies, claw frogs and marine invertebrates (Lecuyer et al, 2007), is required for several biological processes including embryonic axis setup, tissue differentiation, and neuronal function (St Johnston, 2005; St Johnston & Ahringer, 2010) .

Localization of mRNAs has several advantages over localization of proteins. The major one is that a single mRNA can theoretically be translated multiple times to generate many copies of a protein at specific location in the cell, making this process more economical regarding intracellular transport. Secondly, spatial restriction of gene expression can be achieved and regulated. Finally, local distribution of a message helps cells in preventing unwanted and deleterious expression of the protein in other regions (Martin & Ephrussi, 2009).

mRNA localization has been studied in various organisms such as *Drosophila melanogaster, Xenopus laevis, Saccharomyces cerevisiae* and in mammalian cells (Figure 1). In Drosophila, *oskar* and *nanos* mRNAs that localize to the posterior pole of the oocyte and their encoded proteins, Oskar and Nanos respectively are essential for posterior development of the embryo (Wilhelm & Smibert, 2005). *bicoid* is an mRNA that localizes to the anterior pole where it is translated and is responsible for anterior development of the embryo (Johnstone & Lasko, 2001). *Vg1* mRNA, a member of the TGF-β (transforming growth factor β) family, is involved in mesoderm induction and localizes to the vegetal cortex of *Xenopus* oocytes (Birsoy et al, 2006). In mammalian cells, *β-actin* mRNA is targeted to the leading edge of fibroblasts, where the

corresponding protein is essential for local actin polymerization and cell migration (Condeelis & Singer, 2005).

Asymmetric mRNA distribution is mainly achieved by the active transport using motor proteins (Gagnon & Mowry, 2011) that move along microtubules or actin filaments (Bullock, 2007). *Saccharomyces cerevisiae*, although a simple eukaryote, is an excellent model system to understand the basic mechanisms of mRNA localization. In this organism, *ASH1* mRNA localization has been studied in much detail (see below). *ASH1* mRNA is localized to the bud tip and the corresponding Ash1p is involved in mating-type switching (Beach & Bloom, 2001).

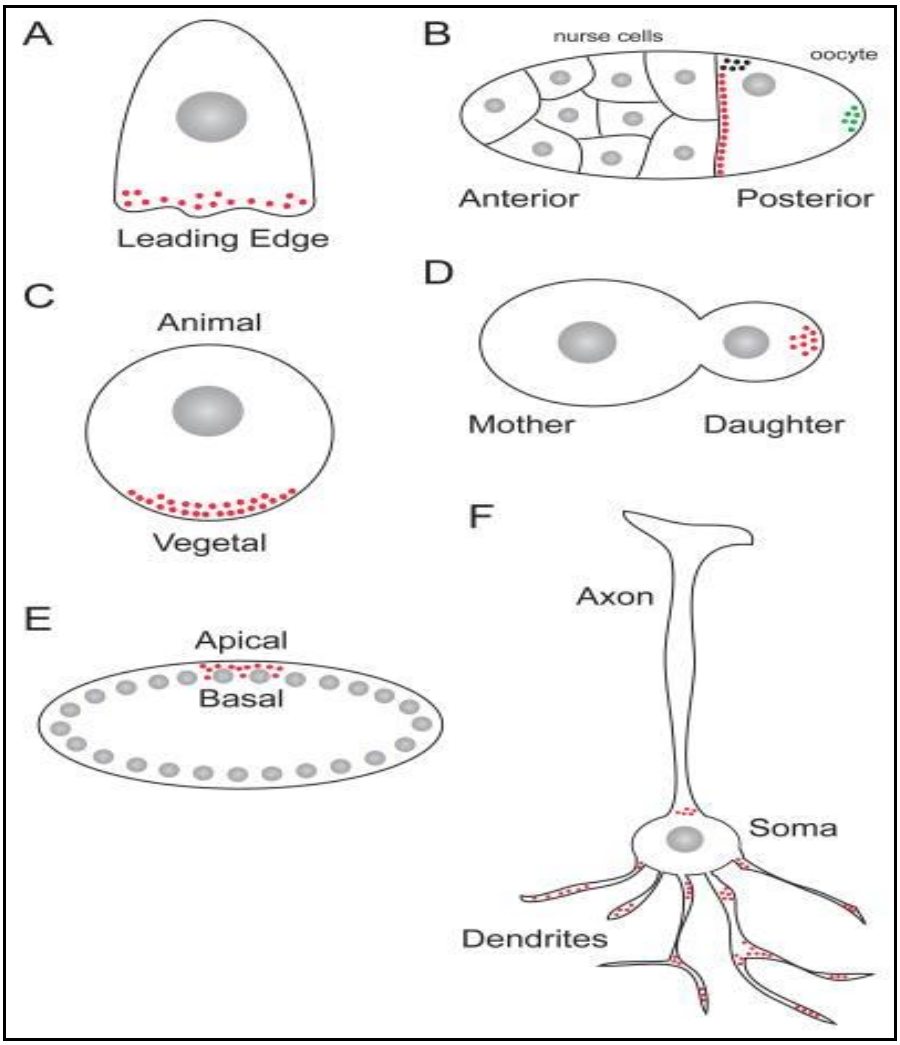


Figure 1: Examples of mRNA localization: (A) *β-actin* mRNA (red stipples) is localized to the leading edge of chick embryo fibroblasts by a myosin V motor. (B) Localization of RNAs in the *Drosophila* oocyte. *Oskar* mRNA (green stipples) is transported to the posterior pole of oocyte by kinesin-1. *bicoid* mRNA (red stipples) is transported to the anterior of the oocyte by dynein. Dynein also localizes *gurken* mRNA (black stipples) to the dorso-anterior corner of the

oocyte. (C) *Vg1* mRNA (red stipples) is transported to the vegetal pole of the *Xenopus* oocyte by kinesin-1 and kinesin-2. (D) Localization of *ASH1* mRNA (red stipples) to the daughter cell in budding yeast is mediated by a myosin motor. (E) Localization of the pair-rule mRNAs (red stipples) to the apical side of nuclei in the *Drosophila* syncytial blastoderm embryo mediated by dynein. (F) mRNAs localized to growth cones and dendrites of neurons mediated by kinesin. For all panels, nuclei are shown in gray. Figure and text adapted from Gagnon and Mowry, 2011.

Localized mRNAs contain specific signals that they require for reaching their destination. These signals are called as "localization elements" (LEs) or "zipcodes" (Jambhekar et al, 2005). In most cases, LEs are present in the 3' untranslated region (UTR) of the mRNA but can also be found in the coding region (Jansen et al, 1996; Jambhekar et al, 2005). They can differ greatly in size, ranging from 11 nucleotides to several hundred nucleotides, which indicates that there is little or no consensus sequence. Examples for short LEs include the RNA trafficking signal in myelin basic protein mRNA (Munro et al, 1999) or a repetitive six nucleotide signal in *Xenopus laevis* Vg1 mRNA (Gautreau et al, 1997). Both appear to function on the nucleotide sequence level. It is known that most other LEs can form secondary or tertiary structures, mainly stem-loop structures that are critical for localization (Jambhekar et al, 2005; Martin & Ephrussi, 2009). The proteins that recognize and bind to these structural elements usually contain well known RNA-binding domain such as RNA recognition motif (RRM) (Maris et al, 2005) or hnRNP K-homology (KH) domain (Valverde et al, 2008). Zipcodes can be simultaneously bound by two or more RNA-binding proteins, thereby generating a large ribonucleoprotein (RNP) complexes which are then transported to the target site (Arn et al, 2003).

1.2. mRNA localization in *Saccharomyces cerevisiae*

As mentioned above, *S. cerevisiae* serves as one of the best model systems for studying mRNA localization. Yeast cells multiply by a process called budding, where bud growth is initiated at specific site, the bud enlarges and finally pinches off from mother cell to form a new daughter cell. This asymmetric cell division gives rise to two cells with different fates which is achieved by localization of mRNAs and proteins (Herskowitz, 1989; Nasmyth, 1993). Most of the mechanistic aspects of mRNA localization were derived by looking at a single mRNA, *ASH1* which is transported to

the tip of daughter cell during mitosis (Beach & Bloom, 2001). Besides, research has been focussed on mRNA localization to cell organelles such as mitochondria in yeast, which encouraged for looking similar phenomena in mammalian cells (Marc et al, 2002).

1.2.1. *ASH1* mRNA localization

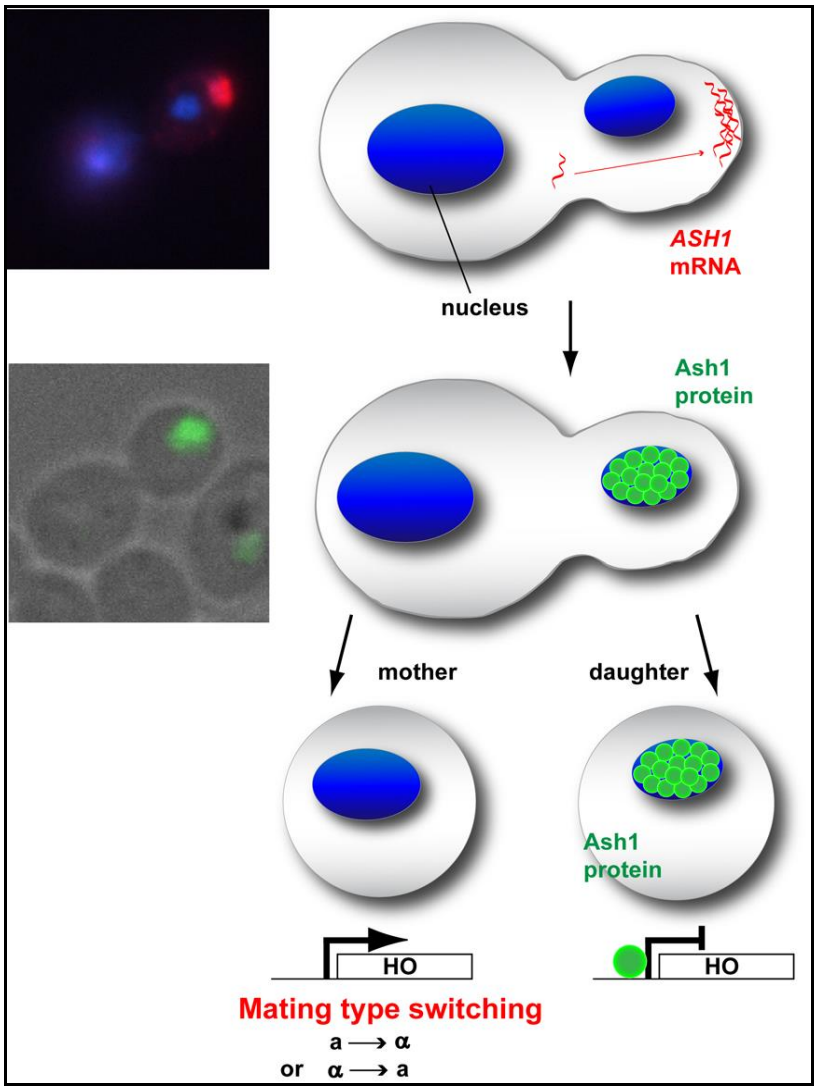


Figure 2: *ASH1* mRNA localization and mating type switching in *S. cerevisiae*: *ASH1* mRNA is localized to the tip of daughter cells during late anaphase which results in expression of Ash1p exclusively in daughter cells and thereby inactivating the mating-type switching by supressing the *HO endonuclease* expression. Image adapted from Maria Schmid's doctoral thesis, AG Jansen, 2008 (Schmid, 2008).

Haploid yeast cells can exist in two mating types, "a" or "α". In homothallic strains, the mating type is stable over the cell cycle but switches after each cell division ("mating-type switching"). Switching is a genomic recombination event at the *MAT* locus and initiated by introducing a double strand break by an endonuclease encoded by *HO* gene, which is then repaired by using a silenced version of the *MAT* locus (*HMR or HML*) where the genetic information of the opposite mating type is stored. Only mother cells can switch mating type whereas daughter cells cannot (Strathern & Herskowitz, 1979; Herskowitz, 1989; Nasmyth, 1993). This is because in daughter cells, the expression of *HO* endonuclease is specifically repressed by Ash1p, a transcriptional repressor (Figure 2) (Bobola et al, 1996; Jansen et al, 1996; Sil & Herskowitz, 1996).

1.2.1.1. Cis-acting factors

Localization of *ASH1* mRNA is a multi-step process and involves many factors in the form of cis-acting elements and trans-acting factors (RNA-binding proteins) (Martin & Ephrussi, 2009). *ASH1* mRNA has four cis-acting localization signals: E1 (115bp), E2A (118bp), E2B (250bp) and E3 (118bp) (Figure 3). Three of the LEs, E1, E2A and E2B are present within the coding region whereas the fourth one, E3 extends into 3'UTR. The location of these LEs is exceptional since they are generally present in the 3'UTR of the mRNA. Although each LEs has the potential to target a reporter mRNA or *ASH1* mRNA to the bud, presence of all four LEs is required for efficient targeting of *ASH1* mRNA which indicates that these LEs function in a cooperative manner (Chartrand et al, 1999; Gonzalez et al, 1999).

There is no sequence homology between the four LEs but structure prediction and mutational studies suggest that they share common structural features. They serve as binding sites for several RNA-binding proteins (RBPs) by forming secondary RNA structures or stem-loop structures (Chartrand et al, 1999; Gonzalez et al, 1999; Chartrand et al, 2002). The function of these LEs is severely impaired if the double strand that forms the more distal region of the stem is disrupted. A conserved RNA motif that consists of a CGA triplet in a loop was identified which is essential for bud localization of *ASH1* and other two mRNAs namely *IST2* and *EAR1* (Jambhekar et al, 2005; Olivier et al, 2005).

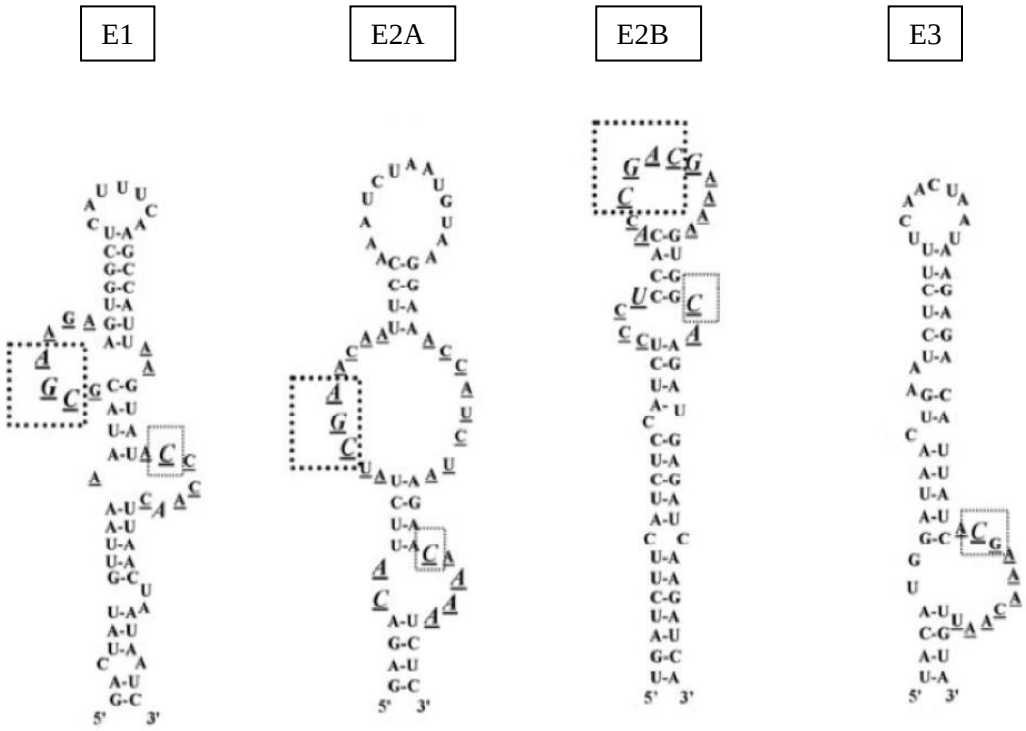


Figure 3: Zipcodes or Localization elements (LEs) in *ASH1* mRNA: E1, E2A, E2B and E3. Secondary structures of four LEs in *ASH1* mRNA are shown. Conserved nucleotides are indicated in capital letters and italicized. Boxes with large dotted line show the conserved CGA sequence whereas the conserved cytosine in E1, E2A, E2B and E3 is boxed with small dotted lines. Figure modified from Olivier et al, 2005.

1.2.1.2. Trans-acting factors

A genetic screen was carried out by Jansen and co-workers in order to select mutants that are defective in asymmetric expression of *HO* endonuclease. They found out five genes namely, *SHE1-5* (Jansen et al, 1996). Among them three genes, *SHE1* (also called *MYO4*), *SHE2* and *SHE3* together form the core complex called "locasome", which is essential for *ASH1* mRNA transport. Localization of *ASH1 in vivo* was first observed by Singer and colleagues, using an MS2 RNA tagging system (Bertrand et al, 1998). This approach, is based on specific interaction between the coat protein of the bacteriophage MS2 (MS2-CP) and RNA stem-loop structures (MS2L) in the viral RNA (Peabody & Lim, 1996). Green fluorescent protein (GFP) fused MS2-CP is co-expressed with the RNA of interest bearing MS2L in the 3'UTR (Urbinati & Long, 2011). The resulting

mRNPs can be visualized by fluorescence microscopy. From the *in vivo* imaging of moving *ASH1-MS2L* particles, it was revealed that localization of a specific mRNA relies on active transport (Bertrand et al, 1998). It requires a functional actin cytoskeleton (Long et al, 1997; Takizawa et al, 1997) together with a type V myosin motor protein (Jansen et al, 1996; Long et al, 1997; Takizawa et al, 1997) and RNA-binding proteins (She2p, She3p, Puf6p, Khd1p and Loc1p) for fulfilling different roles during the *ASH1* mRNA localization, ranging from mRNP assembly to translational inhibition (Long et al, 1997; Takizawa et al, 1997; Bohl et al, 2000; Long et al, 2000; Irie et al, 2002; Gu et al, 2004).

1.2.1.2.1. She2p

She2p is the most extensively studied among the RNA-binding proteins involved in *ASH1* mRNA localization. The *SHE2* gene encodes a 28 KDa protein product. Although it is more abundant in cytoplasm, it shuttles between nucleus and cytoplasm. It bears a non-canonical nuclear localization sequence at its carboxy terminus that is essential for nuclear import whereas nuclear export only happens after binding to RNA (Kruse et al, 2002; Shen et al, 2010). She2p binds specifically to all four LEs within *ASH1* mRNA, albeit with weak affinity (Bohl et al, 2000; Niessing et al, 2004). Crystal structure of the She2p showed that its polypeptide folds into a single globular domain comprising of five anti-parallel α-helices. It is a dimeric protein, containing two RNA-binding domains of an unusual basic helical hairpin type. These two dimers come together in solution in a head-to-head manner to form a tetrameric complex, thereby generating two RNA binding sites (Niessing et al, 2004; Muller et al, 2009). A short α-helix that protrudes at right angle from remaining helices plays a key role in binding to LEs and for interaction with She3p (Muller et al, 2011). Besides *ASH1*, She2p associates with more than 50 additional mRNAs (Shepard et al, 2003; Oeffinger et al, 2007). Furthermore, it has been recently shown that She2p acts like a peripheral membrane protein, which might explain the observed co-transport of mRNAs coding for membrane proteins and endoplasmic reticulum (ER) (Schmid et al, 2006; Fundakowski et al, 2012; Genz et al, 2013).

Unlike *ASH1*, which is expressed during mitosis, several other bud-localized mRNAs are expressed at various stages of cell cycle or throughout the entire cell cycle (Shepard

et al, 2003). The majority of them encode membrane or membrane associated proteins and others include secretory proteins, kinases, GTPases, extracellular proteins (Shepard et al, 2003; Aronov et al, 2007). Similar to *ASH1*, localization of most of them to the bud depends on She2p.

1.2.1.2.2. She3p

She3p is known as adaptor protein as it interacts with She2p via its carboxy terminal while its amino terminal half (amino acids 1-234) binds to Myo4p (Bohl et al, 2000). She3p can form a stable trimeric complex with She2p and *ASH1* mRNA *in vitro* by directly binding to LE of *ASH1*. RNA binding occurs within a 20 amino acid region in the carboxy terminal domain and mutations in this region disturb *ASH1* mRNA localization (Landers et al, 2009; Muller et al, 2011).

1.2.1.2.3. Myo4p

Myo4p is an actin based motor required for mRNA localization in yeast. It is also known as She1p and is one of the two class V myosin proteins present in *S. cerevisiae* (Bobola et al, 1996; Jansen et al, 1996). It has been shown that in live yeast cells, Myo4p is involved in active transport of *ASH1* cargo along the actin cables (Bertrand et al, 1998). Unlike all other class V myosins, Myo4p exists in a monomeric form at its physiological concentration but long distance movement of Myo4p requires protein or RNP-complex mediated oligomerization (Heuck et al, 2007; Chung & Takizawa, 2010; Krementsova et al, 2011). By using single-particle experiments, it has been demonstrated that a minimal complex consisting of Myo4p, She3p, She2p and RNA constitutes a motile mRNP (Chung & Takizawa, 2010).

1.2.1.2.4. Puf6p

Puf6p is one of the six budding yeast RNA-binding proteins that belong to the pumilio-like protein family (Gerber et al, 2004; Gu et al, 2004). Like She2p, it shuttles between nucleus and cytoplasm but in contrast to She2p it is found more in the nucle(ol)us where it participates in 60s ribosomal subunit biogenesis (Li et al, 2009). It has been shown that Puf6p acts as a translational repressor in *ASH1* mRNA localization. Puf6p binds to a region in the E3 localization element of *ASH1* mRNA containing a PUF consensus tetranucleotide UUGU and blocks the assembly of 80s ribosome on the *ASH1* ATG,

thereby keeping the transcript in translationally repressed state (Gu et al, 2004; Deng et al, 2008). Once the *ASH1* mRNA reaches its destination, Puf6p is phosphorylated by casein kinase 2 and gets released from mRNA (Deng et al, 2008).

1.2.1.2.5. Khd1p

Khd1p belongs to the family of RNA-binding proteins that contain heterologous nuclear ribonucleoprotein K (hnRNP K)-like homology (KH) domains. Khd1p is also known as Hek2p (heterogeneous nuclear RNP-like protein) (Hasegawa et al, 2008). The protein has three KH-domains (Irie et al, 2002; Valverde et al, 2008). Besides Puf6p, Khd1p also acts as translational repressor during the *ASH1* mRNA transport (Paquin et al, 2007). It has been reported that Khd1p interacts with the E1 localization element of *ASH1* mRNA and with the C-terminal domain of eIF4G1 for the translation regulation of this transcript. Similar to Puf6p, Khd1p gets phosphorylated at serine and threonine residues in the carboxyterminus by yeast casein kinase 1 (Yck1p) leading to its dissociation from *ASH1* transcript (Paquin et al, 2007). Combining co-immunoprecipitation and bioinformatics tools, Hasegawa and co-workers demonstrated that Khd1p binds to a potential recognition motif containing CNN repeats that is present at 5' of the E1 element in *ASH1* mRNA (Hasegawa et al, 2008). Under nitrogen starvation conditions, diploid yeast cells undergo special growth program that results in the formation of filaments (Gimeno et al, 1992). *ASH1* is known to regulate the filamentous growth by activating *FLO11* expression, which encodes a cell wall protein required for the filamentous growth form (Lo & Dranginis, 1998; Pan & Heitman, 2000). By repressing *ASH1*, Khd1p is involved in regulation of filamentous growth by regulating *FLO11* transcript. While the repression of *FLO11* is *ASH1* dependent, Khd1p represses the Flo11p expression independent of *ASH1* (Wolf et al, 2010).

ASH1 was the only known target of Khd1p until two studies showed that Khd1p could bind to several hundred mRNAs (Hasegawa et al, 2008; Hogan et al, 2008). Among the targets of Khd1p were mRNAs encoding proteins localized to cell periphery including cell wall, plasma membrane were significantly enriched. Furthermore, Khd1p associates with mRNAs that code for signalling proteins, transcription factors and RNA-binding proteins (Hogan et al, 2008). In contrast to its function in *ASH1* mRNA localization, the role of this protein is less understood for its other targets but might involve regulation of

mRNA stability. Khd1p positively regulates the expression of *MTL1* mRNA (encoding a membrane sensor in the cell wall integrity pathway) by stabilizing it. Khd1p stabilizes the *MTL1* transcript by binding to cis-acting elements within the mRNA, thereby preventing it from degradation. Under glucose deprivation conditions, Khd1p localizes to processing-bodies (P-bodies) which are the potential site for mRNA degradation (Mitchell et al, 2013). This suggests that Khd1p is involved in preventing the degradation of *MTL1* at P-bodies (Mauchi et al, 2010). Though Khd1p associates with numerous mRNAs that encode cell wall and membrane proteins, deletion of *KHD1* gene has no drastic phenotype on cell wall synthesis or function. However, *KHD1* deletion results in severe cell lysis along with the deletion of *CCR4* gene, which codes for a cytoplasmic deadenylase suggesting a possible role for Khd1p in cell wall integrity pathway (Ito et al, 2011).

Furthermore, Khd1p is also known to be involved in regulation of telomere length and telomere position effect. Deletion of *KHD1* gene inhibits the Telomeric Rapid Deletion (TRD) pathway, a mechanism for controlling the size of telomeres in *S. cerevisiae* (Li & Lustig, 1996). By using chromatin immunoprecipitation assay and PCR, Khd1p was detected in complex with sub-telomeric regions suggesting a direct role in the telomere maintenance (Denisenko & Bomsztyk, 2002). In addition, Khd1p is found to be present at the *HMR (Hidden MAT Right)* locus, which suggests a direct involvement of Khd1p in the gene-silencing process. Khd1p also regulates the recruitment of Sir2/3 proteins to the *HMR* locus and telomeres (Denisenko & Bomsztyk, 2008).

1.2.1.2.5.1. K-homology (KH)-Domain

Since RNA-binding proteins are involved in key roles in post-transcriptional regulation of gene expression, characterization of these proteins led to the identification of several RNA-binding domains. The KH-domain is first identified in heterogeneous nuclear ribonucleoprotein K (hnRNP K) and hence named hnRNP K-homology (KH) domain (Burd & Dreyfuss, 1994; Siomi et al, 1994). KH-domains are ubiquitous in eukaryotes, eubacteria and archea (Siomi et al, 1993a; Grishin, 2001). From various studies, it has been shown that KH-domains are involved in the recognition of single-stranded DNA (ssDNA) and RNA, and that the corresponding ssDNA- or RNA-binding proteins are essential for various biological functions. A typcial KH-domain consists of ~70 amino

acids and contains a conserved and functionally important signature sequence comprising the GXXG loop [(I/L/V)IGXXGXX(I/L/V)] close to the center of the domain. All KH-domains are structurally organized to form a three-stranded β-sheet packed against three α-helices (Figure 4).

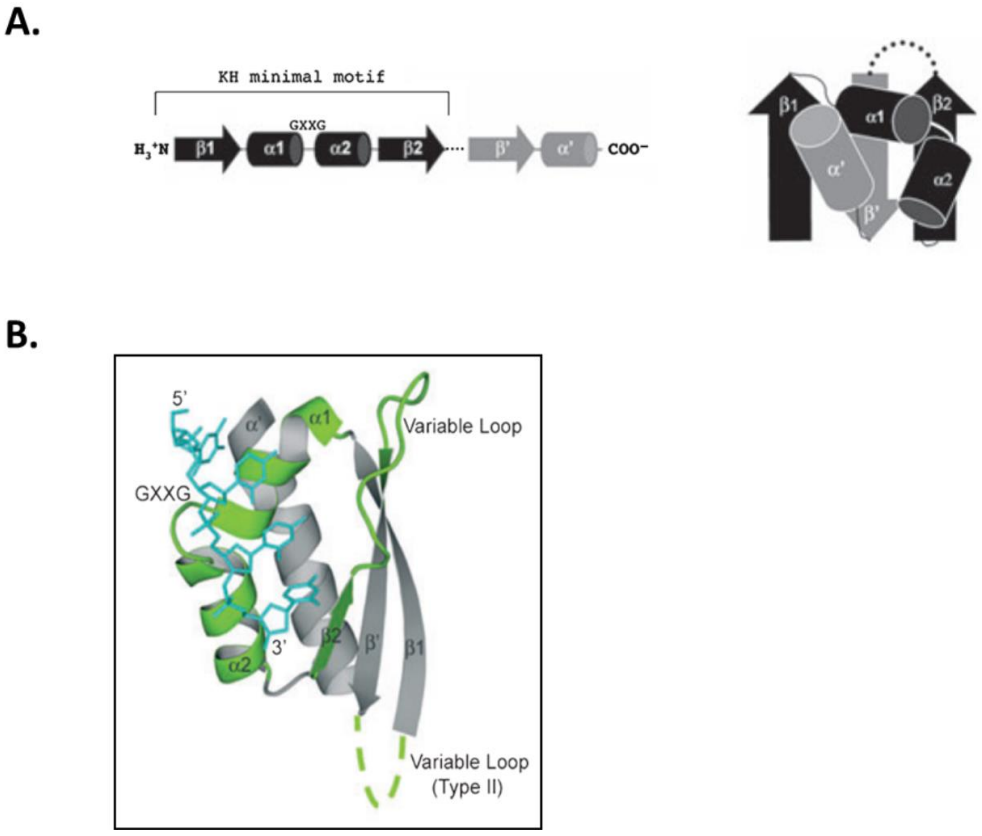


Figure 4: KH-domains: (A) Stylized representation of type I KH-domain. The dotted line connecting the β2 –strand and β‘ strand represents the variable loop. The line connecting the α1 and α2 helix represents the GXXG loop. (B) Type I KH-domain; the binding cleft comprises the secondary structural elements α1-helix, GXXG loop, α2-helix, β2 –strand, variable loop (colored green) and recognizes four nucleotides (cyan sticks). The green dotted line represents the location of the variable loop in type II KH-domains. Image modified from Valverde et al, 2008.

Based on topology, KH-domains can be grouped into two subfamilies: type I domains (present in eukaryotic proteins) have β1α1α2β2β3α3 topology, whereas type II domains (present in prokaryotic proteins) have α1β1β2α2α3β3 fold (Grishin, 2001; Lunde et al,

2007). The nucleic acid-binding cleft in type I domains is composed of the structural elements helix α1, GXXG loop, helix α2, strand β2 and the variable loop between β2 and β3. RNA-binding properties of KH-domains were initially studied in Fragile X Mental Retardation 1 (FMR1), the gene product of human fragile X syndrome, and hnRNP K (Ashley et al, 1993; Siomi et al, 1993b; Lunde et al, 2007). KH-domains are often found in multiple copies; two in fragile X mental retardation protein (FMRP), three in hnRNP K, and 14 in chicken vigilin (Valverde et al, 2008)(Valverde et al, 2008) . On the other hand, there are also proteins with single KH-domain (E.g. Mer1p, a RNA-binding protein required for meiosis specific mRNA-splicing) (Valverde et al, 2008). The significance of KH-domains arises from its conserved hydrophobic residue (Isoleucine or Leucine). A single point mutation in second KH-domain of FMR1 (I304N) alters its structure and thereby impairs RNA binding activity and results in mental retardation (Siomi et al, 1993b; Chen et al, 1997; Lunde et al, 2007). As stated above (section 1.2.1.2.5), Khd1p has three KH-domains, which are responsible for RNA-binding.

1.2.1.2.6. Loc1p

Loc1p is an exclusively nucleolar protein that binds to the E1 and E3 zipcodes of *ASH1* transcript and is required for its localization (Long et al, 2001). It also participates in biogenesis and nuclear export of 60S ribosomal subunits, similar to Puf6p (Harnpicharnchai et al, 2001; Urbinati et al, 2006). Deletion of *LOC1* gene shows complete delocalization of the *ASH1* mRNA. Studies from Komili and colleagues showed that Loc1p is involved in translational regulation of *ASH1* mRNA as Ash1p expression is increased in *loc1Δ* cells. However, Loc1p function at its molecular level is not well understood. It has been speculated that Loc1p might have an indirect effect on *ASH1* mRNA translation by assembling special set of ribosomes ("specialized ribosomes") that will translate *ASH1* mRNA (Komili et al, 2007). Apart from *ASH1*, Loc1p also binds to other endogeneous RNAs, preferably to double-stranded RNA motifs (Long et al, 2001).

An overview of the *ASH1* mRNA localization process has been depicted below (See Figure 5)

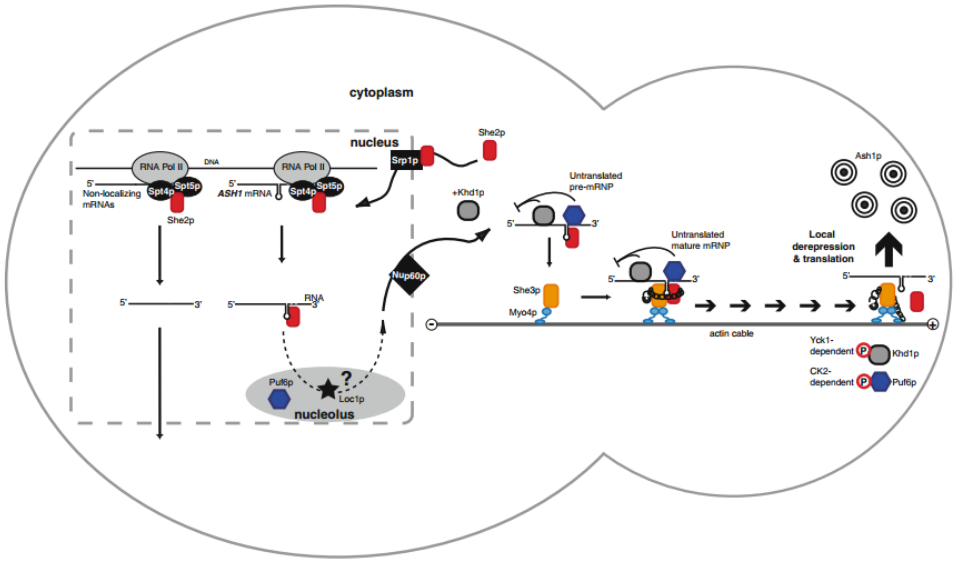


Figure 5: Model of *ASH1* mRNA localization: She2p associates with nascent mRNAs via RNA polymerase II machinery after its nuclear import via Srp1p. Prior to nuclear export, the *ASH1* mRNA-She2p complex enters the nucleolus where the loading of Loc1p and Puf6p on *ASH1* mRNA takes place. Khd1p, one of the translational repressors joins the *ASH1* mRNA-She2p co-complex in the cytoplasm, which is followed by formation of stable complex by binding to Myo4p-associated She3p. With the help of actin cables, the mature ribonucleoprotein (mRNP) reaches the bud tip. The translational repressors Khd1p and Puf6p are released from *ASH1* mRNA by phosphorylation. Figure and text from Heym and Niessing, 2012.

1.3. mRNA localization and endoplasmic reticulum (ER)

1.3.1. ER – Structure and functions

One of the distinct features of eukaryotic cell is the internal compartmentalization. The cytoplasm has many membrane surrounded organelles which play an important role in cell shape and organization. The ER is one such organelle required for the smooth and efficient functioning of the cell. ER is known to be close associated with all other

organelles in cells like plasma membrane, Golgi, vacuoles, peroxisomes, mitochondria, late endosomes and lysosomes (Voeltz et al, 2002). On a morphological and functional basis, ER can be split into two types: rER (rough ER) and sER (smooth ER). The difference between the rER and sER is the presence of ribosomes on rER. rER is involved in biogenesis of membrane and secretory proteins, whereas sER is responsible for synthesis of cholesterol, phospholipids and steroids. Furthermore, it can serve as a reservoir for Ca^{2+} (Baumann & Walz, 2001; Halic & Beckmann, 2005; Rapoport, 2007). In general, ER is made up of network of interconnected membranes with a common intraluminal space and is composed of sheets (or cisternae) and arrays of tubules. By its intracellular distribution, ER can be further classified into two types: perinuclear ER and peripheral ER. Perinuclear ER is the one which surrounds nucleus and consists of membrane sheets whereas peripheral ER forms a network of inter-connected tubules spreading in the entire cytoplasm. Peripheral ER in *S. cerevisiae* is called cortical ER since it is present beneath the cell cortex (Prinz et al, 2000; Voeltz et al, 2002).

1.3.2. mRNA targeting mechanisms to ER

1.3.2.1. SRP-dependent targeting or co-translational targeting

The process of mRNA localization is utilized by almost all types of cells in order to attain cellular asymmetry and spatial control of protein synthesis. Targeting of mRNAs encoding membrane and secretory proteins to the ER remains as one of the best-studied examples (Cui & Palazzo, 2014).

The classical mechanism for mRNA targeting to ER is dependent on the signal recognition particle (SRP) and its receptor (SRP receptor). SRP recognizes the signal sequences of the nascent peptide that emerges from the ribosome. Thus, the SRP pathway works in a co-translational manner i.e. that targeting occurs while the protein is being synthesized on the membrane-bound ribosome, which consequently directs the mRNA together with the nascent chain and ribosome to the ER. In *S. cerevisiae*, SRP is composed of one 7S RNA molecule and six protein subunits. The subunits are encoded by *SRP14*, *SRP21*, *SRP68*, *SRP72*, *SEC65* and *SRP54*, whereas the RNA is encoded by *SCR1* (Hann & Walter, 1991; Brown et al, 1994). SRP54 contains two domains; M-domain and G-domain. SRP binds to signal sequences through M-domain and a GTP-binding site is present in G-domain (Siegel & Walter, 1986; Siegel & Walter, 1988;

Zopf et al, 1990; Keenan et al, 2001). After SRP attachment, translation elongation is paused until the ribosome-mRNA-nascent peptide chain complex reaches the cytoplasmic surface of the ER where it comes in contact with SRP receptor present in the ER membrane. This interaction is GTP-dependent. Upon GTP hydrolysis, SRP is released from the ribosome-nascent chain complex to the cytosol to engage in a new targeting cycle. Translation resumes after SRP release and the elongating peptide is translocated in to ER lumen or the ER membrane via the Sec61p translocon. As the translocation proceeds, the signal sequence is cleaved by the signal peptidase and the polypeptide is eventually released into the ER lumen (Figure 6). Thus, mRNA is targeted to ER along with ribosome and nascent peptide in a SRP dependent manner (Keenan et al, 2001; Rapoport, 2007; Gerst, 2008; Hermesh & Jansen, 2013; Cui & Palazzo, 2014).

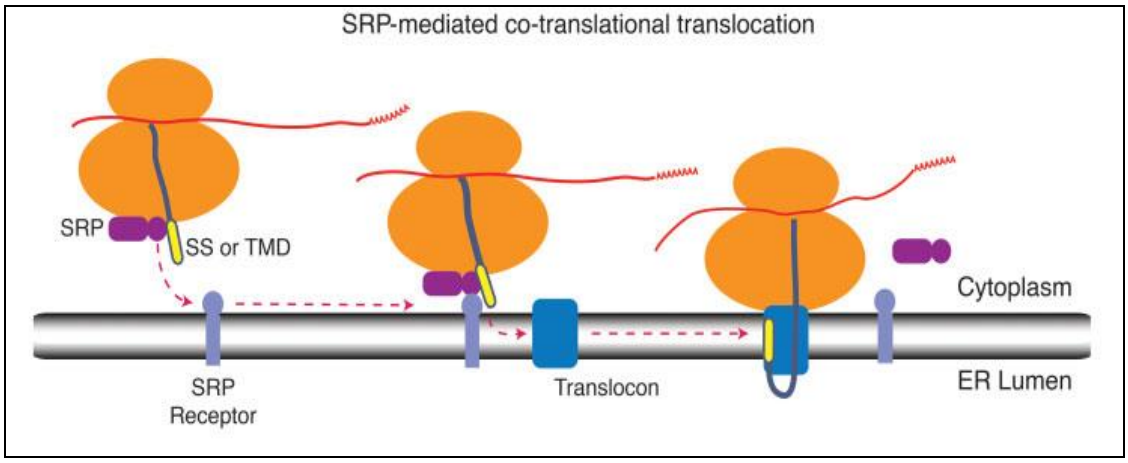


Figure 6: SRP-mediated targeting of mRNA to ER: In this pathway, mRNAs encoding secretory proteins and membrane proteins are translated by cytoplasmic ribosomes. After the N-terminal hydrophobic signal sequence exits the ribosome, the cytosolic signal recognition particle (SRP) binds to it, halts the translation, and then delivers the ribosome-nascent peptide–mRNA complex to the ER by interacting with the SRP receptor (SR). At the ER, the signal sequence is then inserted into the Sec61 translocon, and translation resumes. Image and text adapted from Cui and Palazzo, 2014.

1.3.2.2. SRP independent targeting: Alternate route

Although the SRP pathway is still the most widely accepted pathway for the ER targeting, several reports have pointed to the existence of additional pathways for mRNA targeting to ER. It was first shown in budding yeast that cells were viable even after depleting SRP components (SRP 54 and 7SL) (Hann & Walter, 1991). Later similar studies were carried out in mammalian cells. Using siRNA-mediated knockdown techniques, SRP components were depleted with little effect on membrane protein synthesis and cell viability. *GRP94*, an mRNA coding for an ER luminal protein is localized to ER even in the absence of a start codon or lacking its coding region for a signal sequence. These observations underscored on the existence of pathways that could target mRNA to ER independent of SRP and its components (Ren et al, 2004; Lakkaraju et al, 2007; Pyhtila et al, 2008; Hermesh & Jansen, 2013).

Using various cell fractionation assays, mRNA partitioning between the cytosol and membrane fractions were used for characterizing the mRNAs bound to these two fractions. As one would expect, the cytosol fraction was enriched with mRNAs that codes for cytosolic or nuclear proteins and the ER fraction was enriched with membrane proteins or secretory proteins-encoding mRNAs (Diehn et al, 2000; Diehn et al, 2006; Pyhtila et al, 2008; Reid & Nicchitta, 2012). However, a small group of mRNAs encoding cytosolic or nuclear proteins were present in the ER fraction suggesting an SRP-independent pathway in mRNA targeting to ER. These findings were extended to HeLa cell lines where the knockdown of SRP54 had only moderate effect on mRNA partitioning to ER fraction (Pyhtila et al, 2008). According to the model proposed by Potter and Nicchitta (Potter & Nicchitta, 2000; Nicchitta et al, 2005), all mRNAs, regardless of whether they code membrane or cytosolic proteins, might be initially translated at ER bound ribosomes. Later, the ribosomes that were involved in synthesis of cytosolic proteins would be ejected from ER to complete the translation in cytosol whereas the ribosomes involved in synthesis of membrane proteins remain bound to ER. This process has been named as the elongation-coupled ribosome release (E-CRR) pathway (Figure 7) (Nicchitta et al, 2005; Reid & Nicchitta, 2012)

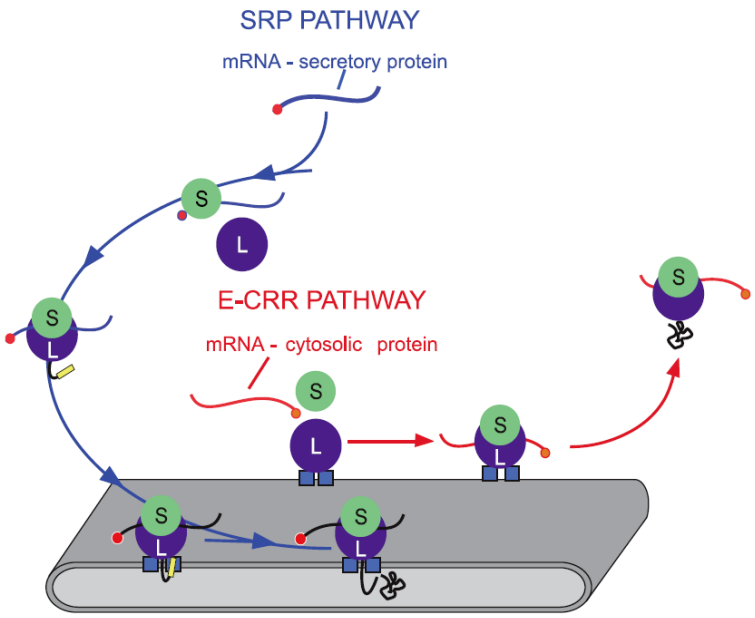


Figure 7: Pathways for mRNA partitioning between ER-bound and free polysomes: The SRP pathway directs mRNAs encoding secretory or membrane proteins to the ER. The elongation-coupled ribosome release (E-CRR) pathway directs mRNAs encoding cytosolic proteins, the translation of which is initiated on the ER, to the cytosol compartment. For both pathways, mRNA partitioning requires translation. Image taken from Nicchitta et al, 2005.

Recent experimental data have come in support of earlier observations. By studying the ribosome loading of ER-bound mRNAs on a global scale in HEK293 cells, Reid and Nicchitta showed that the translational efficiency of ER-bound mRNAs encoding both cytosolic and ER proteins is similar (Reid & Nicchitta, 2012). In addition, they showed ribosome loading on ER-bound transcripts is higher than their cytosolic counterparts. Another study confirmed these results and in addition visualized ER-bound mRNAs in the presence and absence of translational inhibitors (Cui & Palazzo, 2012). Lately, a study by the Gerst group showed that the localization of yeast *SUC2* mRNA (coding for a soluble secreted enzyme) and *USE1* mRNA (encoding a tail-anchored membrane protein) was not affected when the translation initiation codon or signal sequence was removed. In addition, cycloheximide treatment did not alter the localization of these two transcripts to ER (Kraut-Cohen et al, 2013). Altogether, these experiments suggest that mRNA can be targeted to, and remain associated with, ER independent of translation. Such ribosome-independent mRNA localization to ER might depend on certain features within the mRNA (cis-acting elements) that are recognized by a potential mRNA

receptor (trans-acting factor) on the surface of ER (Figure 8). One such mRNA receptor, the mammalian ER membrane protein p180, has been recently found (Cui et al, 2012). An 87 aminoacids lysine-rich region in the protein is responsible for the direct interaction with RNA *in vitro*. Over-expression of this protein resulted in increased mRNA association with ER, whereas its depletion decreased the ER-association of *ALPP* and *CALR* mRNAs, both in translation-dependent and independent manner (Cui et al, 2012). In yeast, overexpression of p180 showed increase mRNA-ER association which suggests that it could guide the mRNA to ER (Becker et al, 1999).

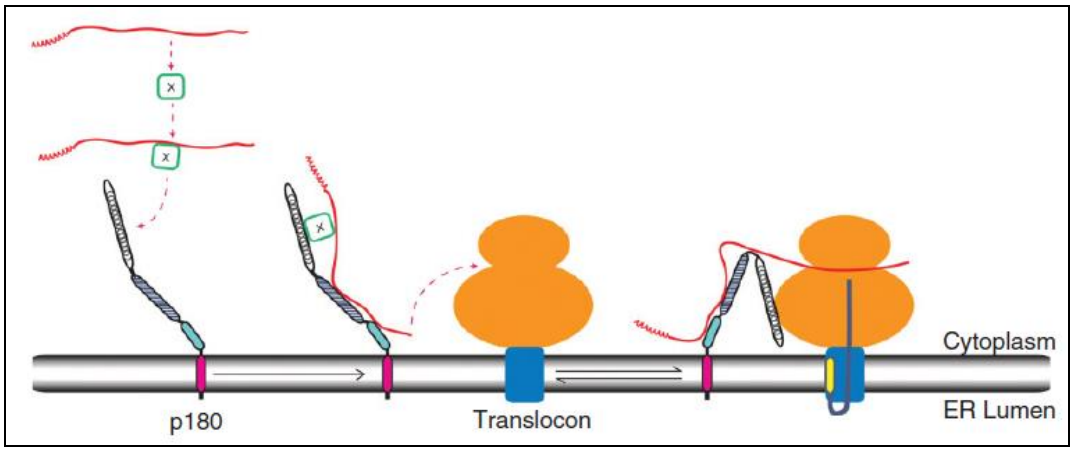


Figure 8: Translation-independent mRNA targeting to ER: p180, an mammalian ER resident RNA binding protein, directs the mRNAs to the ER via cis-signals present in the mRNA. "X" represents unidentified cytosolic RNA-binding protein which could distinguish the targeting mRNAs from non-targeting mRNAs. Several reports suggest that p180 could interact directly with ribosomes or indirectly with translocons, and thereby facilitating the synthesis of secretory and membrane proteins. Image and text adapted from Cui and Palazzo, 2014.

1.4. Aim of this work

The aim of this Ph.D. thesis is to investigate the function of two trans-acting factors (RNA-binding proteins) that are involved in *ASH1* mRNA localization. (1) Khd1p and (2) Loc1p.

1. **Khd1p:** Although the predominant mechanism of targeting mRNAs to ER is dependent on SRP, there are data indicating that mRNAs can be localized to ER in SRP-independent manner. Reports suggest that, in yeast, RNA-binding proteins could adopt the role of partitioning mRNAs to ER by associating with ER. There are four RNA-binding proteins to be tested as potential candidates for mediating ER association. One of the candidates that I have picked for my thesis was Khd1p. As already mentioned (Section 1.2.1.2.5), Khd1p binds to several hundreds of transcripts, most of them coding cell wall proteins, membrane proteins and secretory proteins. Since synthesis of these proteins occurs at ER, I speculated that Khd1p might associate with ER. In addition, I wanted to address whether Khd1p has an active role in targeting mRNAs to ER.
2. **Loc1p:** In collaboration with Prof. Dierk Niessing's group, Institute of Structural biology, Helmholtz Zentrum München, we aimed to find out the exact role of Loc1p in *ASH1* mRNA localization process.

2. Results

2.1. Khd1p

2.1.1. Association of Khd1p with ER

2.1.1.1. Co-migration of Khd1p with ER marker proteins

The localization of Khd1p in living yeast cells is predominantly cytosolic (Huh et al, 2003). In order to check whether Khd1p co-migrates with ER fractions, I performed sucrose gradient assays. For this, whole cell extracts were prepared from a strain that was genomically tagged with 9 myc epitopes at the C-terminus of Khd1p (Khd1p-9xmyc). Addition of tags at the C-terminus does not impair the function of Khd1p as previously reported (Hasegawa et al, 2008). Cells were gently ruptured by making spheroplasting followed by douncing in order to maintain the integrity of membrane structures.

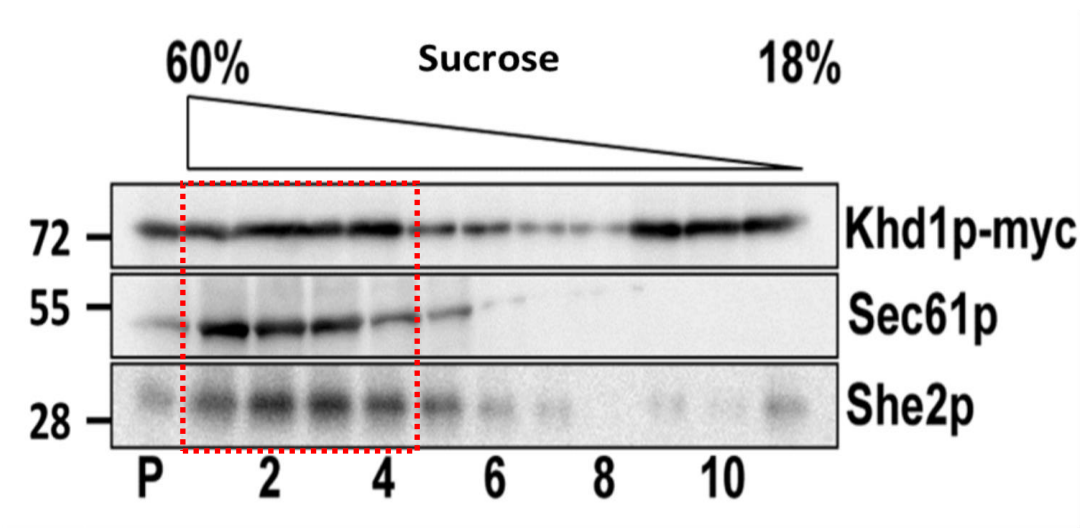


Figure 9: Khd1p co-fractionates with ER marker protein: Whole cell extract from RPY 2662 (Khd1-9xmyc) was separated on a linear sucrose gradient; fractions were collected and treated as described in materials and methods. Aliquots of 11 fractions (1 as bottom and 11 as top fraction) were analyzed by Western blotting against myc, Sec61p and She2p. Khd1p co-migrates with ER marker protein Sec61p and RNA-binding protein She2p, which is known to co-migrate with ER (fractions 1- 4 highlighted by red rectangular box).

Cell extracts were applied onto 18% - 60% continuous velocity sucrose gradient and separated by ultracentrifugation. Fractions of 1ml were collected from the bottom of the tube and the distribution of Khd1p was analysed by immunoblotting along with an ER marker protein, Sec61p. Sec61p migrates close to the bottom of the tube, which represents the high-density fractions. Previous studies from our lab have shown that the RNA-binding protein She2p co-migrates with ER membranes (Schmid et al, 2006; Genz et al, 2013). Thus, She2p could serve as an internal control.

Although Khd1p is known to be a cytosolic protein (Figure 9; fractions 9 - 11) a sub-population of it co-migrates with the ER marker protein Sec61p and RNA-binding protein She2p (Figure 9; fractions 1 - 4), thus providing the first indication for its possible association with ER.

2.1.1.2. Recombinant Khd1p binds to synthetic liposomes

As the initial evidence from sucrose gradient assay showed partial co-fractionation of Khd1p with ER marker protein (Sec 61p), I wanted to investigate if Khd1p can directly interact with the lipid membranes. For this, I purified GST tagged Khd1p using Glutathione fast flow sepharose beads (Figure 10A) as described in section 5.2.7. As a control, GST was purified by the same method (Figure 10B).

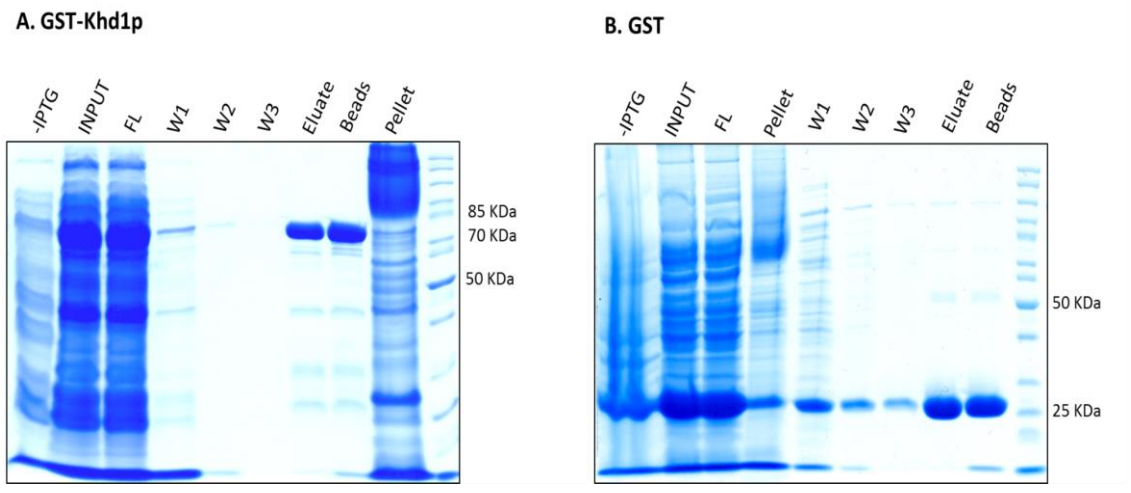


Figure 10: SDS-PAGE of GST –Khd1p and GST: Protein aliquots from input, flow through (FL), different washing steps (W1, W2 and W3), eluate, boiled beads and pellet were run on 10% SDS-PAGE for (A) GST-Khd1p and (B) GST. –IPTG refers to the protein sample without IPTG induction.

Synthetic liposomes of two different sizes, 80 nm and 200 nm were prepared as described in the 'Methods' section (Section 5.3.7.1). Floatation assays were carried out according to Schmid and Genz (Schmid et al, 2006; Genz et al, 2013). If the protein of interest has the potential to interact directly with lipids, it should be detected in the floated fraction after ultracentrifugation (Figure 11A; schematic representation). Therefore, purified GST-Khd1p was incubated with liposomes and subjected to ultracentrifugation. As a control, purified GST was treated in the same manner. Following the ultracentrifugation, the „float" fraction was analyzed using immunoblotting to detect the presence of Khd1p.

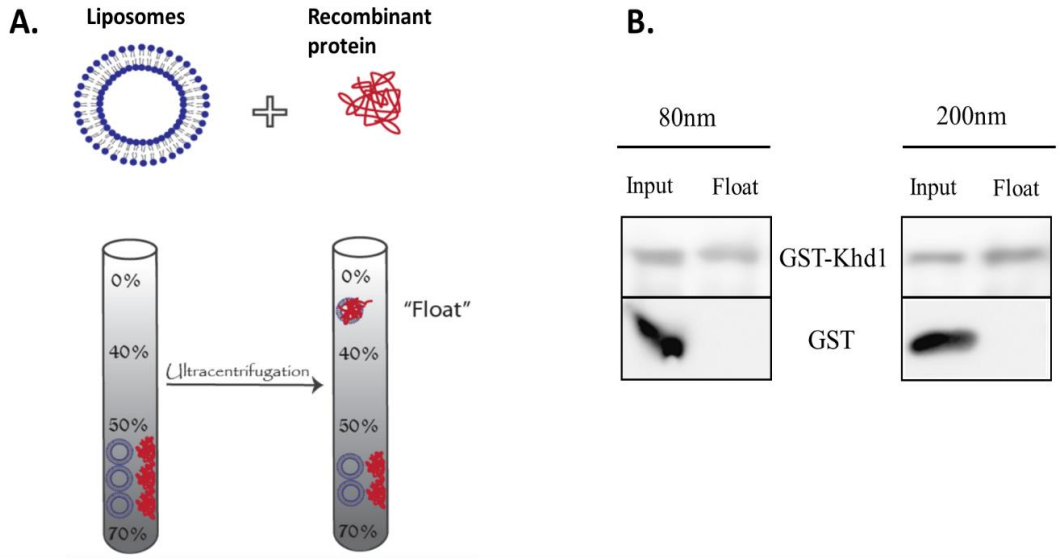


Figure 11: Khd1p floats with artificial liposomes: (A) Schematic illustration of floatation assay. (B) Recombinant Khd1p (GST-Khd1) was incubated with artificial liposomes of two difference sizes (80 nm and 200 nm) and subjected to ultracentrifugation. Input and float fractions were analyzed by western blotting against GST.

I found Khd1p co-floating with the liposome fraction (Figure 11B), whereas GST was completely absent in the „float" fraction. This observation supports the sucrose gradient result (Figure 9). Unlike She2p, which was recently shown to bind ER membranes in a curvature-dependent manner (Genz et al, 2013), association of Khd1p with membranes does not differ between differently sized liposomes and therefore does not depend on the membrane curvature.

2.1.1.3. Targeting a viral peptide to ER

2.1.1.3.1. Khd1p could target a viral peptide to ER whereas control proteins could not

Plant viruses are capable of encoding one or more movement proteins that promote cell-to-cell movement and these proteins generally localize at specific structures called plasmodesmata that allow movement of macromolecules in plant cells (Lucas, 2006). One such protein is TGBp3 (Triple-gene-block 3 protein) from Bamboo mosaic potexvirus. When expressed in yeast, it is localized to cortical ER (Wu et al, 2011). If split, its N-terminus comprising of amino acids 1-30 localizes to perinuclear ER, whereas the expression of the C-terminus (25-52 amino acids) resulted in cytoplasmic localization (Figure 12). This indicates that C-terminal region of TGBp3 requires a transmembrane domain for its cortical ER localization (Wu et al, 2011). The RNA-binding protein She2p has been shown to target this viral peptide to ER *in vivo* (Genz et al, 2013). I wanted to check whether Khd1p behaves in the same way by adapting the role of membrane-associated protein module and thereby localizing the peptide to ER.

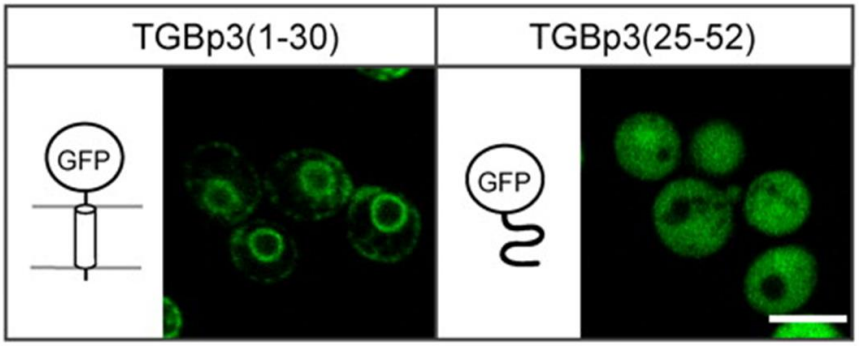


Figure 12: Localization of GFP-TGBp3 fusion proteins: N-terminal (amino acids 1-30) fused to GFP localized to perinuclear ER (left panel) whereas C-terminal (amino acids 25-52) fusion protein found to be diffused in cytoplasm (right panel). Figure adapted from Wu et al, 2011.

For this, I fused the *KHD1* ORF to the GFP-TGBp3 construct and transformed it into the yeast strain which expresses DsRed-HDEL (Bevis et al, 2002) as an ER-marker.

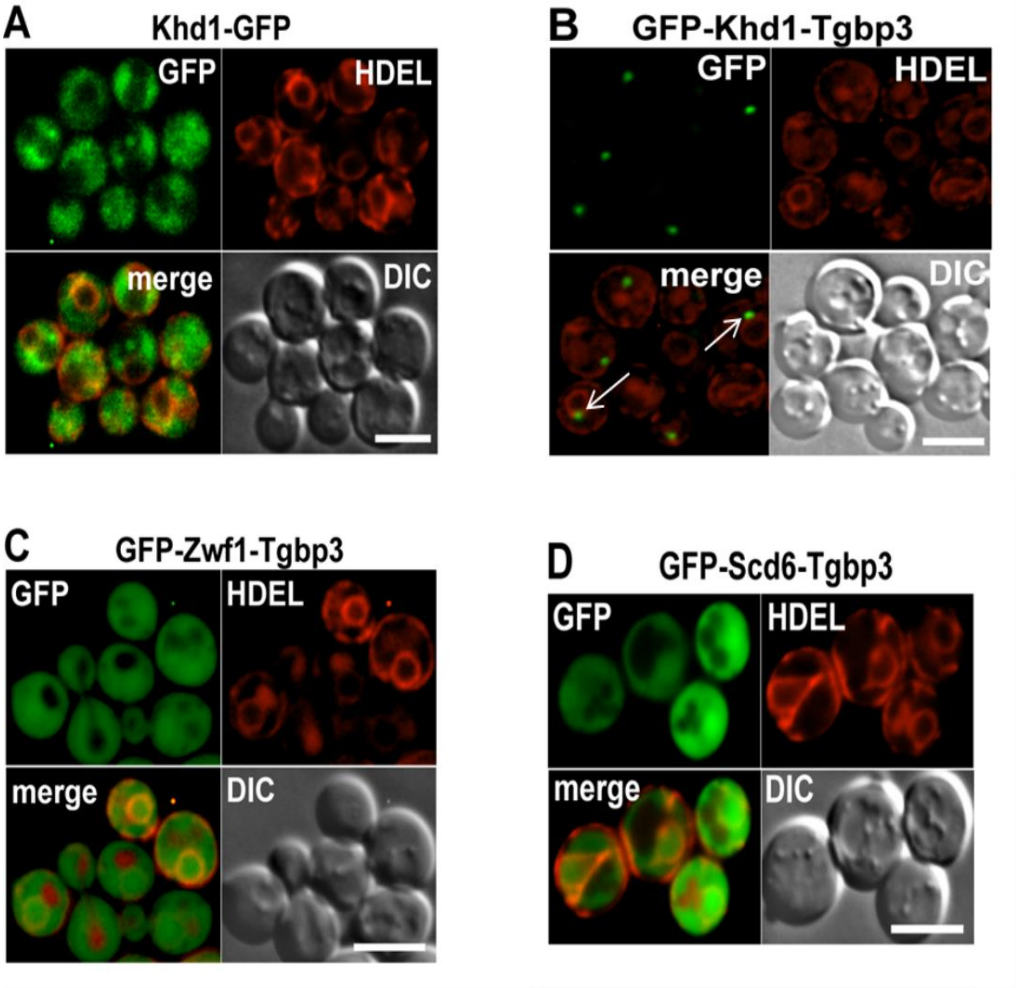


Figure 13: Localization of Khd1p (with and without Tgb3p), Zwf-Tgb3p and Scd6-Tgb3p *in vivo*. Representative images of cells showing the localization of wild type (without Tgb3p) Khd1p (A), Khd1p fused to Tgb3p (B), Zwf-Tgb3p (C) and Scd6-Tgb3p (D). Images represent DIC, GFP, mCherry channels and overlay of GFP-mCherry channels. White arrows in (B) show the co-localization of GFP-Khd1p-Tgbp3 with ER. White bar corresponds to 4 µm.

In order to verify that the targeting of viral peptide to ER is specific to Khd1p, I cloned two control proteins (see below) to the GFP-TGBp3 construct and observed them under microscope.

2.1.1.3.1.1. *ZWF1*

Zwf1p is the cytoplasmic enzyme, glucose-6-phosphate dehydrogenase that is involved in pentose phosphate pathway. The enzyme catalyzes the initial step of the pathway by converting glucose-6-phosphate to 6-phosphogluconolactone, thus regenerating

NADPH from NADP+ through an oxidation/reduction reaction (Nogae & Johnston, 1990; Jarori & Maitra, 1991; Kumar et al, 2002; Huh et al, 2003).

2.1.1.3.1.2. *SCD6*

Scd6 is a cytoplasmic protein known to be a translational repressor. It binds to eIF4G via its RGG domains and inhibits the formation of the pre-initiation complex (Rajyaguru et al, 2012). It has been identified as an RNA-binding protein and binds to more than thousand mRNA targets (Tsvetanova et al, 2010). Being a RNA-binding protein by itself, Scd6 serves as a better control as it possesses similar characteristics as Khd1p.

I verified the distribution of wild type Khd1p (without Tgbp3) *in vivo* by imaging a GFP fusion protein. As expected, wild type Khd1p shows a cytoplasmic localization (Figure 13A) (Huh et al, 2003). The fusion of Khd1p with the Tgbp3 construct resulted in foci overlapping with the ER in the mother cell and the bud (Figure 13B; co-localization shown by white arrows). In contrast to Khd1p-Tgbp3 fusion protein, neither of the control fusion proteins leads to targeting of the viral peptide to ER (Figure 13C and 13D respectively). This indicates that the observed targeting effect is specific and not a general mechanism. With the evidences so far (sucrose gradient assay, floatation assay and Tgbp3 targeting), it suggests that Khd1p has the potential to associate with ER.

2.1.2. Role of Khd1p in mRNA partitioning

2.1.2.1. Khd1p affects distribution of *MID2* and *SLG1* but not other tested mRNAs

As the results from previous sections suggest that Khd1p has the ability to associate with ER, I next wanted to investigate whether Khd1p has role in targeting bound mRNAs to ER. For this, I selected eight targets of Khd1p and analyzed for their distribution between membrane fraction and cytosolic fraction, in a wild type strain and in a *khdΔ* strain. *Actin* mRNA was used as a control. The details of the selected target mRNAs are as follows:

Target Name	**Alternative Name**	**Short description / Function of the encoded protein**
MID2	*KAI1*	O-glycosylated plasma membrane protein; acts as a sensor for cell wall integrity signaling and activates the pathway (Philip & Levin, 2001; Green et al, 2003).
SLG1	*WSC1, HCS77*	Sensor-transducer of the stress-activated PKC1-MPK1 kinase pathway; involved in maintenance of cell wall integrity (Lodder et al, 1999; Mao et al, 2011).
SCW11	-	Cell wall protein with similarity to glucanases (Cappellaro et al, 1998; Zeitlinger et al, 2003).
EGT2	-	Glycosylphosphatidylinositol (GPI)-anchored cell wall endoglucanase (Kovacech et al, 1996; Pan & Heitman, 2000; Terashima et al, 2003).
KRE1	-	Cell wall glycoprotein involved in beta-glucan assembly (Boone et al, 1990).
DSE2	-	Daughter cell-specific secreted protein with similarity to glucanases (Colman-Lerner et al, 2001; Doolin et al, 2001).
GPH1	-	Glycogen phosphorylase required for the mobilization of glycogen (Hwang et al, 1989; Francois & Parrou, 2001).
ZWF1	*MET19, POS10*	Glucose-6-phosphate dehydrogenase (G6PD); catalyzes the first step of the pentose phosphate pathway (Nogae & Johnston, 1990).
ACT1	*END7, ABY1*	structural protein involved in cell polarization and other cytoskeletal functions (Pruyne & Bretscher, 2000).

Table 1: Names of the target mRNAs of Khd1p, control mRNA (*ACT1*) and their function according to Saccharomyces Genome database (http://www.yeastgenome.org/).

In order to check the distribution of these mRNAs between membrane and cytosol, I adapted the subcellular fractionation method (Frey et al, 2001) with slight modifications followed by qRT-PCR (Section 5.3.8 to 5.3.10).

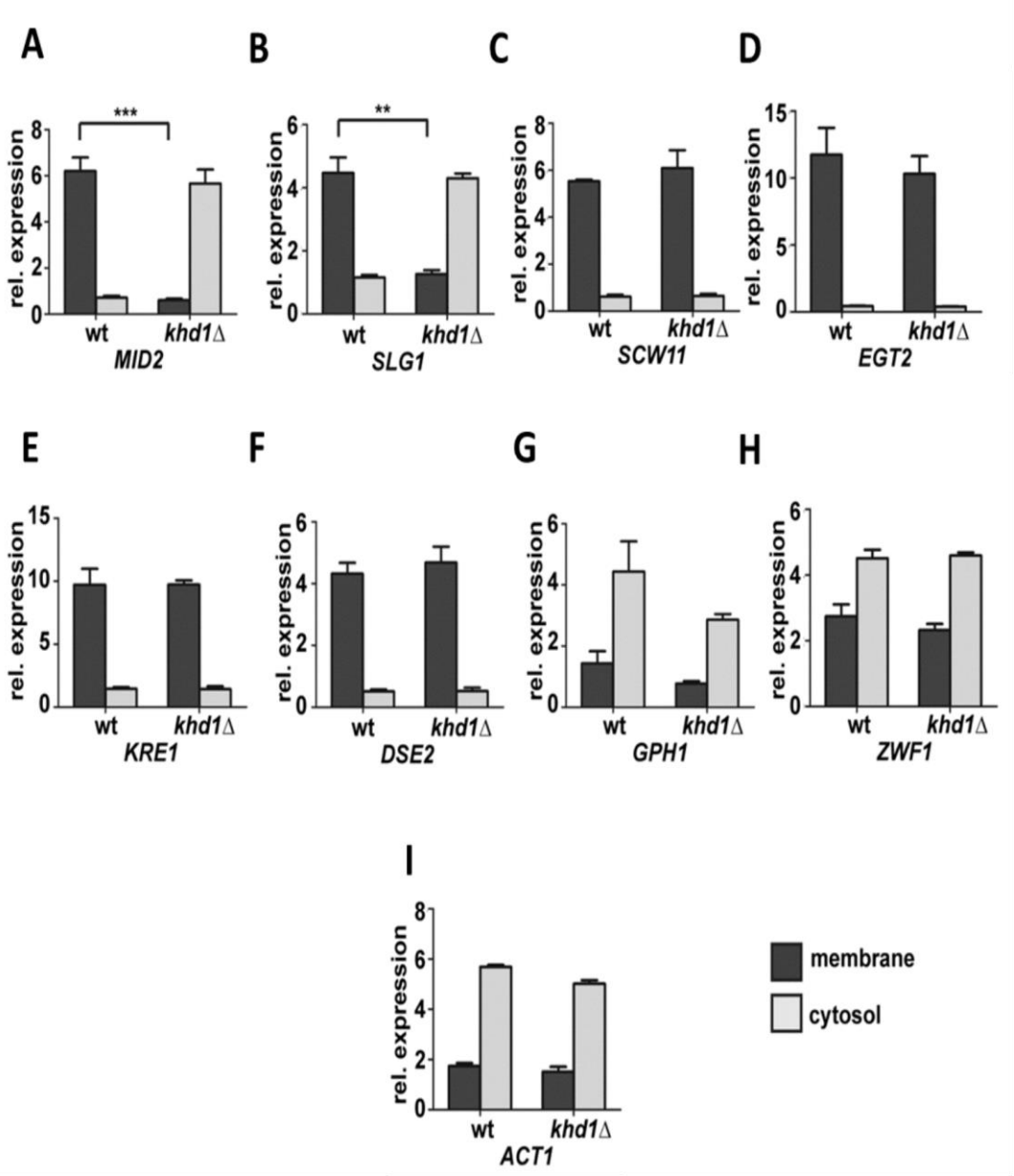


Figure 14: Effect of Khd1p on distribution of its target RNAs between membrane and cytosolic fractions: Wild type Strain (RJY358) and *khdΔ* (RJY 2368) were subjected to subcellular fractionation followed by RNA isolation, cDNA synthesis and qRT-PCR (section 5.3.8 to 5.3.10) for analyzing the distribution of Khd1p target mRNAs. Relative expression levels were calculated by $2^{-\Delta C}{}_{T}$, where ΔC_T is the C_TMembrane - C_TInput or C_TCytosol - C_TInput. (A) *MID2*, (B) *SLG1*, (C) *SCW11*, (D) *EGT2*, (E) *KRE1*, (F) *DSE2*, (G) *GPH1*, (H) *ZWF1* and *ACT1* (I) as a control. All data are presented as mean ± SEM (error bars), n=3. Statistical significance for *MID2 (P=0.0007) & SLG1 (P=0.003)* membrane distribution in WT and *khd1Δ* is indicated.

Among the eight targets used, six of them coding for cell wall proteins or secretory proteins and are expected to be present in membrane fraction. The other two targets, *GPH1* and *ZWF1* code for cytosolic proteins (See Table 1).

Data from qRT-PCR showed that, in wild type cells, the membrane protein encoding mRNAs (*MID2, SLG1, EGT2, DSE2* and *KRE11*) were enriched in the membrane fraction whereas the cytosolic targets (*GPH1* and *ZWF1*) were present in the cytosolic fraction along with *ACT1* mRNA. Upon deletion of *KHD1*, the distribution between membrane and cytosolic fraction of two mRNAs (*MID2* and *SLG1*) was altered (Figure 14A and 14B). Both mRNAs were enriched in the cytosolic fraction of *khd1Δ* cells. Such difference in the distribution pattern was not observed in other targets (Figure 14C-14H). *ACT1* mRNA which is not a target of Khd1p serves as a negative control, did not show any change in distribution pattern between wild type and *khd1Δ* (Figure 14I).

2.1.2.2. ER targeting of *MID2* does not depend on translation

2.1.2.2.1. Co-translational targeting is not required for *MID2* distribution

mRNAs can be targeted to ER in a co-translational manner by using SRP pathway (section 1.3.2.1). Since absence of *KHD1* had shown to affect the distribution of *MID2*, I next wanted to check whether co-translational targeting is also involved in the case of *MID2*. In other words, I wanted to investigate whether the *MID2* targeting is SRP-dependent or independent.

For this, the *MID2* gene including promoter and 3'UTR was cloned in a yeast centromeric plasmid and expressed in cells lacking *MID2*. By site-directed mutagenesis, a mutant version of *MID2* plasmid (*MID2noATG*) was generated in which the start codon (ATG) was mutated to CGG and also introduced into a *mid2Δ* strain. The signal sequence in *MID2noATG* cannot be translated due to the lack of start codon and therefore this mRNA cannot use the SRP-dependent pathway for targeting. The strains were then used to perform the subcellular fractionation experiment followed by qRT-PCR (Figure 15A; schematic illustration).

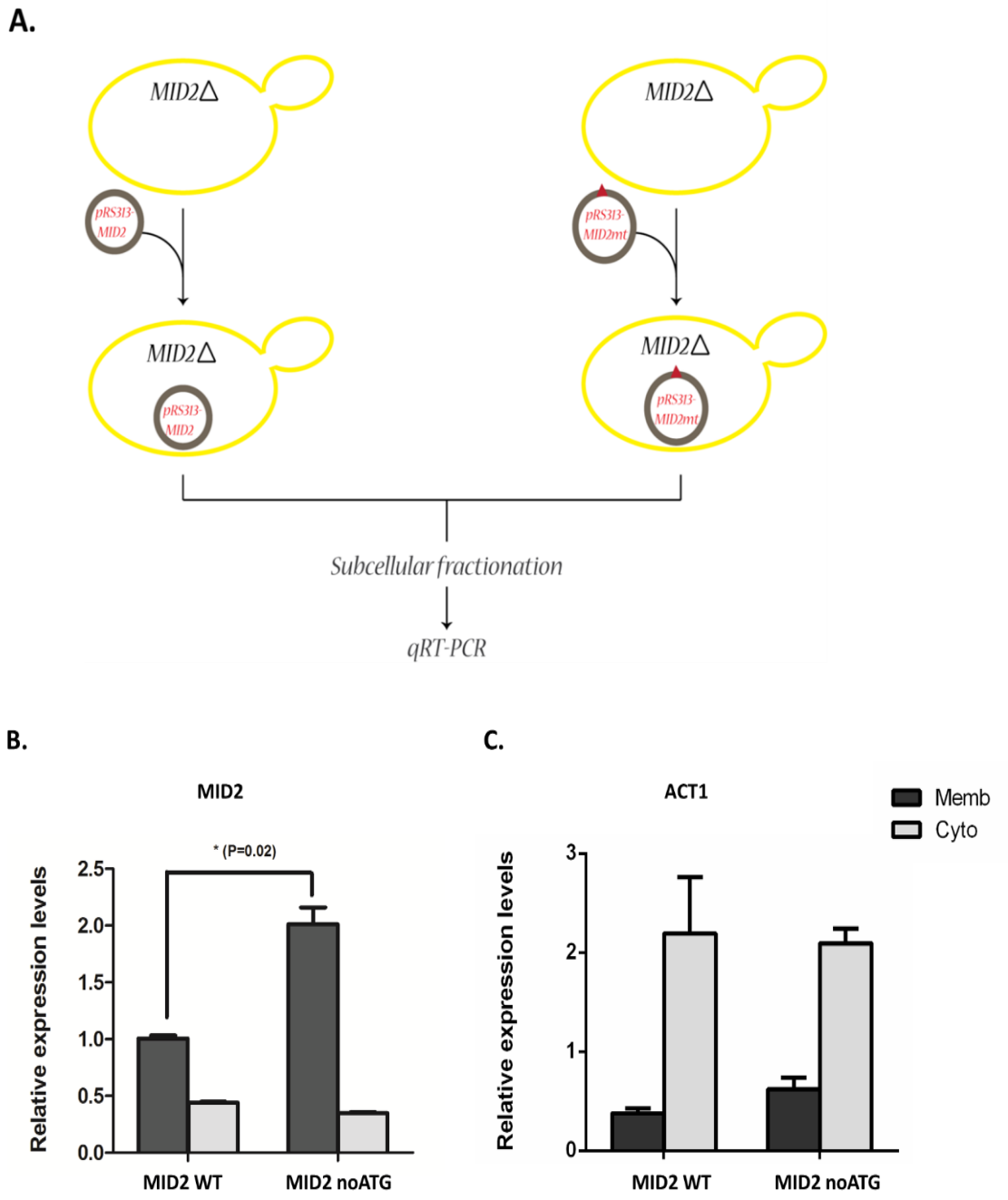


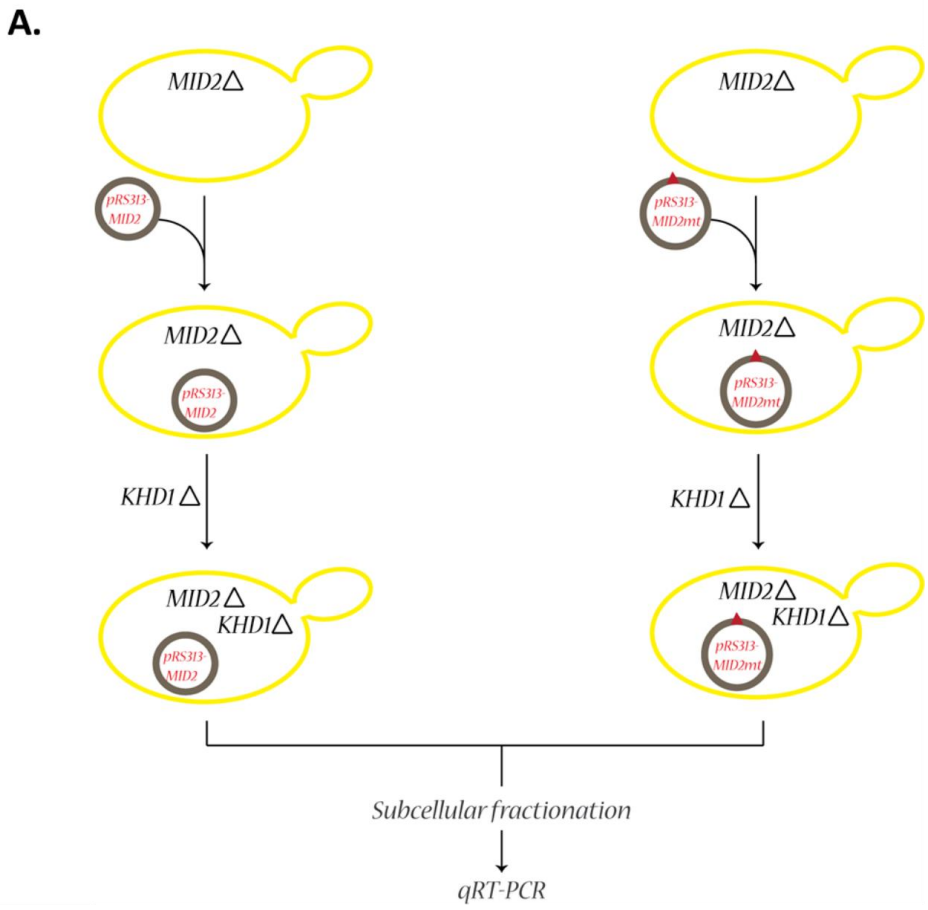
Figure 15: Translation is not required for *MID2* co-fractionation with membranes. (A) Graphical illustration of the experimental process. Subcellular fractionation followed by RNA isolation, cDNA synthesis and qRT-PCR for analyzing the distribution of *MID2* mRNA (B) and *ACT1* mRNA (C) between membrane and cytosolic fraction was carried out for *MID2* WT and *MID2noATG* in the presence of Khd1 (RPY4500 and RPY4506 respectively). Relative mRNA expression levels were calculated by $2^{-\Delta C_T}$, where ΔC_T is the C_TMembrane - C_TInput or C_TCytosol - C_TInput. Data are presented as mean ± SEM (error bars), n=3. Statistical significance compared to the membrane fraction of *MID2 WT* and *MID2noATG* in the presence of Khd1p is indicated: *, P<0.05.

The data show that the relative abundance of *MID2noATG* in the membrane fraction was significantly increased (almost 2-fold) when compared to the wild type *MID2* level (Figure 15B). In contrast, the levels of *ACT1* was not altered (Figure 15C). Therefore, this result indicates that *MID2* targeting to ER does not occur co-translationally.

2.1.2.2.2. *MID2* targeting is dependent on Khd1p, not SRP-pathway

As it was clear from qRT-PCR data that *MID2* mRNA lacking start codon was targeted to ER independent of SRP pathway (Figure 15B), this led to the question whether Khd1p is involved in targeting it to the ER. If Khd1p is involved in *MID2* noATG mRNA targeting, then absence of Khd1p should result this mRNA in cytosol fraction.

In order to test this hypothesis, *KHD1* was deleted according to Janke et al. (Janke et al, 2004) from both the strains (*MID2* wt and *MID2noATG* mutant) and subcellular fractionation was carried out (Figure 16A; schematic illustration).



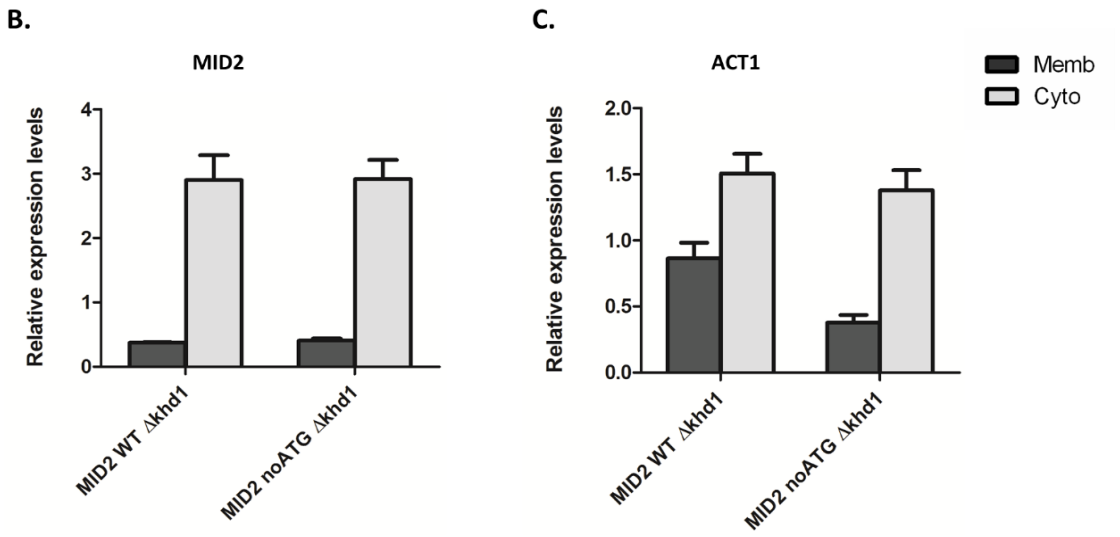


Figure 16: Co-fractionation of *MID2* mRNA with membrane fraction depends on Khd1p. (A) Graphical illustration of the experimental process. Subcellular fractionation followed by RNA isolation, cDNA synthesis and qRT-PCR for analyzing the distribution of *MID2* mRNA (B) and *ACT1* mRNA (C) between membrane and cytosolic fraction was carried out for *MID2WT* and *MID2noATG* in the absence of Khd1 (RPY4545 and RPY4546 respectively). Relative mRNA expression levels were calculated by $2^{-\Delta C}{}_T$, where ΔC_T is the C_TMembrane - C_TInput or C_TCytosol - C_TInput. Data are presented as mean ± SEM (error bars), n=3.

Upon deletion of *KHD1*, the distribution of both, *MID2* wild type and the one lacking start codon (Figure 16B), was clearly shifted to cytosolic fraction from membrane fraction, which was similar to the pattern observed earlier (Figure 14A). The control mRNA, *ACT1* was found to be in cytosol in both the strains (Figure 16C). Thus, this result suggests that Khd1p is required for targeting *MID2noATG* to ER.

2.1.3. Protein levels of *MID2* are not affected by Khd1p

The results from the subcellular fractionation and qRT-PCR studies indicated that Khd1p affects the distribution of *MID2* mRNA between membrane and cytosol. So, this led to the question whether Khd1p also has similar effect on *MID2* at the protein level. To investigate this, I tested the expression of Mid2p in wild type and *khd1Δ* strains using immunoblotting and fluorescence microscopy.

For Western blotting, cell lysates were made by alkaline lysis method. Mid2p-GFP was detected using anti-GFP antibody and Pgk1p was used as a loading control. The expression of Mid2p in both wild type and cells lacking Khd1p are comparable (Figure

17A). In order to check Mid2p expression *in vivo*, I used fluorescence microscopy technique. A yeast strain where green fluorescent protein (GFP) was C-terminally fused to *MID2* gene and expressed in wild type and *khd1Δ* strain. Live imaging showed the similar expression of Mid2p in mother cells (large cell) of both wild type (wt) and *khd1Δ* cells, whereas in the daughter cells of *khd1Δ*, Mid2p expression was found to be slightly weaker (Figure 17B).

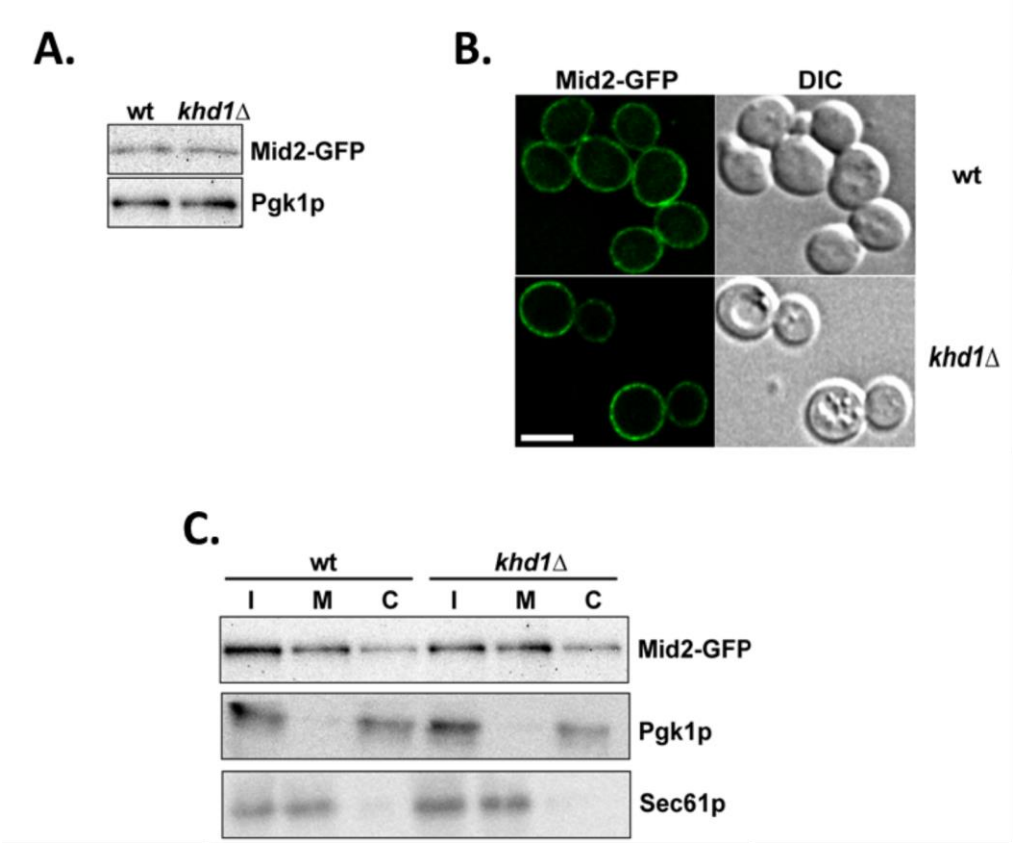


Figure 17: Khd1p does not affect *MID2* at protein level: (A) Alkaline lysis was performed for strains RPY4544 and RPY4553 and western blotting was carried out against GFP to check the expression of Mid2p in wild type (wt) and *khd1Δ* cells. Pgk1p was used as a loading control. (B) Images representing the expression of Mid2-GFP in wild type (wt) cells (RPY4544) and in *khd1Δ* (RPY 4553). Left panel indicates GFP channel and right panel is DIC Nomarski. White bar corresponds to 4 µm. (C) Subcellular fractionation was performed as described in methods for RPY4544 and RPY4553 strains. I – Input, M – Membrane fraction, C – Cytosolic fraction. Aliquots from different fractions were subjected to immunoblotting against GFP, ER marker protein Sec61p and cytosolic marker protein Pgk1p.

Subcellular fractionation method was carried out to check the distribution of Mid2p between membrane and cytosolic fractions *in vitro*. In wild type cells, Mid2p was found to co-fractionate mostly in the membrane fraction along with the ER marker protein Sec61p. *khd1Δ* cells did not show any difference in the distribution of Mid2p between membrane and cytosolic fractions indicating that Khd1p does not affect the distribution of *MID2* at protein level. Pgk1p was used as cytosol marker protein (Figure 17C).

2.1.4. Calcofluor white (CFW) assay

Mid2p is an integral membrane protein that localizes to the plasma membrane. It acts as a potential cell wall integrity sensor and initiates a cellular response by activating the PKC1-MPK1 cell wall integrity pathway (Heinisch et al, 1999). Also, it is known to be an activator of the Skn7p transcription factor, which is involved in number of cellular processes, particularly in cell wall biogenesis (Ketela et al, 1999; Levin, 2005).

Calcofluor white is a fluorochrome dye that intercalates with nascent chains of cellulose and chitin in the cell walls of fungi and other organisms, thereby disrupting the assembly of chitin microfibrils (Roncero & Duran, 1985). In *S. cerevisiae*, exposure to calcofluor white results in abnormally thick septa because of incomplete separation of mother and daughter cells. Calcofluor white exposure increases the chitin production and is lethal to the cells at high concentration (Roncero & Duran, 1985).

Loss of *MID2* affects chitin synthesis during stress conditions (Ketela et al, 1999) and cells lacking *MID2* display resistance to calcofluor white at concentrations of 20 μg/ml. For the synthesis of basal chitin, Mid2p was not required whereas it is required for the production of supplemental chitin, although to a limited extent (Ketela et al, 1999). Since there is evidence suggesting that both Mid2p (Ketela et al, 1999) and Khd1p (Ito et al, 2011) are involved in the cell wall integrity pathway, I next wanted to investigate whether Khd1p is also affecting the synthesis of chitin on exposure to calcofluor white and also whether Khd1p-dependent of *MID2* mRNA targeting has any effect on *MID2* function.

For checking this, a yeast strain lacking both *KHD1* and *MID2* was created (Janke et al, 2004). Single and double deletion strains were then allowed to grow till mid-log phase and serial dilution was carried out on a YEPD plate without and with 20 μg/ml

calcofluor white. As controls, wild type and other deletion strains (*zwf1Δ and adh1Δ*) were used. In order to rule out the possibility that calcofluor white resistance is due to the insertion of Kanamycin marker cassette, I used a strain expressing the Kanamycin cassette alone (Figure 18).

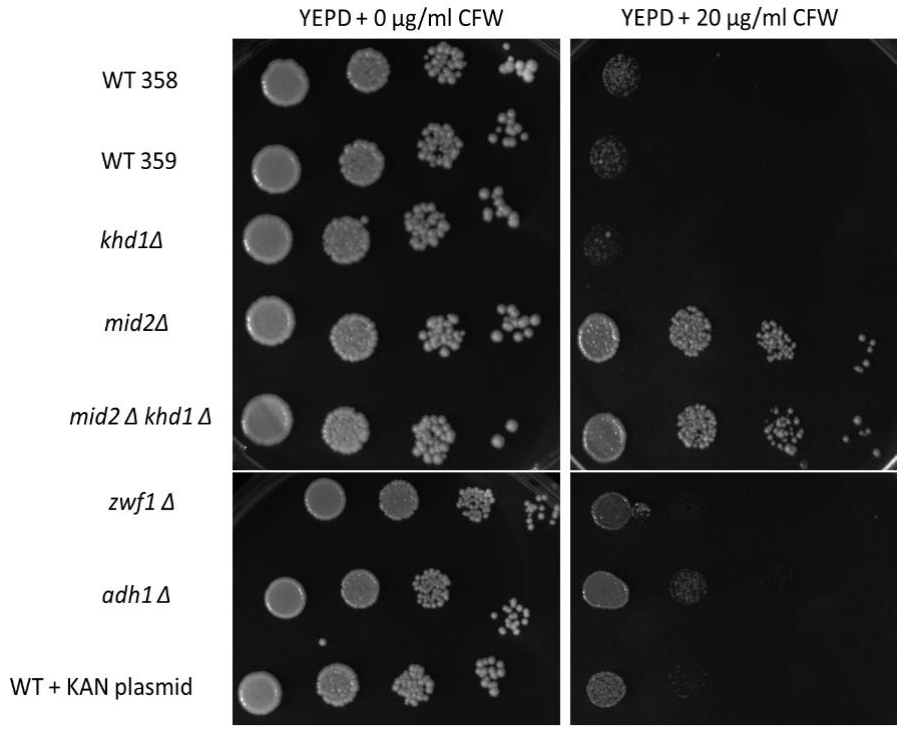


Figure 18: Loss of *KHD1* does not affect function of Mid2p: Cells were grown till mid-log phase and diluted to a concentration of $3x10^6$ cells/ml. 5 µl of this suspension and three subsequent 10-fold serial dilutions of each were spotted onto the YEPD (0 µg/ml CFW) and YEPD + 20 µg/ml CFW and grown at 30 ºC. WT 358 and WT 359 represents the wild type W303 Mat a and Mat alpha strain respectively. WT+KAN plasmid represents the strain with "KAN cassette" that was used in gene knockouts.

2.1.5. Point mutation in one of the KH-domains perturbs RNA-binding and peptide targeting to ER

2.1.5.1. RNA-binding is reduced in KH-domain 1 mutant (I59R)

Khd1p contains three KH-type RNA-binding domains (Irie et al, 2002) (also see section 1.2.1.2.5.1.). Previous reports on KH-domains of Fragile X Mental Retardation protein 1 (Fmr1) showed that a replacement of a single isoleucine residue to asparagine (I304N) shuts down the function of KH-domain (Siomi et al, 1993b). In order to identify

whether the RNA-binding domains in Khd1p contribute to its association with ER indirectly by tethering onto the mRNA translated at the ER, I created a mutant version of *KHD1* (*KHD1-I59R*) by replacing a conserved isoleucine (aaI59) with arginine in KH-domain 1 (Figure 19).

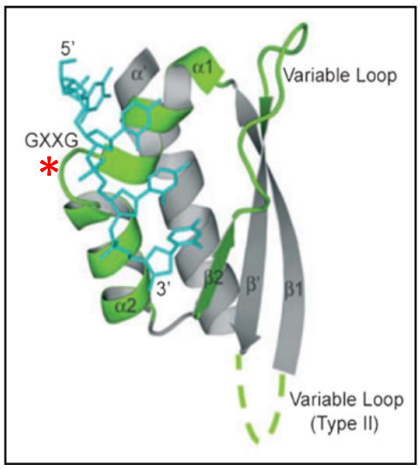


Figure 19: Mutation at KH-domain 1: Isoleucine residue at postion 59 (indicated by asterisk), close to the conserved signature sequence GXXG. Image modified from Valverde, 2008.

To check that this point mutation is deleterious for RNA binding, I used immunoprecipitation (IP) in combination with real-time PCR (qRT-PCR) to test if target mRNAs of Khd1p in wild type and in the *KHD1-I59R* mutant are still associated. HA-tagged versions of both proteins were expressed from a plasmid under the control of a shortened *GAL1* promoter (*GALS*) to avoid over-expression (Mumberg et al, 1994). A *khd1Δ* yeast strain was transformed with either wild type (Khd1p-6xHAWT) or *KHD1-I59R* mutant (Khd1p-6xHA D1 mt). Both the proteins were expressed in yeast and Western blotting confirmed that similar amounts of each protein can be immunoprecipitated (Figure 20A). Mock anti-HA IP was done using the wild type strain. RNA was extracted from the pellet, reverse transcribed and amplified with primers against target mRNAs of Khd1p. Two control mRNAs, *CFT1* and *ACT1*, which are not targets of Khd1p, were also included. Data from qRT-PCR revealed that affinity for target mRNAs was significantly reduced in *KHD1-I59R* mutant version compared to wild type. In contrast, the signal for the control mRNAs *CFT1* and *ACT1* were

expectedly very weak (Figure 20B). This indicates that the mutation of the conserved isoleucine residue in KH-domain 1 reduces the RNA-binding activity of Khd1p.

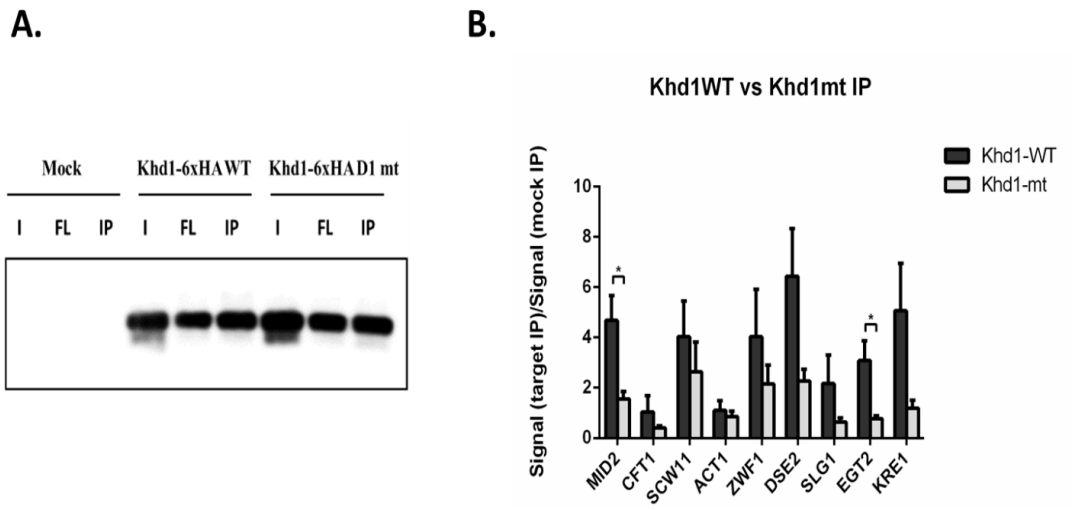


Figure 20: Conserved isoleucine residue in KH-domain 1 is essential for RNA-binding: (A) Western blotting of the immunoprecipitation of Khd1-6xHA wild type (RPY4537) and Khd1(I59R)-6xHA mutant (RPY4358) using anti-HA coated magnetic beads. I – Input, FL – flow through, IP – immunoprecipitate. Strain RJY359 was used for mock IP. (B) Immunoprecipitates from both Khd1-6xHA wild type (Khd1-WT) and I59R mutant (Khd1-mt) were subjected to qRT-PCR and analyzed for bound target RNAs of Khd1 (*MID2, SCW11, ZWF1, DSE2, EGT2, SLG1, KRE1*). *ACT1 mRNA* and *CFT1 mRNA* were used as negative control. Specific enrichment was calculated as ratio of the signal in the target IP to the mock IP (untagged wildtype strain). All data are presented as mean ± SEM (error bars), n=2. * indicates the statistical significance of P<0.01 (in case of *MID2* and *EGT2*)

2.1.5.3. Viral peptide targeting is affected in Domain 1 mutant of Khd1p

To investigate whether RNA-binding is necessary for the viral peptide targeting, a *KHD1* ORF with mutations in different KH-domains were cloned into the GFP-TGBp3 construct and the fusion proteins observed for co-localization with ER. In case of KH-domain 1, the same isoleucine to arginine mutant as described above was used (GFP-Khd1p-Tgbp3 D1 mutant I59R). The conserved isoleucine in KH-domain 2 and leucine residue in KH-domain 3 were mutated to arginine (GFP-Khd1p-Tgbp3 D2 mutant I190R, or GFP-Khd1p-Tgbp3 D3 mutant L284R, respectively).

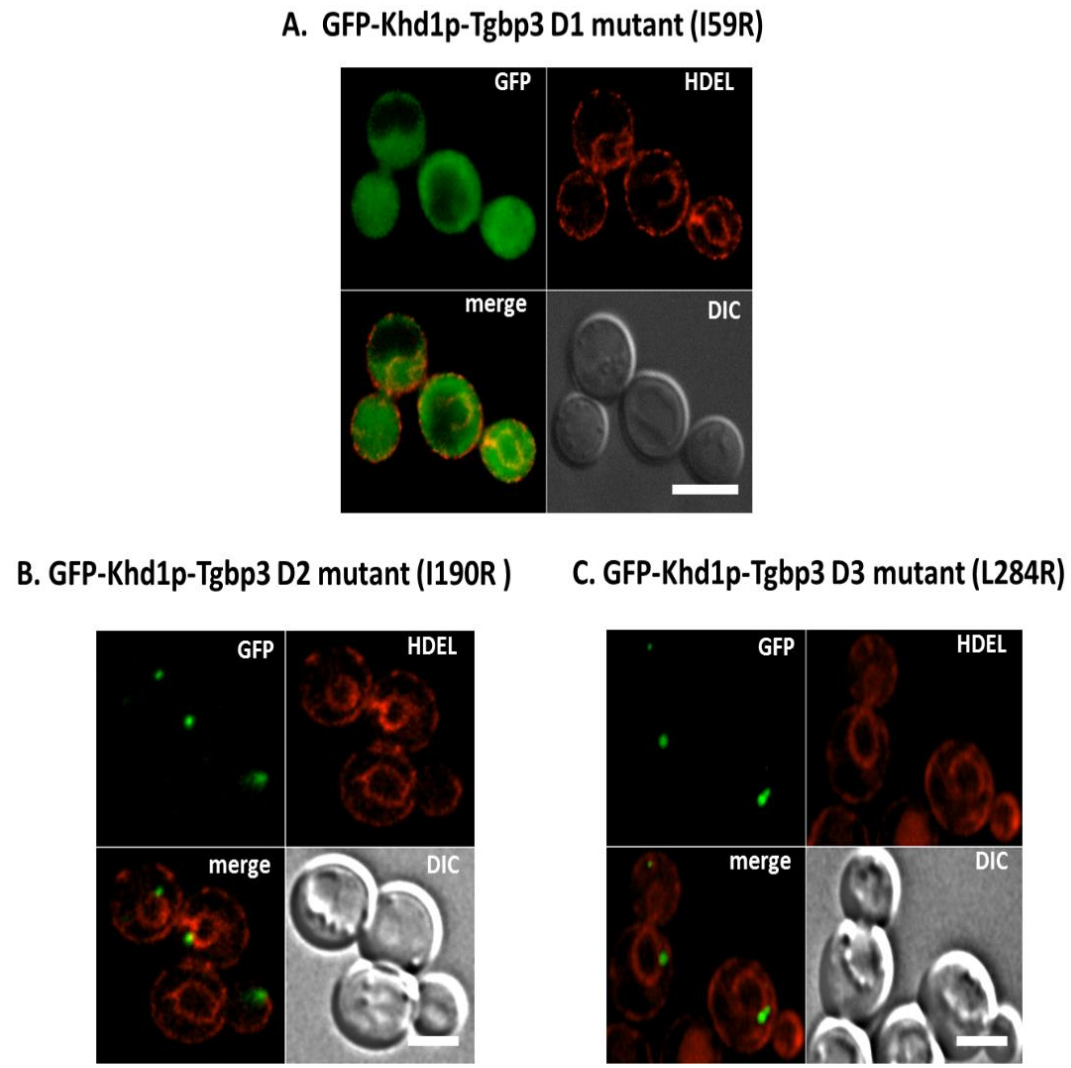


Figure 21: Mutation in KH-domain 1, but not KH-domain 2 and 3 disturbs membrane association of Khd1-Tgb3p fusion protein: Representative images of cells expressing GFP-Tgb3 fusion protein and DsRed-HDEL which labels ER internal lumen are shown. (A) GFP-Khd1p-Tgbp3 fusion protein bearing mutation in KH-domain 1 (I59R), (B) mutation in KH-domain 2 (I190R) and (C) mutation in KH-domain 3 (L284R). White bar corresponds to 4 µm.

Live imaging revealed that the targeting of the viral peptide to ER was affected in the strain that carries the mutation in KH-domain 1 (Figure 21A). In contrast, mutations in KH-domain 2 and KH-domain 3 did not affect the co-localization of fusion protein to ER (Figure 21B and 21C respectively) and found to be similar to wild type (GFP-Khd1p-Tgbp3; Figure 13B).

2.1.5.4. Subcellular fractionation

The above *in vivo* data showed the diffused pattern of KH-domain 1-fusion protein in cytosol. So, I wanted to confirm this observation *in vitro* by using subcellular fractionation (section 5.3.8.). For this I used the strains expressing the wild type Khd1-Tgbp3 fusion protein (GFP-Khd1-Tgbp3 WT) and the one with mutation in KH-domain 1 (GFP-Khd1-Tgbp3 D1 mutant)

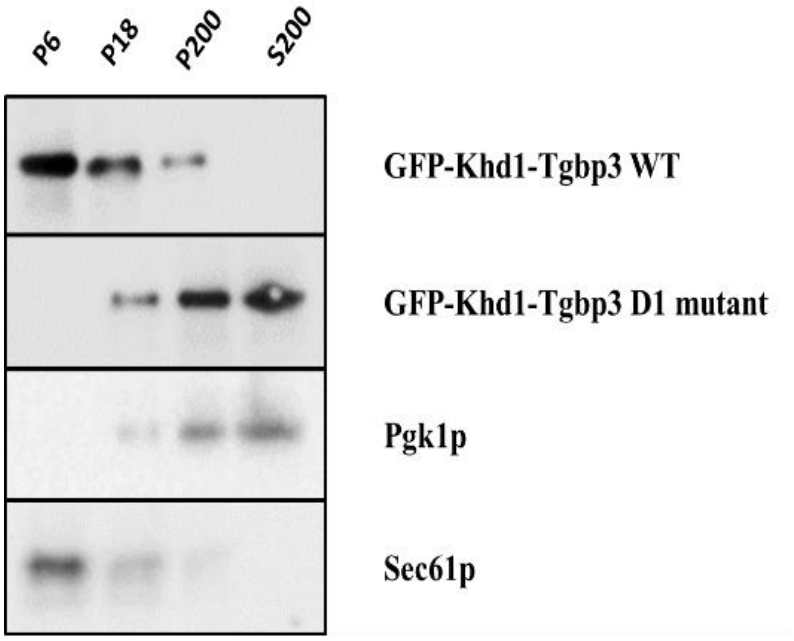


Figure 22: Subcellular fractionation to detect the mutant construct: Lysates from different fractions were measured for protein concentration using Bradford method and 20 μg of protein was used for immunoblotting. Anti-GFP antibody was used for detection of wild type and mutant Khd1-Tgbp3 fusion protein. Sec61p is used as ER marker and Pgk1p as cytosolic marker.

The GFP-Khd1-Tgbp3 D1 mutant protein was enriched in the cytosolic fraction (Figure 22; P200 and S200) which is consistent with live imaging observations (Figure 21A). In contrast, GFP-Khd1-Tgbp3 wild type protein was present in the membrane fraction (Figure 22; P6 and P18). Pgk1p was used as a control for cytosolic fraction, whereas Sec61p was used as an ER marker protein (Figure 22).

Taken together, these results (section 2.1.5.1 to section 2.1.5.4) suggest that KH-domain 1 is required for both mRNA binding and association of Khd1p with ER.

2.1.5.5. *MID2* targeting to ER is independent of the RNA-binding activity of Khd1p

From the aforementioned results (Section 2.1.2.2.), co-translational targeting of *MID2* has been ruled out and Khd1p seems to be involved in targeting *MID2* to ER. In contrast, the RNA-binding mutant (I59R) version of Khd1p (GFP-Khd1-Tgbp3 D1 mutant) failed to target the peptide Tgbp3 to ER *in vivo* (Figure 21A), indicating a requirement of Khd1p on RNA-binding for ER targeting. To address this apparent contradiction, different RNA-binding mutant versions of Khd1p were expressed in yeast and *MID2* mRNA localization analyzed. If Khd1p binding of *MID2* is important, then mutant versions of Khd1p (I59R, I190R, or L284R) should not bind *MID2* mRNA and thereby *MID2* should be present in the cytosol fraction.

In order to test this hypothesis, different mutant versions of Khd1p were generated by site directed mutagenesis. For this, the plasmid with single mutant in KH-domain 1 (I59R) was used as a template to create the double mutant and triple mutant. (D1 mutant – I59R; D1+D2 mutant – I59R+I190R; D1+D2+D3 mutant – I59R+I190R+L284R)

To verify the expression of the generated mutants, alkaline lysis was performed followed by Western blotting (Figure 23A). Expression levels of all the three mutants were found to be similar when compared to the strain expressing 6xHA from endogenous promoter of *KHD1*. These strains were used for the checking the distribution of *MID2* between membrane and cytosol.

To my surprise, none of the RNA-binding mutants of Khd1p affect the distribution of *MID2* mRNA. *MID2* was present in membrane fractions in all the mutants similar to the wild type (Figure 23B), although a slight decrease was observed in the case of triple mutant. *ACT1* mRNA was enriched in cytosolic fraction in wild type as well as the mutants, as expected (Figure 23C).

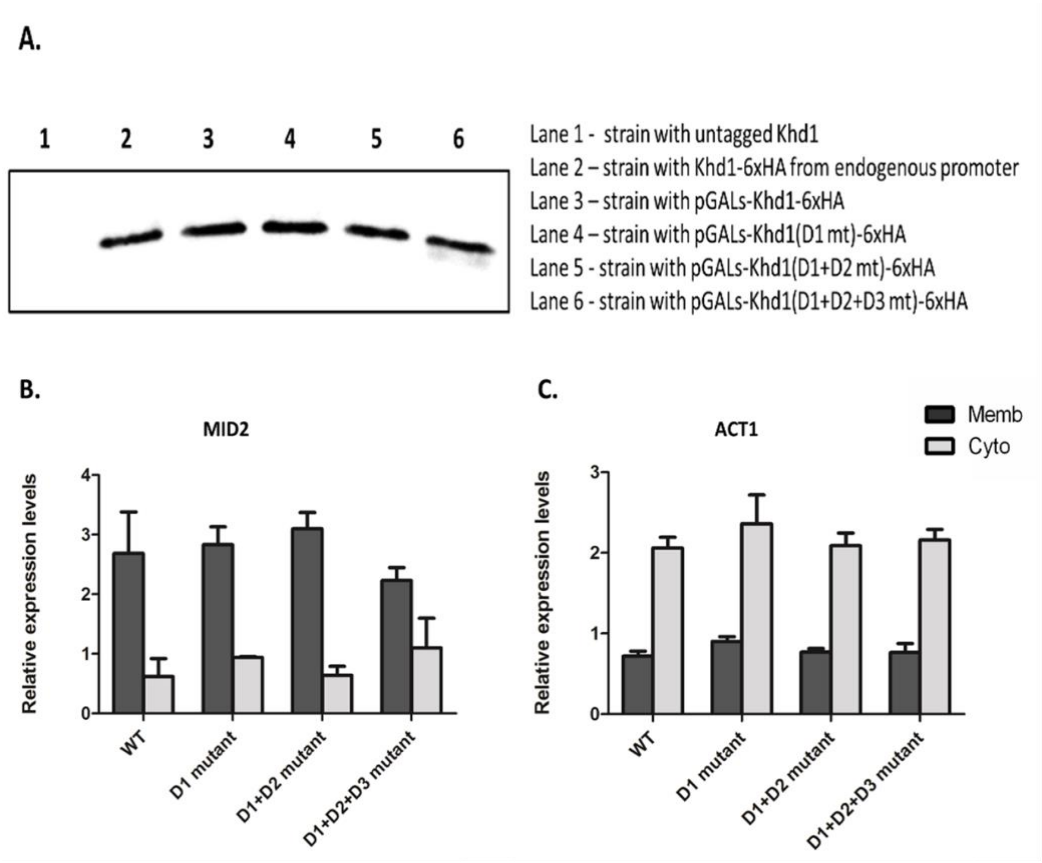


Figure 23: Targeting of *MID2* does not depend on RNA-binding activity of Khd1p: (A) Immunoblotting showing the expression of Khd1-6HA in different strains used for distribution expt. Strains bearing wild type Khd1-6HA (WT), mutation in KH-domain 1 (D1 mt), mutations in KH-domains 1 and 2 (D1+D2 mt) and mutations in KH-domains 1,2 and 3 (D1+D2+D3 mt) on a plasmid were subjected to subcellular fractionation followed by RNA isolation, cDNA synthesis and qRT-PCR for analyzing the distribution of *MID2* mRNA (B) and *ACT1* mRNA (C). Relative expression levels were calculated by $2^{-\Delta C}{}_{T}$, where ΔC_T is the C_TMembrane - C_TInput or C_TCytosol - C_TInput.

In summary, my results on Khd1p suggest that it has the ability to associate with membranes and it is required for the targeting of *MID2* mRNA to the ER.

2.2. Loc1p

Localization of *ASH1* mRNA to the tip of daughter cell is a process involving cis-acting elements and many trans-acting factors in the form of RNA-binding proteins. Loc1p is one of the RNA-binding proteins that are required for localization process (Section 1.2.1.2.6). It is a nuclear protein (Long et al, 2001), enriched in nucleolus (Huh et al,

2003; Du et al, 2008) and participates in assembly of large ribosomal subunit (Urbinati et al, 2006). The exact function of Loc1p in the *ASH1* transport process is not clearly understood except that it is required for the translational regulation of *ASH1* (Komili et al, 2007).

Along with She2p and Puf6p, *ASH1* mRNA is exported to the cytoplasm (Gu et al, 2004; Shen et al, 2009), whereas Loc1p remains in the nucle(ol)us (Long et al, 2001; Du et al, 2008). In the cytoplasm, the *ASH1* containing pre-complex interacts with another sub-complex consisting of Myo4p and She3p. A mature transport complex is formed via the direct interaction between She2p and She3p. There is no evidence so far on the removal of Loc1p from the complex before nuclear export (Bohl et al, 2000; Long et al, 2000; Takizawa & Vale, 2000; Kruse et al, 2002; Heuck et al, 2007; Hodges et al, 2008; Muller et al, 2011).

In collaboration with Dr. Dierk Niessing's group, Institute of Structural Biology, Helmholtz Zentrum München, we aimed at investigating the exact role of Loc1p in *ASH1* localization process.

2.2.1. She2p/She3p complex displaces Loc1p from the RNA *in vitro*

By using pull-down and UV-cross linking experiments, Annika Niedner and Marisa Müller (Prof. Niessing group) had demonstrated a direct and specific interaction between Loc1p and She2p and that both proteins recruit each other onto the *ASH1* mRNA. Being a strict nucleolar protein, Loc1p cannot enter the cytoplasm along with She2p and *ASH1* mRNA complex. So, it has to be stripped off from the RNA before the nuclear export of the complex. One potential candidate to displace Loc1p is the RNA-binding protein She3p.

In order to test the effect of She3p on Loc1p-*ASH1* mRNA association, UV-cross linking of radio-actively labeled *ASH1* E3 zipcode RNA and purified proteins (She2p, She3p and Loc1p) were used. Loc1p was tagged with MBP at its N-terminus whereas She3p was His6-tagged.

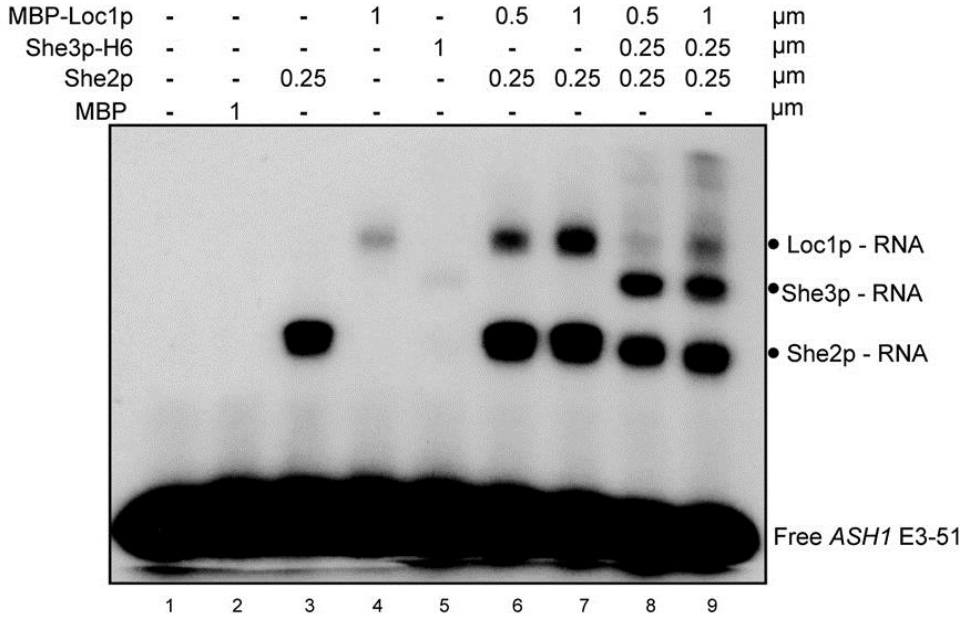


Figure 24: She3p outcompetes Loc1p from the mRNP: UV cross-linking experiment with She2p, She3p-H6, and MBP-Loc1p shows that in the presence of She2p and She3p Loc1p–RNA cross-linking is almost abolished. Both She2p and Loc1p individually interact with *ASH1* E3 RNA (Lanes 3 and 4), whereas the combination of She2p and She3p completely destroys the RNA-crosslinking by Loc1p (Lanes 8 and 9). Image adapted from Niedner et al, 2013.(Niedner et al, 2013) (Figure used with the permission from Annika Niedner, Munich).

From the UV cross-linking experiment, it could be shown that in the presence of both She2p and She3p, RNA cross-linking to Loc1p is almost completely abolished (Figure 24; lanes 8 and 9). This indicates that the She2p-She3p complex, at near-physiological concentrations, can strip off Loc1p from zip-code-containing RNA.

2.2.2. Distribution of Loc1p is altered in *she3Δ* cells

To support the *in vitro* observation that Loc1p is removed from the She2p-*ASH1* complex by She3p, I compared the distribution of Loc1p in wild type and *she3Δ* cells by fluorescence microscopy. For this a yeast strain where Loc1p was tagged with GFP at its C-terminus was generated. *SHE3* was deleted from this strain to create a *she3Δ*. If She3p is involved in displacing Loc1p from the complex, then the nucleolar localization of Loc1p should be altered in *she3Δ* cells. A nuclear pore marker, Nup-120 tagged with mCherry was used to stain the nuclear pores and the demarcation of the nucleus (Aitchison et al, 1995; Skruzny et al, 2009).

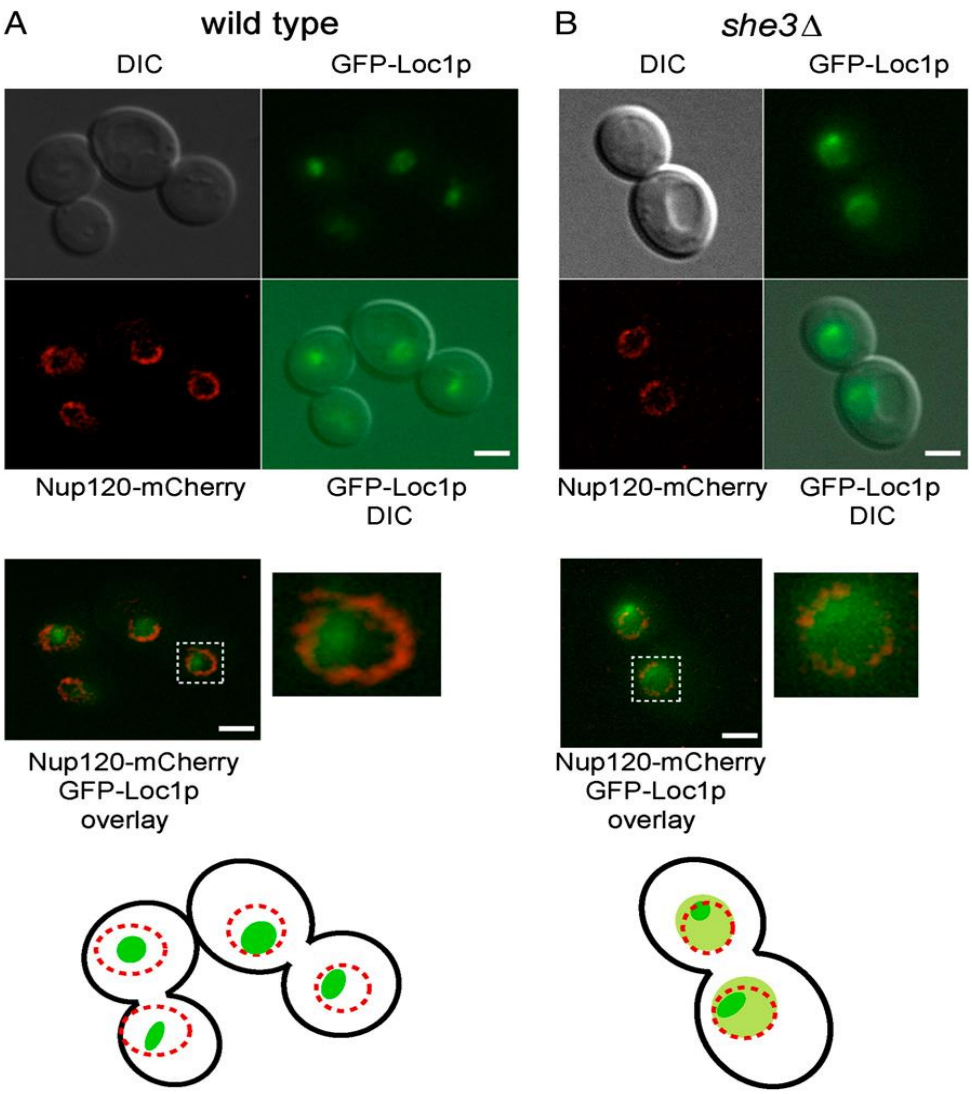


Figure 25: She3p displaces Loc1p from the *ASH1*-mRNP complex in vivo: Localization of Loc1p in dividing wild type (A) and *she3Δ* cells (B). Loc1p is visualized via GFP tag (green) and the nuclear envelope by Nup120-mCherry (red). (A and B, Middle) Overlays of Nup120-mCherry and GFP-Loc1p with magnifications of single cells. (Scale bars, 1 µm.) In wild-type cells (A), GFP-Loc1p shows a distinct localization in the nucleolus. In the *she3Δ* strain (B), part of the nucleolar Loc1p becomes dispersed in the nucleoplasm, demonstrating that cytoplasmic She3p influences the nuclear distribution of Loc1p. Occasionally, we also observed GFP-Loc1p signal in the cytoplasm of *she3Δ* cells. (Bottom) Cartoons illustrate the differences in Loc1p distribution. Image and text from Niedner et al, 2013.

Since *ASH1* mRNA localization takes place during anaphase (Bobola et al, 1996), only anaphase cells were investigated. In addition, *ASH1* was overexpressed from a 2µ plasmid, with the rationale that by increasing the amount of *ASH1* mRNA, more Loc1p

can be re-routed away from ribosome biogenesis inside nucleolus (Urbinati et al, 2006), such that the observable effect would be enhanced.

Images were acquired for GFP and mCherry in the separate channels. To enhance the mCherry signal, the images were deconvolved before overlaying. In the wild type cells, Loc1p localized to the nucleolus as expected (Figure 25A) but in cells lacking *SHE3*, Loc1p was dispersed throughout nucleoplasm (Figure 25B). This observation together with the *in vitro* data from Prof. Niessing group indicated that She3p strips off Loc1p from the complex before the nuclear export.

3. Discussion

3.1. Khd1p and mRNA targeting to endoplasmic reticulum (ER)

3.1.1. Links between ER and mRNA localization

The ER is a versatile organelle in the eukaryotic cell and performs vital functions like translocation of proteins across the ER membrane, integrating proteins into the lipid bilayer, folding and modification of proteins, cell signaling and lipid synthesis (Voeltz et al, 2002).

A conserved mechanism for targeting the mRNAs to the ER is based on the signal recognition particle (SRP) and its receptor. The SRP-mediated pathway or co-translational targeting is the standard mechanism for delivering the membrane and secretory proteins to ER. In this pathway, SRP binds to the hydrophobic signal sequence as it emerges out from the ribosome. Binding of SRP to the signal sequence arrests the translational elongation transiently, transports the complex containing mRNA-ribosome-nascent chain to the membrane where it comes in contact with the SRP receptor. This interaction releases the SRP from mRNA-ribosome-nascent chain complex and resumes the translation (Keenan et al, 2001). Post-translational targeting occurs in the case of tail-anchored proteins. A transmembrane domain is present at the carboxy-terminal of these proteins that emerges from ribosome exit tunnel only upon completion of the translation and they are recognized in the cytosol by GET pathway (Denic, 2012; Cui & Palazzo, 2014). The specific cytosolic chaperons (Get3p/TRC40) recognize the transmembrane domain at the carboxy-terminus of the protein and the protein is subsequently targeted to Get1/Get2p receptor complex in the ER membrane (Hegde & Keenan, 2011; Hermesh et al, 2014). But there are strong evidences for the existence of a translation-independent mechanism in order to get targeted to ER. A sub-population of mRNAs employ such SRP-independent pathway for localizing to ER (Cui & Palazzo, 2014).

Early studies showed that the knockdown of SRP components had mild effect in mRNA partitioning in both yeast and human cell lines suggest the presence of SRP-independent mechanism (Ren et al, 2004; Pyhtila et al, 2008). By using various cell-fractionation

assays, studies had been carried out to analyze mRNA partitioning between cytosol and ER compartments on a global level. All experiments identified mRNAs encoding cytosolic or nuclear proteins in the ER-bound polysomes in addition to the mRNAs encoding membrane and secreted proteins (Diehn et al, 2000; Diehn et al, 2006; Pyhtila et al, 2008; Reid & Nicchitta, 2012). Nicchitta and colleagues reported that all mRNAs, regardless of whether they code for cytosolic or secretory proteins, could be initially translated by ER-bound ribosomes (Nicchitta et al, 2005), suggesting that the initiation of translation at the cytosolic face of ER is the default pathway for all the mRNAs followed by further translation at the ER or in the cytosol depending on the mRNAs coding for membrane or cytosolic proteins. In another study, Coxsackie B virus (CBV) was used as a tool to inactivate the initiation of protein synthesis. This virus infection resulted in the cleavage of eukaryotic translation initiation factor 4G (eIF4G) and poly(A)-binding protein and thereby the translation was suppressed. Although the cytosolic translation is effectively inhibited by the viral infection, translation at the ER is mildly affected (Lamphear et al, 1993; Kerekatte et al, 1999; Lerner & Nicchitta, 2006).

Such SRP-independent targeting of mRNAs was also reported in prokaryotes. In *E.coli*, with the help of cis-acting signals that are present within the transcript, mRNAs are targeted to the plasma membrane independent of translation. For example, the localization of the *bglG* transcript to the poles requires the region encoding the RNA binding domain of BglG protein whereas for localization of the *bglF* transcript to the inner membrane requires the region encoding the membrane domain (Nevo-Dinur et al, 2011).

There are many hints for the connection between mRNA and ER in budding yeast (Schmid et al, 2006; Fundakowski et al, 2012; Genz et al, 2013; Hermesh et al, 2014). However, this phenomenon is not yeast-specific as there are also findings from other multicellular eukaryotic organisms that show a link between ER membranes and mRNA:

In *Xenopus laevis,* an interplay between ER transport and mRNA trafficking occurs during the process of embryonal development. Vg1 mRNA is transported to the vegetal pole of the embryo via ER and the corresponding VgLE binding protein, Vera (VgLE

binding and endoplasmic reticulum association) mediates this mRNA-ER co-transport process by coming in contact with ER membranes (Deshler et al, 1997; Deshler et al, 1998).

In *Ascidians* (sea squirts), maternal mRNAs like *macho1* and *HrPEM* were found to be attached to cortical ER, thereby forming the "cortical ER-mRNA domain" in the developing embryo (Prodon et al, 2005; Sardet et al, 2007).

In case of rat neurons, RNA-binding proteins like Staufen and FMRP co-purify with ER (Ohashi et al, 2002). In *Drosophila*, *gurken* mRNA was localized on the ER at the dorsal side of the oocyte (Herpers & Rabouille, 2004). Another study reported that posterior localization of *oskar* mRNA is affected in mutants that affects ER morphology (Ruden et al, 2000). In addition, the localization of *bicoid* mRNA depends on the endosomal sorting complex required for transport (ESCRT-II) (Irion & St Johnston, 2007), indicating a link between mRNA and membrane trafficking.

In case of rice, two mRNAs encoding seed storage proteins; prolamines and globulin-like glutelins – are destined to different ER sub-domains. Specific mRNA-ER targeting occurs during the segregation of these two transcripts on the ER membranes (Shewry et al, 1995; Herman & Larkins, 1999).

3.1.2. Co-migration of Khd1p with ER

Previous studies from our lab have shed more light on the relationship between ER and mRNA localization (Schmid et al, 2006; Fundakowski et al, 2012; Genz et al, 2013). The initial evidence for ER-mRNA localization relationship arose from fractionation studies on one of the extensively studied RNA-binding proteins acting during *ASH1* mRNA localization, She2p (Schmid et al, 2006; Genz et al, 2013).

Khd1p acts as a translational repressor during *ASH1* localization (Irie et al, 2002; Paquin et al, 2007). By a combination of tandem affinity purification (TAP) and microarray analysis, Hogan et al. (Hogan et al, 2008) revealed that Khd1p binds to more than 500 mRNAs. Especially mRNAs that code for cell wall and plasma membrane proteins were significantly enriched (Hogan et al, 2008). Since ER is the site for synthesis of proteins that are destined for endomembranes, plasma membrane and

secretion (High et al, 1999; Lecomte et al, 2003), we speculated that Khd1p could direct these mRNAs to the ER. A significant fraction of Khd1p was found to co-fractionate with the ER marker protein Sec61p in a sucrose density gradient assay. However, in a global study of protein localization in yeast, GFP-tagged Khd1p was shown to localize mainly in the cytosol (Huh et al, 2003). My observation that Khd1p co-fractionates with ER during cell fractionation is not necessarily contradictory since only a sub-population of Khd1p co-migrates with ER (Figure 9, fractions 1-4). The major present at the top of the gradient represents the cytosolic fractions. This distribution is similar to She2p that was shown to bind membranes (Schmid et al, 2006; Genz et al, 2013).

Membrane binding was supported by co-floatation analysis, where a purified protein is incubated with synthetic liposomes and analysed for its direct association or interaction with the liposomes (Section 5.3.7) (Genz et al, 2013). Purified GST-Khd1p interacts with artificial liposomes, whereas GST alone was unable to float with liposomes (Figure 11B). Similar observations were made for She2p (Schmid et al, 2006; Genz et al, 2013). However, She2p could bind to liposomes in a curvature-dependent manner and thereby has the ability to recognize membrane shape (Genz et al, 2013), whereas such curvature-dependency was not observed for Khd1p, suggesting that the binding mode to membranes varies between the two proteins. Furthermore, the same study (Genz et al, 2013) suggests a specific partner on the ER which mediates this specific interaction between ER and She2p. The specificity of interaction has not yet been addressed for Khd1p and needs further investigation.

Although Khd1p has not been observed at the ER so far, this does not exclude a temporary association. For example, *in vivo* She2p has been visualized at the bud tip (Bohl et al, 2000), at the cytoplasm (Du et al, 2008) or in the nucleus (Du et al, 2008; Shen et al, 2009) but has recently also been detected at the ER. For this, an assay based on Bamboo mosaic potexvirus protein Triple gene block 3 protein (Tgbp3) was implemented in our lab (Genz et al, 2013). The advantage of this assay is that it can detect the protein-ER association even if it is transient. A transmembrane domain is required for the C-terminal region of Tgbp3 (comprising 25-52 aminoacids) for ER association (Wu et al, 2011). I checked whether Khd1p could take on the role of this transmembrane domain and found the fusion protein (GFP-Khd1-Tgbp3) in foci that overlapped with the ER in the mother cell and in the bud (Figure 13B). As mentioned

above, similar co-localization of foci to the ER has been reported for She2p (Genz et al, 2013; Hermesh et al, 2014) and other Tgbp3 fusion proteins (Wu et al, 2011). Interestingly, two other cytoplasmic proteins that I tested, Zwf1p and Scd6p, failed in targeting the viral peptide to the ER. This indicates that the observed targeting effect is not a general mechanism but specific to Khd1p. In addition, co-fractionation of GFP-Tgbp3-Khd1p fusion protein with Sec61p in the fractions consisting of heavy membranes, including ER supports the *in vivo* observation. Taken together, both the *in vivo* and *in vitro* results suggest that Khd1p has the ability to associate temporarily with ER membranes, just like She2p (Schmid et al, 2006; Genz et al, 2013).

3.1.3. Role of Khd1p in mRNA distribution and targeting

Khd1p is known to bind a significant fraction of transcripts encoding cell wall and plasma membrane proteins (Hasegawa et al, 2008; Hogan et al, 2008) and since these messages are generally translated at the cytoplasmic face of ER, I checked for the enrichment of these transcripts in membrane fraction and also the effect of Khd1p on these targets. (For short description about these targets, refer Table 1).

Among the studied targets of Khd1p, the distribution between membrane fraction and cytosol fraction was clearly affected for two mRNAs *MID2* and *SLG1* in the absence of Khd1p. In case of *MID2*, the difference in the membrane/cytosol distribution also suggests direct binding of this mRNA by Khd1p. Hasegawa and co-workers mapped the binding region of Khd1p on several mRNA targets, including *ASH1* and *MID2*. Khd1p associates with the E1 localizing element of *ASH1* mRNA (Irie et al, 2002; Paquin et al, 2007), whereas in the case of *MID2* they identified two Khd1p-binding stretches in the coding region (nucleotides 140-319 and 389-589). By using MEME (Bailey & Elkan, 1994), Khd1p was found to bind to CNN triplet repeats. Altogether, the effect of Khd1p in *MID2* distribution to membrane fraction suggests that Khd1p is involved in guiding this mRNA to ER. The difference in membrane-cytosol distribution of *SLG1* between wild type and *khd1Δ* could be explained similarly.

In case of other targets (*DSE2*, *EGT2*, *SCW11* and *KRE1*) there is no difference in distribution indicating that these mRNAs are taking another pathway to reach the membrane fraction, independent of Khd1p. Most likely this is SRP-dependent pathway

(see Introduction). At this moment, it is not known whether Khd1p directly binds these mRNAs similar to *ASH1* and *MID2*.

Is *MID2* targeting to ER independent of SRP-pathway? The answer is yes. My results suggest that Khd1p could be a new addition into a group of proteins that are involved in mRNA targeting to ER. Based on the results that showed the difference in membrane/cytosol distribution pattern of *MID2* in wild type yeast cell and cell lacking *KHD1*, I looked for the possibility of co-translational targeting of *MID2*. Removal of the start codon did not affect the distribution of this transcript. It was still significantly enriched in the membrane fraction of wild type cells indicating that its association with the membranes does not occur co-translationally (Figure 15B). Upon deletion of Khd1p, *MID2noATG* behaves like normal *MID2* by shifting its distribution to the cytosol (Figure 16B). Taken together, it can be concluded that the targeting of *MID2* transcript to the membrane fraction is dependent on Khd1p, but not on the SRP-pathway. Involvement of RNA-binding proteins in SRP-independent targeting of mRNAs has been recently reported. ER localization of two transcripts was found to be independent of both 3'UTR and signal sequence. The first one being *USE1*, encodes an essential SNARE protein (Dilcher et al, 2003) and other one is *SUC2* coding for the secreted enzyme invertase (Carlson et al, 1983). Upon deletion of two RNA-binding proteins, She2p and Puf2p, localization of these two transcripts to the ER was affected, indicating that RNA-binding proteins are involved in mRNA targeting mechanism to ER (Kraut-Cohen et al, 2013). In case of mammalian cells, the p180 protein was found to be essential for the ER anchoring of *ALPP* and *CALR* mRNAs in a translation-independent manner (Cui et al, 2012). p180 is an abundant membrane-bound protein and its lysine rich domain is essential for RNA-binding. Over-expression of p180 showed an enhancement in ribosome-independent association of *t-ftz* mRNA with the ER (Cui et al, 2012). However, there is no homologue for p180 in yeast.

Khd1p is a three KH-domain containing protein (Irie et al, 2002) (see introduction). The importance of these RNA-binding KH-domains is underscored by the fact that a single mutation of the conserved isoleucine residue (Ile304Asn) in the KH-domain 2 of FMR1 (Fragile X mental retardation protein) results in severe mental retardation. (Chen et al, 1997; Valverde et al, 2008). In my work, a mutant version of Khd1p carrying a point mutation in the KH-domain 1 (I59R) had shown reduced affinity towards RNA targets

(Figure 20B). Similar reduction in RNA binding by Khd1p had been shown earlier for the KH-domain 3 mutation (L284R) by co-localization studies and electrophoretic mobility shift assays (Hasegawa et al, 2008). Imaging studies showed that only the mutation in KH-domain 1 failed to target the viral peptide Tgbp3 to ER, whereas the mutations in other two KH-domains (KH-domain 2 and KH-domain 3) still could target the viral peptide similar to wild type Khd1p. This suggests that the mutation in KH-domain 1 might have an influence on Khd1p's association with ER in addition to its RNA-binding activity. Co-fractionation of Khd1p (I59R)-Tgbp3 fusion protein with cytosolic marker Pgk1p further supported the *in vivo* data suggesting that all the three KH-domains of Khd1p should be functional for its membrane association. This observation is quite contrasting with respect to She2p, as it had been recently shown that mutations that affect the RNA-binding activity of She2p like the deletion of helix E, did not disturb the co-migration of She2p with the ER (Genz et al, 2013).

The effect of mutation (I59R) in KH-domain 1 on both membrane association and RNA-binding activity could be explained by the fact that the isoleucine (I59) is present near the conserved signature sequence of KH-domains, GXXG loop, which connects the α1-helix and the α2-helix (Grishin, 2001; Valverde et al, 2008). Mutation in this particular residue might therefore distort the KH-domain 1 structure and thus abolish the affinity for both membranes and RNA. Although, having less affinity towards RNA, surprisingly the Khd1pI59R did not affect distribution of *MID2* mRNA. *MID2* was clearly distributed to the membrane fraction in all three combinations of mutations in KH-domains (D1 mutant – I59R; D1+D2 mutant – I59R+I190R; D1+D2+D3 mutant – I59R+I190R+L284R). There was no significant difference observed in membrane/cytosol distribution except in triple mutant case, where the distribution is mildly affected. In contrast, upon deletion of *KHD1* completely, *MID2* distribution shifts to the cytosol (Figure 14A). As shown in previous reports, *MID2* is one of the bud-tip localized mRNAs in yeast (Shepard et al, 2003). Also, it is one of the common targets for both Khd1p (Hasegawa et al, 2008) and She2p (Shepard et al, 2003). A very recent study has identified that also Puf6p binds *MID2* as one of its target mRNAs (Shahbabian et al, 2014). Therefore, the observation of *MID2* persisting in the membrane fraction in the Khd1p mutant extracts (all the three cases) could be explained by the presence of other RNA-binding proteins (She2p and Puf6p) that might function

together with Khd1p in mRNA-ER association. A possible scenario would be that the presence of She2p and Puf6p could partially compensate the reduced RNA-binding activity of Khd1p and thereby targeting *MID2* to the membrane fraction, whereas the complete loss of Khd1p might lead to disassembly of a co-complex of the RNA-binding proteins and loss of RNA binding or ER targeting (Figure 26).

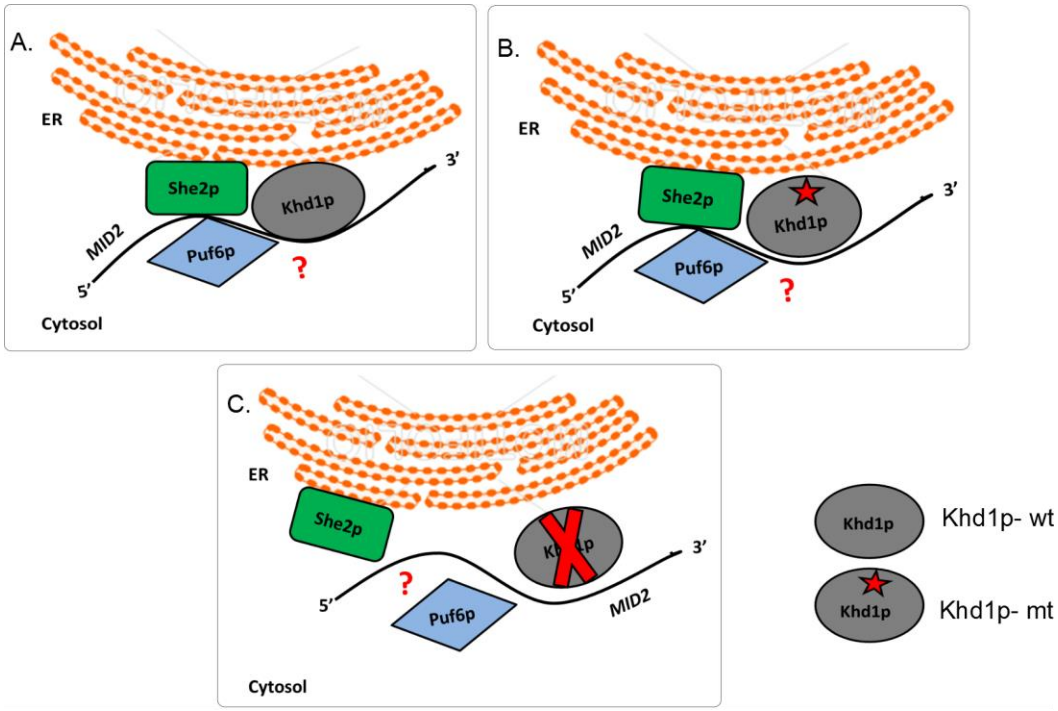


Figure 26: Model for *MID2* mRNA targeting to the ER: *MID2* is a known target mRNA for three RNA-binding proteins (She2p, Khd1p and Puf6p). Association of She2p with the ER has been previously reported. Results from this study suggest the association of Khd1p with ER. Targeting of *MID2* to the ER might therefore involve a complex containing She2p, Khd1p and Puf6p (A). In the case of Khd1p mutants (Khd1p-mt), where the RNA-binding is impaired, She2p and Puf6p could be involved in targeting *MID2* to the ER (B), whereas the deletion of Khd1p might fail to form the co-complex and thereby resulting in loss of *MID2* targeting to the ER.

3.1.4. Khd1p supports asymmetric Mid2 protein distribution

The protein levels of *ASH1* and *SRL1* are strongly reduced upon over-expression of Khd1p indicating that it is involved in translational regulation of these two mRNAs (Irie et al, 2002; Hasegawa et al, 2008). On the other hand, the level of Mtl1 protein was increased. In *khd1Δ* cells, Mtl1p level was strongly reduced, whereas the protein expression of other bud-localized mRNAs did not change significantly (Hasegawa et al, 2008). This indicates that the function of Khd1p on translation and protein levels varies

among its targets. In my case, immunoblotting against Mid2p (Figure 17A) did not reveal any significant change in the expression between wild type and *khd1Δ* cells. As previously mentioned, *MID2* is a bud-tip localized mRNA (Shepard et al, 2003). Since, the technique (immunoblotting) would not differentiate between the Mid2p protein expression in mother cell and daughter cell (bud); *in vivo* imaging was carried out to monitor potential differences in protein levels. I found that the expression of Mid2p in the bud is weaker in *khd1Δ* cells when compared to wild type. This suggests that localization of *MID2* mRNA or local translation in the bud might be affected in *khd1Δ* cells. In case of wild type cells, Mid2p-GFP signal is intense at the cell periphery which is consistent with the previous observations (Ketela et al, 1999; Huh et al, 2003).

In order to investigate whether loss of Khd1p has decreased sensitivity to cell wall stress similar to loss of Mid2p, a calcofluor white assay was used. Calcofluor white is a fluorescent dye which prevents chitin microfibril assembly by intercalating with the nascent chitin chains (Roncero & Duran, 1985). Since Mid2p is known to affect chitin synthesis during stress conditions, *mid2Δ* cells showed higher resistance to calcofluor white at the concentration of 20µg/ml (Ketela et al, 1999). Cells lacking Khd1p did not show any resistance to calcofluor white at the same concentration suggesting that function of Mid2p is not sufficiently disrupted to allow the cells to survive. Furthermore, the double deletion (*khd1Δmid2Δ*) did not exacerbate the growth phenotype of *mid2Δ* on calcofluor white plates. Altogether, these results suggest that Khd1p-mediated ER targeting of *MID2* RNA has little or no effect on Mid2p function.

3.1.5. Other potential candidates for ER association

Hogan et al. in their study reported over 12,000 mRNA-RBP interactions in the yeast transcriptome. They reported that most mRNAs were bound by at least one or more of the estimated 600 RNA-binding proteins. Their data suggested the interaction of each mRNA with different RNA-binding proteins during its life-time and thereby a high complexity in post-transcriptional gene regulation in the cell (Hogan et al, 2008). Although the molecular mechanism and function of most mRNA-RBP interactions are largely unknown, their study showed that individual RNA-binding proteins interact with mRNAs encoding functionally related proteins. For example, RNA-binding proteins Puf1p, Puf2p, Khd1p, Ssd1p, Mrn1p and Scp160p associate with mRNAs that code for

cell wall proteins and plasma membrane proteins. RNA-binding proteins Puf3p, Nsr1p, Pab1p, Npl3p and Nrd1p bind to mRNAs encoding subunits of mitochondrial ribosome (Hogan et al, 2008).

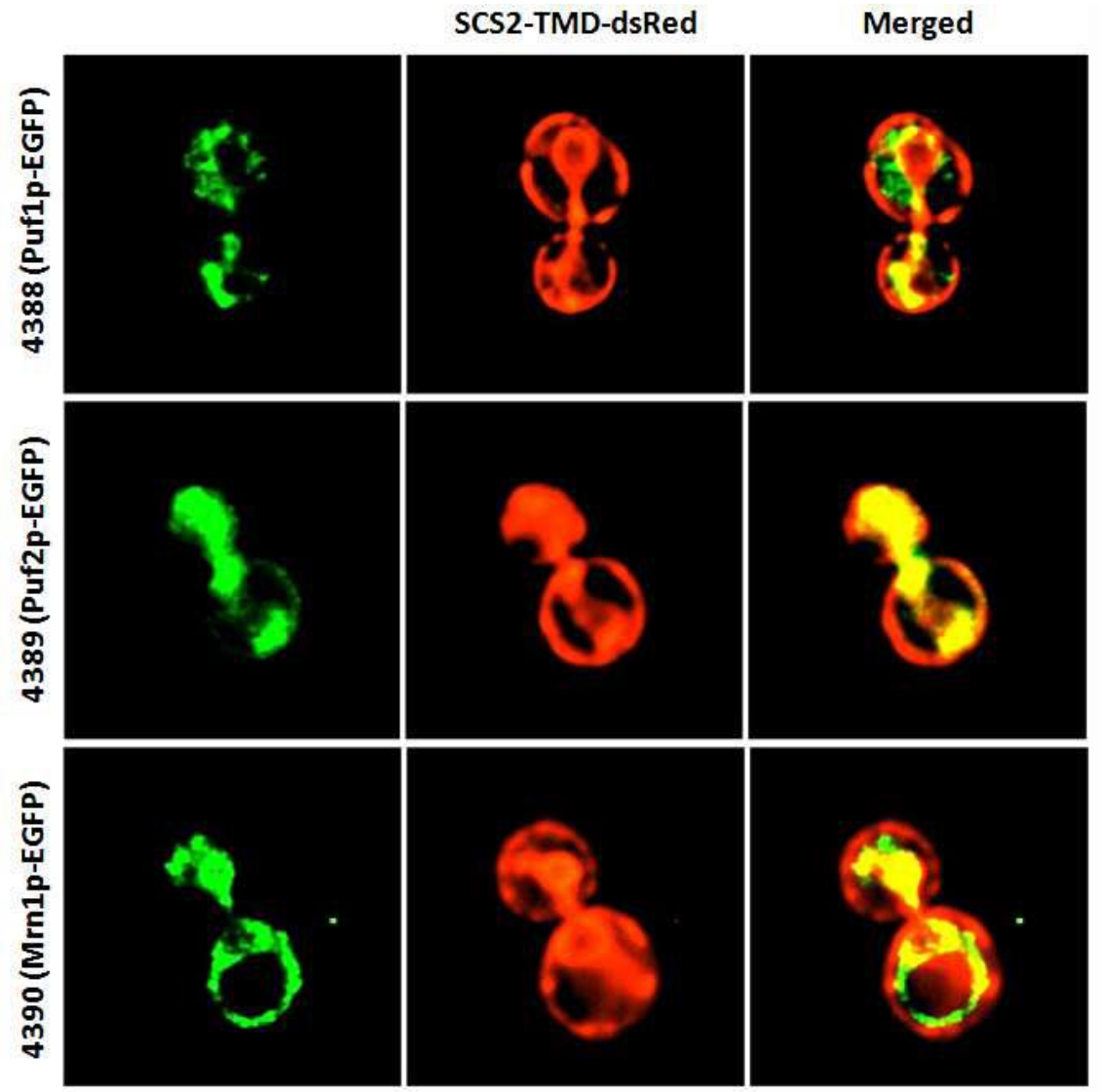


Figure 27: Co-localization of Puf1p, Puf2p and Mrn1p with ER: Representative images of cells expressing the eGFP tagged proteins of interest and plasmid expressing SCS-TMD-dsRed. Images from GFP and Texas red channels are shown separately and also as merged image. Yellow coloured areas in the merge image indicate the co-localization of the proteins of interest with ER. Image from Sabina Vejzovic's Bachelor thesis, AG Jansen, 2013 (Vejzovic, 2013).

At the start of this project, I aimed to investigate the association of other RNA-binding proteins like Puf1p, Puf2p and Mrn1p with ER since they were also interacting with

mRNAs encoding membrane proteins (Hogan et al, 2008). Together with a bachelor student (Sabina Vejzovic), I performed preliminary studies on the membrane association of Puf1p, Puf2p and Mrn1p. Of the three proteins, majority of the Puf2p-GFP signal co-localized with the ER (Figure 27; Vejzovic, 2013). Later, the Gerst group has published that Puf2p indeed has a role in ER targeting of two mRNAs, namely *USE1* and *SUC2* (Kraut-Cohen et al, 2013), whereas further study on other candidates, Puf1p and Mrn1p will be of future interest for investigating their role in ER-mediated targeting of their bound transcripts.

My results on Khd1p add up to an extending list of RNA-binding proteins in budding yeast that have the potential to associate with ER. Whereas co-localization of two RNA-binding proteins, Scp160p and Bfr1p with ER depends on ongoing translation of their target mRNAs (Frey et al, 2001; Lang et al, 2001), association of She2p with ER is independent of polysomes (Schmid et al, 2006; Aronov et al, 2007). For Whi3p, the mechanism of association with ER is currently unknown (Colomina et al, 2008). Although my findings on the loss of ER association upon mutation of an RNA-binding domain of Khd1p suggests that RNA-binding is required for Khd1p, future studies are necessary to reveal the mechanism of Khd1p-ER association.

Presence of Khd1p but not its translation is needed for the co-fractionation of *MID2* mRNA with membranes, providing additional evidence for an SRP-independent targeting of mRNAs to ER in budding yeast. However, despite many reports for such a mechanism from various model organisms, the exact molecular role of SRP-independent targeting of mRNAs to ER is not yet understood. Hopefully, there will be a better understanding of this pathway in future.

3.2. Loc1p

3.2.1. Role of Loc1p in *ASH1*-mRNP assembly

Although the localization process of *ASH1* has been extensively studied, the exact molecular mechanisms guiding the assembly of *ASH1*-mRNP are still not clear. Loc1p is one of the RNA-binding proteins involved in *ASH1* localization. It is a nucle(ol)ar protein and known to function in the biogenesis of large ribosomal subunits (Harnpicharnchai et al, 2001; Urbinati et al, 2006). In the case of *ASH1* localization, it

might act as a repressor of translation (Long et al, 2001; Komili et al, 2007) Similar to Loc1p, the mainly nucleolar protein Puf6p is not only involved in ribosomal biogenesis (Li et al, 2009) but, during *ASH1* localization, promotes translational arrest of the mRNA (Gu et al, 2004; Deng et al, 2008).

At the early stages of *ASH1*-mRNP assembly, a premature mRNP complex, *ASH1*-She2p travels through the nucle(ol)us to reach the cytoplasm (Kruse et al, 2002; Shen et al, 2009). The nuclear assembly of this pre-mRNP is essential for efficient cytoplasmic mRNA localization and translational repression. Such nuclear trespassing has been observed in many cell types. For example, mammalian Staufen2 also traverse through the nucleolus before its nuclear export (Macchi et al, 2004). The interaction of Loc1p and Puf6p to *ASH1* is diminished when She2p is blocked from entering the nucleus (Shen et al, 2009) suggesting the interdependence between these protein-RNA interactions. Although Loc1p is strictly a nuclear protein, it is still needed for the proper transport process in the cytoplasm.

Previous studies had shown that the interaction between She2p and *ASH1* is weak *in vitro* (Muller et al, 2009; Muller et al, 2011). In addtion, She2p is co-transcriptionally recruited onto *ASH1* mRNA (Shen et al, 2010; Muller et al, 2011) and exported to the cytoplasm in an RNA-dependent manner (Kruse et al, 2002). Therefore, the formation of a stable complex involving Loc1p, She2p and *ASH1* is expected to occur in the nuclear compartment prior to nuclear export of *ASH1*- She2p.

In vitro experiments from Niessing group had shown the specific interaction between She2p and Loc1p in a RNA-independent manner. In addition, Loc1p is essential for the stable and specific interaction between She2p and *ASH1* suggesting the co-operative and mutual recruitment of Loc1p and She2p on the RNA, which is consistent with earlier observations that *ASH1* mRNA fails to co-immunoprecipitate with Loc1p in a strain expressing nuclear localization-deficient She2p (Shen et al, 2009). These findings are a hint that Loc1p might come in contact with She2p in or at the nucleolus, where Loc1p is enriched. Another possibility is that Loc1p binds to She2p even before its association with *ASH1* mRNA and helps She2p to tether stably on to *ASH1* mRNA in the nucleoplasm. This complex is not disturbed until She3p joins She2p in the cytosol. As Loc1p is not known to shuttle between nucleus and cytosol (Long et al, 2001), it

should be displaced from the complex before nuclear export. Since Loc1p contains potential nuclear-localization signals, it is possible that an efficient nuclear import recycles the protein to the nucleus. For this, Loc1p must be displaced from *ASH1* by another factor. A possible candidate for removing Loc1p from the nuclear complex (Loc1-She2p-*ASH1*) is She3p. It is a strictly cytoplasmic protein (Muller et al, 2011) and with a molecular weight of 47kDa, it is too large to diffuse freely across the nuclear pore (Gorlich & Kutay, 1999). By using UV-cross linking experiments, data from Niessing group revealed that the formation of She2p-She3p co-complex has the ability to actively displace Loc1p from a zipcode-containing RNA at near physiological concentrations (Figure 24).

In order to provide *in vivo* evidence for this hypothesis, fluorescence microscopy was used to compare the distribution of GFP-Loc1p between wild type yeast strain and a yeast strain lacking She3p. In wild type cells, Loc1p shows the characteristic 'crescent shape' of the yeast nucleolus (Huh et al, 2003; Du et al, 2008). Deletion of *SHE3* alters the localization of Loc1p. It is now found dispersed all over the nucleoplasm and also occasionally in the cytosol (Figure 25). This suggests that She3p is required for recycling or restraining of Loc1p to the nucleolus and indicates that the displacement event of Loc1p by She3p-She2p complex could take place at or close to the nuclear pore.

Together with the data from Niessing group, this allows a refined model for *ASH1* mRNA localization. A stable ternary complex consisting of She2p, *ASH1* mRNA and Loc1p assembles in nucleus and trespasses the nucleolus in a Loc1p-dependent manner. This Loc1p-dependent piggybacking of She2p and *ASH1* mRNA could be the possible mechanism to bring the *ASH1* mRNP close to (nucleolar) Puf6p, thereby allowing it to bind the complex. *ASH1*-mRNP is exported to the cytosol, where the She3p and Myo4p join, thus forming the mature transport complex. Both *in vitro* and *in vivo* data suggests that the co-complex of She2p-She3p strips off Loc1p from the nuclear complex before the export process. Altogether, we report a model for the formation of stable complex in the nucleus and the role of Loc1p in assembly and reorganization of nuclear *ASH1*-mRNP (Figure 28).

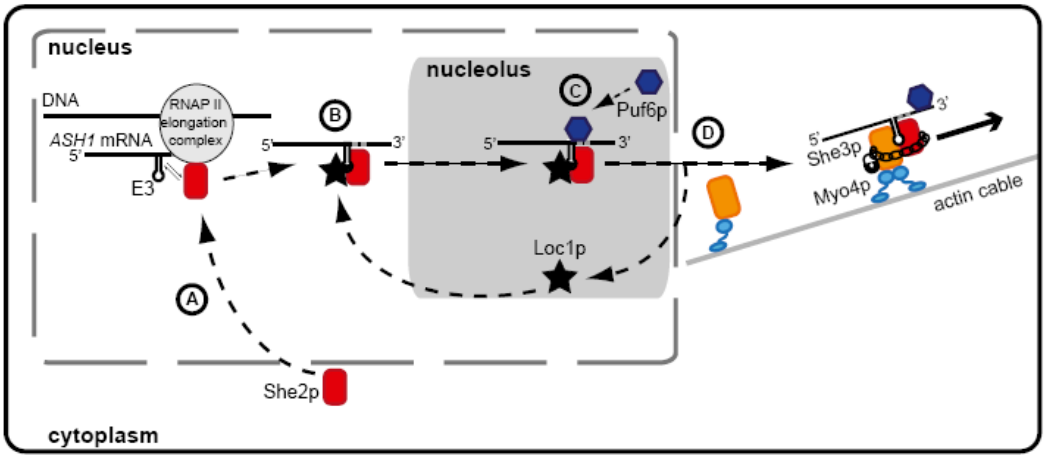


Figure 28: Model for sequential binding and release of Loc1p to the *ASH1* mRNP: She2p enters the nucleus and binds co-transcriptionally to the *ASH1* mRNA (A). Loc1p joins this sub-complex, which leads to stabilization of She2p on the *ASH1* mRNA. Loc1p contains nucleolar localization signals and is strongly enriched in the nucleolus, suggesting that it recruits She2p into the nucleolus through their direct interaction (B). No direct interaction between Puf6p and other core factors could be detected in this study. Nevertheless, in vivo the *ASH1* mRNA association of Puf6p depends on She2p. It is likely that Loc1p and She2p are required to recruit *ASH1* mRNA into the nucleolus, where Puf6p can interact with the transcript (C). Because Loc1p is not part of the cytoplasmic transport complex, it has to be removed from the complex before or during nuclear export (D). The synergistic cocomplex of She2p and She3p outcompetes Loc1p from the mRNP and ensures that Loc1p is absent from the cytoplasmic *ASH1* transport complex Image and text adapted from Nieder et al, 2013.

4. Materials

4.1 Antibodies

Immunoblotting was carried out using Millipore SNAP i.d. system according to the manufacturer instructions. Antibodies were diluted in PBS-T (1x PBS + 0.1% Tween-20) depending on size of the blot and the blot holder. For Single blot holder, 3 ml of antibody solution was used and for double blot holder, 2 ml antibody solution was used.

Name	**Host**	**Dilution (SNAP i.d.)**	**Company**
Primary Antibodies			
Anti-Actin	Mouse	1: 3000 (Western)	Millipore
Anti-Dpm1	Mouse	1: 2000 (Western)	Molecular Probes
Anti-GFP	Mouse	1: 1000 (Western)	Covance
Anti-GST	Rabbit	1: 3000 (Western)	Sigma-Aldrich
Anti-HA	Rat	1: 3000 (Western)	Roche
Anti-myc	Mouse	1: 3000 (Western)	Roche
Anti-Pgk1	Mouse	1: 2000 (Western)	Gift from M. Seedorf, Heidelberg.
Anti-Sec61	Rabbit	1: 2000 (Western)	Gift from M. Seedorf, Heidelberg.
Anti-She2	Rat	1: 2000 (Western)	Jansen Lab
Secondary Antibodies			
Anti-mouse	Sheep	1: 5000 (Western)	Amersham
Anti-rabbit	Donkey	1: 5000 (Western)	Amersham
Anti-rat	Rabbit	1: 5000 (Western)	Dianova

4.1 Chemicals

Acros Organics	D-Glucose monohydrate
AppliChem	Bacto peptone Bacto yeast extract Ethylene Glycol Tetraacetic acid (EGTA)
Biomol	Salmon sperm DNA (ssDNA)

Carl Roth®	Acetic Acid Agarose Amino acids Ammonium persulfate (APS) Bactoagar D-Galactose Dithiothreitol (DTT) Ethylene Diamine Tetraacetic acid (EDTA) Isopropyl-β-D-thiogalactopyranoside (IPTG) Lithium acetate dihydrate (LiOAc) Phenylmethylsulfonyl fluorid (PMSF) Polyethylene glycol 4000 (PEG) Potassium Acetate (KOAc) Potassium dihydrogen phosphate KH_2PO_4 Potassium phosphate K2HPO4 Sodium dodecyl sulfate (SDS) Sorbitol Sucrose Tris base TritonX-100
Fermentas	Deoxynucleotide triphosphate Mix (dNTPs)
Fluca™	Nucleic acids
FORMEDIUM™	Yeast nitrogen base
GENAXXON	GelRed
MERCK	Imidazol Magnesium acetate $MgOAc_2$) Sodium hydroxide (NaOH)
Riedel-de Haën®	Hydrochloric acid (HCL)
Roche	Nonidet P-40 (NP40)

Sigma-Aldrich®	Chloroform Ethanol absolute (ETOH) Isopropyl alcohol β-mercaptoethanol (β-ME) Methanol (METOH)
Serva	Coomassie Brilliant Blue R-250

4.2 Commercial Kits

QIAGEN/ GENAXXON	Gel Extraction Kit Miniprep Kit

4.3 Consumables

BECKMAN COULTER	Thinwall Ultra-Clear™ Thinwall Polyallomer Tubes
BIO-RAD	Poly-Prep® Chromatography Columns
Carl Roth® GmbH & Co.KG	cover slips object slides glass beads (diff. sizes) Rotilabo® - disposable cuvettes
Clontech	TALON ® metal affinity beads
GE Healthcare	Amersham Hybond™ PVDF Transfer Membrane Amersham Hyperfilm™ ECL Gluthation Sepharose Fast Flow beads
KGW-Isotherm GmbH	Dewar Flasks cylindrical Type C
KONTES Glass Co., NJ	Dounce Homogenizer 40 ml
MILLIPORE™	Immobilon-FL Transfer Membrane

Pechiney Plastic Packaging	PM966-Parafilm M®
SARSTEDT AG & Co.	Centrifuge tube 50 ml, 15 ml CryoPure Storage Systems High quality pipette tips High quality serological pipettes Micro Tubes SafeSeal 2 ml, 1.5 ml PCR reaction tubes
Thermo Scientific	Menzel-Gläser® Diagnostika Pierce ECL Western blotting Substrate
WHEATON Industries Inc., USA	Dounce Homogenizer 7 ml
Hellma®	Quarz cuvettes

4.4 Enzymes

Agilent Technologies	Herculase ® II Fusion DNA Polymerase
Amsbio®	Zymolyase® 20T, Zymolyase® 100T
Axon	Taq Polymerase
Biomol®	Lysozyme
Fermentas	Calf Intestine Alkaline Phosphatase (CIP) Conventional restriction enzymes FastDigest® restriction enzymes PageRuler^{TM} Prestained Protein Ladder RiboLock^{TM} RNase Inhibitor RNase A T4 DNA Ligase

4.5 Equipments

Alpha Innotec	FluorChem® FC2
Analyticjena	FlexCycler
Bandelin	SONOREX Super RK 100

BECKMAN COULTER	Ultracentrifuge rotor SW 40 Ti Ultracentrifuge rotor TLA-120.2
BIO ER	ThermoCell Mixing Block MB-102
BIO-RAD	Mini-PROTEAN® Tetra Electrophoresis System PowerPac™ HC Power Supply Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell
BRANSON	Sonifier® S-250A
Eppendorf	Centrifuge 5415R Centrifuge 5702 Centrifuge 5810R Pipettes P1000, P200, P20, P10
GE Healthcare	GeneQuant™ 100 Spectrophotometer
IBS- INTEGRA Biosciences	Pipetboy acu
IKA®	RH basic 2 Vibrax® VXR basic
INFORS HT	Minitron
LI-COR Biosciences	Odyssey® Infrared Imaging System
MILLIPORE™	SNAP i.d.™
Scientific Industries, Inc.	Vortex-Genie® 2
Stuart® Scientific	Gyro rocker
Thermo Scientific	Forma 900 Series

4.5 Oligonucleotides

RPO	**Name**	**Sequence**	**Purpose**
168	pRS_F	GGTAACGCCAGGGTTTTCCC	Sequencing
169	pRS_R	ACTCATTAGGCACCCCAGGC	Sequencing
3674	pGEX-GST-seqfor	TGCTGAAAATGTTCGAAGATCGT	Sequencing
2551	pGEX_Tev_seq_r	CATGTGTCAGAGGTTTTCACCGTC	Sequencing

3017	MID2_600bp_rev	CGGTTGCTGATGGTGTGTA	Cloning
3024	Kan_ORF_rev	GAAACGTGAGTCTTTTCCTTACCC	Colony PCR
4417	Khd1_Sma1_F	TACTcccgggATGTCACAGTTCTTCGAAGCTGCTACTCCCG	Tgb3p cloning
4418	Khd1_EcoR1_R	TGCAgaattcCGACTGCTCCTCTTTGTTATCA	Tgb3p cloning
4445	AcoI SmaI_F	TACTCCCGGGATGCTGTCTGCACGTTCTGCCATCAAG	Tgb3p cloning
4446	AcoI EcoR1_R	TGCAGAATTCTTTCTTCTCATCGGCCTTAA	Tgb3p cloning
4455	zwf1_SmaI_F	TACTCCCGGGATGAGTGAAGGCCCCGTCAAATTCG	Tgb3p cloning
4456	zwf1_EcoR1_R	TGCAGAATTCATTATCCTTCGTATCTTCTGGCTTAGTC	Tgb3p cloning
4488	MID2_qpcr_F	ATGGAGCAAAGCTCCCTTTT	q_PCR
4489	MID2_qpcr_R	GCTCTCCACCGCTATTGGTA	q_PCR
4490	DSE2_qpcr_F	AACTTTGGCGTCATCGTCTT	q_PCR
4491	DSE2_qpcr_R	GCATGGATGTCGTTGCTCTA	q_PCR
4492	SLG1_qpcr_F	GGGAACAAGACAGGATGGAA	q_PCR
4493	SLG1-qpcr_R	AAGAGGGCCCGTGATTAGAT	q_PCR
4494	EGT2_qpcr_F	CATTCCAAAAAGCAACAGCA	q_PCR
4495	EGT2_qpcr_R	TGGAACTTGTTGGCAATGAA	q_PCR
4558	Scd6 SmaI_F	TACTcccgggATGTCGCAGTACATCGGTAAAACTATTTCTTTAATC	Tgb3p cloning
4559	Scd6 EcoRI_R	TGCAgaattcAAATTCAACGTTGGAAGGAGGTTGCGAA	Tgb3p cloning
4571	Khd1 L284R_F	GAATGAACAGAATTAAGAATaggAAAACTTTCACAAAAACC	Site-directed mutagenesis

		AA	
4572	Khd1_L284R_R	TTGGTTTTTGTGAAAGTTTTcctATTCTTAATTCTGTTCATTC	Site-directed mutagenesis
4573	khd1_EcoRI_F	TGCAGAATTCATGTCACAGTTCTTCGAAGCTGCTACTCCC	cloning
4574	khd1_XhoI_R	TAAGctcgagCTACGACTGCTCCTCTTTGTTATC	cloning
4575	MID2_BamHI_F	CTCAggatccTTCAATGAGTTCCACGACGCAC	cloning
4576	MID2_XmaI_R	ACTGcccgggCGTATATCGCGTGAATGCTCTT	cloning
4587	MID2_SLIC_F	ggtggcggccgctctagaactagtggatccTTCAATGAGTTCCACGACGCAC	cloning
4588	MID2_SLIC_R	gacggtatcgataagcttgatatcgaattcCGTATATCGCGTGAATGCTCTT	cloning
4605	MID2triplecl_F	ACTCaagcttTCTTTCACAACCAAGAATAG	cloning
4606	MID2triplecl_R	ACTCaagcttACTATGATTTGCGTATTTTATATGTCC	cloning
4607	Khd16HA1mt_F	CATTGAAAGAGGCTGCCAAGaggATTGGCACTAAGGGCTCCAC	Site-directed mutagenesis
4608	Khd16HA1mt_R	GTGGAGCCCTTAGTGCCAATcctCTTGGCAGCCTCTTTCAATG	Site-directed mutagenesis
4609	Mid2_qp_F	CGTCTTCTACGCAAACGTCA	q_PCR
4610	Mid2_qp_R	GGAGGTTGAAGGTGCAGAAG	q_PCR
4611	Scw11_qp_F	GGCAACACTCAAAGTCAGCA	q_PCR
4612	Scw11_qp_R	GATTCGGTGGACTCCTGAGA	q_PCR
4615	Gph1_qp_f	ATCTGGCCACCCATGAATTA	q_PCR
4616	Gph1_qp_R	GCTAAAGAAGCCGACGTTTG	q_PCR

4617	zwf1_qp_F	TGGGTGACCATTCCAACTTT	q_PCR
4618	zwf1_qp_R	GGTAAATTTCCGGTGTTGGA	q_PCR
4619	Kre1_qp_F	TCCATGTGGGTCACTGTTGT	q_PCR
4620	Kre1_qp_R	AGGGAGAAGCGACTGTTACG	q_PCR
4690	KHI190R_F	ATCAATAAGCACGGCGTTAA GAGAGTGGCTTCCAAGGACT TCTTA	Site-directed mutagenesis
4691	KHI190R_R	TAAGAAGTCCTTGGAAGCCA CTCTCTTAACGCCGTGCTTAT TGAT	Site-directed mutagenesis

4.6 Plasmids

RPJ Plasmid	**Short description**	**Source**
327	pET-23a(+)/GST	Gift from Marc Froeschke, AG Dobberstein
1213	pFA6a-natNT2	Janke et al., 2004
1518	p416-GALS-KHD1-HA6	Stephan, AG Jansen
1745	pGEX-5X-3-KHD1-6xHis	Paquin et al., 2007
1846	p416-ADH1-GFP-TGB3(25-52)	Wu et al., 2011
1867	pRS 314 Nup 120-mcherry	Gift from Prof. Ed Hurt, Heidelberg.
1871	p416-ADH1-GFP-Khd1-TGB3	This study
1877	p416-ADH1-GFP-Zwf1-TGB3	This study
1878	p416-ADH1-GFP--Khd1 (D1 mt) -TGB3	This study
1881	p416-ADH1-GFP--Khd1 (D2 mt) -TGB3	This study
1885	p416-ADH1-GFP--Khd1 (D3 mt) -TGB3	This study
1882	p416-ADH1-GFP--Scd6 -TGB3	This study
1883	pGEX-4T-3 Khd1-D1mutant	This study
1884	pGEX-4T-3 Khd1- D2 mutant	This study
1887	pRS313-MID2	This study

1902	pGALs-Khd1-HA (D1 mutant)	This study
1903	pRS313-Mid2 (noATG)	This study
1913	pGALs-Khd1-HA (D1+D2 mutant)	This study
1914	pGALs-Khd1-HA (D1+D2+D3 mutant)	This study

4.7 Strains

4.7.1. Escherichia coli (E.coli) Strain

Strain	**Genotype**
Top 10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G (Invitrogen™)

4.7.2. Yeast *(S.cerevisiae)* Strains

RPY	**Genotype**	**Origin**
90	Mat alpha, his3, leu2, ade2, trp1, ura3, HO-ADE2, HO-CAN1, she3::URA3	Jansen et al, 1996
358	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+	Rothstein and Sherman, 1980
359	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+	Rothstein and Sherman, 1980
2368	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ khd1::HIS3MX6	Andrade Goncalo AG Jansen
2662	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ Khd1-9xMyc::K.l.TRP	Andrade Goncalo AG Jansen
3506	MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3, GAL, psi+ LOC1-yeGFP::HIS3MX6	Stephan Jellbauer AG Jansen
4290	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+MRN1-6xHA::HIS3MX6	This study
4295	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112,	This study

	his3-11,15, ura3, GAL, psi+MRN1-TAP::HIS3MX6	
4296	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+MRN1-6xHA::HIS3MX6	This study
4297	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+MRN1-9xmyc::HIS3MX6	This study
4298	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+Puf1-9xmyc::HIS3MX6	This study
4324	MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3, GAL, psi+ LOC1-yeGFP::HIS3MX6, she3::URA3	This study
4349	MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3, GAL, psi+ LOC1-yeGFP::HIS3MX6, she3::URA3 +RPJ plasmid 88 (YEplac181-ASH1)	This study
4354	MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3, GAL, psi+LOC1-yeGFP::HIS3MX6 + RPJ plasmid 88 (YEplac181-ASH1) +RPJ plasmid 1868 (pRS 314 Nup 85-mCherry)	This study
4355	MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3, GAL, psi+ LOC1-yeGFP::HIS3MX6, she3::URA3 + RPJ plasmid no. 88 (YEplac181-ASH1) +RPJ plasmid 1868 (pRS 314 Nup 85-mCherry)	This study
4358	MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3, GAL, psi+, LOC1-yeGFP::HIS3MX6 + RPJ plasmid 88 (YEplac181-ASH1) +RPJ plasmid 1867 (pRS 314 Nup 120-mcherry)	This study
4359	MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3, GAL, psi+ LOC1-yeGFP::HIS3MX6, she3::URA3 + RPJ plasmid no. 88 (YEplac181-ASH1)	This study

	+ RPJ plasmid no. 1867 (pRS 314 Nup 120-mCherry)	
4379	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ + RPJ plasmid 1871 p416-ADH1-GFP--Khd1-TGB3(25-52)	This study
4393	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ + RPJ plasmid 1871 p416-ADH1-GFP--Khd1-TGB3(25-52) + RPJ plasmid 1870 (HDEL DsRed)	This study
4394	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ +RPJ plasmid 1877 p416-ADH1-GFP--Zwf1--TGB3(25-52) + RPJ plasmid 1870 (HDEL DsRed)	This study
4436	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ + RPJ plasmid 1882 p416-ADH1-GFP--Scd6--TGB3(25-52) + RPJ plasmid 1870 (HDEL DsRed)	This study
4437	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ +RPJ plasmid 1878 p416-ADH1-GFP-Khd1-D1-mutant--TGB3(25-52) +RPJ plasmid 1870 (HDEL DsRed)	This study
4439	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ +RPJ plasmid 1881 p416-ADH1-GFP-Khd1-D2-mutant--TGB3(25-52) +RPJ plasmid 1870 (HDEL DsRed)	This study
4495	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-	This study

	11,15, ura3, GAL, psi+ mid2::KANMX6	
4496	MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ +RPJ plasmid 1885 - p416-ADH1-GFP-Khd1 domain3 mutant--TGB3(25-52) +RPJ plasmid 1870 (HDEL DsRed)	This study
4500	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ mid2::KANMX6 + RPJ plasmid 1887 (pRS313-MID2)	This study
4501	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ + RPJ plasmid 1518 (p416-GALS-Khd1-6xHA)	This study
4506	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ mid2::KANMX6 + RPJ plasmid 1903 (pRS313-MID2(noATG))	This study
4537	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ khd1::HIS3MX6 +RPJ plasmid 1518 (p416-GALs-Khd1-6xHA)	This study
4538	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ khd1::HIS3MX6 + RPJ plasmid 1902 (p416-GALs-Khd1(I59R)-6xHA domain1 mutant)	This study
4543	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ mid2::KANMX6, khd1::natNT2	This study
4544	MATa his3D1 leu2D0 met15D0 ura3D0 Mid2-GFP::His marker (from invitrogen).	Invitrogen
4545	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ mid2:: KANMX6, khd1:: natNT2 + RPJ plasmid 1887 (pRS313-MID2)	This study
4546	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-	This study

	11,15, ura3, GAL, psi+ mid2:: KANMX6, khd1:: natNT2 +RPJ plasmid 1903 (pRS313-MID2noATG)	
4553	MATa his3D1 leu2D0 met15D0 ura3D0 khd1::natNT2 Mid2-GFP::HIS3MX6	This study
4563	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3- 11,15, ura3, GAL, psi+ khd1::HIS3MX6 + pGALS Khd1-HA D1+D2 mutant	This study
4564	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3- 11,15, ura3, GAL, psi+ khd1::HIS3MX6 + pGALS Khd1-HA D1+D2+D3 mutant	This study

5. Methods

All standard biochemical, microbiological and molecular biology techniques were based on Sambrook J. et al, 2001. Commercial Kits were used according to manufacturer's instructions. Standard buffers were prepared as described (Sambrook, 2001).

5.1 Basic methods

5.1.1 Agarose gel electrophoresis and gel extraction

In most cases, 0.8% agarose gels were used. Agarose was mixed with TAE buffer to the desired concentration, and then heated in a microwave oven until agarose was completely melted. After cooling the solution to about 60°C, 2-3 µl of 10,000x GelRed were added and the gel poured into a casting tray containing a sample comb. The gel was allowed to solidify at room temperature. For PCR product purification, bands were cut out using 70% intensity of the UV illumination at 365 nm. DNA extraction was carried out by Genaxxon gel extraction miniprep kit following the manufacture´s manual.

5.1.2 Restriction digestion

For the digestion of vector and insert DNA the following reaction mixture were used. In most cases, Fast digest enzymes (Fermentas) were used.

Insert DNA mix (20 µl):	*Vector DNA mix (30 µl):*
2 µl (0.2-0.5 µg)	2 µl (~1 µg)
2 µl 10x buffer	2 µl 10x buffer
1 µl enzyme 1	1 µl enzyme 1
1 µl enzyme 2	1 µl enzyme 2
14 µl sterile water	24 µl sterile water

The mixtures were then incubated at the temperature and time suggested by manufacture´s manual. Vector DNA was gel-purified whereas the insert DNA was PCR purified using Genaxxon kits.

5.1.3 Ligation of DNA fragments

The molar ratio between vector and insert DNA should be 1:2 to 1:3 depending on the size of the insert and the presence of blunt ends in the insert. Online ligation calculator tool was used for setting up the ligation reaction (http://www.insilico.uni-duesseldorf.de/Lig_Input.html)

The ligation of digested DNA was performed as the follows:

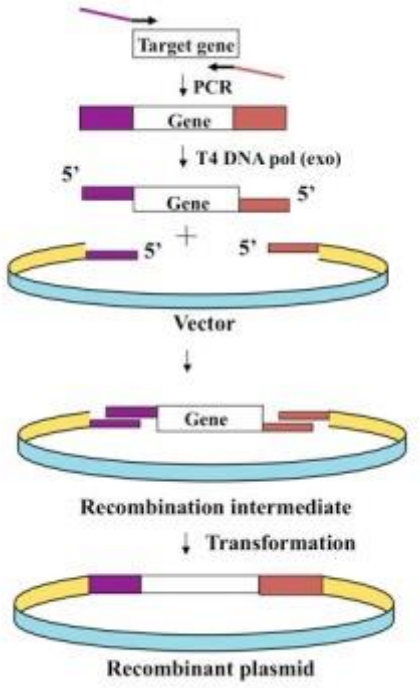
Ligation mix (20 µl):

10x ligation buffer 2 µl
T4 DNA ligase 2 µl
DNA vector 2 µl (~100-200 ng)
DNA insert 7 µl (~450-600 ng)
Sterile water 7 µl

The ligation mix was incubated overnight at 22°C for 30 min (5-10 min in case of 2x Quick Ligation buffer) and afterwards directly used for *E. coli* transformation or stored at -20°C.

5.1.4 SLIC (Sequence and Ligation Independent cloning)

For the construction of some plasmids, a cloning method called SLIC (Sequence and Ligation Independent Cloning) was used. SLIC allows the joining of either single or multiple fragments in a single reaction by using *in vitro* homologous recombination and single strand annealing (Li & Elledge, 2007) (Figure 29). Briefly, the vector was cut open by appropriate restriction enzymes whereas the insert was generated by PCR using primers which contain 30bp homology to the vector and the site of restriction enzyme. Vector was gel purified using Genaxxon gel extraction kit and insert was digested with 1 µl of Dpn1 enzyme (for 50 µl PCR reaction) for 1 h followed by PCR purification and eluted in 20 µl - 30 µl of TE buffer. T4 DNA polymerase reaction was carried out for both vector and insert to create single strand overhangs. 15 µl of vector/insert was used for T4 DNA polymerase reaction which was carried out at room temperature for 30 min. 1µl dNTP was added to stop the reaction and the enzyme was heat inactivated at 65°C for 10 min. Annealing was carried out by mixing vector and insert either with or



without RecA enzyme at 22°C for 30 min. Molar ratio between vector and insert is 1:3. Further, the annealing mixture was used for *E.coli* transformation.

Figure 29: Schematic representation of SLIC cloning method: Target gene was amplified by PCR using primer containing homologous regions to vector. Both vector and insert were subjected to T4 DNA polymerase reaction to create overhangs. Annealing is carried out either by presence or absence of RecA enzyme. Image taken from Li & Elledge, 2007.

5.1.5 SDS-PAGE and Western blotting

5.1.5.1 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Mini-Protean® Terra System (BIO-RAD). In most cases, 8% and 10% separating gel was used with 4% stacking gel. Samples were heated at 65ºC for 10 min before loading. The samples were run at 100 V for 20 min and afterwards at 200 V until the bromophenolblue reaches the bottom of the gel. The gel was removed and either prepared for Coomassie staining or for Western blotting.

5.1.5.2 Coomassie staining

For the staining of polyacrylamide gels using Coomassie Brilliant Blue R-250 the gel was immersed in Coomassie staining solution and warmed in microwave oven for 40 sec to 1 min, then kept on shaker for 30 min. The Destaining was done until no more background was visible (usually overnight on a shaker).

5.1.5.3 Western blotting

Protein transfer onto a polyvinylidene fluoride transfer membrane (PVDF; GE Healthcare) was performed using a semi-dry procedure with the Trans-Blot® SD Semi-Dry Transfer Cell (BIO-RAD). Both gel and Whatman-paper slices were covered with transfer buffer (0.25 M Tris, 1.92 M glycine, pH 8.6 ± 0.2) for 20 min. The PVDF membrane was activated by shortly rinsing in methanol. The transfer sandwich was prepared (2 thin Whatman-papers slices, membrane, gel, 2 thin Whatman-papers slices) and the proteins were transferred 45 min at 12 V. The membrane was blocked with 0.1% blocking buffer (0.1% non-fat dry milk powder in PBS-Tween 20 (PBST) when directly using the SNAP i.d.^{TM} system for immunoblotting. The detection antibodies are listed in section 4.1. After blocking, the membrane was incubated with primary antibody for 10 min at room temperature, and then washed 3x with PBST to remove the unbound primary antibodies. Subsequently, incubation with the secondary antibody was performed for 10 min at room temperature. The membrane was washed 3x with PBST. Visualization of proteins was performed using an enhanced chemiluminescence kit (ECL-kit, GE Healthcare). Signals were detected using the Fluorchem® FC2 (Alpha Innotech) chemiluminescence imaging system.

5.2 *E. coli*-specific techniques

5.2.1 Preparation of chemical competent *E. coli* cells

To prepare chemical competent *E. coli* cells, the strain (*E. coli* top 10 or *E. coli* BL21) was grown in 50 ml Luria Bertani (LB) medium (0.8 g bacto peptone, 0.5 g yeast extract, 0.25 g NaCl pH 7.4) at 37°C in a shaking incubator until the optical density of 0.4-0.6 at 600 nm was reached. Then the cells were chilled on ice for 15 min and harvested by centrifugation (10 min, 4,500 rpm, 4°C). The pellet was resuspended in 1 ml ice-cold 50 mM calcium chloride solution. After spinning down the cells again, the pellet was resuspended in 2 ml of ice cold calcium chloride solution. Finally, cells were pelleted and resuspended in one fourth of the cell volume of 0.1 M $CaCl_2$/10% glycerol. Aliquots of 50 µl were shock-frozen in liquid Nitrogen (LN_2) and stored at -80°C.

5.2.2 Preparation of plasmid-DNA

The preparation of highly pure bacterial plasmid DNA (e.g. for restriction analysis or further transformation into yeast cells) was performed with the Qiagen Miniprep/Midiprep Kit according to the manufacturer's protocol.

5.2.3 Transformation of competent *E. coli* cells

Top 10 *E. coli* competent cells were thawed on ice for about 5-10 min. Then the DNA (5-50 ng of plasmid DNA or 20 µl of the ligation mix) were added to 50 µl of cells and incubated on ice for 10-15 min, then plated on antibiotic containing LB agar plates and incubated at 37°C overnight. After one day the grown bacterial colonies were picked and inoculated in 5 ml of LB medium containing antibiotics (e.g. 100 µg/ml Ampicillin) and grown for 8 h or overnight (37°C, shaker) in order to perform a plasmid preparation (Miniprep).

5.2.4 Colony PCR

Bacterial colony PCR was carried out by picking up a bacterial clone with the help of a 10 µl pipette tip and mixing it with the mastermix containing the components required for the PCR reaction. The components of the mastermix are as follows:

Reaction mixture (total volume 50 µl):		*Program:*
10x PCR buffer S with 15mM $MgCl_2$ (Genaxxon)	2.5 µl	95°C 5 min
25mM dNTPs	0.5 µl	95°C 30 sec
Forward + Reverse primer	2 + 2 µl	55°C 30 sec
25mM $MgCl_2$	2 µl	68°C (~1 min/Kb)
Taq polymerase	0.2 µl	Step 2-4: 30 cycles
Sterile water	15.8 µl	68°C 10 min
		4°C ∞

5.2.5 Sequencing and analysis

Sequencing was done by MWG Eurofins, Munich. The plasmids and primers were prepared and/or chosen as recommended by the company. The analysis of the

sequences was done by using the software *A Plasmid Editor* (Ape; http://www.biology.utah.edu/jorgensen/wayned/ape/).

5.2.6. Glycerol stocks

For long term storage of bacteria, 500 µl of a cell suspension from an overnight culture were taken in a cryo-tube and mixed with 50% (w/v) glycerol to a final concentration of 15% (w/v). The cells were shock-frozen with LN_2 and stored at -80°C.

5.2.7. Purification of GST-tagged proteins

5.2.7.1. Harvesting and Induction

The GST-Khd1p was recombinantly expressed from the *E. coli* strain BL21 (Rosetta 2) using pGEX-5X-*KHD1*-6xHis. 800 ml of LB medium containing 100 µg/ml ampicillin were inoculated with an overnight culture to a starting OD_{600} of 0.2 and grown at 37°C until an OD_{600} of 0.6. 200 ml cold LB medium were added, and expression was induced by the addition of 1 mM IPTG for 3 hours at 30°C. Cells were harvested by centrifugation at 7800g for 10 min. After washing with 30 ml cold water, cells were pelleted by centrifugation at 7800g for 10 min. The pellet was frozen in LN_2 and stored at -20°C.

5.2.7.2. Lysis of the cells

For lysis, cells were resuspended in 30 ml of lysis buffer, (PBS-TritonX100 0.1%, 1 M NaCl, 2 mM DTT, 1 mg/ml lysozyme, antiproteases cocktail) and incubated for 30 min on ice. Subsequently, the cells were lysed by sonication (6 times for 1 min with a pause of 2 min on ice). The lysate was rotated for 30 min at 4°C and cleared by centrifugation for 30 min at 15,000 g to yield the supernatant with the overexpressed soluble protein.

5.2.7.3. Affinity purification

The GST-Khd1-6xHis fusion protein was purified using 500 µl of a slurry of Glutathione Fast Flow Sepharose (Amersham Pharmacia). Beads were pre-washed 3 times with 10 ml of lysis buffer and then added to the lysate. Binding was performed by rotating at 4°C for one hour. After binding, beads were washed with 10 ml PBS. The GST-fusion protein was eluted with 1 mM reduced glutathione (freshly prepared) in

PBS. The eluate was stored in aliquots at -80°C. Same method was used for the purification of GST alone.

5.3 *S. cerevisiae*-specific techniques

5.3.1 Polymerase chain reaction

Gene modification (tagging or deletion) in yeast was done by PCR-based methods (Knop et al, 1999; Janke et al, 2004) (Figure 30; schematic illustration). Two different polymerases were used. First, *Taq* Polymerase (Axon) which is very thermostable and second, Herculase® (Agilent Technologies) for its proof-reading ability and accuracy. PCR products were purified using the Genaxxon PCR purification Kit.

5.3.1.1 Standard analytical PCR

The standard analytical PCR reaction was prepared as follows:

Reaction mixture (total volume 50 µl):		*Program:*
10x Axon Taq buffer	5 µl	95°C 2 min
dNTPs (10 mM)	2.5 µl	95°C 1 min
$MgCl_2$ (25 mM)	1 µl	50°C 30 sec (depending on
Template (50-150 ng/µl)	1 µl	primer length and GC
content)		
Forward primer (10 pmol/µl)	1 µl	72°C 2 min (~1 kb product
Reverse primer (10 pmol/µl)	1 µl	size/sec)
Sterile water	37.5 µl	Step 2-4: 35cycles
Axon Taq Polymerase	1 µl	72°C 10 min
		4°C ∞

5.3.1.2 Yeast colony PCR

In order to check the transformants (after tagging a gene or knocking out a gene), a single yeast colony was scraped off from a fresh plate and suspended in 100 µl of 200 mM lithium acetate containing 1% SDS. The mixture was then incubated at 70°C for 15 min. After adding 300 µl of 100% ethanol and vortexing, the DNA was collected

by centrifugation at 13,000 rpm for 3 min. The precipitated DNA was dissolved in 100 µl TE and cell debris were spun down at 13,000 rpm for 1 min. 1 µl of the supernatant was used for PCR (Looke et al, 2011).

Reaction mixture (total volume 50 µl)		*Program*
10x Axon Taq buffer	5 µl	95°C 2 min
dNTPs (25 mM)	0.5 µl	95°C 1 min
$MgCl_2$ (25 mM)	2.5 µl	50°C 30 sec (depending on
Template	1-10 µl	primer length and GC content)
Forward primer (10 pmol/µl)	1 µl	72°C 2 min (~1 kb product
Reverse primer (10 pmol/µl)	1 µl	size/sec)
Distilled water	38.5-28.5 µl	step 2-4 35x
Axon Taq Polymerase	0.5 µl	72°C 10 min
		4°C ∞

5.3.1.3 Tagging PCR

For the generation of linear inserts (e.g. for yeast transformation) PCR with Herculase® was used. Primers were designed according to Janke et al. (Janke et al, 2004) (Figure 31).

Reaction mixture (total volume 50 µl):		*Program:*
5x Herculase buffer	10 µl	95°C 3 min
dNTPs (10 mM)	2 µl	95°C 1 min
$MgCl_2$ (25 mM)	1 µl	75°C 1 min (depending on
Template (50-150 ng/µl)	1 µl	primer length and GC
content)		
Forward primer (10 pmol/µl)	1 µl	68°C 2 min (~1 kb product
Reverse primer (10 pmol/µl)	1 µl	size/30 sec)
Distilled water	33 µl	step 2-4 35x
Herculase®	1 µl	68°C 10 min
		4°C ∞

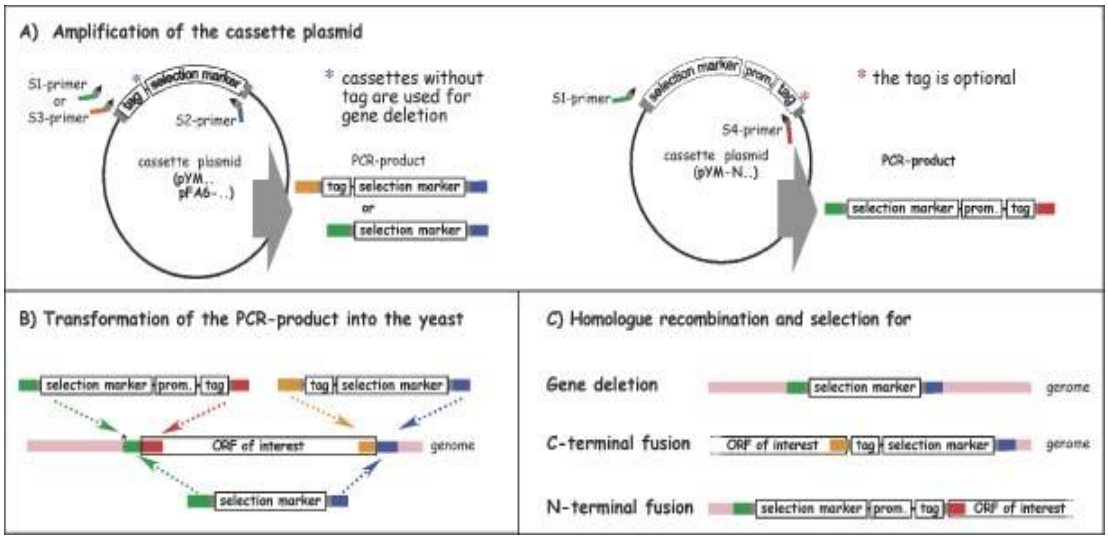


Figure 30: Schematic representation of the principle of genomic manipulation of yeast strains using PCR-based strategies: The plasmid contains a cassette, which consists of a selection marker, and additional sequences, which can be promoter sequences and/or sequences that encode for a tag (e.g. GFP). The S1-, S2-, S3- and S4-primers allow amplification of cassettes (A) and targeting of the respective PCR product to the desired genomic location (B), which becomes defined by the overhangs provided by the S1- S2-S3- and S4-primers (see colour-encoded primers in the figure: the same colours indicate homologous sequences). Depending on whether a gene deletion, a C- or a N-terminal gene fusion should be performed, specific pairs of the S1- S2-, S3- or S4-primers are used to amplify the cassette. Upon transformation, an integration of the cassettes into the yeast genome occurs due to homologous recombination (C). Image and text from Janke et al, 2004.

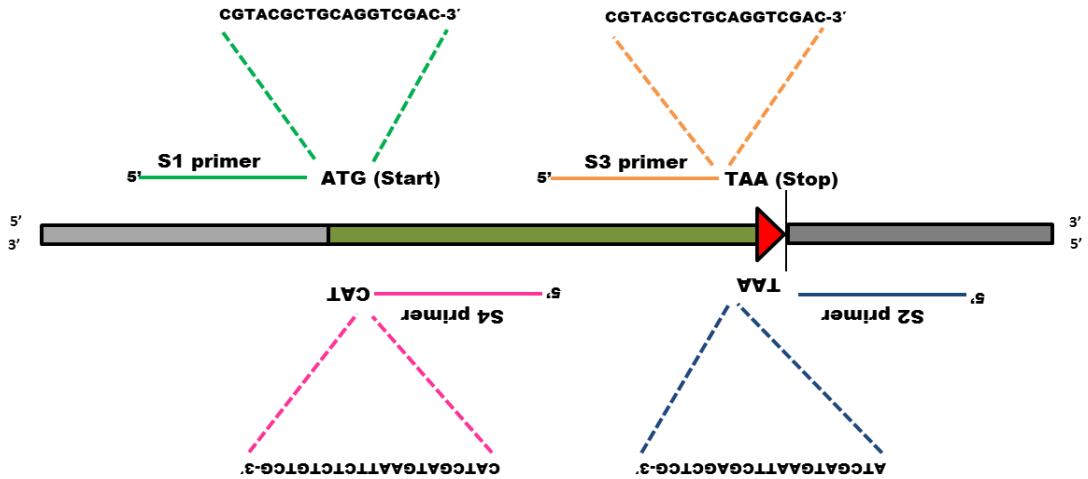


Figure 31: Primer designing strategy for gene deletion, C- or N-terminal tagging of genes: S1-primer, 45–55 bases upstream of the ATG (including ATG = start codon) of the gene, followed by 5′-CGTACGCTGCAGGTCGAC-3′; S2-primer, the *reverse complement* of 45–55 bases downstream of the STOP-codon including STOP) of the gene, followed by 5′-ATCGATGAATTCGAGCTCG-3′; S3-primer, 45–55 bases before the STOP-codon (excluding STOP) of the gene, followed by 5′-CGTACGCTGCAGGTCGAC-3′; S4-primer, the*reverse*

complement of 45–55 bases downstream of the ATG (start-codon) of the gene (excluding ATG), followed by 5′-CATCGATGAATTCTCTGTCG-3′.

5.3.2 Transformation of yeast cell

5.3.2.1 One-step yeast transformation with plasmids

Transformation of yeast with plasmids was done as described (Chen et al, 1992). Cells were inoculated in 5 ml of medium and incubated overnight or used directly by scraping off a fresh plate (one big loop was dissolved in water or medium). The cell suspension (1 ml) was pelleted by centrifugation and the pellet was resuspended by brief vortexing in 100 µl of one-step buffer (0.2 M lithium acetate, 40% PEG 4000, 100 mM DTT). An aliquot of single stranded salmon sperm DNA (ssDNA) was thawed on ice heated for 5 min at 95°C and directly cooled down on ice to make the DNA linear. The amount of 20 µg ssDNA and 100 ng up to 500 ng plasmid DNA was added to the cell-buffer suspension and mixed by vigorous vortexing. Then the mixture was incubated for 30 min at 45°C. After heat shock, cells were resuspended in 1 ml YEPD, resuspended and centrifuged at 10,000 rpm for 15 sec. The supernatant was discarded and the pellet was carefully resuspended in 1 ml YPED medium. 100-200 µl of the suspension were plated onto the corresponding selective plate and incubated at 30°C. Colonies appeared after 2-3 days.

5.3.2.2 High efficiency yeast transformation

Yeast was transformed with PCR products for genomic integration via homologous recombination by high efficiency transformation method (Schiestl & Gietz, 1989). A single colony of yeast was inoculated in 5 ml YEPD medium and rotated on a wheel overnight at 30°C. Subsequently 50 ml of YEPD medium was inoculated with an overnight culture to an OD_{600} of 0.2-0.3. Cells were allowed to grow for two generations until OD_{600} reaches 1. The cells were then harvested by centrifugation at 2,500 rpm for 5 min. The supernatant was discarded, the cells were washed with 25 ml water and the pellet was resuspended in 1 ml of freshly prepared 100 mM lithium acetate. After centrifugation at top speed for 30 sec, the supernatant was removed and the cells were resuspended in 400 µl of 100 mM LiOAc. An aliquot of ssDNA was thawed, boiled for 5 min at 95°C and quickly chilled on ice. For one transformation mix, 50 µl of the

suspension were transferred into a new 1.5 ml reaction tube. After centrifugation lithium acetate was completely removed and the transformation mix (240 µl (50% w/v) PEG, 36 µl of 1 M lithium acetate, 25 µl of ssDNA (2 mg/ml), 50 µl water with 0.1-5 µg linear DNA) was added to the cell pellet as the following:

The mixture was vortexed vigorously until the pellet was completely mixed. The mixture was then incubated for 30 min at 30°C, heat-shock treated for 20-25 min at 42°C and centrifuged at 8,000 rpm for 30 sec. The transformation mix was removed and the pellet resuspended in 1 ml of water or medium. 100-300 µl of the suspension was plated onto the corresponding selective medium.

In the case of Kanamycin and cloNAT markers, cells were recovered on YEPD plate overnight at 30°C and replica plated on to YEPD+G418 (geneticin) or YEPD+cloNAT (nourseothricin) plates. Colonies appeared after 2-3 days.

5.3.3 Preparation of genomic DNA from yeast

In order to use genomic DNA as a template for sequential changes (e.g. tagging or knockout constructs) by PCR, genomic DNA was extracted from yeast as the follows. A big loop of yeast was scraped off the fresh plate and resuspended in 1 ml of water and centrifuged at 1,000 rpm for 5 min at 4°C. The pellet was resuspended in 200 µl of DEB (DNA extraction buffer, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0 and 0.2% TritonX-100). 500 µl of acid-washed sterile glass beads (0.4 mm diameter) and 200 µl of Phenol/Chloroform/Isoamylalcohol (24:24:1) were added to the cells and the mixture vibraxed for 5 min (top speed, 4°C). The lysate was mixed with 200 µl of TE, vortexed for 30 sec and centrifuged at 12,000 rpm for 10 min at RT. After transferring 200 µl of the supernatant to a new Eppendorf cup filled with 750 µl of 100% ethanol and after vortexing the mixture, the genomic DNA was precipitated by centrifugation at 12,000 rpm for 10 min at RT. Ethanol was removed by aspiration. The tube was centrifuged again to remove residual ethanol. The pellet was dried for about 10 min by leaving the tube open and finally resuspended in 50 µl of TE buffer. The DNA was directly used for PCR or stored at -20°C.

5.3.4. Quick yeast alkaline lysis

One loop of cells (preferably from fresh plate) was mixed with 1 ml of water, 150 µl 1.85 M of NaOH and 7.5% β-Mercaptoethanol and chilled on ice for 15 min. 150 µl 50% TCA were added and the mixture centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was discarded and 50 µl of High Urea (HU) buffer (8 M urea, 5% SDS, 20 mM Tris pH 8.8, 1.5% DTT, bromophenolblue) was added to each pellet. The mixture was then shaked on a thermoblock for 10 min at 65°C. The supernatant was collected by centrifugation at 14,000 rpm for 1 min and could be used for further experiments (e.g. Western blotting).

5.3.5. Long term storage of yeast strains

For long term storage of yeast, 500 µl of a cell suspension from an overnight culture were mixed in a cryo-tube with 50% (w/v) glycerol to a final concentration of 25% (w/v). Alternatively, some cells were scrapped off from the fresh plate and transferred to 15% glycerol tubes. The cells were shock-frozen in LN_2 and stored at -80°C.

5.3.6. Sucrose gradient centrifugation

5.3.6.1. Spheroplasting of yeast and cell lysis

For a gentle lysis of yeast, spheroplasts generated from strain RJY2662 (Khd1-9xmyc::*K.l.TRP*) to maximally preserve subcellular integrity, e.g. protein interaction studies, were prepared as described in Schmid et al. (Schmid et al, 2006). Spheroplats are artificially generated cells without a cell wall, which will lyse after transferring into a hypotonic solution. RJY2662 cells were collected upto 530 OD_{600} units and resuspended in 5 ml isotonic buffer SB (1.4 M sorbitol, 50 mM KPi pH 7.5, 10 mM NaN_3, 0.4% β-mercaptoethanol). Cells were spheroplasted by adding 2 mg/ml Zymolyase® 20T and shaking gently for 1 h at 30°C. Intact spheroplasts were collected by pipetting carefully onto an 8 ml sorbitol cushion (1.7 M Sorbitol, 50 mM KPi pH 7.5) and centrifuged at 1,730 rpm for 10 min at 4°C. The pellet was resuspended in 6 ml of HEPES lysis-buffer (20 mM Hepes/KOH, 140 mM KOAc, 1 mM magnesium acetate, 1 mM EDTA, 100 U/ml RiboLock™ RNase Inhibitor and a protease inhibitor cocktail. Lysis was performed by using a 40 ml Dounce homogenizer applying 30-40

strokes. The cell debris was pelleted by centrifugation at 1,400 rpm for 5 x 5 min. The lysate can be used freshly or shock frozen in LN_2.

5.3.6.2. Velocity gradient centrifugation

The velocity gradient centrifugation of cell lysate (RJY 2662) on 18%-60% sucrose gradients was performed as described (Barrowman et al, 2000; Estrada et al, 2003; Schmid et al, 2006). In brief, cells corresponding to 400 OD_{600} units were harvested, spheroplasted, lysed and cleared from cell debris as described above (5.3.6.1). 1 ml of the homogenate (corresponding to 66 OD_{600} units) was loaded onto a linear 18%-60% gradient of sucrose in 20 mM Hepes/KOH pH 6.8, 140 mM potassium acetate, 1 mM magnesium acetate. After centrifugation in a SW40 swing-out rotor for 2.3 h at 17,500 rpm, the tube was punctured by a sterilized needle at the bottom and 12 fractions were collected in 2 ml Eppendorf cups. The remaining pellet was resuspended in 1 ml lysis-buffer (20 mM Hepes/KOH, 140 mM potassium acetate, 1 mM magnesium acetate, 1 mM EDTA, 100 U/ml RiboLock™ RNase Inhibitor and a protease inhibitor cocktail). Fractions were precipitated with trichloroacetic acid (TCA) as described in 5.3.6.3 and resuspended in 100 µl of 5x Laemmli sample buffer. 20 µl were used for Western blot analysis except for the top three fractions where only 7 µl were used. Western blotting was performed as described in 5.1.5.3. The blots were developed by using Fluorchem® FC2 (Alpha Innotech) chemiluminescence imaging system.

5.3.6.3. TCA precipitation from sucrose gradient samples

For the precipitation of protein from sucrose containing protein-samples, TCA and insulin was used. The samples were diluted in 1 ml of sterile water. Insulin (100 µg/2 ml) was added to the mixture and vortexed carefully. TCA was added to the reaction-tubes to a final concentration of 10%-20%, the tubes were inverted and stored overnight at 4°C for precipitation. The next day the samples were centrifuged at 13,000 rpm for 45 min at 4°C. The supernatant was removed carefully and 1 ml of ice-cold acetone was added. The tubes were inverted and centrifuged at 13,000 rpm for 45 min at 4°C. The supernatant was removed again and the pellet was dried at 50°C for 5 min. Subsequently the pellet was resuspended in 100 µl 5x Laemmli sample buffer and boiled at 65°C for 10 min while shaking. If the samples turned orange, the pH was adjusted by using 2 M Tris base. The samples were directly loaded onto the SDS-gel or

stored at 4°C. (1x Laemmli buffer - 60 mM Tris-HCl pH6.8, 2%SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenolblue)

5.3.7. Floatation assay

5.3.7.1. Preparation of ER-like, protein free liposomes:

The lipid mixture was prepared by dissolving phosphatidylcholine (Sigma Aldrich) to the final concentration of 10 mg/ml in degassed liposome buffer (20 mM HEPES pH 7.4, 100 mM NaCl) by gentle swirling at RT for about 20-30 min. The lipid emulsion was then passed 21 times through an 80 and 200 nm pore polycarbonate filter membrane mounted in an extruder ("LiposoFast-Basic", Avestin) in order to create unilamellar liposomes. The liposomes were then aliquoted, used directly or stored at 4°C for maximally one week.

5.3.7.2. *In vitro* binding and floatation of liposomes

In order to assess protein-liposome interaction, 1.36 µg (48 pmol) Khd1p or 1.45 µg GST were combined with 50 µl liposomes and 190 µl binding buffer (50 mM Hepes/KOH, 150 mM potassium acetate, 1 mM magnesium acetate, 1mM EDTA, 1mM DTT) and incubated for 15 min at RT followed by 10 min on ice to allow binding. 40 µl of the sample were kept as "input" for SDS-PAGE and Western blotting. 200 µl were mixed with 3 ml of binding buffer containing 70% sucrose to form the bottom of the gradient in a SW40 ultraclear polycarbonate tube. The sample containing cushion was then over layered with 3 ml of binding buffer containing 50%, 40% and 0% sucrose respectively. After centrifugation (23,000 rpm, 4 h, 4°C, SW40 rotor) the lipid containing fraction of about 1ml was collected at the 0%-40% sucrose interface, TCA precipitated and resolved in 45 µl HU buffer. Flotation samples together with "input" samples were analysed by SDS-PAGE and Western blotting.

5.3.8. Subcellular fractionation

Subcellular fractionation experiments were performed as described (Frey et al, 2001) with slight modifications. 180 ml cultures were grown to OD_{600} 0.5-0.6. Cells were harvested by centrifugation and washed once with sterile water. Pellets were resuspended in 1 ml of ice-cold low-salt buffer (20 mM Hepes-KOH pH 7.6, 100 mM

potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 2 mM DTT, 0.5 mM PMSF and 0.25 mM benzamidine) and vibraxed with 400 µl glass beads at top speed for 5 min (2x with 2 min pause on ice) at 4°C. Cell debris were pelleted by centrifugation (1200 rpm, 2 min) and the supernatant (whole cell extract, WCE) was transferred to a new tube. These lysates were fractionated by consecutive centrifugation steps at 6,000 g (pellet P6), 18,000 g (pellet P18) and 200,000 g (pellet P200, supernatant S200) for 20 min at 4°C. After each centrifugation step, pellets were rinsed twice with ice-cold water and resuspended in 100-150 µl of low-salt buffer. Protein concentration was measured using Bradford's reagent. For SDS-PAGE, 1x Laemmli buffer (60 mM Tris-HCl pH6.8, 2%SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenolblue) was added to the respective samples followed by Western blotting.

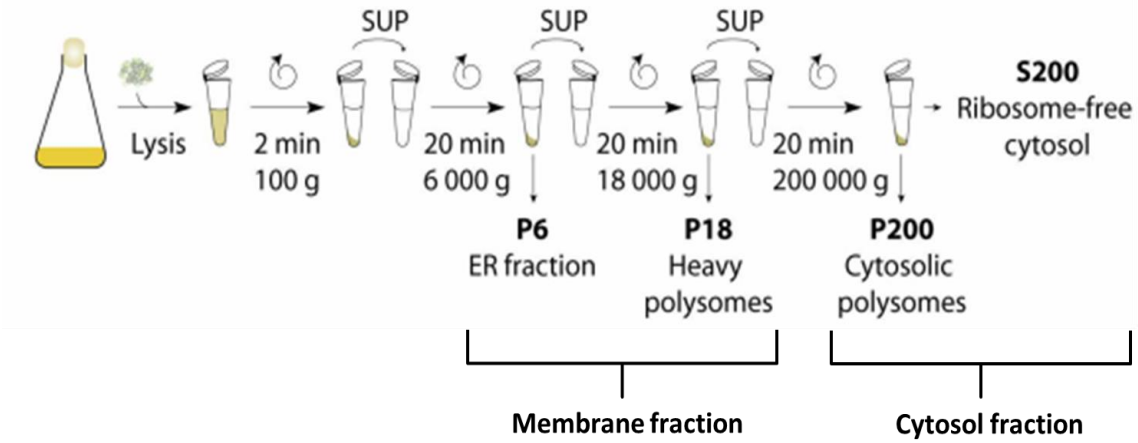


Figure 32: Simplified illustration of sub-cellular fractionation experiment: Whole cell lysate is subjected to series of centrifugation steps to yield various fractions namely P6, P18, P200 and S200. P6 and P18 fractions were pooled up to get membrane fraction and the rest of the lysate used as cytosolic fraction. Image modified from Heidrun Schreck's Ph.D thesis, AG Jansen, 2010 (Schreck, 2010).

For checking the distribution of mRNAs between membrane and the cytosol by qRT-PCR, P6 and P18 are pooled together to get membrane fraction and the cytosolic fraction was obtained by pooling fractions P200 and S200 (Figure 32). P6+P18 were resuspended in 1.5 ml of ice-cold low salt buffer. 50 µl from WCE, membrane fraction (P6+P18) and from cytosolic fraction (P200+S200) were used for RNA extraction.

RNA was extracted by adding 150 µl of DEPC water to the 50 µl aliquots. 200 µl of Phenol/Chloroform/Isoamyl alcohol (25:24:1, Roth) were added and the mixtures vortexed for 10 sec, then centrifuged at 13,000 rpm for 10 min at 4°C. RNA was precipitated from the aqueous phase with 1/10 volume of 3 M sodium acetate pH 5.2 and 3 volumes of 96% ethanol at -20°C for 30 min. Upon centrifugation (13,000 rpm, 30 min), the pellet was washed with 70% ethanol, dried at 37°C and resuspended in 30-50 µl of DEPC water. RNA concentration was measured using Nanodrop instrument.

For digestion of genomic DNA, approximately 1 µg of RNA was used together with 2 µl 10x RQ DNase buffer, 2-3 µl RQ1 DNase (Promega), and 2 µl Ribolock. The reaction mixtures were incubated at 37°C for 30 min. 1 µl of stop solution (Promega) was added and the enzyme was inactivated by heating at 65°C for 10 min. Samples were either directly used for cDNA preparation by RT-PCR or stored at -20°C.

5.3.9. cDNA preparation (RT-PCR)

DNase treated RNA samples were used for generation of cDNA. 10x RT buffer, 10x Random primers, Reverse Transcriptase enzymes (all from Applied biosystems), 25mM dNTPs were mixed with 10 µl of DNAse treated RNA to the total reaction volume of 20 µl. 1 µl Ribolock was added to the reaction. A ‘minus RT’ reaction was carried out by adding all the components except Reverse transcriptase enzyme. Reaction mixtures were placed in the PCR block.

PCR program:

25°C	10 min
37°C	2 h
85°C	10 min
4°C	∞

After PCR, cDNA samples were diluted to 1:10 and 1:100 with HPLC water for qRT-PCR analysis. For long term storage samples were stored at -20°C.

5.3.10. Quantitative real-time PCR (qRT-PCR)

Quantitative RT-PCR (qRT-PCR) was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Reaction mixtures (10 µl final volume) contained 2.5 µl of 1:10 or 1:100 dilutions of the individual cDNA samples, 5 pmol forward and reverse primers and Power SYBR Green PCR Master Mix (Applied Biosystems) as prescribed by the manufacturer. Primers were designed using Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/primer3/input.htm) and tested for mispriming or formation of primer dimers by melting curve analysis of the individual amplification products. The thermocycling profile included an initial denaturation for 10 min at 95°C, followed by 35 cycles of amplification, comprising denaturation at 95°C for 15 sec and annealing/elongation at 60°C for 1 min. After each cycle, a single fluorescence measurement was taken. For melting curve analysis of the amplification products, the temperature was increased in 0.3°C fractions to a final temperature of 95°C (continuous fluorescence measurement). All reactions were run in duplicates and included a negative control (H2O). Relative quantifications were performed by the comparative C_T (threshold cycle) method (Livak & Schmittgen, 2001).

5.3.11. Immunoprecipitation (IP)

Overnight cultures of strains with p416-GALS-KHD1-HA6 (RPJ plasmid 1518) and p416-GALS-KHD1(D1mt)-HA6 (RPJ plasmid 1902) were diluted in 200 ml of SC-HIS medium to OD_{600} 0.25 and allowed to grow till OD_{600} reached 0.6. Galactose was added to a final concentration of 2% and induced for 1 h at 30° C. Cells were harvested at 2500 rpm for 5 min, washed with ice cold DEPC water. The pellet was stored at -80°C until further use. Wild type strain RPY359 was used for mock IP (untagged strain).

IP was carried out by using anti- hemagglutinin (anti-HA) magnetic beads (Thermo scientific, product No.88836) according to manufacturer's instructions. Cells were thawed on ice, resuspended in low salt buffer supplemented with 1U/ µl Ribolock, RNase inhibitor and protease-inhibitor cocktail. Approximately 500 µl of 0.4 µm acid-washed glass beads were added to the cells and the mixture vibraxed for 2x for 5 min with 2 min pauses at 4°C. Meanwhile, anti-HA coated magnetic beads was prewashed with low salt buffer. Aliquots of beads were added to the lysate and the mixture incubated on a rotating wheel for 30 min to 1 h at 4°C. Flow-through was collected,

beads were washed three times with ice cold low salt buffer and divided into two aliquots, one boiled at 65°C for 10 min after adding HU buffer for Western blotting along with 'Input' and 'Flow through' samples; the other aliquot was saved for RNA isolation.

For isolating RNA, the beads were resuspended in 80 µl washing buffer (20 mM HEPES-KOH pH 7.4, 1 mM magnesium chloride, 40 mM sodium chloride) containing 0.8 µl of 10% SDS and 0.8 µl Proteinase K (Thermo Scientific) and incubated on a water bath at 30°C for 30 min. Then one volume (80 µl) of Trizol (TRI®) reagent was added, the mixture vortexed for 30 sec and then incubated at RT for 5 min. 8 µl of bromochloropropane (BCP) were added and mixed well, then incubated at RT for 10 min. Immediately prior to use, Phase Lock gel tubes (PLG, Prime 5, Heavy 2ml) were spun at 12,000 – 16,000 g for 30 sec. Samples were spun shortly and placed on a magnet to capture the beads. The organic and water phases were mixed by pipetting and the supernatant was added to the pre-spun PGL tube (without magnetic beads). Tubes were centrifuged at 12,000 – 16,000 g for 5 min at RT. RNA containing aqueous phase containing was carefully pipetted into a fresh tube; one volume of chloroform was added, mixed and added to pre-spun PGL tube, centrifuged at 12,000 – 16,000 g for 5 min at RT. Again the aqueous phase was carefully pipetted into a fresh Eppendorf tube and added one-tenth volume of 3M sodium acetate, 0.5 µl pellet paint®, 20 µg/ml linear acrylamide and 2.5 volume of cold absolute ethanol. The tube was mixed well and the RNA was precipitated overnight at -20°C. RNA was collected by centrifugation at full speed for 15 min at 4°C. The pellet was washed with 70% ethanol, air dried and resuspended in 10 µl of HPLC-water. For qRT-PCR 1:100 dilution was used.

RNA isolation, DNase treatment, cDNA preparation of Input and Flow through samples were carried out as described in section 5.3.8.

5.3.12. Calcofluor white test

Calcofluor white test was done as described (Ketela et al, 1999). Briefly, mid-log phase cells were diluted to 0.3 OD_{600} and three subsequent 10-fold serial dilution steps were performed. 5 µl of the dilution were spotted onto YEPD plates with and without 20 µg/ml Calcofluor white. Plates were incubated at 30°C for 3 days.

5.3.13. In Vivo Imaging

For imaging, a single yeast colony from a fresh plate grown at 30°C was inoculated in 1 ml of synthetic complete (SC) medium with 2% glucose but lacking leucine and tryptophane. Cells were grown at 30°C for 3-4 h. Cells were collected by short spin and resuspended in 100 µl of SC medium. A thin agarose layer was prepared by dissolving the agarose mixture (2% glucose, 2% agarose) in SC-complete medium. The mixture was dropped onto a microscope slide and covered with another glass slide until the mixture gets solidified. The glass slide was removed and 1 µl of the cell suspension was dropped onto the agarose layer and then covered with a thin cover slip. A single drop of immersion oil was applied onto the coverslip before microscopic observation.

For each cell pair, Z-stacks containing 30-50 images were acquired for GFP, mCherry, and DIC using ZEISS Cell Observer Z1 fluorescence microscope. Due to the weaker signal, mCherry images were deconvoluted before mounting. Image processing and mounting were performed with AxioVision software version 4.8 (Zeiss).

6. Summary

Localization of messenger RNA (mRNA) is a common mechanism for achieving cellular asymmetry and synaptic plasticity. More than 30 mRNAs are localized to bud tip in *Saccharomyces cerevisiae*. Till date, the localization process of a single mRNA, *ASH1* has been well studied. The localization process involves cis-acting elements with the *ASH1* mRNA and various RNA-binding proteins. In the present work, I investigated the functions of two RNA-binding proteins (Khd1p and Loc1p) that bind to *ASH1*. Previous reports demonstrated that Khd1p associates with hundreds of mRNAs and most of them code for cell wall proteins, plasma membrane proteins and secretory proteins. Since ER is the site where the synthesis of such proteins occurs, I investigated the association of Khd1p with ER and if it helps targeting mRNAs to this endomembrane system. My results suggest that Khd1p can associate with ER and directly interacts with artificial liposomes. By using an assay based on a plant viral protein Tgbp3, I showed co-localization of Khd1p with ER *in vivo*. Next, I investigated the role of Khd1p in ER targeting of its bound mRNAs. Although the SRP pathway is the general mechanism for (co-translational) targeting of mRNAs to ER, there is experimental evidence suggesting the existence of an SRP-independent pathway for targeting mRNAs to ER. In budding yeast, two RNA-binding proteins, She2p and Puf2p are involved in ER targeting of mRNAs. By using sub-cellular fractionation and qRT-PCR, I could show that deletion of Khd1p causes re-distribution of two mRNAs (*MID2 and SLG1*) from a membrane fraction to the cytosol. In one case (*MID2*), I found that the ER targeting depends on Khd1p, but not on the SRP pathway. Thus, this study identified yet another yeast RNA-binding protein involved in targeting mRNA to ER.

Loc1p is strictly a nuclear protein and known to participate in ribosome biogenesis. During *ASH1* mRNA localization process, Loc1p has been implicated as a translational regulator. In collaboration with Prof. Niessing's group at the Helmholtz Zentrum München, I could demonstrate a direct and specific interaction between Loc1p and She2p, most likely in the nucleus. Furthermore, Loc1p is stripped off from a nuclear pre-complex (Loc1p-*ASH1*-She2p) complex by the cytoplasmic She3 protein upon nuclear export of the ribonucleoprotein (RNP) complex. This study clarified the role of Loc1p in the assembly of the nuclear ASH1 mRNP.

7. Zusammenfassung

Lokalisierung von Messenger-RNA (mRNA) ist ein verbreiteter Mechanismus zur Etablierung von zellulärer Asymmetrie und synaptischen Plastizität. In der Bäckerhefe *Saccharomyces cerevisiae* werden mehr als 30 mRNAs an die Knospenspitze lokalisiert. Unter all diesen mRNAs ist der Lokalisierungsprozess einer einzigen mRNA, *ASH1* gut untersucht. Er kombiniert cis-agierende Elemente in der *ASH1* mRNA und verschiedene RNA-bindende Proteine. In der vorliegenden Arbeit untersuchte ich die Funktionen von zwei RNA-Bindungsproteinen (Khd1p und Loc1p), die an *ASH1* binden. Frühere Berichte zeigten, dass Khd1p mit Hunderten von mRNAs assoziiert und die meisten von ihnen für Zellwandproteine, Plasmamembran-Proteine und sekretorische Proteine kodieren. Da ER der Ort ist, an dem solche Protein synthetisiert werden, habe ich die Assoziation von Khd1p mit ER untersucht und das Protein bei der Bindung von mRNAs an dieses Endomembransystem beteiligt ist. Meine Ergebnisse legen nahe, dass Khd1p an ER binden kann und direkt mit künstlichen Liposomen interagiert. Durch die Verwendung eines Assays auf der Basis eines Pflanzenvirusprotein Tgbp3 konnte ich die Kolokalisation von Khd1p mit ER *in vivo* nachweisen. Ebenfalls wurde die Rolle des Khd1 Proteins beim ER-*Targeting* seiner gebundenen mRNAs untersucht. Obwohl der Signalerkennungspartikel- (SRP-) Weg den allgemeinen Mechanismus des (co-translationalen) *Targeting* von mRNAs an das ER darstellt, gibt es experimentelle Hinweise auf das Bestehen eines SRP-unabhängigen Wegs. In der Bäckerhefe sind zum Beispiel mindestens zwei RNA-bindende Proteine, She2p und Puf2p am ER-*Targeting* von mRNAs beteiligt. Durch die Verwendung von subzellulärer Fraktionierung und qRT-PCR konnte ich zeigen, dass eine Deletion von Khd1p die Umverteilung mindestens zweier mRNAs (*MID2* und *SLG1*) von einer Membranfraktion in das Cytosol verursacht. In einem Fall (*MID2*) fand ich, dass das ER-*Targeting* von Khd1p, aber nicht vom SRP-Weg abhängt. Damit konnte in dieser Studie ein weiteres Hefe-RNA-Bindungsprotein identifiziert werden, das an der Assoziation von mRNAs mit dem ER beteiligt ist.

Das RNA Bindeprotein Loc1p ist ein Kernprotein und dafür bekannt, in der Ribosomenbiogenese beteiligt zu sein. Während des *ASH1* mRNA Lokalisierungsprozesses wurde Loc1p als Translationsregulator postuliert. In

Zusammenarbeit mit der Arbeitsgruppe von Dr. Niessing am Helmholtz Zentrum München konnte ich eine direkte und spezifische Interaktion zwischen Loc1p und She2p, wahrscheinlich im Zellkern demonstrieren. Loc1p wird vor dem Kernexport aus dem nukleären RNP Prä-Komplex (Loc1p-*ASH1*-She2p) entfernt. Dies geschieht durch die Assoziation von *ASH1* und She2p mit dem zytoplasmatischen She3 Protein. Diese Studie hat die Rolle von Loc1p in der Bildung und Reorganisation des nukleären *ASH1* mRNP aufgeklärt.

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10. Publications

1. The “Loc1p” part of this thesis has been published:

Niedner A, Müller M, **Moorthy BT**, Jansen RP and Niessing D. Role of Loc1p in assembly and reorganization of nuclear *ASH1* messenger ribonucleoprotein particles in yeast.

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2. The major part of the “Introduction” in this thesis has been published as a book chapter:

Moorthy BT and Jansen RP, 2014. mRNA localization.

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